Published in final edited form as:

Mol Pharm. 2013 January 7; 10(1): 307–318. doi:10.1021/mp300445a.

Inhibition of multiple pathogenic pathways by histone deacetylase inhibitor SAHA in a corneal alkali-burn injury model

Xinyu Li¹, Qinbo Zhou³, Jakub Hanus³, Chastain Anderson³, Hongmei Zhang¹, Michael Dellinger², Rolf Brekken², and Shusheng Wang^{3,4,*}

¹Department of Ophthalmology, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75390, USA

²Department of Pharmacology, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75390, USA

³Department of Cell and Molecular Biology, Tulane University, 2000 Percival Stern Hall, 6400 Freret Street, New Orleans, LA, 70118, USA

⁴Department of Ophthalmology, Tulane University, 2000 Percival Stern Hall, 6400 Freret Street, New Orleans, LA, 70118, USA

Abstract

Neovascularization (NV) in the cornea is a major cause of vision impairment and corneal blindness. Hemangiogenesis and lymphangiogenesis induced by inflammation underlie the pathogenesis of corneal NV. The current mainstay treatment, corticosteroid, treats the inflammation associated with corneal NV, but is not satisfactory due to such side effects as cataract and the increase in intraocular pressure. It is imperative to develop a novel therapy that specifically targets the hemangiogenesis, lymphangiogenesis and inflammation pathways underlying corneal NV. Histone deacetylase inhibitors (HDACi) have been in clinical trials for cancer and other diseases. In particular, HDACi suberoylanilide hydroxamic acid (SAHA, vorinostat, Zolinza) has been approved by the FDA for the treatment of cutaneous T-cell lymphoma. The functional mechanism of SAHA in cancer and especially in corneal NV remains unclear. Here, we show that topical application of SAHA inhibits neovascularization in an alkali-burn corneal injury model. Mechanistically, SAHA inhibits corneal NV by repressing hemangiogenesis, inflammation pathways and previously overlooked lymphangiogenesis. Topical SAHA is well tolerated on the ocular surface. In addition, the potency of SAHA in corneal NV appears to be comparable to the current steroid therapy. SAHA may possess promising therapeutic potential in alkali-burn corneal injury and other inflammatory neovascularization disorders.

Keywords

corneal neovascularization; HDAC; HDAC inhibitor; hemangiogenesis; lymphangiogenesis; inflammation; alkali-burn injury

Introduction

Pathological angiogenesis has been implicated in numerous vascular diseases. The cornea is normally devoid of blood and lymphatic vessels, which is essential for good vision acuity.

*To whom correspondence should be addressed: Department of Cell and Molecular Biology, Department of Ophthalmology, Tulane University, 2000 Percival Stern Hall, 6400 Freret Street, New Orleans, LA, 70118, USA. Tel: 504-862-3163. Fax: 504-865-6785. swang1@tulane.edu.

Corneal neovascularization (NV) occurs in response to trauma, alkali burns, viral infection and keratoplasty, and leads to significant vision impairment and even blindness.¹ In response to inflammatory signals, hemangiogenesis and lymphangiogenesis, which involve the outgrowth of blood and lymphatic vessels, respectively, from the limbus into the avascular cornea, break down the “angiogenic privilege” of the cornea and underlie pathogenesis during corneal injury and repair. Angiogenic factors, including vascular endothelial growth factor (VEGF)-A, -C and -D, stimulate hemangiogenesis and lymphangiogenesis in the inflamed corneas through their receptors VEGFR2 and VEGFR3.² Cytokines secreted either from ocular resident cells or recruited inflammatory cells, including Tumor necrosis factor α (TNF- α), Interleukin-1 (IL-1), Interleukin-6 (IL-6) and VEGF, are involved in the pathogenesis and wound healing process of the inflamed cornea.³ Current therapies, like steroid medication, laser photocoagulation and limbal transplantation, are not satisfactory because of their side effects and concerns about clinical safety.⁴ A superior approach would specifically target the hemangiogenic, lymphangiogenic and inflammatory pathways that are critical for corneal NV.

Histone acetylation/deacetylation is a reversible process that is crucial for both developmental processes and disease states.⁵ Histone deacetylases (HDACs) condense chromatin structure and regulate gene expression by removing the acetyl moiety from the lysine tails of histone and non-histone proteins. As a consequence, HDACs regulate multiple pathways critically involved in cell proliferation, migration, differentiation, and cell death. HDACs can be divided into four classes (Class I to IV), which exhibit different subcellular localization and catalytic activities. Broad-spectrum and class-specific HDAC inhibitors (HDACi) have been developed and investigated for therapeutic effects for cancer and numerous other disorders.⁶ In particular, HDACi suberoylanilide hydroxamic acid (SAHA, marketed as vorinostat, brand name Zolinza) has been approved by the FDA for the treatment of cutaneous T-cell lymphoma.⁷ SAHA contains a hydroxamic acid moiety that chelates the active zinc, a carbon linker, and a hydrophobic cap that blocks entrance to the active sites in target proteins. This enables it to target HDACs consisting of a tubular pocket, a zinc-binding site and two Asp-His charge-relay systems, therefore inhibiting HDAC activity.⁸ Later studies show that non-histone proteins can be acetylated by HDAC inhibition, suggesting that HDAC inhibitor targets are broader than expected.⁹ Besides their anticancer properties, HDACi such as SAHA have anti-angiogenic and anti-inflammatory properties, making them ideal candidates for diseases with excessive angiogenesis and inflammation. HDACi block tumor angiogenesis by repressing the expression of pro-angiogenic factors including hypoxia-inducible factor (HIF)-1 α and VEGF.¹⁰ In endothelial cells (EC), SAHA inhibited tube formation in Matrigel, while another HDAC inhibitor Trichostatin A (TSA) repressed VEGF-induced expression of VEGF receptors.¹¹ In addition, intravitreal injection of SAHA repressed oxygen-induced retinal NV and laser-induced choroidal NV in mice.^{11b} HDACi have also shown therapeutic potential in inflammatory disease animal models.¹² The mechanism of HDACi in inflammation is still controversial but seems to involve the regulation of inflammatory mediators, such as nitric oxide or cytokines, through transcription factor NF- κ B.¹³ In the ocular surface, TSA reduced inflammatory and fibrotic responses through repressing TGF β signaling in an alkali-burn corneal injury model.¹⁴ In spite of extensive studies, the mechanism of HDACi action, especially in angiogenesis and inflammation, is not fully understood. Further elucidation of HDACi functional mechanisms is essential for developing superior therapies or broadening the therapeutic window of the existing HDACi.

In this study, we provide evidence that topical application of SAHA inhibits alkali-burn induced corneal NV *in vivo*. Mechanistically, SAHA functions by inhibiting hemangiogenesis, lymphangiogenesis and inflammation in the cornea through regulating hemangiogenic, lymphangiogenic and inflammatory signaling pathways. The potency of

SAHA in corneal NV appears to be at least comparable to the current steroid therapy. In addition, topical SAHA is safe on the ocular surface, without affecting corneal epithelial wound healing or corneal cell survival. We propose that SAHA may have great therapeutic potential in treating inflammatory corneal NV and other similar vascular disorders.

Results

Effect of topical SAHA on alkali-burn injury in the mouse cornea

SAHA, as an FDA approved drug for cutaneous T-cell lymphoma, has been shown to be anti-angiogenic and anti-inflammatory by several studies.^{11, 13, 15} To explore the therapeutic potential of SAHA on inflammatory corneal NV, an alkali-burn corneal injury mouse model was adopted to examine the effect of non-invasive topical SAHA on the neovascularization of mouse corneas. Clinical evaluation of the corneal opacity and neovessel size was performed as described¹⁶ Alkali burn causes corneal epithelial damage and rapid induction of corneal NV. At day 7 after alkali injury, compared to the loss of transparency and significant corneal NV observed under surgery microscope in the saline control treated corneas, the corneas appeared transparent and corneal NV was inhibited when SAHA (10 μ M) was applied topically three times a day (Fig. 1A, Supplemental Fig. 1A). SAHA treatment at 5 μ M concentration also exhibited a repressive effect on corneal NV, although not as potent as 10 μ M SAHA (data not shown). A 14 day time-course quantification of corneal transparency and size of neovessels revealed significant improvement of corneal clarity and inhibition of neovessel growth upon SAHA treatment when compared to the saline control (Fig. 1B–C). Although a repressive effect of SAHA on corneal opacity and neovessel growth was seen as early as 2 days after injury, a reversion of the corneal opacity and neovessel growth was not observed until after 4–5 days of SAHA treatment. After 8–9 days of SAHA treatment, corneal clarity was almost fully recovered and stabilized, and continuous neovessel regression was observed. By histology, at 1 day after alkali injury, although loss of corneal epithelium was observed in both saline and SAHA treated corneas, a reduction of inflammatory cell infiltration into the corneal stroma was observed upon SAHA treatment (Fig. 1D). At day 3, corneal re-epithelialization and decreased inflammatory cell count was observed in both cases, but the cornea stroma appeared less swollen in the SAHA treated corneas compared to the controls, indicating reduced inflammation by SAHA (Supplemental Fig. 1B). At 7 days after injury, corneal epithelium was disorganized in the control cornea. However, in the corneas treated with SAHA, the cornea and corneal epithelium appeared much thinner and phenotypically normal by histology, indicating a recovery of the corneas (Fig. 1D). Moreover, neovessels were readily detectable in the middle region of saline-treated but not SAHA-treated corneas, which confirmed the inhibition of corneal NV by SAHA. Taken together, these results indicate that SAHA potently inhibits corneal NV induced by alkali-burn injury.

Inhibition of alkali burn-induced hemangiogenesis, lymphangiogenesis and inflammation by SAHA *in vivo*

To further characterize the corneal NV phenotype imposed by topical SAHA, neovessels and inflammatory cells in the cornea were stained and quantified at 7 days after alkali-burn injury and SAHA treatment. To distinguish hemangiogenesis and lymphangiogenesis in the cornea, whole mount staining of the corneal neovessels with PECAM-1 (platelet/endothelial cell adhesion molecule-1, a marker for blood ECs) and LYVE-1 (lymphatic vessel endothelial hyaluronin acid receptor-1, a marker for lymphatic vessels) were performed. As shown in Fig. 2A, alkali-burn injury induced significant hemangiogenesis by PECAM-1 staining and lymphangiogenesis by LYVE-1 staining. Topical SAHA treatment dramatically reduced hemangiogenesis and lymphangiogenesis induced by alkali-burn injury. Quantification analyses showed that SAHA reduced PECAM-1⁺ area by ~66%

($249287 \pm 13440 \mu\text{m}^2$ in saline treated corneas and $84821 \pm 3815 \mu\text{m}^2$ in SAHA treated corneas, $N=9$) and LYVE-1⁺ area by ~65% ($186413 \pm 10320 \mu\text{m}^2$ in saline treated corneas and $64249 \pm 4175 \mu\text{m}^2$ in SAHA treated corneas) (Fig. 2B–C). These data illustrate that SAHA inhibits corneal hemangiogenesis and lymphangiogenesis in response to alkali-burn injury.

HDACi have also been shown to repress inflammation in several animal disease models.¹² Our histological analyses suggested that SAHA inhibits corneal inflammation in response to alkali burn injury (Fig. 1D). To further characterize the effect of SAHA in corneal inflammation, macrophages infiltrated into the corneal stroma were stained with F4/80 anti-macrophage antibody at 7 days after injury. F4/80 staining of the corneal sections revealed that extensive macrophage infiltration into the stroma at 7 days after alkali injury (Fig. 2D). Topical SAHA treatment resulted in ~70% decrease in macrophage infiltration (Fig. 2E). These results are consistent with a recent report that systemic administration of a different HDACi TSA reduced inflammation in the alkali-burned mouse cornea.¹⁴

Inhibition of angiogenic signaling pathways by SAHA

SAHA has been shown to inhibit tube formation and cell migration in ECs in Matrigel.^{11b} In addition, TSA was reported to repress VEGF-induced VEGFR1 and VEGFR2 expression at mRNA level.^{11a} As an FDA approved cancer drug, how SAHA regulates angiogenic signaling in ECs is still unclear. During angiogenesis, the binding of VEGF to its receptors induces the activation of several downstream kinases, including mitogen activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and protein kinases C and D (PKC and PKD), which promote EC proliferation, migration and survival.¹⁷ To explore the cell-autonomous effect of SAHA on angiogenic signaling in ECs, human umbilical vein ECs (HUVEC) were treated with SAHA (10 μM) for 24 hours, and tested for its effect on the activation of MAPK, PI3K, and PKC/PKD pathways in response to VEGF by Western blot analyses. We first tested the toxicity of SAHA in HUVECs using a dimethylthiazol tetrazolium bromide (MTT) assay. Treatment of HUVECs with SAHA at 5 μM , 10 μM , and 20 μM for three days resulted in ~12%, ~17%, and 38% decrease in cell growth, respectively (Fig. 3A). These indicate that SAHA at 5–10 μM is tolerated in HUVECs under normal culture conditions. SAHA at 10 μM was then used in the following experiments. Phosphorylation of VEGFR2, ERK1/2, PKC/PKD, and AKT was strongly induced after 20 minutes of VEGF treatment in HUVECs. SAHA treatment dramatically inhibited the VEGFR2, ERK1/2, PKD, and AKT phosphorylation in response to VEGF without affecting their total protein levels (Fig. 3B and data not shown).

Class II HDACs have been shown to shuttle from the nucleus to the cytoplasm in response to VEGF, which is required for EC proliferation, migration and angiogenesis.¹⁸ To further test whether SAHA represses VEGF-induced class II HDAC nuclear export, HUVECs were infected with adenovirus expressing FLAG-HDAC7 and treated with SAHA. HDAC7 subcellular localization in response to VEGF treatment was examined by staining with FLAG antibody. Starvation drove the shuttling of HDAC7 into the nucleus, a process which was reversed by VEGF treatment (Fig. 3C). However, SAHA treatment inhibited the nuclear export of HDAC7 in response to VEGF. Taken together, these data suggest that SAHA functions in ECs by repressing PKC/PKD, MAPK, and PI3K pathways and Class II HDAC nucleocytoplasmic shuttling in response to angiogenic factors, providing a novel mechanism whereby SAHA functions in ECs.

Inhibition of lymphangiogenesis by SAHA *in vitro*

Our novel finding that SAHA inhibits lymphatic EC outgrowth *in vivo* (Fig. 2A) lead us to consider whether HDACi directly regulate lymphangiogenesis. To define a cell-autonomous

role for SAHA in lymphangiogenesis, primary human lymphatic cells (LEC) were treated with SAHA, and tested for proliferation, migration and tube formation in Matrigel *in vitro*. Again, we first tested the toxicity of SAHA in LECs by MTT assay. As shown in Fig. 4A, SAHA was tolerated in LECs, with about 75% survival at 1 day after SAHA treatment with a concentration up to 20 μ M. BrDU incorporation, scratch wound migration and *in vitro* Matrigel assays were then performed as described to test the effect of SAHA on LEC proliferation, migration, and tube formation *in vitro*.¹⁹ Compared to the saline control, SAHA treatment significantly repressed the proliferation of LECs cultured in normal medium as revealed by BrDU incorporation (~30% repression) (Fig. 4B). Moreover, SAHA dramatically inhibited LEC migration (~80% inhibition) after scratch wound (Fig. 4C, Supplemental Fig. 2A) and tube formation (~64% inhibition) as quantified by branch points in Matrigel *in vitro* (Fig. 4D, Supplemental Fig. 2B). Of note, for the wound scratch assay, 1 μ M of 5-fluorouracil was used to block cell proliferation; therefore the effect of SAHA on migration was independent of its repression in cell proliferation. These results suggest that SAHA functions directly on lymphatic ECs to inhibit lymphangiogenesis.

Effect of topical SAHA on inflammatory gene expression *in vivo*

Our results showed that macrophage infiltration into the stroma was strongly inhibited by topical SAHA after alkali injury (Fig. 2D–E). To further confirm whether this phenomenon is associated with decreased inflammatory gene expression in alkali-burned cornea, real-time reverse transcription (RT) PCR was performed to examine the expression of pro-inflammatory and pro-angiogenic cytokines. IL-1 β , IL-6, TNF- α , and VEGF expression was significantly upregulated by alkali-burn injury (data not shown). Compared to the saline control, SAHA application resulted in a dramatic inhibition of the expression of these genes at 3 days, which was further decreased to less than 5% of the controls at 7 days in the cornea after alkali-burn (Fig. 5A, Supplemental Fig. 3A). Considering the ~70% repression of macrophage infiltration by SAHA (Fig. 2D), these data suggest that SAHA inhibits both macrophage infiltration and the expression of inflammatory cytokines in the infiltrated macrophages. To further prove this hypothesis, we treated differentiated THP-1 monocytes with pro-inflammatory agent lipopolysaccharides (LPS) and tested the effect of SAHA on inflammatory gene expression *in vitro*. As shown by real-time RT-PCR, IL-1 β , IL-6, TNF- α , and VEGF expression was induced by LPS, but was dramatically repressed at 24 hours after SAHA treatment (Fig. 5C, and data not shown). The NF κ B pathway plays a key role in inflammation by regulating the expression of inflammatory cytokines.²⁰ In response to pro-inflammatory stimuli, NF κ B translocates into the nucleus and activates the expression of its target genes including IL-1 β , IL-6, TNF- α , and HIF1 α , which can induce VEGF expression. To further dissect the mechanism by which SAHA inhibits inflammation, NF κ B subcellular localization was visualized by NF κ B staining after LPS and SAHA treatment. NF κ B nuclear transport was rapidly induced by LPS in THP-1 cells in 15 minutes, and stronger nuclear staining was observed at 30 minutes and 1 hour, which was then gradually decreased at 4 hours (Supplemental Fig. 3B). SAHA treatment only delayed NF κ B nuclear transport induced by LPS at the 15 minute time point, and NF κ B still translocated to the nucleus at 30 minutes, although less NF κ B nuclear accumulation was observed at 4 hours post treatment. These results indicate that NF κ B nuclear translocation is not a major mechanism for the effect of SAHA on inflammatory gene expression in THP-1 cells. I κ B α degradation and NF κ B p65 phosphorylation are required for NF κ B nuclear translocation. No significant difference in I κ B α degradation and NF κ B p65 phosphorylation was observed in THP-1 cells after LPS and SAHA treatment (Supplemental Fig. 3C), which is consistent with the result that NF κ B subcellular localization induced by LPS is similar with or without SAHA treatment. We did, however, observe a repression of I κ B α phosphorylation and elevation of histone H3 acetylation after SAHA treatment, indicating that SAHA is functional in THP-1 cells. Taken together, SAHA likely affects NF κ B activity in the nucleus, rather than

in the cytoplasm. To test this, chromatin immunoprecipitation (ChIP) experiments were performed to see whether the binding of NFκB to the promoters of its target genes was inhibited by SAHA. As shown by real-time PCR with promoter-specific primers (Fig. 5C), SAHA repressed the binding of NFκB to IL-1β, IL-6, and TNF-α promoter, indicating that SAHA regulates inflammation by repressing the binding of NFκB to its downstream gene promoters.

Comparison of SAHA to steroid therapy and other HDAC inhibitors in corneal NV

Corticosteroids, such as dexamethasone, are the current clinical treatment for corneal NV. To directly compare the effect of SAHA in corneal NV to the current steroid therapy, corneas were treated with topical saline, 10μM SAHA or 0.1% dexamethasone eye drops (Alcon) three times a day after alkali-burn, and evaluated for corneal opacity and neovessel size for 14 consecutive days. The efficacy of SAHA was comparable to dexamethasone in repressing corneal NV as revealed by opacity and neovessel size scores (Fig. 6A–B). In several time points during the course of the treatment, the effect of SAHA appeared significantly better than dexamethasone. To compare the efficacy of different HDAC inhibitors in corneal NV, SAHA was compared side-by-side with class II-specific HDAC inhibitor MC1568 and class I-specific HDAC inhibitor MGCD-0103 using the same molar concentration. SAHA, as a broad-spectrum HDACi, appeared to repress corneal NV more potently than MC1568 or MGCD-0103 after alkali-burn (Supplemental Fig. 4A–B), suggesting that inhibition of both class I and class II HDACs is required for inhibiting corneal NV.

Safety profile of topical SAHA on the ocular surface

To study the safety profile of topical SAHA in the ocular surface, a corneal epithelium wound healing assay was performed.²¹ For this, a central ~1.8mm² area of corneal epithelium was scraped off, and the de-epithelialized area of the cornea was measured by digital imaging after fluorescein staining at 8, 16, and 24 hours after wound. Normally, corneal re-epithelialization and recovery occurs at 24 hours after scraping. Topical application of SAHA at 10μM did not affect the process of recovery of the epithelium, suggesting topical SAHA is safe in the short-term on the ocular surface (Supplemental Fig. 5A). To examine the long-term effect of SAHA on the ocular surface, SAHA was applied topically to the eye at 3 times a day for 7 days, and cell death in the cornea was stained by TUNEL assay. By histology, the corneas appeared normal after 7 days of topical SAHA treatment (Supplemental Fig. 5B). Minimal cell death was observed in the corneal epithelium in the controls, likely reflecting the turnover of the corneal epithelium (Fig. Supplemental Fig. 5C). Importantly, SAHA treatment did not affect cell survival in the cornea. Taken together, these data show that topical SAHA does not affect corneal epithelial wound healing or corneal cell survival *in vivo*, suggesting that topical SAHA is safe on the ocular surface.

Discussion

Our data reveals that topical HDACi SAHA inhibits corneal NV in an alkali-burn corneal injury model. The action of SAHA reflects its specific inhibition of multiple pathogenic pathways critical for corneal NV, namely hemangiogenesis, lymphangiogenesis, and inflammation (Supplemental Fig. 6). The potency of SAHA at inhibiting corneal NV is comparable to the current steroid therapy. Moreover, topical SAHA appears to be safe on the ocular surface, not affecting corneal epithelial wound healing or corneal cell survival.

SAHA is an FDA approved drug for treatment of cutaneous T-cell lymphoma and is under clinical trials for numerous cancers. Our results that SAHA inhibits corneal NV are

consistent with a recent report that SAHA represses oxygen-induced retinal NV and laser-induced choroidal NV in mice.^{11b} Consistently, another HDACi, TSA, was also shown to reduce inflammatory response in the ocular surface.¹⁴ Our data extends these findings by showing that SAHA inhibits alkali-burn induced corneal NV by specifically repressing hemangiogenesis, lymphangiogenesis, and inflammation, which are the critical pathogenic pathways for corneal NV. The repression of corneal hemangiogenesis and lymphangiogenesis by SAHA was demonstrated by specific staining and quantification of blood and lymphatic vessels. Reduced inflammation in the cornea by topical SAHA was shown by histology, staining of infiltrated macrophages with F4/80 antibody, and expression profile of the pro-inflammatory cytokines. As a result, corneas treated with SAHA showed much improved recovery after alkali-burn injury, as demonstrated by time-course clinical assessment of the corneal opacity and neovessel size. Taken together, our data illustrates that SAHA is a multi-functional drug with potent therapeutic effects in inflammatory corneal NV.

HDACi TSA inhibits tumor angiogenesis by repressing HIF-1 α and VEGF.²² The mechanism whereby SAHA regulates angiogenic response in ECs remains unclear. TSA has been shown to inhibit VEGF receptors VEGFR1 and VEGFR2 at the mRNA level, as well as up-regulate the VEGF competitor semaphorin III protein.^{11a} Our results show that SAHA inhibits VEGF induced VEGFR2, PKC/PKD, ERK1/2, and AKT phosphorylation without affecting VEGFR2 protein level. The difference in VEGFR2 expression in our experiments may result from the difference in functional mechanisms between TSA and SAHA, post-transcriptional regulation of VEGFR2, and/or the experimental conditions. Since SAHA also up-regulates the expression of VEGF competitor semaphorin 3A protein and RNA level (Supplemental Fig. 7),^{11a} we speculate that the upregulation of semaphorin 3A may partially account for the repression of VEGF downstream signaling by SAHA. Activation of VEGF signaling through the PKC/PKD pathway results in the phosphorylation of class II HDACs and their shuttling from the nucleus to the cytoplasm.¹⁸ Consistent with the downregulation of PKC/PKD signaling, the shuttling of HDAC7 from the nucleus to the cytoplasm in response to VEGF is also repressed by SAHA. The target proteins of SAHA that repress angiogenic pathways in ECs remain unclear.

Lymphangiogenesis is required for the spreading of immune cells and tumor cells, and therefore involved in the pathogenesis of many diseases.²³ Inflammatory conditions in the cornea caused by chemical burns or herpes infection, and surgical procedures such as penetrating keratoplasty, can lead to both hemangiogenesis and lymphangiogenesis.²⁴ These inflammation-associated processes compromise corneal transparency and can lead to corneal blindness. Outgrowth of lymphatic vessels is primarily triggered by growth factors VEGF-C and -D and their receptor VEGFR3.²⁵ The role of HDACs and HDACi SAHA in lymphangiogenesis is not yet known. We show that SAHA inhibits lymphatic EC proliferation, migration, tube formation *in vitro*, and lymphangiogenesis *in vivo*, suggesting that HDACs play an important role in lymphangiogenesis. It is likely that the repression of inflammation by SAHA in our alkali-burn model is partially attributed to the inhibition of lymphangiogenesis. The identification of SAHA as an inhibitor of lymphangiogenesis may have implications in other diseases, such as corneal transplantation and tumor metastasis. Future studies are warranted to dissect the exact mechanism of HDAC and HDACi function in lymphangiogenesis.

SAHA also functions on inflammatory cells to regulate inflammatory response. Our *in vitro* results reveal that SAHA does not affect NF κ B degradation, NF κ B p65 phosphorylation, or NF κ B nuclear translocation in THP-1 macrophage in response to LPS stimulation. However, SAHA does repress the binding of NF κ B to the promoters of its target genes. In a recent report, SAHA was shown to suppress LPS-induced NF κ B p65 nuclear accumulation

in THP-1 cells.²⁶ The difference between our experiments likely reflects the difference in SAHA concentration, since the effect of SAHA might be cell-type and concentration dependent. NF κ B has been shown to be a central regulator of immune response.²⁷ Activation of NF κ B induces the expression of many inflammatory cytokines. SAHA inhibits the binding of NF κ B to the promoters of its target genes, thereby repressing the expression of IL-1 β , IL-6, TNF- α , and VEGF, which accounts for the repression of inflammation after alkali-burn injury.

Corneal NV induced by trauma, chemical burn, viral infection, and surgical keratoplasty is one of the major causes of vision impairment and corneal blindness. Alkali-burn injury produces extensive damage to the corneal epithelium and the anterior segment of the eye. This triggers a series of events, such as inflammation, hemangiogenesis, and lymphangiogenesis, which leads to corneal damage and repair response. Unlike the current steroid drugs that function as a non-specific inhibitor for inflammation, SAHA specifically inhibits multiple pathogenic mechanisms of corneal NV, namely hemangiogenesis, lymphangiogenesis, and inflammation. Direct comparison of SAHA to the current clinical drug dexamethasone indicates that, with regard to efficacy, topical SAHA is at least comparable to dexamethasone eye drops in repressing corneal NV. In our study, topical SAHA appears to be safe on the ocular surface, not affecting corneal epithelial wound healing or corneal cell survival. Furthermore, topical application of drugs like SAHA represents an ideal route for drug delivery to the cornea due to its non-invasive treatment method and minimal adverse systemic side effects. Therefore, topical SAHA appears to be a viable therapeutic option for corneal NV and potentially other disorders with inflammatory NV. Nevertheless, further work is needed to compare the long-term effects of SAHA with current clinical drugs, especially regarding the potential side effects in cataract and increased intraocular pressure. Further optimization in formulation and delivery is also warranted to develop SAHA into a drug superior to the current steroid agents.

Materials and Methods

Animals

C57BL/6 or 129/Sv strain male mice (8 to 10 weeks of age) were used for the study. The animal studies were conducted in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center.

Alkali-burn Corneal Injury and SAHA Treatment

Alkali-burn corneal injury was performed as described.²⁸ Briefly, mice were anesthetized with intraperitoneal injection of ketamine (40mg/kg) and Xylazine (10mg/kg). Topical application of 0.5% Proparacaine Hydrochloride (Alcon Laboratories, Fort Worth, TX) was placed on their corneal surfaces. A piece of round filter paper (Whatman 1, Whatman International Ltd, diameter at ~2mm) soaked in 1N NaOH was applied on the central cornea of the right eyes for 30 seconds under a surgical microscope. The area of acute alkali-burn was approximately 2 $\times$ 2mm. The eyes were immediately rinsed with 20ml 0.9% sterile saline solution. Stock solution of SAHA (Cayman Chemical), MC1568 (Selleck Chemicals), or MGCD0103 (Selleck Chemicals) was made at 100mM in dimethyl sulfoxide (DMSO), and diluted to 10 μ M in balanced salt solution (BSS) for use. The final DMSO concentration in the solution was less than 0.1%. Dexamethasone (0.1%) eye drops were purchased from Alcon Lab. SAHA was applied topically on the cornea of the mice three times a day for 7 or 14 days immediately after corneal injury. Topical antibiotic ointment was applied to the eyes after surgery. Saline with equivalent concentration of DMSO was included as controls for treatment.

Clinical Assessment of Corneal NV

Clinical assessment of corneal NV was performed as described.¹⁶ The development of corneal NV was observed on a daily basis under stereoscopic microscope in a blinded fashion for 7–14 days. A scoring system was used to evaluate corneal opacity and neovessel size. Corneal opacity was scored on a scale of 0–4 (0 = completely clear; 1 = slightly hazy, iris and pupils easily visible; 2 = slightly opaque, iris and pupils still detectable; 3 = opaque, pupils hardly detectable; and 4 = completely opaque with no view of the pupils). Neovessel size was scored on a scale of 0–3 (0 = no vessel; 1 = vessels detectable under a surgical microscope; 2 = vessels easily seen under a surgical microscope and 3 = vessels easily seen near the center of the cornea under a surgical microscope). The scores by two masked observers were added, and the final score was the average of the two. Photographs were taken at 7 days after treatment using a Canon A650 digital camera attached to the surgical microscope.

Histology and Immunohistochemistry

Histology and immunohistochemistry were performed as described.²⁹ Eyes were removed at 7 days after treatment, and fixed in 4% paraformaldehyde at 4°C for 1 hour. The corneas were excised, washed in PBS, and processed for whole mount immunostaining. For frozen section, the cornea was embedded in optimal cutting temperature (OCT) compound (Sakura Finetek Inc, Torrance), and cut into 8 µm sections. Primary antibodies used include: rat anti-PECAM-1 (1:100, BD Pharmingen), rabbit anti-LYVE-1 (1:500, Abcam), and rat anti-mouse F4/80 (1:200, a gift from Dr. Philip Thorpe at UT Southwestern Medical Center). Alexa-Fluor 488 conjugated goat anti-rat IgG and Alexa-Fluor 590 conjugated goat anti-rabbit IgG (1:800, Invitrogen) were used as secondary antibodies. Corneal cell death was detected using a TUNEL assay kit from Roche. Digital images were taken using a Zeiss Axio Observer D1 inverted microscope with cold black/white camera and color camera. PECAM-1, LYVE-1, and F4/80 positive areas were quantified using a free Image J program (NIH, USA).

Cell culture, Immunocytochemistry, Western blot, RNA analyses and ChIP assays

HUVECs (Lonza) were cultured as described.²⁹ Human adult dermal lymphatic microvascular ECs (Lonza) were cultured using medium from an EGM2 MV Bullet kit. Lymphatic EC proliferation and migration assays were performed as described.¹⁹ Adenovirus expressing FLAG-tagged HDAC7 was infected into HUVECs as described.^{18a} For the treatments, ECs were treated with SAHA for 24 hours, starved overnight, and treated with VEGF for indicated times before they were used for staining, protein, or RNA analyses. The toxicity of SAHA on ECs was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich). Human monocytic cell line THP-1 (ATCC) cells were cultured in IMDM medium supplemented with FBS, Sodium pyruvate, L-glutamine, non-essential amino acids and 2-mercaptoethanol. THP-1 cells were differentiated into macrophage by treating with 10 ng/ml PMA (Sigma-Aldrich), and then with SAHA and LPS before used for NFκB staining. Antibodies used include FLAG (monoclonal; Sigma-Aldrich), IκBα (1:1000, Cell Signaling), Phospho-IκBα (1:1000, Cell Signaling), Phospho-p65 (1:1000 Millipore), Acetyl-Histone H3 (1:1000, Upstate), VEGFR2 (1:1000, Cell signaling), Phospho-VEGFR2 (1:1000, Cell signaling), ERK1/2 (1:1000, Cell signaling), Phospho-ERK1/2 (1:1000, Cell signaling), AKT (1:1000, Cell signaling), Phospho-AKT (1:1000, Cell signaling), Phospho-PKD1 (1:1000, Cell Signaling), PKD1 (1:1000, Cell Signaling), semaphoring (SEMA) 3A (1:1000, Abcam) and GAPDH (1:5000, Polyclonal, Abcam). Primers for real-time PCRs include, mouse β-actin, 5'-GAGATTACTGCTGGCTCCTA-3' and 5'-TCATCGTACTCCTGCTTGCTGAT-3'; mouse IL-1β, 5'-TCCAGGATGAGGACATGAGCAC-3' and 5'-GAACGTCACACACCAGC AGGTTA-3'; mouse IL-6, 5'-

GCTACCAAAGCTGGATATAATCAGGA-3' and 5'-CCAGGTAGCTATGGTACTCCAGAA-3'; mouse TNF- α , 5'-CCCTCACACTCAGATCATCTTCT-3' and "5'-GCTACGACGTGGGCTACAG-3'; mouse VEGF-A, 5'-GGAGATCCTTCGAGGAGCACTT-3' and 5'-GGCGATTTAGCAGCAGATATAAG AA-3'. Human primers used for real-time PCR include, IL-1 β , 5'-GGACAAGCTGAGGAAGATGC-3' and 5'-TCGTTATCCCATGTGTCG AA-3'; IL-6, 5'-CACACAGACAGCCACTCACC-3' and 5'-TTTTCTGCCAGTGCCTCTTT-3'; VEGF-A, 5'-AGTGTGTGCCCACTGAGGA-3' and 5'-GGTGAGGTTTGATCCGCATA-3'; SEMA 3A, 5'-TGCCAAGGCTGAAATTA-3' and 5'-CCTACTCCGTTCCTCAT-3'; TNF α , 5'-AACCTCCTCTCTGCCATCAA-3' and 5'-GGAAGACCCCTCCCAGATAG-3'; CyclophilinA 5'-CCCGTGTCTTTCGACAT-3' and 5'-CCAGTGCTCAGAGCACGAAA-3'.

ChIP assay was performed according to manufacturer's protocol (Millipore). In brief, 1×10^6 THP-1 cells were fixed with 1% formaldehyde for 10min at room temperature. The cells were then washed twice with PBS and collected by scraping and centrifugation. Collected cells were resuspended in the SDS Lysis Buffer containing $1 \times$ Protease Inhibitor Cocktail. DNA was sheared by sonication with 4 set of 10-second pulses using Fisher Scientific Ultrasonic Dismembrator Model 150T set to 50% of maximum amplitude. The sheared and crosslinked chromatin was precleared with Protein G Agarose for 1 hour at 4°C prior to adding 10 μ g of ChIP grade antibody for NF κ B p65 (RelA) (Millipore). As a positive control, 10 μ g of Anti-RNA Polymerase II antibody was used (Millipore). After overnight incubation at 4°C, the samples were then incubated for 1 hour with Protein G Agarose at 4°C followed by collecting the beads by centrifugation and washing with the manufacturer's supplied buffers. Elution of protein/DNA complexes was performed by incubating samples at room temperature for 15min in Elution Buffer (1% SDS, 0.1M NaHCO₃). The samples were incubated for 4 hours at 65°C after adding NaCl to final concentration of 0.2M to reverse crosslink, which was followed by RNase digestion for 30min at 37°C and Proteinase K digestion for 1 hour at 45°C. Freed DNA was purified by the Spin Filter purification system supplied by manufacturer. ChIP samples were analyzed using Real-Time quantitative PCR with following parameters: 1) initial denaturation at 94°C for 10min, 2) denaturation at 94°C for 20sec, 3) anneal and extension at 60°C for 1min. Steps 2–3 were repeated 50 times. Control primer for GAPDH promoter was supplied by the manufacturer, primers to amplify p65 binding region in the promoter region of NF κ B target genes were as follows, IL6 5'-GCTAGCCTCAATGACGACCT-3' and 5'-GCCTCAGACATCTCCAGTCC-3'; TNF α , 5'-GCTTCCTCCAGATGAGCTT-3' and 5'-TGCTGTCCTTGCTGAGGGA-3'; IL1 β , 5'-TCCCTCCCTTAGTGCCAACTATGT-3' and 5'-ACAGTCTCCACAGTTCTGCCATGT-3'.

Corneal Epithelial Wound Healing Assay

Corneal wound healing assay was performed as described to test the safety of SAHA on the ocular surface.²¹ To do so, mice were anesthetized, and the central 1.8-mm² area of corneal epithelium was scraped off using 1.5-mm diameter trephine. SAHA (10 μ M) or Saline control was applied topically to the eye for 3 times in a 12 hour period. Digital images of the corneas were taken after topical application of Fluorescein and washing with PBS to measure the corneal re-epithelialization at 8, 16, and 24 hours after treatment. The epithelial defect area was calculated and quantified using the Image J program.

Statistics

Each experiment was repeated at least three times. Student's T-tests were used to determine statistical significance between groups. P-values of less than 0.05 were considered to be statistically significant.

Abbreviations Used

AKT/PKB: protein kinase B; EGM-2: endothelial cell growth medium-2; ERK: Extracellular signal-regulated kinases; HDAC: histone deacetylase; HDACi: histone deacetylase inhibitors; HIF-1 α : hypoxia induced factor 1 α ; HUVEC: human umbilical vein endothelial cell; IL: interleukin; I κ B- α : nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; LEC: lymphatic endothelial cells; LPS: Lipopolysaccharide; LYVE-1: lymphatic vessel endothelial hyaluronic acid receptor-1; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF κ B: nuclear factor kappa-light-chain-enhancer of activated B cells; NV: neovascularization. PECAM-1: platelet/endothelial cell adhesion molecule-1; PKC/D: protein kinase C/D; SAHA: suberoylanilide hydroxamic acid; TNF- α : tumor necrosis factor α ; TSA: Trichostatin A; VEGF: vascular endothelial growth factor;

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Philip Jarrett for proofreading of the manuscript. We are grateful for Dr. Philip Thorpe from UT Southwestern Medical Center for providing F-4/80 antibody. We would also like to thank Rachel Gallagher for her initial technical help on the project. S.W. was supported by a Startup fund from the Department of Ophthalmology at UT Southwestern Medical Center at Dallas, President's Research Council New Investigator Award, NIH Grants EY021862 and EY020799, an unrestricted grant and a career development award from the Research to Prevent Blindness foundation.

Reference

1. Chang JH, Gabison EE, Kato T, Azar DT. Corneal neovascularization. *Curr Opin Ophthalmol*. 2001; 12(4):242–249. [PubMed: 11507336]
2. (a) Cursiefen C, Chen L, Borges LP, Jackson D, Cao J, Radziejewski C, D'Amore PA, Dana MR, Wiegand SJ, Streilein JW. VEGF-A stimulates lymphangiogenesis and hemangiogenesis in inflammatory neovascularization via macrophage recruitment. *J Clin Invest*. 2004; 113(7):1040–1050. [PubMed: 15057311] (b) Bock F, Onderka J, Dietrich T, Bachmann B, Pytowski B, Cursiefen C. Blockade of VEGFR3-signalling specifically inhibits lymphangiogenesis in inflammatory corneal neovascularisation. *Graefes Arch Clin Exp Ophthalmol*. 2008; 246(1):115–119. [PubMed: 17909835]
3. (a) Sotozono C, He J, Matsumoto Y, Kita M, Imanishi J, Kinoshita S. Cytokine expression in the alkali-burned cornea. *Curr Eye Res*. 1997; 16(7):670–676. [PubMed: 9222084] (b) Planck SR, Rich LF, Ansel JC, Huang XN, Rosenbaum JT. Trauma and alkali burns induce distinct patterns of cytokine gene expression in the rat cornea. *Ocul Immunol Inflamm*. 1997; 5(2):95–100. [PubMed: 9234373] (c) Sotozono C, He J, Tei M, Honma Y, Kinoshita S. Effect of metalloproteinase inhibitor on corneal cytokine expression after alkali injury. *Invest Ophthalmol Vis Sci*. 1999; 40(10):2430–2434. [PubMed: 10476814] (d) Sakimoto T, Yamada A, Kanno H, Sawa M. Upregulation of tumor necrosis factor receptor 1 and TNF-alpha converting enzyme during corneal wound healing. *Jpn J Ophthalmol*. 2008; 52(5):393–398. [PubMed: 18991041] (e) Lu P, Li L, Liu G, Zhang X, Mukaida N. Enhanced experimental corneal neovascularization along with aberrant angiogenic factor expression in the absence of IL-1 receptor antagonist. *Invest Ophthalmol Vis Sci*. 2009; 50(10):4761–4768. [PubMed: 19458323]
4. Gupta D, Illingworth C. Treatments for Corneal Neovascularization: A Review. *Cornea*. 2011

5. Haberland M, Montgomery RL, Olson EN. The many roles of histone deacetylases in development and physiology: implications for disease and therapy. *Nat Rev Genet.* 2009; 10(1):32–42. [PubMed: 19065135]
6. Marks PA, Xu WS. Histone deacetylase inhibitors: Potential in cancer therapy. *J Cell Biochem.* 2009; 107(4):600–608. [PubMed: 19459166]
7. (a) Duvic M, Talpur R, Ni X, Zhang C, Hazarika P, Kelly C, Chiao JH, Reilly JF, Ricker JL, Richon VM, Frankel SR. Phase 2 trial of oral vorinostat (suberoylanilide hydroxamic acid, SAHA) for refractory cutaneous T-cell lymphoma (CTCL). *Blood.* 2007; 109(1):31–39. [PubMed: 16960145]
(b) Marks PA, Breslow R. Dimethyl sulfoxide to vorinostat: development of this histone deacetylase inhibitor as an anticancer drug. *Nat Biotechnol.* 2007; 25(1):84–90. [PubMed: 17211407]
8. Finnin MS, Donigian JR, Cohen A, Richon VM, Rifkind RA, Marks PA, Breslow R, Pavletich NP. Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. *Nature.* 1999; 401(6749):188–193. [PubMed: 10490031]
9. Drummond DC, Noble CO, Kirpotin DB, Guo Z, Scott GK, Benz CC. Clinical development of histone deacetylase inhibitors as anticancer agents. *Annual review of pharmacology and toxicology.* 2005; 45:495–528.
10. Liu T, Kuljaca S, Tee A, Marshall GM. Histone deacetylase inhibitors: multifunctional anticancer agents. *Cancer Treat Rev.* 2006; 32(3):157–165. [PubMed: 16516391]
11. (a) Deroanne CF, Bonjean K, Servotte S, Devy L, Colige A, Clausse N, Blacher S, Verdin E, Foidart JM, Nussgens BV, Castronovo V. Histone deacetylases inhibitors as anti-angiogenic agents altering vascular endothelial growth factor signaling. *Oncogene.* 2002; 21(3):427–436. [PubMed: 11821955]
(b) Kim JH, Oh M, Yu YS, Kim KW, Kwon HJ. N-hydroxy-7-(2-naphthylthio) heptanamide inhibits retinal and choroidal angiogenesis. *Mol Pharm.* 2009; 6(2):513–519. [PubMed: 19718802]
12. Shuttleworth SJ, Bailey SG, Townsend PA. Histone Deacetylase inhibitors: new promise in the treatment of immune and inflammatory diseases. *Curr Drug Targets.* 2010; 11(11):1430–1438. [PubMed: 20583972]
13. Blanchard F, Chipoy C. Histone deacetylase inhibitors: new drugs for the treatment of inflammatory diseases? *Drug Discov Today.* 2005; 10(3):197–204. [PubMed: 15708534]
14. Kitano A, Okada Y, Yamanka O, Shirai K, Mohan RR, Saika S. Therapeutic potential of trichostatin A to control inflammatory and fibrogenic disorders of the ocular surface. *Mol Vis.* 2010; 16:2964–2973. [PubMed: 21203344]
15. Glauben R, Siegmund B. Inhibition of histone deacetylases in inflammatory bowel diseases. *Mol Med.* 2011; 17(5–6):426–433. [PubMed: 21365125]
16. Yoeruek E, Ziemssen F, Henke-Fahle S, Tatar O, Tura A, Grisanti S, Bartz-Schmidt KU, Szurman P, Safety. penetration and efficacy of topically applied bevacizumab: evaluation of eyedrops in corneal neovascularization after chemical burn. *Acta Ophthalmol.* 2008; 86(3):322–328. [PubMed: 17995975]
17. Olsson AK, Dimberg A, Kreuger J, Claesson-Welsh L. VEGF receptor signalling - in control of vascular function. *Nat Rev Mol Cell Biol.* 2006; 7(5):359–371. [PubMed: 16633338]
18. (a) Wang S, Li X, Parra M, Verdin E, Bassel-Duby R, Olson EN. Control of endothelial cell proliferation and migration by VEGF signaling to histone deacetylase 7. *Proc Natl Acad Sci U S A.* 2008; 105(22):7738–7743. [PubMed: 18509061]
(b) Ha CH, Jhun BS, Kao HY, Jin ZG. VEGF stimulates HDAC7 phosphorylation and cytoplasmic accumulation modulating matrix metalloproteinase expression and angiogenesis. *Arterioscler Thromb Vasc Biol.* 2008; 28(10):1782–1788. [PubMed: 18617643]
(c) Ha CH, Wang W, Jhun BS, Wong C, Hausser A, Pfizenmaier K, McKinsey TA, Olson EN, Jin ZG. Protein kinase D-dependent phosphorylation and nuclear export of histone deacetylase 5 mediates vascular endothelial growth factor-induced gene expression and angiogenesis. *J Biol Chem.* 2008; 283(21):14590–14599. [PubMed: 18332134]
19. Zhou Q, Gallagher R, Ufret-Vincenty R, Li X, Olson EN, Wang S. Regulation of angiogenesis and choroidal neovascularization by members of microRNA-23~27~24 clusters. *Proc Natl Acad Sci U S A.* 2011; 108(20):8287–8292. [PubMed: 21536891]

20. Lawrence T. The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harb Perspect Biol.* 2009; 1(6):a001651. [PubMed: 20457564]
21. Bock F, Onderka J, Rummelt C, Dietrich T, Bachmann B, Kruse FE, Schlotzer-Schrehardt U, Cursiefen C. Safety profile of topical VEGF neutralization at the cornea. *Invest Ophthalmol Vis Sci.* 2009; 50(5):2095–2102. [PubMed: 19151400]
22. Kim MS, Kwon HJ, Lee YM, Baek JH, Jang JE, Lee SW, Moon EJ, Kim HS, Lee SK, Chung HY, Kim CW, Kim KW. Histone deacetylases induce angiogenesis by negative regulation of tumor suppressor genes. *Nat Med.* 2001; 7(4):437–443. [PubMed: 11283670]
23. (a) Alitalo K, Tammela T, Petrova TV. Lymphangiogenesis in development and human disease. *Nature.* 2005; 438(7070):946–953. [PubMed: 16355212] (b) Chen L, Hamrah P, Cursiefen C, Zhang Q, Pytowski B, Streilein JW, Dana MR. Vascular endothelial growth factor receptor-3 mediates induction of corneal alloimmunity. *Nat Med.* 2004; 10(8):813–815. [PubMed: 15235599]
24. Cursiefen C. Immune privilege and angiogenic privilege of the cornea. *Chem Immunol Allergy.* 2007; 92:50–57. [PubMed: 17264482]
25. (a) Achen MG, Jeltsch M, Kuk E, Makinen T, Vitali A, Wilks AF, Alitalo K, Stacker SA. Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). *Proc Natl Acad Sci U S A.* 1998; 95(2):548–553. [PubMed: 9435229] (b) Jeltsch M, Kaipainen A, Joukov V, Meng X, Lakso M, Rauvala H, Swartz M, Fukumura D, Jain RK, Alitalo K. Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. *Science.* 1997; 276(5317):1423–1425. [PubMed: 9162011] (c) Joukov V, Pajusola K, Kaipainen A, Chilov D, Lahtinen I, Kuk E, Saksela O, Kalkkinen N, Alitalo K. A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *EMBO J.* 1996; 15(7):1751. [PubMed: 8612600]
26. Choo QY, Ho PC, Tanaka Y, Lin HS. Histone deacetylase inhibitors MS-275 and SAHA induced growth arrest and suppressed lipopolysaccharide-stimulated NF-kappaB p65 nuclear accumulation in human rheumatoid arthritis synovial fibroblastic E11 cells. *Rheumatology (Oxford).* 2010; 49(8):1447–1460. [PubMed: 20421217]
27. Li X, Stark GR. NFkappaB-dependent signaling pathways. *Exp Hematol.* 2002; 30(4):285–296. [PubMed: 11937262]
28. Sosne G, Christopherson PL, Barrett RP, Fridman R. Thymosin-beta4 modulates corneal matrix metalloproteinase levels and polymorphonuclear cell infiltration after alkali injury. *Invest Ophthalmol Vis Sci.* 2005; 46(7):2388–2395. [PubMed: 15980226]
29. Wang S, Aurora AB, Johnson BA, Qi X, McAnally J, Hill JA, Richardson JA, Bassel-Duby R, Olson EN. The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis. *Developmental cell.* 2008; 15(2):261–271. [PubMed: 18694565]

Figure 1.

Inhibition of corneal neovascularization by SAHA in an alkali-induced corneal injury model.

(A). Representative cornea images at 7 days after alkali burn and saline control or topical SAHA treatment (Top view).

(B). Clinical assessment of corneal opacity daily for 14 days after alkali burn and saline control or topical SAHA treatment. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

(C). Clinical assessment of corneal neovessel size daily for 14 days after alkali burn and control or topical SAHA treatment. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

(D). Representative H&E staining at 7 days after alkali burn and saline control or topical SAHA treatment. Red arrows point to the neovessels.

Figure 2.

Repression of hemangiogenesis, lymphangiogenesis and inflammation by SAHA in an alkali-induced corneal injury model.

(A). Representative images showing vascular EC staining by PECAM-1 and lymphatic EC staining by LYVE-1 at 7 days after alkali-burn and topical saline control or SAHA treatment.

(B). Quantification of PECAM-1 positive areas (μm^2) in (A). $p < 0.0001$.

(C). Quantification of LYVE-1 positive areas (μm^2) in (A). $p < 0.0001$.

(D). Representative images showing macrophage staining by F4/80 antibody at 7 days after alkali-burn and topical control or SAHA treatment.

(E). Quantification of F4/80 positive macrophage counts per field in (A). $P < 0.0001$.

Figure 3.

Inhibition of angiogenic pathways by SAHA in ECs.

(A). Toxicity test of SAHA in ECs. HUVECs were treated with SAHA at 5μM, 10μM and 20μM for 3 days, and the cell growth was assessed by MTT assay. **P<0.005, ***P<0.001.

(B). Inhibition of angiogenic pathways by SAHA in ECs. HUVECs were treated with 10 μM SAHA for 24 hours, starved in 0.1% FBS, and treated with VEGF for 20 minutes. The expression of the total and phosphorylated VEGFR2, AKT and ERK1/2 proteins was shown after Western blot analyses.

(C). Inhibition of VEGF-induced HDAC7 nuclear export by SAHA in ECs. HUVECs were infected with adenovirus expressing FLAG tagged HDAC7, treated with 10 μM for 24 hours, starved in 0.1% FBS, and treated with VEGF for 4 hours. HDAC7 subcellular localization was determined by FLAG (green) and DAPI (blue) co-staining.

Figure 4.

Inhibition of lymphangiogenesis by SAHA.

- (A). Toxicity test of SAHA in LEC. Primary LECs were treated with SAHA at 1μM, 5μM, 10μM and 20μM for 3 days, and the cell growth was assessed by MTT assay. *P<0.05.
- (B). Repression of LEC proliferation by SAHA. LECs were treated with SAHA for 24 hours, starved, and tested for BrDU incorporation in LEC full medium for 4 hours. P<0.005.
- (C). Representative images showing inhibition of LEC migration by SAHA in a scratch wound assay. The migration front of cells immediately after scratch wound was indicated by red lines.

(D). Representative images showing inhibition of LEC tube formation in Matrigel *in vitro* by SAHA. Scale bar=200 μ m.

Figure 5.

Repression of inflammatory gene expression by SAHA *in vivo* and *in vitro*.

(A). Inflammatory cytokine expression in the cornea at 7 days after alkali-burn with topical saline or SAHA treatment. P values are indicated.

(B). Inflammatory cytokine expression in the THP-1 cells pretreated with 10 μ M SAHA for 24hr and stimulated with LPS for 24hours. P values are indicated.

(C). ChIP-qPCR analysis of p65 binding to NF- κ B binding site in the promoter region of IL-6, IL-1 β , and TNF- α . Thp-1 cells were pretreated with 10 μ M SAHA and stimulated with LPS for 24 hours. P values are indicated.

Figure 6.

Comparison of SAHA to steroid therapy in corneal NV.

(A). Clinical assessment of corneal opacity daily for 14 days after alkali-burn and topical SAHA or Dexamethasone (0.1%) eye drop treatment. * $p < 0.05$.

(B). Clinical assessment of corneal neovessel size daily for 14 days after alkali-burn and topical SAHA or Dexamethasone (0.1%) eye drop treatment. * $p < 0.05$.