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Recommended Citation

Gronkiewicz KM, Giuliano EA, Sharma A, Mohan RR. Molecular Mechanisms of Suberoylanilide Hydroxamic Acid (SAHA) in the Inhibition of TGF-β1 Mediated Canine Corneal Fibrosis. *Vet Ophthalmol*. 2016;19(6):480-487. doi:10.1111/vop.12331.

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Gronkiewicz KM, Giuliano EA, Sharma A, Mohan RR. Molecular Mechanisms of Suberoylanilide Hydroxamic Acid (SAHA) in the Inhibition of TGF-β1 Mediated Canine Corneal Fibrosis. *Vet Ophthalmol*. 2016;19(6):480-487. doi:10.1111/vop.12331.

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Vet Ophthalmol. Author manuscript; available in PMC 2017 November 01.

Published in final edited form as:

Vet Ophthalmol. 2016 November ; 19(6): 480–487. doi:10.1111/vop.12331.

Molecular Mechanisms of Suberoylanilide Hydroxamic Acid (SAHA) in the Inhibition of TGF-β1 Mediated Canine Corneal Fibrosis

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Abstract

Objective—To investigate molecular mechanisms mediating anti-fibrotic effect of SAHA in the canine cornea using an *in vitro* model. We hypothesized that SAHA attenuates corneal fibrosis by modulating Smad-dependent and, to a lesser extent, Smad-independent signaling pathways activated by TGF-β1, as well as matrix metalloproteinase (MMP) activity.

Methods—Cultured canine corneal fibroblasts (CCF) were incubated in the presence/absence of TGF-β1 (5ng/ml) and SAHA (2.5μM) for 24hrs. Western blot analysis was used to quantify non-phosphorylated and phosphorylated isoforms of Smad2/3, p38 MAP kinase (MAPK), ERK1/2 and JNK1. Real-time PCR and zymography were utilized to quantify MMP1, MMP2, MMP8 and MMP9 mRNA expression and MMP2 and MMP9 protein activity, respectively.

Results—TGF-β1 treatment caused a significant increase in phospho-Smad2/3 and phospho-p38 MAPK. SAHA treatment reduced TGF-β1-induced phosphorylation of Smad2/3 but not of p38 MAPK. TGF-β1 did not modulate the phosphorylation of ERK1/2 or JNK1. SAHA caused a significant reduction in phospho-ERK1/2 expression regardless of concurrent TGF-β1 treatment. Neither SAHA alone nor in combination with TGF-β1 altered phospho-JNK1 expression. TGF-β1 significantly increased MMP1 and MMP9 mRNA expression but did not alter MMP2 mRNA. SAHA treatment attenuated TGF-β1-induced MMP9 mRNA expression while significantly enhancing TGF-β1-induced MMP1 mRNA expression. Zymography detected reduced expression of MMP2 and MMP9 proteins in untreated control CCF. TGF-β1 treatment did not alter their expression but SAHA treatment +/−TGF-β1 significantly increased MMP2 and MMP9 protein expression.

Conclusions—The corneal anti-fibrotic effects of SAHA involve multiple mechanisms including modulation of canonical and non-canonical components of TGF-β1 intracellular signaling and MMP activity.

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Keywords

Canine; cornea; fibrosis; suberoylanilide hydroxamic acid; Smad; mitogen-activated protein kinase; matrix metalloproteinase

Introduction

Corneal wound healing is a complex process that involves the signaling of numerous cytokines, activation of keratocytes, formation of both fibroblasts and myofibroblasts, increased deposition of extracellular matrix (ECM) and up-regulation of matrix metalloproteinases (MMPs) (1–6). Many different cytokines are produced in response to corneal wounding and of these various cytokines TGF-β1 has been shown to play a major role in the formation of corneal fibrosis (2, 7, 8).

The effects of TGF-β1 are facilitated via various intracellular signaling pathways including the Smad-dependent intracellular signaling pathways (9, 10). The Smad protein family is classified based on function and is composed of three categories including receptor-regulated Smads (R-Smads), common mediator Smads (co-Smads), and inhibitor Smads (I-Smads). R-Smads possess ligand specificity and become phosphorylated after directly binding to the TGF-β Receptor. Co-Smads are needed for nuclear entry and consequently allow for signal transduction from the cell membrane to the nucleus. Finally, I-Smads interfere with the activation of R-Smads by inhibiting their phosphorylation. Interactions among these Smad proteins ultimately results in the transformation of quiescent corneal keratocytes into corneal fibroblasts and subsequently myofibroblasts (7, 10, 11).

Although Smad proteins seem to play a primary role in TGF-β1 signal transduction, literature suggest that several other signaling pathways also allow for TGF-β1 to modulate gene expression and cell function. For example, TGF-β1 can activate several mitogen-activated protein kinases (MAPKs) such as ERK, JNK and p38 MAPK (10). These MAPK pathways appear to play to a role in epithelial and endothelial cell migration in the corneas of mice, rabbits and humans. Further they also, alter the expression of ECM genes (12–14).

TGF-β1 is known to induce fibrosis not only through cellular transdifferentiation but also through the modulation of the activity of MMPs (10). MMPs are proteolytic enzymes, which play a major role in extracellular matrix degradation. Physiologically, MMPs allow for remodeling of the extracellular matrix which is a necessary component of the wound repair process. However, excessive and uninhibited activity of MMPs in the cornea such as MMP2 and MMP9 can result in keratomalacia and potentially fibrinogenesis (15). Over activity of MMP1 has been implicated in ophthalmic disease as well, such as superior limbic keratoconjunctivitis and viral keratitis in people (16, 17). Various studies have demonstrated TGF-β1 can cause the up-regulation of the gene and protein expression of MMP1, MMP2 and MMP9 (5, 6, 18–21).

It has been demonstrated that Smad and MAPK mediated gene expression is influenced by histone acetylation (22–25). Histone acetylation is controlled by the interactions of two enzymes, histone deacetylase (HDAC) and histone acetyltransferase (HAT) (26). The

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activities of these two enzymes regulate gene expression and cellular function by modifying core histones or non-histone proteins (26). HDACs have been shown to participate in the development of fibrosis within various organs, including the cornea (27, 28). The over-expression of HDACs and subsequent fibrosis can be prevented by HDAC inhibitors (HDACi) (29–32). Recently, we demonstrated effective inhibition of canine corneal fibrosis *in vitro* using the HDACi, Suberoylanilide Hydroxamic Acid (SAHA) (33). SAHA is an FDA approved drug (Vorinostat®) for human clinical use as a treatment of cutaneous T-cell lymphoma (34).

SAHA efficiently inhibits corneal fibrosis without toxicity; however the precise mechanism by which SAHA decreases corneal fibrosis remains undefined. The purpose of this study was to determine the mechanisms mediating anti-fibrotic effects of SAHA in canine corneal wound healing using an established *in vitro* model. We tested the hypothesis that SAHA inhibits canine corneal fibrosis by modulating Smad and MAPK signaling and attenuating MMP activity.

Materials and methods

Canine corneal fibroblast (CCF) cultures

Primary canine corneal fibroblast cultures were established following the protocol previously described (33, 35). Briefly, full-thickness 6 mm axial corneal buttons were aseptically harvested from 3 dogs euthanized for reasons unrelated to the study. These purpose-bred, university owned, research dogs were being sacrificed for an orthopedic study in which they were enrolled. Eyes were examined by slit-lamp biomicroscopy prior to euthanasia and determined to be free of anterior segment disease. The corneal biopsies were washed with sterile minimal essential medium (MEM, Gibco, Grand Island, NY, USA), and the epithelium and endothelium were removed with careful dissection using a number 10 blade (BD, Franklin Lakes, NJ, USA). The remaining corneal stroma was sub-sectioned and placed in 100×20 mm tissue culture dishes (BD, BioSciences, Durham, NC, USA) containing MEM supplemented with 10% fetal bovine serum. These stromal explants were then incubated in a humidified 5% CO_2 incubator at 37°C to obtain CCF cultures. The primary CCF harvested from the corneal stromal sub-sections were seeded into 100×20 mm tissue culture plates in MEM supplemented with 10% fetal bovine serum and allowed to reach 80% confluence.

TGF-β1 and SAHA treatment

A 10 mM stock solution of SAHA (Cayman Chemical Company, Ann Arbor, MI) was made using dimethylsulfoxide (DMSO) and diluted 4000 times with MEM to achieve a final concentration of 2.5 μM. Upon reaching 80% confluence CCF were exposed to either TGF-β1 (5 ng/ml) or SAHA (2.5 μM) alone or both for 24 hours.

Immunoblotting

Protein lysates were harvested from CCF using a radioimmunoprecipitation assay (RIPA) lysis buffer containing a protease inhibitor cocktail (Santa Cruz Biotechnology, Santa Cruz, CA). The samples were then centrifuged at 10,000 g for 10 min. Following centrifugation,

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samples were suspended in NuPAGE LDS buffer containing a reducing agent (Life Technologies Corporation, Grand Island, NY, USA) and heated at 70°C for 10 min. Proteins were resolved by NuPAGE Novex Bis-Tris mini gels (Life Technologies, Invitrogen, Grand Island, NY, USA) and were transferred onto the polyvinylidene difluoride membranes utilizing overnight wet transfer technique at 25 volts. To detect transferred proteins, the membranes were then incubated with the following primary antibodies: p38 MAPK, phospho-p38 MAPK, JNK1, phospho-JNK1, ERK1/2, phospho-ERK1/2 (Cell Signaling, Beverly, MA, USA), Smad2/3 and phospho-Smad2/3 (Santa Cruz biotechnology Inc., Dallas, TX, USA). Membranes were then washed and incubated with alkaline phosphatase conjugated anti-mouse, anti-goat, or anti-rabbit secondary antibody. After washing three times in Tris-buffered saline for 5 minutes, membranes were developed using the nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate method. All western blots for each protein were repeated at least two times. Digital quantification of western blots was performed using Image J software (NIH, Bethesda, MD, USA).

RNA extraction, cDNA synthesis and PCR

Total RNA was extracted from CCF with RNeasy kit (Qiagen, Valencia, CA). The RNA was then reverse transcribed to cDNA following vendor's instructions (Promega, Madison, WI). Real-time PCR was performed to detect and quantify MMP1, MMP2, MMP8 and MMP9 mRNA using the Step One Plus real-time PCR system (Life Technologies, Grand Island, NY, USA). A 20 μl reaction mixture containing 2 μl of cDNA, 2 μl of forward primer (200 nM), 2 μl of reverse primer (200 nM), and 10 μl of 2X SYBR green super mix (Bio-Rad Laboratories, Hercules, CA) was run at a universal cycle (95 °C for 10 min, 40 cycles at 95 °C for 15 s, and 60 °C for 60 s) in accordance with the manufacturer's instructions as reported earlier.(33) β-Actin was used as the housekeeping gene. Each PCR reaction was run in triplicate and repeated at least 2 times. The primer sequences used in PCR analysis are listed in Table 1.

Zymography

Gelatin zymography was used to quantify pro and active isoforms of MMP2 and MMP9 proteins. The 10% Novex pre-cast SDS polyacrylamide gels (Life Technologies, Novex, Carlsbad, CA, USA) in the presence of 0.1% gelatin were used under non-reducing conditions. A 20 μL zymography sample was prepared by mixing total protein lysates (30 μg), Tris-Glycine SDS sample buffer (10 μL) and deionized water (0–6 μL). Protein standards were run concurrently to identify molecular weight. Samples were loaded for SDS-PAGE with 1X Tris-Glycine SDS Running Buffer and were run at a constant voltage (125 Volt) for 120 minutes without heating. Following completion of electrophoresis, the gel was washed once in 1X Zymogram Renaturing Buffer, which was then decanted, and the gel was next washed twice with 1X Zymogram Developing Buffer. For the second washing, the gel was incubated at 37 °C overnight. For staining, the gel was washed with Simply Blue Safe stain diluted with deionized water (1:2) for 60 minutes. The stain was then decanted and the gel was washed in deionized water for 24 hours. Three separate zymograms were performed for each treatment group. Digital quantification of zymograms was performed using NIH Image J software.

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Statistical analyses

Results were expressed as a mean ± standard error. One-way analysis of variance (ANOVA) and Tukey's test post-hoc were used to statistically analyze results, and $p<0.05$ was considered significant.

Results

Effects of SAHA on TGF-β1-induced Smad phosphorylation

As presented in Fig. 1, TGF-β1 treatment of CCF caused a 2.8 ± 0.28 fold ($p<0.01$) increase in Smad2/3 phosphorylation. Conversely, expression of phospho-Smad2/3 in CCF exposed to both SAHA and TGF-β1 was unchanged and comparable to untreated controls. These results suggested that SAHA significantly ($p<0.01$) attenuated TGF-β1-induced Smad2/3 phosphorylation.

Effects of SAHA on TGF-β1-induced MAPK

In Fig. 2, the effect SAHA and/or TGF-β1 on the phosphorylation of p38 MAPK are displayed. TGF-β1 caused a 3.5 ± 0.66 fold ($p<0.05$) increase in p38 MAPK phosphorylation. SAHA treatment partially attenuated TGF-β1-induced p38 MAPK phosphorylation down to 2.4 ± 0.4 fold; however, SAHA treatment did not significantly attenuate TGF-β1-induced p38 MAPK phosphorylation, suggesting that modulation of p38 MAPK signaling may not be primarily involved in the mechanism of action of SAHA.

Next, the effect of TGF-β1 and SAHA on ERK1/2 and JNK1 signaling were investigated. As demonstrated by Fig. 3, TGF-β1 treatment alone did not alter the phosphorylation of ERK1/2. On the other hand, CCF exposed to SAHA alone or in combination with TGF-β1 showed significantly reduced expression of phospho-ERK1 (2.7 ± 0.6 fold decrease $p<0.01$) and phospho-ERK2 (8.0 ± 3.0 fold decrease $p<0.05$). These results suggested that SAHA treatment inhibited ERK1/2 phosphorylation, regardless of concurrent-treatment with TGF-β1.

Thereafter, we tested the effects of SAHA on JNK1 signaling. Phospho-JNK1 expression was not detected in the untreated control CCF. Similarly, neither TGF-β1 nor SAHA alone or in combination with TGF-β1 showed any change on phospho-JNK1 expression. These results are displayed in Fig. 4.

Effects of SAHA on MMP mRNA and protein expression

Real time PCR was used to quantify the effect of SAHA and/or TGF-β1on MMP gene expression in CCF. As shown in Fig. 5, TGF-β1 caused a 6.0 fold ($p<0.01$) increase in MMP1 mRNA and 10.0 fold ($p<0.01$) increase in MMP9 mRNA expression, but did not have a significant effect on MMP2 mRNA expression. SAHA treatment alone did not alter MMP1, MMP2 or MMP9 expression. However, concurrent SAHA and TGF-β1 treatment caused a significant increase in MMP1 mRNA ($p<0.05$) and a significant decrease in the MMP9 mRNA ($p<0.05$) expression. Real time PCR performed to quantify MMP8 mRNA did detect any change in MMP8 mRNA in untreated control CCF or SAHA or TGF-β1 treated CCF.

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Gelatin zymography was performed to detect the protein isoforms of MMP2 and MMP9; and results are presented in Fig. 6. MMP2 and MMP9 proteins were detected in untreated control CCF and this expression remained unaltered after TGF-β1 treatment. Treatment of SAHA alone or in combination with TGF-β1 caused a significant increase in both MMP2 (1.5 ± 0.01 fold increase $p<0.05$) and MMP9 (3.0 ± 0.77 fold increase $p<0.05$) protein expression.

Discussion

Our group has demonstrated the anti-fibrotic potential of HDACi, specifically trichostatin A (TSA) and SAHA, in various models of corneal fibrosis (33, 36, 37). Sharma *et al.* found that TSA not only inhibited TGF-β1-induced α-SMA, fibronectin and myofibroblast formation in human corneal fibroblasts *in vitro* but also significantly decreased corneal haze in rabbits *in vivo* following photorefractive keratectomy (37). Recently, Bosiack *et al.* determined that SAHA inhibited canine corneal fibrosis *in vitro* (33), and Tandon *et al.* demonstrated the anti-fibrotic properties of SAHA *in vivo,* also utilizing rabbits which had undergone photorefractive keratectomy (36). However, these studies did not detail the mechanism(s) of action of HDACi as corneal anti-fibrotic agents. To the best of our knowledge, the molecular mechanisms of the anti-fibrotic effects of SAHA in the cornea is largely unknown (38).

The results of this study suggest that SAHA prevents canine corneal fibrosis through many different mechanisms including the modulation of TGF-β1-induced pro-fibrotic intracellular signaling pathways. Smad proteins largely mediate the intracellular signaling triggered by TGF-β1. R-Smads, such as Smad2 and Smad3, are considered pro-fibrotic intracellular signaling molecules. Consequently, these Smad proteins represent intracellular therapeutic targets for the manipulation of TGF-β1 mediated fibrosis. The inhibition of TGF-β1-induced phosphorylation of Smad2 and Smad3 has been demonstrated in models of renal, hepatic, cardiac and pulmonary fibrosis (39–42). Previous studies from our laboratory utilizing RNAi-mediated knock down models have shown that TGF-β1-mediated Smad2/3 phosphorylation is an essential step for fibroblast transdifferentiation to myofibroblasts (43). Therefore, it was hypothesized that SAHA may inhibit corneal fibrosis by attenuating TGF-β1-induced phosphorylation of Smad2/3. In the present study, SAHA significantly attenuated TGF-β1-induced phosphorylation of Smad2/3, thereby confirming our initial hypothesis that the inhibition of TGF-β1-induced phosphorylation of these R-Smads plays an important role in the anti-fibrotic effects of SAHA in the canine cornea.

Although Smad intracellular signaling serves as the canonical pro-fibrotic pathway of TGF-β1, it has been demonstrated that TGF-β1 also involves MAPK pathways. Therefore, the effect of SAHA on three MAPK signaling pathways specifically p38 MAPK, ERK1/2 and JNK1 were also investigated. Previous *in vitro* and *in vivo* models of cardiac, renal and corneal fibrosis demonstrated TGF-β1 induces the phosphorylation of p38 MAPK, (44–46) thereby supporting the results of this study, wherein treatment of CCF with TGF-β1 caused a marked increase in the phosphorylation of p38 MAPK. In contrast, treatment of CCF with TGF-β1 for 24 hours did not significantly modulate the phosphorylation of ERK1/2 or JNK1. These results are in accordance with previous studies which demonstrated that TGF-

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β1 caused only a transient increase in ERK and JNK phosphorylation, the expression of which returned to baseline after 1 hour of treatment (46, 47).

In this study, the effects of SAHA on TGF-β1-induced phosphorylation of the various MAPKs appeared to be subtype-specific. For example, SAHA caused a significant reduction in the phosphorylation of ERK1/2. As previously stated, TGF- β1 causes a transient increase in ERK1/2 phosphorylation, and so the observed attenuation of ERK1/2 phosphorylation by SAHA appears to be a means by which SAHA inhibits corneal fibrosis. However, SAHA did not affect the phosphorylation of p38 MAPK or JNK1, suggesting that these two MAPKs are not involved in the anti-fibrotic mechanisms of SAHA in the cornea. These results conflict with those of previous studies which demonstrated the ability of HDACi, including SAHA, to inhibit phosphorylation of p38 MAPK in neoplastic cells, rheumatoid arthritis synovial fibroblast and lens epithelial cells (48–50). It is important to note that these studies utilized cytokines different than TGF-β1 to induce cellular transformation and fibrosis. Therefore, it may be possible that TGF-β1 induces the phosphorylation of p38 MAPK through means which are inaccessible to SAHA, or SAHA may be able to prevent the phosphorylation of p38 MAPK in canine corneal fibroblasts in the presence of a different corneal cytokine, such as interleukin 1β (IL-1β), Platelet Derived Growth Factor (PDGF) or Connective Tissue Growth Factor (CTGF). Future studies would investigate these potential effects of SAHA in an *in vitro* model of canine corneal fibrosis utilizing different inflammatory cytokines.

It has been well established that MMPs play a critical role in the degradation and subsequent remodeling of the extracellular matrix. Past studies have consistently shown that TGF-β1 causes increased expression of MMP1 and MMP9 mRNA in corneal cells *in vitro* (5, 6, 51). Our study's data support previous findings, as TGF-β1 treatment resulted in the increased expression of MMP1 and MMP9 mRNA. While TGF-β1 treatment for 24 hours did not affect MMP2 mRNA in the present study, Donnelly *et al.* demonstrated that TGF-β1 treatment for 5 days caused a significant increase in MMP2 mRNA (52). When analyzing the effects of TGF-β1 on MMP protein expression, no significant changes were noted in the expression of MMP2 or MMP9 proteins. These results suggest that prolonged exposure to TGF-β1 may be necessary to alter MMP protein expression in CCF. Alternatively, it may be possible that TGF-β1 may not be involved in the expression of MMP protein, as previously demonstrated by a study in which both normal and keratoconic human corneal keratocytes were treated with TGF-β1 for 7 days (53).

Several studies have investigated the effects of different HDACi on MMPs in various cell types and the results appear to be drug-, dose- and tissue-specific (54–57). Treatment with SAHA alone did not significantly affect MMP1, MMP2 or MMP9 mRNA in our investigation of CCF. However, treatment with both SAHA and TGF-β1 significantly increased MMP1 mRNA, decreased MMP9 mRNA and had no effect on MMP2 mRNA. Unexpectedly, CCF treated with either SAHA alone or SAHA and TGF-β1 expressed significantly increased expression of MMP9 protein, despite the aforementioned decreased MMP9 mRNA expression within the same treatment group. We speculate that SAHA may affect cellular proteins involved in MMP9 degradation, thus increasing the half-life of this particular MMP. Overall, our data suggest that modulations of MMP expression by HDACi

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is not only isoform specific but also may be affected by other inducible inflammatory cytokines and growth factors.

In summary, this study is the first report to the authors' knowledge investigating both the pro-fibrotic intracellular signaling cascade in canine corneal fibroblast induced by TGF-β1 and the molecular mechanisms of action of SAHA as a corneal anti-fibrotic agent. Results indicate that SAHA affects both canonical and non-canonical components of the TGF-β1 intracellular signaling pathways and pathways independent of TGF-β1. Finally, SAHA alters both gene and protein expression of MMP2 and MMP9. However, the mechanisms of these modulations and their clinical significance need further investigation.

Acknowledgments

The work was primarily supported from the University of Missouri the Ruth M. Kraeuchi Missouri Endowed Chair of Ophthalmology Professor fund (RRM) and partially through the RO1EY017294 National Eye Institute (RRM) and 1I01BX000357–05 Veteran Health Affairs Merit (RRM) grants. The authors gratefully acknowledge the technical support of Ms. Melanie Kunkel, Ms. Jessica Kitchell, Ms. Misha Brown, and Mr. Prashant R. Sinha.

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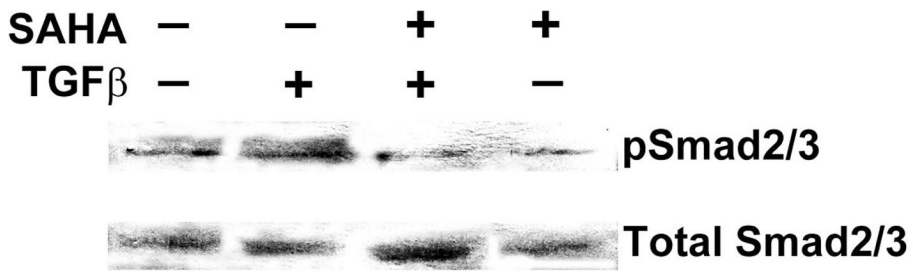

Figure 1.

Representative western blot showing the effect of SAHA +/− TGF-β1 on Smad phosphorylation in canine corneal fibroblast (CCF) cell extracts. TGF-β1 treatment of CCF caused a 2.8 ± 0.28 fold ($p<0.01$) increase in Smad2/3 phosphorylation. SAHA treatment of CCF significantly ($p<0.01$) attenuated TGF-β1-induced Smad2/3 phosphorylation.

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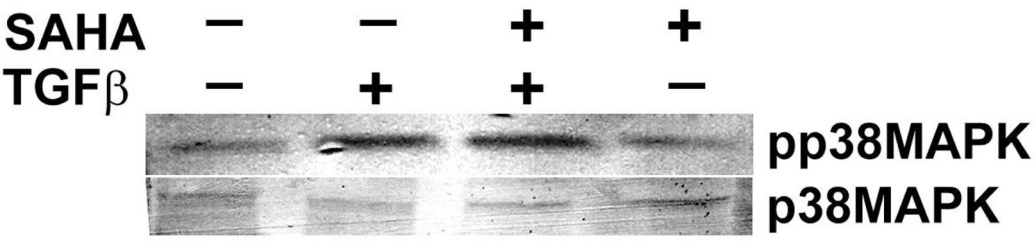

Figure 2.

Representative western blot showing the effect of SAHA +/− TGF-β1 on p38 mitogen-activated protein (MAP) kinase phosphorylation in canine corneal fibroblast (CCF) cell extracts. TGF-β1 treatment of CCF caused a 3.5 ± 0.66 fold ($p<0.05$) increase in p38 MAP kinase phosphorylation. SAHA treatment of CCF did not significantly attenuate TGF-β1-induced p38 MAPK phosphorylation.

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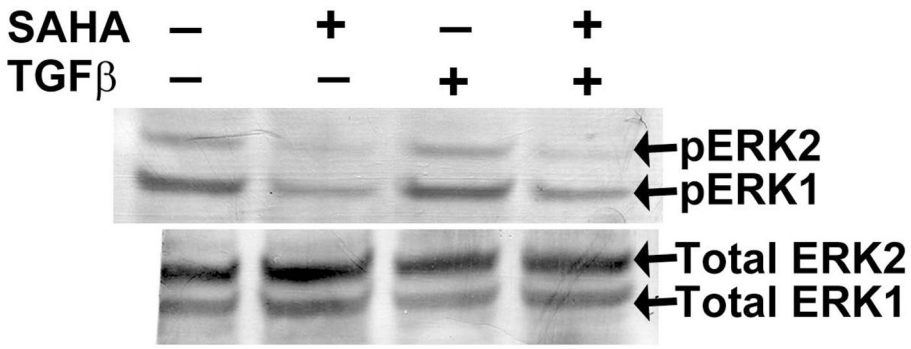

Figure 3.

Representative western blot showing the effect of SAHA +/; TGF-β1 on ERk1 and ERK2 phosphorylation in canine corneal fibroblast (CCF) cell extracts. TGF-β1 alone did not modulate the phosphorylation of ERK1 or ERK2, while exposure to SAHA with or without the concurrent TGF-β1 treatment resulted in significantly reduced expression of p-ERK1 (2.7 ± 0.6 fold decrease, $p<0.01$) and p-ERK2 (8 ± 3 fold decrease, $p<0.05$) which suggests that SAHA treatment inhibited ERK phosphorylation, regardless of TGF-β1 activity.

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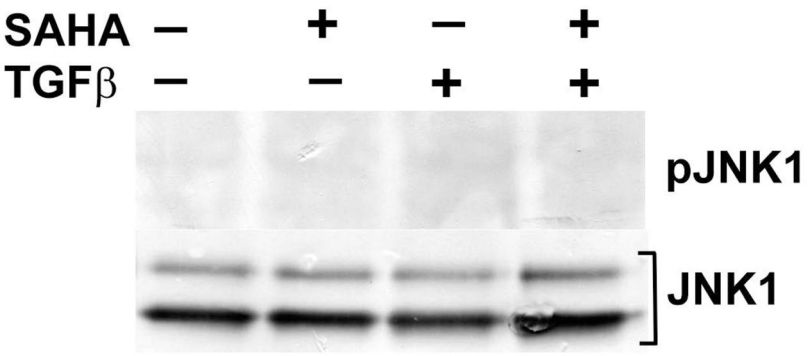

Figure 4.

Representative western blot showing the effect of SAHA +/− TGF-β1 on JNK phosphorylation in canine corneal fibroblast (CCF) cell extracts. As can be seen in the western blot, no p-JNK1 was detected in the untreated control CCF. Neither TGF-β1 nor SAHA alone or in combination showed any effect on p-JNK1 expression.

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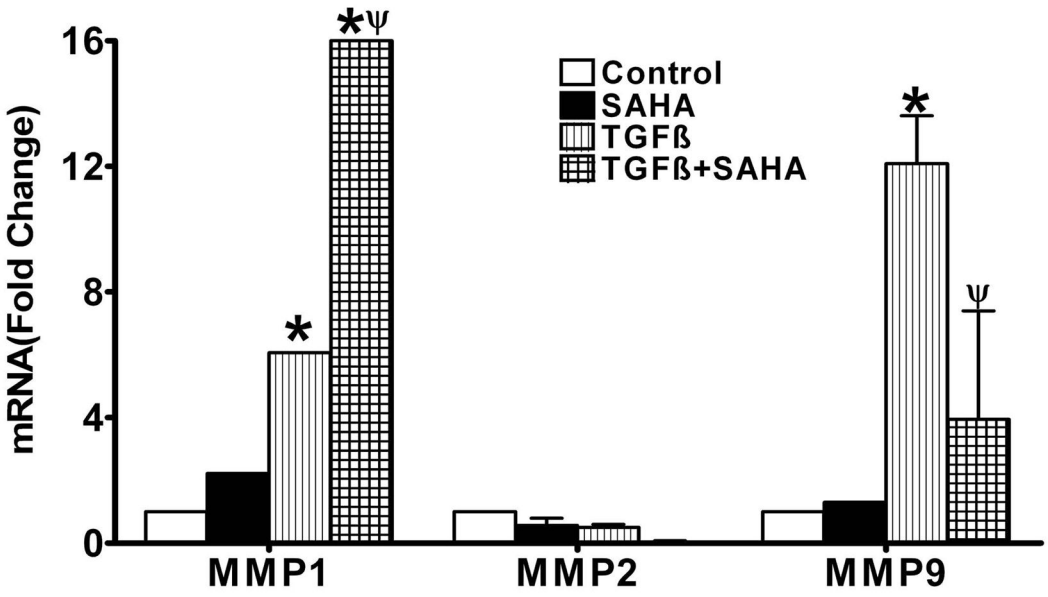

Figure 5.

Graph showing real time qPCR mRNA quantification of matrix metalloproteinases (MMPs) 1, -2 and -9 in canine corneal fibroblasts (CCF). TGF-β1 treatment of CCF caused a 6 fold ($p<0.01$) increase in MMP1 mRNA and 10 fold ($p<0.01$) increase in MMP9 mRNA expression and did not cause any notable change in MMP2 mRNA. SAHA treatment of CCF alone did not cause any notable change in MMP1, MMP2 or MMP9 expression but concurrent treatment of CCF with TGF-β1 caused a significant increase ($p<0.05$) in MMP1 mRNA and a significant decrease ($p<0.05$) in the MMP9 mRNA expression.

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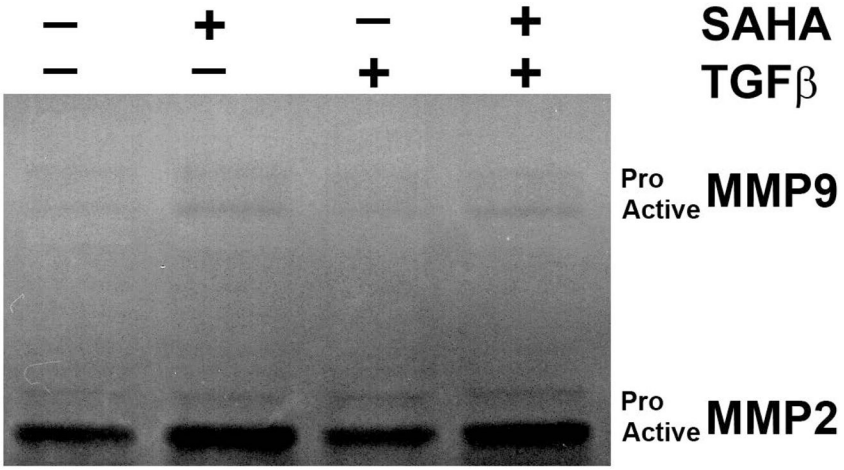

Figure 6.

Representative gelatin zymography demonstrating the effect of SAHA +/− TGF-β1 on MMP2 and MMP9 protein expression in canine corneal fibroblast (CCF) cell extracts. MMP2 and MMP9 proteins were detected in untreated control CCF, and treatment with TGF-β1 alone did not alter this baseline MMP protein expression. However, treatment with SAHA, irrespective of co-treatment with TGF-β1 resulted in a statistically significant increase in MMP2 and MMP9 protein expression.

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Table 1

Sequences of primers for detecting MMP RNA

MMP	Primer	Sequence	Source
MMP1	Forward	5′GGGTCATTCTCTTGGACTTTCT3′	Canine
	Reverse	5′AGCTGGACATTGCCACTATAC3′	Canine
MMP2	Forward	5′CTGACCAAGGGTACAGCTTATT3	Canine
	Reverse	5′CAGACGGAAGTTCTTGGTGTAG3′	Canine
MMP8	Forward	5′CCACACTCCGTGGAGAAATAC3	Canine
	Reverse	5′GGATGGCCAGAACAGAGAAA3′	Canine
MMP9	Forward	5′CTACGACCAGGACAAACTCTAC3′	Canine
	Reverse	5′TTGCCCAGGAAGATGAAGG3′	Canine