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Glaucoma

ALK5 Inhibition of Subconjunctival Scarring From Glaucoma Surgery: Effects of SB-431542 Compared to Mitomycin C in Human Tenon's Capsule Fibroblasts

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Purpose: The gold standard for managing postoperative ocular fibrosis in glaucoma surgery is the chemotherapeutic mitomycin C (MMC) despite its association with significant adverse effects. This study compares in vitro the antifibrotic efficacy and cytotoxicity of the small-molecule TGFβ1 inhibitor SB-431542 (SB) to MMC.

Methods: To measure collagen contraction, human Tenon's capsule fibroblasts (HTCFs) embedded in a three-dimensional collagen lattice were exposed to 0.2 mg/mL MMC or 20 µM SB followed by incubation with 2 ng/mL TGFβ1. Total protein extracted from experimentally treated HTCFs underwent immunoblotting for α–smooth muscle actin (α-SMA), matrix metallopeptidase 9 (MMP-9), and EDA splice-variant fibronectin (EDA-FN) expression. Cytotoxicity and cell metabolism were assessed using LIVE/DEAD staining, lactate dehydrogenase (LDH) assay, and methylthiazole tetrazolium (MTT) assay.

Results: Collagen lattice contraction in TGFβ1-induced HTCFs was significantly lowered by SB and MMC. Pretreatment with SB and MMC significantly lowered protein expression of α-SMA, MMP-9, and EDA-FN in HTCFs relative to TGFβ1 alone. HTCF viability in collagen lattices was significantly reduced with MMC pretreatment but not SB pretreatment. MMC-pretreated HTCFs had a significant increase in LDH release after 3 hours and a decrease in MTT activity after 20 minutes, while SB-pretreated HTCFs showed no significant changes via MTT or LDH assay during the same treatment period.

Conclusions: SB shows comparable efficacy to MMC in reducing expression of fibrosis-promoting proteins in HTCFs and in vitro scarring activity. SB distinguishes itself from MMC by exhibiting less cytotoxicity in both two-dimensional and three-dimensional in vitro assays.

Translational Relevance: This study demonstrates in vitro the potential of SB as a safer alternative ocular antifibrotic agent.

Introduction

Glaucoma is a progressive optic neuropathy resulting in irreversible vision loss that affects over 80 million people globally.[1] Treatment strategies aim to reduce intraocular pressure (IOP), the only modifiable risk factor for glaucoma development and progression.[2] First-line treatments for glaucoma include laser procedures and topical medications. However, when these treatments fail, surgical approaches such as angle-based or bleb-forming microinvasive glaucoma surgery

or the more invasive bleb-forming trabeculectomy are often employed.[3,4] Bleb-forming surgery aims to create a new outflow channel for aqueous humor drainage into the subconjunctival space, thereby lowering the IOP.[5,6] However, bleb-forming surgery is associated with significant failure rates, often caused by an aggressive postoperative fibrotic response.[1] Excessive fibrosis results in closure of the newly formed outflow channel and reductions in extracellular matrix porosity of the bleb tissues, leading to a decrease in outflow facility, return of elevated IOP, and continued progression of glaucoma. Inversely, insufficient healing may result in excessive fluid leakage, causing hypotony.[1] Therefore, proper management of postoperative wound healing in glaucoma surgery is paramount for optimal surgical outcomes.[1,7]

Corticosteroids continue to be the mainstay for managing fibrosis because of their ability to reduce postoperative inflammation and inflammation-induced scarring.[7–10] However, corticosteroids are associated with problematic adverse events, including corticosteroid-induced ocular hypertension, secondary glaucoma, and increased risk of infection.[7,8,11] In 1983, the chemotherapeutic agent mitomycin C (MMC) was adopted as a new gold-standard antifibrotic therapy. MMC exhibits potent cytotoxic effects by acting as an alkylating agent to cross-link DNA.[12,13] While MMC is effective at reducing fibrosis by inhibiting fibroblast proliferation, the drug has potent off-target cytotoxic effects on surrounding ocular tissue, being associated with decreased wound integrity, hypotony, endophthalmitis, and accelerated cataract progression.[14–16] In addition, MMC is highly unpredictable in its efficacy, making a standard dose and exposure time for treatment ambiguous—further contributing to glaucoma surgery failure rates.[5,12] While corticosteroids and MMC have improved the success rates of filtering surgery, surgical failure is still an issue.[5,9] Since surgical intervention is often the ultimate attempt to control IOP for glaucoma, surgical failure leaves patients in danger of permanent, irreversible vision loss. Thus, there is an ongoing need for novel therapeutic targets addressing the shortcomings of current surgical adjuvants.[1]

One potential alternative is the TGFβ1 inhibitor SB-431542 (SB), which can inhibit activin receptor-like kinases (ALKs), including ALK5.[17] TGFβ1 activation of ALK5 signals for nuclear localization of the SMAD2/3 proteins, which enhance transcription of fibrosis-promoting proteins such as collagen, matrix metallopeptidase (MMP), and fibronectin.[1,18–20] Therefore, upstream inhibition of the TGFβ1 pathway by SB in human Tenon's capsule fibroblasts (HTCFs) may result in disruption of TGFβ1-induced fibrosis.

In the current study, we investigate the effects of SB on HTCFs in comparison to MMC by examining TGFβ1-induced expression of fibrosis-promoting proteins MMP-9, α–smooth muscle actin (α-SMA), and EDA splice-variant fibronectin (EDA-FN), as well as collagen lattice contraction in a three-dimensional (3D) tissue mimetic. Furthermore, we characterize the safety of SB relative to MMC by measuring cell death and cellular metabolic activity by LIVE/DEAD staining, lactate dehydrogenase (LDH) assay, and methylthiazole tetrazolium (MTT) assay. We hypothesize HTCFs pretreated with SB or MMC will express comparable levels of collagen contraction, MMP-9, EDA-FN, and α-SMA post-TGFβ1 treatment, with SB exhibiting significantly lower cytotoxic effects than MMC.

Materials and Methods

HTCF Collection and Culture

The current study followed the tenets of the Declaration of Helsinki and was approved by the office of Human Research Ethics at Western University (REB 106783). After providing informed consent, patients undergoing glaucoma surgery at the Ivey Eye Institute in London, Ontario, had part of Tenon's capsule resected and collected for culture (Table). Tissue was cultured according to previously described methodology,[21] and HTCFs were isolated and stored in a cryogenic chamber prior to experimental use. HTCFs were subsequently cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific) and 1% penicillin/streptomycin (P/S) (Gibco, Thermo Fisher Scientific) until 90% confluency.

Collagen Contraction Assessment

HTCFs were prepared in a delayed-release fibroblast-populated collagen lattice model that was constructed using a previously described methodology,[22] based on the model by Bell et al.[23] The HTCFs were incubated, within the collagen lattices, with 2% FBS, 1% P/S, and 0.25 mM L-ascorbic acid 2-phosphate (Sigma-Aldrich, St Louis, MO, USA) DMEM for 72 hours. After, collagen lattices received experimental treatment of 20 µM SB for 5, 10, or 20 minutes or 0.2 mg/mL MMC for 1, 2, or 4 minutes. Following pretreatment, lattices were detached from

Table. Relevant Information of Anonymized HTCF Cell Line Donors With Open-Angle Glaucoma

Patient No.	Age, y	Sex	Ocular Medications	Surgery	Experimental Usage
1	60	M	Avastin, PGA, BB, AA, CAI	Seton implant	LDH, MTT, densitometry
2	87	F	CAI	Trabeculectomy	LDH, MTT, densitometry
3	72	F	BB, CAI, PA, MI	Trabeculectomy	LDH, MTT, densitometry
4	67	F	PGA, AA CAI, MI	Trabeculectomy	LDH, MTT, densitometry
5	74	M	AA, BB, CAI, PA, MI	Trabeculectomy	LDH, MTT
6	79	M	PA, AA, BB	Trabeculectomy	LDH, MTT
7	63	M	BB, CAI, PA, MI	Trabeculectomy	LDH, MTT
8	64	F	BB, AA, CAI	Seton implant	Collagen contraction, LIVE/DEAD staining
9	63	M	PGA, BB, AA, CAI	Trabeculectomy	Collagen contraction, LIVE/DEAD staining
10	76	M	PGA, BB, AA, CAI	Trabeculectomy	Collagen contraction, LIVE/DEAD staining
11	78	M	PGA, BB, CAI	Trabeculectomy	Collagen contraction, LIVE/DEAD staining

AA, alpha-agonist; BB, beta-blocker; CAI, carbonic anhydrase inhibitor; MI, miotic; PA, prostamides; PGA, prostaglandin analogue.

the culture well with a sterile spatula. A positive control (PC) group and vehicle control (VC) group each comprising HTCFs within a collagen lattice without pretreatment were also detached. Detached lattices were incubated for 72 hours in 2 ng/mL TGFβ1 or vehicle media. The culture plates were scanned using a digital flatbed scanner (Scanjet 8200; Hewlett-Packard, Palo Alto, CA, USA), starting with an initialization baseline of 0 hours and then at 2, 4, 6, 8, 12, and 24 hours, followed by 12-hour increments for 3 days, up to 96 hours. The area of the collagen lattices was assessed using ImageJ (National Institutes of Health, Bethesda, MD, USA). Each lattice's measured area was standardized to its baseline area and reported as change from the original area in mean percentage of the baseline area. The mean percentage change in area was compared with a two-way analysis of variance (ANOVA) and Dunnett's test for SB and MMC.

Protein Extraction and Western Blots

HTCFs were cultured in 6-well plates until they reached 90% confluency. Twenty-four hours prior to initiation of treatment, culture medium was changed to serum-free 1% P/S DMEM. Following exposure to serum-free media, each well had medium aspirated, and the well was washed with phosphate-buffered saline (PBS) prior to treatment. Pretreatments included 20 µM SB (S4317; Sigma-Aldrich) for 5, 10, or 20 minutes and 0.2 mg/mL MMC (M4287; Sigma-Aldrich) for 1, 2, or 4 minutes. VC and PC wells received a PBS pretreatment for 20 minutes. After pretreatment, each well had its media removed and was washed with PBS. All groups then received 2 ng/mL TGFβ1 in 1% P/S DMEM, except for the VC, which received 1% P/S DMEM alone. The HTCFs were then incubated for 48 hours at 37°C.

After 48 hours, wells were washed with PBS and treated with 1:100 proteinase-lysis buffer solution of a protease inhibitor mix (P2714; Sigma-Aldrich) and PhosphoSafe Extraction Reagent lysis buffer (71296; Sigma-Aldrich). Each well was scraped to detach any adherent cells, and the solution was then homogenized with a 30-gauge needle and syringe. Total protein concentration for each sample was measured with a BCA protein assay (23225; Pierce, Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Protein concentrations were standardized between all samples and then loaded into wells for sodium dodecyl sulfate–polyacrylamide gel electrophoresis. For MMP-9 and α-SMA, 8% gel was used; for EDA-FN, 6% gel was used. Gel electrophoresis was performed at 100 V for 1 hour followed by electro-transfer to a nitrocellulose membrane. Membranes were exposed to 10 mL of 5% bovine serum albumin in Tris-buffered saline blocking buffer for 1 hour. Primary antibodies used for MMP-9, α-SMA, and EDA-FN (ab38898, ab5694, abcam, Cambridge, UK; sc-59826, Santa Cruz Biotechnology, Dallas, TX, USA) were prepared as 1/1000, 1/500, and 1/200 dilutions, respectively. Additionally, antibodies against the housekeeping gene GAPDH (ab8245; abcam) were used. Primary antibody incubation occurred overnight at 4°C for MMP-9, EDA-FN, and α-SMA and for 1 hour for GAPDH at room temperature. Secondary antibodies of goat anti-mouse IgG were used for GAPDH and EDA-FN immunoblotting (AB_228307; Thermo Fisher

Scientific). Secondary antibodies of goat anti-rabbit IgG were used for MMP-9 and α-SMA immunoblotting (AB_228341; Thermo Fisher Scientific). Secondary antibodies were applied for 1 hour at room temperature. Chemiluminescence was performed with a Gel Doc (Thermo Fisher Scientific) to obtain densitometry data. Quantitative analysis of densitometry was performed by using Image Lab Software (Bio-Rad Laboratories, Hercules, CA, USA). Each sample band was normalized to its respective GAPDH band. Expression of protein was calculated as a ratio of sample protein density to the PC. A one-way ANOVA and post hoc Tukey's test was used for statistical analysis to compare densitometry differences between all groups.

Assessment of Cellular Viability in Collagen Lattices

After completion of collagen contraction measurement, the same collagen lattices were used for LIVE/DEAD staining. The collagen lattices underwent cytotoxicity assay by incubation with the 8 µg/mL fluorescein diacetate (F7378; Sigma-Aldrich) and 20 µg/mL propidium iodide (P4170; Sigma-Aldrich) for 5 minutes. After incubation with the LIVE/DEAD dyes, the collagen lattices underwent PBS wash and immediate imaging at 20× magnification with a laser-scanning confocal microscope (A1R HD; Nikon Instruments Inc, Tokyo, Japan). Each technical replicate had 10 representative images captured. Using ImageJ software, the ratio of live cells to the sum of live and dead cells was calculated and standardized to the PC. The LIVE/LIVE + DEAD ratio was compared between groups with a one-way ANOVA and post hoc Tukey's test.

Metabolic Activity and Cytotoxicity Assessment

HTCFs for LDH and MTT assays were cultured in 24-well plates until 90% confluency. Twenty-four hours prior to initiation of treatment, culture medium was changed to serum-free 1% P/S colorless DMEM. All treatment groups were performed in triplicate. Pretreatments included 20 µM SB or 0.2 mg/mL MMC in 1% P/S colorless DMEM for 20 minutes, 3 hours, or 6 hours. Additionally, 0.2 mg/mL MMC in DMEM was used as a pretreatment to replicate the clinically relevant MMC exposure times of 1, 2, or 4 minutes. VC, PC, and total cell death control (TCD) received a treatment of serum-free 1% P/S colorless DMEM solution for 6 hours. After pretreatment, all wells were washed with PBS. All HTCF groups except for VC and TCD received 2 ng/mL TGFβ1 in 1% P/S DMEM. VC received 1% P/S colorless DMEM and TCD received 10% *v*/*v* Triton X-100 (Sigma-Aldrich) in 1% P/S colorless DMEM. The HTCFs were then incubated for 48 hours at 37°C. After incubation, the LDH and MTT assays were performed on the HTCFs.

Following experimental treatment, media were removed and set aside for the LDH assay before cells were incubated in MTT treatment medium (M2128; Sigma-Aldrich) for 2 hours, and sample signal intensity at 575 nm was measured and analyzed relative to the VC absorbance. The LDH assay (11644793001; Roche Diagnostics, Basel, Switzerland) was performed with the removed media following the manufacturer's instructions. The LDH concentration for samples was determined from an LDH standard curve using the sample's signal intensity at dual filter (490 nm and 655 nm). The ratio of sample LDH concentration relative to the LDH concentration of the TCD treatment group was used for analysis.

One-way ANOVA and post hoc Tukey's test was used for the LDH and MTT assays to perform statistical analysis by comparing LDH concentration and MTT absorbance differences, respectively, across all groups.

Results

SB and MMC Demonstrate Significant Antifibrotic Efficacy in Three- and Two-Dimensional In Vitro Assays

TGFβ1 exposure alone promoted a significant increase in contraction relative to untreated fibroblasts embedded in the collagen lattices ($N = 4$, $n = 3$), demonstrating the fibrosis-promoting properties of TGFβ1. Nonetheless, both SB and MMC pretreatments were able to significantly lower TGFβ1-induced contraction compared to collagen lattices exposed to TGFβ1 alone (Fig. 1). Significant differences in contraction started to become evident as early as 3 hours following detachment for SB-exposed HTCFs and at the 12-hour time point for MMC-exposed HTCFs. Four days following detachment, all tested durations of exposure to SB and MMC displayed a significant decrease in scarring in vitro relative to TGFβ1 alone.

The mean densitometry analysis showed a significant increase in α-SMA, EDA-FN, and MMP-9 expression in HTCFs that received TGFβ1 alone compared to HTCFs that received no TGFβ1 ($N = 4$, $n = 1$). In contrast, TGFβ1-induced HTCFs

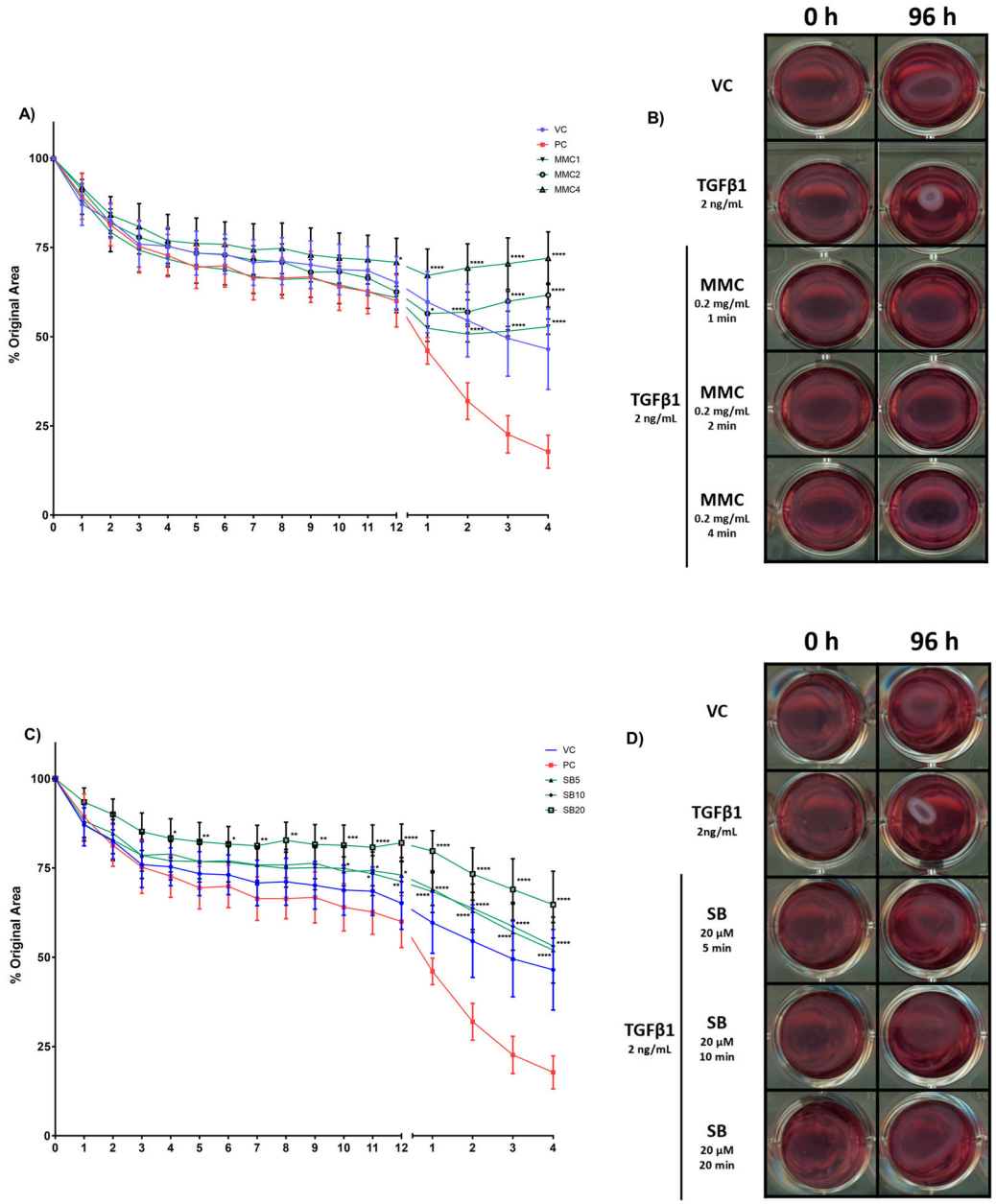

Figure 1. Collagen contraction area of HTCFs in delayed-release fibroblast-populated collagen lattice after SB-431542 (SB) or mitomycin C (MMC) pretreatment. (**A**) Area of contraction by MMC pretreatment. (**B**) Collagen lattice discs pretreated with MMC. (**C**) Area of contraction by SB pretreatment. (**D**) Collagen lattice discs pretreated with SB. VC and PC HTCFs received a PBS pretreatment for 20 minutes. All other HTCFs were pretreated with 20 μM SB in DMEM solution for 5, 10, or 20 minutes or 0.2 mg/mL MMC in DMEM solution for 1, 2, or 4 minutes. After pretreatment, all HTCFs except the VC were incubated in 2 ng/mL TGFβ1 in DMEM solution for 96 hours. VCs were incubated in DMEM solution for 96 hours. Culture plates were scanned after hours 1 to 12 and after days 1 to 4. Collagen lattice area was standardized to the baseline area and expressed as mean percentage change from the baseline. Mean percentage change ± SEM was compared with a two-way ANOVA and Dunnett's test ($N = 4$, $n = 3$). *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$.

pretreated with SB or MMC for all tested lengths of exposure demonstrated a significantly lower expression of α-SMA, EDA-FN, and MMP-9 proteins compared to the TGFβ1-induced HTCFs that received no pretreatment (Fig. 2). Therefore, the densitometry analysis showed that SB and MMC pretreatments were both able to lower TGFβ1-induced promotion of fibrosis-associated proteins.

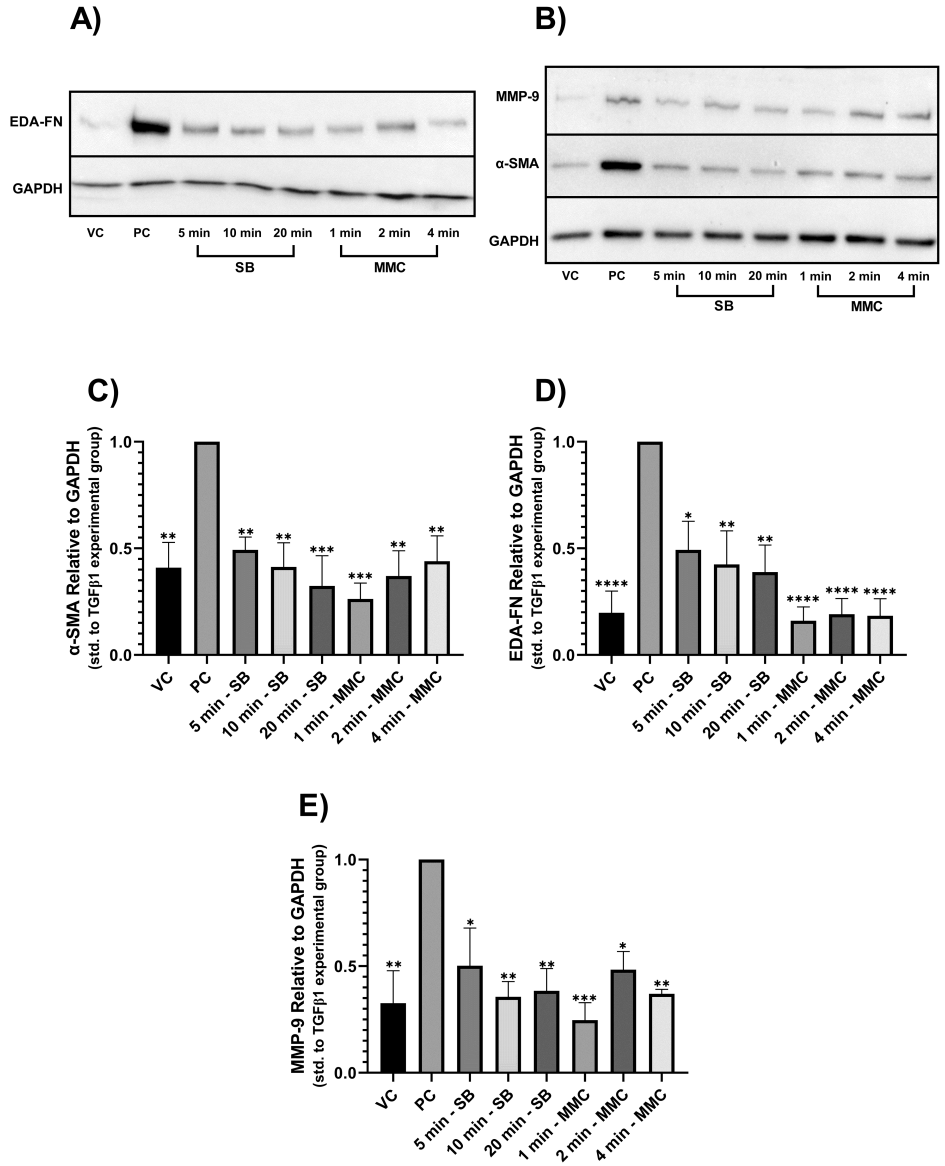

Figure 2. Mean densitometry of sample fibrotic protein expression in HTCFs. (**A**) Representative EDA-FN Western blots with GAPDH on 6% sodium dodecyl sulfate (SDS) gel. (**B**) Representative α-SMA and MMP-9 Western blots with GAPDH on 8% SDS gel. (**C**) Mean densitometry of α-SMA. (**D**) Mean densitometry of EDA-FN. (**E**) Mean densitometry of MMP-9. VC and PC HTCFs received a 20-minute PBS pretreatment. All other HTCFs were pretreated with 20 µM SB or 0.2 mg/mL MMC in PBS solution for varying durations. After pretreatment, all HTCFs except the VC were incubated in 2 ng/mL TGFβ1 in DMEM solution for 48 hours. Protein expression for all groups was standardized relative to the PC protein expression. Significance listed is relative to the PC. Mean protein expression $\pm$ SEM was compared with a one-way ANOVA and post hoc Tukey's test ($N = 4$, $n = 1$). *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$.

MMC Demonstrates Significant Cytotoxicity and Reduction of Cell Metabolic Activity, While SB Shows No Significant Effects on Cell Metabolism or Cytotoxicity

There was no significant difference in LIVE/DEAD cellular viability between TGFβ1-induced HTCFs pretreated with SB compared to the HTCFs that received no pretreatment ($N = 4$, $n = 3$), displaying the minimal effect of SB on HTCF viability. However, MMC pretreatment for 1, 2, and 4 minutes significantly lowered the cellular viability of TGFβ1-induced HTCFs relative to all other experimental groups (Fig. 3), demonstrating the cytotoxicity of MMC.

The extracellular presence of LDH, a marker of cell injury, significantly increased in concentration with TGFβ1-induced HTCFs after exposure to MMC for 3 hours or 6 hours in comparison to all other treatment groups ($N = 7$, $n = 3$). In contrast, even after SB exposure for 6 hours, HTCFs displayed no

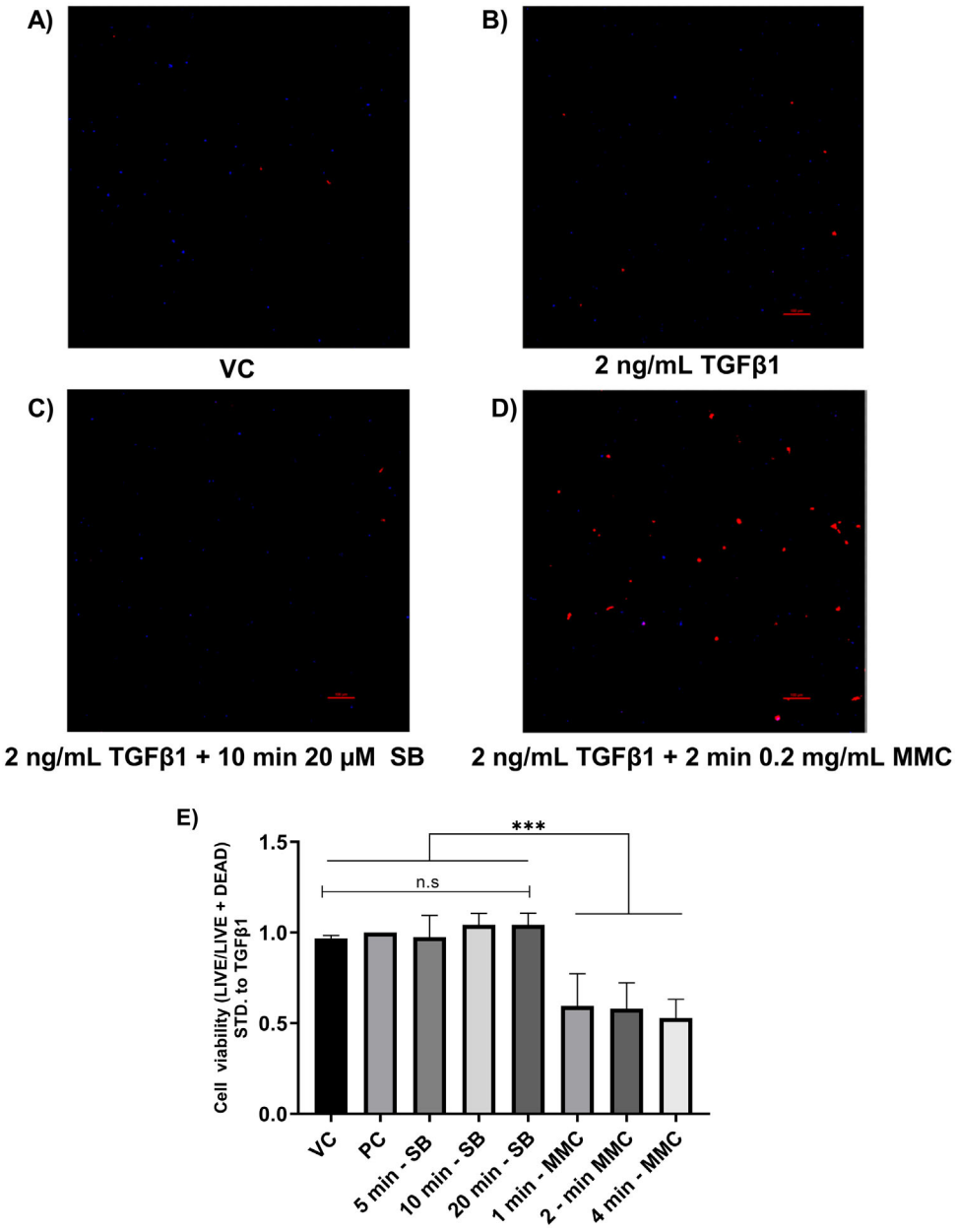

Figure 3. LIVE/DEAD staining of HTCF collagen lattices after SB or MMC pretreatment. (**A**) VC staining. (**B**) PC staining. (**C**) SB staining. (**D**) MMC staining. (**E**) LIVE/LIVE + DEAD signal of stained HTCFs; VC and PC HTCFs received a PBS pretreatment for 20 minutes. All other HTCFs were pretreated with 20 μM SB in DMEM solution for 5, 10, or 20 minutes or 0.2 mg/mL MMC in DMEM solution for 1, 2, or 4 minutes. After pretreatment, all HTCFs except the VC were incubated in 2 ng/mL TGFβ1 in DMEM solution for 96 hours. HTCFs were marked with 20 μg/mL propidium iodide dye signifying dead cells in red and 8 μg/mL fluorescein diacetate dye signifying live cells in blue. The ratio of live cells to the sum total of live and dead cells was measured in ImageJ and standardized to the PC. Mean ratios ± SEM were compared with one-way ANOVA and post hoc Tukey's test ($N = 4$, $n = 3$). ***$P \leq 0.001$.

significant increase in extracellular LDH concentration and had comparable extracellular LDH concentration to HTCFs that received no pretreatment (Fig. 4A), evidencing minimal HTCF injury by SB. Similarly, pretreatments of 2 μM SB and 20 nM SB did not significantly increase LDH release by HTCFs (Supplementary Fig. S1). TGFβ1-induced HTCFs pretreated with MMC for the clinically relevant durations of 1, 2, or 4 minutes had no significant change in LDH concentration in comparison to HTCFs that received no MMC pretreatment (Fig. 4B).

TGFβ1 exposure alone significantly increased HTCF metabolic activity, demonstrating the increased metabolic requirement of the myofibroblast

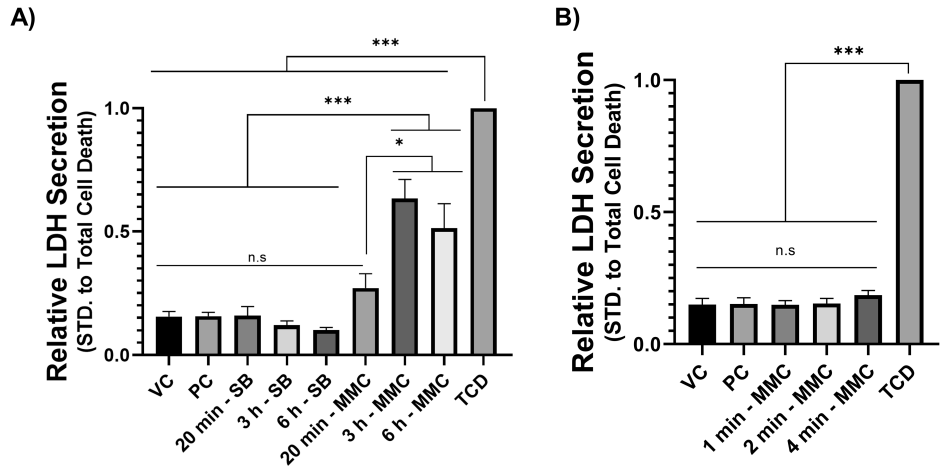

Figure 4. Mean LDH concentration in HTCFs after SB or MMC pretreatment. (**A**) Long exposure of MMC and SB. VC and PC HTCFs received a 20-minute, 3-hour, or 6-hour DMEM pretreatment. All other HTCFs were pretreated with 20 µM SB or 0.2 mg/mL MMC in DMEM solution for varying durations of time. (**B**) Clinically relevant treatment with MMC. VC and PC HTCFs received a 4-minute DMEM pretreatment. All other HTCFs were pretreated with 0.2 mg/mL MMC in DMEM for 1, 2, or 4 minutes. The TCD control consisted of HTCFs given 10% Triton X-100 in DMEM solution. After pretreatment, all HTCFs except the VC and TCD were incubated in 2 ng/mL TGFβ1 in DMEM solution for 48 hours. VCs were incubated in DMEM solution for 48 hours, and TCDs were incubated in 10% *v/v* Triton X-100 in DMEM solution for 48 hours. Supernatant of each well underwent the LDH assay and had LDH concentration measured. LDH concentration was standardized and expressed as a ratio relative to the TCD LDH concentration. Mean LDH concentration ± SEM was compared with a one-way ANOVA and post hoc Tukey's test ($N = 7$, $n = 3$). *$P \leq 0.05$, ***$P \leq 0.0001$.

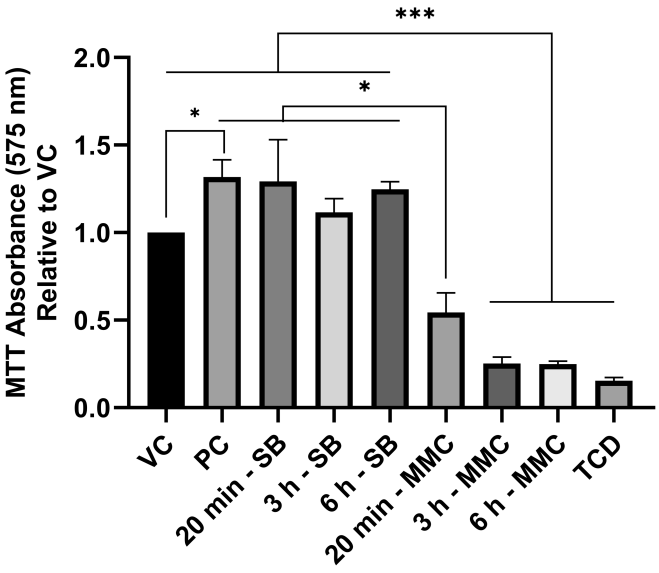

Figure 5. Mean MTT absorbance in HTCFs after SB or MMC pretreatment. VC and PC HTCFs received a 20-minute, 3-hour, or 6-hour DMEM pretreatment. All other HTCFs were pretreated with 20 µM SB or 0.2 mg/mL MMC in DMEM solution for 20 minutes, 3 hours, or 6 hours. The TCD consisted of HTCFs given 10% Triton X-100 in DMEM solution for 48 hours. After pretreatment, all HTCFs except the VC and TCD were incubated in 2 ng/mL TGFβ1 in DMEM solution for 48 hours. VCs were incubated in DMEM solution for 48 hours, and TCDs were incubated in 10% *v/v* Triton X-100 in DMEM solution for 48 hours. HTCFs were incubated in the MTT assay solution for 2 hours and had MTT absorbance measured at 575 nm. MTT absorbance was standardized and expressed as a ratio relative to the VC MTT absorbance. Mean absorbance ± SEM was compared with a one-way ANOVA and post hoc Tukey's test ($N = 7$, $n = 3$). *$P \leq 0.05$, ***$P \leq 0.01$.

phenotype. Pretreatment of TGFβ1-induced HTCFs with MMC for 3 and 6 hours reduced MTT activity to the level of total cell death control, significantly lowering MTT activity in comparison to all other treatment groups ($N = 7$, $n = 3$). In contrast, SB pretreatment slightly lowered TGFβ1-induced metabolic activity in HTCFs after 20 minutes, 3 hours, and 6 hours of exposure to a level within range of HTCFs that received no TGFβ1 (Fig. 5). Metabolic activity of HTCFs pretreated with 2 µM SB or 20 nM SB was comparable to the 20-µM SB pretreatment, with 20 µM showing strongest effects and no notable cytotoxicity (Supplementary Fig. S2).

Discussion

In this study, we investigated the in vitro antifibrotic efficacy of SB and its safety in comparison to MMC using HTCFs. The results suggest that SB is as efficacious at reducing fibrosis as MMC, with significantly less associated cytotoxicity.

The results of the collagen contraction assay demonstrate SB can prevent in vitro fibrosis similar to MMC. These results are supported by previous literature findings for both SB and MMC. Previously, a 25-hour treatment of 1 µM SB had been shown to significantly lower collagen contraction area in TGFβ1-induced human dermal fibroblasts.[24] Type 1 collagen

secretion in rat uterine fibroblasts was significantly lowered by treatment with 5 µg/mL MMC for 48 hours.[25] Our experiments demonstrated significantly lowered collagen contraction with shorter SB and MMC treatment durations compared to those studies. Since the collagen lattice functions as an in vitro 3D tissue mimetic, differences in HTCF-mediated contraction may offer insight into HTCF scarring activity in vivo. Therefore, the significant efficacy of SB at preventing collagen contraction in vitro suggests that SB may be useful to manage postsurgical ocular scarring, but this would require further exploration in vivo.

The impact of TGFβ1-induced expression of α-SMA, MMP-9, and EDA-FN in promoting in vitro scarring activity is evidenced by our research and previously reported findings. Our study showed that SB was able to significantly lower TGFβ1-induced expression of α-SMA, EDA-FN, and MMP-9 in HTCFs with a 5-minute pretreatment and with similar results for 10- and 20-minute pretreatment durations. In addition, this significantly decreased expression of fibrotic markers was comparable to the effects of MMC pretreatment for all proteins measured. These findings are important as α-SMA is a common marker of myofibroblast transformation and is involved in HTCF contractility.[26] As well, MMP-9 is involved in the extracellular matrix remodeling required for the effective regulation of wound healing.[27] Also, EDA-FN, a form of cellular fibronectin with an alternativity spliced domain A, is suggested to promote fibroblast differentiation into the myofibroblast phenotype and, by extension, expression of α-SMA.[28]

The importance of these fibrosis-promoting proteins is also evidenced by their clinical manifestation in glaucomatous individuals. Increased myofibroblast expression of fibrosis-promoting proteins like α-SMA has been reported in glaucomatous compared to nonglaucomatous patients.[29] Furthermore, reduction of myofibroblast activity such as with MMC is important for surgical success.[12,13,15,16] Modulation of myofibroblast activity by MMC affects the expression of fibrosis-promoting proteins and thus clinical scarring outcomes; scar specimens from patients who received MMC showed little α-SMA expression while those from patients who received no MMC showed heavy α-SMA expression.[30] Since SB is able to reduce α-SMA comparably to MMC, SB may have a similar antifibrotic effect on scar tissue.

The antifibrotic efficacy of SB to reduce α-SMA, fibronectin, and MMP-9 expression in TGFβ1-treated fibroblasts has been previously reported.[31–33] Furthermore, α-SMA, some matrix metallopeptidases, and fibronectins are known to have their expression induced by SMAD2/3 localization, a downstream consequence of TGFβ1 activation of ALK5.[18–20] Therefore, it is plausible that preventing SMAD2/3 localization upstream by TGFβ1 inhibition may reduce α-SMA, MMP-9, and EDA-FN expression. MMC is also well documented to lower the expression of fibrosis-related proteins like α-SMA, fibronectin, and MMP-9, which is concurrent with our findings.[34–36] In summary, because TGFβ1-induced expression of α-SMA, MMP-9, and EDA-FN plays an important role in the promotion of scarring, SB-induced inhibition of TGFβ1 could translate positively in the management of postsurgical scarring.

The in vitro safety of SB relative to MMC is reflected in the LIVE/DEAD staining of TGFβ1-treated HTCFs. SB demonstrated no significant change in the percentage of live cells for different exposure times compared to VC or PC groups, unlike the MMC-treated group, which showed significantly increased cytotoxicity. MMC-induced lowering of cell viability is likely due to the ability of MMC to act as a DNA cross-linker, causing cell death.[12,13] Furthermore, a previous LIVE/DEAD staining experiment reported that fibroblast-like synoviocytes cultured in a collagen gel remained viable after exposure to 50 µM SB for 20 hours.[37] Additionally, another previous study using a LIVE/DEAD assay reported MMC to significantly increase cell death in human corneal fibroblasts.[38] Together, these findings as well as our own data from the 3D in vitro tissue mimetic suggest that MMC inhibits contraction via a cytotoxic mechanism. In contrast, as SB does not display any significant cytotoxicity, its anticontractile effects are due to a different mechanism, likely related to the inhibition of procontractile and profibrotic proteins, as demonstrated by immunoblotting.

This study found a significant elevation in LDH concentration associated with MMC pretreatment but not with SB pretreatment. MMC at 0.2 mg/mL was previously shown to increase LDH release in rabbit keratocytes treated up to 24 hours.[39] The lack of significant LDH release with SB suggests it may be safer than MMC across the measured time points. Clinically relevant pretreatment times for MMC were also performed at 1 minute, 2 minutes, and 4 minutes. However, despite 0.2 mg/mL MMC being effective when applied for these times during surgery, 0.2 mg/mL MMC failed to display a significant LDH increase at these durations in vitro. A potential cause of this discrepancy is that MMC is more effectively washed out from cells in vitro after treatment, whereas during surgery, MMC is absorbed into the eye and thus less effectively removed by wash.

The MTT results for SB provide further evidence for the antifibrotic efficacy and potential safety of the

drug. Pretreatment of HTCFs with SB prior to TGFβ1 exposure lowered cellular metabolic activity to within VC range. The decrease in metabolic activity may reflect the inhibition of TGFβ1-induced myofibroblast transformation by SB, as the myofibroblast phenotype is associated with a higher metabolic rate than the fibroblast phenotype.[40] This observation, combined with the lack of significant LDH increase, strengthens the idea that SB is lowering metabolic activity by inhibiting myofibroblast transformation instead of by cytotoxic action. In contrast to SB, MMC significantly lowered cellular metabolic activity for all time points in TGFβ1-induced HTCFs. MMC likely lowered MTT activity by potent cytotoxicity, similar to the LIVE/DEAD staining results for MMC. Furthermore, this result is supported by the current literature as 0.2 mg/mL MMC applied for 3 hours has been previously shown to lower MTT activity in human corneal fibroblasts.[41] In summary, the MTT results provide further evidence supporting SB as a safe and effective antifibrotic drug.

Study Limitations

The primary limitation of this in vitro study is the absence of other inflammatory cytokines present in aqueous humor and the intercellular interactions between immune cells and fibroblasts. Consequentially, the complete inflammatory response seen with ocular filtration surgery is missing, as there can be no immune cells infiltrating into the eye. Therefore, there are restrictions on the conclusions that can be drawn on the efficacy and safety of SB within the entire context of a living system. Going forward, current findings warrant investigations of SB effects in a coculture model of fibroblasts and inflammatory cells, as well as translation of these findings to in vivo animal models.

Conclusions

This study demonstrates the potential of SB to significantly reduce the TGFβ1-induced in vitro fibrotic activity of HTCFs without displaying evidence of cytotoxicity. SB showed comparable efficacy to MMC in mitigating the TGFβ1-induced fibrosis mediated by HTCFs in vitro. These findings strengthen the case for developing SB as a viable alternative therapeutic for managing subconjunctival scarring after bleb-forming glaucoma surgery. Our results strongly support the need for further investigation of SB within an animal model of glaucoma surgery, to determine its in vivo efficacy and potential to ultimately improve patient outcomes at bedside.

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