Bromfenac Inhibits TGF-β1–Induced Fibrotic Effects in Human Pterygium and Conjunctival Fibroblasts

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PREVIOUS STUDIES have shown that pterygium is an invasive and benign overgrowth of fibrovascular conjunctiva over the cornea, which is believed to be related to genetic changes, virus infection, and environmental influences, such as sawdust and UV irradiation exposure.1,2 The characterization of pterygium is proliferation, inflammation, angiogenesis, and extensive deposition of the extracellular matrix (ECM).3 Pterygium tissue expresses higher levels of collagen 1, fibronectin (FN), and alpha-smooth muscle actin (α-SMA) as compared with normal conjunctiva.4 Pterygium can significantly alter the visual functions in advanced cases and thus might reduce the recurrence rate of pterygium,5 but their effectiveness is limited.6,7 There are even risks of corticosteroid-induced ocular hypertension and glaucoma.8 Therefore, it is necessary to investigate the mechanisms of pterygium and to develop more targeted therapies to prevent recurrence.

Excessive wound healing after pterygium excision surgery often leads to fibrosis, which indicates the recurrence of pterygium. A key feature of pathological tissue repair is myofibroblast activation, and α-SMA is a key marker of myofibroblasts.9 Myofibroblasts rapidly synthesize and accumulate redundant amounts of ECM, including collagen, FN, and tenascin-C.10–12 Thus transforming the site into a chronic wound. Transforming growth factor-beta 1 (TGF-β1) activates myofibroblasts, and upregulates the synthesis of ECM in pterygium.13–15 Pterygium excision surgery has been identified as an inducer of myofibroblast activation and induces fibrosis in pterygia.16 Previous studies have shown the overexpression of TGF-β1 in pterygium tissues as compared with normal conjunctiva,17–19 and in recurrent pterygium fibroblasts cultures as compared with primary pterygium fibroblasts cultures.20 TGF-β isoforms
bind to their receptors, which activate multiple intracellular signal-transmitting proteins, including Smads, PI3K/AKT, MEK/ERK, RHO-A, and JNK/P38.23 In the present study, TGF-β1 was used to induce fibrosis in human pterygium fibroblasts (HPFs) and human conjunctival fibroblasts (HConFs). Nonsteroidal anti-inflammatory drugs (NSAIDs) have shown antifibrotic properties in diseases such as breast cancer and skin carcinoma.22,23 The main target of NSAIDs is cyclooxygenase-2 (COX-2). It has been reported that pterygal specimens clearly express COX-2, whereas normal conjunctiva specimens have no COX-2 expression.24 As far as we know, only one study has reported that the NSAID called nimesulide could suppress the proliferation of HPFs.25 NSAIDs have been used during the perioperative period of pterygium for its analgesic and anti-inflammatory effects,26 but the potential preventive effects of NSAIDs on pterygium recurrence remain unclear. Bromfenac is a kind of NSAID, which is approximately three to four times more effective at inhibiting COX-2 in vitro as compared with other ophthalmic NSAIDs amfenac, ketorolac, and diclofenac.26 Bromfenac is usually used to prevent macular edema and inflammation after cataract and complex oculotropic surgeries,27–29 which is less toxic toward corneal epithelial cells.30 Our study intended to explore whether bromfenac has the potential to prevent the excessive wound healing that takes place after the pterygium surgeries.

The aims of the present study were to investigate the antifibrotic effects of bromfenac on HPFs and HConFs, as well as to explore the possible mechanisms involved in these effects.

**METHODS**

**Isolation and Cultivation of Primary HPFs, HConFs, and Human Conjunctival Epithelial Cells (HConEs)**

Twenty-one different pterygium samples were obtained from 21 patients with mean age of 61.7 ± 7.4 years (mean ± SD; range, 49–76 years) who underwent pterygium surgical excision at our hospital. Paraanal pterygium tissues were obtained at the time of excision; the heads and bodies of the pterygium samples were used for cultures. All of the pterygium specimens were classified as grade T3 (fleshy) according to the grade T classification.31 The fleshiness of stroma is significantly associated with pterygium recurrence after excision.32 The heads of these pterygium tissues invaded over the corneal limbus for more than 3 mm. All patients were treated in accordance with the Declaration of Helsinki, and each of them provided informed written consent. This study protocol was approved by the institutional review board of our institute. The information of which patients and donors are summarized in Supplementary Table S1. The cells were grown as communities, cells in a well usually present only one of the two types of morphologies, fusiform or multilateral. If all of the cells in a well presented an elongated and fusiform morphology, the cells were amplified and prepared to be identified as HPFs or HConFs. As long as there were cells presenting typical epithelial multilateral morphology mixed into the elongated and fusiform cells, the corresponding wells were abandoned. If all of the cells in a well presented a typical epithelial multilateral morphology, the cells were also amplified and prepared to be identified as epithelial cells. After the authentications that the fibroblasts were not contaminated with epithelial cells by means of Western blot, immunofluorescence staining assays, and morphology, the fibroblasts were amplified for experiments. Each person’s cells were not mixed with other people’s cells. The HPFs and HConFs were passaged at the ratio of 1:2, cells after the third generation were used for all experiments. The information of which cells were used in each experiment were summarized and tied into the demographics data in Supplementary Table S1.

**Cultivation of Human Foreskin Fibroblasts (HFFs) and HeLa Cells**

HFFs (provided by Stem Cell Bank, Chinese Academy of Science) were used as positive control (standard fibroblasts), and HeLa cells were used as negative control (standard epithelial cells). HFFs were cultivated in DMEM/F-12 containing 20% FBS, 100 U/mL penicillin, and 100 g/mL streptomycin. HeLa cells were cultured in high glucose DMEM (4.5 g/L; Gibco Life Technologies) with 10% FBS, 100 U/mL penicillin, and 100 g/mL streptomycin. All of the cells were maintained in a humidified atmosphere of 5% CO₂ at 37°C.

**Drug Treatments of Cells**

As mentioned above, the cells were incubated with DMEM/F-12 supplemented with 10% FBS before treatments, then the medium was changed to DMEM/F-12 containing 1% FBS to conduct the experiments. But in the wound healing assays, serum-free DMEM/F-12 was used. Bromfenac was used at nontoxic concentrations.33 To assess the antifibrotic effects of bromfenac on TGF-β1–induced cells, the cultured HPFs and HConFs were divided into the following groups and treated for 48 hours: the control group; the TGF-β1 group, in which the cells were exposed to 20 ng/mL TGF-β1 (200-1; PeproTech, Rocky Hill, NJ, USA); the bromfenac group, in which the cells were treated with 90 μg/mL bromfenac (bromfenac active components were from Senju Pharmaceutical, Osaka, Japan); the TGF-β1 + bromfenac group, in which the cells were treated with 20 ng/mL TGF-β1 plus 90 μg/mL bromfenac. For the doze-
effect relationship study, HPFs and HConFs were treated with three concentrations of bromfenac (30, 60, and 90 µg/mL) for 48 hours. For the inhibitors studies, 200 nM wortmannin (Selleck, Houston, TX, USA) (PI3K inhibitor) and 20 µM U0126 (Bio-Rad, Hercules, CA, USA) (MEK inhibitor) were added 2 hours before the TGF-β1 treatment, and the HPFs and HConFs were collected 48 hours after TGF-β1 treatment to evaluate the changes. TGF-β1 was dissolved in DMEM/F-12; bromfenac was dissolved in PBS, then filtered to eliminate the microorganisms; wortmannin and U0126 were dissolved in dimethyl sulfoxide (DMSO). The same volume of solvents DMEM/F-12, PBS, or DMSO was used as controls. The medium was changed every 24 hours to replenish the chemicals.

**Western Blot Assay**

After treatments, cell total proteins were extracted from the cells with the cells to the total protein extraction kit (Sangon, Shanghai, China), which contains lysis buffer (NaCl, SD8138 [1M Tris-buffer solution pH 7.4], 0.5M EDTA, 2Na, NDSB-201, and NP-40), phenylmethanesulfonyl fluoride, protease inhibitor, and phosphatase inhibitor. The total protein concentrations were determined by the bicinchoninic acid Protein Assay Kit (B9643–11; Sigma-Aldrich, St. Louis, MO, USA). The same amounts of proteins were subjected to SDS-PAGE with certain concentrations, then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA), and blocked for 2 hours with 5% BSA in Tris-buffered saline (TBS-T, 10 mM Tris-HCl [pH 7.5], 100 mM NaCl, and 0.1% Tween 20). The primary antibodies were diluted in TBS-T containing 5% BSA. The information of the antibodies used in our study is presented in Supplementary Table S2. The PVDF membranes were then incubated in the primary antibody diluents overnight at 4°C. After that, the PVDF membranes were washed and incubated with horseradish peroxidase–conjugated secondary antibodies for 1 hour at room temperature. Immunodetection was performed with the enhanced chemiluminescence (ECL) Western Blot Detection System (Millipore). The immunoblots were visualized by the ChemiDoc XRS System (Bio-Rad, Hercules, CA, USA). The intensities of the bands were quantified by densitometry; the software Image J (http://image.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) was used. GAPDH served as an internal control.

**Quantitative Real-Time PCR**

HPFs and HConFs having 80% confluency in a 6-cm-dish were treated and harvested, and the total RNA was extracted (Trizol Reagent; Invitrogen, CA, USA). RNA concentrations (200–500 ng/µl) and OD260/OD280 ratios (1.9–2.0) were determined using a NanoDrop 1000 UV spectrophotometer (Thermo Scientific, Waltham, MA, USA); approximately 1000 ng RNA was used per reverse transcription reaction (PrimeScriptRT Reagent Kit; TaKaRa, Shiga, Japan). The SYBR Premix Ex Taq II Kit (TaKaRa) was used for quantitative real-time PCR on a 7500 Fast Real-Time PCR System (ABI, Loma Linda, CA, USA), with a 2-step method. The primers are shown in Supplementary Table S2. The expression levels of the target genes were normalized to that of GAPDH and were calculated using the 2-DDACT method. The data are presented as fold-changes.

**Immunofluorescence (IF) Staining Assay**

HPFs and HConFs were seeded on the cleaned coverslips for 24 hours. Afterward, the medium was changed to DMEM/F-12 with 1% FBS and the indicated drugs, respectively. After being treated for 48 hours, the cells were rinsed with PBS three times and then fixed in 4% formaldehyde solution for 20 minutes. After being rinsed with PBS three times again, the cells were permeabilized with 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) for 15 minutes, and blocked with 10% normal goat serum for 1 hour at room temperature. Subsequently, the cells were incubated with primary antibodies against α-SMA, COL3, pan-keratin, vimentin, or corresponding isotype control overnight at 4°C. Then, after being rinsed with PBS, Alexa Fluor 555 Donkey Anti-Rabbit immunoglobulin (IgG) or Alexa Fluor 555 Donkey Anti-Mouse IgG solution was used to incubate the cells for two hours at room temperature. The nuclei were stained with DAPI (4’,6-diamidino-2-phenylindole) contained in the mounting medium. Eventually, the coverslips were mounted on the slides with a drop of mounting medium with DAPI (H-1200; Vector, Burlingame, CA, USA). Fluorescent images were then observed using a Leica inversion fluorescence microscope (DMi8/DFC 550; Leica, Wetzlar, Germany).

**Wound Healing Assay**

HPFs and HConFs were seeded in 12-well plates. After being cultured for 24 hours, the cell density of each well reached 90% confluency, and then the cells were scratched with a sterile 100-µL pipette tip. The scratched wells were then washed with PBS three times, and the cells were treated with the indicated drugs in serum-free medium. The wounds were photographed at 0 and 24 hours. The cellular migration area in each image was measured using Image J software. The data were quantified based on the area of the wounds at 0 hour, which was considered as 100%.

**CCK-8 Cell Proliferation Assay**

Cell proliferation ability was measured by Cell Counting Kit 8 (CCK-8; Dojindo, Japan). About 5 × 10³ cells/well were seeded in a 96-well plate and cultivated for 24 hours, then treated with indicated drugs in medium free of FBS for 48 hours, respectively. Then, CCK-8 reagent (10 µl) was added to each well and incubated for 4h. Finally, the absorbance value of each well at a wavelength of 450 nm (OD450) was determined using a microplate reader.

**Statistical Analysis**

At least three independent experiments were performed at least in triplicate for each assay. The cells of an independent experiment were from the same batch of the same person. Different independent experiments used different batches of cells. The results of multiple experiments are presented as the mean ± SD. After confirming that all variables were normally distributed using the Kolmogorov-Smirnov test and visualized by Q-Q plot, statistical differences were determined by 1-way ANOVA followed by Bonferroni’s multiple comparison test for comparison among three or more groups. A P value < 0.05 was defined as statistically significant. All data were analyzed using SPSS (version 18.0; IBM Corp., Chicago, IL, USA) statistical software. The statistics are presented using GraphPad Prism 5.

**RESULTS**

**Cultivation and Identification of Primary HPFs and HConFs**

Primary HPFs (n = 21) and HConFs (n = 10) were isolated from human eyes. These cells were expanded in culture plates and their identities were confirmed using Western blotting and indirect IF microscopy for mesenchymal proteins FN, COL3,
vimentin, and absence of the expression of epithelial keratins using the pan-keratin antibody (total keratin 4, 5, 6, 8, 10, 13, and 18). Previous studies have reported the positive expression of pan-keratin in primary human pterygium epithelial cells.34–35 HFFs and HeLa cells were used as positive and negative controls, respectively. As a result, HFFs, HPFs, and HConFs clearly expressed FN and COL3, whereas HeLa cells had negative expression of FN and COL3. HFFs, HPFs, and HConFs expressed higher protein levels of vimentin than HeLa cells. HeLa cells expressed high protein level of pan-keratin, whereas HFFs, HPFs, and HConFs showed negative protein expression of pan-keratin (Supplementary Fig. S1A, S1C).

Cell phenotypes were confirmed by light microscopy. HFFs, HPFs, and HConFs presented elongated and fusiform morphology, whereas primary HConFs and HeLa cells had typical multilayered epithelial cell morphology (Supplementary Fig. S1B).

The Effects of Bromfenac on TGF-β1–Induced ECM Synthesis and Myofibroblast Activation in HConFs and HPFs

HPFs from three patients and HConFs from three donors were treated with TGF-β1 (20 ng/mL) and bromfenac (90 μg/mL) for 48 hours. The effects of bromfenac and TGF-β1 on the synthesis of FN, COL3, and α-SMA were tested by Western blotting. In HPFs, after the treatment of TGF-β1, the protein levels of FN (2.62 ± 0.34 folds, P < 0.001), COL3 (1.98 ± 0.27 folds, P = 0.002), and α-SMA (2.32 ± 0.34 folds, P < 0.001) folds, respectively. In HConFs (Fig. 1A), the results of IF assays indicated that the protein distributions of COL3 and α-SMA were tested by Western blotting. In HPFs, after the treatment of TGF-β1 (20 ng/mL) and the indicated inhibitors (Supplementary Fig. S3B). FN and COL3 are important compositions of ECM.12–13 α-SMA is a reliable marker for myofibroblast activation, which enhances fibroblast contractile activity significantly.38–39 Collectively, these results indicated that bromfenac inhibits TGF-β1–induced ECM synthesis and myofibroblast activation in HConFs and HPFs.

The effects of using bromfenac alone were validated in HPFs and HConFs. The effects of the peptides (Supplementary Fig. S1A) and mRNA levels (Supplementary Fig. S5) expression levels of FN, COL3, α-SMA, and survivin decreased in a dose-dependent manner. Proliferative marker survivin may play a role in the development of pterygium.40 The protein and mRNA levels of matrix metalloproteinase 3 were not changed by bromfenac in our study.

Effects of Bromfenac on the Signaling Pathways in TGF-β1–Treated HConFs and HPFs

HPFs from three patients and HConFs from three donors were treated with TGF-β1 (20 ng/mL) and bromfenac (90 μg/mL) for 48 hours; the effects of bromfenac and TGF-β1 on the signaling pathways were tested by Western blotting. The expression levels of p-AKT and p-GSK-3β-S9 were significantly increased after the treatment of TGF-β1 (Fig. 1B). The basal expression levels of p-Smad 2/3 in HConFs and HPFs were below the linear range for ECL detection in Western blotting, but became detectable after the treatment of TGF-β1. Besides, TGF-β1 rapidly (approximately 30 minutes) activated Smad2/3, p38 MAPK, AKT, and ERK1/2 by phosphorylating them (Supplementary Fig. S2B), as reported before.21 Phosphorylation at serine residue 9 of GSK-3β reduces its activity.41 Our data also showed that TGF-β1 inactivates GSK-3β by phosphorylating GSK-3β-S9.

In HPFs, in comparison with the TGF-β1 group, the TGF-β1 + bromfenac group expressed lower levels of p-AKT (0.66 ± 0.08 folds, P = 0.032), p-ERK1/2 (0.69 ± 0.11 folds, P = 0.003), and p-GSK-3β-S9 (0.65 ± 0.10 folds, P = 0.002); similar results were seen in HConFs (Fig. 1B). The expression of p-Smad2/3, p-p38 MAPK, and the total protein levels of AKT, ERK1/2, GSK-3β, Smad3, and p38 MAPK were not changed by bromfenac in HConFs and HPFs. In addition, at 30 minutes after the treatments, TGF-β1–induced expression of p-AKT, p-ERK1/2, and p-GSK-3β-S9 in HConFs and HPFs was reduced by bromfenac in a dose-dependent manner; however, the increase of p-Smad2/3 and Smad3 induced by TGF-β1 was not changed by bromfenac (Supplementary Fig. S6).

Collectively, these results suggested that bromfenac inhibits TGF-β1–induced ECM synthesis and myofibroblast activation in HConFs and HPFs, and might through dephosphorylating AKT, ERK1/2, and GSK-3β-S9.

HPFs from three patients and HConFs from three donors were taken to validate the effects of using bromfenac alone. After the treatment of bromfenac for 48 hours, the phosphorylated protein levels of AKT, ERK1/2, and GSK-3β-S9 declined with dosage (Supplementary Fig. S4B).

The PI3K/AKT and MEK/ERK Signaling Pathways Mediate TGF-β1–Induced ECM Synthesis and Myofibroblast Activation in HConFs and HPFs

HPFs from three patients and HConFs from three donors were treated with TGF-β1 (20 ng/mL) and the indicated inhibitors for 48 hours; the effects of the inhibitors and TGF-β1 were tested for Western blotting. AKT signaling was blocked with the PI3K/AKT inhibitor wortmannin; ERK signaling was blocked with the MEK/ERK inhibitor U0126. In HPFs, in comparison with the TGF-β1 group, TGF-β1 + wortmannin group had lower expression level of p-AKT (0.41 ± 0.11 folds, P = 0.002), and the TGF-β1 + U0126 group had lower expression level of p-ERK (0.44 ± 0.05 folds, P < 0.001) respectively. Similar results were found in HConFs (Fig. 2A). In HPFs, as compared with the TGF-β1 group, the TGF-β1 + wortmannin group had lower expression level of FN (0.69 ± 0.05 folds, P = 0.001), COL3 (0.64 ± 0.06 folds, P = 0.007), and α-SMA (0.64 ± 0.09 folds, P = 0.002); wortmannin had similar effects on HConFs (Fig. 2B). In addition, in HPFs, TGF-β1 + U0126 group had lower expression levels of FN (0.60 ± 0.09 folds, P = 0.002), COL3 (0.53 ± 0.08 folds, P = 0.001), and α-SMA (0.48 ± 0.05 folds, P < 0.001), in comparison with the TGF-β1 group; U0126 had similar effects on HConFs (Fig. 2B).

Therefore, blocking either PI3K/AKT or MEK/ERK signaling pathway could reduce the TGF-β1–induced ECM synthesis and myofibroblast activation in HConFs and HPFs. From the above, a conclusion can be drawn that bromfenac inactivates the AKT and ERK pathways, both of which could modulate the expression of FN, COL3, and α-SMA.

Effects of Bromfenac on the TGF-β1–Induced Cellular Migration and Proliferation on HConFs and HPFs

The cellular migration ability was detected using wound healing assays. TGF-β1 increased the migration ability.
FIGURE 1. Effects of bromfenac on the TGF-β1-induced HConFs and HPFs. HPFs from three patients and HConFs from three donors were treated with vehicle, TGF-β1 (20 ng/mL) only, TGF-β1 (20 ng/mL) plus bromfenac (90 μg/mL), or bromfenac (90 μg/mL) only for 48 hours. Representative Western blot images of (A) FN, COL3, and α-SMA protein expression and (B) p-AKT, total AKT, p-ERK1/2, total ERK1/2, p-GSK3β-S9, total GSK3β, p-Smad2/3, total Smad3, p-p38 MAPK, and total p38 MAPK expression. Quantitative analysis of immunoblots presents the mean values ± SD of three independent experiments. Total protein levels were normalized to GAPDH for loading control, whereas the phosphorylated proteins were normalized to their total proteins. One-way ANOVA was used to test the variations among groups, and followed by Bonferroni’s multiple comparison test, which was used for comparison between two groups. *P < 0.05, **P < 0.01, ***P < 0.001 versus vehicle; #P < 0.05, ##P < 0.01, ###P < 0.001 versus with TGF-β1.
The recurrence of pterygium is a process of pathological fibrosis, excessive wound healing accompanies the production of large amounts of ECM after pterygium excision surgeries. In our study, bromfenac inhibited the production of α-SMA, FN, and COL3, and suppressed cellular migration and proliferation in TGF-β1–induced HPFs and HConFs. Therefore, bromfenac has nice antifibrotic effects on HPFs and HConFs.

**DISCUSSION**

The recurrence of pterygium is a process of pathological fibrosis, excessive wound healing accompanies the production of large amounts of ECM after pterygium excision surgeries. In our study, bromfenac inhibited the production of α-SMA, FN, and COL3, and suppressed cellular migration and proliferation in TGF-β1–induced HPFs and HConFs. Therefore, bromfenac has nice antifibrotic effects on HPFs and HConFs.

The present study also demonstrated that blocking the PI3K/AKT or MEK/ERK pathway suppressed the synthesis of FN, COL3, and α-SMA induced by TGF-β1. This indicated that the two pathways are essential to pterygium pathogenesis, and that inhibitions of them give promise of other treatment routines for pterygium. TGF-β1 has been identified as an inducer of myofibroblast activation in pterygia; however, knowledge...
about the molecular mechanisms underlying the effects of TGF-β on pterygium is still incomplete. The p38 MAPK pathway has been reported to participate in the TGF-β-induced ECM protein synthesis and myofibroblast activation of HPFs. Previous studies have shown that the canonical Smad2/3 signaling and several other noncanonical pathways participate in the TGF-β1-induced fibrosis in many other diseases, such as breast cancer. TGF-β shows profibrotic effects through activation of the AKT/GSK-3β pathway in liver cancer cells. In TGF-β-induced lung fibroblasts and cutaneous microvascular endothelial cells, ERK/GSK-3β pathway participates in the fibrogenesis.

Our study indicated that bromfenac inhibits the phosphorylated protein levels of AKT, ERK1/2, and GSK-3β-S9 in HPFs and HConFs; meanwhile, blocking the PI3K/AKT or MEK/ERK pathway has similar effects with bromfenac, which leads to a decrease of ECM synthesis and myofibroblast activation. Generally, NSAIDs act by inhibiting COX-2 to block the

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**Figure 3.** Effects of bromfenac on the migration and proliferation ability of TGF-β1-induced HConFs and HPFs. The HConFs and HPFs were treated with vehicle, TGF-β1 (20 ng/mL) only, TGF-β1 (20 ng/mL) plus bromfenac (90 μg/mL), or bromfenac (90 μg/mL) only for 24 hours in wound healing assay and 48 hours in the CCK-8 assay. (A) Light microscopic images of the scratch wound area were taken at 0 and 24 hours. Quantitative analysis of the ratio of cellular migration area to the initial scratch area, and the initial scratch area in each group was set as 100%. (B) The cellular proliferation ability was detected by CCK-8 assays. Forty-eight hours after the treatments, absorbance read at 450 nm was used to compare, and the absorbance of the control group was set as 100%. Values represent mean ± SD of three independent experiments. One-way ANOVA was used to test the variations among groups, and followed by Bonferroni’s multiple comparison test, which was used for comparison between two groups. *P < 0.05, **P < 0.01, ***P < 0.001 versus vehicle; *P < 0.05, **P < 0.01, ***P < 0.001 versus with TGF-β1.
Antifibrotic Effect of Bromfenac on Pterygium

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