Cornea

NK1 Receptor Antagonists as a New Treatment for Corneal Neovascularization

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PURPOSE. To determine whether the inhibition of Substance P (SP) activity can reduce corneal neovascularization (CNV) by means of local administration of high-affinity, competitive, tachykinin 1 receptor (NK1R) antagonists Lanepitant and Befetupitant.

METHODS. We performed a safety and efficacy study by using (1) two different C57BL/6 mouse models of CNV: alkali burn and sutures; (2) different concentrations; and (3) different routes of administration: topical or subconjunctival. Clinical examination endpoints, SP levels, CNV index, and leukocyte infiltration were measured.

RESULTS. Substance P increased after injury in the corneal epithelium of both CNV models, and later in the suture model. Topical Lanepitant was nontoxic to the ocular surface and effective in reducing hemangiogenesis and lymphangiogenesis, corneal SP levels, and leukocyte infiltration, as soon as 4 days later in the alkali burn model. Topical Lanepitant, up to 7 days, was ineffective in the suture model. However, subconjunctival Lanepitant was effective in reducing lymphatic CNV, leukocyte infiltration, and SP levels in the suture model, after 10 days. Additionally, in the alkali burn model, subconjunctival Lanepitant significantly reduced blood CNV, corneal perforation rate, opacity, and leukocyte infiltration, and improved tear secretion. Finally, topical application of Befetupitant reduced CNV in the alkali burn model but was toxic owing to the vehicle (dimethyl sulfoxide [DMSO]); hence, Befetupitant was not tested in the suture model.

CONCLUSIONS. The NK1R antagonist Lanepitant is safe for the ocular surface and effective in reducing both corneal hemangiogenesis and lymphangiogenesis, and leukocyte infiltration. We suggest that inhibition of NK1R may represent an adjunctive tool in the treatment of CNV.

Keywords: corneal neovascularization, Substance P, Lanepitant, NK1 receptor, neuropeptide
Inhibition of Substance P (NK1) Receptor in Cornea

T
cal the normal cornea is one of the few avascular tissues in the human body. Several ocular diseases, however, can cause corneal hemangiogenesis and lymphangiogenesis. Indeed, corneal neovascularization (CNV) affects 4% of the US population, and it is a constant finding in severe corneal diseases, and represents the second cause of blindness worldwide. The growth of vessels into the cornea is clinically relevant because it induces loss of corneal transparency, due to infiltration of calcium, lipids, and inflammatory cells. Additionally, it significantly reduces effectiveness of the key surgical procedure used to restore corneal clarity (i.e., keratoplasty), as it increases the rate of graft rejection.

For these reasons, significant effort is being spent to find novel therapies targeting CNV.

Although totally devoid of vessels, the cornea receives dense sensory innervation. Corneal nerves originate in the trigeminal ganglion, where neural bodies reside. Corneal nerve endings contain several neuropeptides. Between these, Substance P (SP), an 11-amino-acid polypeptide, member of the tachykinin family, is highly expressed. Substance P is derived from preprotachykinin A protein and secreted by both neuronal and nonneuronal cell types (i.e., macrophages and dendritic cells). It exerts its actions by binding to tachykinin receptors (NK1, NK2, and NK3), having the highest affinity for tachykinin 1 receptor (NK1R). This is expressed on a variety of cell types including neurons and immune, endothelial, epithelial, and glial cells. Substance P is well known as a key mediator of inflammation and wound healing. More specifically, SP levels are increased in the murine cornea after alkali burn. Substance P receptor antagonists have been tested in several inflammatory diseases, including inflammatory bowel disease, neurogenic and liver inflammation, and polymicrobial sepsis. In the cornea, inhibition of SP ameliorated Pseudomonas keratitis. Additionally, a recent study has shown that treatment with the SP antagonist Spantide I significantly reduces the severity of herpes simplex virus-1, including reduced angiogenesis.

However, the effect of selective NK1R inhibition in the cornea, and specifically in animal models of CNV, has not been described yet. The aim of this study was to test safety and efficacy of high-affinity, nonpeptide, competitive NK1R antagonists (Lanepitant and Befetupitant) in two different murine models of CNV.

Materials and Methods

Animals
Female, 6- to 8-week-old, C57BL/6 mice (Charles-River, Calco, LC, Italy) were used for all experiments (total: 283 mice). Animals were allowed to acclimatize to their environment for 1 week before experimentation. Each animal was deeply anesthetized with intraperitoneal injection of Tribromoethanol (250 mg/kg) before all surgical procedures. Postoperatively, all animals received a single dose of Carprofen at 5 mg/kg subcutaneous. Carbon dioxide inhalation and subsequent cervical dislocation were applied to euthanize the animals. All experimental protocols were approved by the Animal Care and Use Committee of the San Raffaele Scientific Institute, in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Substance P Quantification in the Cornea
To quantify the amount of SP in healthy, alkali burn, or sutured corneas (before and after Lanepitant treatment), six samples per group (total: 81 mice) were pooled in pairs on days 4, 7, and 10 post injury in 500 μL RIPA buffer (Sigma-Aldrich Corp., St. Louis, MO, USA) containing protease inhibitor cocktail (Sigma-Aldrich Corp.). Each of three pools was homogenized on ice with UltraTurrax T8 (IKA, Wilmington, NC, USA). The samples were then clarified by centrifugation at 10,000g for 5 minutes at 4°C. An aliquot of each supernatant (1:500 dilution) was assayed in triplicate for SP protein by using a commercially available EIA (Enzyme Immunoassay) kit (Cayman Chemical, Ann Arbor, MI, USA), according to the manufacturer’s instruction. Results were expressed as picogram per cornea.

Lanepitant Treatment in the Corneal Alkali Burn Model
A corneal alkali burn was created in the left eye of each mouse by means of a paper disc (3-mm diameter) soaked in 1 N NaOH for 10 seconds, under slit-lamp examination. The ocular surface was then washed with 15 mL normal saline. To increase reproducibility, a single investigator applied the burn to all animals.

Previous literature proposed the use of Lanepitant (LY303870) at 20 mg/kg/d, via intraperitoneal injection, in rats. Considering an average mouse weight of 35 g, we extrapolated a dose of 0.7 mg/d/mouse. We arbitrarily decided to administer topically approximately half of the systemic dose. Specifically, the highest topical dose we used was 6.4 mg/mL, six times per day, in a 10-μL volume (total: 0.384 mg/d). Considering that we treated only one eye, the total amount of Lanepitant we used was approximately half the intraperitoneal dose. We also tested 1:4 scalar dilutions: 6.4, 1.6, and 0.4 mg/mL.

Animals were randomized into four groups (n = 6, each) receiving Lanepitant 0.4, 1.6, or 6.4 mg/mL (MW = 632.66, Dompé S.p.A., L’Aquila, Italy) dissolved in a total volume of 10 μL phosphate-buffered saline (PBS, Sigma-Aldrich Corp.) or 10 μL vehicle as control, topically six times a day for 4 days (Table). A second experiment was designed, extending treatment duration to 7 days with the lower concentrations, 0.4 or 1.6 mg/mL, in comparison to the vehicle.

Topical Lanepitant toxicity was evaluated in two groups of six healthy animals receiving in the left eye 10 μL topical Lanepitant 1.6 mg/mL or PBS, six times a day for 9 days. A third experiment was performed by testing subconjunctival injection of Lanepitant (12.8 mg/mL, in 3 μL, every 2 days for 10 days) versus subconjunctival PBS in the alkali-burned left eye of eight mice.

Befetupitant Treatment in the Corneal Alkali Burn Model
A corneal alkali burn was created as described above for the Lanepitant experiment. Animals were then randomized into three groups (n = 6), receiving 10 μL Befetupitant 0.4 or 1.6 mg/mL (MW = 565.55, Dompé S.p.A.) in 100% dimethyl sulfoxide (DMSO, Sigma-Aldrich Corp.) or 10 μL vehicle (DMSO) as control, topically six times a day for 4 days (Table).
Topical Befetupitant and DMSO toxicity was evaluated in two groups of six healthy animals receiving in the left eye 10 μL topical Befetupitant 0.4 mg/mL or 100% DMSO, six times a day for 9 days.

**Lanepitant Treatment in the Corneal Suture Model**

Mice were anesthetized and three 10.0 nylon sutures were placed intrastromally as previously described with some modifications.30 Briefly, a 2-mm corneal trephine was placed on the cornea and centered on the pupil and then three 10.0 nylon sutures were placed intrastromally 120° apart with knots left unburied. Animals were randomized into four groups (n = 6, each), receiving 10 μL Lanepitant (0.4, 1.6, or 6.4 mg/mL) dissolved in PBS or 10 μL vehicle as control, topically six times a day for 4 days (Table). A second experiment was performed, extending treatment duration to 7 days with topical 0.4, 1.6, 6.4, and 12.8 mg/mL Lanepitant or topical vehicle (n = 6). A third experiment was performed with subconjunctival Lanepitant (12.8 mg/mL in 5 μL, every 2 days for 10 days) or subconjunctival vehicle (n = 10).

**Substance P Treatment in Two Models of Corneal Neovascularization**

Subconjunctival injection of SP (Bachem, Torrance, CA, USA) was performed in the left eye of 10 mice (0.01 mg/mL, in 3 μL, every 2 days for 10 days) and compared with subconjunctival injection of PBS or Lanepitant (12.8 mg/mL) in both models of CNV (alkali burn or suture models), as described in the Table.

All treatments started immediately after caustication or suturing.

**Clinical Endpoints**

Clinical examination was performed to check for drug toxicity and/or gross pathologic changes. Corneas were examined and photographs were taken under the slit-lamp microscope SL 990 (C.S.O., Florence, Italy) every day in a blinded fashion. In vivo corneal fluorescein staining was used to evaluate drug toxicity after Lanepitant and Befetupitant treatment. A scoring system was used to evaluate corneal opacity, as previously described.31 Briefly, corneal opacity was scored on a scale of 0 to 4, where 0 = completely clear; 1 = slightly hazy, iris and pupils easily visible; 2 = slightly opaque, iris and pupils still detectable; 3 = opaque, pupils hardly detectable; and 4 = completely opaque with no view of the pupils. Tear production was quantified with phenol red thread test, as demonstrated before.32 Briefly, tear production was measured with the phenol red thread test (Zone-Quick; Lacrimedics, Eastsound, WA, USA). Under a microscope, the threads were held with jeweler forceps and placed in the lateral canthus of the conjunctival fornix of the left unanesthetized eye for 30 seconds after the excess tears were removed for a standard time of 4 seconds. The tear distance was measured in millimeters under a microscope.

**Immunohistochemical Analysis**

Mouse corneas without (control) or with neovessels (4 days after alkali burn or suture placement) and human corneas affected with keratoconus were frozen in OCT (Optimum Cutting Temperature) compound (Kiliik; Bio-Optica, Milan, Italy) and stored at −80°C until ready for sectioning. Cryosections (7 μm) were fixed with paraformaldehyde 4% (Sigma-Aldrich Corp.), blocked in 2% bovine serum albumin/0.5% Triton X-100 (Sigma-Aldrich Corp.), as previously described.33 Mouse sections were immunostained with rabbit polyclonal antibody binding the entire SP (1:200, AB1566; Chemicon, Temecula, CA, USA) and rabbit anti-TUJ1 (1:200, neuron-specific class III beta-tubulin, 1 mg/mL; Chemicon) at 4°C overnight, and subsequently with Alexa Fluor-488 donkey anti-rabbit IgG, 2 mg/mL (Invitrogen-Molecular Probes, Paisley, UK) in a 1:500 dilution for 2 hours at room temperature.

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**TABLE. Schematic Representation of Different Drug Application Strategies**

<table>
<thead>
<tr>
<th>Corneal Model</th>
<th>Drug</th>
<th>Route of Administration</th>
<th>Concentration, mg/mL</th>
<th>Days of Treatment</th>
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<tr>
<td>Alkali burn</td>
<td>Lanepitant</td>
<td>Topical (six times daily)</td>
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<td>1.6</td>
<td>7</td>
</tr>
<tr>
<td>Sutures</td>
<td>Lanepitant</td>
<td>Subconjunctival (every 2 d)</td>
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<td>10</td>
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<td>Lanepitant</td>
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<td>Subconjunctival (every 2 d)</td>
<td>0.01</td>
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Figure 1. Substance P localization in the neovascularized cornea and its reduction after Lanepitant treatment. In human (A) and mouse (B) corneal cross sections, SP was localized in the epithelium (E) and sporadically in the stroma (S), and it colocalized with TUJ1, principally localized in the
basal layer of the epithelium. The isotype control immunostainings were negative (lower panel). The inserts show a detail at ×2 magnification. (C) Correlation between CD45+ cells and SP levels in both CNV models on days 4 and 7, expressed as fold-change increase with respect to healthy corneas. The two measures had the same trend over time: The amount of SP and leukocyte infiltration was significantly higher in the alkali burn model than the suture model on day 4. (D) Substance P protein level in the cornea was significantly increased in two mouse models of CNV (alkali burn and suture placement) on days 4 and 7. The alkali burn produced the highest increase of SP on day 4. Topical Lanepitant treatment (6.4 mg/mL) significantly reduced the amount of SP only in the alkali-burned cornea. Histograms represent mean values ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (n = 6).

Results

Topical Lanepitant Treatment Reduced Substance P Levels, Increased in the Epithelium of Mouse CNV Models

In avascular human and mouse corneas, the neuropeptide SP was localized in the epithelium and only sporadically in the stroma (Figs. 1A, 1B), as previously reported.27 TUJ1, expressed principally in basal layer of the epithelium, colocalized with SP, suggesting its expression in corneal sensory nerves. Four days after alkali burn and suture placement, the staining for SP was more marked in the corneal epithelium of the mouse.

The increased expression of SP in the cornea after injury was confirmed by ELISA analysis. The alkali burn model induced an early increase of SP level, already on day 4, whereas the suture model stimulated a later production of the neuropeptide in cornea, on day 7 (Fig. 1C). Notably, the amount of CD45+ cell infiltration correlated with SP levels (both expressed as fold-change increase with respect to healthy corneas) after injury. The two measures had the same trend over time. The amount of SP and leukocyte infiltration in the alkali burn model was almost four times that of the control and 2.5 times (P < 0.01 and P < 0.001, respectively) that of the suture model on day 4. After a week, the fold-change increase for SP and CD45+ cells was almost 2.5 times that of the control and similar in both CNV models. The amount of SP in the alkali burn model remained significantly higher (P < 0.05) than in the suture model.

In detail, SP protein level was significantly increased in both neovascularized alkali-burned (P < 0.001) and suture-induced (P < 0.001) mouse corneas (Fig. 1D) on days 4 and 7. The increase was most prominent in the alkali burn model on day 4 (12,455 ± 1,483 pg/cornea), compared to the suture model (4862 ± 418 pg/cornea) and to the control (3255 ± 140 pg/cornea). Substance P protein reached high levels later (day 7) in the suture models (9080 ± 557 pg/cornea). Topical NK1 receptor antagonist (6.4 mg/mL) was significantly effective in reducing the amount of SP only in the alkali burn model on day 4 (5457 ± 319 pg/cornea; P < 0.01) and day 7 (6046 ± 447 pg/cornea; P < 0.05). It was ineffective in the suture model at these time points.

Topical Application of Lanepitant Reduced Corneal Neovascularization in the Mouse Model of Alkali Burn, but Not in the Suture Model

In the alkali burn model (Fig. 2A), topical Lanepitant treatment for 4 days induced a significant 50% reduction (P < 0.01) of blood and lymphatic corneal neovessels (Figs. 2B, 2C) at both concentrations (1.6 and 6.4 mg/mL). Moreover, this treatment preserved corneal transparency, as observed at slit-lamp
examination (Fig. 2A). Finally, topical Lanepitant significantly reduced the CD45+ cell infiltration ($P < 0.05$) on day 4 (Fig. 2D). Lower concentrations (1.6 and 6.4 mg/mL) for a longer time (7 days), confirmed the efficacy of topical Lanepitant in reducing CNV and leukocyte infiltration ($P < 0.05$, data not shown).

In the suture model, topical application of 0.4 and 1.6 mg/mL Lanepitant for 4 or 7 days did not affect CNV (data not shown). Treatment with higher concentrations, 6.4 and 12.8 mg/mL, of Lanepitant for 7 days were similarly ineffective, as observed at the slit-lamp (Fig. 3A) and with immunofluorescence staining for blood vessels and lymphatics (Figs. 3B, 3C). Also, CD45+ cell infiltration was not affected by Lanepitant treatments in the suture model (Fig. 3D).

Topical Lanepitant (1.6 mg/mL for 9 days) treatment was not toxic to the ocular surface as assessed with slit-lamp examination (no opacity or perforations detected); the absence of vital fluorescein staining confirmed that Lanepitant is not toxic to the corneal epithelium (Fig. 4A). Hematoxylin-eosin stain did not detect inflammatory cell infiltration or epithelial damage, confirming Lanepitant safety (Fig. 4B).

**Subconjunctival Application of Lanepitant Reduced SP Levels Only in the Suture Model**

Later, on day 10, the amount of SP was significantly higher in the suture model ($P < 0.001$) than in the alkali burn model (Fig. 5A). Moreover, the suture model showed a significantly higher cell infiltration ($P < 0.05$) than the alkali burn model. In both CNV models, the fold-change increase of SP was significantly lower ($P < 0.05$) than CD45+ cell infiltration (Fig. 5A).

As stated before, topical Lanepitant treatment was ineffective in reducing the amount of SP only in the suture model after 4 and 7 days. Instead, administration of NK1 receptor antagonist was effective in the suture model ($P < 0.01$) after subconjunctival administration on day 10 (Fig. 5B). In the alkali burn, the SP levels were comparable between vehicle and Lanepitant groups.

**Subconjunctival Application of Lanepitant Reduced Corneal Blood Neovascularization in the Alkali Burn Model**

In the alkali burn model (Fig. 5C), Lanepitant (12.8 mg/mL) administered subconjunctivally for 10 days significantly reduced blood vessel growth ($P < 0.05$), compared to the vehicle (Fig. 5D).

Staining for CD45 showed a significant reduction of CD45+ cells in the cornea after Lanepitant treatment ($P < 0.05$) compared to the vehicle (Fig. 5E).

Moreover, Lanepitant was effective in reducing perforation onset and corneal opacity in the alkali burn model, as shown in slit-lamp photographs (Fig. 5C), in comparison to vehicle and SP (0.01 mg/mL). Specifically, the perforation onset was delayed in the Lanepitant group (Fig. 5F), reaching statistical significance ($P < 0.05$) on day 2 (30% of perforations in the Lanepitant group versus 80% to 100% in the vehicle-SP groups, respectively). Corneal opacity was significantly ($P < 0.05$) reduced after Lanepitant treatment (2.50 ± 0.22) in comparison to controls (3.20 ± 0.20), while the SP group showed a higher opacity index (3.90 ± 0.10), which was significantly different from the Lanepitant group (Fig. 5G). Finally, Lanepi-
significant increased tear secretion (1.90 ± 0.07 mm) in comparison to vehicle (1.40 ± 0.16 mm; P < 0.05) and SP (0.75 ± 0.14 mm; P < 0.001) groups (Fig. 5H).

Subconjunctival Application of Lanepitant Reduced Corneal Lymphatic Neovascularization in the Suture Model

In the suture model (Fig. 6A), Lanepitant (12.8 mg/mL) administered subconjunctivally for 10 days reduced blood vessel growth, although not significantly in comparison to the vehicle (Fig. 6B). Substance P treatment induced a significant (P < 0.05) increase of blood CNV in comparison to the Lanepitant group. Lanepitant, however, was significantly effective (P < 0.01) in reducing lymphatic CNV in the suture model in comparison to both vehicle and SP groups (Fig. 6B). Analysis for CD45 staining showed a significant reduction (by half) of CD45+ cells in the cornea after Lanepitant treatment (P < 0.05) compared to the vehicle (Fig. 6C).

Finally, the treatment was effective in reducing corneal opacity, as shown in slit-lamp photographs (Fig. 6A), in comparison to vehicle and to subconjunctival SP (0.01 mg/mL). The corneal opacity index was significantly (P < 0.05) reduced after Lanepitant treatment (1.15 ± 0.11), in comparison to the two groups (vehicle: 1.65 ± 0.15, SP: 1.75 ± 0.13; Fig. 6D).

Figure 3. Topical Lanepitant treatment in the suture models. (A) Topical application of 6.4 and 12.8 mg/mL Lanepitant on sutured corneas for 7 days did not reduce the opacity at slit-lamp examination. (B, C) Moreover, the treatment did not affect CNV, as shown in whole-mount images. (D) The number of CD45+ cells remained unchanged after treatment. Histograms represent mean values ± SEM (n = 10).

Figure 4. Evaluation of Lanepitant toxicity. (A) Topical application of Lanepitant (1.6 mg/mL) on healthy corneas for 9 days was not toxic: no opacity or fluorescein staining was observed at slit-lamp examination (n = 6). (B) Hematoxylin-eosin stain confirmed that Lanepitant was nontoxic to the ocular surface: no inflammatory cell infiltration or epithelial damage was observed.
FIGURE 5. Subconjunctival Lanepitant and Substance P treatments in the alkali burn model. (A) Correlation between CD45+ cells and SP levels in both CNV models on day 10, expressed as fold-change increase with respect to healthy corneas. The levels of SP and leukocyte infiltration were
significantly predominant in the suture model as compared to the alkali burn model on day 10. Moreover, the fold-change increase of CD45+ cells was significantly higher than SP level in both models. (B) On day 10, SP protein levels in the injured cornea remained significantly higher than in the healthy cornea, above all in the suture model. The alkali burn produced the highest increase of SP, on day 4. After 10 days of subconjunctival (s.c.) Lanepitant treatment (12.8 mg/mL), the amount of SP significantly decreased in the sutured cornea. (C) In the alkali burn model, the subconjunctival Lanepitant-treated eyes showed less perforations and opacity at slit-lamp examination, in comparison with subconjunctival injection of vehicle and of SP (0.01 mg/mL). (D) CD31 staining showed a significant reduction of CNV after Lanepitant treatment in comparison to the vehicle. (E) CD45+ cell quantification showed a significant reduction of leukocyte infiltration in the alkali-burned cornea after Lanepitant treatment, compared to the vehicle. (F) Lanepitant treatment significantly reduced the onset of corneal perforation after 2 days of alkali burn. (G) Corneal opacity measure was significantly reduced after Lanepitant treatment, compared to vehicle, while it significantly increased after SP injection, compared to Lanepitant. (H) Tear secretion was significantly higher in the Lanepitant group versus vehicle and SP groups; it significantly decreased in the SP group. Histograms represent mean values ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (n = 10).

**Topical Befetupitant Reduced Corneal Neovascularization in the Alkali Burn Model, but It Was Toxic to the Ocular Surface**

Finally, we tested Befetupitant, a different, highly selective NK1R antagonist, in the alkali burn model (see Supplementary Fig. S1A). Topical application of Befetupitant for 4 days was effective ($P < 0.05$) in reducing hemangiogenesis and lymphangiogenesis (see Supplementary Figs. S1B, S1C) at both concentrations (0.4 and 1.6 mg/mL). Befetupitant and its vehicle DMSO, however, induced corneal opacity even in healthy controls, as observed at slit-lamp examination (see Supplementary Fig. S2A). Moreover, fluorescein and hematoxylin-eosin staining showed epithelial damage and inflammatory cellular infiltration in the stroma, respectively (see Supplementary Figs. S2A, S2B), confirming DMSO toxicity. We limited the experiment just to the alkali burn model and to the topical administration because Befetupitant was toxic.

**DISCUSSION**

The cornea receives the densest sensory innervation of the entire body. Among nerve-secreted peptides, SP, which binds mainly to the NK1R, is one of the most prevalent. Hence, it is tempting to speculate that it may have a key role in modulating corneal inflammation. Since CNV is a constant finding in persistent ocular surface inflammation, we studied the effect of selective NK1R antagonism. Here, we confirmed that SP was expressed in the epithelial layer of avascular cornea and colocatalized with corneal nerve endings, specifically in the subbasal nerve plexus of the epithelium. Further, we showed that SP was significantly increased in two different CNV mouse models (alkali burn and suture models), with a different time course. In the alkali burn model SP peaked earlier (day 4), while in the suture model this occurred later (7–10 days). Since SP is also secreted by macrophages,7–9 SP expression pattern we observed could possibly be due to the early leukocyte infiltration observed in the alkali burn model (peak on day 4), while in the suture model the infiltration was delayed (peak on day 10). Previous literature54 has reported a similar trend of inflammatory cell infiltration in the corneal tissues in these two animal models. The corneal suture model seems to maintain long-term inflammation, which is possibly elicited by long-standing sutures. Notably, we found a good correlation between SP increase in the cornea and CD45+ cell infiltration, in both CNV models. Although the extensive epithelial and stromal disruption induced by the burn may result in ulceration and perforation,55 with consequent loss of epithelium and nerve fibers,56 the alkali burn cornea presented the highest levels of SP, when compared to the suture model.

To evaluate safety and efficacy of SP inhibition in the setting of CNV, we tested two different, highly selective NK1R antagonists: Befetupitant and Lanepitant. These are both potent and selective NK1R antagonists, with a high binding affinity for NK1R in both mice and humans.37–39 Befetupitant was effective in the treatment of CNV, reducing the ingrowth of both corneal blood and lymphatic vessels already after 4 days. However, its topical application was toxic, causing corneal epithelial damage and inflammatory cell infiltration. Similar results were obtained with DMSO, which we used to dissolve Befetupitant, suggesting that toxicity was largely due to the vehicle used, as previously reported.40

Differently from Befetupitant, Lanepitant is freely soluble in water. Topical Lanepitant was effective in reducing SP expression in the alkali-burned cornea, while subconjunctival Lanepitant reduced SP in the suture model. Additionally, Lanepitant application significantly reduced both hemangiogenesis and lymphangiogenesis in the alkali burn model (topical and subconjunctival administration) and only lymphangiogenesis in the suture model (subconjunctival administration only). Interestingly, topical administration decreased leukocyte infiltration in the alkali-burned cornea, while subconjunctival injection reduced the cellular infiltration in both CNV models.

Substance P, through its receptor NK1R, has promitotic and antiapoptotic effects and favors epithelial wound closure.19 For this reason, we tested whether its inhibition with Lanepitant may alter corneal epithelial integrity. Indeed, Lanepitant was not toxic to the epithelium when administered for up to 9 days.

Topical administration of Lanepitant did not affect CNV and inflammatory cell recruitment in the suture model, even at higher concentrations and with longer treatment duration. This was possibly due to the limited drug penetration, as the corneal epithelial barrier remains in place in the suture model, which is not the case after alkali burn. In this regard, it should also be noted that the presence of two strong basic groups induces a decrease in lipophilicity and, hence, poor Lanepitant penetration.41 Additionally, although the suture placement was associated with lower SP levels in comparison to the alkali burn at earlier time points, topical Lanepitant treatment was ineffective. These findings may be explained by the limited penetration of Lanepitant through intact corneal epithelium. On the contrary, SP expression was higher in the suture than in the alkali model at later time points. Since we administered the same dose in the two models, but these exhibited different kinetics in SP expression, it may be possible that a higher concentration/different administration regimen could be effective in reducing hemangiogenesis even in the suture model.

Finally, the different pathophysiologic mechanism inducing CNV in the two models should also be considered. We previously demonstrated that these CNV models had a similar pattern of induction for hemangiogenesis.42 For this reason, we chose the same time points to test treatment efficacy in the two models. Lymphangiogenesis was more pronounced in the suture model; and, the two types of corneal injuries were
associated with different timing in inflammatory cell recruitment. It should be noted that inflammation and necrosis are prevalent by far in the alkali burn model. In this vein, the well-known anti-inflammatory effects of NK1R antagonists may explain the significant CNV inhibition in the alkali burn model. In the suture model, the extension of hemangiogenesis and lymphangiogenesis did not differ significantly. In fact, NK1R promotes inflammation in many ways as it activates the proinflammatory transcription factor NF-κB, inducing expression of cyclooxygenase 2 and production of prostaglandin E2.

In an effort to increase corneal penetration of Lanepitant, we used subconjunctival injection; additionally, we treated for a longer time (10 days) in both models. Indeed, subconjunctival Lanepitant inhibited blood vessel growth in comparison to the vehicle only in the alkali burn model. Moreover, Lanepitant treatment significantly reduced LYVE1+ lymphatic vessels, compared to the other two groups. (C) CD45+ cell quantification showed a significant reduction of leukocyte infiltration in the cornea after Lanepitant treatment. (D) Corneal opacity scores were significantly reduced after Lanepitant treatment, compared to vehicle and to SP. Histograms represent mean values ± SEM. *P < 0.05, **P < 0.01 (n = 10).

Interestingly, SP treatment also induces an increase in VEGF levels in a model of skin wound healing, together with macrophage recruitment and angiogenesis. In this vein, the key role of VEGF in CNV has been established already. Finally, SP, either exogenously applied or released from endogenous sources, can stimulate angiogenesis in the synovium by binding to NK1R.

Subconjunctival Lanepitant improved corneal transparency in the suture model, as opposed to vehicle and to SP administration. Additionally, it was only effective in reducing lymphatic vessel growth, as compared to both vehicle and SP, possibly because of the high amount of SP protein present in the cornea. This may have relevant clinical implications, since lymphangiogenesis is a well-known risk factor for corneal graft rejection, possibly even more relevant than hemangiogenesis. The fact that Lanepitant was ineffective in the suture model, when administered subconjunctivally, may be explained by the higher amount of SP in the sutured cornea at later time points. Interestingly, this was associated with an increased number of inflammatory cells, which are known to produce proangiogenic factors by themselves. Higher concentrations and/or different administration regimen of Lanepitant could possibly reduce even blood CNV in the suture model.
Inhibition of Substance P (NK1) Receptor in Cornea

Intriguingly, leukocyte infiltration in whole mount corneas was reduced by 50% after subconjunctival Lanepitant treatment. This is critical as inflammatory cell infiltration favors corneal angiogenesis, and its inhibition is associated with reduced CNV. In this vein, SP has been described as a potent chemoattractant, inducing oxidative burst in macrophages. Finally, it has been reported that human macrophages express NK1R and are a relevant source of SP.

To the best of our knowledge, this is the first study demonstrating the safety and efficacy of an NK1R antagonist administered locally for the treatment of CNV. This is paramount from a clinical translation perspective as, although previous literature has proposed the use of SP antagonists (Spantide) in mice, limited evidence exists on its human use. Additionally, by antagonizing SP, Spantide can also inhibit NK2 and NK3 receptors—together with their manifold actions—differently from Lanepitant, which blocks NK1R selectively.

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