

Efficacy of the nucleotide-binding oligomerization domain 1 inhibitor Nodinhhibit-1 on corneal alkali burns in rats

Xu Huang¹, Yun Han², Yi Shao³, Jing-Lin Yi¹

¹Affiliated Eye Hospital of Nanchang University, Nanchang 330006, Jiangxi Province, China

²Fujian Provincial Key Laboratory of Ophthalmology and Visual Science, Eye Institute of Xiamen University, Xiamen 361102, Fujian Province, China

³Department of Ophthalmology, the First Affiliated Hospital of Nanchang University, Nanchang 330006, Jiangxi Province, China

Co-first authors: Xu Huang and Yun Han

Correspondence to: Jing-Lin Yi. Affiliated Eye Hospital of Nanchang University, Nanchang 330006, Jiangxi Province, China. yjl0791@126.com

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Abstract

• **AIM:** To evaluate the therapeutic effect of Nodinhhibit-1 on alkali-burn-induced corneal neovascularization (CNV) and inflammation. The nucleotide-binding oligomerization domain 1 (NOD1) is a potent angiogenic gene.

• **METHODS:** The alkali-burned rat corneas (32 right eyes) were treated with eye drops containing Nodinhhibit-1 or phosphate buffered solution (PBS, pH 7.4) only, four times per day. CNV and inflammation were monitored using slit lamp microscopy, and the area of CNV was measured by formula. Vascular endothelial growth factor (VEGF) and pigment epithelium-derived factor (PEDF) was determined by Western blot analysis. The TUNEL assay was used to assess the corneal apoptosis cells.

• **RESULTS:** Alkali-burn-induced progressive CNV and inflammation in the cornea. After treatment for 7d and 14d, there were statistically significant differences in the CNV areas and inflammatory index on that between two groups ($P < 0.05$, respectively). Epithelial defect quantification showed a significant difference between the two groups at days 4 and 7 after the alkali burns ($P < 0.05$). The apoptotic cells on days 1, 4, and 7 between the two groups showed significant differences at all time points ($P < 0.05$, respectively). Compared to that in control group, the protein level of VEGF expression was significantly reduced whereas the PEDF expression was increase in the Nodinhhibit-1 groups on day 14 ($P < 0.05$, respectively).

• **CONCLUSION:** Topical application of 10.0 $\mu\text{g/mL}$ Nodinhhibit-1 may have potential effect for the alkali burn-induced CNV and inflammation. The effect of

Nodinhhibit-1 on CNV may be by regulation the equilibrium of VEGF and PEDF in the wounded cornea.

• **KEYWORDS:** Nodinhhibit-1; inflammation; alkali burn; cornea; neovascularization

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INTRODUCTION

Corneal neovascularization (CNV) as a result of vessel invasion from the limbal arcade to the normally avascular cornea is caused by a wide variety of common pathologic conditions such as infection, trauma, and loss of the limbal stem cell barrier^[1]. Chlamydia trachomatis and herpetic infections are well known major causes of CNV^[2]. The nucleotide-binding oligomerization domain 1 (NOD1) is a cytosolic protein and a member of a family of proteins known as the NACHT and Leucine Rich Repeat domain containing proteins (NLR)/Nod/ CATERPILLER family. The minimal components of bacterial cell walls are recognized by nucleotide-binding oligomerization domain (NOD), which is important for host defense—a mechanism manifested in human corneal cells. NOD1 gene polymorphisms are associated with several human inflammatory disorders, including sarcoidosis, Crohn's disease, asthma, and autoimmune uveitis. Recently, NOD1 has also been implicated in vascular inflammation. Although NOD1 was originally considered to be expressed predominantly in lymphoid tissue, its expression has more recently been documented in non-lymphoid tissue including^[3], eye tissue^[4,5] and human corneal epithelial cells^[6]. In addition, a role for tripeptide L-Ala-g-D-Glu-meso-diaminopimelic acid (Tri-DAP, a NOD1 agonist) in angiogenesis was shown in a recent study that used a mouse model of alkali-induced CNV^[7].

Neovascularization (NV) is mediated by a diverse array of cellular and molecular factors. Vascular endothelial growth factor (VEGF) and pigment epithelium-derived factor (PEDF) are key cytokine in the development of neovessels. In the cornea, the VEGF expression of the epithelial cells, vascular endothelial cells, macrophages, and fibroblasts are significantly upregulated in the inflamed and vascularized

corneas. To date, PEDF is the most potent angiogenic inhibitor found in the mammalian ocular compartment. These observations have led to the hypothesis that the VEGF signaling inhibition may be effective in promoting vessel regression in pathologic CNV.

In the present study, we developed topical Nodinhbit-1 and confirmed its effect on angiogenic properties in CNV. A model of alkali burn in the cornea of rats was used to test the effects of Nodinhbit-1 (like the Nodinitib-1^[8]) as eye drops. The effects of Nodinhbit-1 on angiogenesis, inflammation and corneal epithelial healing were evaluated and compared with those of phosphate buffered solution (PBS). To our knowledge, this is the first study reporting the possible effects of topical Nodinhbit-1 in corneal epithelial cells and CNV.

MATERIALS AND METHODS

Alkali -induced Corneal Neovascularization and Treatment Wistar rat (180-220 g, 2 months old, male), were used in the study. Animal experiments were performed in accordance with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research and the Guidelines of the Animal Experimental Committee of Nanchang University (Jiangxi province, China). Prior to treatment, we confirmed that all rats were free from ocular diseases. Rat corneal alkali burns were conducted as previously reported. In brief, the animals were anesthetized with intraperitoneal ketamine (60 mg/kg), and a filter paper disc (3 mm in diameter) incubated with 1 mol/L NaOH for 60s was then placed on the central cornea of the right eye for 30s. After that the ocular surface was then rinsed with 30 mL PBS. The rats ($n=32$) were randomly divided into two groups of 16 rats per group. The right eyes of each rat was treated with 10 μ g/mL Nodinhbit-1 eye drops (No: CN103800829 A, National Invention Patent to Shao Y) in Nodinhbit-1 group and PBS only in control group. The left eyes were given a same drop respectively as the negative control. Ten microliters of eye drops were administered four times daily for 2wk. Cornea defect area, CNV and inflammatory index were examined under slit lamp microscope by a blinded ophthalmologist. All eyes were harvested on postoperative days (PD) 7 and 14. The expression of VEGF and PEDF in cornea was examined by Western blot assay. The corneal endothelium tissues were subjected to apoptosis detection measurement.

Evaluation of Corneal Neovascularization and Inflammation CNV and inflammatory response were examined by slit lamp every day after the alkali burn. Vessel growth onto the clear cornea was noted in millimeters at each time point. CNV was quantified by calculating the wedge-shaped area (S) of vessel growth with the formula: $S=C/12 \times 3.1416 \times [r^2 - (r-I)^2]$ ^[9], (S is the area, C is time, I is the radius from the center to the border of vessel growth, and r is the radius of the cornea). The inflammatory index was

analyzed as previously described^[10]. Briefly, the inflammatory index was calculated using the following equation: ciliary hyperemia (absent, 0; present but less than 1 mm, 1; present between 1 and 2 mm, 2; present and more than 2 mm, 3); central corneal edema (absent, 0; present with visible iris details, 1; present without visible iris details, 2; present without visible pupil, 3); and peripheral corneal edema (absent, 0; present with visible iris details, 1; present without visible iris details, 2; present with no visible iris, 3). The final inflammatory index result was obtained by summing the crosses of the different parameters divided by a factor of 9^[10].

Measurement of Corneal Epithelium Defect The animals were examined under a slit lamp microscope every day after the alkali burns. The corneal images were taken by an experienced researcher (Shao Y). Fluorescein staining was performed by applying 5 μ L 0.1% fluorescein sodium onto the rat's ocular surface and observed under slit lamp microscope using cobalt blue light. For analysis of re-epithelization ratio, images from sections were processed using Image Pro Plus V6.0 (Media Cybernetics, Silver Spring, USA).

Apoptosis Detection Assay^[11] Cornea tissue after 7d of the Nodinhbit-1 or PBS treatment were subjected to apoptosis detection measurement using terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) staining with the DeadEndTM Fluorometric TUNEL system according to manufacturer's protocol. Cellular nuclei were stained with DAPI, and apoptotic cells were examined under laser confocal microscopy (Fluoview 1000, Olympus, Japan).

Western Blot Assay Western blot assay was performed as previously described^[12]. Briefly, samples of cornea tissues from two groups were washed three times with sterile PBS, sliced into small pieces, and extracted in cold lysis buffer comprising 50 mmol/L Tris-Cl (pH 7.5), 150 mmol/L NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and protease and phosphatase inhibitor cocktails, and then equal amounts of protein extracts (20 μ g) were subjected to western blot analysis using-actin (1:10 000), VEGF (1:200; Santa Cruz) and PEDF (1:200; Santa Cruz) antibodies. The results were visualized using an enhanced chemiluminescence method^[13]. Relative intensity was measured using an ImageMaster VDS (Pharmacia Biotech, San Francisco, CA, USA).

Statistical Analysis Summary data were reported as means \pm SD. Inflammatory index, cornea defect area and CNV area were analyzed using repeated measurements of analysis of variance (ANOVA) followed by the Bonferroni post hoc comparison. Group means were analyzed using the Student's *t*-test, where $P < 0.05$ was considered statistically significant. The statistical analysis was conducted with GraphPad Prism for Windows, ver. 5.00^[14] (GraphPad Software, Inc., USA).

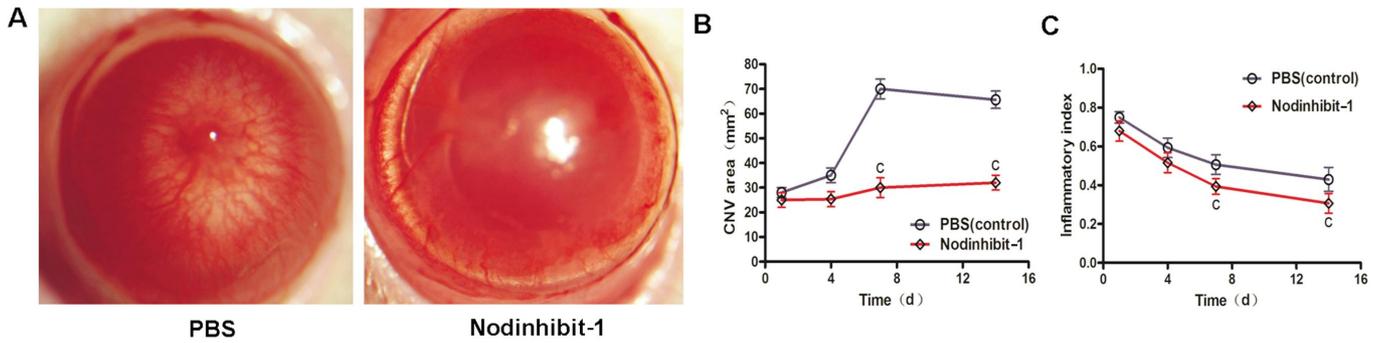


Figure 1 Nodinhhibit-1 inhibits CNV and promotes corneal epithelial wound healing after alkali burns A: On day 14 after the injury, there was only slight new blood vessel formation in the limbal areas in the Nodinhhibit-1 treatment group, and the corneas remained transparent on day 14. In contrast, new blood vessels reached the central corneas in the control group, and there was a remarkable decrease in corneal transparency; B: CNV area in the control group increased from day 1 to day 7, and there was a mild decrease on day 14 after the alkali burns. In contrast, corneas treated with Nodinhhibit-1 showed only a mild increase in CNV area on day 7. There was a significant difference between the two groups on days 7, 14 ($P < 0.05$); C: The inflammatory index of the ocular surface declined from day 1 to day 14 in both groups. However, it was significantly lower in eyes treated with Nodinhhibit-1 on days 7, 14 ($P < 0.05$, respectively).

RESULTS

Effect of Nodinhhibit -1 Eye Drops on Corneal Neovascularization The effect of Nodinhhibit-1 on CNV was evaluated by comparing the CNV area between the Nodinhhibit-1-treated and control groups at different time points. There were no scar and CNV present in normal rat corneas. In the PBS group, the central cornea maintained opaque appearance and there was scar formation on day 14. However, there was only mild edema and no scar formation in corneas treated with Nodinhhibit-1 for 14d (Figure 1A). The average CNV area on day 14 was $26.26 \pm 4.12\text{-mm}^2$ in the Nodinhhibit-1 $10.0 \mu\text{g/mL}$ group and $65.86 \pm 5.36\text{-mm}^2$ in the control group. The CNV areas of $10.0 \mu\text{g/mL}$ Nodinhhibit-1-treated groups was significantly smaller than in the control group ($P < 0.05$ after Nodinhhibit-1 treatment for 7 and 14d; Figure 1B). Topical administration of $10.0 \mu\text{g/mL}$ Nodinhhibit-1 didn't change the limbal vessel congestion on day 1. Vessel growth was suppressed by Nodinhhibit-1 on PD 4, 7 and 10 ($n=16$, respectively). The results showed that the onset and progression of CNV were delayed in $10.0 \mu\text{g/mL}$ Nodinhhibit-1 group, suggesting Nodinhhibit-1 effect on CNV.

Effect of Nodinhhibit -1 Eye Drops on Corneal Inflammatory To determine the effect of Nodinhhibit-1 on corneal inflammation, the inflammatory index was studied at four different time points: 1, 4, 7 and 14d after the two different eye-drop treatment. As shown in Figure 1C, corneas with alkali burns treated with $10.0 \mu\text{g/mL}$ Nodinhhibit-1 showed a significant decrease than in the control group in inflammatory index after 4, 7 and 14d ($P < 0.05$, respectively).

Effects of Nodinhhibit -1 on Corneal Epithelial Wound Healing The effects of topical Nodinhhibit-1 administration on the early re-epithelialization were assessed on PD 1, 4 and 7. The fluorescein staining showed that corneal epithelial defects were completely healed on day 7, if the corneas were treated with $10.0 \mu\text{g/mL}$ Nodinhhibit-1, while the corneas

treated with PBS did not heal (Figure 2A). Epithelial defect quantification showed a significant difference between the two groups at day 4 and day 7 (Figure 2B). These results indicated that Nodinhhibit-1 could promote early re-epithelialization after alkali burns.

Nodinhhibit -1 Reduces the Alkali Burn -Induced Apoptosis of Corneal Cells For the *in situ* detection of apoptotic cells, a TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) assay was performed on corneas treated with $10.0 \mu\text{g/mL}$ Nodinhhibit-1 (Figure 3A) or PBS (Figure 3B) for 7d. As expected, there were no apoptotic cells present in normal rat corneas (Figure 3C). However, the majority of the cells in the central corneas were TUNEL-positive at day 1 after the alkali burns, and the positive cells gradually decreased from day 4 to day 7 in the PBS group. At day 7, there were still many apoptotic cells in the basal epithelia and endothelia. In contrast, when the corneas were treated with $10 \mu\text{g/mL}$ Nodinhhibit-1, there were much fewer apoptotic cells from day 1 to day 7 compared with the PBS group (Figure 3D). Statistical analysis showed a significant difference of apoptotic cells between the two groups at different time points (Figure 3D).

Regulation Vascular Endothelial Growth Factor and Pigment Epithelium -Derived Factor Expression and Corneal Neovascularization To determine whether Nodinhhibit-1's effect on corneal NV is through regulation of the balance of VEGF and PEDF, corneal VEGF and PEDF levels were measured by Western blot. The results demonstrated intensive VEGF signal and reductive PEDF signal in the centre corneas at 14 (Figure 4A) after the alkali burn. In contrast, rats treated with $10.0 \mu\text{g/mL}$ of Nodinhhibit-1 displayed weaker VEGF signals and stronger PEDF signals in the same timepoint under the same exposure intensity (Figure 4A). Densitometry of protein expression showed significant differences between the control group and

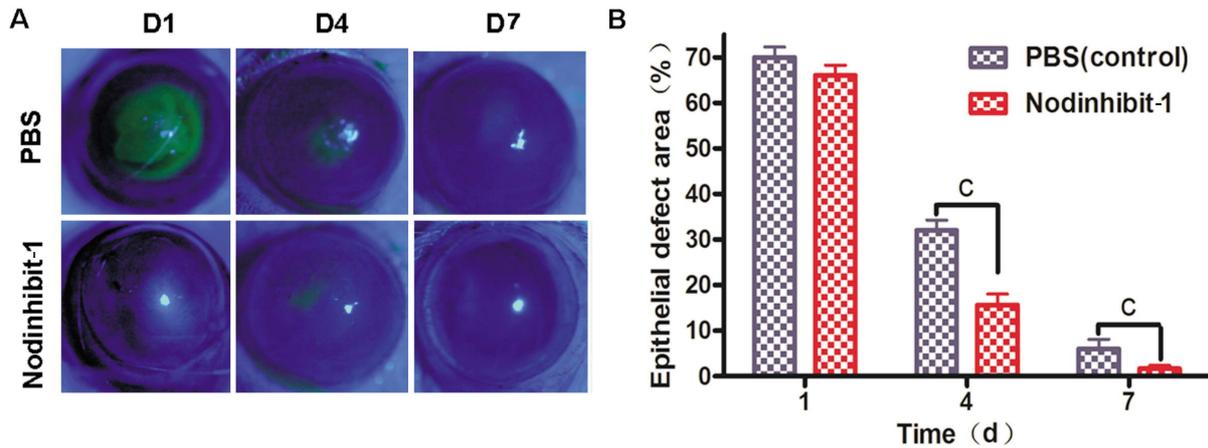


Figure 2 Nodinhhibit-1 promotes corneal epithelial wound healing after alkali burns A: Fluorescein staining showed central corneal epithelial defects at day 1 (D1) after the alkali burns, and there was gradual decrease of the epithelial defect areas at day 4 (D4) and day 7 (D7) in both groups. The epithelial defects were completely healed on day 7, if the corneas were treated with Nodinhhibit-1, while the corneas treated with PBS did not heal. Images at different time points are sequential pictures from the same rat eye treated with either PBS or Nodinhhibit-1; B: Epithelial defect quantification showed a significant difference between the two groups at days 4 and 7 after the alkali burns ($P < 0.05$). $^{\circ}P < 0.05$ vs control.

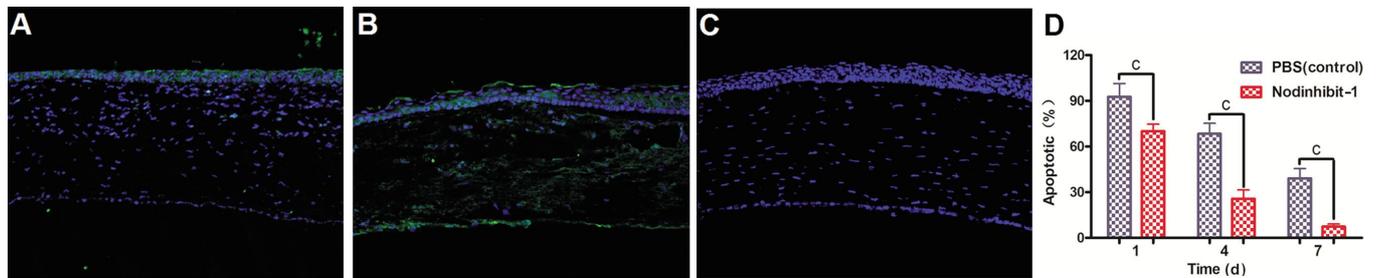


Figure 3 Nodinhhibit-1 reduces alkali burn-induced apoptosis of corneal cells A: A TUNEL assay showed only sporadic apoptotic cells in the corneal epithelia at day 7, in contrast, in the PBS group, there were many apoptotic cells in the epithelia and endothelia (B); C: There were no apoptotic cells present in normal rat corneas; D: A statistical analysis of the apoptotic cells on days 1, 4, and 7 between the two groups showed significant differences at all time points ($P < 0.05$). $^{\circ}P < 0.05$ vs control.

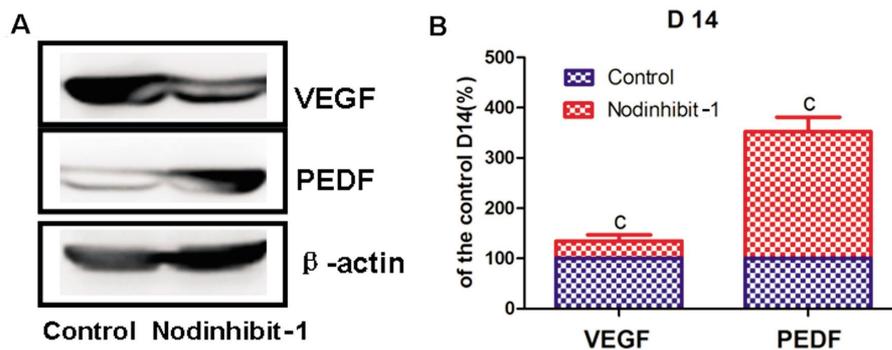


Figure 4 Effect of Nodinhhibit-1 on VEGF and PEDF expression after corneal alkali burns A: Western blot analysis results showed that VEGF was expressed at high levels at day 14 (D14) after alkali burns in the control group. However, in the Nodinhhibit-1 treatment group, there was a dramatic down regulation of VEGF on D14. PEDF was decreased on day 14 after alkali burns, whereas it was restored after Nodinhhibit-1 treatment; B: Densitometry of protein expression showed significant differences between the control group and the Nodinhhibit-1 treatment group in VEGF and PEDF on day 14 ($P < 0.05$). $^{\circ}P < 0.05$ vs control.

the Nodinhhibit-1 treatment group in VEGF and PEDF on day 14 ($P < 0.05$, Figure 4B). These results suggesting that 10.0 $\mu\text{g/mL}$ Nodinhhibit-1 up-regulates PEDF expression and down-regulates VEGF expression.

DISCUSSION

Corneal epithelial cells are non-keratinized, stratified

squamous cells that not only provide a physical barrier, but through sensing invading pathogens, they also contribute to the first line of defense mediated by innate immunity. However, CNV leads to the loss of the corneal immune privilege and destroys the structure of corneal epithelial cells. VEGF and PEDF play critical roles in CNV and

inflammatory^[15]. Nodinhhibit-1, a NOD1 inhibitor, destroys the express balance of VEGF and PEDF induced by the corneal alkali burn. The present study shows that topical Nodinhhibit-1 can effectively reduce established CNV as measured by invasion area, vessel length, and neovascular area in alkali burn-induced rat model.

CNV can occur in many pathologic conditions, including chemical burns, infections and many ocular surface disorders. It has become one of the major reasons for corneal blindness and was a risk factor for rejection after allograft corneal transplantation. New medical and surgical treatments, including nonsteroidal inflammatory agents, argon laser photocoagulation, and photodynamic therapy have been effective in animal models to inhibit CNV and transiently restore corneal "angiogenic privilege". Novel treatment strategies are therefore needed. Based on the research of NLRs in the human corneal epithelial cells, it could act a new target for therapy in the future. NLRs constitute a prominent family of innate immunity proteins found in mammals. NOD1, NLR family members, can recognize the minimal component of bacterial cell walls meso-diaminopimelic acid (iE-DAP) and activate various signaling pathways important for host defense and inflammation, including NF- κ B, stress kinases, interleukin (IL)-1 β and Interferon response factors (IRFs)^[16]. Although various epithelial cells in the skin, kidney, liver, stomach, prostate, intestinal, nasal and oral cavity functionally express NOD1^[17,18], the function of NOD1 inhibitor Nodinhhibit-1 in epithelial cells and CNV have not been clearly defined.

Chlamydia trachomatis, a primary source of infections that result in the new vessels, is one of the gram-negative bacteria that stimulate NOD1, inducing inflammation. The inflammatory immune response is associated with angiogenesis. The previous study demonstrated the NOD1 presented in human corneal epithelial cells and is functionally active as local treatment with its agonist, results in ocular inflammation and CNV in a dose- and time-dependent fashion^[7]. Moreover, NOD1 is reported to traffic between membranes and cytosol, correlating with NF- κ B activation and IL-1 β signaling^[19]. NOD1 could trigger uveitis by acting locally within the eye, especially considering that local injection of its agonist results in ocular inflammation^[20]. It is also possible that leukocytes expressing NOD1 and recruited to the eye could contribute to the propagation of the inflammatory response in the setting of the appropriate ligand. Indeed, we found an increased presence of neutrophils, which are known to express NOD1, within the aqueous, vitreous, and the nerve fiber layer of the retina.

Nodinhhibit-1 eye drops, including the Nodinitib-1 (cayman, ML130; CID-1088438), as a selective NOD1 inhibitors,

which cause conformational changes of NOD1 *in vitro* and provide chemical probes for interrogating mechanisms regulating NOD1 activity^[19]. From the animal experiments, we found that the topical application of Nodinhhibit-1 eye drops can modulated the development of both the alkali-burn, induced CNV and inflammation. Nodinhhibit-1 induced regression of the newly formed vessels in the cornea caused by alkali burn, which is a more interesting clinical end point. In the present study, we chose Nodinhhibit-1 as the first candidate for anti-angiogenesis therapy since Nod-1 is endogenously highly expressed in corneal epithelium. Thus, high dose of Nodinhhibit-1 therapy can be applied not only to prevent CNV and inflammation but also to accelerate the repair of epithelium. The data therefore suggest that high dose of Nodinhhibit-1 is promising as a potential therapeutic drug for CNV. In addition to effect on the cornea angiogenic and inflammatory, Nodinhhibit-1 modulated epithelial healing. Topical application of Nodinhhibit-1 accelerated re-epithelialization on posttherapy days 1, 4 and 7 at the different concentration. However, the effect of Nodinhhibit-1 on epithelial cell is still not clear *ex vivo*.

Nodinhhibit-1 also can modulate the VEGF and PEDF expression in the cornea. It is evident that there is a delicate balance between VEGF and PEDF and that this balance plays a key role in maintaining the homeostasis of angiogenesis. In our study, VEGF are overproduced while PEDF are decreased under alkali-induced CNV. However, 10.0 μ g/mL Nodinhhibit-1 can induce down-regulation of VEGF and up-regulation of PEDF and restore the balance in angiogenesis control and thus, represents a mechanism underlying the inhibitory effect of Nodinhhibit-1 on CNV.

The data therefore suggest that high concentrations of Nodinhhibit-1 is promising as a potential therapeutic drug for CNV. Thus, access to NOD1 inhibitor should empower research on defining the role of NOD1 protein in numerous acute and chronic inflammatory and NV diseases, allowing for an exploration of whether novel therapeutic interventions based on targeting this class of proteins are feasible. This study represents the first documented approach to treat injury-induced corneal NV with an angiogenic inhibitor and provides tools for exploring the roles of NOD1 in various infectious and inflammatory diseases. However, the angiogenic process is complex, and many other signalling pathways beyond VEGF/PEDF are implicated in the formation of new vessels. These include extra-cellular signalling pathways such as the notch/delta, ephrin/Eph receptor, roundabout/slit, and netrin/UNC (uncoordinated) receptor families as well as intracellular proteins such as hedgehog and sprouty^[21].

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