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HL-217, a new topical anti-angiogenic agent, inhibits retinal vascular leakage and pathogenic subretinal neovascularization in *Vldlr*$^{-/-}$ mice

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ABSTRACT

HL-217 is a new synthetic angiogenesis inhibitor. Platelet derived growth factor (PDGF) is a vasoactive factor and has been implicated in proliferative retinopathies. In this study, we examined the mechanism of action and efficacy of topical application of HL-217 on subretinal neovascularization in very low-density lipoprotein receptor knockout (*Vldlr*$^{-/-}$) mice. In three-week-old male *Vldlr*$^{-/-}$ mice, HL-217 (1.5 or 3 mg/ml) was administered twice per day for 4 weeks by topical eye drop instillation. Neovascular areas were then measured. We used a protein array to evaluate the expression levels of angiogenic factors. The inhibitory effect of HL-217 on the PDGF-BB/PDGFRβ interaction was evaluated in vitro. The neovascular area in the *Vldlr*$^{-/-}$ mice was significantly reduced by HL-217. Additionally, HL-217 decreased the expression levels of PDGF-BB protein and VEGF mRNA. Moreover, HL-217 dose-dependently inhibited the PDGF-BB/PDGFRβ interaction (IC_{50} = 38.9 ± 0.7 μM). These results suggest that HL-217 is a potent inhibitor of PDGF-BB. HL-217, when applied topically, is an effective inhibitor of subretinal neovascularization due to its ability to inhibit the pro-angiogenic effects of PDGF-BB.

1. Introduction

Age-related macular degeneration (AMD) is the major cause of blindness in the elderly [1]. AMD is characterized by the growth of blood vessels from the choroid through Bruch's membrane, resulting in choroidal neovascularization (CNV). A visual decline is observed in patients with AMD as a result of CNV [2]. Although angiogenic vessels typically arise from the choroid and invade the subretinal space in neovascular AMD, in a subset of patients with AMD, intraretinal and subretinal neovascularization arise from the inner retinal vessels. This distinct form of neovascular AMD is termed retinal angiomatous proliferation (RAP) [3]. Multiple risk factors for AMD have been reported to date, which independently or in combination may lead to pathological changes in the structural integrity and function of retinal pigment epithelial (RPE) and choroidal endothelial cells [4]. It is well known that the overexpression of vascular endothelial growth factor (VEGF) and its receptors plays a central role in the development of this disease [5]. The interruption of VEGF signaling is a good pharmacological target for the treatment of CNV. Neovascular AMD has been markedly attenuated by anti-VEGF agents, including ranibizumab (Lucentis) and bevacizumab (Avastin) [6]. Although these drugs showed some efficacy in slowing disease progress and improving vision, they require multiple intravitreal injections, which cause serious adverse reactions, such as endophthalmitis, retinal detachment, iatrogenic traumatic cataract, iridocyclitis and injection-site reactions [7,8]. As a route of drug administration, topical application of ocular medication would substantially decrease the risk of endophthalmitis as well as pain and discomfort associated with the intravitreal injection; however intraocular penetration of topical drugs is restricted primarily by the relative impermeability of the corneal epithelium, especially to larger molecules [9]. Therefore, alternative methods of administering drugs for AMD have been a recent focus of research.

Although the detailed mechanism of AMD is largely unclear, a variety of cytokines and growth factors are known to be released in these proliferation areas, suggesting that other factors may be associated with the angiogenesis process [10]. Platelet-derived growth factor (PDGF), which was discovered in 1974 [11], is synthesized and secreted by various cells and has potent mitogenic and chemotactic activities in several different cell types, including

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fibroblasts and vascular smooth muscle cells [12]. The PDGF family consists of five dimeric ligands; PDGF-AA, PDGF-BB, PDGF-AB, PDGF-CC and PDGF-DD [13]. PDGF dimers bind to and activate two membrane receptor tyrosine kinases, PDGF receptor α (PDGFRα) and PDGFRβ [14]. PDGF-BB is one of the important regulators of angiogenesis and is released by endothelial cells, platelets, vascular smooth muscle cells and inflammatory cells at sites of angiogenesis [15]. PDGF-BB binding to PDGFRβ leads to receptor dimerization, autophosphorylation, and activation of downstream signaling pathways, including mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways [16]. Thus, accumulating data suggest that PDGF-BB plays an important role in retinal pathogenic angiogenesis.

(2R,3R,4S)-6-amino-4-[*N*-(4-chlorophenyl)-*N*-(1*H*-imidazol-2ylmethyl)amino]-3-hydroxyl-2-methyl-2-dimethoxymethyl-3,4-dihydro-2*H*-1-benzopyran (referred to as HL-217) is a newly developed low-molecular-weight drug (with a molecular weight of 458.94). This new synthetic agent was shown to exhibit anti-angiogenic activities in endothelial cells in vitro [17] and in a mouse Matrigel plug assay [18]. However, the mechanisms underlying the anti-angiogenic effects of HL-217 are not fully understood. To the best of our knowledge, there have been no reports describing the therapeutic effect of topical application of HL-217 on CNV. To address this, we have studied the effect of HL-217 on subretinal neovascularization in very low-density lipoprotein receptor knockout ($Vldlr^{-/-}$) mice, which is a mouse model for neovascular AMD. In addition, we have tested the hypothesis that unlike larger molecules such as anti-VEGF antibodies, HL-217 may be an effective inhibitor of subretinal neovascularization when administered topically.

2. Methods

2.1. In vitro ligand blocking assay

Purified recombinant human PDGFRβ-Fc chimera (R&D systems, MN, USA) at a concentration of 1 μg/ml in 100 μl phosphate buffered saline (PBS)/well was coated onto the surface of a 96-well ELISA plate overnight at 4 °C. The wells were washed three times, and nonspecific binding was blocked with PBS containing 3% bovine serum albumin for 1 h at room temperature. The blocking solution was removed, and 1 μg of horseradish peroxidase (HRP)-conjugated PDGF-BB (R&D systems, MN, USA) was then added with or without HL-217 at increasing concentrations (0–1000 μM). HL-217 was synthesized and provided by Hanlim Pharm. Co. Ltd. (Seoul, Republic of Korea). HRP-labeled PDGF-BB was prepared using an HRP Labeling Kit (Dojindo Laboratories, Kumamoto, Japan). After incubation for 1 h at 37 °C, free PDGF-BB was removed with three washes. Peroxidase activity was quantified using tetramethylbenzidine. Nonspecific binding was determined in the presence of excess unlabeled PDGF-BB. Blocking of PDGF-BB binding to PDGFRβ was expressed as a percentage of optical density. We calculated the 50% inhibitory concentration (IC_{50}) of ligand binding.

2.2. Animals and experimental design

Breeding pairs of mutant mice with targeted deletion of the *Vldlr* gene (B6;129S7-Vldlrtm1Her/J; $Vldlr^{-/-}$) were obtained from the Jackson Laboratory (Bar Harbor, ME). C57BL/6 mice were used as wild type (WT) controls. The homozygous mutant mice were viable and fertile. All animals were housed with a 12-h light/dark cycle and given food and water ad libitum. Three-week-old male $Vldlr^{-/-}$ mice were randomly divided into three groups. A total of 20 μl of 1.5 or 3 mg/ml (3.27 or 6.53 mM, pH 7.0) HL-217 solution was topically applied to each eye of the $Vldlr^{-/-}$ mice twice daily (at 8:00 AM and 8:00 PM), for 4 weeks. Control $Vldlr^{-/-}$ mice and WT mice received 20 μl of 0.9% NaCl (vehicle). HL-217 or vehicle eye drops were administered directly onto the superior corneal surface of each eye using a syringe without anesthesia, and the mice were held still for 20 s to allow the drop to penetrate the eye. All experiments were approved by the Korean Institute of Oriental Medicine Institutional Animal Care and Use Committee.

2.3. Fluorescein isothiocyanate-dextran microscopy

The mice were anesthetized using zolazepam (Zoletil, Virbac, Carros, France). Subsequently, 0.1 ml of sterile PBS containing 5 mg of fluorescein isothiocyanate (FITC)-dextran (4.4 kDa, Sigma, MO, USA) was circulated through the left ventricle. The eyes were enucleated and fixed in 4% paraformaldehyde for 3 h. The posterior eye cups and retinas were dissected away and incised radially. Flat mounts were generated, and images were captured using an Olympus BX51 microscope and DP72 camera (Olympus, Tokyo, Japan). For quantification of retinal vascular leakage, the mice were perfused with PBS, and the retinas were carefully removed, weighed and homogenized to extract the FITC-dextran in 50 μl of distilled water. Plasma was also collected before perfusion. The fluorescence in each 50-μl sample was measured using a spectrofluorophotometer (Synergy™ HT, Bio-Tek, VT, USA).

2.4. Lectin staining

The flat mounts were incubated with PBS containing 5% Triton X-100 and 1% bovine serum albumin for 3 h at 37 °C. The retinas were then washed 3 times with PBS and labeled with tetramethylrhodamine-conjugated isolectin B4 from *Bandeiraea simplicifolia* (1:50 Sigma, MO, USA) diluted in PBS. The vessels were viewed with an Olympus BX51 microscope (Olympus, Tokyo, Japan). ImageJ software (NIH, MD, USA) was used to measure the CNV area.

2.5. Angiogenesis-related protein array

To investigate the expression levels of 55 angiogenesis-related proteins in retinas, an antibody array analysis (Mouse Angiogenesis Array Kit, R&D Systems, MN, USA) was performed according to the manufacturer's instructions. A list of the 55 antibodies can be found on the manufacturer's web page (http://www.rndsystems.com/index.aspx). Arrays were captured and quantified using an LAS-3000 image analyzer (Fujifilm, Tokyo, Japan).

2.6. Real-time PCR analysis

Total retinal RNA was extracted using TRIzol reagent (Invitrogen, CA, USA), and cDNA was synthesized with 0.5 μg of total RNA using a PrimeScript First Strand cDNA Synthesis Kit (TaKaRa, CA, USA). Quantitative real-time PCR was performed with specific primers for VEGF and GAPDH using an iQ5 Continuous Fluorescence Detector System (Bio-Rad, CA, USA). The primers were as follows: for VEGF, 5′-TTACTGCTGTACCTCCACC-3′ and 5′-ACAGGACGGCTTGAAGATG-3′; for GAPDH, 5′-AACGACCCCTTCATTGAC-3′ and 5′-TCCACGACATACTCAGCAC-3′. All real-time PCR experiments were performed in triplicate. VEGF mRNA expression was normalized to GAPDH gene expression using the iQ5 optical system software (Bio-Rad, CA, USA).

2.7. Statistical analysis

The data are expressed as the mean ± SE. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test or by unpaired Student's *t*-test. Differences with $p < 0.05$ were considered statistically significant.

3. Results

3.1. HL-217 inhibits vascular leakage and CNV in *Vldlr*$^{-/-}$ mice

Topical administration of HL-217 to *Vldlr*$^{-/-}$ *mice* resulted in a significant inhibition of vascular leakage and pathological changes in the inner retina. As shown in Fig. 1, the fluorescence intensity was enhanced, with dye leakage occurring throughout the entire retina in *Vldlr*$^{-/-}$ eyes; however, the fluorescence signal was restricted to the vasculature in WT eyes and in HL-217-treated eyes. In addition, treatment with HL-217 prevented pathogenic neovascularization of the subretina in *Vldlr*$^{-/-}$ mice compared with vehicle treatment (Fig. 2). The average total neovascular area in vehicle-treated *Vldlr*$^{-/-}$ mice was 8.0% of the total retinal area. Topical administration of 1.5 and 3 mg/ml HL-217 prevented neovascularization by 25% and 45%, respectively, compared to vehicle. Thus, HL-217 helps to inhibit retinal vascular leakage and neovascularization in *Vldlr*$^{-/-}$ *mice*.

3.2. HL-217 regulates the expression of angiogenesis-associated factors in *Vldlr*$^{-/-}$ mice

As shown in Fig. 3, HL-217 decreased the expression of pro-angiogenic factors (osteopontin, heparin-binding epidermal growth factor-like growth factor (HB-EGF), leptin, PDGF-AB/BB, tissue factor (TF), insulin-like growth factor binding protein-2 (IGFBP-2), CXC chemokine ligand 16 (CXCL16), IGFBP-3, fibroblast growth factor-1 (FGF-1), IGFBP-3 and matrix metalloproteinase-9 (MMP-9)) in *Vldlr*$^{-/-}$ mice compared with vehicle treatment. Consistent with a previous report [19], pro-angiogenic growth factors such as leptin and FGF-1 were up-regulated in vehicle-treated *Vldlr*$^{-/-}$ mice. By contrast, anti-angiogenic factors such as endostatin and platelet factor-4 (PF-4) were down-regulated by HL-217. Several proteins such as angiopoietin and VEGF were not detected in this protein array in either vehicle- or HL-217-treated retinas, possibly due to the low sensitivity of this antibody on the array. This result suggests that HL-217 exerts anti-angiogenic effects by inhibiting the expression of osteopontin, HB-EGF, leptin, PDGFAB/BB, TF, IGFBP-2, CXCL16, FGF-1, IGFBP-3 and MMP-9. Interestingly, up-regulation of anti-angiogenic factors such as endostatin and PF-4 in vehicle-treated *Vldlr*$^{-/-}$ mice may be a protective feedback mechanism against angiogenesis.

3.3. HL-217 blocks the PDGF-BB/PDGFRβ interaction

To determine the ability of HL-217 to block the binding of PDGF-BB to PDGFRβ in vitro, a ligand binding assay for PDGFRβ was developed. In this assay, HL-217 dose-dependently inhibited the binding of PDGF-BB to PDGFRβ with an IC_{50} value of 38.9 ± 0.7 μM (Table 1).

3.4. HL-217 inhibits the expression of VEGF mRNA in *Vldlr*$^{-/-}$ mice

PDGF is known to increase VEGF expression in human vascular smooth muscle cells [20]. Thus, we examined whether pharmacological inhibition of PDGF-BB by HL-217 decreased retinal VEGF mRNA expression in *Vldlr*$^{-/-}$ mice. Real-time PCR was used to quantify the VEGF mRNA level in the retinas of *Vldlr*$^{-/-}$ mice. It was found that in the *Vldlr*$^{-/-}$ mice, the VEGF mRNA level was increased compared with that in normal WT mice. However, VEGF mRNA expression was significantly decreased in the HL-217-treated *Vldlr*$^{-/-}$ mice (Fig. 4).

4. Discussion

The very low-density lipoprotein receptor mutation in mice induces retinal neovascularization arising from the deep retinal vascular layer [21]. This retinal change can be observed at the end of the second postnatal week and by 4 weeks of age [21,22]. New vessels extend through the avascular photoreceptor layer toward the surface of the retinal pigment epithelium [22]. Similar to the phenotypes observed in neovascular AMD, neovascular lesions form subretinal clusters of abnormal microvessels [23]. However, the neovascular lesions in these mice originate from retinal vessels without initial RPE damage, in contrast to neovascular AMD, in which abnormal vessels grow from the choroid through

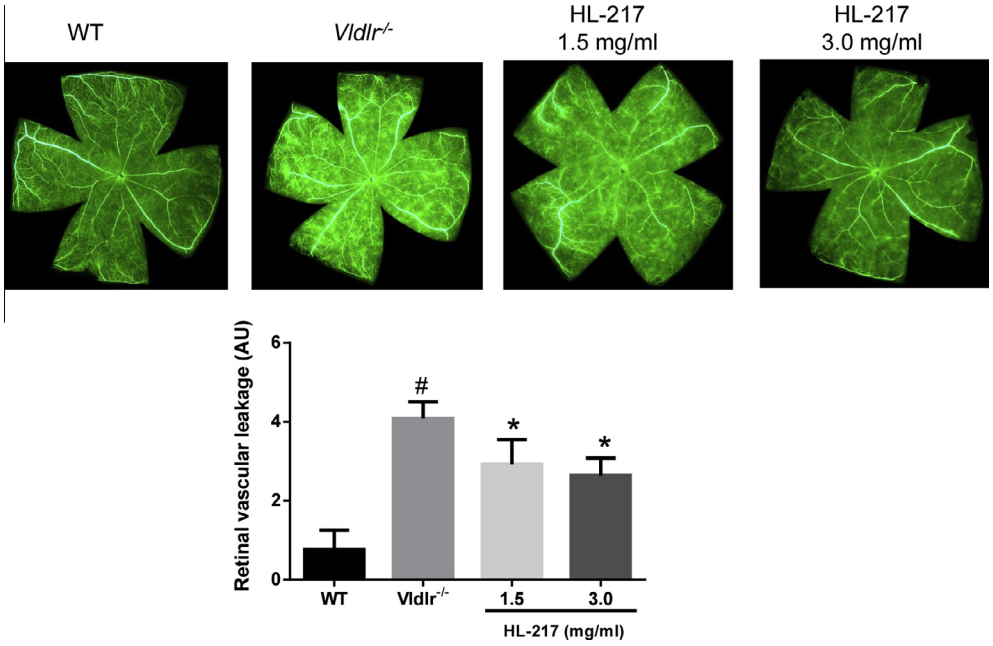

Fig. 1. Effect of HL-217 on retinal vascular leakage in *Vldlr*$^{-/-}$ mice. FITC-dextran perfusion of the retinal blood vessels was observed under a fluorescence microscope. The values in the bar graph represent the mean ± SE, $n = 10$. $^{\#}p < 0.05$ vs. WT mice, $^{*}p < 0.05$ vs. vehicle-treated *Vldlr*$^{-/-}$ mice.

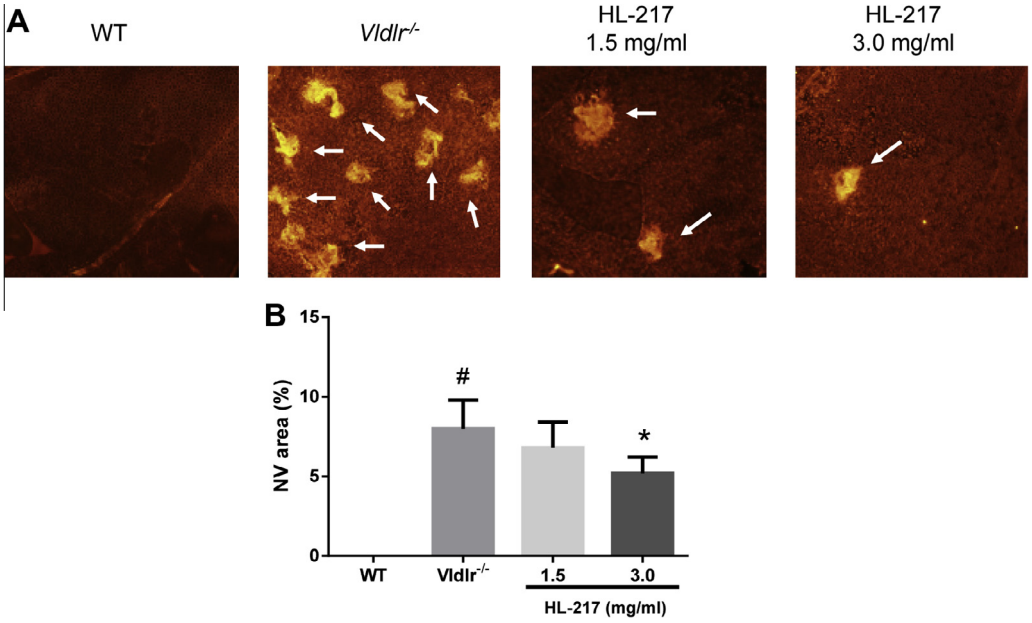

Fig. 2. Effect of HL-217 on subretinal neovascularization in *Vldlr*$^{-/-}$ mice. (A) Isolectin B4-stained flat mounts. Arrows represent neovascularization. (B) Quantification of the neovascular area. The values in the bar graph represent the mean ± SE, $n = 10$. $^{\#}p < 0.05$ vs. WT mice, $^{*}p < 0.05$ vs. vehicle-treated *Vldlr*$^{-/-}$ mice.

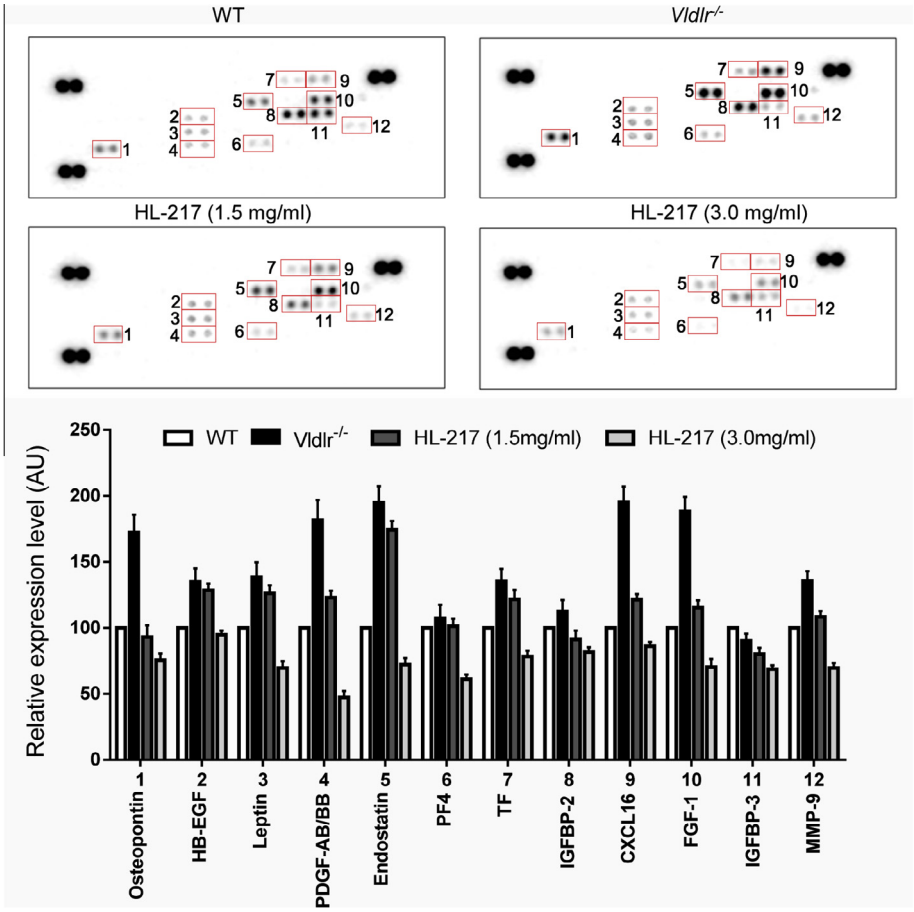

Fig. 3. Expression levels of 55 angiogenesis-related proteins in *Vldlr*$^{-/-}$ mice. The positive controls are located in three corners of the arrays, and the negative control is located in the lower right corner of the arrays. Modulated proteins in retinas treated with HL-217 are highlighted with squares and indicated by numbers. The values in the bar graph represent the mean ± SE, $n = 4$.

defects in Bruch's membrane into the subretinal space [23]. The retinal phenotype in *Vldlr*$^{-/-}$ mice is quite similar to that observed in human patients with RAP AMD [22]. VEGF is elevated in *Vldlr*$^{-/-}$ retinas and is the leading cause of subretinal neovascularization and vascular hyper-permeability [23]. Degeneration of photoreceptor cells is also observed in *Vldlr*$^{-/-}$ mouse retinas [24].

Table 1
Inhibitory effect of HL-217 on the PDGF-BB/PDGFRβ interaction.

Sample	Concentration (μM)	Inhibition (%)	IC_{50} (μM)
HL-217	5	28.9 ± 2.4	38.9 ± 0.7
	10	37.0 ± 8.3	
	50	55.8 ± 11.3	

Inhibitory activity is expressed as the mean ± SE of quadruplicate samples. The 50% inhibitory concentration (IC_{50}) was calculated from the dose-inhibition curve.

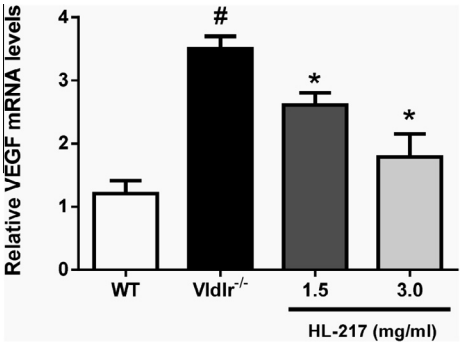

Fig. 4. Real-time PCR analysis of VEGF mRNA levels in *Vldlr*$^{-/-}$ mice. The data are presented as the mean ± SM, $n = 4$. $^{\#}p < 0.05$ vs. WT mice, $^{*}p < 0.05$ vs. vehicle-treated *Vldlr*$^{-/-}$ mice.

Although the precise pathogenic mechanisms leading to these vascular and neuronal lesions in *Vldlr*$^{-/-}$ mice are unknown, the *Vldlr*$^{-/-}$ mouse model has been widely used to study subretinal neovascularization [22]. In this study, we evaluated the therapeutic potential of HL-217, a new synthetic angiogenesis inhibitor, for the treatment of subretinal neovascularization using this animal model. HL-217 could prevent subretinal neovascularization in *Vldlr*$^{-/-}$ mice. We also showed that HL-217 treatment dramatically inhibited the expression of PDGF-BB and VEGF in *Vldlr*$^{-/-}$ mice. Moreover, our in vitro ligand binding assay showed that HL-217 inhibited the binding of PDGF-BB to its receptor, PDGFRβ. These results indicate that HL-217 has anti-angiogenic effects on subretinal neovascularization.

Studies in animal models have indicated that VEGF plays a major role in neovascularization, and this has been validated in clinical trials. VEGF antagonists provide major benefits in patients with neovascular age-related macular degeneration and other types of ocular neovascularization [25]. However, increasing evidence suggests that PDGF-BB has a role in retinal and choroidal neovascularization. Normal vessels show very low to undetectable levels of PDGF-BB, whereas high levels of PDGF-BB and its receptors are produced in vascular cells following injury [26]. Increased expression of PDGF-BB in the retina plays an important role in the pathogenesis of proliferative retinopathy and retinal detachment [27]. Inhibition of PDGFR signaling with antibodies was also shown to enhance the therapeutic effect of anti-VEGF treatment in multiple mouse models of ocular neovascularization [28]. In addition, the PDGF-BB/PDGFR signaling pathway has an important role in supporting vascular integrity and stability [29]. Pericytes are recruited by PDGF-BB secreted from endothelial cells during the process of neovascularization [30]. In pericyte-stripped vessels induced by the inhibition of PDGF-BB, the endothelium becomes more susceptible to VEGF blockade, resulting in regression of the neovascularization [29]. Thus, it has been proposed that PDGF-BB may be a second validated target against retinal neovascularization [25]. In the present study, HL-217 blocked PDGF-BB/PDGFRβ interaction in vitro and prevented subretinal neovascularization through down-regulation of both PDGF-BB and VEGF in vivo. This is the first report demonstrating that HL-217 is a potent inhibitor of PDGF-BB.

HL-217 has been shown to have multifunctional properties in various cells. The anti-angiogenic activity of HL-217 (previously known as KR-31831) has been shown to involve VEGF signaling [17,18]. Our results suggest that the effects of HL-217 are mediated by both VEGF and PDGF-BB. VEGF serves as a major activator of subretinal neovascularization. Other signaling pathways may also be involved in subretinal neovascularization. Several studies have shown that drugs targeting multiple pathways have more potent anti-angiogenic activities. In this regard, a pharmacologic strategy that inhibits both VEGF and PDGF signaling is a more desirable therapeutic approach when targeting angiogenesis [31]. HL-217 is a promising agent that may inhibit subretinal neovascularization via multiple angiogenic pathways. Therefore, it is suggested that the inhibition of subretinal neovascularization by HL-217 may be due to synergic effects of multiple signaling pathways. In a pharmacokinetic study in which eye drops containing HL-217 (0.9 mg/ml) were administered to rats, the concentrations of HL-217 in the aqueous humor, vitreous humor and retina 30 min after topical administration were 1250, 195, and 846 ng/ml, respectively (data not shown). This result suggests that HL-217 contained in the eye drops is able to reach the retina through the cornea.

In conclusion, HL-217 blocked the binding of PDGF-BB to PDGFRß in vitro, and topical administration of 3 mg/ml HL-217 exerted an anti-angiogenic effect in a mouse model of neovascular AMD. The most important finding of our study was that the topical application of HL-217 is effective for the treatment of subretinal neovascularization. Therefore, HL-217 will be clinically useful for the treatment of human neovascular AMD.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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