



OPEN Topical ophthalmic administration of the antiangiogenic peptide VIAN-c4551 protects against experimental diabetic macular edema

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Increased angiogenesis and vascular permeability are hallmarks of microvascular retinal diseases such as diabetic retinopathy and diabetic macular edema (DME). Periodic intravitreal injections of inhibitors of the vascular endothelial growth factor (VEGF) are first-line therapy, but their invasiveness and associated risks often lead to poor compliance and outcomes. Here, we investigate VIAN-c4551, a highly potent antiangiogenic cyclic heptapeptide, as a non-invasive topical ophthalmic alternative to the current standard of care for DME. VIAN-c4551 demonstrated high potency ($IC_{50} = 137$ pM) to inhibit the permeability of human umbilical vein endothelial cell monolayers induced by VEGF. VIAN-c4551 eye drops potently (0.005% minimum effective dose) prevented, for up to 24 h, the retinal vascular leakage induced by VEGF injected intravitreally and reversed the increased retinal vascular permeability due to diabetes in rats and mice. VIAN-c4551 exhibited high penetrability across MDCK epithelium and, after a single eye drop in rabbits, reached the vitreous and the retina-choroid at concentrations several orders of magnitude above its IC_{50} ($C_{max} \sim 239$ nM and ~ 6.7 μ M, respectively, after 6 h) that lasted at least 24 h. In conclusion, VIAN-c4551 is a non-invasive, once-a-day potential intervention for preventing and reversing retinal vascular leakage in DME and other vascular retinopathies and preserving sight.

Keywords Vasoinhibin analog, Therapeutic peptide, Diabetic macular edema, Diabetic retinopathy, Eye drops, Vascular permeability, Efficacy, Pharmacokinetics, Anti-angiogenesis

Diabetes is a common cause of vision loss due to retinal microvascular complications in diabetic retinopathy (DR) and diabetic macular edema (DME). DME is present in 25% of persons with diabetes and 71% of patients with proliferative DR, and it is the clinical condition most closely associated with vision loss¹. In DME, leakage from retinal capillaries causes the accumulation of extracellular fluid and proteins that alter the structure and function of the macula and may lead to blindness if untreated. Vascular endothelial growth factor (VEGF) is a major vascular permeability factor in DME², and the intravitreal injection of agents blocking VEGF has become the first-line treatment for DME with vision loss³. However, intravitreal injections are invasive, and their repeated and long-term use, due to the chronic nature of DME, increases the chance of ocular complications and systemic adverse events⁴. Eye drops are a preferred therapeutic option because they are self-administered, non-invasive, and facilitate long-term therapy. However, reaching the back of the eye through eye drops is challenging due to

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the numerous ocular barriers^{5,6}, and no eye-drop formulation has been approved by the FDA to treat posterior eye diseases, including DME.

VIAN-c4551 is a promising drug candidate for treating DME, DR, and other vascular retinopathies. VIAN-c4551 is an analog of vasoinhibin, an endogenous antiangiogenic protein with significant therapeutic potential. Vasoinhibin inhibits endothelial cell proliferation, migration, survival, and permeability in response to VEGF and other proangiogenic and vascular permeability factors (basic fibroblast growth factor, bradykinin, interleukin 1 β)^{7,8}. It helps restrict retinal angiogenesis under physiological conditions⁹, and the intravitreal administration of vasoinhibin reduces ischemia-induced retinal angiogenesis¹⁰ and prevents and reverses diabetes- and VEGF-induced increase in retinal vascular leakage in rodents¹¹. In humans, the systemic and ocular levels of vasoinhibin are disrupted in retinopathy of prematurity^{12,13} and DR¹⁴, and a prokinetic medication (levosulpiride) that upregulates vasoinhibin levels in the vitreous of patients with proliferative DR¹⁵, improves visual acuity and structural retinal outcomes in patients with DME and vision loss¹⁶.

However, the direct use of vasoinhibin in the clinic is hampered by difficulties in its recombinant production¹⁷. These difficulties have been overcome by the development of VIAN-c4551, a vasoinhibin analog with improved pharmacological properties that conserves the efficacy and potency of vasoinhibin⁸. VIAN-c4551 inhibits VEGF-induced proliferation of endothelial cells with potency like vasoinhibin (IC_{50} = 150 pM) and is orally active to inhibit melanoma tumor growth and vascularization in mice⁸. The oral bioavailability of VIAN-c4551 demonstrates physiochemical properties (solubility, stability, permeability, and potency) that may help VIAN-c4551 overcome the anatomical and physiological barriers that prevent topically administered drugs from reaching the retina^{5,6}. Here, we show the high potency and efficacy of VIAN-c4551 in eye drops to inhibit VEGF- and diabetes-induced retinal vascular leakage in rodents, the in vitro transit of VIAN-c4551 across epithelial cells, and the presence of VIAN-c4551 in the retina-choroid and vitreous at supra-therapeutic concentrations following eye drop administration in rats and rabbits.

Materials and methods

Reagents

VIAN-c4551 (>95% pure), VIAN-c4551 coupled to fluorescein-5-isothiocyanate (FITC), and recombinant human vascular endothelial growth factor-165 (VEGF) were from GenScript (Piscataway, NJ). Basic fibroblast growth factor (bFGF) from Scios, Inc., Mountain View, CA. Recombinant human vasoinhibin of 123 amino acids (Vi1-123) was produced as reported¹⁸. Eye drop vehicle (3% trehalose-0.15% sodium hyaluronate) was from Laboratories Théa (Clermont-Ferrand, France).

Cell culture

Human umbilical vein endothelial cells (HUVEC)¹⁹ were cultured in F12K medium supplemented with 20% fetal bovine serum (FBS), 100 μ g mL⁻¹ heparin (Sigma Aldrich, St, Louis, MO), 25 μ g mL⁻¹ endothelial cell growth supplement (ECGS) (Corning, Corning, NY), and 100 U mL⁻¹ penicillin-streptomycin, and authenticated by CD31 presence. HUVECs were used between passages 4 and 9. The Madin-Darby canine kidney (MDCK) epithelial cell line (ATCC, Rockville, MD) was cultured in 10% FBS-DMEM.

VEGF-induced endothelial cell permeability

HUVEC were seeded at 20,000 cells cm⁻² on a 6.5 mm transwell with a 0.4 μ m pore to form a confluent monolayer. Transendothelial electrical resistance (TEER) was measured with the EVOM² Volt/Ohm meter (World Precision Instruments, Sarasota, FL) as reported¹⁸. Monolayers were incubated with different concentrations of VIAN-c4551 or Vi1-123 for 1 h before adding PBS or 50 ng mL⁻¹ VEGF, and TEER measured over a 150-min period.

Immunofluorescence analysis of F-actin and CD31

HUVEC were seeded at 20,000 cells cm⁻² on 18 mm coverslips placed in a 12-well plate and cultured to confluence. Once the monolayer was established, cells were pretreated with or without 100 nM of VIAN-c4551 for 1 h prior to stimulation with 200 ng mL⁻¹ VEGF for 30 min. Cells were then washed, fixed with 4% paraformaldehyde (PFA) for 30 min, permeabilized with 0.5% Triton X-100 in PBS for 30 min, and blocked with 5% normal goat serum, 1% BSA, 0.05% Triton X-100 in PBS for 1 h at RT. Primary antibody incubation was performed overnight at 4 °C in a humidified chamber using anti-CD31 (1:100, Abcam) diluted in 1% BSA, 0.1% Triton X-100 in PBS. Following washes, cells were incubated for 2 h in darkness with goat anti-mouse Alexa Fluor 488-conjugated secondary antibody (1:500). F-actin was visualized by incubating cells with 160 nM TRITC (Tetramethylrhodamine)-phalloidin for 1 h in darkness (Thermo Fisher Scientific, Waltham, MA). Nuclei were counterstained with 5 μ g mL⁻¹ Hoechst 33,342 (Sigma-Aldrich), and coverslips observed by fluorescence microscopy and merged images obtained using ImageJ-FIJI.

MDCK transit assay

MDCK cells were seeded (40,000 cells cm⁻²) in 200 μ L 10% FBS-DMEM on a 6.5 mm transwell with a 0.4 μ m pore to form a confluent monolayer. Upon confluency, 0.4 nM and 4 nM VIAN-c4551 or Vi1-123 were added to the apical side of the monolayer for 2 h at 37 °C. 600 μ L from the basolateral compartments were then collected and stored at -20 °C. VIAN-c4551 and Vi1-123 were quantified by means of their bioactive properties, as reported²⁰. Recovery levels were expressed relative to those without MDCK cells, and the apparent permeability coefficient (P_{app} , cm s⁻¹) across the epithelial barrier was calculated as reported²¹.

Animals

Male and female Wistar rats (6–7 weeks old, 180–230 g), male CD-1 mice (8–10 weeks old, 20–25 g), and female New Zealand white rabbits (2–2.5 kg) were maintained and treated in adherence to the National Research

Council's Guide for the Care and Use of Laboratory Animals. The Bioethics Committee of the Instituto de Neurobiología (UNAM) approved all animal experiments. The authors confirm that the study is reported in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

VEGF-induced retinal vascular permeability

One drop containing 0.5% [5 mg mL⁻¹ or 6.5 mM] VIAN-c4551 or vehicle was instilled in each eye of male mice (5 µL) and rats (10 µL) manually maintained still for 10 s to allow drop distribution on the ocular surface. After 1 h, animals were anesthetized with 60% ketamine and 40% xylazine (1 µL g⁻¹ body wt, i.p.) and injected intravitreally with 1 µL (mice) or 2 µL (rats) PBS or VEGF (250 ng) and two hours later, retinal vascular leakage was evaluated by the extravasation of albumin stained by the intravenously injected Evans blue dye, as reported³². For the qualitative microscopic imaging of retinal vascular leakage, a single eyedrop of VIAN-c4551 [0.5%] or vehicle was applied to the eye 4 h prior to intravitreal injection of VEGF (300 ng eye⁻¹) or PBS. One hour later, animals were anesthetized (ketamine/xylazine) and fluorescein isothiocyanate-labeled dextran (FITC-dextran, 50 mg mL⁻¹, MW 2 × 10⁶ Da; Sigma-Aldrich) was administered intravenously at a dose of 100 mg kg⁻¹. After allowing one h for systemic circulation, animals were euthanized by CO₂ inhalation and retinas flat-mounted, fixed for 4 h in 4% PFA at room temperature, washed with PBS, mounted on glass slides using 50% glycerol in PBS, and observed under a fluorescence microscope.

Diabetes-induced retinal vascular permeability

Five daily i.p. injections of streptozotocin (STZ) (55 mg kg⁻¹; S0130, Sigma-Aldrich) and a single i.p. STZ injection (60 mg kg⁻¹) induced diabetes in male mice and male rats, respectively^{23,24}. Blood glucose levels > 250 mg dL⁻¹ defined diabetes one week post-STZ. After 5 weeks, non-diabetic and diabetic animals received a daily eye drop of 0.5% VIAN-c4551 or vehicle per eye for 5 days. The Evans blue method evaluated retinal vascular leakage 24 h after the last eye drop.

Ocular pharmacokinetics

Female rats and rabbits were instilled with one eye drop (10 and 50 µL, respectively) containing 0.5% VIAN-c4551, VIAN-c4551-FITC (net peptide), or vehicle, and manually kept still for 10 s to allow drop surface distribution and euthanized by CO₂ inhalation at different intervals for up to 24 h. Eyes were enucleated, and the vitreous, the retina from the rat, and the retina-choroid from the rabbit were collected, snap frozen in liquid nitrogen, and stored at -80 °C until analysis. Tissues were homogenized in RIPA buffer (10 mM Tris-HCl, 140 mM NaCl, 0.5% IGEPAL CA-630, 1mM EDTA, 0.5 mM EGTA, 1% triton X-100, 0.1% SDS, pH 7.4) supplemented with 1:100 protease inhibitor cocktail (Roche). Vitreous samples (125 ng µL⁻¹ protein) and retinas (150 ng µL⁻¹ protein) were added to HUVEC cultures to evaluate VIAN-c4551 levels through its bioactivity, as reported²⁰. For fluorescence emission, a standard curve of VIAN-c4551-FITC from 0 to 100 pmol was prepared in a vitreous matrix (110 ng µL⁻¹ protein) for rat or rabbit or in a retina matrix (2.5 µg µL⁻¹ protein for rat or 750 ng µL⁻¹ protein for rabbit). Fluorescence emission of 100 µL from each standard dilution was quantified (Varioskan Flash, Thermo Fisher Scientific) at excitation and emission wavelengths of 490 nm and 520 nm, respectively, for 100 ms. Values were interpolated from standard curves in the respective matrix and pharmacokinetic analysis was performed using Plotly software²⁵.

Ocular distribution of VIAN-c4551-FITC

Male Wistar rats (~285 g) received three eye drops (one per hour) of either vehicle or VIAN-c4551-FITC (0.5%). Six hours after the first dose, animals were euthanized by CO₂ inhalation, perfused with PBS followed by 4% PFA. The eyes were postfixed in 4% PFA for 2 h, cryoprotected in 15% and 30% sucrose for 24 h each, embedded in TissueTek, and cryosectioned at 10 µm. Sections were mounted on gelatin-coated slides, washed with PBS, permeabilized (0.1% Triton X-100), counterstained with Sytox Red (S34859, Thermo Fisher Scientific, Waltham, MA), mounted in Vectashield, and observed under confocal microscopy (Zeiss LSM 780, Overkochen, Germany). Image analysis was performed in ImageJ-FIJI using Bio-Formats and hyperstacks. For each section, 5–7 optical slices were combined using maximum intensity projection.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism version 10.2.2 for macOS, GraphPad Software (Boston, Massachusetts USA, www.graphpad.com). The threshold for significance was set at $P < 0.05$. For the analysis of two variables over time, we used two-way ANOVA with Dunnett's post hoc test to compare treatment groups to the control at each time point. For grouped column comparisons involving two factors, we used two-way ANOVA with Sidak's post hoc test for pairwise comparisons. For single-variable comparisons across multiple groups, we use one-way ANOVA with Dunnett's post hoc, comparing each group to the control.

Results

VIAN-c4551 potentially inhibits the VEGF-induced permeability of HUVEC monolayers

We evaluated the trans-endothelial electrical resistance (TEER) of HUVEC monolayers as an indicator of vascular permeability (Fig. 1a, b). VEGF rapidly decreased TEER values and kept them low throughout the 150-minute incubation period. VIAN-c4551 prevented the VEGF-induced reduction of TEER, and VIAN-c4551 alone showed no effect (Fig. 1a). TEER inhibition by VIAN-c4551 was dose-dependent with an IC₅₀ of 137.3 pM, like that of the full-length vasoinhibin (Vi1-123) (IC₅₀ = 163.2 pM) (Fig. 1b). Increased vascular permeability due to weakened endothelial cell junctions associates with the redistribution of the actin cytoskeleton that leads to cell contraction²⁶. The actin cytoskeleton was therefore evaluated by the phalloidin-TRITC staining of F-actin in HUVEC monolayers co-stained for the endothelial cell marker CD31 and exposed to VEGF (Fig. 1c). VEGF

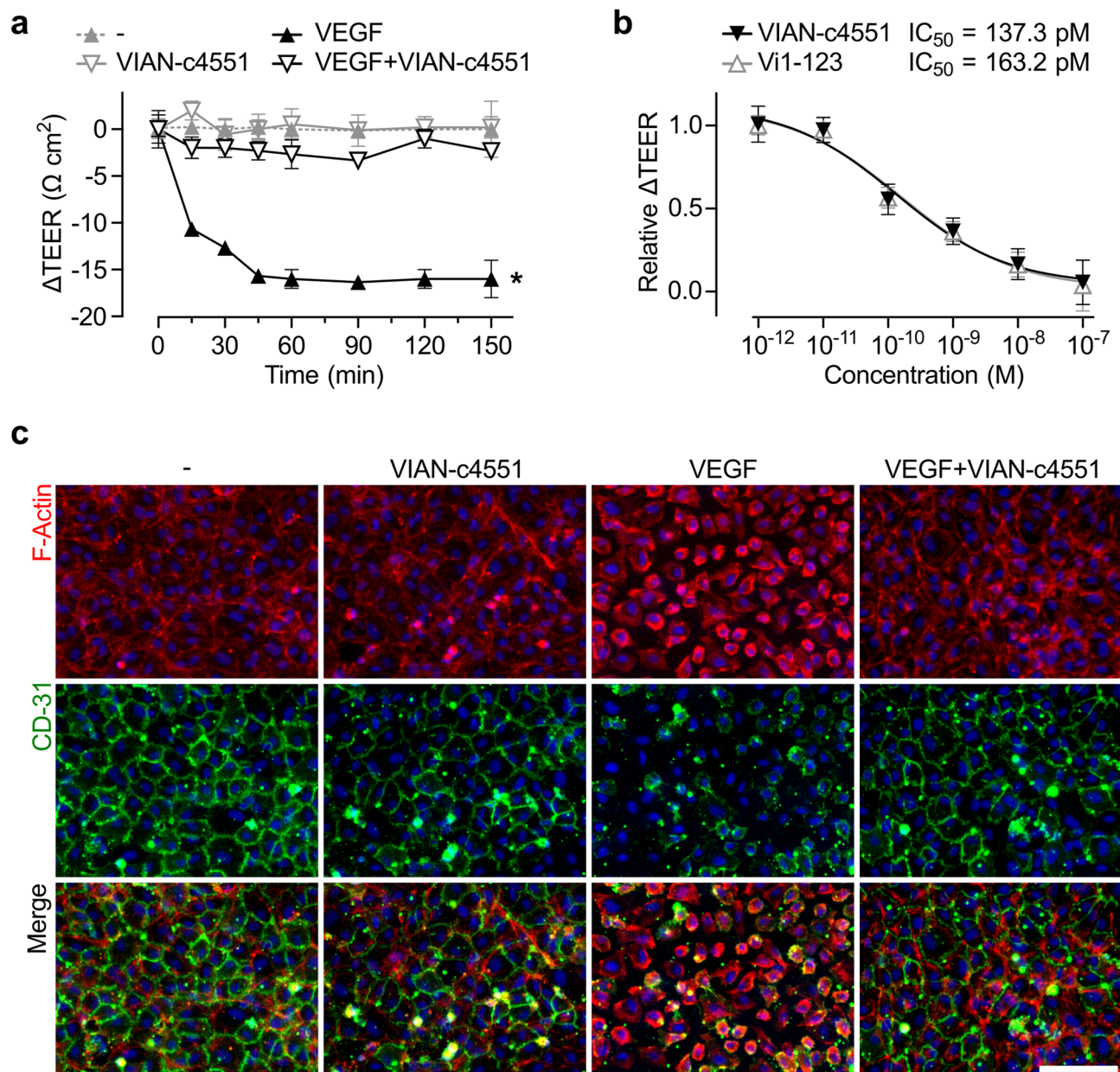


Fig. 1. VIAN-c4551 inhibits VEGF-induced permeability of HUVEC monolayers. **(a)** Changes in the trans-endothelial electrical resistance (Δ TEER) of HUVEC monolayers treated or not with 100 nM VIAN-c4551 throughout a 150-minute incubation period without (-) or with VEGF (50 ng mL⁻¹). Values are means \pm SD, $n = 3$, * $P < 0.006$ vs. (-) for all time points except 0 (2-way ANOVA repeated measures, Dunnett's). **(b)** Dose-response inhibition of the VEGF-induced reduction of Δ TEER at 90 min by different concentrations of VIAN-c4551 or Vi1-123. The IC_{50} of VIAN-c4551 and Vi1-123 are indicated. Δ TEER is relative to that of VEGF alone. Values are means \pm SD, $n = 9$, and the curve represents a nonlinear regression analysis of four parameters (54 points, $r^2 \geq 0.92$). **(c)** Representative fluorescence of HUVEC monolayers stained for F-actin and CD31 pretreated with or without VIAN-c4551 for 1 h followed by VEGF (200 ng mL⁻¹) for 30 min. Nuclei were counterstained with Hoechst 33,342. Scale bar = 100 μ m.

altered the actin cytoskeleton as revealed by perinuclear actin stress fibers, contracted and rounded cell bodies, and lack of CD31 at the surface of cells. These changes were blocked by VIAN-c4551 (Fig. 1c). These findings visualize the protective effect of VIAN-c4551 against the loss of integrity of the endothelial barrier caused by VEGF and thereby confirm and complement TEER measurements.

VIAN-c4551 exhibits high transit across epithelia

We analyzed the capacity of VIAN-c4551 to cross the MDCK epithelial cell monolayer, a well-established model to evaluate the paracellular and transcellular transport of drugs^{27,28}. Around 50% of VIAN-c4551 in two

concentrations (0.4 and 4 nM) crossed the monolayer after 2 h (Fig. 2a). VIAN-c4551 presented a high apparent permeability coefficient ($P_{app} = 1.44 \times 10^{-5} \text{ cm s}^{-1}$) (Fig. 2b), like steroids²⁹. The penetrability of Vi1-123 across the MDCK monolayer was negligible ($P_{app} = 3.86 \times 10^{-7} \text{ cm s}^{-1}$).

Given that VIAN-c4551 is highly potent to inhibit VEGF-induced vascular permeability and highly efficient to cross epithelia, we investigated the effect of VIAN-c4551 in eye drops on VEGF-induced retinal vascular permeability.

VIAN-c4551 eye drops prevent VEGF-induced retinal vascular permeability

A single eye drop containing 0.5% VIAN-c4551 or vehicle was administered one hour before intravitreal injection of VEGF or PBS, and two hours later the retinal vascular leakage was evaluated by the extravasation of albumin. VEGF induced a significant 2-fold increase in retinal vascular permeability relative to PBS, and VIAN-c4551 prevented this effect in both rats (Fig. 3a) and mice (Fig. 3b). The higher basal vascular permeability values in mice vs. rats may be attributed to the greater damage caused by the intravitreal injection given the smaller size of the mouse eye. VIAN-c4551 did not modify the retinal levels of the tracer in the absence of VEGF.

The efficacy of VIAN-c4551, profiled in rats, was dose-dependent, and the minimal effective dose was 0.005% (0.5 µg/eye drop) (Fig. 3c), confirming the high potency of VIAN-c4551. The duration of the effect was also profiled in rats by instilling 0.5% VIAN-c4551 or vehicle at different times before intravitreal injection of VEGF. The protective effect of VIAN-c4551 against the VEGF challenge was evident at 3 h, maximal at 12 h, and lasted up to 24 h after the eye drop (Fig. 3d). The effect of VIAN-c4551 eyedrops on retinal blood vessels was visualized by representative fluorescein angiography images of flat-mounted retinas from rats intravitreally injected with PBS or VEGF and evaluated 6 h after a single eyedrop containing vehicle or VIAN-c4551 (Fig. 4). VIAN-c4551 prevented the VEGF-induced leakage of retinal blood vessels, as evidenced by the reduced extravasation of FITC-dextran (arrows).

The exceptional efficacy and 24-hour duration of VIAN-c4551 in eye drops suggested that VIAN-c4551 reaches the back of the eye at a high and sustained therapeutic concentration and warranted investigating its effect in the DME and DR rodent model induced by STZ, and its pharmacokinetic profile.

VIAN-c4551 in eye drops reverses diabetes-induced retinal vascular permeability

Excessive retinal vascular leakage is evident at 6 weeks of STZ-induced diabetes in rodents (Fig. 5)²³. A single eye drop containing 0.5% VIAN-c4551 delivered once a day for the last five days suppressed the 2-fold increase in retinal vascular permeability due to diabetes in rats (Fig. 5a) and mice (Fig. 5b). The basal level of retinal vascular permeability in non-diabetic mice was smaller than that of non-diabetic rats, thereby, supporting our claim that the intravitreal injection by itself increases retinal vascular permeability in mice and not in rats (Fig. 3a, b).

The reduction of vascular leakage is indicative of the restoration of the blood-retinal barrier function and, thereby, proof-of-concept that VIAN-c4551 represents a potential, non-invasive therapy against DR and DME.

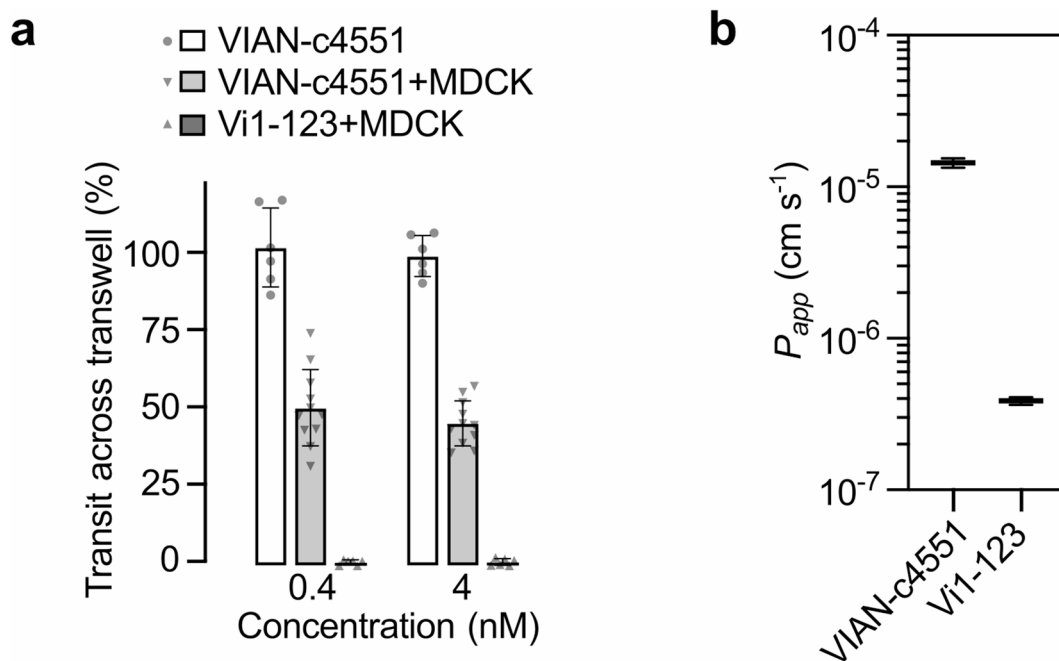


Fig. 2. VIAN-c4551 transit across MDCK epithelial cells. **(a)** Percentage of transit across an MDCK monolayer of two concentrations (0.4 and 4 nM) of VIAN-c4551 or Vi1-123 after 2 h relative to their transit of both compounds in the absence of MDCK monolayers. Bars indicate mean \pm SD, $n \geq 6$. Individual values are shown. **(b)** Permeability coefficient (P_{app}) of VIAN-c4551 and Vi1-123.

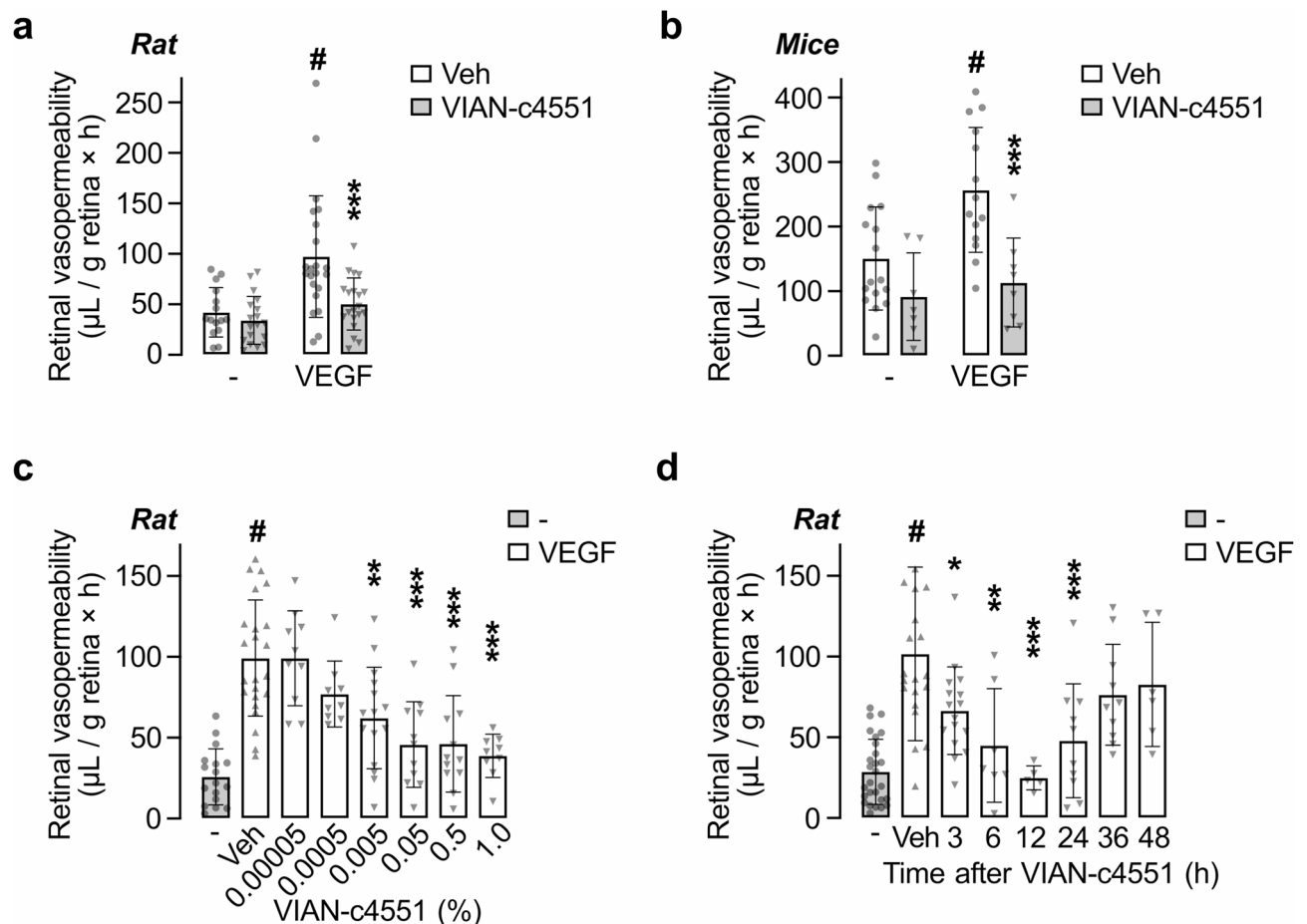


Fig. 3. VIAN-c4551 eye drops potentially inhibit VEGF-induced retinal vascular permeability for up to 24 h. Retinal vascular permeability in rats (**a**) and mice (**b**) treated with a single eye drop containing 0.5% VIAN-c4551 or vehicle administered one hour before the intravitreal injection of 250 ng of VEGF or PBS. (**c**) Dose-response inhibition of eye drops containing different concentrations of VIAN-c4551. (**d**) Duration of the effect of a single 0.5% VIAN-c4551 eye drop administered at different times before the intravitreal injection of VEGF. Quantification of Evans blue-stained albumin extravasation was performed two hours after VEGF administration. Values are means \pm SD. Individual eye values are shown. Sample size (n) was 15–22 (**a**), 7–16 (**b**), 9–23 (**c**), and 5–27 (**d**). # $P < 0.002$ vs. no VEGF (-), * $P < 0.033$, ** $P < 0.002$, *** $P < 0.001$ vs. Veh (-), 2-way ANOVA, Šidák's (**a**, **b**), 1-way ANOVA, Dunnett's (**c**, **d**).

Ophthalmic instillation of VIAN-c4551 leads to high and sustained therapeutic concentration at the back of the eye

After a single eye drop, VIAN-c4551 reached the retina at levels ~ 10 and 30 -fold greater than in the vitreous in rats and rabbits, respectively, and these amounts substantially exceeded the IC_{50} for inhibition of endothelial permeability (Fig. 6a, b). In rats, VIAN-c4551's concentration in the retina was $32.8 \mu\text{g g}^{-1}$ protein or $\sim 4.3 \mu\text{M}$, assuming a tissue density of 1 g mL^{-1} and a retinal protein content of $10\%^{30}$, and in the vitreous 300 ng mL^{-1} or $\sim 391 \text{ nM}$ at 3 h post-dose (Fig. 6a). In rabbits, the pharmacokinetic (PK) profile defined a long absorption phase that peaked in the retina-choroid (C_{max} $52 \mu\text{g g}^{-1}$ protein or $\sim 6.7 \mu\text{M}$) and in the vitreous (C_{max} 184 ng mL^{-1} or $\sim 239 \text{ nM}$) at 6 h and slightly declined thereafter (Fig. 6b). Even after 24 h, VIAN-c4551 exposure in retina-choroid ($3.5 \mu\text{g g}^{-1}$ protein or $\sim 464 \text{ nM}$) and vitreous (10.6 ng mL^{-1} or $\sim 13.88 \text{ nM}$) were still ~ 3 and ~ 2 orders of magnitude higher than its IC_{50} . PK parameters are summarized in Table 1. The PK profile was confirmed with VIAN-c4551-FITC. Despite that the FITC labeling reduced the transit of VIAN-c4551 across MDCK monolayers (Fig. 6c) and lowered its penetration to the retina-choroid and vitreous of rabbits (Fig. 6d), the kinetic profile of VIAN-c4551-FITC in both compartments was like that of the unlabeled compound, i.e., C_{max} in retina-choroid was $1.3 \mu\text{g g}^{-1}$ protein ($\sim 110 \text{ nM}$) at a T_{max} of 6 h and maintained for 24 h at levels beyond IC_{50} . Furthermore, VIAN-c4551-FITC was clearly visible in the retinal pigment epithelium of rats after its ophthalmic instillation (Fig. 7).

Discussion

Excessive vascular leakage is a hallmark of retinal diseases with high prevalence, such as DME, DR, and age-related macular degeneration. The current therapy uses frequent anti-VEGF intravitreal injections, which are

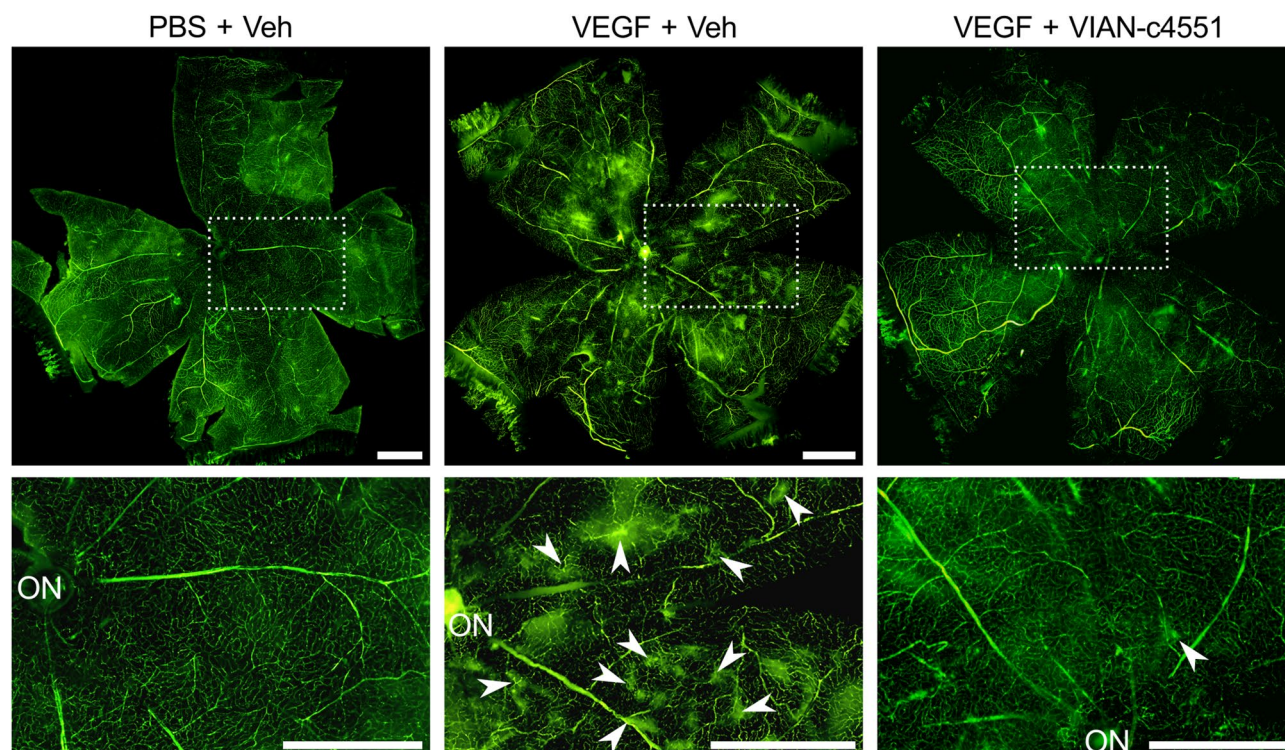


Fig. 4. Visualization of VIAN-c4551 eye drop inhibition of VEGF-induced retinal vascular leakage. Representative fluorescein angiography images of flat-mounted retinas from rats treated with Veh or VIAN-c4551 eyedrops 4 h before intravitreal injection of PBS or VEGF and euthanized 2 h post-intravitreal injections. Retinal vasculature was visualized by FITC-dextran perfusion 1 h after intravitreal injection of VEGF and 1 h of circulation. Vascular leakage areas (arrowheads) and optic nerve (ON) are indicated. Scale bar = 1 mm.

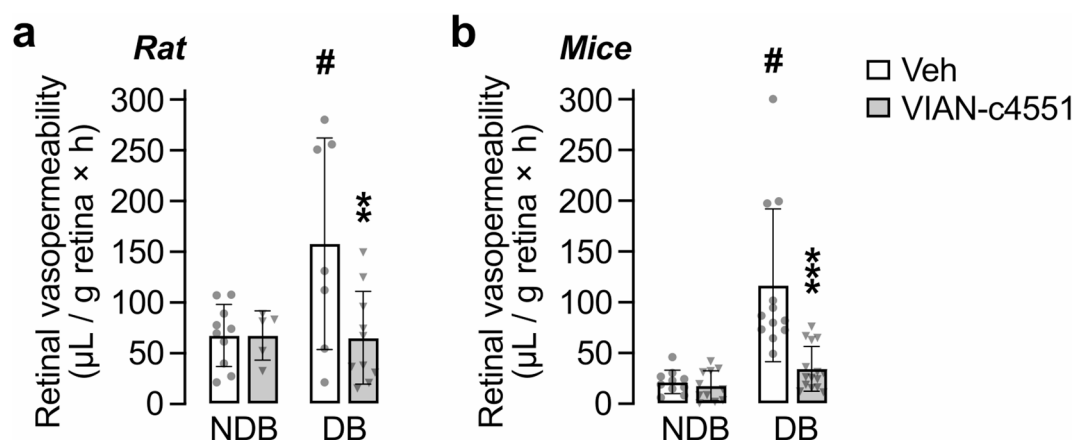


Fig. 5. VIAN-c4551 inhibits diabetes-induced retinal vascular permeability. Retinal vascular permeability in nondiabetic (NDB) and 6-week diabetic (DB) rats (**a**) and mice (**b**) treated daily for 5 days with a single eye drop containing 0.5% VIAN-c4551 or vehicle. The extravasation of Evans blue-stained albumin evaluated retinal vascular permeability 24 h after the last eye drop. Values are means \pm SD. Individual eye values are shown. # $P < 0.002$ vs. NDB, ** $P < 0.002$, *** $P < 0.001$ vs. Veh, (2-way ANOVA, Šidák's).

highly invasive, not always effective, cause complications, and associate with low compliance. Eye drop delivery is much more appealing as it is applied non-invasively by the patients themselves. However, topical administration faces delivery problems since only very low concentrations of drugs reach the retina^{6,31,32}. Here, we report the eye drop delivery of VIAN-c4551, a small peptide derived from an endogenous protein that has outstanding anti-VEGF efficacy and pharmacokinetic properties, and has the potential to eliminate the need for repeated intravitreal injections.

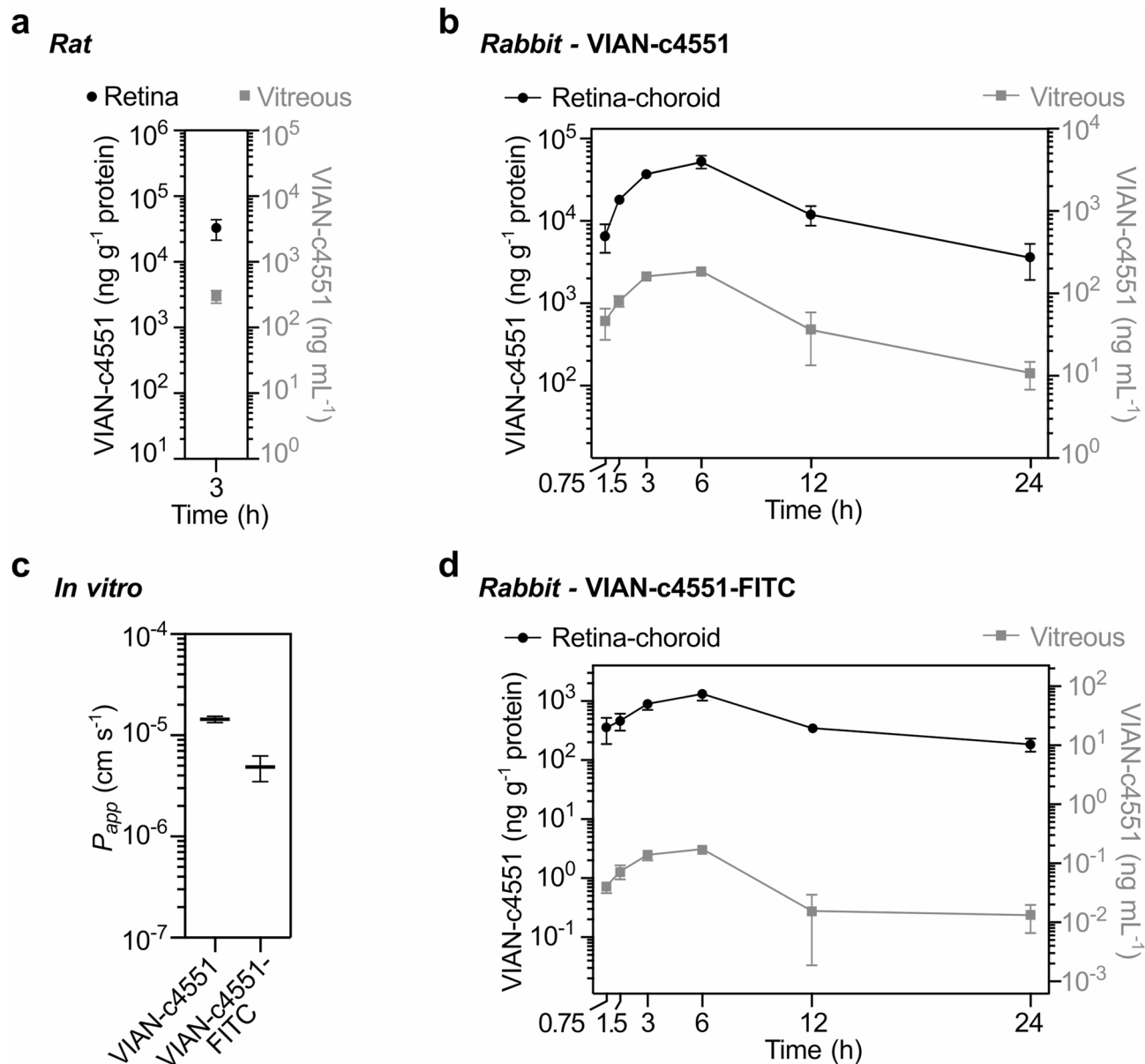


Fig. 6. Ocular penetration and pharmacokinetics of VIAN-c4551. Concentration of VIAN-c4551 in the retina and vitreous of rats three hours after delivery of a single eye drop containing 0.5% VIAN-c4551 (**a**), and in the retina-choroid and vitreous of NZ rabbits at different time points after a 0.5% VIAN-c4551 eye drop (**b**). Values are mean \pm SD, $n \geq 3$; ng g⁻¹ protein is used for retina-choroid (left axis) and ng mL⁻¹ for vitreous (right axis). (**c**) Permeability coefficient (P_{app}) values of VIAN-c4551-FITC across MDCK epithelial cell monolayer compared to that of VIAN-c4551. (**d**) Concentration of VIAN-c4551-FITC in the retina-choroid and vitreous of rabbits at different time points after 0.5% VIAN-c4551-FITC eye drop instillation. Values are mean \pm SD, $n \geq 3$; ng g⁻¹ protein is used for retina-choroid (left axis) and ng mL⁻¹ for vitreous (right axis).

VIAN-c4551 was developed as an effort to translate the anti-angiogenic vasoinhibin family of proteins (5.6 – 18 kDa) into an alternative therapeutic compound of 768 Da with improved pharmacological properties. In VIAN-c4551, the functional domain (His46-Gly47-Arg48) of vasoinhibin is contained within its 7 amino acid sequence (residues 45 to 51 of vasoinhibin) synthesized with D-amino acids in reverse order and cyclized to conserve potency and improve stability. VIAN-c4551 has the same vasoinhibin potency ($IC_{50} = 150$ pM) to inhibit VEGF-induced endothelial cell proliferation and is orally active to inhibit melanoma tumor growth and vascularization in mice⁸. Furthermore, the apoptotic, inflammatory, and fibrinolytic effects of vasoinhibin, which may be undesirable for antiangiogenic therapy, are in a different structural domain (residues 30 to 36) and, thereby, absent in VIAN-c4551³³.

Here, we report for the first time the inhibitory effect of VIAN-c4551 on excessive vascular permeability, another vascular action of vasoinhibin^{11,24}. VIAN-c4551 blocked the VEGF-induced permeability of endothelial cell monolayers with the high potency ($IC_{50} = 137$ pM) of vasoinhibin. Such high potency is key for topical

PK parameters	Retina-choroid	Vitreous
C_{max} (ng g ⁻¹ protein or ng mL ⁻¹)	51,693.81 ± 6678.9	183.66 ± 15.49
T_{max} (hours)	6	6
$AUC_{0-24\text{ h}}$ (hours × ng g ⁻¹ protein or hours × ng mL ⁻¹)	467,434 ± 45,884	1,678 ± 182
$T_{1/2}$ (hours)	5 ± 0.78	4.7 ± 0.58

Table 1. Ocular PK parameters after a single Eyedrop of 0.5% VIAN-c4551 to NZ rabbits. Values are mean ± SEM. *N* = 3 eyes per timepoint. ng g⁻¹ protein is used for retina-choroid and ng mL⁻¹ for vitreous. AUC, area under the curve.

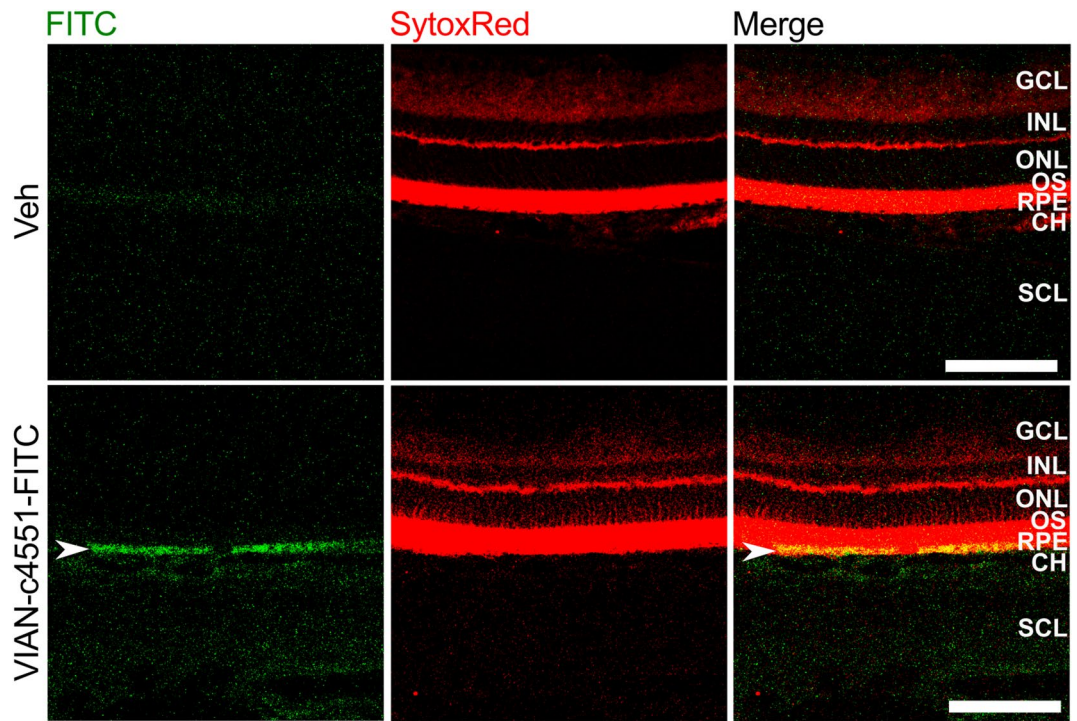


Fig. 7. FITC-labeled VIAN-c4551 localizes in the retinal pigment epithelium of rats after eye drop administration. Representative confocal images of retinal sections from rats treated with 3 eye drops of Vehicle or 0.5% FITC-labelled VIAN-c4551 (VIAN-c4551-FITC) evaluated 6 h after the first eyedrop administration. VIAN-c4551-FITC accumulation is indicated (arrow heads). Retinal layers were stained with SytoxRed. Ganglion cell layer (GCL), inner nuclear layer (INL), outer nuclear layer (ONL), outer segment (OS), retinal pigment epithelium (RPE), choroid (CH), sclera (SCL). Scale bar = 100 μm.

delivery to inhibit excessive retinal vascular leakage, as VIAN-c4551 needs to cross several ocular barriers and reach the retina at therapeutic concentrations. Overcoming ocular barriers depends on physicochemical properties, including size, charge, lipophilicity, solubility in water, stability, and permeability^{5,31}. The oral bioavailability of VIAN-c4551 is indicative of its capacity to cross the intestinal system into the bloodstream and, thereby, of having adequate stability and permeability across epithelial barriers.

Aiming to develop the eye drop delivery of VIAN-c4551, we investigated its transit across MDCK cell epithelial monolayers. The MDCK cell system has been proposed as a model for predicting the penetration of drugs across the cornea²⁷ and for studying the outer blood-retinal barrier²⁸. VIAN-c4551 presented a high apparent permeability coefficient in MDCK cells, with values of magnitude over 10⁻⁵ cm s⁻¹ and an average recovery of more than 50%. Therefore, VIAN-c4551 demonstrated the ability to cross a well-characterized epithelial barrier, and its transport across ocular epithelial barriers (corneal, conjunctiva, sclera, choroid, and retinal pigment epithelium) may not represent a relevant restriction for its eye drop absorption.

Encouraged by its high anti-VEGF potency and high penetrability, our efforts turned toward testing the *in vivo* efficacy of VIAN-c4551 in eye drops against the intravitreal injection of VEGF, a non-diabetic model of DME and DR³⁴. A single eye drop containing 0.5% VIAN-c4551 prevented the increase in retinal vascular permeability induced by VEGF in rats and mice. The effect was dose-dependent and highly potent as the minimal effective dose was 0.005% (0.5 μg/eye/day). This dose is much lower than used for the ophthalmic instillation of antibodies (7.5 mg/eye/day), antibody fragments (0.2–0.5 mg/eye/day), or small molecules (0.3–1% /eye/day)

in preclinical models of retinal microvascular diseases or clinical trials^{31,35,36}. Moreover, the anti-VEGF effect of VIAN-c4551 lasted 24 h, suggesting a successful once-a-day topical ocular treatment.

VEGF is a major contributor to retinal vascular leakage in diabetes². However, the pathologic cascade leading to DME and DR also involves other vasoactive substances like bradykinin³⁷, bFGF³⁸, and interleukin-1b³⁹, all of which are inhibited by vasoinhibin⁷. By interacting with binding proteins (plasminogen activator inhibitor, urokinase, the urokinase receptor, and/or integrin $\alpha 5\beta 1$)^{40,41} in endothelial cell membranes, vasoinhibin inhibits signaling pathways (Ras-Raf-MAPK, Ras-Tiam1-Rac1-Pac1, PI3K-Akt, and PLCg-IP3-eNOS) activated by several proangiogenic and vascular permeability factors (VEGF, bFGF, bradykinin, and interleukin 1b)⁷. Like vasoinhibin, VIAN-c4551 inhibits the proangiogenic and vasopermeability-inducing action not only of VEGF but also of other vasoactive factors such as bFGF and interleukin-1b [8], yet it does not bind to PAI-1, suggesting distinct or partially overlapping pathways³³. In agreement with broad actions, vasoinhibin inhibits the increase in retinal vascular permeability induced by the intravitreal injection of vitreous from patients with DR⁴², and the ocular overexpression of vasoinhibin is more efficient than that of sFlt-1 (the secreted extracellular domain of the VEGF receptor 1) for blocking diabetes-induced retinal vascular permeability²⁴. Addressing only the inhibition of VEGF action may, therefore, be of limited value, and the efficacy of VIAN-c4551 in diabetic conditions could exceed that of agents targeting only VEGF.

To investigate the broader benefit of the topical application of VIAN-c4551, we used the pharmacological induction of diabetes with STZ in rodents, one of the more commonly used DME and DR experimental models. In the STZ model of diabetes, increased retinal vascular permeability can occur as early as after 2 weeks of hyperglycemia³⁴. Daily eye drops of 0.5% VIAN-c4551 for 5 days suppressed the retinal vascular leakage caused by 6 weeks of hyperglycemia in rats and mice. This result indicated that VIAN-c4551 in eye drops reverses the disruption of the blood-retinal barrier in diabetic rodents and, thereby, has potential curative properties in DME, DR, and other microvascular retinal diseases. The efficacy of VIAN-c4551 in the VEGF-non-diabetic model and the STZ-diabetic model is proof-of-concept that its topical delivery reaches the retina at therapeutic concentrations.

In agreement, VIAN-c4551 efficiently migrates to the back of the eye of rats and rabbits. A single 0.5% VIAN-c4551 eyedrop reached micromolar and nanomolar concentrations in the retina/choroid and vitreous, respectively, and the high levels were sustained for 24 h. The maximal concentrations in the retina/choroid ($C_{\max} \sim 6.7 \mu\text{M}$) and vitreous ($C_{\max} \sim 239 \text{ nM}$) were 4 and 3 orders of magnitude higher, respectively, than the VIAN-c4551 IC_{50} (137.3 pM) to inhibit VEGF-induced endothelial cell permeability. These levels position VIAN-c4551 as the installed compound with the highest measured concentration in the retina of rabbits (reviewed here⁶), the next one being dorzolamide hydrochloride, a glaucoma drug yielding a 2.8 μM retinal concentration after a 2% instillation dose⁶. However, pooling together the choroid and retina limits comparisons as it is difficult to conclude exposure in the retina. Along this line, confocal images demonstrated that, despite its low penetrability compared to the unlabeled peptide, VIAN-c4551-FITC eye drops effectively reached the retinal pigment epithelium of rats. Nevertheless, it should also be noted that albino animals confound the more general condition of pigmented animals, where binding to melanin can interfere with the ocular efficacy and distribution of drugs⁴³.

A major question relates to the mechanisms mediating the exceptional ocular pharmacokinetic properties of VIAN-c4551. The fact that the concentration of VIAN-c4551 is lower in the vitreous than in the retina/choroid speaks in favor of migration through the conjunctiva-sclera-choroid-retina route and from the retina to the vitreous. This possibility is consistent with the hydrophilic nature of VIAN-c4551 and with absorption of hydrophilic compounds being facilitated by the larger paracellular pores and surface area of the non-corneal route³¹. However, VIAN-c4551 could also be absorbed through the cornea or through its clearance by the conjunctival blood vessels and subsequent retinal access via the retinal vasculature. Studies evaluating the ocular and systemic distribution of VIAN-c4551 following topical and intravenous delivery should help clarify these mechanisms. The physicochemical properties of VIAN-c4551 should also be addressed. Its small size, solubility in water, high permeability, and resistance to proteolysis very likely contribute to its efficient intraocular migration and 5-hour half-life in the inner ocular tissues. Finally, the relatively high retina and choroid levels of VIAN-c4551 after eye drop merit in-depth toxicological evaluation, including caspase-3 activation and the use of broader markers of cell death or inflammation to rule out putative off-target effects.

In summary, our findings suggest the feasibility of the topical self-administration of VIAN-c4551 in an outpatient setting that would represent a breakthrough in ophthalmology. Currently, no topical ocular formulations are available for treating microvascular retinal diseases. Several small molecules have undergone clinical trials but have not proceeded to the market despite having successful preclinical data^{6,44}. The many reasons for failure include assuming the direct translation of positive results in rodents and rabbits to patients, which is limited by the anatomical and physiological differences between their eyes and the human eye^{31,45,46}. However, the high concentrations of VIAN-c4551 achieved in the posterior segment of the eye indicate that its topical use for treating retinal diseases may be possible. This possibility is further acceptable due to the nature of VIAN-c4551 as an analog of vasoinhibin, an endogenous protein restraining the vascularity of ocular tissues^{9,47} that is downregulated in retinopathy of prematurity¹², DR¹⁴, and DME^{15,16}. Furthermore, the protective action of VIAN-c4551 against a broad range of vasoactive substances could benefit patients unresponsive to VEGF-therapy and its non-invasive route of administration offers the possibility of early intervention, a limitation of the anti-VEGF treatments typically reserved for the more severe stages of the disease⁴⁸. Detailed physicochemical characterization, evaluation of safety profiles, and incorporation of large-scale production requirements warrant further research.

Data availability

All data generated or analyzed during this study are included in this published article.

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Author contributions

EAC and MZ designed, performed most research, and interpreted data. DGC, LMSM, JFGR, FM, and EA performed research. TB and JT contributed reagents. GME supervised and revised the study. JPR and CC conceived research, designed and supervised the study, interpreted the data, and wrote the manuscript. All authors reviewed and approved the manuscript.

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Declarations

Competing interests

JPR, MZ, TB, JT, GME, and CC are inventors of a submitted patent application (WO/2021/098996), which is owned by the Universidad Nacional Autónoma de México (UNAM) and JT. JPR is the CEO and founder of VIAN Therapeutics Inc. MZ and CC are consultants for VIAN Therapeutics. Inc.

Additional information

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