Evaluation of a connexin-based peptide for the treatment of age-related macular degeneration

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ABSTRACT

A critical target in age-related macular degeneration (AMD) is the retinal pigment epithelium (RPE), which forms the outer blood-retina barrier (BRB). RPE-barrier dysfunction might result from the disruption of intercellular tight junctions (TJs). A Connexin43 (Cx43)-based peptide, aCT1, has been shown to prevent VEGF-induced loss of transepithelial resistance, choroidal neovascularization (CNV) and RPE-cell damage via the stabilization of TJs. Here, we probe the relative efficacies of aCT1 alone, anti-VEGF alone, and aCT1 with anti-VEGF in treating AMD pathologies. aCT1 monotherapy administered as topical eye drops with and without a VEGF blocking antibody administered systemically was tested in a mouse model of laser-induced CNV. The CNV mouse is the standard neovascular AMD model, reproducing hallmarks of its pathology. CNV lesion size and fluid accumulation were assessed using optical coherence tomography. During the angiogenesis phase of CNV lesion development, single applications of anti-VEGF or aCT1 reduced lesion and fluid dome size equally. The combinatorial aCT1/anti-VEGF strategy demonstrated lack of additive effects in this model. These data suggest that TJ stabilization by aCT1 is effective in ameliorating RPE dysfunction in a model of AMD-like angiogenesis, and that this strategy is as effective as the current clinical standard of care, anti-VEGF therapy. Critically, aCT1 holds potential as a new neovascular AMD treatment that can be administered using eye drops, which is preferable to the intravitreal injections required for standard anti-VEGF therapy.

1. Precis

AMD is tied to RPE-barrier dysfunction, and ocular injury is reduced by stabilizing tight junctions with a Connexin43 mimetic peptide, aCT1. We tested aCT1 as monotherapy and in combination with anti-VEGF and identified equivalent and non-additive beneficial effects in a mouse model of AMD.

2. Manuscript

Age-related Macular Degeneration (AMD) is a progressive retinal degenerative disease manifested by loss of central vision. AMD is the leading cause of vision loss in individuals >50 years in the United States, with the prevalence of AMD increasing due to increased life expectancy (Pennington and DeAngelis, 2016). Clinically, two forms of AMD exist. While dry AMD is more prevalent, wet AMD is responsible for more than 90% of legal blindness. Dry AMD is characterized by photoreceptor loss in the macula, the deposition of large drusen, and retinal pigment epithelium (RPE) cell damage. Wet AMD is associated with abnormal blood vessels growth through the blood-retina barrier (BRB) into the retina that leak blood and fluid in the macular region. In both forms of AMD, pathological events involve the breakdown of the RPE/Bruch’s membrane. RPE breakdown is thought to be mediated by elevated levels of vascular endothelial growth factor (VEGF) in wet, and possibly in dry AMD (Ablonczy et al., 2014; Cachafeiro et al., 2013).

Current FDA-approved strategies for the treatment of wet AMD are focused on the reduction of VEGF (Khalil et al., 2019); whereas dry AMD is treated using antioxidants with limited success (Ammar et al., 2020; Kowluru and Zhong, 2011). Anti-VEGF drugs require administration by intravitreal injection, which is invasive and uncomfortable for patients, and carries serious short- and long-term risks (Tezel and Kaplan, 2007).

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Connexins (Cxs) are the primary protein component of gap junctions (GJs), which play critical roles in organ development and differentiation, as well as in coordinating cellular activity required for metabolic homeostasis and inflammatory responses (Delvaeye et al., 2018). GJ channels mediate intercellular communication and the diffusion of ions and metabolites between connecting cells (Niessen et al., 2000; Saez et al., 2003), whereas tight junctions (TJs) regulate the flow of molecules between the apical and basolateral compartments, controlling paracellular permeability and communication. αCT1 is a peptide therapeutic that modulates the activity and signaling pathways mediated by the transmembrane GJ protein Connexin43 (Cx43). It is comprised of a compact two-domain design based on linkage of an antennapedia (a cellular membrane transport peptide) internalization domain (1-16aa; RQPKIWFPNRRKPWKK) to the carboxyl-terminal (CT) PDZ-2 binding domain of Cx43 (17-25aa; RPRPDDLEI), that enables cellular translocation following topical application without requirement for excipients (Ghatnekar et al., 2009; Hunter et al., 2005).

αCT1’s proposed mechanism of action is to mimic the C-terminal cytoplasmic regulatory domain of Cx43, thereby disrupting Cx43 interconnection with its binding partners, including the TJ scaffolding protein zonula occludens-1 (ZO-1) (Figure 1A). This leads to a transition of cell-surface Cx43 from non-junctional (hemichannel) to GJ intercellular channels (Rhett et al., 2011). The result is stabilization of GJ (intercellular communication) as well as TJs (intercellular contacts) (Figure 1B) leading to increased coordination of cellular communication, dampened inflammatory responses, reduction in the infiltration of neutrophils, enhanced wound re-epithelialization and reduced formation of excess fibrous connective tissue (Rhett et al., 2011; Ghatnekar et al., 2009; Soder et al., 2009). The ability of αCT1 to restore an efficient and effective wound healing response has been demonstrated in human clinical trials of acute and chronic skin wounds, where treatment with αCT1 accelerated wound closure and prevented fibrosis (Ghatnekar et al., 2015; Grek et al., 2015, 2017).

In a previous publication, we explored the hypothesis that “targeting ZO-1 signaling using αCT1 would maintain BRB integrity and reduce RPE pathophysiology by stabilizing gap- and/or tight-junctions” (Obert et al., 2017). Specifically, we showed in vitro that loss of barrier function in epithelial cells using VEGF in human ARPE-19 cells or calcium-chelation in canine MDCK cells could be prevented by αCT1, and that this effect was based on αCT1 stabilizing TJs (Obert et al., 2017). In addition, in vivo studies using mouse models that mimic aspects of AMD suggest that αCT1 delivered via eye drops reaches the RPE, where it was shown to reduce choroidal neovascularization (CNV) lesion size and fluid accumulation as well as prevent damage in the perilesion area of the RPE surrounding the CNV lesion. Treatment with αCT1 also stabilized the RPE after bright light exposure as determined by ZO-1 analysis in flatmounts (Obert et al., 2017). CNV and bright light exposure are both VEGF-dependent models of RPE damage (Cachafeiro et al., 2013; Campa et al., 2008). Here, we will build upon these data and compare αCT1 monotherapy in combinational strategies with a VEGF blocking antibody in the laser-induced mouse model of CNV.

Pigmented C57BL/6J mice were based on Jackson Laboratory breeding colonies, in housed in the Medical University of South Carolina animal care facility under a 12-hour light/12-hour dark cycle with access to food and water ad libitum. All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, were approved by the Medical University of South Carolina Institutional Animal Care and Use Committee under Protocol # IACUC-2021-01296. The αCT1 peptide (Xequel Bio, Inc., Mount Pleasant, SC) was administered daily, starting on Day 0 (day of CNV lesion) via eye drops (5 mM, 10 μL per mouse eye) formulated in sterile 0.9% NaCl. The control group received the vehicle solution (0.9% NaCl). αCT1 delivered by eyedrops has been shown to successfully reach the RPE in mouse models of AMD (see Figure 1 in Obert et al., 2017). Anti-mouse VEGF neutralizing antibody (Biologend, #512808) was administered as described by Campa and co-workers in their study describing reduction of CNV in mouse after systemic administration (Campa et al., 2008). Anti-VEGF was administered on Days 0, 2, and 4, based on our successful use of other blocking antibodies in the mouse CNV model (Coughlin et al., 2016). The blocking antibody was administered intraperitoneally (5 mg/kg), and equal amounts of an isotype control antibody (Biolegend, #400544) was used as a control. Here, the systemic route for anti-VEGF antibody treatment was chosen to match the non-invasiveness of the αCT1 eyedrops, as feasibility for the intraperitoneal approach has already been established (Campa et al., 2008), whereas intravitreal injections into mouse eyes have been shown to trigger production of growth factors (Faktorovich et al., 1990) or pro-inflammatory cytokines (Naguib et al., 2021). The animal model of

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**Figure 1.** αCT1’s proposed mechanism of action (A) αCT1 disrupts the binding of the tight junction (TJ) protein, zonula occludens-1 (ZO-1), resulting in the translocation of Connexin43 (Cx43) hemichannels from the perinexus region to sequestration in gap junctions (GJs), thereby reducing hemichannel density and availability for activation within the cell membrane. (B) Materials and methods diagram: 1. αCT1 binding to ZO-1 2. Released ZO-1 from Cx43 hemichannels. 3. Resulting in Cx43 sequestration to GJ plaques, reduction in inflammatory signaling, and preserved TJ integrity.
choice is the mouse choroidal neovascularization (CNV) model, which has been used by us (Obert et al., 2017; Rohrer et al., 2009) and many others to evaluate novel treatment strategies (Fabian-Jessing et al., 2022). To induce CNV lesions, 3- to 4-month-old female and male mice (20–25 g of body weight) were anesthetized (20 mg/kg xylazine and 80 mg/kg ketamine). Pups were diluted using 2.5% phenylephrine HCL and 1% atropine sulfate. To avoid cataract formation, mice were treated with Goniovisc (HUB Pharmaceuticals, Rancho Cucamonga, CA) before and after laser treatment. Laser photocoagulation was induced via a 532 nm Argon laser to generate four laser spots per eye bilaterally (100 μm spot size, 0.1 s duration, 100 mW, 4 spots around the optic nerve at a distance of 2–3 optic disk diameters). Inclusion criteria for successful CNV lesion placement included bubble formation and lack of bleeding as described previously by our lab (Parsons et al., 2019). On Day 5, CNV lesion size and size of the fluid dome were determined using optical coherent tomography (OCT) (Parsons et al., 2019; Woodell et al., 2013), using a spectral domain (SD)-OCT system (Bioptigen Inc., Durham, NC) optimized for the use in small animals. Scan parameters were set to 1.6 × 1.6 mm rectangular volume scans, consisting of 100 B-scans (1000 A-scans per B scan). Several horizontal and vertical images were taken per fundus to allow for assessment of the four CNV lesions. Two features were extracted from the images; area of the lesion and fluid dome. First, the center of the lesion was determined (green line in images in Figure 2) by identifying the midline of the RPE/Bruch’s membrane rupture. At the center, the cross-sectional area of the hypo-reflective spot seen in the fundus image (en face) represents the area of the lesion, whereas on the corresponding vertical section the volume of the fluid dome (3-D measurements of the fluid dome based) can be examined. ImageJ software (http://imagej.nih.gov/ij/) was used for quantification. Giani and colleagues have confirmed that the OCT lesion area correlates well with flatmount preparation and ex vivo CNV volume quantification (Giani et al., 2011). Data are presented as mean ± SEM. Single comparisons were analyzed by t-test analysis, accepting a significance level of P < 0.05; multiple comparisons were analyzed by ANOVA post-hoc analysis with Bonferroni correction for multiple comparisons. We have shown previously that aCT1 treatment reduced mouse laser-induced CNV development and fluid leakage as determined by OCT, and that this damage was correlated with disruption in cellular integrity of surrounding RPE cells (Obert et al., 2017). As AMD is associated with increased VEGF secretion and aCT1 reduces VEGF-dependent RPE dysfunction (Obert et al., 2017), we were interested in whether aCT1 would act in synergy with or as an alternative to anti-VEGF treatments. Therefore, we compared aCT1 as monotherapy or in combinatorial strategies with a VEGF blocking antibody. Mice were assigned to one of four groups (n = 6–8 per group), receiving either (1) isotype control antibody + vehicle eye drops; (2) isotype control antibody + aCT1 eye drops; (3) anti-VEGF antibody + vehicle eye drops; or (4) anti-VEGF antibody + aCT1 eye drops. Optimal aCT1 concentration has been evaluated previously (Obert et al., 2017), and blocking antibody concentration was determined from the literature (Campa et al., 2008). On Day 5, OCT was determined from the literature (Campa et al., 2008). On Day 5, OCT was used to assess lesion sizes as published previously (Schnabolk et al., 2018). Previous preclinical studies in animal and cell-based models studying VEGF-dependent RPE pathophysiology in the eye have demonstrated aCT1’s ability to mitigate injury. aCT1 reduced CNV lesion size and fluid leakage in the mouse wet AMD model, and reduced disruption of cell morphology in the perillesion area of the RPE surrounding the CNV lesion in the RPE of the light-damaged mouse (Obert et al., 2017). Likewise, in epithelial cell monolayers in vitro, we confirmed that aCT1’s mechanism of action is to prevent VEGF-mediated loss of barrier function by stabilizing TJs (Obert et al., 2017). In endothelial cells, in addition to aCT1’s effect on TJs, aCT1 has been shown to also stabilize GJs during the wound healing processes, leading to coordination of cellular communication, dampened inflammatory responses, and reductions in neutrophil infiltration and fibroblast proliferation (Ghatnekar et al., 2009; Ghatnekar GS, 2006; Gourdie et al., 2006; Gourdie et al., 2006; Ghatnekar GS, 2006; Gourdie et al., 2006; Rhett et al., 2008). While our previously published results suggest that aCT1 prevents the increase in VEGF-induced RPE damage and thereby reduces the number of newly formed blood vessels entering the eye, the question of whether aCT1 and anti-VEGF would act synergistically has not been evaluated to date. To assess the synergy between aCT1 and anti-VEGF therapeutics, we compared aCT1 as a monotherapy or in combination with a VEGF blocking antibody, using our published effective aCT1 concentration (Obert et al., 2017) and the blocking antibody concentration effective in reducing CNV (Campa et al., 2008). aCT1, anti-VEGF antibody, and aCT1 + anti-VEGF antibody treatments were found to significantly reduce the growth of the CNV lesion as well as fluid accumulation equally, and in a non-additive fashion. We acknowledge that this short study has some limitations, as it relies exclusively on OCT imaging data to analyze mouse CNV and fluid dome size, without adding measurements of actual leakage (e.g., fluorescein angiography) or effects on VEGF. In addition, while we have documented that aCT1 can be detected in the RPE upon eye drop application, we have not yet analyzed where, within the eye aCT1 accumulates, or whether aCT1 is delivered to the RPE via the classical route through the eye or the transscleral route (Loscher et al., 2022). Hence, indirect effects of aCT1 mediated by cells other than RPE, might contribute to the results. However, these data further underscore the therapeutic potential of aCT1 in AMD and support validation of its efficacy in large animal preclinical models, to enable advancement to clinical evaluation.

In terms of limiting AMD pathogenesis, the effects of aCT1 might be two-fold: (1) stabilizing TJs in the RPE appears to prevent growth of the lesions; and (2) modulating Cx43 binding and activity in the choroid might reduce fluid leakage and inflammation. TJs between endothelial cells comprising the inner BRB, as well as RPE cells forming the outer BRB, are essential to retinal homeostasis and have a contributory role in the development of AMD (Hudson et al., 2019; Matusbara et al., 2020). In the choroid, elevated levels of Cx43 have been reported in response to light-damage, where it colocalized with markers of inflammation (Guo et al., 2014). Immune system dysregulation is fundamentally linked to the development and progression of AMD, where accumulation and activation of resident microglia and recruited macrophages contribute to the pathogenesis of AMD, in part through promotion of pro-inflammatory and angiogenic components (Ambati et al., 2013; Fernando et al., 2016; Karlstetter et al., 2015). It is possible that aCT1 binding to ZO-1 releases ZO-1 from intracellular Cx43, possibly promoting ZO-1 to interact with Claudins and occludins by exposing the PDZ-1 domain (Spadaro et al., 2017) (Figure 1). Stabilizing ZO-1 at the plasma membrane prevents TJ degradation in response to injury (Obert et al., 2017). By stabilizing TJs and tempering pro-inflammatory activities of hemichannels, aCT1 targets the underlying molecular and cellular mechanisms that modulate the injury response and mitigate inflammation. However, additional experiments are needed to further delineate these pathways. Treatment with aCT1 eye drop offers the therapeutic opportunity to support barrier function, thereby reducing immune cell infiltration and fluid leakage, as well as precluding induction of pro-inflammatory signaling that initiates and exacerbates AMD pathogenesis. Importantly, the non-additive behavior between aCT1 and
Figure 2. Choroidal neovascularization and fluid leakage in mice treated with aCT1 and anti-VEGF. Animals were analyzed post laser-photocoagulation by SD-OCT. aCT1 eye drops were examined as monotherapy and in combination with systemic anti-VEGF antibody treatment. Vehicle eye drops and isotype control antibodies were co-administered in the indicated group (A) The cross-sectional area of the hyporeflective spot seen in the en face fundus image (left column) as well as the area of fluid accumulation in the outer retina obtained from vertical sections (right column) were determined, and representative OCT images, reflecting the mean of the data taken of the animals treated with vehicle/isotype (1st row), aCT1/isotype (2nd row), vehicle/anti-VEGF (3rd row), or aCT1/anti-VEGF (4th row) are shown. CNV area and fluid accumulation were determined from SD-OCT images. Please note that the green lines depict the center of the lesion; in images with decentralized or no green lines, the green line is from the adjacent CNV lesion in the same focal plane and image (B) Quantification of the cross-sectional areas of the lesions (top graph) as well as area of fluid accumulation (bottom graph) were measured in pixels and converted into μm for the individual treatment groups. CNV lesion size and area of fluid accumulation in the three treated groups were significantly reduced, compared to the vehicle/isotype group. No significant differences were noted between the three treatment groups. Data are expressed as mean ± SEM (n = 6–8 animals per treatment group) (*P < 0.05).
anti-VEGF suggest that aCT1 eye drops alone could be used to substitute for anti-VEGF therapeutics.

Based on these and previous studies, the proposed mechanism of action for aCT1 is based on the modulation of the interaction between Cx43 and its CT-binding partners, including ZO-1 (Jiang et al., 2019; Rhett et al., 2011). ZO-1 regulates the cellular distribution of Cx43, providing a control point for dynamic switching between GJ communication and hemichannel communication, and is also a critical TJ protein (Rhett et al., 2011). aCT1 binds with high specificity to the PDZ-2 domain of ZO-1 and competitively inhibits Cx43 and ZO-1 interactions, thus favoring a transition of Cx43 from hemichannels to GJ channels (Zhu et al., 2005). Cx43 hemichannels play critical roles in providing a paracrine route for intercellular communication, regulating the release of molecules such as NAD+, ATP, glutamate, prostaglandin E2, and glutathione (Decrock et al., 2009). Oxidative stress can induce Cx43 hemichannels to open, leading to a loss of ionic homeostasis, metabolic stress, and cellular apoptosis (Decrock et al., 2009). ATP is a critical extracellular messenger with functions that include promoting proinflammatory responses such as leukocyte chemotaxis, NO generation, cytokine release, and cytotoxicity (Rhett et al., 2014). Furthermore, studies suggest that Cx43 hemichannel activity is reduced by inhibition of the Cx43/ZO-1 interaction with aCT1 (Rhett et al., 2011). The closing of hemichannels leads to a reduction in pro-inflammatory cytokines and cell death (Belousov et al., 2017). Concomitantly, the association of ZO-1 with TJs leads to a coordinated stabilization effect of not only GJs, but also a reinforcement of TJ integrity, that together might reduce the excessive inflammatory remodeling/repair processes and enables a shift towards healthy regenerative healing (Balda and Matter, 2016). Thus, aCT1 presents a novel therapeutic opportunity in neovascular AMD. Because of the challenges associated with anti-VEGF drugs, like patient compliance and severe long-term effects, the translational opportunity for aCT1 eye drops is particularly attractive.

Declarations

Author contribution statement

Elisabeth Obert: Performed the experiments; Analyzed and interpreted the data.

Christina Greš: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Gautam Ghatnekar: Contributed reagents, materials, analysis tools or data.

Bärbel Rohrer: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interest’s statement

The authors declare the following conflict of interests: Funding for this project was partially provided by a subaward to MUSC from Xequel Bio, Inc (formerly FirstString Research, Inc.), as a means of investigating translational research models for the aCT1 peptide and to determine the peptide’s method of action (SBIR grant R43EY028072). Xequel Bio, Inc. holds the exclusive patent for aCT1 (US 7786074; US 7883319; US 8357668). BR and GG are inventors of the patent for composition and methods for use in treating or preventing macular degeneration (US2014018305A1). CG is an employee of Xequel Bio. EO has no financial or non-financial competing interests to disclose.

Additional information

No additional information is available for this paper.

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