Xentry-Gap19 inhibits Connexin43 hemichannel opening especially during hypoxic injury

Frazer P. Coutinho 1,2 · Colin R. Green 2 · Monica L. Acosta 3 · Ilva D. Rupenthal 1,2

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Abstract
Hypoxic injury results in cell death, tissue damage and activation of inflammatory pathways. This is mediated by pathological Connexin43 (Cx43) hemichannel (HC) opening resulting in osmotic and ionic imbalances as well as cytokine production perpetuating the inflammatory environment. Gap19 is an intracellularly acting Cx43 mimetic peptide that blocks HC opening and thus promotes cell survival. However, native Gap19, which must enter the cell in order to function, exhibits low cell permeability. In this study, Gap19 was conjugated to the cell-penetrating peptide, Xentry, to investigate if cellular uptake could be improved while maintaining peptide function. Cellular uptake of Xentry-Gap19 (XG19) was much greater than that of native Gap19 even under normal cell culture conditions. Peptide function was maintained post uptake as shown by reduced ethidium homodimer influx and ATP release due to Cx43 HC block. While XG19 blocked pathologic HC opening though, normal gap junction communication required for cell repair and survival mechanisms was not affected as shown in a dye scrape-load assay. Under hypoxic conditions, increased expression of Syndecan-4, a plasma membrane proteoglycan targeted by Xentry, enabled even greater XG19 uptake leading to higher inhibition of ATP release and greater cell survival. This suggests that XG19, which is targeted specifically to hypoxic cells, can efficiently and safely block Cx43 HC and could therefore be a novel treatment for hypoxic and inflammatory diseases.

Keywords  Cell-penetrating peptide · Connexin43 · Hemichannel · Mimetic peptide · Syndecan-4 · Hypoxia · Xentry · Gap19

Introduction
Hypoxia is a major detrimental factor in ischaemic diseases such as stroke and vascular eye conditions, where the blood flow to tissues and organs is reduced resulting in limited oxygen supply [1]. The events occurring during hypoxia are worsened by sudden reperfusion which is referred to as ischaemia-reperfusion injury [2]. Hypoxia is often associated with the production of pro-inflammatory cytokines as well as the overexpression of proteins such as vascular endothelial growth factor (VEGF), Connexin43 (Cx43) and Syndecan-4 [2–8]. In neovascular age-related macular degeneration (nAMD), for example, unregulated growth of poorly formed blood vessels, known as choroidal neovascularization, results in haemorrhage within the retina leading to tissue ischaemia [9, 10]. To compensate for the disruption in blood/oxygen supply, VEGF is overexpressed by the retinal pigment epithelium (RPE), which contributes to the blood-retinal barrier (BRB) between the vascular choroid and the neural retina [11, 12]. This VEGF overexpression perpetuates the formation of leaky blood vessels [11, 12], which introduces more inflammatory factors to the environment, increases Cx43 expression and causes RPE cell death due to hypoxia, ultimately permitting blood vessel growth into the retina and leading to vision loss.

Cx43 hemichannel (HC) blockers have been shown to prevent vessel leak, support repair of leaky blood vessels and promote tissue repair in numerous animal models [2, 13, 14]. Cx43 is responsible for the formation of gap junctions [15, 16], which mediate communication between cells by
permitting the passage of small molecules for homeostatic processes such as growth, repair and survival. Six connexin monomers form a HC which undocked under normal conditions is closed, while docking of two HC from neighbouring cells results in the formation of a gap junction which opens during physiologic conditions to allow exchange of cellular contents [16–18]. During pathology, however, normally closed, undocked HC are stimulated to open to the extracellular environment eventually resulting in cell death [19–23]. Sudden tissue reperfusion during open Cx43 HC states drastically increases cell death and tissue damage as cells are unable to cope with the rapid ionic influx. In chronic hypoxic or inflammatory conditions, Cx43 HC have been referred to as “pathologic pores” as they are responsible for the activation of the inflammatory cascade via the nod-like receptor family pyrin domain containing 3 (NLRP3) inflammasome complex leading to the production of inflammatory cytokines and thus perpetuating the inflammatory environment [14, 24–26]. Blocking open Cx43 HC during injury using Cx43 mimetic peptides such as Gap27 and Pepide5 has been shown to promote cell survival and tissue repair in cardiac, spinal cord injury and ocular models [27, 28]. However, one concern with these peptides is their action on external motifs of Cx43, potentially affecting gap junction function required for cell survival when used at high concentrations and/or long exposure periods [29–31].

Gap19 is a HC blocker derived from the second cytoplasmic loop of Cx43 which does not interfere with gap junction function. However, it requires entering the cell in order to bind to the corresponding sequence of the cytoplasmic tail of Cx43 [32]. Due to its poor cell penetration, high concentrations have previously been used but with limited efficacy [32, 33]. Cell-penetrating peptides (CPP) are an efficient way of transporting cargo molecules across the cell membrane. The CPP Xentry is derived from the X-protein of the hepatitis B virus and has been shown to efficiently transport a range of molecules into cells via endocytic mechanisms by binding to cell surface–expressed Syndecan-4 [34]. As Syndecan-4 is not expressed on circulating monocytes and erythrocytes, sequestration by the circulation, if delivered systemically, is prevented [34], while uptake into Syndecan-4 overexpressing cells is increased. This study investigated whether conjugation of Xentry to Gap19 (XG19) can increase the cellular uptake of Gap19 to efficiently block Cx43 HC–mediated injury in hypoxic cells at low peptide concentrations.

Materials and methods

Materials

Xentry-Gap19 (XG19) (IclrpvGGKQIEIKFK), Gap19 (KQIEIKFK) and FITC-labelled peptides at 95% purity were obtained from ChinaPeptide Co., Ltd. Peptide5 (VDCFLSRPTEKT) was purchased from Auspep. Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12; GlutaMAX™ medium), EGM-2™ BulletKit™ medium, foetal bovine serum, antibiotic-antimycotic (AA), ethidium homodimer-1 (EthD-1), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Lucifer Yellow were purchased from Thermo Fisher Scientific. Carbenoxolone (CBX), 4’6-diamidino-2-phenylinodole (DAPI) and formaldehyde were obtained from Sigma-Aldrich. Constituents of high calcium solution/Hank’s balanced salt solution (HBSS) (0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na2HPO4, 5.6 mM glucose, 0.44 mM KH2PO4, 1.3 mM CaCl2, 1.0 mM MgSO4 and 4.2 mM NaHCO3 in 100 ml of ultrapure water); low calcium solution (0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na2HPO4, 5.6 mM glucose, 0.44 mM KH2PO4, 1.0 mM MgSO4, 4.2 mM NaHCO3 and 5 mM EGTA in 100 ml of ultrapure water); and hypoxic, acidic, ion-shifted Ringer (HAIR) solution (38 mM NaCl, 13 mM NaHCO3, 3 mM Na-glucanate, 65 mM K-glucanate, 38 mM N-methyl-d-glucamine, 1 mM Na2HPO4 and 1.5 mM MgCl2 in 100 ml of ultrapure water) [35, 36] were of analytical grade. Immortalized human retinal pigment epithelium cells (ARPE-19) were purchased from American Type Culture Collection (ATCC), while primary human retinal microvascular endothelial cells (hRMEC) were obtained from Neuromics.

Cell seeding and imaging

ARPE-19 or hRMEC were harvested with TrypLE™, centrifuged at 1500 rpm for 7 min, resuspended in culture medium (for ARPE-19 cells: DMEM/F-12; GlutaMAX™ medium with 10% FBS and 1% AA; for hRMEC: EGM-2™ BulletKit™ medium containing endothelial basal medium (EBM-2 and EGM-2 SingleQuots™ (excluding VEGF) with 10% FBS and 1% AA) and counted using trypan blue and the Neubauer haemocytometer. Cells were plated into appropriate culture dishes (8-well chamber slides, 6-, 12-, 24- or 96-well plates) at a seeding density of 2 × 105 cells/ml and were incubated with the relevant culture medium over two nights at 37 °C with 5% CO2 before conducting the various assays. Images were acquired using an Olympus BX-10 microscope with a FV-1000 confocal laser scanning system and Olympus FV-10 software with area measurements performed using ImageJ. Statistical analysis was carried out using GraphPad Prism 7 software with the test used stated in each figure caption.

Cellular uptake

FITC-labelled Gap19 and XG19 were dissolved in culture medium and applied to ARPE-19 or hRMEC in 8-well
chamber slides at 5, 10, 20, 50 and 100 μM for 1 h at 37 °C with 5% CO₂. For cell uptake under hypoxic conditions, peptides were mixed with HAIR solution at 5, 10 and 20 μM instead of normal culture medium. After 1 h, the medium was removed for both normal and hypoxic uptake assays, cells were washed with PBS and fixed in 4% formaldehyde in PBS and nuclei were counterstained with DAPI (diluted 1:1000 in 1% PBS). Coverslips were mounted in anti-fade medium (Citifluor™ AF1), and cells were visualized using a confocal microscope.

**Syndecan-4 and Cx43 labelling**

Medium was replaced with either fresh culture medium or HAIR solution, and cells were incubated for 1, 3, 6 or 24 h at 37 °C with 5% CO₂. Cells were then washed with PBS and fixed in 4% formaldehyde/PBS at room temperature for 10 min. Cells were again washed with PBS and labelled with primary antibody (anti-Syndecan-4 raised in goat (1:5; RDSAF2918; R&D systems) or anti-Cx43 raised in rabbit (1:2000; C6219; Sigma-Aldrich) in PBS overnight. The next day, excess primary antibody was washed away with PBS before applying the secondary antibody (anti-goat 488 raised in donkey (1:100; ab150155; Abcam) or anti-rabbit 488 raised in goat (1:500; A11034, Thermo Fisher Scientific) and DAPI (1:1000) in PBS for 1 h at room temperature in a humid box. Coverslips were mounted in anti-fade medium (Citifluor™ AF1), and cells were visualized using confocal microscopy.

**Cell viability post XG19 uptake**

XG19 was mixed with culture medium or HAIR solution and applied to ARPE-19 cells seeded into 96-well plates at 5, 10 or 20 μM for either 1 or 24 h at 37 °C with 5% CO₂. After the incubation period, solutions in each well were replaced with 0.5 mg/ml of MTT in PBS and incubated for 4 h at 37 °C with 5% CO₂. The MTT/PBS solution was then replaced with hydrochloride-isopropanol solution (0.04 M) to dissolve the formed formazan. The intensity of the purple colour was quantified at 570 nm with correction of interference at 650 nm (BioTek Synergy HT). Each group was tested in triplicate (three wells of a 96-well plate) on two separate occasions.

**EthD-1 uptake**

XG19 (5 μM) or FITC-XG19 (5 μM) mixed with culture medium was applied to ARPE-19 cells seeded into 8-well chamber slides and incubated for 1 h at 37 °C with 5% CO₂. High (1.3 mM Ca²⁺, closes Cx43 HC) or low calcium (0 mM Ca²⁺, opens Cx43 HC and allows dye uptake) solution containing 2 μM of EthD-1 fluorescent dye was applied to cells for 40 min. Solutions were removed, cells were washed in PBS and fixed in 4% formaldehyde in PBS and nuclei were counterstained with DAPI. Coverslips were mounted in anti-fade medium (Citifluor™ AF1), and cells were visualized using a confocal microscope.

**ATP release**

XG19 (5, 10, 20 μM), Peptide5 (20 μM) or CBX (100 μM) were mixed with either culture medium or HAIR solution and applied to ARPE-19 cells seeded in 12-well plates for 0, 1 or 24 h at 37 °C with 5% CO₂. The medium was then removed, and low calcium solution was applied to the cells for 35 min at 37 °C with 5% CO₂. Solutions were removed and transferred into a black 96-well plate, and ATP measurements were performed as per the instructions of the ATPlite kit (no. 6016943; Perkin Elmer) using a Victor X Light luminescence plate reader.

**Dye scrape-load gap junction assay**

XG19 and CBX were mixed with culture medium and applied to ARPE-19 cells seeded in 8-well plates at 5 and 100 μM for 1 h at 37 °C with 5% CO₂. Solutions were removed, and cells were exposed to 0.1% Lucifer Yellow in high calcium solution for 15 min at room temperature after a horizontal scrape was created in the cell monolayer using a pipette tip. The Lucifer Yellow solution was removed, cells were washed in PBS and fixed in 4% formaldehyde in PBS and nuclei were counterstained with DAPI. Coverslips were mounted in anti-fade medium (Citifluor™ AF1), and cells were visualized by a confocal microscope. To quantify dye transfer using Image J, image measurement scales were set to pixels and areas of Lucifer Yellow dye spread were selected using the colour threshold feature to separate fluorescent from non-fluorescent cells and background. Statistical analysis was carried out using Prism GraphPad software.

**Results and discussion**

**Cellular uptake**

In nAMD, choroidal endothelial cell damage results in vascular permeability and loss of BRB integrity due to RPE cell death [37, 38]. Primary hRMEC and immortalized ARPE-19 cultures present similar physiological characteristics to the inner and outer BRB, respectively [39–41], while also expressing functional Cx43 HC. They are thus ideal to assess the efficacy of Cx43 HC blockers [25, 42] as a potential treatment for nAMD or similar indications. Cx43 HC inhibition has been shown to reduce vascular permeability, inflammation and cell death in numerous ocular disease models [2, 13, 25, 36, 42–45]. However, in order to block Cx43 HC opening,
Gap19 must enter the cell and gain access to the cytoplasmic tail of Cx43 to inhibit intracellular loop-tail interactions [46]. Since native Gap19 has limited cell permeability, we investigated the uptake of Xentry-conjugate Gap19 (XG19) in comparison with the native peptide (both tagged to FITC) in hRMEC and ARPE-19 cells (Fig. 1a, b, respectively).

As can be seen, XG19 uptake occurred in a dose-dependent manner as observed by increasing FITC fluorescence with increasing XG19 concentrations in both hRMEC and ARPE-19 cells (Fig. 1a, b, respectively). Using native Gap19, no fluorescence was visible at concentrations below 50 μM in hRMEC and 100 μM in ARPE-19 cells suggesting that the native form was unable to enter the cells at low concentrations. The amount of uptake observed at 10 and 20 μM of XG19 was far greater than that of Gap19 at 100 μM in either cell culture, confirming that the addition of the CPP, Xentry, to Gap19 improved peptide cellular uptake even under normal culture conditions.

Previous attempts to observe in vitro function of native Gap19 have necessitated high peptide concentrations with a half maximal Cx43 HC opening effect seen at 142 μM in astrocytes compared with 47 μM in C6 (glioma) cells [32, 46]. Native Gap19 itself has some cell penetration ability due to the four charged lysine residues in its sequence but seemed to better enter C6 cells compared with astrocytes. In a similar manner, native Gap19 was able to enter hRMEC marginally better than ARPE-19 cells. This could be due to the greater surface area available in hRMEC cultures compared with ARPE-19 cells which form tight junctions thus achieving in ARPE-19 compared with that in hRMEC which could be related to the amount of surface-expressed Syndecan-4 present in ARPE-19 cells and normal conditions was responsible for the variations in XG19 uptake observed [34]. ARPE-19 cells and hRMEC also express Cx43 and have been used to assess Cx43 HC opening as well as block with Cx43 mimetic peptides in vitro [25, 42]. Thus, Cx43 levels were also investigated to determine which cell line would be preferable for Cx43 HC functional assays. As shown in Fig. 3, hRMEC and ARPE-19 both expressed Cx43 consistent with previous literature [42, 45, 51] although expression was much greater in ARPE-19 cells. RPE cells naturally form a monolayer in vivo to create the outer BRB between the neural retina and the choroidal vasculature [52, 53]. As a result, ARPE-19 cells also form a more regular monolayer in culture with clearly defined cell boundaries [40, 54]. These boundaries may account for the higher Cx43 gap junction labelling while the homogeneity of the cell structure also renders them more suitable for functional assays.

While Syndecan-4 expression has previously been reported in primary human umbilical endothelial cells, this is the first investigation of Syndecan-4 expression in hRMEC [48]. Syndecan-4 expression was also observed in ARPE-19 cells, consistent with previous literature, and was shown to be higher than in hRMEC [49]. Syndecan-4 plays a diverse role in cell adhesion as well as intracellular and extracellular signalling [55, 56]. In endothelial cells, Syndecan-4 is primarily required for the formation of focal adhesion and endothelial cell alignment to preserve the structure of blood vessels [48, 57]. RPE cells, on the other hand, interact with a number of extracellular molecules such as VEGF, fibroblast growth factor and platelet-derived growth factor to maintain homeostasis of the outer retina [52, 58, 59]. In the RPE, Syndecan-4 can be utilized for cell adhesion by binding fibronectin for the formation of focal adhesions as well as binding of extracellular molecules in order to mediate signalling mechanisms to maintain retinal homeostasis [49, 56, 60]. The diverse function of Syndecan-4 in RPE cells is likely the reason for the higher Syndecan-4 labelling in ARPE-19 cells compared with hRMEC. Overall, being an immortalized cell line that allows for more reliable culturing and with the increased expression of Syndecan-4 and Cx43, ARPE-19 cells were preferred for further uptake and functional studies.

In a second step, Syndecan-4 expression in response to hypoxic injury was assessed over time. In nAMD, RPE cells are exposed to inflammatory cytokines such as TNF-α and IL1-β [25, 61, 62]. Furthermore, vascular permeability results in insufficient perfusion resulting in localized sites of
ischaemia and hypoxia [2, 43]. Both inflammation and hypoxia are known to alter Syndecan-4 expression [8, 48, 63, 64]. In vitro, hypoxia can be induced by the application of HAIR solution which in addition to changes in Syndecan-4 expression also results in higher Cx43 levels as well as increased Cx43 HC opening [35, 36, 42]. As can be seen in Fig. 4a,
Fig. 3 Syndecan-4 and Cx43 labelling as well as XG19 uptake under normal conditions. hRMEC and ARPE-19 cells were labelled for Syndecan-4, as well as Cx43, and were also treated with 50 μM XG19. Syndecan-4, Cx43 and XG19 were labelled in green while nuclei were stained with DAPI (blue). Scale bar, 100 μm

Fig. 2 Uptake of Gap19 and XG19 in normal vs. hypoxic ARPE-19 cells. Normal cells were left untreated or were treated with 100 μM Gap19 or 5 μM XG19. Cells exposed to HAIR solution in order to induce hypoxia were also left untreated or treated with 100 μM Gap19 or 5 μM XG19. Peptides were visualized by a FITC-tag (green), and cell nuclei were stained with DAPI (blue). Scale bar, 50 μm
hypoxic cells expressed higher Syndecan-4 levels from 1 to 6 h compared with normal cultures, with a slight decline at 24 h likely due to cell death caused by over-exposure to HAIR solution, thus reducing the cell density and therefore the overall Syndecan-4 expression levels. Quantitative analysis revealed that Syndecan-4 expression in hypoxic cells was significantly greater than in normal cells at all time points (Fig. 4b), confirming that ARPE-19 cells increase Syndecan-4 expression in response to hypoxia.

Fig. 4 Syndecan-4 expression in normal vs. hypoxic cells over time. ARPE-19 cells were either exposed to normal culture medium for 1, 3, 6 or 24 h or treated with HAIR solution to induce hypoxia for 1, 3, 6, or 24 h (a). Syndecan-4 expression (green) was quantified by measuring the mean fluorescence intensity in four areas of each well (b). Two-way ANOVA was carried out with post hoc Sidak’s test and statistical significance (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001) was represented as a difference in expression to normal cells for each time point (n = 4 mean fluorescence intensity measurements per treatment; mean ± SD). Scale bar, 50 μm
Previous studies have shown that Syndecan-4 expression increased following acute myocardial infarction in humans, especially in repair regions of damaged cardiac tissue compared with undamaged areas, with hypoxia treatment resulting in increased Syndecan-4 gene expression [65]. This suggests that Syndecan-4 upregulation due to hypoxia during myocardial infarction can act as a repair mechanism due to its cell binding and signalling properties [56, 65]. Previous studies have also shown that HIF-1α expression is increased in ARPE-19 cells under hypoxic conditions [7], likely resulting in increased Syndecan-4 expression. HIF-1α is a transcription factor for many angiogenic molecules including VEGF, which is overproduced by RPE cells during ischaemia to promote choroidal blood vessel growth and restore the vascular supply [7, 66, 67]. Furthermore, cultured human RPE cells have been shown to have some resistance to hypoxia-mediated cell death in lowered oxygen environments suggesting a functional role under these conditions [68]. It is likely that overexpression during hypoxia occurs to aid angiogenic pathways for retinal homeostasis [56, 69]. In nAMD, localized sites of ischaemia due to choroidal neovascularization lead to chronic hypoxia in RPE cells stimulating the overproduction of VEGF and perpetuating the disease [1]. Since Syndecan-4 is the target ligand used by XG19 for cellular uptake, increased Syndecan-4 expression in hypoxic RPE cells would allow for targeted delivery to and increased uptake of the peptide into diseased cells [34].

**Cell viability post XG19 uptake**

CPP uptake or storage within cells can cause cytotoxicity due to disruption of the cell membrane during uptake or interference with cellular components post uptake [70, 71]. An MTT assay was performed to observe the short- and long-term metabolic activity and cell viability of ARPE-19 cells post XG19 treatment. Cells were exposed to increasing concentrations (5, 10 or 20 μM) of XG19 for either 1 or 24 h, with untreated cells serving as a positive viability control. As shown in Fig. 5, XG19-treated cells showed no significant difference in viability when compared with untreated cells at both 1 and 24 h, confirming that the initial uptake process of XG19 did have no effect. Furthermore, even 24-h post uptake of XG19, cell viability was not affected, which is consistent with previous literature showing that Xentry uptake does not affect cell viability [34, 72, 73].

In addition to cell viability under normal culturing conditions, we investigated whether Cx43 HC block by XG19 during hypoxia can improve cell survival. As shown in Fig. 6, the viability of cells in HAIR solution was significantly reduced compared with cells in normal medium suggesting that hypoxic cells were experiencing injury due to Cx43 HC opening triggering ionic and osmotic imbalances, ATP release and inflammasome activation [25, 74–76]. It should be noted that the MTT signal does not solely reflect cell viability but may also reflect its metabolic activity [77]. No significant difference in cell viability was seen in XG19-treated hypoxic cells when compared with the untreated cells in normal medium. This confirmed that XG19 prevented a decrease in cell death during hypoxic injury by blocking Cx43 HC opening. The block of ATP release via Cx43 HC inhibition has been shown to reduce retinal injury and promote tissue recovery in rat models of AMD [44, 78]. Therefore, XG19 has great potential to target Syndecan-4 overexpressing cells in nAMD and block Cx43 HC opening and ATP release to promote survival of diseased RPE cells.

**EthD-1 uptake**

The ability of XG19 to inhibit HC opening post cellular uptake was observed using the HC-mediated EthD-1 uptake assay. EthD-1 is a small fluorescent molecule which has classically been used as an indicator of dead cells. It enters dead cells via the compromised cell membrane and binds to the nucleus resulting in a bright fluorescence. When the membrane is intact, small molecules such as EthD-1 can enter cells via open Cx43 HC in a low calcium environment. As such, EthD-1 can be used as an indicator of HC opening by measuring the fluorescence of EthD-1 within intact cells. In addition to native XG19, FITC-labelled XG19 used in the cellular uptake experiments was also tested here to observe if the FITC label itself affects peptide function. Cells exposed to low calcium solution showed increased EthD-1 uptake due to HC opening (Fig. 7a). High calcium solution closed Cx43 HC and therefore inhibited EthD-1 dye uptake. Both XG19 and FITC-XG19 were able to inhibit EthD-1 dye uptake similar to the high calcium control, suggesting that both peptides were available in a biologically active form post cellular uptake. Quantification of mean EthD-1 fluorescence revealed that high calcium (p < 0.0001), XG19 and FITC-XG19 (p < 0.001 for both) resulted in a significant reduction of EthD-1 uptake due to inhibition of HC opening compared with low calcium solution (Fig. 7b). This confirmed that XG19 functioned as a Cx43 HC blocker and was in a bioavailable form post uptake. Previous experiments observing ethidium bromide uptake into brain slices have shown that up to 172 μM of Gap19 had no effect on Cx43 HC function with inhibition only improved when the concentration was increased to 344–688 μM [32], although this may be partially explained by the additional structural barriers to be overcome by the peptide. Nevertheless, our studies have shown that XG19 concentrations as low as 5 μM can block Cx43 HC opening due to the higher uptake efficiency compared with 47 μM and 142 μM of native peptide needed in C6 (glioma) and astrocyte cultures, respectively [32, 46].

**ATP release**

XG19 inhibition of HC-mediated ATP release was compared with the known HC and gap junction blocker CBX [24, 79, 80].
As shown in Fig. 8, low calcium solution alone resulted in maximal ATP release. Statistical analysis revealed that ATP release was significantly reduced in the presence of XG19 ($p < 0.01$) and CBX ($p < 0.0001$) (Fig. 8a). This confirmed that XG19 was able to block ATP release via HC. The greater reduction in ATP release seen with CBX compared with XG19 suggests that CBX is a more efficient Cx43 HC blocker. However, it may also be due to the non-specificity of CBX which blocks multiple channels including other connexin channels as well as pannexins also present in ARPE-19 cells, whereas XG19 is specific to Cx43 HC and thus not interfering with other ATP releasing channels [24, 79, 80]. Previous studies observing blockage of ATP release with Gap19 have used much higher peptide concentrations (142 μM in astrocytes [32] and 47 μM in C6 (glioma) cells [46]) whereas a comparable HC block was achieved with only 5 μM of XG19 in the studies presented here, suggesting improved function of XG19 over Gap19 due to improved cell uptake.

Peptide5, an extracellular acting Cx43 mimetic peptide, has been shown to block the release of ATP via Cx43 HC [25, 36, 78]. Therefore, XG19 inhibition of ATP release was compared with that of Peptide5 (Fig. 8b). In this assay XG19 was applied at the same time as the low calcium solution to observe if XG19 could act immediately, in a similar way to the extracellular acting Peptide5. As shown in Fig. 8b, ATP release was significantly ($p < 0.0001$) reduced in the presence of both XG19 and Peptide5. This showed that XG19 was able to have a similar onset of action as the well-established extracellular loop Cx43 mimetic peptide, Peptide5. This was an interesting finding as Peptide5 immediately acts on the extracellular region of Cx43, whereas the efficiency of XG19 depends on its cell uptake in order to interact with the Cx43 cytoplasmic tail, suggesting that this occurred in a rapid fashion such that intracellular blocking using Xentry as a CPP was as efficient as extracellular blocking [36]. The advantage of XG19 is its ability to maintain gap junction coupling irrespective of dose concentration (discussed in the next section).

To observe if XG19 was still functional several hours post uptake, ATP release was measured 1 and 24-h post uptake. As shown in Fig. 9, XG19 inhibition of Cx43 HC resulted in a significant reduction in ATP release compared with untreated cells at 1 and 24 h. Peptides typically have short half-lives in serum rendering efficient peptide delivery a challenge as the drug must reach its site of action before it is broken down. Therefore, it is interesting to note that XG19 was able to function even 24-h post uptake [81, 82]. Furthermore, Cx43 turnover occurs every few hours depending on the cell type [83].
which alters the kinetics of Cx43 HC blockers [84]. This suggested that XG19 was able to block even newly formed Cx43 HC at the 24-h time point. Taken together, the rapid cell entry may protect XG19 from degradation and internalized XG19 could potentially act as a reservoir until triggered by a Cx43 HC opening event such as hypoxia, thus improving the long-term efficacy of the peptide.

In addition to ATP release under normal culture conditions, we also investigated ATP release during hypoxia. Peptide5, the extracellular loop peptide, has been shown to inhibit ATP release during hypoxia by blocking Cx43 HC [36, 78]. Therefore, XG19 inhibition of Cx43 HC–mediated ATP release during hypoxic injury was also investigated. As shown in Fig. 10, XG19-treated cells showed a significant reduction in ATP release compared with untreated cells in HAIR solution. Furthermore, XG19 was able to efficiently block HC activity at only 5 μM with no further decrease in ATP release at higher XG19 concentrations.

**Dye scrape-load assay**

Intercellular communication via gap junctions is essential for physiological function with long-term gap junction block detrimental to cell health [85, 86]. Some extracellularly acting peptides such as Gap27 and Peptide5 may inhibit gap junction function at high concentrations and/or during prolonged periods of exposure [30, 36, 87]. Gap19, however, is said to have no effect on gap junction function [32, 46, 88]. While Cx43 HC are

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**Fig. 7 EthD-1 uptake.** ARPE-19 cells were treated with low calcium solution alone, high calcium solution, 5 μM XG19 or 5 μM FITC-XG19 in low calcium solution in the presence of EthD-1 (red), and uptake was observed by a confocal microscope (a). EthD-1 uptake was quantified by measuring the mean EthD-1 fluorescence in four areas per treatment group (b). One-way ANOVA was carried out with post hoc Dunnett’s test, and statistical significance (***p < 0.001, ****p < 0.0001) was represented as a difference from the low calcium control (n = 4; mean + SD). Scale bar, 500 μm

**Fig. 8 Inhibition of HC-mediated ATP release with XG19, CBX and Peptide5.** ATP release from ARPE-19 cells in low calcium solution either left untreated or pre-treated with 5 μM XG19 or 100 μM CBX (a). ATP release of ARPE-19 cells in low calcium solution either left untreated or in the presence of 5 μM XG19 or 20 μM Peptide5 (b). Statistical analysis was carried out via one-way ANOVA and post hoc comparisons using Dunnett’s test, and statistical significance (***p < 0.01, ****p < 0.0001) was represented as a difference from untreated cells (n = 3 wells per group; mean + SD)
closed, the cytoplasmic loop and tail are free and as such gap junctions are open. Therefore, XG19 interference with the cytoplasmic tail inhibits interaction with the cytoplasmic loop and keeps HC closed while gap junctions remain open [32]. The dye scrape/load assay is a classic assay to determine gap junction function [89]. Here, the passage of fluorescent Lucifer Yellow from cell to cell via gap junctions can be visualized [78]. As shown in Fig. 11a, non-scraped cells did not take up any dye whereas when the monolayer was scraped, cells adjacent to the scrape take up Lucifer Yellow and spread into neighbouring cells was allowed via open gap junctions. Consistent with previous literature, cells treated with the non-specific HC and gap junction blocker, CBX, took up Lucifer Yellow into cells immediately adjacent to the scrape, but dye did not further spread to any neighbouring cells due to blocked gap junctions [90]. In addition to Lucifer Yellow uptake at the site of the scrape, XG19-treated cells showed dye transfer to neighbouring cells via gap junctions similar to the untreated control. Lucifer Yellow dye spread was quantified by measuring the total number of pixels in areas that had taken up Lucifer Yellow dye in each of the treatment groups (Fig. 11b). Statistical analysis revealed that the untreated and XG19-treated groups showed significantly higher dye spread than the CBX group (\( p < 0.01 \)). There was no significant difference in the Lucifer Yellow dye spread between the untreated and XG19-treated groups which confirmed that gap junction communication was not affected by XG19. This is consistent with previous studies which have shown the ability of Gap19 to maintain gap junction function post uptake in dye scrape-load assays [32]. Overall, these studies confirmed that while XG19 is a Cx43 HC blocker, it does not affect gap junction function at the dose level used here.

**Conclusion**

These studies showed that XG19 uptake was higher than that of native Gap19 under both normal and hypoxic conditions without any effect on cell viability, suggesting that Xentry is a safe CPP aiding the uptake of Gap19 and providing the potential to reduce administration doses. Previous studies have attempted to improve Gap19 uptake using TAT [32, 33]. However, TAT raises safety concerns due to its high positive charge and low cell specificity causing cytotoxicity especially in vivo studies [91–93]. Functional assessments of XG19 post uptake revealed that XG19 was an effective HC blocker without affecting gap junction communication. This suggests that XG19 has the potential to be used for the treatment of
hypoxic and inflammatory diseases where uncontrolled HC opening causes cell death and tissue damage.

As hypoxia alters the expression of many proteins including Syndecan-4, this offers a distinct therapeutic advantage to XG19 as it specifically enters Syndecan-4 overexpressing hypoxic cells. Furthermore, XG19 was functional under hypoxic conditions resulting in Cx43 HC block and thus improved cell survival under hypoxic conditions. In nAMD, XG19 has the potential to target hypoxic RPE cells and the inflamed choroid and retina to shut down the inflammatory cascade, blocking Cx43 HC opening and thus reducing ATP release and activation of the inflammatory cascade via the NLRP3/inflammasome complex [24, 25].

The systemically delivered benefits of XG19 may extend its therapeutic potential to tissues outside the eye. As XG19 is not sequestered by the circulation due to its specificity for Syndecan-4 which is not expressed on circulating monocytes and erythrocytes, systemic delivery would allow for the targeting of several hypoxic and inflammatory conditions such as acute pancreatitis, Kawasaki disease, hypoxic tumours and neurological conditions such as Parkinson’s and Alzheimer’s diseases due to the presence of Syndecan-4 in (and thus a potential entry way across) the blood-brain barrier. In particular, XG19 allows rapid intervention where, for example, the pharmacokinetics and practicality of an orally delivered drug such as the hemichannel blocker tonabersat [14] might constrain rapid intervention. This includes acute events such as heart attack, acute respiratory distress syndrome, retinal artery and vein occlusion and stroke where the need to block HC opening upon rapid reperfusion is critical to the survival of the affected and neighbouring tissue. Taken together, XG19 offers a novel approach to targeting hypoxic and inflammatory disease with distinct advantages over existing therapies for many indications.

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Compliance with ethical standards Conflict of interest C.R.G. is a founding scientist of OcuNexus Therapeutics, Inc. (USA) (and previously CoDa Therapeutics, Inc.) which has intellectual property related to the regulation of connexin channels in the treatment of ocular and other disease. F.P.C. C.R.G. and I.D.R. are inventors on a PCT application regarding Xentry fusion peptides for modulation of connexin gap junction and hemichannel activity, with this technology licenced by OcuNexus Therapeutics, Inc. (USA). M.L.A. declares no conflict of interest.

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