Final report

Title: A novel cell-penetrating peptide for oligonucleotide delivery to the retina – Can we close the gap?

Lay summary:

Retinal disorders, such as age-related macular degeneration (AMD), are the leading cause of vision loss in the developed world. A vast number of targets responsible for retinal degeneration have already been identified, which has resulted in a marked growth in protein and peptide therapeutics. However, the efficacy of such therapies depends largely on the efficient delivery of these macromolecules to the retinal tissues. This study evaluated a novel cell-penetrating peptide (Xentry) for the delivery of specific antisense oligodeoxynucleotides (Cx43 AsODN) to the back of the eye in the treatment of inflammatory retinal disorders. Utilizing such efficient carriers may reduce the effective drug dose required, minimizing the risk of potential side effects and reducing the injection frequency, which ultimately could result in more efficient treatment of retinal conditions.

Aim:

The aim of this study was to evaluate Xentry (X) for efficient retinal delivery of connexin43 antisense oligodeoxynucleotide (Cx43AsODN) in the treatment of inflammatory retinal disorders by determining the optimal ratio of Cx43AsODN:X in ARPE-19 cells and quantifying protein down regulation.

Progress:

Preliminary cell culture experiments revealed that a net positive charge was required for Cx43AsODN:X complexes to cross the cell membrane. In order to observe the charge ratio at which a net positive charge was achieved, Cx43AsODN was complexed with either Xentry-Protamine (XP) or Xentry-KALA (XK) at various charge ratios and zeta potential as well as complex size were measured using a Zetasizer. **Studies revealed that a net positive charge was achieved at a charge ratio of 1:1.2 for Cx43AsODN:XP (434 nm) and 1:2 for Cx43AsODN:XK (1166 nm).**

To optimise the charge ratio at which all free Cx43AsODN efficiently bound to Xentry, gel shift assays were carried out. Cx43AsODN:X complexes were loaded onto a 1% agarose gel, with large complexes remaining in wells without any bands during imaging signifying complete complexation. **This was observed at a charge ratio of 1:2.2 (XP) and 1:2.5 (XK) respectively, suggesting that while an overall positive charge of the complex can be measured by the Zetasizer at lower ratios, higher ratios are actually required for complete complexation and thus improved cell uptake.**

Cellular uptake of Cx43AsODN:X was visualised by fluorescence microscopy. ARPE-19 cells were grown on 8-chamber slides until confluent and treated with 0.5, 1 or 2 µM of Cy3-Cx43AsODN:XP (1:1.5 or 1:2.2) for 4 h. Cells were fixed in 4% PFA and nuclei stained with DAPI. **Optimal cell uptake was observed with 1 µM Cy3-Cx43AsODN at 1:2.2 XP.** Repeating the same experiment with XK (1:2 or 1:2.5), **optimal uptake was observed with 0.5 µM Cy3-Cx43AsODN at 1:2.5 XK.**

Since complete complex formation and thus better uptake was achieved at lower ratios for XP reducing potential toxicity while the size of the resulting complex was also much smaller for Cx43AsODN:XP facilitating cell uptake, further experiments were performed with XP only.

Knockdown of Cx43 in ARPE-19 cells post incubation with Cx43AsODN:XP was measured by Western blots. ARPE-19 cells were grown in a 12-well plate and 0.5, 1 or 2 µM of Cx43AsODN:XP was added

before incubating cells at 37 °C and 5% CO_2 for 4 h. Media was then removed and 7.5 µg of cell lysate was loaded per lane. The gel was run at 300 V for 20 min and transferred onto a PVDF membrane at 170 mA for 60 min, before probing the membrane with the primary rabbit anti-connexin43 and secondary anti-rabbit-HPR antibodies for visualisation by chemiluminescence. **While this assay is still undergoing optimisation, initial results suggested that Cx43 knockdown after addition of Cx43AsODN:XP under normal conditions was negligible.**

Hypoxic conditions generally result in Cx43 hemichannels to open, leading to cell death. The ability of Cx43AsODN:XP to potentially enter cells through open hemichannels was observed by fluorescent microscopy. Cells were grown on 8-chamber slides and treated with either Cy3-Cx43AsODN, Cy3-Cx43AsODN:XP (1:2.2) or Cy3-Cx43AsODN:oligofectamine as control before being incubated at 37 °C and 5% CO_2 in either DMEM/F12 (normal) or hypoxic solution for 2 h. Cells were fixed in 4% PFA and nuclei stained with DAPI. While uncomplexed Cy3-Cx43AsODN was unable to penetrate cells in normal or hypoxic conditions, **Cy3-Cx43AsODN:XP showed increased affinity for cells cultured in hypoxic solution (attached to the cell surface as well as penetrated inside the cell). This effect was not observed with oligofectamine controls suggesting that it was Xentry specific.**

To confirm if this high affinity binding also resulted in significant Cx43 knockdown, Western blot analysis was repeated. Cells were grown in a 6-well plate and were either left untreated or treated with Cx43AsODN:XP (1:2.2) or Cx43AsODN:oligofectamine before being incubated at 37 °C and 5% CO_2 in either DMEM/F12 (normal) or hypoxic solution for 2 h. Cell lysates were then run on a gel and probed as previously described. **While this assay is still undergoing further optimisation, initial results confirmed the negligible Cx43 knockdown in normal conditions (see above). However, cells treated with Cx43AsODN:XP in hypoxic solution showed reduced Cx43 expression when compared to untreated or Cx43AsODN:oligofectamine treated cells.**

Overall, this study has given us great insight into the optimal concentrations of Xentry to be used for efficient cell uptake and Cx43 down regulation. However, due the relative large size of Cx43AsODN (~10 kDa) and XP (16 amino acids) resulting in large variability of the results, future studies will concentrate on conjugating Xentry to a specific Cx43 mimetic peptide which should further facilitate cell uptake and thus efficacy. Frazer Coutinho, who was employed as a technician on this grant, will continue these studies as his PhD project and we hope to report some exciting *in vivo* results in the near future. While tissue permeation and preliminary *in vivo* studies were not carried out as planned due to the many obstacles encountered during the *in vitro* optimization, it is envisaged that the much smaller new construct will result in more consistent results with improved cellular uptake and tissue permeation. Once the concentrations of these new constructs have been optimized *in vitro*, we will evaluate the efficacy of the constructs in CNV-laser induced *in vivo* studies.