Multi-functional cationic dendrimers for connexin43 antisense oligodeoxynucleotide delivery in the treatment of wet age-related macular degeneration

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ABSTRACT

Retinal delivery of genetic material is limited by the molecules’ stability and cellular uptake. This study investigated seven multi-functional dendrimer carriers for their ability to form complexes with connexin43 antisense oligodeoxynucleotides (AsODN) and efficiently deliver these molecules into retinal pigment epithelium cells with minimal cytotoxicity.

INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of vision loss in the developed world and predominantly affects the region of the eye used for sharp, central vision. Currently, repeated intravitreal injections of antibodies against vascular endothelial growth factor (VEGF) are used to treat wet AMD, with frequent injections increasing the infection risk as well as the treatment burden.1, 2 Moreover, recent studies have shown that repeated injections of anti-VEGF drugs may increase the development of geographic atrophy, worsening the condition in the long term.3

An alternative approach to anti-VEGF therapy is the down-regulation of Cx43 gap junction proteins by specific AsODN, which has been shown to reduce inflammatory processes such as vessel leak and edema present in wet AMD. However, unmodified AsODN are easily degraded in biological fluids and can show limited permeability across biological membranes.4

This study investigated seven multi-functional dendrimer carriers in vitro for their ability to complex Cx43 AsODN using a gel shift assay. Moreover, the cytotoxicity of dendrimers and dendrimer-AsODN complexes on ARPE-19 and HeLa cells was determined and the cellular uptake of Cy3-labelled Cx43 AsODN was evaluated using fluorescence activated cell sorting (FACS). Finally, down-regulation of Cx43 in ARPE-19 cells was assessed using immunohistochemistry.

EXPERIMENTAL METHODS

Cx43 AsODN complexation

Multi-functional carriers (cationic dendrimer (D), cell penetration peptide (TAT), fusogenic peptide (HA2) and C14 lipoamino acid combinations) were synthesized by Boc solid-phase peptide synthesis. The optimal charge ratio to achieve complete complexation of dendrimer and AsODN was determined using a gel shift assay. Briefly, carriers were added to Cx43 AsODN with charge ratios ranging from 0.5:1 to 10:1 (dendrimer carrier(+):Cx43 AsODN(-)). Samples were run on a 1% agarose gel in TBE buffer containing 0.1 µg/ml ethidium bromide at 100 V for 20-30 min and nucleic acid bands were viewed under UV light. Finally, particle size and zeta potential of the formed complexes were determined using a Zetasizer.

Cytotoxicity studies

Cell viability was evaluated with calcein-AM using Cx43 expressing ARPE-19 cells with non-Cx43 expressing HeLa cells serving as a functional control. Briefly, cells were seeded in 96-well plates and grown overnight before adding increasing concentrations of AsODN-dendrimer complexes or carrier alone. After 24 h of incubation, cells were washed with PBS and incubated with 1 µM calcein-AM for 75 min, before measuring the fluorescence using a Bio-Tek Synergy microplate reader. Readings were corrected for fluorescence of control wells without cells and were normalized to untreated controls, representing 100% cell viability. To exclude cytotoxic effects caused by Cx43 down-regulation, ARPE-19 and HeLa cells were compared. Moreover, the assay was repeated with a Cx43 sense ODN to confirm that Cx43 down-regulation was not the cause of the decline in cell viability.

Cellular uptake studies

Cellular uptake of Cy3-tagged Cx43 AsODN, with and without complexation to dendrimer carriers, was determined by FACS using oligofectamine as a positive control. In short, ARPE-19 and HeLa cells were seeded in 12-well plates and cultured overnight. Cy3-Cx43 AsODN (0.5 µM) alone or complexed with dendrimer carriers (charge ratio 5:1) was added to the cells and incubated for 24 h. Cells were washed with PBS, harvested with TrypLE and resuspended in PBS. FACS was performed in triplicate counting 10,000 events per sample. Cellular uptake was calculated as the average geometric mean fluorescence and was normalized to the cellular uptake achieved with oligofectamine, representing 100% uptake.

Cx43 down-regulation

Cx43 expression in ARPE-19 cells was determined by Cx43 immunolabelling. Briefly, cells were seeded on collagen coated coverslips in a 12-well plate and treated with 0.5 µM Cx43 AsODN or Cx43 sense ODN alone or complexed with the most effective dendrimer carrier. After 8 h of incubation, cells were washed and quickly fixed with ice-cold methanol for 3 min. To prevent non-specific binding, 10% goat serum in 0.1% Triton X and 100 mM glycine was added for 1 h. Cx43 was then labelled using a polyclonal rabbit anti-Cx43 primary antibody and a goat anti-rabbit Alexa 488-tagged secondary antibody, while cell nuclei were counterstained with DAPI. Coverslips were mounted in Citifluor and images were acquired on an Olympus FV1000 Confocal Laser Scanning Microscope.
RESULTS AND DISCUSSION

Uncomplexed AsODN (labelled with ethidium bromide) was seen to move freely across the gel, whilst dendrimer-AsODN complexes were too large (with an overall positive charge) and thus remained in the wells. AsODN-dendrimer complexation seemed to be complete at a charge ratio of 5:1 for all but one (2C14-DHis) dendrimer (Fig. 1), correlating with a net positive zeta potential at that charge ratio. Further experiments were therefore performed at a charge ratio of 5:1.

Cytotoxicity studies revealed that high dendrimer and dendrimer-AsODN complex concentrations resulted in a significant reduction in cell viability. This enabled a cytoplasmic dose response curve for an unmodified AsODN to be derived for the first time (data not shown).

When comparing the carriers at a charge ratio of 5:1, dendrimers containing the cell penetrating peptide TAT and the fusogenic peptide HA2 showed the highest cytotoxicity in ARPE-19 cells (Fig. 2B). Dendrimers without TAT and HA2 were less toxic, with 2C14-DHis showing the least cytotoxicity. This can be explained by the low pKₐ (6.1) of the side chain amine in histidine used as the terminal amino acid of the dendrimer. Thus, only one of the two amine groups may be protonated at neutral pH, resulting in less binding capacity to AsODN (see Fig. 1), but also reducing the overall positive charges responsible for the cytotoxic effects.

Cy3 Cx43 AsODN uptake was normalized to the uptake achieved with oligofectamine. For HeLa cells there was no significant difference in cellular uptake and cell viability between the different dendrimers whereas carriers containing TAT and HA2 achieved higher Cx43 AsODN uptake in ARPE-19 cells while also showing slightly increased cytotoxicity.

Cx43 immunolabeling revealed that treatment with naked AsODN resulted in hardly any down-regulation (Fig. 3B), and was comparable to treatment with sense ODN delivered by the dendrimer (Fig. 3D). However, dendrimer complexed Cx43 AsODN down-regulated Cx43 expression almost entirely (Fig. 3C).

CONCLUSION

A net positive charge of the AsODN-dendrimer mixture was required to achieve complete nucleic acid complexation and improve cellular uptake required for efficient Cx43 down-regulation. Whilst excessive intracellular concentrations of both dendrimer carrier and ODN can induce cytotoxicity, functionally effective AsODN and dendrimer carrier concentrations are able to provide efficient and safe target protein reduction.

REFERENCES