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Ocular antisense oligonucleotide delivery by cationic nanoemulsion for improved treatment of ocular neovascularization: An *in-vivo* study in rats and mice

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ABSTRACT

The efficacy of an antisense oligonucleotide (ODN17) cationic nanoemulsion directed at VEGF-R2 to reduce neovascularization was evaluated using rat corneal neovascularization and retinopathy of prematurity (ROP) mouse models. Application of saline solution or scrambled ODN17 solution on eyes of rats led to the highest extent of corneal neovascularization. The groups treated with blank nanoemulsion or scrambled ODN17 nanoemulsion showed moderate inhibition in corneal neovascularization with no significant difference with the saline and scrambled ODN17 control solution groups, while the groups treated with ODN17 solution or Avastin® (positive ODN17 control) clearly elicited marked significant inhibition in corneal neovascularization confirming the results reported in the literature. The highest significant corneal neovascularization inhibition efficiency was noted in the groups treated with ODN17 nanoemulsion (topical and subconjunctivally). However, in the ROP mouse model, the ODN17 in PBS induced a 34% inhibition of retinal neovascularization when compared to the aqueous-vehicle-injected eyes. A significantly higher inhibition of vitreal neovascularization (64%) was observed in the group of eyes treated with ODN17 nanoemulsion. No difference in extent of neovascularization was observed between blank nanoemulsion, scrambled ODN17 nanoemulsion, vehicle or non-treated eyes. The overall results indicate that cationic nanoemulsion can be considered a promising potential ocular delivery system and an effective therapeutic tool of high clinical significance in the prevention and forthcoming treatment of ocular neovascular diseases.

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1. Introduction

Antisense oligonucleotides (ODNs) have been extensively studied as therapeutic agents because they can selectively interfere with gene expression in target cells, thus opening promising novel therapeutic approaches [1,2]. ODNs must be taken up by the target cell, tissue or organ in a sufficient quantity to provoke a biological response. However, when ODNs are injected systemically *in-vivo*, most of them concentrate in the kidney and liver where marked rapid degradation occurs, seriously limiting their availability to other tissues [3–5]. Furthermore, ODNs are exposed to exonucleases resulting in rapid degradation during their circulation prior to reaching the target. In addition, following cell internalization, ODNs must reach the mRNA intact, avoiding destruction in lysosomes and degradation by the various endonucleases within the cells [6]. Thus, for the treatment of eye diseases, local delivery of ODNs can overcome some of the above-mentioned drawbacks. For the treatment of neovascularization complicating AMD anti VEGF treatments have shown efficacy not only in preventing progression of diseases but also in gaining visual acuity in a subset of patients [7,8]. Two anti-VEGF agents have been approved for wet AMD: pegaptanib (Macugen®; Eyetech/Pfizer), and ranibizumab (Lucentis®; Genentech/Novartis) whereas Avastin is being used off label [9]. The marketed products are injected intravitreally at 4–6 week intervals for at least 1 year. To improve patient compliance and diminish safety concerns, the number of repeated intravitreal administrations should be markedly reduced. Because the tyrosine kinase VEGF receptor-2 (VEGFR-2) is the major mediator of the mitogenic and angiogenic effects of VEGF, it has become a leading anti-neovascularization target. An antisense ODN directed at VEGFR-2 has been screened and selected based on its ability to prevent the proliferation of HUVEC cells *in-vitro*. However, anti-angiogenic ODN-based therapy is compromised by rapid degradation of ODN in biological fluids and by their inability to efficiently cross cellular membranes due to their hydrophilic and polyanionic character and to their large molecular structure [10]. The major limiting step for the clinical application of ODN strategy for eye diseases is the lack of appropriate and efficient delivery systems which can successfully transport the active molecules to the involved ocular tissues. Topical application of ODNs does not result in efficient intraocular penetration.

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Repeated intravitreal injections may efficiently target non-neural cells of the retina and retinal pigment epithelium. Considering the relatively short half-life value (about 20 h in monkey) of presently available ODNs and their degradation in the vitreous [11], it is difficult to obtain therapeutic concentrations for an extended period of few weeks following multiple intraocular injections over 1–2 week intervals. Therefore, an effective intraocular delivery system is needed to improve the ODN low-efficiency. The potential of the cationic nanoemulsion has been demonstrated in terms of antisense ODN association efficiency, ODN partitioning and protection from degradation in culture medium [10,12]. More recently, we showed that a cationic nanoemulsion prepared with DOTAP was able to protect both *in-vitro*, and *in-vivo* the associated ODN from vitreous degradation over the 72-h test period and that the nanoemulsion yielded apparently therapeutic levels of ODN in the retina over the 72-h post injection [13,14]. The objective of the present study is to evaluate the potential of an antisense ODN cationic nanoemulsion directed at VEGF-R2 to reduce neovascularization in a rat corneal neovascularization model and in a mouse model of retinopathy of prematurity (ROP). This research may lead to the development of a new efficient drug delivery system that could ultimately be a significant clinical tool for the treatment of pathologic ocular neovascularization.

2. Materials

A seventeen-base oligonucleotide (sequence: 5′GsCsAsTsCTCCTT-TTCsTsGsAsC3′) was purchased from Proligo, Boulder, CO, USA. This is a 17-mer partially phosphorothioated ODN with a MW of 5217 Da and is hereby termed ODN17 in the present study. Mid-chain triglycerides (MCT) were purchased from Societé des Oleagineux, Bougival, France. Lipoid E-80 was purchased from Lipoid AG, Ludwigshafen, Germany. Pluronic F-68 was purchased from BASF, Ludwigshafen, Germany. Vitamin E was purchased from Fluka, Taufkirchen, Germany. (N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium salts) DOTAP was purchased from Sigma, St. Louis, MO, USA.

3. Methods

3.1. Nanoemulsion preparation

The blank cationic nanoemulsion was prepared according to a method previously described [15]. Briefly, glycerol and Poloxamer-188 were dissolved in the aqueous phase and the pH was adjusted to 7.4. The Lipoid E-80, α-tocopherol and the cationic lipid, DOTAP were dissolved in the MCT oil phase. Both phases were heated separately to 70 °C, after which the two phases were mixed and stirred with a magnetic stirrer and further heated to a temperature of 80 °C. The resulting emulsion was then mixed by a high shear mixer Polytron™ (Kinematica, Luzern, Switzerland) at 16,000 rpm for 5 min and rapidly cooled to below 20 °C. After cooling, the emulsion was homogenized using a Rannie (APV Gaulin, Hilversum, the Netherlands) at 10,000 psi for 5 min. The pH of the emulsion was adjusted to 7.4 by titration with hydrochloric acid (0.1 N). The emulsion was then filtered through a Track-Etched (TE) membrane filter (Schleicher & Schuell, Dassel, Germany) with a pore size of 0.2 μm. The emulsion was filled under nitrogen atmosphere into siliconized glass bottles and then sterilized by steam autoclave at 121 °C for 15 min. A typical formulation consisted of (w/w %): MCT (1.66), poloxamer-188 (0.425), glycerol (2.25), lipoid E-80 (0.5), DOTAP (0.33), α-tocopherol (0.01) and doubled-distilled water (DDW) up to 100.

3.2. Nanoemulsion characterization

Droplet size measurements were carried out utilizing an ALV Non-invasive Back Scattering High Performance Particle Sizer (ALV-NIBS HPPS, Langen, Germany) at 25 °C using water as the solvent. The sensitivity range was 0.5 nm to 5 μm.

Zeta potential measurements of the nanoemulsions were performed with the Malvern zetasizer (model Nano ZS zetasizer, Malvern, UK) diluted in DDW (150 mV).

3.3. Oligonucleotide association to the blank cationic nanoemulsions

Oligonucleotide association was performed at the end of the manufacturing process as reported previously [13]. Finally, an ODN concentration of 10 μM either in aqueous solution or cationic nanoemulsion was prepared from ODN stock solution. All ODN-associated cationic nanoemulsions were incubated for 12 h at room temperature.

3.4. Pharmacological evaluation

The animal investigation adhered to the Hebrew University Authority for Animal Facilities Ethical Committee resolutions on the use of animals in research, NIH approval number: OPRR-A01-5011 (Ethical committee research number: MD-07-10860-4). This study also followed the tenets of the Statement for the Use of Animals in Ophthalmic and Visual Research of the Association for Research in Vision and Ophthalmology.

3.4.1. Efficacy assessment using the rat corneal neovascularization model

3.4.1.1. Neovascularization protocol used in a rat study. Forty eight adult male albino rats (Sprague Dawley; 300 g) were used in this study. They were randomly divided into 8 groups. Induction of corneal neovascularization was performed using the silver nitrate cauterization technique described by Mahoney and colleagues. [16,17]. All procedures were performed under general anesthesia induced by intraperitoneally-administered ketamine hydrochloride (75 mg/kg) and xylazine (10 mg/kg) combination. Following ocular application of 0.5% proparacaine hydrochloride for eye-surface anesthesia, cauterization was performed by pressing an applicator stick (diameter of 1.8 mm) coated with silver nitrate:potassium nitrate (3:1) to the central cornea of the right eye of each animal for 10 s while the left eye remained untouched. Excess of silver nitrate/potassium nitrate was removed by irrigation with 5 ml of balanced salt solution. A single investigator was responsible for cauterization in all animals to ensure consistency in the corneal cauterization process. Following cauterization, the eyes of the rats were randomized to eliminate any potential bias in the degree of injury within the different groups. There were 8 experimental groups comprising 6 rats per group and consequently, 8 different formulations were evaluated and compared. 50 μl of specific formulations was instilled in the right eye of each rat immediately after cauterization. Group 1 was topically treated with balanced salt solution, group 2 with 10 μM scrambled ODN17 sequence solution, group 3 with blank DOTAP nanoemulsion, group 4 with 10 μM scrambled ODN17 DOTAP nanoemulsion, group 5 with Avastin® that served as a positive control, group 6 with 10 μM ODN17 solution, group 7 with 10 μM ODN17 DOTAP nanoemulsion, and group 8 with 10 μM ODN17 DOTAP nanoemulsion that was injected subconjunctivally. Prior to anesthesia recovery, the rats were injected intraperitoneal with a solution of buprenorphine for analgesia purposes. The rats were kept in their house cages for a 7-day period under a controlled light/dark cycle and constant humidity and temperature conditions prior to initiating the neovascularization assessment.

3.4.1.2. Qualitative and quantitative corneal neovascularization assessment following treatment in rats. The animals were anesthetized as described above 7 days post treatment and their corneas were photographed (SLR digital camera, Nikon D300 with Nikon AF Micro

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