**Topical Application of a G-Quartet Aptamer Targeting Nucleolin Attenuates
Choroidal Neovascularization in a Model of Age-Related Macular Degeneration**

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Abstract:

Choroidal neovascularization (CNV) associated with the 'wet' form of age related macular degeneration (AMD) is one of the most common causes of central vision loss among the elderly. The 'wet' form of AMD is currently treated by intravitreal delivery of anti-VEGF agents. However, intravitreal injections are associated with complications and long-term inhibition of VEGF leads to macular atrophy. Thus, there is currently an unmet need for the development of therapies for CNV that target molecules other than VEGF. Here, we describe nucleolin as a novel target for the 'wet' form of AMD.

Nucleolin was found on the surface of endothelial cells that migrate from the choroid into the subretinal space in the laser-induced model of 'wet' AMD. AS1411 is a previously described G-quartet oligonucleotide that has been shown to bind nucleolin. We found that AS1411 inhibited the formation of tubes by human umbilical vein endothelial cells (HUVECs) by approximately 27.4% *in vitro*. AS1411 co-localized with the site of laser induced CNV *in vivo*. Intravitreally injected AS1411 inhibited laser-induced CNV by 37.6% and attenuated infiltration of macrophages by 40.3%. Finally, topical application of AS1411 led to a 43.4% reduction in CNV. Our observations have potential implications for the development of therapies for CNV and specifically for the 'wet' form of AMD.

1. Introduction

Age related macular degeneration (AMD) is the leading cause of central vision loss in developed nations among individuals 65 years of age and older (Lim et al., 2012). An advanced form of AMD known as 'wet' AMD involves the growth of new blood vessels from the choroid into the subretinal space (choroidal neovascularization or CNV) (Bhutto and Luty, 2012). These new blood vessels are generally immature and hence leak fluids into the retina, causing macular edema (Bhutto and Luty, 2012). CNV in AMD patients is associated with an elevation of vascular endothelial growth factor (VEGF) and thus, anti-VEGF antibodies including *ranibizumab* and *bevacizumab* are the current standard of care for the treatment of 'wet' AMD (Funk et al., 2009; Saint-Geniez et al., 2009; Ferrara, 2010; Martin et al., 2011). However, anti-VEGF antibodies need to be injected into the ocular compartment via intravitreal injection every four to twelve weeks (Keane and Sadda, 2012). Repeated intravitreal injections are associated with endophthalmitis, retinal detachment and increased intraocular pressure (Sampat and Garg, 2010). Although the frequency of these complications is relatively low per injection, these frequencies become relevant over the lifetime of the patient. Moreover, given that AMD affects primarily an aged population, the relatively high frequency of intraocular injections negatively impacts patient compliance (Goldstein et al., 2012).

Recent clinical studies confirm prior studies in animals suggesting that long-term inhibition of VEGF is deleterious to the retina (Nishijima et al., 2007; Papadopoulou et al., 2009; Sacu et al., 2011; Kurihara et al., 2012; Campochiaro et al., 2014).

Specifically, an anti-VEGF mediated regression of CNV can lead to macular atrophy (Campochiaro et al., 2014). This observation is not surprising given that VEGF is involved in a large variety of cellular processes including maintenance of tissue

homeostasis (Saint-Geniez et al., 2008). Delivery of VEGF-inhibitors to the eye can also lead to systemic complications in some individuals due to anti-VEGF antibodies traveling from the ocular compartment into systemic circulation (Heiduschka et al., 2007; Ueta et al., 2009; Ueta et al., 2011). Thus, there is currently an unmet need for the development of inhibitors of CNV that target molecules other than VEGF. There is also an unmet need for a mechanism of drug delivery for anti-CNV agents that is less invasive than intravitreal injections.

The most commonly used animal model for the 'wet' form of AMD is laser induced CNV. In this model, a laser burn of the Bruch's membrane causes a local elevation of VEGF and a subsequent migration of endothelial cells from the choroid into the subretinal space, features observed in 'wet' AMD patients (Lambert et al., 2013). Despite some limitations, this model of 'wet' AMD is widely used for the testing and development of novel inhibitors of CNV.

Nucleolin is a ubiquitously expressed phosphoprotein involved in numerous cellular activities, including rRNA maturation, ribosome assembly and mRNA metabolism (Abdelmohsen and Gorospe, 2012). Despite being a nuclear and cytoplasmic protein, nucleolin is also found on the surface of various cancer cells (Hovanessian et al., 2000; Hovanessian et al., 2010; Abdelmohsen and Gorospe, 2012). Surface nucleolin serves as a receptor for various growth factors such as midkine and lactoferrin (Legrand et al., 2004; Hovanessian, 2006). AS1411 is a G-quartet quadruplex DNA aptamer formed from a single strand G-rich phosphodiester oligonucleotide (Bates et al., 1999). AS1411 has been found to have various anti-proliferative effects including cell cycle arrest of cancer cells via binding to cell surface nucleolin (Bates et al., 2009). We wished to test the hypothesis that nucleolin is expressed on the surface of endothelial cells migrating

from the choroid into the subretinal space following laser induced injury. We also wished to test the hypothesis that AS1411 may inhibit the formation of such CNV when delivered to the intravitreal compartment. Finally, we wished to test the hypothesis that topical application of AS1411 to the ocular surface may also inhibit laser induced CNV. If successful, our studies will have identified a drug target not previously implicated in an animal model of AMD. Equally important, our results will address the clinically relevant need of development of drugs that can inhibit CNV without the need for an invasive intravitreal injection.

2. Materials and Methods:

2.1 Materials:

The oligodeoxynucleotides AS1411, (5'-d(GGTGGTGGTGGTTGTGGTGGTGGTGG)-3', and inactive control oligonucleotide CRO, 5'-d(CCTCCTCCTCCTTCTCCTCCTCCTCC)-3' were synthesized by the Tufts Core DNA Synthesis facility (Tufts University, Boston, MA). AlexaFluro594 labeled AS1411, 5'-FL-AS1411, and AlexaFluro594 labeled CRO, 5'-FL-CRO, were purchased from Invitrogen and used for localization studies. All oligonucleotides were dissolved in nuclease free water. Neutralizing goat antibody against mouse VEGF-A (AF-493) was purchased from R&D Systems (Minneapolis, MN) and 2pmol were administered via intravitreal injection for lasered mice.

2.2 Endothelial Tube Formation Assay

Tube formation assays were completed according to the Endothelial Tube Formation Assay (*In Vitro* Angiogenesis) protocol as per manufacture's instructions (Life Technologies Corporation, Carlsbad, CA). 4.5×10^4 HUVECs were seeded per well of a 24-well plate on Geltrex™ (Reduced Growth Factor Basement Membrane Matrix, Life

Technologies Corporation, Carlsbad, CA) coated wells. Incubations were performed by adding CRO or AS1411 to 200PRF/LSGS medium to a final concentration of 500nM. 200PRF/LSGS medium was used as a positive control. 18 hours post-incubation, microscopic images of each well were captured (IX51, Olympus, Center Valley, PA) and the number of master junctions, master segments and meshes determined using the “HUVEC angiogenesis analyzer plugin” for ImageJ software. Master segments are defined as tube segments between two junctions each of which contain 2 additional branches. Master junctions are defined as junctions linking at least 3 master segments and meshes are defined as areas enclosed by segments/master segments. Further detail on this approach have been published elsewhere (Birke et al., 2013; Lipo et al., 2013).

2.3 Intravitreal Injections

All animal studies were conducted in accordance with ARVO and European Union guidelines for the use of animals in ophthalmic and vision research. This study was approved by the Tufts University Institutional Animal Care and Use Committee. C57BL/6J (Jackson Laboratories) were maintained in accordance with federal, state and local regulations. Six- to ten-week old mice were anesthetized via an intraperitoneal injection of ketamine/xylazine mixture. Intravitreal injections were performed using a 32-gauge needle attached to a 5µl syringe (Hamilton, Reno, NV) to deliver a 1µl injection.

2.4 Laser Induced Choroidal Neovascularization

Laser induced CNV was performed as described by us previously (Cashman et al., 2011). Briefly, mice were sedated with an intraperitoneal injection of ketamine (0.1g/kg)/xylazine (0.01g/kg) and pupils were dilated with one drop of 2.5% phenylephrine HCl (Bausch & Lomb Incorporate, Tampa, FL) and one drop of 1%

Tropicamide (Bausch & Lomb Incorporate, Tampa, FL) per eye. To minimize corneal injury, one drop of 2.5% Hypromellose (Goniovisc, Wellhead UK) was used and a coverslip attached. Four laser spots were created per eye at a spot size of 75µm in diameter, 150mW power and 100ms pulse time using an argon laser (532nm, IRIS Medical Light Solutions, IRIDEM, IRIDEX, Mountain View, CA).

2.5 Topical Application of Aptamers

1 nmole of AS1411 or CRO was diluted in 0.9% NaCl to a total volume of 20µl. A total of 20µl of the diluted AS1411, CRO or 0.9% NaCl (empty vehicle control) was administered per eye one day prior to laser treatment and every subsequent 24hrs until the mice were sacrificed on day 7 post-laser injury.

2.6 GSL-I Lectin, F4/80 and Nucleolin Staining

Mice were sacrificed by CO₂ inhalation, followed by cervical dislocation. Optic nerve, cornea, lens, iris and retina were removed and the posterior eyecups (RPE/sclera/choroid) were fixed overnight in 4% paraformaldehyde. Following fixation, eyecups were incubated at 37°C in 2.5 mg/ml BSA in PBS for 30 minutes. Eyecups were then incubated at 37°C in 100 mg/ml FITC-conjugated *Griffonia Simplicifolia* Lectin I (GSL-I, isolection B4, Vector Labs) in PBS for one hour. For nucleolin staining, eyecups were blocked in 6% normal goat serum for 30 minutes and then incubated with nucleolin antibody (NB600-241, Novus Biologicals, Littleton, CO)(1:50 dilution). Cy3 goat anti-rabbit (1:200) was used for 1 hour at room temperature for secondary detection. Rat monoclonal anti-mouse F4/80 antibody (Cl:A3-1; Abcam) followed by Cy3 goat anti-rat secondary antibody. Control staining of the posterior eyecup using both an isotype control primary antibody followed by secondary antibody, as well as secondary antibody alone, were performed.

2.7 Measurement of laser spot size and statistics

For determining localization of FL-AS1411/FL-CRO following intravitreal injection, RPE/choroid/sclera (with retina removed) of treated eyecups were flatmounted and coverslipped. Images for each lesion were taken using an Olympus IX51 microscope with Retiga 2000R camera at the same exposure time. Images were exported to ImageJ (National Institutes of Health; Bethesda, MD, USA) for quantification of fluorescence intensity of image pixels. Background pixel intensity was determined for non-CNV lesion regions and subtracted from each image using ImageJ. For determining CNV area, images for each lesion were taken as described above and the area of staining was selected following subtraction of background pixel intensity using the freehand selection tool. Confocal images were captured using a Leica TCS SPE microscope (Leica Microsystems; Wetzlar, Germany). Statistical analysis was done for all assays using dual-tailed unpaired t-tests and performed using Prism 5 (GraphPad Software Inc, La Jolle, CA).

3. Results:

3.1 AS1411 attenuates the formation of tubes by HUVECs *in vitro*

Prior to the formation of CNV, endothelial cells migrate from the choroid into the subretinal space and form neovascular membranes in response to elevated levels of cytokines such as VEGF (Grossniklaus et al., 2010). In order to determine whether AS1411 can inhibit endothelial cell migration and tube formation *in vitro*, we utilized the human umbilical vein endothelial cell (HUVEC) tube formation assay, a widely used model of *in vitro* angiogenesis. HUVEC cells were incubated with AS1411 or a negative control aptamer CRO at a concentration of 500nM and the formation of master junctions, master segments and meshes was determined 20 hours post incubation using the

'HUVEC angiogenesis analyzer' plugin for Image J. Media lacking CRO or AS1411 was used as an additional negative control. We determined a significant reduction in all three parameters for HUVECs incubated with AS1411 relative to CRO. Specifically, HUVECs incubated with AS1411 exhibited a $30.96\% \pm 6.78\%$ ($p=0.0038$); $24.26\% \pm 6.95\%$ ($p=0.013$) and $27.04\% \pm 9.36\%$ ($p=0.0277$) reduction in the number of master junctions, master segments and meshes respectively (Figure 1A-D). We conclude that AS1411 attenuates the formation of tubes by HUVECs *in vitro*.

3.2 Cell surface nucleolin is expressed in laser-induced CNV

AS1411 has been found to bind nucleolin receptors on the cell membrane, and to be preferentially taken up by cells expressing high levels of surface nucleolin (Xu et al., 2001). While surface nucleolin expression has been reported on proliferating endothelial cells of tumor- or matrigel induced angiogenic vessels, expression on 'active' endothelial cells of the retina or choroid have not been previously reported (Huang et al., 2006). We investigated the expression of cell surface nucleolin on migrating endothelial cells post laser injury. Specifically, we utilized an argon laser to generate 4 laser burns in the Bruch's membrane of 6 week old C57BL/6J mice and harvested their eyecups four days post-laser injury and stained for endothelial cells using GSL-1 lectin or cell surface nucleolin using an anti-nucleolin antibody. As expected, GSL-I strongly stained the surface of cells at the center of the CNV lesion (Fig. 2A). Co-staining of eyecups with an antibody against nucleolin demonstrated co-localization of GSL-I and nucleolin. Confocal imaging and generation of serial images of the co-stained CNV lesions confirmed co-localization of the GSL-I and nucleolin staining (Fig. 2B). Additionally, confocal microscopy confirmed staining of cell surface nucleolin of the endothelial cells, and not nucleolin in either the cytoplasm or nucleus. We conclude that nucleolin is expressed on the surface of migrating endothelial cells in the laser-induced model of 'wet' AMD.

3.3 AS1411 localizes to the site of laser-induced CNV

In order to determine whether AS1411 may target cells within a CNV *in vivo*, 0.2 nmol of Alexa594 conjugated AS1411 (FL-AS1411) or a negative control aptamer (FL-CRO) was injected into the vitreous of mice at 4 days post-laser injury. Eyecups were harvested 2 hours post-injection and stained for endothelial cells using FITC-labeled GSL-I. Analysis of Alexa594 fluorescence indicated a marked increase in fluorescence in the region of the GSL-I stained CNV lesion in those eyes injected with FL-AS1411 relative to those injected with FL-CRO (Fig. 3A). Quantification of the average Alexa594 fluorescence intensity in the CNV lesions of injected eyes demonstrated a 4.47 ± 0.69 fold increase ($p < 0.0001$) in intensity in FL-AS1411 injected mice relative to that of FL-CRO injected mice (Fig. 3B). We conclude that AS1411 localizes to migrating endothelial cells in the laser-induced model of CNV.

3.4 Intravitreal Injection of AS1411 attenuates laser induced CNV

The aptamer AS1411 is known to bind to cell surface nucleolin, which in turn transports it to the nucleus and induces cell senescence (Xu et al., 2001). Since we found both increased expression of cell surface nucleolin as well as localization of intravitreally injected AS1411 to the site of laser-induced CNV, we hypothesized that AS1411 may be an effective inhibitor of CNV through inhibition of endothelial cell proliferation. To assess the potential of AS1411 as an anti-angiogenic agent, immediately following laser injury, mice were injected in the vitreous with 1 μ l of PBS only, or PBS containing either 0.1 or 0.2 nmol of AS1411 or CRO. As a positive control for attenuation of laser-induced CNV, an additional set of mice were administered 2 pmol of a neutralizing antibody against mouse VEGF-A by intravitreal injection. This antibody has previously been shown to attenuate CNV formation when administered via subretinal or intravitreal injection

(Nozaki et al., 2006; Liu et al., 2011). 7 days following laser injury, eyecups were harvested and stained for endothelial cell growth, using FITC-conjugated GSL-I. Although no significant attenuation of CNV growth was noted in eyes injected with the 0.1nmol dose of AS1411, a significant reduction in CNV size was observed in those eyes injected with 0.2nmol of AS1411 relative to eyes injected with either CRO or PBS (Fig. 4A). Specifically, measurement of the area of GSL-I positive staining indicated a $29.53 \pm 12.58\%$ reduction ($p=0.0207$) and a $37.61 \pm 12.63\%$ reduction ($p=0.0035$) in the size of CNV lesions in eyes injected with 0.2nmol AS1411 relative to eyes injected with PBS or 0.2nmol CRO respectively (Fig. 4B). Thus we conclude that 0.2nmol is the minimally effective intravitreally injected dose of AS1411 required to attenuate formation of CNV. GSL-I staining indicated a $66.06 \pm 12.56\%$ reduction ($p<0.0001$) in CNV size in eyes injected with the anti-VEGF antibody compared to those injected with PBS. Additionally, no significant difference in the size of CNV lesions was noted between 0.1 or 0.2nmol CRO injected eyes relative to those injected with PBS. We conclude that intravitreal injection of AS1411 following laser injury attenuates choroidal neovascularization in the laser-induced model of 'wet' AMD and that this effect is not due to a non-specific response to oligonucleotides.

3.5 Intravitreal injection of AS1411 reduces infiltration of macrophages following laser induced CNV

Elevated levels of macrophages are present in the eyes of AMD patients as well as at the site of laser induced CNV (Penfold et al., 2001). Depletion of macrophages in the laser-induced model of 'wet' AMD results in an attenuation of CNV (Penfold et al., 2001; Espinosa-Heidmann et al., 2003; Sakurai et al., 2003). Expression of cell surface nucleolin has been documented on monocytes and macrophages (Hirano et al., 2005).

We hypothesized that AS1411 may impact the number of macrophages infiltrating the site of laser-induced injury in the model of wet AMD. Hence, we stained laser-induced CNV lesions of eyes injected with either AS1411 or CRO with an antibody against murine F4/80, a marker for macrophages (Fig. 5A). The average area of F4/80 staining observed in mice injected with AS1411 was $41689 \pm 6711 \mu\text{m}^2$ relative to $69840 \pm 13459 \mu\text{m}^2$ in mice injected with CRO, resulting in a $40.3\% \pm 19.8\%$ ($p=0.0485$) reduction in the area of macrophage infiltration (Fig. 5B). We conclude that AS1411 directly or indirectly inhibits macrophage infiltration in the murine laser-induced model of 'wet' AMD.

3.6 Topical Application of AS1411 attenuates laser induced CNV

The current standard of care for delivery of anti-VEGF molecules to the eye involves the use of an intravitreal injection (Keane and Sadda, 2012). As mentioned above, intravitreal injection is associated with a variety of complications, including patient discomfort and reduced compliance (Casten and Rovner, 2013). Hence, we wished to examine whether AS1411 may act as an inhibitor of CNV *in vivo* when delivered via topical application to the eye. We applied 1nmol of AS1411 or CRO or normal saline buffer once daily for eight days topically to the eye, initiating one day prior to laser-induced injury. Posterior eyecups were harvested on day 7 post-laser injury and stained with GSL-I (Fig. 6A). The average size of CNV lesions was $14909 \pm 1995 \mu\text{m}^2$ in eyes treated topically with AS1411, $26325 \pm 3494 \mu\text{m}^2$ in eyes treated topically with CRO and $28200 \pm 2991 \mu\text{m}^2$ in saline treated eyes (Fig. 6B). Topical treatment with AS1411, therefore, resulted in a $47.15\% \pm 12.58\%$ ($p=0.0003$) and $43.37\% \pm 15.57\%$ ($p=0.0066$) reduction in CNV size relative to saline treated or CRO treated eyes, respectively (Fig. 6B). CNV size between saline and CRO treated eyes was not significantly different

($p=0.6893$). We conclude that topical application of AS1411 inhibits laser-induced CNV in a murine model of 'wet' AMD.

4. Discussion:

This is the first study to examine the potential efficacy of a G-quartet aptamer targeting nucleolin in an animal model of 'wet' AMD. We report that expression of nucleolin is elevated on endothelial cells migrating in response to laser induced injury and that AS1411 co-localizes with nucleolin *in vivo* at the site of laser injury. Intravitreal delivery of AS1411 attenuates laser induced CNV and suppresses the infiltration of macrophages. Importantly, topical application of AS1411 also attenuates laser induced CNV. Our results have potential implications for the treatment of 'wet' AMD and possibly other ocular diseases involving choroidal neovascularization.

Due to the mounting evidence that VEGF plays a critical role in retinal homeostasis, one motivation for this study was to identify a therapeutic that did not directly target VEGF. While anti-VEGF therapeutics can prevent loss of vision for a significant proportion of 'wet' AMD patients, recent evidence from clinical studies indicates that long-term inhibition of endogenous VEGF negatively influences the ocular architecture and exacerbates macular degeneration (Sacu et al., 2011; Campochiaro et al., 2014). Unlike ranibizumab or bevacizumab that act through VEGF, AS1411 acts through nucleolin. Cell surface nucleolin is not only present on mitotic cells; nucleolin has also been reported on the surface of bovine and C57BL/6J murine photoreceptors (Hollander et al., 1999; Conley and Naash, 2010). There is a potential, therefore, for binding and internalization of AS1411 by photoreceptors. However, as photoreceptors are post-mitotic and AS1411 acts by inducing cellular senescence through inhibition of DNA

replication, the likelihood of AS1411 producing adverse off-target effects is reduced (Xu et al., 2001).

Comparison between current anti-VEGF treatments and intravitreal injection of AS1411 is not possible, since both *bevacizumab* and *ranibizumab* have been shown to be ineffective in the murine laser model of CNV (Lu and Adelman, 2009). Additionally, *pegaptanib sodium*, an aptamer targeting VEGF, did not exhibit any therapeutic effect in the murine laser induced model of CNV (Lu and Adelman, 2009). (Lu and Adelman, 2009) (Xu et al., 2001; Birke et al., 2013) Unlike these anti-VEGF therapies, AS1411 acts through nucleolin, which exhibits a high degree of evolutionary conservation, possibly allowing function of the aptamer across species. Due to the impracticality of testing equivalent concentrations of antibody to aptamer, the relative potency of the anti-VEGF antibody used in this study cannot be directly compared with the AS1411 aptamer. We can say, however, that our study indicates a more efficient minimum effective dose required for the anti-VEGF antibody than required for the nucleolin targeting AS1411. This could reflect the greater accessibility of a diffusible VEGF protein target by the intravitreally injected antibody than the surface bound nucleolin targeted by the AS1411 aptamer. This study warrants further investigation of nucleolin as a potential target for treating choroidal neovascularization

Our studies were also motivated by the current limitations associated with administration of therapeutics for the treatment of 'wet' AMD via intravitreal injection every 4 to 12 weeks (Keane and Sadda, 2012). Each injection carries a risk for retinal detachment, hemorrhage and endophthalmitis (Sampat and Garg, 2010). Sustained elevation in intraocular pressure in normotensive eyes following intravitreal injections was noted to occur in patients treated with *bevacizumab* at <8 week intervals and intravitreal

injections of *ranibizumab* and *bevacizumab* have been associated with ocular inflammation (Tolentino, 2011; Mathalone et al., 2012). Unlike some monoclonal antibodies associated with allergic infusion reactions, DNA aptamers, such as AS1411, are not significantly immunogenic, reducing the risk of such a reaction (Esposito et al., 2011). The significant burden placed on patients receiving injections every 4-12 weeks causes a reduction in quality of life and coupled with increased incidence of depression among this population can lead to decreased compliance (Goldstein et al., 2012). A therapeutic that could be administered noninvasively by the patient would overcome many of the issues associated with intraocular injections.

Due to the above unmet needs, the potential for inhibition of CNV through topically delivered drugs has been examined previously (Kiuchi et al., 2008; Sheu et al., 2009; Cloutier et al., 2012; Birke et al., 2013). In our studies, we found an approximate 45% reduction in CNV when 1nmol (8.5µg) of AS1411 was applied once daily one day prior and 7 days post-laser treatment. When 86µg aganirsen, an antisense oligonucleotide that inhibits insulin receptor substrate (IRS)-1 expression, was topically applied two days prior and 14 days post-laser treatment, it was observed to result in almost complete inhibition of high grade laser induced CNV in African green monkeys (Cloutier et al., 2012). A nicotinic acetylcholine receptor antagonist, mecamylamine, was reported to reduce laser induced CNV by 35% when topically applied twice daily at 100µg over the course of 7-14 days (Kiuchi et al., 2008). Topical application of 50ng of vasostatin three times daily for 21 days was also shown to reduce laser induced CNV by approximately 50% in rats (Sheu et al., 2009). Additionally, we have recently found that topical application of 80ng of PPADS, a P2X receptor antagonist, once daily for 3 days post laser treatment resulted in a 40% reduction in laser-induced CNV (Birke et al., 2013). We envisage that AS1411 may offer some unique advantages over other molecules in

that it has already gone through clinical trials as a potential chemotherapeutic and was found to be well-tolerated in patients enrolled in a phase II trial in whom it was administered intravenously at 40mg/kg/day over 1-4 days (Rosenberg et al., 2014). Another advantage of AS1411 is that DNA aptamers have a relatively low immunogenicity profile and synthesis of DNA aptamers is cost effective, reducing batch variability and potential viral or bacterial contamination associated with therapeutic antibody production (Esposito et al., 2011)(Esposito et al., 2011). While future studies examining the pharmacokinetic and toxicity profile of AS1411 will be required, in this study we have provided proof of concept that nucleolin is a target worthy of further investigation and that AS1411 has potential as a future treatment of AMD and possibly other ocular diseases involving choroidal neovascularization.

Figure Legends:

Figure 1: AS1411 attenuates the formation of tubes by HUVECs *in vitro* A)

Representative phase contrast images of tubes formed in the absence of supplements (negative control) or in the presence of 250nM of CRO or 250nM of AS1411. **B-D)**

Quantification of mean number of master segments, master junctions and number of meshes formed in the presence of AS1411 or CRO. Studies were performed twice in duplicate and statistical significance was determined using two-tailed unpaired t-test.

Figure 2: Cell surface nucleolin is expressed in laser-induced CNV A)

Representative images of murine eyecup harvested at 4 days post-laser captured with an inverted microscope. **B)** Representative confocal images of murine eyecup harvested at 4 days post-laser. Eyecups were stained for endothelial cell infiltration with GSL-I (green) and for surface nucleolin (red). Co-localization of GSL-I and nucleolin is shown in the overlay (yellow). N=15 spots (5 eyes).

Figure 3: AS1411 localizes to the site of laser-induced CNV A)

Representative images of GSL-I stained CNV in a mouse eye intravitreally injected with 0.2 nmol of FL-AS1411 or FL-CRO at day 4 post laser and harvested two hours post injection. **B)** Quantification of fluorescent signal per CNV area for FL-CRO (32 spots/10 eyes) and FL-AS1411 (34 spots/10 eyes) injected eyes (**** $p < 0.0001$)[two-tailed unpaired t-test].

Figure 4: Intravitreal Injection of AS1411 attenuates laser induced CNV A)

Representative images of GSL-I stained CNV at day 7 post laser in a mouse eye intravitreally injected with PBS, 0.1 nmol of CRO, 0.1 nmol of AS1411, 0.2 nmol of CRO, 0.2 nmol of AS1411, or 2pmol anti-VEGF antibody immediately following laser. **B)** Mean

values $\pm$ SEM of CNV area of PBS injected eyes (44 laser spots/16 eyes); 0.1nmol CRO injected eyes (28 laser spots/10 eyes); 0.1nmol AS1411 injected eyes (36 laser spots/10 eyes); 0.2nmol CRO injected eyes (60 laser spots/20 eyes); 0.2nmol AS1411 injected eyes (65 laser spots/20 eyes), or anti-VEGF Ab injected eyes (29 laser spots/10 eyes); * $p=0.0335$; ** $p=0.0035$; **** $p<0.0001$ [two-tailed unpaired t-test].

Figure 5: Intravitreal injection of AS1411 reduces infiltration of macrophages

following laser induced CNV A) Representative laser-induced CNV spots in murine eyecups stained with an antibody against the macrophage marker F4/80 in eyes intravitreally injected with 0.2 nmol CRO or 0.2 nmol AS1411. **B)** Quantification of the area of F4/80 staining indicated a significant reduction ($40.3\% \pm 19.8\%$, $p=0.0485$) in the area of macrophage staining in laser-induced CNV spots of eyes injected with 0.2 nmol of AS1411 (24 laser spots/6 eyes) relative to those injected with 0.2 nmol of CRO (17 laser spots/6 eyes)[two-tailed unpaired t-test].

Figure 6: Topical Application of AS1411 attenuates laser induced CNV A)

Representative images of GSL-I stained CNV at day 7 post laser in a mouse eye topically treated one day prior to and 7 days post laser with 0.9% NaCl; 1 nmol CRO or 1 nmol AS1411. **B)** Mean values $\pm$ SEM of CNV area of eyes topically treated daily with 0.9% NaCl (39 laser spots/12 eyes); 1 nmol CRO (45 laser spots/13 eyes) or 1 nmol AS1411 (42 laser spots/12 eyes). (** $p=0.0066$; *** $p=0.0003$)[two-tailed unpaired t-test].

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Figure 1

Figure 2

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Figure 3

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Figure 4

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Figure 5

Figure 6