Research Article

Novel Endogenous Glycan Therapy for Retinal Diseases: Safety, *In Vitro* Stability, Ocular Pharmacokinetic Modeling, and Biodistribution

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Abstract. Asialo, tri-antennary oligosaccharide (NA3 glycan) is an endogenous compound, which supports proper folding of outer segment membranes, promotes normal ultrastructure, and maintains protein expression patterns of photoreceptors and Müller cells in the absence of retinal pigment epithelium support. It is a potential new therapeutic for atrophic age-related macular degeneration (AMD) and other retinal degenerative disorders. Herein, we evaluate the safety, *in vitro* stability, ocular pharmacokinetics and biodistribution of NA3. NA3 was injected into the vitreous of New Zealand white rabbits at two concentrations viz. 1 nM (minimum effective concentration (MEC)) and 100 nM (100XMEC) at three time points. Safety was evaluated using routine clinical and laboratory tests. Ocular pharmacokinetics and biodistribution of [³H]NA3 were estimated using scintillation counting in various parts of the eye, multiple peripheral organs, and plasma. Pharmacokinetic parameters were estimated by non-compartmental modeling. A 2-aminobenzamide labeling and hydrophilic interaction liquid chromatography were used to assess plasma and vitreous stability. NA3 was well tolerated by the eye. The concentration of NA3 in eye tissues was in the order: vitreous>retina>sclera/choroid>aqueous humor>cornea>lens. Area under the curve (0 to infinity) (AUC_∞) was the highest in the vitreous thereby providing a positive concentration gradient for NA3 to reach the retina. Half-lives in critical eye tissues ranged between 40 and 60 h. NA3 concentrations were negligible in peripheral organs. Radioactivity from [³H]NA3 was excreted via urine and feces. NA3 was stable at 37°C in vitreous over a minimum of 6 days, while it degraded rapidly in plasma. Collectively, these results document that NA3 shows a good safety profile and favorable ocular pharmacokinetics.

KEY WORDS: age-related macular degeneration (AMD); NA3 glycan; pharmacokinetics; safety.

INTRODUCTION

Globally over 60 million people are crippled by blindness. The leading causes of irreversible blindness include age-related macular degeneration, retinal vascular diseases, and glaucoma. Visual impairment leads to a significant deterioration of the quality of life of an individual by negatively affecting daily living activities (1,2).

Age-related macular degeneration (AMD) is defined as an abnormality of the retinal pigment epithelium (RPE) that leads to degeneration of the overlying photoreceptors in the macula and consequent loss of

central vision (2–4). There is a decline in central vision due to the damage to the macula. Our central vision is essential for seeing objects clearly and for visualizing colors. AMD causes no pain. The diagnosis of AMD is based on visual dysfunction and characteristic macular findings. In some cases, AMD advances so slowly that people notice little change in their vision. In others, the disease progresses faster and may lead to a loss of vision in both eyes (reviewed in (5)).

AMD usually occurs in individuals over 50 years of age and is the most common cause of vision loss in elderly individuals throughout the developed world (2,6–12). It is estimated that over nine million people in the USA suffer from intermediate or advanced forms of AMD (2,13). Approximately 10–18% of individuals between 65 and 75 years old have lost some central vision as a result of AMD (5,12,14). Although neovascular AMD is the most damaging form of the disease, atrophic AMD accounts for approximately 90% of all cases (reviewed in (15)).

The atrophic form of AMD is characterized by the accumulation of membranous debris underneath the RPE basement membrane, which forms small, yellowish deposits called drusen (2). As reviewed succinctly (5), it is

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understood that RPE degeneration severely impairs the function of retinal photoreceptors. RPE thus plays a major role in normal retinal health. With age, there is a change in the pigmentation pattern, reduction of melanosomes, and cell density of RPE and an increase in the number of lipofuscin granules. There are also reports on accumulation of advanced glycation end products in RPE and Bruch's membrane that might contribute toward oxidative stress in the ECM environment (16–18). Hence, in atrophic AMD, there is an increase in oxidative stress due to compromised protective mechanisms of RPE. When RPE support is eliminated or reduced—as in the case in atrophic AMD/retinal detachment—the retinal health is compromised. Thus, there is a dire need to develop a therapy that ensures support to the retinal components in the absence of RPE support.

During the past decade, there has been a radical change in the treatment regimen of AMD with monoclonal antibody therapies emerging as the main line of therapy for neovascular AMD. While no Food and Drug Administration (FDA)-approved macular degeneration treatment options lead to a complete restoration of vision lost to the eye disease, a few possibilities such as ranibizumab, aflibercept, and some other investigational drug approaches may preserve or even improve the remaining vision. There are also reports on novel drug delivery approaches that are being investigated to retain the drug in the eye for a longer period of time both by injections and topical applications (19–21). Most of these approaches, both FDA and non-FDA-approved, however, are for the neovascular form of AMD. There is a huge technological gap and an untapped market potential for drug development for atrophic AMD (20). Over the last years, a significant amount of research has been focused on the physiopathology of atrophic AMD and new treatment approaches. However, research has been hampered by some issues, such as the multifactorial nature of atrophic AMD, its complex physiopathology, the lack of a suitable animal model, and the lack of *in vitro* systems for testing new drugs (reviewed in (15)). Currently, the only approved treatment for atrophic AMD is the use of the Age Related Eye Disease Study formulation. However, this multivitamin complex does not prevent AMD, and its positive effects are modest as it only slows down the progression for patients at high risk for advanced AMD (15).

We have convincing evidence (22) that administration of “permissive glycans” to the retina promotes the ability of photoreceptors to assemble outer segment membranes correctly in an RPE-deprived state, promotes normal ultrastructure, and maintains normal patterns of protein expression of photoreceptors and Müller cells, thus making the glycans a potential therapeutic option for atrophic AMD and retinal detachments (22–25).

In our current endeavor, we study the preclinical safety, biodistribution, and pharmacokinetics of NA3 glycan (molecular weight, 2,006.82 Da) in New Zealand white (NZW) rabbits by delivering the compound in a sterile solution form via the intravitreal route, a possible route explored by us for the delivery of this molecule. Understanding the pharmacokinetics and safety is very critical for development of new drug delivery systems of NA3.

MATERIALS AND METHODS

Materials

NA3 was procured from QA-Bio, San Mateo, CA, USA. Tritiated sodium borohydride, Ultima GoldTM cocktail, and SolvableTM were procured from Perkin Elmer, Waltham, MA, USA. Sodium borate, sodium hydroxide, acetic acid, and methanol were all of the highest purity available from Sigma Aldrich, St. Louis, MO, USA. Ketamine hydrochloride was procured from Fort Dodge, Inc., Fort Dodge, IN, USA; xylazine was procured from Phoenix Scientific, Inc., St Joseph, MO, USA; proparacaine hydrochloride, phenylephrine hydrochloride 2.5% (Akorn, Inc., Decatur, IL, USA), and tropicamide 1% (Alcon, Inc., Humacao, Puerto Rico) were gifts from the Hamilton Eye Institute clinics. 2-Aminobenzamide (100 pmol) was purchased from Prozyme Inc., Hayward, CA, USA. HPLC column viz. TSK-gel amide-80 column—4.6 mm i.d., 150 mm length, and 3 μ column packing—and amide-80 guard column—3.2 mm i.d., 15 mm length, and 3 μ column packing—were procured from TOSOH Biosciences, King of Prussia, PA, USA. Five percent low-melt agarose was procured from Sigma, St. Louis, MO, USA. DeadEndTM Fluorometric terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) system kit procured from Promega, Madison, WI, USA was used for Müller cell activation studies.

Methods

Animals

NZW rabbits (procured from Myrtle Rabbitry, Thompsons Station, TN, USA) were maintained and treated according to guidelines set forth by the Association for Research in Vision and Ophthalmology for the use of animals in ophthalmic and vision research and were approved by our Institutional ACUC. A total of 15 rabbits of either sex weighing approximately 2.0–3.0 kg each were used in the safety evaluations of NA3 glycan. The safety of NA3 glycan was assessed after intravitreal injection at two concentration levels of NA3 glycan solution (1 and 100 nM) at three time points each, with three rabbits per group. They were housed under 12:12-h light/dark cycle and were allowed free access to water and food. Before any procedure, animals were anesthetized with a mixture of ketamine hydrochloride (35 mg/kg) IM and xylazine (5 mg/kg) intramuscularly (i.m.). Following general anesthesia, an additional local anesthesia in the form of proparacaine hydrochloride (0.5% ophthalmic drops) was used topically on the eye. The pupils were dilated with one drop each of phenylephrine hydrochloride 2.5% (Akorn, Inc., Decatur, IL, USA) and tropicamide 1% (Alcon, Inc., Humacao, Puerto Rico). For safety estimations of NA3 glycan, each rabbit underwent clinical inspections by slit-lamp biomicroscopic examination, fundus photography, intraocular pressure (IOP), electroretinography (ERG), and optical coherence tomography (OCT) recordings prior to study and on 1, 3, 7, and 14 days after intravitreal injection to detect possible damage to the eye.

A total of 15 NZW rabbits of either sex weighing approximately 1.0–2.0 kg were used for the pharmacokinetic

estimations. The animals were conditioned and maintained as described above. Due to the abundant availability of endogenous NA3 glycan/other glycans, which could interfere with the analytical estimation of NA3 after intravitreal injection, NA3 was radiolabeled with [^3H] to trace the disposition of NA3 in the eye and other organs. Pharmacokinetics of NA3 was assessed using injections of [^3H]NA3 at following time points based on our pilot studies: 2 h, 6 h, 1 day, 2 days, 3 days, 5 days, 7 days, 10 days, 12 days, and 14 days.

Radiolabeling of NA3 Glycan

We labeled NA3 glycan with [^3H] to enable sensitive analytical estimation. The labeling reaction involved reduction with tritiated [^3H]sodium borohydride at the reducing end of NA3 glycan. Tritium labeling was performed using a reported method for tritium labeling of carbohydrates with slight modifications (26). Briefly, NA3 glycan (20 μg) was dissolved in borate buffer, pH 9.5, and radiolabeled using [^3H]sodium borohydride (100 mCi; 5–15 Ci/mmol; 5 mCi/reaction). Sodium borohydride was used to complete the reaction while acetic acid in methanol and methanol washings was used to remove unreacted [^3H]. The sample obtained was dissolved in 400 μL of water, which was dialyzed six times using Float-a-lyzer, 500–1,000 Da against 900 mL of phosphate buffer saline, 24 h each time. The collected dialysate was checked for [^3H] counts after every step. The dialysis was continued until the [^3H] counts in the dialysate were less than 100 dpm. After dialysis, the solution from the dialysis bag was transferred to a sterile vial. This was the final [^3H]NA3 glycan solution used for the intravitreal injections. Aseptic conditions were maintained throughout the process.

Intravitreal Injections

Safety Assessments. At aforementioned time points, depending on the animal group, either 1 or 100 nM of NA3 solution (formulated using sterile phosphate-buffered saline (PBS) buffer, pH 7.4) was injected intravitreally in both eyes. All procedures were performed under aseptic conditions using an operating microscope for visualization. Intravitreal injection was performed transconjunctivally, in the superior-temporal quadrant, using a 31G needle attached to BD insulin syringe inserted bevel up approximately 1 mm posterior to the limbus. The tip was pointed at the center of the vitreous cavity. A volume of 30 μL (either 1 or 100 nM NA3) was then slowly injected. At the end of the procedure, topical Tobradex (tobramycin and dexamethasone) was administered to each eye, once a day until sacrifice. All observations were compared to control rabbits (un-injected). Slit-lamp biomicroscopic and fundoscopic examinations were performed prior to injection and thereafter during the follow-up period as described in the section for clinical exams. The animals were observed for 14 days for detection of possible retinal injury, inflammation, or cataract formation. All clinical and ocular observations were recorded on a chart. The rabbits were euthanized by intravenous injection of an overdose of pentobarbital sodium (EuthasolTM) at the predetermined time of sacrifice.

Pharmacokinetic and Biodistribution Estimations. Rabbits were grouped in three sets, with each group comprising of five NZW rabbits. To ensure robustness, repeatability, and accuracy of the analyses, the groups were identical and received the same dose of [^3H]NA3, following the same protocol. [^3H]NA3 glycan solution (as obtained in aseptic conditions from the radiolabeling reaction; 1.311 μCi) was injected and ocular tissues harvested at ten time points viz. 2 h, 6 h, 1 day, 2 days, 3 days, 5 days, 7 days, 10 days, 12 days, and 14 days. The time points were optimized based on our pilot study results of disposition of [^3H]NA3 glycan. Each eye received a fixed injection volume (30 μL) and dose (1.311 μCi) of [^3H]NA3. The clinical procedure and setup for intravitreal injections were similar to that followed for the injection of NA3 glycan. As we injected in both eyes, we considered double the amount [^3H]NA3 for biodistribution studies in extra-ocular/peripheral tissues.

Clinical Exams for Safety Estimation

For assessment of safety in rabbits after intravitreal injections of NA3 glycan, we conducted follow-up clinical examinations that included color fundus photography, IOP measurement, OCT, and ERG analysis. Rabbits were anesthetized prior to examinations as described previously. Baseline examinations were performed 1 day before the first intravitreal injection, and follow-up examinations were performed at days 1, 3, 7, and 14 after the first injection. We also conducted slit lamp biomicroscopy randomly to see if there are any adverse effects during the follow-up period.

Fundus Photography. To assess if there were any gross structural abnormalities of the posterior parts of the eye, we used a hand-held color fundus photographic device (ClearView[®] optical imaging system, Optibrand). The images were captured by a portable digital fundus camera system after the pupil was dilated. The fundoscope was connected to a computer, and the device was adjusted to focus on the posterior segment of the eye. After adjustment, multiple images of each eye were taken and analyzed for abnormalities such as inflammation, retinal detachment, and optic nerve head changes. The examinations were conducted at 1 and 14 days after the intravitreal injections either at 1 or 100 nM concentration level of NA3 glycan.

Intraocular Pressure. To assess whether intravitreal NA3 causes any change in IOP, the pressure was measured in all animals at aforementioned time points. Rabbits were anesthetized prior to examinations as described previously. IOP was measured using a tonometer (Icare Tonolab[®], Icare Finland Oy) for each eye. Anesthetized animals were placed in a restrainer prior to IOP measurement. An average of three consecutive readings was considered for the analysis. Pre-injection measurements were compared with the final measurements after designated time points up to 14 days at both dose levels, i.e., 1 and 100 nM of NA3 glycan. Statistical analyses were performed using a paired *t* test (Prism 6 software, La Jolla, CA, USA).

Optical Coherence Tomography. An SD-OCT system (Bioptigen Spectral Domain Ophthalmic Imaging System; Bioptigen, Inc., Durham, NC, USA) was used to assess the retinal health post-intravitreal injection in each rabbit at the specified time periods. OCT evaluation allows for non-invasive detection of retinal architecture, including quantitative measurements of retinal thickness and longitudinal observation of the retinal architecture (27). OCT examination demonstrated that the structure of retina was intact and there was no difference in the retina after intravitreal injection. Rabbits were anesthetized and the pupils dilated with tropicamide, 1% solution. Corneas were lubricated frequently (Systane Ultra ophthalmic lubricant; Alcon Ltd., Fort Worth, TX, USA) during the imaging session. Rabbits were placed in a restrainer for stability, and a pediatric probe was used for OCT analysis. We used 6-mm horizontal line scans in the inferior retina below the optic disk for capturing the retinal cross section. The safety was estimated using the highest used concentration of NA3, i.e., 100 nM, for 14 days.

Electroretinography. To assess functional alterations in sensorineural retina, ERG analysis was performed before and after intravitreal injections of NA3 glycan after specified time periods. Dark-adapted ERG responses were elicited by flashes of different intensities (from -4.0 Log units to 2.88 Log units) during each recording session.

The ERG responses of experimental/NA3-injected eyes (7 and 14 days after injection) were compared to the ERG response of the control/pre-injected eye at a concentration of 100 nM at 7 and 14 days post-injection and compared statistically using ANOVA. Rabbits were dark adapted for at least 1 h and were anesthetized 20 min before ERGs were obtained. The cornea was anesthetized with topical 0.5% proparacaine hydrochloride, and the pupils were dilated with 1% tropicamide. Gold ring electrodes with diameter of 10 mm were put on both corneas (Roland Consult, Stasche & Finger GmbH). The reference and ground electrodes were stainless steel surgical needles. The reference electrodes were placed subcutaneously in the middle of forehead, and the ground electrode was on the back of the rabbit. ERG recordings were obtained in both eyes simultaneously, with the head in the upright position. The ERG signals were amplified, and light stimuli were obtained from a Ganzfeld light source. The wave tracings were plotted using the raw data generated by the instrument. The amplitude of the a-wave is measured from the baseline to the first negative slope, and the amplitude of the b-wave is measured from the most negative aspect of the a-wave slope to the most positive aspect of the following slope. ERG changes were considered significant if the maximal amplitude of a- and b-waves were decreased by more than 30% compared to the pre-injection values.

Slit Lamp Biomicroscopy. Rabbit eyes were dilated with one drop each of 0.5% tropicamide ophthalmic solution prior to examination. The animals were restrained manually with a standard rabbit holder during the examination. Eyes were evaluated at baseline and different time points after injection using slit-lamp biomicroscopy. Anterior segment was examined for aqueous flare, iris hyperemia, and fibrin.

Müller Cell Activation and Apoptosis Estimations

We performed conventional immunohistochemistry on control eyes as well on the NA3-injected eyes at specified time periods and tested for Müller cell activation using an anti-glial fibrillary acidic protein (GFAP) antibody. We also tested for apoptotic cell death using TUNEL staining. The technique is based upon detection of fragmented DNA, a well-recognized characteristic of apoptosis. We combined immunofluorescent GFAP labeling and subsequent TUNEL assay on the same tissue sections using our previously published methods (24,28) and followed DeadEnd™ Fluorometric TUNEL system protocol provided by Promega, Madison, WI, USA (29). Sections were obtained using a Vibratome (Leica VT 1000S, Leica Biosystems Nussloch GmbH, Germany). Images of the sections were then viewed and digitally captured on a laser scanning confocal microscope (C1 Plus Nikon confocal microscope, Tokyo, Japan).

Ex Vivo Plasma and Vitreous Stability of NA3 Glycan

A 2-aminobenzamide labeled-NA3 glycan solution (2-AB NA3) was incubated with rabbit plasma and excised vitreous at final concentration of 10 pM for 24 h and up to 6 days, respectively. At each sampling point, a fixed aliquot of the sample was removed, and the proteins in the samples were precipitated by addition of acetonitrile followed by centrifugation at 10,000 rpm at 4°C . The supernatant was diluted suitably with a dilution fluid (acetonitrile/ammonium acetate buffer, 50 mM; 70:30 v/v) to obtain a final analyte concentration of 10 pmol. The control samples consisted of incubating 2-AB NA3 with PBS buffer, pH 7.4, and subsequently followed the aforementioned steps of sample preparation to obtain a final concentration of 10 pmol. The samples and controls were analyzed by a slightly modified version of the reported protocol of Prozyme Inc. (Hayward, CA, USA). Briefly, samples (20 μL) were injected in an HPLC system using the following conditions: column: TSK-gel amide-80 column—4.6 mm i.d., 150 mm length, 3 μ packing; method: gradient, column temperature $30^{\circ}\pm 2^{\circ}\text{C}$, excitation λ_{max} 330, emission λ_{max} 420 nm; guard column: amide-80—3.2 mm i.d., 15 mm length, 3 μ column packing; mobile phase: solvent A—acetonitrile; solvent B—50 mM ammonium formate, pH 4.4 adjusted with formic acid, gradient program: as presented in Supplementary Table 1.

The peak areas within the chromatograms were used for computing NA3 content in each sample based upon areas from the standard NA3 solution (10 pmol). The stability behavior was studied using a control in PBS. We evaluated statistical differences using a paired *t* test (Prism 6 software, La Jolla, CA, USA).

Sample Preparation and Scintillation Counting Analysis for Ocular Kinetics and Bio-distribution Analyses

Rabbits were grouped into three sets, with each group comprised of five NZW rabbits. Rabbits were sacrificed at designated time points viz. 2 h, 6 h, 1 day, 2 days, 3 days, 5 days, 7 days, 10 days, 12 days, and 14 days. After anesthesia, blood was collected from the ear vein or heart, prior to

sacrifice using Euthasol® injection. Eyes were enucleated immediately after sacrifice. Under a dissecting microscope, the aqueous humor was collected using an insulin syringe with a 31-awg needle. The cornea and lens were collected separately. Using a 1-mL syringe, vitreous humor was excised. Sclera, choroid, and RPE were obtained as one-single component because the rabbit was non-pigmented; hence, it was difficult to separate these parts. Retina was then isolated. All tissues were preserved in -80°C until further processing.

Blood collected from the heart was centrifuged at 10,000 rpm at 4°C to separate plasma as the supernatant. Plasma was stored at -80°C until further use. Similarly, vital rabbit organs, namely kidneys, spleen, liver, lungs, heart, and brain, were isolated and stored in sterile containers at -80°C until further processing.

The tissue samples were thawed prior to sample preparation for liquid scintillation counting. Approximately 50–100 mg of each ocular tissues (except vitreous) was transferred to 20 mL clear glass scintillation vials. Vitreous humor was sampled using a graduated micropipette and approximately 200 mg was transferred in 20 mL clear glass scintillation vials. Except for cornea and retina, all the samples were prepared in triplicate for all three groups of rabbits. As for cornea and retina due to small sample size, duplicate samples were prepared. The samples were prepared as per the protocol provided by Perkin Elmer for biological tissue preparation using slight modifications. Briefly, to the weighed ocular tissues in 20 mL clear glass scintillation vials, 1 mL of Solvable™ was added. The vial was vortexed for 1 min using vortex mixer. The vial was then placed in thermostatic oven at 60°C for 1 h for solubilizing the tissues. After 1 h, the samples were allowed to attain ambient temperatures, and 200 μL of hydrogen peroxide (30%) was added for bleaching, if any. The vials were then kept at 60°C for 2 h for complete bleaching and solubilization of the samples. After 2 h, the samples were allowed to cool to ambient temperature, and 10 mL Ultima Gold™ liquid scintillation cocktail was added. The vials were then allowed to dark adapt in a box under gentle swirling for 2 h before being read on a TriCarb™ scintillation counter (Perkin Elmer, Waltham, MA, USA) with appropriate controls. The analysis was carried out in triplicate.

Similar methods were used for tritium estimation in plasma, urine, feces, and other tissues, except 200 μL of plasma sample, and approximately 50–100 mg of extra-ocular tissue samples was used. A qualitative measure was performed for urine (mixed with hay) and feces.

Readings obtained from the scintillation counter were used to calculate the disintegrations per minute values of the radioactivity from the tissue samples. The counting methods for the tissue samples were optimized and validated using appropriate standards. The radioactivity values were then converted to nanocuries per gram of the tissue and further processed using *in silico* pharmacokinetic tools.

Pharmacokinetic Modeling

The pharmacokinetic profile of radiolabeled NA3 glycan was analyzed from radioactivity/g of tissue—time data after intravitreal administration by non-compartmental modeling using Phoenix WinNonlin 6.2 (Pharsight Corporation,

Mountain View, CA, USA). Briefly, mean radioactivity values (from triplicate samples in each group) from vitreous humor, cornea, lens, retina, sclera/choroid, and RPE at pre-determined time points were analyzed to determine the mean half-life (in hours) and area under the curve (0 to infinity) ($\text{AUC}_{0-\infty}$) (in hour nanocuries per gram) (30–33). A model assuming linear kinetics with instantaneous input into the first compartment (i.e., vitreous) was used to assess the pharmacokinetic parameters after a single intravitreal dose of NA3 glycan. Results were expressed per gram of tissue. In ocular tissues, the terminal-phase elimination rate constant (K_v) was determined by linear regression of the natural logarithms of the concentrations against time for the log-linear elimination phase as follows:

$$K_v = \ln C_t - \ln C_0 / \Delta t$$

where C_t =concentration at the specified time period, Δt =time difference between the two points in consideration, and the terminal half-life ($t_{1/2}$) was calculated from the equation:

$$t_{1/2} = 0.693 / K_v$$

The area under the radioactivity–time curve from time 0 to the time t (AUC_{0-t}) for each tissue/component was calculated by the trapezoidal rule and extrapolated to infinity according to the following equation:

$$\text{AUC}_{0-\infty} = \text{AUC}_{0-t} + (C_t / K_v)$$

where C_t is the last drug concentration available, taken at time t (14 days). Similar models were used to compute the pharmacokinetic parameters from other non-ocular tissues.

RESULTS

Based on our previous studies on retinal degenerative diseases and potential therapies (22–25), NA3 glycan has clearly emerged as one of the forerunners for atrophic AMD, retinal detachments, and other retinal diseases. To test if NA3 is safe after intravitreal injection, we used routine clinical exams. Our objective was to ascertain that there are no adverse effects seen after the injection for a sustained time period. We confirmed these results by testing for microglia activation and via GFAP immunohistochemistry and apoptosis via TUNEL. Overall, we observed that NA3 glycan was well tolerated at both dose levels 1 and 100 nM at 14 days post-injection. No clinical signs or changes in food consumption or body weight attributable to the drug were observed.

Clinical Examinations for Assessment of Safety of NA3 Glycan

Clinical exam for evaluation of safety of NA3 after intravitreal injections included color fundus photography, IOP measurements, and OCT and ERG analyses.

Fundoscopic Examination Reports

Figure 1a, c shows the fundus image of non-injected rabbits (controls). There were no signs of inflammation or adverse effects noted on these animals during the course of the study. After intravitreal injection, as well as during the follow-up period of 14 days, no inflammation, uveitic reactions, cataracts, or apparent disease of the vitreous, retina, or choroid were observed by ophthalmoscopy or fundus photography in all eyes (Fig. 1b, d). The vitreous demonstrated no opacities or degeneration. The conjunctiva and cornea were clear, and there were no signs of anterior or posterior chamber inflammatory response. The lens appeared clear and the fundus was intact in both the NA3 glycan and non-injected eyes (Fig. 1a–d). Images from Fig. 1a, b are from the same rabbit and so are from Fig. 1c to d. No adverse effects were seen after intravitreal injection of 100 nM NA3, that is, 100 times the physiological dose, with the optic nerve head and visual streak clearly visible compared similar to the non-injected group.

Intraocular Pressure

There was no statistically significant difference ($p > 0.05$) in intraocular pressures after injection of 1 nM ($p = 0.0618$) or 100 nM ($p = 0.571$) NA3 glycan. The average IOP value at baseline (pre-injection) was 11.1 ± 0.9 mmHg. The values post-injection over a period of 14 days at two dose levels of 1 and 100 nM ranged from 9.5 ± 0.7 to 11.7 ± 3.4 mmHg. All post-injection IOP values were within the normal physiological ranges (Fig. 2).

OCT Examination

Figure 3a elucidates the OCT examination result before injection, while Fig. 3b gives the OCT analysis results 14 days post-100 nM injection on the same rabbit. The thickness and structure of different retinal layers viz. RPE, inner segment/outer segment, outer nuclear layer, inner nuclear layer, and ganglion cell layer remains unchanged after injection of 100 times the physiological dose at 14 days post-injection.

ERG Examination

Figure 4a shows no statistical significant differences ($p > 0.05$) when using ANOVA and Bonferroni correction in the amplitude between the rabbits' eyes before injection and 1, 7, and 14 days after 100 nM NA3 injection. Figure 4b shows a representative ERG graph before 100 nM NA3 injection, while Fig. 4c represents the ERG tracings 1 day after injection of 100 nM NA3. In Fig. 4a, the x-axis represents the average and standard error of the mean before injection and 1, 7, and 14 days after injection. In Fig. 4b, c, x-axis shows time in milliseconds and y-axis shows amplitude in nanovolts. There is a slight increase in the amplitude after 1 day post-injection of 100 nM NA3.

Slit Lamp Biomicroscopy

Slit lamp examinations on random animals revealed that the corneas of all the eyes were clear (not shown). There were no signs of ocular irritations, and anterior chamber cells could be normally identified upon ophthalmic examination.

Fig. 1. Fundoscopic images before and after intravitreal injection of NA3 glycan: **a** rabbit 1, day 0, before injection; **b** rabbit 1, day 1 after 100 nM NA3 glycan injection; **c** rabbit 2, day 0, before injection; and **d** rabbit 2, day 14 after 100 nM NA3 glycan injection

Fig. 2. Intraocular pressure assessment after intravitreal injection of NA3 glycan: The x-axis represents the days post-injection up to 14 days at two concentration levels of 1 and 100 nM

GFAP and TUNEL Staining

GFAP expression in Muller cells is a widely used, highly sensitive molecular indicator of retinal stress (34). As shown in Fig. 5a–c, no GFAP expression was seen at 3 days post-injection of 1 nM NA3 (Fig. 5a), or at 1 day post-injection of 100 nM NA3 (Fig. 5b). However, as shown in Fig. 5c, we did see some weak staining for GFAP immunoreactivity in the ganglion cell layer in the retina at 7 days post-injection of 100 nM NA3 (white arrow), which is hundred times the physiological dose. The expression seen was not pronounced and was present only in a few areas of the retina. There was also no activation of Müller glia in the deep retinal layers after all the injections and subsequent follow-up. This confirmed the non-inflammatory/non-toxic nature of NA3 glycan in the eye after intravitreal injection.

TUNEL staining in rabbit retinas is shown in Fig. 5a–c, while Fig. 5d provides positive control for comparison. No TUNEL-positive cells were seen at 3 days post-injection of 1 nM NA3 (Fig. 5a), or at 1 day post-injection of 100 nM NA3 (Fig. 5b) or at 7 days post-injection of 100 nM NA3 glycan. No TUNEL-positive cells are noted in the retinas of any animal injected with NA3 glycan during the follow-up period.

Ex Vivo Plasma and Vitreous Stability of NA3 Glycan

We developed a robust hydrophilic interaction liquid chromatography (HILIC) method for estimation of commercially available 2-AB NA3. The method was highly sensitive and reproducible (data not shown). As seen in Fig. 6a, NA3 content dropped significantly by over 80% over a period of 24 h in plasma samples as compared to the control samples in PBS ($p=0.0340$). This analysis clearly shows that NA3 glycan degrades when incubated *ex vivo* in plasma. In

the next step, we determined the stability of NA3 glycan in excised rabbit vitreous. We followed a similar methodology as followed for plasma stability of NA3 glycan. As seen in Fig. 6b, NA3 glycan was stable in vitreous for the entire 6-day stability study. NA3 content in vitreous under simulated biological conditions does not differ significantly from control PBS samples during the 6-day testing period ($p=0.2822$). The NA3 content was found to range from 94% to 111% showing the stable behavior of NA3 in vitreous. For representative purposes, blank plasma chromatogram, chromatogram of NA3, chromatogram of plasma spiked with NA3, and chromatogram of vitreous spiked with NA3 are shown in Supplementary Figure 1.

Pharmacokinetics

NA3 glycan pharmacokinetics in rabbits after a single intravitreal injection in each eye was modeled using non-compartmental analysis (30–33). The NA3 glycan concentration (in terms of radioactivity) in various eye tissues was in the following order from the highest to lowest: vitreous > retina > sclera/choroid > aqueous humor > cornea > lens. A concentration (in nanocuries per gram tissue)–time profile for critical eye tissues over a period of 14 days is presented in Fig. 7. AUC_{∞} was the highest in the vitreous ($51,678 \pm 5,771$ h nCi/mL) and was the lowest in the lens ($3,074 \pm 749$ h nCi/mL, not shown). As presented in Fig. 8, the second highest AUC_{∞} was observed in the retina, which is also our proposed site of action (22–25). Our studies indicate a positive concentration gradient between the eye tissues which is likely very important for the movement of NA3 glycan between eye tissues and subsequent equilibration.

The half-lives of NA3 glycan in critical eye tissues are presented in Table I. Half-lives in critical eye tissues were between 40 and 60 h, with the longest half-life of 58.2 ± 13.6 h seen in the aqueous humor. Estimated vitreal half-life was 48.9 ± 11.9 h. Negligible amounts of NA3 glycan were found in other systemic organs (Fig. 9). This corroborates our earlier finding of degradation of glycan in plasma. Radioactivity from the tritiated NA3 glycan was excreted via urine and feces as detected qualitatively by tritium counts (data not shown).

DISCUSSION

Over the last few years, a significant amount of research has been focused on the physiopathology of atrophic AMD and possible new treatment approaches. The multifactorial nature of atrophic AMD, its complex physiopathology, the lack of an animal model for atrophic AMD, and the lack of *in vitro* systems for testing new drugs make atrophic AMD a

Fig. 3. Optical coherence tomography analysis after intravitreal injection of NA3 glycan: **a** rabbit 3, day 0, before injection and **b** rabbit 3, day 14, 100 nM NA3 glycan injection. RPE retinal pigment epithelium, OS/IS outer segment/inner segment, ONL outer nuclear layer, INL inner nuclear layer, GCL ganglion cell layer

Fig. 4. ERG analysis after intravitreal injection of NA3 glycan: **a** measurement of a- and b-wave amplitudes (in nanovolts) before and after injection, **b** representative ERG graph before injection, and **c** ERG tracings after day 1 of 100 nM NA3 injection

very challenging disease to treat (15). With no effective drug treatment options, atrophic AMD represents a huge technological gap for new drug development (15,19).

In our previous research, it was convincingly shown (22) that administration of “permissive glycans” to the retina promotes the ability of photoreceptors to assemble

the outer segment membranes normally in an RPE-deprived state, promotes normal ultrastructure, and maintains normal patterns of protein expression of photoreceptors and Müller cells. Based on these previous studies on retinal degenerative diseases and potential therapies (22–25), NA3 glycan has clearly emerged as one of the

Fig. 5. GFAP and TUNEL estimations after intravitreal injection of NA3 glycan: **a** day 3 after 1 nM NA3 glycan injection, **b** day 1 after 100 nM NA3 glycan injection, **c** day 7 after 100 nM NA3 glycan injection, and **d** positive control for TUNEL. Blue color indicates nuclei, red color indicates GFAP expression, and green color indicates TUNEL staining. ONL outer nuclear layer, INL inner nuclear layer, GCL ganglion cell layer

Fig. 6. Stability estimation of NA3 glycan: **a** *ex vivo* plasma stability of NA3 glycan compared to PBS (control) at day 0 and day 1 and **b** *ex vivo* stability of NA3 glycan in vitreous compared to PBS (control) at day 0, day 5, and day 6

forerunners for atrophic AMD, retinal detachments, and other retinal diseases.

In these previous studies, we demonstrated that galactose and structurally related simple sugars support outer segment membrane assembly, outer retina ultrastructure, and protein expression profiles in RPE-deprived *Xenopus laevis* tadpole eyes at 10^{-3} M (23–25,35–37). Subsequently we tested the ability of this complex oligosaccharide with three terminal galactose residues (i.e., NA3 glycan), to support outer segment assembly. A3 family glycans, to which NA3 glycans (asialo tri-antennary oligosaccharide, a permissive glycan) belong, are naturally occurring N-linked oligosaccharides conjugated to a diverse group of glycoproteins (38). NA3 was applied to isolated RPE-deprived *Xenopus* tadpole eyes at concentrations ranging from 10^{-7} to 10^{-11} M. These results demonstrated that the increased valency of the glycan dramatically reduced the effective concentration necessary

Fig. 7. NA3 glycan concentration (expressed as radioactivity)–time profile in ocular tissues after intravitreal injection of NA3 glycan

Fig. 8. Area under the curve (AUC_∞; in hour nanocuries per gram) 14 days after intravitreal injection of NA3 glycan in critical eye tissues

to support outer segment assembly compared to univalent sugars (unpublished finding). Because NA3 supports outer segment folding and outer retinal protein expression levels in the RPE-deprived state, in other ongoing parallel studies we are evaluating it as a possible treatment for retinal conditions in which RPE support of the retina has been compromised such as atrophic AMD and retinal detachment. We demonstrated a novel function of permissive glycans in the regulation of photoreceptor outer segment assembly and adherens junction formation. Because of our extensive data documenting the neuroprotective and neurosupportive nature of NA3, we are evaluating it as a possible treatment for atrophic AMD, retinal detachment, and other retinal diseases.

It is imperative that a molecule must be safe and possess favorable pharmacokinetic parameters to enable its further use in the clinic. It is also important that the drug molecule reaches its proposed site of action at the required amount. In our current endeavor, we tested the safety of NA3 glycan in rabbits after intravitreal injection at predetermined time points over a period of 14 days. Follow-up clinical examinations including IOP, OCT, and ERG analysis are important tools for observing any abnormal reactions during the course of the study. Our follow-up studies were free from any adverse reactions even at 100 times the MEC, which clearly demonstrated the safe nature of NA3 glycan. We have thus factored in a wide range of NA3 dose for our safety analyses. Adverse effects like high IOP, inflammation, loss/degeneration of retinal layers/cells, reduction of retinal function, increased GFAP expression, etc. are detrimental to the retinal health and may lead to reduction in visual acuity in the long run.

We found that the IOP was well within the normal range at all conditions including up to 14 days after 100 nM NA3 glycan injection suggesting that the intravitreal injection does

Table I. Half-Life Estimates in Critical Eye Tissues After Intravitreal Injection of NA3

Compartment of the eye	Mean half-life (h)	SE
Vitreous	48.9	11.9
Retina	46.2	8.9
Aqueous humor	58.2	13.6
Sclera, choroid, and RPE	43.3	5.7

SE standard error, RPE retinal pigment epithelium

Fig. 9. Biodistribution (concentration expressed as radioactivity; in nanocuries per gram tissue) of NA3 glycan in various critical organs in NZW rabbit up to 14 days after intravitreal injection of NA3 glycan

not cause any transient or chronic increase in IOP. OCT examination post-intravitreal injection revealed a three-dimensional in-depth picture of the retina, in which we did not observe any abnormalities. The retinal layers were intact without any swelling or edema after a period of 14 days further indicating the safe nature of NA3 glycan. After the intravitreal injection of NA3 glycan, we did not find a change in the a- or b-wave after 7 days at both 1 and 100 nM; however, we found a slight increase in the amplitude after 14 days after a 100-nM injection possibly due to a protective effect. Slit lamp biomicroscopy examinations on random animals did not reveal any inflammation or adverse effects, corroborating with other studies concluding the safety of NA3.

We assessed the toxicity of NA3 glycan at the cellular level by glia activation and apoptosis estimation. Astrocytes present in the central nervous system, retina, and optic nerve head were previously thought to be bystander cells, but clearly in the past few years various studies have indicated that they play important roles such as regulation of ionic balance, neurotransmission, synaptic plasticity, and neurodegeneration (39). GFAP is an intermediate filament that is normally expressed in astrocytes but not Müller cells in the retina (34,40). However, in a variety of retinal injuries such as retinal detachment (41), ischemia (42), and increased intraocular pressure (43), GFAP expression in retinal Müller cells became evident (34). Because of this, GFAP expression in Müller cells has been widely used as a molecular indicator of retinal stress. It has also been suggested that GFAP immunostaining is a more sensitive tool for detecting retinal stress than ERG (34). Reactive glial cells also express various inflammatory cytokines such as TNF- α , interleukin-1b, and endothelin-1 that lead to death of retinal ganglion cells (39). In our study, we did not see any signs of GFAP

immunoreactivity after 3 days post-NA3 injection. However, very weak immunoreactivity was documented after 7 days at a 100 times physiological dose level, and there was no activation of Müller glia in the deeper retinal layers after all the injections and subsequent follow-up; hence, the mild reaction was not considered significant (Fig. 5). Also, the GFAP-positive reaction in Müller cells and feet was a rare occurrence and did not exist across retina.

Apoptosis is the process of an intracellular death program leading to characteristic biochemical and morphological changes within a cell that consequently result in cell death (44). A hallmark of late apoptosis is extensive genomic DNA fragmentation that generates a multitude of DNA double-strand breaks with accessible 3'-hydroxyl (3'-OH) groups. This unique characteristic forms the basis for a well-established apoptosis detection method known as TUNEL assay (45). Apoptosis occurs in the retina in common degenerative diseases such as retinitis pigmentosa, anterior ischemic optic neuropathy, and glaucoma (46). It was important for us to confirm that there are no degenerative signs after intravitreal injection of NA3 glycan. No TUNEL-positive cells were present in the retinas of any animal injected with NA3 glycan during the follow-up period.

For a drug to elicit its action, it must be stable *in vivo* at the site of action and administration. We thus tested the *ex vivo* stability of NA3 glycan in excised plasma and vitreous. As NA3 glycan is endogenously present, we chose a chemically modified form of NA3 glycan that would enable analytical estimation. We chose 2-aminobenzamide labeled NA3 glycan (100 pM) since it was studied extensively. To the best of our knowledge, commercially available standards (supplied by Prozyme Inc./QA-bio/Ludger) for 2-AB NA3 are all supplied in 100 pM vials only. Hence, we used this concentration which is lower than the targeted dose. We have

optimized a 2-AB labeling method for NA3; however, we chose to use commercially available 100 pM vials for the highest purity and robust assessment. Interestingly, NA3 concentration dropped rapidly after incubation in plasma with almost a complete degradation within a few hours. There are no reports on the factors affecting the chemical stability of NA3 glycan. In our study, we did not study the detailed mechanism of degradation in plasma. In contrast, NA3 glycan was stable in vitreous throughout the study period of 6 days. This behavior of differential stability serves as a useful property for a targeted effect in the eye. We supported these stability findings with the biodistribution studies carried out *in vivo* on NZW rabbits, wherein we found negligible amounts of NA3 glycan in peripheral organs and plasma, while high concentrations in various parts of the eye over a period of 14 days.

NA3 glycan was radiolabeled to enable *in vivo* tracking after intravitreal injection. Chemical/radio labeling of sugars like NA3 glycan is carried out at the reducing terminal of the side chain and is an established reaction used for many decades. Labels like 2-AB (2-aminobenzamide) and 2-AA (2-aminobenzoic acid) are also attached by a similar coupling mechanism. NA3 glycan is a multivalent non-metabolizable ligand. We have converted the disintegrations per minute values to nanocuries per gram tissue for pharmacokinetic studies. Our pharmacokinetic estimates are based on radioactivity values and not on concentration of NA3. We have not been able to convert the disintegrations per minute values to amount of NA3. However, since we have used a large excess of sodium borohydride in our reaction (26), we could assume that all the NA3 used in the reaction is labeled. With that assumption, the concentration is well within the dose range. However, further confirmatory quantitative studies must be performed to assess the exact amount of NA3.

For the assessment of NA3 pharmacokinetics, we injected radiolabeled NA3 glycan in both eyes based on reports from similar pharmacokinetics studies where either both eyes were injected (32,47). It is noted that drugs injected in one eye enters the contralateral eye through the systemic circulation (48). Additionally, we observed that NA3 glycan was highly unstable in plasma. Hence in our study, we believe there is a limited possibility that NA3 injected in one eye would enter the fellow eye. In summary, we did not expect any contralateral eye effect analytically. Negligible amounts of NA3 glycan in peripheral organs strongly corroborated our targeted retina effect hypothesis of NA3.

We found the highest NA3 concentrations in vitreous followed by retina; hence, a concentration gradient was always maintained during the study. The injected NA3 glycan equilibrated within the first 2 days of the injection, after which it was cleared from the vitreous during the course of the study. The graphical pattern for clearance of NA3 glycan from the various eye tissues was found to be similar, which suggested that there was no accumulation of NA3 in the unexamined tissues. We believe that a positive concentration gradient from the vitreous to the retina is very important for the movement of NA3 glycan (molecular weight 2,006.82 Da) by diffusion and subsequent equilibration. The vitreal half-life value was consistent with the half-life values of small molecular weight drugs after an intravitreal injection (49). Laude *et al.* provide a concise review on the subject matter

and summarize various factors affecting the intravitreal pharmacokinetics with relevant examples of small and large molecule pharmacokinetics in different species (49). Contrary to the common belief, vitreous is a complex matrix system with a total solid content of approximately 1% (w/w). However, the alignment of collagen and glycosaminoglycans is sufficient to maintain the rigidity and stability of the gel and more critically, to act as a molecular barrier to diffusion. Hence, it is reported that the diffusion of materials through the vitreous deviates sharply from that in water. This was confirmed by treatment of the eye with hyaluronidase, wherein the movement of materials from mid-vitreous to choroid was significantly faster suggesting that structural elements constituted by the vitreous components do contribute to the overall rate of material transport (49).

We also performed autoradiography studies (data not shown, manuscript in progress) after intravitreal injection of [³H]NA3 glycan and found punctated pattern in the retina suggesting localization of [³H]NA3 in the retina. As discussed before, we also established the stability of NA3 glycan in vitreous and PBS our sensitive using HILIC method and found that NA3 glycan is stable in both vitreous and PBS (Fig. 6). Moreover, we did not detect any free label over a period of 6 days on the sensitive HILIC method. These results lend a strong support for establishing the intact nature of [³H]NA3 after intravitreal injection. Moreover, we have separated the unreacted [³H]sodium borohydride, by dialysis of the reaction mixture with multiple cycles of dialysis using fresh medium after every 24 h. We also observed a gradual decrease of radioactivity of the radiolabeled NA3 glycan inside the dialysis bag indicating the purification of [³H]NA3.

Most importantly, if tritium was separated from NA3 glycan after intravitreal injection, it would have been eliminated within the first few hours owing to its size and physicochemical properties; however, we found a slow elimination of [³H]NA3 glycan with half-lives over 45 h in critical areas. These findings strongly corroborate our results and indicate that the label is intact in NA3 glycan during its course of elimination. However, a detailed discriminatory analytical study may be required to conclude beyond this, which is also beyond the scope of our current paper. Even though non-specific and qualitative, we also detected radioactivity in both urine and feces suggesting excretion of NA3 glycan or its metabolites via these mechanisms.

CONCLUSIONS

NA3 glycan—a potential novel therapeutic for atrophic AMD—was found to be safe in the preclinical studies using NZW rabbits over a period of 14 days at two concentration levels. NA3 glycan degraded rapidly in plasma, while it remained highly stable in vitreous in simulated biological conditions, thereby providing a potential breakthrough targeted therapy for atrophic AMD and other retinal diseases. Ocular pharmacokinetics confirmed the movement of NA3 glycan from the vitreous to the retina. There is negligible accumulation of NA3 glycan in extra-ocular organs as confirmed by bio-distribution studies. Collectively, these results document that NA3 is safe and shows favorable ocular pharmacokinetics.

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