Abstract

Purpose: Determine if a novel hybrid compound SA-2 can be delivered to the retina in a nanoparticle formulation and have protective effects on retinal ganglion cells (RGCs) following an optic nerve crush (ONC) model of RGC death.

Methods: Pattern Electroretinography (PERG) was performed on six- to twelve-week-old female (C57BL/6) mice (n = 1-8 mice per group) prior to performing ONC on the left eye to promote RGC death similar to that seen in normotensive glaucoma. Mice were dosed topically for seven or fourteen days either with SA-2 in polylactic glycolic acid (PLGA) nanoparticles, or empty PLGA nanoparticles. Subsequent PERG was performed at seven day following ONC to reassess RGC function after the optic nerve injury and treatments. The mice were subsequently euthanized and both eyes we enucleated and fixed with paraformaldehyde. The retinas were removed, flat mounts were prepared and immunostained with RBPMS antibody to quantify surviving RGCs.

Results: Our study demonstrated that SA-2 can be delivered to the retinal tissue with PLGA nanoparticles. However, following optic nerve crush in mice, at the selected doses and delivery regimen of SA-2, neuroprotective effects determined by RGC counts and PERG analysis were not statistically significant.

Conclusion: Following ONC in mice, topically delivered SA-2 loaded nanoparticles demonstrated some trend in neuroprotection without statistical significance. Further investigation is required to delineate the efficacious delivery mode and dose.

NEUROPROTECTIVE EFFECTS OF TOPICALY DELIVERED SA-2 NANOPARTICLES IN A MOUSE MODEL OF RGC INJURY

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Neuroprotective Effects of Topically Delivered SA-2 Nanoparticles in a Mouse Model of RGC Injury

Practicum Report

Medical Science Research Track Cohort 2020

Department of Pharmacology and Neuroscience

North Texas Eye Research Institute

Graduate School of Biomedical Science

UNT Health Science Center

For the Degree of

MASTER OF BIOMEDICAL SCIENCE

By

Jonathan Ferguson

Fort Worth, Texas

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Preface

This year of research has been the most fulfilling and enriching time I have had in my seven years of higher education. I joined the second-year medical science program to gain a deeper understanding of how research takes place and the intricacies behind and beyond the experiments themselves. I learned so much more than I had thought possible not only knowledge, but I also developed lab skills that I thought were beyond me. Some of the knowledge I learned this past year was how to plan out research, starting with selecting a topic that you think people can benefit from and reading background literature on the topic, delineating a future direction and breaking your research goals down into small manageable experiments that build on one another. Unfortunately, with the COVID-19 pandemic in full force at the beginning of my research year the start of my lab work was delayed for more than three months. Nevertheless, over the course of this project I was able to learn and refine many research skills. Some of the skills and techniques I learned are pertained to my passion molecular biology these skills were PCR, qPCR and gel electrophoresis. I had the pleasure of refining the qPCR protocol that will be used to identify genetic changes in future experiments. I also had the opportunity to become proficient at working with rodents performing various techniques involved in our study. I became very familiar with the collection and processing of various tissues including retina, optic nerve and the brain and their dissection. As well, I had ample practice of immunofluorescence technique; many rounds of starting with a live specimen for tissue collection then going through the immunohistochemistry staining process, taking florescent images of the slides. Then quantifying each photo taken collecting the data so I could process it into a graph and present the findings to my lab mates. It is my belief that had my lab not lost the summer semester I would have been

able to do even more experiments collect more data and make a greater contribution not just to my lab or a publication but vision science itself.

Acknowledgments

I would like to thank all of my lab mates Julie (Wei) Zhang, Charles Amankwa, Gretchen Johnson, Linya Li, Sudershan Gondi, Biddut Deb Nath, and Arlene Funk for helping to develop as a student and a scientist this past year. I would like thank Dr. Suchismita Acharya and Dr. Raghu Krishnamoorthy for helping me to better understand research and the thinking that takes place outside of the lab. I would like to extend my appreciation to my committee members Drs. Tara Tovar-Vidales and Cameron Millar for helping develop my research skills and definitely helping me with presenting my research findings. I would to than Dr. Raghu Krishnamoorthy for multiple contributions to my project and helping me not to get discouraged when and experiment did not go as I expected. As well, Dr. Suchismita Acharya and Dr. Dorette Ellis for their efforts throughout this past year and for allowing the opportunity to participate in their collaborative projects. Also, I would like to thank my wife Makayla and my two wonderful children Reegen and Archer for helping me day in and day out and for being my inspiration.
I would like to my give my deepest thanks to Dr. Dorota Stankowska, without her guidance and patience this past year I would never have been able to complete this project and all that it entails. I would be remorseful if I did not thank Dr. Stankowska and Dr. Krishnamoorthy for fostering a desire to continue with research in the future. I would also like to thank my dear friend Ian Dadeboe for helping me throughout the past two years whose contribution to my success is unmeasurable.

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Chapter 1

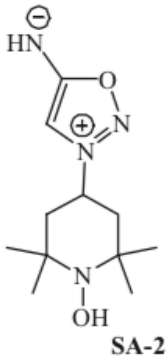
Introduction

Glaucoma is the second leading cause of vison loss worldwide and is described as a progressive optic neuropathy characterized by axonal degeneration (1) accompanied by loss of retinal ganglion cells (RGCs) beyond typical age-related decline in function and cell loss (2). Glaucoma is subdivided into various categories, including closed angle glaucoma and open angle glaucoma (6). Closed angle glaucoma, also known as acute angle-closure glaucoma (AACG), is different from primary open-angle glaucoma (POAG) in terms of the gonioscopic appearance of angle of the anterior chamber. In AACG, the resistance to aqueous humor outflow is due to mechanical obstruction of the outflow pathway by the iris. In primary open angle glaucoma, there is a functional and structural change to the trabecular meshwork (TM) that causes an occlusion of TM tissue, producing the restriction to outflow and a rise in intraocular pressure. However, both types of the disease will lead to irreversible damage to RGCs, even though the time frame is different. AACG can cause damage to the retina and optic nerve leading to permanent vision loss in a matter of hours and sometimes is called an “ocular stroke”. Unlike AACG, POAG has a slow gradual onset and may not be recognized by the afflicted person until several years later, following more advanced stages of the disease. Primary open angle glaucoma can further be divided into subcategories, including one that is simulated in the animal model of optic nerve crush described in this proposal, the pathology of which has similarities to normal-tension glaucoma (6). The cause of normal-tension glaucoma is still widely unknown; one potential cause is ischemia due to compromised circulation at the optic nerve head causing neuronal death. Another proposed cause of normal tension glaucoma is imbalance in

translaminar pressure. Translaminar pressure is the difference between the intraocular pressure and intracranial pressure (IOP-ICP) the difference in this pressure is hypothesized to cause the lamina cribrosa to apply pressure on the unmyelinated axons where they pass into the sub arachnoid space (19). The pressure applied to the axons from the lamina cribrosa side leads to axonal damage similar to the damaged caused by increased intraocular pressure glaucoma. Regardless of the cause, the outcome is the same: retinal ganglion cells are depleted, and vision is lost permanently. Generally, diagnosis of glaucoma is defined by characteristic changes in the optic disc and visual field such as optic disc cupping and "blind spots" in the visual field typically starting at the periphery.

The current glaucoma treatments used clinical work exclusively by lowering the intraocular pressure (IOP) through various mechanisms (2). At this time lowering IOP is the only modifiable risk factor in treating glaucoma, even in patients that do not have an increased IOP. Studies show that lowering IOP can significantly slow the rate at which vision is lost but fails in fully preventing it (2). Furthermore, nearly 30 to 40% of primary open angle glaucoma patients have 'normal' intraocular pressure (less than 21 mm Hg) (6). Therefore, drugs that prevent vision loss by means other than only lowering IOP need to be investigated. There are currently few FDA approved therapies for neuroprotection of central nervous system diseases but not for glaucoma and more need to be developed for this blinding disorder. Glaucoma is projected to afflict more people in the future, particularly as the percentage of people above the age of forty years has been steadily increasing globally (5). For this reason, neuroprotective drugs are being explored to be developed for the long-term treatment of glaucoma. Many compounds are being developed and some are now under investigation in clinical trials. Some of the tested compounds such as Tempol (7) and SA-2 (3) have shown promising neuroprotective effects in rodent models

of glaucoma, while some novel compounds such as SA-21 and SA-22 remain to be tested. These compounds work through various pharmacological mechanisms, including, reducing the injury caused by ischemia, or scavenging radical oxygen species and some by additionally lowering IOP. The drug of interest in this study (SA-2, **Figure 1**) works on the aforementioned principles and it is composed of two components.



SA-2

Figure 1. A chemical structure of the SA-2 compound. *SA-2 compound synthetized in Dr. Acharya's laboratory, the compound is composed of a six-member ring (bottom ring) and the five-member ring (top ring) that contains an amide and imine functional group. (11).*

The first component is a superoxide dismutase mimetic which scavenges reactive oxygen and nitrogen species (**Figure 2**, in red) (11). The second component releases small amounts of nitric oxide, an endogenous neuromodulator that increases blood vessel diameter via smooth muscle relaxation (3) (**Figure 2**, in blue). The intravitreally delivered SA-2 (not encapsulated) compound has been proven to have neuroprotective properties in two distinctive mouse models of RGC death, ONC and ischemia reperfusion model (3). ***Here, we propose to test if SA-2 delivered topically to the eye via a nanoparticle formulation will protect RGCs from cell death following optic nerve crush (ONC) in mice.*** The current study attempted to elucidate the neuroprotective effects of the hybrid SA-2 compound delivered as an eye drops (ED) via a poly lactic-co-glycolic acid (PLGA) nanoparticle (SA-2-NPs). The goal of the study was to

investigate if SA-2-NPs via eye drops (ED) would be delivered to the retina and would protect RGCs in a mouse optic nerve crush (ONC) model of RGC injury.

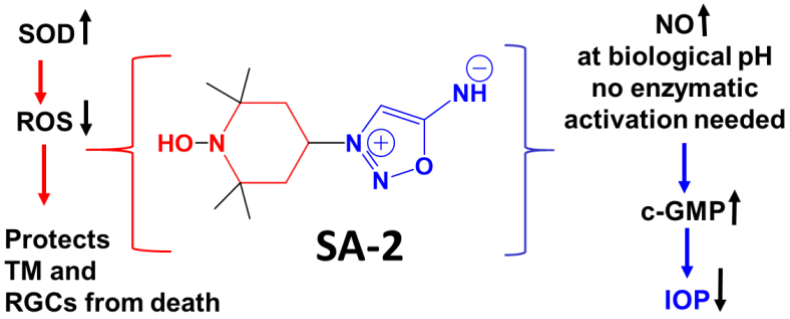


Figure 2. Possible mechanism via which compound SA-2 lower IOP, protects TM cells and RGC death*. IOP leads to mitochondrial dysfunction and increased formation of ROS, which increase TM cell death and further increases in IOP. Compound SA-2 rescues RGCs against glaucomatous death possibly via upregulation of SOD activity and scavenging of ROS.*

While the intravitreal injection method is suitable for laboratory experiments and hospital settings, it is not amenable to everyday application for those afflicted with glaucoma. The ideal method is to deliver the compound directly to the eye in an eye drop, as many glaucoma drugs are already on the market in this form (2). The half-life of SA-2 was determined to be 24 hours in a previous study when animals were treated by intravitreal injection (3). The bio-distribution study of SA-2 NPs delivered as a 1% of eye drop (ED) was previously performed in rat eyes and it was estimated that SA-2 reaches various ocular tissues including: cornea, aqueous humor, vitreous humor, retina, choroid and sclera (30 µL topical ocular eye drop of 1% w/v SA-2 NPs) (14) The study also showed that when delivered encapsulated in the PLGA NP, the action of the compound was extended and had the ability to significantly lower IOP for thirty hours (14). The SA-2 was delivered via a poly lactic-co-glycolic acid (PLGA) nanoparticle vehicle that is already

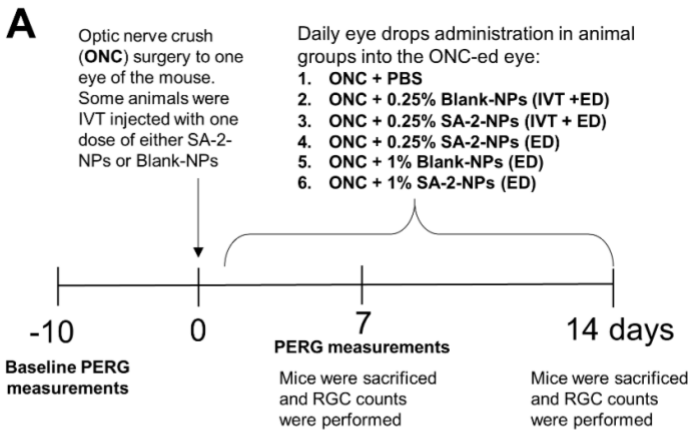
FDA approved for drug therapy. In this proposal the SA-2-NPs delivered topically (ED) was investigated for neuroprotection to RGCs in ONC mouse model of RGC injury.

Chapter 2

Methods

Animals: The animals used in the study were mice (*Mus musculus*) strain C57BL/6J that were ten to twelve weeks in age. The animals were used in accordance with the protocol approved by the IACUC at the University of North Texas Health Science. The animals were housed in the Department of Laboratory Animal Medicine in the Integrated Research and Education Building at UNTHSC, and all studies and procedures were conducted at the same location.

Experimental design: The **Figure 3** below indicates all major steps involved in the procedures described below.



B

Experimental groups	Description	7 days	14 days
		mice	mice
1	ONC +PBS	4	4
2	ONC + 0.25% Blank-NPs (IVT +ED)	np	3
3	ONC + 0.25% SA-2-NPs (IVT + ED)	np	3
4	ONC + 0.25% SA-2-NPs (ED)	np	3
5	ONC + 1% Blank-NPs (ED)	1	np
6	ONC + 1% SA-2-NPs (ED)	5	np

Figure 3. Experimental scheme. (A) *Design to delineate the effect of SA-2-NPs on neuroprotection following optic nerve crush (ONC) in C57BL6 mice.* ***(B)*** *Table describing experimental groups used for the study with the indication of n= animal number per each group. All animals used in this study were females. ONC surgery was performed by Linya Li and majority of the PERG analysis was performed by Wei Zhang.*

*Drug administration was performed by Jonathan Ferguson and Charles Amankwa. The majority of the tissue collection, immunostaining and microscopic analysis using RBPMS antibody to detect surviving RGCs was performed by Jonathan Ferguson, Wei Zhang and Linya Li. Cell counts were performed by Jonathan Ferguson and Wei Zhang. "**np**"- ONC was not performed on that experimental group, "**IVT**"- intravitreal administration (once at the time of ONC), "**ED**" – eye drop administration daily.*

Pattern Electroretinography: Baseline Pattern Electroretinography (PERG) (Jorvec Inc., Miami, FL). The PERG measurements were made in a dark room environment, and the animals were anesthetized with ketamine/xylazine (100/10 mg/kg) that was injected intraperitoneal. Two electrodes were placed sub-dermal on the dorsal side, one in the nasal region, a second electrode was placed interparietal, a ground electrode was placed at the base of the tail, and a thermometer

was placed in the rectum. The mouse was placed on stage with the light panels placed 11cm from each eye respectively at a 45^0 angle, multiple readings were taken for each eye. The experiment consisted of two reversals per second, 300 averaged signals with cutoff filter frequencies of 1–30 Hz, 98% contrast, 800 cd/m^2 average monitor illumination intensity (3). Base line PERG data of 89.6 milliseconds for latency and an average base line amplitude of 19 microvolts was recorded prior to experimental procedures carried out on the mice.

Optic Nerve Crush: Following the baseline PERG measurements, the mice underwent the optic nerve crush procedure (ONC) on the left eye only. The mice were anesthetized with ketamine/xylazine (100/10 mg/kg) injected intraperitoneally. The left eye was numbed with Alcain, then an incision was made in the corner on the eye socket. The optic nerve was located and crushed for 4 seconds approximately 1mm behind the globe of the eye with self-closing forceps. The wound was dressed with Bacitracin/Polymyxin B ointment. The animals were monitored throughout the study to ensure an infection did not develop as well to ensure the animals were not exposed to unnecessary stress.

Drug delivery: Animals were anesthetized with an intraperitoneal injection ketamine/xylazine (95mg/mL 5mg/mL; 100μL/100g). The SA-2 compound loaded into the PLGA nanoparticle was suspended in PBS. The animals were intravitreally injected with 2μL of the compound and eye drops delivery (ED) dosed daily for time course of the experiment (either 7 or 14 days). Some animals were given SA-2-NPs by eye drops (ED) delivery only. The animals were divided into treatment groups and each animal served as its' own control *via* the contralateral eye. A control group for the insult was comprised of mice that underwent ONC procedure and received phosphate-buffered saline (PBS) vehicle. All ED treated animals received five microliter drops to the left eye. The animals were treated with SA-2-NPs while under isoflurane anesthesia

(2%/ O_2 1L/min) which allowed to permeate the tissue for two minutes. Following treatments, the mice were placed in an isolated recovery cage.

Retinal Flat mounts preparation: After treatment, the mice were euthanized with an intraperitoneal injection of pentobarbital sodium (120mg/kg). The eyes were enucleated and placed in 0.5ml of paraformaldehyde (4% PFA) for two hours in ice on a rocker at medium speed of agitation. Following two hours of fixation the paraformaldehyde was removed with three five minute rinses of 1ml of 1xPBS. Afterwards, the eyes were dissected to remove the retinal tissue from the globe. After the retinas were separated from the eye, the vitreous humor was carefully removed with a small brush under microscopic observation, then the retinas were carefully flat mounted.

Immunohistochemistry: The flatmounts were rocked in tubes in a 0.3% Triton/1xPBS permeabilization solution for 1 hour on a rocker table. After permeabilization, the retinas were washed with PBS for 15 minutes, changing the PBS twice. Following this, the retinas were blocked with 10% donkey serum (DNS) in a 0.3%Triton/PBS for 1 hour on a rocker to prevent non-specific binding. After blocking, the retinas were washed in 1ml of 1xPBS for three 5-minute cycles on a rocker. The primary antibody incubation was carried out with mouse monoclonal anti-RNA Binding Protein with Multiple Splicing (RBPMS) antibody (GTX118619 GeneTex Irvine, California) at 1:250 dilution by rocking at 4˚C for 48 hours. Following 48 hours of primary antibody exposure the retinas were washed for three times 15 minutes respectively in 1xPBS to remove the unbound primary antibody. The flatmounts were subsequently treated with a 1: 1000 dilution of a secondary antibody Donkey-anti-mouse IgG (Alexa Fluro 546 Thermo Fisher Waltham, Massachusetts) in a dark box at 4^0C for 24 hours. Following overnight treatment with the secondary antibody, retinas were rinsed for 45 minutes in 1xPBS, changing

the PBS every 15 minutes. The retinas were then placed on a glass slide with the RGC layer facing up, and a drop of flurosave reagent (Calbiochem San Diego, California) was added to a cover slip that was placed on the retina. After the addition of the flurosave and coverslip, the retinas were placed in a dark immunostaining moisture chamber for 24 hours before being imaged.

Microscopy: Retinal flat mounts were imaged with a Biotek Cytation5 automated digital microscope. Eight pictures were taken per retina, two pictures in each quadrant at two different eccentricities located either in the mid-peripheral or peripheral region of the retina from the optic nerve head. Imaging started in the left leaflet of the retina at the peripheral edge, followed by the center of the leaflet (180 μm from the edge) for the first picture, then moving towards optic disc (50 μm) for the second picture, then repeating this pattern for each individual leaflet as outlined in the **Figure 5** below.

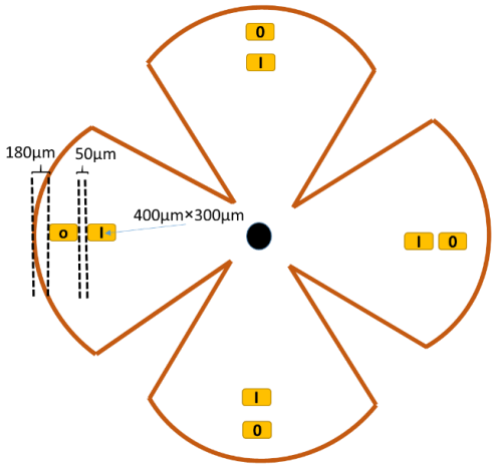


Figure 4. Imaging of the retinal flatmounts. *The images used for RGCs counts were are taken from the eight regions indicated as yellow rectangles (0 and I) located at all quadrants of retinal flat mounts. The cells were counted and then averaged to obtained representative cell counts for the specimen.*

Assessing RGC survival: RGCs from obtained pictures were counted in a masked manner using the *Image-J* software available through the National Institute of Health website. After all cells were counted in the visual field, the number was recalculated to cells per millimeter square (RGC/mm^2). The equation of (total cells counted/0.114564) was used to calculate the RGC density (RGC/mm^2) as described by the Cytation5 microscope imaging manual. The data was reported as the average numbers of RBPMS positive (viable) RGCs per mm^2 and SEM for each group.

Results

Rationale

Previous studies performed in our laboratory addressed the pharmacochemical properties of SA-2 compound and demonstrated the ability of SA-2 to protect cells in *in vitro* studies (3). In addition, SA-2 was found to be non-toxic to retinal tissue and thus would be safe to use in animal models. **SA-2** was shown to be effective in three separate models of RGC death, including, hypoxia, ONC and I/R model, and the neuroprotective activity was demonstrated to be mediated through upregulation of SOD levels in the retinas following ONC. The results from our laboratory also demonstrated that in human primary TM cells, SA-2 increased levels of cGMP and SOD protecting them from tert-Butyl hydroperoxide (TBHP) induced oxidative stress. The nano-encapsulated SA-2NPs (1%) eye drops were detected in the retina, vitreous humor, aqueous humor, as well as corneal and choroidal tissue in pg/mg level 1 h post-dosing. A single dose of eye drops containing 1% SA-2-NPs reduced IOP in both the Morrison ocular hypertensive rat and the Ad5.CMV.hTGF$\beta_2^{C226/228S}$ ocular hypertensive mouse models with IOP-lowering effect

lasting up to 30 h in mice and 72 h in rats. We wanted to further assess the neuroprotective potential of SA-2 administered in nanoparticles in a rodent model of optic nerve injury.

Encapsulated SA-2 (SA-2-NP) 0.25% demonstrated a trend in increased RGC survival (14 days' post ONC) and improvement of RGC function (7 days' post ONC) following acute axonal injury (ONC) in mice.

To assess the neuroprotective effects of SA-2-NPs, 12-week-old female mice underwent ONC, an acute injury model of the axons of RGCs and were treated with either PBS, 0.25% SA-2-NPs (w/v) or unloaded Blank-NPs. The drug and vehicles were administered as single intravitreal injection (2µL) at the time of ONC surgery along with daily eye drops (ED) or ED only for the time of experiment. Un-operated eyes served as contralateral controls for ONC. **Figure 5A** shows microscopic images of RBPMS-positive RGCs from 14 days' post ONC. As expected, there was a statistically significant decline in number of RBPMS-positive RGCs in all the eyes subjected to optic nerve crush, compared to the contralateral eyes (neon blue; 1350 ± 146 RGCs/mm^2, ****$p<0.0001$, **Figure 5B**). We did not observe any statistical differences when comparing ONC+PBS group (in red) with ONC-ed treated with Blank-NPs or SA-2-NPs. There was a slight trend in RGC survival detected in ONC-ed and SA-2-NPs (0.25 %,) treated group (373 ± 76.6 RGCs/mm^2, in teal). RGC function (**Figure 5 C**) was measured using PERG. The PERG amplitude was significantly greater as expected in contralateral eyes (neon blue, 17.56 ± 1.66 µV). ONC-ed eyes demonstrated statistically significant decrease in amplitude (****$p<0.0001$, red bar; 2.87 ± 1.08 µV) (**Figure 5C**). There was a trend in improvement of visual function ($p=0.177$) in SA-2-NP ED (in teal; 6.31 ± 1.11 µV) group in comparison with PBS treated group (in red, 2.87 ± 1.08 µV) following ONC.

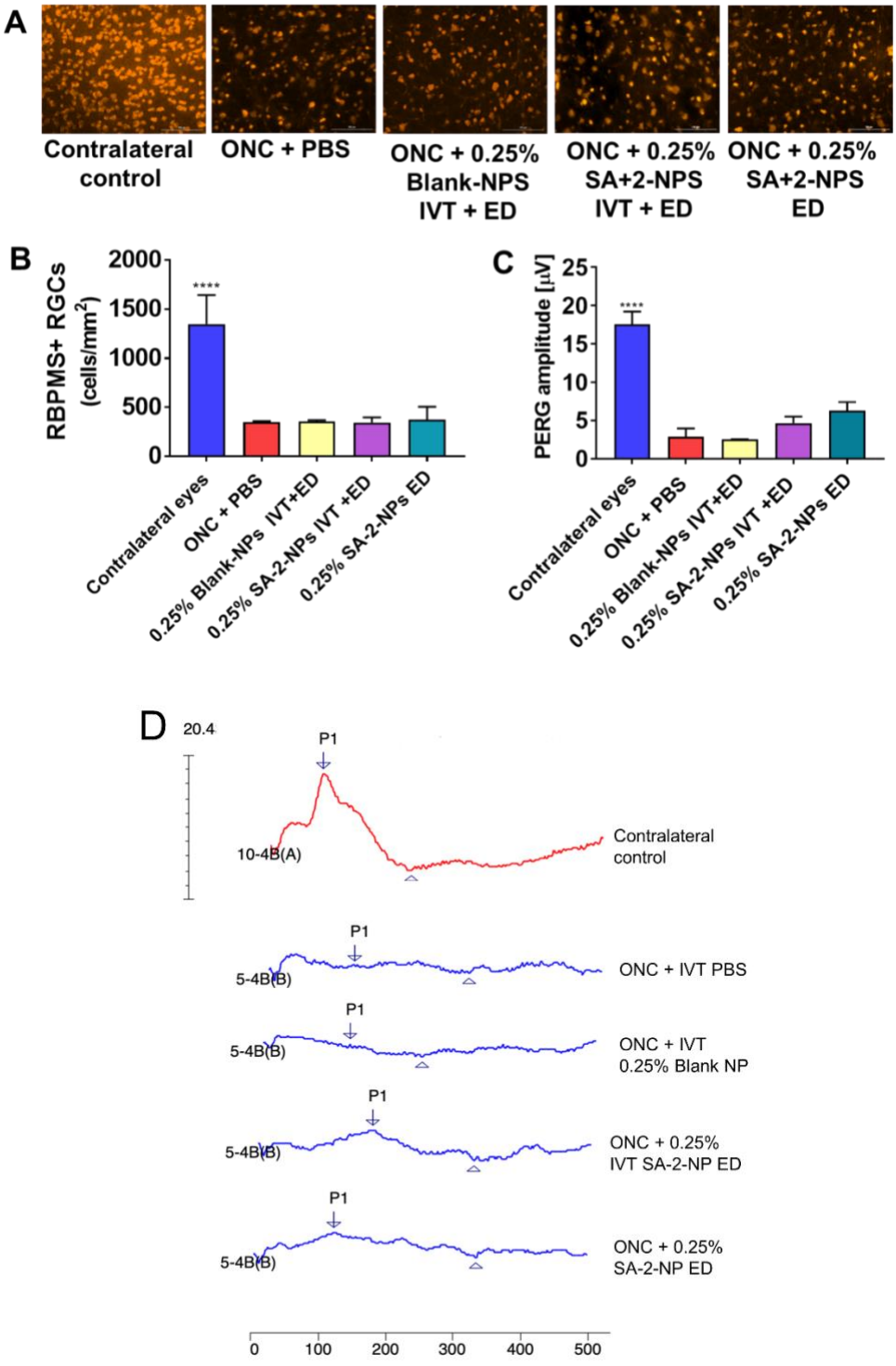


Figure 5. Effects of eye drops administration of 0.25% SA-2 in C57BL/6J mouse following ONC. *12-week-old C57BL/6J mice subjected to ONC in the left eye were administered 0.25%*

SA-2NPs, Blank-NP, PBS via intravitreal injection and eye drops (ED) or eye drops alone. ***A:*** *Representative fluorescent images of RBPMS labeled RGCs in contralateral and ONC mouse retinas. Scale bar = 100 µm.* ***B****: RBPMS positive retinal ganglion cells count 14 days of ONC experiment. Data presented as mean +/- SEM, n=2-5.* ***C:*** *PERG was conducted 7 days following ONC. N = 2-8. One-way ANOVA followed by Tukey's multiple comparisons test; ****p<0.0001.* ***D:*** *Representative PERG traces from all experimental groups seven days following ONC. (P1)-indicates major positive wave and (△)-indicates major negative wave of each trace. Peak (P1)-to-trough (△) value represents PERG amplitude in µV.*

1% SA-2 NPs did not demonstrate a protection of RGC and RGC function 7 days following ONC in mice.

Because the administration of 0.25% SA-2-NPs delivered through eye drops (ED) revealed a neuroprotective trend (345 ± 30), we further investigated a higher (1%) dose SA-2-NPs in ONC mouse model. Moreover, the PERG amplitudes for the 0.25% SA-2-NPs ED (2.49 ± 0.05 µV) at day 7 showed an improvement in PERG amplitude, hence we decided to investigate both the RGC survival and PERG analysis following the same time frame (7/8 days) as PERG was previously performed. The 1% dose for SA-2-NPs was chosen based on the *in vitro* data performed previously in the laboratory. The percent cell survival was measured by a cell proliferation assay to detect NTM-5 cell viability after treatment with 350 µM TBHP for 18 h. Treatment with TBHP (350 µM) significantly decreased the NTM-5 cell proliferation, which was significantly improved, by more than 50% (***p<0.001, One-way ANOVA, data not shown) following treatment with 1% SA-2-NPs. Similarly, to previous experiment we used 12-week-old female mice which underwent ONC. The 1% SA-2-NPs and Blank-NPs were administered as daily eye drops (ED) for 7 days (1113 ± 40 RGCs/mm^2).

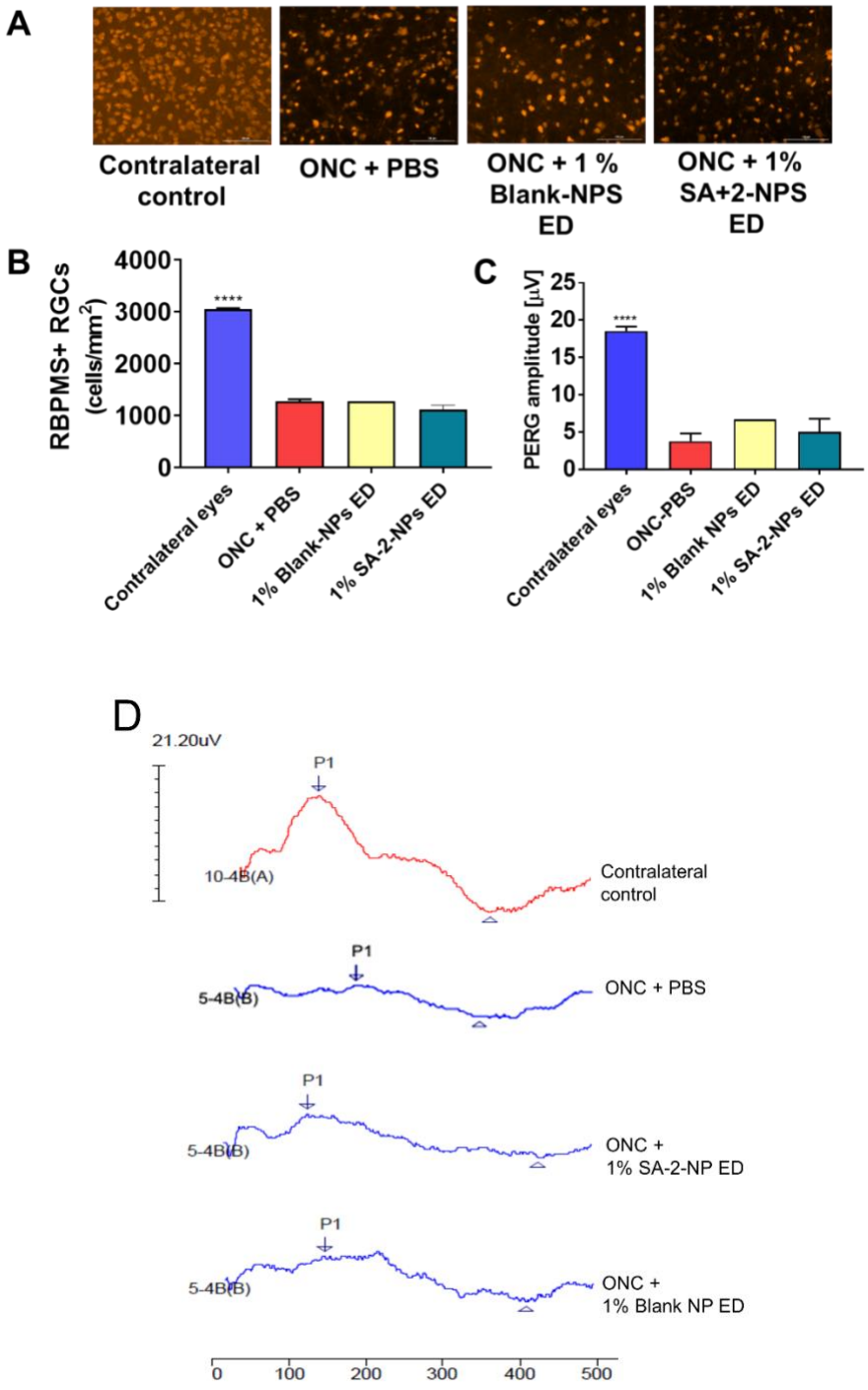


Figure 6. Effects of eye drops administration of 1% SA-2 nanoparticles in C57BL/6J mouse following ONC. *12-week-old C57BL/6J mice subjected to ONC in the left eye were administered 1% SA-2NPs, Blank-NP, or PBS via eye drops (ED).* ***A:*** *Representative fluorescent images of*

RBPMS labeled RGCs in contralateral and ONC mouse retinas. Scale bar = 100 µm. ***B****: RBPMS positive retinal ganglion cells count after 8 days of ONC experiment. Data presented as mean +/- SEM, n=2-5.* ***C:*** *PERG was conducted 7 days following ONC. n = 2-8. One-way ANOVA followed by Tukey's multiple comparisons test; ****p<0.0001.* ***D:*** *Representative PERG traces from each experimental group seven days following ONC. (P1)-indicates major positive wave and (△)-indicates major negative wave of each trace. Peak (P1)-to-trough (△) value represents PERG amplitude in µV.*

Contralateral eyes served as internal controls for ONC-ed groups. **Figure 6A** shows representative images obtained in Cytation5 of RBPMS-positive RGCs 7 days following ONC. After obtaining RGC counts, we observed statistically significant higher numbers of RBPMS-positive RGCs in the contralateral control eyes (neon blue color; 3060 ± 7.24 RGCs/mm^2) in comparison with ONC-ed and PBS, SA-2-NPs or Blank-NPs groups (****p<0.0001, **Figure 6B**). We did not observe any statistical differences between ONC+PBS group (in red; 1279 ± 36.51 RGCs/mm^2) with ONC-ed treated with 1% Blank-NPs (1277 ± 0.5 RGCs/mm^2) or 1% SA-2-NPs (1113 ± 39.90 RGCs/mm^2). We did not observe the trend previously noted with using a lower (0.25%) dose of drugs. We observed a statistically insignificant trend in improvement of RGC function in SA-2-NPs treated group (**Figure 6C**, teal bar; 5.06 ± 0.77 µV) when compared with ONC-ed PBS treated group (red bar; 3.8 ± 1.02 µV). The PERG amplitude was significantly greater as expected in contralateral eyes (neon blue; 18.55 ± 0.59 µV). ONC-ed eyes in all treatment groups demonstrated expected statistically significant decrease in amplitude (****p<0.0001, red bar).

Discussion

Present therapies for glaucoma include the use of prostaglandin analogs, β-adrenergic antagonists, rho kinase inhibitors, α-2 agonist, and nitric oxide donors as IOP lowering tools. However, about 10 to 15% of glaucoma patients do not respond sufficiently to these therapies and patients can develop tolerance to medications over time. Tolerance to IOP treatments can lead patients to undergo surgery. This tolerance is also a major driving force for the research of new classes of more effective IOP lowering drugs. Similar to IOP lowering drugs surgery can only lower IOP and addresses vision loss through the mechanical action of the disease and cannot restore lost RGCs. Together with the increase in IOP, there is a natural and glaucoma-induced age-related decline in antioxidant enzymes, resulting in increased ROS formation, which contribute to the cell death of RGCs and TM cells. Long term deficits in vision are not appropriately addressed by current glaucoma treatment therapies. It is well known that SOD declines with age in the trabecular meshwork tissue (15). Similarly, *in vitro* experiments using primary RGCs have shown that RGC death is induced by ROS and which can be prevented by use of various antioxidant substances. It is known that SOD-deficient mice demonstrate an increased in oxidative stress in the RGC layer (16). SA-2 compound possesses a superoxide dismutase (SOD) mimetic antioxidant moiety (**Figure 2**, red ring) and is well positioned to play a role of an activator of SOD that could generate antioxidant protective effects, which will in turn increase the rate of RGC survival and TM tissue protection in glaucoma patients. The work from our laboratory has recently demonstrated that, non-encapsulated compound SA-2 was able to increase SOD levels in mouse retina and reduce RGCs death in two RGC death models in mice (3). Our latest work showed that SA-2 compound can increase cyclic GMP (cGMP) and SOD levels in primary human TM cells and can protect them from TBHP-induced oxidative

stress (14). Apart from being an antioxidant and ROS scavenger, the SA-2 compound is a spontaneous (not requiring activation by enzymes), pH-dependent NO donor (**Figure 2**, blue ring). Nitric oxide is involved in the IOP regulation and recently a new drug Latanoprostene bound (a NO-donating analog of prostaglandin) was approved by FDA in 2017 for glaucoma therapy to lower IOP. The Latanoprostene bound has not yet been proven to act as neuroprotectant. One NO donor and anti-glaucomatous drug Nipradilol (having nitrate ester as an NO donor), has been demonstrated to have neuroprotective effects in RGCs after optic nerve axotomy in rat (17). Targeting the NO system can enhance aqueous outflow facility by relaxing the TM cells and thereby lower IOP, which was demonstrated in our lab's recent publication (14) both in a rat and mice glaucoma models. The original SA-2 drug has a short aqueous half-life of $t_{1/2}$ < 1d at pH7.4. To alleviate that shortcoming, our lab prepared PLGA encapsulated nanoparticles of SA-2 (SA-2-NPs). The originating SA-2-NPs have size of average of 200-500 nm. SA-2-NPs were shown to be stable in serum and saline ($t_{1/2}$ >3 days at 23˚C) and demonstrated sustained SA-2 release profile over 30 days (9).

In this proposal, we have performed an eye drop (ED) administration or ED administration combined with intravitreal delivery of SA-2-NPs to the mice retinas to demonstrate the effects of SA-2-NPs on RGCs following ONC. Contrary to our expectations, we observed more promising neuroprotective results when using low dose of SA-2 (0.25%, **Figure 5**) than when four-fold higher dose of SA-2-NPs (1%, **Figure 6**). PERG amplitudes in animals treated with 0.25% SA-2-NPs eye drop (ED), daily showed the most positive trend in preserving visual acuity following ONC (**Figure 5 C**, teal bar vs red bar). That suggests that SA-2 released from NPs does provide some neuroprotection when the drug is administered with an eye drop (ED). We know that 1% SA-2 NPs delivered as an eye drop (ED) reaches cornea, aqueous humor, vitreous humor, retina,

choroid and sclera (14). Unexpectedly, when dosing 1% of SA-2-NPs, the effect of improving the visual acuity was diminished in comparison with the lower dose of 0.25% (**Figure 6C**, teal bar vs red bar showing no differences). This phenomenon emphasized the need to carefully estimate efficacious dose for future experiments. We did not observe any improvement in RGC survival nor increase in PERG amplitudes with 1% dose despite encouraging *in vitro* results using 1% of SA-2-NPs on TBHP stressed NTM5 cells. This possibly brings us back to systematic testing of efficacy first *in vitro* and then *in vivo* for longer duration of time. We know that the PLGA nanoparticles are FDA approved and safe and there are many FDA-approved PLA/PLGA-based drug products available on the US market (14). Some safety tests of intravitreal delivery of PLGA were performed on primates on high doses of 3, 10, or 12.5 mg/eye of PLGA microspheres (17) and demonstrated inflammatory response in primate eyes. The highest dose of SA-2-NPs we tested in this experiment was 1% (2 µl injected intravitreally) equals 0.002 mg/eye of PLGA microspheres (a 150-fold less than the lowest dose tested in primates). We did not observe any inflammatory response, redness or eye scratching behavior following ED administration of SA-2-NPs in mice up to 14 days. Reassuringly, some PLGA nanoparticles have been use for the ocular disease. For example, a biodegradable PLGA implant carrying dexamethasone (Posurdex) has been already tested in patients with macular edema in phase 2 trials (DEX-PS-DDS from Allergan) but not yet been approved by the FDA. To the best of our knowledge, currently there is no PLGA based drug approved for intravitreal delivery or eye drop delivery for ocular diseases. If SA-2-NPs will be developed for that purpose, we will be one of the first to achieve that goal. The results obtained in this study did not demonstrate expected results and did not confirm our hypothesis. Every aspect of the study was reevaluated, and it was determined that we might need to prepare new improved formulations and

characterize them by conducting drug loading efficacy, drug release profile in saline prior to next round of experiments on animals. Overall, the study produced results that lack statistical significance, yet they lead to a conclusion that some neuroprotective effect of 0.25% of SA-2-NPs was observed in mice following ONC. To what degree the different variables in the study exert a direct effect on RGC survival needs to be evaluated in the future studies. Due to the exemplary data produced in the cellular assays (NTM-5 cells) conducted by Charles Amankwa, we can conclude that the results can be potentially transferred from *in vitro* to *in vivo* studies, but this will require more standardization and more "n" numbers of animals tested per group. After replication, the future experiments can advance to the next stage that would test varied concentrations of SA-2-NPs to find the effective dose for preventing RGC death.

Conclusions

SA-2-NPs have shown some promising results as a neuroprotective agent in ONC model when used at the low dose of 0.25% (**Figure 5C**). 1% SA-2-NPs was previously shown to be effective to lower IOP in rodent models of glaucoma (14) but in current study it did not demonstrate efficacy for protecting RGCs from dying following ONC. This study needs to be duplicated using newly synthetized SA-2 reloaded to new formulations of PLGA NPs in various ratios. Future studies should include a new SA-2-NP as well as an increased number of tested animals per group that will be sufficiently powered to generate statistically meaningful data (10). This work lays the groundwork that will help future studies avoid pitfalls and work towards testing the SA-2-NP compound and assess its full capability of neuroprotection.

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