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NANOLIPOSOMAL MINOCYCLINE FOR OCULAR DRUG DELIVERY

A Thesis in
Pharmacology
by
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ABSTRACT

The leading cause of preventable blindness among working-age adults in the industrialized world is Diabetic Retinopathy (DR). Currently, there is no FDA approved drug labeled for prevention or therapy in DR, though various anti-inflammatory drugs are often used off-label in combination with stringent glycemic control, with limited success. Delivery of these pharmaceuticals to the retina, across the various barriers present in the eye, poses a significant hurdle that inhibits the full therapeutic potential of many drugs. Nanoliposomal technology is a promising drug delivery system that could be employed to improve the pharmacokinetic properties of clearance and distribution in ocular drug delivery to the retina. We developed a nano-scale version of an anionic, cholesterol-fusing liposome that can encapsulate therapeutic levels of minocycline capable of drug delivery. We demonstrate that size-extrusion followed by size-exclusion chromatography can form a stable 80nm liposome that encapsulates minocycline at a concentration of 450µM ± 30µM, which is 2-3% of loading material. More importantly, these nontoxic nanoliposomes can then deliver 40% of encapsulated minocycline to the retina after a subconjunctival injection in the STZ-model of diabetes. Efficacy of therapeutic drug delivery was assessed via a transcriptomic biomarker panel previously validated as a surrogate endpoint for this animal model of DR. Although this liposomal drug combination displays efficacy similar to minocycline alone, the data presented here show the promise of nanoscale liposomal delivery systems to cross the tissues of the eye and deliver increased amounts of a drug directly to the retina. Moreover, this study validates the use of a transcriptomic biomarker panel as a means to assess the utility of an ocular drug delivery vehicle.
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Key words: minocycline, diabetic retinopathy, liposomes, transcriptomics, inflammation
**Abbreviations:** Diabetic Retinopathy (DR), Food and Drug Administration (FDA), Streptozotocin (STZ), Fluorescent Resonance Energy Transfer (FRET), Poly Ethylene Glycol (PEG), Support Vector Machine Classification Analysis (SVMCA), Enzyme Linked Immunosorbent Assay (ELISA), 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS)
INTRODUCTION

Diabetic retinopathy (DR) is a progressive chronic disease that is the leading cause of preventable blindness among working age adults within developed countries. Although many different types of drugs are currently being used off label for the treatment and prevention of diabetic retinopathy, there are no FDA approved pharmacotherapies labeled for the prevention or treatment of DR specifically [1]. Because of the prevalence and debilitating effects of advanced diabetic retinopathy, it is necessary to expand on the current pharmacotherapeutic options, as well as to make advances toward novel pharmacological delivery approaches. Liposomes and other nanotechnologies have been used as delivery vehicles for therapeutic agents in ocular drug delivery as they improve pharmacokinetic profiles and reduce toxicity [2, 3]. The potential for these improved properties has spurred the scientific investigation of liposomal formulations of various drugs from nearly every topically or subconjunctivally administered ophthalmic drug class [4]. In general, local administration of liposomal-encapsulated drug was found to significantly decrease the rate of clearance from the injection site and avert systemic toxicities[2-4]. Yet, it has also been observed that these liposomal formulations can sometimes collect within and cloud the vitreous. Frequently, these various nanoscale formulations are heterogeneous in size and agglomerate to 500nm or more. Thus, encapsulation of drugs within a stable nanoscale homogenous formulation may have the potential to improve delivery of therapeutic doses.

The Streptozotocin (STZ)-induced Sprague Dawley rat model of DR is known to phenotypically display an increased rate of neuronal apoptosis and an increase in retinal vascular permeability [5]; both hallmarks of human DR. Also, a highly reproducible panel of gene expression biomarkers has recently been described for the STZ-diabetic Sprague Dawley rat retina [6, 7]. These transcriptional biomarkers of DR code for proteins linked to dysfunctional microvascular, neuronal and inflammatory phenotypes associated with DR. The present manuscript utilizes these biomarkers as surrogate endpoints to test the efficacy of a locally-administered nanoscale therapeutic.
It is well established that molecular events underlying DR, like other pathological consequences of type I diabetic complications, are proinflammatory in nature [5]. Minocycline is an FDA approved antibiotic that binds to and inhibits the 30s ribosomal subunit of prokaryotes and inhibits protein synthesis. However, it also has multiple anti-inflammatory modes of action in eukaryotic systems. Known eukaryotic molecular targets of minocycline are: matrix metalloproteinase-9 [8, 9], vascular endothelial growth factor A [10], arachidonate 5-lipoxygenase [11, 12], cytochrome c [13, 14], interleukin 1β [15, 16], and caspase 1 and 3 [15, 17]. Systemic administration of minocycline has been shown to be efficacious in DR by reducing proinflammatory cytokine expression, microglia activation, and caspase-3 activation in the STZ Sprague Dawley rat model [18]. However, as with most antibiotics, there are significant toxicities associated with long term systemic administration of minocycline [19-22]. Local injections of minocycline in nanoliposomal form may afford the patient therapeutic benefit without needless exposure to high systemic minocycline serum levels that may potentially induce adverse reactions. In this study, we explore the utility of nano-liposomal encapsulated minocycline as a subconjunctivally-administered pharmacotherapy for DR.
METHODS

Liposomal Preparations

To prepare a minocycline-encapsulated nanoscale liposomal formulation, we significantly adapted previously described methods for encapsulation of tetracycline derivatives [23-25]. Chloroform-based stock solutions of egg phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL), dihexadecyl phosphate and cholesterol (Sigma Aldrich, St. Louis MO) were mixed together in borosilicate glass test tubes at a molar ratio of 7:2:1. The solvent was completely removed via a constant flow of nitrogen gas at room temperature. Liposomal formulations were hydrated with 800µl of sterile isotonic 0.9%NaCl hydration solution (Baxter Healthcare Corporation, Deerfield, IL) at 60°C and briefly vortexed every 10-15 minutes for 2 hours. Formulations were sonicated for 1 minute at 60°C and then 200µl of 100mM minocycline, pH 7.2 (Sigma Aldrich, St. Louis MO) was added to the preparation and incubated with the liposomes for 30 minutes. The formulation was then extruded 13-times through a 100nm polycarbonate membrane utilizing an Avanti mini-extruder. Encapsulated minocycline was separated from non-encapsulated free drug via size exclusion chromatography with Sepharose® CL-4B (Sigma Aldrich, St. Louis MO).

For imaging experiments, identical ghost (no minocycline) formulations were “spiked” with 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl). For Fluorescent Resonance Energy Transfer (FRET) experiments, similar formulations were engineered that were “spiked” with FRET lipid pairs. Specifically, we utilized ((1,2 Dipalmitoyl-sn-Glycero-3-phosphoethythio)-3’-Succinimide)-N-(3’-Flouranthy1) (DSP-F), which exhibits an excitation of 365nm and an emission of 465nm and 1-oleoyl-2-(6-((7-nitro-2-1,3-benzoxadiazol-4-yl)amino)hexanoyl)-sn-glycero-3-phosphoethanolamine (PS-NBD), which has an excitation of 465nm and an emission of 534nm. Controls for the FRET liposomes were nanoliposomes that contain only one of the fluoreprobes in the FRET pair. To differentiate FRET liposomes from control liposomes, we excited the liposome at 365nm and
visualized emission at 534nm. The phenomenon of FRET will determine if liposomes are transported to the retina in an intact state. If disassembled, the liposomal components would not be detectable by FRET. In all experiments, fluorescent lipids were added so that they constituted less than less than 0.5% of the total lipids as measured by molar percentage.

**Liposomal Evaluation**

The size (nm) and zeta potential (mV) of newly synthesized minocycline nanoliposomal formulations were evaluated over time using a Malvern Dynamic Light Scattering instrument. Briefly, liposomes recently purified from a CL-4b column were then diluted 10-fold and placed into a cuvette and observed using quasi elastic light scattering. To evaluate the mass of minocycline encapsulated into nontoxic, anionic, nanoscale liposomes we utilized a MDS/Sciex 4000 QTrap mass spectrometer using a C-18 HPLC column. The signal intensity of the mass to charge ratio detected from the Qtrap was then compared to a standard curve of minocycline concentrations to allow extrapolation of the quantity of minocycline present in the sample.

**STZ-induced Diabetic Model**

All rats were maintained under specific pathogen free conditions in compliance with protocols approved by the Institutional Animal Care and Use Committee of Penn State College of Medicine and monitored by quarterly sentinel testing. Male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) were purchased at 200-225g. Experimental type I diabetes was induced after 7 days of acclimation and an overnight fast, with an intraperitoneal (IP) injection of 65mg/kg streptozotocin (Sigma Aldrich, St. Louis, MO) in 10mM Na Citrate, pH 4.5 (vehicle). Non-diabetic age-matched control rats were given comparable volume of vehicle only. Food and water were available to the rats ad libitum and they were housed under a 12 hour light/dark cycle. Body weights and blood glucose levels
were first monitored seven days post-induction and once a week thereafter. At no time throughout the experiment was exogenous insulin administered to attain glycemic control. Glycemic levels of control rats were 122.0mg/dl ±8.4 and diabetic rats were 495.0mg/dl ±17.6 on average throughout the experiment. Twelve weeks after diabetic induction, rats were given 20µl, 10µl and 5µl of subconjunctival, topical and intravitreal volumes, respectively, of the various treatments once a day for four days. To aid in the administration of the subconjunctival injections, the rats were briefly sedated with the use of a gas mixture consisting of 70%CO₂/30%O₂. The rats were placed in a Plexiglas box then flooded with the gas mixture and allowed to breathe the gas for no more than 1.5 minutes. After this exposure to the 70%CO₂/30%O₂, the rats were removed from the box and would typically remain anoxicated for 45-60sec during which time both eyes could be injected. No signs of distress were observed in rats upon their waking. At the conclusion of the experimental treatment protocol, the rats were given a lethal dosage of sodium pentobarbital (100mg/kg) via intraperitoneal injection and retinas were harvested and snap frozen in liquid nitrogen, or rat eyes were dissected and cornea, lens, vitreous, and retina were isolated. Minocycline levels in tissue were quantified after extraction by mass spectroscopy. Minocycline was extracted from the tissue by homogenization in a solution of water, methanol, and formic acid 79:20:1 v/v/v and then centrifugation at 14000rcf followed by quantifying the supernatant concentration using a MDS/Sciex 4000 QTrap mass spectrometer.

DNA Fragmentation Analysis

A cell death detection ELISA protocol (Roche, Mannheim, Germany) was employed to detect fragmented retinal DNA, indicative of late stage apoptosis. Briefly, retinas were thawed on ice and then homogenized in 200µl of the provided lysis buffer and allowed to stand for 30min at room temperature. The lysates were centrifuged for ten minutes at 500rcf at room temperature. 20µl of each sample supernatant, was added into separate wells of the ELISA plate. 80µl of the provided Immunoreagent
was then added to each well and the micro plate was covered and incubated at room temperature for two hours on an orbital rocker at 300rpm. The immunoreagent was removed and the wells were washed three times with incubation buffer. 100µl of ABTS solution was then added to each well and incubated in the same manner for 10-20 minutes. DNA fragmentation was quantified as follows.

\[
\text{DNA fragmentation} = \frac{(OD_{\text{sample}} - OD_{\text{negative control}})}{(\text{retinal weight})}
\]

**qPCR**

Quantitative Polymerase Chain Reaction (qPCR) analysis was conducted with the use of a 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA), using a 384-well optical format, with Assay-On-Demand (Applied Biosystems) gene specific primers and probes. SDS 2.2.2 software using the \(2^{\Delta\Delta Ct}\) analysis method was used to quantitate the differential amounts of product using β-actin as an endogenous control [26]. β-actin mRNA transcript expression was found to be statistically equivalent in an absolute quantitation experiment (data not shown). Primer and probe sets have previously been reported [6]. Statistical tests for qPCR data are a one way ANOVA Kruskal-Wallis test followed by a Dunn’s Multiple Comparison test and a standard two-tailed parametric t-test with an \(\alpha < 0.05\).
RESULTS

Characterization of minocycline-encapsulated nanoliposomes

Quasi elastic light scattering characterized size-excluded nanoliposomes to be unilamellar vesicles with an average diameter of 80nm ±20nm. Analysis of the zeta potential determined that the nanoliposomes were slightly anionic at -21mV. The shelf-life stability of minocycline-encapsulated nanoliposomes was determined as a function of time, temperature and determined not to change from 1 hour to two days or from +4 to 30°C. Stability was maintained in various biological mediums including DMEM +/- 5% fetal bovine serum. Encapsulation efficiencies of nanoliposomal minocycline formulations were evaluated via mass spectroscopy. 100mM stock solution was mixed with lipids prior to sonication and extrusion for 30min to formulate a 20.3mM encapsulation buffer. After sonication and size-extrusion, this was then purified by CL-4B size exclusion chromatography to form the purified nanoliposomal fraction with a concentration of minocycline of 451.0µM ± 30µM. Therefore, the final encapsulation efficiency was shown to be approximately 2 to 3% of the starting loading material as determined by mass spectroscopy.

Delivery of the minocycline-encapsulated nanoliposomes to the retina.

Confocal analysis of rhodamine-labeled nanoliposomes was utilized to assess ocular localization after intravitreal, subconjunctival injections and topical applications to Sprague Dawley rats. Fluorescent staining of the inner and outer plexiform layers and the inner and outer segments as well as the pigmented epithelium layer of the retina was visible after 15 min of administration for all delivery modalities (Figure 1). A small degree of auto-fluorescence indicative of frozen retinal sections can be visualized in control sections, but the greater intensity of staining in rhodamine-labeled liposome-treated eyes shows definitive positive staining. Liposomes that did not contain rhodamine and sections from the contralateral eye served as negative controls. Subconjunctival injections have the most intense
fluorescence followed by topical administration and then intravitreal injections at 15 min. Similar results were observed after 1 hour for Subconjunctival injection and topical administration with little fluorescence observed for intravitreal injection (data not shown), possibly reflecting more rapid clearance for intravitreal administration.

To validate if intact nanoliposomes reach the retina, we employed a FRET liposome via subconjunctival injections. FRET pairs must remain in close proximity (i.e., as components of an intact liposome) to exhibit FRET staining in vivo. As controls, we administered liposomes containing only the first fluorophore DSP-F (Figure 2B), only the second fluorophore PS-NBD (Figure 2C), or no fluorophore at all (Figure 2A) and compared them to the liposome that contained both DSP-F and PS-NBD (Figure 2D). When retinal segments were excited at 365 nm and imaged at 534 nm, fluorescence was only observed with the FRET pair formulation (Figure 2D). The pattern of fluorescence was similar to what was observed with the rhodamine-spiked liposomal formulation (Figure 1). Taken together, these results indicate that the anionic nanoliposomes remain intact as a consequence of transit to the retina.

To conclusively show that intact nanoliposomes can deliver encapsulated cargo to the retinal segments, we utilized mass spectrometry to assess minocycline mass delivered to ocular tissue as a function of subconjunctival administration. Untreated rats and rats treated with liposomes that did not contain minocycline served as negative controls, while non-encapsulated minocycline served as a positive control for the nano-encapsulated minocycline formulation. Retinas were excised at various time points and extracted minocycline was quantified via mass spectroscopy as described in methods (Figure 3). Nanoliposomal minocycline delivery increased the mass of minocycline in the retina at all time points as compared to free (non-liposomal) delivered minocycline. At one and six hours, an approximate one- and four-fold increase was observed for the subconjunctival-administered minocycline liposome verses free minocycline. Moreover, given a concentration of encapsulated minocycline of 451.0 µM, nearly 40% of the minocycline is localized within the retina six hour after
subconjunctival administration. Significant cornea localization was also noted, with little localization in the vitreous or the lens. At 24 hours after administration, minocycline has largely been cleared from the retina, but trace amounts near the detection limit of our mass spectrometer still point to localization within the retina.

**In vivo toxicity of minocycline liposomes in control rats.**

We next evaluated the toxicity of nanoliposomal minocycline as a function of once a day for four days subconjunctival-administered dose. Fragmented DNA, a marker of apoptosis, was utilized to assess retinal toxicity of nanoliposomal minocycline formulations purified via size exclusion chromatography (Figure 4). Concentrations of encapsulated minocycline (316 and 31.6µM) were chosen consistent with the retinal delivery experiments (451µM). A 10mM mixture of free and nanoliposomal minocycline that did not undergo CL-4B size exclusion chromatography was utilized as a positive control for toxicity. The purified nanoliposomes did not exhibit toxicology as assessed by retinal DNA fragmentation at either of the two pharmacological doses.

**Transcriptomic Biomarker Panel can be used as a Measure of Pharmacological Effects**

We evaluated the efficacy of minocycline-encapsulated nanoliposomes by utilizing a biomarker panel as a surrogate endpoint for therapeutic efficacy [6, 7]. We analyzed extracts of mRNA from retinal tissue of three month diabetic or control rats treated once a day for four days subconjunctival with vehicle, free or encapsulated (316µM) minocycline. The relative expression of 7 of the 14 gene products was changed by free minocycline administration returning them to non-diabetic levels (Figure 5). These gene products (LAMA5, Gpb2, Chl3L1, Icam1, C1s, C1Inh, and Carhspb1) are all associated with pro-inflammatory phenotypes [6, 7]. In contrast, nanoliposomal minocycline failed to produce such a statistically significant change in all but two out of the fourteen gene targets analyzed (Lama5 and
Icam1). Analysis of individual animals by support vector machine classification analysis (SVMCA) revealed that both free and nanoliposomal minocycline can reverse the biomarker panel in individual rats. This unbiased approach determined that three of the eight animals in the free minocycline treated group and 2 of the eight in the nanoliposomal minocycline treated group exhibited a normal non-diabetic transcript phenotype (Table 1). Surprisingly, despite increases in mass of minocycline delivered via encapsulation (Figure 3), different efficacies were noted between free and nanoliposomal minocycline as free minocycline preformed better than the nanoliposomal form. However, the fact that both free minocycline and nanoliposomal minocycline demonstrated a positive impact on select "pro-inflammatory" end points suggests efficacy after local delivery, which could potentially improve with an optimization of dosage and duration of treatment.
Discussion

The goal of this study was to elucidate whether local administration of nanoliposomal or free minocycline can deliver a pharmacologically relevant dose of a drug to the retina via a local injection. In addition to results from Figure 2 noting increased fluorescent imaging with subconjunctival injection compared to intravitreal or topical administration, we chose to assess delivery, toxicology and efficacy by subconjunctival injections for the following medical reasons. Subconjunctival injections are less invasive than intravitreal injections and also afford a consistent reproducible dose unlike topical administration that can vary in dose before absorption due to blinking and tear washing. It has also been demonstrated in the clinic that patient compliance with eye drops is inadequate [27].

The unique aspect of the present approach is documentation of encapsulation of a therapeutic dose of minocycline within nontoxic, fusigenic, anionic, nanoscale liposomes that can be used for ocular delivery to posterior segments of the eye. Even though minocycline has been encapsulated in liposomal formulations before [23, 28-30] for non-ocular uses, our formulation is unique in that size exclusion chromatography and size extrusion were utilized to generate a nanoliposome devoid of surface minocycline contamination. In fact, without "clean-up" these formulations were toxic (Data not shown). Moreover, shortly after a subconjunctival injection, minocycline nanoliposomes can be effectively trafficked intact to the retina and deliver minocycline in higher more sustained amounts than when free minocycline is given alone. This ability to remain intact during trafficking is a novel aspect of the formulation and possibly is responsible for the higher concentration of encapsulated minocycline delivered to the retinas compared to free minocycline.

We also demonstrate that subconjunctival injections of ether nanoliposomal minocycline or free minocycline are capable of reversing a portion of the aberrant expression gene profile documented in STZ-induced diabetic rat retinas. Explanations for the lack of an enhanced effect on the gene panel with minocycline-encapsulated nanoliposomes, compared with free minocycline, despite the improved
kinetic delivery, might reflect non-optimized minocycline dosage and duration of treatment protocols. Alternatively, this lack of efficacy for liposomal minocycline to reduce all of the inflammatory biomarkers on the panel that free minocycline did may reflect liposomal trafficking of minocycline to lysosomes where it is degraded. The use of less fusigenic liposomal formulations (i.e., less cholesterol) could remedy this putative liposomal trafficking. However, the fact that both free minocycline and nanoliposomal minocycline demonstrated efficacy for these particular "inflammatory" end points suggests efficacy after local delivery.

Many drugs that have potential therapeutic utility in DR are ultimately limited due to adverse outcomes on other systems when the drug is given systemically [19-21]. In order to treat the retina with a systemic treatment regimen of minocycline, the blood plasma levels of the drug would have to be very high over long periods of time. This may needlessly expose the entire body to the high doses of the drug and increase the chance of adverse reactions. This is especially problematic for minocycline, as an adverse side effect of systemic high dose therapy for teenage acne is pharmacological induction of lupus. Subconjunctival local administration of nanoliposomal minocycline can potentially be used to administer these high therapeutic doses to the retina without inducing systemic toxicities. In fact, clearance mechanisms within the eye, by definition, would dilute the minocycline concentration as it clears into the systemic circulation. Finally, nanoliposomal or free minocycline can be administered directly to the site of action and thus avoid first pass metabolism as well as side affects involving depletion of normal flora in the gut. Taken together, the engineering of a nontoxic nanoliposomal formulation that remains intact during ocular delivery has the potential to deliver therapeutic doses of anti-inflammatory agents to combat diseases such as diabetic retinopathy.
Future Directions

Going forward, we plan to optimize the dose and duration of treatment of the current formulation. In the current study, we tested the efficacy of nanoliposomal minocycline after four days of treatment, in rats, after three months of diabetes. It may be necessary to test much longer durations of treatment. For example we could test one, two, and four weeks of treatment. Treatments on this timescale may have the potential to elucidate the long term potential of this nanoliposomal formulation to outperform free minocycline. Within each of these treatment durations, dosage could be varied to help us understand what dose would be best at a given duration of treatment.

Recently, a paper was published reporting that minocycline binds calcium. This information may help us obtain much higher encapsulation rates [31]. Minocycline chelates divalent cations and through this interaction becomes more lipophilic. Thus, future formulations may utilize calcium thereby increasing encapsulation concentration and efficiencies. Poly Ethylene Glycol (PEG) bound to a lipid can give a liposome increased half-life in the blood or interstitial space. Our attempts at nanoliposomal minocycline formulations containing PEG proved not to encapsulate the drug at concentrations useful in our experiments. Therefore, we abandoned the prospect. We then learned that other groups have successively prepared pegylated minocycline nanoliposomes [29]. The key factor was the addition of calcium to the liposomal buffer. In the future, we would keep in mind the possibility of specific ionic species modulating the lipophilicity of drugs and altering drug loading equilibriums. However, we have concern with this method because delivery of Ca$^{2+}$ may be toxic due to possible induction of pathways such as Ca$^{2+}$-mediated apoptosis [32].

One question that needs to be answered is: what aspect of minocycline is providing efficacy for DR in the rat retina. At the level of its anti-neuroinflammation, anti-apoptotic effects, minocycline has a complex multimodal mechanism of action. It has been previously documented that minocycline inhibits many pathways involving apoptosis, inflammation, and vascular permeability. These pathways are
known to be pathologically perturbed in DR. However, while some molecular targets of minocycline are being affected positively to reduce neuroinflammation and or decrease apoptosis, others may be causing undesired side effects. As stated previously; known eukaryotic molecular targets of minocycline are: matrix metalloproteinase-9 [8, 9], vascular endothelial growth factor A [10], arachidonate 5-lipoxygenase [11, 12], cytochrome c [13, 14], interleukin 1β [15, 16], and caspase 1 and 3 [15, 17]. Though minocycline can therapeutically affect multiple targets, the potency of minocycline at these targets is most likely not the same. Additionally, there may be other undocumented molecular targets of minocycline that can be deleterious to the retina and thereby narrow the therapeutic window. Thus, a more efficacious and less toxic approach may be to individually target pro-inflammatory and anti-apoptotic pathways with more selective agents. This individualized targeting approach may allow for more efficacious combinatorial therapies. To ensure timely clinical development, the choice of these drugs would reflect efficacy against targets that are dysregulated in DR and that can respond to minocycline. Below I list specific examples for each molecular target of minocycline that might benefit from nanoscale ocular delivery modalities.

The angiotensin-converting enzyme inhibitor captopril also inhibits matrix metalloproteinase-2 and 9 [33, 34] and therefore may be a good candidate drug to test the role of matrix metalloproteinase-9 inhibition as a therapeutic target in DR. Orally administered captopril has been demonstrated to be partially therapeutic in DR [35, 36]. This efficacy is theoretically achieved by reducing micro albuminuria produced as a consequence of diabetic nephropathy, but direct inhibition of matrix metalloproteinase-9 in the retina may also play an integral role. Captopril has an experimentally derived LogP of hydrophobicity of 0.6 and is not known to cross the blood brain barrier significantly [37]. Therefore it may not passively cross the various barriers in the eye necessary to treat the site of action, the retina, if it is taken orally or injected directly into the blood stream. However, the pharmacological inability to cross such barriers, may not pose such a problem in a diabetic retina that possesses the leaky
vasculature typical of DR. Also, it is plausible to obtain local inhibition of matrix metalloproteinase-9 in the retina via a subconjunctival injection of free or nanoliposomal captopril. The LogP of captopril is lower than one and the drug is said to be freely soluble in aqueous solutions of physiological pH and isotonic strength. Typically, chemical properties such as these cause liposomal encapsulation to be difficult. This is in fact the case with minocycline (LogP 0.5). Yet, 25-30% of captopril has been found to be bound to plasma proteins primarily albumin. Potentially, this property may be exploited to gain higher encapsulation efficiencies, possibly for use in a nanoliposomal delivery vector.

Vascular endothelial growth factor A, along with a multitude of other pro angiogenic factors are known to be over expressed in DR and thus contribute to the aberrantly high levels of angiogenesis in the retina that cause the leaky vasculature phenomenon synonymous with the pathogenesis of DR [38, 39]. Bevacizumab and Ranizumab, inhibitors of vascular endothelial growth factor, have been studied in DR and are both being investigated in clinical trials [40-43]. These drugs are recombinant IgG fragments that bind to vascular endothelial growth factor A, at the receptor binding region of this ligand, and thereby prevent it from activating it’s receptor, competitively. Usually, this medication is administered via an intravitreal injection. These IgG fragments could potentially be conjugated to a nanoliposomal vector that could then be injected subconjunctivally. This would improve the risk to benefit ratio of this treatment by eliminating the risks that are associated solely with intravitreal injections, and replacing them with the relatively innocuous subconjunctival injection. Another drug gliclazide is a small molecule that has been shown to bind directly to the vascular endothelial growth factor receptor and inhibit ligand mediated activation [44, 45]. Although it is primarily known as an inhibitor of the sulfonylurea receptor, an inward-rectifier potassium channel, that primes β-cells for insulin release [46], the antagonistic effects on vascular endothelia growth factor receptor may allow this drug to be useful in the treatment of DR. Testing systemic versus local administration of gliclazide may elucidate whether local administration has any additional benefit. However, discerning the broad therapeutic benefit of
improving insulin secretion, from inhibition of vascular endothelial growth factor A signaling in the retina would be difficult.

Arachidonate 5-lipoxygenase is an enzyme responsible for converting arachidonic acid into leukotriene \( \text{A}_4 \), an unstable product that is rapidly metabolized into one of the following products leukotriene \( \text{B}_4, \text{C}_4, \text{D}_4 \) and \( \text{E}_4 \) all being proinflammatory leukotrienes, that each have specific receptors, physiological consequences, and are thought to exacerbate pathogenesis in DR \([47, 48]\). Inhibitors of arachidonate 5-lipoxygenase prevent the production of these proinflammatory cytokines in one of two ways. Veliflapon can inhibit the 5-lipoxygenase activating protein while zileuton can directly inhibit arachidonate 5-lipoxygenase \([49, 50]\). Both of these drugs have been studied in diabetes \([51, 52]\). Both of these drugs are hydrophobic with Log P of hydrophobicity for veliflapon and zileuton being 4.78 and 2.01, respectively. This property could potentially improve the efficiency of encapsulation of these drugs into a nanoliposomal vector as compared to minocycline if such encapsulation is necessary for ocular delivery. Moreover, the specificity of these drugs to preferentially act at arachidonate 5-lipoxygenase as opposed to drugs that broadly inhibit cyclooxygenase, prostaglandin synthase, thromboxane synthase, as well as leukotriene synthase, should in theory cause less adverse side effects than broad spectrum inhibitors of arachidonic acid metabolism like the salicylic acid, the nutraceutical hyperforin, or the anthelmintic diethylcarbamazine \([53-55]\). Alternatively, a cysteinyl leukotriene receptor antagonist may be a better candidate for the local inhibition of arachidonate 5-lipoxygenase signaling. This would especially be the case if a significant amount of leukotrienes are constantly perfusing into the retina from the blood, causing local inhibition of leukotriene production to be largely ineffective. With a ‘-lukast’ class drug, the receptors for leukotrienes would be blocked and signaling through this inflammatory pathway would be decreased no matter where the leukotrienes were produced. Montelukast is a cysteinyl leukotriene receptor 1 antagonist with wide use in the treatment
of asthma [56, 57]. With a Log P of 7.9 it may incorporate into a nanoliposomal vector well. This may be necessary if the oral preparation is found not to readily cross the blood-retinal barrier.

Minocycline has been shown to inhibit various members of pro-apoptotic signaling cascades such as cytosolic cytochrome c, caspase 1, and caspase 3 [13-15, 17]and is being investigated for its possible neuroprotective capabilities [58, 59]. Many other drugs have been shown to have neuroprotective properties and have been studied as a treatment in diabetic retinopathy [60, 61]. Methozolamide is a broad spectrum carbonic anhydrase inhibitor, it is indicated for ocular diseases where the reduction of the intraocular pressure is likely to be beneficial, such as in glaucoma [62]. Modifications of this drug are being tested topically [63]. Other groups have prepared formulations of nanoliposomal and calcium phosphate nanoparticles of methozolamide for intraocular delivery of this drug [64, 65]. Additionally, methozolamide has recently been shown to be neuroprotective by inhibiting the release of cytochrome c from isolated mitochondria, and through this mechanism prevent neuronal apoptosis in mouse models of Huntington’s disease [66]. It would be interesting to test nanoliposomal methozolamide in our STZ induced rat models of early DR. Also, an observational clinical trial to compare the progression of DR between matched control patients with DR only, to patients who had been treated with methozolamide for glaucoma, but are co-morbid with DR could be very informative.

In diabetic retinopathy the retinal vasculature is exposed to increased levels of Interleukin 1-β. This increase in Interleukin 1-β is thought to modulate the permeability of the microvasculature and contribute to neovascularization in the retina [67]. The recombinant human monoclonal IgG canakinumab is a interleukin 1-β inhibitor that binds Interleukin 1-β and prevents it from interacting with the interleukin 1 receptor [68, 69]. In theory, canakinumab would work as a treatment for diabetic retinopathy against interleukin 1-β the same way bevacizumab and ranizumab works against vascular endothelial growth factor A.
Conducting such a study is complicated by the fact that there are multiple candidate drugs for each of the molecular targets of minocycline as mentioned above. In theory *in vitro* and *pane in vivo* (retinal explants) models of DR could be employed to identify putative combinations of these drugs with higher potential to demonstrate minimal toxicity with maximal efficacy before moving to expensive and labor-intensive animal trials. However, the complexity of the diabetic retina is not easily modeled, and it may in fact, be more economical and faster to assess toxicity and efficacy in fully diabetic rats at the age and time point after diabetic induction desired. Also, complicating this approach would be the independent pharmacokinetic nature of each compound. Because of inevitable differences in clearance and elimination, possible bioconjugation, and ability to cross various barriers in the eye; variables like dosage, frequency of administration, and even route of administration will have to be optimized for each drug before the optimal synergistic efficacy could be documented. However, using a cocktail of drugs that, as individual agents are more specific for their intended molecular targets than minocycline, should provide a larger therapeutic window, by eliminating the undesired off target effects observed when using high doses of minocycline over a long period of time. These drugs could then theoretically be formulated into a nanotechnological drug delivery platform that helps to control the trafficking and release of the cocktail in the eye. Each individual compound could theoretically be dosed to maximize the safety and efficacy at each intended pathway.
Appendix

Figures for Nano-Liposomal Minocycline for Ocular Drug Delivery

(Figure 1)

Intravitreal injection, 15min time-point

<table>
<thead>
<tr>
<th>Liposome</th>
<th>Rhodamine-Liposome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contralateral Eye</td>
<td>Contralateral Eye</td>
</tr>
</tbody>
</table>

Subconjunctival injection, 15min time-point

<table>
<thead>
<tr>
<th>Liposome</th>
<th>Rhodamine-Liposome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contralateral Eye</td>
<td>Contralateral Eye</td>
</tr>
</tbody>
</table>

Topical administration, 15min time-point

<table>
<thead>
<tr>
<th>Liposome</th>
<th>Rhodamine-Liposome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contralateral Eye</td>
<td>Contralateral Eye</td>
</tr>
</tbody>
</table>
Rhodamine-labeled liposomes image the retina fifteen minutes after administration via different methods. Ten micrometer thick sections of the retina are shown to demonstrate fluorescent imagery. In each panel, the top left section is treated with non-fluorescently labeled liposomes as a negative control. The Bottom left is treated with liposomes labeled with rhodamine. The right panels top and bottom are sections from the contralateral eye of that individual. Shown here are representative pictures from intravitreal injection (a) subconjunctival (b) and topical (c) administration methods. In these pictures blue is nuclear staining and red is rhodamine. The various layers of the retina are labeled in the top right section of (a).
Sprague Dawley rats were administered 20µl subconjunctival injections with FRET nanoliposomes or appropriate controls. After 15 minutes retinas were extracted and prepared for frozen sectioning. Visualizing FRET by exiting at 365nm and observing emissions at 534nm. a) Nanoliposomes containing no fluoroprobe. b) Nanoliposomes containing only DSP-F. c) Nanoliposomes containing only PS-NBD. d) Nanoliposomes containing both DSP-F and PS-NBD. Although the change in retinal fluorescence is subtle, the signal seen in panel d treated with the complete FRET liposome displays a fluorescent signal that is brighter than background auto fluorescence seen in panels a, b and c.
Figure 3.

(a) 30min

(b) 6hr

(c) 24hr

(d) 24hr
Nanoliposomal encapsulation increases the concentration of minocycline in various tissues of the eye. Thirty minutes and six hours after administration, nanoliposomal minocycline delivers more minocycline to the denoted tissues than when free minocycline is administered. Twenty four hours after administration the drug has largely cleared yet a statistically non-significant difference can still be observed panels c and d. * Denotes statistical significance between the marked column and the same tissue treated with free minocycline alone, as determined by a one-tailed non-parametric Mann-Whitney t-test α=0.05
Figure 4.

Level of fragmented DNA found in rat retinal lysates as detected with the Roche Cell Death Detection ELISA®. There was no increase in fragmented DNA detected when the animals are treated only with control nanoliposomes or nanoliposomal minocycline at concentrations of 316µM, 31.6µM minocycline as compared to animals treated with 0.9%NaCl irrigation fluid. The control liposomal and the 10mM liposomal minocycline treatments were not exposed to CL-4B size exclusion chromatography for purification of nanoliposomal minocycline amongst non-encapsulated ‘free’ minocycline denoted by (+). In previous experiments, data not shown, this 10mM free/nanoliposomal minocycline preparation was observed to induce apoptosis and here serves as a positive control. However, the equivalent preparation of liposomes without minocycline causes no such increase in DNA cleavage and demonstrates no retinal toxicity, intrinsic to this nanoliposomal formulation, at this concentration and treatment regimen. 316µM and 31.6µM nanoliposomal minocycline preparations were exposed to purification via CL-4B size exclusion chromatography (-). This step eliminates non-encapsulated minocycline and greatly reduces the concentration of minocycline in the preparation. In this experiment no increase in DNA cleavage was detected in rat retinal lysates from rats treated with these preparations. *Denotes statistical significance between the marked column and the retinal samples treated with 0.9%NaCl irrigation fluid as determined by a one-tailed non-parametric Mann-Whitney t-test α=0.05
Figure 5.
Relative expression of various mRNA transcripts previously determined by Freeman et al. [6, 7] to be differentially expressed in the three month STZ induced diabetic rat retina are detailed here next to the effects of administration of free and nanoliposomal minocycline in the STZ rat retina. For each transcript the relative expression of STZ, free minocycline, and nanoliposomal minocycline groups are normalized to the control group. * Denotes transcripts having significantly different means as determined by a Kruskal-Wallis one-way ANOVA where the asterisks appear over the groups that were determined to differ significantly from the control group as determined by Dunn’s post test \( P < 0.1 \). # Denotes the free minocycline treated groups that were found to differ significantly from the STZ group as determined by a one-tailed Mann Whitney t-test \( P < 0.05 \).
Table for Nano-Liposomal Minocycline for Ocular Drug Delivery

Table 1.

<table>
<thead>
<tr>
<th>Test Set</th>
<th>Control</th>
<th>Diabetic</th>
<th>Diabetic + Free Minocycline</th>
<th>Diabetic Nanoliposomal Minocycline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Classified as Control</td>
<td>7</td>
<td>1</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Classified as Diabetic</td>
<td>1</td>
<td>8</td>
<td>5</td>
<td>6</td>
</tr>
</tbody>
</table>

Support Vector Machine Classification Analysis with polynomial kernel scale factor of 1. Both nanoliposomal minocycline and free minocycline treated groups are treated with the subconjunctivally administered 20µl of 316µM minocycline once a day for four days after the three months of STZ induced diabetes.
References


43. Deissler, H.L., H. Deissler, and G.E. Lang, Inhibition of vascular endothelial growth factor (VEGF) is sufficient to completely restore barrier malfunction induced by growth factors in microvascular retinal endothelial cells. British Journal of Ophthalmology.