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Use of Clofibrate to Bypass Nuclear Targeting in the Development of Non-Viral Gene Delivery Systems

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USE OF CLOFIBRATE TO BYPASS NUCLEAR TARGETING IN THE DEVELOPMENT OF NON-VIRAL GENE DELIVERY SYSTEMS

by

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A thesis submitted in partial fulfillment of the requirements
for graduation with Honors in the Health and Human Physiology

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All requirements for graduation with Honors in the
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ABSTRACT

Clofibrate is a commercially available chemical that has been found to induce increased cell division in the hepatocytes of rodents. Previous experiments in our lab have investigated the use of clofibrate to bypass the need for nuclear targeting in non-viral gene delivery systems, since the large size of the gene delivery vector limits its ability to enter the nucleus of hepatocytes. We decided to replicate these experiments, with the intention of seeing higher levels of gene expression by using targeted DNA nanoparticles that contained additional targeting for mice hepatocytes. Mice were dosed with corn oil, clofibrate, clofibrate and non-targeted DNA nanoparticle, or clofibrate and targeted DNA nanoparticle over the course of a 5-day clofibrate dosing protocol. Body weight gain, liver weight gain, and levels of luciferase gene expression was compared across treatment groups. It was found that only the treatment group dosed with non-targeted DNA nanoparticles exhibited a 10-fold increase in gene expression compared to the control group ($p = 0.0108$). Therefore, we decided to further explore this result by determining if a dose-dependent response would be seen from non-targeted DNA nanoparticles over the course of a 14-day clofibrate dosing protocol. Mice were dosed with clofibrate, clofibrate and 10 μg non-targeted DNA nanoparticle, or clofibrate and 100 μg non-targeted DNA nanoparticle with changes in body weight, liver weight, and luciferase gene expression being recorded. The data suggested that non-targeted DNA nanoparticles did not exhibit a dose-dependent response in luciferase gene expression when dosed in the presence of clofibrate.

ACKNOWLEDGEMENTS

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I would also like to thank Raghu Ramanathan for helping me carry out these experiments and making this project a reality. I greatly appreciate all that he has done for this project, including going over with me anything that I was unsure about, answering any and all calculation questions I had when I kept second-guessing myself, and giving me the confidence to dose (and face my fear of) mice.

AN INTRODUCTION TO GENE THERAPY

Hemophilia is a rare, genetic disorder that affects an estimated 400,000 people worldwide.[1] This disorder prevents the blood from clotting normally, because the liver is unable to produce functioning blood-clotting proteins (clotting factors). Therefore, bleeding cannot be stopped because a blood clot cannot form. Currently, hemophilia is treated by injecting the missing clotting factor into the bloodstream. A person with hemophilia that has a simple cut or scrape can be given this replacement therapy until the bleeding is stopped. However, individuals can suffer from internal bleeding, caused by something such as a bump to the knee or in severe cases, be due to spontaneous bleeding that occurs within the body. Internal, spontaneous bleeding is very difficult to detect and can have life-threatening complications.

Gene therapy can provide an alternative solution for those with hemophilia. Rather than using a replacement therapy to treat the primary symptom, gene therapy can potentially treat the disorder itself. Gene therapy treats disease by introducing genetic material into cells. This would mean that for individuals with hemophilia, new DNA would be delivered to hepatocytes, thereby allowing for the continuous production of normal, functioning clotting factors without the need for replacement therapy.

Gene therapy has the ability to treat several diseases using a variety of approaches. Viral gene therapy is one of these approaches, where an inactivated virus is used to deliver genetic material to cells. In December 2017, LUXTURNA™ became the first FDA-approved prescription gene therapy, which uses a benign form of the adeno-associated virus (AAV) to treat inherited retinal disease.[2] Unlike most viral gene therapies, which can be compromised by the potential for immunogenicity, LUXTURNA™ is successful because the eyes are immune-privileged.[2] Usually viral gene therapy can only be performed under *in vitro* conditions, where gene delivery takes place outside of a living organism, such as in a test tube or a cell culture dish. Viral gene therapy is difficult to use under *in vivo* conditions, because the living organism can develop an immune response to the viral delivery vector.

Non-viral gene therapy provides an alternative method, because it is potentially non-immunogenic when delivered under *in vivo* conditions. Rather than using a viral delivery vector, non-viral gene therapy utilizes natural and synthetic nanoparticles to deliver genetic material. In August 2018, ONPATTRO™ became the first FDA-approved prescription non-viral gene therapy, with its ability to deliver small interfering ribonucleic acid (siRNA) to treat a rare, peripheral nerve disease.[3] This genetic disorder is characterized by the buildup of amyloid proteins in peripheral nerves, the heart, and other organs. ONPATTRO™ encases siRNA into a lipid nanoparticle, therefore allowing therapeutic levels of siRNA to be delivered to hepatocytes.[3] Once it reaches the hepatocyte the siRNA is able to interfere with protein production, thereby reducing the accumulation of amyloid deposits.[3]

Despite its recent success, several obstacles still stand in the way of the application of non-viral gene delivery to all diseases. One of the most significant barriers is delivering DNA to the nucleus, the site of DNA transcription that eventually leads to protein translation. Due to the large size of the non-viral delivery vector, only limited amounts of DNA can be actively transported into the nucleus. Consequently, DNA delivery and expression are often inefficient. Therefore, the focus of this thesis was to develop a non-viral delivery system that can deliver therapeutic levels of DNA to the nucleus.

CREATING THE NON-VIRAL DELIVERY VECTOR

Packaging DNA

DNA must be packaged into a nanoparticle in order to prevent metabolism and to promote entry into the hepatocyte. This process involves the chemical interaction between DNA and a peptide molecule. The structure of peptides offers several advantages when creating a DNA nanoparticle, including more precise and flexible chemical modifications, use in both *in vivo* and *in vitro* transfection, and a low molecular weight to avoid detection by the immune system.[4] Despite these advantages, the smaller size of peptides results in decreased DNA binding affinity.[4] Therefore, the peptide underwent chemical modification in order to restore binding affinity.

First, several cysteine residues were added onto the terminal ends of the peptide.[4] Then polyethylene glycol (PEG), an important polymer that aids in systemic delivery, was attached to one of these cysteine residues.[4] The DNA nanoparticle remained PEGylated in circulation until it reached the hepatocyte.[4] Once the nanoparticle entered the hepatocyte, the PEG layer dissociated and the nanoparticle could now better interact with the cell.[4]

Primary targeting

The positive charge of the DNA nanoparticle prevents its diffusion across the cellular membrane of the hepatocyte. Therefore, galactose residues were added onto the peptide in order to target the asialoglycoprotein receptor (ASGP-R) present on the hepatocyte.[4] Targeting the ASGP-R facilitated entry of the DNA nanoparticle into the hepatocyte.[4]

Secondary targeting

Upon entry into the hepatocyte, the DNA nanoparticle is immediately trafficked into the endosome before being delivered to the lysosome for metabolism.[4] To avoid this automatic degradation, histidine residues present on the nanoparticle acted to buffer the endosomal pH. This increased the acidity of the endosome, consequently encouraging the osmosis of water across the endosomal membrane.[4] The endosome started to swell and eventually burst, allowing the nanoparticle to undergo endosomal escape and be released back into the cytosol.[4]

Tertiary targeting

Nuclear pore complexes (NPCs) perforate the nuclear envelope, thereby determining which particles are allowed to pass between the cytosol and the nucleus. Due to the large size of the DNA nanoparticle, NPCs prevent its entry from the cytosol into the nucleus.[4] Therefore, a nuclear localizing sequence (NLS) was installed onto the nanoparticle to encourage interaction with the NPCs.[4] This allowed the nanoparticle to enter the nucleus and carry out DNA transcription.

CHAPTER 1: USE OF CLOFIBRATE TO BYPASS NUCLEAR TARGETING

Introduction

Clofibrate is a commercially available drug that was intended for use in humans to control high cholesterol and triacylglyeride levels.[5] However, a study in 1965 revealed that clofibrate caused hepatomegaly in rodents and the drug was withdrawn from the market.[6] Over the next several years, numerous clinical trials were done to investigate differences in response between rodents and primates. These studies found that primates have less peroxisome proliferation compared to rodents and hepatomegaly is consequently not observed.[7]

Previous experiments in our lab have investigated the use of clofibrate in non-viral delivery systems.[8] Because of its mitogenic effects, clofibrate could potentially be used to bypass the need for nuclear targeting. Diffusion of the DNA nanoparticle through dividing hepatocytes would allow for re-formation of the nuclear envelope around the nanoparticle, essentially delivering the genetic material into the nucleus without having to install nuclear targeting sequences onto the peptide. These experiments showed a significant increase in gene expression when using non-targeted DNA nanoparticles.[8] However, no significant increase was observed with primary-targeted DNA nanoparticles.[8]

We decided to replicate this experiment, with the possibility of seeing more gene expression by using targeted DNA nanoparticles that now contained additional secondary and tertiary targeting. We hypothesized that these fully targeted DNA nanoparticles will exhibit increased gene expression in hepatocytes compared to non-targeted DNA nanoparticles.

Materials and Methods

Animal model. Twelve, 20 g ICR male mice were purchased from Envigo Labs. The mice were assigned to one of four treatment groups, with each group containing three mice (n = 3) that were dosed with the designated treatment protocol (Figure 1-1).

Dosing clofibrate. Clofibrate (Sigma-Aldrich, St. Louis, MO), with a density of 1.14 mg/μl, was dissolved in corn oil (Sigma-Aldrich, St. Louis, MO) prior to dosing. Mice were weighed and dosed at 500 mg/kg in a 200 μl intraperitoneal (IP) injection of corn oil with or without the presence of clofibrate. Mice were dosed daily for five days. Mice were weighed every day.

Dosing the DNA nanoparticle. Previously synthesized non-targeted and targeted peptides were obtained from the lab. Non-targeted peptides lacked targeting specific for hepatocytes. Targeted peptides contained primary, secondary, and tertiary targeting for hepatocytes. In order to form the DNA nanoparticle, 0.4 nmol of the peptide underwent heat shrinking at 100°C for 10 minutes before being complexed with 10 μg CMV promoted luciferase plasmid DNA (gWiz Luc) obtained from Alta Biotech Labs. Only mice from Treatment Group III and Treatment Group IV were dosed with the non-targeted or targeted DNA nanoparticle on Day 4 via intravenous (IV) injection of their tail vein.

Treatment Group	Dosing Protocol
Group I	1. Corn oil IP injection
Group II	1. Clofibrate in corn oil IP injection
Group III	1. Clofibrate in corn oil IP injection 2. Non-targeted DNA nanoparticle IV injection
Group IV	1. Clofibrate in corn oil IP injection 2. Targeted DNA nanoparticle IV injection

Figure 1-1. Treatment protocols of each treatment group used in the experiment. Corn oil with or without the presence of clofibrate was dosed intraperitoneally (IP). DNA nanoparticles were dosed intravenously (IV). Each treatment group contained three mice (n = 3).

Measuring gene expression. Mice were imaged for bioluminescence activity on Day 5 and Day 8 to determine gene expression from non-targeted and targeted DNA nanoparticles. Mice were sacrificed on Day 8 and their livers were weighed to confirm mitogen activity in the mice that received clofibrate. Statistically significance was determined by t-test or one-way analysis of variance (ANOVA) with Dunnet's Multiple Comparisons Test on GraphPad Prism. A p-value $\leq$ 0.05 between treatment groups was to be considered statistically significant.

Results

Body weight gain. No significant difference in body weight gain of the mice was seen across treatment groups over the course of the 5-day dosing period (Figure 1-2). In the previously performed experiments in our lab, clofibrate also did not significantly affect the overall weight of the mice.[8]

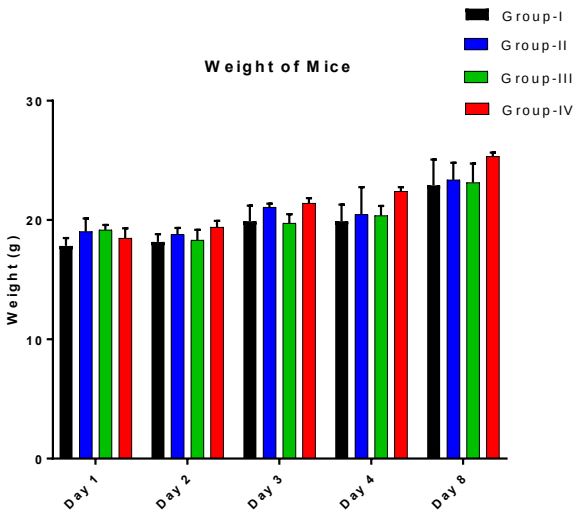

Figure 1-2. Mice were weighed every day and the average body weight was obtained from each treatment group (n = 3). No significant difference in body weight was observed across treatment groups after GraphPad Prism ANOVA analysis (p = 0.8791 for G-I vs. G-II; p = 0.9817 for G-I vs. G-III; p = 0.4834 for G-I vs. G-IV).

Liver weight gain. No significant difference in liver weight gain of the mice was seen across treatment groups (Figure 1-3). Given the mitogenic effects of clofibrate, mice were expected to exhibit hepatomegaly over the course of the dosing period.[8]

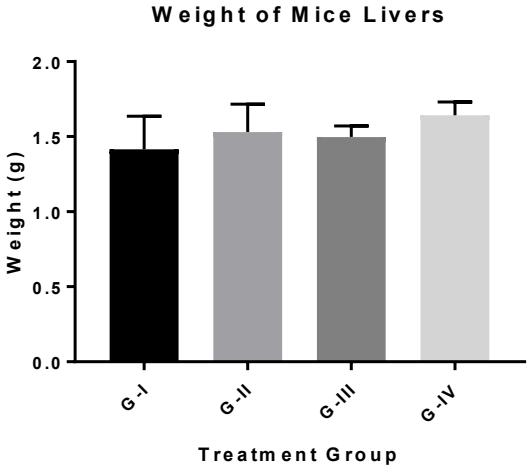

Figure 1-3. Mice were sacrificed and livers were surgically removed and weighed. Liver weights were obtained from all mice and the average liver weight was determined from each treatment group (n = 3). Groups II, III, and IV were dosed with the liver mitogen, clofibrate. No significant difference in liver weight was seen across treatment groups (p = 0.7031 for G-I vs. G-II; p = 0.8555 for G-I vs. G-III; p = 0.2469 for G-I vs. G-IV).

Despite no significant difference in liver weight gain, visual inspection of the livers did reveal hepatomegaly in the mice that received clofibrate compared to corn oil only (Figure 1-4). Previous experiments also indicated a difference in liver appearance upon visual inspection.[8]

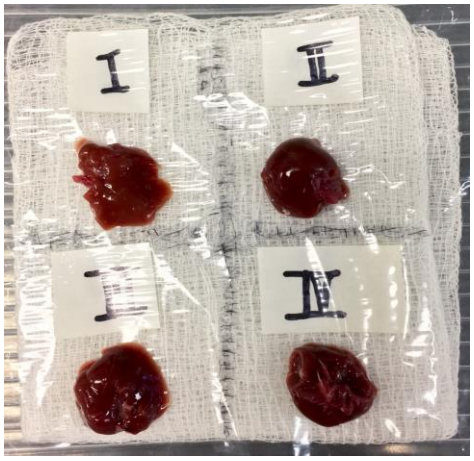

Figure 1-4. Livers of mice were inspected for differences in physical appearance across treatment groups. Group I received corn oil and Groups II, III, and IV received clofibrate.

Gene expression. Compared to the corn oil only control group, mice that received the non-targeted DNA nanoparticle dosed in clofibrate had a 10-fold increase in luciferase gene expression

(Figure 1-5). This result was statistically significant. However, as seen in previous experiments, no significant different was seen in gene expression across the remaining experimental groups.[8]

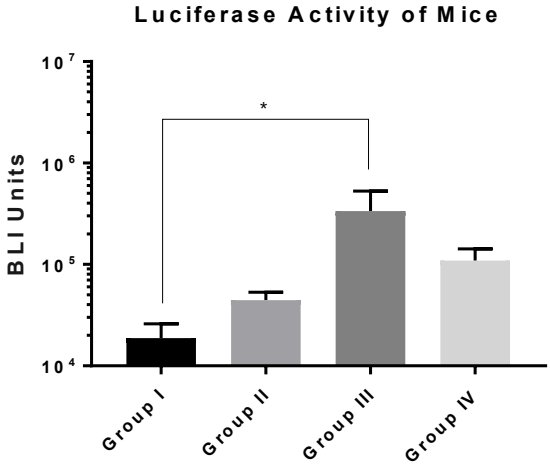

Figure 1-5. Group III mice (clofibrate + non-targeted DNA) had a statistically significant increase in luciferase gene expression compared to Group I mice (corn oil only) ($p = 0.9763$ for G-I vs. G-II; $p = 0.0108$ for G-I vs. G-III; $p = 0.5620$ for G-I vs. G-IV).

Discussion

This experiment was done to reproduce results from a previous experiment done in the lab that suggested the use of clofibrate to bypass the need for nuclear targeting in non-viral delivery systems.[8] Because previous results counterintuitively showed a significant increase in gene expression from non-targeted DNA nanoparticles, but not targeted DNA nanoparticles, we hypothesized that gene expression would increase with DNA nanoparticles that contained additional targeting for mice hepatocytes.

Over the course of the five-day dosing period, no significant body weight gain was seen across the treatment groups. This was expected because clofibrate only induces mitogenic activity in the liver, rather than all cells within the body.[6] In addition, no significant body weight gain was observed in the previously performed clofibrate experiments in our lab.[8]

No significant liver weight gain was seen across treatment groups that were dosed with clofibrate. Since clofibrate is a liver mitogen, we expected to observe significant liver weight gain in the mice that received clofibrate compared to the mice that received corn oil only.[5] A dosing protocol of 500 mg/kg has been shown to increase liver weight gain in experiments done in our lab as well as across literature data investigating the effects of clofibrate on liver metabolism.[8] Although there was no significant weight gain, visual inspection of the livers from all clofibrate-treatment groups appeared to exhibit hepatomegaly.

Despite the addition of more targeting, targeted DNA nanoparticles did not show a significant increase in luciferase gene expression. Instead, only non-targeted DNA nanoparticles exhibited a statistically significant increase, with a 10-fold increase in gene expression in the non-targeted group compared to the control group. This indicates that more DNA was delivered to the nuclei of hepatocytes in the presence of clofibrate when using non-targeted DNA nanoparticles compared to targeted DNA nanoparticles. Increased gene expression from non-targeted DNA

nanoparticles compared to targeted DNA experiments was also observed in previous experiments in our lab.[8]

A significant limitation to this experiment was the design of the targeted DNA nanoparticle. We have found that these targeted peptides, despite the installation of additional targeting, do not bind well to DNA during nanoparticle formation. Therefore, due to its bulky structure, limited amounts of the DNA nanoparticle are able to enter the nuclei of hepatocytes. Further experiments are currently being done to improve DNA binding efficiency to these targeted peptides.

In conclusion, non-targeted DNA nanoparticles exhibited significantly more gene expression compared to targeted DNA nanoparticles in mice hepatocytes when dosed in the presence of clofibrate. Despite increased gene expression, there was no significant increase in mice liver weight over the course of the experiment. Considering gene expression was higher in non-targeted DNA nanoparticles, further experiments should be performed to determine if non-targeted nanoparticles exhibit a dose-dependent response in hepatocyte gene expression when in the presence of clofibrate.

CHAPTER 2: DOSE-DEPENDENT RESPONSIVENESS OF NON-TARGETED DNA NANOPARTICLES IN THE PRESENCE OF CLOFIBRATE

Introduction

Recent experiments on the use of clofibrate to bypass the need for nuclear targeting found that luciferase gene expression was greater in non-targeted DNA nanoparticles. Therefore, we wanted to determine if these nanoparticles were able to exhibit a dose-dependent response in gene delivery to hepatocytes. If a dose-dependent response is observed, this could support the use of non-targeted DNA nanoparticles in non-viral gene delivery systems.

In addition, no significant increase in liver weight gain was observed in mice dosed with clofibrate. Since the literature typically utilizes a 14-day dosing protocol to induce hepatomegaly in rodents, we decided to modify our original clofibrate dosing protocol for this experiment.[9]

Due to non-targeted DNA nanoparticles exhibiting increased gene expression, we hypothesized that a dose-dependent response would be seen in mice hepatocytes. In addition, we hypothesized that when dosed with clofibrate over a 14-day protocol, a significant weight gain would be observed in the livers of mice.

Materials and Methods

Animal mode. Nine, 20 g ICR male mice were purchased from Envigo Labs. The mice were assigned to one of three treatment groups. Each group contained three mice (n = 3) that were dosed with the designated treatment protocol (Figure 2-1).

Dosing clofibrate. Clofibrate (Sigma-Aldrich, St. Louis, MO), with a density of 1.14 mg/μl, was dissolved in corn oil (Sigma-Aldrich, St. Louis, MO) prior to dosing. Mice were weighed and all mice were dosed at 500 mg/kg in a 200 μl intraperitoneal (IP) injection of corn oil with clofibrate. Mice were dosed daily for 14 days. Mice were weighed every day.

Dosing the DNA nanoparticle. Previously synthesized non-targeted peptides were obtained from the lab. The peptides were to be complexed with either 10 μg or 100 μg gWizLuc (Figure 2-1). First, gWizLuc underwent heat shrinking at 100°C for 10 minutes. Then, 0.4 nmol of non-targeted peptide was added to the heated plasmid, forming the DNA nanoparticle. All treatment groups were dosed with clofibrate. Only Treatment Group II and Treatment Group III were dosed with the non-targeted DNA nanoparticle on Day 12 via intravenous (IV) injection of their tail vein.

Treatment Group	**Dosing Protocol**
Group I	1. Clofibrate in corn oil IP injection
Group II	1. Clofibrate in corn oil IP injection 2. Non-targeted DNA nanoparticle containing 10 ug gWiz Luc IV injection
Group III	1. Clofibrate in corn oil IP injection 2. Non-targeted DNA nanoparticle containing 100 ug gWiz Luc IV injection

Figure 2-1. Treatment protocols of each treatment group used in the experiment. Corn oil in clofibrate was dosed intraperitoneally (IP). DNA nanoparticles were dosed intravenously (IV). Each treatment group contained three mice (n = 3).

Measuring gene expression. Mice were imaged for bioluminescence activity on Day 14 to determine gene expression. Mice were sacrificed and their livers were weighed to confirm mitogen

activity of hepatocytes via visual inspection. Statistically significant differences were determined by t-test or one-way analysis of variance (ANOVA) with Dunnet's Multiple Comparisons Test on GraphPad Prism. A p-value $\leq$ 0.05 between treatment groups was to be considered statistically significant.

Results

Body weight gain. No significant difference in body weight gain was seen across treatment groups over the course of the 14-day dosing period (Figure 2-2). Previously performed experiments also did not see a significant increase in weight gain.[8]

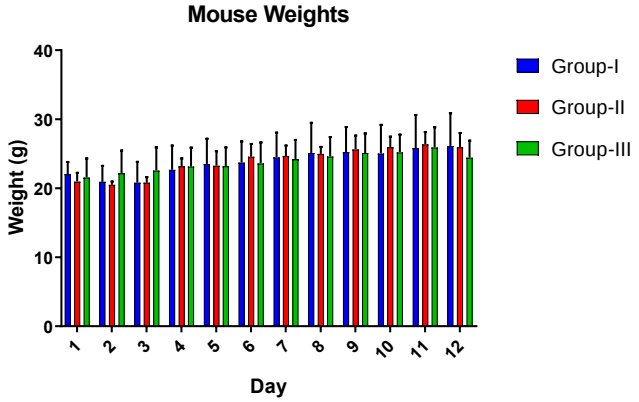

Figure 2-2. Mice were weighed every day and the average body weight was obtained from each treatment group (n = 3). No significant changes in body weight was observed after GraphPad Prism ANOVA analysis (p = 0.9833 for G-I vs. G-II; p = 0.9996 for G-I vs. G-III).

Liver weight gain. No significant difference in liver weight gain was seen across treatment groups (Figure 2-3). Mice were expected to have an increase in liver weight over duration of the longer dosing period based on literature data.[9]

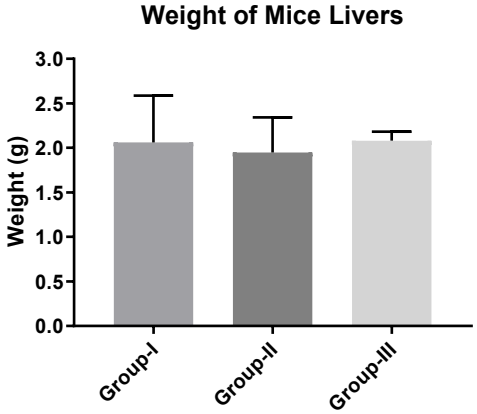

Figure 2-3. Mice were sacrificed and livers were surgically removed and weighed. Liver weights were obtained from all mice and the average liver weight was determined from each treatment group (n = 3). All treatment groups received the liver mitogen, clofibrate. No significant difference in liver weight was seen across treatment groups (p = 0.9089 from G-I vs. G-II; p = 0.9976 for G-I vs. G-III).

Despite no significant difference in weight gain, visual inspection of the livers did reveal hepatomegaly in all mice (Figure 2-4). Appearance of hepatomegaly from visual inspection is consistently seen across previous experiments performed in our lab.[8]

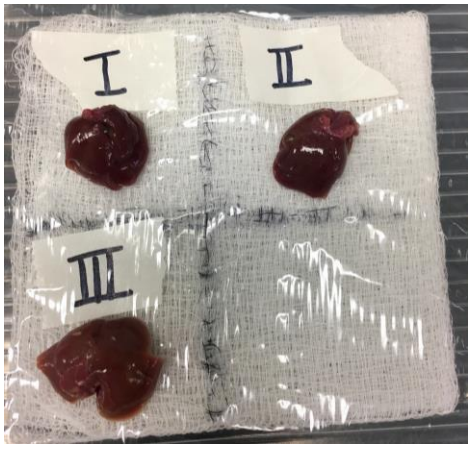

Figure 2-4. Livers of mice were inspected for differences in physical appearance across treatment groups. Groups I, II, and III all received the clofibrate dissolved in corn oil for a duration of 14 days.

Gene Expression. No significant difference in luciferase gene expression was observed across all treatment groups (Figure 2-5). This result contradicts previous experiments, where mice that received non-targeted DNA nanoparticles (Groups II and III) displayed a significant increase in gene expression compared to the control group (Group I).[8]

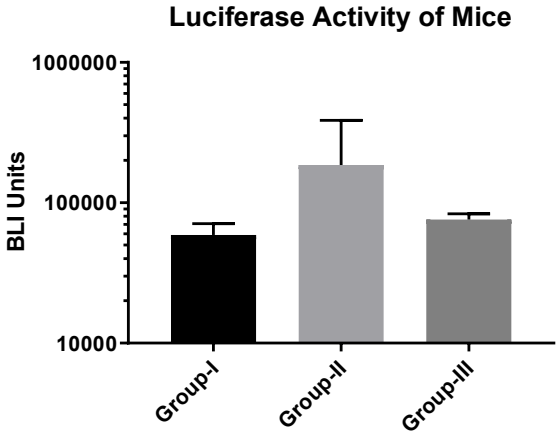

Figure 2-5. Group I treatment group mice received clofibrate only. Groups II and III treatment group mice received 10 μg and 100 μg respectively of gWiz Luc plasmid complexed to non-targeted DNA nanoparticles. No statistically significant difference in gene expression was seen across treatment groups ($p = 0.3672$ for G-I vs. G-II; $p = 0.9771$ for G-I vs. G-III).

Discussion

This experiment was done to determine if non-targeted DNA nanoparticles displayed a dose-dependent response in luciferase gene expression. If a dose-dependent response was seen,

non-targeted DNA nanoparticles could potentially be implemented in non-viral delivery of DNA to the hepatocytes of mice.

No significant increase in the body weight of the mice was observed across treatment groups. This result is supported by previous experiments as well.[6,8]

No significant increase in liver weight was seen in the mice, however visual inspection of the livers did reveal apparent hepatomegaly. With a 500 mg/kg dosing protocol over the duration of 14 days, we expected to see an increase in liver weight based on literature data.[6,8]

Despite previous experiments showing increased gene expression from non-targeted DNA nanoparticles, no significant increase in expression was seen in this experiment.[8] This contradicted what was expected, because the Group II mice received the same 10 μg dose of gWizLuc shown in previous experiments to increase gene expression.[8] In addition, the Group III mice received a higher, 100 μg dose of gWizLuc but also displayed no significant increase in expression. Therefore, due to the lack of a dose-dependent response, non-targeted DNA nanoparticles cannot be relied upon to induce consistent increases in gene expression from mice hepatocytes dosed with clofibrate.

One limitation was in the experimental modification that had to be used. Given the small size of mice, the IV dose of the DNA nanoparticle must be limited to 200 μl in total volume. Therefore, less buffer and water had to be used than usual for a 100 μg dose of DNA. As a result, this may have affected the structure of the DNA as well as the ability of DNA to bind to the peptide when forming the non-targeted DNA nanoparticle. Another possible limitation was due to the mitogenic effects of clofibrate on the mice hepatocytes. Since no significant increase in liver weight was seen consistently across both experiments, it is possible that not enough clofibrate was delivered to hepatocytes in order to induce mitogenic activity. This would have affected liver weight gain as well as limited the ability of the DNA nanoparticle to enter the nucleus of dividing hepatocytes.

In conclusion, because non-targeted DNA nanoparticles lack a dose-dependent response, it cannot be determined that clofibrate can be used to bypass the need for nuclear targeting in non-viral gene delivery systems. Therefore, it was decided that the use of clofibrate would not be further explored in the development of a non-viral gene delivery system.

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