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Stopping Parkinson's Disease with Phenylbutyrate: The Science, the History, and the Miles Left to FDA Approval

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Stopping Parkinson's disease with phenylbutyrate: The science, the history, and the miles left to FDA approval

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by

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The first gulp from the glass of natural sciences will make you an atheist,

but at the bottom of the glass, God is waiting for you.

−Werner Heisenberg, father of quantum physics

Beyond Textbook: An Introduction

Of the most important things I've learned at Regis, science came second. Science, in its beautiful complexity, was straightforward. For a nine-year-old just learning English, science was a language that I understood just as well as the other English-speakers. I did not come from a background that prepped me for reading, writing, or American history, but none of that mattered when I was conducting fourth-grade science experiments. I welcomed the sense of community I felt when I was immersed in science, and for the next ten years of my life, I made science my home.

Nevertheless, like science, we all must evolve. In my elementary understanding of it, I saw science as a dissection of the world, a systematic way to break down, analyze, and define my surroundings. If science was a brick, I wanted to take my microscope and probe every detail of it. I was ready to commit the rest of my life to that one brick—to study it, to test it, to carve my initials onto every surface of it.

In my four years here at Regis, I spent a lot of time studying bricks, both metaphorical and physical. When I was building houses in Pinellas County on a Habitat for Humanity trip last spring, I spent an entire week adding bricks to the exterior walls of half-finished houses. The bricks came in different shades of beige, cream, and brown, and it was up to our job to cement the slabs together to create a sturdy, reinforced wall. Alone, the bricks were not that useful, but when cemented together, they represented the boundaries of a small office, an outline of a private bedroom, or a framework of a future

dining area. Eventually, I learned that science was not much different from those concrete bricks in Pinellas County. Science, I came to accept, was a building block that helped support a larger, more important design.

As my understanding of science matured, I recognized its applications in economics, healthcare, politics, and public regulations. In turn, these factors feedback to affect the current scientific research process we know today. Science cannot and should not be isolated to a single brick, because its significance protrudes into boundaries well outside of the scientific community. It is no longer satisfactory to simply study science without regard to the context in which the science is applied, which is why, for this thesis, I will also be exploring the history and impacts of federal regulations on scientific research.

Science, while already a compelling field on its own, is exponentially more influential and intriguing when studied in context of a larger picture. This project, at its core, is an exploration of science and detail. For many reasons, the questions I'm exploring in this research needs to be asked. How does this Parkinson's disease-fighting drug work? What does the drug do? How does it fight Parkinson's disease? But even more importantly, we have to ask what this science means in the larger scope. How soon can the drug be used? What are the long-term side effects? And in what ways can it help people? Mirroring the way my education developed in my four years at Regis, this thesis will first explore the science behind the phenylbutyrate mechanism. Then, it will take a step back and put the science into the context of historical and current drug research policies, FDA regulations on drug development, and previous Parkinson's disease

research in hopes of identifying potential directions of the phenylbutyrate drug.

Like the Pinellas County brick, science belongs to a greater infrastructure. The brick is not meant to be broken down and studied on its own; instead, it should serve as a building block. I have learned engage with science, to chisel it and recognize the ways it influences and builds the world. After years of narrowing my research in the laboratory, I'm left to ask myself in what ways I can expand—and that, is beyond textbook.

CHAPTER 1: The History

Parkinson's Disease Research

Parkinson's disease was first described by James Parkinson in 1817, who depicted the condition as a shaking palsy syndrome (Goetz, 2011). He wrote of a slowly progressing ailment that initially caused a "slight sense of weakness" in patients who had "a prone to trembling… in the hands or arms" that eventually developed into a complete "submission of the limbs" (Freed & Levay, 2002, p. 7-9). "As the disease proceed[ed] towards its last stage", he wrote, "the trunk is almost permanently bowed, the muscular power [was] more decidedly diminished, and the tremulous agitation becomes violent" (Freed & Levay, 2013, p. 7-9). Although Parkinson did not know what he was observing at the time, his strikingly accurate descriptions of the disease's symptoms became the iconic snapshot of Parkinson's disease for centuries to come.

Now, Parkinson's disease is known as one of the most common and devastating neurological disorders in the world. Currently, over 10 million people worldwide are living with Parkinson's disease and as many as 60 million Americans are newly diagnosed each year (Parkinson's Disease Foundation). The disease is a gradual, chronic, and progressive brain-degenerating condition characterized by uncontrollable tremors, stooped posture, and movement rigidity—archetypal symptoms that Parkinson depicted in his original writings (Fahn & Sulzer, 2004; Goetz, 2014). Sadly, the effects of Parkinson's disease are not limited to just motor impairments. Later stages of the disease

results in mental decline such as dementia, impaired language, and decreased levels of higher reasoning (Aarsland et al., 2004; Goetz, 2011).

Although James Parkinson's essay was not recognized at the time of its publication, Jean-Martin Charcot reintroduced Parkinson's disease in the 1870s after stumbling across James Parkinson's old notes. With this, Charcot ignited a fascination for the shaking palsy. Almost immediately following Charcot's publication, several researchers conducted separate long-term studies of Parkinson's disease patients, reporting symptoms of the disease in its progressive stages (Freed & Levay, 2002; Goetz, 2011). Soon after, scientists associated Parkinson's disease with damages in the substantia nigra and the midbrain. Then, in 1960, Austrian scientist Oleh Hornykiewicz began measuring dopamine levels in postmortem Parkinson's disease patients. He published an article six years later detailing the correlation between dopamine depletion in the substantia nigra and the motor deficits observed in Parkinson's disease (Fahn, 2008). From this moment forward, Parkinson's disease and dopamine deficiency became irreversibly linked.

We now know that Parkinson's disease is induced by the death of dopamine-releasing neurons in the substantia nigra, a region in the midbrain that plays an important role in movement control and fine motor coordination. When the chemical dopamine is released, it relays motor commands from the substantia nigra to the rest of the body. Without the message relay via dopamine, the body lacks the ability to inhibit inappropriate movements or initiate correct movements (Olanow, 2015). But despite the well-known importance of dopamine in motor control, the exact cause of the dopamine-

releasing cell death was—and is—still unclear. However, by simply mapping the dopamine motor control pathway, Hornykiewicz introduced the possibility of a cure. He launched the world on a mission to find out why dopamine neurons were dying and what can be done to prevent such destructive cell death (Fahn, 2008; Freed & Levay, 2002; Goetz 2011).

Up until Hornykiewicz, most of the pioneering research in Parkinson's disease had been exploratory. However, as Parkinson's disease research shifted towards a pharmacological path, researchers had to follow a different set of research regulations. During the time scientists were studying the long-term effects and clinical presentations of Parkinson's disease in the late 1800s and early 1900s, both the United Kingdom and the United States began reforming their ethical standards for drug research (Rägo & Santoso, 2008). With the new revolutions in medicine and technology in the 1900s, there grew a need for government intervention to ensure public safety as new drugs entered the market in large quantities. The United Kingdom introduced the Committee of Drug Safety in 1963, and following that, a public drug adverse reaction reporting system. The United States, similarly, passed the Drug Amendments Act of 1962, stating that the government had to approve all new drugs entering the market (Rägo & Santoso, 2008). In terms of Parkinson's disease research, this created restrictions for scientists who were looking for a cure to Parkinson's disease. Not only was Parkinson's disease research redirecting its focus for the first time towards treatment research, it was doing so under newly formed drug research guidelines.

During the time that these drug research regulations were forming, Hornykiewicz took his research into his own hands. After mapping out the dopamine motor pathway, Hornykiewicz speculated that replacing the brain's depleted dopamine reserve could alleviate the motor symptoms of Parkinson's disease (Freed & Levay, 2002). Unfortunately, the dopamine molecule itself was too big to enter the brain, so dopamine had to be replenished indirectly. Hornykiewicz theorized that its precursor, the smaller dihidroxyphenylalanine, or D,L-DOPA, was small enough to cross the blood-brain barrier. It then could be converted into dopamine once it reached the brain by the body's natural enzymes (Fahn, 2008; Freed & Levay, 2002; Goetz 2011). In 1961, two years shy of the United Kingdom's introduction of the Committee of Drug Safety, Hornykiewicz sent a vial of D,L-DOPA to his friend, Walther Birkmayer, a physician at a Viennese neurology facility, to test the effects of D,L-DOPA on the Parkinson's disease patients housed at Birkmayer's neurology facility (Fahn, 2008; Freed & Levay, 2002; Goetz 2011).

Without the scrutiny of government drug research regulations, Birkmayer administered D,L-DOPA to his late-stage Parkinson's disease patients, taking advantage of their incapacitated states to test the effects of Hornykiewicz' mystery drug. By today's standards, this would have been a clear violation of research's ethical code of conduct. Birkmayer did not know the drug's side effects, did not perform safety checks on the drug, and did not test the drug on non-human models beforehand, but because those restrictions did not exist in 1961, neither Hornykiewicz or Birkmayer were held liable for their questionable research methods. But regardless of Birkmayer and Hornykiewicz'

controversial protocols, their results changed the world. D,L-DOPA seemed to cure even the most severe cases of Parkinson's disease, pulling bedridden patients back onto their feet once more (Freed & Levay, 2002; Goetz 2011). Perhaps a cure was possible after all.

However, the drug was not perfect. After Birkmayer and Hornykiewicz published their findings, D,L-DOPA went under high levels of scientific scrutiny, partly influenced by the increasing standards of drug research and drug quality in the public market. For one, the right-hand versions of DOPA was toxic and produced horrible side effects, and only L hand DOPA, or L-DOPA, was actually helpful in alleviating Parkinsonian symptoms. Furthermore, subsequen studies showed that high doses of L-DOPA were needed before enough of the drug reached the brain to effectively treat Parkinsonian motor symptoms. Unfortunately, these high doses left large concentrations of the drug circulating inside the body, causing side effects such as nausea, vomiting, GI disturbances, dyskinesia, rashes, etc. (Fahn, 1999; Goetz, 2011). In response to this, later versions of L-DOPA treatments were mixed with carbidopa, a dopa decarboxylase inhibitor, to delay the conversion of L-DOPA to dopamine until after L-DOPA passed the blood brain barrier and increase the percentage of the drug that reached the brain (Fahn, 1999; Goetz, 2011).

Even after the Carbidopa/L-DOPA combination (commonly marketed as Sinemet), the drug was not still not foolproof, continuing to produce side effects with prolonged use. In the U.S., the first recorded review of Sinemet by the FDA was in 1977, but since its introduction, Sinemet's dosing, labeling, manufacturing has been adjusted over 50 times with the last FDA-published revision in 2015 (U.S. Food &

Administration, 2017). To this day, the carbidopa/L-DOPA combination continues to be frequently revised for better drug efficacy. Unfortunately, all research thus far seems to suggest that L-DOPA therapy is not a permanent fix for Parkinson's disease (Fahn, 1999; U.S. Food & Administration, 2017).

Over the years, the climate of Parkinson's disease research evolved. For one, as Parkinson's disease research evolved, so did government standards for conducting research, impacting the way Parkinson's disease research can be carried out, especially in the United States. Secondly, there was a push to move away from L-DOPA, since it became widely known as merely a temporary solution for an irreversibly progressive problem. Instead, research began exploring gene therapy, misfolded protein targets, and alternative drugs as potentially better treatments for Parkinson's disease. My research, for example, investigates an alternative drug called phenylbutyrate that could prevent the onset and progression of Parkinson's disease in the brain. To further explore this search for the cure, the next chapter of this thesis is a research paper that will highlight the process of new drug proposals, drug testing, and mapping of drug mechanisms to determine drug efficacy. Together, as research and research regulations developed side by side, it shaped the search to find a better cure for Parkinson's disease.

II. CHAPTER 2: The Science

Does TAF1 activity regulate DJ-1 gene production in response to phenylbutyrate treatment?

Abstract

Parkinson's disease is a disruptive brain-degenerating disease caused by aging, abnormal protein build-up in the brain, and genetic predisposition. While the drug L-DOPA is currently the most effective treatment for Parkinson's disease symptoms, long-term use of the drug creates adverse effects. One alternative solution is to treat Parkinson's disease at the genetic level. For example, malfunctions in the gene PARK7, or DJ-1, are associated with early-onset Parkinson's disease. On the other hand, enhancing production of the DJ-1 gene is protective against the development of Parkinson's disease. In 2011, Zhou and colleagues found that treatment with a drug called phenylbutyrate increases DJ-1 gene production in neurons, thus protecting neurons from the onset of Parkinson's by manipulating the protective genetic material already present in the normal cells. While the mechanism which the drug phenylbutyrate acts upon DJ-1 gene production remains unclear, previous research in the Freed lab shows that the Sp1 transcription factor, a protein on the DJ-1 promoter that initiates DJ-1 gene production when bound, activates in response to phenylbutyrate treatment. This protein activates through a method that adds an acetyl group to the Sp1 transcription factor, changing the transcription factor's structure by switching it "on". We believe that TAF1, a protein previously known to add acetyl groups to various other transcription factors, is

involved. To illustrate the importance of TAF1 activity in Sp1 activation, we treated cells with the drug phenylbutyrate and compared the response of cells without the TAF1 proteins to normal cells with TAF1 proteins intact. However, there was no significant differences between TAF1-intact and TAF1-deficient cells.

Research Introduction

Currently, the most effective symptomatic treatment for Parkinson's disease is L-DOPA, a drug that is converted to dopamine in the brain to substitute for the dopamine loss that causes Parkinson's disease. However, L-DOPA is not effective in long-term use as the body builds tolerance to increasing levels of the drug and because L-DOPA works by increasing dopamine production in existing dopamine neurons; as the dopamine neurons degenerate through the course of Parkinson's disease, there are not enough dopamine neurons left for L-DOPA to target (Olanow, 2014). Additionally, studies suggest that levodopa has toxic effects after prolonged exposure, prompting researchers to pursue novel treatments for Parkinson's disease (Lesser et al., 1979).

One novel treatment approach is to manipulate the genetic components of the disease. Although the exact cause of dopamine cell death is still unclear, there are genetic components that predispose the onset of Parkinson's disease (Fahn & Sulzer, 2004; Olanow, 2015). Severe cases of Parkinson's disease are associated with many factors, including mutations in the PARK genes that cause the genes to stop working properly (Lücking et al., 2000; Mullin & Schapira, 2015). Particularly, mutations that damage the PARK7 gene, or the DJ-1 gene, are linked to early-onset, autosomal recessive forms of

Parkinson's disease. On the other hand, increasing production of the DJ-1 gene has protective effects against the development of Parkinson's disease (Inden et al., 2006). When neurons are exposed to a toxic chemical called 6-hydroxydopamine, a substance designed to mimic brain deterioration in Parkinson's disease by selectively killing dopamine neurons, it stimulates DJ-1 production, which is able to increase the body's natural defenses to preserve and save the brain cells (Zhou & Freed, 2005).

If we can increase concentrations of the DJ-1 gene in a Parkinson's disease patient, we potentially prevent the progression of the disease. Phenylbutyrate naturally boosts DJ-1 production. Phenylbutyrate increases DJ-1 by 300% in cultured neurons after 48 hours of treatment and almost double in mice models after 3 months of treatment (Zhou et al, 2011). In doing so, phenylbutyrate enhances DJ-1 protective properties, preventing dopamine neurons from dying even after exposure to toxic levels of oxidative stress.

While phenylbutyrate is a promising drug for increasing cellular DJ-1 levels and for preventing the onset of Parkinson's disease, the mechanism which phenylbutyrate increases the DJ-1 gene production still remains unclear. My previous research in the Freed lab with dopamine neurons suggest that phenylbutyrate treatment activates the Sp1 transcription factor, a protein that binds to the DJ-1 promoter to initiate the protein synthesis in the cell (Freed, 2016). Sp1 transcription factor activation occurs when Sp1 is acetylated, or when an acetyl group is added to Sp1 to change the transcription factor's shape and turn it on. I was able to show that as the cells were treated with increasing phenylbutyrate concentrations, Sp1 acetylation increased correspondingly (Freed, 2016).

However, the rate of increased Sp1 transcription factor acetylation did not directly correlate with the concentrations of phenylbutyrate used, suggesting that there are intermediate steps in the mechanism. I believe that in one intermediate step, phenylbutyrate is attaching an acetyl group to the Sp1 transcription factor inside the cell. Recent studies show that TAF1 proteins assist Sp1 transcription factor acetylation and activation in cyclin D1 promoters of hamster cells. The TAF1 protein transfers acetyl groups from a donating molecule and attaches it to the Sp1 transcription factor, changing its shape and launching it into action. Moreover, in the absence of the TAF1 protein, Sp1 transcription factor acetylation does not take place and gene production halts as the cyclin D1 promoter is not activated (Hilton & Wang, 2003; Hilton, Li, Dunphy, & Wang, 2005). Sp1 acetylation activated cyclin D promoter activity, but this mechanism is not necessarily universal to the DJ-1 gene promoter since every promoter is unique to the gene. Furthermore, Hilton, Li, Dunphy and Wang did not test the effects of Sp1 acetylation in response to the phenylbutyrate drug.

My thesis project expands on the findings of Hilton, Li, Dunphy, and Wang (2005), applying their TAF1 Sp1 transcription factor acetylation model to the context of DJ-1 gene production in response to phenylbutyrate. While their project largely investigated cellular reproduction as a whole, my project will specifically compare the levels of DJ-1 gene production in wild-type cells with intact TAF1 activity and mutant cells without the TAF1 activity, both before and after phenylbutyrate treatments. I want to isolate the effects of the phenylbutyrate drug on TAF1-dependent Sp1 acetylation and clarify the unknown steps of phenylbutyrate's mechanism from my previous research.

Additionally, in the past, I studied the Sp1 acetylation in response to phenylbutyrate in dopamine neurons. In Hilton, Li, Dunphy, and Wang's experiments and in this thesis project, I will use temperature-sensitive mutant cells, called ΔSTU, that retained normal TAF1 activity at low temperatures but turned off TAF1 activity at high temperature.

I hypothesize that at low temperatures, because both the normal wild type and the mutant cells would be functioning regularly, phenylbutyrate will affect both the normal wild type and the mutant cells in the same way and increase DJ-1 gene production in both cell types. However, I predict that, at higher temperatures, phenylbutyrate will increase DJ-1 gene production in the normal wild-type cells, but not the mutant cells because the TAF1 activity in the mutant cells will have ceased under these conditions. Ideally, this project will illustrate the importance of TAF1 activity in Sp1 activation and DJ-1 gene production such that, without TAF1 activity, Sp1 activation and DJ-1 gene production will discontinue. My results will either reaffirm my proposition that phenylbutyrate acetylates the Sp1 promoter to increase DJ-1 gene production or offer a chance for us to readdress the mechanism on which phenylbutyrate acts on DJ-1 with a different approach.

Method

Culturing wild-type ts13 cells and mutant TAF1-deficient ts13 cells

Wild-type ts13 cells, or temperature-sensitive baby hamster kidney cells, exhibit late-stage cellular arrest at 39.5 °C such that the cells stop producing the materials necessary for continued growth and multiplication (Hilton, Li, Dunphy, & Wang, 2005). Additionally, mutants of the ts13 cells, ΔSTU ts13, turn off the TAF-1 genes above 37.5

°C. Essentially, at these temperatures, these cells are unable to make TAF-1 proteins and therefore cannot assist in acetylation activities. However, at a lower temperature of 33.5 °C, TAF-1 protein functions are restored (Hilton, Li, Dunphy, & Wang, 2005).

Both wild-type and mutant cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with L-glutamate, which has high concentrations of amino acids and vitamins to stimulate cell growth (Sigma Aldrich, 2016). Following the Wang lab's protocol for optimal ts13 cell culture, we additionally added in 10% Hyclone Fetal Clone III as supplements for cell growth, 1% penicillin/streptomycin to reduce chances of bacterial contamination, and 2mM of L-glutamine with 200 µg/mL of G418 for added amino acid supplementation to increase the rates of cell growth (Thermo Scientific, 2016). Both cell lines were incubated in 100 mm plates at 33.5 °C, 37.5 °C, and 39.5 °C for 72 hours before treatment.

Phenylbutyrate treatment of wild-type and mutant ts13 cells

Wild-type and TAF-1 deficient mutant ΔSTU ts13 cells were treated with 150 µM of phenylbutyrate for 96 hours. 150-300 µM of phenylbutyrate most effectively increases DJ-1 production in rat dopamine neurons (Zhou et al., 2011). There are no reports of DJ-1 expression in temperature-sensitive baby hamster kidney cells.

Protein extraction and Western blotting

After the wild-type and mutant ts13 cells were cultured and treated, they were scraped from culture plates, washed, and lysed using a buffer made of 50 mM Tris-Cl at pH 7.4, 10 mM NaCl, and 1% Tris, which induces rupture of the cell membrane for protein extraction. We additionally added a protease inhibitor tablet to protect the

proteins from degrading once they were removed from the cell (Thermo Scientific, 2016). Proteins were kept on ice during the extraction and were stored at -80˚C afterwards.

We ran the proteins through an electric current and separated them by atomic molecular weight. We separated 40 µg of cellular protein on a 15-well 10% Mini Protean TGX gel. Proteins were then transferred to a nitrocellulose 0.45 µm membrane, a special paper that readily catches and holds on to protein. The membranes were incubated with rabbit DJ-1 antibody for a minimum of 12 hours. We added rabbit β-actin antibody as a loading control. Membranes were incubated with donkey anti-rabbit secondary antibodies for another 24 hours to amplify the first antibody signal.

Western blotting quantification and statistical analyses.

Western blots were scanned and quantified using ImageJ software. DJ-1 protein densities were normalized to the β-actin protein densities per sample to reduce background noise and protein loading errors. Each experiment was repeated at least 6 times. Outliers were calculated and removed using the Thompson tau technique. The data was analyzed using a 3-way ANOVA in SPSS for significance and Eta-squared (η^2) measurement for effect size. Significance was set at $p < 0.05$. Values are shown as mean ± SE (See Figure 1).

Results

The averages and standard deviations of the protein band densities of the different cells, different culture temperatures, and different treatments are present in Table 1. There was a significant main effect of temperature on DJ-1 protein densities ($F(2, 12) = 6.62$, $p > .01$, $\eta^2 = .17$) and there was a significant main effect of drug treatment on DJ-1

protein densities ($F(1, 12) = 4.40$, $p = .04$, $\eta^2 = .06$). However, there was no significant main effect of ts13 strain on the DJ-1 protein densities ($F(1, 12) = 0.54$, $p = .47$, $\eta^2 = .01$). Additionally, there was a significant interaction of ts13 strain and drug treatment on DJ-1 protein densities ($F(1, 12) = 5.21$, $p = .03$, $\eta^2 = .07$). There was no significant interaction of ts13 strain and temperature on DJ-1 protein densities ($F(2, 12) = 2.09$, $p = .132$, $\eta^2 = .06$) and no significant interaction of temperature and drug treatment on DJ-1 protein densities ($F(2, 12) = 0.47$, $p = .63$, $\eta^2 = .01$). Finally, there was no significant interaction of ts13 strain, temperature, and drug treatment on DJ-1 protein densities ($F(2, 12) = 0.241$, $p = .79$, $\eta^2 = .01$).

Discussion

I hypothesized that at low temperatures, wild type and mutant cells would be the same and phenylbutyrate would be able to increase DJ-1 production in both cell lines. I also hypothesized that at higher temperatures, phenylbutyrate would increase DJ-1 in wild-type cells but not the mutant cells. My results only partially supported these hypotheses. In agreement with Zhou et al. (2005) and my initial predictions, treatment with the drug phenylbutyrate increased DJ-1 concentrations. Also in agreement with the original hypothesis, DJ-1 protein densities were lower at 39.5 °C than at 33 °C and at 37.5 °C, suggesting that DJ-1 production may have been inhibited when TAF1 activity was turned off at high temperatures. Unfortunately, there was no main effect of cell type on DJ-1 concentrations, which indicates that cells with TAF1 activity and cells without TAF1 activity largely produced the same amount of DJ-1 protein densities.

Mutant ts13 cells reportedly turned off TAF1 activity without affecting the large

remainder of cellular functioning. However, because the mechanism of how phenylbutyrate is increasing DJ-1 production is largely unclear, there is no way to ensure that inhibition of ts13 cell processes at high temperatures are not turning off other critical components of the phenylbutyrate mechanism at this time. As a result, differences between wild type and mutant ts13 cells may not be reliable. Additionally, errors in Western blot handling left background noises and air bubbles in the protein transfer, creating splotchy lanes (see Figure 2). These interferences, although small when looking at the blot, can amount to large differences in protein densities when examined with sensitive detecting equipment like ImageJ. For future technical changes, I will repeat the experiment with more trials and larger groups to improve the reliability of the results and minimize the differences due to Western blotting technique.

In Wenbo and Freed's experiments in 2005, DJ-1 production and its protective effects were stimulated when dopamine brain cells were stressed or exposed to toxic chemicals that threatened to kill the cell. In these experiments, the ts13 cells were not stressed because I originally thought that changes in temperature would be enough turn on or off cellular processes. Regrettably, this lack of stress could have affected the rate of DJ-1 production. Future replications of this experiment will include treatments of phenylbutyrate by itself and treatments of phenylbutyrate paired with the neurotoxin 6-hydroxydopamine to maximize DJ-1 production.

Similar to the Freed Lab's previous experiments showing that phenylbutyrate increased Sp1 acetylation and with Hilton, Li, Dunphy, and Wang's (2005) findings that showed TAF1 activity playing an important role in Sp1 acetylation, I was able to show

that phenylbutyrate increased DJ-1 protein concentrations and DJ-1 was more abundant at low temperatures. My results offer more insight to mapping out the mechanism in which phenylbutyrate increases the production of the DJ- gene. With this data, the Freed Lab further supports the benefits of phenylbutyrate against preventing Parkinson's disease, building a stronger portfolio when they seek FDA approval for this drug. Evidently, phenylbutyrate is a revolutionary drug that has the potential to change the way we treat Parkinson's disease. Hopefully with the FDA's blessing, it will rightfully be available to the public in the near future.

Tables and Figures

Table 1

Average DJ-1 Protein Densities Normalized to β-Actin Control and Standard Deviations (SD) After Exposure to Different Temperatures and Phenylbutyrate (PB) treatments

Temperature cultured	33°C n = 34	37.5°C n = 33	39.5°C n = 12
Ts13 wild types − PB	.62 (.35)	1.0 (.38)	1.1 (.39)
Ts13 wild types + PB	1.2 (.53)	1.5 (.25)	1.3 (.82)
TAF1-deficient mutants – PB	.99 (.42)	1.4 (.34)	.78 (.34)
TAF1-deficient mutants + PB	1.03 (.50)	1.3 (.27)	.76 (.74)

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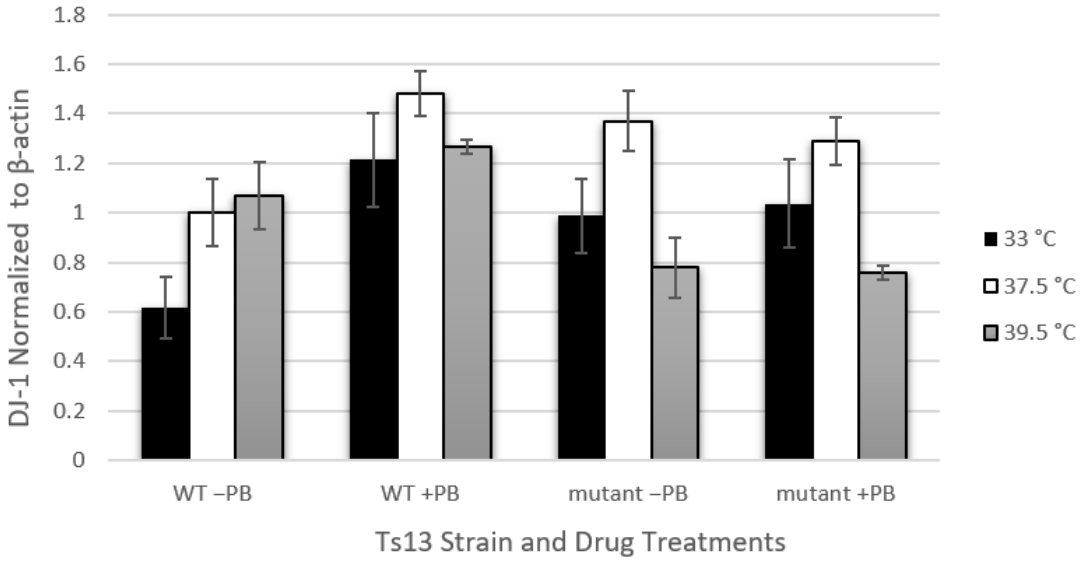

Figure 1. Mean DJ-1 protein densities in wild type and mutant ts13 cells after normalized to β-actin control density bands. Cells were incubated with drug treatments for 72 hours. Error bars represent standard error.

Tables and Figures

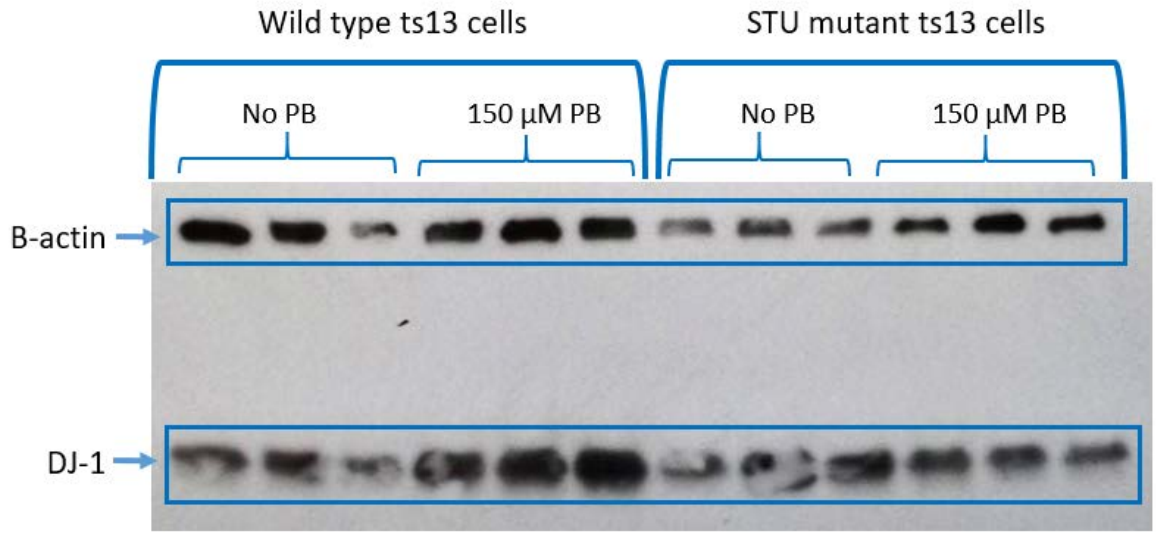

Figure 2. Western blot depicting DJ-1 and β-actin protein bands from wild type and mutant ts13 cells treated after phenylbutyrate treatment. Cells were incubated at 37.5 °C prior to protein extraction.

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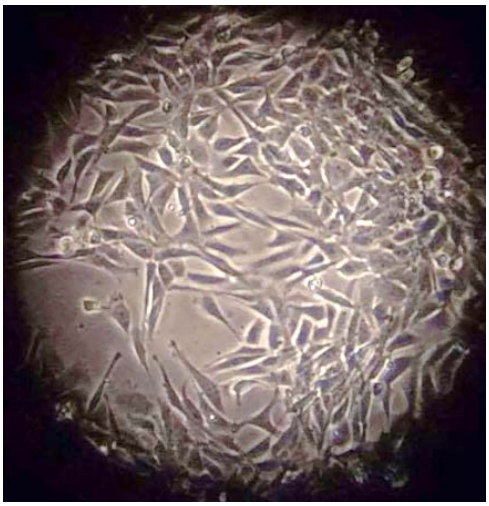

Figure 3. Wild type ts13 cells after 72 hours of incubation with 150 µM phenylbutyrate. Cells were cultured at 37.5 °C.

III. CHAPTER 3: The miles left until FDA approval

Developments in FDA and Drug Research

As a student researcher investigating the potential use of phenylbutyrate in future treatments of Parkinson's disease, I was introduced to the Food and Drug Administration (FDA) very early on in my project. The FDA is a branch of the U.S. Department of Health and Human Services that regulates the production, marketing, and distribution of food, drugs, biomedical devices, and consumer goods to protect Americans from harmful or misleading products (Hamburg & Sharfstein, 2009). Under President Theodore Roosevelt in 1906, the FDA started as the Pure Food and Administration Act under the Bureau of Chemistry of the Department of Agriculture in response to multiple complaints of contaminated food and water. Now, the FDA regulates over 2 trillion dollars' worth of food, consumer goods, and drugs, including the phenylbutyrate drug that is being explored in this thesis (Hamburg & Sharfstein, 2009; Schacter, 2006).

The FDA began in the early 1900s after a wave of medical malpractices in the late 1800s made it clear that there needed to be some form of government regulation over drugs available to the public market. Before drug regulations existed, pharmaceutical companies produced drugs laced with cocaine, opium, and alcohol, or sold products that were diluted down to ineffective doses. Unknowing physicians and pharmacies would then prescribe these products to their patients, unaware of the harmful side effects the drugs would produce (Schacter, 2006). In response to the tragic deaths of the diethylene glycol poisoning in 1937 from physician-prescribed drugs, the Federal Food, Drug, and

Cosmetic Acts was passed in 1938. This gave the U.S. government the authority to regulate contents of new drugs before they entered the market (Rägo & Santoso, 2008). For the first time, the government was overseeing drug distribution.

But even under early federal regulation, drug safety still posed a problem. The red clauses of patent laws protected pharmaceutical companies from legal consequences from their false drug advertisements (Schacter, 2006). Furthermore, drugs were still released into the public before thorough screening, leading to catastrophic events like the thalidomide-induced birth defects in 1956, a case where pregnant mothers were given thalidomide as a form of anti-anxiety medication, only to find that the pills caused the mothers to give birth to over 10,000 crippled babies shortly after (Rägo & Santoso, 2008). The government was trying to establish a foundation for drug review, but their review process was still hasty and hindered by a desire to produce more drugs. It was not until after several more years of disease outbreaks, severe reactions, fatalities, and birth abnormalities that the U.S. government passed the Drug Amendments Act of 1962, declaring that all new drugs entering the market will be subjected to a *long-term* review process. (Rägo & Santoso, 2008; Schacter, 2006; Stepp, 1999).

Still, the 1962 Drug Amendment Act did not finalize the drug review system; the FDA continued to refine their drug review process to keep up with modern changes in drug production. In 1997, the United States government passed the FDA Modernization Act, which made the screening process even more rigorous for companies that wanted to introduce new drugs to the public. While the 1962 Drug Amendment Act allowed drugs into the public after a few months of review, the FDA Modernization Act demanded an

average of 12 years, multiple phases, and multiple review cycles to get FDA approval for a new drug. With each phase of the review process, the percentage of drugs moving on to the next stage decreases (Ross, Dzara, Downing, 2015; Schacter, 2006).

The first step of getting FDA approval for a potential drug, while time consuming and costly, is just the beginning. After discovery of a potential drug in the laboratory—which itself can take multiple years—the researcher must make the decision to develop the drug as a marketable item in the future. Here, the researcher must find sponsors, grants, or other sources of funding, sometimes costing up to hundreds of thousands of dollars, for further laboratory and animal testing of the drug to determine the safety of the drug, the dosage which it is most useful, and the efficacy of the drug. (Adams & Brantner, 2006; Schacter, 2006). At this point, the drug has not left the laboratory.

After the results of the first stage have been reviewed multiple times by the government and review boards, the drug moves onto clinical human testing, which is composed of 3 phases. Phase 1 of clinical trials tests whether the drug has negative effects in the human body, how quickly the drug enters and leaves the human body, and how effectively does the drug act on its targeted cells (Schacter, 2006). Basically, this steps translates the results found in the animal testing stages into a dosage and schedule that is appropriate for human patients. Then, we move into Phase 2 of clinical trials, where we start to see double blind trials, randomization, and more targeted populations of the drug used in controlled studies. This a process is a continuation of Phase 1, and can take from several months to several years to complete (Schacter, 2006). Finally, Phase 3 of clinical trials are long-term studies of the drug, where the number of participants are

often increased to the hundreds or thousands, the selections are more randomized, and the experiments are even more extensively controlled. On average, this phase lasts 5 years and can cost up anywhere from hundreds of thousands to millions of dollars (Adams & Brantner, 2006; Schacter, 2006).

At last, the drug proposal can be submitted to the FDA for review, supported by years of research, hundreds of data sheets, and an average investment of 800 million dollars per drug (Schacter, 2006). Even so, most drug proposals will be sent back for another round of revision on the basis of insufficient data, safety concerns requiring additional analysis, unsatisfactory marketing and distribution plans, or inadequate drug labels/consumer information sheets (Ross, Dzara, Downing, 2015). 17% of returned drug proposals are approved within 3 years and 47% are approved within 10 years (Patridge, Gareiss, Kinch & Hoyer, 2015). Unfortunately, only 20% of the drugs entering Phase 1 of clinical trials ultimately get used by the public (Schacter, 2006).

Efficacy of FDA drug regulation—An analysis.

Given the increasingly lengthy review process, the practicality of the FDA has grown controversial, criticized for hindering the productivity of drug research and development. Still, the chief purpose of the FDA is still to protect its people from exploitation by pharmaceutical companies or from accidental side effects of poorly developed drugs. For one, side effects of novel drugs may not manifest right away as some drugs take longer to metabolize and integrate itself into the body's system (Lesser, Fahn, Snider, Cote, Isgreen & Barrett, 1979). Many drugs require constant dosages to

build up a large enough chemical gradient to change the way an organ system is behaving. If we only relied on data from short-term studies, we potentially dismiss promising drugs that benefit the patient in the long-run, but do not show any immediate results. Vice versa, if the drug is not acting where it is intended or if it is causing as overreaction in its targeted system, we would not know how the body would truly react until much later on when the drug has accumulated to dangerous levels inside the body.

For example, L-DOPA can improve motor impairments in some Parkinson's patients, but intensify the severity of the same motor impairments in others. However, after a few years of L-DOPA use, some of the same patients who originally benefited from L-DOPA develop dramatic side effects to the drug while others largely remained unchanged (Lesser, Fahn, Snider, Cote, Isgreen & Barrett, 1979). All of these factors influence the efficacy of drugs, translating into changes in drug dosages, drug schedules, and drug regimen—components which are monitored and adjusted in Phases 2 and 3 of Clinical Trials during the FDA approval process (Schacter, 2006). In these cases, short-term data is too narrow. We have to know that a drug will improve a majority of the public's conditions and these improvements outweigh the potential risks that the drugs cause. It is nearly impossible to fully capture the benefits and harms of novel drugs without years of extensive observation.

On the other hand, since the FDA's introduction, the culture of drug research and development has shifted. In 1962, the number of new chemical entities entering the public market dropped by nearly half and continued to gradually decline over several decades (Thomas, 1990). With the increasing number of regulations by the FDA, drugs

are forced to stay in the research and development phase longer than ever before, costing pharmaceutical researchers more money to develop and delay the monetary rewards for their long years of research. In the U.S., an estimated 820 million dollars was invested by companies into drug research and development in the 1990s, a number projected to increase in the 21st century (Schacter, 2006).

In terms of Parkinson's disease research, the National Parkinson's Disease Foundation—one of the many large organizations funding Parkinson's disease research—has invested over $115 million since they were founded in 1957 (Parkinson's Disease Foundation, 2017). Another big supporter, the Michael J. Fox Foundation, has funded more than $650 million dollars since 2000 to search for a cure (Michael J. Fox Foundation). These are, of course, the funding giants in Parkinson's disease research; the numbers do no justice to the amount of money invested by private donors, researchers, and family members of those suffering from Parkinson's disease. Research is costly.

With costs in the millions, the means for research is often dominated by big firms and corporations. Individual researchers and university affiliates have to apply for government funding or request sponsorship from wealthy donors in order to sustain their drug research. I, myself, have had to apply for multiple grants for my research projects and I have watched two different small labs shut down from lack of funding. The hunt for funding is greatly stressful and markedly hinders research progress. Under the FDA reform, small players in the pharmaceutical industry are at a disadvantage; the system becomes exclusive to those who can afford the investment rather.

Even within big corporations and governmental funding, the chances of producing

a successful drug are slim. There are thousands of drug proposals every year, but only a limited amount of funding available. Thus, companies and government grant programs are forced to screen for which potentially successful drugs, discarding less promising projects at an early stage (Schacter, 2006). As mentioned previously, early estimates of a drug's efficacy are inaccurate. But because each project needs sufficient funding to progress *into* late clinical trials, in-depth analyses of many potential drugs cannot afford to be explored. In an unforgiving cycle, drug research needs to be funding for reliable results, but conversely, there needs to be reliable results for funding. Under this system, the productivity of drug research is hindered, shackled by desires for profit.

Hand in hand with decreased novel drug production, more people with severe illnesses are denied potentially life-changing treatment. On average, the FDA approved 50 new drugs every year from 1955 to 1960. However, this number dropped to an average of 17 a year between 1965 and 1970 (Gieringer, 1985). Although these numbers are steadily decreasing in the 21st century, novel drug shortages are still a relevant concern (Eichler, Pignatti, Flamion, Leufkens, & Breckenridge, 2008). If a new drug saves 1,000 lives per year, a delay in its introduction would result in 1,000 deaths *every single year* that the drug is withheld from the market. In 2005, the World Health Organization projected that chronic illnesses will be responsible for 41 million deaths in 2015 if these illnesses are not controlled appropriately (Abegunde, Mathers, Adam, Ortegon, Strong, 2007). Without intervention, these numbers will not change.

Even in less fatal illnesses, people living with mental impairments, physical handicaps, or any type of impaired functioning are still subjected to the effects of their

diseases until treatment is available. In end-stage Parkinson's disease, patients begin to suffer the effects of mental deterioration as they start to lose the ability move on their own, care for themselves, and recall their loved ones (Aarsland et al., 2004). In fact, these mental deficits, paired with the chronic motor impairments, extensively interfere with a patient's quality of life, often resulting in a high prevalence of depression and other mental illnesses (Cummings, 1992). Without adequate treatment, quality of life will still decline physically, mentally, and emotionally. Right now, there is an astounding amount of research dedicated to finding treatment for chronic illnesses like Parkinson's disease. However, many of these treatments will not be available to the public market for another several years to decades under the current FDA system (America's BioPharmaceutical Research Companies, 2014; Schacter, 2006).

Another contributing factor to patients' quality of life are the long-term expenses of treating Parkinson's disease from diagnosis to departure. Outside of the money spent on Parkinson's disease research, Parkinson's disease, on a macro level, costs the U.S. economy $60 billion for direct and indirect expenses in patient care, a value that will increase in 2020 if left unaddressed (America's BioPharmaceutical Research Companies, 2014). These costs include revenue lost from patients that are too sick to work and patients that require health benefits for their chronic conditions. On the micro level, a Parkinson's disease patient spends an average of $2,500 medication treatment per year and an average of $100,000 therapeutic surgery costs once they enter the severe end stages of Parkinson's disease (America's BioPharmaceutical Research Companies, 2014). These out-of-pocket expenses contribute to the quality of life diagnosed patients can live.

We have a moral and social obligation to minimize death and suffering for as many people as possible. At the root of drug research is the desire to improve the conditions of mankind. In the past, efforts in drug development were corrupted by greed and power from pharmaceutical companies, calling the need for some form of drug regulation; however, trends in recent years suggest that over-regulation of drug development can also cripple drug research productivity as it denies drug access to people who urgently need it. As a prospective medical student and a student researcher, I have seen both the need for drug regulation as well as its complications. Particularly, my phenylbutyrate research with Dr. Freed has given me a more personal understanding of how FDA drug regulation impacts a new drug proposal.

Phenylbutyrate in the Freed Lab

The phenylbutyrate project started in the Freed Lab in 2006, discovered by a summer student studying the effects of histone deacetylase inhibitors on the DJ-1 gene. At the time, the neuroprotective effects of the DJ-1 gene had already been established and the lab was trying to find ways to increase DJ-1 gene production. During the process of protein expression, DNA is copied into strands of RNA, which are then used as templates to generate proteins that the cell can use. When the cell is not making new proteins, DNA strands are wrapped around histone proteins. In order for protein production to start, DNA strands must acetylate and uncoil from the histone proteins before it can be copied into strands of RNA. Histone deacetylase inhibitors encourage the acetylation and uncoiling of the DNA strands from the histones, therefore promoting protein production

(Ryu et al., 2013). Of course, different histone deacetylase inhibitors target different genes and different histones, which was why finding the right histone deacetylase inhibitor to target the DJ-1 gene was necessary.

Out of all the histone deacetylase inhibitors tested, phenylbutyrate was the most effective at increasing cellular levels of DJ-1 (Freed, 2016). These DJ-1 increases were even further amplified when the cells were treated with both phenylbutyrate and 6-hydroxydopamine to induce stress in the cells. Eventually, this evolved into extensive tissue culture and rat studies whose results were published by Zhou, Bercury, Cummiskey, Luong, Lebin, and Freed in 2011. By the time the 2011 was published, the Freed Lab had enough data to suggest that phenylbutyrate could be a promising treatment for Parkinson's disease. And so, the drug continued into human clinical trials.

When we started pursuing phenylbutyrate as an alternative treatment for Parkinson's disease, the drug had already been FDA approved to treat urea cycle disorders and was marketed as Ravicti or Buphenyl (Freed, 2016; Leonard, 1995). Aside from the market cost of the drug, there were very little restrictions against us using phenylbutyrate for our experiments. Thus, getting approval to start phenylbutyrate clinical trials was relatively easy; the lab was exempt from the extensive FDA approval process and was instead allowed to move on after completing a smaller scale proposal. Basically, as long as the lab promised to not perform studies that will disprove its efficacy in urea cycle disorders, change the description of the drug on FDA-approved labels, and only use the drug at already approved doses or lower, we could largely bypass the FDA screening process (Freed, 2016; Schacter, 2006). In the end, getting FDA

approval to transition from bench work to clinical trials only took two weeks (Freed, 2016).

During Phase 1 of clinical trials, the Freed Lab focused on finding the side effects of phenylbutyrate. Forty participants between 40 and 75 years old were selected for this study, as Parkinson's disease typically manifests in this age range. Participants were age matched between a group with existing Parkinson's disease and a group without Parkinson's disease diagnoses. Over three weeks, all 40 participates took daily phenylbutyrate pills at previously FDA-approved doses and monitored for side effects and reactions to the drugs (Freed, 2016). As it turned it turned out, because phenylbutyrate is a fatty oil substance, many people developed gastrointestinal side effects, such as stomach irritation, heartburn, nausea, and vomiting. Ten percent of the participants developed rashes. Fortunately, all of these side effects were consistent with FDA-published labels for Buphenyl, showing that using phenylbutyrate as a Parkinson's disease treatment is not significantly harmful (Freed, 2016; Leonard, 1995). The studies were largely considered successful.

However, as the Freed Lab moved into Phase 2 of clinical trials, things became more complicated. Now, we were looking at phenylbutyrate dose tolerability in Parkinson's patients. In Phase 2, we were trying to find a dose that will be the most effective long-term while producing the least amount of side effects in the patients. To do so, we implemented a double-blind system, using a fatty, oil-based placebo pill as a control. We then gave participants the maximum FDA-approved dose, two-thirds of the maximum FDA-approved dose, one-third of the maximum FDA-approved dose, or a

placebo pill that contained no drug at all and monitored the type and severity of the side effects each dosage produced (Freed, 2016). Neither the researchers in the Freed lab, our study coordinators, or the patients knew who received the phenylbutyrate treatment and who received a blank pill until the conclusion of the study.

We are currently still in Phase 2 of clinical trials. Ideally, each participant will take these pills for one year from the time they enroll into the study. Given the commitment that the study requires (i.e. the constant check-ups, the secrecy and frustration of the double blind trials, and the potential side effects that both the placebo and the drug will have), we predict that 50% of the participants will drop out by the end of the study (Freed, 2016; Freed and Levay, 2002). Thus, to ensure that we have sufficient data by the end of the study for effective analyses, we have to start with a large enough population. We are hoping for 25 participants per group, 100 participants in all, with the expectation that we will have at least a dozen patients per group that are still participating by the end (Freed, 2016).

We started Phase 2 in early 2016. Currently, Gabrielle Lemicke, the coordinator for the study, is still interviewing participants from clinics across the United States for the first rounds of testing. She is reaching out to promising candidates, informing about the purposes of our study, and interviewing them, in a process that can take several months per patient. For the population size we want, recruitment will take an estimated 2 to 4 years (Freed, 2016). As patients enroll into the study, we individually start them on their 1-year phenylbutyrate course as we continue to recruit more potential participants and age-matching them to the participants we already have. The study as a whole will take the

Freed Lab an estimated 5 years if events unfold according to plan. We expect to have results by 2021 (Freed, 2016).

Reflections and Conclusions

When I first read about the phases of clinical testing, I imagined that each phase was self-contained, conducted independently from the phase prior. Realistically, time and money does not allow for this type of separation. For one, I was surprised to find that recruitment for clinical trials took so long, stretching out to years and sometimes even decades for a drug trial that only lasts a year itself. When I was merely reading about clinical trials, I imagined that recruitment, initiation of treatment, data collection, and data analysis occurred sequentially and in clearly defined time frames. However, I now realize that many of these steps overlap to accommodate for participant preferences, recruitment difficulties, changes in participants' health, technical issues, etc. Research with human participants is not as straight-forward as the textbook depicts.

For example, in the case of phenylbutyrate testing in the Freed lab, Phase 2 of our clinical trials, in theory, should only take one year. After all, patients are only taking the drug for twelve months once they agree to participate in the study. Unfortunately, recruiting participants requires time to search for potential patients, money to fly out to meet the patients, and thoroughness to make sure that patients have well-informed consent. With the staggered enrollment, each participant will start and end their trial at different times and the study will not technically be finished until the last participant finishes his or her phenylbutyrate course, dragging out the clinical trials five times longer than the actual time needed to conduct the trial. In writing, these issues are simplified.

The textbook does no justice to the time and effort that is actually invested in conducting these clinical trials.

Secondly, what surprised me even more was how the bench work did not stop, even when clinical trials were taking off. Originally, I thought that a drug had to be finalized and perfected before it was handed off to the next stages of testing, like a baton in a relay race. From the way the FDA approval process was described in my books, I assumed that research teams would first devote 100% of their energy in cell and animal research, submit their research for approval, then redirect all of their energy into clinical testing once their proposals have been approved. In reality, this idea is extremely inefficient. Realistically, as a drug moves through clinical trials, researchers often choose to simultaneously continue their drug investigations in hopes of refining it, learning more about it, or finding a better substitute for it, so that clinical trials will have a higher chance of succeeding.

This is the case in the Freed Lab and, in fact, was the reason why this thesis project was possible. Although phenylbutyrate was halfway into its clinical trials, my colleagues and I were still working behind the scenes to try and identify its mechanisms. My project is one of many exploratory phenylbutyrate research. There are others in my lab that are looking at how phenylbutyrate is affecting mitochondria, how phenylbutyrate is changing cellular communication, etc. More specifically to the clinical trials, we are still feeding rats phenylbutyrate and observing cellular responses to phenylbutyrate treatment so that we can better understand how phenylbutyrate works in humans. If anything, my two years in the Freed Lab has taught me that science is less of a relay race

and more of a brick-building contest, open to modification by any of its team-members. There is just so much that I have learned in practice that the textbook has failed to fully capture.

In truth, phenylbutyrate still has a long way to go before it reaches the public as a treatment for Parkinson's disease. As of 2016, 2 million dollars has been invested into the phenylbutyrate project, $600,000 of which from the Michael J. Fox Foundation, $400,000 from individual sponsors, and $1 million from Dr. Freed himself, who truly believes that we can cure Parkinson's disease within the next few decades. However, there is no doubt that this project will need more funding, more experiments, and more FDA reviews in phenylbutyrate's future, before phenylbutyrate can change the world in the way that we envision. But for now, the Freed lab is grounded on the science, refining phenylbutyrate and expanding our scientific understanding of it. We are looking for new ways to explore the phenylbutyrate mechanism, to test its effects in Parkinson's disease patients, and to detail its chemical actions in the body. The phenylbutyrate story, though started over a decade ago, is far from finished.

My work in the Freed lab is, literally, quite small, focused on finding the molecular mechanism in which phenylbutyrate affects the Parkinson's disease-fighting DJ-1 gene. When I joined the team, the phenylbutyrate project was already halfway into its clinical trials. The brick that I picked up from Dr. Freed had long been part of the mission to the cure for Parkinson's disease, built from an idea created many years ago. And when I leave the lab, the building will surely continue until we have constructed an

answer. My only hope now is that the research that I have done—the brick that I have chiseled—has contributed in some meaningful way.

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