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A Small Molecule Screen to Identify a Potential Treatment for MTDPS3

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A Small Molecule Screen to Identify a Potential Treatment for MTDPS3

by

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A thesis submitted to the Medical University of South Carolina faculty in partial fulfillment of the requirements for the degree of Master of Science in the College of Graduate Studies.

Department of Regenerative Medicine and Cell Biology

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To my mother, thank you for always having my back and constantly pushing me to be the best version of myself. To my father, thank you for reminding me to be authentic. To my brother, thank you for being the other side of the coin. To the most patient lab technician I know, Paige, thank you for the copious hours you spent working with me on this thesis. To the Duncan Lab, it's been a pleasure working, learning, and laughing with you all. To my committee and mentor, thank you for the scholarly insight and direction. To the drug discovery core, thank you for your help and support. To the IMSD group, thank you for your companionship and priceless resources.

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ABBREVIATIONS

ActA - Activin A (ActA)

ADP - Adenosine Diphosphate

ATP - Adenosine Triphosphate

B27 $^{-}$ - B27 with insulin

B27^{+} - B27 without insulin

BMP-4 - Bone Morphogenetic Protein

CAC – Citric Acid Cycle

CoQ – Coenzyme Q

dAMP - deoxyadenosine monophosphate

dGMP - deoxyguanosine monophosphate

dNTPs - Deoxyribonucleotides

DGUOK – Deoxyguanosine Kinase

ESCs - Embryonic Stem Cells

ETC - Electron Transport Chain

FGF2 - Fibroblast Growth Factor 2

HCM - Hepatocyte Culture Medium

HGF - Hepatocyte Growth Factor

iPSCs - Induced Pluripotent Stem Cells

mtDNA - Mitochondrial DNA

MTDPS3 - Mitochondrial DNA Depletion Syndrome 3

MTDPS - Mitochondrial DNA Depletion Syndrome

NAD - Nicotinamide adenine dinucleotide

OSM - Oncostatin M

OXPHOS – Oxidative Phosphorylation

RNR - Ribonucleotide Reductase

SC3 - South Carolina Compound Collection Library

zbFGF - Zebrafish Growth Factor

CONNOR JADE JOCELYN GRAHAM. A small molecule screen to identify a potential treatment for MTDPS3. (Under the direction of STEPHEN DUNCAN).

ABSTRACT

Mitochondrial DNA Depletion Syndrome 3 (MTDPS3) is the most common mitochondrial DNA (mtDNA) depletion syndrome. MTDPS3 is an autosomal recessive disorder caused by a mutation in the deoxyguanosine kinase (*DGUOK*) gene in which a reduction in mtDNA nucleotides results in decreased levels of Adenosine Triphosphate (ATP). The *DGUOK* phenotypes vary; however, liver dysfunction tends to be consistent for all pediatric patients. Liver transplants can be beneficial; however, it's only a palliative treatment at best, and the affected population rarely reaches adulthood. This thesis describes a high-throughput drug screen to identify a potential therapy for MTDPS3. MTDPS3 mutant hepatocytes derived from induced pluripotent stem cells (iPSCs) were utilized in the drug screen of the South Carolina Compound Collection (SC3) library. From the primary screen of 10,000 compounds, 63 compounds increased ATP levels at a sample number of one. The primary hits were used to perform biological replicates with the knockout cells, and six can increase ATP levels consistently. Two of the six compounds increased ATP production at eight different drug concentrations. My results show six small molecules with the therapeutic potential to treat an MTDPS3 phenotype in-vitro.

CHAPTER 1: INTRODUCTION

Mitochondrial Biology

The complexity of mitochondrial biology begins with the theory of endosymbiosis (Martin & Mentel, 2010). Once believed to be an independent free-roaming cell, the mitochondria created a mutualistic relationship with a larger prokaryotic cell (Martin & Mentel, 2010). Millions of years later, the doubled membraned organelle produces Adenosine Triphosphate (ATP) for every cell in the human body (Figure 1). The mitochondria are the only organelle that contains DNA, known as the mitogenome. Unlike nuclear DNA, however, mtDNA is circular and haploid. Instead of being inside a nucleus, mtDNA is in a nucleoid anchored to the mitochondria's inner membrane (Almannai, El-Hattab, & Scaglia, 2018). There are 37 genes in the mitogenome, and 13 of them encode the Electron Transport Chain (ETC) complexes, while the remainder codes for mtDNA replication machinery. THE ETC consists of five complexes embedded in the inner mitochondrial membrane (Cooper, 2000) (Figure 2). The ETC is one of two primary components of oxidative phosphorylation (OXPHOS). Together with the mechanism of chemiosmosis, the ETC produces ATP.

Oxidative Phosphorylation

OXPHOS is the mechanism that produces the majority of ATP in a cell, with the help of glycolysis and the citric acid cycle (CAC) (Cooper, 2000). The first molecule, NADH, is relieved of two electrons which enter Complex I of the ETC to begin the process. The negative subatomic particles pass through Coenzyme Q (CoQ), also known as ubiquinone. The second molecule produced by the CAC cycle, FADH2, also releases electrons that flow through Complex II and

CoQ. The electrons leave ubiquinone and enter Complex III and Cytochrome C. Finally, the electrons are passed through Complex IV (cytochrome oxidase), where they bond to the hydrogen $^{+}$ ions in the mitochondrial matrix O_2 to form two water molecules. This entire process is known as the ETC. Naturally occurring hydrogen + ions in the mitochondrial matrix are pumped across the membrane and into the intermembrane space throughout the electron transfer process. Consequently, creating a proton gradient, with an accumulation of hydrogen + ions in the intermembrane space. This gradient fuels ETC complex V (ATP synthase) by going from a high-energy state to a lower one. This equilibrium process creates a conversion where a phosphate group is attached to each adenosine diphosphate (ADP), creating the final ATP product. Therefore, the primary responsibility of the mitogenome is to produce energy for cellular activities.

Mitochondrial DNA Depletion Syndromes

Despite having DNA, the mitochondria still depends on nuclear DNA to help with replication and maintenance (Bogenhagen, 2012) (Figure 3). The replication process is uninterrupted, requires copious proteins, a constant supply of nucleotides, and is independent of cell division (Almannai, El-Hattab, & Scaglia, 2018). Defects in the nuclear genes causes abnormal mtDNA maintenance (Table 1). These defects create depletions and deletions in mtDNA, and those disorders are known as Mitochondrial DNA Depletion Syndromes (MTDPSs). MTDPSs are diseases caused by disruptions in mtDNA's function and maintenance and manifests as various multi-organ system malfunctions (Shimura, et al., 2020; Almannai, El-Hattab, & Scaglia, 2018) (Table 1).

DGUOK Deficiency

Mitochondrial DNA Depletion Syndrome 3 (MTDPS3), an autosomal recessive disorder, accounts for one-fourth of all MTDPSs (Sezer, Ozçay, Balci, & Alehan, 2015). MTDPS3 is caused by the nuclear gene deoxyguanosine kinase (*DGUOK*) mutation. The transcription and translation of the *DGUOK* gene produce the protein that travels to the mitochondria to assist in mtDNA replication (Van Goethem, Dermaut, Löfgren, Martin, & Van Broeckhoven, 2001; Sarzi, et al., 2007). Specifically, the *DGUOK* protein phosphorylates mtDNA nucleosides, deoxyadenosine monophosphate (dAMP), and deoxyguanosine monophosphate (dGMP) (Gower, Carr, & Ives, 1979) (Figure 4). The nucleotides are the building blocks for mtDNA needed to produce ATP. Without mtDNA, the human cell will lose its primary energy source, leading to apoptosis or necrosis (Tsujimoto, 1997). A mutated *DGUOK* protein causes a depletion in mtDNA, which ultimately leads to a depletion in ATP (Figure 4).

The most well-known cases of MTDPS3 involve infants suffering from hepatic failure. This disease is known for its hepatocerebral dysfunction in most patients due to the accompanying neurological aberrations. This disease was first characterized in the late twenty-century when a 9-month-old infant died of liver failure (Boustany, Aprille, Halperin, Levy, & DeLong, 1983).

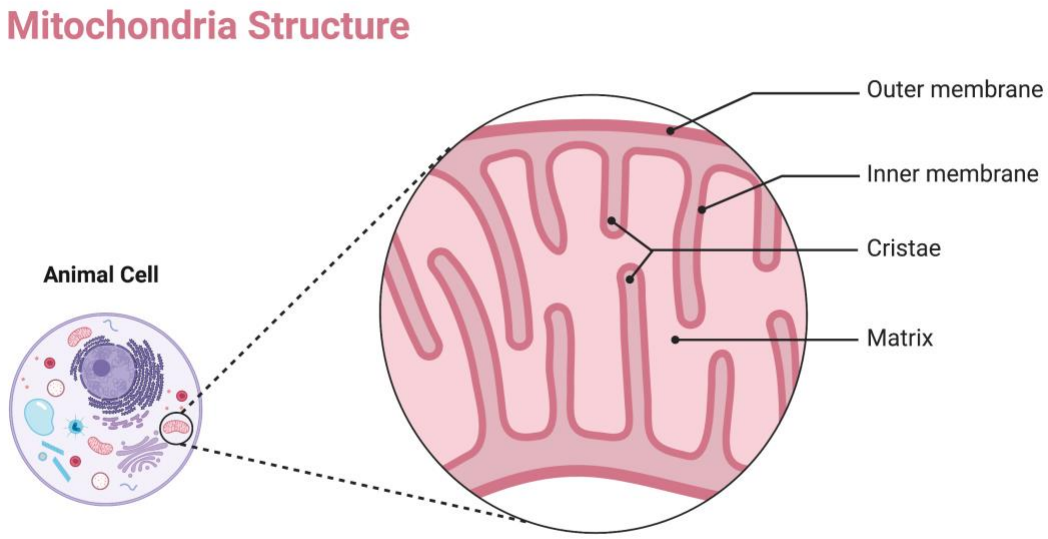


Figure 1. Mitochondrion Structure

A mitochondrion is a double-membraned organelle that contains a genome and produces most energy in a cell. (Created using Biorender.)

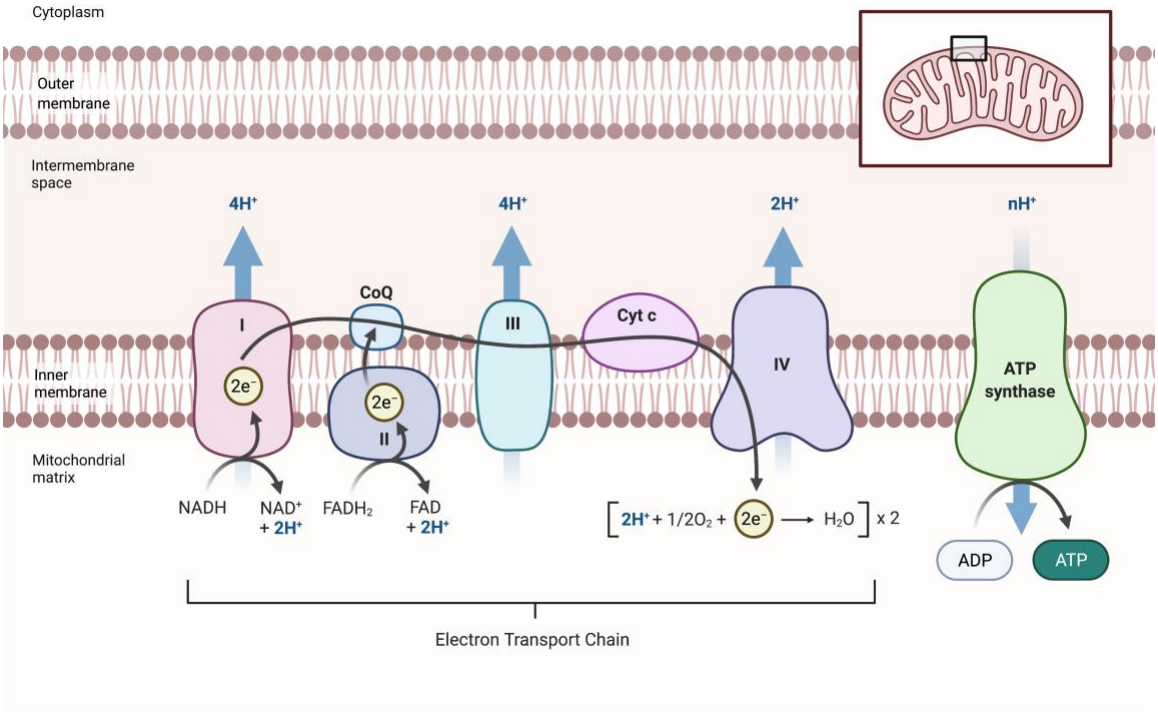


Figure 2. Oxidative Phosphorylation.

Oxidative phosphorylation(OXPHOS) produces approximately 24-32 ATP molecules per one molecule of glucose. This process consists of the electron transport chain (ETC) and chemiosmosis. (Created using Biorender.)

Table 1. mtDNA maintenance defects

Gene	Phenotype	MtDNA
Disorders associated with defects in MtDNA replication machinery		
POLG	Childhood myocerebrohepatopathy spectrum (MCHS) Alpers–Huttenlocher syndrome (AHS) Myoclonic epilepsy-myopathy-sensory ataxia (MEMSA) Ataxia neuropathy spectrum (ANS) Progressive external ophthalmoplegia (PEO) Mitochondrial neurogastrointestinal encephalopathy (MNGIE)	Depletion Depletion Multiple deletions Multiple deletions Multiple deletions Depletion/multiple deletions
POLG2	Myopathy	Multiple deletions
TWNK	PEO Infantile-onset spinocerebellar ataxia (IOSCA) Encephalohepatopathy Perrault syndrome	Multiple deletions Depletion Depletion NA
TFAM	Encephalohepatopathy	Depletion
RNASEH1	Encephalomyopathy	Depletion/multiple deletions
MGME1	Myopathy	Depletion/multiple deletions
DNA2	Myopathy Seckel syndrome	Multiple deletions NA
Disorders of mtDNA nucleotides supply		
TK2	Myopathy PEO	Depletion Multiple deletions
DGUOK	Encephalohepatopathy Myopathy	Depletion Multiple deletions
SUCLG1	Encephalomyopathy with methylmalonic aciduria	Depletion
SUCLA2	Encephalomyopathy with methylmalonic aciduria	Depletion
ABAT	Encephalomyopathy with elevated GABA	Depletion
TYMP	Mitochondrial neurogastrointestinal encephalopathy (MNGIE)	Depletion/multiple depletions
RRM2B	Encephalomyopathy with renal tubulopathy PEO	Depletion Multiple deletions
SLC25A4	PEO Autosomal recessive cardiomyopathy Autosomal dominant cardiomyopathy	Multiple deletions Multiple deletions Depletion
AGK	Sengers syndrome	Depltion
MPV17	Encephalohepatopathy Encephalomyopathy	Depletion Multiple depletions

(Almannai, El-Hattab, & Scaglia, 2018) Defects in nuclear genes that maintain and help replicate mtDNA lead to depletions in mtDNA and ultimately multi-organ system failure.

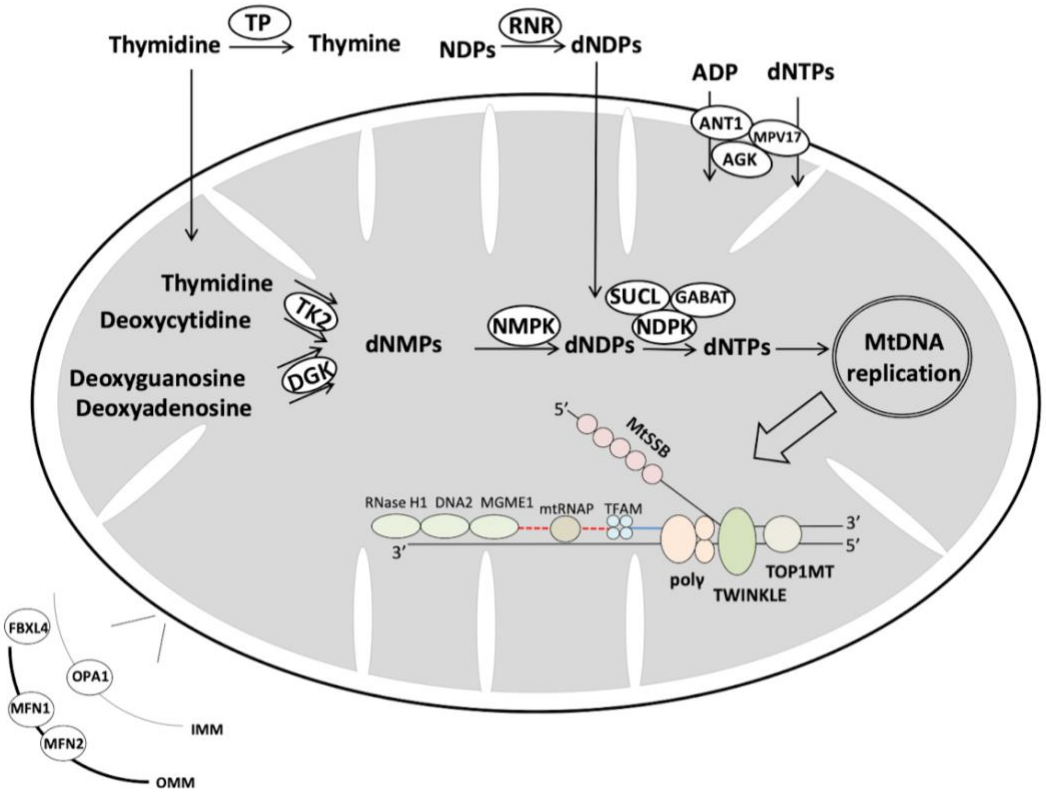
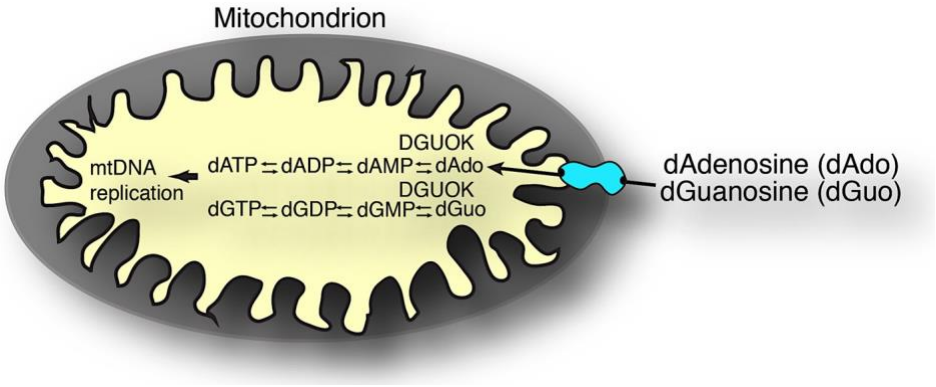


Figure 3. mtDNA replication proteins

(Almannai, El-Hattab, & Scaglia, 2018). Above are the proteins associated with mtDNA replication. The functions of the proteins are in Table 1.

A.



B.

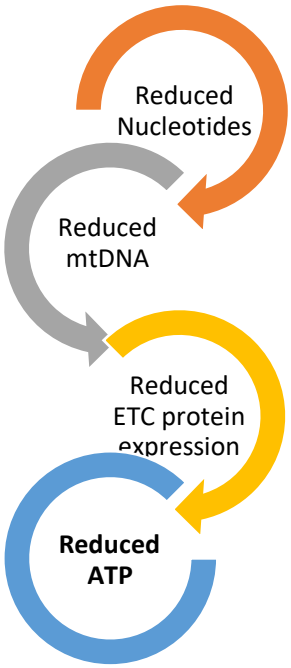


Figure 4. Consequence of DGUOK protein.

(A). The DGUOK protein enters the mitochondria through an intermembrane channel. It phosphorylates purine deoxyribonucleosides. Deoxyribonucleotides (dNTPs) are the foundation of mtDNA. (B). The implications of a DGUOK mutation which results in a depletion in ATP.

The symptoms vary per patient but include the two mentioned above as well as hypoglycemia and increased lactate accumulation (Mandel, et al., 2001; Salviati, et al., 2002; Mancuso, et al., 2005; Dimmock, et al., 2008; Jing, et al., 2018). The most common current therapy is a liver transplant; however, due to the onset of declined brain functions and low survival rate, this option is palliative at best (Dimmock, et al., 2008; Jing, et al., 2018).

Disease Modeling and Therapy with iPSCs and Drug Screens

Induced pluripotent stem cells (iPSCs), reprogrammed somatic cells, are becoming the leading tool in modeling diseases for therapeutic applications (Si-Tayeb, et al., 2010). The benefit of iPSCs is their ability to become almost any cell type. Discovered by Dr. Yamanaka in 2006, these revolutionary stem cells have changed the way we study and treat diseases. Somatic cells are reprogrammed when introduced to Yamanaka factors (OCT4, SOX2, NANOG, or LIN28 and KLF4 or MYC) (Bellin, Marchetto, Gage, & Mummery, 2012). An essential transcription factor for this induction is Oct4, which indicates pluripotency (Liu, David, Trawczynski, & Fessler, 2020). Transcription factors help turn genes "on" and "off." In this case, the Yamanaka factors manipulate the genes in the somatic cells to induce pluripotency. The idea of iPSCs arose due to the challenges of using embryonic stem cells (ESCs) for clinical application.

Human ESCs were created in the later 20th century with the hope of using cell therapies in numerous human ailments and injuries (Yamanaka, 2020). ESCs are pluripotent stem cells from a human embryo, and similarly to iPSCs; they can differentiate into all three germ layers (Evans & Kaufman, 1981) (Figure 4 and 5). Utilizing ESCs became problematic during the early 2000s due to

ethical issues using human embryos (Yamanaka, 2020). The production of new cell lines even became a national political issue polarizing the country between medical advancement and principled beliefs. The problem of tissue rejection was also a challenge for cell-based therapies using ESCs. Consequently, the idea of using patients' somatic cells to generate differentiating stem cells came to be. The advantages of using iPSCs include proliferation potential, controlling genomic variants, and autologous transplantation (Yamanaka, 2020). Although relatively new, iPSC technology can recapitulate various diseases, fortifying their role in clinical studies (Elitt, Barbar, & Tesar, 2018). With other therapeutic techniques such as high throughput drug screens and gene editing, iPSCs have become the leading disease modeling tool.

An iPSC-based drug screen requires four stipulations: assay development, primary screen, secondary screening, and ultimately in vivo testing (Elitt, Barbar, & Tesar, 2018). Assay development involves creating or following a robust differentiation protocol with the iPSCs recapitulating the disease of study's phenotype (Elitt, Barbar, & Tesar, 2018). For the primary screening finding a screening library that meets the project's needs is imperative. Identifying the screening dose, controls, and eventual hit selection criteria is also important for this second stage (Elitt, Barbar, & Tesar, 2018). The secondary screening requires validating the hit compounds from the primary screen by performing biological replicates and a dose-response assay (Elitt, Barbar, & Tesar, 2018). The final component of an iPSC-based drug screen demonstrates the efficacy and toxicity of the compounds in live animal models (Elitt, Barbar, & Tesar, 2018). Along with comprehending the steps of a high throughput drug screen, defining what type of

screen will be executed is also essential. When performing a phenotypic screen, the objective is to identify chemical compounds that modify the cell's phenotype (Moffat, Vincent, Lee, Eder, & Prunotto, 2017). Unlike a target-based screen, no defined molecular target allows for a larger target space (Henry & Wlodkowic, 2019) (Figure 7). While target-based drug discovery (TDD) has been the standard approach in this space for 30 years, phenotypic drug discovery (PDD) screens provide illuminating awareness in unpredicted consequences of compounds and the ways they alter a cell's phenotype (Moffat, Vincent, Lee, Eder, & Prunotto, 2017).

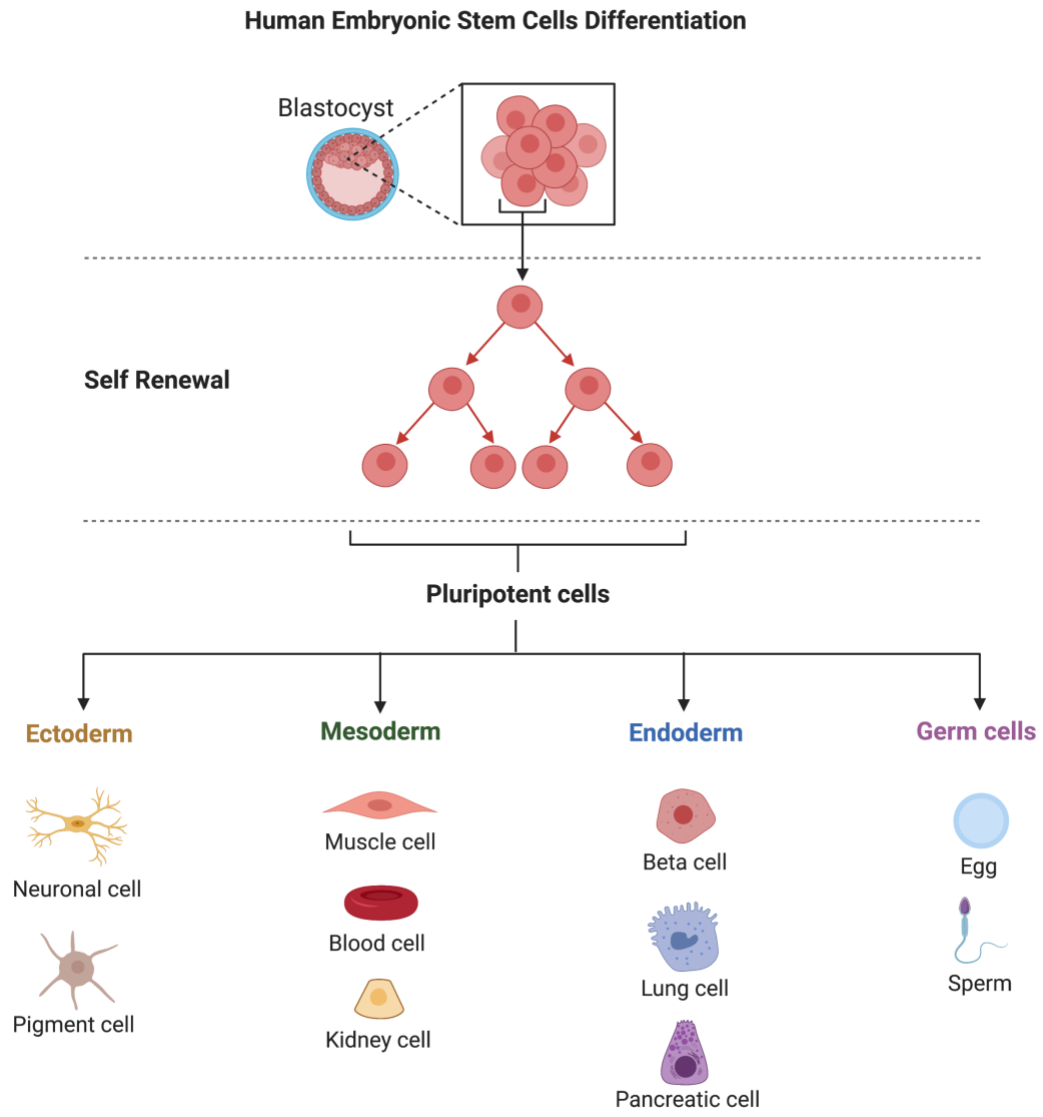


Figure 5. Embryonic Stem Cell Differentiation

ESCs are the inner mass of an embryonic blastocyst, and they can become every cell type depending on the cellular microenvironment. (Created using Biorender.)

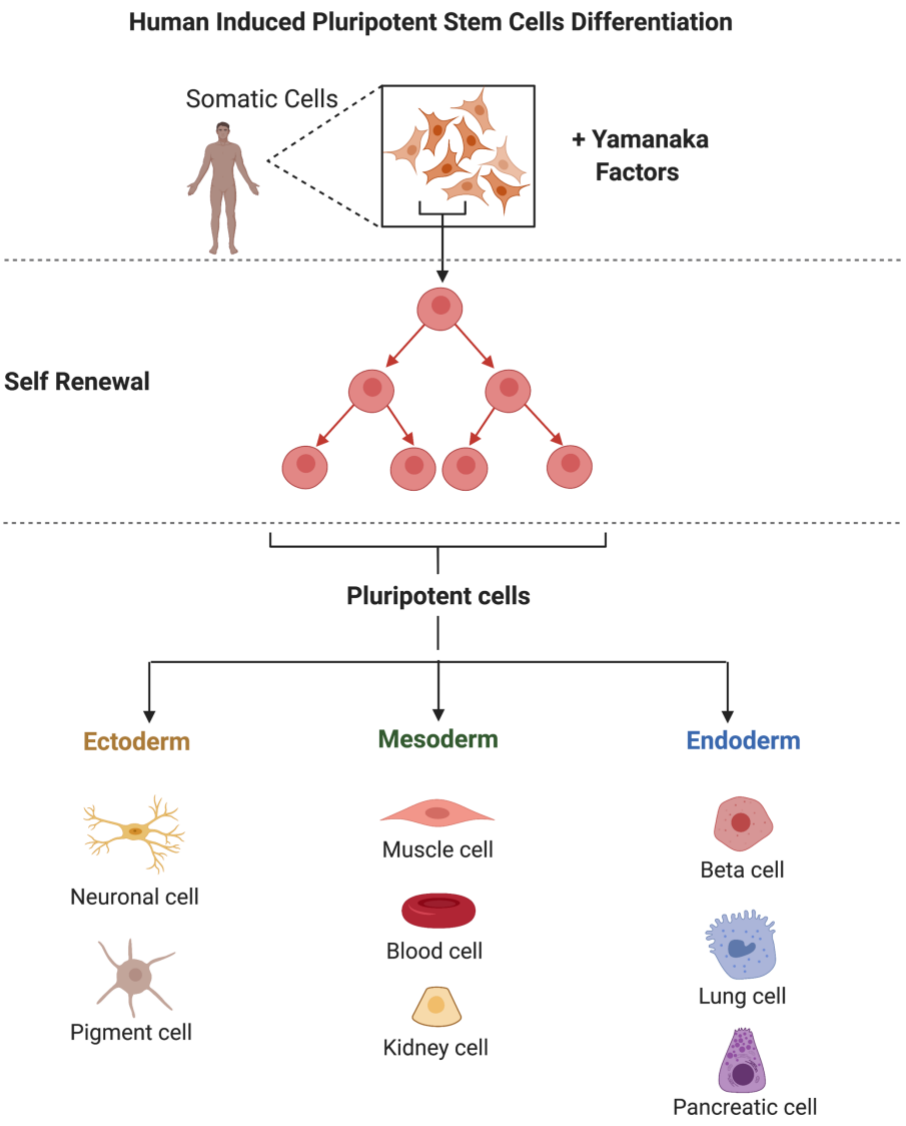


Figure 6. Induced Pluripotent Stem Cell Differentiation

iPSCs are somatic cells that were treated with specific transcription factors. They can become every cell type except a germ cell. (Created using Biorender.)

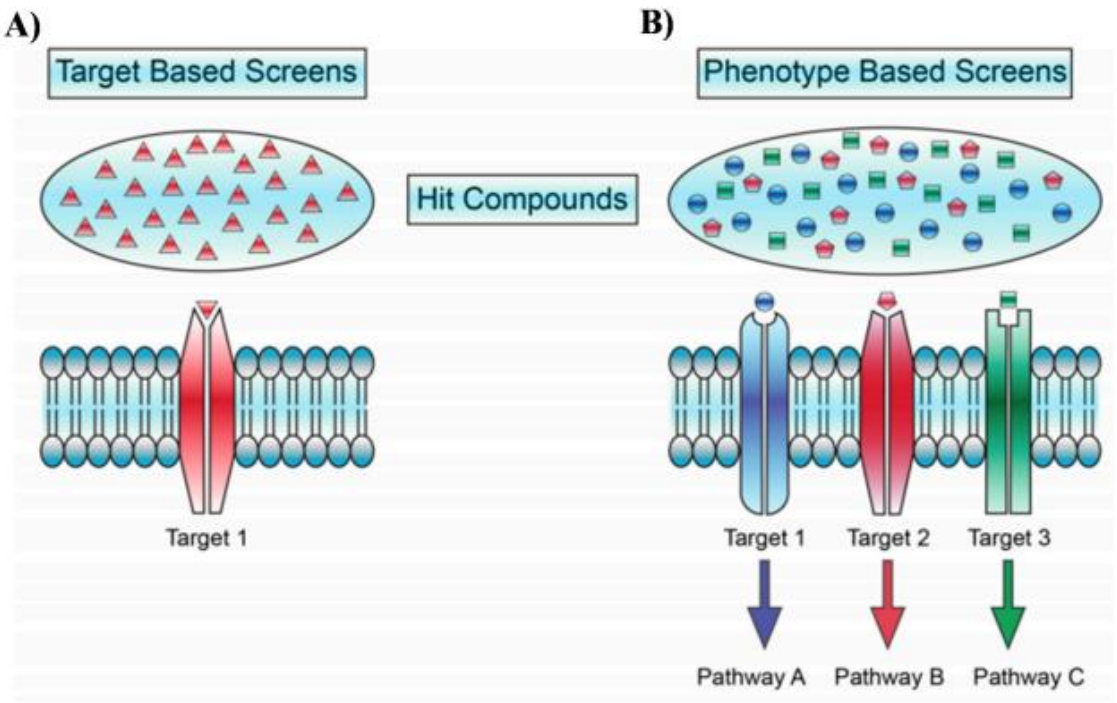


Figure 7. Target-Based vs. Phenotype-Based Screens

(Henry & Wlodkowic, 2019) (A). Target-based drug discovery (TDD) involves screening for compounds interacting with a specific target. (B). Phenotypic drug discovery (PDD) allows a broader target margin while also providing insight into the diverse effects of specific compounds.

Drug Screen using *DGUOK* $^{-/-}$ iPSCs-derived hepatocytes

The Duncan lab previously published article an iPSC-based phenotypic drug screen designed to identify a treatment for MTDPS3 (Jing, et al., 2018). Following a previously published protocol (Si-Tayeb, et al., 2010), the stem cells were differentiated, and their mitochondrial respiration was measured. This experiment was to ensure that the differentiated cells could model mitochondrial function. This protocol concluded that the hepatogenesis did not disturb the natural metabolic progression in the cells. Later, an MTDPS3 model was generated utilizing CRISPR/Cas9 to manipulate the *DGUOK* exon 4. The selected exon resembles patients having similar deletions in this location (Jing, et al., 2018). The cell line contained frameshift deletions of 14 and 5 bp and successfully differentiated into hepatocytes (Figure 8). MTDPS3 patient cells tend to have severe and specific mitochondrial dysfunction, including depletions in mtDNA, ATP production, ETC protein expression, membrane potential, and overall oxygen consumption rate. These phenotypic qualities produced an MTDPS3 phenotype in an iPSC differentiated hepatocyte-like cell (Jing, et al., 2018).

In this previously published article, with a functional *DGUOK* -/- iPSC cell line, a small molecule screen was performed using the SPECTRUM library. The SPECTRUM library consists of 2,400 repurposed compounds that have been previously tested and approved to treat humans (Jing, et al., 2018). Using a luciferase assay to measure ATP production, 34 primary hits were identified, with 15 of them producing at least 20% more energy than the control cells (Figure 10). This experiment utilized the z-factor, a statistical test known for its aptness in high throughput screens (Zhang, Chung, & Oldenburg, 1999). Out of the 15

compounds, one increased ATP levels and ETC genes' expression, nicotinamide adenine dinucleotide (NAD). NAD restores ATP production by activating peroxisome proliferator-activated receptor-gamma coactivator 1-alpha (PGC1-α) through deacetylation by Sirtuin 1 (Sirt 1). NAD increased gene expression of several mitochondrial transcription factors (Jing, et al., 2018). Another compound that increased ATP levels in the *DGUOK* -/- cells was Trimipramine Maleate (TM), which was used as a positive control for the later experiments in this thesis. Ultimately to cure MTDPS3, it is necessary to increase mtDNA content, which NAD could not do and ultimately had a similar response to the *DGUOK* -/- cells not treated with NAD. Therefore, this drug is not an ideal treatment for MTDPS3, but also because the mechanism of how it enters the cell is still unknown, and its inconsistencies in the iPSC model system (Jing, et al., 2018).

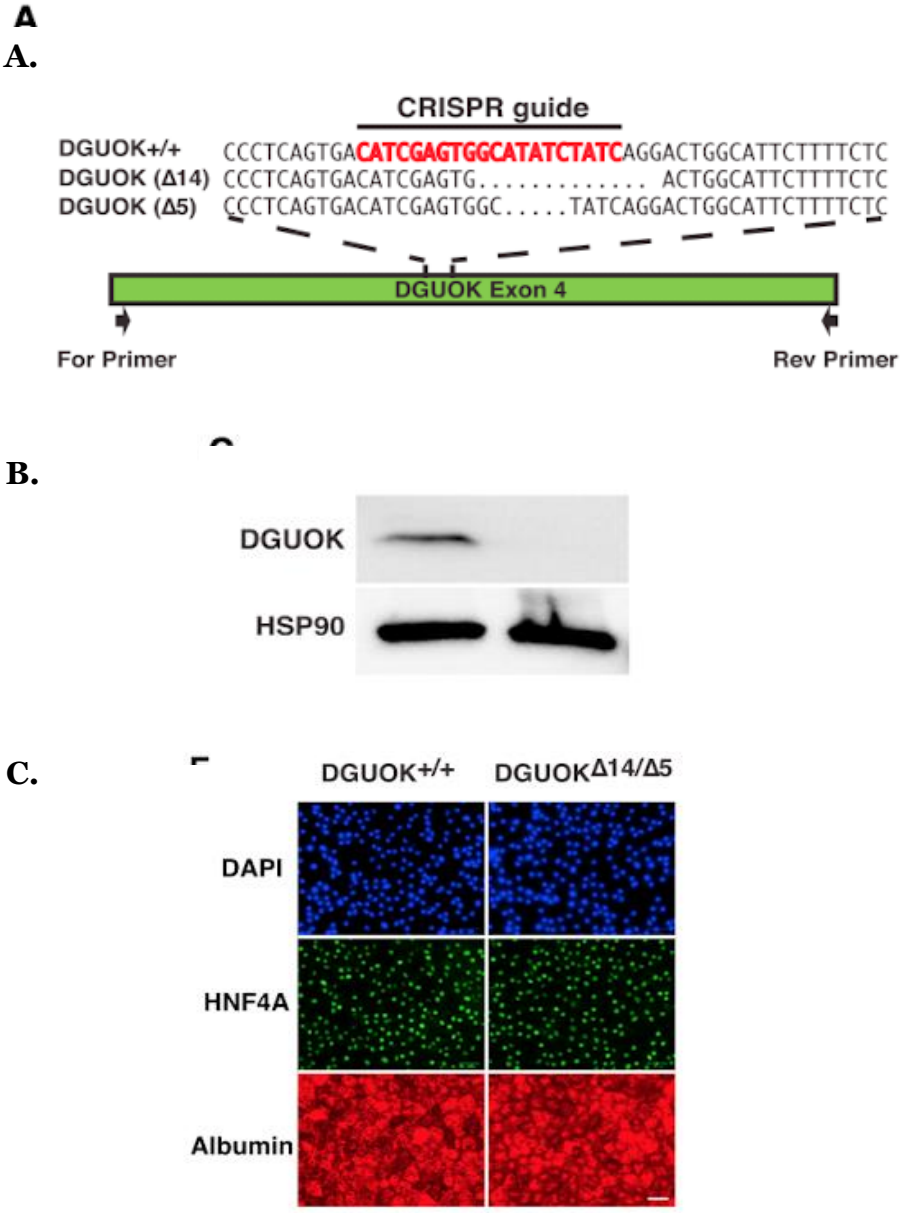


Figure 8. Generation of DGUOK $^{-/-}$ iPSC-derived hepatocytes

(Jing, et al., 2018) (A). A schematic of the knockout cell line that contained frameshift deletions of 14 and 5bp in Exon 4 using CRISPR/Cas9. (B). Immunoblot analysis of cell extracts from *DGUOK* +/+ vs. *DGUOK* Δ14/Δ5 (C). Immunofluorescence showed that the *DGUOK* Δ14/Δ5 cells could differentiate to hepatocyte-like cells.

HSP90: Heat Shock Protein: conserved chaperone protein. HNF4A: Hepatocyte Nuclear Factor: hepatic differentiation regulator. Serum Albumin: Protein produced by the liver.

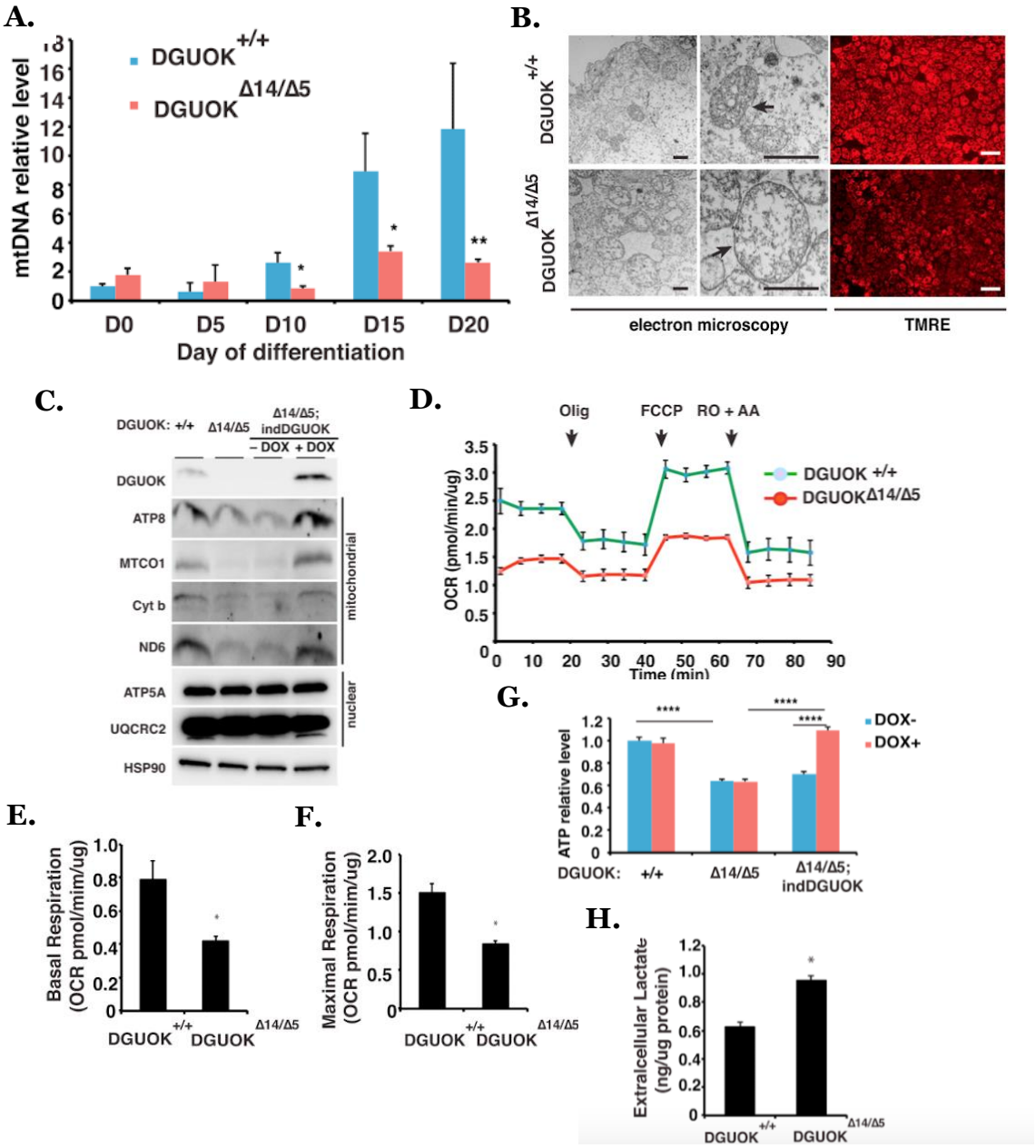


Figure 9. Recapitulating MTDPS3 Phenotype

(Jing, et al., 2018) (A). In the *DGUOK* -/- hepatocyte-like cells, there was a decrease in mtDNA content (B). disruption in mitochondrial morphology and membrane potential, (C). depletion in ETC protein expression (D, E, F) decrease in oxygen consumption rate (oxidative phosphorylation) (G). depletion in ATP levels (H). and an increase in lactate production.

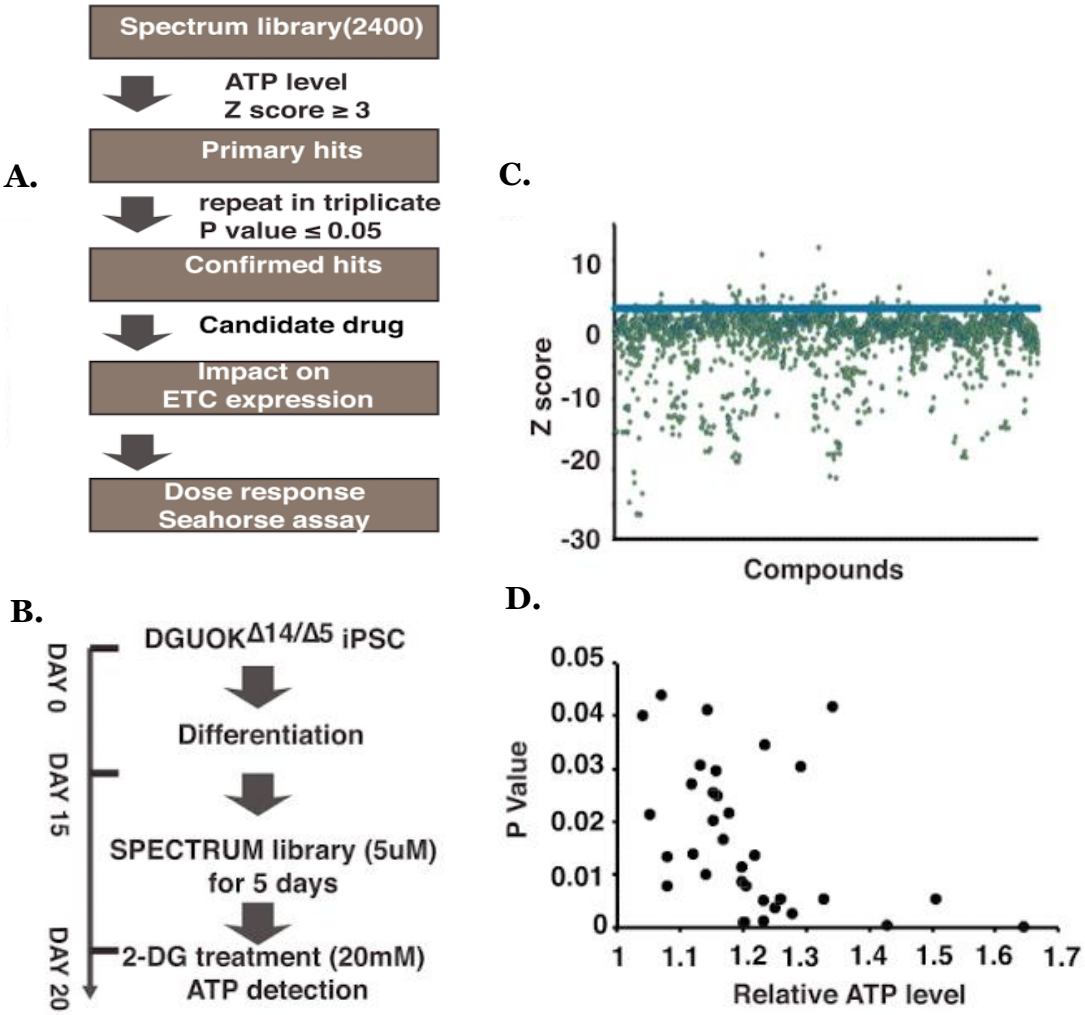


Figure 10. Drug Screen using DGUOK -/- iPSC-derived hepatocyte-like cells

(Jing, et al., 2018) (A). The approach to identifying drugs from the SPECTRUM library. (B). Experimental overview to identify compounds. (C). The results of the primary screen. The blue line indicates a z-score ≥ 3. (D). Relative ATP levels of confirmed hits ($p \leq 0.05$).

Table 2. The 15 Compounds from the Spectrum Library that increased ATP levels

Drug name	Releative ATP level (normalized to DMSO)	P value	Bioactivity
AMINACRINE	1.64	0.0002	local antiseptic
ACRISORCIN	1.51	0.0054	antifungal
NAD	1.43	0.0005	alcohol and narcotic antagonist
ALLYLISOTHIOCYANATE	1.34	0.0416	counterirritant
DEFLAZACORT	1.33	0.0054	antiinflammatory
TRIMIPRAMINE MALEATE	1.29	0.0304	antidepressant
FOSCARNET SODIUM	1.28	0.0025	antiviral
MILRINONE	1.26	0.0053	cardiotonic
THIOTEPA	1.25	0.0038	antineoplastic, alkylating agent
METARAMINOL BITARTRATE	1.23	0.0346	antihypotensive
PROTIRELIN	1.23	0.0011	prothyrotropin
FURAZOLIDONE	1.23	0.0050	antibacterial
METHYLDOPA	1.22	0.0136	antihypertensive
PYRITHIONE ZINC	1.20	0.0080	antibacterial, antifungal, antiseborrheic
IRBESARTAN	1.20	0.0010	angiotensin 2 receptor antagonist

The compounds increased ATP levels by greater than 20% compared to the negative control. The red box highlights NAD, a drug that consistently increased energy production in the *DGUOK* -/- iPSC-derived hepatocytes.

Project Overview

Similarly to Jing, et al., 2018, an iPSC-based phenotypic high throughput drug screen was performed. The South Carolina Compound Collection (SC3) library was selected to complete this project. The hypothesis for this thesis was that compounds from the SC3 screening library would restore ATP production in the differentiated DGUOK -/- cell line. There are approximately 130,000 repurposed chemically diverse compounds adapted and organized for the sole purpose of drug screening. The entire collection, known as the master library, is broken into two sub-libraries, the Representative Set (RS) and the Representatives Set Lite (RSL). The RS set is 10,000 small molecules that are chemically representative of the master library, and the RSL set is the first 1,000 compounds of the RS set. As a proof of concept, the RSL set was tested first. The remainder of the RS set was completed after confirming that small molecules from the SC3 library could be used to drug treat *DGUOK* -/- iPSC-derived hepatocytes. Specific aim 1 was to screen for compounds in the library that could increase energy levels in the mutant iPSC-derived hepatocyte. Specific aim 2 confirmed the hit compounds from the primary screen and identified the candidates that most effectively improved mitochondrial function in the model cells.

CHAPTER 2: MATERIALS AND METHODS

Maintaining iPSCs

Materials

Non-treated P-100 culture dishes (Corning) were prepared for iPSCs by adding 5mL of prepared E-cad ((E-cadherin-Fc) (Nagaoka & Duncan, 2012)) to the plates and storing it at 37 °C for one hour up. The E-cad was aspirated from the dishes, and 2ul/ml of zbFGF to mTeSR1 (Ludwig, et al., 2006) was placed on the p-100. iPSCs were passaged by adding 5mL of room temperature Versene/EDTA (Gibco) to the passaged plate and placed in hypoxic conditions (4%O2/5%CO2) at 37 °C for 4 minutes. After retrieving the p-100, the Versene/EDTA (Gibco) was aspirated off. The cells were washed with 5ml of warm 2ul/ml of zbFGF to mTeSR1 (Ludwig, et al., 2006), and then the aliquoted cell suspension was placed a newly prepared p-100 with media. The dishes were then placed in 4%O2/5%CO2 at 37 °C. The cells were maintained by removing the media every day and feeding each p-100, new 2ul/ml of zbFGF to mTeSR1 (Ludwig, et al., 2006). Due to the rapid growth of the iPSCs, they were passaged to new p-100s every time the dish was around 60-75% confluency, which usually took 3-4 days to occur.

Plating for Cell Differentiation

Once the confluency of the dish was around 75-80%, the p-100s (Corning) were ready for plating. The p-100 to 96-well plate ratio is 1:1. The sterile 96-well plate (Corning) was prepared by adding 60uL of the Matrigel coating (Geltrex and DMEM/F12, 2mg/ml, Invitrogen) to the plate placed at 37 °C. for 1 hour. The media was aspirated off, and 2ml of Accutase (Stemcell Tech) was added to each

p-100. The dishes were then placed in hypoxic conditions (4%O2/5%CO2 at 37 °C) for 2 minutes. Once the p-100s were retrieved, they were washed with warm 2ul/ml of zbFGF to mTeSR1 (Ludwig, et al., 2006) to detach the cells. The cell suspension was collected and centrifuged at 300RCF for 5 minutes in a centrifuge-safe conical tube. After the centrifuge cycle, the media was aspirated, which left the cell pellet untouched at the bottom. The pellet was resuspended with 1ml + 2ul/ml of zbFGF to mTeSR1 (Ludwig, et al., 2006) per 96-well plate. 100ul of cell suspension was pipetted into the 96-well plate and stored in 4%O2/5%CO2 at 37 °C.

Hepatocyte Differentiation

On days 1-2, the recently plated 96-well plate was fed 95uL of RPM1 media (Invitrogen) containing B27 - (20ul/ml, Invitrogen), FGF2 (20ng/ml, Invitrogen), ActA (100ng/ml, R and D systems), and BMP-4 (10ng/ml, R and D Systems) and stored at 37 °C in normoxic conditions (O2/5% CO2). On days 3-5, the cells were fed 95uL of RPMI with B27 - (20ul/ml, Invitrogen) and ActA (100ng/ml, R and D Systems) and stored back at O2/5% CO2 in 37 °C. On day 5, due to the tendency to overgrow, causing cell death, the cells were treated with 60ul Versene/EDTA (Gibco) for 15 seconds before the RPMI media (Invitrogen) was replaced gently. On days 6-10, the differentiating cells were fed 95uL of RPMI media with B27 + (20ul/ml, Invitrogen), FGF2 (20ng/ml, Invitrogen), and BMP-4 (20ng/ml, R and D Systems) and stored in hypoxic conditions (4%O2/5%CO2) at 37 °C. On days 11-15, the cells were fed 95uL of RPMI media (Invitrogen) with B27 + (20ul/ml, Invitrogen) and HGF (20ng/ml, Peprotech) and stored in 4%O2/5%CO2 at 37 °C. On days 16-20, the last five days, the

immature hepatocyte-like cells were fed 95uL of HCM media (Lonza) with OSM (20ng/ml, R and D Systems) and stored in normoxic conditions (O2/5% CO2) at 37 °C.

South Carolina Compound Collection (SC3)

Dr. Charles Beam's donation of 3,000 chemical compounds founded the South Carolina Compound Collection (SC3). Due to the continual contribution from chemists everywhere, this library now contains over 130,000 diverse small chemical molecules. The SC3 is MUSC's first proprietary screening library maintained by Dr. Yuri Peterson (MUSC Drug Discovery Core). The library, as mentioned before, was broken into two smaller libraries using a biochemical algorithm that arranged them by chemical structure (Figure 12). The RS library was utilized for this project, which are 10,000 compounds chemically representative of the entire SC3 master library. All compounds and their chemical properties can be found in a secure and highly organized database.

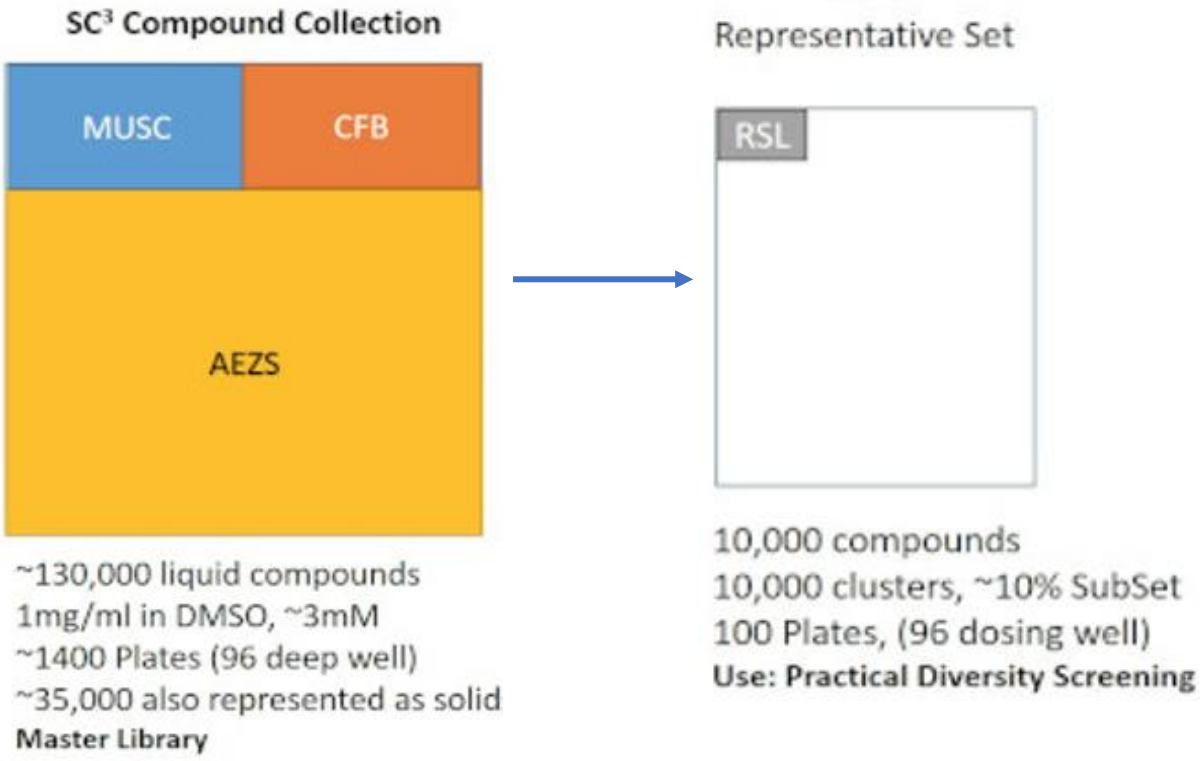


Figure 11. The South Carolina Compound Collection Library

The master library consists of 130,000 chemically diverse small molecules. The representative set (RS) is 10,000 compounds representing the entire master library. The representative lite set (RSL) are the first 1,000 compounds of the RS set.

Drug Treatment

The 10,000 compounds, stored in the MUSC Drug Discovery Core, are currently diluted in a DMSO solution, making their concentration 1mg/ml (calculated by Dr. Yuri Peterson). The lab currently has diluted samples of the compounds in sterile Hanks's Balanced Salt Solution (HBSS) on 96-well plates organized by their RS number and diluted to 100ug/ml, and this has been named the master plate. From the master plate, the "working stock" plate was created so that compounds could be easily accessed. The compounds in the working plate were a 1/5th dilution in HBSS (20ug/ml concentration).

Primary Screen: From days 16-20, the DGUOK -/- iPSC-derived hepatocytes were treated with the 5uL of the small molecules (RS library) and 95uL of HCM media with OSM, which created a final concentration of 1ug/ml concentration. The negative control was 0.1% DMSO, and the positive control was 5uM TM. To accurately measure ATP produced by aerobic respiration with added compounds alone, glycolysis was inhibited by adding 20mM 2-deoxy-D-glucose (2DG) to the cells on the 20th day of feeding and drug treating for 24 hours (Figure 13).

Confirming Hits: The 63 compounds that increased ATP were used to treat day 16 *DGUOK* -/- hepatocyte-like cells at a concentration of 1ug/1ml with replicates (N=8). The rest of the drug treating protocol followed what was mentioned above in the primary screen.

Dose-Response: Six compounds were found to consistently increase ATP levels in the *DGUOK* -/- iPSC-derived hepatocyte. Two of the six compounds were selected to perform an 8-point dose-response assay. Utilizing a two-fold dilution,

the concentrations ranged from 40uM to 0.3125uM. The cells were treated like previously stated above on day 16 and for five days, treated with 2DG on day 20 for 24 hours. On day 21, a luciferase assay was performed to measure ATP production.

Luciferase Assay

ATP production was measured on day 21 DGUOK -/- hepatocyte-like cells using a CellTiter-Glo kit (Progema). The experiment was performed per the manufacturer's instructions. The CellTiter-Glo reagent was taken out of the freezer and placed in a dark location for two hours. An hour and thirty minutes later, the 96-well plates were removed from normoxic conditions (O2/5% CO2) at 37 °C. Once the reagent and plates were at room temperature, the cells were washed with 60ul of Phosphate Buffer Saline (PBS) three times, leaving the third wash on the cells. After the third wash, 60ul of reagent solution was added to the plates. The 96-well plates were immediately covered in aluminum foil and placed on a shaker for 20-30 minutes. The reagent will lyse and interact with the endogenous ATP in the cells. 100ul of the now homogenous solution was transferred to opaque-walled plates and read on a plate reader. This bioluminescent assay works when the ATP in the cells interacts with the luciferin in the CellTiter Glo-reagent. This reaction produces oxyluciferin and releases energy (light) detected by a microplate reader.

Z-Score Analysis

The z-score is a standard statistic test, where the values are measured a certain number of standard deviations away from the average mean of the data set. For this project, compounds that could increase ATP levels in the DGUOK-/-

hepatocyte-derived cells with a z-score of 3 were selected. This means that at least 99% of the remaining compounds are not as significant and are "covered" within three standard deviations of the mean. The z-scores were calculated using this formula, = 1-$\frac{3(\sigma_{S+}+\sigma_{DMSO})}{|\mu_S-\mu_{DMSO}|}$, where σ_S and σ_{DMSO} is the standard deviation of the sample (S) and the DMSO control (DMSO) respectively and μ_S and μ_{DMSO} is the mean of the sample and DMSO.

CHAPTER 3: RESULTS

Experimental Overview

The RSL library was screened first, which is the first 1,000 compounds of the RS library, as a proof of concept (Figure 14). Once the RSL was completed, we moved on to the RS library. Due to the size of the library, we performed our experiment with N=1. Since a reduction of ATP is one of MTDPS3's side effects, we decided to measure if the small molecules could increase ATP in the *DGUOK* $^{-/-}$ hepatocyte-like cells. The cells were differentiated in 96-well plates and treated with compounds with a concentration of 1ug/1ml on day 16, for five days. On day 20 20mM 2-DG was added to inhibit glycolysis for one day. This was to prevent the production of non-mitochondrial ATP (Jing, et al., 2018). ATP was measured by following the directions of the ATP assay kit. This assay uses the enzyme luciferase to lysis and react with endogenous ATP molecules in the cells to produce a light emission that can be read on a plate reader. From this data, I performed a z-factor analysis to identify primary hits (Figure 15). Out of 10,000 small molecules, 63 hit compounds had z-scores The RSL library was screened first, which is the first 1,000 compounds of the RS library, as a proof of concept (Figure 14). Once the RSL was completed, we moved on to the RS library. Due to the size of the library, we performed our experiment with N=1. Since reducing ATP is one of MTDPS3's side effects, we decided to measure if the small molecules could increase ATP in the *DGUOK* $^{-/-}$ hepatocyte-like cells. The cells were differentiated in 96-well plates and treated with compounds with a 1ug/1ml on day 16 for five days. On day 20, 20mM 2-DG was added to inhibit glycolysis for one day. 2-DG was to prevent the production of non-mitochondrial ATP (Jing, et al., 2018). ATP was measured by following the directions of the ATP assay kit.

This assay uses the enzyme luciferase to lyse and react with endogenous ATP molecules in the cells to produce a light emission that can be read on a plate reader. A z-factor analysis was performed to identify primary hits (Figure 15). Out of 10,000 small molecules, 63 hit compounds had z-scores ≥ 3.

Confirming Hits

Following a similar experiment to the primary screen, the 63 hits compounds were tested with replicates, N=8. This experiment confirmed if the compounds were naturally increasing ATP levels in the *DGUOK* $^{-/-}$ differentiated cells. An analysis was performed to determine the validity of the 63 hits compounds, along with a student's t-test to determine significance between the compounds and negative control, DMSO. The drugs needed to have a normalized ratio average ≥ 1 and p-value of ≤ 0.05 to be considered for the final selection. From the 63 compounds, six increased ATP levels at a 1ug/ml concentration (Figure 16). The negative control was 0.1% DMSO, and the positive control was 5uM TM.

The Final Six

Out of 10,000 compounds, six consistently increased ATP levels in the *DGUOK* $^{-/-}$ hepatocyte-like cells (Figure 16). All the compounds had z-scores greater than or equal to 3, normalized ratio averages greater than or equal to 1, and p-values less than or equal to 0.05 (Figure 17).

Dose-Response Assay

Following the final requirement of an iPSC-based drug screen, a dose-response assay was performed to assess the compounds' maximal concentration

effect and toxicity levels. (Elitt, Barbar, & Tesar, 2018). Due to time restrictions, only two compounds were selected to be tested with an 8-point dose-response assay. The concentrations ranged from 40uM and 0.3125uM with a two-fold dilution. The first small molecule chosen was D-09311 due to the initial z-score of the primary screen. The dose-response graph shows the expected sigmoidal curve with increasing effects and increasing concentration (Figure 18). However, around 40uM, ATP production began to decline. This data could be the outcome of drug toxicity; fewer cells make fewer ATP molecules. Regardless, it would be best to explore more concentration levels, specifically higher than 40uM and lower than 20uM.

The second compound, D-88920, was selected due to drug availability. The dose-response graph showed that ATP production was increased around 50% for all eight concentrations compared to the negative control, DMSO (Figure 19). It could be assumed that this small molecule reached the maximum effect range at the lowest concentration, 0.3125uM, if it followed the schematic of the dose-response graph. It can also be concluded that this specific compound has a low concentration range where ATP production is increasing. To further analyze this, it would be wise to explore drug concentrations in the nanomolar range.

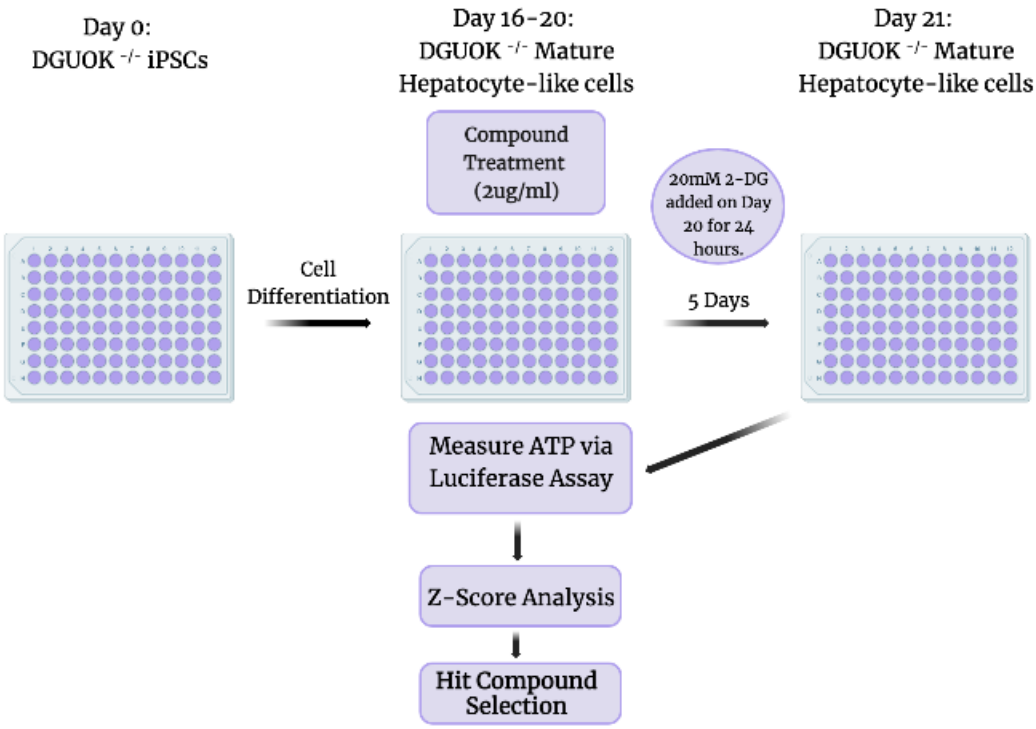


Figure 12.Thesis Experimental Design

An overview of this thesis project. Using compounds from the SC3 library to increase ATP production in *DGUOK* $^{-/-}$ iPSC-derived hepatocytes.

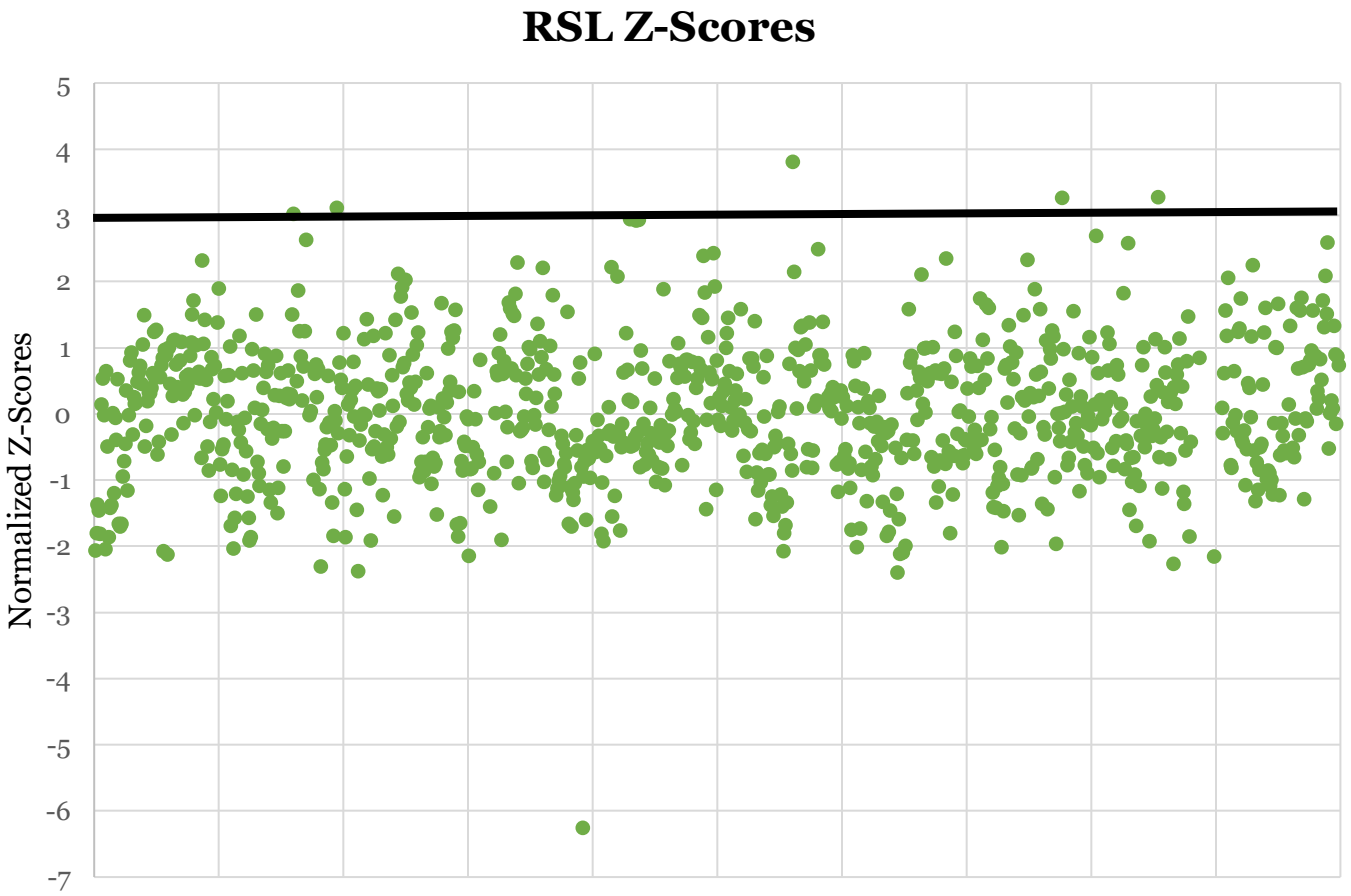


Figure 13. RSL Z-Scores

As a proof of concept, 1,000 compounds of the RSL set were tested (N=1) to see if they could increase ATP levels in the *DGUOK* $^{-/-}$ iPSC-derived hepatocytes. These are the z-scores of the first 1,000 compounds of the RS set. The emission score detected from the plate related were converted to Z-scores and normalized to the negative control, DMSO. The black line indicates the cutoff at a z-score of 3. The small molecules with negative z-scores were assumed to have killed the cells.

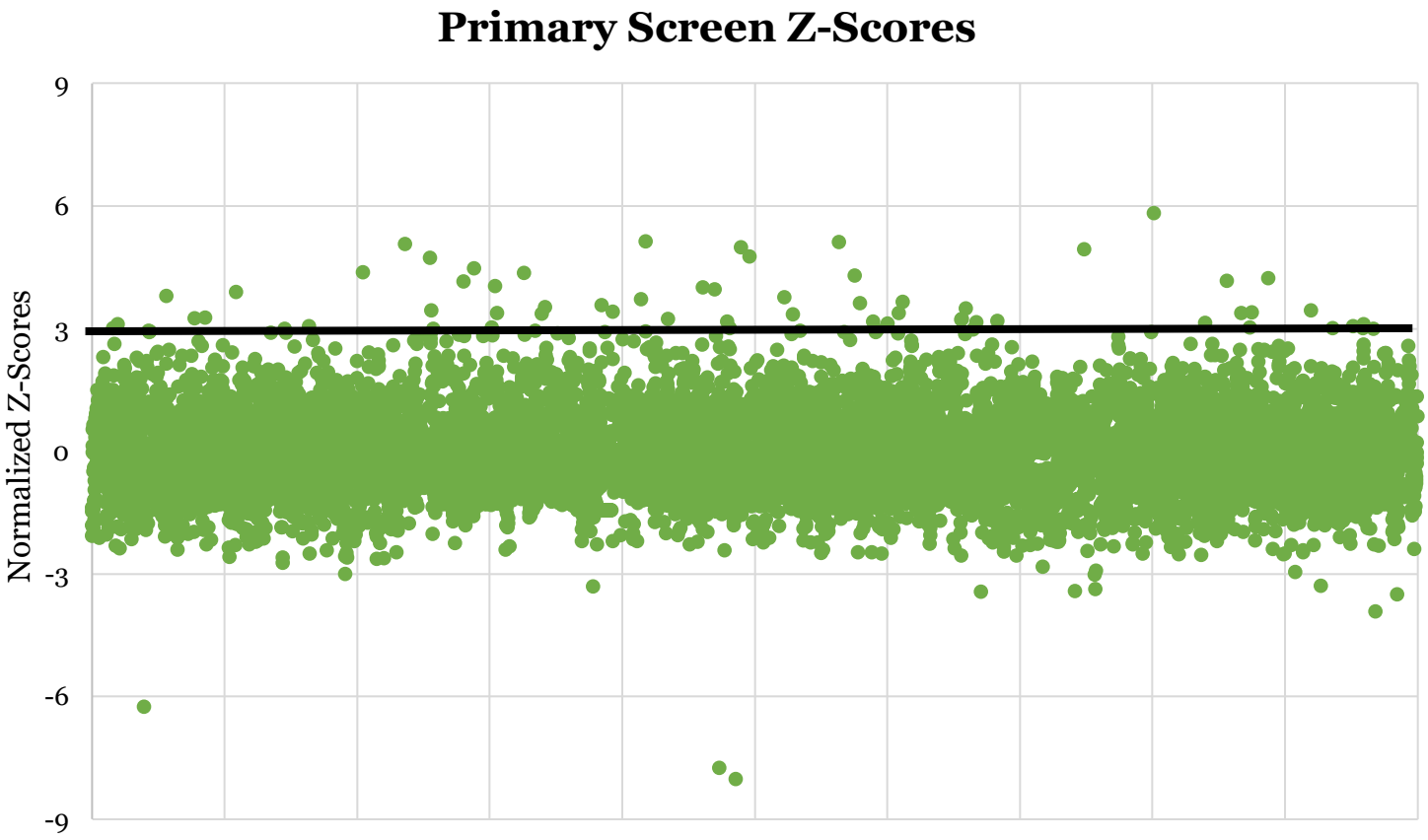


Figure 14. Primary Screen of the SC3 Library

Day 16 DGUOK -/- iPSCs derived hepatocytes were treated with each compound (N=1) at an average 2ug/ml concentration for five days. The result of the primary screen is the z-scores of the 10,000 compounds from the SC3 library. The z-scores were normalized to the negative control DMSO. The black line indicates the cutoff at a z-score of 3.

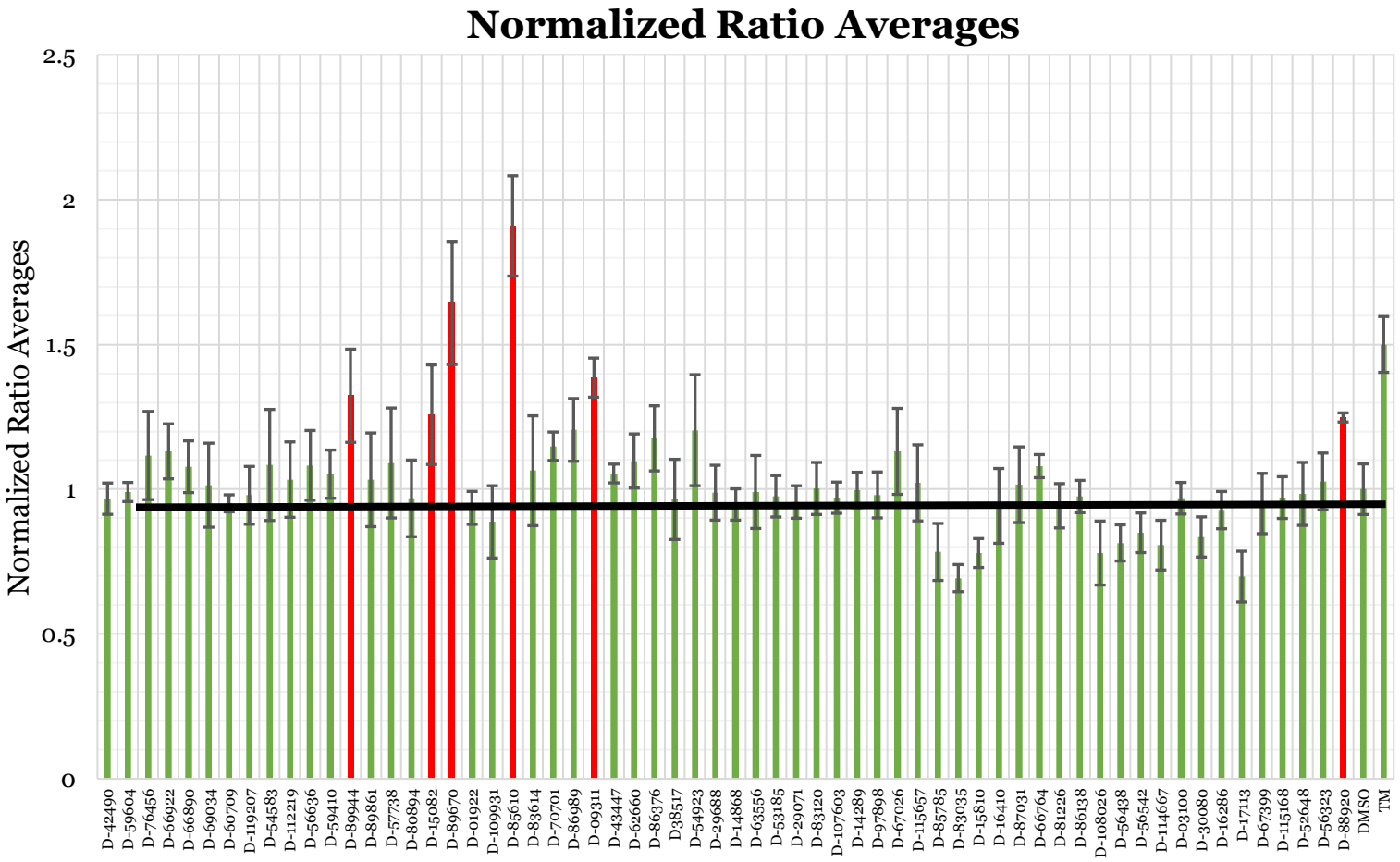


Figure 15. Confirming the hits of the Primary Screen

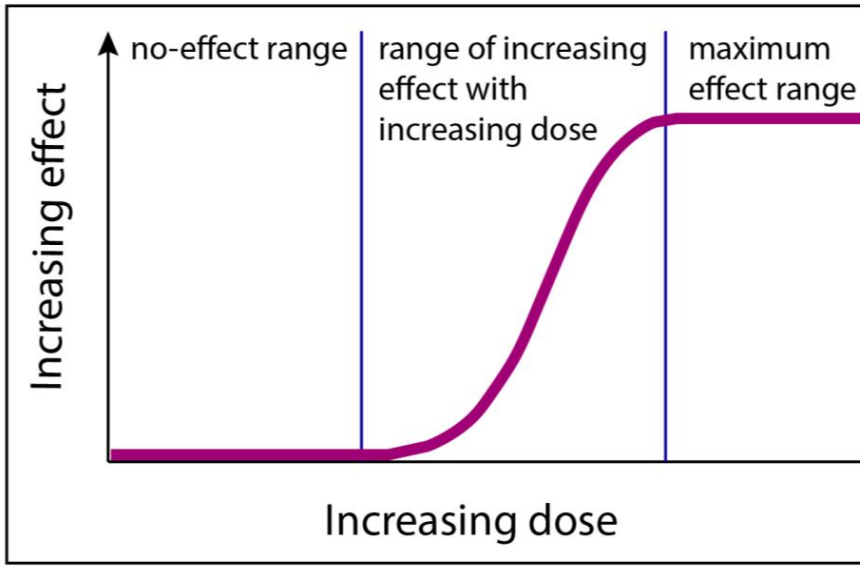
Biological replicates were performed with the 63 compounds (N=8) following the same experimental design as the primary screen. Out of the 10,000 small molecules, 63 successfully increased ATP levels compared to the negative control (DMSO) (Z-scores ≥ 3). The black line indicates the cutoff at a ratio average greater than one. The red bars indicate the compounds selected for moving forward, ratio averages ≥ 1 and $p \leq 0.05$

Table 3. Final Six Compounds

Compounds	Initial Z-Scores	Ratio Average Values	P-Values
D-89944	4.007	1.323	0.0005
D-15082	3.763	1.257	0.0034
D-89670	4.978	1.642	2.22E-05
D-85610	4.372	1.910	2.33E-07
D-09311	5.058	1.385	4.83E-08
D-88920	4.763	1.248	0.0508

These are the six small molecules that can increase ATP levels consistently.

A.



B.

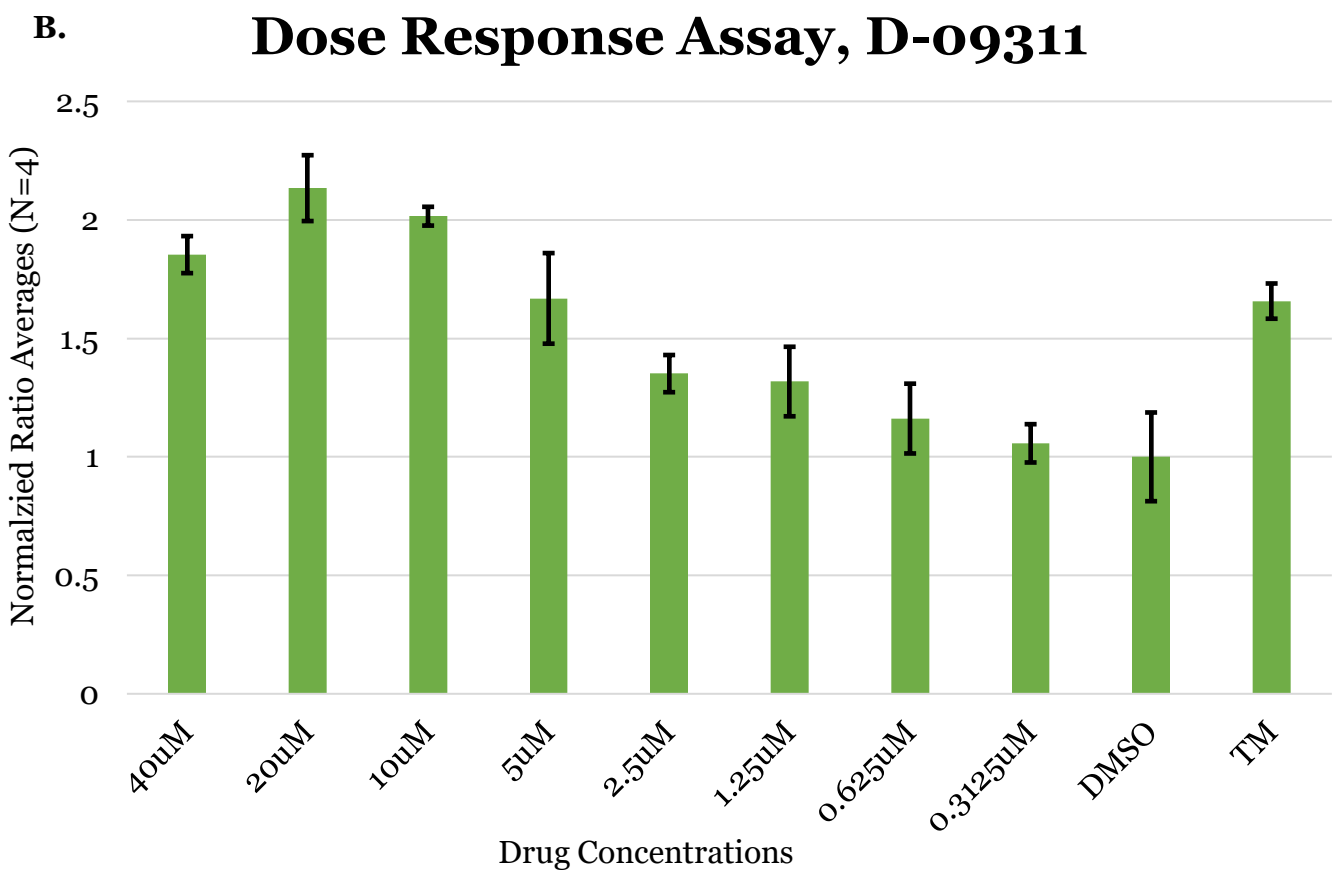
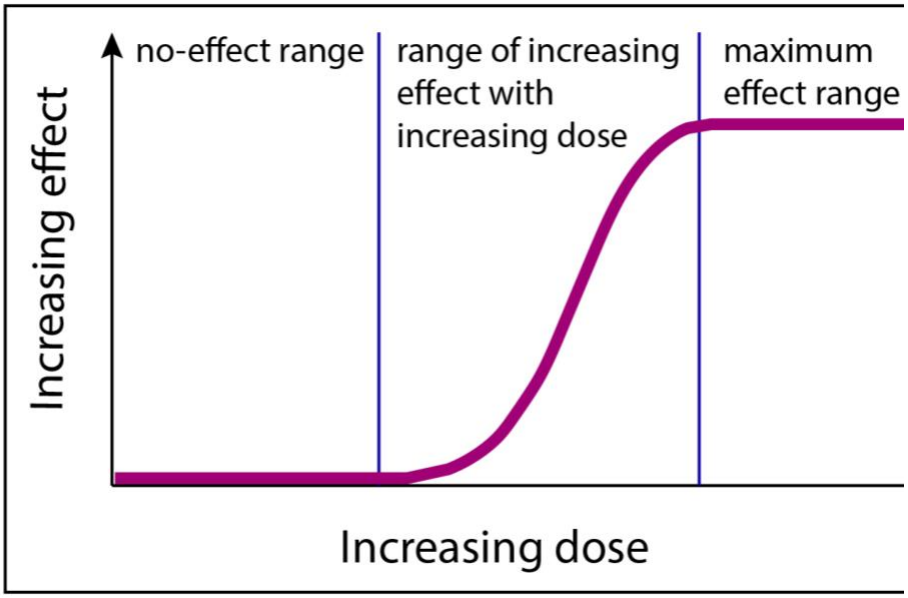


Figure 16. 8-Point Dose Response Assay, D-09311

(A). A dose-response schematic. (B). Compound D-09311 had an increasing effect on ATP production with increasing drug concentration.

A.



B.

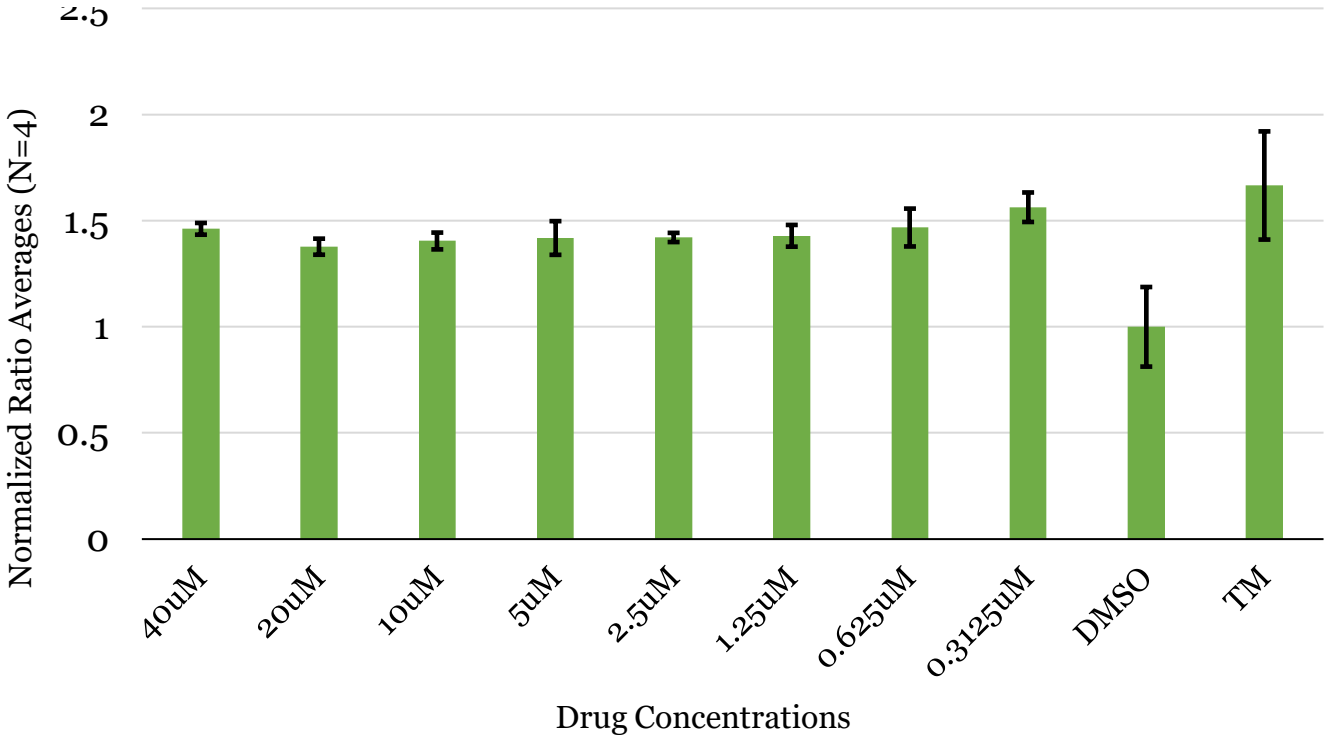


Figure 17. 8-point Dose Response Assay, D-88920

(A). A dose-response schematic. (B). Compound D-88920 reached the maximum effect of ATP production with all drug concentrations.

CHAPTER 4: DISCUSSION

MTDPS3 is fatal to its patients, and a novel treatment is needed despite its rarity. This thesis project aimed to use a high throughput drug screen to find said treatment. Utilizing iPSCs that were differentiated into hepatocyte-like cells containing the *DGUOK* mutation as models, the SC3 library's RS sub-library was narrowed down to six compounds. These six compounds D-89944, D-15082, D-09311, D-89670, D-85610, and D-88920, increased ATP levels in the knockout cells. This thesis is the first step in finding a treatment that can treat MTDPS3 in-vitro. Two out of the six compounds are similar in chemical structure, reassuring us that they have similar activity in the differentiated cells. Since the RS set was representative of 120,000 compounds, it was decided to find the structurally related compounds of our candidate hits, and 148 small molecules were at least 60% percent similar to our final six. It would be interesting to see if these molecules can increase ATP and affect this disease's pathology.

The following steps in this project will confirm the small molecules as potential treatments for *DGUOK* deficiency. Along with an increase in ATP production, the most vital assay would be to measure mtDNA content in the knockout cells with the hit compounds. Measuring mtDNA is crucial to finding the cure to MTDPS3. There is a possibility that some or all the compounds will affect the production of mtDNA. If so, then the mitochondria in those cells must have the purine nucleotides needed to produce mtDNA; in this case, that would explain the increased production in ATP because, as mentioned above, the mitogenome codes for the ETC complexes. However, just like NAD, the compounds could not positively influence mtDNA content, but there is a possibility that they can.

It is known that mtDNA needs a continuous supply of dNTPs for mitochondria homeostasis (Almannai, El-Hattab, & Scaglia, 2018). This is through specific nucleotide salvage pathways, including *DGUOK* and *TK2* and mitochondrial transport proteins (Copeland, 2008; Spinazzola, 2011). Cytosolic nucleotides are also imported to the double-membraned organelle by two primary enzymes: thymidine phosphorylase and ribonucleotide reductase (RNR) (Spinazzola, 2011; Almannai, El-Hattab, & Scaglia, 2018; Thelander, 2007; El-Hattab, Craigen, & Scagila, 2017). The MTDPS3 phenotype has a depletion in purine ribonucleotides. Therefore, if the compounds could increase mtDNA content, it would not interact with thymidine phosphorylase. RNR, however, has been seen to supply all dNTPs to the mitochondria for mtDNA replication (Thelander, 2007). The candidate compounds have successfully increased ATP production in *DGUOK* -/- iPSC-derived hepatocytes, which could be due to the rescue of mtDNA through RNR.

It will also be imperative to measure mitochondrial morphology and membrane potential. The DGUOK mutation causes detrimental effects on mitochondrial morphology. In similar studies, *DGUOK* -/- cells and tissue samples have disruptions in the mitochondrial structure, the matrix, and cristae formation (Dimmock, et al., 2008; Jing, et al., 2018). Along with mitochondrial morphology disruption, there seems to be a consistent decrease in mitochondrial membrane potential in the cells (Jing, et al., 2018). Membrane potential is the product of chemiosmosis during oxidative phosphorylation; restoring it could have effects on MTDPS3 pathophysiology as well. Hopefully, this project is just the beginning of a revolutionary approach to treating mitochondrial depletion

syndromes. In the future, once the mechanism of action is known, and the compounds can successfully restore the cells to wild-type mitochondrial functions, animal studies and ultimately clinical trials are the only stages away from improving the lives of MTDPS3 patients.

CHAPTER 5: APPENDIX

Additional Dose-Response Assays

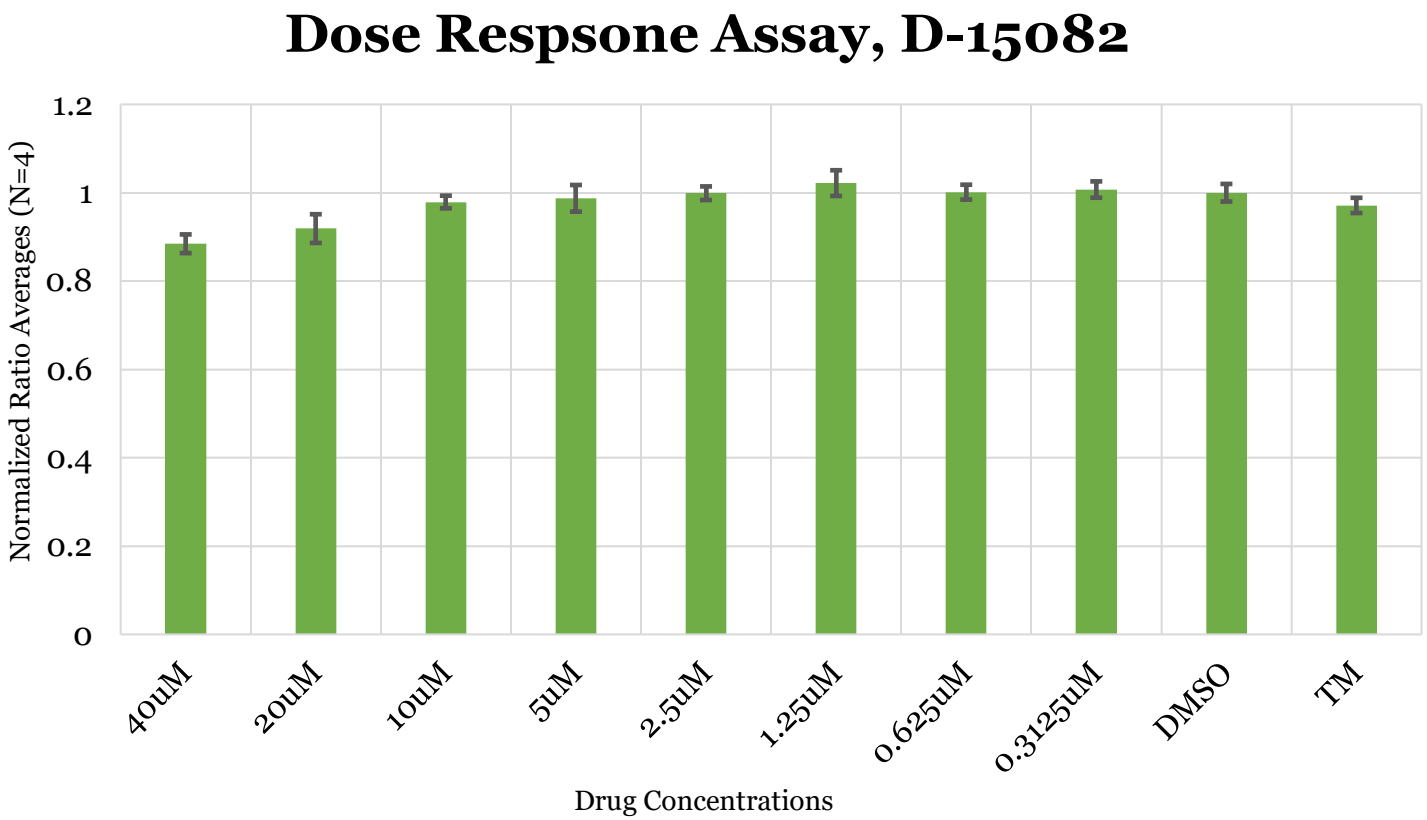


Figure 18. Dose Response Assay, D-15082

The first attempt of a dose-response assay using compound D-15082 was unsuccessful. The positive control, TM, did not have the anticipated increased ATP production response. This result was due to the constant freezing and thawing of this chemical, and over time it lost some of its chemical properties that increased ATP levels. Therefore, this data is unusable and undecipherable. It was expected that the 5uM concentration would increase ATP production due to its similarity to the concentration used in the primary screen. There is the possibility that the *DGUOK* -/- iPSC-derived hepatocytes did not differentiate properly, or there was unnoticeable cell death. This could be due to human error or the cells being a higher passage number, resulting in spontaneous and unexpected differentiation.

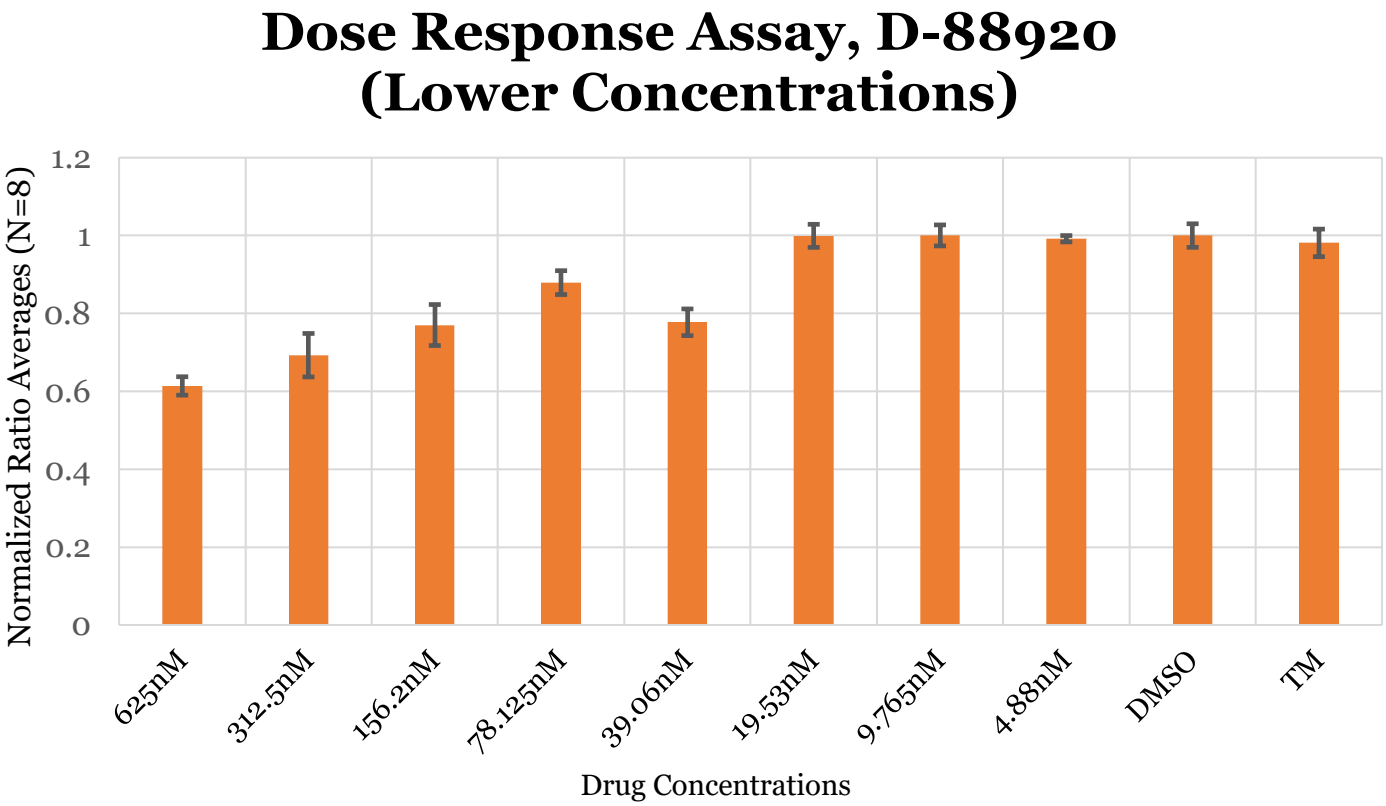


Figure 19. Dose Response Assay, D-88920 Lower Concentrations

The second attempt of a dose-response assay using compound D-88920 was unsuccessful. The positive control, TM, did not have the anticipated increased ATP production response. This result was due to the constant freezing and thawing of this chemical, and over time it lost some of its chemical properties that increased ATP levels. Therefore, this data is unusable and undecipherable. It was expected that the 5uM concentration would increase ATP production due to its similarity to the concentration used in the primary screen. This could be due to human error or the cells being a higher passage number, resulting in spontaneous and unexpected differentiation.

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