Neopeltolide-inspired compound mediated transdifferentiation of Mesenchymal Stem cells (MSC) to neuron-like cells

Dissertation submitted in partial fulfillment for the degree of

Master of Science in Applied Microbiology

Submitted By

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CERTIFICATE

This is to certify the dissertation entitled **"Neopeltolide-inspired compound mediated transdifferentiation of Mesenchymal Stem cells (MSCs) to neuron-like cells"** Submitted by **Samarpita Tarafder**in partial fulfilment of the requirement for the degree of Master of Science in Applied Microbiology, KIIT School of Biotechnology, KIIT to be deemed University, Bhubaneswar bearing Roll No. **1662014** & Registration No. **16675158073** is a bonafide research work carried out by her under my guidance and supervision from **16th January 2018** to **12th May 2018**.

Dr. Subhadra Dravida

Name and signature

CERTIFICATE

This is to certify that the dissertation entitled "**Neopeltolide-inspired compound mediated transdifferentiation of Mesenchymal Stem cells (MSC) to neuron-like cells"** submitted by name **Samarpita Tarafder** Roll No. **1662014** Registration No. **16675158073** to the KIIT School of Biotechnology, KIIT to be deemed University, Bhubaneswar-751024, for the degree of Master of Science in **Applied Microbiology** is his original work, based on the results of the experiments and investigations carried out independently by her during the period from **15th January 2018** to **12th May 2018** of study under my guidance.

This is also to certify that the above-said work has not previously submitted for the award of any degree, diploma, fellowship in any Indian or foreign University.

Date: 12-05-18 Dr. Madhu Mohan Rao

Place: Hyderabad *Supervisor name & signature*

DECLARATION

I hereby declare that the dissertation entitled "**Neopeltolide inspired compound mediated transdifferentiation of Mesenchymal Stem cells (MSC) to neuron-like cells**" submitted by me, for the degree of Master of Science to KIIT University is a record of bonafide work carried by me under the supervision of Dr. **Madhu Mohan Rao**, Chief Scientist, Tran-scell Biologics, ALEAP industrial estate, Plot No- 64, Road No- 5, Hyderabad, Telangana 500090.

Date: 12-05-18 Samarpita Tarafder

Place: Hyderabad *Name & Signature*

ABSTRACT

For a range of conditions, the neurodegenerative disease is an umbrella term in which primarily the neurons are affected in the brain. Neurons are basically the building blocks of the central nervous system (CNS) which includes the brain and the spinal cord (Khanna *et al.*, 2016)(Weninger *et al.*, 2016). Generally, neurons cannot be replaced by the body when they become damaged or die cause they don't reproduce or replace themselves.

There are lots of examples of neurodegenerative diseases like Parkinson's, Alzheimer's, Huntington's disease etc. Neurodegenerative diseases are exhausting conditions that are incurable and as a result, there is progressive degeneration and /or death of neuron cells and it causes problems with patient movement (ataxias), or mental functioning (dementia) (Dimos *et al.*, 2011)(Engelender and Isacson, 2017)(Lee *et al.*, 2011)(Narayan, Ehsani, Lindquist, 2014)(Dobson, 2017).

Dementias are mainly responsible for the greatest burden of disease with Alzheimer's representing 60-70% of cases approximately. As opposed to modifying these diseases existing treatments tend to address symptoms. Research in (target identification, mechanism of action based) drug development has been stung by the recent setbacks of several novel compounds in Phase III trials. There is no pipeline of novel drug candidates discovered for targeted Neurodegenerative disease cure. So consequently, there is an urgent need for new and next generation drug candidates with identified regeneration properties addressing the degeneration pathologies of the disease in the patients' context. The development of novel nature-inspired hybrid complex small molecules and the importance of screening new libraries built on patient-derived precise/predictive progenitor cellular platforms are supposed to be the first step to discover new drug candidates, while proposing combinatorial drug regime, the only approach available to the scientific community in treating Neurodegenerative diseases, that this proposal aimed at.

We stumbled upon a novel Neopeltolide inspired scaffold with Neurogenerative and Neuroprotective properties identified in the labs on an adult Stem Cell-based predictive platform. The next generation sequencing of the Stem cell platform genome after exposing to pure scaffold chemistry has revealed sets of neuron-specific genes regulated highlighting some of the genes involved in Neurodegeneration disease pathology.

ACKNOWLEDGEMENTS

I am grateful to KIIT School of Biotechnology, KIIT to be deemed University for providing me the opportunity to work as a Project Trainee as a compulsory part of our academic curriculum. It helped me to witness research work closely and boosted my interest towards research. I also thank **Dr. Mrutyunjay Suar** (Head of the Department), for recommending me to work in Tran-scell Biologics Pvt Ltd with the Chief scientist Dr**. Madhu Mohan Rao**. My honest gratitude to **Dr. Subhadra Dravida**, Founder, CEO of Tran-scell Biologics Pvt Ltd for providing me the opportunity for training in this renowned company with an esteemed status in the field of neurological drug discovery.

I express my sincere gratitude to **Dr. Madhu Mohan Rao, Chief Scientist, IDI, IICB** for thinking me worthy enough to work in his lab for a period of five months (January, 2018-May, 2018). He has served as a wonderful guide and working with him inspired me to learn a lot. His loving and caring nature made me very comfortable.

My special thanks to the person **Dr. Raveendra Babu as immediate supervisor,** who has supported me both technically and morally in completing my training. He is a very experienced scientist, has a lot of working knowledge and maintains a strict working schedule. I am truly honored to work with his and want to thank him for supporting me always and teaching me skills and techniques that will surely be beneficial for me in future. My heartfelt gratitude to the Vice-President and Scientist **Mr. Leela Krishna Gorthi** for his support and encouragement.

My project would have been incomplete without the help of my staff members of the department **Ms.A. Sushma, Mr. Thirupati Reddy**and**, Naveen Kumar.**

I also convey my thanks and regards to my family for their constant support, wishes, understanding, motivation, which are an invaluable possession to me than anything else and which made my journey so fruitful at Tran-scell Biologics Pvt Ltd. Above all, I would like to thank **God** for being with me always without whose support I could do nothing. I shall always cherish these memories in life forever. Thank you all!!!

DATE: 12-05-18 (Samarpita Tarafder)

PLACE: Hyderabad name and signature

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ABBREVIATIONS

MSC	Mesenchymal Stem cell
NSC	Neuronal Stem cell
BBB	Blood-Brain Barrier
ALS	Amyotrophic lateral sclerosis
CNS	Central nervous system
BM	Bone Marrow
WST-1	Water Soluble Tetrazolium-1
Neo-O	Neuron- Orange
RT- PCR	Real-time PCR
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco Modified Eagle Medium
FBS	Fetal Bovine Serum
HPMSC	Human Pluripotent Mesenchymal stem cells

LIST OF FIGURES

INTRODUCTION

Neurological disorders are the break down of the nervous system (Dimos *et al.*, 2011)(Engelender and Isacson, 2017)(Lee *et al.*, 2011)(Narayan, Ehsani, Lindquist, 2014)(Dobson, 2017) and these could arise from either biochemical abnormalities or due to abnormalities in the brain, spinal cord. This could finally lead to muscle weakness, memory loss, poor coordination, forgetfulness, loss of sensation, depression, seizures, confusion, mood changes and altered levels of consciousness (Khanna *et al.*, 2016)(Weninger *et al.*, 2016)(Brunden *et al.*, 2014). Parkinson's, Alzheimer's, Amyotrophic lateral sclerosis (ALS), Huntington's disease etc are the example of neurodegenerative diseases(Gitler, Dhillon and Shorter, 2017).

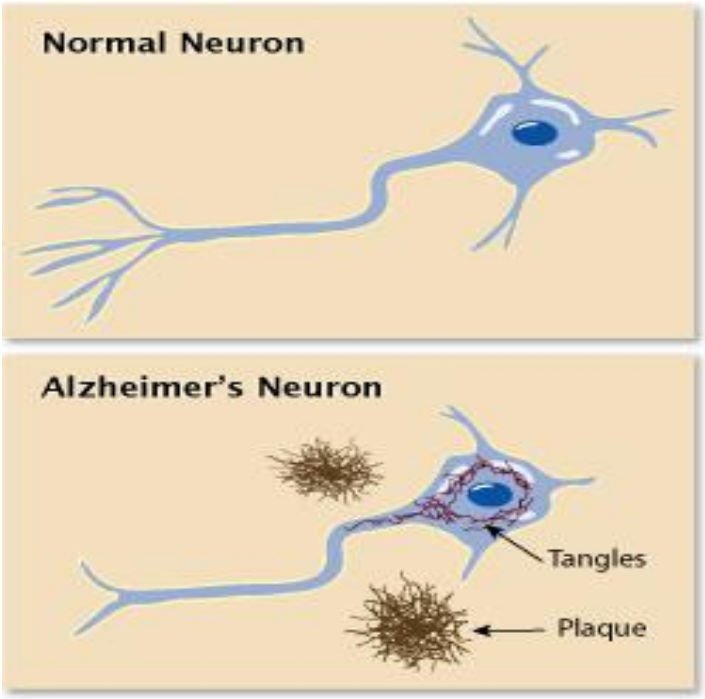


Figure 1: Comparison between a normal neuron and Alzheimer's neuron (*Getting Alzheimer's - Alzheimer's Project*, no date).

Alzheimer's disease is a very common chronic neurodegenerative disease that usually slowly destroys memory and worsens over time. Even 60% to 70% of cases of dementia could be the result of neurodegenerative disorders (*Amyotrophic Lateral Sclerosis (ALS) Fact Sheet | National Institute of Neurological Disorders and Stroke*,

no date). Advanced brain imaging technique helps to see the spread and development of abnormal amyloid and tau proteins in the living human brain, as well as changes in brain structure and function (Kocahan and Doğan, 2017)(*ALZHEIMER'S DISEASE: MOLECULAR MECHANISMS*, no date). There is a genetic component to some cases of early-onset Alzheimer's disease. Late-onset Alzheimer's arises from a complex series of brain changes which happens to people age 65 and older. As it is a progressive disease more and more brain cells die and Alzheimer's leads to brain shrinkage significantly. Two types of hallmarks of Alzheimer's are Plaques and Tangles. Clumps of a protein called beta-amyloid are plaques which may damage and destroy brain cells. Tangles are formed when a microtubule-associated protein known as tau becomes hyperphosphorylated causing it to aggregate, or group, in an insoluble form. In Alzheimer's, inside brain cells threads of this tau protein twist into abnormal tangles, leading to failure of the transport system and also strongly implicated in the decline and death of brain cells (Crews and Masliah, 2010).

Parkinson's Disease, occur in people over age 60. Dopamine, a substance released from neurons acts as a messenger chemical between two brain areas - the substantia nigra and the corpus striatum - to send signals to other nerve cells and produce smooth movements (Dauer and Przedborski, 2003)(Maiti, Manna and Dunbar, 2017). Most of the movement-related symptoms of Parkinson's disease are caused by a lack of dopamine due to the loss of dopamine-producing cells in the substantia nigra. When the amount of dopamine becomes too low, communication between the two brain areas (substantia nigra and corpus striatum) becomes ineffective, and movement becomes impaired; the greater the dopamine loss increases, the worse the movement-related symptoms. Many other cells in our nervous system also degenerate to some degree which may result in non-movement related symptoms of Parkinson's disease. In addition, abnormal clumps called Lewy bodies, which associated with deposits of a protein called alpha-synuclein in the brain of individuals with Parkinson's disease. The function of these clumps in regards to Parkinson's disease is not fully understood. In general, scientists suspect that dopamine loss is due to a combination of environmental and genetic factors (*Discovery medicine on the leading edge.*, no date).

Amyotrophic Lateral Sclerosis (ALS), motor neurons are found in the brain and spinal cord and are responsible for controlling voluntary muscle movement. As ALS

progresses, these cells degenerate and die meaning the symptoms get worse over time (Turner *et al.*, 2013)(Cluskey and Ramsden, 2001). They stop sending messages to muscles. Eventually, the brain no longer controls voluntary movement, and the muscles gradually weaken and waste away (atrophy). ALS can be caused by the disorganized immune response, the immune system may attack some of the body's cells and possibly killing nerve cells. People with ALS often have higher levels of glutamate. Normally glutamate is cleared from the nerve cell junctions to keep the messages brief. Molecules called transporters aid in keeping glutamate in proper concentrations near the motor neurons. Prolonged excitation of glutamate is toxic to nerve cells (Paez-Colasante *et al.*, 2015).

Neuronal stem cell (NSC) transplantation is one of the major strategies proposed replacing the damaged neurons with potential for treatment of such neurological disorders (Li *et al.*, 2016). However, the NSC transplantation will be hampered by the limited number of brain donors and the toxicity of allogeneic transplantation. These limitations may be avoided if NSCs can be generated from bone marrow (BM), peripheral blood cells and embryonic stem cells that are suitable for autologous transplantation(Pawitan, 2011).

Consequently, there is an urgent need for new and next generation drug candidates with identified regeneration properties addressing the degeneration pathologies of the disease in the patients' context. Existing treatments tend to address symptoms as opposed to modify disease course. Research in (target identification, mechanism of action based) drug development has been stung by the recent setback of a novel compound. The development of novel nature-inspired hybrid complex small molecule and the importance of screening new libraries built on patient-derived precise/predictive progenitor cellular platforms is the first step to discover new drug candidates, while proposing combinatorial drug regime, the only approach available to the scientific community in treating Neurodegenerative diseases, that this proposal aims to build (Martin *et al.*, 2015).

Due to the lack of biologically relevant research model, mainly when it comes to the study of various neurological disorders, the use of human-derived (healthy donors) stem cells (for example, mesenchymal stem cells) and their use in the generation of

neuronal cells for studying specific neurological disorders is gaining serious attention. This is an attractive research direction because the use of stem cells (ranging from healthy donors to neuro-patients) in inducing the differentiation into neuronal cells is opening new opportunities in this highly complex disease area (Li *et al.*, 2014). Although there are several examples of differentiating stem cells to neurons by the use of different types of induction media, another emerging direction is the use of small molecule for directly causing this effect.

Recent studies showed that mesenchymal stem cells (MSCs) were able to trans-differentiate into functional neuronal cells, opened up the possibility of using MSCs to replace damaged neurons, in order to repair the central nervous system. Mesenchymal stem cells are multipotent stromal cells that can differentiate into a variety of cell types, including osteoblasts (bone cells), chondrocytes (cartilage cells), myocytes (muscle cells) and adipocytes (fat cells which give rise to marrow adipose tissue). Mesenchymal stem cells are transdifferentiated by small molecule into neuron-like cells (Nichols *et al.*, 2013)(Divya *et al.*, 2012).

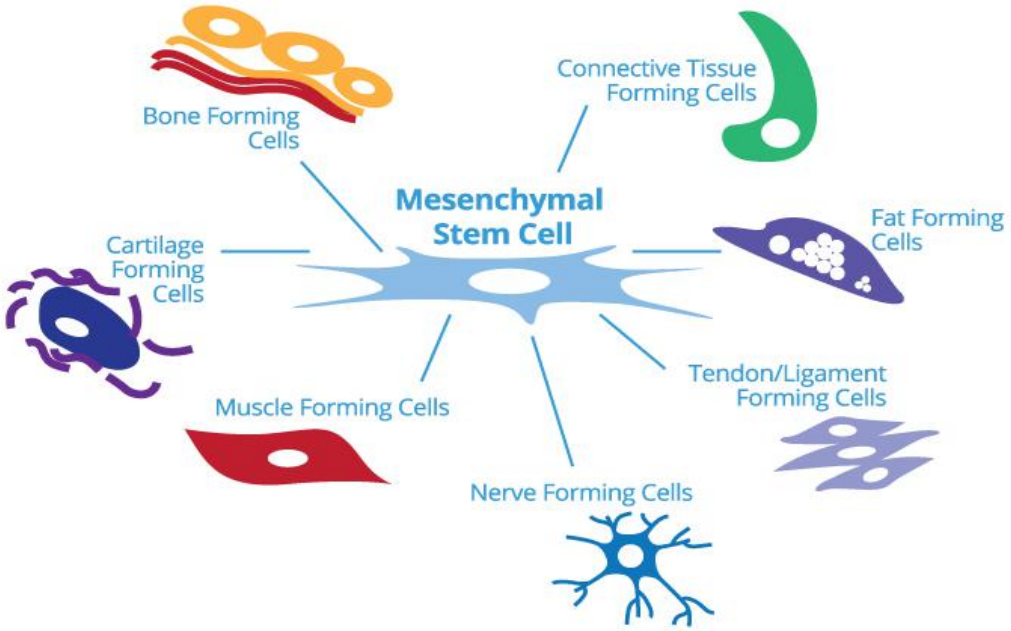


Figure 2: Differentiation of mesenchymal stem cell to different types of cells.

Small molecules are small in size, low molecular weight molecules which include monosaccharides, lipids, second messengers, metabolites, other natural products as

well as drugs and other xenobiotics. Small molecules are small in size and easy to enter into the human body find applications in pluripotent stem cell derivation, maintenance, production, and differentiation and can be used as a drug for treating neurodegenerative diseases wherein other treatment procedure interference occurs (Lu and Atala, 2016)(Dimos *et al.*, 2011). They can be used in vitro to facilitate production and expansion of adult stem cells. They are different from macromolecules such as proteins. Unlike the use of biological or chemical cocktails that are generally utilized in causing trans-differentiation effect, the use of single, well-defined small molecule offers several advantages and some of these include: (i) the ease of reproducibility in seeking results, (ii) the greater possibility of targeted mechanistic understanding, (iii) the newer selection criteria in strengthening the pipeline of candidates for neurodegenerative diseases, and (iv) the possibility of placing these findings with chemical entities onto the translational drug discovery path. While proposing combinatorial drug regime, the only approach available to the scientific community in treating neurodegenerative diseases, that this proposal aims to build. For building a novel chemical collection from natural product-inspired compounds or small molecules for this program was focused on natural products: neopeltolide, a novel biologically active marine-derived complex macrolide compound (Bai, Davis and Dai, 2014). We have studied the effect of Neopeltolide inspired small molecules on trans-differentiation of human mesenchymal stem cells to neurons and further exploring their applications as neuroprotective and neurogenesis agents.

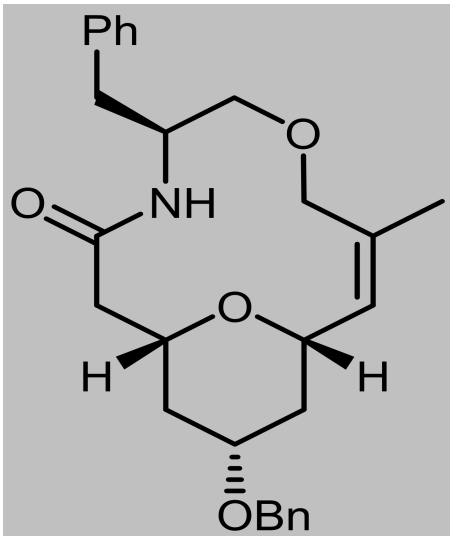


Figure 3: **Structure of neopeltolide-inspired small molecule.**

Signaling pathway in Alzheimer's Parkinson's and ALS diseases:

A: Alzheimer's Disease:

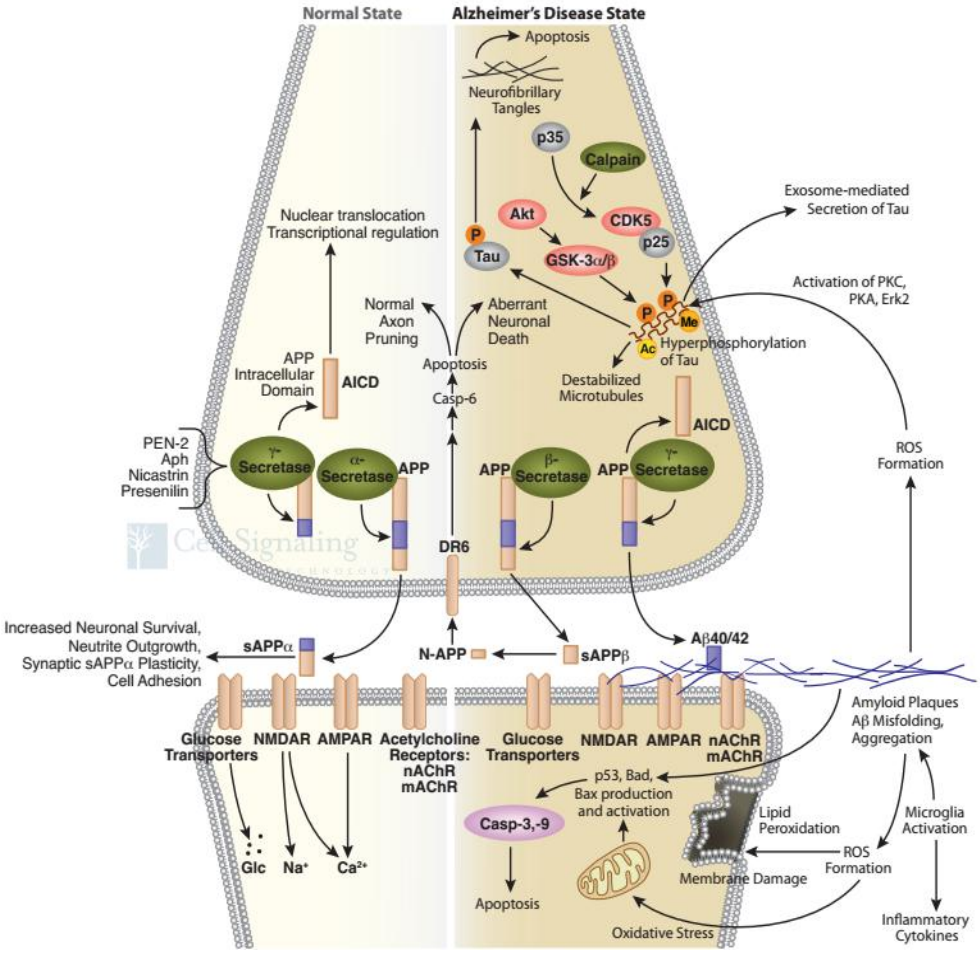


Figure 4: Schematic diagrammatic representation of di-erential processing of Amyloid Precursor protein (APP) in the normal vs. diseased state in Alzheimer's disease.(Bossy-Wetzel, Schwarzenbacher and Lipton, 2004)(Chen and Yan, 2010).

Alzheimer's disease is one of the most common neurodegenerative diseases worldwide. Clinically, the hallmarks of Alzheimer's disease are the extracellular amyloid plaques and intracellular neurofibrillary tangles. Central to this disease is the di-erential processing of the integral membrane protein APP (Amyloid Precursor Protein) in the normal versus disease state. In the normal state initially, APP is cleaved

by α-secretase to generate sAPP and a C83 carboxy-terminal fragment and this sAPP is results in learning and memory, synaptic plasticity, emotional behaviors, and neuronal survival. APP is sequentially cleaved by β-secretase and γ-secretase for releasing an extracellular fragment called Aβ40/42 in the diseased state. The aggregation of this neurotoxic fragment results in Aβ40/42 oligomerization and plaque formation as well as blocked ion channels, impaired energy metabolism, disruption of calcium homeostasis, mitochondrial oxidative stress and abnormal glucose regulation, and ultimately neuronal cell death. Another hallmark of Alzheimer's disease is the presence of neurofibrillary tangles. These tangles are formed as a result of hyperphosphorylation of the microtubule-associated protein Tau. The kinases GSK-3α/β and CDK5 are primarily responsible for phosphorylation of Tau, although other kinases such as PKC, PKA, and Erk2 are also involved. As a result, the Tau protein dissociates from the microtubule, leading to apoptosis of the neuron (Bossy-Wetzel, Schwarzenbacher and Lipton, 2004) (Chen and Yan, 2010).

B: Parkinson's Disease:

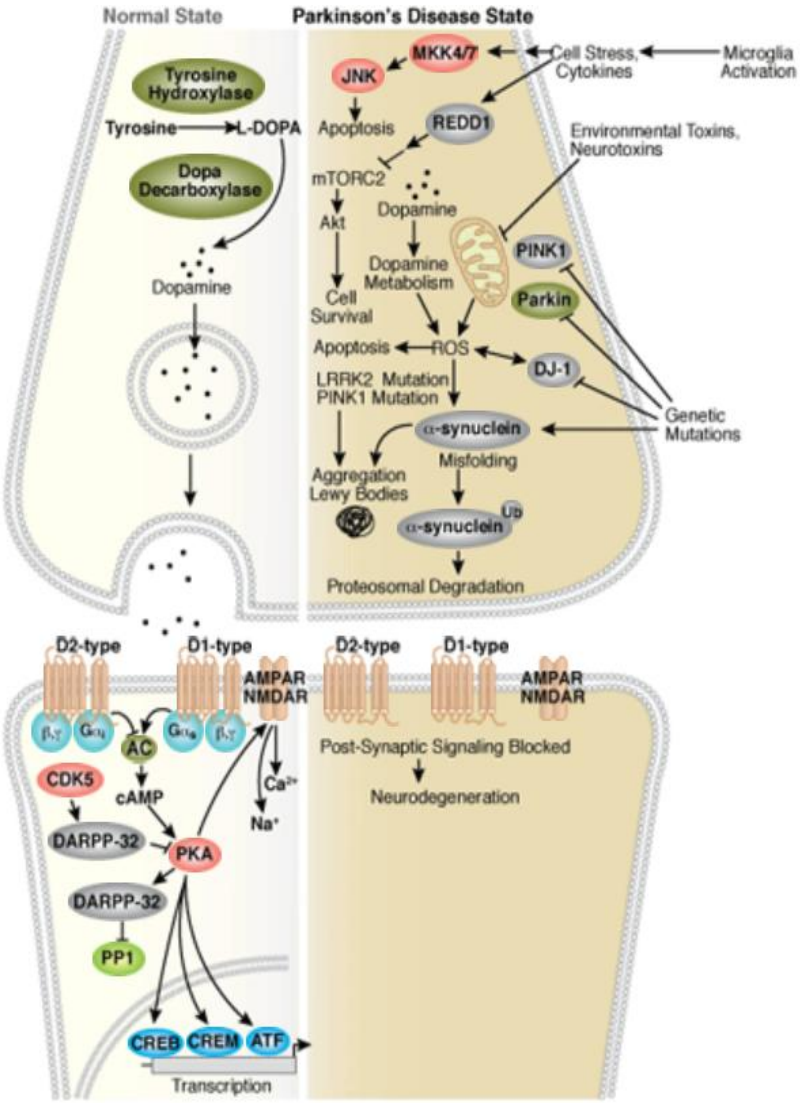


Figure 5: Schematic diagrammatic representation of dopamine signaling in the normal vs. diseased state in Parkinson's disease (Dauer and Przedborski, 2003)(Girault and Greengard, 2004).

Parkinson's disease is the second most neurodegenerative disorder. This disease is caused due to the loss of dopaminergic neurons within the substantia nigra section of ventral midbrain and characterized by bradykinesia, resting tremors etc. In the normal state, the presynaptic neuron release this neurotransmitter dopamine results in signaling in the postsynaptic neuron through D1- and D2-type dopamine receptors. By the signaling through G protein, D1 receptors activate adenylate cyclase, causing cAMP formation and activation of PKA and the D2-type receptors block this

signaling by inhibiting adenylate cyclase. In the diseased state, Parkinson's disease can occur through both genetic mutations, exposure to environmental and neurotoxins. The altered mitochondrial function is caused by loss-of-function mutations in parkin, DJ-1, and PINK1 and accumulation of reactive oxidative species (ROS). Missense mutations in the gene encoding α-synuclein and LRRK2 affecting protein degradation pathways which lead to protein aggregation and accumulation of cytoplasmic eosinophilic inclusions called Lewy bodies are the common cause of autosomal dominant in Parkinson disease. By releasing of ROS, can also cause mitochondrial dysfunction when exposing to environmental and neurotoxins which lead to disruption of protein degradation pathways and apoptosis. The activation of microglia and the release of inflammatory cytokines cause apoptosis through the JNK pathway and Akt signaling pathway blocked via REDD1.

C: Amyotrophic lateral sclerosis:

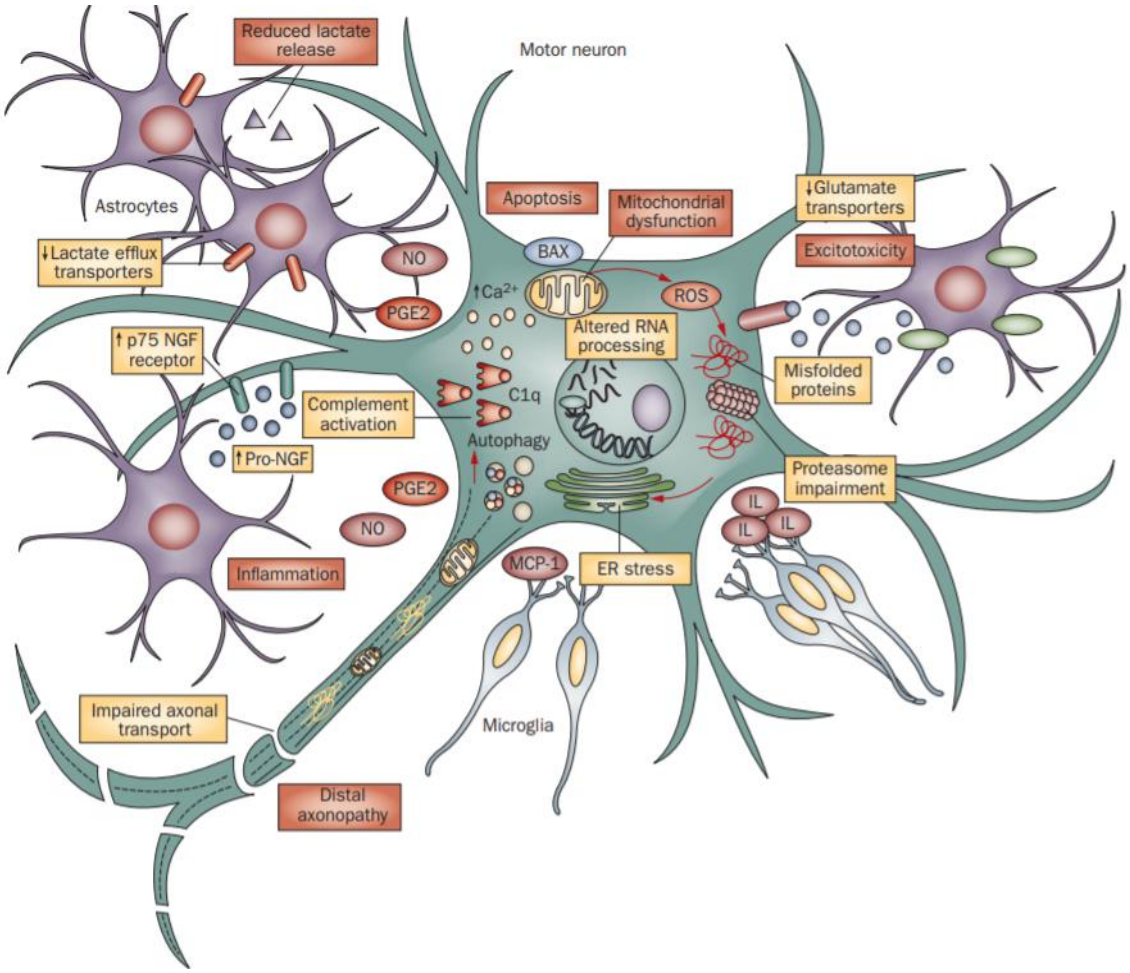


Figure 6: Mechanism of Amyotrophic lateral sclerosis (Ferraiuolo *et al.*, 2011).

ALS is a progressive complex neurodegenerative disease can be characterized by loss of motor neuron in the central nervous system. This involves activation of several cellular pathways in motor neurons, dysregulated interaction with neighboring glial cells. An inflammatory cascade is activated by Microglia leading to secretion of MCP-1 and other cytokines. Another supporting cell of CNS, Astrocytes releases inflammatory mediators such as NO and PGE2 which contribute to motor neuron injury. Lactate release and activation of pro-NGF–p75 receptor signaling is the result of reduced expression and activity of the glutamate reuptake transporter EAAT2,

situated in neighboring glial cell and presynaptic membrane. Transcriptional dysregulation and abnormal RNA processing in motor neurons leading to overproduction of ROS, causing protein misfolding can form aggregates which lead to proteasome impairment, ER stress, and ultimately activating apoptotic pathways. Mitochondrial impairment and dysregulation of calcium handling lead to activation of the apoptotic pathways which are two major components of motor neuron injury. In ALS, a mutation in messenger protein involved in axonal transport may contribute to energy deficit and the dying-back axonopathy. Difect in the different regulatory protein complement activation pathway contributes to the formation of active complement fragments that act as an inflammatory mediator to the neighboring cells.

1.1 Background and Context:

The Neurodegenerative disease is a malfunctioning which primarily affects the neurons in the human brain. Neurons are the building blocks of the nervous system which include the brain and spinal cord. Neurons normally don't replace by themselves, so when they become damaged or die they cannot be reproduced by the body (Engelender and Isacson, 2017)(Lee *et al.*, 2011). Neurodegenerative diseases include Parkinson's, Alzheimer's, Huntington's disease etc. Neurodegenerative diseases are debilitating conditions that result in progressive degeneration and /or death of nerve cells.

The blood-brain barrier (BBB) is a membrane that controls the passage of substances from the blood into the central nervous system (CNS) is a physical barrier between the local blood vessels and most parts of the central nervous system itself and stops many substances from traveling across it (Daneman and Prat, 2015). The blood-brain barrier (BBB) represents a major obstacle to the delivery of drugs to the central nervous system (CNS). But many drugs are unable to pass the barrier since 98% of them are heavier than 500 daltons. So many drugs for the neurodegenerative disease cannot enter the blood-brain barrier. In that case, small molecules are widely used as research tools to regulate cell fates in vitro. Small molecule drugs are found to have regenerative activities other than their approved use. These drugs may be repurposed for regenerative medicine. Most of these small molecules function through regulating adult stem cells. There is a nature-inspired small molecule named Neopeltolide-inspired compound which has an effect on trans-differentiation of human mesenchymal stem cells to neuron-like cells and thus their applications as a drug in neurodegenerative disorders (Kartika, Gruffi and Taylor, 2008).

1.2 Scope and Objectives:

Scope: The scope of the research proposal covers molecular profiling of phenotype i.e transdifferentiation of adult human sourced mesenchymal stem cell-based predictive, a responsive platform to neuron-like cells under the exposure of neopeltolide-inspired small molecule analogs.

Objectives:

- Cellular characterizations of predictive adult stem cell-based platforms before and after treatment with Neopeltolide-inspired small molecules.
- Molecular characterizations of predictive adult stem cell-based platforms before and after treatment with Neopeltolide-inspired small molecules.

1.3 Achievements:

- Isolation of mesenchymal stem cells from healthy donors (source - umbilical cord cells).
- Phenotypic validations of trans-differentiation of stem cells to neuron-like cells.
- Validation of neuronal cells by Neu-O Staining method.
- Testing the cytotoxicity of the neopeltolide-inspired compound by WST-1 Assay (Water Soluble Tetrazolium Assay) on mesenchymal stem cell platform.
- Validation of neuronal cells by specific neurogenesis biomarkers using PCR methods.
- Omics of mesenchymal stem cell platform exposed to neopeltolide-inspired compound using genomic tools (next-generation sequencing).

1.4 Overview of Dissertation

Material and Method:

A: Cell culture

The HPMSCs derived from umbilical cord cell (E-106/369/2016/CHE patent reference) were seeded in plates or dishes at a seeding density/well in DMEM medium supplemented with 10% FBS and antibiotics. Growth factors were not added to the medium to allow for the unbiased trans-differentiation of HPMSCs into neuron-like cells. The following day when the cells were ~70% confluent, growth medium was replaced with medium containing the test compounds (final concentration 10.0 μ M) and medium containing DMSO (final concentration 0.1%) to the corresponding wells. The plate was returned to the incubator with the constant supply of 5% CO_2 at 37 ^oC. The medium in the wells was changed for every 48 hrs until the end of the induction period. The visual inspection of phenotypic changes in the cells was monitored on a daily basis and the results documented. Towards the end of the induction period (~7 days), the medium in the wells was removed, following which the cells were washed in PBS to remove any residual medium.

B. WST-1 assay

Reagents: Premix WST-1 Cell Proliferation Assay System.

The current assay is designed to screen the cytotoxicity effect of small molecules on MSCs using WST-1 Assay system. The idea of performing the WST-1 (Water Soluble Tetrazolium1) assay is to determine cell viability, cytotoxicity, and proliferation. WST-1 is a tried and tested method owing to its ease of handling of reagents and simplicity of assay.

The stable form of tetrazolium is cleaved into formazan by an intricate cellular mechanism. The bioreduction of Tetrazolium to Formazan in the cell is totally dependent on the amount of

NADH that is produced during glycolysis. The amount of the formazan dye formed is directly proportional to the number of viable cells. The Tetrazolium salt is cleaved to formazan by a Succinate-Tetrazolium-reductase system which is part of the mitochondrial respiratory chain. This chain is only active in live cells or viable cells. The OD value measured at 450nm is directly proportional to the viability of the cells in the medium.

MSCs were seeded at the cell densities of 1000 cells/well in DMEM Complete Medium (with 10% FBS, growth factors and antibiotic-antimycotic) respectively in each well of a 96-well plate and placed in a 37℃ incubator with 5% CO2. After 24 hours, media was changed and fresh new complete media with compound (neopeltolide inspired small molecule) was added to the cells (MSCs) in triplicate. 48 hours later, the medium was changed and fresh medium was added. After 5 days of incubation, the previous medium was changed and 100µl of the transparent complete medium was added in each well. 10µl of WST-1 premix was added to all the wells. The Neuro induction medium was positive control here and the only medium (DMEM + 10% FBS + antibiotic) is negative control here. At 450 nm wavelength, the absorbance was measured in an ELISA plate reader after 30 minutes, 1 hour, 2 hours. The values were plotted on the excel sheet.

C: Neu-O staining

A fluorescent chemical probe which has the ability to label and image live neuron selectively over the other cells in the brain when Mesenchymal Stem cells (MSCs) are transdifferentiating to neuron-like cells induced by the neopeltolide-inspired small molecule.

Neu-O represents the first selective fluorescent dye for specific live neuron labeling. Neu-O stains selectively neurons over other non-neuronal cells in the background with a distinct cytoplasmic perinuclear staining pattern. High magnification imaging of Neu-O stain primarily extends to the fine processes of neurites with continuous dye staining throughout some parts of the neurite or otherwise being localized or discrete granules along the branches. Neu-O exhibit excellent cell permeability due to its ability to diffuse rapidly and stain neuronal cells passively upon loading into the cell media which is valuable for prospective studies in various areas of neuron development, networking, degeneration.

Culture medium was aspirated and 1ml of fresh labeling medium (DMEM media + Neu-O stain) was added to it. It was incubated at 37℃ for 1 hour. Then labeling medium was

removed. 1ml of fresh medium without Neu-O was added. After that, It was incubated at 37℃ for 2 hours. Finally, Neu-O labeling was visualized using a fluorescent microscope (Floid) with the green filter (Neu-O Ex/Em: 468/557nm).

D: RNA isolation

The RNA from the cells was isolated using Nucleospin Plus RNA isolation kit. Cells were scraped off in the wells with scrapper as we need cells to be detached first so that we can go for further processing. Then 2ml vials were taken and cells with media were put in the 2ml vials for spinning at 3500 rpm for 5mins. After discarding supernatant, sterile PBS was added in each 2ml vials. Then all the tubes were short spinned for 1min for stress removal. 250µl RNA Later was added in each vial, labeled properly and stored at -80 °C. The RNA concentration and integrity were checked using Qubit® RNA HS Assay Kit and a Qubit Fluorometer.

E: PCR

After confirming that the RNA concentration and quality were within the required range, the complementary DNA (cDNA) was generated using Verso cDNA synthesis kit. The obtained cDNA was used as the template to check for the expression of specific neuronal biomarkers. The images from the wells were captured on a daily basis and annotated. Test Compounds: Neopeltolide inspired small molecule.

F. RNA Sequencing

RNA sequencing experiments were performed using Neopeltolide-inspired compound versus DMSO (i.e. used as solvent) treated cells in triplicate. In brief, HPMSCs were exposed to Neopeltolide inspired **(**10.0 µM) for 7 days and then subjected to RNA sequencing analysis.

The total RNA (150 ng) was utilized for RNA sequencing experiments. The mRNA was fragmented and then converted to cDNA according to the protocol. The cDNA was end-repaired and further purified using Ampure XP beads. The cleaved DNA was adapter ligated

and also purified using Ampure XP beads. These adapter-ligated fragments were then subjected to 12 cycles of PCR using primers provided in the kit. The PCR products were purified using Ampure XP beads. The quantification and size distribution of the prepared library was determined using Qubit fluorometer and Agilent Tape station D1000 Kit (Agilent Technologies) according to the manufacturer's instructions.

G. Data Analysis:

The transcriptome libraries (mRNA) were constructed using the NEB adapters and were sequenced on Illumina HiSeq at 150 not read length using the paired-end chemistry. The raw data obtained was then processed for the low-quality bases and adapters contamination. We performed biological interpretation of RNA seq data using clustering and pathway analysis.

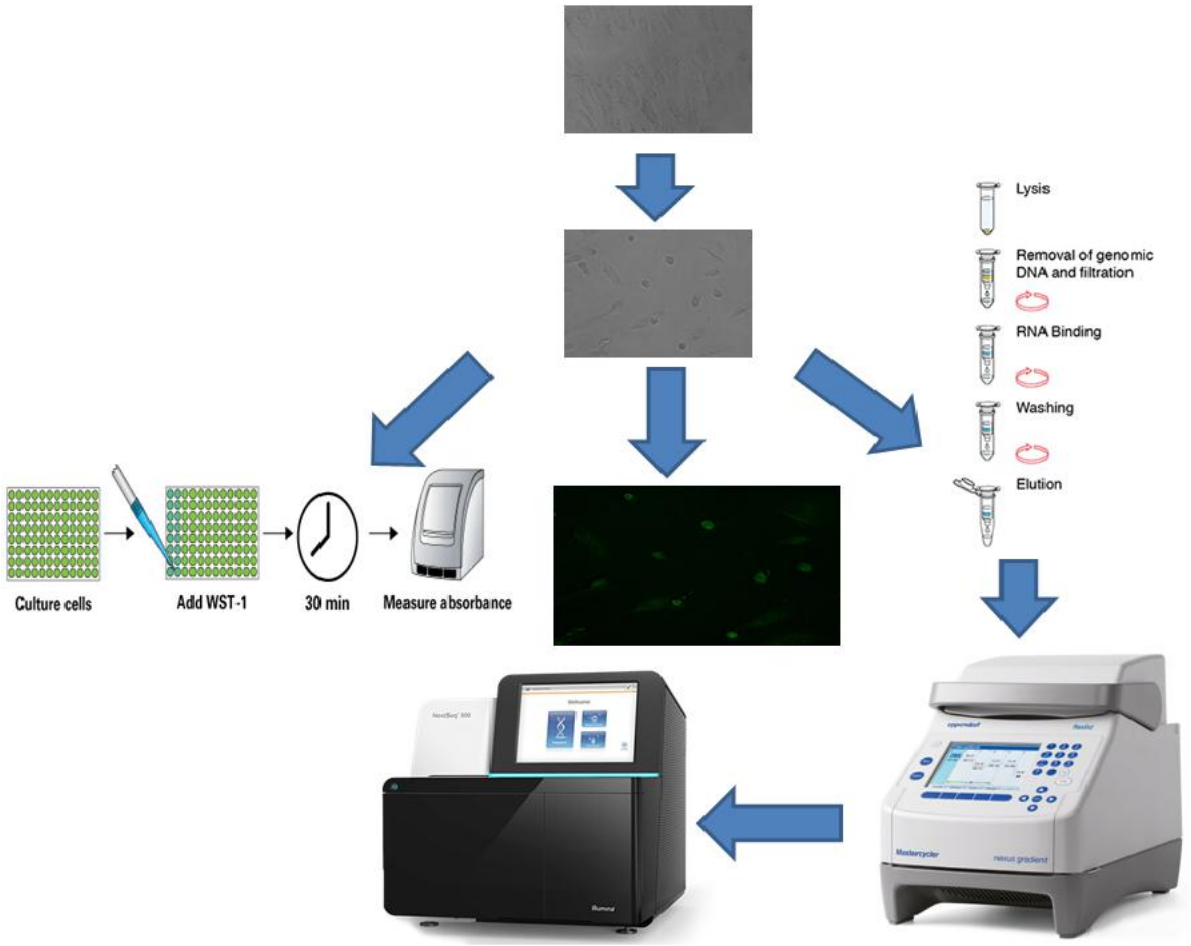


Figure 7: Flowchart of my work plan

Result and discussion:

A: WST-1 Assay

We measured cytotoxicity of this neopeltolide-inspired compound on human MSCs for checking how many cells were viable after treating with this compound.

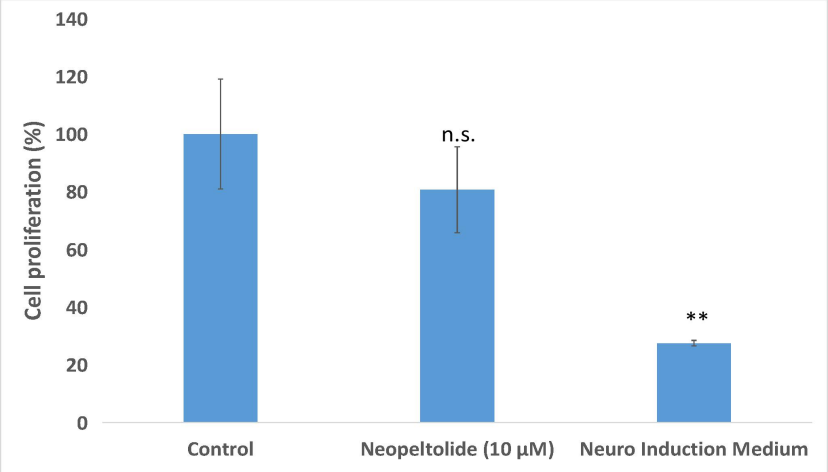


Figure 8: Cytotoxicity of the neopeltolide-inspired small molecule.HPMSCs were treated with 10 uM of the neopeltolide-inspired compound and neuro induction medium for 5 days and the WST-1 assay was done. Cell proliferation was plotted against control as 100%. Data indicated as mean ± S.D. (**P < 0.01).

The decrease in cell proliferation upon treatment with neopeltolide-inspired small molecule was only ~20%, which is also not significant. There was ~70% decrease in cell proliferation when treated with neuro induction medium (Figure: 8). This result suggests that neopeltolide-inspired small molecule is non-toxic to MSCs, whereas neuro induction medium is showing significant toxicity. So Neopeltolide-inspired compound is safe for neurodegenerative disease treatment.

B: Neu-O Staining

Neu-O is a membrane-permeable fluorescent probe that selectively labels neurons in live cultures. Cells labeled with Neu-O can be visualized using fluorescent imaging. Labeling with this probe is non-permanent; it can be washed off, providing unlabeled, viable cells for downstream applications.

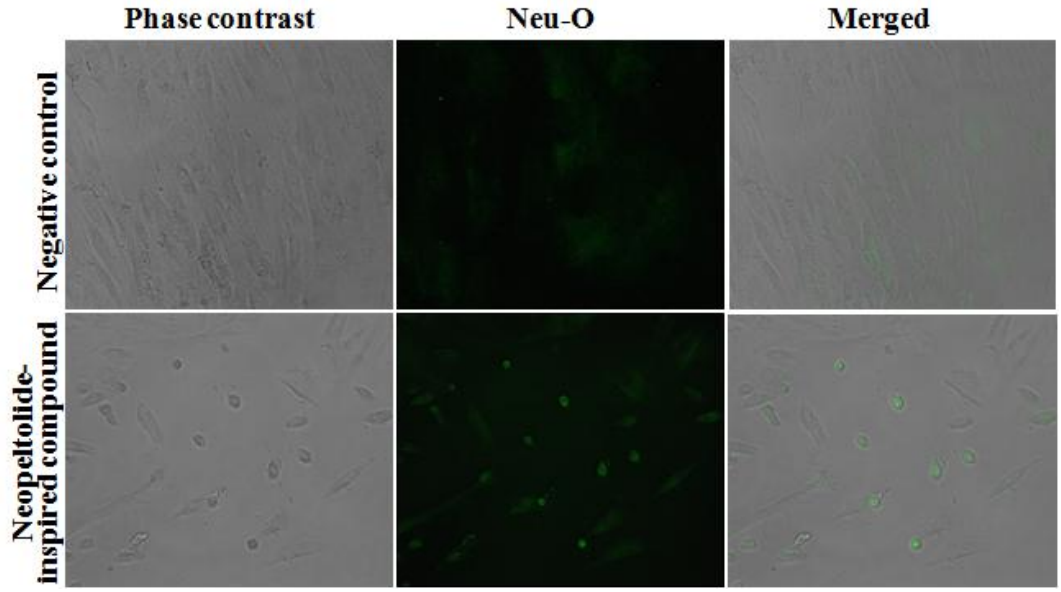


Figure 9: Functional characterization of Neurons. MSCs were treated with the neopeltolide-inspired small molecule for 5 days, stained with Neu-O and images were obtained using Floid fluorescent microscope.

Neu-O staining was observed only upon treatment with the neopeltolide-inspired small molecule, whereas there was no staining in untreated cells (Figure 9). This suggests that neopeltolide-inspired small molecule might be transdifferentiating MSCs to neuron-like cells, as Neu-O specifically stains neurons.

C: PCR

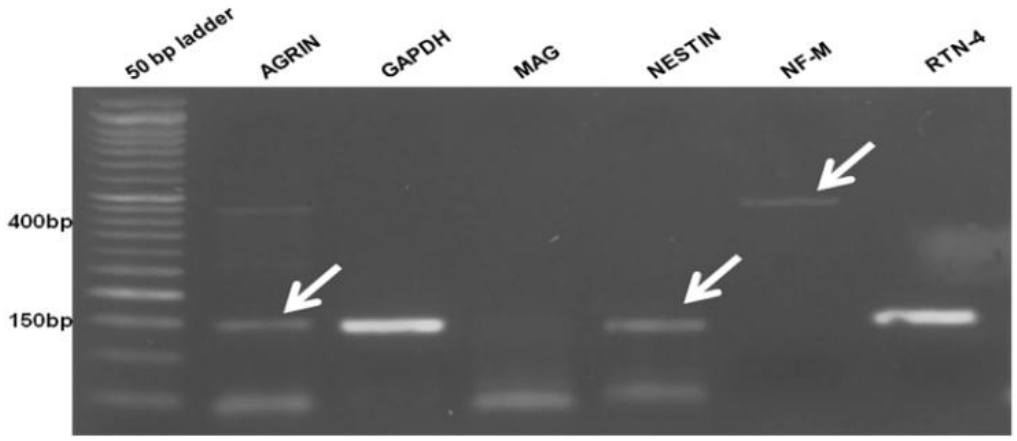


Figure 10: PCR gel image. MSCs were treated with the neopeltolide-inspired small molecule for 7 days, RNA was isolated, cDNA was prepared and semi-quantitative RT-PCR was performed for different neuronal markers. GAPDH was used as loading control.

Shown in **Figure 10** are the phenotypic effects after treatment with small molecule whereas, the identified neural specific biomarkers, such as AGRIN [505152], NESTIN [535455], NF-M[5657] and RTN4 [585960] in this study, are widely reported to be playing a crucial role in various stages of the neuronal development. Based on the semi-quantitative RT-PCR data, we proceeded to go ahead with the Next Generation Sequencing (NGS) genomic study to look for the differential gene expression across the whole genome upon exposure to the neopeltolide-inspired compound.

D: Neuronal Markers

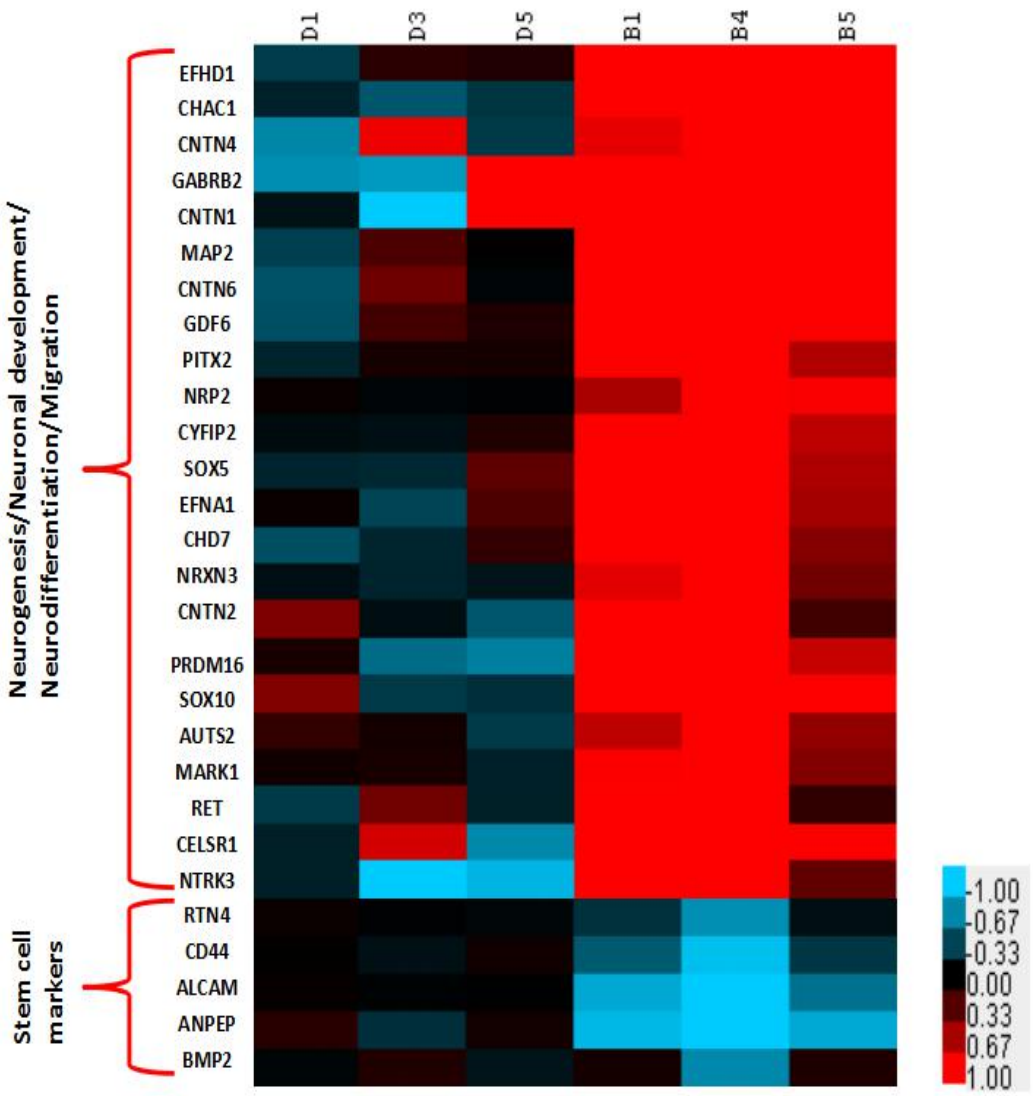


Figure 11: Neopeltolide-inspired small molecule upregulated Neuronal Markers. Unsupervised hierarchical clustering of Neuronal specific genes and stem cell markers upon Neopeltolide treatment and DMSO control. Red (upregulation), Blue (down-regulation) and black (No change).

Genome-wide characterization of Neopletide induced trans-differentiated neurons:

We investigated the molecular mechanisms underlying the ability of neopeltolide-inspired small molecule in inducing neural differentiation using advanced NGS experiments. The biological interpretation of RNA sequencing data showed that different stages of

neurogenesis-related genes involved in neurogenesis, axonogenesis, neurodifferentiation, neurodevelopment, neuronal migration, neurotransmitter transport and neuronal cellproliferation were enriched in upon treatment with Neopeltolide-inspired compound (Figure. 11)

2. BACKGROUND AND RATIONALE

Neurodegeneration is a malfunctioning and is a feature of many incurable and debilitating diseases that are rapidly rising in prevalence, such as Alzheimer's disease, Parkinson's disease which primarily affect the neurons in the human brain. Normally neurons don’t reproduce or replace themselves, so when they die they cannot be replaced by the body.

The blood-brain barrier (BBB) is a membrane that separates the blood from the cerebrospinal fluid which selectively excludes most blood-borne substances from entering the brain. Many drugs are heavier in size and unable to pass the blood-brain barrier and there is an urgent need to develop new and more effective therapeutic strategies to combat these devastating diseases. So we are focused on small molecules to combat this condition and our goal is to convey the sense of excitement and hope in the neurodegenerative disease research field as well as to acknowledge the challenges ahead. Small molecule drugs are found to have regenerative activities other than their approved use. These drugs may be repurposed for regenerative medicine. Most of these small molecules function through regulating adult stem cells. There is a nature-inspired small molecule named Neopeltolide-inspired compound which has an effect on trans-differentiation of human mesenchymal stem cells to neuron-like cells and thus their applications as a drug in neurodegenerative disorders.

3 WHAT DID YOU LEARN?

- Phenotype screening as the new primary inclusion criteria for selecting new chemistry in drug discovery.
- Use of adult stem cell-based platform technologies in drug discovery as a novel approach.

In carrying out the research project we faced lots of barrier and limitations.

- Availability of homogenous required yield of stem cell cultures.
- Integration of real-time cell-based high throughput microscopic screening to evaluate phenotype variation.

The scope for further research based on the results.

- Still, need to increase the number of performing more patient sample.
- Evaluate the targets using CRISPER techniques.
- Investigate the effect of the small molecule Alzheimer's Disease and Parkison Disease animal model.

3.1 Summary

- Transdifferentiation of mesenchymal stem cells to neuron-like cells using small molecule neopeltolide.
- Mode of action of neopeltolide.
- Genome-wide changes or molecular mechanism underlying transdifferentiation of MSCs to neuron-like cells.

3.2 Evaluation

The present study led to the discovery of novel, first-in-class compounds that are capable of differentiating HPMSCs into neuron-like cells. Given the simplicity of using a single small molecule over the conventional biological cocktails, our work demonstrates the potential of discovering powerful chemical tools for probing neurodevelopment. The long-term goal of this work is to explore the value of these functional chemical candidates in treating neurodegeneration-based disorders, such as Alzheimer's disease and Parkinson's disease. For this study, we isolated mesenchymal stem cells from healthy donors (source - umbilical cord cells), phenotypically validated the trans-differentiation of stem cells to neuron-like cells and validation of the neuronal cells by Neu-O Staining method. We have tested the cytotoxicity of the neopeltolide-inspired compound by WST-1 Assay (Water Soluble Tetrazolium Assay) on mesenchymal stem cell platform and validated the neuron-like cells by specific neurogenesis biomarkers using PCR method and finally exposed neopeltolide-inspired compound using genomic tools (next-generation sequencing).

REFERENCES

1. *ALZHEIMER'S DISEASE: MOLECULAR MECHANISMS* (no date). Available at: http://www.benbest.com/lifeext/Alzheimer.html (Accessed: 9 May 2018).

2. *Amyotrophic Lateral Sclerosis (ALS) Fact Sheet | National Institute of Neurological Disorders and Stroke* (no date). Available at: https://www.ninds.nih.gov/Disorders/Patient-Caregiver-Education/Fact-Sheets/Amyotrophic-Lateral-Sclerosis-ALS-Fact-Sheet (Accessed: 9 May 2018).

3. Bai, Y., Davis, D. C. and Dai, M. (2014) 'Synthesis of tetrahydropyran/tetrahydrofuran-containing macrolides by palladium-catalyzed alkoxycarbonylative macrolactonizations.', *Angewandte Chemie (International ed. in English)*. NIH Public Access, 53(25), pp. 6519–22. doi: 10.1002/anie.201403006.

4. Brunden, K. R. *et al.* (2014) 'Microtubule-stabilizing agents as potential therapeutics for neurodegenerative disease', *Bioorganic & Medicinal Chemistry*, 22(18), pp. 5040–5049. doi: 10.1016/j.bmc.2013.12.046.

5. Cluskey, S. and Ramsden, D. B. (2001) 'Mechanisms of neurodegeneration in amyotrophic lateral sclerosis.', *Molecular pathology : MP*. BMJ Publishing Group, 54(6), pp. 386–92. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11724913 (Accessed: 9 May 2018).

6. Crews, L. and Masliah, E. (2010) 'Molecular mechanisms of neurodegeneration in Alzheimer's disease.', *Human molecular genetics*. Oxford University Press, 19(R1), pp. R12-20. doi: 10.1093/hmg/ddq160.

7. Daneman, R. and Prat, A. (2015) 'The blood-brain barrier.', *Cold Spring Harbor perspectives in biology*. Cold Spring Harbor Laboratory Press, 7(1), p. a020412. doi: 10.1101/cshperspect.a020412.

8. Dauer, W. and Przedborski, S. (2003) 'Parkinson's disease: mechanisms and models.', *Neuron*, 39(6), pp. 889–909. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12971891

(Accessed: 9 May 2018).

9. Dimos, J. T. *et al.* (2011) 'Induced Pluripotent Stem Cells as Human Disease Models', *Annual Reports in Medicinal Chemistry*. Academic Press, 46, pp. 369–383. doi: 10.1016/B978-0-12-386009-5.00005-9.

10 .Discovery medicine medicine on the leading edge. (no date). Discovery Medicine. Available at: http://www.discoverymedicine.com/Christopher-M-Tolleson/2013/01/28/advances-in-the-mechanisms-of-parkinsons-disease/ (Accessed: 9 May 2018).

10. Divya, M. S. *et al.* (2012) 'Umbilical cord blood-derived mesenchymal stem cells consist of a unique population of progenitors co-expressing mesenchymal stem cell and neuronal markers capable of instantaneous neuronal differentiation', *Stem Cell Research & Therapy*, 3(6), p. 57. doi: 10.1186/scrt148.

11. Dobson, C. M. (2017) 'The Amyloid Phenomenon and Its Links with Human Disease.', *Cold Spring Harbor perspectives in biology*. Cold Spring Harbor Laboratory Press, 9(6), p. a023648. doi: 10.1101/cshperspect.a023648.

12. Engelender, S. and Isacson, O. (2017) 'The Threshold Theory for Parkinson's Disease', *Trends in Neurosciences*, 40(1), pp. 4–14. doi: 10.1016/j.tins.2016.10.008.

13. *Getting Alzheimer's - Alzheimer's Project* (no date). Available at: https://sites.google.com/a/beaufortschools.org/cade-saenz-and-gavin-smiley-google-site/home/getting-alzheimer-s (Accessed: 9 May 2018).

14. Gitler, A. D., Dhillon, P. and Shorter, J. (2017) 'Neurodegenerative disease: models, mechanisms, and a new hope.', *Disease models & mechanisms*. The Company of Biologists Ltd, 10(5), pp. 499–502. doi: 10.1242/dmm.030205.

15. Kartika, R., Gruffi, T. R. and Taylor, R. E. (2008) 'Concise Enantioselective Total Synthesis of Neopeltolide Macrolactone Highlighted by Ether Transfer', *Organic Letters*. American Chemical Society, 10(21), pp. 5047–5050. doi: 10.1021/ol802254z.

16. Khanna, M. R. *et al.* (2016) 'Therapeutic strategies for the treatment of tauopathies: Hopes and challenges', *Alzheimer's & Dementia*, 12(10), pp. 1051–1065. doi: 10.1016/j.jalz.2016.06.006.

17. Kocahan, S. and Doğan, Z. (2017) 'Mechanisms of Alzheimer's Disease Pathogenesis

and Prevention: The Brain, Neural Pathology, N-methyl-D-aspartate Receptors, Tau Protein and Other Risk Factors.', *Clinical psychopharmacology and neuroscience : the official scientific journal of the Korean College of Neuropsychopharmacology*. Korean College of Neuropsychopharmacology, 15(1), pp. 1–8. doi: 10.9758/cpn.2017.15.1.1.

18. Lee, V. M.-Y. *et al.* (2011) 'Developing Therapeutic Approaches to Tau, Selected Kinases, and Related Neuronal Protein Targets', *Cold Spring Harbor Perspectives in Medicine*, 1(1), pp. a006437–a006437. doi: 10.1101/cshperspect.a006437.

19. Li, X. *et al.* (2016) 'Human Neural Stem Cell Transplantation Rescues Cognitive Defects in APP/PS1 Model of Alzheimer's Disease by Enhancing Neuronal Connectivity and Metabolic Activity', *Frontiers in Aging Neuroscience*, 8, p. 282. doi: 10.3389/fnagi.2016.00282.

20. Li, Y. *et al.* (2014) 'Role of autophagy and mTOR signaling in neural differentiation of bone marrow mesenchymal stem cells', *Cell Biology International*, 38(11), pp. 1337–1343. doi: 10.1002/cbin.10320.

21. Lu, B. and Atala, A. (2016) 'Small Molecules', in *In Situ Tissue Regeneration*. Elsevier, pp. 87–110. doi: 10.1016/B978-0-12-802225-2.00006-4.

22. Maiti, P., Manna, J. and Dunbar, G. L. (2017) 'Current understanding of the molecular mechanisms in Parkinson's disease: Targets for potential treatments', *Translational Neurodegeneration*. BioMed Central, 6(1), p. 28. doi: 10.1186/s40035-017-0099-z.

23. Martin, S. K. *et al.* (2015) 'Brief Report: The Differential Roles of mTORC1 and mTORC2 in Mesenchymal Stem Cell Differentiation', *STEM CELLS*, 33(4), pp. 1359–1365. doi: 10.1002/stem.1931.

24. Narayan, P., Ehsani, S. and Lindquist, S. (2014) 'Combating neurodegenerative disease with chemical probes and model systems', *Nature Chemical Biology*, 10(11), pp. 911–920. doi: 10.1038/nchembio.1663.

25. Nichols, J. E. *et al.* (2013) 'Neurogenic and neuro-protective potential of a novel subpopulation of peripheral blood-derived CD133+ ABCG2+CXCR4+ mesenchymal stem cells: development of autologous cell-based therapeutics for traumatic brain injury', *Stem Cell Research & Therapy*, 4(1), p. 3. doi: 10.1186/scrt151.

26. Paez-Colasante, X. *et al.* (2015) 'Amyotrophic lateral sclerosis: mechanisms and

therapeutics in the epigenomic era', *Nature Reviews Neurology*. Nature Publishing Group, 11(5), pp. 266–279. doi: 10.1038/nrneurol.2015.57.

27. Pawitan, J. A. (2011) 'Prospect of cell therapy for Parkinson's disease', *Anatomy & Cell Biology*, 44(4), p. 256. doi: 10.5115/acb.2011.44.4.256.

28. Turner, M. R. *et al.* (2013) 'Mechanisms, models and biomarkers in amyotrophic lateral sclerosis.', *Amyotrophic lateral sclerosis & frontotemporal degeneration*. NIH Public Access, 14 Suppl 1(0 1), pp. 19–32. doi: 10.3109/21678421.2013.778554.

29. Weninger, S. *et al.* (2016) 'Collaboration for Alzheimer's Prevention: Principles to guide data and sample sharing in preclinical Alzheimer's disease trials', *Alzheimer's & Dementia*, 12(5), pp. 631–632. doi: 10.1016/j.jalz.2016.04.001.

30. Ulanovskaya, O.A., Janjic, J., Suzuki, M., Sabharwal, S.S., Schumacker, P.T., Kron, S.J., and Kozmin, S.A. (2008). Synthesis enables identification of the cellular target of leucascandrolide A and neopeltolide. Nat Chem Biol *4*, 418-424.

31. Woo, S.K., Kwon, M.S., and Lee, E. (2008). Total Synthesis of (+)-Neopeltolide by a Prins Macrocyclization. Angewandte Chemie International Edition *47*, 3242-3244.

32. Custar, D.W., Zabawa, T.P., Hines, J., Crews, C.M., and Scheidt, K.A. (2009). Total Synthesis and Structure−Activity Investigation of the Marine Natural Product Neopeltolide. Journal of the American Chemical Society *131*, 12406-12414.

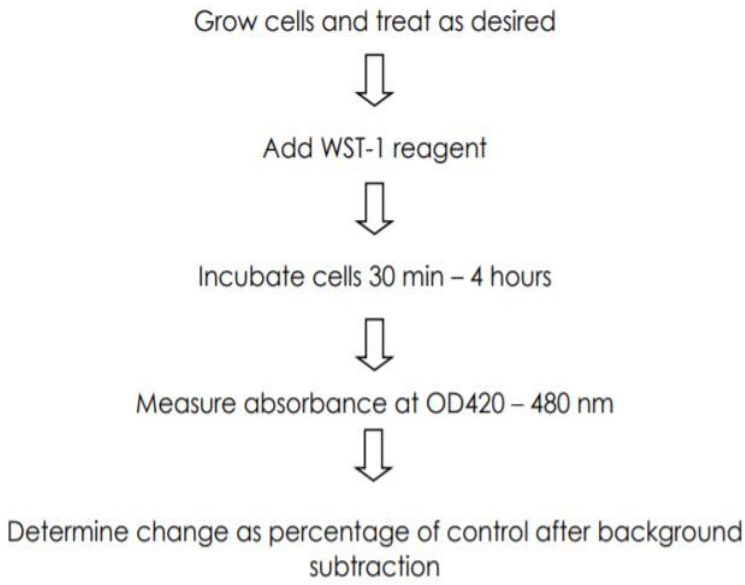
33. Guinchard, X., and Roulland, E. (2009). Total synthesis of the antiproliferative macrolide (+)-neopeltolide. Org Lett *11*, 4700-4703.

34. Fuwa, H., Saito, A., and Sasaki, M. (2010). A Concise Total Synthesis of (+)-Neopeltolide. Angewandte Chemie International Edition *49*, 3041-3044.

35. Hartmann, E., and Oestreich, M. (2010). Asymmetric Conjugate Silyl Transfer in Iterative Catalytic Sequences: Synthesis of the C7–C16 Fragment of (+)-Neopeltolide. Angewandte Chemie International Edition *49*, 6195-6198.

Appendix

- **For WST-1 Assay:** In 15ml of DMEM Media (without growth factor) 15µl of the Neopeltolide-inspired small molecule was added and 100µl media+small molecule was added on each well in triplicate in 96 well plate.
- **WST**-1 **Assay:** Water-soluble tetrazolium- 1 Assay.



- **Labeling medium preparation for Neu-O staining:**

  This should be done in a light free environment.

  The concentration of Neu-O -100µM.

  Desired final concentration in 37℃ medium without growth factor

  0.15µM.(Volume-8ml).

  12µl of 100µM stock of with 8ml of 37℃ DMEM without growth factor.

- **Neuroinduction Medium:** Basal media with Supplement (9:1 ratio).

- **Neu-O green light:** Green light- 532/482 (Emission/Excitation)

  Blue light- 446/390 (Emission/Excitation)

  Red light- 646/586 (Emission/Excitation).

- **Library preparation for NGS:** DNA sample was collected and was fragmented by sonication. Ends were repaired into the blunt end for adapter ligation and sequences were amplified. Two types of adapter were there P5 and P7.
- **Abbreviations:** EAAT2, excitatory amino acid transporter 2; ER, endoplasmic reticulum; IL, interleukin; MCP-1, monocyte chemoattractant protein 1; NGF, nerve growth factor; NO, nitric oxide; PGE2, prostaglandin E2; ROS, reactive oxygen species.