

SCREENING MARINE COMPOUNDS AGAINST NURR1 DOMAIN AS POTENTIAL NEUROREGENERATION AND REPAIR

Submitted in partial fulfillment of the requirements for the award of
**Bachelor of Technology Degree
in Biotechnology**

By

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**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

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DEPARTMENT OF BIOTECHNOLOGY

BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of SUBATHRA .D (37230086) and PRATHYANGIRA .R (37230058) carried out the project entitled **"Screening Marine Compounds Against Nurr1 Domain as Potential Neuroregeneration and Repair"** under our supervision from September 2019 to March 2020

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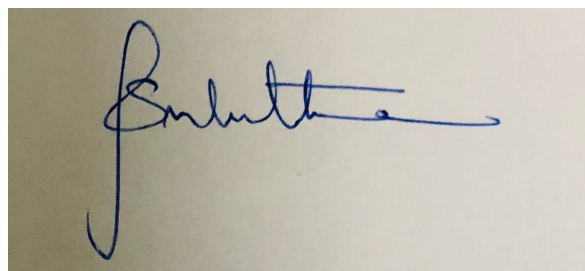
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DECLARATION

We, SUBATHRA.D (37230086) and PRATHYANGIRA.R (37230058) hereby declare that the Project Report entitled "Screening Marine Compounds Against Nurr1 Domain as Potential Neuro Regeneration and Repair" done by us under the guidance of Dr. JAYSHREE NELLORE and Dr. SWETHA.S at Sathyabama Institute of Science and Technology; Jeppiar Nagar, Rajiv Gandhi Road, Chennai - 600119 is submitted in partial fulfilment of the requirements for the award of Bachelor of Technology degree in Biotechnology.



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ABSTRACT

This current study aims to identify the active principle from marine compounds that target Nurr1 protein progression in Parkinson's Disease(PD) patients. A disease-modifying therapy for PD has been long sought-after and will fundamentally change the care of PD patients. In order to accomplish this aim we have employed a bioinformatics approach using drug discovery tools such as Biovia, Patchdock and Pymol along with compound identification database such as CMNPD. The Nuclear receptor related 1 protein (NURR1), is a member of the nuclear receptor family of intracellular transcription factors and is essential for the development and maintenance of Dopaminergic neurons in the adult brain. Mutations in this gene have been associated with disorders, including Parkinson's disease, schizophrenia and manic depression. Marine plants have been identified as a useful source of bioactive small molecules with promising neuroprotective capabilities. However, further studies are required to identify the active principles responsible for the significant neuroprotective effect. In order to validate that MPP⁺ (1-methyl-4-phenylpyridinium) is a cause for the degeneration of DA neurons experimentally, we developed a zebra fish model which was used to study the disease progression. Hence, in the present report, we propose to establish proof of principle that small molecules targeting the Nurr1 can be used as a mechanism-based and neuroprotective strategy for PD.

Keywords: Neurodegenerative disorders, Parkinson's disease, NURR1, Neuroprotective, Marine plants

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ABBREVIATIONS

NURR 1	- Nuclear Receptor Related 1 Protein
ADP	- Adenine Diphosphate
ATP	- Adenine Triphosphate
DA	- Dopaminergic neurons
L-DOPA	- Levodopa
MAP	- Microtubule Associated Protein MPP+ (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)
MPTP	- 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
PD	- Parkinson's Disease
SNpc	- Substantia Nigra pars compacta
TF	- Transcription Factor
VTA	- Ventral Tegmental Area
CMNPD	- Comprehensive Marine Natural Products Database
PDB	- Protein Data Bank
LRRK2	- Leucine-Rich Repeat Kinase 2
PARK 7	- Parkinson Disease Protein 7
AdP	- Adenosine diphosphate
MDV	- Mitochondrial Derived Vesicle
SMILES	- Simplified Molecular-Input Line-Entry System
SDF	- Structure Data File
HPF	- High Power Field

CHAPTER – 1

INTRODUCTION

1.1 DOPAMINERGIC NEURONS

Dopaminergic neurons of the midbrain are the main source of dopamine in mammalian central nervous system. Loss of these are associated with the most prominent neurological disorder-PARKINSON'S DISEASE. And one of the reasons why dopamine neurons are more vulnerable is due to the reason that they have extensive branching, and that they have a high energy requirement. Since dopamine neurons are pace making neurons they constantly discharge and recharge, using up high amounts of energy, but when these neurons don't have sufficient energy they start to degenerate (Mamelak, *et.al.*,2018).

The major clinical symptoms of PD includes loss of dopamine neurons in the substantia nigra pars compacta but there is widespread neuropathology and the SNC only becomes involved toward the middle stages of the disease. Lewy bodies (LBs) and dystrophic neuritis (Lewy neuritis) are a round eosinophilic inclusions composed of a halo of radiating fibrils and a less defined core which are considered to be the hallmark of PD (Ted *et.al.*,2003).

After several years of treatment with levodopa and other drugs, one motor fluctuations ranging from bradykinesia to hyperkinesia develop in many patients with Parkinson's disease. No drug therapy has eliminated these fluctuations. However, the implantation of embryonic dopamine neurons into the brain may improve motor control. It has been reported that transplanted dopamine neurons survive and that patients may have progressive clinical improvement over a period of three to four years.

1.2 DOPAMINERGIC PATHWAYS

There are 4 major Dopamerigic pathways in the brain.

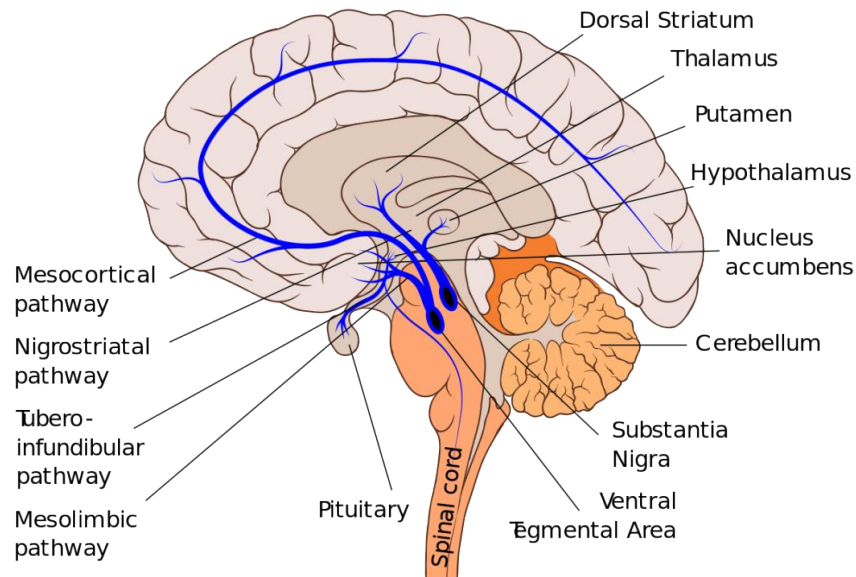


Fig: 1.1 Dopaminergic pathways

1.2.1 Nigro -Striatal Pathway

In this pathway the dopamine neurons project from the SN and end in the dorsal striatum (Luo *et.al.*, 2016). In this pathway dopamine plays a significant role in movement (the control of motor function and in learning new motor skills). Degeneration of the nigrostriatal system causes Parkinson's disease (Ayano *et.al.*,2016). Although in the nigrostriatal system, the presynaptic neurons to the dorsal putamen were the more significantly affected than that of SN. The nigrostriatal pathway analysis demonstrated a severe reduction in connectivity between ipsilateral SN and ipsilateral dorsal putamen in PD patients with respect to control (Caminiti *et.al.*,2017). Dopaminergic neurons in this pathway form highly branched networks within their striatum and they take the role of autonomous pace makers generating 2–4 Hz action potentials (Tsui *et.al.*,2011).

1.2.2 Mesolimbic Pathway

The neurons of the ventral tegmented area projecting to limbic areas follow the mesolimbic pathway (Björklund *et.al.*, 2007). Dopaminergic neurons in the limbic area mediate the pleasure in the brain. Dopamine in these neurons is released during pleasurable events and stimulates the person to do any pleasurable activity. This involves food, sex, and many drugs which stimulants of dopamine release

in the brain are especially in the mesolimbic pathway (Ayano *et.al.*, 2016). Virtually all drugs of abuse increase dopaminergic transmission in the limbic area, and this is thought to contribute to the acute rewarding effects of the drugs leading to addiction (Pierce *et.al.*, 2006).

1.2.3 The Mesocortical pathway

In which the DA arise from the VTA region and project to the frontal cortex and septal hippocampal regions. Mesocortical dopamine mediates cognitive and emotional behavior, especially in helping with improved working memory and attentions (Ayano *et.al.*, 2016). During the phase of learning the conditioning task, reward-related activation of DA neurons first occurs in response to the primary reward (Lapish *et.al.*, 2006). Positive symptoms of schizophrenia are associated with malfunctioning mesolimbic circuits; the neurotransmitters that regulate mesolimbic neuronal functioning include dopamine (DA), which plays a predominant regulatory role (Tsuyoshi *et.al.*, 2011).

1.2.4 The Tuberoinfundibular Pathway

The DA originates in the hypothalamus and projects to pituitary gland (Ayano *et.al.*, 2016). In this pathway it inhibits and regulates the prolactin release. The tuberoinfundibular pathway is present in the hypothalamus region and regulates some functions of the anterior pituitary through the release of dopamine into the portal vessels (Cousins *et.al.*, 2009). Dopamine is also called as the prolactin inhibiting hormone or the prolactin inhibiting factor (Ayano *et.al.*, 2016).

1.3 PARKINSON'S DISEASE

Parkinson's disease (PD) is a chronic escalating neurodegenerative disorder influenced by the premature death of dopaminergic neurons - leading to dopamine deficiency in the Substantia Nigra pars compacta (SNpc), a region in the midbrain present in the central nervous system. PD is characterised by motor and non-motor symptoms such as tremor, rigidity, slowness of movement (bradykinesia)

and poverty of movement (hypokinesia). From studies, it has been understood that about 2 percent of the global population above the age of 65 have been affected by PD.

Thus far, the causative factors of PD still lack proper recognition as it is unknown whether inheritance (genetic factors) or environmental factors play a role. Treatment for PD has been given to only subside the symptoms in the patients and not cure the disease itself. Primary level of treatments include drugs such as Dopamine agonists, MAO-B inhibitors, levodopamine and COMT inhibitors. Levodopa was first synthesised by Casmir Funk in 1911 and was the first drug to be used for the treatment of PD. It is known to increase the dopamine levels in a PD patient by crossing the blood-brain barrier. Although, these medications become less potent as the level of disease progresses and causes side effects. The surgeries performed for PD involve placing microelectrodes within the deep brain for stimulation of the neurons to decrease the motor symptoms in patients for whom drugs are found to be ineffective.

The disease was first analysed thoroughly by an English doctor named James Parkinson who published a detailed report in *An Essay on the Shaking Palsy* in the year 1817. Many other neurologists have made further additions to the observation of the disease. In 1912, it was proclaimed that microscopic particles were found to affect the brain, these particles are called “Lewy bodies” and were named after Fredric Lewy who first identified them.

1.4 NEUROPATHOLOGY OF PD

For 100 years the pathological backgrounds of PD remained a mystery although the clinical features and symptoms have been well described. SN was discovered in 1786 by Felix Vicq Azyr, it is a part of mesencephalon and an important element in the basal ganglia system as well. It is named so because of the native content of neuromelanin pigment found in them. Only after a century the connection between SN and PD was established by Paul Blocq and Georges Marinesco (Parent *et.al.*, 2010). This discovery came from the study of a tuberculosis patient who was suffering from unilateral Parkinsonian tremor. The autopsy of this patient revealed

an encapsulated tumour which lead to the conclusion that in that case tremor is due to midbrain lesion. This work which was published in 1893, led Edouard Brissaud (Parent *et.al.*,2010) to frame a hypothesis stating that the SN is the major pathological site in PD.

It was only the 1938 Tretiakoff work was confirmed by Rolf Hassler a German pathologist who showed evidence suggesting that the striatum and globus pallidus were mostly unaffected and only the SN showed considerable loss of neurons and held abundant of Lewy bodies (Parent *et.al.*,2010). It was also supported by the discovery of dopaminergic neuron in the SN by Arvid Careissons in 1958 (Dauer *et.al.*,2003).The underlying key morpho pathological feature is their progressive loss of DA neuron in the SN of the midbrain, with intracytoplasmic inclusions in the remaining intact nigral neurons (Braak *et.al.*,2004). The symptoms of PD starts to appear when there is about 50-60% of DA in SN and 70-80% if their projection in the striatum are lost (Deumen *et.al.*,2002). In PD degeneration caused by aging in normal peoples. The DA neuron loss in PD is mainly uneven and occurs mainly in the ventral-lateral part of SN also the rate of degeneration of DA neuron in PD is 10 times faster than people not suffering from PD (Alexander *et.al.*,2004).

1.5 PREVALENCE AND EPIDEMIOLOGY OF PARKINSON'S DISEASES (PD)

Parkinson's disease (PD) is a common neurodegenerative disorder α synucleinopathy with a prevalence of 5 to 35 new cases per 100,000 individuals every year (Poewe *et.al.*,2017). PD affects 1-2 per 1000 of the population at any given time (Tysnes *et.al.*,2017). It has been studied that the prevalence of PD increases with age it : 41 per 100,000 at the age of 40 to 49 years;173 per 100,000 at the age of 55 to 64 years; 1,087 per 100,000 at the age of 70 to 79 years; and 1,903 per 100,000 in people over age 80 (Pringsheim *et.al.*,2014). More than half a million people suffer from PD in USA alone and it is expected to increase 3 folds in the next 50 years as the average age of the population increases (Pringsheim *et.al.*, 2014). In a study it was seen that the incidence of Parkinson's disease among men was higher than the incidence among women, although this differed for asians (Van *et.al.*,2003). There was no significance in incidence rates between men

and women younger than 50 years, whereas it was over 1.6 times higher in men compared to women older than 80 years (Moisan *et.al.*,2016). Among asians however Parkinson's disease incidence was highly lower among men than women, the overall incidence rate for men was 19 per 100,000 individuals and for women it was 9 per 100,000 individuals (Van *et.al.*,2003).

1.6 SYMPTOMATOLOGY OF PD

Parkinson's disease was first identified by James Parkinson's in 1817. He identified the basic triad of symptoms as rigidity-an abnormal stiffness in a limb or a part of the body, tremor a shaking of hand, legs especially when the limb is at rest and bradykinesia - gradual loss and slowing down of spontaneous movement. It was noted that Hypomimia, dysarthria, dysphagia, dialorrhea are also associated with the disease (Jankovic, *et.al.*,2008). The symptoms of PD are usually asymmetric and is receptive to dopaminergic treatment. These symptoms are primarily due to loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and due to the intracellular protein accumulation in the brain(Tysnes*et.al.*,2017). Apart from these primary symptoms there are a lot of secondary symptoms which includes unwanted acceleration in movements or in speech Dyskinesia-prolonged muscle contractions that can cause twisting of body parts or repetitive movements, akathisia, etc. (Jankovic *et.al.*,2005). Cognitive impairment, bradyphrenia, depression, apathy, anhedonia, fatigue are some of the other non-motor symptoms that are associated with Parkinson's disease (Jankovic *et.al.*,2008) .

1.7 GENETIC ETIOLOGY FOR PD

Though we do not know the exact cause of the disease mechanisms, it has been recognised that PD aggregates in families, with about 15-20% of patients with PD reporting a positive family history (Sellbach *et.al.*,2006). Along the course of this observation, the past twenty years of genetics research have shown in the DNA sequence variants, especially single nucleotide polymorphism that play a substantial role in disease development (Lill *et.al.*,2016).Approximately 5-10%of all patients

suffer from a monogenic form of PD, which are caused by highly penetrant rare mutations segregating with the disease in families (De Lau *et.al.*,2006). However, the vast majority of PD cases are caused by the combined action and likely of dozens to potentially hundreds of DNA sequences variants of moderate to weak effect sizes, which are also present in the general populations, environmental and lifestyle exposures as well as possibly epigenetic factor (Lill *et.al.*,2016).

1.8 PROTEINS INVOLVED IN PARKINSON'S DISEASE

There are many different proteins that are associated with PD. Each type of protein is related to a specific type of PD caused. PINK1 and Parkin regulate mitophagy and ROS-induced mitochondrial-derived vesicle (MDV) transport to late endosomes. PINK1 is kinase stabilised at the surface of damaged mitochondria, where it phosphorylates both ubiquitin and Parkin, promoting the recruitment of mitophagy receptors (Matheoud *et.al.*,2016). Mutations in leucine-rich repeat kinase 2 (LRRK2) are strongly associated with late-onset autosomal dominant Parkinson's disease. The modification of this protein occurs due to the G2019S mutation in the LRRK2 gene. This mutation of the protein and its involvement in late-onset autosomal dominant PD occurs in relatively high frequency in specific populations (Bardien *et.al.*,2011).

DJ-1, the protein transcribed from the PARK7 gene, is a ubiquitous protein associated with Parkinson's disease. Studies have shown that mutations or deletion in DJ-1 gene are linked to autosomal recessive early onset PD. The function of DJ-1 includes a chaperone with protease activity, transcriptional regulator, to antioxidant scavenger and redox sensor (Denis *et.al.*,2013). Tau protein is a part of the microtubule associated protein (MAP) family, which stabilize and regulate the MT assembly. Tau protein is encoded by a single gene located on chromosome 17q21. The tau protein in relation to PD has been studied during the interaction between tau and α -synuclein that could lead to both a toxic gain of function, evidenced by protein deposition, and loss of function by inhibiting axonal transport. The axonal transport deficits lead to an accumulation of α -synuclein, signs of which have been seen in certain forms of PD (Lei *et.al.*,2010).

1.9 FACTORS AFFECTING DEGENERATION OF NEURONS

Protein aggregation made up of α -synucleins, a common pathological factor in PD. These aggregated proteins are due to a change in their conformations or that newly synthesized polypeptides failing to fold properly (Ross *et.al.*,2004). Incompletely or incorrectly folded proteins have an exposed hydrophobic amino acid side chains on their surfaces that are usually buried in the interior of the native state. Thus, the proteins become prone to aggregation that can function as nuclei that recruit additional monomers (Brundin *et.al.*,2010). One of the reasons for the protein misfolding and aggregation is due to point mutation or a gene addition that causes gain of function mutation in α synuclein gene in autosomal PD (Ross *et.al.*,2004).

In Parkinson's disease, nigral cells are seen under a heightened state of oxidative stress, that is indicated by elevated levels of lipid by-products, protein and DNA oxidation, and by an increase in antioxidant systems (Lotharius *et.al.*,2002). These are also due to the fact that there are some mutations in the α synuclein gene, which causes an increase in cytoplasmic dopamine levels that in turn increase the oxidative stress in the neurons (Stefanis *et.al.*,2011). Dopamine-induced oxidative stress, impaired synaptic vesicle function and mis folding of α -synuclein, due to mutations or to oxidative damage to the protein, can all be the components that have interrelated functions that results in the death of dopaminergic neurons (Lotharius *et.al.*,2002).

1.10 TRANSCRIPTION FACTORS

Developmental transcription factors important in early neuron specification and differentiation often remain expressed in the adult brain. However, how these transcription factors function to maintain appropriate neuronal identities in adult neurons and how transcription factors dis regulation may contribute to disease remain largely unknown. It is noted that there is an onset of symptoms only occur after there is more than 70% decrease in dopamine neurons in SN. And many of the drugs available for treatment today only reduce the symptoms, without curing the disease. There is a need to find markers to detect the progression of PD in the early

stage. Thus, the transcription factor Nurr1 being associated with Parkinson's disease and an essential TF for the development of DA neurons in SN, became an important factor for novel treatments.

1.11 NURR1 IN PD

Mapped to chromosome 2q22-23, the human Nurr1 gene includes eight exons and seven introns; the total length is 9.822 kb. Nurr1 gene has promoter and transcription regulatory element (Jankovic *et.al.*, 2005). Nurr1, as a transcriptional factor, seems to express earlier than numerous phenotypic markers of DA neurons. There is an age dependent reduction in Nurr1 expression in the midbrain DA neurons, which arouses much interest because it may possibly explain the high morbidity of PD in the elderly people (Dong *et. al.*, 2016).

NURR1 is one of the key regulators for development of DA neurons, which is also considered as a crucial regulator for the expression of several genes involved in PD pathology (Liu *et.al.*, 2012). One of the main functions of Nurr1 is the control over synthesis, vesicle packaging, axonal transport, and re uptake of dopamine, thereby critically determining the neurotransmitter phenotype of DA neurons. Surprisingly, this control is exclusive to this group of DA neurons and is not required in any other TH-expressing cell population (Alavian *et.al.*, 2008). In the nucleus, α -synuclein binds directly to histones, thereby inhibiting acetylation by histone acetyltransferases (Decressac *et.al.*, 2013). Nurr1 gene when it was down regulated or in a case where it was knocked out there was an increase in the expression of α -synuclein. Whereas an over expression or an up regulation of the Nurr1 gene a slight decrease in α -synuclein was observed (Yang. *et.al.*, 2008).

Experimental studies in Nurr1 knock-out mice indicate that Nurr1 deficiency results in impaired dopaminergic function and increased vulnerability of those midbrain dopaminergic neurons that degenerate in PD. Decreased Nurr1 expression is found in the autopsied PD midbrains, particularly in neurons containing Lewy bodies, as well as in peripheral lymphocytes of patients with parkinsonian disorders. Several variants in Nurr1 gene have been reported in association with PD. All these studies suggest that Nurr1 is not only essential in the development of

mesencephalic dopaminergic neurons and maintenance of their functions, but it may also play a role in the pathogenesis of PD (Jankovic et.al.,2005).

1.12 MPTP(1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)

MPTP is obtained as a byproduct when synthetic opioid meperidine named 1-*methyl-4- phenyl-4-propio* piperidine was synthesized in a home laboratory. When the MPTP was injected into humans as well as animal models, it was observed that it produces an irreversible parkinsonian syndrome characterised by all of the features of PD, including tremor, rigidity, slowness bradykinesia etc.. (Bové et.al.,2012). It is seen that the dopaminergic neurons in zebrafish are especially

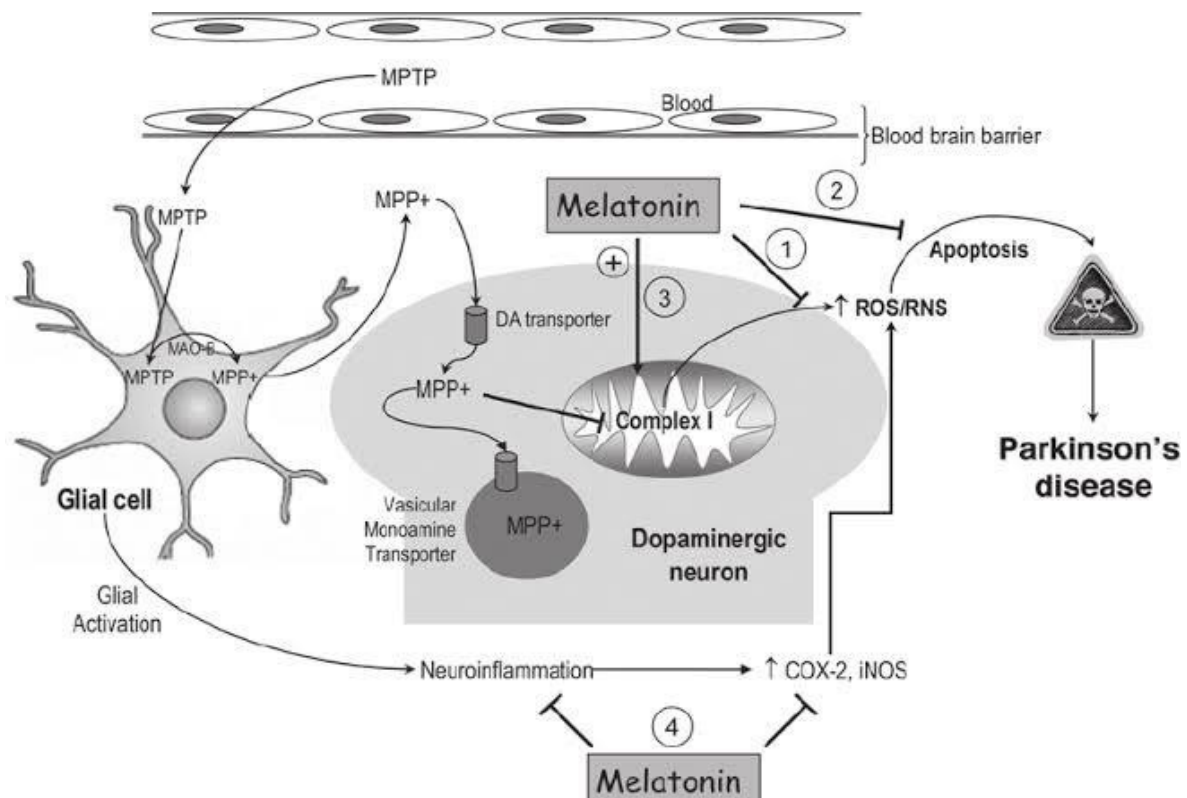


Fig: 1.2 Mechanism of MPTP action

affected when exposed to mutualise mammals, the zebrafish do not possess a midbrain dopaminergic system instead the neurons expressing the dopamine transporter are located in the posterior tuberculum of the diencephalon. The exposure of embryos to MPTP leads to a drastic reduction in the number of dopaminergic neurons in the diencephalon (Chen et.al.,2015).

The mechanisms by which MPTP toxicity occurs is that, MPTP being highly lipophilic easily penetrates the blood—brain barrier and enters the brain. Because of its amphiphilic property, it is captured directly by acidic organelles, and lysosomes especially astrocytes. Since MPTP is lipid soluble it binds mainly to astrocyte lysosomes and there is general agreement that astrocytes convert MPTP to its toxic metabolite, the 1-methyl-4-phenylpyridinium (MPP⁺)(Meredith *et.al.*,2011). MPTP itself does not appear to be toxic, but its oxidized product, methyl-4-phenylpyridinium (MPP⁺), is toxic (Sian *et.al.*,1999). After the MPP⁺ has entered the neuron, it starts accumulating within the mitochondria and acts by inhibiting the electron transport system of the mitochondrial complex I. Once the complex becomes impaired the production of ATP decreases, an elevation of the intracellular calcium concentration occurs and generation of free radicals, resulting in cellular energy failure (Porrás *et.al.*,2011). Low dose exposure produces a pattern of nigrostriatal degeneration which is characteristic to that seen in PD. In addition to the striatal dopamine loss, some of the extrastriatal DA, noradrenaline and serotonin also change, a case often seen in the brain of patients with idiopathic PD (Wang *et.al.*,2014)

2. ZEBRAFISH AS A MODEL

The zebrafish (*Danio rerio*) has now become an emerging new model organism for various studies of neurological, behavioral, genetic studies. (Alderton *et.al.*,2008) As early as the 1930s, the zebrafish was being used as a classical developmental and embryological model. This was due to the combination of the optical clarity of the zebrafish embryos and larvae and the ease of manipulation of the embryos to suit each study (Lieschke *et.al.*,2007).

The zebrafish shows a striking balance between practical simplicity and system complexity. It has numerous practical features that are in its favor, such as being only 4 cm long when fully grown and preferring to stay in close proximity to its conspecifics, a behavior called shoaling. This behavior allows researchers to study the brain functions associated with social behaviors, and also making it possible for the investigator to house a large number of these fish in small fish tanks, with lower costs and ease of maintenance. The similarities between the mammals and

zebrafish behavioral suggests that there are evolutionarily conserved nature of many behaviors across species, implying face and construct validity of zebrafish models(Robert et.al.,2014).



Fig: 1.3 Danio rerio

2.1 HOMOLOGY OF ZEBRAFISH AND HUMAN

The utility of both adult and larval zebrafish in neuroscience has grown markedly in the past decades because it is a vertebrate species with high physiological and genetic homology to humans, and because of the ease of genetic manipulation and similar central nervous system (CNS) morphology. Due to increase in its shelf life and rapid development it is being used as a model for various brain disorders. The availability of zebrafish strains have enabled the study of strain difference in brain function behavior and drug responses. Hence, they may represent an ideal species for medium- and high-throughput screen for genetic mutations and small molecules (Allan *et.al.*,2014).

Zebrafish models for genetic disorder: The attributes that make the zebrafish as a model system are its ability to apply efficient invertebrate style genetics to vertebrate-specific questions. External fertilization, high creativity, rapid development and high stocking densities mean that zebrafish are particularly genetically tractable. However, considerable genomic resources exist for zebrafish, including several high-density genetic maps for mapping induced mutations and the ongoing sequencing of the zebrafish genes. Furthermore, genetics approaches, the

main tool-of-trade for zebrafish biologists is a core group of mutants generated by random mutagenesis and selected on the basis of a common phenotype that can be used to dissect a particular process of interest (Graham *et.al.*,2007).

Humans resemblance with zebra fish species are in accordance to the same cell types, of which the same gene dictate the identity and functions. There are about 25 pairs of chromosomes containing about 26,000 protein-coding genes (Howe *et.al.*,2013). As zebrafish models have high intervening value, it supports the genetic homology to mammals and humans. It's been observed that zebrafish shows 70% homology of nucleotide sequence and 84% of genes are involved with human disease having zebrafish as an analogue (Howe *et.al.*,2013).

Recently zebrafish are the kingpin of neuronal behavior studies since larvae display learning, sleep, neuronal phenotypes and drug addiction that are quantifiable and are correlated with humans. However, zebrafish being subtle to neurotropic drugs includes mood stabilizers, stimulants, anxiolytics, antidepressants, anesthetic/analgesics, cognitive enhancer, antipsychotics they are paragon model for neurological studies (Alderton *et.al.*,2008).

3. MARINE COMPOUNDS

The bioactive compounds derived from marine sources could produce numerous pharmacological effects and could prove to be potent for the development of new drugs. A few marine compounds have entered clinical trials as novel drugs targeting PD. The potential candidates from marine-derived compounds could be Archaea, Bacteria, Fungi, Algae, Coral, Sponge, Sea Cucumber, Mollusk, Conus.

4.MOLECULAR MODELLING AND DOCKING

Molecular docking studies have become an important component to achieving the discovery of a drug. Molecular modelling is basically performed to analyse how two or more molecules interact among themselves and can display the most suitable orientation of a ligand (small drug-like molecule) that may bind to the target

molecule if found to be suitable. As docking is of low-cost and is simple in use it is the most sought after methods in industries and academic communities. The process of identifying a target, synthesising an active compound and then developing is a time consuming process. With the help of molecular modelling, a target is first identified that is the cause of the diseases. On identifying the target-disease link, the next step is to screen for suitable compounds (in the case of this project, a marine compound) that will terminate the progression of the disease. Screening is the process of going through various compounds from natural sources and online databases for biological activity in high-throughput assays.

4.1 MOLECULAR DOCKING

Molecular docking aids in predicting the intermolecular framework among a protein and a ligand, small molecule or protein and protein as two molecules can interact in various ways and also suggest the binding modes that are responsible for inhibition of the protein. To precisely carry out docking studies high-resolution X-ray, NMR or homology modelled structure with a predicted or known binding site in the biomolecule is required. Currently in the marine database (CMNPD -

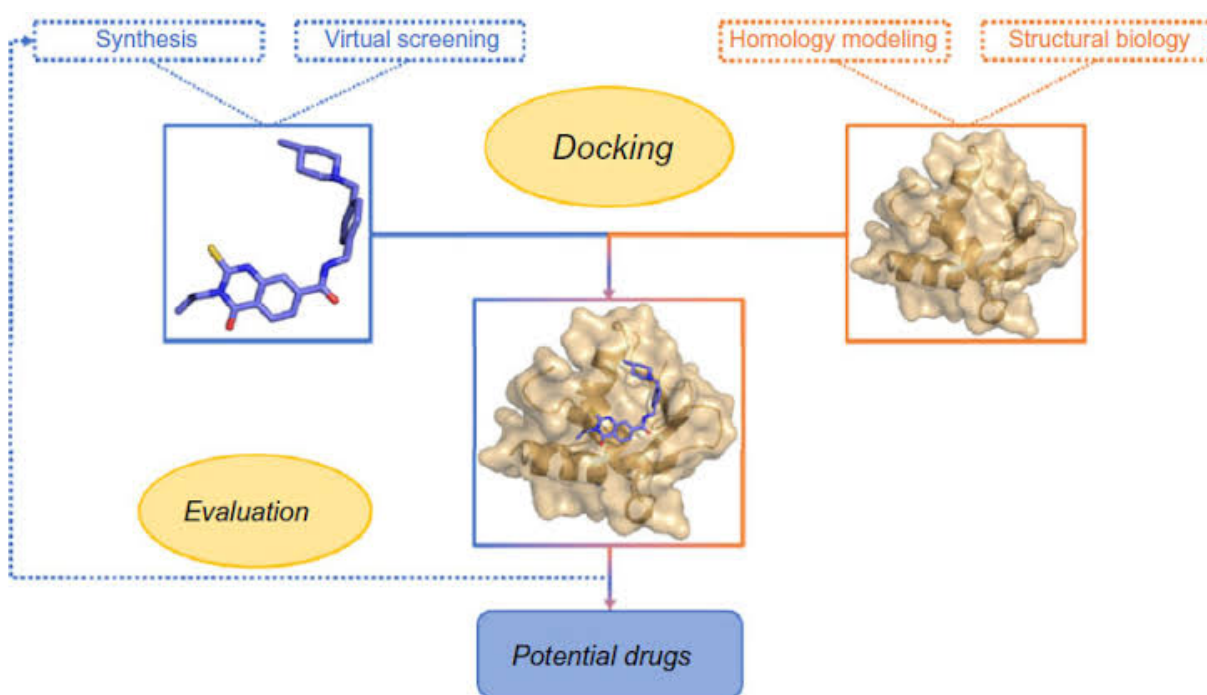


Fig: 1.4 Process of Molecular Docking

Comprehensive Marine Natural Product Database) there are about 32,000 compounds. The docking methods fit a ligand into a binding site by optimising and combining variables such as hydrophobic, steric and electrostatic complementarity and also estimates the free binding energy (scoring).

4.2 SCORING FUNCTIONS

Through the course of the docking process, search algorithm explore a great number of ligand conformations. The scoring functions are used to assess the standard of these docking poses, leading the different search methods towards pertinent ligand conformations. The main purpose of the scoring function is to be able to differentiate the experimentally observed binding modes. Alternatively, scoring functions are also used to properly classify inactive and active ligands (virtual screening). They also predict the affinity constants and perfectly rank many compounds according to their potency.

CHAPTER – 2

LITERATURE REVIEW

Studies indicate that the level of TH expression in DA neurons can change over time and vary in response to changes in hormonal status and functional demand. Moreover, it seems clear that the presence of the TH enzyme at immunohistochemically detectable levels is not always a reliable (let alone necessary) condition for identifying DA neurons, even if it remains perhaps the most sensitive and consistent single marker currently available (Björklund et.al.,2007).

Revealed the different neurotoxin-based animal models of PD that are presently available, the 6-hydroxydopamine (6-OHDA) and the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) models have been established and validated as useful models for the development of therapeutic strategies aimed to treat motor symptoms and to study alterations of the basal ganglia that occur in this disease. The 6-OHDA rat model and the MPTP primate model have contributed enormously to translate animal experimentation into clinical practice, including pharmacological treatments and deep brain stimulation of the subthalamic nucleus. These models, along with the MPTP mouse model, are helping to elucidate the pathogenic mechanism of neurodegeneration in PD. The roles of oxidative stress, apoptosis, mitochondrial dysfunction, inflammation, and impairment of the protein degradation pathways have also come under careful consideration (Bové et.al.,2012).

A study depicts that the protein misfolding can result from native proteins changing their conformations or newly synthesized polypeptides failing to fold properly. Incompletely or incorrectly folded proteins expose hydrophobic amino acid side chains on their surfaces that are normally buried in the interior of the native state. Thus, they become prone to self-association into aggregates that can function as nuclei that recruit additional monomers.

Such protein aggregates can become infectious and are called prions (Brunden et.al.,2010).

It was observed that most familial-associated mutations in Parkinson's are defective in E3 ubiquitin protein ligase activity making it a cause of autosomal recessive PD. DA neurons seem vulnerable to proteasome inhibition where in over expression of α - synuclein inhibits the proteasome and sensitises cells to toxicity induced by proteasomal inhibitors. It was also noticed that Parkin is neuroprotective agent that allows the cell to mitigate proteasomal impairment. Loss of parkin function inhibits the cells ability to handle proteasomal dysfunction (Dawson et.al.,2003).

One of the first functions to be described to 14-3-3 proteins was the regulation of tyrosine hydroxylase activity and its involvement in PD. SOD1 has also been directly linked with PD. It has been noted that SOD1 may form toxic aggregates by using proteins important for neuronal survival or transport. Evidence shows that SOD1 may block UPS and lysosomal protein degradation pathways causing degeneration of the neurons (Gebriel et.al.,2013).

Investigates the expression of DJ-1 which starts at early developmental time point, and in adult zebrafish brain DJ-1 protein is present in dopaminergic neurons. DJ-1 has no proper enzymatic activity, it functions as a redox-sensitive modulator of antioxidative and antiapoptotic signaling pathways and a transcriptional modulator. When the DJ-1 is knockdown the zebrafish become highly sensitive to oxidative stress. MPP+ treatment removed the cytoprotective of DJ-1 in neuroblastoma cells (Kahle et.al.,2009).

The review showed that zebrafish models are becoming an important tool in neuroscience due to its sensitivity to all major neurotropic drugs and the ability to respond to them in a manner similar to humans. The sophisticated zebrafish behavioural repertoire, including its highly social nature and that it relies heavily on

vision similar to humans, make it an excellent tool for analysis of brain function and dysfunction (Kalueff *et.al.*, 2014).

In this research embryos were exposed to medium containing both MPTP and deprenyl. Deprenyl inhibits the activity of MAOB, a substance that converts MPTP to its active metabolite, and has been used as a neuroprotective agent as it protects dopaminergic neurons from damage by oxidative radicals. When the embryos the treated with both deprenyl and MPTP, the number of DN in the PTN was the same and at 72 hpf., there was almost a complete recovery of DN when compared with embryos treated with MPTP only. In embryos treated with only MPP+ at 48 hpf., there was a reduction in the number of DN. Prolonged incubation of the embryos to MPP+ till 72 hpf led to DN being completely destroyed (Lam *et.al.*,2005).

The studies examined Tau protein which is a member of the microtubule associated protein family, that stabilises and regulates microtubule assembly. Tau is a component that is incorporated into filaments in Lewy bodies. In PD brains there is an increased tau phosphorylation at Ser396 has been discovered in synapse-enriched fractions, along within the phosphorylated alpha synuclein, thereby impeding axonal transport as seen in PD (Le *et.al.*,2010).

Investigations revealed some instances, where the mutant phenotypes in zebrafish have striking similarity to the disease pathology in humans. Once a zebrafish disease model is generated, the embryo manipulation becomes easy and enables further investigation of the molecular and cellular basis of the disease. Zebrafish also gives an opportunity to study genetic influences on complex, integrated neurological functions. Several chemical or pharmaceutical agents that alter synaptic transmission and neural membrane stability in humans show analogous activities in zebrafish (Lieschke *et.al.*,2007).

The studies showed quantitative proteomic analysis showed a total of 78 proteins as differentially regulated between the control and MPTP treated zebrafish brain. A western blot analysis was done, and the result observed that there was a significant

up-regulation of PARK8 protein. It was also noticed that DJ1 and SNCA/B were upregulated. Similarly based on immunofluorescence analysis, a high-level expression of SNCA/B protein was observed on the MPTP treated zebrafish (Sarath *et.al.*,2016).

The analysis had shown 96 proteins identified from the Zebrafish Brain Based on Matrix-Assisted Laser Desorption, in the zebrafish brain alone. Out of the 96 proteins identified 68 protein identities were found to be associated with various brain-related genetic disorders. ANXA2, CA13, EEF1A1, PCNA, PEBP1, PGK1, PRDX2, RAN,SNCB, SOD1, STMN1, and UCHL1 were the 13 different proteins identified and found to be associated with Parkinson's disease (Singh *et.al.*,2010)

This study showed that the PD gene uch-L1 and the dopaminergic neuron marker tyrosine hydroxylase are co-expressed in the developing nervous system. This hints at the fact that Uch-L1 may have an important role in differentiation and maintenance of dopaminergic neurons in the ventral diencephalon. The zebrafish uch-L1 transcripts were detected in DN at the first day of embryonic development. In the brain of developing zebrafish embryo at 48 hpf, uch-L1 mRNA expression was detected in DN present in diencephalon and ventral regions of the brain (Son *et.al.*,2003).

The studies revealed that oxidative stress plays an important role in dopaminergic neuronal degeneration. Since the maintenance of redox potential is an important factor for survival of neurons, the oxidative stress produced will affect the potential and cause its death. ROS comes mainly from dopamine metabolism, mitochondrial dysfunction and neuroinflammation. They have also observed a correlation between oxidative stress the proteins involved in PD such as α synuclein, pink 1, parkin, and DJ 1, leading to the pathophysiology of the disease (Vera *et.al.*,2013).

This study has found two proteins that are involved familial PD, namely DJ-1 and UCH-L1, although there was no direct association between UCH-L1 and Parkinson's disease. And both DJ-1 or UCHL1 levels had no impact in idiopathic PD. It was also demonstrated that increased levels of GST enzymes are neuroprotective in a drosophila model of PD. The study also revealed another protein with

neuroprotective properties: galectin 1, was overexpressed in PD samples. A particular finding was that the redox mechanism was greatly altered but the alteration was very distinct (Werner *et.al.*,2008).

Many studies have demonstrated that Nurr1 plays an essential role in the development, differentiation, maintenance, and survival of DA neurons in the SN that are mostly involved in PD. Also being an early marker for DA neurons, Nurr1-activating compounds, Nurr1 modulators, and Nurr1-based gene therapy are considered as therapeutic options for PD treatment. The pharmacological effects of Nurr1-based treatment against PD are, an increase in the expression of DA-related genes, protect DA neurons against neurotoxins, and to inhibit the microglia activation and suppress neuroinflammation. Thereby providing a promising treatment for PD (Dong *et.al.*,2016).

Numerous studies have documented that Nurr1 is essential for both the survival and differentiation of the mesencephalic dopaminergic precursor cells. Nurr1 gene has promoter and transcription regulatory element. While it is highest in the embryonic stage than other stages, its expression remains high in dopaminergic neurons through life. Several studies have found that abnormalities in Nurr1 gene might be risk factors for both familial PD and sporadic PD. Nurr1 gene contains at least four single nucleotide polymorphisms. These variants could cause dysfunction of dopaminergic neurons and lead to PD. Suppression of Nurr1 expression lead to the decrease of mRNA levels of DA neuron associated genes TH, DAT, AADC, and VMAT2 by 50–72% (Jankovic *et. al.*,2005).

A polymorphism in NURR1 intron 6 (NI6P) was found to confer a greater risk for PD and diffuse Lewy body disease. At least three point mutations in NURR1 gene have been identified in association with PD. Patients with familial history have been noted to correlate with greater reduction in PBL NURR1 expression; (4) levels of NURR1 gene expression seem to be lower in women, in patients of 60 years old or older, and in subjects of Caucasian origin; and (5) anti-PD medications have limited or no impact on NURR1 expression (Le *et.al.*,2008).

At present, molecular docking is a commonly used computational tool that has been righteously employed in drug design and discovery studies, Nevertheless, some theoretical and computational challenges are yet to be overcome; doing so would enhance the predictive power and broaden the applications of this important computational tool.

Adequate docking results can be acquired when relatively small ligands with less rotatable bonds are docked against protein binding pockets in which flexibility does not play an important role. Although it is worth noting that even when dealing with simple docking problems, some factors related to ligand and protein preparation can lead to unsuccessful docking predictions. Moving forward, present docking predictions for highly flexible ligands are less reliable, and more sophisticated search strategies may be required.

Protein flexibility and the certainty that protein behaviour is not most likely related to a single conformation but rather to a group of conformations and are important challenges to conquer by further docking methodology developments. Binding energy estimates and binding site analyses of all ligands displayed that binding positions of the amino acids mostly vary in their sites at which they formed hydrogen bonds. These hydrogen bonds are needed to maintain the structural stability of the protein-ligand complex. Furthermore, the free energy landscape profile, i.e. multiple ligand binding modes linked with multiple minima with various shapes and energy barriers, ought to be addressed in scoring more function developments to improve binding affinity estimation (Mobley and Dill 2009; Marsh et al. 2012).

Marine compounds have been found to be a potent molecule against PD due to their anti-PD factors and their ability to produce PC12 cells against MPP⁺ and peroxynitrite-induced neuronal death by reversing mitochondrial complex 1 dysfunction.

CHAPTER - 3

AIM AND SCOPE

3.1 AIM

To identify the small molecule from marine sources to target Nurr1 protein to regenerate the dopaminergic neurons

3.2 OBJECTIVE

- To study the Molecular function of Nurr1 towards dopamine neurodegeneration
- To identify the small molecule from the source of marine organisms database.
- To experimentally validate the molecular docking approach in PD model to predict the binding of small drug-like molecules to target Nurr1 protein and verify if the compound can be used as a novel strategy
- To target the structure-based virtual screening, which employs protein-small molecule docking to identify potential ligand.

CHAPTER-4

MATERIALS AND METHODS

4. MATERIALS

4.1. EQUIPMENTS AND GLASSWARES

Refrigerated Centrifuge, Autoclave, Water bath, Microscope, Eppendorf, Tissue Culture Flasks, Deep Freezer, Measuring Cylinders, Laminar Hood, and Personal Protective Equipment.

4.2. CHEMICALS

MPP⁺, Trizol Reagent, E3 medium

4.3 ANIMALS

Zebra fish was selected as the animal model for PD as it expresses PD symptoms more faster in comparison to other models.

4.4 SOFTWARE

The softwares used for docking are:

- 1.CMNPDP
- 2.OPENBABEL
- 3.Swiss ADME
4. Pubchem
5. Biovia
- 6.PyMoL
7. Patchdock

4.5 STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS for multi group analysis, Student's t-test and one-way analysis of variance (ANOVA) was used followed by multiple comparison. Regression and Pearson correlation was used for correlation analysis and the P-values were adjusted with the Benjamini and Hochberg method to control the false discovery rate. Quantitative data was represented in Mean $\pm$ SEM and Statistical significance was defined at 95% level ($P < 0.05$) (Jayshree et.al., 2020).

5. METHODOLOGY

5.1 MAINTENANCE OF ZEBRA FISH

Wild type adult Zebrafish were reared and kept at aquatic research laboratory at Sathyabama Institute of Science and Technology under standard laboratory conditions of temperature $28 \pm 0.5^\circ \text{C}$, pH 7.2 ± 0.2 on a 14:10 Dark/light photoperiod according to the standard breeding protocols (Westerfield et.al., 2007]. Collected eggs were stored in embryo medium and fertilized embryos were staged under stereo microscope (ZEISS) according to the description of (Sallinen et.al., 2009).

5.1.2 DEVELOPMENT OF ZEBRA FISH EMBRYOS

The fertile embryos once collected were transferred to 8 separate culture flasks, each containing about 100 embryos in about 150ml of E3 medium. In order to avoid contamination of the embryos and to provide proper nutrition, the embryos were placed in E3 medium. E3 medium is an embryogenic culture medium that contains the necessary ions for growth, such as magnesium, sodium chloride, calcium, and potassium. E3 medium also contains methylene blue that acts as an antifungal agent and staining agent. The E3 medium in the flasks was changed for

every 24 hrs with fresh medium. The dead embryos were also removed, and the live embryos were continuously grown and treated.

5.1.3 TREATMENT PARADIGM FOR DA REGENERATION

To determine the optimum point at which DA neurons degenerate and induce functional impairments in response to MPP⁺ toxicity, briefly zebrafish embryos were maintained in EM containing 1 mM MPP⁺ between 18 Hpf to 96 Hpf. At 96 Hpf, significantly ablated larvae were removed from MPP⁺ and allowed to recover in EM. The evidence of regeneration/neurogenesis/functional recovery was documented for 1 week every 24 h or 87 days. For the control group same volume of EM only was added. The solution of each group was disposed of daily and replaced with fresh EM. Exposures were carried out as three independent experiments and results were combined. MPP⁺ exposure was performed as described by with slight modifications and disposal according to current safety protocols. Embryos showing proper and sequential development when they reached 18 hours post fertilization (hpf) were selected for exposures. MPP⁺ exposure was performed as described by (McKinlry *et.al.*,2005)with slight modifications. Experiments performed previously from our laboratory established that MPP⁺(1mM) exposure during DAergic neuron development (18Hpf-96Hpf), manifested PD in zebrafish larvae (96Hpf) with a significant decrease in locomotor activity and 40-50% lesioning/injury of DA neurons. Thus, we analyzed in the current vertebrate model the early molecular events specific to DAergic neurons that triggered substantial loss of DA neurons which later developed into PD like functional impairments.

Briefly, zebrafish embryos from the spawning mentioned above were subjected to 1mM MPP⁺ in EM (Embryo Medium) from 18Hpf to 96Hpf and for the control group same volume of EM only was added. The solution of each group was disposed of completely daily and replaced with fresh EM. Exposures were carried out as three

independent experiments and results were combined. MPP⁺ exposures were carried out and toxins were disposed of according to current safety protocols (Przedborski *et.al.*,2001). The embryos/larvae that had developed normally between 24Hpf-96Hpf were selected for the following experiments.

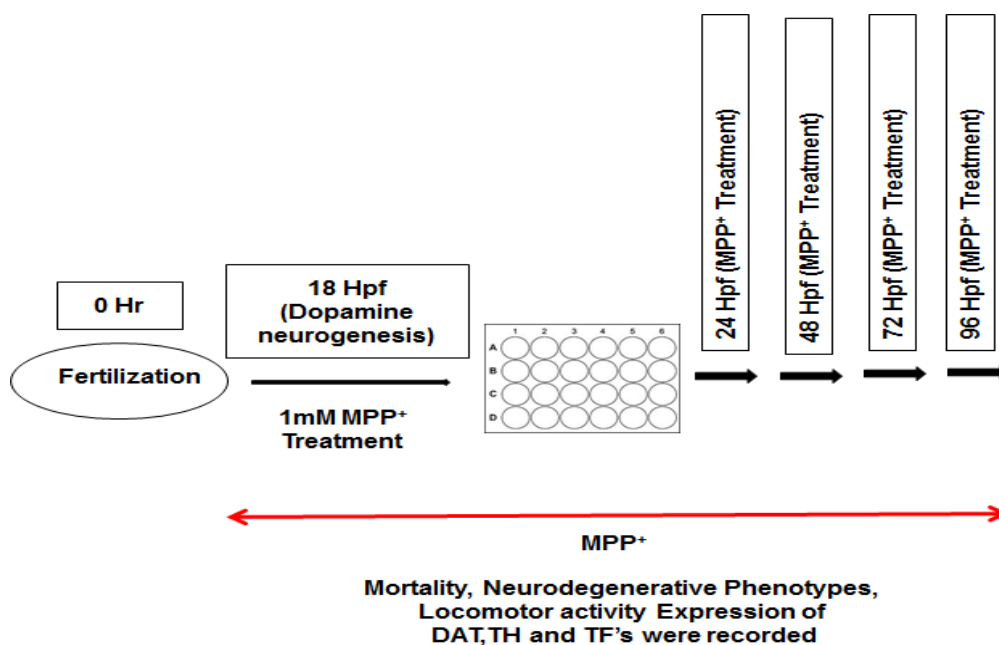


Fig 4.1 Experimental paradigm and construct design. Timeline of experimental manipulation used in the study. Controls were included for each of these groups

5.1.4 RNA ISOLATION AND REAL TIME PCR

The gene expression was performed during both the adult and embryonic stages. For the embryonic stage a total of 50 embryos were taken to form the control, which will also form the adult fish brain tissue. Then, about 10 brain tissues were extracted and subjected to study the Nurr1 expression by qPCR. Total RNA was extracted using TRIZOL (Invitrogen) reagent according to the standard procedures. One microgram of RNA was converted into the complementary DNA (cDNA) by using high capacity cDNA synthesis kit from Applied Biosystem. The fluorescent real time PCR reaction was carried out in “Applied Biosystem Step

one” instrument with Applied Biosystem power SYBR green with specific primers targeting of Nurr1 following the PCR conditions.

PROCEDURE

Trizol reagent was used for RNA isolation. 50 embryos were taken in to a DEPC treated 2 mL tube and triturated using 500 µl of Trizol by inverting the pipette up and down. To these homogenate mixture, 500 µl of Trizol was added and mixed by inverting 3 times followed by incubation at room temperature for 15 min. To these homogenates 400 µl of Chloroform was added and vortexed followed by the centrifugation at 12,000 rpm for 15 min at 4°C. Then supernatant was then transferred into a fresh 2mL tube and 500 µl of 2-Propanol followed by mixing and incubation at -20°C for 2hr. Then the samples were kept for centrifugation at 12,000 rpm for 15 min at 4°C. After supernatant was discarded and pellet was washed with 75% Ethanol. Finally, pellet was dissolved in 30 µl of nuclease free water. Initial denaturation 95°C for 1 min and followed by 40 cycles of denaturation 95°C for 30 sec, annealing with 52- 59.6°C for 45 second extension with 72°C for 1 min, followed by a final extension at 72°C for 10 min. β-actin was used as internal control, assay was performed in duplicates with 25 µL reaction. The data interpretation of Real time PCR data was quantitatively analysed by using the formation of $2^{-\Delta Ct}$, in which $^{-\Delta Ct}$ represents the ratio between the number of cycles (Ct) of the target genes with the endogenous control.

Table 4.1: Primer sequences used for amplification of gene fragments

Component	Volume(µL)
10X RT Buffer	2.0
25X100mM dNTP Mix	0.8
RT Primers	2
MultiScribe™ Reverse Transcriptase	1
RNase Inhibitor	1
Nuclease free water	3.2
Total Volume	10

Table 4.2: qPCR Reaction preparation

Reaction Components	Volume((μ L)
Power SYBR green (2X)	12.5
Forward Primer (5(μ M)	1.25
Reverse Primer (5(μ M)	1.25
Nuclease free water	8
cDNA	2
Total volume	25

The primers were designed using the Primer Express (Applied Biosystem, USA), 6X6 matrix reactions were used to optimize the primer concentration and followed by standard curve analysis with serial dilution of transcribed RNA to check the efficiency of primers. Following primers were used for gene expression studies.

Table 4.3: Primer Sequence Details

PRIMER NAME	PRIMER DETAILS	SIZE OF AMPLICON
<i>Nurr1</i>	Sense primer: TGAAGCCTCCCTGCCTCTAC	103bp
	Antisense primer: GATGGTGCTGCTGCTGATG	
<i>ACT B</i>	Sense primer: GGACTCTGGTGATGGTGTGA	129bp
	Antisense primer: GCTCGGTCAGGATCTTCAT	

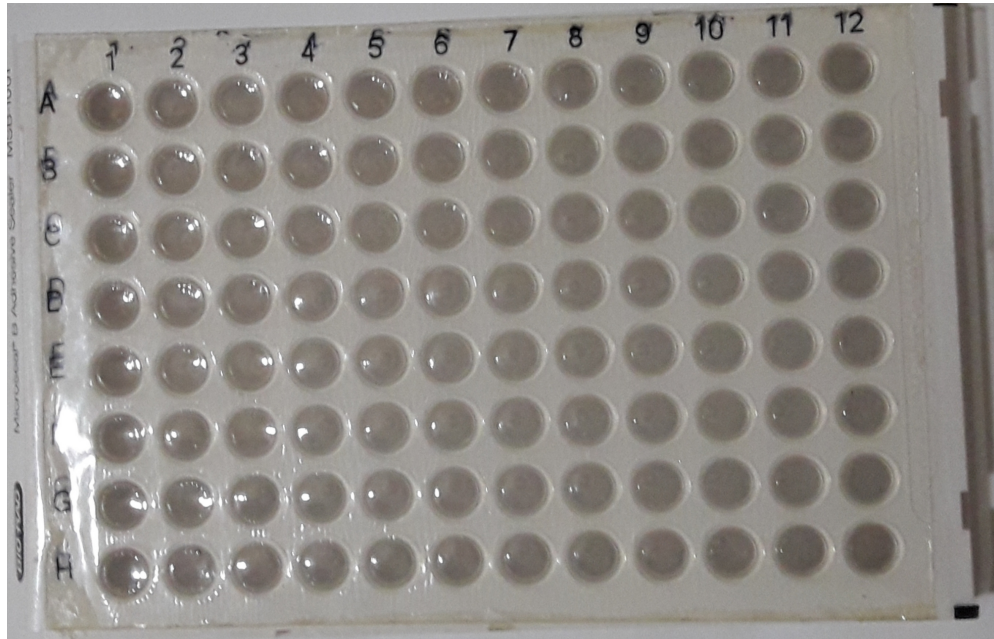


Fig 4.2 96 well plate loaded with samples for RT-PCR

Data interpretation

Relative quantification of gene expression was calculated after normalizing with the reference genes. The level of gene expression was assessed by $2^{-\Delta\Delta C_t}$ formulae. Data was analyzed using the 2-factorial ANOVA, the main effects (Age and treatment) and their interactions were calculated using the SPSS 15ver software.

Calculation of $2^{-\Delta\Delta C_t}$

Arithmetic mean of C_t value were recorded from the qPCR and values were normalized by using C_t value of internal control gene.

$$\Delta C_t (\text{Control}) = C_t (\text{Target Gene}) - C_t (\text{internal Control})$$

$$\Delta C_t (\text{Test}) = C_t (\text{Target Gene}) - C_t (\text{internal Control})$$

$$\Delta\Delta C_t = \Delta C_t (\text{Test}) - \Delta C_t (\text{Control})$$

Relative quantity or fold change in gene expression of target genes with respect to the control group was calculated.

$$\text{Fold Change} = 2^{-\Delta\Delta C_t}$$

5.2 COMPREHENSIVE MARINE NATURAL PRODUCT DATABASE

It is a manually curated open access knowledge base dedicated to marine natural products. CMNPD is an integrated platform for structure replication of marine natural products, discovery of lead compounds, data mining of structure activity and investigation of chemical ecology. Some of the sources of marine compounds include PubChem, ChEMBL, ZINC database and CMNPD.

5.2.1 PROTEIN DATA BANK

PDB is a database that contains three dimensional (3D) structural data of large molecules such as proteins and Nucleic acids. This data can be freely accessed throughout the world by using websites of its member organisations. wwPDB (World Wide Protein Data Bank) can be accessed from anywhere in the world.

5.2.2 PUBCHEM

Pubchem is a database that contains chemical molecules and their activities against biological assays. It mostly contains small molecules (ligands), although large molecules such as carbohydrates, peptides, nucleotides, lipids and chemically modified macromolecules can also be found. The 2D and 3D structure, chemical formula, molecular weight and other related information to the selected molecule can be obtained from the database.

5.2.3 OPENBABEL

Openbabel is a computer software it is a chemical expert system mainly used to interconvert chemical file formats. Openbabel is designed to support molecular modeling, chemistry and many related areas, including interconversion of file formats and data

5.2.4 PATCHDOCK

Patchdock is an algorithm for molecular docking. Patchdock method is performed

to predict the structure of protein-protein and protein-small molecule complexes.

5.2.5 SWISS ADME

Swiss ADME is an online tool employed to evaluate the drug-likeness, pharmacokinetics and medicinal friendliness of the selected small molecules. To obtain the results, the SMILES structure of the small molecule is taken from PDB database and then pasted in the input dialog box.

5.2.6 BIOVIA

Biovia discovery studio provides the complete toolset to explore of protein chemistry and catalyse the discovery of small and large molecule therapeutics from target id to lead optimisation. BIOVIA discovery studio is a software for simulating small molecules and macromolecules system.

5.2.7 PyMoL

PyMoL is an open source molecular visualisation system created by warren Lyford DeLano. Pymol can produce high quality 3D images of small molecules and biological macromolecules such as proteins. Pymol is one of the open source model visualisation tool used in structural biology.

5.3 METHODOLOGY

The entire process of obtaining the suitable molecule and verifying the ligand-drug binding is given below:

STEP: 1

Initially, the crystal structure ligand-bound Nurr1 in *Homo sapiens* (5Y41) was obtained from Protein Data Bank (PDB) and saved as a pdb file.

STEP: 2

Next, the screening of marine compounds was performed employing

Comprehensive Marine Natural Products Database(CMNPD) through the process of selection of various molecules according to the user defined filter and also by referring a variety of literature works.

STEP: 3

The filters were applied based on factors such as Taxonomy – Fungi, Molecular Weight – 200 to 400 and Compound Bioactivity.

STEP: 4

On application of these filters, the most suitable molecules according to the search query will appear.

STEP: 5

Randomly select the different available compounds. Obtain the SMILES format (copy) from the compound and open another website called Openbabel online converter.

STEP: 6

Two different dialog boxes will appear – input and output, in the input box paste the obtained SMILES sequence and change the input format to Structure Data File (SDF).

STEP: 7

The results will be sent to the given e-mail address. On opening the results, the highest docking score will be displayed at the top of the table as result1.

STEP: 8

Download the results. Now, with the help of drug discovery software – Biovia, the receptor-ligand interaction can be viewed in the 3D structure (coil).

STEP: 9

The various hydrogen bonds [(H-bond) (green)] can be seen – under 2D ligand structure to verify whether the bonding occurs in the target area of the protein.

STEP :10

Through literature reference it was found that the Nurr1 bonding site for various proteins in the target molecule was in the range between 500 to 550.

STEP :11

Based on the results it can be finalised whether to shortlist this compound or not.

STEP :12

The screening of nearly 500 compounds was performed before shortlisting the most suitable candidates. The shortlisted compounds can be viewed in table 5.1.

Structure of Nurr1

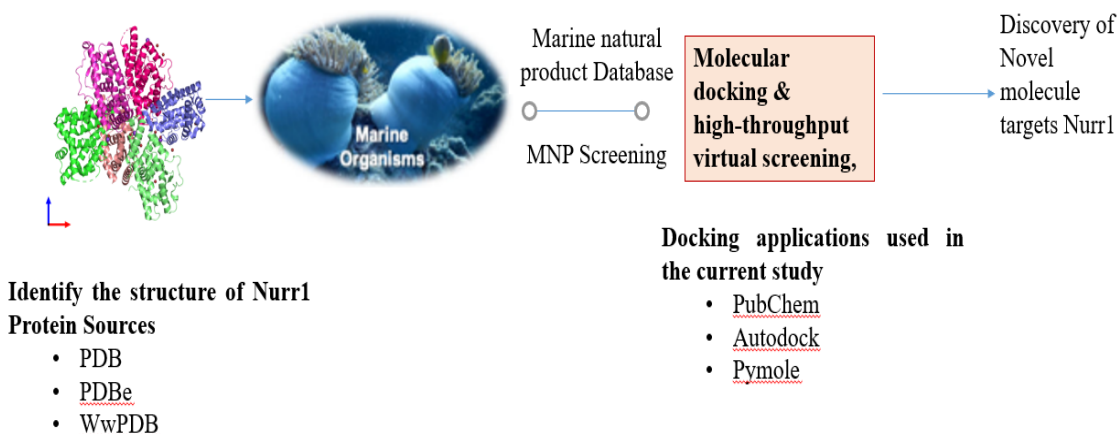


Fig 4.3 Flow chart diagram representing flow of work performed

CHAPTER-5

RESULTS AND DISCUSSIONS

5. SCREENING MARINE NATURAL COMPOUNDS AGAINST NURR1 USING BIOINFORMATICS TOOLS

5.1 TARGET IDENTIFICATION Nurr1 - PDB

Current study profiles the screening of marine compounds and suitable molecule was identified by docking it against the target *Nurr-1* protein. However, the effectiveness of a compound cannot be solely based on the homology modelling but must also be verified from a series of *in-vivo* studies and experiments.

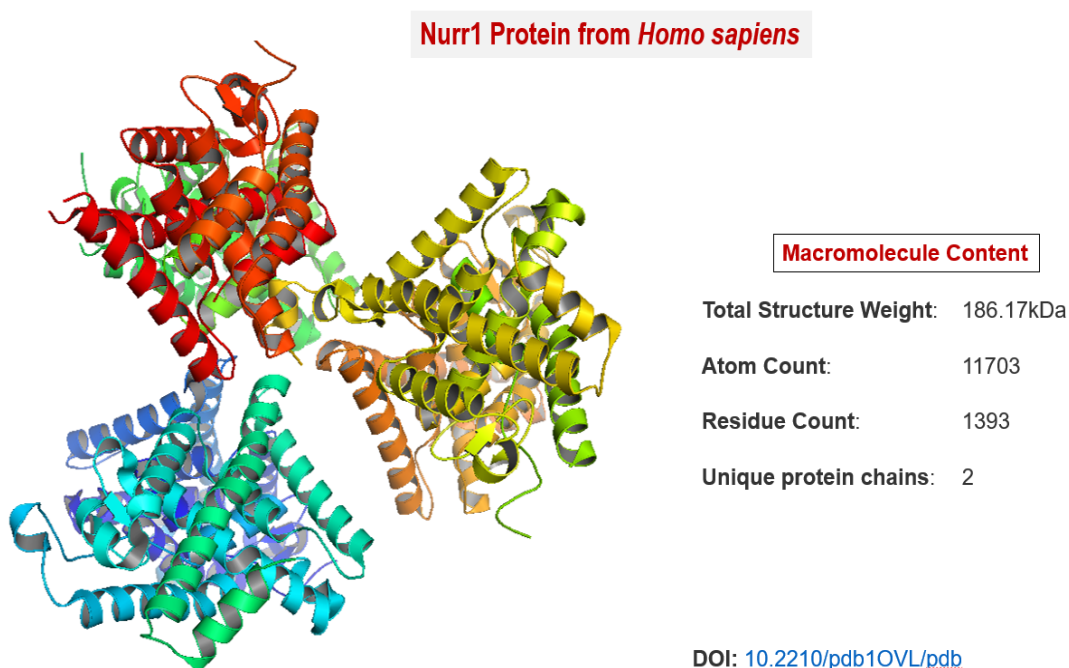
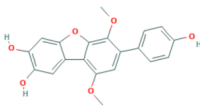
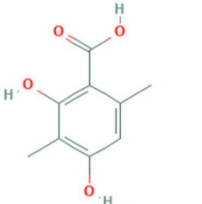
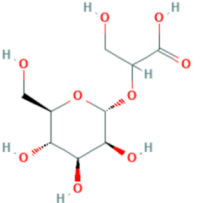
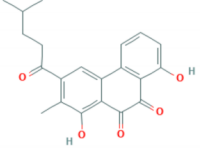
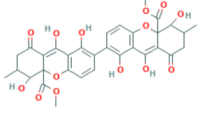


Fig 5.1: Structure of Nurr-1 protein from homo sapiens

5.2 COMPOUND IDENTIFICATION

The compounds were identified from Comprehensive Marine Natural Database(CMNPD) and PubChem. The selected compounds along with their PubChem id, molecular weight, molecular formula and species were found. These compounds were selected based on the binding with Nurr1 and the drug likeliness. The results can be interpreted from Table 5.1.

Table 5.1 Details of suitable marine compounds with their name and classification

Compound Name	Pubchem ID	Molecular Formula	Molecular Weight	Species	Structure
Candidusin A	24011606	C ₂₀ H ₁₆ O ₆	352.3g/mol	A.candidus	
3-Methylorsellinic acid	736228	C ₉ H ₁₀ O ₄	182.17g/mol	A.terreus	
Mannosylglycerate	443262	C ₉ H ₁₆ O ₉	268.22g/mol	R.marinus	
Piloquinone	624669	C ₂₁ H ₂₀ O ₅	352.4g/mol	S. variabilis	
Secalonic acid	423972	C ₃₂ H ₃₀ O ₁₄	638.6g/mol	G. corda	

5.3 OPENBABEL

Based on the selected compounds from the marine and protein databases, the SMILES sequence was downloaded and converted to PDB format using the online tool Openbabel. The SMILES sequence of each compound can be found in Table 5.2. Upon obtaining the SMILES sequence, it was pasted in an input box to obtain the PDB sequence which is then used in Patchdock.

TABLE 5.2 CANONICAL SMILES OF THE COMPOUNDS

S.NO	COMPOUNDS	CANONICAL SMILES
1.	Candidusin-A	<chem>COC1=C2C3=CC(=C(C=C3OC2=C(C(=C1)C4=C C=C(C=C4)O)OC)O)O</chem>
2.	3-Methylorsellinic	<chem>CC1=CC(=C(C(=C1C(=O)O)O)C)O</chem>
3.	Mannosylglycerate	<chem>C(C1C(C(C(C(O1)OC(CO)C(=O)O)O)O)O)O</chem>
4.	Piloquinone	<chem>CC1=C(C=C2C3=C(C(=CC=C3)O)C(=O)C(=O)C 2=C1O)C(=O)CCC(C)C</chem>
5.	Secalonic acid	<chem>CC1CC(=O)C2=C(C3=C(C=CC(=C3O)C4=C(C5= C(C=C4)OC6(C(C(CC(=O)C6=C5O)C)O)C(=O)OC)O)OC2(C1O)C(=O)OC) O</chem>
6.	Fucoesterol	<chem>CC=C(CCC(C)C1CCC2C1(CCC3C2CC=C4C3(CCC(C4)O)C)C)C(C)C</chem>
7.	Xyloketal	<chem>CC1(CC2C3=CCC4C(C3(CCC2(CC1O)C)C) (CCC5C46CCC(C5(C)C)(OC6)O) C)C</chem>
8.	Neoechinulin	<chem>CC1C(=O)NC(=CC2=C(NC3=CC=CC=C32)C(C) (C)C=C)C(=O)N1</chem>
9.	Astaxanthin	<chem>CC1=C(C(CC(C1=O)O) (C)C)C=CC(=CC=CC(=CC=CC=C(C)C=CC= C(C)C=CC 2=C(C(=O)C(CC2(C)C)O)C)C)C</chem>

5.4 PATCHDOCK

With the help of the online tool patch dock, the obtained PDB file from openbable was uploaded to patch dock server along with the Nurr1 protein. The results were

run and the docking score was interpreted. The docking score for each compound can be seen in Table 5.3.

TABLE 5.3 PATCHDOCK DOCKING SCORE

S.NO	COMPOUNDS	DOCKING SCORE
1.	Candidusin A	5090
2.	3-Methylosellinic acid	3438
3.	Mannosylglycerate	3978
4.	Piloquinone	5038
5.	Secalonic acid	6190
6.	Fucosterol	5438
7.	Xyloketal	5354
8.	Neoechinulin	5538
9.	Astaxanthin	6988
10.	6-hydroxy-N- acetyl-beta-oxotryptamine	5773

5.5 SWISS ADME

The online tool SWISS ADME was employed to identify whether the selected compounds had the properties to behave like a drug and if they could be used for further progression involved in drug identification and preparation. It also helps to study the pharmacokinetics of the selected compound. The results can be interpreted from the spider-web structure and understand the pink regions within it. The unfavourable compounds were further disregarded for further studies in this project. The results we obtained from SWISS ADME are given in Table 5.4.

TABLE 5.4 LIST OF COMPOUNDS SHOWING DRUG-LIKENESS PROPERTIES

S.NO	COMPOUND NAME	DRUG-LIKENESS
1.	Candidusin A	Yes
2.	3-Methylosellinic acid	Yes
3.	Mannosylglycerate	Yes
4.	Piloquinone	Yes
5.	Secalonic acid	No
6.	Fucosterol	Yes
7.	Xyloketal	Yes
8.	Neoechinulin	Yes
9.	Astaxanthin	No
10.	6-hydroxy-N- acetyl-beta-oxotryptamine	No

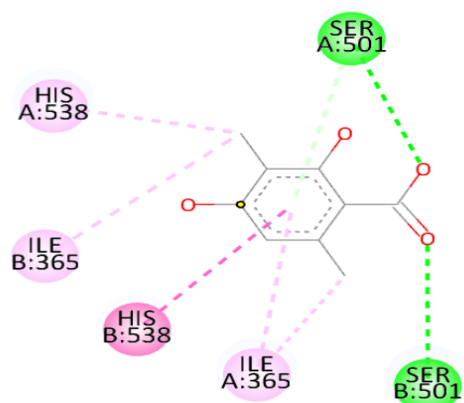
5.6 VISUALISATION OF LIGAND-RECEPTOR BINDING USING BIOVIA DISCOVERY TOOL

With the help of Patchdock and upon retrieving the results, the drug discovery tool Biovia was used to analyse whether binding occurred between the ligand and receptor molecule. The major protein binding area between the receptor molecule and the ligand are Serine, Histidine, Isoleucine, Cysteine, Proline, Asparagine, Valine and Arginine. The binding site of the ligand with the protein was analysed and the identification of hydrogen bonding was found. Only if there are hydrogen bonds present will it determine that the ligand is even more suitable to be used as a drug, this can be understood from Table 5.5. The results obtained from the visualiser can also be viewed below.

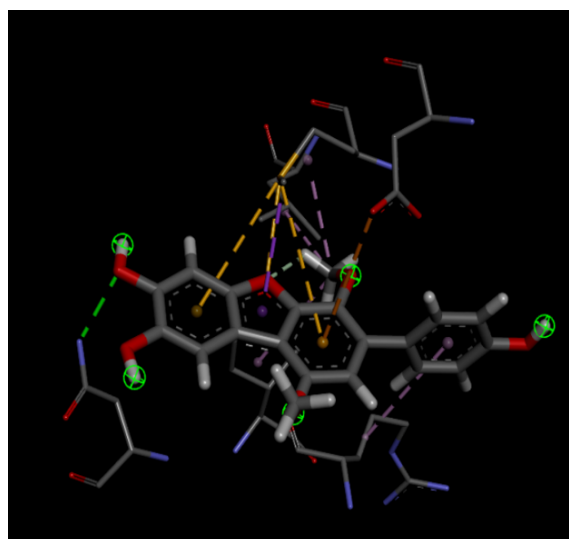
TABLE 5.5 COMPOUNDS SHOWING HYDROGEN BOND

S.NO	COMPOUNDS	HYDROGEN BONDING
1.	Candidusin A	Present
2.	3-Methylosellinic acid	Present
3.	Mannosylglycerate	Present
4.	Piloquinone	Present
5.	Secalonic acid	Present
6.	Fucosterol	Absent
7.	Xyloketal	Absent
8.	Neoechinulin	Absent
9.	Astaxanthin	Absent
10.	6-hydroxy-N- acetyl-beta-oxotryptamine	Absent

Fig 5.2: Representing the binding between Nurr-1 (receptor) and Candidusin A (ligand)



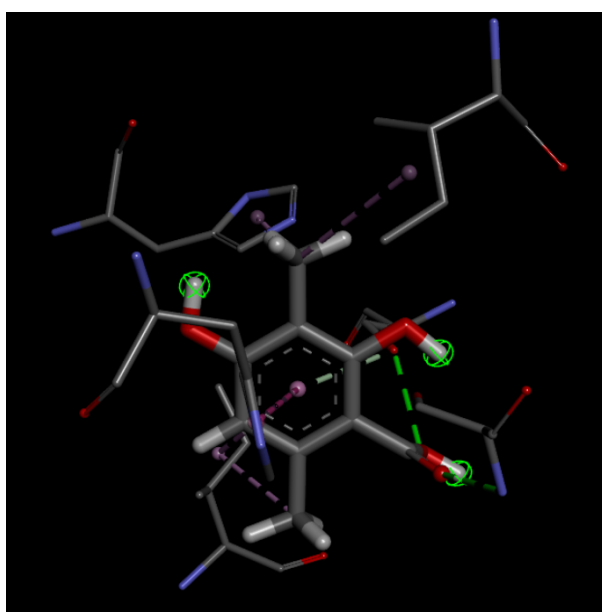
(A) 2D ligand structure



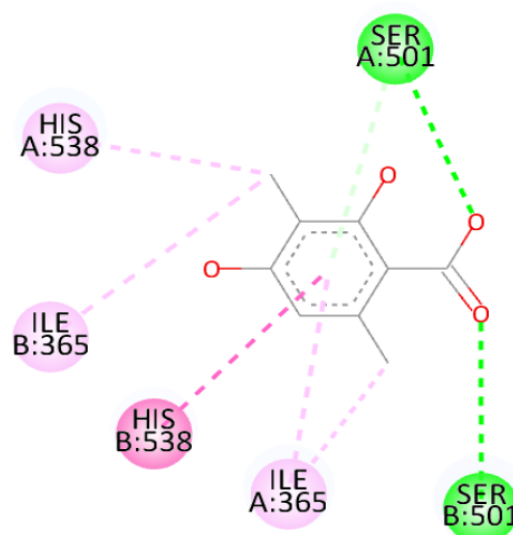
(B) 3D ligand structure



Fig 5.3 : Representing the binding between Nurr-1 (receptor) and 3-Methylorsellinic Acid (ligand)



(A) 3D ligand structure



(B) 2D ligand structure

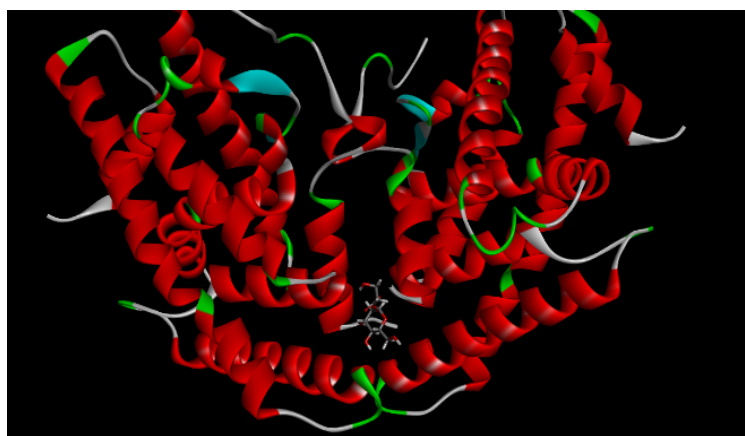
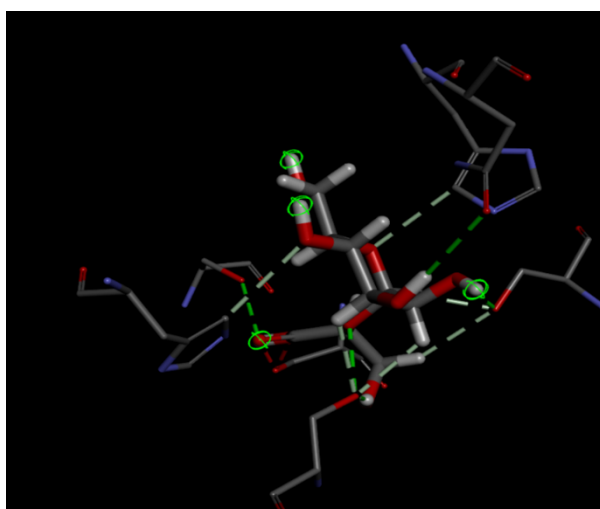
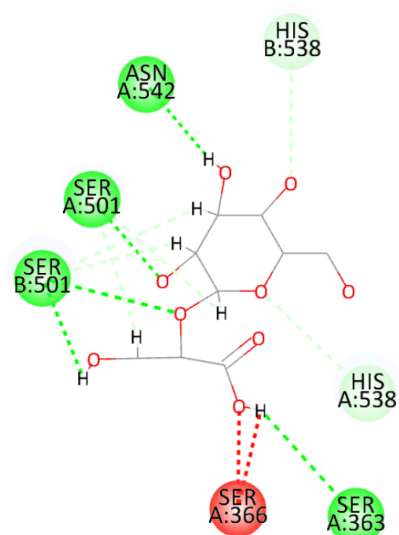


Fig 5.4 : Representing the binding between Nurr-1 (receptor) and Mannosylglycerate (ligand)



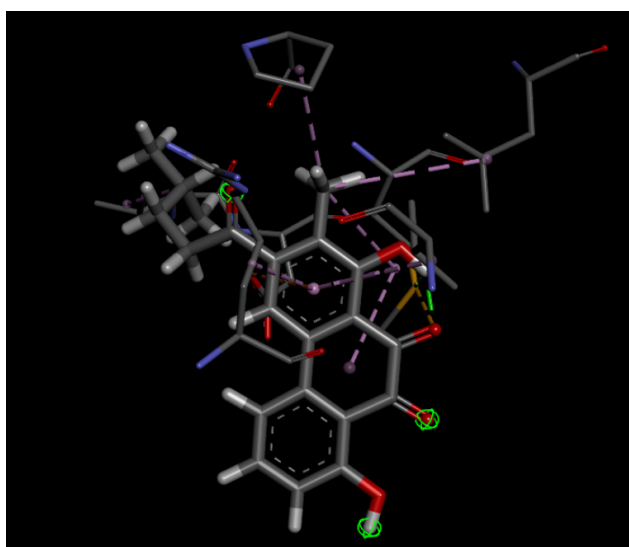
(A) 3D ligand structure



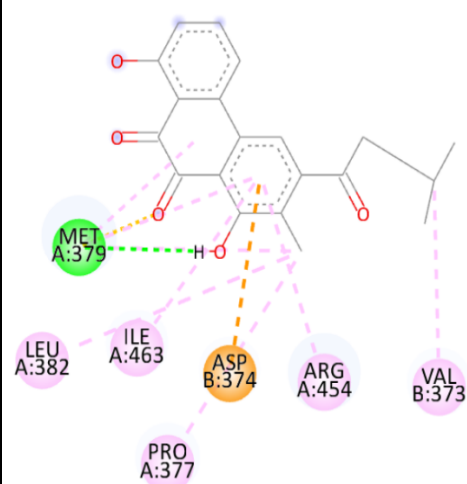
(B) 2D ligand structure



Fig 5.5: Representing the binding between Nurr-1 (receptor) and Piloquinone



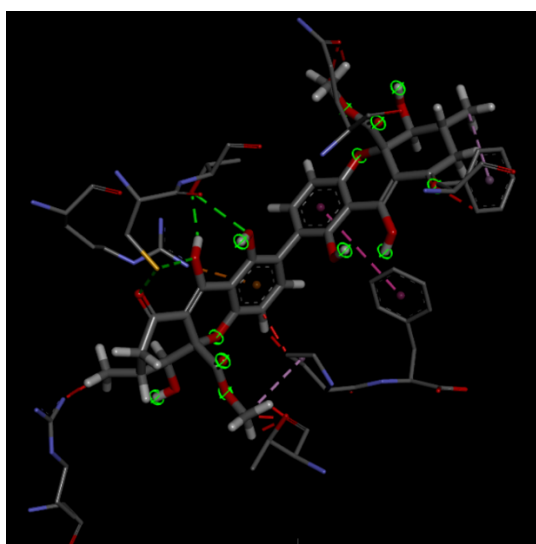
(A)3D ligand structure



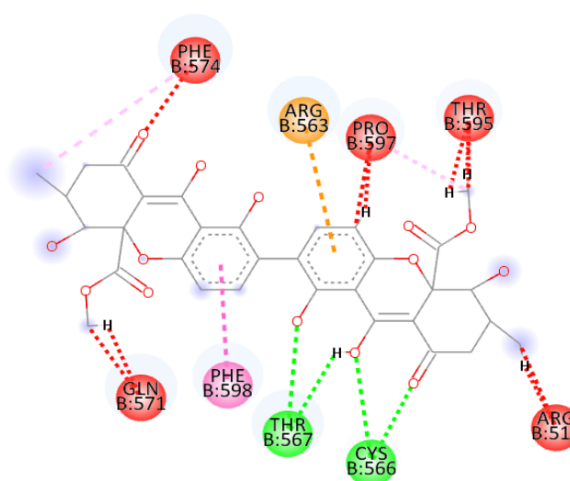
(B) 2D ligand structure



Fig 5.6: Representing the binding between Nurr-1 (receptor) and secalonic acid (ligand)



(A) 3D ligand structure



(B) 2D ligand structure

6. RT-PCR

The figures below represent the results obtained from performing real time PCR.

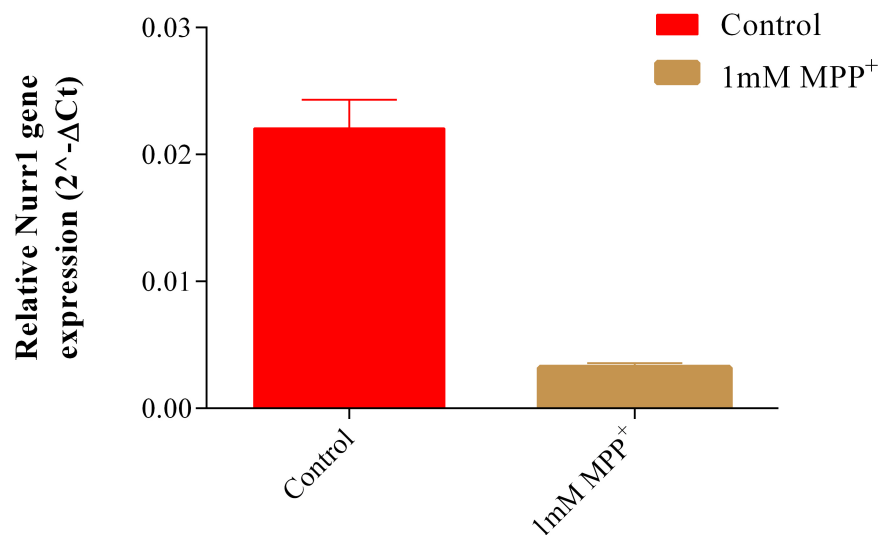


Fig 5.7: Relative Nurr1 gene expression chart

Table 5.6: RT-PCR Result

Gene		
	Control	1mM MPP ⁺
Nurr1	0.022±0.00042	0.003±0.00011

From these figures it can be interpreted that at 96 hpf a significant down regulation expression of Nurr1 protein in the ventral diencephalon and midbrain-hindbrain boundary was elucidated in MPP⁺ exposure. The RT-PCR result confirmed the dynamic changes in the expression of TFs observed fro 96hpf for both control and MPP⁺ embryos. The genes were down regulated in. Time dependant manner during the course of MPP⁺.(Jayshree et al.,2020)

Nurr 1, an orphan nuclear receptor belonging to the superfamily of ligand activated transcription factors. When mes DA progenitors exit the cell cycle and start to differentiate, they initiate the expression of Nurr1 (Zetterstrom *et.al.*,1997). It is required for the differentiation and maintenance of dopaminergic neurons. Hence in the current study we analysed Nurr1 expression patterns at 96 hours post fertilization (hpf) of MPP⁺ treated embryos. At 96 hpf, Nurr 1 is expressed in the ventral diencephalic region of control embryos while MPP⁺ significantly reduced the expression. It is interesting to point out that many interconnections between Nurr 1, and PD-linked genes such as α -synuclein, Pink1 or Parkin have been recognized (Yang *et.al.*,2008). Conversely, decreased expression of Nurr 1 reported in PD patients could transcriptionally induce expression of α -synuclein (Yang *et.al.*,2008). Our results are in concurrence with these discussed findings, suggesting a general role of NURR1 in DA neuron physiology and PD progression consequent to its impairment.

CHAPTER – 6

SUMMARY AND CONCLUSION

Parkinson's disease was induced in zebrafish embryos by treating them with MPP⁺ from the 18 hpf till 96 hpf, as this is critical window period for development of DA neurons. The induction of PD in zebrafish embryo by MPP⁺ causes morphological changes, such as flat head, small eyes, pinched midbrain-hindbrain boundary, thin yolk extension, and curved-up body, whereas the control embryos showed normal growth.

In conclusion, the developed of a treatment paradigm that mimics PD pathology without having to wait for many months to generate an age related disease can be observed. This system shows here that redeploying the developmental transcriptional cascades is critical for DA degeneration. Finally, targeting TFs can be used to activate pro-regenerative mechanism in mammals as well which will lead to generation and functional integration of new dopaminergic neurons.

Amongst the 500 molecules screened for a novel compound, the final 5 compounds were selected based on the target binding capability and the presence of hydrogen bonds along with the protein and area of binding between the receptor and ligand.

The approach of using marine compounds as a potential drug to regenerate the lost dopaminergic neurons and not only to target the symptoms or relieve the pain of PD will hopefully be a novel identification. Previously, many studies involving plant compounds have been carried out although, no definite drug has been identified. The finalised compounds ought to be subjected to many other intricate studies to be able to be selected as a potent drug and process further.

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