Parkinson’s disease (PD) is a pathological condition that has been known for centuries through traditional Indian texts, Greek scientific descriptions and ancient Chinese sources. The prime clinical symptoms of PD are tremor at rest, rigidity, bradykinesia (slowness of movement), postural abnormalities, freezing phenomenon and these symptoms have been compiled by Agnivesha (2500 BC) of Hindu University and re-edited in second century BC (Stern, 1989). Parkinson’s disease is the second most common neurodegenerative disorder after Alzheimer’s disease, affecting between 0.5 to 1% of the population aged 65–69 years and 1–3% of the population over 80 years of age (De Lau and Breteler, 2006). In 1817, Dr. James Parkinson assorted the core symptoms of PD and published a classic monograph “Essay on Shaking Palsy” which was later renamed as Parkinson’s disease by Jean-Martin Charcot. Eighty five percent of newly diagnosed PD cases occur in patients above 65 years of age. Most developed countries are already experiencing an aging phenomenon, where population over 65 years are increasing more rapidly than the other age groups, which results in the increase in incidence of PD and cost of the health care system. It has been estimated €798 billion as the total cost of brain disorders in Europe (2010) of which percentage of direct health care cost, non-medical cost and indirect cost were 37%, 23% and 40% respectively (Olesen et al., 2012). Pritchard et al. (2004) reported that rate of death related to neurological disorders dramatically increased by one to two fold between 1979 and 1997 in Western countries, which brings out the importance of brain-related disorders in our society. Since PD is a progressive disease, the social and economic impact increases over time, not only to the patients but also to caregivers. Current medical cost of PD patient per year is estimated to be US$ 24,000 and further, increase in cost was found to be six times higher for patient in the later stages of PD (Keränen et al., 2003; Lindgren et al., 2005). Epidemiological studies have found that Parkinsonism is prevalent to a varying degree in all countries. It is estimated that 4 million people worldwide have PD, and the incidence increases day by day. Symptoms of PD were reported to be reduced by treatment at early stages of the disease and it worsens as the disease progresses. Complete cure for Parkinson’s disease has not yet been worked out since current pharmacological and surgical therapies are futile.
inhibiting the progression of PD and supports neurodegeneration. Hence, the main aim of pharmacotherapy is to slow down the disease progression.

The population of elderly Indians is projected to increase from 8 percent in 2010 to 19 percent in 2050 (http://www.prb.org/Publications/Reports/2012/india-older-population.aspx). This increasing life expectancy results in increase of Parkinson’s disease incidence and other age-related diseases. Ragothaman et al. (2008) reported that nearly one-third of the 493 residents (Banglore, India) living in elderly homes had movement disorders, of which PD is most common (24%), followed by essential tremors (4.5%). Most of the manganese mine workers in India suffer from manganese-induced Parkinsonism. These patients walk on their toes and fall frequently, as they have severely impaired postural reflexes and typically have a pathological laughter and deep pigmentation of gums, palate, and uvula (Dhatrak and Nandi, 2010). Moreover, Parsi community of Mumbai (India) have a PD prevalence rate of 328.3 per 100,000 populations which is almost equal to Nebraska (USA) and Amish Community (Switzerland, North America, Canada) which has the incidence of 329.3 and 407 per 100,000 respectively. The Parsi follows Zoroastrianism religion and as a part of their religion, they burn Aspand seeds (Peganum harmala) which contain harmine and harmaline (alkaloids) to rid their children of the Evil Eye and the fumes are often inhaled. Harmine and harmaline are identified as monoamine oxidase (MAO) inhibitors, which are also used in treatment of Parkinson’s disease. But, long-term use of MAO inhibitors eventually has the opposite effect, and so this custom may be the cause of high PD prevalence amongst the Parsi (Bharucha et al., 1998).

The underlying pathological finding in PD is the loss of nigrostriatal dopaminergic (DA-ergic) neurons with a resultant loss of neurotransmitter dopamine in the corpus striatum (region in brain responsible for motor control). Clinical symptoms appear when approximately 50% of dopamine (DA) neurons in the substantia nigra pars compacta (SNpc) and 80% of the striatal dopamine are lost. Post-mortem studies revealed that presence of Lewy bodies to be an important hallmark for PD. Lewy bodies are intracytoplasmic eosinophilic proteinacious
inclusions made up of alpha-synuclein aggregation and are toxic to DA neurons. Lewy bodies are not only restricted to the SNpc but can also be found in other brain regions and in the peripheral autonomic nervous system as the disease progresses (Braak et al., 2003)

Oxidative stress and mitochondrial dysfunction are underpinned for initiating a cascade of toxic events leading to dopaminergic neuronal death in Parkinson’s disease (PD) and identified as vital target for therapeutic intervention. It is possible to reduce the risk of chronic neurodegenerative disease by either enhancing the body’s natural antioxidant defense or by supplementing with proven dietary antioxidants. This is the main reason why discovery and synthesis of novel antioxidants is a major active area. Curcumin, a polyphenol isolated from Curcuma longa has been reported to have potent antioxidant, anti-apoptotic, anti-inflammatory properties and protects dopaminergic neurons through its mitochondrial action (Cole et al., 2007). Though curcumin has excellent biological properties, it lacks the ability to inhibit excitotoxicity and has less bioavailability and stability with higher EC_{50} value. These drawbacks are overcome by CNB-001[4-((1E)-2-(5-(4-hydroxy-3-methoxystyryl)-1-phenyl-1H-pyrazol-3-yl)vinyl)-2-methoxy-phenol)], a novel hybrid pyrazole derivative from two neuroprotective compounds namely, Curcumin and Cyclohexyl bisphenol A. It has been reported to have various neuroprotective properties and is superior to its parental compounds (Liu et al., 2008). Hence, this study was aimed to analyze the neuroprotective properties of CNB-001 by utilizing Parkinson disease models.

![Chemical structure of CNB-001](image)

**Figure 1. Chemical structure of CNB-001**
Aim of the Research

The general objective of the present study is to analyze the neuroprotective effect of CNB-001 on Parkinson’s disease.

The specific objectives of the study were as follows:

- To identify potent inhibitor of alpha-synuclein aggregation and pharmacokinetic evaluation of antioxidants using in silico approach.
- To analyze in vitro antioxidant potential and DNA protecting activity of CNB-001.
- To evaluate the neuroprotective effect of CNB-001 using rotenone induced SK-N-SH cellular model of PD.
- To elucidate the therapeutic efficacy of CNB-001 in MPTP induced Parkinson mouse model.
3. Parkinson’s disease

The clinical symptoms of PD was first defined by James Parkinson (1817) as “Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellects being uninjured” and he named the disease as Paralysis Agitans. Later it was observed that apart from motor symptoms, non-motor symptoms also arise during disease progression. It was then the work done by the great French neurologist Jean-Martin Charcot (1825-1893) who exclusively brought great attention to Parkinson’s description and coined the term maladie de Parkinson. In 2003, Fahn defined Parkinson as any combination of six specific motor features: tremor at rest, bradykinesia, rigidity, loss of postural reflexes, flexed posture and the freezing phenomenon. Apart from cardinal symptoms, various secondary symptoms arise as the disease progresses (figure 2).

Figure 2. Schematic representation of symptoms of Parkinson’s disease.
• Dementia - impaired intellectual functioning that interferes with normal activities and relationships.
• Loss of ability to solve problems and maintain emotional control, personality changes, and behavioral problems such as agitation, delusions, hallucinations, and memory loss.
• Anhedonia (inability to experience pleasure from activities formerly found enjoyable, e.g., hobbies, exercise, social interaction, or sexual activity).
• Apathy - a state of indifference or the suppression of emotions such as concern, excitement, motivation, and passion.
• Psychosis - loss of contact with reality.
• Restless Leg Syndrome (RLS) - irresistible urges to move one’s body to stop uncomfortable or odd sensations.
• Periodic Limb Movements of Sleep (PLMS) - sudden jerking movements of the legs that occur involuntarily during sleep and of which the affected individual may remain unaware.
• Paresthesiae - sensation of tingling, pricking, or numbness of a person’s skin with no apparent long-term physical effect.

Until now, no biomarkers have been identified to diagnose PD and it purely depends on clinical diagnosis. Structural imaging of the central nervous system (CNS) and functional imaging of neurotransmitter activities using SPECT and PET supports diagnosis of PD (Tolosa et al., 2007). PD is classified as Sporadic and Familial PD where sporadic PD is defined as PD in a patient without any known first or second degree relative affected with parkinsonism and familial PD is defined as PD in a patient with at least one first or second degree relative with parkinsonism.

Parkinson’s disease occurs due to degeneration of dopaminergic neurons in Substantia nigra pars compacta (SNpc) and resultant loss of dopamine in the striatum (ST). Dopamine is an important neurotransmitter responsible for transmitting signals between SNpc and corpus striatum to produce smooth and purposeful muscle activity. During dopamine loss due to neurodegeneration, patients can’t direct or control their movements in a normal manner (Fahn, 2003).
3.1. Basal ganglia

Basal ganglia motor circuitry has been identified to play an important role in various motor functions. Hence, understanding its anatomy, circuitry and physiological functions is essential to understand PD pathogenesis. Basal ganglia consist of four important structures: the striatum (caudate nucleus, putamen and nucleus accumbens), the pallidum (external and internal segments of the globus pallidus and ventral pallidum), the subthalamic nucleus (STN) and the substantia nigra (pars compacta and pars reticulata). Globus pallidus has two important compartments, the internal segment of globus pallidus (GPi) and external segment of globus pallidus (Gpe) separated by medullary lamina. Basal ganglia internal serve as the output structure of basal ganglia. Similarly, substantia nigra has two segments: the ventral substantia nigra pars reticulata (SNr) and the dorsal part Substantia nigra pars compacta (SNe) that covers the SNr (Marsden, 1982; Lee, 1987).

Basal ganglia circuitry which plays an important role in dopamine transmission and movements has two major pathways (direct and indirect pathway) that process cortical information and feed the processed information back to the cortex (Alexander et al., 1986; Segawa, 2000). The striatum is connected to the internal segment of the GPi and SNr through direct pathway. The GPi and SNr are the output nuclei of the basal ganglia (GPi/SNr) and project to the brainstem and the thalamus. The influence of the GPi and SNr on the thalamus is inhibitory, whereas the thalamic projection to the cortex is excitatory. The indirect pathway also connects the striatum to the output nuclei of the basal ganglia but these fibers first pass through synaptic connections in the GPi and then to STN. Output from the STN to the GPi/SNr is excitatory. At the level of the output structures of the basal ganglia, these two pathways oppose each other in controlling the excitatory output of the VA/VL complex to the motor and premotor cortices (Romo and Schultz, 1992; Exner et al., 2002).

3.1.1. Role of dopaminergic neurons in basal ganglia

Dopaminergic neurons play an important role in regulating the neurotransmission within the basal ganglia. These neurons are divided into four types: nigrostriatal, mesolimbic, mesocortical and tuberoinfundibular. In mesolimbic
pathway, dopamine is transmitted from the ventral tegmental area (VTA) to nucleus accumbens which are located in midbrain and limbic system respectively. Mesocortical pathway transfers dopamine from VTA to frontal cortex whereas; in tuberoinfundibular pathway dopamine is transmitted from hypothalamus to pituitary gland. Previous reports showed that severe death of neurons occur in nigrostriatal pathway in PD while other pathways are less affected (Uhi et al., 1985; Hirsch et al., 1988). Degeneration of neurons in other pathways leads to other cognitive and psychological impairments such as dementia which occurs in almost 30% of PD cases (Aarsland et al., 1996). Normally, dopaminergic neurons in SNpc communicate with neurons of basal ganglia by liberating dopamine and it helps in motor function. In PD, almost 80% of the dopamine depletes in the corpus striatum resulting in movement disorders by altering basal ganglia circuitry (Leenders and Oertel, 2001).

![Diagram of brain pathways](image)

**Figure 3.** Nigrostriatal, Meso-limbic and Meso-cortic pathways. Dopaminergic neurons are present in the midbrain structures connecting substantia nigra (SN) and the ventral tegmental area (VTA). Axons of dopaminergic neurons project to the striatum, the ventral and dorsal prefrontal cortex (Arias-Carrion et al., 2010).
3.2. Dopamine metabolism

The process involved in the formation of catecholamines (dopamine) has been characterized and knock out studies using mice model proved the importance of dopamine in various physiological processes. Tyrosine hydrolase (TH) is the rate-limiting enzyme for the formation of dopamine and it is found in all cells that can synthesize catecholamines by using tyrosine and molecular oxygen as its substrates and biopterin as its co-factor (Shiman et al., 1971). This enzyme is a heterotetramer with a molecular weight of approximately 60,000 daltons and it helps in the formation of 3,4-dihydroxy-L-phenylalanine (L-Dopa) by catalyzing the addition of hydroxyl group to meta position of tyrosine. Various immunohistochemical reports proved that 50% of reductions in TH immunoreactivity and correlated with behavioral pattern (Genc et al., 2001). Tyrosine hydrolase can also hydroxylate phenylalanine to tyrosine which then converted to L-Dopa and this synthetic route can be given to patients suffering from phenylketonuria (phenylalanine hydroxylase activity is depressed) (Siegel et al., 2006). DOPA decarboxylase (DDC) which also called as aromatic amino acid decarboxylase (AADC) is a pyridoxine dependent enzyme that has low $k_{m}$ and high $V_{max}$ when compared to L-Dopa which aids in the conversion of L-Dopa to dopamine. AADC is found throughout the body in both neuronal and non-neuronal tissues including kidney and blood vessels. This enzymatic conversion is the final step in the synthesis of dopamine and hence it is being exploited clinically for treatment of PD (Lerner, 1987).

Newly synthesized dopamine is sequestered into vesicles by vesicular monoamine transporter 2 which is an ATP dependent process linked to proton pump. The concentration of dopamine inside the vesicles is approximately 0.5 molar/L and they exist as a complex with ATP and chromogranins (acidic proteins). The vesicles help in mediating the process of catecholamine (dopamine) release and they maintain a ready supply of catecholamines at the nerve terminals. Variations in the level of VMAT2 result in bipolar disorders and lithium treatment modulates the expression of VMAT2 (Zubieta et al., 2000; Cordeiro et al., 2002).
Figure 4: Dopamine pathway. TH: Tyrosine Hydrolase; L-Dopa: L-3, 4-dihydroxy-phenylalanine; DDC: DOPA decarboxylase; MAO: Monoamine oxidase; DAT: Dopamine transporter; DOPAC: 3, 4-dihydroxyphenlacetic acid; DRD: Dopamine receptor; COMT: catechol-O-methyltransferase; HVA: Homovanillic acid; 3-MT: 3-methoxytyramine (Quaak et al., 2009).

When action potential reaches the nerve terminals, Ca$^{2+}$ channel opens resulting in increased intracellular calcium which facilitates fusion of vesicles with the neuronal membrane and excocytosis of the neurotransmitter (Weinshilboum et al., 1971). Dopamine released into the synaptic space either binds to pre or postsynaptic neurons or taken back to presynaptic neuron via dopamine transporter (DAT) or degraded. Intracellular dopamine gets degraded by monoamine oxidase to form 3,4-dihydroxyphenylacete. Extracellular dopamine is degraded by Catechol-O-methyl-transferase (COMT) to form 3-methoxytyramine (3-MT) and homovanillic acid from DOPAC. Extracellular MAO converts DA to DOPAC and 3-MT in HVA (Li and Chin, 2003).

3.3. Underlying pathogenesis of PD

Emerging challenges in understanding the pathogenesis of PD include abnormalities in cellular protein transport, interaction between proteins and protein aggregation, environmental toxins and mutations. Studies on toxic models and
functions of genes implicated in inherited forms of PD suggest two hypotheses regarding pathogenesis of PD. One hypothesis posits that misfolding and aggregation of proteins are instrumental in the death of SNpc dopaminergic neurons, while the other proposes the culprit is mitochondrial dysfunction and the consequent oxidative stress. Recent advances in molecular genetics and neurochemistry have shown the involvement of excitotoxicity and oxidative stress in cell death. Strong evidence now exists to support a role of aberrant mitochondrial form and function as well as increased oxidative stress in pathogenesis of PD (Schapira, 2008). A complex interplay occurs between mitochondria and other cellular machinery that affects cell survival, as mitochondria not only have the key role in the electron transport chain and oxidative phosphorylation but are also the main source of free radicals (Henchcliffe and Beal, 2008). Mitochondrial dysfunction leads to increased oxidative stress such as oxidative damage to lipids, proteins, and DNA as well as decrease in levels of important antioxidant enzymes leads to cell death. Post-mortem studies have shown increased levels of oxidative stress and decreased level of glutathione, the brain’s main anti-oxidant in PD patients (Dexter et al., 1989). The possibility that an oxidative phosphorylation defects play a role in the pathogenesis of PD was fueled by the discovery that MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) blocks mitochondrial electron transport chain by inhibiting complex I (Nicklas et al., 1987). Invitro studies indicate that mitochondrial complex I defect may subject cells to oxidative stress and energy failure (Schapira et al., 1990). Nearly 100% of molecular oxygen is consumed by the mitochondrial respiration, and powerful oxidants are normally produced as byproducts, including hydrogen peroxide and superoxide radicals. Inhibition of complex I increases the production of superoxide radicals, which may form toxic hydroxyl radicals or react with nitric oxide to form peroxynitrite. One target of these reactive species may be the electron transport chain itself, leading to mitochondrial damage and further production of Reactive Oxygen Species (ROS) (Cohen, 2000). Many markers of oxidative damage are increased in SNpc of PD patients. The presence of ROS causes misfolded proteins to further misfold, thereby increasing the demand on the ubiquitin-proteosome system to remove them. Dopaminergic neurons may be a
particularly fertile environment for generation of ROS as the metabolism of DA produces hydrogen peroxide and superoxide radicals, and auto-oxidation of DA produces DA-quinone, a molecule that damage proteins by reacting with cysteine residues (Graham, 1978). In cells, including neurons, there is a necessary fine-tuned balance between the production and removal of oxidants, and it is this balance that keeps ROS and Reactive Nitrogen Species (RNS) constantly at low, nontoxic levels. Furthermore, oxidative damage to DNA (Zhang et al., 1999), proteins (Alam, 1997) and lipids (Dexter et al., 1994) has been found in SNpc of PD patients. Oxidative stress contributes to the cascade, leading to dopamine cell degeneration in Parkinson’s disease. However, oxidative stress is intimately linked to other components of the degenerative process, such as mitochondrial dysfunction, excitotoxicity, nitric oxide toxicity, and inflammation. A study was conducted by Nikam et al. (2009) in 40 PD patients in the age group of 40-80 years where oxidative stress was assessed by estimating: lipid peroxidation product in the form of thiobarbituric acid reactive substances; nitric oxide in the form of nitrite and nitrate; enzymatic antioxidants in the form of superoxide dismutase, glutathione peroxidase, catalase, ceruloplasmin, and non-enzymatic antioxidant vitamins, e.g., vitamin E and C in either serum or plasma; and trace elements, e.g., copper, zinc, and selenium; and found that plasma thiobarbituric acid-reactive substances and nitric oxide levels were significantly high but superoxide dismutase, glutathione peroxidase, catalase, ceruloplasmin, vitamin E, vitamin C, copper, zinc, and selenium levels were significantly low in Parkinson’s disease when compared with control subjects. This study states that elevated oxidative stress is playing a role in dopaminergic neuronal loss in substantia nigra pars compacta and involved in pathogenesis of Parkinson’s disease. The decreased activity of these antioxidant enzymes is indirectly responsible for neuronal loss, which led to the conclusion that oxidative stress and antioxidants might be playing an important role in PD.

3.4. Inflammatory and apoptotic mechanisms in pathogenesis of PD

Inflammatory and apoptotic mechanisms are underpinned to initiate self-propelling neurodegeneration in PD. Due to the presence of Blood-Brain Barrier
(BBB) and lack of lymphatic system, brain was initially considered immune-privileged but later various studies reported the presence of cytokines, chemokines and other immune factors produced by microglia and astroglia which leads to brain edema. Inflammatory mechanisms in brain is being described as a ‘double-edged sword’ since in acute conditions, short-lived mechanisms helps to maintain brains micro-environment and when chronically sustained at high levels, inflammatory mechanisms damages host tissue (McGeer and McGeer, 2004). Microglia plays an important role in initiating innate immune response to pathogens and activates rapidly during pathological events (Wersinger and Sidhu, 2002). Apart from microglia, astrocytes and oligodendrocytes play a vital role in inflammatory response. Astrocytes maintains brain’s extracellular environment by regulating glutamate uptake. Astrocytes can also be activated by various chemicals and physical damage and leads to a process called reactive gliosis which can be identified by the up-regulation of glial fibrillary acidic protein (GFAP) and the gap junction protein Connexin 43 (Haupt et al., 2007). Activation of oligodendrocytes induces production of various pro-inflammatory cytokines, nitric oxide, prostaglandins and upregulates chondroitin sulfate proteoglycans like NG2 which inhibits axonal growth during CNS injury (Rhodes et al., 2006). Role of inflammation in pathogenesis of PD have been proved from various post-mortem studies where higher number of activated microglia were found in substantia nigra when compared to control subjects (McGeer et al.,1988). Similar studies reported enhanced expression of numerous inflammation related enzymes like tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), cyclooxygenase 1 and 2 (COX-1 and COX-2) and inducible nitric oxide synthase (iNOS) in substantia nigra, striatum and cerebrospinal fluid of PD patients (Hirsch et al., 1998; Knot et al., 2000; Tansey et al., 2007). These inflammatory mechanisms lead to neuronal death by binding to “death receptors” and activate extrinsic and intrinsic cell death pathways. Intrinsic cell death pathway initiates neurodegeneration by activating down-stream apoptotic cascade. Neurons undergo apoptosis based on its internal and external environment. Internal environment include cell type, cell maturity and developmental history and external environment being growth factors, cytokines, appearance and disappearance
of hormones. Various apoptotic cascades such as intrinsic, extrinsic, mitochondrial and death receptor mediated p53 dependent and independent pathways as well as caspase dependent and independent pathways are induced in neurodegeneration based on the environmental factors. In extrinsic signaling pathway, apoptosis is initiated via transmembrane-receptor interactions which involve members of tumor necrosis factor receptor gene family (FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5) (Locksley et al., 2001). These members bind to the death domain and transmits signal from the cell surface to intracellular signaling pathways. Upon these response, clustering of receptors takes place and the ligand binds to the receptor resulting in formation of death-inducing signaling complex which activates procaspase 8 (Kischkel et al., 1995).

![Intrinsic and extrinsic pathway leading to apoptosis](image)

**Figure 5. Intrinsic and extrinsic pathway leading to apoptosis** (Favaloro et al., 2012).

Similarly, positive and negative signals regulate intracellular signaling pathway. Negative signals include production of cytokines, hormones, absence of growth factors and positive signal includes exposure to toxins, radiations, free radicals and hypoxia. These toxic stimulus causes depolarization of mitochondrial membrane and release of pro-apoptotic proteins (cytochrome C, Smac/DIABLO, serine protease HtrA2/Omi) into the cytosol and it in turn activates caspase dependent mitochondrial pathway (Chinmaiyan, 1999). Caspase mediated apoptotic cascade plays an important role in PD pathogenesis by disruption of cellular machinery, inhibiting the synthesis of new proteins, DNA fragmentation,
degradation of cytosolic and nuclear proteins, formation of apoptotic bodies and finally taken up by phagocytic cells (Susan, 2007).

3.5. Current treatments for Parkinson’s disease

Present therapeutic modalities have some initial success, but the beneficial effects wear off with long-term use, and many of the alternative therapies have severe side effects. Current therapies alleviate the symptoms but do not halt the progression of the disease. Broadly, treatment of PD are classified as

- Pharmacological treatment
- Surgical treatment

3.5.1. Pharmacological treatment

The most commonly used treatment for PD is the dopamine precursor, L-Dopa. Unfortunately approximately 50% of patients using L-Dopa develop complications within 5 years of treatment (Ahlskog and M uenter, 2001). These complications are mostly severe motor fluctuations (wearing-off effect) and dyskinesia (drug-induced voluntary movements including choreiform and dystonic movement). Various strategies have been proposed to provide a uniform dopamine supply, including new delivery methods like transdermal delivery, increased frequency of delivery, and new sustained-release formulations (Stocchi, 2005; Steiger, 2008). Monoamine oxidase inhibitors are sometimes useful as monotherapy for early PD and also can be used with L-Dopa therapy. Their use is sometimes associated with a worsening of L-Dopa’s side effects in some patients (Singh et al., 2007). Though dopamine receptor agonists are potent in controlling primary motor symptoms, because of their side effects (hallucinations, confusion, and psychosis) they often are contraindicated, especially in older patients (Fahn, 2003). The limited efficiency over the course of the disease and incapacity to stop or reinstate neurodegeneration makes these drugs less than optimal choice for treatment.

3.5.2. Surgical treatment

Prior to L-Dopa, surgical ablation of deep brain structures was performed. Thalamotomy was an effective treatment for reduction of contralateral tremor, while
pallidotomy was found to improve motor symptoms with variable degrees of success (Burchfield, 1995; Guridi and Lozano, 1997). Although these therapies were completely abandoned with the introduction of L-Dopa, recently pallidotomy has re-emerged as an option in treatment. Although they are effective in controlling cardinal symptoms, they have serious limitations. Unilateral pallidotomy has no long-lasting effects on gait and balance problems. Bilateral pallidotomy has severe cognitive and psychiatric side effects (Walter et al., 2005). Hence, these therapies are used to treat only the symptoms of PD, but none of them protects the neurons from damage or can repair or replace the damaged neurons. Recently, deep brain stimulation, a new surgical treatment has emerged which involves the passage of high-frequency electrical pulses through electrodes implanted into deep brain structures. The Food and Drug Administration (FDA) approved Deep Brain Stimulation (DBS) as a treatment for essential tremor in 1997, for PD patients in 2002 and for dystonia in 2003. Despite its advantages, deep brain stimulation has been associated with worsening of some PD symptoms, including dyskinesia and speech and gait disturbances (Umemura et al., 2003). Neuroprotection offers a way of preserving the remaining neurons and fights the cause of neuronal death.

3.6. Neuroprotective therapy

The therapies described above are used to treat only the symptoms of PD, but none of them protects the neurons from damage or can repair or replace the damaged neurons. Neuroprotection offers a way of preserving the remaining neurons and fights the cause of neuronal death.

Antioxidant: The key to prevention and control

Researchers are working on a number of antioxidants, like selenium, vitamin C, vitamin E, and coenzyme Q10, but contradictory results have made these less than optimal. It is thought that racemic forms found in vitamin E supplements and other synthetic antioxidant compounds might have a lower bioactive potential than naturally occurring forms (Zhang et al., 2002).
3.6.1. Nicotine

It was observed that cigarette smoking is inversely associated with the risk of developing PD. These studies suggest that chemicals present in cigarette smoke might provide some form of neuroprotection (Allam et al., 2004). The protection depends upon the dose of nicotine which is a major alkaloid in tobacco. It binds to nicotine acetylcholine receptors that are present in striatum and partly overlaps with the dopaminergic system and modulates dopamine release (Zhang and Sulzer, 2004). However, clinical trials in PD patients produced conflicting data showing improvement, worsening, or no change in motor symptoms (Kelton et al., 2000). Previous study illustrates the difficulty of delivering an efficient dose, because smoking is clearly not an option, gum chewing is insufficient, and transdermal patches are associated with side effects (Lemay et al., 2003). Despite promising results, these studies remain to be confirmed in clinical trials. Development of receptor subtype-specific agonists and antagonists may pave the way for future nicotine-based neuroprotective therapeutic strategies.

3.6.2. Monoamine oxidase B inhibitors

Monamine oxidase (MAO) inhibitors catalyze the oxidative deamination of monoamine neurotransmitters such as dopamine, noradrenaline, and serotonin in the central and peripheral nervous system. Two isoforms have been described, MAO-A and MAO-B, which differ in their substrate specificity and localization (Collins et al., 1970). MAO-A is responsible for the deactivation of circulating catecholamines and the deamination of the monoamine neurotransmitters. Its inhibition has some benefits in treatment of psychiatric disorders but has various contraindications in the treatment of PD because of its numerous side-effects (Chen and Ly, 2006). MAO-B is the predominant isoform in the basal ganglia, where it is mainly expressed by glial cells and is particularly important for the breakdown of dopamine. However, the deamination produces toxic aldehydes and ROS; hence, this treatment is not highly recommended (Youdim and Bakhle, 2006). Selegiline and rasagiline are among the monoamine oxidase inhibitors. Overall, the neuroprotective effects of MAO
inhibitors have been difficult to identify and may remain elusive, as the symptomatic benefits will be nearly impossible to segregate.

Interestingly, the body poses defense mechanisms against free radical–induced oxidative stress which involve preventive mechanisms, repair mechanisms, physical and antioxidant defenses. Enzymatic antioxidant defenses include superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), etc. Non-enzymatic antioxidants includes ascorbic acid (vitamin C), alpha-tocopherol (vitamin E), glutathione (GSH), carotenoids, flavanoids, etc. All of these act by various mechanisms, including reducing activity, free-radical scavenging, potential complexing of pro-oxidant metals, and quenching of singlet oxygen. It is possible to reduce the risk of chronic neurodegenerative disease by either enhancing the body’s natural antioxidant defense or by supplementing with antioxidants (Stanner et al., 2004). This is the main reason why discovery and synthesis of novel antioxidants is a major active area.

3.7. Models of Parkinson’s disease

Apart from post-mortem brain tissues, various genetic models of PD are developed but failed to create the exact pathobiochemical alterations as seen in PD and hence neurotoxic models remained the cornerstone for understanding the pathogenesis of PD and development of novel drugs (Bove et al., 2006). Over the years, many chemical toxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone, 6-hydroxydopamine (6-OHDA), paraquat were widely used to create PD models. The potent neurotoxin, MPTP mimics the same pathobiochemical alterations as seen in PD patients are extensively used to create PD model (Burns et al., 1980). MPTP undergoes two-step biotransformation via mono-amine oxidase B (MAO-B) initially in astrocytes and then oxidized to form MPP⁺ (1-methyl-4-phenylpyridinium), a decisive neurotoxin which is taken up into dopaminergic neurons through the dopamine transporter (DAT) and sequestered in mitochondria (Przedborski et al., 1993). MPP⁺ inhibits cellular respiration by blocking the electron transport enzyme NADH: ubiquinone oxido-reductase (complex I). Various reports have also showed that MPP⁺ can inhibit complexes III (ubiquinol: ferrocytochrome c oxidoreductase) and IV (ferrocytochrome c:oxygen
oxidoreductase or cytochrome c oxidase) of mitochondrial ETC (Speciale, 2002). The toxic effect of MPTP is mainly due to the mitochondrial impairment, disturbance in redox homeostasis and inflammatory responses (Ojha et al., 2012). MPTP is susceptible to humans and many other animals and are vitally used to study behavioral impairments as seen in PD patients. Various techniques (probenecid with MPTP) and regimens are used to modulate the effect of MPTP in brain tissues (Petroske et al., 2001). Rotenone, an insecticide is isolated from the roots of Derris elliptica and Lonchocarpus urucu plant species. Because of its hydrophobic nature, it can easily cross blood brain barrier (BBB) and does not depend upon dopamine transporter to access neuronal cytoplasm (Greenamyre et al., 2001). Rotenone exposure inhibits respiratory complex I of ETC which results in accumulation of electrons and ROS formation. Various reports showed that inner mitochondrial Permeability Transition Pore (PTP) opening initiates complex I conformational change which results in increased ROS (Batandier et al., 2004). Moreover, rotenone inhibits phosphorylation of Peroxiredoxin-2 (Prx2) and alleviates Prx2 peroxidase activity resulting in increased ROS (Przedborski, 2007).

Figure 6. Mechanism of toxicity induced by neurotoxins on mitochondria. BBB: Blood-brain barrier; AAT: amino acid transporter; PAT: polyamine transporter; CI & CII: Complex I & II; ROS: Reactive oxygen species; PQ: paraquat radical; 6-OHDA; 6-hydroxyphe dopamine; DAT: Dopamine transporter; MAO-B: Monoamine oxidase B; MPP⁺: 1-methyl-4-phenylpyridinium (Keane et al., 2011).
A study conducted by Betarbet et al. (2000) showed that rats which received rotenone via jugular vein cannulation reproduced the same behavioral anomalies and biochemical alterations as seen in PD patients. The difference between rotenone and MPTP is that rotenone inhibits complex I of mitochondria throughout the brain and MPTP selectively inhibits complex I of dopaminergic neurons. The major disadvantage of rotenone model is variability between animals and bilaterally lesioned animals are difficult to maintain as compared to MPTP treated animals. 6-hydroxydopamine (6-OHDA) is the first identified neurotoxin which is known to degenerate cholinergic system (Sachs and Jonsson, 1975). As dopamine and norepinephrine, 6-OHDA induces neurodegeneration via inhibiting catecholamine transport system. Since 6-OHDA does not have the capability to cross blood-brain barrier, systemic administration cannot be achieved. This toxin is injected into substantia nigra, striatum via stereotaxic injection for specific toxicity and dopaminergic neurons degenerate within 24 h of the first injection followed by loss of striatum in 3 days (Faul and Laverty, 1969). The magnitude of lesion produced depends upon the site of injection, amount of toxin injected and sensitivity between animals. Usually 6-OHDA is injected in one hemisphere while to other serves as control but this leads to asymmetric circling behavior due psychological imbalance between unlesioned and lesioned hemisphere (Ungerstedt, 1968). Similar to MPTP and rotenone, 6-OHDA inhibits complex I of ETC and produced superoxide radicals in the presence of iron. Prior administration of iron chelating agents, antioxidants prevented the neurotoxic effect of 6-OHDA (Ben-Shachar et al., 1991). The limitations of 6-OHDA model is that it does not from cytoplasmic inclusions and it does not cause progressive degeneration of dopaminergic neurons as seen in PD. Apart from their limitations, 6-OHDA has been used to evaluate the efficacy of cell transplantation, support of neurotrophic factors and to analyze the ability of compounds which promotes cell survival (Dunnet et al., 1981).

Paraquat (1,1’-dimethyl-4,4’-bipyridinium) is one of the agricultural chemicals which is known to affect the nigrostriatal system. Since paraquat is structurally similar to MPP⁺, the mechanism of toxicity is believed to be the same as MPP⁺ but the ability of paraquat to cross blood-brain barrier is limited (McCormack
et al., 2002). The only difference between paraquat and MPP\textsuperscript{+} structurally is that paraquat has N-methyl-pyridinium group and MPP\textsuperscript{+} has phenyl group. Although it causes striatal dopaminergic innervation and produces ambulatory movements, higher systemic toxicity profiles leads to higher mortality in experimental animals (Prasad et al., 2009).

3.8. CNB-001

CNB-001[4-((1E)-2-(5-(4-hydroxy-3-methoxystyryl)-1-phenyl-1H-pyrazol-3-yl)vinyl)-2-methoxy-phenol]] is a hybrid molecule synthesized from Curcumin and Cyclohexyl Bisphenol A (CBA) (Liu et al., 2008). Curcumin, a polyphenol isolated from turmeric (Curcuma longa) has been known to possess various biological properties invitro and invivo. Despite curcumin’s persuasive pharmacological properties, its activity is limited because of its poor bioavailability and its failure to inhibit excitotoxicity and amyloid toxicity (Anand et al., 2007). Cyclohexyl Bisphenol A, a neurotrophic molecule has greater EC\textsubscript{50} value which is toxic to the cell.

A recent study by Narumoto et al., (2012) proved that CNB-001 possessed superior inflammatory properties by suppressing active SERpine1, IL-6, TNF-\textalpha, IL-13 expression in Normal human bronchial epithelial (NHBE) cells when compared to its parental compounds. Moreover CNB-001 has been proved to be neuroprotective in cell culture assays for trophic factor withdrawal, oxidative stress, excitotoxicity, glucose starvation, as well as toxicity from both intracellular and extracellular amyloid with a lower EC\textsubscript{50} value when compared to Curcumin and CBA (Liu et al., 2008). Alzheimer’s disease (AD) is the most common neurodegenerative disorder followed by PD which is due to accumulation of \textbeta-amyloid protein and its resultant toxicity. CNB-001 has been proved to inhibit \textbeta-amyloid aggregation by enhancing eIF2\textalpha phosphorylation, HSP90 and ATF4 levels in MC65 cells. It also maintains expression of synapse associated proteins and improves memory in transgenic animal model of AD (Valera et al., 2013).
Figure 7. Molecular structure of CNB-001 synthesized from Curcumin and Cyclohexyl bisphenol A (Liu et al., 2008).

Most of the neurodegenerative disorders results in loss of memory which affects the social life of the patients. Ca$^{2+}$/calmodulin dependent protein kinase II (CaMKII) plays an important role in long-term potentiation (LTP), memory and its expression is decreased in Alzheimer’s and Parkinson’s disease. Administration of CNB-001 to adult male Wistar rats facilitated induction of LTP and memory in object recognition test (Maher et al., 2010). Apart from these diseases, acute ischemic stroke is a major risk of mortality in ageing population and currently only one drug known as thrombolytic tissue plasminogen activator is approved by U.S Food and drug administration to treat stroke. A recent study conducted by Lapchak et al. (2011) using rabbit ischemic stroke model revealed that CNB-001 maintained levels of ATP, and phosphorylated ERK and Akt which are reduced in ischemic stroke. Pharmacokinetic properties of CNB-001 showed that it has the ability to cross blood-brain barrier and the maximal brain concentration reached 1 h after oral gavage and the maximum plasma concentration reached 2 h after
administration (Liu et al., 2008). These reports provide concrete evidence that CNB-001 possessed various neuroprotective properties. However, the protective effect of CNB-001 on Parkinson’s disease has not been elucidated. Hence, this study was aimed to analyze the effect of CNB-001 on behavioral deficits, biochemical, neurochemical and protein modulations associated with PD.
4.1. Computational feature

All insilico analysis were performed using HP Workstation Z220 with Next-generation 22 nm processors, including the Intel Xeon processor E3-1200v2 family with 16 GB RAM, 1 TB Hard disk, NVIDIA Quadro 2000, Windows 7 Ultimate 64 bit. Accelry’s Discovery Studio 3.5 (AD 3.5), CLC Genomic Workbench 5.1 and Biosolve IT softwares were used for insilico analysis.

4.2. Chemicals

CNB-001 was obtained as a gift from Dr. Dave Schubert, Cellular Neurobiology Lab, Salk Institute of Biological Sciences, La Jolla, USA. MTT, 2-7-diacetyl dichlorofluorescein (DCFH-DH), Rhodamine 123 (Rh 123), Ethidium Bromide (EtBr), acridine orange (AO), Sodium Dodecyl Sulphate (SDS), β-mercaptoethanol, bromophenol blue, TEMED, 2,4-dinitrophenylhydrazine (DNPH), heat inactivated bovine serum albumin (BSA), trypsin EDTA, agarose, phosphate buffered saline (PBS), Dulbecco’s modified Eagle’s medium (DMEM), glutamine, penicillin, streptomycin, gentamicin, DPPH, ABTS, 2-deoxyribose, MPTP, rotenone, PMS, Osmium tetraoxide and propylene oxide were purchased from Sigma, Co., St. Louis, USA. Potassium ferricyanide, trichloroacetic acid, sulfanilamide, naphthylethylene diamine dihydrochloride, riboflavin, EDTA, NADH, Butylated hydroxyl toluene (BHT), xylene orange, curcumin, thiobarbituric acid (TBA), nitroblue tetrazolium (NBT) and DTNB were purchased from Merck Inc, USA. Anti-TH, anti-VMAT2, anti-IL-1β, anti-IL-6, anti-TNF-α, anti-iNOS, anti-synuclein and β-actin antibodies were purchased from SantaCruz Biotechnology Inc, USA. Anti-DAT antibody was purchased from Chemicon, USA. Anti-Bcl-2, anti-Bax, anti-cytochrome C, anti-caspase-3 (p17), anti-cyclooxygenase 2 and anti-GFAP antibodies were obtained from Cell Signaling (USA). Anti-mouse, anti-rabbit secondary antibodies and Plasmid DNA (pUC19) were purchased from Genei, Bangalore, India. Enhanced chemiluminescence (ECL) kit was purchased from Gen Script ECL kit, USA. All other chemicals used were of analytical grade.
4.3.1. Ligands selected for the study

The ligands used for this study have been selected based on various literatures and given in table 1. Two-dimensional structure of the compound have been downloaded from Pubchem compound and [www.chemicalize.org](http://www.chemicalize.org). Marvin Sketch software developed by Chemaxon was used to draw 2D and optimize the 3D structure of CNB-001 which was not available in the databases. 3D structure of the ligands were optimized and used for the docking study.

Table 1. Structure of Ligands with their molecular properties

<table>
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<tr>
<th>S.No</th>
<th>Compound Name</th>
<th>Derivative</th>
<th>Properties</th>
<th>2D Structure</th>
<th>Source</th>
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</table>
| 1    | CNB-001       | Curcumin and Cyclohexyl bisphenol A | Molecular weight: 442.5063  
Molecular formula: C25H22N2O8 | ![2D Structure](image1.png) | Marvin sketch |
| 2    | 7,8 dihydroxyflavone | Vitis vinifera | Molecular weight: 254.2375  
Molecular Formula: C16H10O6 | ![2D Structure](image2.png) | Chemicalize |
| 3    | Curcumin | Curcuma longa | Compound ID: 969516  
Molecular Weight: 368.3799  
Molecular Formula: C21H20O9 | ![2D Structure](image3.png) | Pubchem |
| 4    | Riboflavin | Malus domestica | Compound ID: 493570  
Molecular Weight: 376.3639  
Molecular Formula: C17H22O7 | ![2D Structure](image4.png) | Pubchem |
| 5    | Epigallocatechin gallate | Camellia sinensis | Compound ID: 65064  
Molecular Weight: 458.3712  
Molecular Formula: C33H30O11 | ![2D Structure](image5.png) | Pubchem |
| 6    | Resveretrol | Vitis vinifera | Compound ID: 445154  
Molecular Weight: 228.24328  
Molecular Formula: C11H12O3 | ![2D Structure](image6.png) | Pubchem |
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<th>Molecular Formula:</th>
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| 7   | Ascorbic acid          | Citrus reticulata | Compound ID: 54670067  
Molecular Weight: 176.12412  
Molecular Formula: C6H8O6 |                  |                             | Pubchem |
| 8   | Alpha tocopherol       | Brassica napus  | Compound ID: 14085  
Molecular Weight: 430.7061  
Molecular Formula: C30H48O2 |                  |                             | Pubchem |
| 9   | Coenzyme Q10           | Synthetic      | Compound ID: 5281915  
Molecular Weight: 586.3435  
Molecular Formula: C47H55O5 |                  |                             | Pubchem |
| 10  | Lycopene               | Lycopersicon lycopersicum | Compound ID: 446925  
Molecular Weight: 536.87264  
Molecular Formula: C39H58O7 |                  |                             | Pubchem |
| 11  | Lutein                 | Spinacia oleracea | Compound ID: 6433159  
Molecular Weight: 568.87144  
Molecular Formula: C47H56O4 |                  |                             | Pubchem |
| 12  | Butylated hydroxytoulene | Spilanthes acmella | Compound ID: 31404  
Molecular Weight: 220.35046  
Molecular Formula: C9H14O4 |                  |                             | Pubchem |
| 13  | Propyl gallate         | Synthetic      | Compound ID: 4947  
Molecular Weight: 212.19928  
Molecular Formula: C10H12O3 |                  |                             | Pubchem |
| 14  | Apigenin               | Matricaria recutita | Compound ID: 5280443  
Molecular Weight: 270.2369  
Molecular Formula: C15H10O5 |                  |                             | Pubchem |
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Materials and Methods

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Neuroprotective effect of CNB-001 against PD 28
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4.3.2. Drug likeliness evaluation

The drug likeliness property of the compounds was studied with the help of Lipinski drug filter using Accelrys Discovery Studio 3.5 which rejects false-positive compounds. Lipinski rule of five [1. Molecular mass less than 500Da, 2. Less than 5 hydrogen bond donors, 3. Less than 10 hydrogen bond acceptors and 4. High lipophilicity (expressed as Log P less than 5)] identifies whether the compound satisfies all the rules based on the 2D structure and provides valuable result regarding the use of compound as a drug (Lipinski et al., 2001).

4.3.3. ADME-Toxicity investigation

ADME-Toxicity studies were executed through Accelrys Discovery Studio 3.5. The Absorption, Distribution, Metabolism and Excretion (ADME) studies provide insight into the pharmacokinetic property of the compounds. Aqueous solubility, Blood brain barrier level, CYP 2D6, Hepatotoxicity and Plasma Protein Binding level were studied. Toxicity profile of the compounds were predicted using TOPKAT which uses a range of robust, cross-validated, Quantitative Structure-Toxicity Relationship (QSTR) models for identifying specific toxicological activity.
4.3.4. Target protein

The structure of the target protein (alpha-synuclein) was retrieved from Protein Data Bank [PDB ID-1XQ8]. Most favored regions of the protein structure were evaluated through Ramachandran plot analysis via PROCHECK (Laskowski et al., 1993) from EBI server (www.ebi.ac.uk/thornton-srv/software/PROCHECK/) to investigate the quality of the target protein structure.

4.3.5. Protein sequences analysis

Physicochemical properties such as sequence information, half life, extinction coefficient, atomic composition, count of hydrophobic and hydrophilic residues, count of charged residues, amino acid distribution, secondary structure analysis and plot of hydropathy were computed using the CLC Genomic workbench 5.1.

4.3.6. Molecular simulation studies of alpha-synuclein

X-Ray crystallography structure of α-synuclein was retrieved from protein data bank (PDB). Alpha-synuclein was further processed by applying CHARMM force field. Potential energy of a specified structure was evaluated by using calculate energy protocol of DS 3.5. The calculate energy protocol can be used to compare the relative stability of different configurations of the same structure; or as a prelude to lengthy simulations to confirm the availability of appropriate force field parameters. Energy minimization of 3-D modeled protein structure was done with the help of standard dynamics cascade protocol of DS 3.5 which performs the following steps: minimization with steepest descent method, minimization with conjugate gradient, dynamics with heating, equilibration dynamics, and production dynamics. The minimization protocol minimizes the energy of a structure through geometry optimization. For the simulation cascade, following parameters were used: steepest descents minimization (500 steps, RMS gradient 0.1) in first minimization step and in second steepest descents minimization (500 steps, RMS gradient 0.0001), heating (2000 steps, initial temperature 50 K, final temperature 300 K), equilibration (120 ps, 1fs time step, coordinates saved every 1000 steps) and production (120 ps, 1fs
4.3.7. Active site prediction

The active site has been identified as 64-100 amino acid residues since it is the critical binding region in the alpha-synuclein molecule responsible for its self-association and aggregation (El-Agnaf et al., 2004). Hence this active site sequence of alpha-synuclein was taken for docking study.

4.3.8. Molecular docking

The possible interaction between the ligands and the target protein (alpha-synuclein) were studied using LeadIT software. Ligand minimization was performed using CHARMM and MMF forcefield using AD 3.5 before docking. The algorithm used for docking in LeadIT is a FlexX based docking approach which flexibly places ligands into the active site with an incremental buildup algorithm which starts with selecting a base fragment, which is placed into the active site based on superposing interaction points of the fragment and the active site (Rarey et al., 1996). FlexX algorithm generates and uses up to 200 poses for each ligand and the best pose will be selected and scored.

4.4. Invitro antioxidant assays

The antioxidant activity of CNB-001 was analyzed by various invitro antioxidant assays. All the tests were carried out in triplicate and ascorbic acid was used as the standard.

4.4.1. DPPH radical scavenging activity

DPPH radical scavenging activity of CNB-001 was detected by following the method of Blois (1958).

Principle

DPPH is a stable free radical which accepts electron or hydrogen radical from other molecules to become a stable diamagnetic molecule. This assay is based
on electron transfer reaction. Hence the antioxidant or radical scavenger turns from violet to yellow by the presence of hydrogen or electron donation.

Reagents

- 0.1 mM Diphenyl-2-picrylhydrazyl (DPPH)
- Ethanol

Procedure

One ml of 0.1 mM DPPH radical solution was mixed with 3 ml of different concentrations of CNB-001 (20, 40, 60, 80 and 100 μg/ml) dissolved in methanol. The mixture was then thoroughly vortexed and incubated in dark for 30 min at 40 °C. DPPH solution without CNB-001 was used as control and the absorbance measured at 517 nm. Percentage of inhibition was calculated as ([A0−A1]/A0) × 100 where A0 was the absorbance of the control and A1 was the absorbance in the presence of the compound.

4.4.2. 96-Multiwell plate assay of DPPH

Aliquots of 0.1 ml of 0.04 mM DPPH solution in methanol was added into 96-multiwell plate. Hundred microlitres (20, 40, 60, 80 and 100 μg/ml concentration) of CNB-001 was added into each well except that of the control. The plate was incubated for 10 min in room temperature and photographs were taken. Ascorbic acid was used as standard (Mon et al., 2011).

4.4.3. ABTS radical scavenging activity

ABTS radical scavenging activity of CNB-001 was detected by following the method of Miller and Rice-Evans, (1997).

Principle

ABTS is an improved version of Trolox Equivalent antioxidant capacity assay. ABTS+ is a blue chromophore produced by the reaction between ABTS and potassium persulfate. This assay is used to understand the oxygen radical scavenging capacity.
Reagents

- 5 mM phosphate buffered saline (PBS)
- 5 mM 2,2,6,6-tetramethylpiperidine-N-oxyl (Tempo) (ABTS)

Procedure

Radical cation scavenging capacity of CNB-001 was observed against ABTS$^+$ (radical) generated by chemical methods. In brief, ABTS$^+$ was prepared by oxidizing a 5 mM aqueous solution of ABTS diammonium salt with manganese dioxide at room temperature for 30 min. The reaction mixture contained 1.0 ml of ABTS$^+$ with an absorbance of 0.7 at 734 nm, 100 μl of CNB-001 with various concentrations and 1 ml of 5 mM phosphate buffered saline (PBS) (pH 7.4). The absorbance was read after 1 min at 734 nm and the percentage inhibition was calculated. Percentage of inhibition was calculated as ((A0−A1)/A0) × 100, where A0 was the absorbance of the control (without CNB-001) and A1 was the absorbance in the presence of the CNB-001.

4.4.4. Determination of reducing power

Reducing power of CNB-001 was analyzed by following the method of Oyaizu (1986).

Principle

The presence of antioxidant causes the reduction of the Fe$^{3+}$/ferricyanide complex to the ferrous form. Yellow color of the test solution changes to various shades of green and blue depending upon the reducing power of the compound. Therefore, the Fe$^{2+}$ can be monitored by measuring the formation of Prussian blue at 700 nm.

Reagents

- 200 mM sodium phosphate buffer, (pH 6.6)
- 1% potassium ferricyanide
- 10% trichloroacetic acid (TCA)
- 0.1% ferric chloride
Procedure

Different concentrations of CNB-001 were mixed with 1.0 ml of 200 mM sodium phosphate buffer (pH 6.6) and 1 ml of 1% potassium ferricyanide. The mixture was incubated for 30 min at 50 °C. Later 1 ml of 10% trichloroacetic acid (w/v) was added and the mixture was centrifuged at 1431 x g for 10 min. The supernatant (2 ml) was mixed with equal volume of deionized water and 0.4 ml of 0.1% of ferric chloride was added. The absorbance was measured spectrophotometrically at 700 nm. Control was prepared using distilled water instead of CNB-001. Higher absorbance of the reaction mixture indicated greater reductive potential. The experiment was carried out in triplicate. Percentage of inhibition was calculated as \( ([A0-A1]/A0) \times 100 \) where A0 was the absorbance of the control (without CNB-001) and A1 was the absorbance of the mixture in presence of CNB-001.

4.4.5. Nitric oxide radical scavenging activity

Nitric oxide scavenging activity of CNB-001 was detected by following the method of Sreejayan and Rao, (1997).

Principle

In this assay, sodium nitroprusside at physiological pH spontaneously generates nitric oxide (NO), which interacts with oxygen to produce nitrite ions. The nitrite ions formed can be estimated using Griess Illosvosy reaction. Scavengers of NO compete with oxygen, leading to reduced production of NO and formation of pink colored chromophore.

Reagents

- 5 mM Sodium nitroprusside
- 1% sulfanilamide
- 0.1% naphthylethylene diamine dihydrochloride
- 2% H₃PO₄

Procedure

Nitric oxide radical scavenging activity was measured spectrophotometrically. Sodium nitroprusside (5 mM, 1.5 ml) in PBS was mixed in different concentration of
CNB-001 and incubated at 25 °C for 30 min. Sodium nitroprusside without test CNB-001 but with an equal amount of methanol was taken. Thirty minutes after incubation, 1.5 ml of the incubated solution was diluted with 1.5 ml of greiss reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride in 2% H₃PO₄). The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and subsequent coupling napthyl ethylene diamine was measured at 546 nm. Percentage radical scavenging activity of CNB-001 was calculated as follows:

\[ \text{Percentage of inhibition} = \left( \frac{[A_0-A_1]}{A_0} \right) \times 100 \]

where A0 was the absorbance of the control (without CNB-001) and A1 was the absorbance in the presence of the CNB-001.

4.4.6. Superoxide (O₂) radical scavenging activity

Superoxide (O₂) radical scavenging ability of CNB-001 was estimated by following the method of Beauchamp and Fridovich, (1971).

Principle

The assay was based on the capacity of the sample to inhibit blue formazan formation by scavenging the superoxide radical generated using riboflavin-light-nitroblue tetrazolium (NBT) system.

Reagents

- 50 mM phosphate buffer (pH 7.6)
- Riboflavin
- 12 mM ethylenediaminetetraacetic acid (EDTA)
- Nitro blue tetrazolium (NBT)

Procedure

Three microlitre of reaction mixture contained 50 mM phosphate buffer (pH 7.6), 20 µg riboflavin, 12 mM EDTA, 0.1 mg NBT and various concentrations (20-100 µg/ml) of CNB-001. Reaction was started by illuminating the reaction mixture for 90 sec. Immediately after illumination the absorbance was measured at
590 nm. The entire reaction was carried out under dark condition. Identical tubes with the reaction mixture except CNB-001 served as control. The percentage inhibition of superoxide anion generation was calculated as: Percentage of inhibition = \( \frac{(A0-A1)}{A0} \times 100 \) where A0 was the absorbance of the control (without CNB-001) and A1 was the absorbance in the presence of CNB-001.

4.4.7. Superoxide anion scavenging activity assay

Superoxide anion scavenging activity of CNB-001 was analyzed by following the method of Liu et al. (1997).

**Principle**

Superoxide radicals are generated in PMS-NADH systems by oxidation of NADH and assayed by the reduction of nitroblue tetrazolium (NBT).

**Reagents**

- Nitro blue tetrazolium salt (156 μM)
- Nicotine amide adenine dinucleotide solution (468 μM)
- Phenazine methosulfate (60 μM)

**Procedure**

The reaction mixture contains 156 μM NBT, 468 μM NAAD in 2 ml of 100 mM phosphate buffer (pH 7.4) with different concentrations of CNB-001. The reaction was initiated by adding 100 μl of PMS in 100 mM phosphate buffer, (pH 7.4) to the mixture. The reaction mixture was incubated at room temperature for 5 min and the absorbance was measured at 560 nm. The superoxide anion scavenging activity was calculated by the following equation. Percentage of inhibition was calculated as \( \frac{(A0-A1)}{A0} \times 100 \) where A0 is the absorbance of the control (without CNB-001) and A1 is the absorbance in presence of CNB-001.

4.4.8. \( \text{H}_2\text{O}_2 \) radical scavenging activity

Hydrogen peroxide radical scavenging activity of CNB-001 was estimated by following the method of Long and Evans, (1999).
Reagents

- FOX reagent (9 volumes of 4.4 mM BHT in methanol with 1 volume of 1 mM xylanol orange and 2.56 mM ammonium ferrous sulfate in 0.25 M H2SO4)

Procedure

The reaction mixture of CNB-001 and 50 mM H2O2 (1:1 v/v) was incubated for 30 min at room temperature. After incubation, 10 µl methanol and 0.9 ml of freshly prepared FOX reagent was added. The reaction mixture was vortexed and incubated at room temperature for 30 min. The absorbance of the ferric-xylanol orange complex was measured at 560 nm.

Percentage of inhibition = ([A0–A1]/A0) × 100 where A0 was the absorbance of the control (blank, without compound) and A1 was the absorbance in the presence of the CNB-001.

4.4.9. Hydroxyl radical (•OH) scavenging activity

Hydroxyl radical (•OH) scavenging activity of CNB-001 was estimated by following the method of Chung et al. (1997).

Principle

Quantification of the degradation product of 2-deoxyribose by condensation with TBA is the basic principle behind the assay. Hydroxyl radical was generated by the Fe³⁺-ascorbate-EDTA-H2O2 system which is known as the Fenton’s reaction.

Reagents

- 2-deoxyribose (10 mM)
- Ferrous Sulfate Heptahydrate (10 mM)
- Hydrogen peroxide (10 mM)
- 2.8% Trichloroacetic acid (TCA)
- 1% Thiobarbituric acid (TBA)
Materials and Methods

- Fenton’s reagent [0.2 ml FeSO₄·7H₂O (10 mM), 0.2 ml EDTA (10 mM) and 0.2 ml 2-deoxyribose (10 mM) mixed with 1.2 ml phosphate buffer (0.1 M, pH 7.4)]

Procedure

CNB-001 was added to Fenton’s reagent followed by the addition of 0.2 ml H₂O₂ and incubated at 37 °C for 4 h. Later, 1 ml TCA and TBA was added to the reaction mixture and placed in a boiling water bath for 10 min. The resultant mixture was brought to room temperature and centrifuged at 395 ×g for 5 min and absorbance was obtained at 532 nm. Hydroxyl radical scavenging activity of CNB-001 is calculated using Percentage of inhibition = (A0–A1/A0) × 100 where A0 was the absorbance of the control (without CNB-001) and A1 was the absorbance in the presence of the CNB-001.

4.4.10. IC₅₀

IC₅₀ value was determined for all the assays from the plotted graph of scavenging activity versus the concentration of CNB-001 and Ascorbic acid using GraphPad Prism 5 software, GraphPad Software, Inc., La Jolla, USA. Triplicate measurements were carried out and their activity was calculated by the percentage of radical scavenged.

4.4.11. DNA protecting activity

DNA protecting activity of CNB-001 was determined by following the method of Devi et al. (2012).

Procedure

DNA protecting activity was performed to determine the ability of CNB-001 to protect DNA against hydroxyl radicals (•OH). Fenton’s solution was prepared by adding (1 mM •OH) was prepared by adding 1.6 µl of 30% H₂O₂ into 50 ml of 1 mM FeSO₄. Total reaction solution consists of 5 µl plasmid DNA (pUC19), 5 µl of CNB-001, 5 µl of Fenton’s reagent and irradiated in ultraviolet (UV) by placing on UV transilluminator (MX-1280-01, Medox-Bio, Chennai, India). The reaction solution was incubated in dark for 1 h. Later, 3 µl of gel loading buffer (30 mM
EDTA, 36% [v/v] glycerol, 0.05% [w/v] bromophenol blue) was added and the reaction products were electrophoresized in 1% agarose gel for 1 h under 50 V condition. The gel was then stained with 0.05% (w/v) ethidium bromide and documented (Alpha Imager mini, Alpha Innotech, USA). Densitometric analysis of supercoiled DNA was performed using ‘Image J’ software.

4.5. Cell culture

SK-N-SH neuroblastoma cells were obtained from National Centre for Cell Science (NCCS), Pune, India. Cells were grown in DMEM medium supplemented with Gentamicin (100µg/ml), 2 mM Glutamine, Streptomycin and penicillin (100 U/ml) and 10% (v/v) heat-inactivated fetal bovine serum. Cells were maintained at 37 °C under 5% CO2/ 95% humidity and the medium was changed thrice a week. For all the experiments, CNB-001 and Rotenone were freshly prepared in DMSO (0.05%). All experiments were carried out when cells attain 90% confluency. CNB-001 was added 2 h prior to rotenone treatment.

4.5.1. Measurement of cell viability (MTT assay)

Cell viability was analyzed using conventional MTT reduction assay. Cells were treated with CNB-001 and the viability was assessed based on the detection of mitochondrial dehydrogenase activity in viable cells (Mosmann, 1983).

Principle

In this assay, MTT reduction is catalyzed by NAD-dependent dehydrogenase (in active mitochondria) to form a dark blue formazan product.

Reagents

- 0.5 mg/ml MTT
- Dimethyl sulfoxide (DMSO)

Procedure

Cells were cultured in 96-multiwell plates and seeded at 3×10^3 cells/well. Initially, to optimize the LD_{50} value of rotenone, cells in medium were preincubated with different concentration of rotenone (0.5, 5, 50, 100, 200 nM) for 2 h. Twenty
Neuroprotective effect of CNB-001 against PD

Materials and Methods

four hours later; cells were incubated with MTT (5mg ml\(^{-1}\)) at 37 °C for 4 h. After incubation, the medium was removed and the cells were suspended with DMSO (200 µl). The absorbance of formazan reduction product was measured at 570 nm in a plate reader (Bio-Rad, Hercules, CA, USA).

Percentage viability was calculated as follows:

\[
\text{% Cell Viability} = \frac{\text{Test optical density}}{\text{Control optical density}} \times 100
\]

In a separate study, cells were pre-treated with different concentrations of CNB-001 (0.5, 1, 2, 4 µM) for 2 h and then incubated with 100 nM rotenone for 24 h. The same procedure as mentioned above was followed to identify the optimum dose of CNB-001 against rotenone toxicity.

4.5.2. Invitro experimental design

To evaluate the protective effect of CNB-001 against rotenone toxicity, cells were divided into four groups. Group I served as control, Group II were treated with CNB-001 (2 µM). Group III were treated with 100 nM rotenone and Group IV were treated CNB-001 (2 µM) followed by treatment with rotenone (100 nM).

4.5.3. Measurement of intracellular ROS formation

The intensity of intracellular peroxides was quantified by loading the cells with 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA). The esterified fluorescent probe penetrated into the intracellular matrix of the cells, reacts with ROS and oxidized to form fluorescent dichlorofluorescein (DCF). Intracellular ROS content was measured by following the method of Halliwell and Whiteman, 2004.

Reagents

- Phosphate buffered saline (PBS)
- 10 µM 2-7-diacetyl dichlorofluorescein diacetate (DCFH-DA)
Procedure

Control and treated cells (3x 10³ cells/ml) were incubated with 100 µl DCFH-DA (10 µM) for 30 min at 37 °C. Cultures were rinsed twice with PBS and photographed on Fluorescent microscope (450 - 490 nm; blue filter, Nikon, Eclipse TS100, Japan). Fluorescence estimations were done with excitation and emission filters set at 485 ± 10 nm and 530 ± 12.5 nm respectively using spectrofluorometer (Shimadzu RF-5301 PC). Initial fluorescent values (time 0) were found to differ from each other by less than 5%. Results were calculated using the formula [(Ft30-Ft0)/(Ft0X100)] and the values are expressed in percentage increase in fluorescence intensities at 0(Ft0) and 30(Ft30) min.

4.5.4. Measurement of mitochondrial membrane potential ΔΨ_M

Mitochondrial membrane potential was determined fluorometrically using Rhodamine 123 (Rh-123), a highly lipophilic cationic dye which readily enters cells and stains mitochondria and exhibits high fluorescent intensity. Polarized mitochondrion emits orange-red fluorescence and depolarized mitochondrion emits green fluorescence. This assay was performed by following the method of Scaduto and Grotyohann, 1999.

Reagents

- 1.1% PBS
- Fluorescent probe –Rhodamine-123.
- Rhodamine-123 stock –1mg/ml of 1% PBS.
- Working -10 µl from stock and made up to 1 ml with PBS

Procedure

After incubation with test compound and rotenone for 24 h, cells were incubated with 1 µl Rhodamine 123 (5 mM/l) for 15 min in PBS. Cells were then rinsed with PBS and fluorescence was observed under fluorescence microscope using blue filter (450-490 nm).
4.5.5. Apoptosis analysis using dual staining

Fluorescent probes acridine orange (AO) and ethidium bromide (EtBr) were used to analyze apoptosis by fluorescence microscope. Acridine orange, a permeable dye stains all the cells and ethidium bromide, a non permeable dye enters into the cell only when the cells membrane disintegrates and it intercalates with DNA forming an orange-red complex. Apoptosis assay was performed by following the method of Kasibhatla et al., (2006).

Reagents

- Phosphate buffered saline (PBS)
- 100 µg/ml (v/v) Acridine orange (AO) and ethidium bromide (EtBr)

Procedure

After treatments, medium was removed from the plates; cells were washed with PBS twice and stained with AO and EB. These cells were incubated for 20 min at room temperature and washed with warm PBS to remove excess dye. Cellular morphology was observed using fluorescent microscope (λEx / λEm=490 nm/530 nm) and photographed. Fluorescent intensity was measured at 535 nm using spectrofluorimeter.

4.5.6. Preparation of protein extract

Twenty four hours after treatment, cells were lysed in 100 ml of ice-cold radio immunoprecipitation assay (RIPA) buffer (1% Triton, 0.1% SDS, 0.5% deoxycholate, 20 mM Tris HCl, pH 7.4, 150 mM NaCl, 10 mM NaF, 1 mM Na3VO4, 1 mM EDTA, 1 mM EGTA, and 0.2 mM PMSF) followed by centrifugation at 10,000 x g at 4 °C for 10 min to remove insoluble material. The supernatant was used for estimation of protein and immunoblotting.

4.5.7. Estimation of protein

Protein in the cells/tissue/enzyme extract was determined after trichloroacetic acid precipitation by the method of Lowry et al. (1951).
Materials and Methods

Principle

Blue purple color complex will be produced by phenolic group of tyrosine and tryptophan residues in a protein with absorption at 660 nm in presence of Folin’s reagent which consists of sodium tungstate molybdate and phosphate. Thus, the intensity of the color formed depends upon the amount of protein present.

Reagents

- Alkaline copper reagent:
- Reagent A: 2% sodium carbonate in 0.1 N NaOH
- Reagent B: 0.5% copper sulphate
- Reagent C: 1% sodium potassium tartarate. 50 ml of reagent A was mixed with 0.5 ml of reagent B plus 0.5 ml of reagent C just before use.
- Folin’s Ciocalteu reagent (FCR): The commercial reagent was diluted in the ratio of 1:2 with distilled water.
- Stock standard: 1 mg of bovine serum albumin/1 ml of water.
- 10% TCA

Procedure

To 0.5 ml of protein extract, equal volume of 10% TCA was added and centrifuged for 10 min and precipitate was dissolved in 1 ml of 0.1 N NaOH. To this aliquot, 4.5 ml of alkaline copper reagent was added and allowed to stand for 10 min under dark at RT. To this mixture, 0.5 ml of FCR was added and the blue color developed was read at 640 nm after 20 minutes. A standard curve was obtained with standard bovine albumin and was used to estimate the tissue protein level for enzyme activity and the values are expressed as mg/dl.

4.5.8. Western blot analysis

Principle

Western blot analysis was used to identify specific proteins from complex tissue homogenate/cells. The proteins were separated based on the size/charge and transferred to a solid support (PVDF/Nitrocellulose membranes). Following transfer,
the protein of interest can be detected by incubation of the membrane with antibodies (primary) specific to the target protein followed by detection with an enzymatically labeled secondary antibody. Secondary antibody is then detected by adding an appropriate substrate for the enzyme conjugated to the secondary antibody.

Procedure

Proteins were electrophoresed in 10% SDS-polyacrylamide gels and electrotransferred onto PVDF membrane by semi-dry transfer (Trans-Blot®, BIO-RAD, 350 mA, 2 h). After blocking of the membrane with 5% non-fat dry milk in TBS at 25 °C for 1 h, blots were incubated with primary antibody against Bcl-2, Bax at a dilution of 1:500, cytochrome C (1:250), caspase-3 p17 (1:200) and β-actin at 1:2000 overnight at 4 °C. After washing thrice with TBST, the membranes were incubated with anti-rabbit HRP conjugated secondary antibody (1:2000) and bands were detected by chemiluminescence staining using ECL detection kit. Densitometry analysis was done using ‘Image J’ software.

4.6. Experimental animals and ethics statement

Adult male C57BL/6 mice weighing between 25-27 g purchased from National Institute of Nutrition (NIN), Hyderabad were used in this study. Since male C57BL/6 mice are more sensitive to MPTP intoxication than females and PD is observed frequently in males, female mice were avoided (Antzoulatos et al., 2010). Animals were maintained at ambient conditions (22 ± 1 °C, 60% humidity and 12 h diurnal cycle) and had ad libitum access to food and water. All experiments were performed in accordance to National Guidelines on the Proper Care and Use of Animals in Laboratory Research (Indian National Academy, New Delhi, 2009) and approved by Institutional Animal Ethics Committee (1085/ac/07/PU-IAEC/2012/13).

4.6.1. Experimental induction of Parkinsonism with MPTP

To evaluate the neuroprotective effect of CNB-001, sub-acute MPTP paradigm was used which represents one of the most stable toxin based PD models.
Parkinsonism was induced by administrating intraperitoneal injection of MPTP (30 mg/kg b.w, once daily) for five consecutive days. Safety precautions for the use and preparation of MPTP were strictly followed according to the instructions (Lau et al., 2005).

4.6.2. CNB-001 dosage and preparation

CBN-001 (1mg/40µl) was dissolved in 100% ethanol followed by 10 fold dilution with 1% (v/v) Tween 80/Saline (Narumoto et al., 2012). A lead study (Phase I) was carried out with four doses of CNB-001 (6, 12, 24 and 48 mg/kg, i.p) to evaluate the dose-dependent effect of CNB-001 in MPTP induced PD mice. The effective dose was identified and administered for seven days of the experimental period (Phase II).

4.6.3. Sample size

The entire study was divided into two phases, phase I (n=21) was used to identify the effective dose of CNB-001 (HPLC) and phase II (n=40) was conducted to analyze the behavioral, biochemical, protein expressions (Western blotting and immunohistochemistry) and ultrastructural analysis (Transmission electron microscope) by administering the animals with effective dose of CNB-001.

PHASE I

Animals were randomly divided into four groups with 3 animals per group to identify the optimum dose of CNB-001. CNB-001 (6, 12, 24 and 48 mg/kg) was dissolved as described previously and administered by intraperitoneal injection for seven days.

Group I: Served as control and was given intraperitoneal injection of saline and ethanol (10 fold dilution with 1% (v/v) Tween 80/Saline) which were vehicles for MPTP and CNB respectively.

Group II: Animals received MPTP (30 mg/kg, intraperitoneal) from the 4th to the 7th day of the experimental period.
Group III: Animals were administered with CNB-001 (6 mg/kg) from 1st to 7th day of the experimental period and injection of MPTP from 4th to 7th day as described in Group II.

Group IV: Animals were administered with CNB-001 (12 mg/kg) from 1st to 7th day of the experimental period and injection of MPTP from 4th to 7th day as described in Group II.

Group V: Animals were administered with CNB-001 (24 mg/kg) from 1st to 7th day of the experimental period and injection of MPTP from 4th to 7th day as described in Group II.

Group VI: Animals were administered with CNB-001 (48 mg/kg) from 1st to 7th day of the experimental period and injection of MPTP from 4th to 7th day as described in Group II.

Group VII: Animals received only CNB-001 (48 mg/kg) for seven consecutive days starting from 1st to 7th days of the experimental period.

PHASE II

Animals were randomly divided into four groups (n=10) and the experimental study was performed by administering the optimum of CNB-001 as mentioned below. After acclimatization and seven days before the experiment, behavioral training was performed.

Group I: Served as control and administered with saline and ethanol (10 fold dilution with 1% (v/v) Tween 80/PBS) which were vehicles for MPTP and CNB respectively. Group II and III mice received MPTP (30 mg/kg) from the 4th to the 7th day of the experimental period. Group III received CNB-001 (24 mg/kg) intraperitoneally for 7 successive days starting from the 1st to the 7th day of the experimental period. Group IV received CNB-001 alone (24 mg/kg) for 7 days. Two days after last MPTP dosage, mice were tested for behavioral studies and animals were sacrificed on the 10th day. Schematic representation of treatment and behavioral paradigm are shown in Figure 8.
4.6.4. Estimation of dopamine and its metabolites

The wet weights of the tissue was recorded and homogenized in 1 ml of 0.17 M perchloric acid containing DHBA as internal standard in the range of 25 ng/ml, using polytron homogenizer. Samples were centrifuged at 35,000 x g for 20 min at 4 °C and the supernatant was used for the analysis. The sample (20 μl) was injected via a HPLC pump (Model 1525, Binary Gradient Pump, Waters, Milford, MA, USA) into a column (Spherisorb, RP C18, 5 μm particle size, 4.6 mm id x 250 mm at 30 °C) connected to an electrochemical detector (Model 2465, Waters, Milford, MA, USA). Oxidation potential was fixed at 0.80 V using a glass carbon working electrode versus an Ag/AgCl reference electrode. The mobile phase consisted of 32 mM citric acid, 12.5 mM disodium hydrogen orthophosphate, 1.4 mM sodium octanyl sulphonate, 0.05 mM EDTA and 16 per cent (v/v) methanol. The pH of the mobile phase was adjusted to 4.05 and separation was carried out at a flow rate of 1.2 ml/min. The neurotransmitters were quantified using Breeze software version 3.2. The levels were expressed in ng/mg wet weight of the brain tissue. Quantification was made by comparing peak heights of the samples to the corresponding standard curve.

4.6.5. Behavioral parameters

To analyze the neuroprotective effect of CNB-001 and since PD is a motor disorder; behavioral assessments were performed in experimental animals. Behavioral tests were carried out in such a way that the order of tasks did not affect the outcome of our results.
4.6.5.1. Open field test

Open field test was widely used to assess the acclimatization activity, mental stress and motor activity of mice. The animals were placed in a large illuminated box and evaluated for their exploratory behavior, immobilization, and motor activity like grooming/ rearing relating to emotional status of the animal.

Apparatus

Open field test was carried out in a wooden apparatus (W100 X D100 X H4 cm). The floor was covered with a resin cloth and divided into 25 (5 X 5) equal squares (Plate 1). A 100 watt bulb was placed 150 cm above the centre of the field to provide illumination.

Procedure

The animals were placed at one end of the apparatus and its behavior was observed for 5 min. Central and periphery movements were calculated based upon number of peripheral (sixteen) and central (nine) squares crossed by the animals respectively (Plate 1A and B). One count was made only when the animal enters a square with both its fore-limbs. Further, number of rearing activity (exploratory behavior) and grooming (licking the fur, scratching behavior or washing face) were also manually scored in the open field for 5 min (Plate 1C and D). The entire study was conducted in a blinded manner (Rajasankar et al., 2009).

4.6.5.2. Narrow beam walking test

The narrow beam walking test was performed to analyze muscular coordination, balance and vestibular integrity. This test was performed by allowing the animals to work on a narrow but stationary wooden beam. Mice were trained to traverse through the runway for 10 trials per day with 1 min interval.

Procedure

Narrow beam test (NBT) consists of a start-and-goal box with a runway of 100 x 1 cm (L x W) placed at a height of 100 cm from the floor. NBT is vital to evaluate foot slips and latency to traverse the beam. One day before the
experimental period, mice were allowed to explore NBT (Plate 2A and B). The time of journey between the start and goal box and foot slip errors were counted as described previously (Rajasankar et al., 2009). Food pellets were kept on the goal box for motivation and as a reward for animals.

4.6.5.3. Grid hang test

The effect of CNB-001 on neuromuscular strength was analyzed by grid hang test. Animals were placed on a horizontal grid and supported until they held the grid with all their four paws. The grid was then kept in an inverted position allowing the animals to hang upside down and the maximum hanging time was noted. The grid was mounted 50 cm above a hard surface, to discourage falling or injury in case of falling (Plate 2C). Proper care was taken to prevent injury/damage to animals in case of falling. Maximum latency time was fixed as 300 seconds (Tillerson and Miller, 2003).

4.6.5.4. Rotorod test

This test was performed to assess muscular co-ordination, strength and balancing ability of animals using rotorod apparatus (Gerlai et al., 1996). Initially, the animals were trained to walk/run on the rotating rod which revolves around the longitudinal axis at different rpm. In the stationary beam the mice must maintain balance while walking on a horizontal surface. The animals were placed in opposite direction of the rotating rod (25 mm diameter; 25 cm height) so that fall could be prevented by forward locomotion. Animals were habituated and trained on rotating rod (5, 10 and 15 rpm) with a maximum cut off time of 180 seconds and the average retention time of the experimental animals were noted. The apparatus consists of five compartments which allow performing this test in five mice at a time (Plate 2D).

4.6.5.5. Catalepsy

Catalepsy test was performed to analyze the incapability of an animal to rectify an externally enforced posture. This test was carried out by placing the animal in a half rearing position on a horizontal wooden bar, placed 5 cm above the
platform on which the animals were kept. The duration of time the animals maintained this position was recorded (Plate 3A). Maximum cut off limit was fixed to 180 seconds (Muralikrishnan and Mohanakumar, 1998). This experiment was repeated six times for each animal and mean value was taken.

4.6.5.6. Akinesia

Akinesia was monitored by observing the latency of the animals to shift all four limbs and the test was lapsed if the latency time transcends 180s. Initially, individual animals were acclimatized for 5 min on a raised wooden platform (40 cm x 40 cm x 30 cm). The duration taken by the animals to shift their four limbs was noted (Muralikrishnan and Mohanakumar, 1998). The exercise was repeated six times for each animal (Plate 3B).

4.6.5.7. Swim test

Swim test was performed to analyze the motor impairments by placing animals in water basin (40 cm L x 25 cm W x 16 cm H) and rated based on their swimming ability (Muralikrishnan and Mohanakumar, 1998). Each animal was placed in the basin and swim score was measured (Plate 4A). The temperature of the water was strictly maintained at 27 ± 2 °C since lower temperature could induce stress. Immediately after the experiment, animals were thoroughly wiped using a dry towel. Scores were given as follows

0- hind part sinks with head floating
1- Occasional swimming using hind limbs while floating on one side
2- Occasional floating / swimming only
3- Continuous swimming

4.6.5.8. Stride length measurement

This test was performed by allowing the animals to walk on a runway (4.5 cm wide, 50 cm long with borders of 12 cm height) by following the method of Fernagut et al. (2002). Animals fore and hind limbs were wetted with commercially available color ink (different colors for fore limbs and hind limbs) and allowed to trot on a strip of paper (4.5 cm wide, 48 cm long) down the brightly lit runway
towards the goal box (Plate 4B). Once the paper is dried, the distance between steps on the same side of the body, from the middle toe of the first step to the heel of the second step were measured manually. The three longest stride lengths (corresponding to maximal velocity) were measured from each run. Paw prints made at the beginning (7 cm) and the end (7 cm) of the run were excluded because of velocity changes. Runs in which the mice made stops or obvious decelerations observed by the experimenter were excluded from analysis. The animals were immediately put back into their home cage upon their completion of the task.

4.6.6. Perfusion and tissue processing

At the end of the experiments, animals were sacrificed by terminal anesthesia and perfused via intracardial infusion with 0.9% saline followed by 4% paraformaldehyde (pH 7.4). Substantia nigra (SN) and striata (ST) was removed after intracardinal perfusion and post-fixed in 4% paraformaldehyde for 24 h at 4 °C. The tissues were embedded in paraffin wax and sliced into 5 µm coronal sections containing entire SN and ST (antero-posterior levels: Bregma -2.92 to -3.64 mm and +0.02 to +0.86 mm) with the reference of mouse brain atlas (Franklin and Paxinos, 2007) for immunohistochemical study. For biochemical analysis, brain tissue (SN) were homogenized in Tris HCl buffer (20 mM, pH 7.4) and centrifuged (9861 x g) at 4 °C for 20 min. The supernatant was used for further studies. SN and ST were dissected and rapidly frozen on dry ice and stored at -80 °C for immunoblotting. Brain sections (SN and ST) were fixed in 3% gluteraldehyde at 4 °C, 24 h for Transmission Electron Microscopy processing.

4.6.7. Lipid peroxidation products

4.6.7.1. Estimation of TBARS

The activity of Thiobarbituric acid reactive substances was analyzed by following the method of Bhattacharya et al. (2001).

Principle

Thiobarbituric acid reactive substances are formed as a result of lipid peroxidation. Since reactive oxygen species have extremely short half-life, the
products formed due to damage caused by oxidative stress such as malondialdehyde can be measured. In this method, the product reacts with thiobarbituric in acidic condition to form a pink color chromophore which was read at 535 nm.

Reagents

- 5% TCA
- 0.67% TBA
- Stock standard: 0.48 M solution of stock was prepared from 1,1,3,3-Tetramethoxpropane purchased commercially.
- Working standard: stock solution was diluted to get a concentration of 48 nmol/ml.

Procedure

The tissue extracts (0.2 ml) were incubated in water bath at 37 °C and one hour after incubation, 0.4 ml of 0.67% thiobarbituric acid and 0.4 ml of 5% tricarboxylic acid were added. The reaction mixture was centrifuged at 1431 x g for 15 min and the resultant supernatant was boiled for 10 min. The samples were cooled and read at 535 nm. The rate of lipid peroxidation was expressed as nmol of TBARS formed per hour/g tissue.

4.6.8. Enzymatic antioxidants
4.6.8.1. Assay of Superoxide Dismutase (SOD)

Superoxide dismutase activity was measured by following the method of Oberley and Spitz, (1984).

Principle

Superoxide dismutase inhibits the formation of formazan, a reduction product of NBT and the rate of reaction is measured by recording changes in the absorbance at 550 nm.

Reagents

- Xanthine (1 mM)
- NBT (57 μM)
Materials and Methods

- Xanthine oxidase (50 mU)
- Phosphate buffer (0.5 M, pH 7.4)

Procedure

Reaction mixture (1 ml) consists of 0.5 M phosphate buffer, 0.1 ml PMS, 1.0 mM xanthine, 57 μM NBT. The mixture was incubated at RT for 15 min and reaction was initiated by the addition of 50 mU xanthine oxidase. The absorbance was measured spectrometrically at 560 nm and the activity was expressed as units/min/mg protein.

4.6.8.2. Estimation of Catalase

Catalase activity was assayed by following the method of Aebi (1984).

Principle

The assay was based on the ability of catalase to consume H₂O₂/min/mg protein. Changes in the absorbance were recorded at 240 nm.

Reagents

- Phosphate buffer (0.05 M, pH 7.0)
- H₂O₂ (0.019 M)

Procedure

In this assay, the reaction mixture consisted of 0.05 M phosphate buffer pH 7.0, 0.019 M hydrogen peroxide and 20 μl of the supernatant. Changes in absorbance were recorded at 240 nm. Enzyme activity was expressed as nmol of H₂O₂ decomposed/min/mg protein.

4.6.8.3. Estimation of Glutathione peroxidase

Glutathione peroxidase (GPx) activity was measured according to the procedure described by Yamamoto and Takahashi, (1993).
Principle

This assay was based on the disappearance of NADPH at 340 nm recorded at room temperature. The enzyme activity was calculated as 1 nmol NADPH oxidized/min/mg protein.

Reagents

- Phosphate buffer (0.05 M, pH 7.0)
- EDTA (1 mM)
- Sodium azide (1 mM)
- Glutathione reductase (1 eU/ml)
- Glutathione (1 mM)
- NADPH (0.2 mM)
- H₂O₂ (0.25 mM)

Procedure

In this assay, the total reaction mixture consisted of 1.44 ml phosphate buffer (0.05 M, pH 7.0), 0.1 ml of EDTA, 0.1 ml of sodium azide, 0.05 ml of glutathione reductase (GR), 0.1 ml of glutathione, 0.1 ml of NADPH, 0.01 ml of hydrogen peroxide, 0.1 ml of PMS (10% w/v) and 10 µl of brain homogenate. The disappearance of NADPH at 340 nm was recorded at room temperature. The enzyme activity was calculated as 1 nmol of NADPH oxidized per min.

4.6.9. Non enzymatic antioxidants

4.6.9.1. Estimation of Reduced Glutathione (GSH)

Reduced glutathione in the mid brain was determined by following the method of Jollow et al. (1974).

Principle

The assay was based on the development of yellow color when 5, 5’-dithio-
bis (2-nitrobenzoic acid) (DTNB) was added to compounds containing sulphydryl groups.
Reagents

- Phosphate buffer (0.1 M, pH 7.4)
- Sulfosalicylic acid (4%)
- DTNB (40 mg/10 ml of phosphate buffer).

Procedure

In this assay, 0.5 ml of homogenate was precipitated with 0.5 ml of sulfosalicylic acid and incubated at 4 °C for 1 h. To this 0.2 ml DTNB and 0.3 ml of phosphate buffer was added and centrifuged at 201 x g for 5 min at room temperature. The yellow color developed was read immediately at 412 nm and the results were expressed as mg/g tissue.

4.6.10. Estimation of nitrite

Nitrite content in the tissue sample was estimated by following the method of Raddassi et al. (1994).

Principle

The amount of nitrite is detected by the formation of red pink color due to reaction between NO₂⁻ present in the sample with the Griess reagent. The nitrite reacts with sulphanilic acid to form a diazonium salt which reacts with N-alpha-naphthyl-ethylenediamine to form a red pink color.

Reagents

- Griess reagent (sulfanilamide, N-(1-naphthyl)-ethylenediamine dihydrochloride and H₃PO₄ in the ratio of 1%, 0.1% and 2.5% respectively)

Procedure

The concentration of nitric oxide was estimated by evaluating nitrite, a stable metabolite of NO using Griess reagent. In brief, 100 µl of the supernatant was mixed with 100 µl of Griess reagent and nitrite reacts with Griess reagent to form a purple azo colour which is read at 540 nm. Sodium nitrite was used as a standard. Results were expressed as mM of nitrite accumulated/mg protein.
4.6.11. Estimation of citrulline

Citrulline content in the sample was estimated by following the method of Boyde and Rahmatullah, (1980).

Procedure

Nitric oxide synthase activity was also estimated by analyzing citrulline content in the tissue sample. Chromogenic solution was prepared by mixing 0.5% (w/v) diacetylmonoxime and 85% phosphoric acid with 0.1% (w/v) thiosomocarbazide in the proportion of 2:1 immediately before use. Protein samples were precipitated using 30% zinc sulfate and centrifuged at 358 x g for 10 min. Forty microlitre of the supernatant was mixed with 1.5 ml of chromogenic solution and 460 µl of 0.1N HCl. The mixture was boiled for 5 min and absorbance was measured at 530 nm. The concentration of citrulline in the samples was expressed as mM of citrulline/mg of protein using a standard plot of citrulline (0.125 – 1 mM).

4.6.12. Immunoblotting

Immunoblot analysis was performed to determine the expression of vital pro-inflammatory factors (TNF-α, IL-6, IL-1β, iNOS, GFAP, COX-2), apoptotic markers [Bax, Bel-2, cytochrome C, caspase-3 (p17)] and monoamine transporter expressions (TH, DAT and VMAT2). Tissue samples were processed according to the method of Wright et al. (1998). Brain tissues (striatum and substantia nigra) were disrupted by homogenization in an ice-cold RIPA buffer and the cellular debris was cleared by centrifugation (12,879 x g for 15 min at 4 °C). Samples were analyzed for their protein concentration by the method of Lowry et al. (1951). Semi-dry transfer method was used to transfer proteins from the gel to the membranes followed by electrophoresis. Briefly, blotting papers and sponges were soaked in ice-cold buffer before the transfer and transfer sandwich was then placed in the blotting apparatus and subjected to an electric current of 20 V for 1 h. After the transfer, ponceau-S dye (0.5 % ponceau-S in 5% glacial acetic acid) was applied to check the transferred protein and then washed with distilled water. Membranes were then blocked with blocking buffer containing 5% non-fat dry milk powder in TBS at 25 °C for 1 h to prevent the non-specific protein binding and probed primarily
against TNF-α (mouse polyclonal; 1:700), IL-6, IL-1β (rabbit polyclonal; 1:500), α-synuclein (mouse monoclonal; 1:1000), GFAP, iNOS (mouse monoclonal; 1:500), Bax, Bcl-2, cytochrome C, caspase-3 p17 (rabbit polyclonal; 1:750), anti-mouse TH (1:1000), anti-mouse DAT (1:500) and anti-mouse VMAT2 (1:1000) and β-actin (rabbit polyclonal; 1:500) overnight with gentle shaking at 4 °C. After washing the membranes three times with TBST (10 min/wash, RT), corresponding secondary antibodies (anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase) were added to the membrane and incubated for 2 h at room temperature. Immunoreactive bands were visualized by chemiluminescence protocol (GenScript ECL kit, USA). Densitometry was done using ‘Image J’ analysis software. The blot intensities were normalized with that of β-actin as loading control.

4.6.13. Immunohistochemistry

Principle

Immunohistochemistry, a widely used technique refers to the detection of proteins in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues. This technique allows visualization of antigen via sequential application of a specific antibody (primary antibody) to the antigen, a secondary antibody to the primary antibody and an enzyme complex with a chromogenic substrate with interposed washing steps. The enzymatic activation of the chromogen results in a visible reaction product at the antigen site.

Procedure

Substantia nigra and striatum were serially sectioned and the slides were deparaffinized using xylene followed by rehydration in graded series of ethanol. The slides were boiled for 10 min in 10 mM citrate buffer (pH 6) for antigen retrieval. The sections were then incubated with H2O2 (0.3%) for 10 min at RT for release of endogenous peroxidase. The slides were then placed in blocking buffer [10% Normal Goat Serum (NGS) with 0.2% Triton X-100 in 0.01 M PBS] at 37 °C for 30 min. In each treatment, slides were washed three times with 0.01 M PBST for 5 min. Sections were incubated primarily with anti-mouse dopamine transporter (1:500), anti-mouse TH (1:1000) in 2% NGS, 0.2% Triton X-100 and 0.02% sodium azide in
PBS for 24 h. After washing with PBST, the sections were incubated with anti-mouse IgG-HRP conjugated secondary antibody (1:1000 in 1.5% NGS) for one hour followed by washing with PBST. The sections were then incubated with diaminobenzidine (DAB) to analyze TH immunoreactivity.

4.6.13.1. Quantification of Tyrosine hydrolase and Dopamine transporter immunoreactivity

The intensity of TH and DAT immunoreactivity in ST was measured using Micro Computer Imaging Device software (Elite 7.0, Imaging research Inc., Canada) and results were expressed as percentage of control. Mouse brain atlas was initially used to delineate SN at low magnification and the numbers of TH immunoreactive neurons in SN were counted at higher magnification (4X) by persons who were blind to the treatment. Cell counts were determined every sixth section (total 8-10 sections) through SN corresponding to the bregma -2.92 to -3.64 mm from each animals. The analyses of TH and DAT positive neurons were restricted to SN and thus excluded the ventral tegmental area.


After fixation, brain sections were cut into approximately 1 mM cubes and post fixed in 1% osmium tetraoxide for 2 h at 4 ºC. The cubes were then dehydrated in series of ethanol and treated twice with propylene oxide for 10 min (RT). The tissues were infiltrated with EPON mixture and propylene oxide (1:1 v/v for 2 h at RT] and embedded in EPON mixture containing Taab/812, followed by polymerization at 60 ºC for 24 h. Ultra microtome was used to cut tissues at 0.5μm sections and stained with 0.5% toluidine blue to confirm the presence of neurons. Then the 60 nm ultra thin sections were cut and mounted on Nickel grids (300 mesh). The sections were double stained with uranyl acetate and lead citrate and then examined by Transmission Electron Microscope (Philips CM10) and photographed.

4.6.14.1. Morphological analysis of mitochondria within SN and ST

Measurements were generated from three randomly acquired TEM images from SN and ST with a magnification of 16,000X and coded for blinded analysis.
The perimeter length of each mitochondrion was analyzed by a single trained technician using MeasureIT software (Olympus soft imaging solutions). Mitochondria were identified by presence of identifiable cristae and distinct double membrane. Mitochondria with unclear morphology were avoided.

4.6.15. Statistical analysis

All data were expressed as mean ± Standard deviation (SD) of number of experiments. The statistical significance was evaluated by one-way analysis of variance (ANOVA) using SPSS version 11.5 (SPSS, Cary, NC, USA) and the individual comparison were obtained by Duncan’s Multiple Range Test (DMRT). A value of P< 0.05 was considered to indicate a significant difference between groups. Values not sharing common superscript are significant with each other at P< 0.05.
Insilico identification of inhibitors of alpha-synuclein aggregation

Postmortem results of PD brain samples revealed prevalence of triggered microglia and intracytoplasmic eosinophilic proteinaceous inclusions termed Lewy bodies made up of alpha-synuclein in SN neurons (Ouchi et al., 2005). Moreover the role of α-synuclein in PD is solidly proven by Dauer et al. (2002), he reported that deletion of gene coding for α-synuclein protects dopaminergic neurons against MPP⁺ and MPTP toxicity in invitro and invivo models. Small molecules/ligands that can block/slow down or reverse α-synuclein aggregation, especially at its early stage can provide an attractive and effective therapeutic approach for inhibiting PD disease progression. El-Agnaf et al. (2004) cornered that amino acid residue 64-100 of α-synuclein is responsible for its self-association and blocking those residues prevented proteotoxicity. Ono and Yamada, (2006) showed that antioxidants compounds have been proven to show anti-fibrillogenic and fibril-destabilizing effects for α-synuclein fibrils invitro. However there is less computational based molecular docking studies to understand the binding ability of natural and synthetic compounds against α-synuclein. The present study is aimed to evaluate the pharmacokinetic properties and binding patterns of ligands against 64-100 amino acid residues of α-synuclein. As of our knowledge, docking studies were not performed for CNB-001 till now. The results obtained will provide insights in understanding the activities of compounds as inhibitors based on docking scores.

5.1. RESULTS

5.1.1. Drug likeness evaluation

The Lipinski rule of five for the compounds was predicted using Lipinski drug filter. These results showed that out of the fifty compounds selected for the study, only 38 satisfied Lipinski rule of five and only these compounds can be robustly recommended to be used as a drug. The compounds which passed Lipinski rule of five and their properties are depicted in table 2.