Effect of CNB-001 on MPTP induced Parkinson mice model

Experimental and post-mortem studies reported that oxidative stress and inflammation involving microglial and astroglial activation fueling apoptotic mechanisms might be crucial in the progression of PD. Various reports showed that incidence of PD is mainly due to degeneration of neurons in nigrostriatal pathway and whereas mesolimbic pathway is less affected (Uhi et al., 1985; Hirsch et al., 1988). 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a potent neurotoxin mimics the same pathobiochemical alterations as seen in PD and are extensively used to create PD models (Burns et al., 1980). The toxic effect of MPTP is mainly due to the mitochondrial impairment, disturbance in redox homeostasis and inflammatory responses, leading to neurodegeneration in substantia nigra and depletion of dopamine in the striatum (Ojha et al., 2012). MPTP activates microglia through a process called ‘reactive microgliosis’ and triggers the production of pro-inflammatory cytokines which initiates caspase mediated apoptosis in dopaminergic neurons (Anandhan et al., 2013). These concrete evidence propose that apoptosis, neuroinflammatory mediated microglial and astroglial activation is harmful to the DA neurons. Hence it is rationale to presume that anti-inflammatory and anti-apoptotic regiments which target multiple toxic pathways might provide tangible benefit for treatment of PD. The intent of this study is to evaluate the potency of CNB-001 against MPTP induced Parkinson mice model.

5.4. Results

5.4.1. CNB-001 dampens MPTP-induced striatal dopamine, DOPAC and HVA loss

The present study reveals that administration of MPTP caused a significant reduction in striatal dopamine and its metabolite levels when compared to control (p<0.05). Alternatively, this amendment was reinstated significantly upon CNB-001 (6, 12, 24 and 48 mg/kg) pre-treatment when compared to MPTP group. From these results, it was noted that CNB-001 at a dose of 24 and 48 mg/kg showed similar dopamine levels but distinctly higher than 6 and 12 mg/kg. Thus, an optimum dose of 24 mg/kg was used for further studies. There was no significant variation of dopamine, DOPAC, HVA between control and CNB-001 treated groups (table 10).
The representative HPLC trace of dopamine, DOPAC and HVA in control, MPTP, CNB-001 (24 mg/kg) + MPTP and CNB-001 (24 mg/kg) are depicted in figure 33.

Table 10. Effect of CNB-001 on the levels of dopamine, DOPAC and HVA in experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>DA (ng/mg of tissue weight)</th>
<th>DOPAC (ng/mg of tissue weight)</th>
<th>HVA (ng/mg of tissue weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14.237±1.13(^a)</td>
<td>2.369±0.18(^b)</td>
<td>1.46±0.11(^c)</td>
</tr>
<tr>
<td>MPTP</td>
<td>3.92±0.31(^b)</td>
<td>1.08±0.10(^b)</td>
<td>0.38±0.04(^b)</td>
</tr>
<tr>
<td>CNB (6mg/kg)+MPTP</td>
<td>4.26±0.38(^b)</td>
<td>1.15±0.12(^b)</td>
<td>0.40±0.04(^b)</td>
</tr>
<tr>
<td>CNB (12mg/kg)+MPTP</td>
<td>4.66±0.40(^c)</td>
<td>1.28±0.13(^c)</td>
<td>0.44±0.05(^c)</td>
</tr>
<tr>
<td>CNB (24mg/kg)+MPTP</td>
<td>7.683±0.72(^d)</td>
<td>1.846±0.14(^d)</td>
<td>0.73±0.05(^d)</td>
</tr>
<tr>
<td>CNB (48mg/kg)+MPTP</td>
<td>7.88±0.75(^d)</td>
<td>1.90±0.15(^d)</td>
<td>0.78±0.06(^d)</td>
</tr>
<tr>
<td>CNB</td>
<td>14.837±1.33(^a)</td>
<td>2.301±0.19(^a)</td>
<td>1.41±0.11(^a)</td>
</tr>
</tbody>
</table>

Results represent the dopamine and its metabolite levels measured in control and experimental mice. Values are represented as mean ± SD (n= 6), values not sharing common superscript are significant with each other p< 0.05, ANOVA followed by DMRT.

Figure 33. HPLC-ECD chromatographic profiles of Dopamine, DOPAC and HVA in experimental animals. 1: DOPAC; 2: Dopamine and 3:HVA.

5.4.2.1. Effect of CNB-001 on MPTP induced locomotor activity

Locomotor and exploratory behavior of experimental animals is assessed by Open field test. Locomotor and exploratory behaviors are intricate in physiological and behavioral functions. MPTP intoxication caused a significant decline in peripheral, central movements, rearing and grooming activities compared to control
group (p<0.05) (figure 34 I-IV). However, these impairments were reduced when pre-treated with CNB-001 as compared to MPTP group (p<0.05).

![Graphs showing control and experimental mice results](image)

Figure 34. Open field test of control and experimental mice. Values are expressed as mean ± SD for six animals in each group. *p<0.05 compared to control, #p<0.05 compared to MPTP.

5.4.2.2. Effect of CNB-001 on MPTP induced beam walking performance

Narrow Beam test was performed to evaluate the motor coordination ability and balance in mice. MPTP intoxicated mice significantly exhibited more number of foot slip errors and higher time to cross the runway when compared to control (p<0.05). CNB-001 treatment significantly abrogated this behavioral impairment when compared to MPTP per se group (p<0.05) (figure 35 I and II).
5.4.2.3. Effect of CNB-001 on MPTP induced neuromuscular strength

Hang test was performed to analyze the neuromuscular strength of control and experimental animals. Compared to control group, the average hanging time of MPTP intoxicated mice were significantly lower (p<0.05) (figure 36). CNB-001 treatment distinctly enhanced neuromuscular strength compared to MPTP group as evinced by improved hanging time.

Figure 36. Effect of CNB-001 on neuromuscular strength. Values are represented as mean \pm SD for six mice per group. *p<0.05 compared to control, #p<0.05 compared to MPTP group.
5.4.2.4. Effect of CNB-001 on MPTP induced motor coordination

Motor coordination ability of experimental animals was evaluated by performing rotorod test. As shown in figure 37, MPTP treatment showed neuromuscular incoordination and significant reduction in retention time as compared to control (p<0.05). Moreover none of the MPTP treated animals could balance themselves on the rotating rod for full cut off time (180 seconds). Pre-treatment with CNB-001 distinctly enhanced balancing ability, retention time and inhibited disorientation as compared to MPTP treated mice (p<0.05).

![Rotorod performance of control and experimental mice. Values are expressed as mean ± SD for six animals in each group. *p<0.05 compared to control, #p<0.05 compared to MPTP.](image)

5.4.2.5. Effect of CNB-001 on movement coordination ability

Movement coordination ability was recorded in control and experimental mice (figure 38 I and II). Acute administration of MPTP showed fixative posture of animal (catalepsy) and inability to perform voluntary movement (akinesia) when compared to control mice (p<0.05). Co-treatment of CNB-001 markedly alleviated MPTP induced catalepsy and akinesia when compared to MPTP group (p<0.05). There was no significant variation between CNB-001 and control cohorts.
5.4.2.6. Effect of CNB-001 on MPTP induced motor impairment

MPTP administration caused a marked decrease in swim score as compared to control animals (p<0.05). Whereas, pretreatment with CNB-001 to MPTP intoxicated mice enhanced the swim score than those of MPTP alone treated mice. No significant differences in swim scores were observed between CNB-001 and control mice (figure 39).

Figure 39. Swimming ability of control and experimental animals. Values are expressed as mean ± SD for six animals in each group. *p<0.05 compared to control, #p<0.05 compared to MPTP.
5.4.2.7. Effect of CNB-001 on stride length

Shortened stride length is one of the important characteristics of abnormal gait in PD. The result of this study showed that administration of MPTP caused a significant decrease in forelimb and hind limb stride length when compared to control animals (figure 40). In contrast, pretreatment with CNB-001 increased the stride length than those of MPTP animals. In addition no significant differences in stride lengths were observed between CNB-001 and control animals.

![Stride length measurement](image)

Figure 40. Gait performance of control and experimental animals. Values are expressed as mean ± SD for six animals in each group. *p<0.05 compared to control, #p<0.05 compared to MPTP.

5.4.3. Effect of CNB-001 on lipid peroxidation and antioxidants

Intraperitoneal injection of MPTP caused a significant increase in the levels of TBARS, activities of SOD, CAT, decreased the levels of GSH and activity of GPx when compared to control (p<0.05). Alternatively, CNB-001 inhibits MPTP induced oxidative stress significantly by attenuating enhancement of TBARS, SOD and CAT activities and increasing the levels of GSH and GPX activities compared to MPTP group (p<0.05) (table 11).
Table 11. In vivo antioxidant status of control and experimental mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>MPTP</th>
<th>CNB-001+MPTP</th>
<th>CNB-001</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBARS(nmols/g tissue)</td>
<td>0.86±0.90 a</td>
<td>2.54±0.20 b</td>
<td>1.79±0.13 c</td>
<td>0.82±0.09 a</td>
</tr>
<tr>
<td>GSH(mg/g of tissue)</td>
<td>9.31±1.05 a</td>
<td>4.9±0.39 b</td>
<td>7.92±0.71 c</td>
<td>10.1±1.02 a</td>
</tr>
<tr>
<td>GPx (U A/mg protein)</td>
<td>9.6±0.94 a</td>
<td>4.31±0.32 b</td>
<td>7.36±0.75 c</td>
<td>9.9±0.90 a</td>
</tr>
<tr>
<td>SOD(U B/mg protein)</td>
<td>1.32±0.19 a</td>
<td>4.6±0.50 b</td>
<td>3.36±0.23 c</td>
<td>1.27±0.21 a</td>
</tr>
<tr>
<td>Catalase (U C/mg protein)</td>
<td>1.98±0.17 a</td>
<td>3.96±0.34 b</td>
<td>2.82±0.14 c</td>
<td>1.94±0.11 a</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD for six animals in each group. Values not sharing common superscript are significant with each other P<0.05.

A nmol NADPH oxidized/minute/mg protein.
B Amount of enzyme required to inhibit 50% of NBT reduction.
C nmol H2O2 consumed/minute/mg protein.

5.4.4. Effect of CNB-001 on nitrite and citrulline

Results showed a significant increase in nitrite and citrulline (13.4 mM/mg and 16 mM/mg protein respectively) in MPTP treated mice when compared to control (p<0.05). CNB-001 treatment dampened copious accumulation of nitrite and citrulline when compared to MPTP treated group (p<0.05) (table 12). There was no significant difference between control and CNB-001 treated groups.

Table 12. Nitrite and citrulline content in control and experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Nitrite (mM/mg protein)</th>
<th>Citrulline (mM citrulline/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.2±0.8 a</td>
<td>7±0.7 a</td>
</tr>
<tr>
<td>MPTP</td>
<td>13.4±1.3 b</td>
<td>16±1.5 b</td>
</tr>
<tr>
<td>CNB+MPTP</td>
<td>8.3±1 c</td>
<td>10.2±0.9 c</td>
</tr>
<tr>
<td>CNB</td>
<td>4.9±0.7 a</td>
<td>6.8±0.5 a</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD for six mice per group. Values not sharing common superscript are significant with each other p<0.05, ANOVA followed by DMRT. b, p<0.05 compared with Control. c, p<0.05 compared with the MPTP.
5.4.5. Effect of CNB-001 on MPTP induced inflammatory response

Administration of MPTP significantly increased production of pro-inflammatory factors (TNF-α, IL-6, IL-1β and iNOS) and activates astroglial cells as indicated by enhanced GFAP & COX-2 expressions when compared to control (p<0.05). These toxic pro-inflammatory cytokine amendments and glial activation were markedly diminished upon treatment with CNB-001 when compared to MPTP group (p<0.05) (figure 41 I and II).

![Figure 41](image)

Figure 41. Effect of CNB-001 on MPTP induced inflammation and glial activation (I). Histogram attributes densitometric analysis and protein expressions are quantified using β-actin as an internal standard (II). Values are expressed as arbitrary units and given as mean ± SD of three animals in each group. *p<0.05 compared to control,#p<0.05 compared to MPTP group.

5.4.6. Effect of CNB-001 on MPTP induced apoptotic response

MPTP significantly enhanced the expression of pro-apoptotic (Bax, cytochrome C and Caspase-3) markers and diminished the expression of anti-apoptotic protein Bcl-2 when compared to control (p<0.05). CNB-001 treated group dramatically prevented the expression of pro-apoptotic markers when compared to MPTP group by reinstating the expression of Bcl-2 (p<0.05) (figure 42).
Figure 42. Effect of CNB-001 on MPTP induced apoptosis (I). Quantitative analyses of western blots are depicted in histogram (II). Values are expressed as arbitrary units and given as Mean ± SD of three animals in each group. *p<0.05 compared to control, #p<0.05 compared to MPTP group.

5.4.7. Effect of CNB-001 on alpha-synuclein expression in SN and ST

Results explicated that MPTP administration significantly elevated the α-synuclein expression in SN and ST when compared to control mice (p<0.05) (figure 43). In contrast, pretreatment with CNB-001 distinctly diminished α-synuclein expression compared to MPTP group (p<0.05).

Figure 43. Effect of CNB-001 on α-synuclein expression in control and experimental mice (I). Densitometric analysis of protein expression were quantified using β-actin as an internal standard (II). Values are expresses as arbitrary units and given as mean ± SD of six animals per group. *p<0.05 compared to control, #p<0.05 compared to MPTP group.
5.4.8. Effect of CNB-001 on TH, DAT and VMAT2 expressions

To determine the protective effect of CNB-001 against MPTP induced neurodegeneration, the expression pattern of phenotypic markers (TH, DAT and VMAT2) in SN and ST were analyzed by western blot. As depicted in figure 44, intraperitoneal injection of MPTP significantly alleviated the expression of TH, DAT and VMAT2 in both SN and ST compared to control (p<0.05). Meanwhile, treatment with CNB-001 reinstated these protein expressions distinctly as compared to MPTP group (p<0.05). There were no significant changes in control and CNB-001 treated groups.

![Western Blot Images](image)

Figure 44. Immunoblotting observation of TH, DAT and VMAT2 in SN and ST of control and experimental mice (I and III). Protein expressions were quantified using β-actin as an internal standard and values are expressed as arbitrary units and given as mean ± SD (III & IV). *p<0.05 compared to control, #p<0.05 compared to MPTP.

5.4.9. Effect of CNB-001 on MPTP induced TH immunoreactivity in ST and SN

Acute administration of MPTP resulted in a significant decrease in TH density and TH-positive neurons in ST and SN respectively when compared to control (p<0.05). However, treatment with CNB-001 prior to MPTP administration
distinctly cosseted neurons by increasing TH expression in ST (figure 45) and SN (figure 46) compared to MPTP group (p<0.05).

Figure 45. Photomicrographs of ST sections depicting TH-immunoreactive (TH-ir) fibers (4X) (I). TH-immunoreactivity of A. Control, B. MPTP, C. CNB+MPTP and D. CNB was analyzed by measuring the optical density in ST (II). Mean value of TH-ir was determined for each group and expressed as percentage control. Values are expressed as mean ± SD for three animals in each group. *p<0.05 compared to control, #p<0.05 compared to MPTP.

Figure 46. Photomicrograph of SN sections illustrating TH-immunoreactive (TH-ir) neurons (4X) (I). Quantification (II) of TH positive neurons of E. Control, F. MPTP, G. CNB+MPTP and H. CNB was performed by counting number of TH-ir neurons and values are expressed as mean ± SD of three animals per group. *p<0.05 compared to control, #p<0.05 compared to MPTP.
5.4.10. Effect of CNB-001 on MPTP induced DAT immunoreactivity in ST and SN

Immunohistochemical analysis was performed to analyze the loss of DAT positivity in SN and ST. Intoxication with MPTP caused drastic reduction in DAT immunoreactivity (DAT-ir) and density in SN and ST respectively when compared to control (figure 47 I and 48 I).

Figure 47. (I) Immunohistochemical expression (4X) of DAT positivity in control and experimental animals. (II) DAT intensity of A. Control, B. MPTP, C. CNB+MPTP and D. CNB was analyzed by measuring the optical density in ST. Mean value of DAT was determined for each group and expressed as percentage control. Values are expressed as mean ± SD for three animals in each group. *p<0.05 compared to control, #p<0.05 compared to MPTP.

Figure 48. Immunohistochemical expression (4X) of DAT in nigrostriatal neurons of control and experimental animals (I). Quantification (II) of DAT positive neurons of E. Control, F. MPTP, G. CNB+MPTP and H. CNB. DAT positive cells in SN were counted in experimental animals for DAT immunoreactivity. Results are expressed as total number of cells in SN (contralateral and ipsilateral sides). Values are presented as mean ± SD of three animals per group.
However, treatment with CNB-001 prior to MPTP administration distinctly cosseted neurons by increasing DAT expression in SN and ST compared to MPTP group (p<0.05) (figure 47 II and 48 II). There was no significant DAT neuronal loss in control and CNB-001 treated groups.

5.4.11. Effect of CNB-001 on ultrastructural changes induced by MPTP in ST and SN

Electron microscopic studies were performed to analyze whether the antioxidant potential of CNB-001 were strong enough to protect mitochondria morphology against MPTP toxicity. Striking structural changes were observed in mitochondria of ST (figure 49) and SN (figure 50) in MPTP treated mice.

![Figure 49. Photomicrographs of striatum viewed under transmission electron microscope. All panels demonstrate representative striata at 2000X. Control (A) and CNB-001 (D) alone treated group showed normal ultrastructural morphology with enhanced mitochondrial population with membrane stability and cytoplasmic contents. Strikingly, MPTP treated group (B) showed mitochondria greatly enlarged and swollen rather than normal rod shaped. Moreover, there were also distinct loss of mitochondrial cristae and permeabilization of mitochondrial membrane. These toxicity changes were distinctly diminished upon CNB-001 treatment (C) showing normal nuclear morphology with increased mitochondrial population with structural stability.](image)

MPTP intoxication resulted in distorted cristae, permeabilization and enlargement of mitochondria in SN and ST (788 and 1032 nm respectively) (figure 51). In contrast, pre-treatment with CNB-001 protected mitochondria from MPTP toxicity by
maintaining normal mitochondrial morphology and size (660 and 825 nm) with distinct nucleus and cristae compared to MPTP group (p<0.05).

Figure 50. Transmission electron micrographs of SN showing ultrastructural changes in control and experimental mice. All panels demonstrate SN neurons at 2000X. In control (A) and CNB-001 group (D) increased mitochondrial population with prominent cristae were seen. In MPTP group (B), mitochondria showed abnormal structure with distorted cristae and number of mitochondria was greatly reduced. In contrast CNB-001 treatment (C) dampened these apoptotic changes and improved mitochondrial morphology and population as compared to MPTP group.

![Figure 50](image)

Figure 51. Analysis of mitochondrial perimeter length in SN and ST of experimental animals. Mitochondrial perimeter length/mitochondrion was significantly increased in MPTP group than control. Pre-treatment with CNB-001 significantly maintained mitochondrial size as compared to MPTP group. No significant changes were observed in control and CNB-001 treated groups. *p<0.05 compared to control, #p<0.05 compared to MPTP.

![Figure 51](image)
Discussion

In the present study, we substantiate that neuroprotective action of CNB-001 against MPTP induced neurodegeneration is by i) attenuation of characteristic parkinsonian impairments ii) inhibition of lipid peroxidation and enhancement of antioxidant response iii) Attenuation of inflammatory and apoptotic response iv) inhibition of alpha-synuclein over expression v) prevention of DA-ergic neuronal loss by reinstating expression of TH, DAT and VMAT2 in SN and ST; and vi) protection of mitochondrial morphology in SN and ST against MPTP toxicity.

This study utilizes an MPTP (mitochondrial complex I inhibitors) model of PD which selectively interferes with nigrostriatal pathway that connects SN with ST which resulting in dopamine depletion and subsequently leads to behavioral disturbances as seen in PD (Hanakawa et al., 1999; Crocker et al., 2003). Ultimately, all neuropathological changes and neuroprotective action of drugs are reflected in animal behavior. Behavioral assessments are imperative to analyze motor impairments in MPTP model of PD. Consequently, this study was initially aimed to analyze behavioral alterations by subjecting animals to open field, Narrow beam walk (NBT), Hang, rotord, catalepsy, akinesia, swim test and stride length measurement. Open field test indicates acclimatization activity, mental stress and motor activity of mice. The narrow beam walking test was performed to analyze muscular co-ordination, balance and vestibular integrity (Fleming et al., 2004). Hang test was performed to analyze the neuromuscular strength and rotorod tests are widely used to analyze motor learning and motor-coordination ability by allowing the animals to walk on a rotating rod (Tillerson and Miller, 2003). Catalepsy and akinesia are vital behavioral parameters which mimic gait and limb akinesia impairments due to neurochemical alterations in mid brain and basal ganglia as seen in PD. In our experiments, we found that intraperitoneal injection of MPTP induced behavioral impairments as evinced by behavioral assessments. These are ultimately linked to dopaminergic degeneration which might be partially due to proteotoxicity induced by alpha-synuclein over expression and deterioration of motor performance. Similar to our results, a recent study reported that geraniol, an acyclic monoterpene prevented α-synuclein expression invivo and ameliorated neuromuscular impairment.
(Rekha et al., 2013a). Moreover, α-synuclein has 40% similar amino acid sequence with molecular chaperone 14-3-3 which might result in two proteins with binding properties. Surprisingly, α-synuclein binds to many proteins [protein kinase C (PKC), BAD and extracellular regulated kinase (ERK)] which are similar to molecular chaperone 14-3-3 (El-Agnaf et al., 2004). Hence, these interactions inhibit the activities of PKC, BAD and ERK and make less protein available to hinder apoptosis and enhance cellular stress. Thus, at normal state α-synuclein acts as a chaperone supporting cell survival but over expression of α-synuclein inhibits functions of proteins involved in signal transduction and supports neurodegeneration. These results suggest that enhanced motor coordination ability in animals is due to inhibition of alpha-synuclein over expression by CNB-001 which results in decreased toxicity to dopaminergic neurons and enhanced biosynthesis of dopamine. Dopamine is a vital neurotransmitter involved in motor co-ordination and amendments in brain dopamine levels results in behavioral anomalies as seen in PD. In the present study, administration of MPTP results in depletion of brain dopamine and its metabolite levels which is due to neuronal loss. These results reciprocate to the efficacy of CNB-001 in reinstating dopaminergic neurons as assessed by striatal dopamine levels and findings are consistent with previous reports (Kadoguchi et al., 2008; Rekha et al., 2013b).

Antioxidant activity of CNB-001 might be the possible mechanism involved in neuroprotection due to the presence of electron donating styryl groups at 3,5-position of pyrazole and methoxyphenol group accountable for iron chelation. To strengthen this property, our results revealed that administration of MPTP increased the levels of TBARS due to lipid peroxidation, reduced GSH and GPx levels along with increased activities of SOD and CAT. Pre-treatment with CNB-001 modulated free radical toxicity by alleviating TBARS levels along with increased activities of antioxidant enzymes. Increased SOD and CAT in MPTP animals is due to adaptive response since MPTP mediated mitochondrial impairment results in leakage of superoxide anions (Thiffault et al., 1995). Moreover depletion of GSH and GPx might be an early mechanism involved in initiation of oxidative stress and provides sensitivity to DA-ergic neurons against toxin (Toffa et al., 1997). Our results
showed that CNB-001 protected SK-N-SH cells against rotenone toxicity by inhibiting accumulation of intracellular ROS formation. Thus, neuroprotective activity of CNB-001 may be partially due to the regulation of antioxidant enzymes which is depleted upon MPTP treatment. Further, the neuroprotective mechanism of CNB-001 may correlate well with the report of Liu et al. (2008) where CNB-001 protected HT-22 cells against glutamate induced oxidative stress.

Apart from free radicals, astroglia and microglia are key glial cells which play an important role in initiating toxic neuroinflammatory process due to oxidative stress. Various reports hypothesized that neuroinflammatory response is initially arbitrated by nitric oxide (NO) and citrulline which are lethal reaction products of iNOS (He et al., 2013; Thakur and Nehru, 2013). Moreover iNOS activation results in inhibition of neuronal respiration which causes depolarization, glutamate release from neurons and cytochrome oxidase inhibition ultimately directing to excitotoxicity (Bal-Price and Brown, 2001). As seen in the present study, MPTP intoxication significantly elevates the production of nitrite and citrulline levels and it is bolstered by enhanced expression of iNOS. These amendments were diminished when animals were pre-treated with CNB-001. Our study harmonizes with a report that minocycline, a second generation tetracycline analogue exerts neuroprotection by suppressing iNOS expression as well as IL-1β upregulation possibly by inhibiting phosphorylation of p38 MAPK (Du et al., 2001).

Several reports showed that oxidative stress activates microglial cells which upregulates production of pro-inflammatory cytokines (IL-6, IL-1β, TNF-α) and self-propels degeneration of cholinergic system (Du et al., 2001; Anandhan et al., 2013). Pott et al. (2010) reported that removal of IL-1β attenuated lipopolysaccharide mediated neurotoxicity in neuronal cultures and also inhibited motor impairments. Tumor necrosis factor alpha mediated cytotoxicity is arbitrated by activation of pro-apoptotic domains either by binding to TNF receptor 1 on dopaminergic neurons or through inflammatory action. Distinct from other cytokines, TNF-α has long term impact on inflammatory potentiation in PD and activation of TNF-α triggers iNOS expression strengthening inflammatory response
and ultimately leads to apoptosis (Guadagno et al., 2013). Our current results showed distinct down regulation of IL-6, IL-1β, TNF-α expression upon CNB-001 treatment. Thus, CNB-001 might have the ability to normalize neuroinflammatory stress induced by MPTP. In concordant with the present study, Wu et al. (2002) reported that microglial activation precedes dopaminergic degeneration in PD and this inhibition prevents apoptosis. Pro-inflammatory cytokines such as IL-6 and IL-1β are mainly involved in astroglial activation by accelerating GFAP release, a initial protein marker that favors neuronal loss and inflammation in PD (Brahmachari et al., 2006). Our results demonstrated over expression of GFAP in striatum of MPTP intoxicated mice and its expression was mitigated by pre-treatment with CNB-001 which proves that CNB-001 dampened astrogliosis.

Cyclooxygenase-2 (COX-2) is reported to play a significant immunomodulatory role in the brain tissues of PD patients (Reksidler et al., 2007). MPTP induced COX-2 cytotoxicity is mediated through oxidative damage mechanisms duringconversion of prostaglandins-G2 to prostaglandins-H2 which results in ROS generation and in turn produces pro-inflammatory prostaglandins causing microglial activation (Litteljohn et al., 2011). Moreover, dopamine is oxidized to dopamine-quinone in the presence of hydrogen peroxide by COX-2 invitro (Hastings, 1995). Thus, down-regulation of COX-2 by CNB-001 as seen in the present study accounts for preventing the loss of dopamine and inhibition of pro-inflammatory cytokine production (Feng et al., 2002).

In pathological conditions like PD and in MPTP toxicity, over expression of inflammatory cytokines leads to direct activation of apoptotic pathways conferring neurodegeneration (Litteljohn et al., 2011). Chemical inhibition of caspase-3/7 hindered microglial activation and prevented apoptosis invitro which supports the involvement of inflammation mediated apoptosis in PD (Burguillos et al., 2011). Hence, the present study was also aimed to evaluate anti-apoptotic property of CNB-001 by analyzing expressions of apoptotic markers through western blotting. Under pathological conditions, apoptosis is mediated by regulation of Bax and Bcl-2 which inturn activates downstream apoptotic pathways. An initial event in apoptosis due to
cytotoxic stimuli is mediated by alterations in mitochondria. Apoptosis is regulated by release of cytochrome C into the cytosol and this event is supported by the expression of Bax which destabilizes mitochondrial membrane either by binding with mitochondrial channel proteins or by forming pores (Mallet et al., 2005). Released cytochrome C forms a multiprotein complex termed ‘apoptosome’ and it activates pro-enzyme caspase-9 which subsequently leads to activation of executioner caspase-3. This active caspase-3 promotes apoptosis by endonucleases activation resulting in cleavage of cellular substrates and ultimately neuronal death as seen in Parkinson disease (D’Amelio et al., 2012). Bcl-2, a potent anti-apoptotic protein hinders apoptosis by inhibiting cytochrome C release via blockade of mitochondrial transition pore opening and mediates cell survival (Adams and Cory, 1998). It is evident from our results that CNB-001 distinctly upregulates the expression of anti-apoptotic protein Bcl-2 and alleviated pro-apoptotic expressions proposing CNB-001 might offer neuroprotection by regulating the function of Bcl-2 family. Based on results, this study proposes the neuroprotective action of CNB-001 is due to inhibition of inflammatory and apoptotic response as shown in figure 52.

![Image](image.jpg)

Figure 52. Illustration of anti-apoptotic and anti-inflammatory action of CNB-001.

Ultimately, neuroprotection offered by CNB-001 is strongly supported by immunohistochemical and western blot studies. Administration of MPTP significantly reduced the expression of TH, DAT and VMAT2 in SN and ST of mice. These proteins play a vital role in nigrostriatal pathway and are involved in dopamine biosynthesis. Tyrosine hydrolase is a rate-limiting enzyme responsible for
the conversion of tyrosine to 3,4-dihydroxyphenylalanine (DOPA). Further, aromatic amino acid decarboxylase removes carboxylic acid moiety from L-DOPA to form dopamine. Synthesized dopamine is sequestered in VMAT2 and released in synaptic space and further transmitted. VMAT2 also plays an important role in sequestering neurotoxins and prevents toxicity (Fon et al., 1997). Non-transmitted dopamine is further recycled into pre-synaptic neuron via dopamine transporter. Previous reports showed that diminished expression of TH, DAT (Heikkila and Sonsalla, 1992) and VMAT2 (Miller et al., 1999) in SN and ST are thought to underlie PD. Our results were in concordant with various reports which showed that MPTP intoxication selectively targets and destroys DA-ergic neurons as evinced by TH-immunoreactivity which is responsible for inactivation of TH and resultant dopamine depletion (Anandhan et al., 2012, Rekha et al., 2013b). Apart from the role of DAT in dopamine biosynthesis, it also acts as a gateway for several neurotoxins including MPTP for DA-ergic toxicity (Dauer and Przedborski 2003). Hence, immunoblotting results in our study showed decrease in DAT expression in SN and ST of MPTP treated mice due to loss of nigrostriatal neurons and fibers respectively. Similar to TH and DAT expressions, MPTP injection significantly depleted VMAT2 expression in brain. VMAT2 play an important role in inhibiting dopamine mediated toxicity by sequestering cytosolic dopamine and it has also been reported that VMAT2 deletion cause mice lethality (Wang et al., 1997). Accumulation of cytosolic dopamine support formation of oxyradical stress and excess cytosolic dopamine causes proteotoxicity to DA-ergic neurons and autophagy block (Martinez-Vicente, 2008; Mosharov et al., 2009). Therefore enhanced expression of VMAT2 could potentially contribute to neuroprotection. Consistent to these reports TH, DAT and VMAT2 protein levels were reduced distinctly in patients affected by PD (Wilson et al., 1996). Pre-treatment with CNB-001 enhanced TH-ir and expression of TH, DAT and VMAT2 which might contribute to enhanced dopamine synthesis and mitigation of behavioral impairments caused by MPTP.

Clinical reports demonstrate modest reduction in mitochondrial complex I activity in PD patients (Izuno et al., 1989, Parker et al., 1989). Further, MPP⁺

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selectively blocks complex I of mitochondria and inhibits flow of electrons across electron transport chain and resultant ATP depletion with increased ROS production. Electron microscopic results from our studies showed that MPTP intoxication enhanced mitochondrial pathology in SN and ST which reflect the sequelae of injury to mitochondria. Furthermore mitochondria from PD patients and MPTP treated animals have also been described to be swollen and enlarged possessing distorted mitochondrial cristae with discontinuous outer membrane rather than ordered cristae with normal rod shaped morphology which are similar to our results. To confirm the toxicity and to address difference in mitochondria structure, we measured mitochondrial perimeter length of experimental animals because increase in mitochondrial perimeter length as a result of mitochondrial swelling is a well-accepted hallmark in organelle dysfunction (Trimmer et al., 2000). MPTP treatment increased mitochondrial size when compared to the control group whereas, pre-treatment with CNB-001 protected the mitochondria and the ultrastructural changes were less prominent as compared to MPTP group. Our findings were also similar to those reported in apoptosis mediated cell death in SN of PD (Anglade et al., 1997). These results confirm the action of CNB-001 on mitochondria alleviating neuronal pathology. Our qualitative structural study showed that dendritic (ST) mitochondria are larger than axonal (SN) mitochondria which are similar to previous reports (Binukumar et al., 2010). We have also observed that CNB-001 prevented DA-ergic neuronal loss by attenuating inflammatory and apoptotic response in MPTP model of PD. Further results are substantiated by previous reports were CNB-001 has been shown to possess neuroprotective effects against intracellular amyloid toxicity, trophic factor withdrawal and excitotoxicity (Liu et al., 2008).

In summary, CNB-001 protects DA-ergic neurons against MPTP toxicity by regulating various molecular and cellular events. The therapeutic potential of CNB-001 is supported by its ability to inhibit behavioral impairments, oxidative stress, apoptotic and inflammatory response, alpha-synuclein aggregation, mitochondrial deficits and by enhancing expression of TH, DAT and VMAT2 in animal model of PD (figure 53).
Figure 53. Illustration showing proposed mechanism of anti-parkinson effect offered by CNB-001 in experimental PD models. **GREEN** - CNB-001 and **RED** - MPTP.
Parkinson’s disease is the second most chronic progressive neurodegenerative disorder of unknown etiology. Currently there is no medication to treat PD and the symptoms aggravates as the disease progresses leading to death. Oxidative stress, mitochondrial dysfunction and proteotoxicity (alpha-synuclein) are the three major hypotheses responsible for neurodegeneration in PD. Hence any drug with potent antioxidant activity remains to be a baseline property for treatment of PD.

Fifty potent antioxidant compounds (ligands) were selected and structure of the compounds has been downloaded followed by molecular simulation. Marvin sketch software was used to draw and optimize the 3-D structure of CNB-001, which was not available in the databases.

Fifty compounds were analyzed and out of which only 38 compounds satisfy “Lipinski rule of five” and pharmacokinetic properties like Adsorption, Distribution, Metabolism, Excretion and Toxicity (ADMET) were evaluated using Accelry’s Discovery Studio 3.5 software.

Thirty eight compounds were docked against the active site (64-100 amino acid) of alpha-synuclein using Biosolve LeadIT software. Based on the docking score, CNB-001 was found to be the potent compound against alpha-synuclein aggregation.

Various free radical scavenging (DPPH, ABTS, Reducing power, Nitric oxide radical scavenging, Superoxide radical scavenging, Superoxide anion scavenging, Hydrogen peroxide radical scavenging, Hydroxyl radical scavenging) and DNA protecting assays were performed to analyze the antioxidant potential of CNB-001. These results revealed that CNB-001 possessed good free radical scavenging and DNA protecting activity.

The neuroprotective activity of CNB-001 was assessed in rotenone induced SK-N-SH cellular model of Parkinson’s disease. The dose dependent effect of CNB-001 was analyzed using MTT assay and it was found that 2 μM of
CNB-001 protected SK-N-SH cells effectively against 100 nM rotenone toxicity.

Rotenone insult significantly reduced cell viability (MTT assay) due to intracellular ROS formation, depolarisation of mitochondrial membrane and apoptosis. Whereas, pre-treatment with CNB-001 diminished intracellular ROS formation, apoptosis and maintained mitochondrial membrane potential.

The partial mechanism behind neuroprotection offered by CNB-001 against rotenone toxicity was identified as inhibition of downstream apoptotic cascade by increasing the expression of vital antiapoptotic protein Bcl-2 and decreasing the expression of proapoptotic markers (Bax, caspase-3, and cytochrome C).

Brain dopamine levels of mice were assessed to analyze the dose-dependent effect of CNB-001 (6, 12, 24 and 48 mg/kg) against acute MPTP (30 mg/kg) intoxication. CNB-001 at a dose of 24 & 48 mg/kg showed similar dopamine levels but distinctly higher than 6 and 12 mg/kg. Thus, an optimum dose of 24 mg/kg was used for our study.

Behavioral alterations were assessed by subjecting animals to the following tests: Narrow beam walk, Catalepsy, Akinesia, Open field, Rotorod, Hang, Swim and Stride length measurement. Results showed that MPTP intoxication significantly caused behavioral impairments and these alterations were diminished upon pre-treatment with CNB-001.

Invivo antioxidant results revealed that administration of MPTP increased the levels of TBARS due to lipid peroxidation, reduced GSH and GPx levels along with increased activities of SOD and CAT. Pre-treatment with CNB-001 modulated free radical toxicity by alleviating TBARS levels along with increased activities of antioxidant enzymes.