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In silico identification of potent inhibitors of alpha-synuclein aggregation and its in vivo evaluation using MPTP induced Parkinson mice model

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Abstract

Parkinson disease is a progressive neurodegenerative disorder characterized by the presence of Lewy bodies with dense α-synuclein self-aggregation which is responsible for its toxic effect on Substantia nigra pars compacta and resultant neuronal death. Hence, blocking alpha-synuclein aggregation is a new channel to cure PD. This study initially investigates drug likeness and ADMET properties of CNB-001, 7,8 dihydroxyflavone, curcumin, naringenin and emodin and its inhibitory effect on alpha-synuclein (PDB:1XQ8) aggregation via molecular docking (LeadIT). Results revealed that the ligands satisfy drug likeness and ADMET properties and best-fit ligands were associated with VAL95, GLU83 and ALA91 as major amino acid residues of receptor site. Moreover, CNB-001 showed potent inhibitory effect than other compounds with a docking score of −13.6158. Further, we investigated the inhibitory effect of CNB-001 against alpha-synuclein expression using MPTP induced Parkinson mice model. Results explicated and confirmed that CNB-001 inhibited α-synuclein expression significantly when compared to MPTP group as evidenced by western blotting. Therefore, these results attribute that CNB-001 can be further developed as a promising therapeutic candidate for PD treatment.

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for CNB-001 till now. The results obtained will provide insights in understanding the activities of compounds as inhibitors based on docking scores. The potent compound was taken further for in vivo studies to confirm the pattern of α-synuclein expression upon treatment using a murine MPTP model of PD.

1. Experimental

1.1. Materials

MPTP was purchased from Sigma-Aldrich, Bangalore, India. Primary antibodies against alpha-synuclein and β-actin were purchased from Santa Cruz Biotechnology, Inc., USA. Anti-rabbit and anti-mouse secondary antibodies were procured from Genei, Bangalore, India. Enhanced chemiluminescence kit was purchased from GenScript ECL kit, USA. All other chemicals used were of analytical grade and obtained from Merck (Germany).

1.1.1. Computational feature

All the works 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. Softwares used were Accelrys’ Discovery Studio 3.5 (AD 3.5) and Biosolve IT.

1.2. In silico analysis

1.2.1. Ligand

The ligand used for this study has been selected based on various literatures and given in Table 1. Three-dimensional structure of the compound have been downloaded from PubChem compound and www.chemialalyze.org. Marvin Sketch software developed by Chemaxon is used to draw and optimize the 3-D structure of CNB-001, which was not available in the databases. A 3-D structure of the ligands was optimized and used for the docking study.

1.2.2. Drug likeness evaluation

The drug likeness 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 500 Da. 2. Less than 5 hydrogen bond donors. 3. Less than 10 hydrogen bond acceptors. 4. High lipophilicity (expressed as Log P less than 5) identifies whether the compound satisfies all the five rules based on the 2-D structure and provides valuable result regarding the use of compound as a drug [16].

1.2.3. ADME-Toxicity investigation

ADME-Toxicity studies were executed through Accelrys Discovery Studio 3.5. The absorption, distribution, metabolism and excretion (ADME) studies provides 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 is predicted using TOPKAT, which uses a range of robust, cross-validated, quantitative structure-toxicity relationship (QSTR) models for identifying specific toxicological activity. Toxicity profile includes NTP carcinogenicity, mutagenicity, developmental toxicity and skin irritation test.

1.2.4. Molecular simulation studies of 1XQ8

X-Ray crystallography structure of α-synuclein (1XQ8) (Fig. 1) 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

Table 1

<table>
<thead>
<tr>
<th>Properties</th>
<th>CNB-001</th>
<th>7.8 DHF</th>
<th>Curcumin</th>
<th>Naringenin</th>
<th>Emodin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
<td>442.506</td>
<td>254.237</td>
<td>368.38</td>
<td>272.253</td>
<td>270.237</td>
</tr>
<tr>
<td>H-bond donor</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>H-bond acceptor</td>
<td>6</td>
<td>4</td>
<td>6</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>A log P</td>
<td>4.985</td>
<td>2.652</td>
<td>3.554</td>
<td>2.373</td>
<td>2.568</td>
</tr>
<tr>
<td>AQ SOL LEV</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>BBB LEV</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>CYP 2D6</td>
<td>–7.5138</td>
<td>–5.77583</td>
<td>–4.36961</td>
<td>–4.28486</td>
<td>0.90881</td>
</tr>
<tr>
<td>HEPATOX</td>
<td>–7.2741</td>
<td>–1.28197</td>
<td>-6.64822</td>
<td>–1.46247</td>
<td>–2.26419</td>
</tr>
<tr>
<td>PBP LEV</td>
<td>5.63659</td>
<td>2.10687</td>
<td>3.38794</td>
<td>3.75955</td>
<td>-5.89908</td>
</tr>
</tbody>
</table>

Note: Solubility: 0-2 highly soluble; BBB: 2- medium penetration and 3- low penetration; CYP2D6: -ve - non-inhibitors & +ve - inhibition; HEPATOX: 0-1: non-toxic; PBP: greater the value greater the binding capacity.
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 time step, 300 K, NVT ensemble, non-bond cutoff 14A, switching function applied between 10 and 12A, coordinates saved every 1000 steps).

1.2.5. Active site prediction

Based on the study by El-Agnaf et al., 2004, the active site has been identified as 64-100 amino acid residues since it is the critical binding region in the α-syn molecule responsible for its self-association and aggregation [12]. Hence this sequence was taken as active site for this study.

1.2.6. Molecular docking

The possible interaction between the ligands and the target protein (1XQ8) 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. The detailed procedure is described elsewhere [17]. FlexX algorithm generates and uses up to 200 poses for each ligand and the best pose will be selected and scored.

1.3. In vivo studies

1.3.1. Animals and experimental design

Adult male C57BL/6 mice (25–30 g) were procured from National Institute of Nutrition, Hyderabad and maintained at ambient conditions. Animals were kept in vivarium and fed with standard laboratory diet and water ad libitum. All experiments were carried out strictly in accordance to National guidelines on the proper care and use of animals in laboratory research (Indian National Science Academy, New Delhi, 2000) and approved by Institutional Animal Ethics Committee (1085/ae/07/PU-IAEC/2012/13).

After acclimatization (1 week), mice were randomly divided into four groups (n = 6). Group I mice were treated with saline (vehicle) and served as control. Group II and III mice received MPTP (30 mg/kg, i.p) for 4 consecutive days initiating from 4th to 7th day of the experimental period [18]. Group III received CNB-001 (24 mg/kg) intraperitoneally for 7 successive days starting from 1st to 7th day of the experimental period. CNB-001 was dissolved in 100% ethanol (40 μl for 1 mg) followed by 10-fold dilution with 1%(v/v) Tween 80/Saline [19]. Group IV received CNB-001 alone (24 mg/kg, i.p) for 7 days.

1.3.2. Tissue preparation

Animals were sacrificed by terminal anaesthesia and perfused by means of intracardial infusion with saline. Striatum and substantia nigra was immediately dissected out on ice and stored at –80 °C for further analysis.

1.3.3. Western blotting

Immuno blot analysis was performed to determine the expression of α-synuclein [20]. Briefly, brain tissues (striatum and substantia nigra) were disrupted by homogenization in an ice-cold RIPA buffer (1% Triton 0.1% SDS, 0.5% deoxycholate, 1 mM/L EDTA, 20 mM Tris (pH 7.4), 10 mM NaCl, 50 mM NaF and 0.1 mM/L phenylmethylsulfonyl fluoride (PMSF)) and the cellular debris was cleared by centrifugation (12,000 rpm for 15 min at 4 °C). Samples were analyzed for their protein concentration by the method of Lowry et al. (1951) and resolved on 10% SDS gel [21]. The gel was then electrotransferred onto PVDF membrane by semi-dry transfer (BIORAD). Membranes were then blocked with blocking buffer containing 5% non-fat dry milk powder in TBS at 25 °C for 1 hr and probed primarily against α-synuclein (mouse monoclonal, 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 hr at room temperature. Immunoreactive bands were visualized by chemiluminescence kit (GenScript ECL kit, USA). Densitometry was done using ‘ImageJ’ analysis software.

1.4. Statistical analysis

All statistical analysis were carried out using SPSS 15.0 and results were expressed as mean ± SD for six animals per group. Statistical significance were analyzed by one way analysis (Anova) followed by Duncan’s multiple range test (DMRT). P < 0.05 was considered statistically significant.

2. Results and discussion

Since natural compounds can act as an anti- Parkinson drug by inhibiting the fibril formation of α- synuclein protein and thereby hindering Lewy body formation, we simulate the theoretical binding of the ligands (chemicals) towards the active site of α- synuclein protein.

2.1. In silico studies

2.1.1. Drug likeness evaluation

The Lipinski rule of five for the compounds was predicted using Lipinski drug filter (Table 1). These results showed that all these compounds obey Lipinski rule of five and it can be robustly recommended to be used as a drug. One of the most known factor for developing a drug is the “Lipinski rule of five”, which is abstracted empirically from the analysis of Worlds drug index and it helps in screening the drug which possess all the properties to be used as an orally active drug. In the present study, all the five compounds satisfied Lipinski properties.

2.1.2. ADME investigation

Analysing ADMET properties of ligands are vital in understanding the pharmacokinetic property of the drug for therapeutic intervention. In this study, the drug likeness of selected molecules were analysed by calculating ADMET properties using “TOPKAT” module of AD 3.5. Important parameters analysed for screening were aqueous solubility (AQ SOL), blood brain barrier (BBB) penetration levels, cytochrome 450 2D6 (CYP 2D6) inhibition, Hepatotoxicity (HEPATOX), and plasma protein binding (PPB) levels. The obtained ADME results (Table 1) were within the acceptable range which are as follows: AQ SOL, 0-2; BBB, 2-3; CYP 2D6, negative for non-inhibitors; HEPATOX, <1 and PPB, greater the value, greater the binding capacity. Toxicity profiles in this study include mutagenicity, NTP carcinogenicity and developmental toxicity assays. Toxicity prediction studies serve as an important preclinical examination and help to economically reduce time and cost during clinical trials. Skin irritation test helps in providing information on the use of compound for topical applications and all the compounds showed negative result for skin irritation except naringenin. TOPKAT features a patented algorithm (US Patent 6, 036,349, issued March 14, 2000), which determines whether a query structure lies within the optimum prediction space (OPS) to determine toxicity. If it ranges between 0 and 0.29 the compound is non-toxic, if it is between 0.3 and 0.69 the result is indeterminate, and if the score is between 0.7 and 1, the compound is toxic.
Table 2
Toxicity analysis using TOPKAT.

<table>
<thead>
<tr>
<th>Compound</th>
<th>NTP carcinogenicity</th>
<th>Developmental toxicity potential (DTP) (v3.1)</th>
<th>Skin irritation (v6.1)</th>
<th>Ames mutagenicity (v3.1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNB-001</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>7,8 DHF</td>
<td>1.000</td>
<td>0.999</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Curcumin</td>
<td>0.000</td>
<td>1.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Naringenin</td>
<td>0.989</td>
<td>0.921</td>
<td>0.999</td>
<td>0.000</td>
</tr>
<tr>
<td>Emodin</td>
<td>0.976</td>
<td>1.000</td>
<td>0.000</td>
<td>1.000</td>
</tr>
</tbody>
</table>

Note: 0: Negative result, 1: Positive result.

Table 3
Energy values of protein (1XQ8) before and after minimization.

<table>
<thead>
<tr>
<th>Alpha-synuclein (1XQ8)</th>
<th>Forcefield</th>
<th>Potential energy (kcal/mol)</th>
<th>Van der Waals energy (kcal/mol)</th>
<th>Electrostatic energy (kcal/mol)</th>
<th>RMS gradient (kcal/mol Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>After minimization</td>
<td>CHARMM</td>
<td>–6355.0577</td>
<td>–540.0159</td>
<td>–6604.72327</td>
<td>0.89223</td>
</tr>
</tbody>
</table>

In our present study, CNB-001 and curcumin do not show any carcinogenic effect but other compounds showed positive results for carcinogenesis. All the compounds showed developmental toxicity effect except CNB-001 but none of them showed mutagenic and skin irritation effect (Table 2). Thus, these results substantiate theoretically the use of these compounds to be orally active for human use.

2.1.3. Molecular simulation studies
CHARMm is a highly flexible molecular mechanics and dynamics program. It is derived from the program CHARMM (Chemistry at HARvard Molecular Mechanics). CHARMM performs various calculations and simulations, including calculation of geometries, interaction and conformation energies, local minima, barriers to rotation, time-dependent dynamic behavior, and free energy [22]. Energy minimization for ligands is performed prior to dynamics to relax the conformation and remove steric overlap that produces bad contacts between the ligand and the protein. The results obtained before and after minimization of protein are shown in Table 3.

2.1.4. Molecular docking simulation
Molecular docking plays an efficient and important role in the field of computer aided drug design (CADD) which helps
in identifying small molecules by docking towards the active binding site protein. Using these results, novel ligands for receptors of known receptor structure were designed and their interaction energies were calculated using the scoring function [23]. The aim of our study was to analyze the possibility of molecules to inhibit a specific protein based on docking score. Molecular docking studies were performed using LeadIT software. The results of interaction between α-synuclein protein with the compounds (a) CNB-001, (b) 7,8 dihydroxyflavone, (c) curcumin (d) naringenin and (e) emodin are shown in Fig. 2. The observed results of the drug-receptor interaction for the compounds are tabulated in Table 4. The results show that a good interaction occurs between the protein and the Ligand. CNB-001 showed more binding capacity than the other compounds. The build in program ProToss analyses the hydrogen bond network of the active site and maximizes the number of hydrogen bonds with a scoring function [24]. The LeadIT suite uses FlexX scoring function, to identify initial best 200 poses. For final evaluation of the poses, the scoring function HYDE was used [25]. The best score from the best pose for each compound was taken and compared to the scores of the other compounds. The compounds, which show highest negative LeadIT score, show greater capability to bind strongly with the protein and inhibit α-synuclein fibrilisation. In this present study, CNB-001 showed greater binding energy (~13.6158) and the amino acid involved in interaction were VAL95, GLU83 and ALA91 followed by 7,8 dihydroxyflavone (~13.6499) involving PHE94, VAL9 and ALA90, curcumin (~12.0386) interacts with VAL95, ALA91 and GLU83, naringenin (~11.1311) interacts with SER87, ALA91 and emodin (~8.8539) with SER87, ILE88 of alpha-synuclein. All these compounds had 3 hydrogen bonds with varying hydrogen bond length.

2.2. Alpha-synuclein expression

Immunoblotting was performed to evaluate the expression of α-synuclein upon MPTP and CNB-001 treatment. Results explicated that MPTP administration significantly elevated the α-synuclein expression when compared to control mice (P < 0.05) (Fig. 3). Whereas pretreatment with CNB-001 distinctly alleviated α-synuclein expression compared to MPTP group (P < 0.05). A recent study reported that geraniol, an acyclic monoterpene prevented α-synuclein expression in vivo and ameliorated neuromuscular impairment [26]. Similarly, El-Agnaf et al. showed that α-synuclein inhibitors specifically binds to 64-100 amino acid sequence of α-synuclein and prevented proteotoxicity by reducing the expression pattern of pro-apoptotic proteins and resultant neuronal death. These results were in concordance with the present study. 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 14-3-3] [12]. 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 stage α-synuclein acts as a chaperone supporting cell survival but overexpression of α-synuclein inhibits functions of proteins involved in signal transduction and supports neurodegeneration. Thus, prevention of α-synuclein overexpression might be a vital target to inhibit PD progression.

3. Conclusion

Comprehensively, this study stands unique in its consent that CNB-001, a novel pyrazole curcumin derivative possessed acceptable drug likeness and ADMET properties. Moreover, CNB-001

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has the ability to inhibit α-synuclein aggregation at a dosage of 24 mg/kg in an MPTP induced Parkinson mice model. Thus CNB-001 could be further developed as a potential drug for treatment of synucleopathies influenced disease such as PD.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

Acknowledgment

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References

In vitro antioxidant potential and deoxiribonucleic acid protecting activity of CNB-001, a novel pyrazole derivative of curcumin

Abstract

Background: Free radicals are underpinned to initiate cascade of toxic events leading to oxidative stress and resultant cell death in many neurodegenerative disorders. Now-a-days antioxidants have become mandatory in the treatment of various diseases apart from the drug’s modes of action. CNB-001, a novel hybrid molecule synthesized by combining curcumin and cyclohexyl bisphenol A is known to possess various biological activities, but the antioxidant property of the compound has not yet been elucidated. Aim: The present study is aimed to analyze various free radicals scavenging by employing in vitro antioxidant assays and to evaluate the deoxiribonucleic acid (DNA) protecting the ability of CNB-001 against hydroxyl radicals. Materials and methods: The in vitro antioxidant potential of CNB-001 was evaluated by analyzing its ability to scavenge DPPH, ABTS, nitric oxide, superoxide, hydrogen peroxide, superoxide anion, hydroxyl, hydrogen peroxide radicals and reducing power using spectroscopic method. The DNA protecting activity of CNB-001 was also evaluated on pUC19 plasmid DNA subjected to hydroxyl radicals using standard agarose gel electrophoresis. Results: From the assays, it was observed that CNB-001 scavenged free radicals effectively in a dose dependent manner. CNB-001 scavenged 2,2-diphenyl-1-picrylhydrazyl (IC50 = 44.99 µg/ml), 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (IC50 = 17.99 µg/ml), nitric oxide (IC50 = 1.36 µg/ml), superoxide radical (IC50 = 77.17 µg/ml), hydrogen peroxide (IC50 = 492.7 µg/ml), superoxide (IC50 = 36.92 µg/ml) and hydroxyl (IC50 = 456.5 µg/ml) radicals effectively and the reducing power was found to be 11.53 µg/ml. CNB-001 showed considerable protecting activity against plasmid DNA (pUC19) strand scission by OH at dose dependent manner. Conclusion: Results from these assays concluded that CNB-001 has a good antioxidant potential by reducing reactive oxygen and reactive nitrogen radicals and it showed significant protecting activity against DNA scission by hydroxyl radicals. Hence, CNB-001 can be further developed as potential drug for free radical induced neurodegenerative disorders.

Key words: 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid), 2,2-diphenyl-1-picrylhydrazyl, antioxidant, -001, deoxiribonucleic acid protecting, free radicals

Introduction

Atoms or molecules having a retiring unpaired electron in the outer shell are called as free radicals. The tendency of free radicals is that they react chemically with other molecules to capture electron in order to pair the radicals unpaired electron.[1] Free radicals in biological systems were first investigated by Denham Harmon in 1956. His first proposal was the role of free radicals in ageing.[2] Later, based on the importance of free radicals in human health, various researches have been carried out. Superoxide dismutase, an important antioxidant enzyme first discovered in the year 1969 by McCord and Fridovich gave strong evidence on free radicals in living system.[3] Mitochondrial oxidative phosphorylation, reactive oxygen species (ROS) like superoxide radicals (O2—), hydrogen peroxide (H2O2).

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hydroxyl radical (•OH), peroxynitrite (ONOO−) and reactive nitrogen species (RNS) are free radicals that are byproducts of various metabolism occurring within the body systems[4,5] possessing beneficial as well as deleterious effects. Beneficial effects include energy production, phagocytosis, intracellular signaling and cell growth regulation.[6] Though the beneficial effects of ROS occurs at lower concentration, the body cells are prone to the destructive effect by free radicals, which is termed as oxidative stress and nitrosative effect causing various biological damage.[7] Naturally, biological systems have the capability to counteract free radical production with the help of a mechanism called “redox regulation”. During this mechanism, redox homeostasis is maintained[8] with the help of free radical detoxifying antioxidant enzymes namely superoxide dismutase, glutathione peroxidase, catalase, glutathione-S-transferase and also by the support of non-enzymatic antioxidants like ascorbic acid, α-tocopherol, glutathione, carotenoids and flavanoids. When there is a disturbance in prooxidant/antioxidant reactions, these free radicals cause disorientation of the cell membrane, Protein damage, attack lipids in cell membranes, causes deoxyribonucleic acid (DNA) damage by means of single strand breaks and double strand breaks leading to cancer,[9,10] cardiovascular diseases, neurodisorders[11] and reduces immunity. Mitochondrion is the primary source of ATP production involving complex I-IV. During energy transduction, small amount of electrons react with oxygen to form oxygen free radical “superoxide” and this free radical generation and impairment of normal metabolism leads to decreased ATP synthesis and ultimately cell death. Free radicals initiated mitochondrial dysfunction is proposed to be a major mechanism underlying many neurodegenerative disorders.[12] These toxic effects are balanced by antioxidants by donating electrons to free radicals, making them harmless molecules and preventing radical-chain reaction.[13] Hence antioxidants play an important role in maintaining harmony between the body and mind. Sometimes free radical scavenging activity of the system are alone not sufficient to fight free radicals and hence the need in search of drugs with antioxidant activity are of prime importance.[14]

CBN)-001[4-((1E)-2-(5-(4-hydroxy-3-methoxy styryl)-1-phenyl-1H-pyrazol-3-yl)vinyl)-2-methoxy-phenol)] [Figure 1] is a hybrid molecule synthesized by combining cyclohexyl bisphenol A (CBA), a molecule with neurotrophic activity and curcumin, a spice with potent neuroprotective activity.[15] This compound has been studied for enhancement of memory,[16] anti-inflammatory activity[17] and its protection against ischemic stroke[18] but the antioxidant potential of the compound has not been proved in vitro. The focus of the current study is to analyze various free radical scavenging ability of CBN-001 by employing 2,2-azinobis (3-ethylbenothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), H2O2 scavenging activity, hydroxyl radical scavenging activity, nitric oxide scavenging activity, reducing power, superoxide radical scavenging activity and superoxide scavenging activity. Since free radicals cause DNA nicks, we also analyzed the DNA protecting activity of CBN-001 against FeSO4 and H2O2 induced DNA damage on plasmid DNA (pUC19).

### Materials and Methods

**DPPH radical scavenging activity**

DPPH radical scavenging activity was determined according to the method of Blois.[19,20] Briefly, 1 ml of 0.1 mM DPPH radical solution was mixed with 3 ml of different concentrations of CBN-001 (20, 40, 60, 80 and 100 μg/ml) dissolved in methanol. The mixture was then thoroughly vortexed and left in dark for 30 min at 40°C. For the baseline control, 3 ml of methanol was used. The absorbance was measured at 517 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 compound.

**96-Multiwell plate assay**

Aliquots of 0.5 ml of 0.04 mM DPPH solution in methanol was added into 96-multiwell plate. 0.5 ml (20, 40, 60, 80 and 100 μg/ml concentration) of CBN-001 was added into each well except that of the control. The concentrations of samples were increasing sequentially as shown in Figure 1. The plate was incubated for 10 min in room temperature and photographs were taken. Ascorbic acid was used as a standard.[21]

**Radical cation 2,2′-azinobis (3-ethylbenothiazoline-6-sulfonic acid) diammomium salt (ABTS+)** scavenging activity

Radical cation scavenging capacity of CBN-001 was observed against ABTSd + generated by chemical methods.[22,23] In brief, ABTS+ was prepared by oxidizing a 5 mM aqueous solution of ABTS diammomium 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 CBN-
001 with various concentrations and 1.0 ml of 5 mM phosphate buffered saline (PBS) buffer (pH 7.4). The absorbance was read after 1 min at 734 nm and the % inhibition was calculated. The experiment was performed in triplicate. Percentage of inhibition = \((\text{A}_0 - \text{A}_1) / \text{A}_0\) × 100, where A0 was the absorbance of the control (blank, without compound) and A1 was the absorbance in the presence of the compound.

**Determination of reducing power**

The reducing power of CNB-001 was evaluated by the method of Oyaizu.

In brief, various concentrations of CNB-001 (0.2 ml) were mixed with 1.0 ml of 200 mM sodium phosphate buffer (pH 6.6) and 1.0 ml of 1% potassium ferricyanide. The mixture was incubated for 30 min at 50°C. Later 1.0 ml of 10% trichloroacetic acid (w/v) was added and the mixture was centrifuged at 4000 rpm for 10 min. The supernatant (2 ml) was mixed with 2 ml 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 inhibition was calculated using, percentage of inhibition = \((\text{A}_0 - \text{A}_1) / \text{A}_0\) × 100 where A0 was the absorbance of the control (blank, without compound) and A1 was the absorbance of the mixture in presence of CNB-001.

**Nitric oxide radical scavenging activity**

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. Control without test compound but with an equal amount of methanol was taken. 30 min after incubation, 1.5 ml of the incubated solution was diluted with 1.5 ml of gues reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride in 2% H₃PO₄). The absorbance of the chromophore formed during diazotization of nitrite with sulfanilamide and subsequent coupling naphthyl ethylene diamine was measured at 546 nm. Percentage radical scavenging activity of the sample was calculated as follows:

Percentage of inhibition = \((\text{A}_0 - \text{A}_1) / \text{A}_0\) × 100 where A0 was the absorbance of the control (blank, without compound) and A1 was the absorbance in the presence of the compound.

**Superoxide (O₂⁻) radical scavenging activity**

The assay was based on the capacity of the sample to inhibit blue formazan formation by scavenging the superoxide radicals generated using riboflavin-light-nitroblue tetrazolium (NBT) system. Briefly, 3 ml reaction mixture contained 50 mM phosphate buffer (pH 7.6), 20 µg riboflavin, 12 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mg NBT/3 ml and various concentrations (20-100 µg/ml) of CNB-001. Reaction was started by illuminating the reaction mixture for 90 s. Immediately after illumination the absorbance was measured at 590 nm. The entire reaction assembly was enclosed in a box lined with aluminum foil. Identical tubes with the reaction mixture except CNB-001 were kept in dark and served as blank. The percentage inhibition of superoxide anion generation was calculated as: Percentage of inhibition = \((\text{A}_0 - \text{A}_1) / \text{A}_0\) × 100 where A0 was the absorbance of the control (blank, without compound) and A1 was the absorbance in the presence of the compound.

**Superoxide anion scavenging activity assay**

Superoxide anion scavenging activity of CNB-001 was validated. Briefly, the reaction mixture contains 1 ml of nitro blue tetracosilum solution (156 µM in 100 mM phosphate buffer, pH 7.4), 1 ml nicotine amide adenine dinucleotide solution (468 µM in 100 mM phosphate buffer, pH 7.4) and 0.1 ml of different concentrations of CNB-001. The reaction was initiated by adding 100 µl of phenazine methosulfate solution (60 µM) 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 according to the following equation. Percentage of inhibition = \((\text{A}_0 - \text{A}_1) / \text{A}_0\) × 100 where A0 is the absorbance of the control (blank, without compound) and A1 is the absorbance in presence of compound.

**H₂O₂ radical scavenging activity**

This activity was determined according to a method described elsewhere with minor modifications. Various concentrations (20, 40, 60, 80 and 100 µg/ml) of CNB-001 and aliquot of 50 mM H₂O₂ were mixed (1:1 v/v) and incubated for 30 min at room temperature. After incubation, 10 µl methanol and 0.9 ml FOX reagent (prepared in advance by mixing 9 volumes of 4.4 mM BHT in HPLC grade methanol with 1 volume of 1 mM xylenetol orange and 2.56 mM ammonium ferrous sulfate in 0.25 M H₂SO₄) was added to 90 µl of the incubated mixture. The reaction mixture was vortexed and incubated at room temperature for 30 min. The absorbance of the ferricyclenol orange complex was measured at 560 nm. All tests were carried out in triplicate and ascorbic acid was used as the standard.

Percentage of inhibition = \((\text{A}_0 - \text{A}_1) / \text{A}_0\) × 100 where A0 was the absorbance of the control (blank, without compound) and A1 was the absorbance in the presence of the compound.

**Hydroxyl radical (•OH) scavenging activity**

Hydroxyl radicals generated by the Fenton reaction were measured as per the method of Chung et al. Fenton reaction mixture constituted of 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). CNB-001 at various concentrations (20, 40, 60, 80 and 100 µg/ml) was added to Fenton reaction mixture followed by the addition of 0.2 ml H₂O₂ (10 mM) and incubated at 37°C for 4 h. Later, 1 ml TCA (2.8%) and 1 ml TBA (1%) were 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 the compound is calculated using 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 compound.

**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. IC50 is defined as the amount of antioxidant necessary to decrease the initial radical concentration by 50%. Triplicate measurements were carried out and their activity was calculated by the percentage of radical scavenged. The IC50 values obtained are shown in Table 1.

**DNA protecting activity**

This assay was performed to analyze the ability of CNB-001 to protect DNA against hydroxyl radicals (•OH). In brief, damage 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 (20, 40, 60, 80 µg/ml), 5 µl of damage solution and irradiated in ultraviolet (UV) by placing on UV transilluminator (300 nm). Curcumin (20, 80 µg/ml) was used for comparison. 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 analyzed using gel documentation system (Alpha imager mini, Alpha Innotech, USA).

**Results**

**DPPH radical scavenging activity**

CNB-001 has an antiradical activity by inhibiting DPPH radical with an IC50 value of 44.99 µg/ml compared with that of reference standard, ascorbic acid of 90.81 µg/ml. The inhibiting potential of CNB-001 is shown in Figure 2.

**96-Multiwell plate assay**

The color reaction shows the ability of the compound to reduce DPPH radical. Pink color (high intensity) indicated the presence of DPPH radical and yellow color (low intensity) indicates the scavenging capacity of compound in a dose dependent manner [Figure 3]. Lower the intensity, higher the DPPH radical scavenging activity.

**ABTS radical scavenging activity**

The antioxidant activity of CNB-001 was calculated by decolorization of ABTS radical. Radical scavenging activity of CNB-001 is expressed by percentage inhibition. IC50 value was found to be 17.99 µg/ml. These values are superior to standard antioxidant compound ascorbic acid (45 µg/ml). The percentage of inhibition corresponding to the concentration is shown in Figure 4.

**Reducing power**

To investigate the reducing potential of CNB-001, the reduction of Fe³⁺ to Fe²⁺ was studied in the presence of CNB-001. Observed results showed that CNB-001 and ascorbic acid have a dose dependent quenching ability [Figure 5]. Similarly the IC50 values were closer which was found to be 11.53 and 9.55 µg/ml respectively.

**Nitric oxide scavenging activity**

The concentration of CNB-001 is directly proportional to the nitric oxide scavenging property [Figure 6]. The ability of CNB-001 to quench 50% of nitric oxide was found to be 1.36 µg/ml and ascorbic acid was found to be 8.13 µg/ml. Hence the test compound has higher nitric oxide scavenging activity at a very low dose.

**Superoxide radical scavenging activity**

The ability of the compound to scavenge superoxide radical generated from dissolved oxygen by phenazine methosulfate-NADH coupling can be measured by their capability to reduce NBT. Superoxide anion is one of the important radical generated in living systems. Hence the ability of test compound to scavenge superoxide anion were studied. The results indicate that CNB-001 has superoxide scavenging activity at a very low dose and as the dose increases, the scavenging activity increases.

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**Table 1: IC50 values of CNB-001 and ascorbic acid. Values are represented as mean ± SD**

<table>
<thead>
<tr>
<th>IC50 µg/ml</th>
<th>DPPH</th>
<th>ABTS</th>
<th>TRAP</th>
<th>Hydroxyl radical</th>
<th>Superoxide radical</th>
<th>Superoxide scavenging</th>
<th>Nitric oxide</th>
<th>Reducing power</th>
<th>Hydrogen peroxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNB-001</td>
<td>44.99±1.28</td>
<td>17.99±1.35</td>
<td>128.4±1.38</td>
<td>456.5±3.20</td>
<td>77.17±1.05</td>
<td>36.92±0.75</td>
<td>1.36±0.04</td>
<td>11.53±0.29</td>
<td>492.7±1.14</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>90.81±0.58</td>
<td>45±0.23</td>
<td>64.53±0.49</td>
<td>4.088±0.04</td>
<td>739.5±2.54</td>
<td>1301±0.78</td>
<td>8.13±0.13</td>
<td>9.55±0.28</td>
<td>450±2.25</td>
</tr>
</tbody>
</table>
Jayaraj and Elangovan: *In vitro* antioxidant activity of CNB-001

Figure 2: 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity of CNB-001 at different concentration. Data are presented as mean ± SD of each of three replicates (n = 3)

Figure 3: Part of 96 multi-well plate showing the gradually change in color from deep purple to pink to yellow for comparative study of the antioxidant activities of compounds at different concentrations after addition of 0.4 mM 2,2-diphenyl-1-picrylhydrazyl solution in methanol

Figure 4: 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonyl acid) radical scavenging activity of CNB-001 at different concentration. Data are presented as mean ± SD of each of three replicates (n = 3)

Figure 5: Reducing power of CNB-001 at different concentration. Data are presented as mean ± SD of each of three replicates (n = 3)

Figure 6: Nitric oxide radical scavenging activity of CNB-001 at different concentration. Data are presented as mean ± SD of each of three replicates (n = 3)

Figure 7: Superoxide radical scavenging activity of CNB-001 at different concentration. Data are presented as mean ± SD of each of three replicates (n = 3)
decreases [Figure 7]. The IC50 values of CNB-001 and ascorbic acid were found to be 77.17 and 739.5 µg/ml respectively.

Superoxide anion scavenging activity
Similar to superoxide radical scavenging activity, CNB-001 has higher superoxide anion quenching effect at a low dose when compared to standard [Figure 8]. IC50 values of CNB-001 and ascorbic acid were found to be 36.92 and 1301 µg/ml respectively.

H₂O₂ scavenging activity
H₂O₂ scavenging activity was assayed by FOX reagent method. The scavenging effect of CNB-001 on H₂O₂ was in a concentration dependent manner [Figure 9]. CNB-001 and reference standard both had inhibitory percentage of 22 at a concentration of 20 µg/ml. The IC50 value of CNB-001 and ascorbic acid were 492.7 and 450 µg/ml respectively.

Hydroxyl radical scavenging activity
Hydroxyl radical scavenging activity of the test compound was comparatively lesser than ascorbic acid [Figure 10]. Similarly IC50 value of CNB-001 was found to be 456.5 µg/ml and ascorbic acid was found to be 4.088 µg/ml. Hence ascorbic acid has superior hydroxyl radical scavenging activity.

DNA protecting activity
CNB-001 was found to protect plasmid DNA (pUC19) from hydroxyl radicals in a dose dependent manner [Figure 11]. Plasmid DNA has three forms when run of gel electrophoresis namely supercoiled circular DNA, Open circular form and linear DNA. UV radiation and hydroxyl radicals cleaved supercoiled form into open and linear DNA (Lane B, C, D) as shown in Figure 11, CNB-001and curcumin rescued the plasmid DNA from H₂O₂ and UV induced DNA scission by scavenging hydroxyl radicals in a dose-dependent manner.
Discussion

Free radicals are the main culprits in the initiation of various diseases.⁴¹ Hence any drug of interest is nowadays analyzed for its antioxidant property.⁴¹ CNB-001, a pyrazole derivative is synthesized from curcumin, a neuroprotective compound⁴⁵ and CBA, a neurotrophic molecule. Though, both the parental compounds have excellent biological properties, its activities are limited. Curcumin, a potent antioxidant fails to inhibit excitotoxicity in cortical neurons and has poor bioavailability.⁴³ Recent cell culture work reported by Liu et al. showed that CNB-001 was far more superior than curcumin and CBA as evinced by trophic factor withdrawal, excitotoxicity, glucose starvation and amyloid toxicity assays. CNB-001 also showed neurotrophic factor like activity many folds higher than CBA. Neurotrophic factors play an important role in maintaining and regulating brains microenvironment during CNS injury. Moreover, both the parental compounds have higher EC50 value compared to CNB-001. Hence CNB-001 was synthesized to enhance the effectiveness of curcumin and CBA. Insilico analysis done in our lab (data not shown) also showed that CNB-001 is non-toxic, has good ADMET properties and protected SK-N-SH cells in vitro against rotenone toxicity.⁴⁵ Therefore, this compound is tested for antioxidant and DNA protecting activity, which stands as a baseline property for a drug. It is believed that the present work would enhance and highlight the potentiality of CNB-001 for further drug targeted studies. Stereo selectivity of radicals affects the interaction between the compounds which in turn affects the scavenging activity of the compound.⁴⁷ A study by Wang et al. concluded that compound which has good ability to scavenge ABTS + did not scavenge DPPH radical.⁴⁸ Contradictory to this result, CNB-001 was found to scavenge different radicals in different systems.

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.⁴⁹ This is one of the most common antioxidant assays to analyze the free radical scavenging activity of the test compound. In our study the EC50 of test compound was found to be 44.99 μg/ml whereas standard ascorbic acid was found to be 90.81 μg/ml. This scavenging ability was in turn proved using 96 multi well plate assay. Free radicals were of pink color (high intensity) and when CNB-001 was added, the radicals were quenched in a dose dependent manner and the color changes to yellow (low intensity). This assay was supported by the work done by Mon et al.⁵¹

ABTS is an improved version of Trolox Equivalent antioxidant capacity assay.⁴⁶ 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. Our experiment revealed that CNB-001 has an excellent radical scavenging capacity.

Ferric reducing power is an electron transfer based assay where Ferric salt, Fe(III)(TPTZ)2C1 is used as an oxidant. It shows the ability of antioxidants to reduce Fe³⁺ to Fe²⁺. The principle behind this assay is depicted below

Oxidant + electron from antioxidant → reduced oxidant + oxidized antioxidant.

The reducing capacity of antioxidant is directly proportional to the color change. CNB-001 has a good redox potentiality than ascorbic acid. Antioxidant capacity of CNB-001 involves the reduction of ferricyanide complex to ferrous form, which is indicated by the formation of Pearl’s Prussian blue color at 700 nm.⁴³ The reducing power of CNB 001 was observed to be in a dose-dependent manner, which is measured in terms of inhibiting potential. CNB-001 was found to be highly potent in reducing Fe³⁺ than the standard antioxidant, ascorbic acid.

Nitric oxide radical has different roles in a biological system.⁴⁵ Peroxynitrite (ONOO—) is one of the important RNS causing oxidative damage in biological systems. Biological system has a self-defense mechanism to convert harmful ROS to harmless species. Radicals like O₂•— and H₂O₂ can be converted to non-radicals by SOD and Catalase respectively. But few radicals like ROO•, HO•, 1O₂ and ONOO— cannot be scavenged by enzymatic defense mechanism and hence the body purely depends upon non enzymatic antioxidants and supplements. CNB-001 has a good RNS scavenging activity than ascorbic acid.

Superoxide radical scavenging and superoxide anion scavenging assays are based upon singlet oxygen scavenging capacity. Superoxide anion indirectly initiates lipid oxidation⁴³ and reduces certain iron complexes such as cytochrome.⁴⁴ Immune cells, during Inflammation process generate superoxide radicals where NADPH oxidase initiates vascular complications.⁴⁶ Narumoto et al. proved that CNB-001 showed superior anti-inflammatory property in vivo compared to curcumin by suppressing the expression of vital inflammatory proteins interleukin-6 and tumor necrosis factor-alpha possibly by scavenging superoxide radicals and inhibiting NF-κB and p38MAPK activation.¹⁷ This result is supported by the superoxide radical scavenging property in the present study which showed potent superoxide radical scavenging activity of CNB-001 even ate a very low dose of 20 μg/ml.

Lipids present in cell membrane are prone to peroxidation by hydroxyl radicals resulting in lipid hydroperoxide free radicals.¹³ Hydroxyl radicals have a short life time and

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[50] Chronicles of Young Scientists
can hydroxylate biomolecules very rapidly. The conversion of $\text{H}_2\text{O}_2$ to $\text{H}_2\text{O}$ and $\text{O}_2$ is done by Catalase and when this defense mechanism fails, antioxidants play a vital role in scavenging hydroxyl radicals. These radicals are known to be highly mutagenic and carcinogenic. $\text{H}_2\text{O}_2$ readily crosses cell membrane and reacts with $\text{Fe}^{2+}$ and $\text{Cu}^{2+}$ ions which results in many toxic effects.\(^{[45]}\) The scavenging capacity of CNB-001 and ascorbic acid were higher at a lower concentration and decreases slightly as the concentration increases. This might be because the interference substance(s) won’t be able to donate protons at critical higher concentration. The other reason for prooxidant effect may be the formation of phenoxy radicals which participates in radical chain propagation as corroborated by others.\(^{[46,47]}\)

Hydroxyl radicals has the capacity to interfere with the DNA by degrading deoxyribose using $\text{Fe}^{2+}$ salt as a catalytic complex which results in mutation and carcinogenesis.\(^{[48,49]}\) DNA damage was performed by exposing DNA to damage solution (1.6 $\mu\text{l}$ of 30% $\text{H}_2\text{O}_2$ and 1 mM $\text{FeSO}_4$) for radical nicking and UV for photodegradation (Lane B-D). Treatment with CNB-001 at different concentrations (20, 40, 60, 80 $\mu\text{g}$/ml) (Lane E-H) and curcumin (20, 80 $\mu\text{g}$/ml) (Lane I and J) showed DNA protecting activity by reducing hydroxyl radical and inhibiting DNA strand scission. DNA protection was observed to be in a dose dependent manner. CNB-001, a pyrazole derivative of curcumin lacks labile dicarboxyl group of curcumin with 1H-Pyrazole as a basic nucleus. The presence of substituted styril groups at 3,5-positions of pyrazole has electron donating/releasing groups like -OH, -OCH3 which might be responsible to its free radical scavenging property. Moreover, CNB-001 has two methoxyphenol group separated by $\beta$-diketone bridge which is accountable for iron chelation. These structural modifications accounts for its various biological properties and can be effectively developed as a novel drug for therapeutic intervention.

**Conclusion**

Etiological factors of many diseases reveal that free radicals are basic culprit in initiation of various toxic events and resultant cell death. To combat the disease progression, drugs with antioxidative property will be an effective way of treatment. The results from this study show that CNB-001 has an excellent antioxidative property by scavenging ROS and RNS. The protective role of CNB-001 in various in vivo experiments may be catalyzed by the antioxidative property of the compound and can be a superior target for therapeutic potential.

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Neuroprotective Effect of CNB-001, a Novel Pyrazole Derivative of Curcumin on Biochemical and Apoptotic Markers Against Rotenone-Induced SK-N-SH Cellular Model of Parkinson’s Disease

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Abstract 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. Curcumin, a potent antioxidant has been reported to display diverse neuroprotective properties against various neurodegenerative diseases including PD. In this present study, we investigated the protective effect of CNB-001, a pyrazole derivative of curcumin on rotenone-induced toxicity and its possible mechanisms in neuroblastoma SK-N-SH cells. Rotenone insult significantly reduced cell viability (MTT assay) and resulted in 78 % apoptosis (dual staining) by altering Bcl-2, Bax, caspase-3, and cytochrome C expression. Moreover, rotenone enhanced ROS production and disrupts mitochondrial membrane potential. These resultant phenotypes were distinctly alleviated by CNB-001. Pretreatment with CNB-001 (2 μM) 2 h before rotenone exposure (100 nM) increased cell viability, decreased ROS formation, maintained normal physiological mitochondrial membrane potential, and reduced apoptosis. Furthermore, CNB-001 inhibited downstream apoptotic cascade by increasing the expression of vital antiapoptotic protein Bcl-2 and decreased the expression of Bax, caspase-3, and cytochrome C. Collectively, the results suggest that CNB-001 protects neuronal cell against toxicity through antioxidant and antiapoptotic properties through its action on mitochondria. Therefore, it may be concluded that CNB-001 can be further developed as a promising drug for treatment of PD.

Keywords CNB-001 · Rotenone · Oxidative stress · Mitochondria membrane potential · Apoptosis

Introduction

The major etiology of Parkinson’s disease (PD) is the loss of nigral dopaminergic neurons and its associated decline in striatal dopamine (Narumoto et al. 2012). One of the major hypotheses posits that mitochondrial dysfunction and subsequent oxidative stress are the main culprits for neuronal cell death in PD (Schapira 2008). Mitochondria are the major source of free radicals apart from their role in electron transport chain and oxidative phosphorylation. Enhanced free radical production may result in inhibition of respiratory chain components, cessation of ATP production, and formation of reactive oxygen species (ROS). ROS is largely derived from two mitochondrial respiratory chain complexes (I and II) with superoxide as a primary product followed by hydrogen peroxide as secondary product (Pirnia et al. 2002). Moreover, free radicals interact with physiological signaling mechanisms and instigate apoptosis which further activates proapoptotic regulators (Bcl-2 family, cytochrome c, caspases, Smac/Dialbo, and AIF) (Sherer et al. 2003; Perier et al. 2003).

Many mitochondrial complex I (NADH dehydrogenase) inhibitors such as rotenone, isoquinoline, tetrahydroisoquinoline, and MPP⁺ are shown to enhance free radical production and initiates cell death that are used to create models of PD (Li et al. 2003). Rotenone, an insecticide, is isolated from the roots of Derris and Lonchorcarpus plant species. Because of its hydrophobicity nature, it can easily cross the blood–brain barrier and does not depend upon dopamine transporter to access the neuronal cytoplasm (Greenamyre et al. 2001).

Various therapeutic efforts by using antioxidants are aimed to quench and inhibit free radical production which
may be beneficial in PD. Curcumin is the potent antioxidant, antiapoptotic, and antiinflammatory functions which is reported to protect dopaminergic neurons through its mitochondrial action (Cole et al. 2007). Curcumin lacks the ability to inhibit excitotoxicity and also has less stability. These drawbacks are overcome by CNB-001, and it shows protective activities against glucose starvation assay, excitotoxicity, increased stability, and antiinflammatory properties (Narumoto et al. 2012; Maher et al. 2010). CNB-001[4-((1E)-2-(5-(4-hydroxy-3-methoxystyr1-)1-phenyl-1H-pyrazol-3-yl)vinyl)-2-methoxy-phenol] (Fig 1) is a novel hybrid molecule synthesized by combining cyclohexyl bisphenol A, a neurotrophic molecule, and curcumin, a molecule with neuroprotective activity (Yuanbin et al. 2008). Recently, our investigation showed that CNB-001 has the ability to scavenge various free radicals and protected DNA against H₂O₂-induced oxidative stress in vitro. Since CNB-001 has various biological activities, the present study is designed to examine its neuroprotective action against rotenone-induced toxicity in SK-N-SH cellular model by evaluating ROS formation, mitochondrial membrane potential (ΔΨm), and apoptosis. Apoptosis due to oxidative stress initiates regulation of Bcl-2 proteins, release of cytochrome C, and activation of caspases. As a part of antiapoptotic studies, we also analyzed changes in the expressions levels of these key apoptotic proteins.

Materials and Methods

Chemicals

Rotenone, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2',7'-dichlorodihydrofluorescin diacetate (DCFH-DA), rhodamine 123 (Rh-123), heat-inactivated fetal calf serum, Dulbecco’s modified Eagle’s medium (DMEM), glutamine, penicillin–streptomycin, ethylenediaminetetraacetic acid (EDTA), and trypsin were purchased from Sigma Chemicals Co., St. Louis, USA. Anti-Bcl-2, Bax, cytochrome C, and caspase-3 p17 antibodies were obtained from Cell Signaling (USA), and β-actin antibodies were purchased from Santa Cruz Biotechnology, Inc, USA. Anti-mouse and anti-rabbit secondary antibodies were purchased from Genei, Bangalore, India.

Cell Culture

SK-N-SH neuroblastoma cells were obtained from the National center for Cell Science, Pune, India. Cells were grown in DMEM medium supplemented with gentamicin (100 µg/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 dimethyl sulfoxide (DMSO) (0.05 %). All experiments were carried out after 3 days incubation, at which 90 % of the cells had differentiated. CNB-001 was added 2 h prior to rotenone treatment.

MTT Assay

Cell viability was analyzed using conventional MTT reduction assay (Mosmann 1983). Briefly, 96-multiwell plates were seeded at 3×10⁴ cells/well. Cells in medium were preincubated with CNB-001 (0.5, 1, 2, 4 µM) for 2 h, and later rotenone 100 nM was added. Twenty four hours later, cells were incubated with MTT (5 mg ml⁻¹) at 37 °C for 4 h. After incubation, the medium was removed, and the cells were dissolved with DMSO. The absorbance of formazan reduction product was measured at 570 nm in a plate reader.

Measurement of Intracellular ROS Formation

The intensity of intracellular peroxides was quantified by loading the cells with DCFH-DA as described elsewhere (Halliwell and Whiteman 2004). The esterified fluorescent probe penetrated into the intracellular matrix of the cells, reacts with ROS, and oxidized to form fluorescent dichlorofluorescein (DCF). In brief, 1 ml aliquot of control and treated cells 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 (Nikon, Japan). Fluorescence estimations were done with excitation and emission filters set at 485±10 m and 530±12.5 nm, respectively, using spectrofluorometer (Shimadzu RF-5301 PC). All initial fluorescent values (time 0) were found to differ from each other by less than 5 %. Results were calculated using the formula as follows: ([Ft30–Ft0]/Ft0X100)], and the values are expressed in percentage increase in fluorescence intensities at 0 (Ft0) and 30 (Ft30) min.

Fig. 1 Structure of CNB-001
Measurement of Mitochondrial Membrane Potential $\Delta \psi_m$

Mitochondrial membrane potential was determined fluorometrically using fluorescent dye Rh-123 which enters the mitochondrial matrix resulting in photoluminescent quenching which depends on mitochondrial membrane potential (MMP). After incubation with test compound and rotenone for 24 h, cells were incubated with 1 μL rhodamine 123 (5 mMol/l) for 15 min (Scaduto and Grotyohann 1999). Cells were then rinsed with PBS, and fluorescence was observed under fluorescence microscope using blue filter (450–490 nm). Polarized mitochondrion emits orange–red fluorescence, and depolarized mitochondrion emits green fluorescence. Fluorescent intensity was measured at 535 nm using spectrofluorometer.

Apoptosis Analysis Using Dual Staining

Fluorescent probes acridine orange (AO) and ethidium bromide (EB) was used to analyze apoptosis by fluorescence microscopy inspection (Kasibhatla et al. 2006). After treatments, medium was removed from the plates; cells were washed with PBS twice and stained with 100 μg/mL 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 ($\lambda_{Em}/\lambda_{Em}=$490/530 nm) and photographed. Fluorescent intensity was measured at 535 nm using spectrofluorometer.

Western Blotting

Western blot analysis was used to study protein expression of Bcl-2, Bax, Cytochrome C and Caspase-3. Twenty-four hours after treatment, cells were lysed in 100 ml of ice-cold radioimmunoprecipitation assay buffer (1 % triton, 0.1 % SDS, 0.5 % deoxycholate, 20 mM Tris, pH 7.4, 150 mM NaCl, 10 mM NaF, 1 mM Na$_3$VO$_4$, 1 mM EDTA, 1 mM EGTA, and 0.2 mM PMSF) followed by centrifugation at 10,000×g at 4 °C for 10 min to remove insoluble material. Protein concentration was quantified using Bradford method (1976) and electrophoresed in 10 % SDS-polyacrylamide gels and electrotransferred onto PVDF membrane by semidy transfer (Bio-Rad). After blocking of the membrane with 5 % nonfat 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 1:2,000 overnight at 4 °C. After washing, the membrane was incubated with anti-rabbit HRP conjugated secondary antibody (1:2,000), and bands were detected by chemiluminescence staining using ECL detection kit. Densitometry was done using ‘Image J’ analysis software.

Data Analysis

Results were expressed as Mean ± SD for four experiments in each group. One-way ANOVA followed by Duncan’s multiple range test (DMRT) was performed using SPSS 12.0. P<0.05 was considered statistically significant.

Results

Effect of CNB-001 on Cell Viability

We first fixed the optimal dose of CNB-001 on rotenone-induced cytotoxicity in SK-N-SH cells using MTT assay. MTT is reduced by living cells, and the resultant formazan product is proportional to the cell viability. Figure 2 represents the percentage of viable cells treated with CNB-001 (0.5, 1, 2, 4 μM) 2 h before rotenone exposure (100 nM). One hundred nanomolar rotenone treatment significantly decreased cell viability by 55±4 % compared to control cells (p<0.05). However, incubation with CNB-001 (0.5, 1, 2, 4 μM) mitigated rotenone-induced toxicity dose-dependently, and the maximum viability was observed at a concentration of 2 μM. Hence, this concentration was taken for further studies.

Effect of CNB-001 on Intracellular ROS Formation

To analyze antioxidant activity of CNB-001 against rotenone-induced toxicity in SK-N-SH cells, the cells were treated with 2 μM CNB-001, and 2 h later, the cells were exposed to rotenone (100 nM). The levels of intracellular ROS formed were monitored by DCFH-DA fluorescence. The fluorescence intensity expressed is directly proportional to intracellular ROS formation.

![MTT Assay](image_url)

Fig. 2. Preventive effect of CNB-001 against rotenone-induced cytotoxicity in SK-N-SH cells. Cells were incubated with CNB-001 2 h before 100 nM rotenone exposure. Cell viability was determined by measuring MTT reduction. Rotenone exposure decreased cell viability to 55±4 %, but the toxicity is abrogated by CNB-001, and maximum protection was found at 2 μM. Values are given as mean ± SD of four experiments in each group. *p<0.05 compared to control, #p<0.05 compared to rotenone groups.
ROS content. As shown in Fig 3, rotenone treatment revealed significant increase in ROS level (379±24 %) which is reflected by enhanced green fluorescence when compared to control (100±8 %) (p<0.05). In contrast, pretreatment of rotenone exposed cells with CNB-001 sharply attenuated intracellular ROS formation indicated by the decrease in green color intensity (137.73±8.3 %) (p<0.05) when compared to rotenone-treated cells pointing out the antiradical activity of CNB-001 against ROS.

Effect of CNB-001 on Mitochondria Membrane Potential

The stability of mitochondria was analyzed using rhodamine-123, a lipophilic positively charged fluorochrome. Rh-123 steadily enters the cells, stains mitochondria, and exhibits high fluorescent intensity. Figure 4 shows rotenone (100 nM) exposure reduced MMP rapidly, which was reflected by weakening in fluorescence intensity of mitochondrial fluorescent probe 123 (12±0.5 %) compared to control (55±1.2 %) (p<0.05). However, pretreatment with CNB-001 prevented mitochondrial membrane depolarization which is revealed by increase in fluorescent intensity (37±0.9 %) (p<0.05).

Effect of CNB-001 on Apoptosis

Acridine orange/ethidium bromide staining reveals distinctive characteristic of apoptotic morphology in SK-N-SH cells. This method discriminates viable cells with uniform bright green nuclei and nonviable cells with orange to red nuclei. The results obtained from AO/EB staining are presented in Fig 5. Control cells fluoresced brightly with green nuclei and showed normal morphology. In contrast, at 100 nM rotenone exposure cells revealed orange luminescent apoptotic body formation, and the percentage of apoptotic cells were found to be 78±1.5 % when compared to a trifling 9±0.5 % in control (p<0.05). Distinctly, treatment with CNB-001 increased cell viability and decreased apoptotic cell death (18±0.8 %) when compared to cells exposed solely to rotenone (p<0.05).

Effect of CNB-001 on Bcl-2 and Bax Levels

Bcl-2 and Bax are important members of cytoplasmic proteins engrossed in apoptosis. To analyze the protective effect of CNB-001 on rotenone-induced apoptosis, we determined the expression of these pro- and antiapoptotic markers. Figure 6 depicts the expression of Bcl-2 and Bax proteins by Western blot analysis. Densitometric analysis revealed that rotenone treatment manifested significant increase in Bax and depletion in Bcl-2. Meanwhile, these amendments were abrogated by pretreatment with CNB-001. Our results showed that CNB-001 distinctly regulated pro- and antiapoptotic markers.

Effect of CNB-001 on Cytochrome C Release and Caspase-3 Levels

Cytochrome C is an important mitochondrial outer membrane protein released from the mitochondria into the cytosol during apoptosis, which in turn activates downstream caspases causing neuronal death. Our results attributed that rotenone treatment significantly increased expression of cytochrome C and active caspase-3 levels when compared to control. This trend was alleviated when treated with CNB-001. Cells treated with

Fig. 3 CNB-001 reduced ROS formation as stained by DCFH-DA. (I) Photomicrograph showing the preventive effect of CNB-001 (2 μM) against rotenone-induced ROS generation. A Control, B CNB-001, C rotenone, and D rotenone + CNB. (II) Rotenone (100 nM) treatment significantly increased the levels of ROS as compared to control cells, while CNB-001 (2 μM) pretreatment significantly decreased the levels of ROS as compared to rotenone alone treated cells. Values are given as mean ± SD of four experiments in each group. *p<0.05 compared to control and #p<0.05 compared to rotenone group (DMRT)
Discussion

SK-N-SH, a neuroblastoma cell line shows neuronal phenotype with expression of numerous biochemical markers, and they respond to various toxic insults indicating to be used as a suitable model for neurotoxic and neuroprotective studies. In this study, we used SK-N-SH cells to analyze the protective effect of CNB-001 against rotenone-induced toxicity. We performed MTT assay to analyze whether CNB-001 exerted a concentration-dependent protective effect against rotenone toxicity in SK-N-SH cells. Two micromolars showed maximum neuroprotection concomitant with 100 nM rotenone. Cellular growth was analyzed by MTT reduction catalyzed by NAD-dependent dehydrogenase (in active mitochondria) to form a dark blue formazan product (York et al. 1998). In concordant with this, our results proved that CNB-001 has a direct effect on mitochondria.

rottenone along with CNB-001 reinnstate rotenone toxicity distinctly by diminishing the expression of key executioner apoptotic proteins (Fig. 7).
Mitochondria are one of the principle sources of ROS and an important target of oxidative stress (Han et al. 2013; Fiskum et al. 2004). Reactive oxygen species are formed as an earlier response to oxidative stress. Rotenone exposure inhibited respiratory complex I which results in the accumulation of electrons and ROS formation. Various reports showed that inner mitochondrial permeability transition pore 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). In this study, DCFH-DA staining is used to assess the overall oxidative stress of a cell (Wang and Joseph 1999). The DCF fluorescent intensity formed is directly proportional to the intracellular ROS content. Results show that ROS insult to the cells is inhibited by pretreatment with 2 μM CNB-001. This is due to the presence of aromatic hydroxyl groups which has the ability to quench free radicals. Moreover, curcumin and its derivative, CNB-001 has two methoxyphenol group separated by β-diketone bridge, which is responsible for iron chelation, and free iron is responsible for various neurodegenerative disorders (Gottlieb et al. 2000). Our result is consistent with the work done by Schubert et al., where CNB-001 protected HT22 cells against glutamate-induced oxidative stress (Yuanbin et al. 2008). Excess extracellular glutamate interferes with normal cysteine uptake mechanism via cysteine/glutamate antiporter and results in decreased intracellular GSH synthesis which leads to oxtosis (Tan et al. 2001).

Mitochondria are a complex organelle for ATP production, engaged in oxygen consumption, and ROS generation (Green and Kroemer 2004). Excess ROS attacks membrane phospholipids causing matrix swelling and results in loss of mitochondrial membrane potential (ΔΨm). The protective effect of CNB-001 against mitochondrial toxin is corroborated by its ability to maintain ΔΨm, an important factor for ATP production. MMP depolarization and succeeding protection by CNB-001 was demonstrated by Rh-123 staining, providing visual evidence that CNB-001 protects SK-N-SH cells by alleviating mitochondrial function. An imperative initial event in apoptosis is the disruption of mitochondrial membrane potential forming transition pores on the mitochondrial membrane followed by release of proapoptotic factors from mitochondrial intermembrane space (Mallet et al. 2005). In the current study, this process is abrogated by the action of CNB-001 against rotenone toxicity, and it bolsters cytosolic energy state.

Apoptosis may be initiated either by the activation of death receptors (extrinsic) or by intracellular stimuli (intrinsinc). In both these events, caspase-3 gets activated which results in morphological changes in the cell (Li et al. 2007). Our results provided supportive microscopic evidence for antiapoptotic property of CNB-001 by performing AO/EB staining. Acrylaline orange is a permeable dye, and it stains all the cells. Viable cells will be
uniformly green. During apoptosis, cell membrane becomes permeable to EB, and the cells turn orange red after intercalating of EB with DNA. During apoptosis, an imbalance between deoxyribonuclease and the enzymes responsible to maintain DNA integrity occurs, which results in chromatin condensation and resultant cell death (Pirnia et al. 2002). Following rotenone exposure, cells succumbed to apoptosis, but CNB pretreatment rescued the cells. This result was in concordant with previous experimental evidence (Reus et al. 2005).

Various apoptotic pathways are initiated by oxidative stress (Francoa et al. 2009). The two important apoptotic related genes Bcl-2 and Bax play an important role in regulating programmed cell death by initiating cytochrome C release into the cytosol which in turn activates caspase-3 and amends mitochondrial function during various pathological conditions (Ullah et al. 2012; Antonsson and Martinou 2000). To analyze the mechanism of neuroprotection offered by CNB-001 against rotenone toxicity, we examined changes in the expression of these two apoptotic-related gene products using western blotting. From our study, it is found that rotenone exposure downregulated Bcl-2 expression and upregulated Bax expression showing that these proteins are imperative in apoptotic process (Langena et al. 2005). Bcl-2 family proteins play an important role in apoptosis by cytochrome c release via membrane transition pore into the cytosol. This is supplemented by the expression of Bax, a proapoptotic protein, which depolarizes ΔΨm either directly by forming pores or by binding with mitochondrial channel proteins (voltage-dependent ion channel and adenosine nucleotide transporter). Moreover, Bax reacts with Bcl-2 forming a heterodimer, which is translocated from cytosol to mitochondria-mediating release of cytochrome C, an important apoptotic initiating factor (Mallet et al. 2005). Cytochrome C binds with apoptotic protease activating factor 1 along with procaspase-9 forms “apoptosome”. Apoptosome is engrossed in the activation of caspase-9, which in turn activates caspase-3, a member of cysteine-aspartic acid protease. Caspase-3 executes apoptosis by activating endonucleases resulting in impairment of nucleic acids leading to neuronal death (D’Amelio et al. 2012). This cascade of events is inhibited by Bcl-2 which is present in cytoplasmatic face of nuclear envelope and in mitochondrial outer membrane by interfering with proapoptotic membrane aggregation and maintains membrane integrity. Our results shows that CNB-001 pretreatment significantly attenuated the expression of proapoptotic proteins and supports the expression of antiapoptotic protein Bcl-2, suggesting that the protective mechanism of CNB-001 against rotenone toxicity might include both its antioxidant property and its regulatory function on Bcl-2 family. Our results were in concordance with Zhaohui et al. Zhaohui et al. reported that curcumin-protected rotenone induced toxicity by inhibiting ROS formation and regulating apoptotic cascade (Zhaohui et al. 2013).

Many reports conclude that overproduction of ROS via initiation of ubisemiquinone and subsequent oxidative stress leading to apoptosis is involved in rotenone toxicity (Sherer et al. 2003). CNB-001 is reported to have neuroprotective in vitro studies that encompass models of intracellular and extracellular amyloid toxicity, trophic factor withdrawal, and excitotoxicity (Yuanbin et al. 2008). But the activity of CNB-001 against mitochondrial impairment involved cell death, and its protective strategy has not yet been elucidated. From the present study using fluorescent microscopy and Western blotting, we conclude that CNB-001 manifested protection against rotenone-induced toxicity in SK-N-SH cells by inhibiting mitochondrial ROS generation, retains ΔΨm, and prevents apoptosis. These results indicate that CNB-001 offers neuroprotection by its antioxidant, mitochondrial protective, and antiapoptotic properties. In the future, it can be used as a multitarget drug which holds the therapeutic potential in treatment of Parkinson’s disease.

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Conflict of Interest No conflict of interest

References


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PLATE-1

A. Peripheral  B. Central

C. Rearing  D. Grooming

Open field test
PLATE-2

A. Narrow beam walking

B. Foot slip error

C. Grid hang test

D. Rotorod test
PLATE-3

A. Catalepsy

B. Akinesia
3 - Continuous swimming          2 - Occasional floating/swimming only

1 - Occasional swimming using hind limbs while floating on one side.

A. Swim Test

0- Hind part sinks with head floating

B. Stride length measurement