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https://doi.org/10.31925/farmacia.2026.3.11 **ORIGINAL ARTICLE**

NEUROPROTECTIVE EFFECTS OF HISTAMINE H3 RECEPTOR LIGANDS CIPROXIFAN AND CLOBENPROPIT THROUGH ANTIAPOPTOTIC AND ANTIOXIDANT MECHANISMS: EVIDENCE FROM *IN VIVO* AND COMPUTATIONAL STUDIES

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Abstract

Histamine H3 receptors (H3Rs) located on presynaptic neurons function as both autoreceptors and heteroreceptors, regulating neurotransmitter release. This dual role makes them compelling targets for drug discovery, particularly for the treatment of neurodegenerative conditions. Therefore, in this work, two structurally distinct H3R antagonists, ciproxifan (CIP) and clobenpropit (CLO), were evaluated for neuroprotection using a lipopolysaccharide (LPS) treated animal model. CIP and CLO were assessed at doses of 1 and 3 mg/kg, respectively (oral, 30 days). Neuroinflammation and neurotoxicity were induced by intraperitoneal LPS administration (1 mg/kg) during the final four days of the protocol (days 27 - 30). To capture the mechanistic breadth of any neuroprotective response, brain tissue analyses included apoptotic signalling proteins and oxidative stress markers. It includes B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax), Caspase-3, malondialdehyde (MDA), reduced glutathione (GSH) and catalase activity. The treatment with CIP and CLO resulted in reduced Bax, Caspase-3 and MDA levels, accompanied by increased Bcl-2, GSH and catalase levels. Molecular docking studies revealed favourable binding of CIP and CLO with Bcl-2, Bax and Caspase-3. Collectively, the combined *in vivo* and computational findings suggest that CIP and CLO may exert neuroprotective effects against LPS-induced neuronal injury.

Rezumat

Acest studiu a evaluat potențialul neuroprotector al unor antagoniști ai receptorului H3R, cu structuri chimice distincte, ciproxifan (CIP) și clobenpropit (CLO), utilizând un model animal de neuroinflamație indusă de lipopolizaharide (LPS). CIP și CLO au fost administrate oral, pe o perioadă de 30 de zile, în doze de 1 mg/kg, respectiv 3 mg/kg. Inducerea neuroinflamației și a neurotoxicității s-a realizat prin administrarea intraperitoneală de LPS (1 mg/kg) în ultimele patru zile ale protocolului experimental (zilele 27 - 30). Pentru a surprinde complexitatea mecanismelor implicate în răspunsul neuroprotector, analizele efectuate la nivelul țesutului cerebral au inclus atât markeri ai apoptozei, cât și ai stresului oxidativ. Printre aceștia s-au numărat proteinele implicate în reglarea apoptozei, B-cell lymphoma 2 (Bcl-2), proteina asociată Bcl-2 X (Bax) și caspaza-3, precum și indicatori ai stresului oxidativ, respectiv malondialdehida (MDA), glutationul redus (GSH) și activitatea catalazei. Rezultatele au evidențiat faptul că tratamentul cu CIP și CLO a determinat scăderea nivelurilor Bax, caspazei-3 și MDA, concomitent cu creșterea nivelurilor Bcl-2, GSH și a activității catalazei. În plus, studiile de andocare moleculară au demonstrat o afinitate favorabilă de legare a celor doi compuși față de Bcl-2, Bax și caspaza-3. În concluzie, rezultatele obținute sugerează că CIP și CLO pot exercita efecte neuroprotectoare semnificative împotriva leziunilor neuronale induse de LPS.

Keywords: ciproxifan, clobenpropit, apoptosis, oxidative stress, molecular docking simulation

Introduction

Histamine H3 receptor antagonists (H3RAs) are promising candidates for treating several CNS disorders, including neurodegenerative diseases, sleep disorders, such as narcolepsy and insomnia, and psychiatric conditions like schizophrenia, with potential neuroprotective effects in cerebral ischemia (1, 2). Among histamine receptors (H1 - H4), H3Rs are primarily coupled to Gi/o proteins and inhibit adenylate cyclase, reducing intracellular cyclic AMP and downstream signalling. H3Rs are mainly located presynaptically as autoreceptors that regulate histamine release, and postsynaptically, H1 and H2 receptors are involved in wakefulness, attention and cognition. They also act as heteroreceptors that modulate the release of neurotransmitters, including acetylcholine, norepinephrine, dopamine and serotonin (3-5). H3Rs are expressed in brain regions such as the cerebral cortex and hippocampus, which are associated with cognitive functions (6). Modulation of histaminergic

signalling may therefore provide therapeutic benefits in neurodegenerative disorders by influencing neurotransmission, synaptic plasticity, oxidative stress and neuroinflammation (7). Oxidative stress and apoptosis-induced neuronal damage are major contributors to neurodegeneration and are linked to mitochondrial dysfunction, lipid peroxidation and protein aggregation (8).

Apoptosis is a programmed cell death process that maintains cellular homeostasis by removing damaged or abnormal cells without causing inflammation (9). Oxidative stress-induced apoptosis is closely associated with ROS production, which affects both primary apoptotic pathways: the extrinsic and intrinsic pathways (10). The extrinsic pathway is initiated through the stimulation of cell-surface death receptors belonging to the TNF superfamily, including receptors for TNF-α, CD95/APO-1/Fas and the TRAIL receptor. Binding specific ligands, such as TNF, Fas ligand (FasL) and TRAIL, to their receptors triggers the recruitment of adaptor proteins, leading to Caspase-8 activation and initiating the cascade that results in apoptosis (9, 11, 12). The intrinsic pathway is triggered by stress signals such as hypoxia, protein misfolding and DNA damage, leading to increased permeability of the mitochondrial outer membrane and the release of pro-apoptotic mediators, including cytochrome c, apoptosis-inducing factor and Smac/DIABLO, thereby activating Caspase-9 and subsequently releasing Caspase-3 (13). Notably, proteins of the Bcl-2 family play a central role in the events described above. Proapoptotic proteins such as Bax, Bid, Bak and Bad are involved in increased membrane permeability and the release of cytochrome c. In contrast, anti-apoptotic proteins such as Bcl-2 and Bcl-XL are responsible for preserving membrane integrity (10, 12, 14).

Ciproxifan (CIP) and clobenpropit (CLO) are well-established H3R ligands. CIP is a potent H3R antagonist and inverse agonist. CIP has been demonstrated to improve the symptoms of schizophrenia, Parkinson's disease (PD) and working memory, in addition to being explored for neuroprotection (15-18). The CLO is known as a H3R blocker and a partial agonist of the histamine H4 receptor (H4R). Reports highlight the anti-inflammatory role of H4R agonists in neuroinflammation (19, 20). Therefore, the dual action of CLO *via* H3R and H4R broadens its therapeutic potential in neurodegenerative disorders. Further, CLO has demonstrated modulation of H3R and H4R located in the substantia nigra and striatum of the PD model (16).

Lipopolysaccharide (LPS) treated animals represent established models for demonstrating the neuroprotective effects of small molecules. Notably, LPS does not cross the blood-brain barrier; however, its intraperitoneal administration causes peripheral immune activation, leading to inflammation, cytokine release and the generation of reactive oxygen species (ROS), which induce oxidative stress, neuroinflammation and neuronal injury (18, 20). Therefore, the observed neurotoxic effects in LPS-treated animal models are not due to the LPS penetration into the CNS but rather to the inflammation-driven oxidative damage. Therefore, in this work, CIP and CLO were evaluated for their effects on oxidative stress-mediated neuronal apoptosis. The brain homogenate was analysed for the expression of apoptotic proteins (Bcl-2, Bax and Caspase-3) and oxidative stress markers (MDA, GSH and catalase). These assessments were conducted within an LPS-induced neurotoxicity mouse model, expanding on the markers studied in our previous research (4). Furthermore, we evaluate the molecular binding affinities of both drugs for their target enzymes using molecular docking studies.

Materials and Methods

Drugs and chemicals

Ciproxifan maleate (CIP) and lipopolysaccharide (LPS) from Escherichia coli O128:B12 were obtained from Sigma-Aldrich (St. Louis, MO, USA). At the same time, clobenpropit hydrobromide (CLO) was purchased from Cayman Chemical (Ann Arbor, MI, USA). ELISA kits for mouse B-cell lymphoma 2 (Bcl-2), Bcl-2–associated X protein (Bax), Caspase-3, malondialdehyde (MDA), glutathione (GSH) and catalase were procured from MyBioSources Inc. (San Diego, CA, USA). The remaining chemicals and solvents were of analytical grade and procured from local suppliers.

Animals

A total of 36 adult male ICR mice, aged 8 - 12 weeks and weighing 25 - 35 g, were obtained from the institutional animal facility and randomly assigned to six groups, with six animals *per* group. The experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the College of Pharmacy (2020-CP-7). All procedures for animal care and use complied with the Guide for the Care and Use of Laboratory Animals issued by the National Research Council (USA). Furthermore, the research was reported in line with the ARRIVE 2.0 guidelines, with particular attention to the Essential 10 items, to promote clarity, transparency and reproducibility (21). Mice were housed in polypropylene cages, with three animals *per* cage, and had unlimited access to food and water throughout the study.

Experimental design

The study consisted of six groups with distinct treatment protocols (Figure 1). Group 1 (CON) received vehicle (normal saline (NS), 10 mL/kg, p.o.) daily for 30 days, with additional NS injections (10 mL/kg, i.p.) during the last four days (days 27 - 30). Group 2 (LPS) was treated with vehicle (NS, 10 mL/kg, p.o.)

for 30 days and subjected to neurotoxicity using four doses of lipopolysaccharides (LPS, 1 mg/kg, i.p.) on days 27, 28, 29 and 30, based on prior studies (22, 23). Groups 3 and 4 (CIP1 + LPS and CIP3 + LPS) were orally treated with ciproxifan (1 or 3 mg/kg, respectively) for 30 days and induced with LPS neurotoxicity as *per* the LPS protocol. Similarly, Groups 5 and 6 (CLO1 + LPS and CLO3 + LPS) were treated with oral clobenpropit (1 or 3 mg/kg, respectively) for 30 days, followed by induction of LPS-induced neurotoxicity. The selected doses of CIP and CLO were based on previous studies (18, 20). LPS was administered during the final phase of the experiment (days 27 - 30) to produce an acute inflammatory and oxidative stress response, allowing assessment of the neuroprotective effects of CIP and CLO after sustained pre-treatment (22, 23). On day 30, all animals were sacrificed, and brain tissues were collected for ELISA-based analysis of biochemical markers.

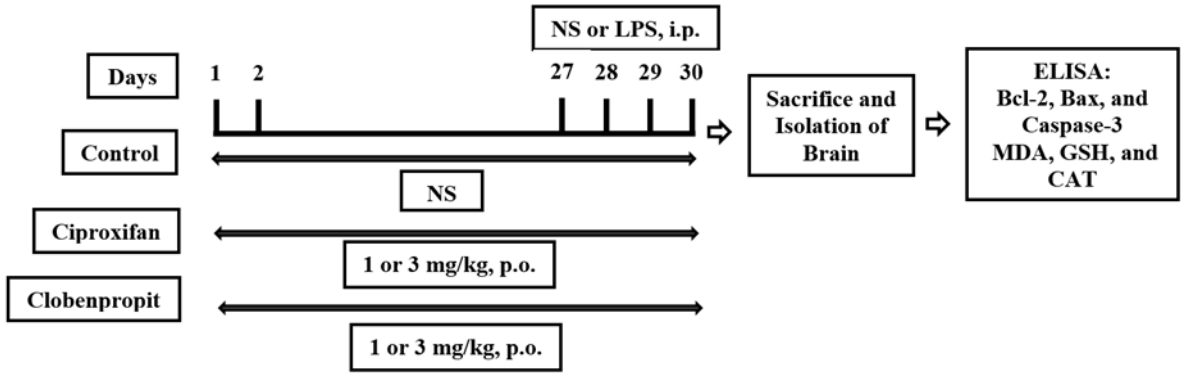

Figure 1.
Experimental design and day-by-day treatment schedule

Collection of brain homogenate

At the end of the 30-day treatment period, all mice were sacrificed by cervical decapitation under anaesthesia (ketamine, 100 mg/kg, and xylazine, 10 mg/kg; i.p.). Whole brain tissues were collected and homogenized in ice-cold phosphate buffer (4°C, pH 7.4) using a homogenizer. The homogenates were centrifuged at 4000 rpm for 10 minutes, and the supernatant was collected for analysis. Total protein concentration was determined using the biuret colorimetric method with a commercial assay kit (Crescent Diagnostics, Saudi Arabia), based on the formation of a violet-coloured complex between peptide bonds and copper ions under alkaline conditions. Bovine serum albumin was used to generate the standard curve, and absorbance was measured at 540 nm according to the manufacturer's instructions. All biochemical parameters were normalized to total protein content to reduce variability. Homogenization was performed using a consistent tissue-to-buffer ratio under identical conditions. ELISA assays were conducted according to the manufacturer's instructions, with concentrations determined from standard curves, and all samples were analysed in duplicate.

Quantification of apoptosis proteins

Apoptosis-associated proteins, including the anti-apoptotic Bcl-2, the pro-apoptotic Bax and Caspase-3, were measured using ELISA kits (MyBioSource Inc., San Diego, CA, USA). The Bcl-2 protein was quantified using a Bcl-2 ELISA kit (Catalog no. MBS265203; detection range: 15.6 - 1000 pg/mL; sensitivity: 5 pg/mL), which employs a double-antibody sandwich ELISA technique. The pre-coated antibody is a monoclonal anti-mouse Bcl-2 antibody, and the detection antibody is a biotinylated polyclonal antibody. Bax levels were determined using a sandwich ELISA kit (Cat. No. MBS457867). The assay has a detection range of 62.5 - 4000 pg/mL with a minimum sensitivity of 24.5 pg/mL, according to the manufacturer's specifications. Caspase-3 levels were assessed through a competitive enzyme immunoassay using a polyclonal anti-Caspase-3 antibody and a Caspase-3-HRP conjugate (Catalog no. MBS733100; detection range: 1.56 - 100 ng/mL; sensitivity: 0.5 ng/mL). This assay quantifies total Caspase-3 protein and does not distinguish between pro-caspase-3 and cleaved (active) Caspase-3. All assays were performed following the manufacturer's protocols, and absorbance was measured at 450 nm using a Microplate Reader (BioTek Instruments, Santa Clara, CA, USA).

Quantification of oxidative stress markers

Oxidative stress was assessed by measuring malondialdehyde (MDA) and the antioxidant markers glutathione (GSH) and catalase (CAT) using mouse-specific ELISA kits (MyBioSource Inc., San Diego, CA, USA). The MDA (Catalog no. MBS269473; detection range: 0.156 nmol/mL - 10 nmol/mL; sensitivity: 0.05 nmol/mL) and GSH (Catalog no. MBS161633; detection range: 15 - 1800 mg/L; sensitivity: 5.02 mg/L) kits both used the Double Antibody Sandwich ELISA method. Each assay employed a pre-coated anti-Mouse monoclonal antibody specific to MDA or GSH, respectively, along with a

biotinylated polyclonal detection antibody. Catalase was quantified using the CAT kit (Catalog no. MBS160589; detection range: 0.2 - 70 ng/mL; sensitivity: 0.093 ng/mL), in which the assay plate was pre-coated with a Mouse CAT-specific antibody. Catalase in the samples bound to the antibodies immobilized on the plate wells. All procedures followed the manufacturer's instructions.

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Group comparisons were performed using one-way ANOVA followed by the Tukey-Kramer post hoc test to determine significance between pairs of groups. Statistical analyses were conducted using GraphPad Prism version 9 (GraphPad Software Inc., USA). A p-value of < 0.05 was considered statistically significant.

Molecular modelling

Molecular docking analyses were performed using AutoDock Vina (24) (version 1.1.2, CA, USA) to investigate the molecular level interaction with target proteins Bcl-2 (PDB: 4IEH; PDB DOI: 10.2210/pdb4ieh/pdb) (25), Bax (PDB: 1F16; PDB DOI: 10.2210/pdb1f16/pdb) (26) and Caspase-3(PDB: 1GFW; PDB DOI: 10.2210/pdb1GFW/pdb) (27). The preparation of input files was performed using AutoDock Tools, part of MGL Tools (v1.5.7, La Jolla, CA, USA) (28). Protein structures were obtained from the RCSB Protein Data Bank (29), and the three-dimensional structure of CLO and CIP was retrieved in SDF format from the PubChem database (30). Prior to docking, each protein structure was pre-processed by removing non-relevant chains and all non-protein HETATM records, including co-crystallized ligands (Co-CL), ions and water molecules. Polar hydrogens were added, and Kollman charges were assigned before saving the proteins in pdbqt format. The ligands CLO and CIP were subjected to energy minimization using the universal force field to optimize their geometry. Additionally, its torsional bonds were set to be flexible, and Gasteiger charges were assigned before conversion to the pdbqt format. The active site of Bcl-2 was targeted by centring a docking grid at Lys17 (x = 5.566 Å, y = 21.393 Å, z = 29.022 Å), while for Bax, the docking grid was centred at Lys21 (x = 12.125 Å, y = 7.381 Å, z = -7.061 Å) located at the trigger site. For the Caspase-3, the docking grid was defined by centring on the Co-CL ligand (x = 37.453 Å, y = 34.057 Å, z = 27.751 Å). It is pertinent to note that the grid size used for Bcl-2 and Bax was 20 Å, whereas that for Caspase-3 was 30 Å. Configuration files were prepared to specify receptor, ligand and grid settings. Docking simulations were run, and binding affinities were calculated in kcal/mol. The binding pose with the lowest binding energies) were selected for further analysis. The key interactions between ligands and essential amino acid residues in the protein binding sites were visualized using the freely available Discovery Studio Visualizer.

Results and Discussion

Protective effects of CIP and CLO on apoptosis in the mouse brain

Figure 2 illustrates the impact of CIP and CLO on key apoptosis-related proteins, including the anti-apoptotic marker Bcl-2 and the pro-apoptotic markers Bax and Caspase-3.

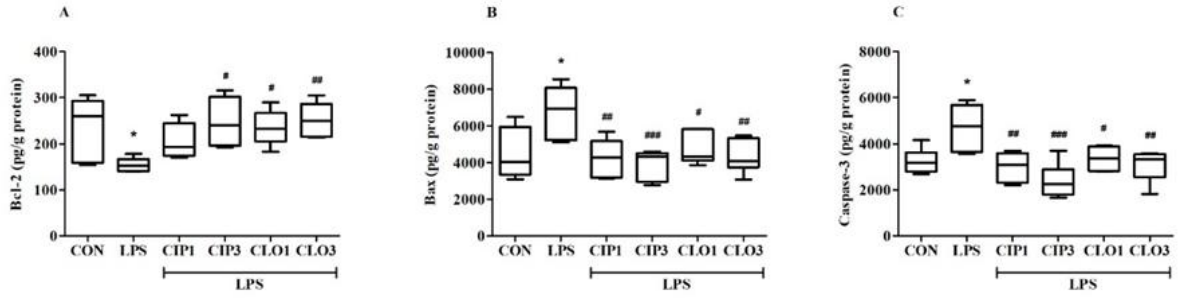

Figure 2.

The impact of ciproxifan (CIP) and clobenpropit (CLO) on apoptosis-associated protein levels: (A) Bcl-2, (B) Bax and (C) Caspase-3 in LPS-induced mice

The results are reported as the mean ± SEM (n = 6); *p < 0.05 *vs*. control; #p < 0.05, ##p < 0.01 and ###p < 0.001 *vs*. LPS

Analysis of an anti-apoptotic, Bcl-2 levels (Figure 2A) using one-way ANOVA revealed significant differences among the treatment groups ($p = 0.0049$). Post hoc comparisons showed that LPS treatment significantly reduced Bcl-2 levels ($p < 0.05$) compared to the control group. CIP treatment at the higher dose (3 mg/kg) significantly restored Bcl-2 levels ($p < 0.05$) in LPS-induced mice. Furthermore, both doses of CLO significantly elevated Bcl-2 levels, with improvements noted at 1 mg/kg ($p < 0.05$) and 3 mg/kg ($p < 0.01$).

Refer to pro-apoptotic Bax levels (Figure 2B), which showed a significant difference among treatment groups ($p = 0.0001$). LPS treatment significantly increased

Bax levels ($p < 0.05$) compared to control mice. Both doses of CIP attenuated the LPS-induced increase in Bax, with reductions observed at 1 mg/kg ($p < 0.01$) and 3 mg/kg ($p < 0.001$). Similarly, CLO treatment resulted in a significant reduction in Bax levels, with declines noted at 1 mg/kg ($p < 0.05$) and 3 mg/kg ($p < 0.01$). Further, Caspase-3 levels followed a pattern similar to Bax (Figure 2C). Overall, significant differences among treatment groups were observed ($p = 0.0001$). LPS treatment significantly increased Caspase-3 levels ($p < 0.05$). Treatment with CIP reduced Caspase-3 levels in a dose-dependent manner, with significant reductions at 1 mg/kg ($p < 0.01$) and 3 mg/kg ($p < 0.001$). CLO treatment also decreased Caspase-3 levels, with reductions at 1 mg/kg ($p < 0.05$) and 3 mg/kg ($p < 0.01$).

Apoptosis-induced cell death is a tightly regulated process crucial for maintaining cellular homeostasis. However, in neurodegenerative diseases such as AD, PD and HD, dysregulated apoptosis contributes to progressive neuronal loss and cognitive decline. Among the apoptotic pathways, the intrinsic pathway plays a central role, as mitochondrial dysfunction triggers the activation of pro-apoptotic proteins such as Bax and Bak. These proteins compromise mitochondrial membrane integrity, leading to cytochrome c release and the activation of the Caspase cascade, ultimately resulting in neuronal death (12). In detail, in the Bcl-2 family, the Bcl-2 protein plays a crucial role in protecting neurons from apoptosis by stabilizing mitochondrial membrane integrity and preventing cytochrome c release (31). Our results showed that CIP and CLO significantly upregulated Bcl-2 expression, suggesting that these compounds enhance neuronal survival mechanisms. The increase in Bcl-2 levels indicates a shift toward cell survival, counteracting the apoptotic signalling triggered by oxidative stress and neuroinflammation (32).

During apoptosis, a key pro-apoptotic protein from the Bcl-2 family facilitates mitochondrial outer membrane permeabilization, leading to cytochrome c release and activation of downstream Caspases. In our study, CIP and CLO significantly reduced Bax expression, indicating an inhibition of apoptotic signalling. This suggests that these compounds can effectively suppress neuronal apoptosis by preventing mitochondrial membrane disruption (32). Caspase-3 is a key mediator of apoptotic signalling. In the present study, ELISA measurements reflect changes in total Caspase-3 protein levels, which may indicate modulation of apoptotic pathways, but do not directly measure cleaved Caspase-3 activation. Increased Caspase-3 activation has been widely reported as a hallmark of neurodegenerative diseases (33). Our findings demonstrated that CIP and CLO significantly downregulated Caspase-3 levels, suggesting a modulatory effect on apoptotic signalling pathways. It should be noted that the ELISA assay quantified total Caspase-3 protein levels and did not differentiate between pro-Caspase-3 and cleaved active Caspase-3; therefore, the results indicate modulation of apoptotic signalling rather than direct measurement of caspase activation. This observation further supports the potential neuroprotective effects of these compounds in LPS-induced neurotoxicity.

Protective effects of CIP and CLO on oxidative stress in the mouse brain

Figure 3 illustrates the effects of CIP and CLO on oxidative stress markers, including the oxidative marker MDA and the antioxidant markers GSH and catalase, in mouse brain tissues.

Significant alterations in MDA levels were observed across all treatment groups ($p = 0.0001$). As expected, LPS treatment significantly increased MDA levels ($p < 0.01$) compared to the control group, indicating elevated oxidative stress in the brain (Figure 3A). However, both CIP and CLO treatments effectively mitigated this increase. CIP significantly reduced MDA levels at doses of 1 mg/kg ($p < 0.01$) and 3 mg/kg ($p < 0.001$), while CLO showed reductions at 1 mg/kg ($p < 0.05$) and 3 mg/kg ($p < 0.01$).

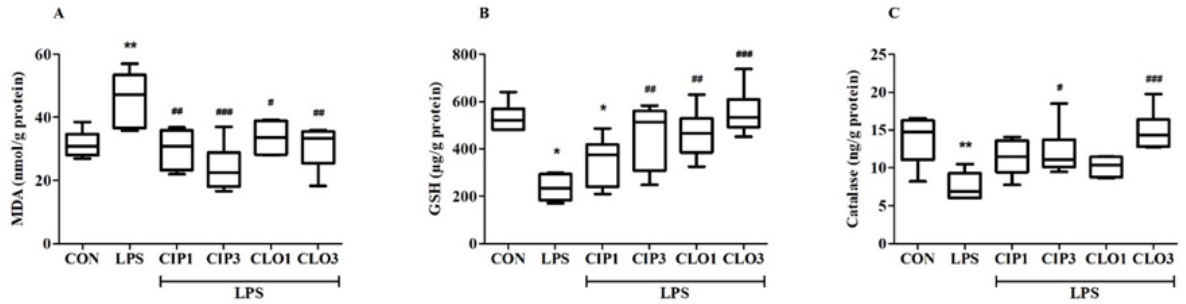

Figure 3.

The effects of ciproxifan (CIP) and clobenpropit (CLO) on oxidative parameters: (A) MDA, (B) GSH and (C) CAT in LPS-induced mice

The results are reported as the mean ± SEM (n = 6); *p < 0.05 and **p < 0.001 *vs*. control; #p < 0.05, ##p < 0.01 and ###p < 0.001 *vs*. LPS

The antioxidant GSH levels varied significantly among treatment groups ($p < 0.0001$) (Figure 3B). LPS administration resulted in a significant reduction in GSH levels ($p < 0.05$) compared with the control group. CIP treatment reversed this decline, with significant increases at doses of 1 mg/kg ($p < 0.05$) and 3 mg/kg ($p < 0.01$). CLO treatment also restored GSH levels, with significant improvements noted at 1 mg/kg ($p < 0.01$) and 3 mg/kg ($p < 0.001$), thereby protecting against LPS-induced neurotoxicity. Additionally, brain catalase levels were also significantly altered by the treatments ($p = 0.0003$) (Figure 3C). LPS injection reduced catalase levels ($p < 0.01$) compared to the control group, indicating oxidative stress. CIP treatment at 3 mg/kg ($p < 0.05$) and CLO treatment at 3 mg/kg ($p < 0.001$) effectively restored catalase levels, protecting against LPS-induced oxidative damage.

It is important to emphasize that the LPS-induced oxidative stress and apoptotic changes observed in brain tissue are not due to direct passage of LPS across the blood-brain barrier. Instead, intraperitoneal LPS administration elicits systemic inflammation, resulting in elevated circulating pro-inflammatory cytokines and oxidative mediators that indirectly affect the central nervous system. This peripheral-to-central inflammatory signalling cascade is widely recognized as a key mechanism underlying LPS-induced neurotoxicity and supports the interpretation of our findings (18, 20).

Since oxidative stress is a key contributor to neurodegeneration and apoptosis, we also assessed oxidative stress parameters, including MDA, GSH and catalase, to understand the antioxidative potential of CIP and CLO. It is well known that MDA is a biomarker of LPO, indicating the extent of oxidative damage in neurons. Increased MDA levels reflect membrane lipid damage due to ROS (34). Our study showed that CIP and CLO treatment significantly reduced MDA levels, suggesting that these compounds effectively mitigate oxidative damage and protect neuronal membranes from peroxidative stress.

A major antioxidant, GSH is a critical intracellular antioxidant that neutralizes ROS and maintains redox homeostasis. Depletion of GSH is associated with increased oxidative stress and neuronal vulnerability (35). Our findings demonstrated that CIP and CLO significantly increased GSH levels, indicating their role in restoring antioxidant defence mechanisms and reducing oxidative burden in neurons. An endogenous antioxidant enzyme, catalase, is essential for detoxifying hydrogen peroxide (H_2O_2), a major ROS, into water and oxygen, thereby preventing oxidative damage (36). Our study revealed that CIP and CLO enhanced catalase levels, suggesting an improved antioxidant response that helps counteract oxidative stress-induced neuronal damage.

Molecular docking

Molecular docking was conducted to predict the binding affinity of CLO and CIP with Bcl-2, Bax and Caspase-3. It is pertinent to mention that there was no Co-Cl ligand present in the Bcl-2 and Bax crystal structure, while the crystal structure of Caspase-3 carried a small molecule as a co-crystal. Therefore, the docked score of CLO and CIP in the case of Bcl-2 and Bax was not compared with any standard ligand, while the docked score of CLO and CIP in the case of Caspase-3 was compared with the docked score of Co-Cl ligand. Figure 4 shows the superimposed binding mode of Co-Cl ligand and redocked Co-Cl ligand into the caspase-3 active site. The RMSD of heavy atoms between the crystal and redocked pose was 1.28 Å.

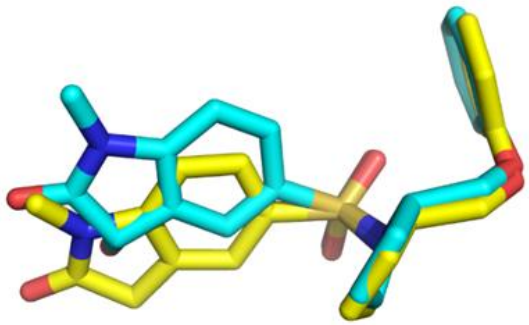

Figure 4.
Superimposed binding mode of Co-CL ligand (cyan) and redocked Co-Cl ligand (yellow, RMSD = 1.28 Å)

Table I
Docking score (kcal/mol) CIP and CLO against Bcl-2, Bax and Caspase-3

Protein / Ligand	Bcl-2	Bax	Caspase-3
Co-CL	N.A	N.A	-8.8
CIP	-5.3	-5.5	-6.2
CLO	-5.4	-5.4	-6.1

The molecular docking results for the selected compounds against Bcl-2, Bax and Caspase-3 are summarized in Table I. Docking studies targeting Bcl-2 were specifically performed at the BH4 binding site, which is associated with its anti-apoptotic function. For Bax and Caspase-3, ligands were docked into their respective inhibitory binding sites, both of which play key roles in regulating the proteins' apoptotic activity. Binding energies, reported in kcal/mol, revealed that Co-CL displayed the most favourable interaction with Caspase-3 (-8.8 kcal/mol). The compounds CIP and CLO showed moderate predicted binding affinities for Bcl-2 at the BH4 site (-5.3 and -5.4 kcal/mol, respectively), as well as for Bax (-5.5 and -5.4 kcal/mol, respectively) and Caspase-3 (-6.2 and -6.1 kcal/mol, respectively). These findings suggest that CIP and CLO exhibit relatively similar binding

profiles across all three proteins. The absence of data for Co-CL with Bcl-2 and Bax reflects the limitation posed by the lack of available Co-CL ligand structures for these proteins.

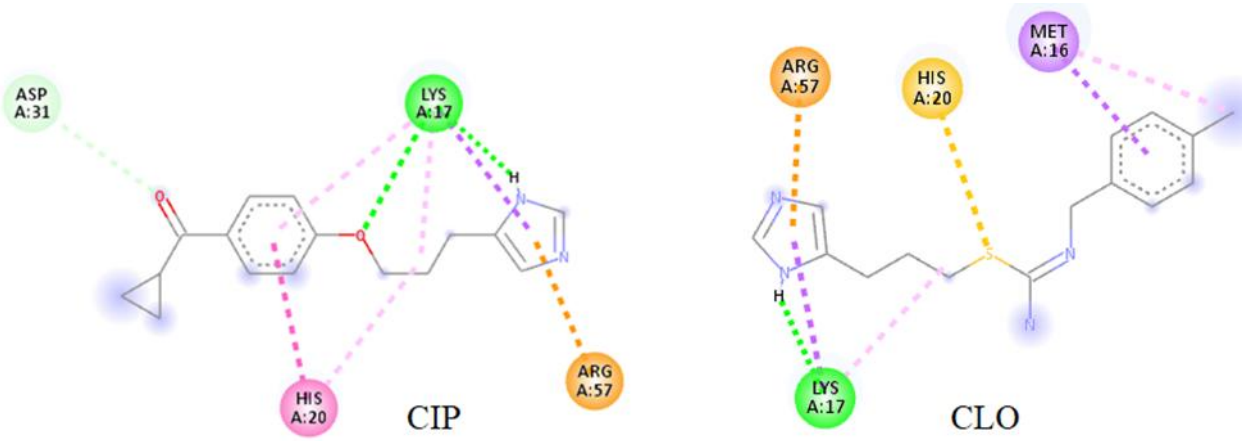

Figure 5.
Binding pose of CIP and CLO in the BH4 domain of Bcl-2

Figure 5 shows the potential binding orientation of CIP and CLO in the BH4 domain of Bcl-2. Both CIP and CLO display hydrogen bond interaction with Lys17, pi-cation interaction with Arg57 and hydrophobic interaction with His20. CIP also showed a weak hydrogen bond with Asp31 and CLO showed an additional hydrophobic interaction with Met16. These interactions may collectively stabilize the native pro-survival conformation of the Bcl-2 protein and are potentially responsible for the observed neuroprotective effect.

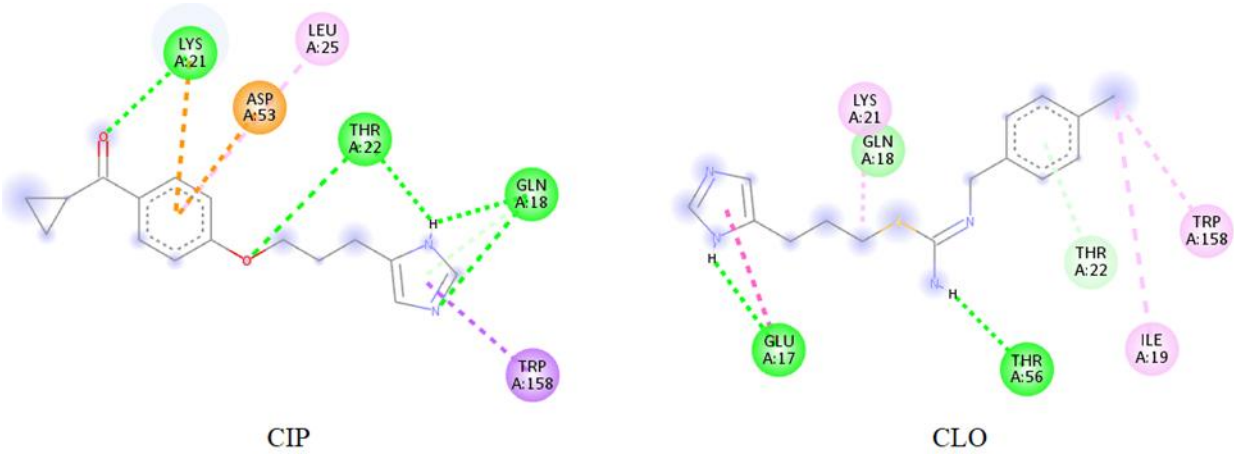

Figure 6.
Binding pose of CLO and CIP in the trigger site of Bax

Figure 6 shows the potential binding modes of CIP and CLO at the Bax trigger site, interacting with residues involved in Bax activation. The molecular docking results show CIP displaying hydrogen bonds with Lys21, Thr22, Gln18, pi-cation interactions with Asp53 and pi–pi stacking or hydrophobic contacts involving Leu25 and Trp158. CLO demonstrates hydrogen bonds with Glu17 and Thr56, in addition to the hydrophobic interactions with Lys21, Gln18, Thr22, Ile19 and Trp158. These interactions collectively stabilize CIP and CLO in the trigger site, thus restricting the conformational change of Bax and forbidding the pro-apoptotic state.

Figure 7 shows the binding pose of CIP and CLO in the active site of Caspase-3. The molecular docking analysis revealed that both CIP and CLO occupy the active site through a network of stabilizing interactions with specific amino acid residues. For CIP, the binding pose shows hydrogen-bond interactions with Arg207 and Ser251, along with pi–pi contacts involving Trp206, Tyr204 and Phe256. Similarly, CLO establishes the hydrogen bonds with Arg207 and Ser209, as well as pi–pi stacking interactions with Trp206 and Tyr204. Additional hydrophobic contacts with Phe250 further support CLO's orientation within the pocket. These residue-specific interactions anchor the ligands securely within the ac-

tive site of Caspase-3, suggesting that CLO and CIP may inhibit its proteolytic activity by directly occupying and stabilizing the enzymatic pocket. Such inhibition could modulate key apoptotic pathways, highlighting the potential of CLO and CIP to regulate Caspase-3 activity in the context of neurodegenerative or apoptosis-related pathological conditions.

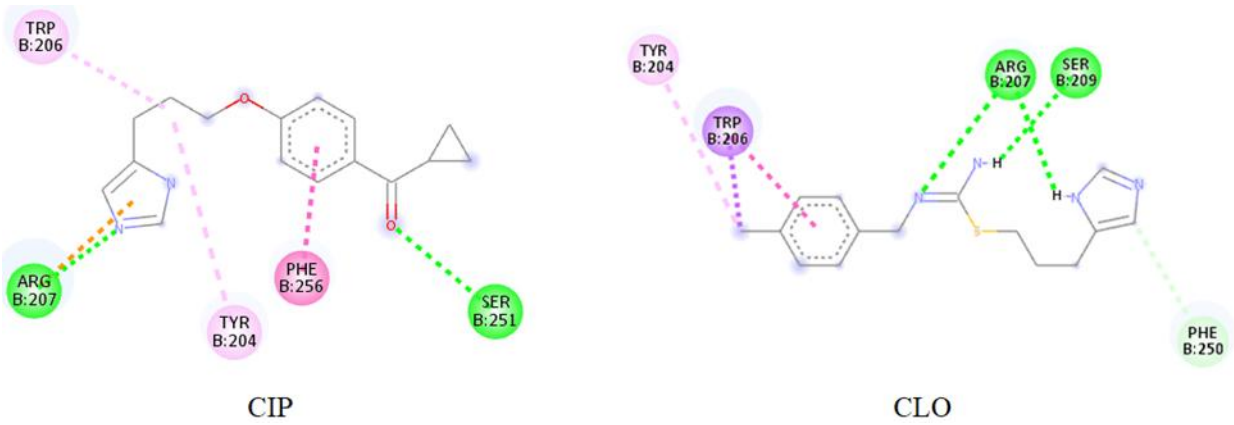

Figure 7.
Binding pose of CLO and CIP in the active site of Caspase-3

In this work, molecular docking was employed to investigate the binding interactions of CIP and CLO with key apoptotic regulators, offering insights into their potential neuroprotective mechanisms. For Bcl-2, Bax and Caspase-3, the ligands were docked into the BH4 domain, the trigger site and the catalytic site, respectively. The comparable binding affinities of CIP and CLO for these apoptotic targets suggest that both compounds may act as broad-spectrum modulators of cell death mechanisms. Although the absence of Co-CL ligands in the Bcl-2 and Bax structures limited the scope for direct comparative analysis, the moderate affinities observed for CIP and CLO at these sites still support their potential relevance. Further, the binding mode analysis of three docked complexes showed interaction with critical residues. The lowest-energy conformations of CLO and CIP in the BH4 domain of Bcl-2 showed hydrogen-bond interactions with Lys17 and electrostatic or aromatic contacts with His20 and Arg57. The literature review shows that the mutation of the Lys17 residue reduces the Bcl-2's neuroprotective effect by weakening IP3R interaction, directly highlighting its critical role (37). Further systematic alanine-scanning mutagenesis by Monaco *et al*. has shown that residues Lys17, His20, Tyr21, or Arg26 are critical for the binding of IP3R to the BH4 domain (38). It should be noted that the BH4 is a noncanonical regulatory domain that contributes to the stabilization of the anti-apoptotic conformation of Bcl-2. Therefore, small molecule binding to the BH4 domain may either stabilize the native pro-survival state or disrupt it, depending on the ligand's functional effect; therefore, BH4 docking should be interpreted as a hypothesis about regulatory modulation rather than proof of stabilization. The binding pose of docked ligands into the trigger site of Bax showed interactions with Gln18, Lys21, Thr22 and Trp158. In addition, CIP showed interactions with Leu25 and Asp53, while CLO showed interactions with Glu17, Thr56 and Ile19. The collective evidence from structural, mutational and docking studies underscores the critical roles of specific trigger site residues, namely Lys21, Thr22, Gln18, Asp53, Leu25 and Trp158 in Bax regulation. Various studies have suggested that Lys21 is the critical residue for Bax activation. Molecular docking showed that both CIP and CLO interact with Lys21, potentially stabilizing the inactive state of Bax (39, 40).

For Caspase-3, the binding poses of CIP and CLO each formed a hydrogen bond with Arg207. Further, CIP and CLO displayed additional hydrogen bonds with Ser251 and Ser209, respectively. The hydrophobic interactions for CIP and CLO were observed with Tyr204 and Trp206, in addition to Phe256 and Phe250, respectively. It is pertinent to note that Trp206 and Phe250 are essential for substrate interaction, while Tyr204 and Phe256, along with Trp206, are responsible for selective substrate binding, as they are unique to Caspase-3 and 7 (41, 42). The effects of CIP and CLO on apoptosis-related proteins are likely mediated indirectly, through modulation of histaminergic neurotransmission and associated downstream signalling pathways, rather than through direct interactions with these proteins. Therefore, the molecular docking results should be considered supportive *in silico* observations rather than direct evidence of *in vivo* target engagement.

Finally, as H3R antagonists/inverse agonists, CIP and CLO may enhance histaminergic and cholinergic neurotransmission, which play crucial roles in cognitive function, synaptic plasticity and neuronal survival. By altering the neurotransmission system, the CIP and CLO support improvements in memory and learning

function, as well as neuroprotection. Our findings from this study support the potential of CIP and CLO for the treatment of neurodegenerative disorders through a multi-layered mechanism (1). It should be noted that this work was intended to document the neuroprotective potential of CIPs and CLOs in the LPS-treated mouse model. However, we acknowledge that including CIP- and CLO-only treated groups would have provided additional mechanistic details. It is pertinent to note that the number of animals was limited due to ethical considerations and financial constraints for the biochemical analysis. In the future, experimental designs will include drug-alone treatment groups, which will further strengthen the results of studies demonstrating the neuroprotective potential of drug molecules.

Conclusions

This study demonstrates CIPs' and CLOs' ability to modulate apoptotic and oxidative stress pathways in LPS-treated mice and hence their potential against neurodegenerative disorders in addition to substantiating the reports of multifaceted mechanisms of the above-mentioned drug molecules. Future studies will include validating the neuroprotective potential in behavioural and clinical-relevance models. Future work will also include MD simulations to assess the dynamic stability of CIP and CLO in the active site of Bcl-2, Bax and Caspase-3.

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Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article.

Ethics approval and consent to participate

All experimental procedures were approved by the Institutional Animal Ethical Committee of the College of Pharmacy (approval No. 2020-CP-7).

AI use disclosure

During the preparation of this manuscript, the authors used ChatGPT for editing assistance and language improvement, specifically to improve spelling, grammar, word choice, readability and clarity. The authors reviewed and revised all outputs from these tools and take full responsibility for the final content of the work.

Conflict of interest

The authors declare no conflict of interest.

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