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RESEARCH ARTICLE

Cyclotide Prevents Neuronal Cell Death Induced by 6-Hydroxydopamine Neurotoxin

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ABSTRACT

Neuronal cell death is a major factor in the initiation and progression of Parkinson's disease (PD). Reactive oxygen species (ROS) overproduction is one of the primary causes of neuronal death, hence drugs that decrease oxidative stress-dependent neuronal death as a preventive measure against Parkinson's disease (PD) may be promising. However, cyclotide's preventive effects have not been studied using both in vitro and in vivo models of Parkinson's disease. Therefore, in order to investigate the efficacy of cyclotide against 6-hydroxydopamine (6-OHDA)-dependent neuronal cell death, we employed immortalized hypothalamic neurons (GT1-7 cells). First, we found that cyclotide prevents 6-OHDA-dependent neuronal cell death by inhibiting ROS overproduction in GT1-7 cells. The cytoprotective effect of cyclotide was largely abolished by verapamil, an OCTN1 inhibitor that inhibits cyclotide absorption. According to these results, cyclotide or foods containing it may be a helpful tactic to postpone the start and progression of Parkinson's disease (PD).

Keywords: Cyclotide; neuronal cell death; 6-OHDA; oxidative stress; functional food

INTRODUCTION

Parkinson's disease (PD) is one of the most common progressive neurodegenerative illnesses. Parkinson's disease (PD) affects both the central nervous system and the body parts that are innervated by diseased nerves. This results in altered postural reflexes, stiffness in the muscles, akinesia, and resting tremor. In the initial stages of Parkinson's disease (PD), sluggish movements and trouble walking are frequently observed. People who have the disease gradually become bedridden or unable to move freely, requiring them to use a wheelchair [1,2].

Amantadine hydrochloride, anticholinergic drugs, and the dopamine precursor L-dopa are commonly used in the treatment of Parkinson's disease (PD) patients in order to compensate for the dopamine depletion brought on by the death of dopaminergic neurons. Despite the moderate efficacy of these drugs [3], there is now no established method to stop the onset and progression of Parkinson's disease. Parkinson's disease (PD) has no known exact etiology, although elevated inflammatory response and loss of dopaminergic neurons in the substantia nigra of the midbrain are established risk factors. Lesion-related microglia are thought to be the main cause of Parkinson's disease (PD) [4,5]. Treating mice or neurons with 6-hydroxydopamine (6-OHDA) or N-methyl-4-phenylpyridinium iodide (MPP+) has been used in basic research because these substances can produce models that mimic oxidative stress-mediated dopaminergic neuronal degeneration and enhanced microglial inflammation [6,7]. Moreover, disturbance of the hypothalamic-pituitary-adrenal axis is said to have an impact on the development and progression of Parkinson's disease (PD) and govern the synthesis of numerous hormones [8]. The comparison of the plasma concentrations of adrenocorticotropic hormone and nocturnal growth hormone in Parkinson's disease (PD) patients with those of healthy controls indicates possible abnormalities in the hypothalamus [9].

A reduction in the quantity of neurons in the hypothalamus of Parkinson's disease patients was found in one study [10]. Furthermore, a strong positive correlation has been observed between the clinical stage of Parkinson's disease and a decrease in orexin-containing neurons, a hormone that concentrates melanin in the hypothalamus [11]. These preliminary findings suggest that inhibiting neuronal death in the hypothalamus may prevent the onset and progression of Parkinson's disease. One of these potentially beneficial herbs is *Clitoria ternatea*, a key ingredient in the brain tonic medhya rasayan, which is used to treat neurological illnesses. Ayurvedic medicine has been used traditionally in India from ancient times. This study supports Indian medicine by highlighting the plant's significance as a brain remedy. *Clitoria ternatea* has a potent brain-stimulating effect, unlike other plants [12]. Moreover, C. ternatea has long been used in traditional medicine, particularly as a supplement to improve cognitive function and lessen the symptoms of numerous illnesses like fever, inflammation, discomfort, and diabetes [13]. This study assessed the antiparkinson's activity of cyclotide, the active ingredient in *Clitoria ternatea.* Previous work on the analysis of cyclotide insilico and invitro served as the foundation for the current investigation. Despite the fact that cyclotide has been linked to a number of biological processes, its ability to prevent 6-OHDA-dependent hypothalamic neuronal cell death has not been studied. Therefore, we used immortalized hypothalamic neurons and GT1-7 cells in this investigation to investigate the effectiveness of cyclotide on 6-OHDA-dependent neuronal cell death. Additionally, we ascertained cyclotide's impact on the generation of ROS in response to 6-OHDA as well as its effectiveness in preventing neuronal cell death caused by 6-OHDA.

MATERIALS AND METHODS

Chemicals and Reagents

MPP+ was supplied by Cayman Chemical. Promega Corporation was the CellTiter-Glo® 2.0 provider. Fujifilm Wako Pure Chemical Corporation in Tokyo, Japan was the seller of DMEM/Ham's Nutrient Mixture F-12 (Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture). The 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) was supplied by Merck KGaA. The FastGeneTM RNA Basic kit was provided by Nippon Genetics Co., Ltd.; the PrimeScriptTM RT master mix (Perfect Real Time) was provided by Takara Bio; and the THUNDERBIRD® Next SYBR® qPCR mix was provided by Toyobo.

Culture of Cells

For the investigation, immortalized hypothalamic neurons, or GT1-7 cells, were employed. 10% fetal bovine serum was added to DMEM/Ham's-F12, the growth media used for GT1-7 cells. After being treated with trypsin (Fujifilm Wako Pure Chemicals), the cells were suspended in a serum-free medium, plated, and cultured at 37◦C in a humidified incubator with 7% CO2 [15].

Determination of cell viability.

Cell viability was evaluated, same as it was in our previous studies [20, 21]. To put it briefly, 3.0 x 104 GT1-7 cells were seeded per well onto 96-well growth plates using 200 μL of culture medium. After a 24-hour preincubation period, the medium was supplied with either 6-OHDA (final concentrations ranging from 0 μmol/L to 80 μmol/L) or MPP+ (final concentrations ranging from 0 mmol/L to 8 mmol/L). As an alternative, the cells were preincubated for 24 hours before being treated with cyclotide (final concentrations ranging from 0 mmol/L to 1.0 mmol/L) for 10 min. The medium was subsequently supplemented with either MPP+ (final concentration: 4 mmol/L) or 6-OHDA (final concentration: 40 mmol/L). The cells' vitality was evaluated using the luminous reagent CellTiter-Glo® 2.0, which quantifies intracellular ATP, following a 24-hour incubation period. Assuming that cyclotide is ingested as a daily meal or supplement, it was pretreated to GT1-7 cells in this investigation. Its efficacy was evaluated. Furthermore, despite discrepancies in reporting, the blood concentration of cyclotide in humans is roughly 0.2 mmol/L, which is comparable to the concentration of cyclotide used in this experiment [22].

ROS Level Measurement

GT1: Seven cells were cultured in black 96-well microplates with 3.0 × 104 cells per well for a whole day. Next, the cells were exposed to the ROS indicator H2DCFDA (10 μmol/L) for 60 minutes. Subsequently, cyclotide was added to the media (final concentration: 40 μmol/L) and the cells were treated with it (final concentrations ranging from 0 mmol/L to 1.0 mmol/L). After an hour, the ROS levels were measured using a microplate reader (excitation: 480 nm, emission: 530 nm).

Instantaneous Analysis of Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from GT1-7 cells according to the manufacturer's instructions using the FastGeneTM RNA Basic kit. Samples were reverse-transcribed using the PrimeScript RT master mix. The resulting cDNA was analyzed in real-time PCR experiments with THUNDERBIRD Next SYBR qPCR mix using CFX ManagerTM software (Version 3.1) on a Bio-Rad CFX96TM realtime system (Hercules, CA, USA). The specificity was verified by electrophoretic analysis of the reaction products with template- or reverse transcriptase-free controls. Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) cDNA was employed as an internal standard to adjust the total RNA concentration in every experiment. Primers were designed using the Primer-BLAST website (https://www.ncbi.nlm.nih.gov/tools/primer-blast/).Primer sequences are shown in Supplementary Figure S1.

Statistical Analysis

All data are expressed as mean ± using the standard error of the mean (S.E.M.). One-way analysis of variance (ANOVA) was used to examine differences between three or more groups, or between two groups. For unpaired data, Dunnett's test or Student's t-test were used, respectively. A difference was considered significant if it was $p < 0.05$ (* or # $p < 0.05$, ** or ## $p < 0.01$). Details on the symbols are provided by the figure legends. The number of samples (n) is also indicated in each image.

RESULTS

Efficacy of Cyclotide on 6-OHDA-Induced Neuronal Cell Death

The development and course of Parkinson's disease are significantly influenced by neuronal cell death [4,5]. In addition to producing a variety of Parkinson's disease-like symptoms when injected into the brains of test animals, 6-OHDA also acts as a neurotoxic in cellular experimental setups, leading to the death of neurons [6,23]. We started by evaluating the survival of GT1-7 cells, an immortalized line of mouse hypothalamus neurons, that were only exposed to 6-OHDA. Cell viability decreased in a concentration-dependent manner in response to treatment of 20, 40, 60, and 80 μmol/L concentrations of 6-OHDA (Figure 1A). The groups' respective post-treatment viabilities were 73.7 ± 2.7, 47.4 ± 2.7, 33.2 ± 2.3, and 23.9 ± 1.6% (mean ± S.E.M., n = 4). All groups shown a noteworthy decrease in comparison to the ultrapure water-treated control group. Next, we investigated the effect of cyclotide pretreatment using a 40 μmol/L final concentration of 6-OHDA on the 6-OHDA-induced decrease in GT1-7 cell viability. Pretreatment with

0.5 mmol/L and 1.0 mmol/L of cyclotide restored cell viability to 77.1 ± 2.5% and 82.8 ± 0.8% (mean ± S.E.M., n = 4) respectively, effectively mitigating the 6-OHDA-dependent decrease in cell viability. The protective effect was concentration-dependent. Under these circumstances, cyclotide therapy alone had little effect on cell viability (Figure 1C). Likewise, MPP+ functions as a neurotoxic in experimental settings that are both in vivo and in vitro [7]. We looked into if cyclotide also prevented the death of neurons triggered by MPP+. Reduced cell viability was seen in GT1-7 cells treated with 1.0–8.0 mmol/L MPP+ (Figure 1D).The MPP+-dependent loss in cell viability was considerably mitigated by cyclotide; after pretreatment with 0.5 mmol/L and 1.0 mmol/L of cyclotide, respectively, cell viability reached 53.0 ± 1.3% and 55.9 ± 0.5% (mean ± S.E.M., n = 4). (Figure 1E). Numerous investigations suggest that the endoplasmic reticulum (ER) stress response plays a crucial role in the neuronal cell death caused by 6-OHDA [24, 25]. Our lab has previously shown that enhanced ER stress-related gene expression in GT1-7 cells produced by 6-OHDA may be suppressed by the antioxidant peptide carnosine and an antioxidant protein–thioredoxin–albumin fusion [26,27]. Treatment with 40 μmol/L of 6-OHDA in this study elevated the expression of genes linked to ISR, especially the significant elevation of growth-arrest and DNA-damage-inducible gene 34 (Gadd34) and CCAAT-enhancer-binding protein homologous protein (Chop). In particular, compared to the control group, Chop and Gadd34 mRNA increased by 6.72 ± 0.08-fold and 4.60 ± 0.22-fold (mean ± S.E.M., n = 3), respectively. In addition, 6-OHDA treatment significantly increased the levels of ER degradation-enhancing α mannosidase (Edem), binding immunoglobulin protein (Bip), activating transcription factor 4 (Atf4), inositol-requiring transmembrane kinase/endoribonuclease 1α (Ire1α), protein disulfide isomerase (Pdi), and glucose-regulated protein 94 (Grp94). On the other hand, the overexpression of these genes was inhibited by cyclotide pretreatment, especially Chop (to 2.50 ± 0.15-fold) and Gadd34 (to 2.14 ± 0.04-fold) mRNA (mean ± S.E.M., n = 3). According to these results, cyclotide suppresses the elevated expression of genes linked to ER stress, hence preventing 6-OHDA-induced neuronal cell death (Figure 2).

Efficacy of Cyclotide on 6-OHDA-Induced Reactive Oxygen Species (ROS) Production

In recent studies, it was found that the antioxidant peptide carnosine and the antioxidant protein-thioredoxin-albumin fusion both successfully reduced the increased expression of genes linked to ER stress that 6-OHDA caused in GT1-7 cells [26, 27]. In the present investigation, 40 μmol/L of 6-OHDA induced an upregulation of ISR-related gene expression, most notably CCAAT-enhancer-binding protein homologous protein (Chop) and growth-arrest and DNA-damage-inducible gene 34 (Gadd34). In particular, compared to the control group, Chop and Gadd34 mRNA levels increased by 6.72 ± 0.08-fold and 4.60 ± 0.22-fold (mean ± S.E.M., n = 3), respectively. Furthermore, after 6-OHDA treatment, there were notable increases in the following processes: binding immunoglobulin protein (Bip), ER degradation enhancing α mannosidase (Edem), activating transcription factor 4 (Atf4), inositol-requiring transmembrane kinase/endoribonuclease 1α (Ire1α), protein disulfide isomerase (Pdi), and glucose-regulated protein 94 (Grp94). On the other hand, cyclotide pretreatment inhibited the overexpression of these genes, specifically reducing the mRNA levels of Gadd34 (down to 2.13 ± 0.04-fold) and Chop (down to 2.52 ± 0.15-fold) (mean ± S.E.M., n = 3). These results clearly suggest that cyclotide has the ability to inhibit the overexpression of genes linked to ER stress that is brought on by 6-OHDA.

Involvement of OCTN1 in Cytoprotective Effects of Cyclotide

The protein known as OCTN1, a membrane transporter, is in charge of identifying and moving endogenous or exogenous substances into and out of cells. OCTN1 is essential for cyclotide absorption, as demonstrated by a number of investigations on mice and cultured cells [15,16]. Therefore, we looked into whether verapamil, a well-known OCTN1 inhibitor, would neutralize the protective effect of cyclotide on cells [30]. As expected, cyclotide successfully counteracted the cell viability loss caused by 6-OHDA (Figure 4). However, the protective effect of cyclotide was greatly diminished when cells were pretreated with verapamil. Notably, verapamil administration by itself did not significantly affect ROS generation or cell viability in this experimental setup (Supplementary Figure S2). These results strongly imply that the cytoprotective action of cyclotide in GT1-7 cells is dependent on its entry into the cell through OCTN1.

DISCUSSION

In this study, we examined the protective effect of cyclotide against 6-OHDA-induced neuronal cell death using GT1-7 cells as immortalized hypothalamic neurons. Our results show that cyclotide significantly reduces the overexpression of ER stress-related proteins, including Chop and Gadd34, in GT1-7 cells as well as 6-OHDA-induced cell death. The pathophysiology and exacerbation of a number of diseases, including cancer, pneumonia, and neurological disorders, are significantly influenced by the ER stress response, which is mediated by protein kinase R-like ER kinase (PERK), inositol-requiring enzyme-1 (IRE1), and ATF6 [33, 34]. When these sensors are activated, the cell begins to undergo signaling processes, such as PERK phosphorylating eIF2's α subunit, which affects ATF4 translation in turn.ATF4 in turn initiates the transcription of GADD34 and CHOP, which are involved in the induction of cell death [33, 34]. We suggest that cyclotide prevents 6-OHDA-dependent neuronal cell death by inhibiting the expression of proteins linked to ER stress and cell death, specifically Gadd34 and Chop, as well as the transcription factor that is upstream of these factors, Atf4. In conclusion, our groundbreaking discovery emphasizes how cyclotide suppresses the ER stress response, hence inhibiting 6-OHDA-induced neuronal cell death and suggesting the drug's potential as a PD preventative. Since 6-OHDA is known to cause apoptosis in neuronal cells [26, 29], we will be investigating a more thorough way of triggering cell death in subsequent studies.

In our work, the OCTN1 inhibitor verapamil inhibited the cytoprotective effect of cyclotide; nevertheless, it is important to highlight that this protective effect was not completely eliminated by verapamil pretreatment. Furthermore, verapamil by itself demonstrated cytotoxicity at values higher than 100 μmol/L (Supplementary Figure S2), which raises questions about its suitability for use in research. As a result, future research should identify additional pathways that contribute to cyclotide's cytoprotective action in addition to OCTN1. One of the main causes of neuronal cell death and exaggerated inflammatory responses in Parkinson's disease (PD) is oxidative stress, which is marked by elevated generation of reactive oxygen species [28,29,35]. Clinical research has shown a direct link between the generation of ROS and Parkinson's disease. Cyclotide appears to be a promising treatment option for Parkinson's disease (PD) since it inhibits the creation of excessive ROS or oxidative stress. This idea is in line with earlier research that shown the effectiveness of antioxidants, including N-acetylcysteine and coenzyme Q10, in reducing Parkinson's disease symptoms in both human and animal trials. Moreover, cyclotide has proven effective in stroke and Alzheimer's disease animal models, exhibiting neuroprotective and antioxidant properties [17, 18]. Its neuroprotective qualities have also been shown in cultured cells, where it prevents amyloid-beta and anticancer medications from injuring neuronal cells. While cyclotide's effectiveness in treating a variety of neurological conditions has been studied, there have been no reports of studies examining its effectiveness in PD animal models or 6-OHDA-induced neuronal cell death, with the exception of one clinical study that found PD patients' blood levels of cyclotide were lower than those of controls. These results highlight the potential importance of adding cyclotides to meals or supplements as a critical prophylactic against the onset and progression of Parkinson's disease.

Through its antioxidant qualities, cyclotide, we found in our inquiry, protects against 6-OHDA-dependent neuronal cell death. Based on these findings, we propose that cyclotide may be a viable treatment option for delaying the onset and progression of Parkinson's disease (PD). In the future, we want to investigate more thoroughly how well cyclotide works in animal models of Parkinson's disease. GT1-7 cells were treated with the indicatedconcentrations of 6-hydroxydopamine (6-OHDA) (μmol/L) or N-methyl-4-phenylpyridinium(MPP+) (mmol/L) and incubated for 24 h (A,D). GT1-7 cells were pretreated with cyclotide (0.06–1.0 mmol/L) and then incubated in the absence (Control) or presence of 6-OHDA (40 μmol/L)or MPP+ (4 mmol/L) for 24 h (B,E). GT1-7 cells were treated with cyclotide (0.06–1.0 mmol/L)alone and cultured for a further 24 h (C). Cell viability was measured using CellTiter-Glo® 2.0. Valuesrepresent mean ± S.E.M. (n = 4). ** $p < 0.01$, vs. Control; ## $p < 0.01$, vs. 6-OHDA (40 μmol/L) aloneor MPP+ (4 mmol/L) alone. GT1-7 cells were pretreated with cyclotide (1.0 mmol/L) and then incubated in theabsence (Control) or presence of 6-OHDA (40 μmol/L) for 24 h. After total RNA extraction fromGT1-7 cells, cDNA was synthesized, and real-time RT-PCR was performed using primer pairs thatspecifically amplify Chop, Gadd34, Atf4, Bip, Ire1α, Pdi, Edem, and Grp94. Values were normalizedto Gapdh and expressed relative to control. Values represent mean ±

S.E.M. (n = 3). * p < 0.05, vs.Control; ** p < 0.01, vs. Control; # p < 0.05, vs. 6-OHDA (40 μmol/L) alone; ## p < 0.01, vs. 6-OHDA(40 μmol/L) alone. GT1-7 cells were pretreated witha reactive oxygen species (ROS) indicator, 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA)(10 μmol/L) for 60 min (A,B). Cells were then treated with 6-OHDA (20–80 μmol/L) and culturedfor 1 h (A). GT1-7 cells were pretreated with cyclotide (0.06–1.0 mmol/L) and then incubated inthe absence (Control) or presence of 6-OHDA (40 μmol/L) for 1 h (B). GT1-7 cells were treatedwithcyclotide (0.06–1.0 mmol/L) alone and cultured for 1 h (C). ROS levels were measured usinga fluorescence microplate reader. Values represent mean } S.E.M. (n = 4). ** p < 0.01, vs. Control;## p < 0.01, vs. 6-OHDA (40 μmol/L) alone. GT1-7 cells were pretreatedwith verapamil (100 μmol/L) for 60 min. After replacement with fresh medium, cells werethen pretreated with cyclotide (0.5 or 1.0 mmol/L) and incubated in the absence (Control) or presenceof 6-OHDA (40 μmol/L) for 24 h. Cell viability was measured using CellTiter-Glo® 2.0. Valuesrepresent mean ± S.E.M. (n = 4). Not Significant (n.s.), ** p < 0.01, Control vs. verapamil (100 μmol/L).

REFERENCES

1. Pagano, G.; Ferrara, N.; Brooks, D.J.; Pavese, N. Age at onset and Parkinson disease phenotype. Neurology 2016, 86, 1400–1407.[CrossRef]
2. Kalia, L.V.; Lang, A.E. Parkinson's disease. Lancet 2015, 386, 896–912. [CrossRef]
3. Zahoor, I.; Shafi, A.; Haq, E. Pharmacological Treatment of Parkinson's Disease. In Parkinson's Disease: Pathogenesis and ClinicalAspects; Stoker, T.B., Greenland, J.C., Eds.; Codon Publications: Brisbane, Australia, 2018.
4. Erekat, N.S. Apoptosis and its Role in Parkinson's Disease. In Parkinson's Disease: Pathogenesis and Clinical Aspects; Stoker, T.B., Greenland, J.C., Eds.; Codon Publications: Brisbane, Australia, 2018.
5. Macchi, B.; Di Paola, R.; Marino-Merlo, F.; Felice, M.R.; Cuzzocrea, S.; Mastino, A. Inflammatory and cell death pathways in brainand peripheral blood in Parkinson's disease. CNS Neurol. Disord. Drug Targets 2015, 14, 313–324. [CrossRef] [PubMed]
6. Konnova, E.A.; Swanberg, M. Animal Models of Parkinson's Disease. In Parkinson's Disease: Pathogenesis and Clinical Aspects;Stoker, T.B., Greenland, J.C., Eds.; Codon Publications: Brisbane, Australia, 2018.
7. Zeng, X.; Chen, J.; Deng, X.; Liu, Y.; Rao, M.S.; Cadet, J.L.; Freed, W.J. An in vitro model of human dopaminergic neurons derivedfrom embryonic stem cells: MPP+ toxicity and GDNF neuroprotection. Neuropsychopharmacology 2006, 31, 2708–2715. [CrossRef][PubMed]
8. Ham, S.; Lee, Y.I.; Jo, M.; Kim, H.; Kang, H.; Jo, A.; Lee, G.H.; Mo, Y.J.; Park, S.C.; Lee, Y.S.; et al. Hydrocortisone-induced parkinprevents dopaminergic cell death via CREB pathway in Parkinson's disease model. Sci. Rep. 2017, 7, 525. [CrossRef] [PubMed]
9. Bellomo, G.; Santambrogio, L.; Fiacconi, M.; Scarponi, A.M.; Ciuffetti, G. Plasma profiles of adrenocorticotropic hormone, cortisol,growth hormone and prolactin in patients with untreated Parkinson's disease. J. Neurol. 1991, 238, 19–22. [CrossRef] [PubMed]
10. Giguere, N.; Burke Nanni, S.; Trudeau, L.E. On Cell Loss and Selective Vulnerability of Neuronal Populations in Parkinson'sDisease. Front. Neurol. 2018, 9, 455. [CrossRef] [PubMed]
11. Thannickal, T.C.; Lai, Y.Y.; Siegel, J.M. Hypocretin (orexin) cell loss in Parkinson's disease. Brain 2007, 130, 1586–1595. [CrossRef][PubMed]
12. Sivaranjan VV, Balachandran I. Ayurvedic drugs and their plant sources. New Delhi: Oxford and IBHPublishing Company; 1994. pp. 425–428.
13. Mukherjee, P. K., Kumar, V., Kumar, N. S., and Heinrich, M. (2008). The Ayurvedic medicine Clitoriaternatea-from traditional use to scientific assessment. J. Ethnopharmacol. 120, 291–301. doi:10.1016/j.jep.2008.09.009
14. Muneeswari, M., Gangasani Narasimha Reddy., Arivukodi, D., Usharani, B., Shobana, C., (2022) Analysis of potentiality of Cyclotide, A Major Compound from Clitoria Ternatea as Anti-Parkinsonism Drug: A Pilot In Silico Study. NeuroQuantology 20(10):2758-2773. doi:10.14704/nq.2022.20.10.NQ55237
15. Kawahara, M.; Kato-Negishi, M.; Kuroda, Y. Pyruvate blocks zinc-induced neurotoxicity in immortalized hypothalamic neurons.Cell. Mol. Neurobiol. 2002, 22, 87–93. [CrossRef] [PubMed]

16. Tanaka, K.I.; Shimoda, M.; Sugizaki, T.; Ikeda, M.; Takafuji, A.; Kawahara, M.; Yamakawa, N.; Mizushima, T. Therapeutic effectsof eperisone on pulmonary fibrosis via preferential suppression of fibroblast activity. Cell Death Discov. 2022, 8, 52. [CrossRef]
17. Nakano, Y.; Shimoda, M.; Okudomi, S.; Kawaraya, S.; Kawahara, M.; Tanaka, K.I. Seleno-l-methionine suppresses copperenhancedzinc-induced neuronal cell death via induction of glutathione peroxidase. Metallomics 2020, 12, 1693–1701. [CrossRef]
18. Cheah, I.K.; Halliwell, B. Cyclotide; antioxidant potential, physiological function and role in disease. Biochim. Biophys. Acta2012, 1822, 784–793. [CrossRef]
19. Park, S.Y.; Kim, D.Y.; Kang, J.K.; Park, G.; Choi, Y.W. Involvement of activation of the Nrf2/ARE pathway in protection against 6-OHDA-induced SH-SY5Y cell death by alpha-iso-cubebenol. Neurotoxicology 2014, 44, 160–168. [CrossRef]
20. Elmazoglu, Z.; Ergin, V.; Sahin, E.; Kayhan, H.; Karasu, C. Oleuropein and rutin protect against 6-OHDA-induced neurotoxicityin PC12 cells through modulation of mitochondrial function and unfolded protein response. Interdiscip. Toxicol. 2017, 10, 129–141.[CrossRef] [PubMed]
21. Kim, Y.; Li, E.; Park, S. Insulin-like growth factor-1 inhibits 6-hydroxydopamine-mediated endoplasmic reticulum stress-inducedapoptosis via regulation of heme oxygenase-1 and Nrf2 expression in PC12 cells. Int. J. Neurosci. 2012, 122, 641–649. [CrossRef][PubMed]
22. Kubota, M.; Kobayashi, N.; Sugizaki, T.; Shimoda, M.; Kawahara, M.; Tanaka, K.I. Carnosine suppresses neuronal cell death and inflammation induced by 6-hydroxydopamine in an in vitro model of Parkinson's disease. PLoS ONE 2020, 15, e0240448.[CrossRef] [PubMed]
23. Sakakibara, O.; Shimoda, M.; Yamamoto, G.; Higashi, Y.; Ikeda-Imafuku, M.; Ishima, Y.; Kawahara, M.; Tanaka, K.I. Effectivenessof Albumin-Fused Thioredoxin against 6-Hydroxydopamine-Induced Neurotoxicity In Vitro. Int. J. Mol. Sci. 2023, 24, 9758.[CrossRef] [PubMed]
24. Bernstein, A.I.; Garrison, S.P.; Zambetti, G.P.; O'Malley, K.L. 6-OHDA generated ROS induces DNA damage and p53- andPUMA-dependent cell death. Mol. Neurodegener. 2011, 6, 2. [CrossRef] [PubMed]
25. Kim, D.W.; Lee, K.T.; Kwon, J.; Lee, H.J.; Lee, D.; Mar, W. Neuroprotection against 6-OHDA-induced oxidative stress andapoptosis in SH-SY5Y cells by 5,7-Dihydroxychromone: Activation of the Nrf2/ARE pathway. Life Sci. 2015, 130, 25–30.[CrossRef] [PubMed]
26. Zhang, H.Y.; Wang, Z.G.; Lu, X.H.; Kong, X.X.; Wu, F.Z.; Lin, L.; Tan, X.; Ye, L.B.; Xiao, J. Endoplasmic reticulum stress: Relevanceand therapeutics in central nervous system diseases. Mol. Neurobiol. 2015, 51, 1343–1352. [CrossRef]
27. Grootjans, J.; Kaser, A.; Kaufman, R.J.; Blumberg, R.S. The unfolded protein response in immunity and inflammation. Nat. Rev.Immunol. 2016, 16, 469–484. [CrossRef]
28. Trist, B.G.; Hare, D.J.; Double, K.L. Oxidative stress in the aging substantia nigra and the etiology of Parkinson's disease. AgingCell 2019, 18, e13031. [CrossRef]
29. Agil, A.; Duran, R.; Barrero, F.; Morales, B.; Arauzo, M.; Alba, F.; Miranda, M.T.; Prieto, I.; Ramirez, M.; Vives, F. Plasma lipidperoxidation in sporadic Parkinson's disease. Role of the L-dopa. J. Neurol. Sci. 2006, 240, 31–36. [CrossRef] [PubMed]
30. Alam, Z.I.; Jenner, A.; Daniel, S.E.; Lees, A.J.; Cairns, N.; Marsden, C.D.; Jenner, P.; Halliwell, B. Oxidative DNA damage inthe parkinsonian brain: An apparent selective increase in 8-hydroxyguanine levels in substantia nigra. J. Neurochem. 1997, 69,1196–1203. [CrossRef] [PubMed]
31. Yoritaka, A.; Kawajiri, S.; Yamamoto, Y.; Nakahara, T.; Ando, M.; Hashimoto, K.; Nagase, M.; Saito, Y.; Hattori, N. Randomized,double-blind, placebo-controlled pilot trial of reduced coenzyme Q10 for Parkinson's disease. Park. Relat. Disord. 2015, 21,
32. 911–916. [CrossRef] [PubMed]
33. Monti, D.A.; Zabrecky, G.; Kremens, D.; Liang, T.W.; Wintering, N.A.; Bazzan, A.J.; Zhong, L.; Bowens, B.K.; Chervoneva, I.;Intenzo, C.; et al. N-Acetyl Cysteine Is Associated With Dopaminergic Improvement in Parkinson's Disease. Clin. Pharmacol. Ther.2019, 106, 884–890. [CrossRef] [PubMed]

34. Haleagrahara, N.; Siew, C.J.; Mitra, N.K.; Kumari, M. Neuroprotective effect of bioflavonoid quercetin in 6-hydroxydopamineinducedoxidative stress biomarkers in the rat striatum. Neurosci. Lett. 2011, 500, 139–143. [CrossRef] [PubMed]
35. Houldsworth, A. Role of oxidative stress in neurodegenerative disorders: A review of reactive oxygen species and prevention byantioxidants. Brain Commun. 2024, 6, fcad356. [CrossRef]
36. Reddy, V.P. Oxidative Stress in Health and Disease. Biomedicines 2023, 11, 2925. [CrossRef]
37. Hatano, T.; Saiki, S.; Okuzumi, A.; Mohney, R.P.; Hattori, N. Identification of novel biomarkers for Parkinson's disease bymetabolomic technologies. J. Neurol. Neurosurg. Psychiatry 2016, 87, 295–301. [CrossRef] [PubMed]

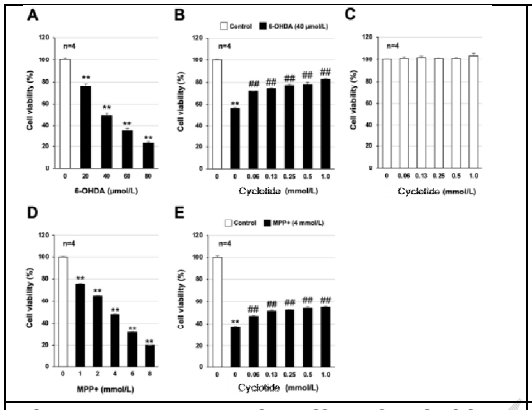

Figure 1. Neuroprotective effect of cyclotide

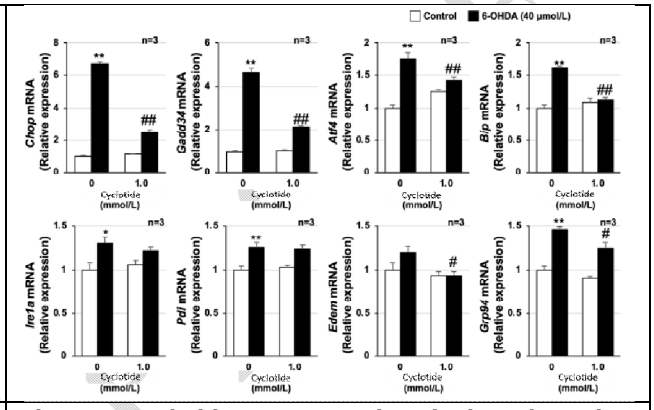

Figure 2. Cyclotide suppresses the 6-hydroxydopamine-dependent endoplasmic reticulum stress response

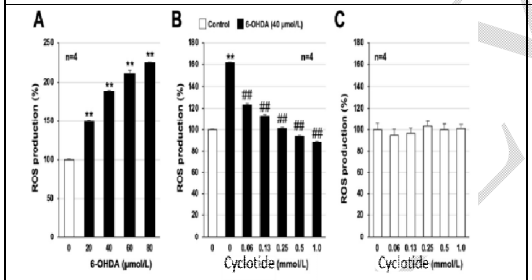

Figure 3. Antioxidant effect of cyclotide in GT1-7 cells

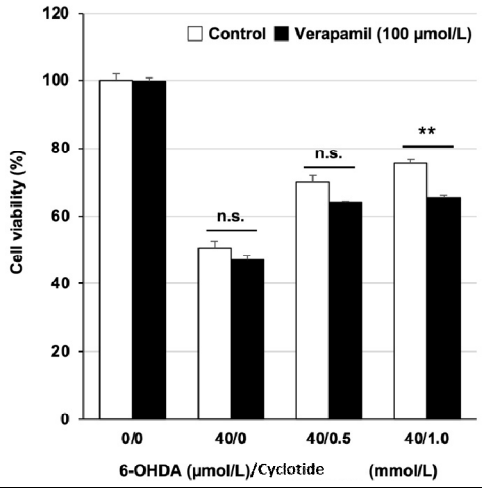

Figure 4. Involvement of OCTN1 in the cytoprotective effect of cyclotide.