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Research Article

Antioxidant and Neuroproliferative Effects of THL-3-PTD5 Peptide Derived from Hydramacin-1 Antimicrobial Peptide

Ping-Chien Lee, Ching-Chun Lin, Hui-Hsuan Hsu, and Feng-Di T. Lung

Department of Chemistry, Tunghai University, Taichung, Taiwan

Correspondence should be addressed to Feng-Di T. Lung; fdlung@thu.edu.tw

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Parkinson's disease (PD) is a neurodegenerative disorder characterized by reduced dopamine levels in the brain, affecting over 6 million people worldwide. While current treatments for patients primarily focus on symptom relief, there is a necessity for the development of novel therapeutic agents due to the presence of side effects and declining effectiveness. Functional peptides, known for their high selectivity, specificity, and diverse bioactivities, have shown the potential to develop therapeutic candidates for neurodegenerative diseases. Notably, the macin family of proteins exhibits potent antimicrobial activity and nerve repair effects. In this study, we investigated the antioxidant activity and neuroproliferative effects of a series of truncated peptides (THL, THL-1, THL-2, and THL-3) derived from the C-terminal sequence of hydramacin-1. Results showed that THL-3 exhibited the strongest antioxidant activity (EC_{50} of 22.5 μM) in ABTS-radical scavenging assays, with amino acid residues Cys^5, Pro^6, Leu^7, Lys^9, and Lys^{10} playing critical roles in its activity. Upon conjugation with the cell-penetrating PTD5 peptide to form THL-3-PTD5, it exhibited a significant dose-dependent neuroproliferative effect, increasing the viability of SH-SY5Y cells by 118% at 100 μM. However, it did not exhibit neuroprotective effects under *in vitro* conditions of oxidative stress induced by an exogenous oxidizing agent. This study suggests that THL-3-PTD5 may serve as a potential candidate for developing therapeutic agents against neurodegenerative diseases.

1. Introduction

Parkinson's disease (PD), a neurodegenerative disease, is characterized by reduced dopamine levels in the brain due to abnormal apoptosis of dopaminergic neurons in the substantia nigra [1, 2]. Dopamine plays a crucial role in neuromodulatory systems, primarily regulating executive functions, motor control, motivation, and emotions [3]. In addition, the long-term low dopamine levels in the brain may lead to the development of language and physical disorders in patients [3, 4]. Although the exact pathogenesis of PD remains unclear, several factors have been associated with the development of the disease, such as the accumulation of α-synuclein [5], mitochondrial dysfunction [6, 7], and high levels of reactive oxygen species (ROS) [8, 9] in neurons. Unfortunately, PD has affected more than 6 million people worldwide, becoming a significant global health issue, especially in countries with aging populations [10].

Current medications for PD primarily focus on alleviating symptoms in patients [11, 12], such as levodopa, dopamine agonists, catechol-O-methyl transferase inhibitor, and monoamine oxidase B inhibitor. On the other hand, drug repurposing strategies have led to the development of potential therapeutic candidates with neuroproliferative or neuroprotective effects from several clinical drugs, particularly in antibiotics. For instance, minocycline and beta-lactams have been reported to exhibit neuroprotective effect on both *in vitro* and *in vivo* disease models [13–15]. However, these drugs may have significant side effects or diminishing efficacy over time [12, 16, 17]. Therefore, there is an urgent need to develop novel therapeutic agents that could improve efficacy and reduce side effects for treating or delaying the symptoms in patients with PD.

Functional peptides have been reported to exhibit various bioactivities, such as antimicrobial, antivirus, anticancer and neuroprotective effects, alone with their high selectivity and specificity for the targets [18]. For instance, lumbricusin, an 11-mer antimicrobial peptide (NH_2-RNRRWCIDQQA), is extracted from earthworms and has been identified to exhibit neuroprotective effects, reduce neuroinflammatory activity, and improve dopaminergic neurogenerative effects *in vivo* [19–21]. Tripeptide MSP8 (ARW-COOH) isolated from monkfish swim bladders (MSBH) has been shown to exhibit potent antioxidant effects in radical scavenging assays and demonstrate cytoprotective effects *in vitro* [22]. Furthermore, it has been shown to upregulate the expression of Nrf2 and HO-1 proteins to activate the Nrf2 pathway for reducing excessive ROS levels and alleviating cellular oxidative stress [23]. Pituitary adenylate cyclase-activating polypeptides (PACAPs), have been shown to exhibit neuroprotective effect on nerve cells through reducing the intracellular ROS or increasing antioxidant enzyme activity [24, 25]. In addition, the macin family of antimicrobial proteins, including hydramacin-1, neuromacin, and theromacin, has been reported to not only exhibit potent antimicrobial activity but also display nerve repair effects [26–28]. It is worth mentioning that a cluster of six amino acid residues (Ser^{42}, Leu^{51}, Asn^{53}, Asn^{54}, Lys^{55}, and Gln^{56}) within the C-terminal sequence of neuromacin and theromacin associated with nerve repair effects [27]. As a result, these peptides have considerable potential for the development of therapeutic candidates for neurodegenerative diseases.

In this study, we investigated the bioactivities of the C-terminal sequence .in hydramacin-1 from Arg^{30} to Ala^{54} and shortened it into overlapping 11-residue peptides (THL, THL-1, THL-2, and THL-3). A series of truncated THL peptides contains the amino acid cysteine (Cys) in their sequence, while cysteine is an effective hydrogen donor and can increase the antioxidant activity of peptides [29]. In order to determine the antioxidant activity and neuroproliferative effects of these peptides, they were synthesized using solid-phase peptide synthesis (SPPS), purified by reversed-phase high-performance liquid chromatography (RP-HPLC), and identified via matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). Their antioxidant activities were measured using the ABTS and DPPH radical scavenging assays. Moreover, the neuroproliferative and neuroprotective effects of the peptides were evaluated in human neuroblastoma SH-SY5Y cells, a suitable *in vitro* model for Parkinson's disease due to their dopaminergic neuron-like characteristics [30]. Circular dichroism and the I-TASSER server [31, 32] were used to analyze the secondary structure of the peptides. In conclusion, the findings could potentially realize the structure-activity relationship of hydramacin-1 in its C-terminal sequence, providing a novel candidate for developing therapeutic agents against neurodegenerative diseases.

2. Materials and Methods

2.1. Materials. The N_α-Fmoc and side chain protected derivatives of amino acids for solid phase peptide synthesis were purchased from Anaspec Inc. (Fremont, CA, USA). Peptide amide linker (PAL) resin, piperidine, phenol, trifluoroacetic acid (TFA), and 1,2-ethanedithiol (EDT) were purchased from Merck (Darmstadt, Germany). N,N-diisopropylethylamine (DIPEA) and thioanisole were purchased from Alfa Aesar (Heysham, Lancashire, UK). Hydroxybenzotriazole (HOBt) and (1H-benzotriazol-1-yloxy) (dimethyl-amino)-N,N-dimethylmethaniminium hexafluorophosphate (HBTU) were purchased from AgeneMax Co. (Taipei, Taiwan). Dimethylformamide (DMF) was purchased from Duksan Co., Ltd (Ansan city, Kyunggi, Korea). Dichloromethane (DCM) and acetonitrile (ACN, HPLC grade) were purchased from Avantor (Center Valley, PA, USA). Semipreparative scale Nucleodur C_{18} HTec column (250×10 mm, particle size 5.0 μm) was purchased from Macherey-Nagel (Düren, Germany). 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), potassium persulfate, and tert-butyl hydroperoxide (t-BHP) were purchased from Merck Millipore (Burlington, MA, USA). 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) and sodium dodecyl sulfate (SDS) were purchased from USB corporation (Cleveland, OH, USA). Dulbecco's modified Eagle medium (DMEM), HEPES buffer, penicillin, and streptomycin (PS) were purchased from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Biological Industries (Cromwell, CT, USA). Dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS, pH = 7.4), lactate dehydrogenase (LDH) cytotoxicity assay kit, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl- tetrazolium bromide (MTT) cell proliferation reagent were obtained from Thermo Scientific (Waltham, MA, USA).

2.2. Synthesis and Characterization of Truncated THL and Alanine-Scanning THL-3 Series Peptides. A series of truncated THL and alanine-scanning THL-3 peptides were synthesized using solid-phase peptide synthesis [33] with Fmoc/tBu chemistry [34] and our own manual procedures [28, 35]. Briefly, the PAL resin was swelled in DMF for 5 min at room temperature, and the Fmoc-protecting group was removed from the resin using 20% piperidine in DMF for 15 min and repeated four times. The N_α-Fmoc, side-chain-protected amino acid was activated by mixing with the coupling reagent (HOBt/HBTU/DIPEA, 1 : 1 : 2) for 5 min and added to the reaction vessel for coupling with the resin at room temperature for 1.5 h. The coupling reactions and removal of the Fmoc group were monitored by the ninhydrin test [36]. Cycles of deprotection of Fmoc and coupling with the subsequent amino acids were repeated to synthesize the desired peptide-bound resin. After peptide synthesis was completed, the peptide-bound resin was cleaved from the resin using a TFA cleavage solution (TFA/water/phenol/thioanisole/EDT, 82.5 : 5 : 5 : 2.5). The crude

peptides were purified by reverse-phase high-performance liquid chromatography (RP-HPLC; Hitachi, Chiyoda-ku, Tokyo, Japan) on a C_{18} column using a linear gradient of 0 to 90% ACN in 0.05% TFA for 50 min. Each peptide was characterized by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS; Bruker, Billerica, MA, USA).

2.3. ABTS and DPPH Radical Scavenging Assay. To determine whether the designed peptides have antioxidant activity, we used the ABTS assay [37] and the DPPH assay [38] to analyze the radical scavenging activity of each peptide. In the ABTS assay, ABTS solution (7 mM) was prepared by dissolving ABTS powder in distilled-deionized water. The working ABTS solution was prepared by mixing 7 mM of ABTS solution with 2.45 mM of potassium persulfate and mixing in the dark at room temperature for 12 to 16 h. The working ABTS solution (180 μL) was reacted with 20 μL of PBS (blank) or different concentrations of peptide/ascorbic acid (2.5, 5, 10, 20, 40, 70, and 100 μM) for 10 min in the dark. After the reaction was completed, the absorbance of each reaction mixture was detected at a wavelength of 734 nm by using an ELISA microplate reader. The percentage of ABTS-radical scavenging activity was determined with the following equation:

$$\text{Scavenging (\%)} = \frac{Abs_{blank} - Abs_{sample}}{Abs_{blank}} \times 100. \quad (1)$$

In the DPPH radical scavenging assay, DPPH powder was dissolved in methanol to prepare a 200 μM of DPPH solution. PBS buffer (20 μL, blank) or different concentrations of peptide/ascorbic acid (2.5, 5, 10, 20, 40, 70, and 100 μM) were mixed with 80 μL of Tris-HCl buffer (100 mM, pH = 7.4). Then, the mixture reacted with 100 μL of 200 μM DPPH solution for 30 min in the dark. The absorbance of each reacted mixture was detected using an ELISA microplate reader with a detection wavelength of 517 nm. The percentage of scavenging activity on DPPH-induced free radicals was determined with the following equation:

$$\text{Scavenging (\%)} = \left(1 - \frac{Abs_{sample}}{Abs_{blank}}\right) \times 100. \quad (2)$$

After evaluating the antioxidant activities of the peptides using the ABTS and DPPH radical scavenging assays, we determined the half-maximal effective concentration (EC_{50}) of each sample, which represents the concentration of ascorbic acid or peptides required to achieve a 50% reduction in the initial concentration of DPPH and ABTS free radicals [39].

2.4. Measurement of Cell Proliferation in SH-SY5Y Cells. To determine whether the human neuroblastoma SH-SY5Y cells were influenced by the truncated THL series peptides, we measured the cell proliferation of the peptides by the MTT assay [19, 40]. Briefly, 4×10^4 SH-SY5Y cells/well in DMEM containing 5% FBS were seeded in a 96-well culture plate for 24 h at 37°C in 5% CO_2. The cells were treated with culture medium (control) or different concentrations of the peptides (25, 50, 75, and 100 μM) for 24 h at 37°C in 5% CO_2. After the culture medium containing the peptides was removed, 50 μL of MTT solution (1.0 mg/mL) was added to each well and further incubated for 2 h at 37°C. The formazan salt reaction product of MTT was dissolved in 100 μL of DMSO for 5 min. The optical density (OD) of each solution was detected at 550 nm by using an ELISA microplate reader (Spectramax, Sunnyvale, CA, USA).

Human neuroblastoma SH-SY5Y cells (ATCC, CRL-2266) were kindly provided by Dr. Teng-Nan Lin (Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan). SH-SY5Y cells were cultured in DMEM containing 5% FBS at 37°C in 5% CO_2. The complete culture medium was replaced every 3 days.

2.5. Neuroprotective Effects of the Designed Peptides in SH-SY5Y Cells. To determine whether the designed peptides with antioxidant activity exert neuroprotective effects in SH-SY5Y cells, we used tert-butylhydroperoxide (t-BHP) as an exogenous oxidizing agent to induce a high level of intracellular reactive oxygen species (ROS) in SH-SY5Y cells [6]. We measured the cell viability of the peptides by lactate dehydrogenase (LDH) activity. Specifically, 4×10^4 SH-SY5Y cells/well in DMEM containing 5% FBS were seeded in a 96-well culture plate for 24 h at 37°C in 5% CO_2. The cells were then treated with t-BHP (100 μM) alone or in combination with the peptides (100 μM) for 24 h at 37°C. The neuroprotective effects of each peptide in the ROS-induced SH-SY5Y cells were measured using the LDH assay kit.

$$\text{LDH release (\%)} = \frac{Abs_{sample\ (490nm)} - Abs_{sample\ (680nm)}}{Abs_{blank\ (490nm)} - Abs_{blank\ (680nm)}} \times 100. \quad (3)$$

2.6. Prediction of Preliminary Structure of the Designed Peptides Using I-TASSER. A preliminary three-dimensionalmodel for the truncated THL series peptides and THL-3-PTD5 peptide was determined by analysis of the peptide amino acid sequence with version 5.1 of the I-TASSER program [31, 41], using the automated I-TASSER web servicer (https://zhanggroup.org/I-TASSER/) [32]. I-TASSER is a homology algorithm that models specific regions of the protein using the molecular coordinates of multiple PDB depositions. The output for a predicted three-dimensional structure was a PDB file, and the accuracy of these models was estimated using such parameters as C-score that is a confidence score for estimating model quality.

2.7. Secondary Structure of the Peptides Using Circular Dichroism (CD). A stock solution of 1.0 mM of the peptide was prepared by dissolving in PBS or 30 mM SDS, and then diluted to 10 μM. The CD spectrum of each peptide was detected by using Jasco J-715 CD spectrometer (Jasco Inc., Easton, MD, USA). The CD spectrum of peptide was recorded at room temperature, and then scanned three times

at the range from 190 to 240 nm with a bandwidth of 1.0 nm, a response time of 2 sec, a scan speed of 100 nm/min and a resolution of 1.0 nm.

2.8. Statistical Analysis. All experiments were conducted in triplicate unless otherwise specified. Data are presented as the mean ± standard deviation (SD).

3. Results and Discussion

3.1. Design and Characterization of the Truncated THL Series Peptides. Hydramacin-1, a protein of the macin family, exhibits potent antimicrobial activity and increases the viability of the murine neuroblastoma cells. Based on sequence analysis of the macin family, a cluster of six amino acid residues (Ser^{42}, Leu^{51}, Asn^{53}, Asn^{54}, Lys^{55}, and Gln^{56}) within the β-loop of neuromacin and theromacin is associated with nerve proliferation and repair effects [27, 42]. It is evident that the neuroproliferative effects of the macin family may be related to the amino acid residues in its C-terminal sequence. To investigate whether the C-terminal sequence of hydramacin-1 exhibits neuroproliferative effects, we designed a series of truncated peptides based on the C-terminal sequence of hydramacin-1 from Arg^{30} to Ala^{54} and further shortened it into overlapping 11-residue peptides (THL, THL-1, THL-2, and THL-3). Moreover, the truncated THL series peptides contained the antioxidant amino acid of cysteine (Cys), which has been reported to be an effective hydrogen donor and can increase the antioxidant activity of peptides [29, 43]. Thus, we determined their antioxidant activity and investigated which amino acid residues played crucial roles in peptides using alanine-scanning analysis, thereby further exploring the relationship between neuroproliferative effects and antioxidant activity.

A series of the truncated THL peptides were successfully synthesized and purified. The purity (>90%) and identity of the peptides were verified by RP-HPLC and MALDI-TOF MS (Figures S1 and S2). In addition, the free cysteine in the truncated THL series peptides did not spontaneously form disulfide bonds and multimers in the experimental condition of PBS or methanol solutions because their measured and theoretical molecular weights were the same in their mass spectra (Figure S3). The measured mass, retention time, and sequence of the peptides are summarized in Table 1.

3.2. Antioxidant Activity of the Truncated THL and Alanine-Scanning THL-3 Peptides. The antioxidant activity of truncated THL series peptides, including their half-maximal effective concentration (EC_{50}), was evaluated using the ABTS and DPPH radical scavenging assays, which are commonly used to measure the antioxidant activity of the sample in water- and oil-soluble conditions, respectively [44, 45]. According to the results (Table 2), THL-3 exhibited optimal antioxidant activity in the ABTS assay (EC_{50} is 22.5 μM), while THL showed superior antioxidant activity in the DPPH assay (EC_{50} is 53.8 μM) compared to other truncated series peptides. Although THL had lower EC_{50} value in the DPPH assay than others, its radical scavenging efficiency obviously reached saturation above 40.0 μM in the ABTS assay (Figure 1). In contrast, the EC_{50} value of THL-3 in the ABTS assay was close to ascorbic acid (EC_{50} is 22.6 μM), a natural antioxidant, and had similar radical scavenging efficiency with THL at 100 μM in DPPH assay. In addition, several studies have reported that the specific amino acids with positively charged and hydrophobic characteristics, such as Arg, Lys, Pro, Leu, Val, and Ala, can enhance the antioxidant activity of peptides through their efficient radical scavenging abilities [46–49]. Therefore, THL-3 exhibited the strongest antioxidant activity compared to other truncated THL series peptide, which can be attributed to a high percentage of positively charged and hydrophobic amino acids in its sequence.

To determine which amino acid residues are influenced on the antioxidant activity of THL-3, we performed the alanine-scanning analysis by individually replacing each residue in the sequence of THL-3 with alanine, and further synthesized THL-3-A1 to THL-3-A10 for evaluating their antioxidant activity in the ABTS and DPPH assays. In addition, we calculated the total difference in EC_{50} values between THL-3 and the series of THL-3-A peptides to effectively identify the residues that influenced the antioxidant activity of THL-3. A total difference in EC_{50} value less than −20 indicated that the amino acid residue significantly influenced the antioxidant activity of THL-3. Results (Table 3 and Figure 2) showed that the total difference in EC_{50} value of the following peptides (THL-3-A5, -A6, -A7, -A9 and -A10) was less than −20, indicating that Cys^5, Pro^6, Leu^7, Lys^9, and Lys^{10} as essential residues for the antioxidant activity in THL-3. In addition, the thiol group of Cys can interact with the reactive oxygen species, thereby enhancing the antioxidant activity of peptides and protecting cells from oxidative stress [47]. As a result, the substitution of the fifth amino acid residue with alanine resulted in a loss of the antioxidant activity of THL-3. Obviously, cysteine (Cys), with the sulfhydryl (SH) group played the most important role in antioxidant activity on THL-3.

Moreover, THL-3 was further conjugated with the cell-penetrating PTD5 peptide, which is commonly utilized for protein transduction [50], to ensure successful transport of THL-3 into the cells. The antioxidant activity in EC_{50} values of THL-3- PTD5 were 27.1 μM and 40.5 μM in the ABTS and DPPH assay, respectively. Results indicated that PTD5 did not influence the radical scavenging efficiency of THL-3 and even enhanced that of antioxidant activity. Therefore, THL-3- PTD5 was used for the following experiment to explore its bioactivities in the nerve cells.

3.3. Neuroproliferative Effect of THL-3-PTD5 and Truncated THL Series Peptides. To investigate the neuroproliferative effect and cytotoxicity of THL-3-PTD5 and truncated THL series peptides, the cell viability of human neuroblastoma SH-SY5Y cells was determined in treatment with various peptide concentrations (25, 50, 75, and 100 μM) using MTT

Table 1: Physicochemical characterization of truncated THL series peptides.

Type	Peptide	Sequence	Theoretical mass (Da)[a]	Measured mass (Da)[b]	RP-HPLC (R_t, min)[c]	Purity (%)[d]
Truncated peptide of hydramacin-1	THL	RCKELGRKRGQCEEKPSRCPLSKKA-NH_2	2887.45	2887.6	14.23	98.3
	THL-1	RCKELGRKRGQ-NH_2	1329.60	1329.7	12.69	97.8
	THL-2	KRGQCEEKPSR-NH_2	1316.51	1316.7	12.05	95.6
	THL-3	KPSRCPLSKKA-NH_2	1213.51	1213.8	13.25	98.6
Alanine-scanning of THL-3	THL-3-A1	APSRCPLSKKA-NH_2	1156.41	1157.7	8.74	99.7
	THL-3-A2	KASRCPLSKKA-NH_2	1187.46	1188.7	10.18	99.9
	THL-3-A3	KPARCPLSKKA-NH_2	1197.50	1198.8	7.71	99.7
	THL-3-A4	KPSACPLSKKA-NH_2	1128.39	1129.6	9.01	99.4
	THL-3-A5	KPSRAPLSKKA-NH_2	1181.43	1179.8	4.95	95.1
	THL-3-A6	KPSRCALSKKA-NH_2	1187.46	1188.7	6.11	95.7
	THL-3-A7	KPSRCPASKKA-NH_2	1171.42	1170.2	4.26	92.4
	THL-3-A8	KPSRCPLAKKA-NH_2	1197.50	1198.0	8.57	99.3
	THL-3-A9	KPSRCPLSAKA-NH_2	1156.41	1156.8	9.47	99.0
	THL-3-A10	KPSRCPLSKAA-NH_2	1156.41	1156.8	9.41	99.9
Cell-penetrating	PTD5	YARAARRAARR-NH_2	1316.54	1316.5	9.98	99.2
	THL-3-PTD5	KPSRCPLSKKA-YARAARRAARR-NH_2	2513.02	2513.5	12.10	98.0

[a]Theoretical molecular weight of the peptides was calculated using the software provided in the website: https://www.bachem.com/knowledge-center/peptide-calculator/. [b]Molecular weight of peptides were determined by MALDI-TOF MS. [c]Retention time (R_t) was determined by RP-HPLC using the C_{18} column. [d]Purity of the peptides was calculated using the following formula on HPLC: peak area of peptide/peak area of all.

Table 2: Antioxidant activity of truncated THL series peptides.

Peptide	EC_{50}[a,b] (μM)	
	ABTS	DPPH
THL	40.0 ± 3.8	53.8 ± 3.0
THL-1	42.3 ± 5.6	71.3 ± 7.5
THL-2	31.4 ± 6.8	78.3 ± 9.1
THL-3	22.5 ± 3.7	67.8 ± 4.6
THL-3-A1	36.4 ± 5.8	53.0 ± 4.8
THL-3-A2	28.8 ± 4.2	52.1 ± 5.7
THL-3-A3	27.1 ± 4.7	75.0 ± 8.6
THL-3-A4	13.7 ± 3.5	59.1 ± 6.8
THL-3-A5	N.D.[c]	N.D.[c]
THL-3-A6	73.8 ± 7.6	50.1 ± 9.8
THL-3-A7	78.8 ± 4.6	84.6 ± 4.2
THL-3-A8	37.2 ± 5.2	67.6 ± 7.2
THL-3-A9	57.2 ± 3.9	60.0 ± 4.8
THL-3-A10	60.1 ± 3.0	50.5 ± 4.6
PTD5	70.0 ± 6.7	N.D.[c]
THL-3- PTD5	27.1 ± 3.2	40.4 ± 4.3
Ascorbic acid	22.6 ± 2.25	24.5 ± 5.1

[a]EC_{50} was determined as the half-maximal effective concentration of peptide that scavenged 50% reactive oxygen species. [b]All data were indicated that means ± SD obtained in the experiment in triplicate. [c]N.D. is meaning that antioxidant activity of peptide is not detected in the experiment dose.

assay. According to the results (Figure 3(a)), none of the peptides exhibited cytotoxicity in SH-SY5Y cells, while THL and THL-3-PTD5 showed cell proliferative effect on SH-SY5Y cells. Compared to the control, treatment with THL at concentrations of 25 and 100 μM increased the viability of SH-SY5Y cells to 38% and 70%, respectively. In addition, when THL-3 was conjugated with the cell-penetrating PTD5 peptide to form THL-3-PTD5, the viability of SH-SY5Y cells enhanced by 118% at a concentration of 100 μM, while THL-3 and PTD5 alone did not show the neuroproliferative effect on SH-SY5Y cells. It also displayed a significant dose-dependent effect at the experimental doses. Moreover, Cell-penetrating PTD peptides have been demonstrated to successfully transport the protein or peptides into mammalian cells *in vitro* and *in vivo*, which can interact with membrane phospholipids through their α-helical structures [50–52]. Then, one of the PTD peptides, PTD4, has exhibited neuroprotective effect on the model of acute ischemic stroke *in vitro* by protecting against mitochondrial dysfunction [52]. This suggests that PTD5 may have effectively transported THL-3 into the cells, enabling THL-3 to exert its maximal antioxidant activity in the cells for enhancing its neuroproliferative effect on SH-SY5Y cells. Therefore, our findings demonstrate that THL-3-PTD5 had the strongest neuroproliferative effect compared to other truncated series peptides.

3.4. Neuroprotective Effect of THL-3-PTD5 and Truncated THL Series Peptides. To evaluate whether the peptides have a neuroprotective effect on SH-SY5Y cells through their antioxidant activities, t-BHP was used as an exogenous oxidizing agent to induce a high level of intracellular ROS in SH-SY5Y cells. In addition, the LDH assay was used to measure the neuroprotective effect of the peptides. Based on the results (Figure 3(b)), treatment with t-BHP at a concentration of 100 μM significantly damaged the nerve cells and increased the percentage of LDH release by 116% in SH-SY5Y cells. However, the series of truncated THL peptides and THL-3-PTD5 did not reduce the percentage of LDH release in the t-BHP-induced SH-SY5Y cells. Although THL-3-PTD5 exhibited potent antioxidant activity and promoted neuroproliferative effect on SH-SY5Y cells, it did not demonstrate the neuroprotective effects under the high levels of ROS induced by the exogenous oxidizing agent t-BHP. This suggests that the antioxidant activity of the peptides might not directly translate into a neuroprotective effect on SH-SY5Y cells to enhance the viability of nerve cells. On the other hand, several neurotoxic models, such as 6-hydroxydopamine (6-OHDA) and serum deprivation, have been shown to increase intracellular oxidative stress *in vitro* [53, 54]. Furthermore, the expression of cyclin D1 can be promoted by the activation of the P13 K/Akt or ERK1/2 pathway, which increases nutrient utilization, such as glucose and glutamine, in the cells, thereby enhancing the cell viability [55, 56]. It is important to note that the mechanism by which THL-3-PTD5 has neuroproliferative effects is unclear in the present study. Therefore, future studies will further investigate the relationship between THL-3-PTD5 and nerve cell viability, as well as use other neurotoxic models to induce intracellular oxidative stress *in vitro*. This may enable a comprehensive understanding of the cell signaling pathways for the neuroproliferative effects of THL-3-PTD5 on nerve cells.

3.5. Structure Analysis of THL-3-PTD5 and Truncated THL Series Peptides. To investigate the relationship between the antioxidant activities of truncated THL series peptides and their secondary structures, CD spectroscopy was used to analyze the secondary structure of the peptides. In addition, the I-TASSER server was used to predict their three-

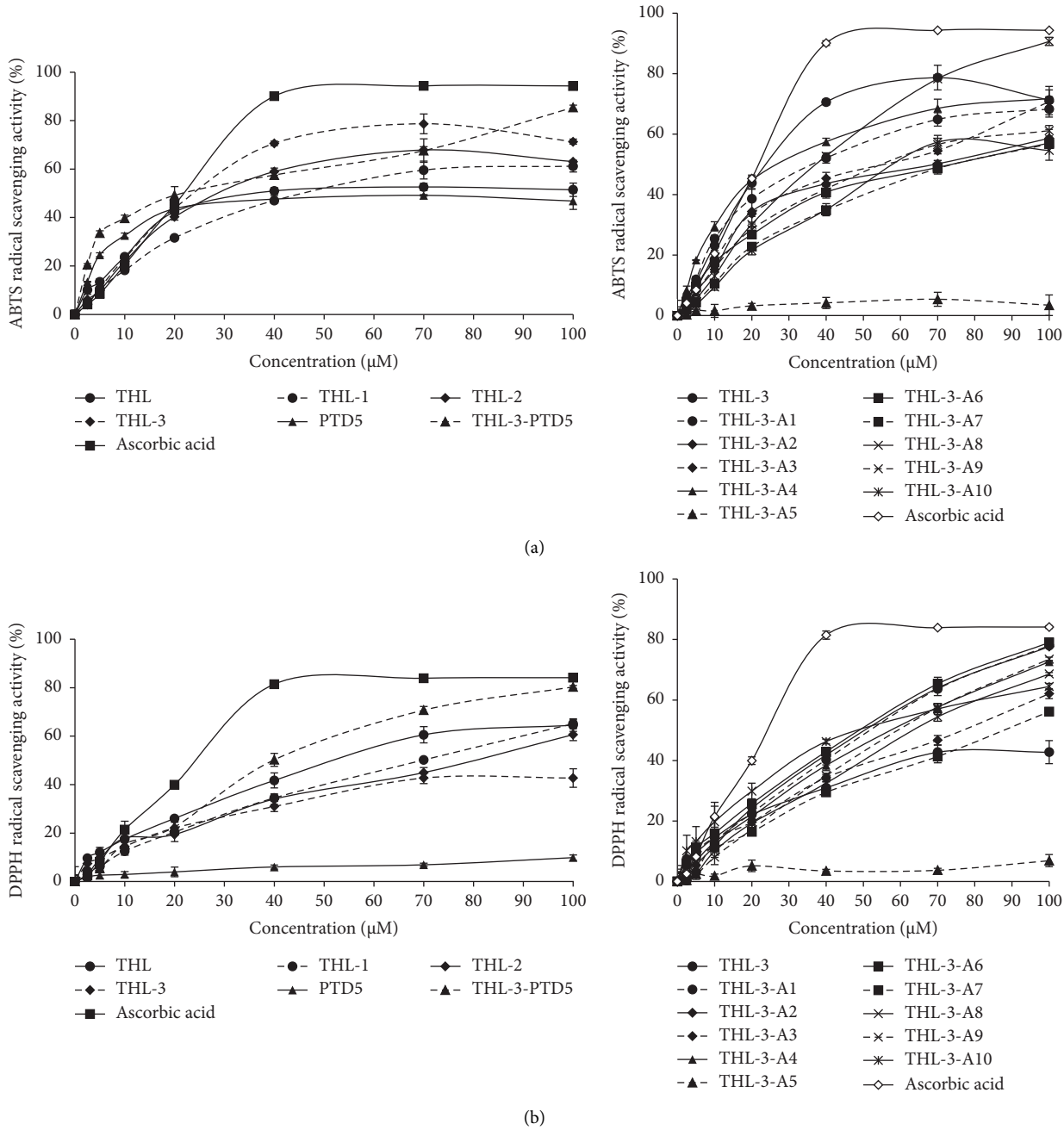

Figure 1: Antioxidant activity of a series truncated THL and alanine-scanning THL-3 peptides in (a) ABTS and (b) DPPH radical scavenging assay. The results were indicated that means ± SD obtained in the experiment in triplicate.

dimensional structures. According to the results, all truncated THL series peptides exhibited a negative absorption peak at 198 nm in the CD spectra (Figure 4(a)), indicating that all peptides shared the same secondary structure in PBS solution. Subsequently, Selcon-3 software was utilized to calculate the secondary structure content of the peptides. The ratios of random coil for THL, THL-1, THL-2, and THL-3 were 68.1%, 69.2%, 75.3%, and 50.3%, respectively. Furthermore, the three-dimensional structure of the peptides, as predicted by the I-TASSER server (Figure 4(b)), confirmed that the truncated THL series peptides did not have a specific secondary structure. However, the predicted three-dimensional structure of THL-3-PTD5 demonstrated that the secondary structure of THL-3 maintained a random coil structure when conjugated with PTD5. In addition, several studies have indicated that the amino acids in the sequence are a major factor for influencing the antioxidant activity of peptides, as well as secondary structure may be a minor factor due to their low molecular weight [57]. Therefore, this suggests that the antioxidant activity of truncated THL series peptides is related to their amino acid composition rather than their secondary structures.

Table 3: Comparison of antioxidant activity of the alanine-scanning THL-3 peptide in both the ABTS and DPPH radical scavenging assay.

Peptide	Difference of EC_{50}[a] (μM)		Total[c]
	ABTS	DPPH	
THL-3	0	0	0
THL-3-A1	−13.9	14.8	0.9
THL-3-A2	−6.3	15.7	9.4
THL-3-A3	−4.6	−7.2	−11.8
THL-3-A4	8.8	8.7	17.5
THL-3-A5	N.D.[b]	N.D.[b]	−100
THL-3-A6	−51.3	17.7	−33.6
THL-3-A7	−56.3	−16.8	−73.1
THL-3-A8	−14.7	0.2	−14.5
THL-3-A9	−34.7	7.8	−26.9
THL-3-A10	−37.6	17.3	−20.3

[a]Difference of EC_{50} was calculated using the following formula: EC_{50} (THL-3) − EC_{50} (sample). [b]N.D. indicates that the antioxidant activity of the peptide was not detected, and a value of −50 was used to calculate the total difference. [c]The total difference value of less than −20 indicates that the original amino acid plays more important roles in the antioxidant activity of THL-3.

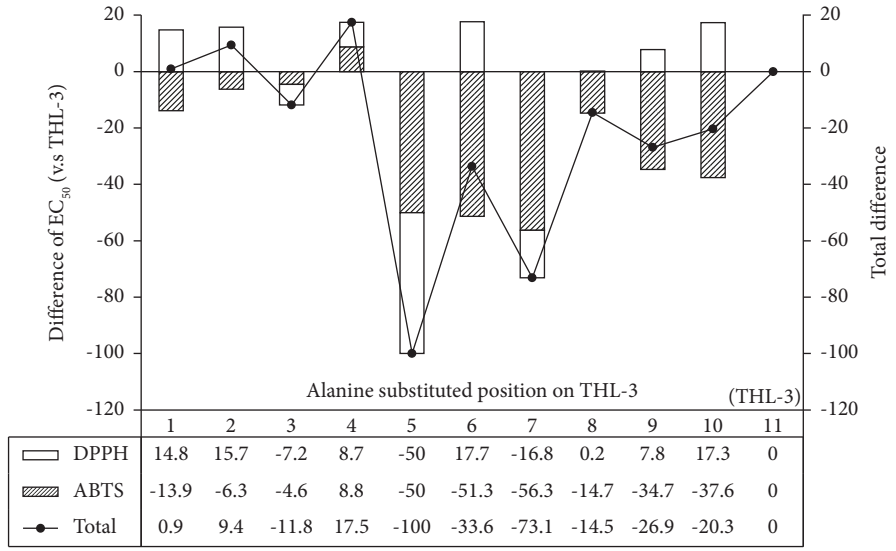

Figure 2: Relationship between amino acid position and antioxidant activity in THL-3 peptide by the total difference of ABTS and DPPH radical scavenging activity.

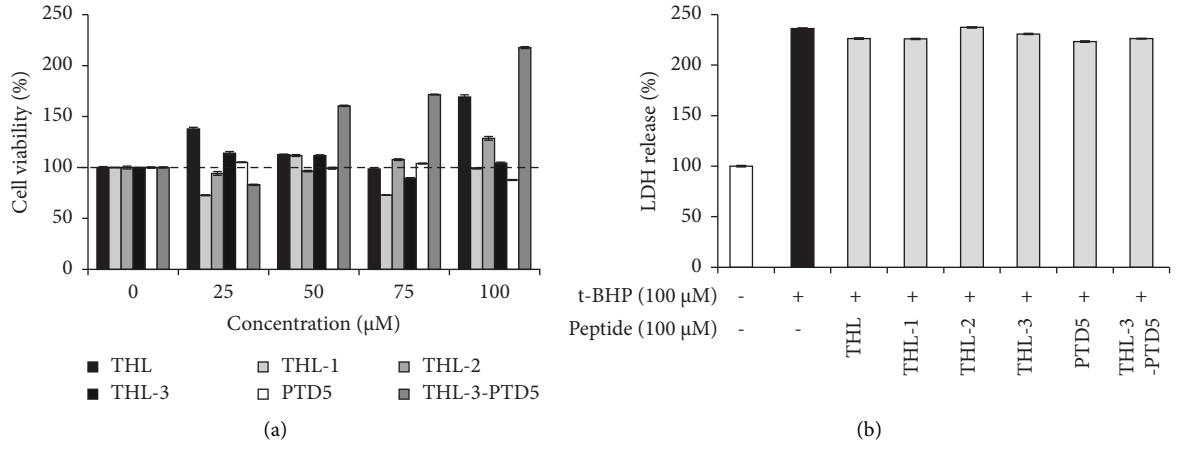

Figure 3: Bioactivity of truncated THL series peptides on (a) neuroproliferation and (b) neuroprotection in human neuroblastoma SH-SY5Y cells. The results indicate that the means ± SD obtained in the experiment in triplicate.

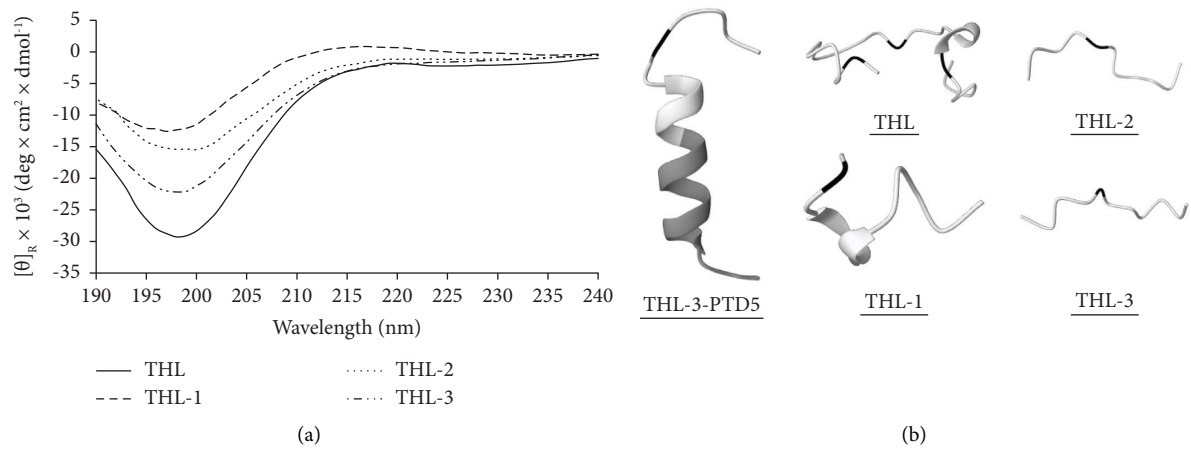

Figure 4: Structural analysis of truncated THL series peptide. (a) Circular dichroism spectra of the peptides in PBS solution using circular dichroism. (b) Predicated three-dimensional structure of the peptides using I-TASSER [32]. The residue of cysteine and the sequence of PTD5 is color coded as black and grey, respectively.

4. Conclusion

In this study, we demonstrated that THL-3-PTD5 exhibited strong antioxidant activity in the ABTS and DPPH radical scavenging assays and promoted neuroproliferative effects on SH-SY5Y cells, making it a potential candidate for developing therapeutic agents for neurodegenerative diseases. However, the present study only evaluated the activity and safety of the peptides *in vitro*, and the mechanisms of action for THL-3-PTD5 have not been clearly identified in this study. Further investigation into the effects of THL-3-PTD5 on nerve cell viability and associated signaling pathways will help to improve the application for the animal models *in vivo*.

Data Availability

The data that support the findings of this study are available from the corresponding author, Fend-Di T. Lung, upon reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

PCL and FDTL conceptualized the study. PCL and CCL proposed the methodology; PCL, CCL, and HHH performed formal analysis. PCL, CCL, and HHH investigated the study. FDTL was responsible for the resources. PCL wrote the original draftPCL, CCL, HHH, and FDTL performed writing, review, and editing. FDTL was responsible for funding acquisition.

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Supplementary Materials

Chromatograms and mass spectra of all designed peptides were obtained using RP-HPLC and MALDI-TOF MS, respectively, and are provided as supplementary materials. Supplementary Figure 1: Qualitative analysis of THL series peptides. (A) Retention time of each peptide was determined by RP-HPLC on C_{18} column, (B) Molecular weight of each peptide was detected by MALDI-TOF MS. Supplementary Figure 2: Qualitative analysis of alanine-scanning/cell-penetrating THL-3 peptides by using RP-HPLC on C_{18} column and MALDI-TOF MS. Such as (A) PTD5, (B) THL-3-PTD5, and (C)~(L) THL-3-A1 to THL-3-A10. Supplementary Figure 3: Analysis of THL series peptide was dissolved in PBS or methanol by using MALDI-TOF MS. (*Supplementary Materials*)

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