Preparation of Nereid oligopeptide and investigation of the mechanism underlying the induction of apoptosis in human lung cancer H1299 cells

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Abstract. In the present study, oligopeptides from Nereid (Perinereis aibuhitensis) were prepared via enzymatic hydrolysis, and the mechanism underlying the induction of apoptosis in H1299 cells was investigated. According to the analysis of the inhibition rate on proliferation, alkaline protease demonstrated the best enzymatic efficiency. The optimal conditions for hydrolysis were as follows: 50˚C and pH 10 for 6 h; a material-to-liquid ratio of 1:1 (g/ml); and addition of 400 U/g enzyme. The hydrolysates were purified using ultrafiltration, anion chromatography, gel filtration chromatography, and high-performance liquid chromatography. The nereid oligopeptide (noP), with a molecular weight of 841 kDa and an amino acid sequence of glutamine-isoleucine-asparagine-glutamine-histidine-leucine, was obtained. noP inhibited the proliferation of H1299 cells in a time- and dose-dependent manner. Morphological changes and apoptosis were also induced by noP in H1299 cells. The western blot analysis revealed that the B-cell lymphoma 2/Bcl-2 associated X (Bcl-2/Bax) ratio was reduced by 24.7% in the noP treatment group compared with the control group. The relative expression levels of cleaved caspase-9 (cleaved-casp9) and cleaved caspase-3 (cleaved-casp3) in the noP treatment group were 2.55- and 1.71-fold higher than those measured in the control group, respectively. These results suggested that noP exerts antitumor effects by influencing the proliferation and apoptosis of H1299 cells.

Introduction

Lung cancer is one of the most common malignancies worldwide, and associated with high rates of morbidity and mortality (IARC, 2020). There are few early symptoms, and their onset is relatively insidious. Therefore, most cases are confirmed at mid and late stages of lung cancer. Currently, chemotherapy and surgery are the most commonly used treatment modalities, which can increase the survival rate of patients. However, long-term chemotherapy also severely impacts important organs and induces drug resistance in tumors (1). The marine environment, characterized by high salt content, high pressure, and low temperature, markedly differs from the terrestrial environment. Numerous marine organisms can produce massive active substances with novel structures that differ from those synthesized by terrestrial organisms. Recent research on marine drugs has found that numerous bioactive substances of marine origin can significantly inhibit tumor growth in various ways (2,3). Therefore, there is increasing interest in discovering new anticancer drugs with low toxicity and high efficacy from marine organisms. It is expected that a diverse array of active substances of marine origin, such as peptides, polysaccharides, alkaloids, terpenes, and macrolides, will be developed into new antitumor drugs (4). For example, dolastatin-10 (obtained from the sea hare) has been used in clinical trials (5).

Nereids are invertebrates of the polychaete family (Nereididae). They are widely distributed in the Sea of Japan and the Pacific coast, and endemic to Japan and China. In recent years, the interest in the bioactive substances produced by the Nereids has gradually increased. Japanese scholars have isolated biologically active polypeptides from the Nereids (6). These polypeptides can strongly shrink the esophagus of annelids. The first isolation and purification of a protease from the Nereids was achieved at Bethune School of Medicine, Jilin University (Jilin, China). This protease is a serine protease with a molecular weight of 29 kDa, an isoelectric point of 4.4, and an optimal temperature of 60˚C. The protease exerts a strong effect on the degradation of fibrin and fibrinogen. The order of hydrolysis is as follows: Aα, Bβ, and γ chains. Studies suggested that the protease isolated from the Nereids can significantly inhibit various leukemic cells in vitro (7). The protease Nereis virens proteinase (aSP) also has strong antitumor activity in leukemic cells and lung cancer.

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Abbreviations: noP, Nereid oligopeptide; casp3, caspase-3; casp9, caspase-9; IR, inhibition rate

Key words: Perinereis aibuhitensis Grube, oligopeptides, antitumor, H1299 cells, apoptosis
SC-P-A-1 cells (8-12). However, there is limited knowledge on the enzymatic hydrolysis of Nereid oligopeptides (NOP), and their inhibitory effects on non-small cell lung cancer H1299 cells. The objective of the present study was to investigate the preparation of an NOP and its effects on the growth of H1299 cells. Moreover, the induction of cell apoptosis and the mechanism of action are preliminarily discussed, thereby providing an experimental basis for the clinical application of NOP.

Materials and methods

Samples and reagents. The Nereid was purchased from Zhoushan Jusha Aquaculture Co., Ltd., and was identified as Perinereis aibuhitensis Grube by Professor Zhao Shenglong from Zhejiang Ocean University (Zhoushan, China). Trypsin, papain, alkaline protease, neutral protease, and pepsin were purchased from YTHX Biotechnology Co., Ltd. Fetal bovine serum was obtained from Hangzhou Sijiqing Biological Engineering Co., Ltd. RPMI-1640 powder and medium were purchased from Gibco; Thermo Fisher Scientific, Inc. Methyl thiazolyl tetrazolium (MTT) and dimethyl sulfoxide were purchased from Gibco; Thermo Fisher Scientific, Inc. Fetal bovine serum was obtained from Hangzhou Sijiqing Biological Engineering Co., Ltd. rPMi-1640 nutrient solution containing 10% bovine serum. All reagents were of analytical grade.

Cells and cell culture. Human lung cancer cell lines [A549 (cat. no. SCSP-503), H1299 (cat. no. SCSP-589) and 95C (cat. no. SNL-168)] and neuroblastoma [Pc-12 (cat. no. SCSP-517), H1299 (cat. no. SCSP-589) and 95c (cat. no. Snl-168)] and were purchased from Sigma-Aldrich; Merck KGaA. The other reagents were of analytical grade.

Equipment and instruments. The equipment and instruments included the following: refrigerated centrifuge (NUAIRE), carbon dioxide incubator and microplate reader (both from Thermo Fisher Scientific, Inc.), protein electrophoresis and western blotting system (Bio-Rad Laboratories, Inc.), Odyssey Imager (LI-COR Biosciences), horizontal shaker (Hangzhou Miu Instruments Co., Ltd.), clean bench (Suzhou Cleaning Equipment Co., Ltd.), 80°C ultra-low temperature incubator (Haier Group), ice machine (Changshu Xueke Electric Co., Ltd.), and electric-heated thermostatic water bath (Changzhou Guohua Electric Appliance Co., Ltd.).

Preparation of Nereid enzymatic hydrolysates and NOP separation and purification

Selection of enzyme species. The Nereid was rinsed and homogenized. Homogenized samples (13.14) (10.0 g each) were obtained for enzymatic hydrolysis with five enzymes (i.e., trypsin, alkaline protease, neutral protease, pepsin, and papain) at certain temperatures and pH values (Table I). The optimal conditions for hydrolysis were as follows: 50°C and pH 10 for 6 h, a material-to-liquid ratio of 1:1 (g/ml), and addition of 400 U/g enzyme. Following hydrolysis, the samples were inactivated in a water bath at 100°C for 10 min, and centrifuged at 10,000 x g, at 4°C for 20 min in the refrigerated centrifuge. The supernatant was obtained and examined using the MTT method to determine the inhibition rate (IR) on the proliferation of H1299 cells. This process permitted the recognition of the optimal enzyme species.

Optimization of enzymatic hydrolysis conditions. The optimal protease was used for enzymatic hydrolysis to determine the optimal temperature and pH. The relevant literature detailing single factor experiments (15-17) involving temperature, pH, material-to-liquid ratio, duration, and the amount of enzyme added during the enzymatic hydrolysis, were reviewed. Subsequently, an orthogonal test (Table II) involving the aforementioned parameters was designed, using the IR as an indicator to determine the optimal conditions for enzymatic hydrolysis.

NOP separation using ultrafiltration. Enzymatic hydrolysate <3 kDa was separated using the membrane ultrafiltration technique, cooled down, and dried. The MTT method was used to detect the IR in A549, H1299 and 95C cells after 24 h of intervention with different components at 4,000 mg/l. Next, the cell lines demonstrating more pronounced anti-lung cancer effects after treatment were identified for further separation.

NOP separation through diethylaminoethanol (DEAE) Sepharose Fast Flow chromatography. The ultra-filtrated components at 0.25 g/ml were further separated using DEAE Sepharose Fast Flow (Beijing Asia-Pacific Hengxin Biological Technology Co., Ltd.) chromatography. Following centrifugation (10,000 x g, 4°C, 20 min), the supernatant was collected, filtered with a filter membrane (pore size, 0.22 µm), and eluted. The separation conditions were as follows: Column size, 3.6x20 cm; packing material, DEAE Sepharose FF; column particle diameter, 45-165 µm; sample volume, 2 ml; eluent, 0-0.4 mol/l NaCl solution; gradient elution, ~50 ml for each column; flow rate, 1 ml/min; detection wavelength, 280 nm; and collected volume, 5 ml per tube. The peaks were collected, concentrated, and freeze-dried. The MTT method was applied to select the peak components with the highest IR (inhibition rate) for further separation.

NOP separation using Sephadex G-25. The components with the highest IR collected in the previous step were further separated and purified by chromatography using the Sephadex G-25 column (Beijing Asia-Pacific Hengxin Biological Technology Co., Ltd.). The mass concentration of the sample was 0.25 g/ml. Following centrifugation (10,000 x g, 4°C, 20 min), the supernatant was filtered with a filter membrane (pore size, 0.22 µm) and eluted. The conditions for separation were as follows: Column size, 2.6x60 cm; packing material, Sephadex G-25; column particle diameter, 50-150 µm; sample volume, 2 ml; eluent, distilled water; flow rate, 1.0 ml/min; detection wavelength, 280 nm; and collected volume, 5 ml per tube. The peaks were collected, concentrated, and freeze-dried. The MTT method was used to identify the peak components with the highest IR (inhibition rate) for further separation.

Separation and purification using high-performance liquid chromatography (HPLC). The peak components with the highest IR in H1299 cells collected in the previous step were further separated and purified. The chromatographic...
conditions were as follows: Chromatographic column, Luna 10u C18(2) analytical column (10x250 mm; 5 µm); detection wavelength, 280 nm; flow rate, 1.0 ml/min; mobile phase A, acetonitrile; mobile phase B, ultrapure water (containing 0.05% trifluoroacetic acid); gradient elution, 20-100% elution A for 5-20 min; column temperature, 25°C; and automatic sampling with a sample volume of 100 µl. The most active components were collected, freeze-dried, and sequenced as described below.

**Determination of molecular weight and N-terminal sequencing of target peptides.** The collected components with the highest activity were freeze-dried for the measurement of the relative molecular weight and N-terminal sequencing of target peptides at APTBIO.

**MTT assay for the detection of the anti-proliferative activity of NOP.** H1299 cells were seeded into 96-well culture plates (density, 1x10^5 cells/ml) and cultured in RPMI-1640 medium (200 µl per well) under conventional conditions. After 24 h, the culture medium was removed, and a blank control (no drug treatment, 250, 500, or 1,000 mg/l) for another 24 h. The cell culture was continued for 4 h. The cell absorbance at 450 nm was detected using a microplate reader. The IC₅₀ which is a measure of the potency of a substance when the half maximal inhibitory concentration is achieved was calculated as follows: IC₅₀=lg⁻¹[Xm-i(ΣP-0.5)].

**Observation of cell morphology using hematoxylin and eosin (H&E) staining.** Acid-treated coverslips were placed onto 96-well plates, and A549 cells were routinely cultured (density, 1x10⁵ cells/ml) for 24 h. The culture medium was subsequently removed, and a blank control group and a drug group were prepared. The cells in the drug group were treated with NOP (250, 500, or 1,000 mg/l). After 24 h of culture, the coverslips were removed, and the cells were fixed in 95% ethanol for 20 min at room temperature. The samples were stained using hematoxylin for 3-5 min at room temperature, immersed in tap water, developed with eosin for 1 min, dehydrated with ethanol, treated with xylene to become transparent, mounted in neutral gum, and images were captured under an optical microscope (BX2-FLB3 fluorescence microscope; Olympus Corporation).

**Morphology of tumor cells after treatment with NOP using a transmission electron microscope.** H1299 cells (1x10⁵ cells/ml) cultured for 24 h were digested and transferred to a 10-ml centrifuge tube. This was followed by centrifugation at 800 x g at 4°C for 6 min, washing with PBS (800 x g, 4°C for 6 min), and removal of the supernatant. Subsequently, 2.5% dialdehyde was added, and the cell suspension was incubated overnight at 4°C. The cell suspension was treated at the Biological Laboratory of Zhejiang University Medical College (Hangzhou, China). The cells were fixed using 2.5% glutaraldehyde at 4°C for 24 h. Sections were cut to a thickness of 100 nm and uranyl acetate and lead citrate were used to stain cells at room temperature for 15 min. The cells were embedded in resin at 37°C for 4-6 h, and 60°C for 40 min. Finally, it was observed and images were captured under a transmission electron microscope.

**Cell apoptosis analysis.** Apoptotic rates were measured by flow cytometry using Annexin V-Flourescein isothiocyanate/propidium iodide (FITC/PI) staining. H1299 cells (1x10⁵ cells/ml) were firstly seeded in six-well flat-bottomed plates and incubated for 24 h at 37°C. Cells were subsequently treated with NOP (250, 500, or 1,000 mg/l) for another 24 h at 37°C and harvested. After digestion with trypsin, the cells were suspended in PBS and harvested by centrifugation at 800 x g at room temperature for 10 min. Next, the cells were incubated with Annexin V-FITC (5 µl) and PI (10 µl) at room temperature for 15 min in the dark. After staining, cells were immediately analyzed using flow cytometry (Easy Cyte6 HT-2L Flow Cytometer; LumineX Corporation).

**Western blot analysis.** Total protein was extracted with radioimmunoprecipitation assay (RIPA) lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.), and protein concentrations were determined using a bicinchoninic acid kit. Proteins

### Table I. Optimal temperature and pH value for enzymatic hydrolysis.

| Enzyme       | Temperature (°C) | pH value |
|--------------|-----------------|----------|
| Trypsin      | 50              | 8.0      |
| Alkaline protease | 45              | 10.0     |
| Neutral protease   | 45              | 7.0      |
| Pepsin       | 37              | 3.0      |
| Papain       | 45              | 6.0      |

A, temperature, °C; B, pH value; C, material-to-liquid ratio, g/ml; D, time, h; E, amount of enzyme added, U/g.

### Table II. Orthogonal test design.

| Level | A  | B  | C  | D  | E  |
|-------|----|----|----|----|----|
| 1     | 35 | 7  | 1:1| 2  | 400|
| 2     | 40 | 8  | 1:2| 4  | 600|
| 3     | 45 | 9  | 1:3| 6  | 800|
| 4     | 50 | 10 | 1:4| 8  | 1,000|

**Cell Counting Kit-8 (CCK-8) assay for the detection of the anti-proliferative activity of NOP.** Cell culture and drug administration were designed as above. CCK-8 reagent (Shanghai Biyuntian Biotechnology Co., Ltd.) (10 µl) was added, and the cell culture was continued for 4 h. The cell absorbance at 450 nm was detected using a microplate reader. The proliferation IR (%) was calculated using the following equation: IR (%)=[1-(A treated/A control)] x100%.

**Cell apoptosis analysis.** Apoptotic rates were measured by flow cytometry using Annexin V-Flourescein isothiocyanate/propidium iodide (FITC/PI) staining. H1299 cells (1x10⁵ cells/ml) were firstly seeded in six-well flat-bottomed plates and incubated for 24 h at 37°C. Cells were subsequently treated with NOP (250, 500, or 1,000 mg/l) for another 24 h at 37°C and harvested. After digestion with trypsin, the cells were suspended in PBS and harvested by centrifugation at 800 x g at room temperature for 10 min. Next, the cells were incubated with Annexin V-FITC (5 µl) and PI (10 µl) at room temperature for 15 min in the dark. After staining, cells were immediately analyzed using flow cytometry (Easy Cyte6 HT-2L Flow Cytometer; LumineX Corporation).

**Western blot analysis.** Total protein was extracted with radioimmunoprecipitation assay (RIPA) lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.), and protein concentrations were determined using a bicinchoninic acid kit. Proteins
(20 µl) were subsequently separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a 12 or 15% polyvinylidene difluoride membrane. The membranes were rinsed once with PBS containing 0.05% Tween-20 (PBST) and blocked in 5% skimmed milk powder at 4˚C for 2 h. After two rinses in PBST (10 ml) and one rinse in PBS (10 ml), the membranes were placed into a hybridization bag containing the primary antibodies B-cell lymphoma 2 (Bcl-2; 1:1,000; cat. no. a00040-2), Bcl-2 associated X (Bax; 1:1,000; cat. no. Ba0315-2), caspase-9 (caSP9; 1:1,000; cat. no. BM4619), and caspase-3 (caSP3; 1:500; cat. no. BM4620) (all from Wuhan Boster Biological Technology, Ltd.). The antibodies were diluted with 1% bovine serum albumin solution according to the instructions provided by the manufacturer. Following incubation overnight at 4˚C, the membranes were placed on a shaker at room temperature. After 1 h, the membranes were rinsed thrice with PBST (10 min per rinse). The membranes were incubated with fluorescent secondary antibodies goat anti-rabbit (1:10,000; cat. no. A00100-2), Bcl-2 associated X (Bax; 1:1,000; cat. no. BA0315-2), caspase-9 (CAS9P; 1:1,000; cat. no. BM4619), and caspase-3 (CAS3P; 1:500; cat. no. BM4620) (all from Wuhan Boster Biological Technology, Ltd.). The antibodies were diluted with 1% bovine serum albumin solution according to the instructions provided by the manufacturer. Following incubation overnight at 4˚C, the membranes were placed on a shaker at room temperature. After 1 h, the membranes were rinsed thrice with PBST (10 min per rinse). Following incubation, the membranes were washed thrice with PBST (10 min per rinse). β-actin was used as an internal reference, and protein bands were quantified using the Odyssey Imager (LI-COR Biosciences).

One-way ANOVA with Dunnett's post hoc test was used to determine the difference between different groups. The data are expressed as the mean ± standard deviation (n=3). P<0.05 was considered to indicate a statistically significant difference.

Results

Optimization of the enzymolytic process of NOP. In H1299 cells, the IRs were 69.94±1.35% for alkaline protease, 62.25±2.03% for trypsin, 53.76±1.43% for pepsin, 49.62±1.09% for neutral protease, and 45.27±2.25% for papain. In addition, the order of activity was as follows: alkaline protease > trypsin > pepsin > neutral protease > papain (Fig. 1). According to the IR, alkaline protease was selected for further screening.

Orthogonal test results. The IR was determined under different enzymolysis conditions, such as temperature, pH value, material-to-liquid ratio, g/ml; D, time, h; E, amount of enzyme added, U/g; IR, inhibition rate.

| Number | A  | B  | C   | D   | E    | IR (%) |
|--------|----|----|-----|-----|------|--------|
| 1      | 1  | 7  | 1:1 | 2   | 400  | 90.34±1.23 |
| 2      | 1  | 8  | 1:2 | 4   | 600  | 40.11±1.45 |
| 3      | 1  | 9  | 1:3 | 6   | 800  | 51.43±0.88 |
| 4      | 1  | 10 | 1:4 | 8   | 1000 | 38.78±2.45 |
| 5      | 2  | 1  | 3   | 4   | 4    | 56.41±3.02 |
| 6      | 2  | 2  | 1   | 4   | 3    | 58.47±2.41 |
| 7      | 2  | 3  | 4   | 1   | 2    | 42.12±0.88 |
| 8      | 2  | 4  | 3   | 2   | 1    | 88.71±1.69 |
| 9      | 3  | 1  | 3   | 4   | 2    | 41.58±2.45 |
| 10     | 3  | 2  | 4   | 3   | 1    | 42.01±3.21 |
| 11     | 3  | 3  | 1   | 2   | 3    | 41.89±1.12 |
| 12     | 3  | 4  | 2   | 1   | 4    | 43.25±2.47 |
| 13     | 4  | 1  | 4   | 2   | 3    | 49.64±0.89 |
| 14     | 4  | 2  | 3   | 1   | 4    | 54.11±2.11 |
| 15     | 4  | 3  | 2   | 4   | 1    | 55.54±2.55 |
| 16     | 4  | 4  | 1   | 3   | 2    | 89.32±1.76 |

A, temperature, °C; B, pH value; C, material-to-liquid ratio, g/ml; D, time, h; E, amount of enzyme added, U/g; IR, inhibition rate.

Statistical analysis. Three parallel experiments were conducted for all groups. Experimental data were analyzed and processed using SPSS version 19.0 statistical software (IBM Corp.).
an ultrafiltration membrane. The components were collected and freeze-dried, and the proliferation of various cancer cells (A549, H1299, 95C) was examined using MTT. After a 24-h intervention with the components at 2,000 mg/l, the IR of the aforementioned cells was 73.10±2.70%, 85.44±1.81%, and 73.02±2.71%, respectively (Fig. 2). These findings revealed that H1299 cells had the best IR, and could be selected for further screening.

**DEAE Sepharose Fast Flow chromatography.** Chromatography using a DEAE Sepharose Fast Flow column yielded two elution peaks from the enzymatic hydrolysate <3 kDa, namely noP‑1 and noP‑2 (Fig. 3A). After 24 h of intervention with these two peptides at 2,000 mg/l, the IR of H1299 cells was 36.50±3.08% and 64.47±1.25%, respectively. At a concentration of 4,000 mg/l, these values were 52.88±1.64% and 86.23±2.71%, respectively (Fig. 3B). Therefore, the second peptide was selected for further separation and purification.

**Sephadex G-25 separation.** The second peptide yielded in the above step was freeze-dried and separated using a Sephadex G-25 column to obtain two elution peaks, namely noP‑2‑1 and noP‑2‑2 (Fig. 3C). Following treatment of H1299 cells with these two peptides at a concentration of 1,000 mg/l, the IR was 53.97±3.40% and 69.33±2.27%, respectively. At a concentration of 2,000 mg/l, these values were 74.77±2.61% and 88.10±1.60%, respectively (Fig. 3D). Therefore, the second peptide with the highest activity was used for further separation and purification using HPLC.

**HPLC separation.** After elution and purification through HPLC, two elution peaks (noP‑2‑2‑1 and noP‑2‑2‑2) were noted (Fig. 3E). After treatment of H1299 cells with these two peptides at a concentration of 500 mg/l, the IR was 56.50±3.64% and 77.10±2.09%, respectively. At a concentration of 1,000 mg/l, these values were 70.83±1.45% and 87.97±1.89%, respectively (Fig. 3F). Thus, the second peptide with the highest activity was termed NOP. The molecular weight of the NOP was determined, and N-terminal amino acid sequencing was performed.

**Molecular weight measurement and N-terminal sequencing of target peptides.** The molecular weight was 841.0768 kDa, as identified by mass spectrometry at APTBIO (Fig. 4). The amino acid sequence was glutamine-isoleucine-asparagine-glutamine-histidine-leucine (Gln-Ile-Asn-Gln-His-Leu). The molecular weight of the peptide obtained by amino acid sequencing was 841.91 kDa; this finding was completely consistent with the results of molecular weight determination.

**Anti-lung cancer H1299 cell activity**

**Proliferation of H1299 cells.** After 12 h of treatment with 100 mg/l NOP, the IR of H1299 was 43.77±3.84%. At a concentration of 1,000 mg/l, this value was 76.00±2.67%. After 24 h of intervention with 100 mg/l and 1,000 mg/l NOP, this value was 58.47±2.44% and 90.07±1.56%, respectively (Fig. 5A). The 12- and 24-h IC50 values were 172.5 and 107.8 mg/l, respectively (Fig. 5B). Therefore, compared with control, the IR was significantly elevated in parallel with the increase in mass concentration and action time (P<0.01).

**H&E staining results.** H1299 cells grew well in the normal group and were tightly arranged, exhibiting good morphology. The cytoplasm was uniformly stained, the nucleus was in uniform size, and numerous nucleoli were detected (Fig. 6A). Following 24 h of treatment with NOP at low concentration (250 mg/l; Fig. 6B), the number of H1299 cells per field of view...
decreased, and numerous vacuoles appeared in the cytoplasm. The number of nucleoli decreased, and the intercellular space appeared. The cell contour was blurred, the area of plasma membrane was enlarged, the cytoplasm was vacuolated, the nucleus appeared nuclear pyknosis and the number of nucleoli was reduced (500 mg/l; Fig. 6c), after 24 h of treatment with NOP at a high concentration (1,000 mg/l; Fig. 6d), the cell contour was blurred, with irregular shape and numerous protrusions. Morphological changes, such as chromatin condensation and pyknosis, were also observed (Fig. 6D).

Cell ultrastructural changes. Under the transmission electron microscope, H1299 cells in the control group showed dense microvilli on the surface and had rich cytoplasm without swelling. Moreover, the mitochondria were relatively intact, there were no obvious effects on the endoplasmic reticulum, and the plasma and nuclear membranes were relatively intact (Fig. 7A and B). After intervention with 1,000 mg/l NOP, the cells were almost round in shape without microvilli, but with obvious cytoplasmic swelling. In addition, the cell membrane was disrupted, swelling of the nuclear membrane was observed, the endoplasmic reticulum was severely vacuolated, and a large number of vacuoles appeared in the cytoplasm (Fig. 7C). After treatment with NOP at a high concentration (1,000 mg/l; Fig. 7D), H1299 cells had broken plasma and nuclear membranes, a shrunken nucleus, heterochromatin aggregation, extended smooth endoplasmic reticulum, and swollen mitochondria. More obvious changes were observed as the concentration of NOP increased. Under the transmission electron microscope, H1299 cells had obvious ultrastructural changes. In the control group, the endoplasmic reticulum was relatively intact, the nuclear membrane was intact, the mitochondria were intact, and the cytoplasm was rich in dense microvilli (Fig. 7A). After intervention with NOP at a high concentration (1,000 mg/l; Fig. 7D), the endoplasmic reticulum was severely vacuolated, the nuclear membrane was disrupted, the mitochondria were swollen, and the cytoplasm was vacuolated (Fig. 7D).
microscope, H1299 cells exhibited apoptotic characteristics after 24 h of exposure to NOP.

Detection of the apoptotic rate of cells by flow cytometry. In order to quantitatively measure the NOP-induced apoptosis in H1299 cells, Annexin V-FITC and PI double staining was used to determine the number of apoptotic cells. As revealed in Fig. 8, for the control, the percentage of Annexin V-FITC-stained H1299 cells was 7.08±0.65%. After 24 h of exposure to 250, 500, and 1,000 mg/l NOP, the percentage of apoptotic cells increased to 11.93±0.75%, 12.52±1.16%, and 24.36±1.53%, respectively (Fig. 8). The apoptotic effect on H1299 cells was significantly enhanced with the increasing concentration of NOP compared with that observed in the control group. Therefore, NOP was able to effectively induce apoptosis in H1299 cells.

Western blotting. The protein bands of Bcl-2, Bax, cleaved-CASP9, and cleaved-CASP3 in samples obtained from H1299 cells treated with different concentrations of NOP for
was significantly reduced by 24.72% compared with the control group. The relative expression of cleaved-CASP9 and cleaved-CASP3 was 2.55- and 1.71-fold higher than that in the control group, respectively. Therefore, at a certain concentration range, NOP can significantly upregulate the protein levels of cleaved-CASP9, cleaved-CASP3, and Bax, and significantly downregulate those of Bcl-2.

Activity on the proliferation of PC12 and SK-N-SH cells. Following treatment of PC12 and SK-N-SH cells with 250 mg/l NOP for 24 h, the IR was 106.52±0.92% and 102.53±1.24%, respectively. At a concentration of 1,000 mg/l, these values were 119.38±1.10% and 110.41±0.52%, respectively (Fig. 10). Higher mass concentration indicated higher IR. The differences between the control and NOP groups were statistically significant (P<0.05). These findings indicate that NOP did not exert a toxic effect on PC12 and SK-N-SH cells; in contrast, it promoted cell proliferation.

Discussion

Bioactive peptides are composed of multiple amino acids linked by peptide bonds (18). They exert several beneficial effects on the human body (e.g., anti-swelling, analgesic, immune-modulatory, anti-viral, and anti-hypertensive). Currently, there are three main methods utilized for the extraction of bioactive peptides, namely direct extraction, synthesis, and hydrolysis. The direct extraction method is only suitable for laboratory extraction of some natural bioactive peptides with low content and complex structure in animals and plants. Although it is easy to obtain pure bioactive peptides using the synthetic method, its large-scale use is limited by the high cost and numerous adverse reactions. Hydrolysis can be divided into two types, namely chemical and enzymatic. The latter is a controllable process, in which the proteins are hydrolyzed using appropriate proteases (19,20). It is characterized by mild conditions, a controllable process, high yield, and good safety. Enzymatic hydrolysis has been used to extract peptides from marine organisms, which have demonstrated favorable antioxidant activity. For example, Zhou (21) used the compound enzymatic hydrolysis method to extract fucoidan that was deproteinized using the Sevag method. In their study, homogeneous components with reducing ability and quenching lipid peroxidation ability were obtained through chromatography using the DEAE Sepharose Fast Flow ion exchange column and Sephadex-G150 column. Zhu et al (22) used a two-step enzymatic hydrolysis method to extract polysaccharides from Urechis unicinctus, reporting a yield of 6.2%. Visceral polysaccharides from Urechis unicinctus are glycosaminoglycan-like, with a molecular weight of approximately 4.1x10^3 kDa. A preliminary study investigating in vitro antioxidant activity revealed that visceral polysaccharides possess significant lipid peroxide scavenging activity, with a half scavenging mass concentration of 2.47 mg/ml (22). Huang et al (23) and Ding et al (24) used enzymatic hydrolysis to extract a polypeptide from cuttlefish ink. They found that this cuttlefish polypeptide could inhibit prostate cancer cells DU-145, PC-3, and LNCaP in a time- and dose-dependent manner. Therefore, in the present study, trypsin, papain, alkaline protease, neutral protease, and pepsin were used. Considering the IR as an indicator, alkaline protease was

Figure 6. H&E staining images of H1299 cells after treatment with NOP (x400). (A) The negative control group (normal H1299 cells) grew well, had full morphology, clear borders, and a large number of nucleoli. (B) Treatment of H1299 cells with 250 mg/l NOP. The cell morphology was irregular, the intercellular space was enlarged, and vacuoles were detected in the cytoplasm (red circles). (C) Treatment of H1299 cells with 500 mg/l NOP. The cell morphology was irregular, the cell outline was blurred, the plasma membrane area was enlarged, vacuoles were detected in the cytoplasm, pyknosis was observed in the nucleus, and the number of nucleoli was decreased (red circles). (D) Treatment of H1299 cells with 1,000 mg/l NOP. The cell morphology was irregular, the cells shrank and became rounder and smaller, the volume was decreased, and pyknosis was observed in the nucleus (red circles). H&E, hematoxylin and eosin; noP, nereid oligopeptide.

Figure 7. Ultrastructure changes in H1299 cells. (A) Magnification of the ultrastructure of normal H1299 cells (x2,500), revealing microvilli around them, less heterochromatin, and one nucleolus (red arrow). (B) Magnification of the ultrastructure of normal H1299 cells (x5,900), demonstrating more obvious surrounding slender microvilli (red arrow). (C) Following treatment of H1299 cells with 1,000 mg/l NOP for 24 h, their ultrastructure was visualized through magnification (x2,500). The nuclear volume was reduced, the morphology was abnormal, heterochromatin edge aggregation was obvious, numerous vacuoles were detected in the cells, and the cell microvilli disappeared (red arrow). (D) Magnification of the cell ultrastructure (x5,900) after treatment with 1,000 mg/l NOP for 24 h showed an apparent increase in apoptotic bodies (red arrow). NOP, Nereid oligopeptide.
determined to be the most effective protease for NOP. According to the results of the single factor test, the enzymatic hydrolysis process was optimized through an L16 (45) orthogonal test. The optimal conditions for the extraction of NOP were as follows: Enzymatic hydrolysis at 50˚C and pH 10 for 6 h, a material-to-liquid ratio of 1:1, and addition of 400 U/g enzyme. The purified oligopeptide was termed NOP, and its amino acid sequence is Gln-ile-asn-Gln-His-leu.

The MTT assay is a colorimetric assay for measuring cell survival and proliferation, involving the conversion of yellow-dyed MTT to bluish-purple formazan by the action of mitochondrial reductase. However, this conversion does not occur in dead cells (25). The MTT method is often used to detect inhibitory effects on cell proliferation. Ma et al. (26) applied the MTT method to detect the proliferation of human glomerular mesangial cells treated with different concentrations of Lumbricus's active ingredients. They found that the middle dose (40 µg/ml) exerted the best effect. Using the MTT assay, Chen et al. (27) reported that phycoerythrin had an inhibitory effect on human cervical cancer HeLa cells in a dose-effect manner. The results of flow cytometry showed that phycoerythrin arrested the cell cycle of HeLa cells from the G2/M to the S phase, thereby playing an inhibitory role. In the present study, the MTT method was used to detect the inhibitory effect of NOP on the proliferation of H1299 cells. It was determined that NOP could inhibit the activity of H1299 cells in a time- and dose-dependent manner. In other words, the IR of cells was significantly increased in parallel with the increase in the mass concentration of the NOP and time extension. The discovery of new anticancer drugs from marine organisms, including peptides, glycosaminoglycans, and macrolides, is a new goal of anticancer drug research. Marine peptides are characterized by low molecular weight, high activity, and low toxicity, and have become a research hotspot worldwide. In 1987, Pettit et al. (28) and Cao and Song (29) isolated dolastatin-10, a small linear peptide from the sea hare. In a Phase II clinical trial, the combination of dolastatin-10 and other anticancer drugs exerted an obvious anticancer effect. Its derivatives, such as TZT-1027 (auristatin PE) and auristatin PYE, also showed significant clinical therapeutic effects (30). Chi et al. (31) isolated two polypeptides, namely BCP-A (Trp-Pro-Pro) and BCP-B (Gln-Pro), from Tegillarca granosa Linnaeus. BCP-A exhibited a favorable effect on the IR of PC-3, DU-145, H-1299, and HeLa cells in a time- and dose-dependent manner. Following treatment with 15 mg/ml BCP-A, the early apoptotic rate of PC-3 cells increased from 11.22 to 22.78%. Yao et al. (32) extracted a type of polypeptide from Tegillarca granosa Linnaeus, which induced cell cycle arrest at the G2/M and G0/G1 phases in A549 and Ketr-3 cells, respectively; these polypeptides could significantly inhibit transplanted tumors in mice. Wang et al. (33) extracted and isolated the polypeptide Mere15 from clams through ion exchange and gel filtration. Mere15 could inhibit the growth of A549 cells in a dose- and time-dependent manner. With the increase in treatment time, cell cycle arrest at the G2/M phase was induced in A549 cells; this effect was related to

![Figure 8. Apoptotic effect of NOP on H1299 cells.](image)

(A) Flow cytometric analysis of H1299 cells using double staining with Annexin V fluorescein isothiocyanate (FITC) and propidium iodide (PI). Quadrants: lower left, live cells; upper left, necrotic cells; lower right, early apoptotic cells; upper right, late apoptotic cells. The percentage of early apoptotic cells was (a) 7.08% in the negative control cells; (b) 11.93% in the 250 mg/l NOP-treated cells; (c) 12.52% in the 500 mg/l NOP-treated cells and (d) 24.36% in the 1,000 mg/l NOP-treated cells. (B) Percentage of apoptotic cells. All data are presented as the mean ± standard deviation of measurements obtained from three experiments. *P<0.05 and **P<0.01 vs. the control. NOP, Nereid oligopeptide.
In the present study, the MTT method was used to detect the inhibition of H1299 cells treated with NOP. It was determined that H1299 cells treated with 250 mg/l NOP for 12 h exhibited apoptotic characteristics. After treatment with 1,000 mg/l NOP for 24 h, the IR of H1299 cells was increased to 90.07%.

The morphological changes strongly indicated the occurrence of apoptosis, which corresponded with a previous study (34). The characteristic morphology of early apoptotic cells could be directly detected using H&E staining under a transmission electron microscope. H1299 cells exhibited obvious cell morphological changes after intervention with NOP, such as increased intercellular space, irregular shape, decreased size, vacuoles, and apoptotic bodies in some cells. These changes became more detectable as the concentration increased. Overall, NOP extracted by alkaline protease can effectively inhibit the proliferation of H1299 cells in vitro.

In addition, H1299 cells showed obvious apoptotic characteristics after 24 h of intervention with NOP. Thus, Annexin V-FITC was used to detect cells undergoing apoptosis, and PI was used to determine the number of necrotic cells. Flow cytometry revealed that the percentage of apoptotic cells was significantly increased in parallel with the increasing concentration of NOP. The results of the present study indicated that NOP exhibits anti-lung cancer activity through the induction of apoptosis.
Apoptosis refers to programmed cell death that is controlled by genes; it includes two classical pathways, namely the exogenous and endogenous (35,36). The endogenous apoptotic pathway is the main process of apoptosis. In the present study, the expression of related proteins, including intracellular Bax and Bcl-2, was detected using western blotting assays. The aim of this analysis was to determine whether NOP induces apoptosis in H1299 cells through the endogenous pathway. The Bcl-2 family plays a vital role in regulating the initiation of the endogenous pathway. During apoptosis, Bax and other pro-apoptotic proteins are translocated from the cytoplasm to the outer mitochondrial membrane, altering the permeability of the mitochondrial membrane. Bcl-2 and Bax regulate the release of cytochrome c (Cyt-C) by forming homodimers or heterodimers (37-39), and the ratio of Bcl-2/Bax is used to indicate the regulatory effect of apoptosis. Specifically, an increase in the Bcl-2/Bax ratio denotes inhibition of apoptosis. In contrast, a decrease in the Bcl-2/Bax ratio indicates promotion of apoptosis. The findings of the present study revealed that treatment with 250, 500, and 1,000 mg/l NOP significantly increased the protein expression of Bax and decreased that of Bcl-2. Moreover, the Bcl-2/Bax ratio was decreased. These effects promote the release of Cyt-C and initiate the endogenous apoptotic pathway from the upstream. Changes in the intracellular levels of activated CASP9 and CASP3 were also detected. In the mid and late stages of endogenous apoptosis, Cyt-C that enters the cytoplasm is combined with the WD repeat sequence at the carboxyl end of apoptotic protease-activating factor 1 (Apaf-1) in a 2:1 ratio to form an Apaf-1/Cyt-C complex. This complex can change the conformation of Apaf-1, inducing the formation of apoptotic bodies, triggering caspase cascade reactions, and eventually resulting in cell apoptosis (40,41). The present study showed that the protein content of activated CASP9 and CASP3 was significantly increased in parallel with the increase in the concentration of NOP. This observation indicated that activated CASP9 induces the downstream activated CASP3. CASP3 is the key executor in the downstream process of apoptosis. Activated CASP3 can degrade poly ADP-ribose polymerase, activate endonuclease, and cleave DNA strands between nucleosomes by hydrolyzation; these effects lead to DNA segmentation that is unique to apoptosis (42). The results of the present study showed that the protein levels of cleaved-CASP9 and cleaved-CASP3 were significantly increased. This indicates that NOP can activate the key downstream caspases via the endogenous apoptotic pathway, thereby inducing apoptosis in H1299 cells.

In summary, in the present study, alkaline protease was selected to enzymatically hydrolyze a Nereid homogenate at 50°C and pH 10 for 6 h. The material-to-liquid ratio was 1:1 and the amount of enzyme added was 400 U/g. An NOP with a molecular weight of 847 kDa was obtained after separation and purification through ultrafiltration, ion exchange chromatography, gel filtration chromatography, and HPLC. The amino acid sequence of the NOP was Gln-Ile-Asn-Gln-His-Leu. Collectively, the present findings indicated that NOP could inhibit the proliferation of H1299 cells in a dose- and time-dependent manner. Apoptosis in H1299 cells may be triggered by downregulation of Bcl-2 protein, upregulation of Bax protein, and stimulation of the cascade reaction of the caspase family. NOP did not exert a toxic effect on PC12 and SK-N-SH cells; in contrast, it promoted cell proliferation. The limitation of the present study is that the yield of separated and purified samples was relatively low, and it is hoped to improve the yield of samples in the future. In subsequent studies, the precise action pathway and specific action compounds will be further studied.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Authors’ contributions**

LS conceived and designed the experiments. GZ, HL, SL, ML and LT performed the experiments and statistical analysis of the data. GZ and HL contributed equally to this study and share first authorship. All authors contributed to the writing of the manuscript and read and approved the final manuscript. LS and GZ confirm the authenticity of all the raw data.

**Ethics approval and consent to participate**

Not applicable.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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