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Anticancer activity of peptide W-0803 derived from *Anoplophoa glabripennis*

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Abstract

Natural compounds are known as a resource of anti-cancer agents. Anti-cancer capacity toward human epithelial lung cancer cell lines (A549, H460) was examined with and without treatment of trypsin on the extract of *Anoplophora glabripennis*. IC50 values without trypsin treatment were about 21.3 and 25.0 µg/mL for H460 and A549, respectively. When the extract was treated with trypsin, the IC_{50} values were 16.0 and 15.6 µg/mL for H460 and A549, respectively, indicating that the trypsin treatment increased the anti-cancer capacity. Because trypsin treatment increased the capacity, the extract was treated with trypsin to isolate the peptide W-0803 which has lysin (K) in C-terminal and α-helix structures. With treatment of W-0803, the cell viability decreased dose-dependently for H460 and A549 cells. Apoptosis analysis showed that the cell death with the treatment of peptide W-0803 was mainly by apoptosis. The wound-healing assay also showed that the peptide W-0803 has an inhibitory capability on cell migration of H460 and A549 cells. All these results suggest that the peptide W-0803 is an anti-cancer agent for lung cancer cell treatment.

Keywords *Anoplophora glabripennis*, Anti-cancer peptide, Human epithelial lung cancer cells, Trypsin treatment

Introduction

Cancer is one of the worldwide health problems. Cancer can be developed by oncogene, mutation, DNA damage, or carcinogen. Lung cancer is one of the most common cancers worldwide. It occupied 10% of total cancer development in 2020 in the USA [1]. Traditional chemotherapy has problems like the induction of drug resistance, side effects, or cytotoxicity. Targeted therapy for cancer is known to have a relatively low cytotoxicity. However, the targeted therapy is known to be costly and difficult to mass-produce [2]. Thus, chemotherapy is the first line of cancer therapy in parallel with radiation therapy [3].

Anoplophora glabripennis was known to cause critical damage to living plants and financial damage to farmhouses [4]. The insect is known as a source of bioactive materials such as protein, lipids, and fatty acids. The long-horned beetle was also used as a drug to treat various diseases like bleeding, pain, and menstrual irregularity. This fact suggests that the beetle has a variety of sources of natural compounds having anti-cancer effects [5, 6].

Some insects are known to have antimicrobial peptides (AMPs) as one of the main components of their innate immune system [7]. Some AMPs proved to have anticancer capability which thus are called anticancer peptides (ACPs) [8]. ACPs could be considered an efficient substitute for chemical anti-cancer drugs. This is because

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ACPs were known to have a low cytotoxicity compared to cancer treatment chemicals [9]. In addition, ACPs are short peptides of less than 50 amino acids in length that can be easily synthesized by chemical methods or cloning. ACPs have common properties like short amino acid sequences, and amphipathic charges on the peptide. ACPs are known to have a net positive charge for targeting the membrane of cancer cells [10]. It is suggested that ACPs target cancer cell membrane that has a negative charge to penetrate the cell and mitochondria membranes, disrupting them [11]. Finally, due to the disrupted mitochondria, programmed cell death and apoptosis might be induced to release cytochrome c [12, 13]. In this present study, from *Anoplophora glabripennis*, we have screened and isolated peptide W-0803 that has an anticancer effect and was evaluated for its anti-cancer efficiency.

Materials and methods

Chemicals

A peptide W-0803 (NH_2-VISNASCTTNCLAK-COOH) was synthesized by Peptron (Daejeon, Korea). EZ-3000 for cell viability assay was purchased from Sigma-Aldrich (St. Louis, MO, USA). The FITC Annexin V Apoptosis Detection kit was purchased from BD Biosciences (San Diego, CA, USA).

Cell cultures

A549, H460, and HDF cells were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). A549 and HDF were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Gibco; New York, NY, USA), 100 U/mL penicillin (Gibco), and 100 mg/mL (Gibco) in a humidified 5% CO2 atmosphere at 37 ℃. H460 cells were maintained in the same condition while using Roswell Park Memorial Institute (RPMI)-1640 as a medium (Wellgene; Gyeongsan, Korea).

Preparation of extract of *Anoplophoa glabripennis*

Anoplophoa glabripennis sample was washed with methanol for 1 min. After washing, the sample was homogenized with extraction solution [900 mL of 0.3% acetic acid solution + 100 mL of Radioimmunoprecipitation assay buffer (RPPA buffer, Sigma-Aldrich)]. The homogenized sample was sonicated for 4 min with Q125 sonicator (QSonica; Newtown, CT, USA) and subjected to centrifugation (2500×*g*, 40 min, 4 °C). The supernatant was collected, and freeze-drying was conducted for 2 days. The freeze-dried powder was suspended in a buffer (Dulbecco's phosphate-buffered saline, DPBS, Sigma-Aldrich). One mg of the freeze-dried powder in the buffer (FP extract) was treated with 1 mL of 0.25% trypsin–EDTA for 30 min at 30 °C [14]. After reacting, the solution was treated with protease inhibitor cocktail (BD Biosciences) of 10 µL for inhibiting trypsin to obtain the trypsin-treated FP extract (TF extract).

Protein separation from extract of *Anoplophoa glabripennis*

The FP extract and TF extract were denatured in a solution containing 950 µL of 5×sample buffer (Bio-Rad; Hercules, CA, USA) and 50 µL of β-mercaptoethanol by heating for 10 min at 99 °C to obtain the denatured samples. The denatured FP extract and TF extract were loaded in the 10% acrylamide gel for sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then, the two samples were run at 80 V for 30 min and 100 V for 60 min. Fixation was conducted for 4 h on the shaker. After fixation, the gel was stained with Coomassie brilliant blue (CBB) solution for 1 h. Gel was then destained for confirmation of bands.

3D modeling of protein and peptide

α-Fold 2.0 (https://github.com/google-deepmind/alphafold) and Pymol 2.5 (https://pymol.org/) were used for 3D modeling of protein and peptide.

Cell viability assay

Two human epithelial lung cancer cell lines (A549, H460) and human dermal fibroblast cells (HDF) were seeded in 96 well plates. Cell concentration was 1×10^3 cells/well for H460 cells, 5×10^3 for A549 cells, and 5×10^4 for HDF cells. Plates were incubated for 1 day and treated with FP extract, TF extract, or peptide W-0803. After 72 h, cell viability was measured using a PreMix water-soluble tetrazolium salts-1 (WST-1) and Cell proliferation assay system (TaKaTa; Tokyo, Japan) according to the manufacturer's protocol. Each well was treated with the WST-1 solution (10% WST-1 and 90% medium) and the absorbance was measured immediately and then every 30 min at 450 nm.

Cell apoptosis assay by PI/Annexin V staining

The cells were seeded in 6 well plates. A549 cells were seeded at 5×10^4. H460 cells were seeded at 1×10^4. Plates were incubated for 1 day and treated with peptide W-0803 at IC50 concentration for A549 and H460 cells. Cells were harvested with 0.25% trypsin and centrifuged at 286×*g* for 3 min. Washing was conducted twice with 1 mL of DPBS by centrifugation at 372×*g* for 5 min. After washing, the cells were suspended with 100 µL of 10×binding buffer (BD Biosciences) and treated with 5 µL of PI solution and Annexin V solution (BD Biosciences). Cells were reacted in a dark room for 15 min. After the reaction, the solution containing the cells was treated with 10×binding buffer of 300 µL. Stained cell

samples were analyzed with a fluorescence-activated cell sorting machine, FACSverse (BD Biosciences).

Wound healing assay

The cells were seeded in 6 well plates. A549 cells were seeded at 5×10^5. H460 cells were seeded at 1×10^5. Plates were incubated for 1 day and treated with peptide W-0803. SPL scratcher was used for forming wounds. After 72 h, the plate was washed with DPBS twice. The cells were fixed with a fixation solution (Cell Biolabs; San Diego, CA, USA) for 2 min and permeabilized with methanol for 20 min at room temperature. After washing twice, the cells were stained with the staining solution (Cell Biolabs) for 15 min at room temperature in a dark room. After washing twice with DPBS, the cell migration was pictured with a phase-contrast microscope (Olympus CK 40; Waltham, MA, USA).

Results

Effect of the two extracts on the viability of H460 and A549cells

When A549 and H460, human epithelial lung cancer cell lines, were treated with FP extract, cell viability gradually decreased with increasing concentration of FP extract (Fig. 1A and 1B). One of the controls had no extract and was without trypsin treatment. The other control had no extract and was with trypsin treatment. IC50 values were about 21.3 μg/mL and 25.0 μg/mL for H460 and A549, respectively, for FP extract. As shown in Fig. 1A and 1B, when the FP extracts (50.0 μg/mL and 20.0 μg/mL for

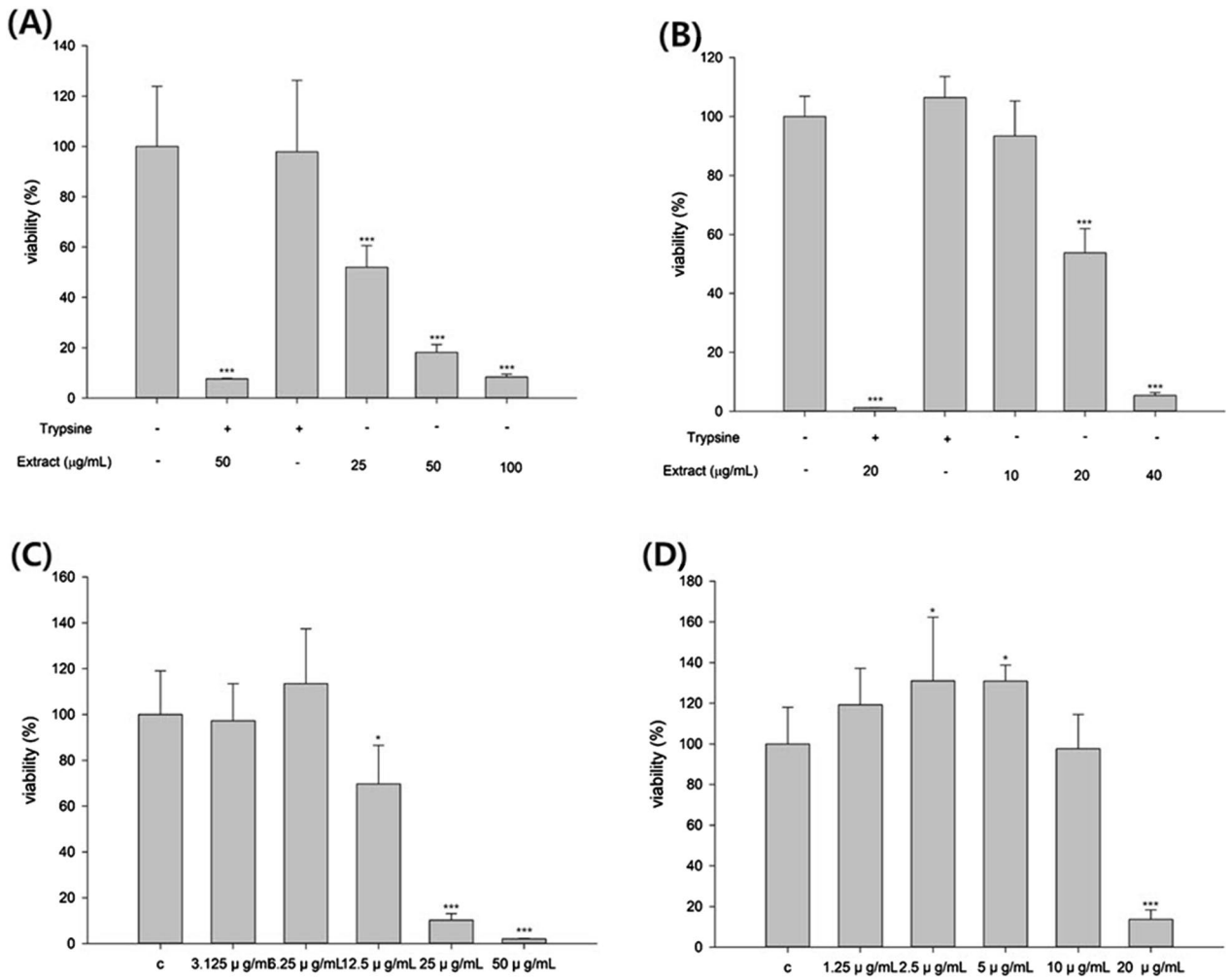

Fig. 1 Cell viability in A549 and H460 with the freeze-dried powder in the buffer (FP extract) and the trypsin-treated FP extract (TF extract). **A** Cell viability of A549 cells with treatment of FP extract. **B** Cell viability of H460 cells with FP extract. **C** Cell viability of A549 cells with treatment of TF extract. **D** Cell viability of H460 cells with treatment of TF extract. Data was expressed by mean ± standard error of the mean. *$p < 0.1$, **$p < 0.01$, and ***$p < 0.001$

A549 and H460, respectively) were treated with trypsin (in other words on the treatment with TF extract), the cell viability considerably decreased compared to the controls. Thus, we examined the cell viability with increasing concentrations of TF extract (Fig. 1C and 1D). The IC50 values were about 16.0 μg/mL and 15.6 μg/mL for H460 and A549, respectively. This indicates that the anti-cancer capability of the extract increased with trypsin treatment. No cytotoxicity was found with trypsin treatment in both cell lines (data not shown). These results showed that trypsin treatment to the extract increases the anti-cancer effect of the extract.

Isolation of anti-cancer peptide W-0803

The TF extract was denatured by 2×sample buffer containing β-mercaptoethanol. The protein concentration in the treated extract was about 1 mg/mL. The treated extract was loaded onto 12% acrylamide gel. After running the gel, the protein bands were fixed with a fixation solution for 4 h. After fixation, the gel was stained with CBB solution for 30 min. The stained gel was washed with the washing solution. Figure 2A showed that 1 band disappeared between 35 and 48 kDa after trypsin treatment. The part of the bands was cut out from the gel for FP and put into the washing solution to obtain a peptide mixture with vortexing. The various peptides were analyzed to obtain their sequences through the services of EMASS (Seoul, Korea).

We selected a few peptide sequences that had a positive charge on C-terminals and could form α-helix structures that were constructed by α-Fold 2.0 and Pymol 2.5 (Fig. 2B). To select the peptides that have anti-cancer capabilities, the peptides were examined to find the peptides having a net positive charge. The net positive charge of the peptides was one of the requirements for the anti-cancer peptides [9]. Finally, we have chosen 1 peptide that has 17 amino acids with a positive net charge and α-helix structure. We named the peptide W-0803 (NH_2-VISNASCTTNCLAK-COOH) and predicted that it has an anti-cancer effect. Peptide W-0803 has a molecular weight of 1706.1 g/mol, 1 basic net charge, 44% of hydrophobic/hydrophilic ratio, and α-helix structure.

Anti-cancer effect of peptide W-0803

To confirm the anti-cancer effect of the synthesized peptide W-803, the cell viability assay was performed (Fig. 3). While the cell viability was not affected much with normal cell line HDF cells, the cell viabilities of A549 and H460 cells decreased with increasing concentration of peptide W-0803 up to 50 μM. Thus, the cell viability decreased dose-dependently for A549 and H460 cells,

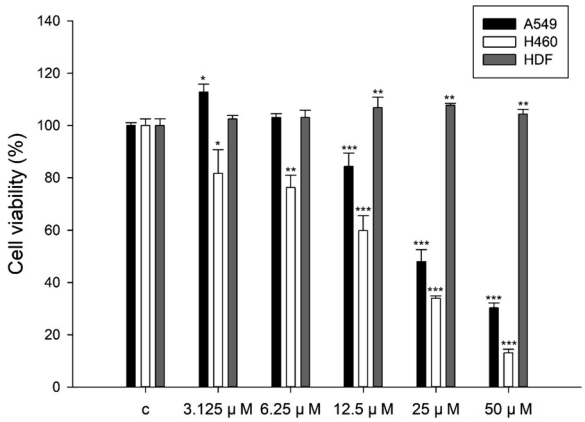

Fig. 3 Cell viability of A549, H460, and HDF cells with treatment of peptide W-0803. Cell viability was compared with two lung cancer cell lines (A549, H460) and a normal cell line (HDF). Cell viability of H460 and A549 cells was decreased dose-dependently, compared to that of HDF cells. Data was expressed by mean ± standard error of the mean. (*$p < 0.1$, **$p < 0.01$ and ***$p < 0.001$)

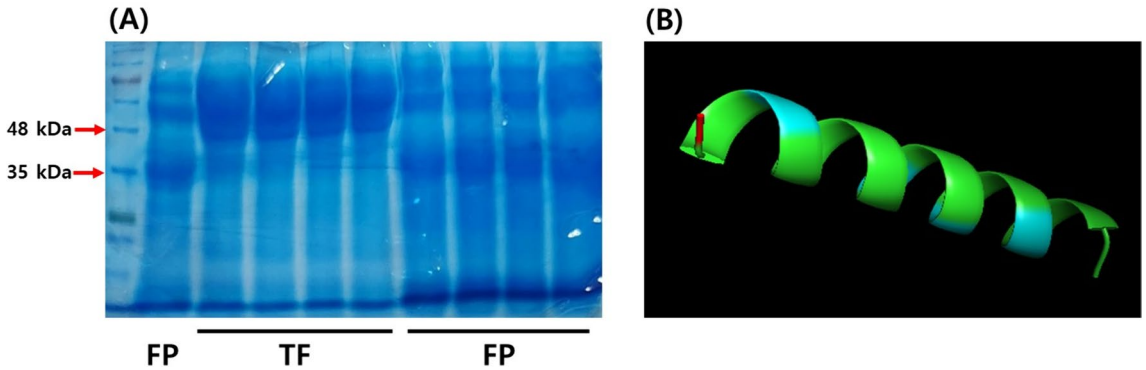

Fig. 2 Isolation and characterization of anti-cancer peptide W-0803. **A** SDS-PAGE with the freeze-dried powder in the buffer (FP extract) and the trypsin-treated FP extract (TF extract). **B** 3D modeling of peptide W-0803. Peptide W-0803 has molecular weight of 1706.1 g/mol, 1 basic net charge, 44% of hydrophobic/hydrophilic ratio, and α-helix structure

lung cancer cell lines. IC50 values were 50.0 μM and 17.3 μM for A549 and H460, respectively (Fig. 3). This result showed that peptides W-0803 have an anti-cancer effect on lung cancer cell lines of A549 and H460 cells. It was found that the cell viability of A549 and H460 cells noticeably decreased from 12.5 μM and 3.13 μM, respectively. Overall, these results show that while peptide W-0803 has no cytotoxicity in a normal cell, it has a significant anti-cancer effect on the H460 and A549 cells, lung cancer cell lines.

Cell death analysis by PI/Annexin V staining

To analyze the cell death phenomenon, PI/Annexin V staining was conducted by using FACS analysis (Fig. 4). In A549 cells, the extent of apoptosis was increased by 12.3% with the treatment of peptide W-0803 (Fig. 4A and 4B). In H460 cells, the extent of apoptosis was increased by 24.2% with the treatment of peptide W-0803 (Fig. 4C and 4D). However, a significant change was not observed in the extent of necrosis for both A549 and H460 cells. Although the extent of apoptosis was lower than we expected, there was almost no necrosis with the treatment of peptide W-0803, indicating that the major anti-cancer mechanism is apoptosis. A high extent of necrosis could be problematic due to the inflammation during cancer therapy. This result demonstrates the peptide W-0803 can inhibit the proliferation of both human epithelial lung cancer cell lines by inducing apoptosis.

Wound healing assay

In cancer therapy, the inhibition of cell migration and metastases is one of the important points to be considered. In the microenvironment in tumor tissue, angiogenesis can induce cancer cells which can migrate to other tissue from primary tissue. Therefore, a wound-healing assay was conducted to confirm that W-0803 has an inhibitory capability on cell migration and metastases of A549 and H460 cells. A549 and H460 cells were treated with peptide W-0803 at a concentration of 6.25 μM and 3.13 μM, respectively. As shown in Fig. 5, peptide

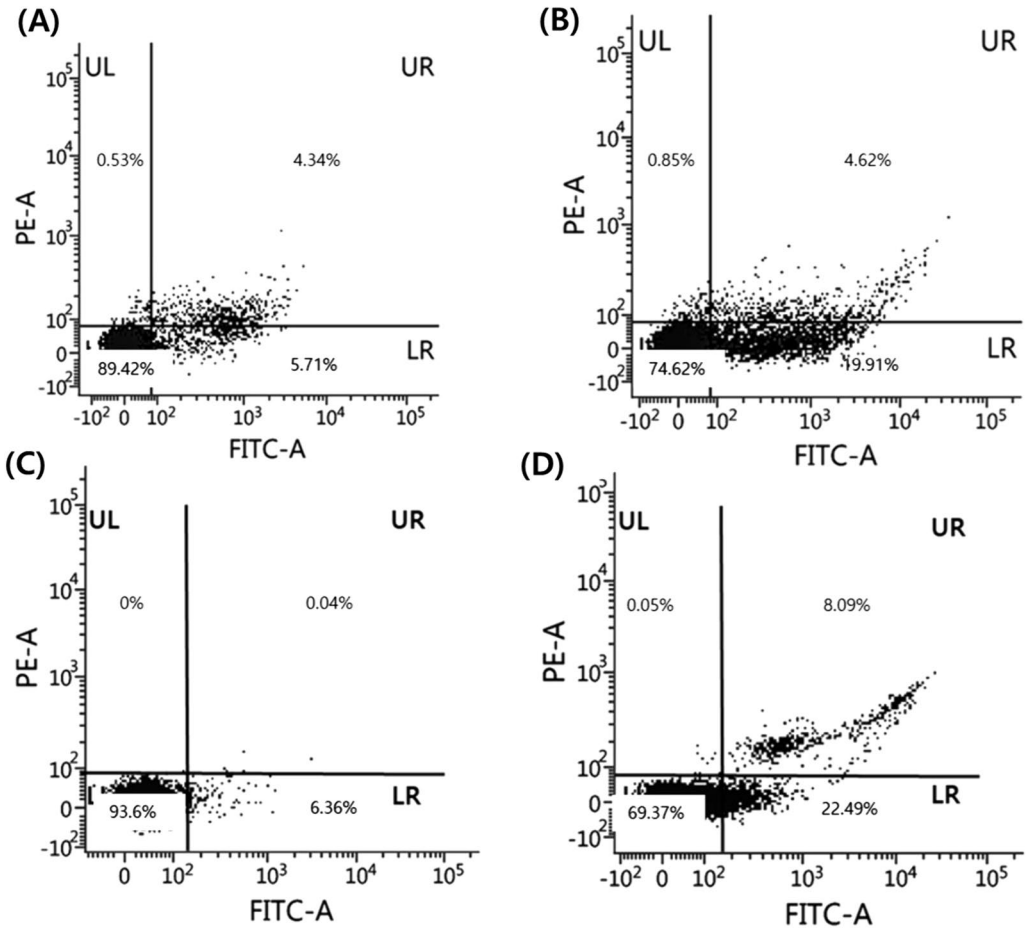

Fig. 4 Cell death analysis by PI/Annexin V staining of lung cancer cell lines. **A** Control of A549 cells. **B** Cell death analysis of A549 cells with treatment of peptide W-0803. **C** Control of H460 cells. **D** Cell death analysis of A549 cells with treatment of peptide W-0803

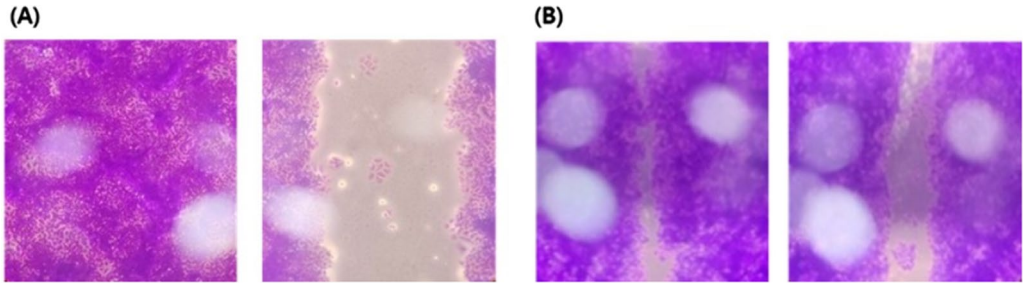

Fig. 5 Wound-healing assay of human epithelial lung cancer cell lines. **A** Wound-healing assay with A549 (control, left; peptide W-0803 treatment, right). **B** Wound-healing assay with H460 (control, left; peptide W-0803 treatment, right). In both cases, peptide W-0803 had an extent of inhibition capacity for cancer cell migration

W-0803 has an inhibitory capability on cancer migration of H460 and A549 in the treated concentrations. This result suggests that potential therapeutic interventions might be obtained for the treatment of lung cancer cells including A549 and H460 cells.

Discussion

It is known that natural compounds are great sources of anti-cancer agents. However, massive production is difficult in most cases due to the complexity of the compound. Depending on the choice of resources, the compound might be synthesized or easily obtained [8]. In our present study, we obtained an extract from *Anoplophora glabripennis* often called the Asian long-horned beetle [4–6]. The extract may contain a variety of biological compounds including many forms of anti-cancer proteins or peptides [15–17]. One of the kinds of anti-cancer proteins or peptides is known to have a net positive charge and α-helix structures. In this study, an anti-cancer peptide W-0803 was isolated by trypsin treatment of the protein fraction of the extract (Fig. 2). We expected that the anti-cancer effect could be increased by trypsin treatment of the extract (Figs. 1 and 2). This is because the anti-cancer proteins are cleaved behind arginine (R) or lysine (K), positively charged amino acids, by trypsin treatment so that cleaved peptides have a positive charge on the C-terminal. It means an increase in the amount (+) charge on peptides such as ACPs. For this reason, trypsin treatment could increase the extent of net positive charge on the peptide. It is known that the positive charge on ACPs increases the penetrating capability toward the cell membrane [18].

In this present study, we discovered peptide W-0803 which has the potential to be a new anticancer drug with low cytotoxicity. To confirm the anti-cancer effect of peptide W-0803, WST-1 assay was conducted to measure the cell viability of A549 and cells H460 (Fig. 3). The result shows that the viability of cancer cells decreased in a dose-dependent manner and that IC50 values were lower with H460 cells (17.3 μM) than with A549 cells (50.0 μM), indicating that H460 cells are more sensitive to W-0803 than A549 cells (Fig. 3). It was found that the extent of A549 viability slightly increased in a low concentration (3.125 μM). We thought that the small increase in cell viability might be induced by serine (S) residue in peptide W-0803. This is because serine was known to be involved in the increase of some tumor tissue volume [19]. However, the extent of A549 viability decreased from 6.25 μM down to 50 μM of peptide, suggesting that the increase is a temporary phenomenon. Next, we conducted a cell death experiment with PI/Annexin V staining to confirm whether the cell death by peptide W-0803 is apoptosis or necrosis (Fig. 4). The kind of cell death can be explained by various ratios of apoptosis and necrosis. We confirmed that peptide W-0803 induced programmed cell death mainly by apoptosis for both cell lines, regardless of the differentially affected cell viability between A549 and H460.

To confirm the inhibition of migration and metastasis, we conducted a wound-healing assay which is often employed to observe the inhibition of cancer cell migration by cancer drugs (Fig. 5). In the present study, the result shows that peptide W-0803 inhibited the recovery of the gap for both cell lines. It was found that the extent of cell migration was higher with A549 cells than with H460 cells, although the employed concentration of peptide W-0803 was higher for A549 cells than for H460 cells. This indicates that the sensitivity to peptide W-0803 is higher with H460 cells than with A549 cells. These results show that peptide W-0803 has an inhibitory capacity for tumor migration and metastasis.

The peptide W-0803 might be a choice of anti-cancer drug that has no side effects. This is because the peptide W-0803 has no cytotoxicity to HDF cells, the normal cell line, up to 50 μM (Fig. 3). Further experiments need to be performed with a higher concentration over 50 μM to make sure that the peptide W-0803 is not cytotoxic at the concentration for a clinical application. The

peptide could be easily degraded in vivo. This means that the peptide has a short half-life due to the digestion by various peptidases and removal from the kidney. To overcome this limitation, the peptide W-0803 needs to be modified by attachment of biostable molecules or peptide engineering by which amino acid sequence might be altered without changing the anti-cancer capability. All these findings suggest that more studies still need to be performed to prove that peptide W-0803 has an anti-cancer effect in vivo.

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Author contributions
JHY and JWL: Data curation, formal analysis, investigation, writing—original draft. SMH and DJL: Resources, software, validation. DCP and NC: Conceptualization, supervision, writing—review and editing. All the authors have read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Declarations

Competing interests
Namhyun Chung is an Executive Editor of Applied Biological Chemistry. Executive Editor status has no bearing on editorial consideration.

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