Anti-cancer Effect of Luminacin, a Marine Microbial Extract, in Head and Neck Squamous Cell Carcinoma Progression via Autophagic Cell Death

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Anti-cancer Effect of Luminacin, a Marine Microbial Extract, in Head and Neck Squamous Cell Carcinoma Progression via Autophagic Cell Death

Directed by Professor Eun Chang Choi

The Doctoral Dissertation
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of Doctor of Philosophy

Yoo Seob Shin

June 2015

This certificates that the Doctoral Dissertation of Yoo Seob Shin is approved.

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Thesis Committee Member : Chul-Ho Kim :

The Graduate School
Yonsei University

June 2015

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My family, who always had unwavering confidence in me, and have encouraged me on this academic journey.

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ABSTRACT

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The Graduate School, Yonsei University

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To determine whether Luminacin, a marine microbial extract from the marine Streptomyces species, has anti-tumor effects on head and neck squamous cell carcinoma (HNSCC) cell lines via autophagic cell death. The inhibition of cell survival and increased cell death was measured using cell viability, colony forming, and apoptosis assays. Migration and invasion abilities of head and cancer cells were evaluated using wound healing, scattering, and invasion assays. Changes in the signal pathway related to autophagic cell death were investigated. Drug toxicity of Luminacin was examined in *in vitro* the HaCaT cells and an *in vivo* zebrafish model. Luminacin showed potent cytotoxicity in HNSCC cells in cell viability, colony forming and FACS analysis. *In vitro* migration and invasion of HNSCC cells were attenuated by Luminacin treatment. Combined with Beclin-1 and LC3B, Luminacin induced autophagic cell death in head and neck cancer cells. In addition, in a zebrafish model used for toxicity testing, embryonic or neural toxicity was not resulted in by Luminacin treatment with a cytotoxic concentration for HNSCC cells. Taken together, these results demonstrate that Luminacin induces the inhibition of growth and cancer progression via autophagic cell death in HNSCC cell lines, indicating a possible alternative chemotherapeutic approach for the treatment of HNSCC.

Keywords: Luminacin; Head and neck cancer; Autophagy; Streptomyces; Cytotoxicity

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I. INTRODUCTION

Cancer is a major cause of death, and head and neck squamous cell carcinoma (HNSCC) occupies the seventh place in annual incidence worldwide.[1,2] The treatment of HNSCC involves surgery, external beam radiation or platinum-based chemotherapy.[3] In cases of locally advanced HNSCC, these three modalities should be administered concurrently or sequentially. Despite intense treatments, over 50% of patients with advanced HNSCC eventually experienced locoregional or distant failure within 2 years.[4,5] Although there have been a number of technical advances in chemotherapeutics, including targeted therapy for epidermal growth factor receptor (EGFR), the survival rates of HNSCC have not improved significantly.[6] Therefore, efforts to develop alternative an anti-cancer agent should be continued. The limited efficacy of current synthetic chemotherapeutics has necessitated the search for and evaluation of novel bioactive drugs, especially from natural compounds.[7]

Compounds derived from natural sources have played an important role in the development of novel anticancer therapeutics. From the 1940s to 2010, 112 naturally-derived anti-cancer drugs were discovered, purified and finally approved, out of a total of 206 approved anti-cancer drugs in the same period.[8] The ocean is one of the best sources of

bioactive natural products. Until now, over 22,000 marine natural compounds have been identified and reported in the literature.[9] Some of these compounds showed considerable tumor suppressing activity and currently are being investigated in clinical trials or used as lead compounds to develop new anti-cancer drugs.[7] We hypothesized that Luminacin (L512C2, Fig.1), which is a metabolite from the marine Streptomyces species, is a promising alternative therapeutic agent for HNSCC.

In this investigation, we determined whether a marine micro-organism extract, the metabolic product of Streptomyces species, has anti-proliferative and anti-progression effects on HNSCC cell lines, especially via the autophagic cell death pathway.

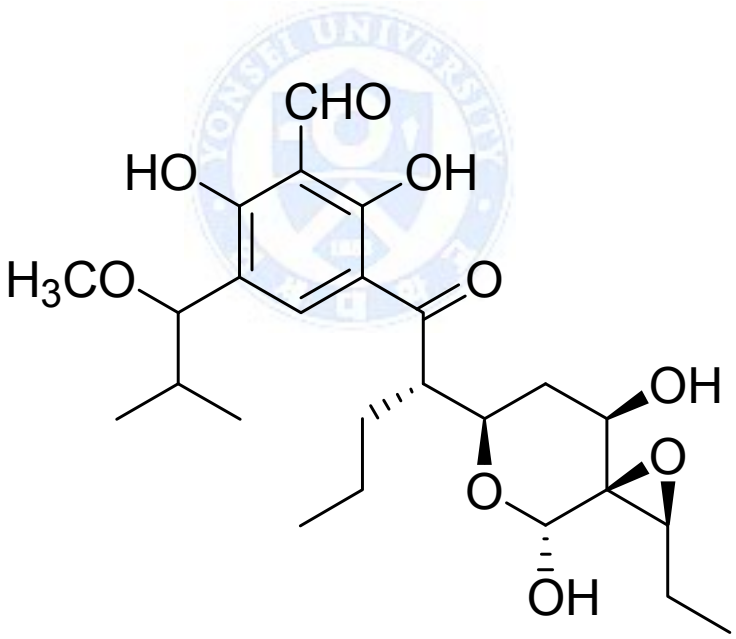


Fig. 1. Structure of Luminacin (L512C2).

II. MATERIALS AND METHODS

1. Cytotoxic effects on zebrafish

Freshly fertilized embryos (6 h post-fertilization) were treated with Luminacin (0, 0.1, 1, or 10 μg/mL). The hatching rate of embryos was assessed visually at 24 h intervals up to 3 days post fertilization (dpf) by light microscopy. Mortality was identified by the lack of a heartbeat, coagulation of the embryos, a non-detached tail and failure to develop somites. The morphology was assessed visually using an Axiovert 200 light transmission microscope (Carl Zeiss, Göttingen, Germany) at a magnification of 60–100X. Hair cell lateral line neuromasts were labeled using 2 μM YO-PRO1 (Molecular Probes, Eugene, OR, USA) for 30 min. The zebrafish were rinsed three times (5 min per wash) in embryo medium and anesthetized with 8 μg/mL 3-aminobenzoic acid ethyl ester methanesulfonate salt (MS-222; Sigma-Aldrich, St. Louis, MO, USA). The zebrafish were mounted with methylcellulose on a depression slide for observation under a fluorescence microscope.

2. Cell lines

Seven established human HNSCC cell lines – SCCQLL1, SCC15, SCC25, SCC1483, MSKQLL1, HN6 (oral cancer cell lines), and HNE1 (nasopharyngeal cancer cell line) – were obtained from American Type Culture Collection (Manassas, VA, USA) and Korean Cell Line Bank (Seoul, Korea). The cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and penicillin-streptomycin at 100 U/mL (GIBCO, Carlsbad, CA) at 37˚C in a humidified atmosphere with 5% CO_2 and 95% air. Luminacin were dissolved in autoclaved water as a stock solution for *in vitro* studies.

3. Cell viability assay

To determine cell viability, the HNSCC cell lines were seeded onto 96-well plates at densities of 5×10^3 cells/well in 1 mL complete medium with various concentrations of Luminacin (0 – 50 μg/mL). MTT, also known as 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (Sigma-Aldrich), was added to 40 μL of the cell suspension for 4 h. After three washes with phosphate buffered saline (PBS, pH 7.4), the insoluble formazan product was dissolved in dimethyl sulfoxide (DMSO). The optical density (OD) of each culture well was measured using a microplate reader (Bio-Tek, Winooski, VT, USA) at 540nm.

4. Colony forming assay

Colony forming assays were performed as previously described.[10] To determine long-term effects, HNE1 cells were untreated or treated with 30 ng/mL hepatocyte growth factor (HGF) after being treated with Luminacin (0, 0.1, or 1 μg/mL) in 24-well plates. After being rinsed with fresh medium, cells were allowed to grow for 3 days to form colonies, which were stained with 4% crystal violet (Sigma-Aldrich). More than 2 mm of the cells were counted.

5. Wound healing assay

Cell migration ability was measured using the wound healing assay as previously described.[10] Briefly, cells were grown to confluent monolayers. The monolayers were wounded by scratching the surface, as uniformly as possible, with a 1 mL pipette tip. MSKQLL1 cells were pre-treated with HGF (10 ng/mL) and then were treated with Luminacin (0, 0.05, 0.1, or 0.5 μg/mL). The images of the wound area were captured on day

0 (day of scratching) and on day 2 using an Olympus SC 35 camera (Tokyo, Japan) connected to an inverted microscope.

6. Scattering assay

The scattering assay was performed as previously described.[11] Briefly, MSKQLL1 cells were seeded in 24-well tissue culture dishes at a density of 1 X 10^5 per well and incubated under serum deprivation conditions for 24 h. Afterwards, the cells were cultured with or without Luminacin (0, 0.05, or 0.1 μg/mL) and HGF (10 ng/mL). After 24 h, the cells were stained 0.1% crystal violet solution (dissolved in 20% of methanol). The number of cell colonies, their sizes, and the degree of scattering were observed, and representative images were captured using light microscopy.

7. Invasion assay

The invasion assay was carried out as previously described[12] using 24-well transwell filters with an 8-μm pore size and coated collagen in filters. MSKQLL1 cells (2 X 10^4) in the upper chamber were pretreated with Luminacin (0, 0.05, or 0.1 μg/mL) and then with or without 10 ng/mL HGF. Both inserts and lower wells were treated with vehicle control (DMSO), Luminacin and HGF. The chambers were incubated for 16 h at 37 °C in an atmosphere containing 5% CO_2. After 16h, the cells in the insert were gently removed using a cotton swab. Cells on the lower surface of the filter were fixed and stained by using hematoxyin and eosin staining solutions. The number of invading cells was counted in four representative fields per membrane using light microscopy at 40X magnification.

8. Western blot assay

Cells were pre-treated with HGF (30 ng) or PBS for 10 min, and then the cells were washed twice with cold PBS. Subsequently, the cells were treated with Luminacin (0, 1, 5, or 10 μg/mL). Total proteins were extracted using the ProteoExtract® Subcellular Proteome Extraction Kit (Calbiochem, La Jolla, CA, USA) following the manufacturer's instructions. Protein concentrations were measured using the BCA assay (Pierce, Rockford, IL, USA). The proteins were separated by electrophoresis on 12% and 10% sodium dodecyl sulfate polyacrylamide (SDS) gels. An equal amount of protein (10 μg) was loaded in each lane. After electrophoresis, the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. The membrane was blocked in Tris-Buffered Saline Tween-20 (TBST) containing 5% non-fat milk for 1 h, and then were incubated overnight at 4°C with primary antibodies. All primary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). After washing the membrane extensively, incubation with horseradish peroxidase-conjugated secondary antibody (1:1000, Cell Signaling Technology) was performed for 1 h at room temperature. Protein bands on the blots were visualized using ECL Plus Western Blot detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

9. Annexin V-fluorescein isothiocynate (FITC)/propidium iodide (PI) double staining

Quantitative analysis of apoptotic cell death caused by Luminacin was performed using the FITC Annexin V Apoptosis Detection kit II (Becton Dickinson, Franklin Lakes, NJ, USA), following the manufacturer's protocols. Briefly, cells were plated at 1×10^6 cells/well in a 6-well plate, incubated for 16 h, and then treated with Luminacin (0, 1, 2.5, 5, 10, or 20 μM) for 24 h. The cells were harvested, washed with cold PBS, and subjected to Annexin V-FITC and PI staining in binding buffer at room temperature for 10 minutes in the dark. The

stained cells were analyzed by fluorescence-activated cell sorting (FACS ARIA3, BD Biosciences, San Jose, CA, USA) using WinMDI 2.9 Software.

10. Immunocytochemistry (ICC)

The SCC15 cells (5 x 10^4) were incubated on a coverslip in a12 well plate and then treated 10 μg/mL Luminacin for 24 h. Cells were washed twice with PBS. Cells were incubated with 4 % paraformaldehyde for 20 min at room temperature and then treated with 0.075 % Triton X-100 for 5 min to allow the antibody to permeabilize the cell membranes. After washing twice with PBS and blocking with 2 % BSA/PBS for 30 min, the slides were incubated with primary antibody, anti-LC3B I/II antibody (1: 100 dilution, Cell Signaling Technology, Danvers, MA, USA), overnight at 4 °C. Then, the slides were washed three times with PBS and incubated with secondary antibody (Alexa Flouor 488, goat anti-rabbit IgG, Invitrogen) for 30 min at room temperature. The digital images of stained cells were captured with the confocal microscope A1R-A1 (Nikon, Japan) and randomly selected.

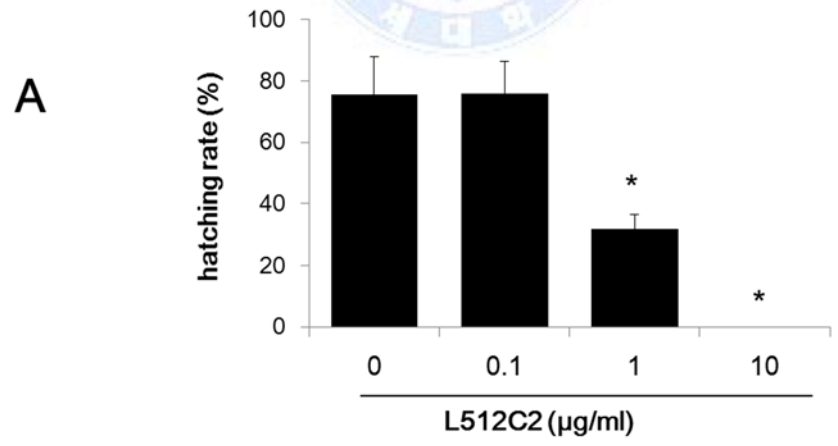
11. Statistical analyses

All values were expressed as mean ± standard deviation and statistical analysis was performed using the Kruskall-Wallis Test and the Mann-Whitney U Test (SPSS, version 17, Chicago, IL, USA). A *P*-value <0.05 was regarded as statistically significant.

III. RESULTS

1. Luminacin-induced in vivo *and* in vitro *toxicity in zebrafish embryos and HaCaT cells*

To determine *in vivo* toxicity of Luminacin, we exposed zebrafish embryos at 6 hours post fertilization to Luminacin (0, 0.1, or 1 μg/mL) and measured the effects of this treatment on their morphologic appearance and survival up to 3 dpf of development. Luminacin decreased hatching rates of the zebrafish embryos only at 1 μg/mL concentration (Fig. 2A, $P<0.05$). Fig. 2B shows the distribution of neuromasts in live 3 dpf zebrafish, as detected by staining with YO-PRO1. Luminacin exposure did not result in a significant loss of YO-PRO1 staining in neuromasts and there were few morphological changes in zebrafish embryos treated with 0.1 μg/mL Luminacin, but 1 μg/mL concentration of Luminacin caused total loss of neuromasts and definite morphological changes (Fig. 2C). As shown in Figure 2D, Luminacin treatment did not decrease the viability of the HaCaT cells, except in high concentration (5 μg/mL).



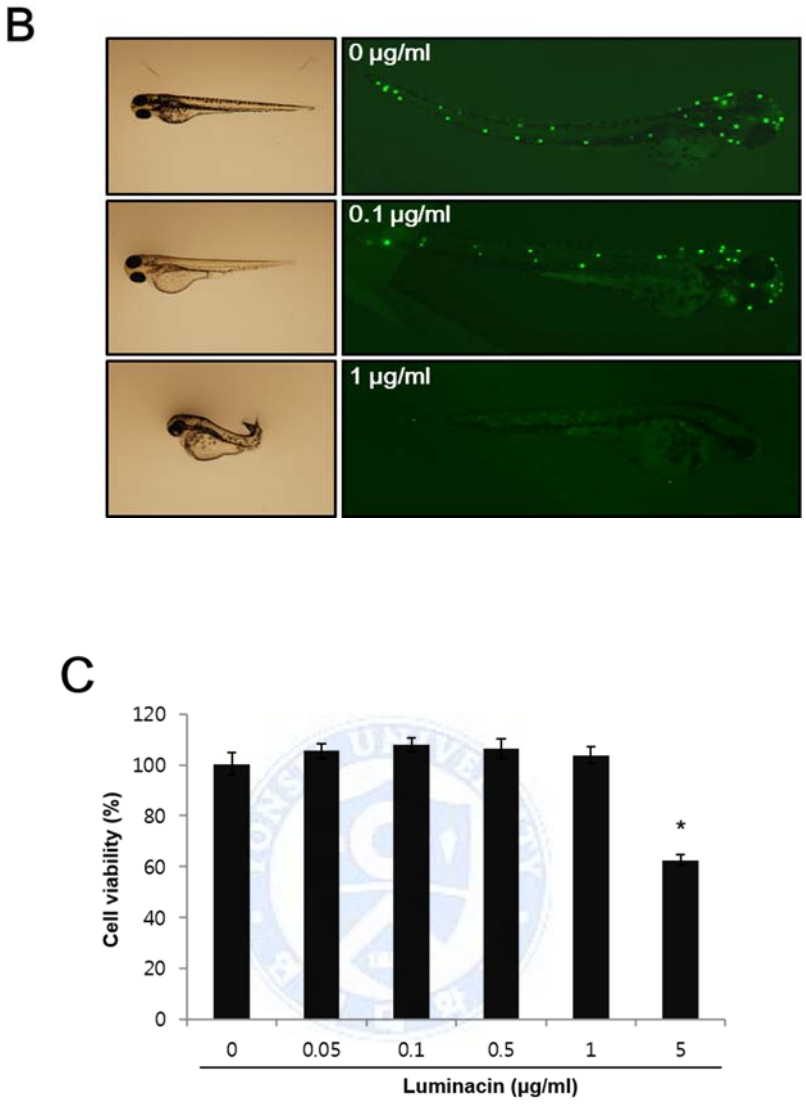


Fig. 2. *In vivo* and *in vitro* toxicity of Luminacin tested on zebrafish embryos and HaCaT cells.

Embryos were exposed to Luminacin at 6 h post-fertilization. (A) Hatching rate of zebrafish embryos. (B) Staining of neuromasts in zebrafish embryos with YO-PRO1 after treatment with Luminacin. Neuromasts were stained as white dots. Treatment with 0.1 µg/mL Luminacin did not decrease the number of neuromasts. (C) The HaCaT cells were exposed to various concentration of Luminacin (0 – 5 µg/mL). Cell viability was measured by MTT assay. Luminacin decreased viability of HaCaT cells only in a concentration of 5 µg/mL. The data represent the mean ± SD of three independent experiments. * $P<0.05$.

2. Luminacin inhibited cell growth in various head and neck cancer cells

As shown in Figure 3, treatment with Luminacin decreased the viability of various HNSCC cells. We examined the effects of different concentrations of Luminacin on HNSCC cells and found that Luminacin significantly inhibited HNSCC cell growth in a dose-dependent manner (Fig. 3).

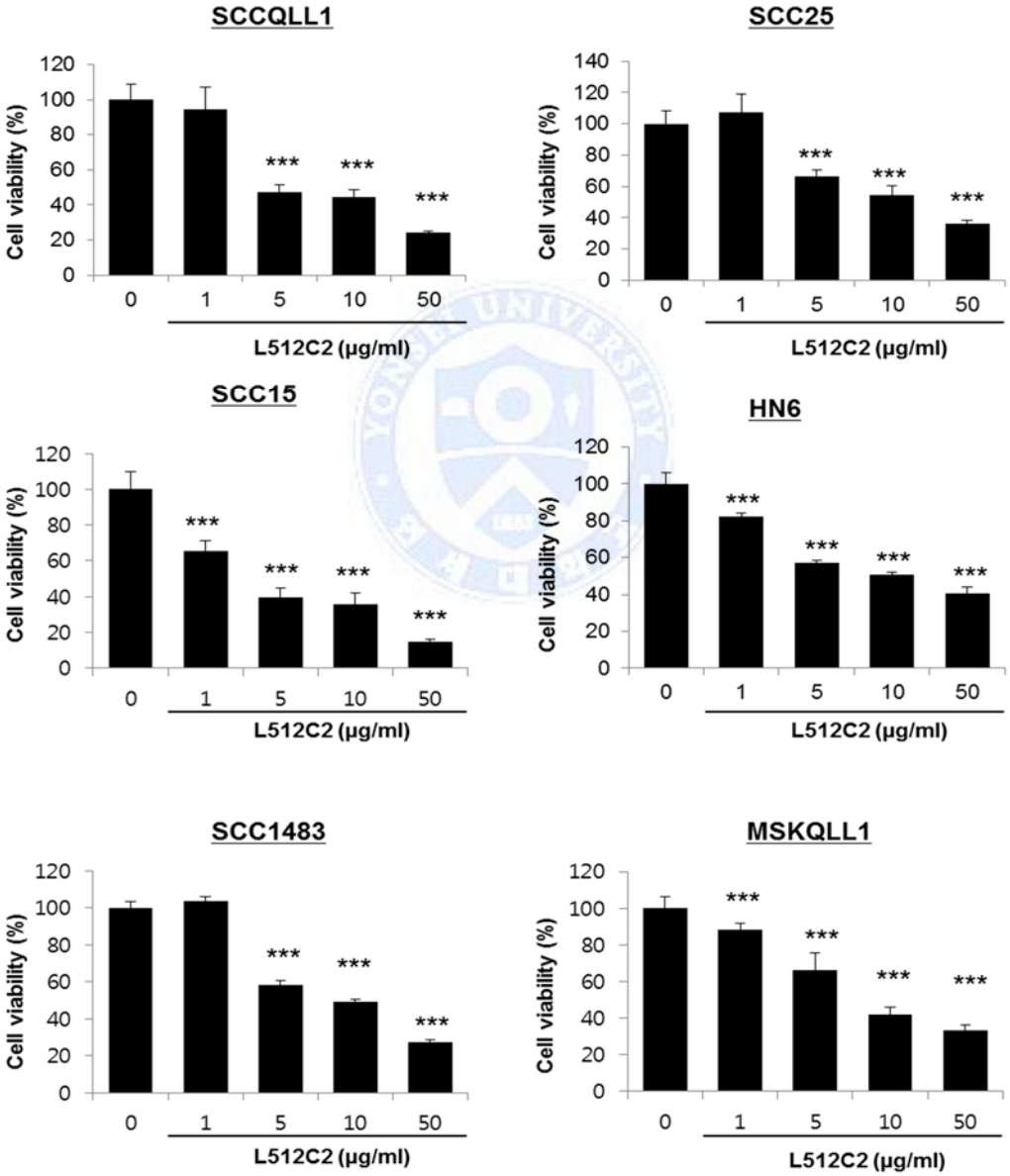


Fig. 3. Effects of Luminacin on proliferation in various head and neck carcinoma cell lines.

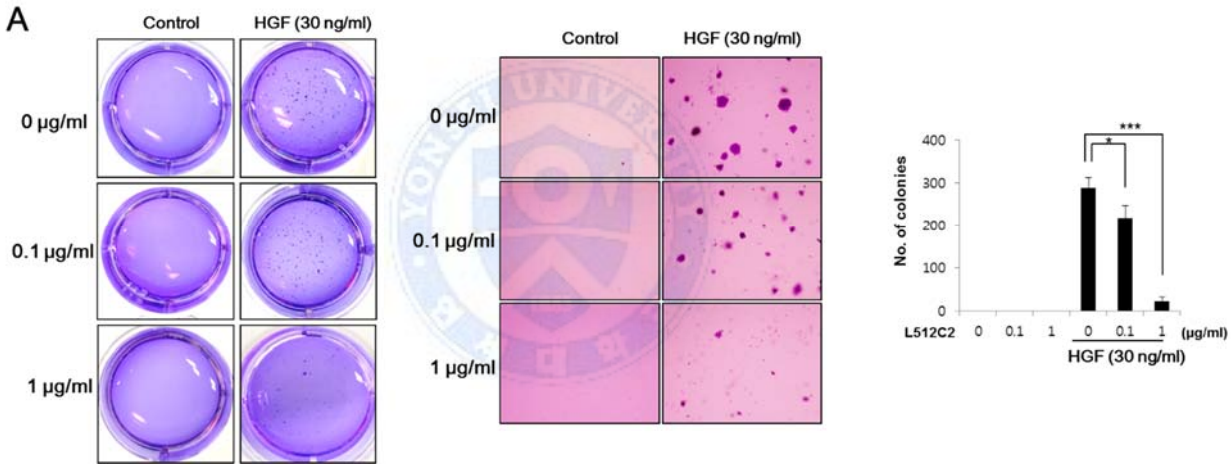
Results of the cell proliferation assay. The head and neck cancer cells were exposed to various concentrations of Luminacin (0 – 50 μg/mL). At 5 days after treatment, cell viability was measured by MTT assay. Luminacin significantly inhibited the proliferation of head and neck carcinoma cell lines. The data represent the mean ± SD of three independent experiments. *** $P<0.001$ compared to control.

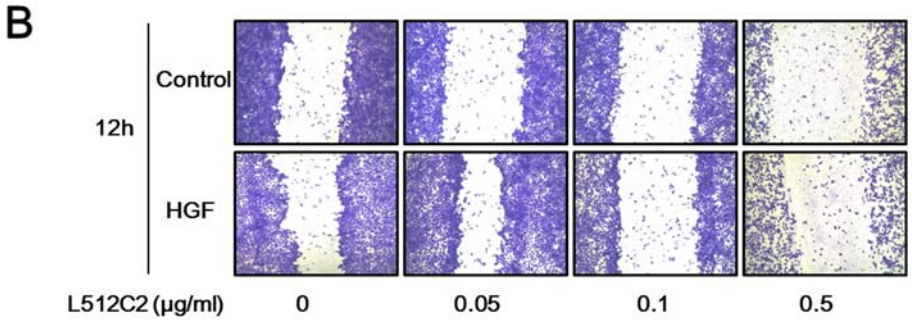
3. Luminacin decreased viability, migration, and invasion capability induced by HGF in head and neck cancer cells

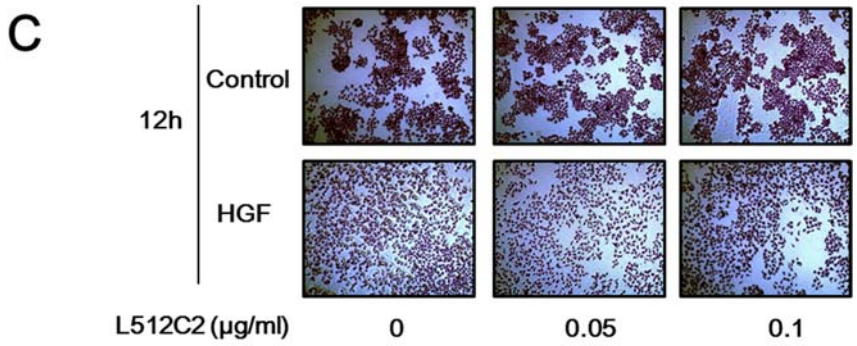
HGF is widely accepted as a potential enhancer of invasive growth in HNSCC. To analyze the suppressive effect of Luminacin on the survival of the HNSCC cell lines, we performed a colony-forming assay. As shown in Figure 4A, pre-treatment with Luminacin completely suppressed the survival of HNSCC cells that were not treated with HGF. Furthermore, the survival rate of cells co-treated with HGF and Luminacin was statistically significant (Fig. 4A). The wound healing assay was used to determine the migration capability of HNSCC cells. HGF enhanced the proliferation and migration abilities of the cells; this enhancement was successfully inhibited by Luminacin treatment (Fig. 4B). The degree of scattering in HNSCC cells treated with or without HGF/Luminacin was observed. HGF-treated cells showed increased scattering behavior, while HGF/Luminacin co-treated cells exhibited a discernible reduction in HGF-induced cell scattering (Fig. 4C). We further addressed the effect of Luminacin on HNSCC cell invasion using transwells, and found that co-treatment with Luminacin and HGF significantly inhibited cell invasion compared to treatment with

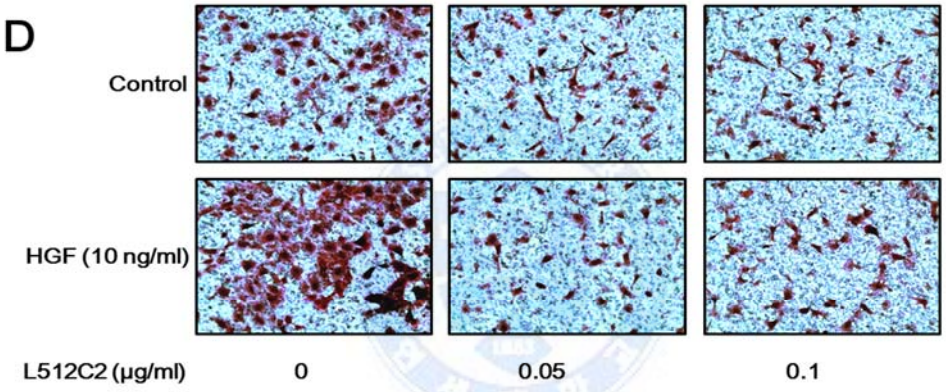
HGF alone (Fig. 4D), indicating that Luminacin inhibited HGF-induced HNSCC cellular migration and invasion.

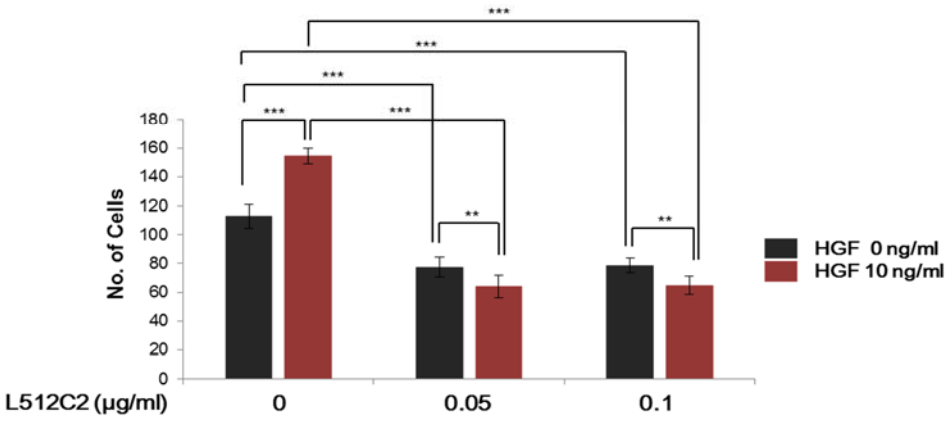
We next determined whether HGF-induced changes at the protein level were restored by Luminacin. A representative western blot is shown in Figure 4E; the results confirmed increased protein levels or phosphorylation of p-Met and p-Erk after HGF treatment. Luminacin inhibited the HGF-induced increase in phosphorylation of p-Met and the downstream target, p-Erk (Fig. 4E). These results suggest that the suppressive mechanisms of Luminacin and the decreased progression may correlate with reduced phosphorylation of Met.






C
12h
Control
HGF
L512C2 (μg/ml)
0
0.05
0.1


D
Control
HGF (10 ng/ml)
L512C2 (μg/ml)
0
0.05
0.1


No. of Cells
180
160
140
120
100
80
60
40
20
0
***
**
HGF 0 ng/ml
HGF 10 ng/ml
L512C2 (μg/ml)
0
0.05
0.1

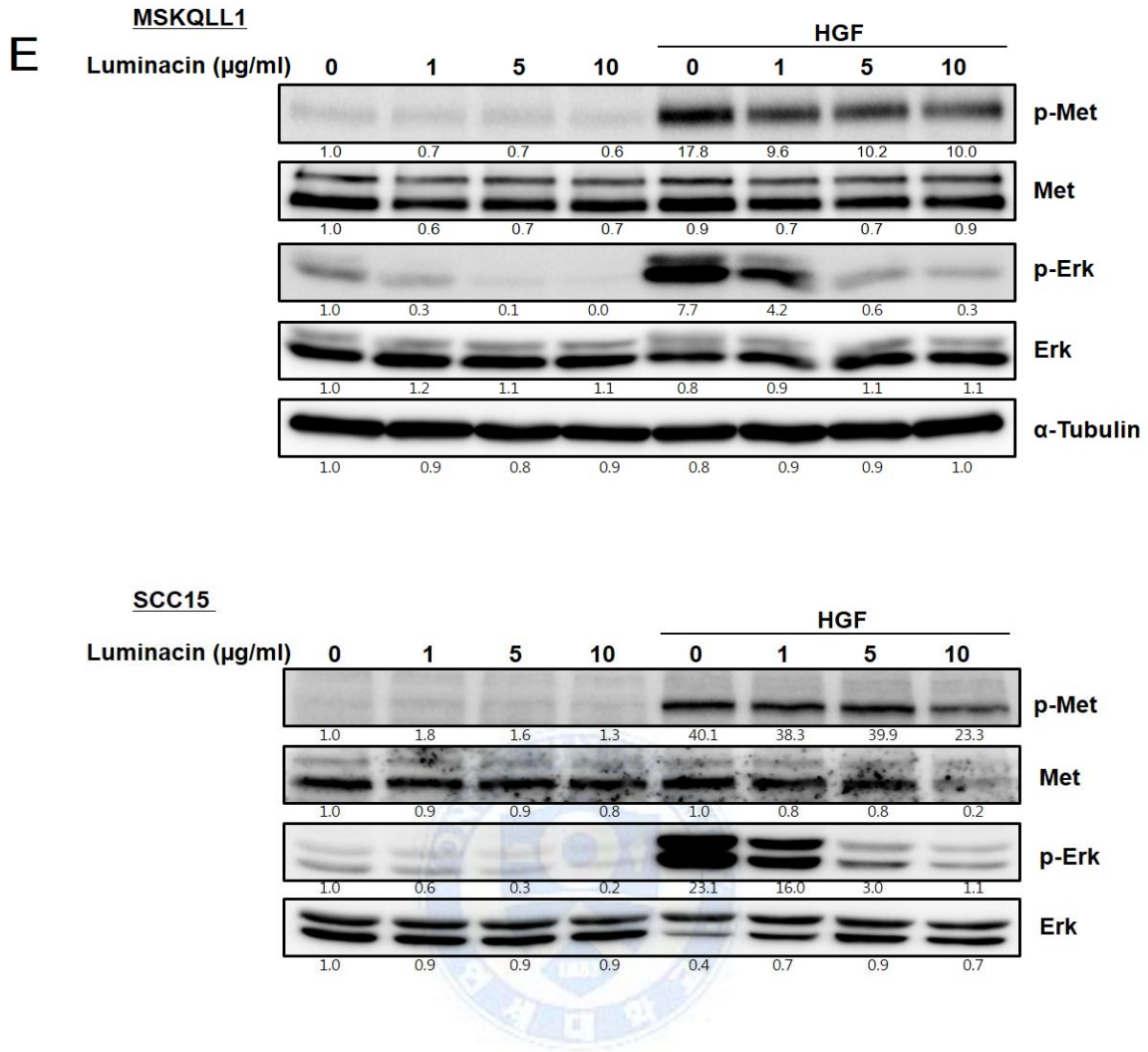


Fig. 4. Effect of Luminacin on HGF-induced viability, migration, and invasion capability in head and neck carcinoma cells

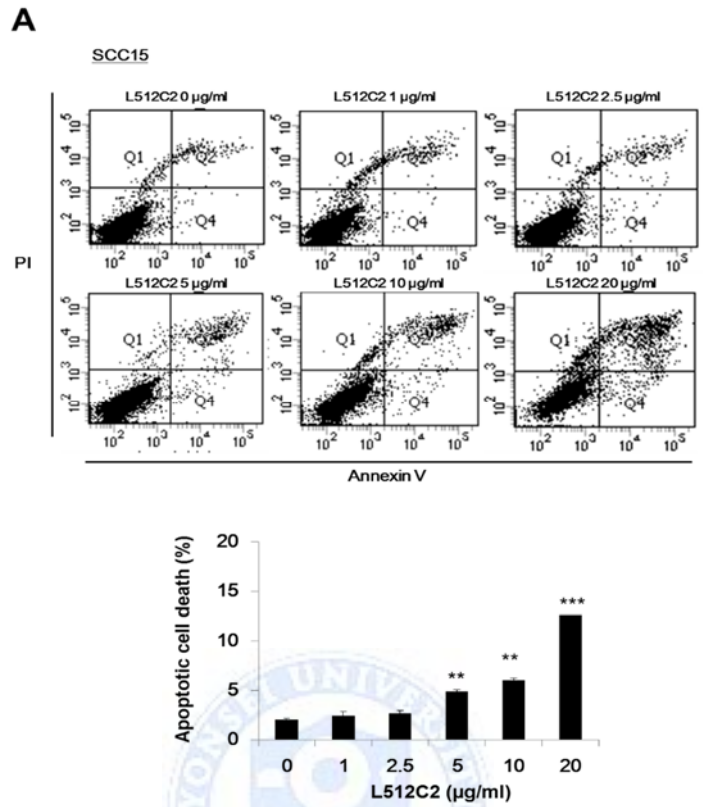
Investigation of cell viability, migration, and invasion capability after HGF/Luminacin treatment using colony forming, wound healing, cell scattering, and invasion assays. (A) HNE1 cells were treated or untreated with 30 ng/mL HGF and Luminacin (0, 0.1, 1 μg/mL) and then were incubated for 3 days to form colonies. After 4% crystal violet staining, more than 2 mm of the colonies were counted. Luminacin treatment significantly inhibited the survival rates of cells treated with HGF. (B) Confluent monolayers of MSKQLL1 cells were wounded by scratching the surface as uniformly as possible with a 1 mL pipette tip. Cells

were treated or untreated with 10 ng/mL HGF and Luminacin (0, 0.05, 0.1, 0.5 μg/mL), and then were cultivated for another 24 h. Percent closure of wound areas was measured. Luminacin treatment significantly inhibited HGF-induced enhancement of cell proliferation and migration, dose-dependently. (C) MSKQLL1 cells were cultured with or without Luminacin (0, 0.05, or 0.1 μg/mL) and HGF (10 ng/mL). After 24 h, the cells were stained in a 0.1% crystal violet solution. The number of cell colonies, their sizes, and the degree of scattering were observed. HGF/Luminacin co-treated cells exhibited a discernible reduction in HGF-induced cell scattering. (D) MSKQLL1 cells (2×10^4) in the upper chamber were pretreated with Luminacin (0, 0.05, or 0.1 μg/mL) and then with or without 10 ng/mL HGF.

After 16 h, the number of invading cells was counted in 4 representative fields per membrane. Co-treatment with Luminacin and HGF significantly inhibited cell invasion compared to treatment with HGF alone. (E) Immunoblot of Luminacin/HGF treated cells stained with antibodies against Met, p-Met, Erk, and p-Erk. Luminacin inhibited HGF-induced enhancement of phosphorylation of p-Met and the downstream target, p-Erk. The data represent the mean ± SD of three independent experiments. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

4. Luminacin induced cytotoxicity through autophagic cell death.

To determine whether Luminacin induced apoptosis in HNSCC cells, cells were treated with Luminacin (0, 1, 2.5, 5, 10, and 20 μg/mL) before being subjected to Annexin/PI FACS apoptosis analysis. As shown in Fig.5, the percentages of apoptotic cells in Luminacin-treated groups did not exceed 15% in SCC15 or 40% in MSKQLL1 cells, even when treated with 20 μg/mL Luminacin. Our results demonstrated that apoptosis, or necrosis, is not involved in Luminacin-induced cell death.


A
SCC15
L512C2 0 μg/ml
L512C2 1 μg/ml
L512C2 2.5 μg/ml
L512C2 5 μg/ml
L512C2 10 μg/ml
L512C2 20 μg/ml
Q1
Q2
Q4
PI
Annexin V
Apoptotic cell death (%)
L512C2 (μg/ml)
0
1
2.5
5
10
20
*
**
***

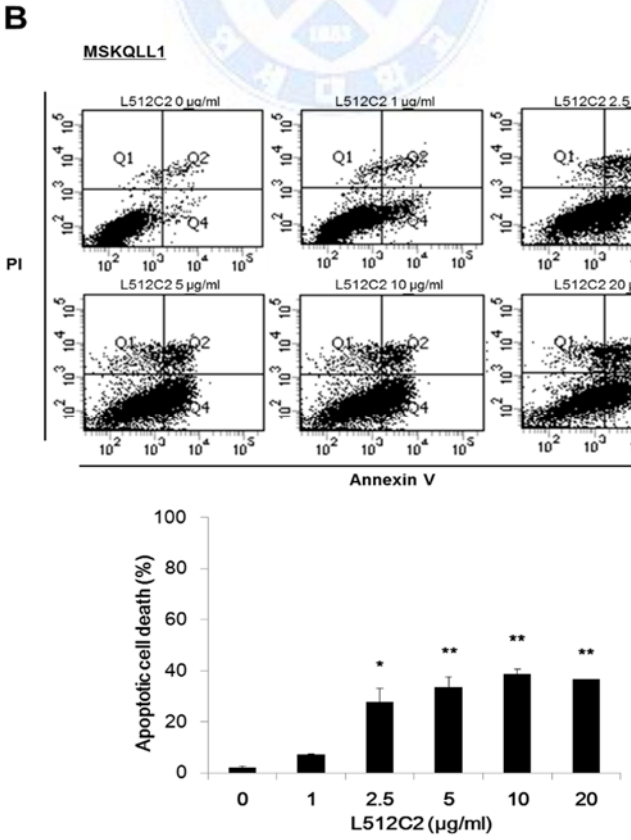
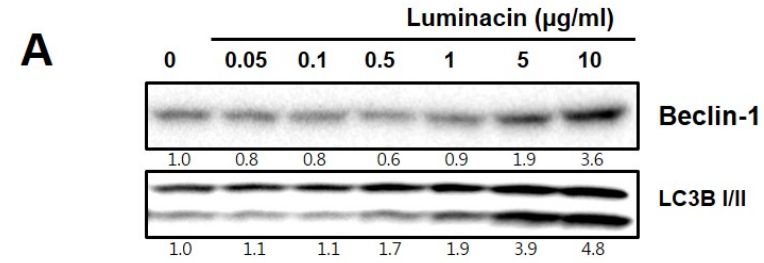
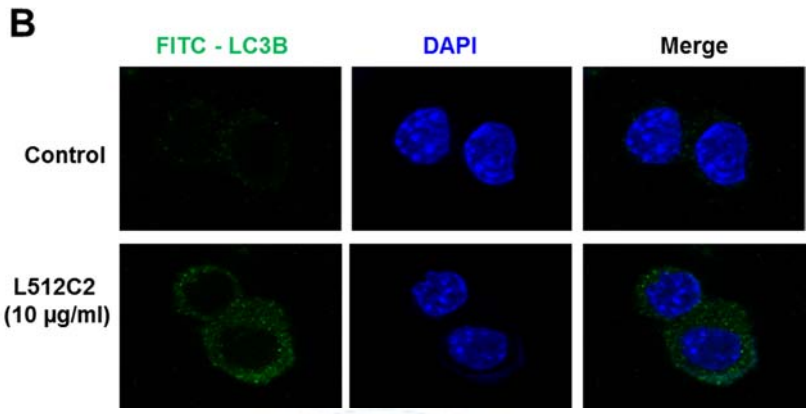

B
MSKQLL1
L512C2 0 μg/ml
L512C2 1 μg/ml
L512C2 2.5 μg/ml
L512C2 5 μg/ml
L512C2 10 μg/ml
L512C2 20 μg/ml
Q1
Q2
Q4
PI
Annexin V
Apoptotic cell death (%)
L512C2 (μg/ml)
0
1
2.5
5
10
20
*
**

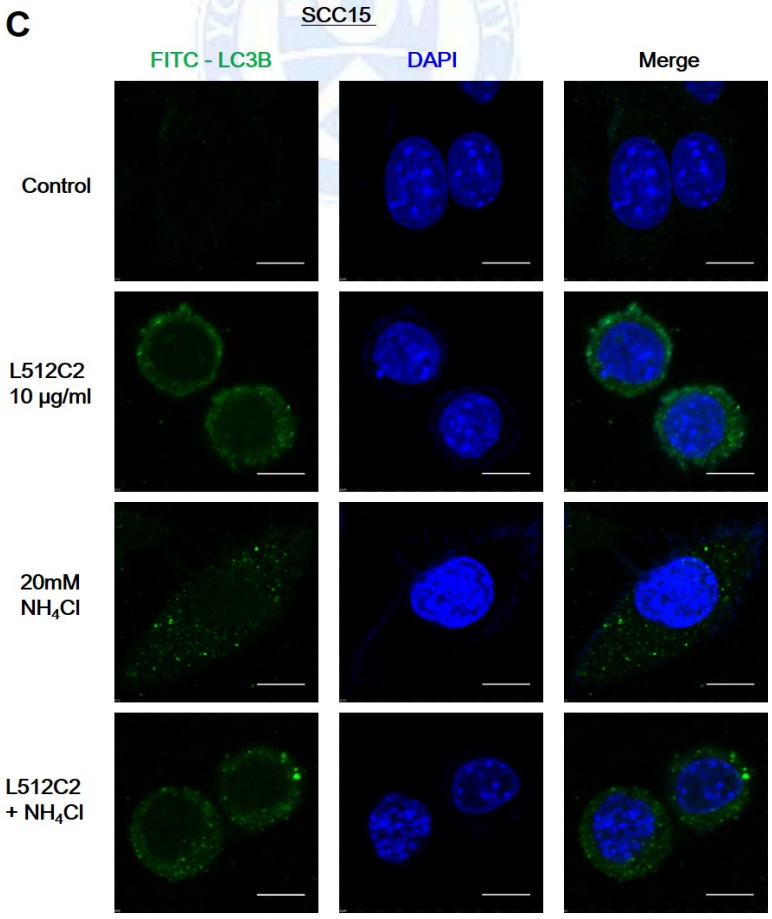
Fig. 5. Apoptotic cell death induced by Luminacin treatment in head and neck cancer cells.

Cells were treated with Luminacin (0 - 20 μg/mL). To quantify the Luminacin-induced apoptosis, we used flow cytometry: Annexin V-FITC and propidium iodide staining were used to analyze the percentage of apoptotic cells treated with Luminacin. The percentages of apoptotic cells in Luminacin-treated groups did not exceed (A) 15% in SCC15 cells and (B) 40% in MSCQLL1 cells, even when treated with 20 μg/mL Luminacin. The data represent the mean ± SD of three independent experiments. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

We next determined whether the cell death induced by Luminacin treatment was mediated through autophagy. As determined by western blot analysis, Luminacin increased the expression of Beclin-1 and LC3B I/II, which are proteins required for autophagosome formation (Fig. 6A). In addition, confocal microscopy showed that Luminacin-treated cells expressed LC3B, indicating that cells underwent autophagic cell death (Fig. 6B). When we treated the cells with NH_4Cl, well-known autophagy inhibitor, expression of LC3B was decreased (Fig. 6C), and consequently survival of the cells also increased (Fig. 6D). We next performed western blot analysis to determine whether Akt and MAPK signaling, which contribute to autophagy, were blocked by Luminacin. As shown in Figure 6E, Luminacin treatment increased p-JNK and p-p38 protein levels subsequently resulted in decreased p-Akt phosphorylation. These results suggest that the mechanism by which Luminacin induces autophagic cell death may involve the Akt and MAPK signaling pathways.


A
Luminacin (μg/ml)
0
0.05
0.1
0.5
1
5
10
Beclin-1
1.0 0.8 0.8 0.6 0.9 1.9 3.6
LC3B I/II
1.0 1.1 1.1 1.7 1.9 3.9 4.8


B
FITC - LC3B
DAPI
Merge
Control
L512C2
(10 μg/ml)


C
SCC15
FITC - LC3B
DAPI
Merge
Control
L512C2
10 μg/ml
20mM
NH4Cl
L512C2
+ NH4Cl

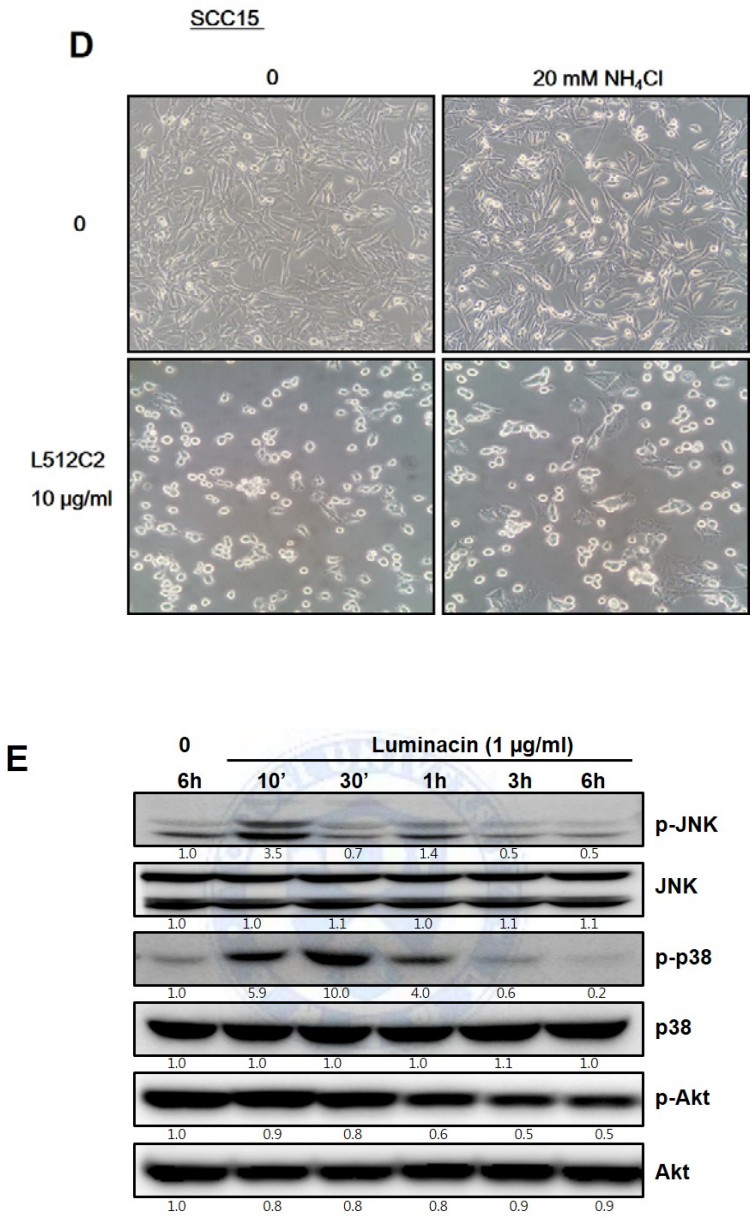


Fig. 6. Autophagic cell death in head and neck cancer cells induced by Luminacin treatment.

(A) Cell lysates were collected, electrophoresed through an SDS–polyacrylamide gel, and subjected to immunoblot analysis with antibodies against Beclin-1 and LC3B I/II. Luminacin increased the expression of Beclin-1 and LC3B I/II, which are known as autophagosome formation proteins. (B) Immunocytochemistry of LC3B was performed after Luminacin (10

μg/mL) treatment for 24 h. Results from confocal microscopy showed that Luminacin-treated cells expressed LC3B, indicating that cells underwent through autophagic cell death process. (C) NH_4Cl (20 mM) treatment decreased the expression of L3B expression which was increased by Luminacin treatment. (D) Survival of SCC15 was restored by autophagy inhibitor, NH_4Cl. (E) Representative western blot analysis of Akt and MAPK signaling indicated Luminacin-induced cell death was involved with these signaling pathway.

IV. DISCUSSION

Nature-derived products have infinite potential for use in biomedical and pharmaceutical applications, so they also can be applied to diverse biotechnological indications.[13-15] The biomedical and pharmaceutical application of a natural substance is related to its anti-viral, anti-tumor, anti-microbial, and general cytotoxic properties.[16] Natural product drug might have advantages of shorter development periods and smaller research costs than those of completely synthetic compounds.[9] From 1981 to 2010, over 50% of new chemical entities were developed from natural substances, either natural products themselves or natural products that were modified in one way or another.[8] In anti-cancer drug category, only 20.2% of anti-cancer drugs were developed using completely synthetic methods.

As sources of novel bioactive substances, several marine microbes have come into the spotlight in the field of drug development. One of the most promising groups of microbes is in the genus Streptomyces, the members of the Actinomycetes, which are known to produce several antibiotics and bioactive compounds.[17,18] Some Streptomyces have biosynthetic gene groups that produce over 30 secondary metabolites.[19-21] Thus, only a single Streptomyces strain can be a source of more than 30 bioactive substances. Luminacin, which is one of the metabolites produced by Streptomyces, was originally found in the course of screening for brand-new angiogenesis inhibitors. Luminacins are composed of fourteen components with similar structures, A1, A2, B1, B2, C1, C2, D, E1, E2, E3, F, G1, G2, and H[18]. Since, Luminacin C2 (L512C2) was available in the largest quantity and had the strongest bioactive potency, investigators mainly focused on this component. Several subsequent studies have demonstrated the biological efficacy of Luminacin, but little has been revealed to date, especially regarding the anti-cancer capability of this component. Atatreh *et al* reported that Luminacin suppresses non-tyrosine kinase interactions that play a role in growth factor signal

transduction and cell adhesion in human colorectal carcinoma cells.[22] We hypothesized that Luminacin could be a promising alternative anti-cancer molecule for HNSCC. The aim of the present study was to determine the safety and effectiveness of Luminacin on proliferation, migration, and invasion of HNSCC cell lines *in vitro* and *in vivo* in a zebrafish model.

In this study, Luminacin did not show cytotoxic effect on the survival and neural development of zebrafish embryos in toxic concentration to HNSCC cell lines. Luminacin-induced cell death was detected by cell viability assay in a number of HNSCC cell lines. Luminacin successfully inhibited HGF-induced proliferation, migration, and invasion of HNSCC cell lines. HGF-induced Met and downstream Erk activation were also blocked by Luminacin treatment. Regarding the type of cell death, Luminacin increased Beclin-1 and LC3B expression, indicating involvement of autophagic cell death. The JNK, p38 MAPK and Akt pathways were involved in the cell death mechanism induced by Luminacin treatment. These findings suggest that anti-tumor effects of Luminacin on HNSCC cell lines might be achieved via autophagic cell death.

Autophagy is one of the most important types of cell death, a cellular degradation process in response to stressed conditions. Cellular proteins and cytoplasmic organelles are degraded within autophagosomes and eventually by lysosomal enzymes.[23-25] Double- or multi-layered, large-scale autophagic vacuolization is observed without chromatin condensation.[25] However, the role of autophagic cell death in cancer development, progression, or treatment is still unclear and controversial. On one hand, autophagy might operate as a protective mechanism that inhibits uncontrolled cell proliferation.[23] Supporting this hypothesis, some cancers respond to agents that trigger autophagy, showing the possibility of anti-cancer therapy that targets autophagic cell death induction.[23] Several anti-cancer drugs, such as 5-fluorouracil, rapamycin, and tamoxifen are known to induce autophagic cell death.[26-28] Another point of

view in cancer biology is that autophagy is a pro-survival mechanism. Growing evidence indicates that autophagy enables cancer cell survival under stressed conditions induced by hypoxia, starvation or cancer treatment.[29,30] Inhibition of autophagy facilitates the anti-cancer efficacy of chemotherapeutic agents including oxaliplatin or doxorubicin.[31,32] In this study, Luminacin consistently showed cytotoxic effects on different HNSCC cell lines, and in most cases, cell death was not caused by apoptosis or necrosis. Given the increased expression of key autophagy proteins such as Beclin-1 or LC3B, autophagy may play an important role in the cytotoxicity of Luminacin.

Meanwhile, several studies have shown that some agents exert their cytotoxicity not only through autophagy, but also through apoptosis, depending on the cell type and experimental conditions.[33] Many reports show an inter-relationship of apoptosis and autophagy, because activation of these two death modalities involves the same molecular pathway and occurs simultaneously.[34,35] In this study, apoptosis by Luminacin treatment was responsible for 15% of SCC15 and 40% of MSCQLL1 cell death. Our results demonstrated an anti-cancer potential for Luminacin by showing that autophagy works with the help of apoptosis to induce cell death. Further investigations should be performed to determine which conditions can induce autophagy or apoptosis for cancer therapy with Luminacin.

There are several molecular pathways involved in the induction of autophagy. Deregulation of Akt serine/threonine kinase is a well-known cause of anti-apoptosis, and uncontrolled cell proliferation.[36] Hennesy *et al* reported that autophagy is induced by regulation of the PI3K/Akt/mTOR pathway.[37] Baicalin, a possible anti-cancer agent derived from a natural substance, is reported to trigger autophagy in human bladder cancer cells via modulation of the Akt pathway.[36] In addition, MAPK, including JNK and p38 MAPK, also has been reported to regulate autophagy.[33] Recent studies showed that activation of JNK is

closely involved in the induction of autophagy.[38,39] p38 MAPK, which regulates cell differentiation, migration, and invasion, is also strongly associated with autophagy.[40] In this investigation, increased expression of p-JNK, p-p38, and decreased expression of p-Akt phosphorylation by treatment with Luminacin demonstrated the possible involvement of Akt and the JNK, p38 MAPK pathways in the induction of autophagy and cell death.

V. CONCLUSION

Growth and progression inhibition via autophagic cell death were successfully induced in HNSCC cells by treatment with Luminacin, a metabolite of the marine Streptomyces. The in vivo zebrafish model confirmed acceptable toxicity of Luminacin, even at cytotoxic concentrations to HNSCC. Our results demonstrate that Luminacin is a plausible chemotherapeutic agent for HNSCC, even though further characterization is necessary.

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ABSTRACT (IN KOREAN)

신규 해양미생물 추출물, Luminacin의 Autophagic cell death 를 통한 두경부암 항암효과분석

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신 유 섭

종양의 발생과 진행에 있어서 중요한 역할을 하는 것이 신생혈관의 생성(angiogenesis), 종양의 침습(invasion)과 전이(metastasis)이고 이 과정에는 종양과 숙주(host)사이의 여러 반응(tumor and host interaction)이 필요하며 이에는 간질 변화(stromal alterations)이 종양의 악성화에 앞서 선행되고 이어 종양세포와 간질세포간의 반응을 통해 세포외 기질의 변화, 신생혈관생성, 종양의 전이 및 침습에 관여한다는 것이 알려졌다. 이러한 일련의 과정에 관여하는 원인인자를 밝혀내는 것은 병의 생태병리에 대한 이해뿐 아니라 궁극적으로 암의 진단과 치료를 위한 기초가 될 것이다. 이러한 원인인자의 하나인 HGF(hepatocyte growth factor)는 hepatocyte의 성장에 관여하는 것으로만 알려져 있다가 최근 이 물질이 종양을 포함한 다양한 세포에서 세포의 성장뿐 아니라 종양의 활동(motility)과 침습을 일으키고 형질변경과 신생혈관의 생성에 관여함이 증명되면

서 주목받기 시작했다. 한편 천연추출물로 해양미생물 Streptomyces species 로부터 기원한 Luminacin (분자식: C25H36O9)은 여러 연구를 통해 항암 및 면역억제, 항균, 항골다공증 등에 생리활성을 보이는 것으로 밝혀져 있는 물질이다. 이에 Luminacin의 두경부암에서의 항암효과에 대해 알아보고자 하였다. 본 연구는 기존의 연구를 통해서 c-met oncogene이 상피세포에서 발현되며 상피에서 유래한 두경부 암종에서 종양의 진행과 연관되어있음을 기초로 하여, 두경부암에서의 신규 해양미생물 추출물의 HGF억제와 autophagic cell death에 관하여 연구하고자 하였다. Luminacin의 독성에 관해서는 zebrafish embryo와 normal keratinocytes cell line 에서 일반적인 세포독성을 확인하였고, 중간정도의 독성을 보임을 확인하였다. 본 연구에서는 Cell viability assay, Annexin stain, FACS analysis 를 통해 Luminacin에 의해 유도되는 세포사멸현상을 확인하였다. 또한, Luminacin이 두경부암 세포주에서 HGF에 의해 유도되는 증식, 분산, 이동과 침습을 억제하는 것을 wound healing, scattering, 그리고 invasion assays 를 통해 확인하였다. 세포사멸현상을 분석하고 대표적인 세포사멸경로에 대해 조사하였고, Beclin-1 과 LC3B 의 증가를 western blot 과 immucocytochemical stain 에서 확인하여 autophagic cell death mechanism 의 연관을 확인하였다. 결론적으로 Luminacin의 Autophagic cell death 를 통한 두경부암 항암효과를 확인할 수 있었다.

핵심되는 말: Luminacin; 두경부암; Autophagy; Streptomyces; 세포독성