CEO *Clinical and Experimental Otorhinolaryngology* Vol. 3, No. 4: 217-225, December 2010 DOI 10.3342/ceo.2010.3.4.217

Original Article

Growth Inhibition and Apoptosis with H31 Metabolites from Marine *Bacillus SW31* in Head and Neck Cancer Cells

Young Chang Lim, MD[1,2] · Ki Woong Cho, PhD[3] · Hak Cheol Kwon, PhD[4] · Sung Un Kang, MS[5,6]
Jung Hee Pyun, MS[5,6] · Mi Hye Lee, MS[5,6] · Hye Sook Hwang, MS[5,6] · Jang Hee Kim, MD[7] · Ha Neul Lee, MS[5,6]
Eun Chang Choi, MD[8] · Chul-Ho Kim, MD[5,6]

[1]Department of Otorhinolaryngology-Head and Neck Surgery, [2]Research Institute of Medical Science, Konkuk University School of Medicine, Seoul; [3]Department of Marine Biotechnology, Anyang University, Anyang; [4]Korea Institute of Science and Technology, Gangneung; [5]Department of Otolaryngology, [6]Center for Cell Death Regulating Biodrug, [7]Department of Pathology, Ajou University School of Medicine, Suwon; [8]Department of Otorhinolaryngology, Yonsei University College of Medicine, Seoul, Korea

Objectives. To determine whether a novel marine micro-organism with anticancer properties, H31, the metabolic product of *Bacillus SW31*, has anti-tumor effects on head and neck cancer, and potential for apoptotic-enhancing anti-cancer treatment of affected patients.

Methods. The cell viability and apoptosis assays were performed. Changes in the signal pathway related to apoptosis were investigated. Then, the therapeutic effects of H31 were explored in mouse xenograft model and drug toxicity of H31 was examined in zebrafish model.

Results. We identified the anticancer activity of H31, a novel metabolic product of *Bacillus SW31*. *Bacillus SW31*, a new marine micro-organism, has 70% homology with *Bacillus firmus* and contains potent cytotoxic bioactivity in head and neck cancer cells using MTT assay. Combined with c-JUN, p53, cytochrome C, and caspase-3, H31 induced apoptosis of KB cells, a head and neck cancer cell line. In a separate *in vivo* model, tumor growth in C3H/HeJ syngeneic mice was suppressed by H31. In addition, in a zebrafish model used for toxicity testing, a considerable dose of H31 did not result in embryo or neurotoxicity.

Conclusion. Growth inhibition and apoptosis were achieved both *in vitro* and *in vivo* in head and neck cancer cells after exposure to H31, a metabolite from the marine *Bacillus* species, without any significant toxicity effects even at considerable H31 dose concentrations.

Key Words. *Apoptosis, Bacillus, Marine toxins, Head and neck cancer, Cytotoxicity*

INTRODUCTION

Head and neck cancer is the fifth most common cancer worldwide, accounting for over 35,000 new cases of cancer each year in the United States alone (1). Despite technical advances in surgery, radiotherapy, and chemotherapy, survival rates have remained virtually unchanged over the last 50 years (2). Recently, the primary treatment modalities have increasingly shifted from surgery to chemotherapy and/or radiotherapy (3). A number of anti-tumor agents for chemotherapy have been developed and used; however, the considerable toxic effects of the current drug therapies have decreased the quality of life of the patients treated with these agents (4). The limitations of current therapy have motivated the investigation and evaluation of novel bioactive compounds, especially from natural products (5).

• Received July 10, 2010
Accepted after revision November 1, 2010

• Corresponding author: **Chul-Ho Kim, MD**
Department of Otolaryngology, Ajou University School of Medicine, Woncheon-dong, Yeongtong-gu, Suwon 443-721, Korea
Tel: +82-31-219-5269, Fax: +82-31-219-5264
E-mail: ostium@ajou.ac.kr

The ocean has always been a source for new bioactive metabolites, especially from micro-organisms (6). Over 200 novel metabolites were identified from marine micro-organisms in 2005 alone (7). Recent studies have reported that natural products from marine micro-organisms have potent cytotoxic characteristics, potentially useful for future pharmaceutical and biomedical applications (8).

One key mechanism associated with cancer cell growth is the control of apoptosis. Apoptosis is defined as programmed cell death, which occurs in response to disruption of normal homeostatic mechanisms; it is critical to the surveillance of tumor and/or malfunctioning cells (9). Induction of apoptosis among cancer cells has been considered one potential novel approach to the treatment of head and neck cancer (10).

Therefore, the goal of this study was to determine whether a novel marine micro-organism with anticancer properties, H31, the metabolic product of *Bacillus SW31*, has anti-tumor effects on head and neck cancer, and potential for apoptotic-enhancing anti-cancer treatment of affected patients.

MATERIALS AND METHODS

Isolation and identification of cytotoxic *Bacillus SW31*

Seawater samples were collected from Kanghwa Island (western coast of Korea). The seawater samples (0.1 mL) were streaked onto the surface of 1/10 marine agar (bacto tryptone 5 g, yeast extract 3 g, bacto agar 10 g, glycerol 3 mL, dissolved in 75% seawater, pH 7.5). After incubation at room temperature for 24 hours, all colonies were chosen for isolation.

Among 753 samples (from 188 strains) isolated, the individual strain that showed the strongest cytotoxic effects on head and neck cancer cells (FaDu, KB, SNU 899, SNU1066) by 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was selected. We then performed biochemical tests to analyze the physiological and metabolic characteristics to identify the specific bacterial strain. This strain was grown at 25°C under aerobic conditions for 3 days in sea water complete medium (bacto tryptone 5 g, yeast extract 3 g, glycerol 3 mL, dissolved in 75% seawater, pH 7.5). Extraction of genomic DNA and 16S rRNA gene amplification were carried out according to the method reported by Rainey et al. (11). The resulting polymerase chain reaction (PCR) product was ligated into a pGEM-T easy vector (Promega, Madison, WI, USA), sequenced using a Termination Sequencing Ready Reaction kit (Perkin Elmer, Boston, MA, USA), and analyzed using an ABI 377 genetic analyzer (Perkin Elmer). The complete 16S rDNA sequence (1,485 bp) was aligned using the CLUSTAL W software program (12). The 16S rDNA sequences used for the phylogenetic analyses were derived and compared with those of other bacterial 16S rDNA sequences available in the DDBJ/EMBL/GenBank database. Kimura's two-parameter model was used for the calculation of evolutionary distance (13). Our 16S rDNA sequencing of the strain characterized it to be most similar to *Bacillus firmus* (71.1% homology). We named the new marine strain *Bacillus SW31* and added it to the Korean Collection of Type Cultures under accession number KCTC 11135BP. Table 1 shows the results of the biochemical tests of *Bacillus SW31* using the Easy 24E Plus Kit (Komed, Seoul, Korea).

Bacterial culture and isolation of single pure cytotoxic compounds

For the production of secondary metabolites, the *Bacillus SW31* was cultured in Erlenmeyer flasks containing sea water complete medium. The flasks were then incubated on a shaker at 150 rev min^{-1} for 3 days at 25°C. The culture broth was centrifuged (10,000 rpm for 5 minutes at 4°C) to remove the cells, and then hexane 100 mL was added. The hexane was concentrated by a rotary vacuum evaporator and the new metabolic product obtained was named H31. The H31 (3 g) was further purified by reverse phase HPLC (Shimadzu, Tokyo, Japan; Cosmosil 5C18-MS column, 10×250 mm; linear gradient of MeOH in H_2O containing 0.05% trifluoroacetic acid [TFA], 80–100% in 50 minutes; flow rate, 1.5 mL min^{-1}; UV detection at 210 nm) to yield a pure cytotoxic compound (PCC).

Cell lines

Human FaDu and KB, SNU 899, SNU1066 (head and neck

Table 1. Biochemical characteristics of marine *Bacillus SW31*

	Test	Substrates utilization	*Bacillus SW31*
1	ARA	Arabitol	-
2	LAC	Lactose	-
3	SUC	Sucrose	-
4	MAN	Mannitol	-
5	DUL	Dulcitol	-
6	ADO	Adonitol	-
7	SOR	Sorbitol	-
8	CEL	Cellobiose	-
9	RAF	Raffinose	-
10	RHA	Rhamnose	-
11	INO	Inositol	-
12	MAL	Maltose	-
13	G-side	Glucopyranoside	-
14	ESC	Esculin	+
15	ONPG	O-Nitrophenyl-β-D-galactopyranoside	-
16	IND	Tryptophan	-
17	PDA	Phenylalanine	+
18	NIT	Potassiumnitrate	-
19	URE	Urease	+
20	H_2S	H_2S production from Sodium thiosulfate	+
21	LYS (LDC)	Lysine decarboxylase	-
22	ARG (ADH)	Arginine dehydrogenase	+
23	ORN (ODC)	Ornithine decarboxylase	+
24	F	Glucose	-

cancers), AGS (gastric cancer), HepG2 (liver cancer), and HT29 (colon cancer) were obtained from the American Type Culture Collection (Rockville, MD, USA) and Korean Cell Line Bank (Seoul, Korea). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) or RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS) with penicillin-streptomycin at 37°C in a humidified 5% CO_2 atmosphere. SCC VII/SF cell lines were grown in RPMI 1640 medium supplemented with 10% FBS and antibiotics.

Cell viability assay

To determine cell viability, the cells were seed in 96-well plates at densities of 5×10^3 cells/well in 1 mL complete medium after the cells were exposed to various concentrations of H31. Then, MTT was added to 40 μL of cell suspension for 4 hours. After three washes with PBS, the insoluble formazan product was dissolved in DMSO. The optical density (OD) of each culture well was measured using a microplate reader (Bio-Tek, Winooski, VT, USA) at 540 nm.

DNA fragmentation analysis

We performed DNA fragmentation tests with the G-DEX II Genomic DNA Extraction Kit (Intron, Seoul, Korea). Briefly, the cells were plated in 6-well plates at 3×10^5 cell/well, incubated for 24 hours, and then treated with different H31 concentrations for 24 hours in the absence of serum. After the cells were washed twice with PBS, they were harvested then lysed in 150 μL of cell lysis buffer with 1 μL RNAase A solution for 30 minutes at 37°C. The cell lysates were cleared by centrifugation. After centrifugation, the supernatant was collected and treated with 150 μL of 100% isopropanol by gently mixing. Centrifuge was performed at room temperature for 5 minutes at 13,000 rpm. The supernatant was discarded and treated with 1 mL of 75% EtOH by gently mixing. After centrifugation, the supernatant was discarded and the pellet was air dried. DNA was obtained by adding precipitation solution. The DNA pellet was dissolved in 50 μL of TE buffer (100 mM Tris-Cl pH7.4 and 10 mM EDTA pH8.0) containing RNase and incubated for 1 hour at 37°C. The fragmented DNA was resolved on 2% agarose gels in the presence of ethidium bromide and electrophoresed for 30 minutes at 100 V and then the bands were detected by UV light.

Cell cycle analysis

Cells were plated at 1×10^6 cell/well in 6-well plates, incubated for 24 hours, and then treated with different concentrations of H31 for 24 hours. Trypsinized cells were washed with PBS and fixed in 70% ethanol. After fixation, the cells were incubated for 30 minutes with 200 mg/mL of RNase A and stained with 5 mg/mL propidium iodide (PI). The stained cells were analyzed using a flow cytometry cell sorter (Becton Dickinson, Franklin Lakes, NJ, USA). In addition, the cells were conjugated with Annexin V-FITC using a TACS Annexin V-FITC kit (Trevigen Inc., Gaithersburg, MD, USA) according to the manufacturer's protocol and analyzed by flow cytometry.

Terminal deoxyuncleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) assay

The apoptotic effects of H31 were also determined by the TUNEL method using an *in situ* cell detection kit (Roche Molecular Biochemicals, Mannheim, Germany). KB cells were glass coverslipped in 24-well dishes containing growth medium. After 60-70% cell confluence was achieved, the cells were exposed to different H31 concentrations. The cells were incubated with 50 μL of TUNEL reaction mixture at 37°C for 1 hour in a humid atmosphere. The cells were then stained with 5 μg/mL Hoechst 33258 for 5 minutes, and the stained cells were visualized under a fluorescence microscope.

Western blot analysis

The level of protein expression was evaluated using Western blot analysis. The KB cells were washed with PBS and isolated in RIPA (radioimmunoprecipitation) buffer. The mixture was centrifuged at 12,000 rpm for 5 minutes at 4°C. Then, 20 μg of total proteins per well were separated by SDS-PAGE, and were transferred to a nitrocellulose membrane (Amersham, Arlington Heights, IL, USA). The membrane was incubated with specific antibodies overnight at 4°C. The membrane was reacted with peroxidase-conjugated donkey anti-rabbit antibodies (Amersham, Piscataway, NJ, USA) or donkey anti-mouse antibodies (Amersham, Piscataway). The membrane was developed using the Enhanced Chemiluminescence (ECL) Detection System (Amersham, Piscataway) and X-ray film.

In vivo syngeneic mouse C3H/HeJ model

The animal experiments were approved by the Animal Care and Use Committee of Ajou University. For the *in vivo* study, we used a syngeneic mouse model (C3H/HeJ mice, SCC VII/SF cell) that is commonly used for an *in vivo* immunocompetent murine model of head and neck cancer (14, 15). Six-week-old female C3H/HeJ syngeneic mice (about 20 g weight) were obtained from Orient (Orient Bio, Seoul Korea) and were acclimated in pathogen-free conditions for 1 week. All mice were then injected subcutaneously with 5×10^5 viable SCC VII/SF cells into the right flank. Three mice were given daily intraperitoneal injections of distilled water (D.W, control group), and the other three mice received intraperitoneal injections of 0.2 mg H31 (treatment group) 5 days a week. The tumor size, in two perpendicular diameters, was measured with calipers every second day. The tumor volume was calculated by the formula $V=(\pi/6)\times(L\times W^2)$ (L: large diameter, W: smaller diameter) (16).

Growth curves were plotted using the average tumor size within each experimental group at the same points in time. The animals were euthanized and the tumors were collected and weighed at 22 days after implantation. The mice were sacrificed

by cervical dislocation, and the tumors were excised and divided into two parts. For each sample, the final tumor size and weight were calculated.

Immunohistochemistry (IHC)

Active caspase-3 IHC was performed on paraffin-embedded mouse squamous carcinoma tumor sections collected on polylysine-coated slides. After paraffin removal, using xylene, the sections were rehydrated and incubated with 0.3% hydrogen peroxide for 10 minutes to eliminate endogenous peroxidase activity. The specimens were incubated with anti-active caspase-3 mouse primary antibodies (Cell signaling Technology, Danvers, MA, USA) diluted 1:400 in blocking solution overnight at 4°C. The sections were incubated for 2 hours at room temperature with the streptavidin–biotin–peroxidase complex (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA). The immunolabeling was revealed after three washes in PBS using 2,3′ diaminobenzidene as substrate diluted 1:10 in buffer according to the manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany). Staining was performed by incubation with the 3,3-diaminobenzidine (DAB) substrate-chromogen, which results in a brown-colored precipitate at the antigen site. Measurements of active caspase-3-positive cells were performed on 10–15 images/slide identified by an independent observer blinded to the experiment and normalized to the total cell count by DAPI staining. A minimum of five sections were randomly selected for the analysis of the apoptotic index, and the number of apoptotic cells were viewed under the light microscope (Leica, Wetzlar, Germany).

Embryotoxicity and neurotoxicity evaluation *in vivo* using a zebrafish model

Wild-type zebrafish (*Danio rerio*) was maintained at 28.5°C on a 14 hours light/10 hours dark cycle. For the embryotoxicity evaluation, fertilized eggs were exposed to various concentrations of H31 or 100 μM cisplatin diluted with Danieu's Solution, and maintained in an incubator at 28.5°C for 1 hour. After H31 treatment, evaluation for egg death, organogenesis, hatch rate, and any abnormal features was performed. For the neurotoxicity evaluation, 4 days post fertilization (dpf) larvae were placed at a density of 50 per 100 mm^2 in a petri dish with embryo medium (1 mM $MgSO_4$, 120 μM KH_2PO_4, 74 μM $NaHPO_4$, 1 mM $CaCl_2$, 500 μM KCl, 15 μM NaCl, and 500 μM $NaHCO_3$) and placed in an incubator at 28.5°C. Then, 4 dpf zebrafish larvae were exposed to different concentrations of H31 diluted with Danieu's Solution, and maintained in an incubator at 28.5°C for 1 hour. After the larvae were washed with 0.3×Danieu's Solution and cultured at 28.5°C for 3 hours, and labeled using 2 μM YO-PRO1 (Molecular Probes, Eugene, OR, USA) for 1 hour, performed three times. The zebrafish was then anesthetized with 8 μg/mL 3-aminobenzoic acid ethyl ester methanesulfonate salt (MS222, Sigma Chemical Co., St Louis, IL, USA). After fixation, the zebrafish was mounted with methylcellulose on a slide and photographs were taken using a fluorescence microscope.

Statistical analysis

The Student *t*-test and one-way ANOVA were used for the statistical analyses with SPSS ver. 10.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was defined at $P<0.05$.

RESULTS

H31 inhibited cell growth in head and neck cancer cells

Our primary aim was to investigate whether H31 had strong cytotoxic activity in four head and neck cancer cell lines. As shown in Fig. 1, the MTT assay shows that the cell viability dropped by 40% or more when the cells were treated with 10 μg/mL of H31, among all four cell lines. The antitumor activity of H31 was strongest in the FaDu cells and KB cells among the four cell lines; therefore KB cells were used for further experiments.

H31 induced apoptosis of KB cells

To investigate the mechanism of H31-induced growth inhibition of KB cells, we studied internucleosomal DNA cleavage by DNA fragmentation analysis for the detection of apoptosis. DNA fragmentation was observed at 24 hours after H31 treatment, a characteristic feature of apoptosis, which was not noted in the ethanol-treated control group (Fig. 2A). The TUNEL assay also showed that H31 increased the unclear condensation, DNA fragmentation, and perinuclear apoptotic bodies in the KB cells

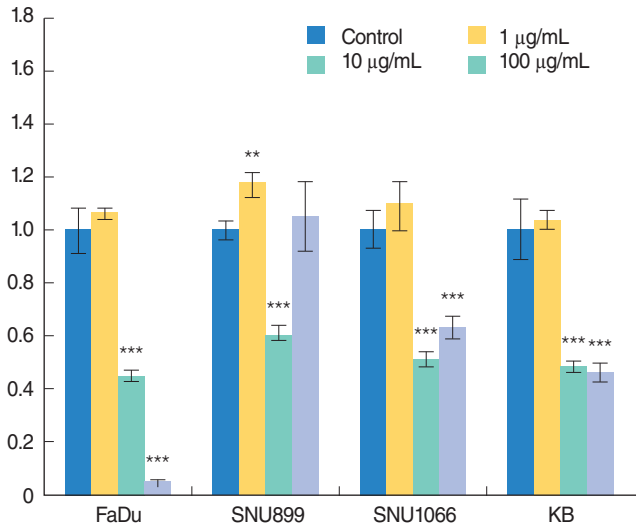

Fig. 1. Four head and neck cancer cell lines (FaDu, SNU 899, SNU 1066, and KB) were incubated with different concentrations (1, 10, 100 μg/mL) of H31 for 24 hours, and cell viability was evaluated by the MTT assay. The data represent the mean±SD of three independent experiments; $^{**}P<0.01$, $^{***}P<0.001$ by student *t*-test, compared to medium alone.

as detected by Hoechst 33258 staining (Fig. 2B). In addition, the amount of sub-G1 DNA was investigated, which contained less DNA than the G1 cells, to quantify the amount of apoptotic cells. Flow cytometric analysis showed that H31 treatment increased accumulation during the sub-G1 phase in a dose-dependent manner (Fig. 2C). Furthermore, annexin V analysis showed that the percentage of apoptotic cells (annexin V-positive, PI-negative, bottom right quadrants) increased in a dose-dependant manner (Fig. 2D). Therefore, H31 induced apoptosis in the KB cells

Apoptosis by H31 was associated with involvement of c-jun, p53, cytochrome C, and caspase-3

To study the mechanism of H31-induced apoptosis in the KB

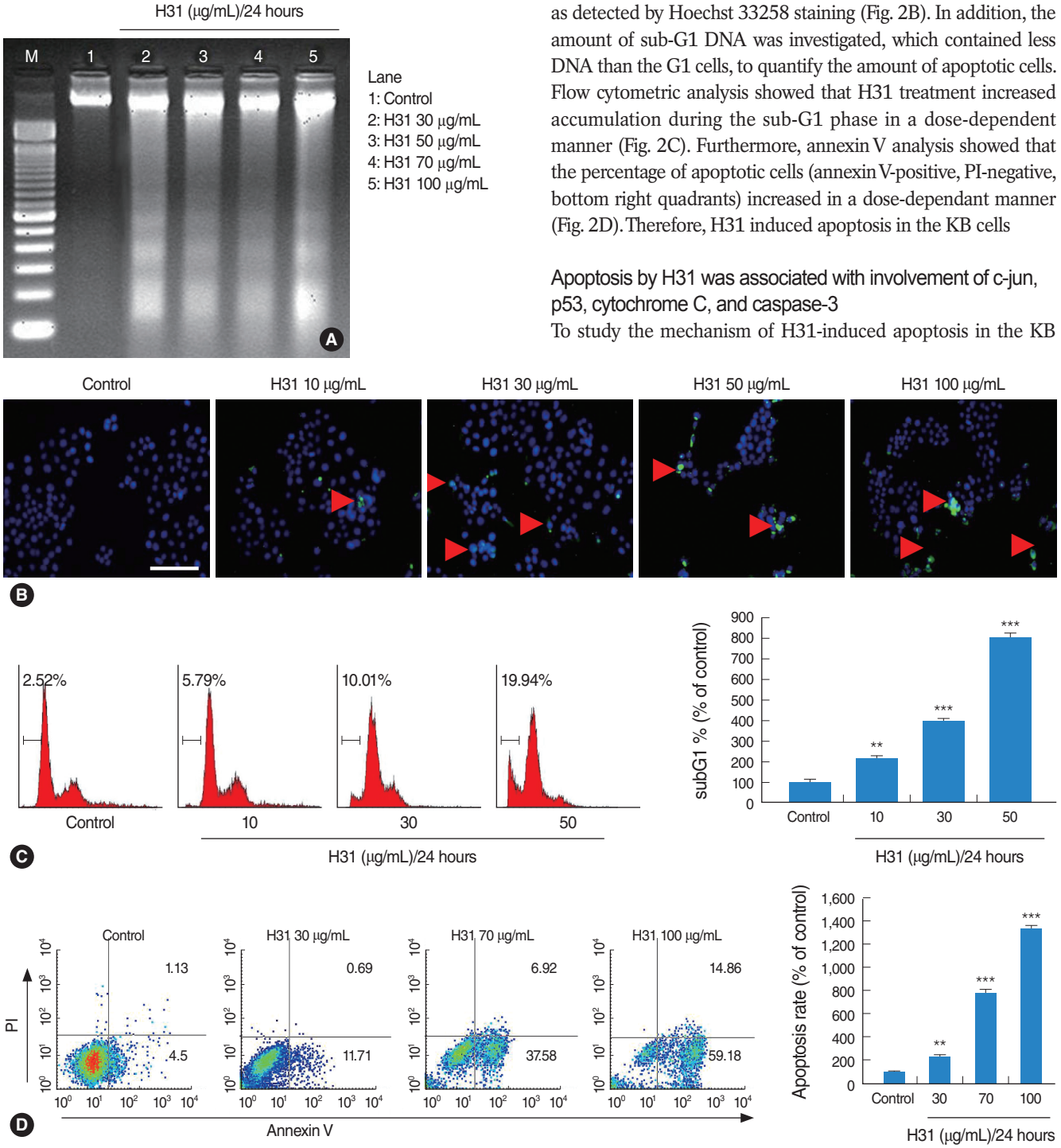

Fig. 2. Effect of H31 on apoptosis and the cell cycle in KB cells. (A) DNA fragmentation analysis: KB cells were grown for 24 hours at different concentrations of H31; a DNA fragmentation assay was performed with an ApopLadder EX DNA fragmentation assay kit. DNA fragmentation was visualized by electrophoresis on a 2% agarose gel containing ethidium bromide. lane 1, control; lane 2, H31 30 μg/mL; lane 3, H31 50 μg/mL; lane 4, H31 70 μg/mL; lane 5, H31 100 μg/mL. (B) TUNEL study: apoptosis in KB cells was determined by the TUNEL method using an in situ cell detection kit. The cells were then incubated with 50 μL of TUNEL reaction mixture (TdT and fluoresccin–dUTP) and stained with Hoechst 33258 (5 μg/mL). The stained cells (arrowhead) were observed under a fluorescence microscope. Scale bar=50 μm. (C) Flow cytometry: KB cells were incubated in 6-well plates with various concentrations of H31, treated with 200 mg/mL RNase A, and stained with 5 mg/mL PI. The stained cells were then analyzed with a FACS caliber instrument. (D) Annexin V and PI staining. To quantify the effects of H31 on apoptosis, annexin V-FITC and propidium iodide staining were used to analyze the percentage of apoptotic cells treated with H31. The data represent the mean±SD of three independent experiments. $^{**}P<0.01$, $^{***}P<0.001$ by student *t*-test, compared to medium alone.

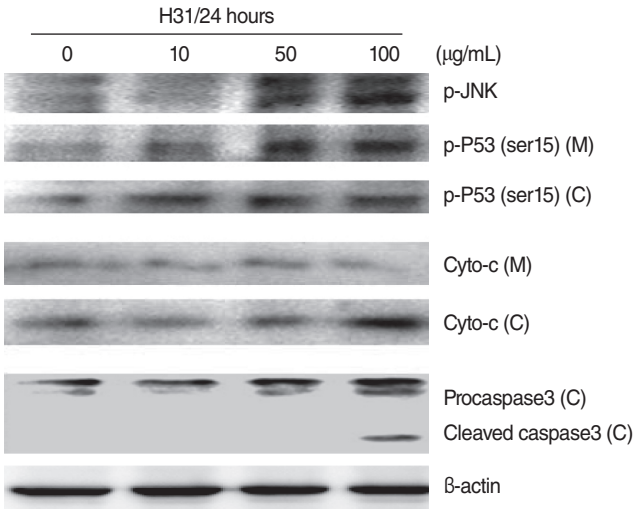

Fig. 3. Expression of apoptosis-associated proteins: KB cells were treated with H31 at the indicated concentrations. Serum-starved KB cells were treated with different concentrations of H31, and then Western blotting was performed with various antibodies, including p-JNK, p-p53, cytochrome C, procaspase 3, and cleaved caspase-3.

cells, we investigated several factors related to apoptotic pathways, including *c-jun*, p53, cytochrome *C*, and caspase-3. As shown in Fig. 3, the expression of phosphorylation of JNK and p53 was increased in a dose-dependent manner. Cytochrome C, which triggers the mitochondrial apoptotic pathway, was increasingly released from mitochondria into the cytosol by H31. In addition, the expression of the active form of caspase-3 was increased at a concentration of 100 μg/mL.

H31 suppressed tumor growth in C3H/HeJ syngeneic mice

To determine whether these results could be confirmed *in vivo*, we used the immunocompetent syngeneic mouse model (C3H/HeJ mice, SCC VII/SF cell) and randomly divided the animals into two equal groups (control and treatment group, 0.2 mg/kg) after injection of SCC VII/SF cells. As shown in Fig. 4, the final volume (A), final weight (B), and time course volume change (C) of the SCC VII/SF tumors were significantly inhibited at 21 days after treatment with 0.2 mg H31 compared to the control group (D). For the TUNEL staining (Fig. 4E) and immunohistochemical study (Fig. 4F) of caspase-3, which was performed to observe the apoptosis induced by H31 in the tumor tissue removed from the C3H/HeJ syngeneic mice, the apoptotic index in the tumor tissue from the H31 injected mice was statistically higher than in the controls ($P<0.01$).

Evaluation of embryotoxicity and neurotoxicity of H31 in the zebrafish model

We used the zebrafish model for the toxicity assays of H31. First, we examined the embryotoxicity of H31. As shown in Fig. 5A, normal organogenesis in eggs was observed with up to 100 μM of H31, while death of all eggs was noted with 100 μM of cisplatin. Next, we investigated the neurotoxicity of H31 using the zebrafish neuromast. As shown in Fig. 5B, doses of H31 that were increased up to 100 μM did not decrease the number of hair cells of the neuromast; by contrast, there was almost total loss of the hair cells of the neuromast with 100 μM cisplatin.

Extraction of a pure single compound using the HPLC method

We have extracted several PCCs from the crude extract of H31 using the HPLC method. Two PCCs demonstrated strong cytotoxicity among the cell lines: FaDu, KB AGC, HepG2, and HT-29 by the MTT assay (data not shown). Therefore, additional HPLC studies were carried out on one PCC among them. The MTT assay of KB cells was performed using several substances that showed possible tumor suppression. As shown in Fig. 6, the PCC that showed the highest peak in the HPLC study showed strong cytotoxicity in the KB cells at a concentration of 10 μg/mL. Therefore, this single compound appears to possess the anti-tumor capabilities of H31.

DISCUSSION

Marine micro-organisms are increasingly used as a source for anti-cancer agents (17). Cytarabine, the extract of nucleosides from the Caribbean sponge and *Cryptotheca crypta*, are now routinely used for the treatment of patients with leukemia and lymphoma (17). Jeong et al. (18) reported that a compound from *Bacillus vallismortis* showed direct cytotoxic and apoptotic effects on colon cancer cells in a dose and time dependent manner. In an attempt to identify a novel compound for cancer therapy, the results of this study show that H31, from the *Bacillus SW31*, had cytotoxic and apoptotic properties in head and neck cancer cells.

One therapeutic approach to the control of cancer cells involves the induction of apoptosis (19). Whether H31 could induce apoptosis in head and neck cancer cells was studied, as a potential method of growth inhibition. The results of this study showed that H31 suppressed KB cells and SCC VII/SF cell growth *in vitro* and *in vivo*, respectively, and the mechanism involved was mediated by up-regulation of p53 expression and activation of caspase-3.

Apoptosis is caused by the activation of cysteine protease, known as caspases, and the subsequent cleavage of certain cellular substrates (20-22). Of the 14 currently known human caspase family members, caspase-3 is the key factor associated with apoptosis; its active form is pivotal in the breakdown of cellular activity connected with DNA repair and regulation (23). The findings of this study showed that caspase-3 expression was up regulated when the cells were treated with H31, suggesting that the apoptotic-action of H31 was associated with caspase-3 involvement.

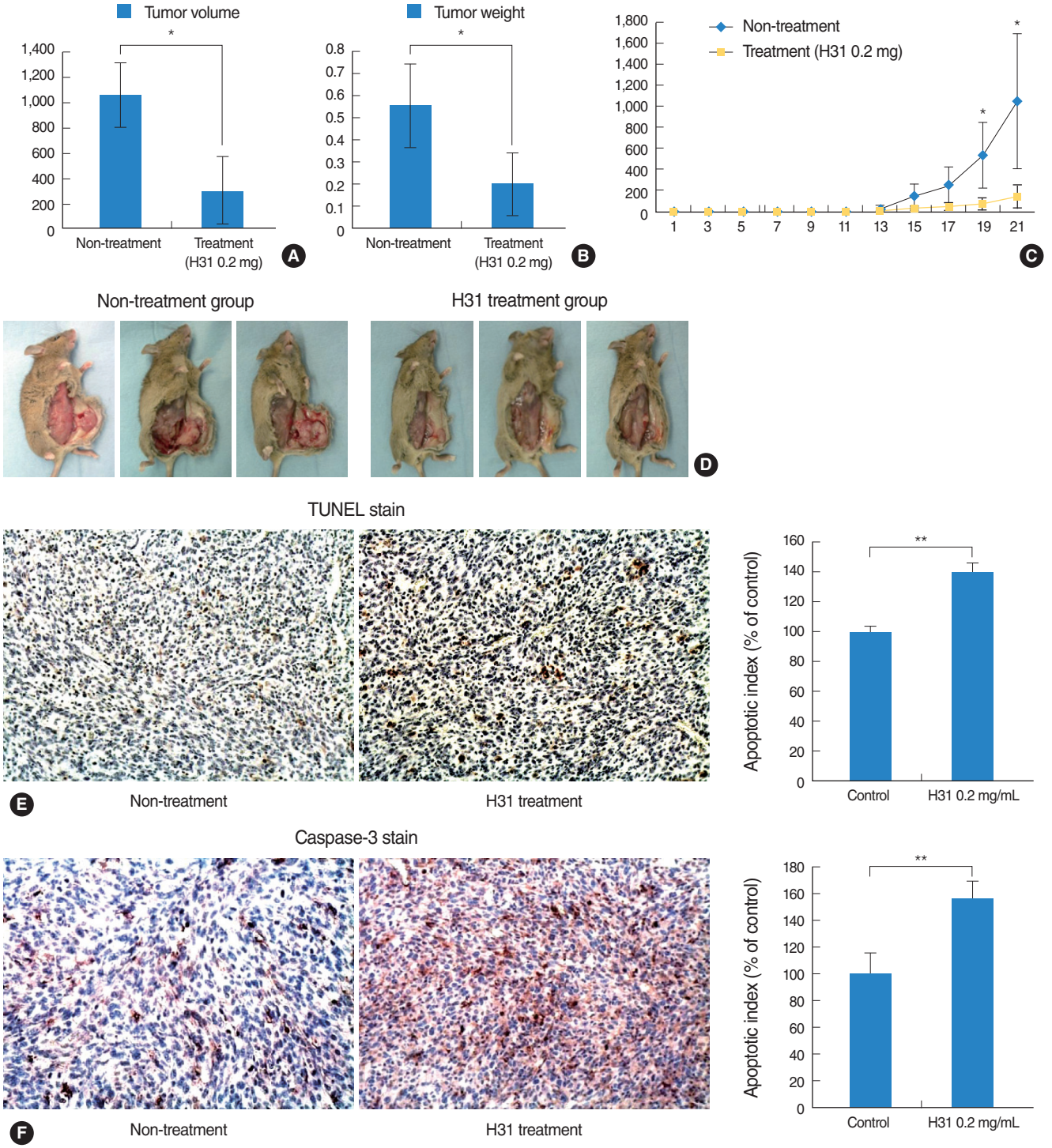

Fig. 4. Effect of H31 on tumor growth and apoptosis in C3H/HeJ syngeneic mice. Six C3H/HeJ syngeneic mice with subcutaneous injections of 5×10^5 SCC VII cells were divided into two groups. Three were then given daily intraperitoneal injections of distilled water (control) and the other 3 mice were treated with 0.2 mg H31. After 22 days, the mice were sacrificed and tumors were removed. (A) Effect of H31 on tumor volume. (B) Effect of H31 on tumor weight. (C) Effect of H31 on tumor growth (time course). (D) Gross appearance. (E) Tissue TUNEL staining. (F) Immunohistochemistry for caspase-3. *P<0.01, **P<0.05.

The tumor suppressor protein, p53, is a central controlling modulator that receives specific signals, interprets complex input, and determines regulatory output (24). The loss of p53 in HNSCC has been observed to result in a relative lack of apoptosis (25). Targeting p53 may be one approach to reduce the anti-apoptotic features associated with HNSCC. Gene therapy using

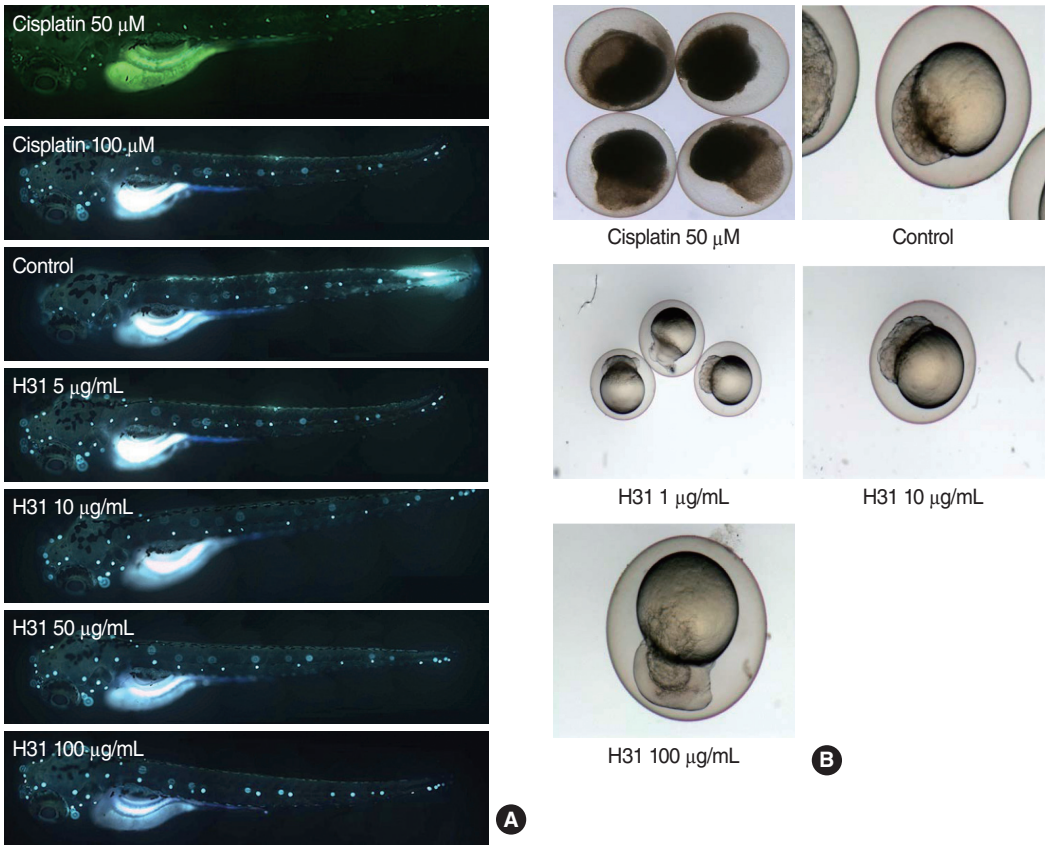

Fig. 5. Zebrafish assay for the evaluation of embryotoxicity and neurotoxicity of H31: (A) Embryotoxicity: wild type zebrafish were maintained at 28.5°C on a 14 hours light/10 hours dark cycle, then fertilized eggs were exposed to the indicated concentrations of H31 or 100 μM cisplatin, and maintained in a incubator at 28.5°C for 1 hour. After H31 treatment, egg death, organogenesis, hatch rate, and any abnormal features were evaluated; (B) Neurotoxicity: The 4 dpf zebrafish were treated with cisplatin (50 μM, 100 μM) or increasing concentrations of H31 for 24 hours and then were stained with YO-PRO1. White dots on the body of zebrafish indicate neuromasts.

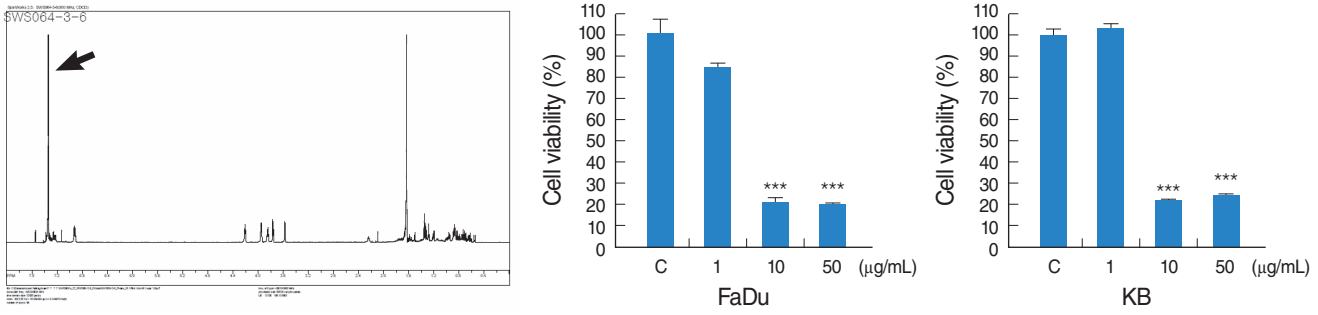

Fig. 6. Extraction of a pure cytotoxic compound (PCC) of H31 using the HPLC method and results of the MTT assay: The PCC showed the highest peak (black arrow) and the HPLC showed strong cytotoxicity to KB cells in the concentration of 10 μg/mL. This single compound appears to possess the anti-tumor capabilities of H31. ***P<0.001.

adenoviral transfer of p53 is being aggressively investigated as adjuvant therapy for head neck cancer cells (26). Therefore, H31 may be an additional candidate for enhancing apoptosis of head and neck cancer cells.

Chemotherapy agents are often limited by drug toxicity (27). A new agent's toxicity profile is a very important characteristic that should be defined during the drug discovery process. Recently, the animal model using zebrafish (*Danio rerio*) has become a useful technique to screen for cardiotoxicity, neurotoxicity, hepatotoxicity, nephrotoxicity and genotoxicity of new candidate drugs (28-30). The results of this study showed that treatment of zebrafish with 100 μM cisplatin resulted in nearly total loss of the neuromast by immunofluorescence, while 100 μM of H31 did not decrease the number of neuromast hair cells. In addition, normal zebrafish organogenesis occurred with 100 μM H31 treatment.

In conclusion, the results of this study demonstrated that growth inhibition and apoptosis was achieved both *in vitro* and *in vivo* in head and neck cancer cells after exposure to H31, a metabolite from the marine Bacillus species. The zebrafish model did not reveal any significant toxicity effects even at considerable H31 dose concentrations. Although further characterization of the chemical structure and biological activities of HPLC purified PCC is necessary, our results suggest that H31 appears to be a safe candidate for apoptosis-inducing anti-cancer treatment of head and neck cancer.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

ACKNOWLEDGEMENT

This research was supported by CCRB through the "GRRC" Project of Gyeonggi Provincial Government, Korea (GRRC-Ajou-2009-A03).

REFERENCES

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, et al. Cancer statistics, 2008. CA Cancer J Clin. 2008 Mar-Apr;58(2):71-96.
2. Argiris A, Karamouzis MV, Raben D, Ferris RL. Head and neck cancer. Lancet. 2008 May 17;371(9625):1695-709.
3. Forastiere AA, Goepfert H, Maor M, Pajak TF, Weber R, Morrison W, et al. Concurrent chemotherapy and radiotherapy for organ preservation in advanced laryngeal cancer. N Engl J Med. 2003 Nov 27; 349(22):2091-8.
4. Boulikas T, Vougiouka M. Recent clinical trials using cisplatin, carboplatin and their combination chemotherapy drugs (review). Oncol Rep. 2004 Mar;11(3):559-95.
5. Bernan VS, Greenstein M, Maiese WM. Marine microorganisms as a source of new natural products. Adv Appl Microbiol. 1997;43:57-90.
6. Isnansetyo A, Kamei Y. MC21-A, a bactericidal antibiotic produced by a new marine bacterium, Pseudoalteromonas phenolica sp. nov. O-BC30(T), against methicillin-resistant Staphylococcus aureus. Antimicrob Agents Chemother. 2003 Feb;47(2):480-8.
7. Blunt JW, Copp BR, Hu WP, Munro MH, Northcote PT, Prinsep MR. Marine natural products. Nat Prod Rep. 2007 Feb;24(1):31-86.
8. Kim J, Park EJ. Cytotoxic anticancer candidates from natural resources. Curr Med Chem Anticancer Agents. 2002 Jul;2(4):485-537.
9. Singh B. Molecular pathogenesis of head and neck cancers. J Surg Oncol. 2008 Jun 15;97(8):634-9.
10. Gastman BR. Apoptosis and its clinical impact. Head Neck. 2001 May;23(5):409-25.
11. Rainey FA, Ward-Rainey N, Kroppenstedt RM, Stackebrandt E. The genus Nocardiopsis represents a phylogenetically coherent taxon and a distinct actinomycete lineage: proposal of Nocardiopsaceae fam. nov. Int J Syst Bacteriol. 1996 Oct;46(4):1088-92.
12. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994 Nov 11;22(22):4673-80.
13. Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol. 1980 Dec;16(2):111-20.
14. O'Malley BW Jr, Cope KA, Johnson CS, Schwartz MR. A new immunocompetent murine model for oral cancer. Arch Otolaryngol Head Neck Surg. 1997 Jan;123(1):20-4.
15. Zou J, Qiao X, Ye H, Yang Y, Zheng X, Zhao H, et al. Antisense inhibition of ATM gene enhances the radiosensitivity of head and neck squamous cell carcinoma in mice. J Exp Clin Cancer Res. 2008 Oct 26;27:56.
16. Ciardiello F, Bianco R, Damiano V, De Lorenzo S, Pepe S, De Placido S, et al. Antitumor activity of sequential treatment with topotecan and anti-epidermal growth factor receptor monoclonal antibody C225. Clin Cancer Res. 1999 Apr;5(4):909-16.
17. Adrian TE. Novel marine-derived anti-cancer agents. Curr Pharm Des. 2007;13(33):3417-26.
18. Jeong SY, Park SY, Kim YH, Kim M, Lee SJ. Cytotoxicity and apoptosis induction of Bacillus vallismortis BIT-33 metabolites on colon cancer carcinoma cells. J Appl Microbiol. 2008 Mar;104(3):796-807.
19. Lim YC, Lee SH, Song MH, Yamaguchi K, Yoon JH, Choi EC, et al. Growth inhibition and apoptosis by (-)-epicatechin gallate are mediated by cyclin D1 suppression in head and neck squamous carcinoma cells. Eur J Cancer. 2006 Dec;42(18):3260-6.
20. Los M, Wesselborg S, Schulze-Osthoff K. The role of caspases in development, immunity, and apoptotic signal transduction: lessons from knockout mice. Immunity. 1999 Jun;10(6):629-39.
21. Thornberry NA, Rano TA, Peterson EP, Rasper DM, Timkey T, Garcia-Calvo M, et al. A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. J Biol Chem. 1997 Jul 18;272(29):17907-11.
22. Rano TA, Timkey T, Peterson EP, Rotonda J, Nicholson DW, Becker JW, et al. A combinatorial approach for determining protease specificities: application to interleukin-1beta converting enzyme (ICE). Chem Biol. 1997 Feb;4(2):149-55.
23. Schultz DR, Harrington WJ Jr. Apoptosis: programmed cell death at a molecular level. Semin Arthritis Rheum. 2003 Jun;32(6):345-69.
24. Bode AM, Dong Z. Post-translational modification of p53 in tumorigenesis. Nat Rev Cancer. 2004 Oct;4(10):793-805.
25. Ritta M, De Andrea M, Mondini M, Mazibrada J, Giordano C, Pecorari G, et al. Cell cycle and viral and immunologic profiles of head and neck squamous cell carcinoma as predictable variables of tumor progression. Head Neck. 2009 Mar;31(3):318-27.
26. Gurnani M, Lipari P, Dell J, Shi B, Nielsen LL. Adenovirus-mediated p53 gene therapy has greater efficacy when combined with chemotherapy against human head and neck, ovarian, prostate, and breast cancer. Cancer Chemother Pharmacol. 1999;44(2):143-51.
27. Rosenthal DI, Lewin JS, Eisbruch A. Prevention and treatment of dysphagia and aspiration after chemoradiation for head and neck cancer. J Clin Oncol. 2006 Jun 10;24(17):2636-43.
28. Beis D, Stainier DY. In vivo cell biology: following the zebrafish trend. Trends Cell Biol. 2006 Feb;16(2):105-12.
29. den Hertog J. Chemical genetics: Drug screens in Zebrafish. Biosci Rep. 2005 Oct-Dec;25(5-6):289-97.
30. Mandrekar N, Thakur NL. Significance of the zebrafish model in the discovery of bioactive molecules from nature. Biotechnol Lett. 2009 Feb;31(2):171-9.