

Manumycin A from a new *Streptomyces* strain induces endoplasmic reticulum stress-mediated cell death through specificity protein 1 signaling in human oral squamous cell carcinoma

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Received May 11, 2015; Accepted July 10, 2015

DOI: 10.3892/ijo.2015.3151

Abstract. Manumycin A (Manu A) is a natural antibiotic produced by new *Streptomyces* strain, exhibiting antitumor and anticancer effects. However, the anticancer effects of Manu A on oral squamous cell carcinoma (OSCC) have not been reported. OSCC is an aggressive type of cancer because of its poor prognosis and low survival rate despite advanced medical treatment. We observed that Manu A reduced cell growth and Sp1 protein levels in OSCC cell lines (HN22 and HSC4) in a dose- and time-dependent manner. We also observed downregulation of Sp1 downstream target genes such as p27, p21, Mcl-1 and survivin. Moreover, nuclear staining with DAPI showed that Manu A was able to cause nuclear

condensation and further fragmentation. Flow cytometry analyses using Annexin V and propidium iodide supported Manu A-mediated apoptotic cell death of OSCC cells. Furthermore, Bcl-2 family such as mitochondrial pro-apoptotic Bax, anti-apoptotic Bcl-xl and Bid were regulated by Manu A, triggering the mitochondrial apoptotic pathway. In conclusion, these results indicate that Manu A is a potential to treat human OSCC via cell apoptosis through the downregulation of Sp1.

Introduction

Oral cancer, which occurs in oral cavity and oropharynx, is a leading cause of cancer-related death and approximately 263,900 new cases were reported and approximately 128,000 of the patients died of oral cancer in 2011, in USA (1). Oral cancer is one of most common types of cancer and over 500,000 patients suffer from it every year (2). Oral squamous cell carcinoma (OSCC) which occurs in the lining of the epithelial cell represents approximately 95% of head and neck cancer and is the sixth most common malignant neoplasm worldwide (3-5). This aggressive epithelial malignancy has a poor diagnosis and the incidence rate of oral cancer has been elevated up to 50% over the past two decades, with only a 50% 5-year survival rate in patients with OSCC despite advanced medical treatment (6-10). There are many chief factors for OSCC, including tobacco, alcohol, and HPV infection (11-13). In addition, it was reported that bacterial infections are associated with tumor site of OSCC because of their ability to induce chronic inflammation.

Manumycin A (Manu A), a product of *Streptomyces parvulus*, is a natural antibiotic and is known to be a potential tumoricide. Many studies have demonstrated that Manu A inhibits cell viability and induces cell apoptosis in many cancers, such as prostate cancer, multiple myeloma, anaplastic thyroid cancer and colon cancer (14-17). Taking into consideration of possible correlation of bacterial infection to OSCC,

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Abbreviations: OSCC, oral squamous cell carcinoma; Manu, manumycin; Sp1, specificity protein 1; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; Mcl-1, myeloid cell leukemia-1; PARP, poly(ADP-ribose) polymerase; P/S, penicillin and streptomycin; MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium); DAPI, 4'-6-diamidino-2-phenylindole; PI, propidium iodide

Key words: manumycin A, apoptosis, specificity protein 1, oral squamous cell carcinoma, endoplasmic reticulum stress-mediated cell death

it is proposed that capability of Manu A to directly suppress some prevalent bacteria (18) has also anticancer effect on OSCC.

To induce apoptosis of cancer cells by targeting the specific signal-transduction pathway could be an effective anticancer therapy. Therefore, we investigated whether the Manu A-induced cell apoptosis is related to Specificity protein 1 (Sp1), a transcription factor that binds to a specific DNA sequence, overexpressed in many cancer cells, such as bladder cancer (19), breast cancer (20,21), pancreatic cancer (22), gastric cancer (23) and oral cancer (24). Specificity protein 1 (Sp1) has already been examined and plays important physiological roles such as cell cycle regulation, cell proliferation, and cell apoptosis (25). However, the relationship between Manu A treatment and downregulation of Sp1 in OSCC cells has not been studied yet. If Manu A can reduce Sp1 expression, it will be a potential candidate material for OSCC therapy. In order to verify its therapeutic effect of Manu A, we investigated the apoptotic effect of Manu A by downregulation of Sp1 levels using the OSCC cell lines HN22 and HSC4.

Materials and methods

Reagents. All the solvents used in the experiments were of extra pure grade. Hexane, ethyl acetate and acetonitrile were purchased from J.T. Baker (Phillipsburg, NJ, USA). Silica gel for Thin layer chromatography, precoated silica gel plate (Kieselgel 60F254, Merck, NJ, USA) was used. Silica gel for silica gel column, Kieselgel 60 (70-230 mesh, Merck) was used to purify manumycin.

Purification of manumycin A. *Streptomyces* sp. CS392 was grown on rotary shaker at 180 rpm in Emerson media for 2-3 days at 28°C. Culture broth (3L) was centrifuged at 6,000 rpm for 20 min. Supernatant was extracted two times with ethyl acetate (1:1, v/v). The extracted ethyl acetate fraction was evaporated and dried using a rotary evaporator at 50°C under reduced pressure. Purification of antibiotic was carried out by silica gel column chromatography (0.8x15 cm). After washing the column with hexane, active material was eluted from the column with hexane-ethyl acetate (4:1). Active fractions were collected and rechromatographed, using a reverse phase-C18 silica gel column (1.0x15 cm) with 0.01% formic acid-acetonitrile (4:6) to give manumycin A.

Cell culture. HN22 and HSC4 are human oral squamous cancer cell lines. HN22 cells were provided by Dankook University (Cheonan, Korea) and HSC4 cells were provided by Hokkaido University (Hokkaido, Japan). HN22 and HSC4 cells were cultured in DMEM containing 10% heat-inactivated FBS and 100 U/ml each of penicillin and streptomycin at 37°C under 5% CO₂ and humidified condition.

3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. The result of the Manu A on HN22 and HSC4 cell viability was observed using the Cell Titer 96[®] Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The cells were seeded in 96-well plates, grown for 24 h and treated with

various concentration of Manu A. After treatment with Manu A for 24 and 48 h, MTS solution was added to each well and the plates were incubated for 2 h at 37°C. Its absorbance was read using an Enspire Multimode Plate reader (Perkin-Elmer, USA) at 490 nm.

Cell cycle analysis. HN22 and HSC4 cells were seeded and treated with Manu A (0, 2.5, 5, and 10 μM) for 48 h. The harvested cells were washed with 1 ml PBS and 150 μl of Muse™ Cell cycle reagent (EMD Millipore Corp. USA) was added. Additionally, cells were incubated at RT for 30 min in the dark. Samples were analyzed by Muse Cell Analyzer (Merck Millipore, Billerica, MA, USA) with Muse Cell cycle kit (Merck Millipore).

(4',6-diamidino-2-phenylindole) DAPI staining. After treatment with Manu A, the cells were harvested by trypsinization. The cells were washed with cold PBS, and fixed in 100% methanol at room temperature for 20 min. The cells were deposited on slides, stained with DAPI solution (2 μl/ml) and observed through a FluoView confocal laser microscope (FluoView FV10i, Olympus Corp., Tokyo, Japan).

Reverse transcription-polymerase chain reaction (RT-PCR). To analyze the effect of Manu A on OSCC cell lines, we performed RT-PCR using total RNAs and primers designed for the specific gene. Total RNAs were harvested from OSCC cells treated with or without Manu A using the Total RNA extraction (Life Technologies, Carlsbad, CA, USA). With 2.5 μg of RNA, RT-PCR was done using First-strand cDNA synthesis kit (Bioassay Co., Ltd., Korea) according to the kit instructions. We obtained cDNA using actin-specific and Sp1-specific primers under the following PCR condition (30 cycles: 1 min at 95°C, 1 min at 56°C and 1 min at 72°C). The actin primers used were: forward, 5'-GTG GGG CGC CCC AGG CAC CA-3'; and reverse, 5'-CTC CTT AAT GTC ACG CAC GAT TTC-3'; and the Sp1 primers were: forward, 5'-ATG CCT AAT ATT CAG TAT CAA GTA-3'; and reverse, 5'-CCC TGA GGT GAC AGG CTG TGA-3'. Actin was used as an internal control. The RT-PCR products were visualized with ethidium bromide staining under UV light, after electrophoresis on a 2% agarose gel.

Annexin V. HN22 and HSC4 cells were seeded and grown for 24 h. After 48 h from treatment with various concentration of Manu A, cells were harvested by trypsinization for analysis. The cells were analyzed by Muse Cell Analyzer (Merck Millipore) with the Muse Annexin V & Dead Cell kit (MCH100105, Merck Millipore). The whole process of analysis followed the instructions of the kit. The percentage of apoptotic and necrotic cells was calculated from each triplicate sample by statistical analysis of the dot plot using Muse 1.1.2 analysis software (Merck Millipore).

Western blotting. The lysates of treated cells were generated using PRO-PREP™ Protein Extraction Solution (iNtRON Biotechnology, Korea), followed by centrifugation and supernatant collection. Proteins were separated using SDS-PAGE gel electrophoresis and transferred onto a polyvinylidene fluoride (PVDF) membrane. After blocking with PBS containing

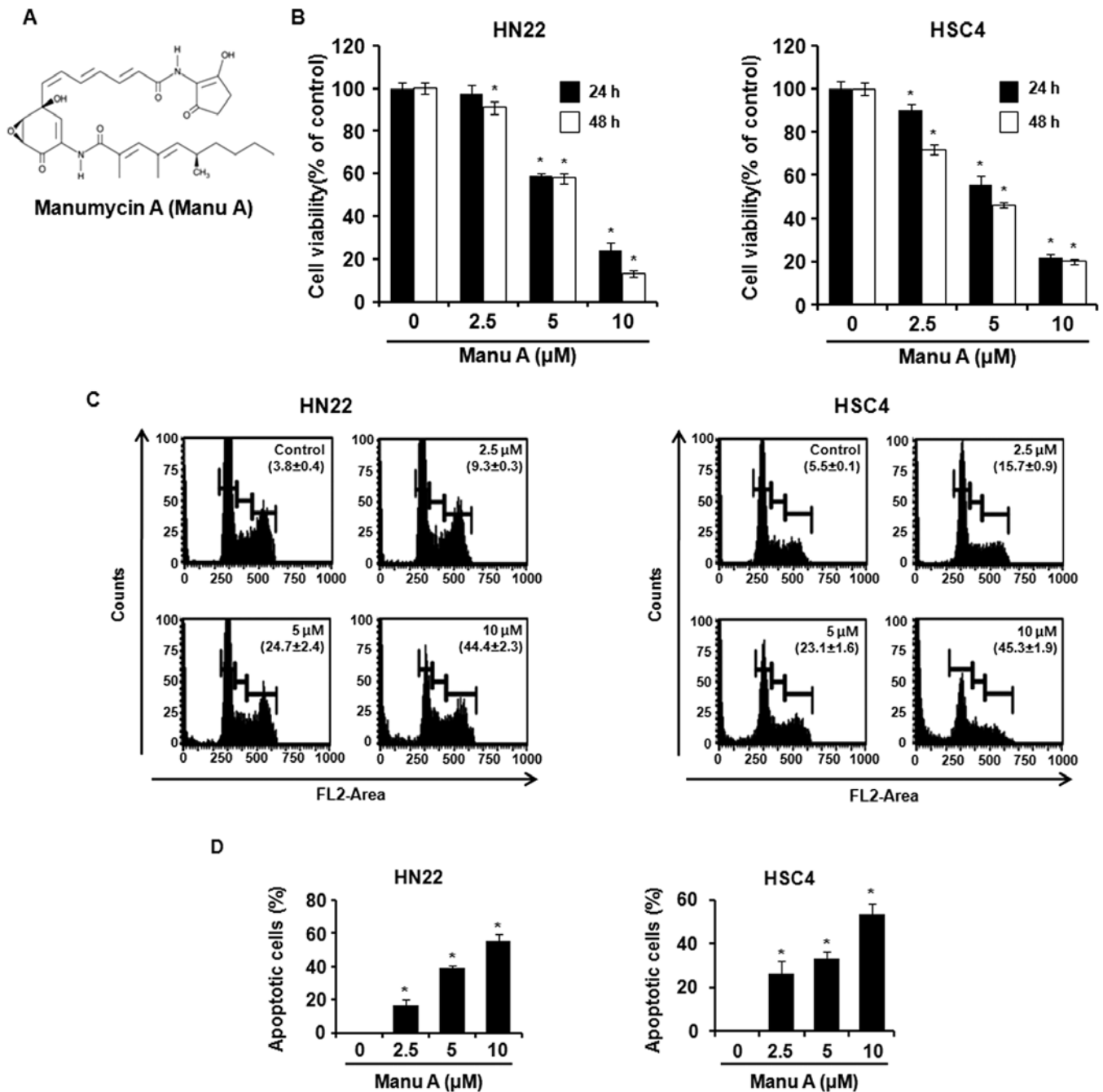


Figure 1. Effect of Manu A on cell viability of OSCC cells. (A) Chemical structure of Manu A. (B) Cell viabilities of HN22 and HSC4 treated with Manu A (2.5, 5 and 10 μM). Cell viability was measured by MTS assay kits. (C) HN22 and HSC4 cells were treated with 2.5, 5 and 10 μM of Manu A, and the representative of sub-G₁ population was measured by Muse analysis after PI staining. (D) DNA fragmentation and nuclear condensation were quantified using DAPI staining. The data represent the mean percentage levels \pm SD (n=3); *p<0.05, significant difference compared with DMSO-treated control cells by paired t-test.

0.1% Tween-20 and 5% skim milk, membrane probed with primary antibody was shaken at 4°C overnight and then incubated with the secondary antibody. The protein bands were detected using ECL Plus Western Blotting Detection System (Santa Cruz Biotechnology, USA).

Multi-Caspase. The process of the analysis followed the instructions of the Muse Multi-Caspase kit (Muse Cell Analyzer, Merck Millipore). OSCC cells including control and treatment groups were incubated for appropriate time to induce caspase activity and harvested. Cell samples in 1X

caspase buffer with 50 μl of Muse Multi-Caspase reagent working solution were incubated at 37°C for 30 min, then 150 μl of 7-AAD working solution was added to each sample and triplicate samples were analyzed by Muse Cell Analyzer (Merck Millipore).

Mitochondrial membrane potential assay (MMP). The whole process of the analysis followed the instructions of the Muse MitoPotential kit (Merck Millipore). Control cells and Manu A-treated (2.5, 5 and 10 μM) cells were harvested. To investigate the mitochondrial membrane permeability,

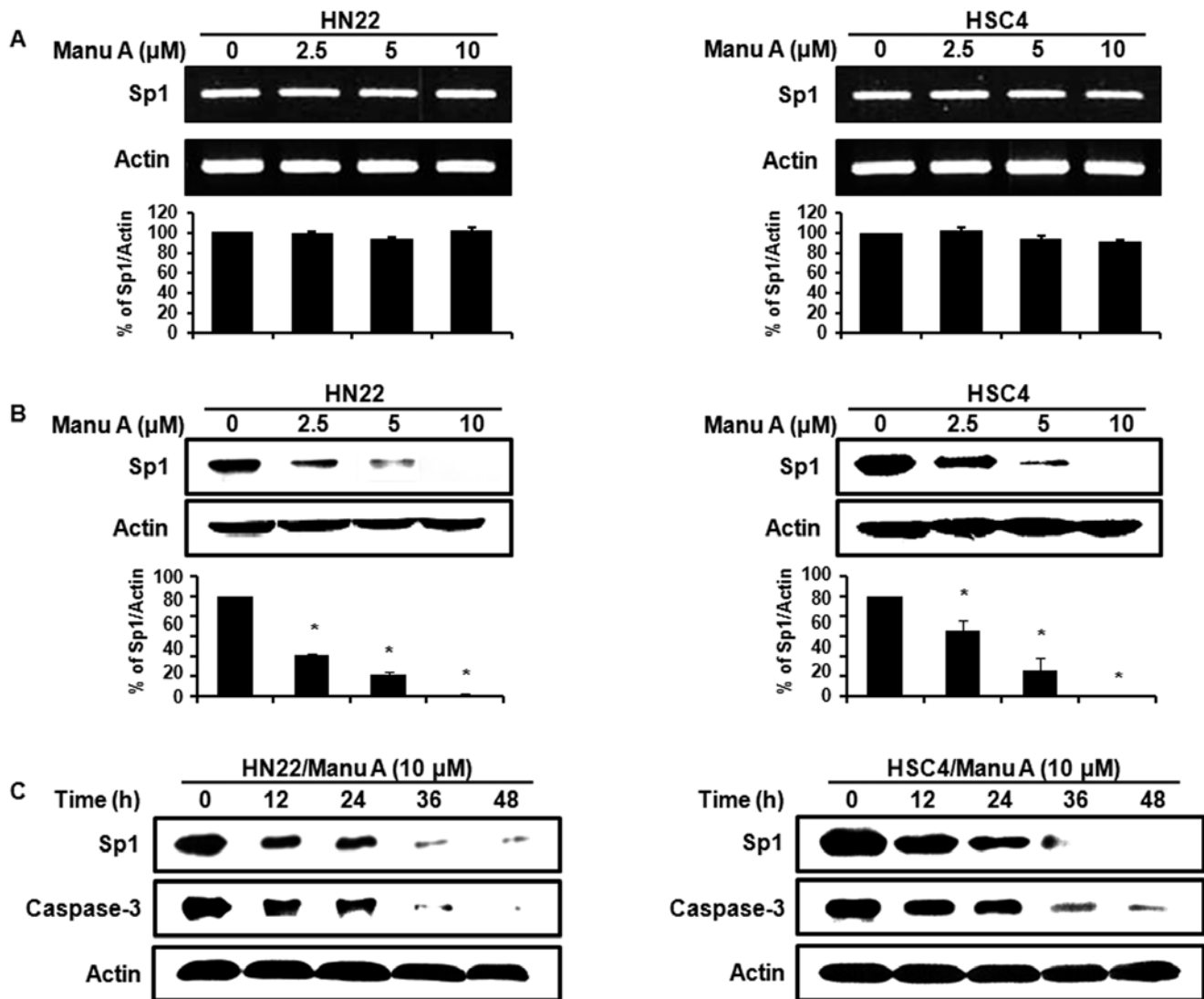


Figure 2. Manu A suppresses Sp1 proteins in OSCC cells and induces cell apoptosis. HN22 and HSC4 cells were treated with 2.5, 5 and 10 μM of Manu A for 48 h. RT-PCR and western blot analysis were employed. The graphs indicate the mRNA (A) and protein (B) expression levels of Sp1 normalized to actin. (C) To examine the time-dependent effects of Manu A on Sp1 and caspase-3, HN22 and HSC4 cells were treated with 10 μM Manu A for 12, 24, 36 and 48 h. Actin was included as the loading control.

these cells were incubated with 95 μl of Muse MitoPotential working solution that is diluted with 1X assay buffer (1:1,000) for 20 min in dark. Then, 5 μl of Muse 7-AAD was added and samples were incubated at 37°C for 5 min. Finally, all samples were analyzed by Muse Cell Analyzer (Merck Millipore).

Statistical analysis. Using Student's t-test, the statistical significance was assessed. The results with a P-value <0.05, was considered as statistically significant.

Results

Manu A inhibits human OSCC cell viability. Two OSCC cell lines HN22 and HSC4 were grown to investigate the effect of Manu A on OSCC cells. Manu A treatment significantly decreased cell viability in a dose- (2.5, 5, and 10 μM) and time- (24 and 48 h) dependent manner (Fig. 1B). Forty-eight hours

after Manu A treatment, the viabilities of HN22 cells were, respectively, 91, 57.8 and 13.3% at 2.5, 5 and 10 μM compared with control group and it showed significant decrease of cell viability in a dose-dependent manner. Similarly, the viabilities of HSC4 cells were, respectively, 71.8, 46, and 19.9% at 2.5, 5, and 10 μM compared with control cells and it also showed the same dose-response as that in HN22. The IC_{50} values of Manu A for cell viability were 6.38 and 4.6 μM in HN22 and HSC4, respectively.

Manu A induces apoptosis in human OSCC cells. We tested if Manu A could induce apoptosis of HN22 and HSC4 cells, using cell cycle analysis, DAPI staining, and double-staining of 7-AAD and Annexin V. The OSCC cells were treated with 2.5, 5 and 10 μM of Manu A for 48 h. Cell cycle analysis showed that Manu A induced sub- G_1 phase in HN22 and HSC4 cells in a dose-dependent manner (Fig. 1C). Especially, both of HN22 and HSC4 showed a significant increase in

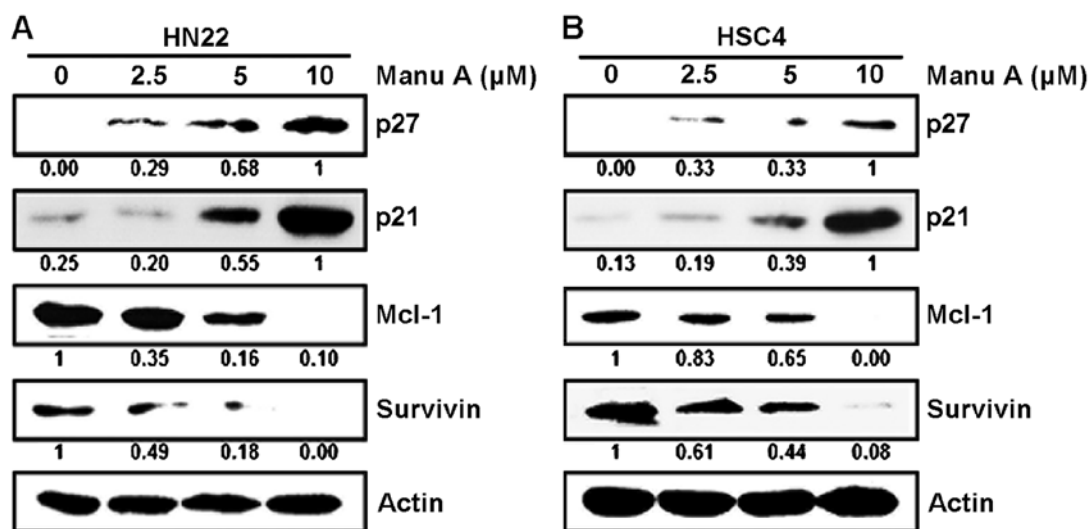


Figure 3. The effect of Manu A on Sp1 downstream target proteins in: (A) HN22 and (B) HSC4 cells treated with 2.5, 5 and 10 μ M of Manu A. The effects of Manu A on p27, p21, Mcl-1 and survivin protein expression levels were determined by western blotting. The blots were re-probed with actin as a loading control.

sub-G₁ phase and a decrease in G₁ phase at 10 μ M of Manu A, as compared to control groups. The proportion of sub-G₁ phase increased from 3.8 ± 0.4 (control) to $44.4 \pm 2.3\%$ (10 μ M) in HN22 cells (Fig. 1C, left) and also increased from 5.5 ± 0.1 (control) to $45.3 \pm 1.9\%$ (10 μ M) in HSC4 cells (Fig. 1C, right). Further DAPI staining revealed the presence of nuclei condensation and apoptotic bodies in Manu A-treated OSCC cells (Fig. 1D). Moreover, 7-AAD and Annexin V double-staining displayed an increased percentage of apoptotic cells after treatment with Manu A for 48 h in a dose-dependent manner (Fig. 5C and D).

Manu A regulates Sp1 and its target protein levels in human OSCC cells. Sp1 is a transcription factor of various genes that are essential to the regulation of cell survival, cell growth, cell cycle, and apoptosis (26-28). To investigate whether the Sp1-mediated apoptosis of OSCC cells might be caused by Manu A treatment or not, we used RT-PCR and western blotting in OSCC cells treated with Manu A (2.5, 5, and 10 μ M). As shown in Fig. 2A, there were no significant changes in the expression of Sp1 mRNA. However, the Sp1 protein levels in HN22 and HSC4 cells were decreased in a dose-dependent manner (Fig. 2B). We also monitored the protein levels of Sp1 and caspase-3 in OSCC cells (HN22 and HSC4) treated with 10 μ M of Manu A for various times (0, 12, 24, 36 and 48 h). The amounts of Sp1 were downregulated and also caspase-3 levels were significantly decreased with time by Manu A (Fig. 2C). Sp1 regulated the expression of its downstream targets such as p27, p21, Mcl-1, and survivin. The protein levels of cell cycle arrest proteins including p27 and p21 were elevated in HN22 (Fig. 3A) and HSC4 (Fig. 3B) by increasing doses of (2.5, 5 and 10 μ M) Manu A. On the contrary, cell proliferation- and survival-related proteins like Mcl-1 and survivin were decreased in HN22 (Fig. 3A) and HSC4 (Fig. 3B).

Manu A induces cell stress and controls the mitochondrial membrane permeability during apoptosis. We investigated

possible relationship between Manu A-induced cell stress and mitochondrial integrity. CHOP, death receptor 4 (DR4), and death receptor 5 (DR5) are related to endoplasmic reticulum (ER) stress. In a previous study, CHOP upregulated DRs (DR4 and DR5) by cell stress (29). The expression levels of CHOP, DR4, and DR5 were significantly increased in HN22 cells (Fig. 4C) and HSC4 cells (Fig. 4D) by Manu A. Changes of mitochondrial membrane permeability (MMP) are the common pathway of stress, triggering cell apoptosis (30). The members of Bcl-2 family regulate cell death by controlling the permeability of mitochondrial membrane (31). As judged from changes in MMP, total depolarized cell proportions were 5.6 ± 0.2 (2.5 μ M), 33.7 ± 0.76 (5 μ M), and $80.0 \pm 0.61\%$ (10 μ M) in HN22 cells (Fig. 4A). In HSC4 cells (Fig. 4B), total depolarized cell proportions were 7.1 ± 1.0 (2.5 μ M), 36.3 ± 0.4 (5 μ M) and $75.2 \pm 0.2\%$ (10 μ M).

Manu A modulates apoptosis-related proteins in OSCC cells. It has been reported that suppression of Sp1 induces apoptosis of cancer cells (32-34). To investigate molecular mechanism of Sp1-mediated apoptosis in HN22 cells (Fig. 5A) and HSC4 cells (Fig. 5B), we carried out western blot analysis of apoptosis-regulating proteins. Consequently, there was a decrease in levels of Bcl-2, Bid, Bcl-x1, and PARP in Manu A-treated OSCC cells. The levels of Bax and cleavage of PARP were elevated in a dose-dependent manner by Manu A. As shown in Fig. 5E and F, there was an increase of multi-caspase activity in both HN22 and HSC4 cells. As shown in Fig. 5E, the proportion of Multi-Caspase-positive HN22 cells was increased from 7.7 ± 0.4 (2.5 μ M) to $15.5 \pm 1.2\%$ (10 μ M) and the population of caspase-positive/dead HN22 cells was increased from 18.9 ± 1.0 (2.5 μ M) to $73.7 \pm 1.6\%$ (10 μ M). In HSC4 (Fig. 5F), the proportion of caspase-positive cells were 4.2 ± 2.6 , 7.0 ± 0.9 and $2.1 \pm 0.2\%$ of control cells while caspase-positive/dead cells were 12.1 ± 0.6 , 29.5 ± 1.4 and $90.2 \pm 3.2\%$ of control cells at 2.5, 5 and 10 μ M of Manu A, respectively. As a whole, suppression of Sp1 by Manu A induces apoptosis in OSCC cells.

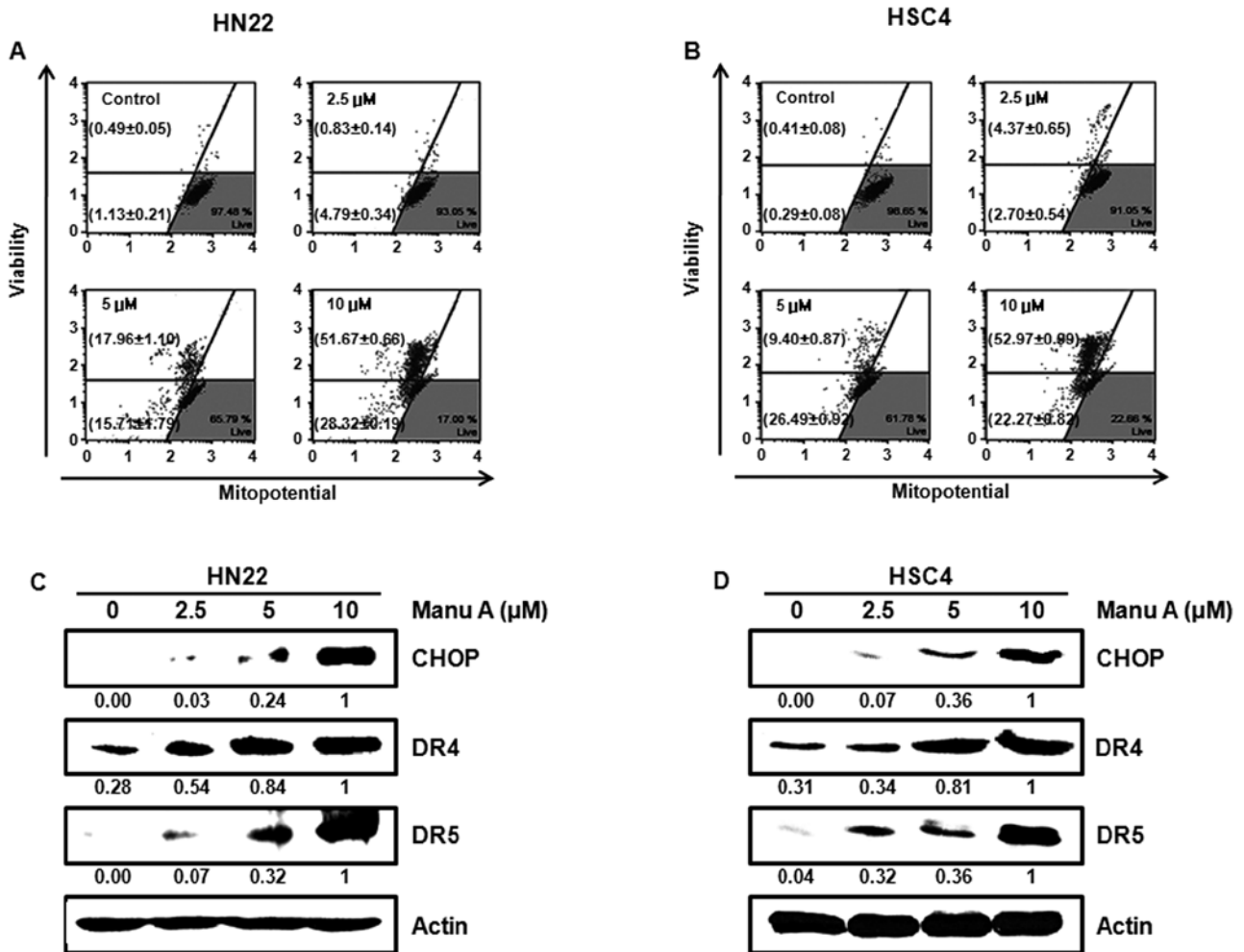


Figure 4. Increased expression levels of the death receptor proteins and induction of mitochondrial dysfunction by Manu A induced cell apoptosis. Depolarization profiles of the mitochondrial membrane are shown in plots (A and B). The data represent the mean percentage levels \pm SD ($n=3$). The expression levels of CHOP and death receptor proteins (DR4 and DR5) were analyzed by western blotting in HN22 (C) and HSC4 (D) cells. Actin was used as the loading control.

Discussion

Oral cancer is a subtype of head and neck cancer and its 5-year survival rate has been slightly improved over the last few decades in spite of advanced cancer diagnosis or therapies (radiotherapy, chemotherapy, and surgery) (35). Recent studies revealed that some antibiotics not only reduce cell proliferation but also induce apoptosis on human cancer cells (36,37). We examined Manu A, a natural antibiotics and tumoricide, as a new potential candidate substance for OSCC chemotherapy. In our study, we investigated whether Manu A could reduce cell proliferation and induce apoptosis through Sp1 regulation in OSCC cell lines (HN22 and HSC4). First of all, we tested anti-proliferation effect of Manu A using MTS assay. Treatment of cells with various concentration of Manu A exhibited a significant decrease in cell viability in a dose-dependent manner. PI staining was performed to find any link of Manu A-mediated cell cycle regulation to cell apoptosis. We observed remarkable increase in proportion of sub- G_1 in a dose-dependent manner. Furthermore, both cell analyses of cells stained with 7-AAD and Annexin V and measurement of caspase activity demonstrated dose-dependent apoptotic

effects of Manu A. Taken together, the data described above, Manu A has biological effects on OSCC cells with respect to cell growth and death.

Sp1 is a zinc finger transcription factor that binds to GC-rich motifs of many promoters (38) and has been reported to affect the tumorigenesis of many cancers including angiogenesis, cell cycle progression and inflammation (38). To prove that the cell apoptosis by Manu A is mediated by Sp1 regulation, we performed RT-PCR and western blotting. Although the expression of Sp1 mRNA was not decreased, the Sp1 protein levels were significantly downregulated by Manu A in a dose- and time-dependent manner. To further investigate molecular mechanism of Sp1-mediated cell apoptosis, we also examined the expression levels of Sp1 target proteins such as p21, p27, Mcl-1, and survivin. It was demonstrated that regulators of cell cycle progression such as p21, p27 (39,40) were increased. It is known that p27 binds to and prevents the activation of cyclin E-cyclin-dependent kinase 2 (CDK2) or cyclin D-cyclin-dependent kinase 4 (CDK4) complexes (41). During the cell division, p27 acts as a cell cycle inhibitor. Similarly, p21 protein binds to and inhibits the complexes of cyclin-CDKs (CDK2, CDK3, CDK4, or CDK6) and thus acts

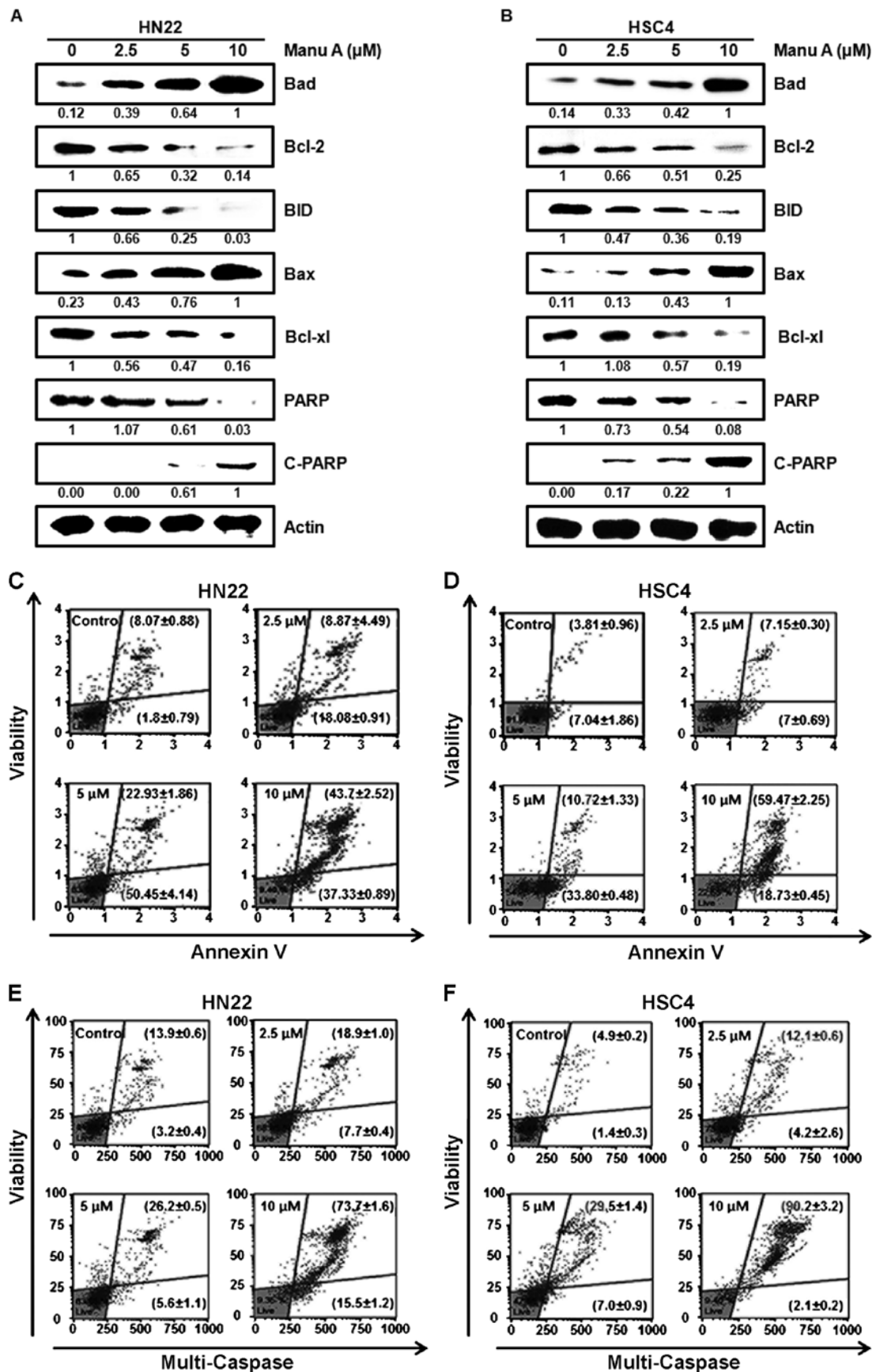


Figure 5. The effects of Manu A on expression of proteins associated with apoptosis in OSCC cells. HN22 (A) and HSC4 (B) cells were treated with 2.5, 5 and 10 μM of Manu A for 48 h and analyzed by western blotting using antibodies against Bad, Bcl-2, BID, Bax, Bcl-xl, PARP and C-PARP. Actin was used to normalize the protein bands. Induction of apoptosis in OSCC cells by Manu A was quantitated as determined by Muse Cell Analyzer. The plots indicate HN22 (C and E) and HSC4 (D and F) cells after exposure to 2.5, 5 and 10 μM of Manu A for 48 h. The analyzer profiles were made after Annexin V (C and D) and Multi-Caspase staining (E and F). (C and D) The number represents the percentage of early to mid apoptotic cells (lower right quadrant) and late apoptotic/dying cells (upper right quadrant). (E and F) Each quadrant indicates caspase-positive, caspase-positive/dead and dead in the counter-clockwise rotation. The results shown are representative of three independent experiments. The data represent the mean percentage levels ± SD (n=3).

as a cell cycle inhibitor (42). Anti-apoptosis factors such as Mcl-1, and survivin were diminished by Manu A. As a member of Bcl-2 family, Mcl-1 is overexpressed in many human cancers and plays an important role in acquiring resistance to apoptosis (43). The inhibitors of apoptosis (IAP), survivin suppresses the apoptosis and its overexpression is associated with development of human cancer (44). Accordingly, it can be summarized that Manu A induces apoptotic pathways in OSCC cells through regulation of Sp1 and its target proteins (p21, p27, Mcl-1 and survivin).

In response to stress, cell initiates cell death signaling through the intrinsic and the extrinsic pathways. Intrinsic pathway involves mitochondrial involvement when exposed to death stimuli. Mitochondria produce energy that the cell needs by oxidative phosphorylation process on the inner membrane. In many pathophysiological context, cell fate is dependent on Bcl-2 family members (45). Bax is a pro-apoptotic Bcl-2 family member and accelerates the opening of voltage-dependent anion channel (VDAC) and Bad, pro-apoptotic Bcl-2 family member, allowing Bax-triggered apoptosis interacting with Bcl-2 and Bcl-x1 (46-48). Opening VDAC pore activates death-driving proteolytic proteins known as caspase (30) beginning with cleavage of PARP (49). Whereas, anti-apoptotic Bcl-family members such as Bcl-2 and Bcl-x1 inhibit opening the VDAC pore (50). Manu A treatment facilitated pro-apoptotic proteins (Bax and Bad) and cleaved PARP while a decrease in anti-apoptotic proteins (Bcl-2 and Bcl-x1) was also observed by Manu A treatment. Considering the data associated with mitochondrial membrane potential, Manu A induces apoptosis through the intrinsic pathway in OSCC cells, whereas, the extrinsic pathway starts with stimulation of tumor necrosis factor (TNF) receptor superfamily including TNF-related apoptosis-inducing ligand (TRAIL) receptor (51). DR4 (TRAIL-R1 for TNF-related apoptosis-inducing ligand receptor-1) and DR5 (TRAIL-R2) interact with its cognate ligand and share common signaling mechanism that activates caspase-8 (52) and increased C/EBP homologous protein (CHOP) elevates DR5 expression (53). Because caspase-8 catalyzes cleavage of BH3-only protein (Bid) to t-Bid that facilitates the release of mitochondrial proteins into cytosol and it has been reported that CHOP downregulates Bcl-2 expression and sensitizes the cell to ER stress (29), extrinsic pathway incorporating the part of intrinsic apoptotic pathway (54). We found that CHOP, DR4, and DR5 were overexpressed in a dose-dependent manner while Bid was downregulated in Manu A-treated OSCC cells. These results revealed that Manu A induces cell apoptosis through not only the intrinsic pathway, but also the extrinsic pathway.

Based on the effects of Manu A in OSCC cells, we conclude that Manu A downregulates Sp1 protein levels, which in turn induces cell apoptosis of OSCC cells (HN22 and HSC4) through both the intrinsic and the extrinsic pathways. Therefore, the use of Manu A may be a novel therapy for OSCC patients with overexpression of Sp1 protein.

Acknowledgements

This study was supported by Basic Science Research program through the National Research Foundation Korea (NRF) funded by the Ministry of Education, Science and

Technology (2014R1A1A2053500) and the Next-Generation BioGreen 21 Program (PJ01116401) from Rural Development Administration, Republic of Korea.

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