Mithramycin A Inhibits Myeloid Cell Leukemia-1 to Induce Apoptosis in Oral Squamous Cell Carcinomas and Tumor Xenograft through Activation of Bax and Oligomerization\textsuperscript{\textcopyright} 

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ABSTRACT

In several human malignancies, overexpression of myeloid cell leukemia-1 (Mcl-1) confers resistance to induction of apoptosis; however, Mcl-1-mediated inhibition of apoptosis in oral squamous cell carcinoma (OSCC) is not fully understood and has been investigated in this study. The Mcl-1 promoter activators (TPA) and epidermal growth factor (EGF) enhanced neoplastic transformation of JB6 cells and this response was accompanied by enhanced expression of Mcl-1, and knockdown of Mcl-1 by RNA interference (RNAi) decreased JB6 cell transformation. In the same cell line, we also demonstrated that mithramycin A (Mith) decreased TPA-induced JB6 cell transformation and Mcl-1 expression. Mcl-1 was overexpressed in human oral tumors compared with normal oral mucosa and also in several OSCC cell lines including HN22 and HSC-4 cells. Treatment of these cells with Mith also decreased Mcl-1 expression and neoplastic cell transformation, and this was accompanied by induction of several markers of apoptosis. Knockdown of Mcl-1 by RNAi also induced apoptotic cell death. The downregulation of Mcl-1 by Mith and RNAi increased pro-apoptotic protein Bax, resulting in the Bax translocation into mitochondria and its oligomerization. Mith also suppressed tumor growth in vivo and induced apoptosis in tumor by also regulating expression of Mcl-1 and Bax proteins. These indicate a critical role for Mcl-1 in the growth and survival of OSCC and demonstrate that Mith may be a potential anticancer drug candidate for clinical treatment of OSCC.

Introduction

Human oral squamous cell carcinoma (OSCC) is the most common neoplasm of oral cavity cancers, and the incidence of this carcinoma has been increasing (Silverman, 2001; Liang et al., 2008). Most patients are diagnosed with OSCC only after the disease has reached an advanced stage and the 5-year survival rate of oral cancer patients remains relatively low. The optimal treatment or therapy for OSCC remains controversial. Surgery is an effective but complex method for treatment of OSCC, and the development of novel therapeutic regimens to prevent and treat OSCC is critically important.

Myeloid cell leukemia-1 (Mcl-1) is a member of the B-cell lymphoma-2 (Bel-2) family protein and has been characterized as a critical survival factor for hematopoietic-derived cells such as multiple myeloma cell lines (Opferman et al., 2005). The prosurvival functions of Mcl-1 are due to interacting with proapoptotic members of the Bel-2 family such as Bim, Bak, and Bax, and this results in the inhibition of cytochrome c release from the mitochondria and decreased apoptosis (Kazi et al., 2011; Zhang et al., 2011). Recently, it has been reported that Mcl-1 is overexpressed in tumor cells of human primary OSCC and cultured SCC cell lines, suggesting that Mcl-1 may be an important drug target for treatment of human OSCC cases (Nagata et al., 2009). Our group also recently reported that downregulation of Mcl-1 by the non-steroidal anti-inflammatory drug (NSAID), tolfenamic acid induced apoptosis in YD-15 mucoepidermoid carcinoma cells, a major malignant carcinoma of the salivary gland (Choi et al., 2011b). However, these studies did not identify the molecular mechanisms and linkages between tolfenamic acid-induced apoptosis and Mcl-1 inhibition.

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ABBREVIATIONS: Bcl-2, B-cell lymphoma-2; BMH, bismaleimide; Cox4, cytochrome c oxidase polypeptide IV; DAPI, 4',6-diamidino-2-phenylindole; EGF, epidermal growth factor; Mcl-1, myeloid cell leukemia-1; Mith, mithramycin A; MTS, 3-(4,5-dimethylthiazol-2-yl)-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium; NOM, normal oral mucosa; NSAID, non-steroidal anti-inflammatory drug; OSCC, oral squamous cell carcinoma; PARP, poly (ADP-ribose) polymerase; RNAI, RNA interference; RT-PCR, reverse transcription-polymerase chain reaction; TPA, 12-O-tetradecanoylphorbol-13-acetate; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.
acid-dependent downregulation of Mcl-1 and induction of apoptosis in YD-15 cells.

Mith is a chemotherapeutic compound that has been used in the therapy of several types of cancer (Koller and Miller, 1986). Mith binds to GC-rich DNA sequences and regulates expression of Sp1, other Sp proteins, and Sp-regulated genes including surviving cyclin D1 and VEGF, which also have GC-rich promoter sequences (Abdelrahim et al., 2002, 2004; Chadalapaka et al., 2008). Most Mith-related studies have been focused on the anti-cancer activity of Mith due to its suppression of Sp1 protein (Jia et al., 2010; Seznec et al., 2011). Recently, we reported that Mith can modulate expression of both Sp1 and Mcl-1 proteins, indicating that this drug may be important for targeting Mcl-1 overexpressing tumors where Mcl-1 play an antiapoptotic function (Choi et al., 2011b). In the present study, we sought to determine the role of Mcl-1 in OSCC and the antitumor activity of Mith in OSCC cells in vitro. Materials and Methods

Chemicals and Antibodies. Mith and 12-O-tetradecanoylphorbol-13-acetate (TPA) were supplied by Sigma-Aldrich (St. Louis, MO). Epidermal growth factor (EGF) was obtained from Invitrogen (Carlsbad, CA). PARP and Bax (6A7) antibodies were purchased by BD Pharmingen (San Jose, CA). Sp1 and actin antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against Mcl-1, Bax, cleaved caspase-3, and cleaved caspase-9 were supplied by Cell Signaling Technology, Inc. (Charlottesville, VA). Cox4 antibody was obtained from Abcam (Cambridge, UK).

Materials. Fourteen normal oral mucosa (NOM) tissues and twenty-five oral squamous cell carcinoma (OSCC) tissues were obtained from adult patients who visited the Pusan National University Dental Hospital (Pusan, Korea). Samples were obtained during the third molar removal. Twenty-five cases of OSCC with good preservation of paraffin tissue and hematoxylin/eosin-stained slides were obtained from files of the Department of Pathology, Pusan National University School of Medicine between January 2002 and December 2007. The ethics committee of Pusan National University School of Medicine and Dentistry approved the methods used in the present study.

Cell Culture and Chemical Treatment. HN22 cells were kindly provided by Dankook University (Cheonan, Korea), and HSC4 and HSC2 cells were provided by Hokkaido University (Hokkaido, Japan). Cells were cultured in DMEM supplemented with 10% FBS and antibiotics at 37°C in a 5% CO2 incubator. Cells were treated with DMSO or various concentrations of Mith A (15, 30, or 60 nM) for different time points. JB6 mouse skin epidermal cells were obtained from American Tissue Culture Collection (Manassas, VA), and cells were obtained from the Department of Pathology, Pusan National University School of Medicine. Western Blot Analysis. Whole cell lysates were extracted with lysis buffer and protein concentrations were measured using a DC protein assay (Bio-Rad Laboratories, Hercules, CA). Samples containing equal amounts of protein were separated by SDS-PAGE and then transferred to Immun-Blot PVDF membranes (Bio-Rad Laboratories). The membranes were blocked with 5% skim milk in TBST at room temperature for 2 hours, and incubated overnight at 4°C with primary antibodies against PARP, cleaved caspase-3, cleaved caspase-9, Sp1, Mcl-1, Bax, Bax (6A7), COX4, or actin, followed by incubation with HRP-conjugated secondary antibodies. Antibody-bound proteins were detected using an ECL Western Blotting Luminol reagent (Santa Cruz Biotechnology, Inc.). DNA Interference. On TARGETplus SMARTpool siRNA sequences targeting Mcl-1 and nontargeting control were purchased from Dharmacon Research (Lafayette, CO). Briefly, cells were seeded on 6-well plates and transiently transfected with 50 nM siRNA for 72 hours using a DharmaFECT2 transfection reagent (Thermo Scientific, Lafayette, CO). After transfection, cells were analyzed by soft agar assay, DAPI staining, and Western blot analysis.

Immunohistochemistry. Tissue sections, 4-μm thick, were deparaffinized, treated in 100% alcohol, and stained with DAPI solution (2 μg/ml). The cell morphology and visualized by ethidium bromide staining. To analyze sections, five nonoverlapping fields per slide were randomly selected and images were captured with a light microscope attached to a digital camera (Olympus, BX51T, Tokyo, Japan, 100×). The captured images were examined independently in a blinded manner by two experienced oral pathologists. The expression levels of Mcl-1 were categorized into four easily reproducible subgroups as follows: (a) no detectable expression (point 0); (b) positive expression in less than 30% of cells (point 1); (c) positive expression in 30–50% of cells (point 2); (d) positive expression in greater than 50% of cells (point 3).

Reverse Transcription-Polymerase Chain Reaction. Total RNA was extracted from easy-BLUE Total RNA Extraction Kit (iNTRON, Daejeon, Korea). cDNA was prepared from 1 μg of total RNA using the ImProm-II Reverse Transcription System (Promega, Madison, WI). Mcl-1 and β-actin transcripts were amplified by PCR using specific primers; Mcl-1 sense 5′-TGC TGG AGT TGG TCG GGAA-3′, Mcl-1 anti-sense 5′-TGG TAA GTG CTC GCG CTG CT-3′, β-actin sense 5′-GTG GGG CCC GGG ACC CAC CA-3′, β-actin anti-sense 5′-CTC CTT AAT GTC ACG CAG CTT-3′. Mcl-1 amplification was done for 28 cycles (1 minute at 95°C, 1 minute at 60°C, and 1 minute 30 seconds at 72°C), and β-actin amplification was done for 25 cycles (1 minute at 95°C, 1 minute at 60°C, and 1 minute 30 seconds at 72°C). PCR products were analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

MTS Assay. The effects of Mith on cell viability of HN22 and HSC4 cells were determined by the CellTiter 96 Aqueous One Solution cell proliferation assay kit (Promega) according to the manufacturer's instructions. Briefly, cells were seeded in 96-well plates and then incubated with various concentrations (15, 30, 60 nM) of Mith for different times (24 or 48 hours). MTS (3-(4,5-dimethylthiazol-2-yl)-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium) solution was added to each well and maintained for 2 hours at 37°C. The absorbance was measured at 490 and 690 nm (background) using an ELISA microplate reader (BIO-TEK Instruments, Inc., Madison, WI).

DAPI Staining. Detection of nuclear fragmentation and chromatin condensation in nuclei of apoptotic cells was carried out using a fluorescent nuclear dye, DAPI (4′,6-diamidino-2-phenylindole; Sigma). HN22 and HSC4 cells treated with Mith or transfected with Mcl-1 siRNA were harvested by trypsinization and fixed in 100% methanol at room temperature for 10 minutes. Cells were deposited on slides and stained with DAPI solution (2 μg/ml). The cell morphology was observed under a fluorescence microscope.
Preparation of Cytosolic and Mitochondrial Fractions. Cytosolic and mitochondrial fractions were isolated by digitonin or Triton X-100 permeabilization. Briefly, cells were washed with ice-cold PBS, and cell pellets were resuspended for 1 minute at room temperature in plasma membrane extraction buffer containing 0.05% digitonin. Following a centrifugation step at 15,000g at 4°C for 5 minutes, the supernatant was separated from the pellet consisting of cellular debris. The supernatant containing cytosolic proteins was collected, and the mitochondrial pellet was harvested by centrifugation at 15,000g at 4°C for 5 minutes. The pellet consisting of mitochondrial proteins was resuspended by plasma membrane extraction buffer containing 0.5% Triton X-100. The supernatant containing mitochondrial proteins was collected from the last centrifugation.

Detection of Bax Activation. HN22 and HSC4 cells treated with Mith or transfected with Mcl-1 siRNA were harvested and then whole cell lysates were extracted by lysis buffer. The extracted proteins were analyzed by Western blot. Bax activation was detected using primary antibody recognizing only the active form of Bax (6A7).

Crosslinking. HN22 and HSC4 cells were treated with DMSO or Mith for 48 hours and then cells were harvested. For Bax oligomerization, the cells were suspended by conjugation buffer with 10 mM EDTA. The cell lysates were incubated with 0.2 mM Bismaleimide (Thermo Scientific) at room temperature for 1 hour and then extracted by lysis buffer for Western blot analysis.

Nude Mouse Xenograft Assay. Female nude mice were purchased from Orient Ltd (Suwon, Korea), and maintained in accordance with the Institutional Animal Care Use Committee guidelines. HN22 cells were implanted with s.c. injection into the flanks of the mice. Tumor growth was measured 4 days later, and tumor volumes were calculated weekly.

TUNEL Assay. The tumor tissues were analyzed by Dead-End Colorimetric TUNEL System (Promega) according to the manufacturer’s instructions. Briefly, paraffin-embedded slides were deparaffinized and rehydrated. The sections were treated with proteinase K for 15 minutes at room temperature and then the endogenous peroxidase was blocked with 0.3% H2O2 in PBS for 5 minutes. The digoxigenin-dUTP end labeled DNA was detected using an antidigoxigenin-peroxidase antibody followed by peroxidase detection with 0.05% H2O2. The sections were counterstained with methyl green. The brown-colored apoptotic bodies in the tumor sections of the control and Mith-treated mice were counted using a Nikon Eclipse E800 microscope (Nikon Inc., Melville, NY) at 20× magnification.

Statistical Analysis. Data are reported as means ± S.D. of triplicate samples from at least three independent experiments. Statistical significance was evaluated using a Student’s t test or one-way analysis of variance and considered significant when P < 0.05.

Results

Normal mouse epidermal JB6 cells readily undergo neotransformation after treatment with EGF or TPA known as Mcl-1 promoter activator. Thus, the JB6 cells treated with EGF or TPA serve as initial model cells for investigating the relationship between Mcl-1 expression and neoplastic cell transformation. Results in Fig. 1A show that in the absence of these mitogens, a minimal number of colonies were observed in an anchorage-independent cell transformation assay. In contrast, treatment with 5–20 ng/ml TPA or EGF significantly increased anchorage-independent growth. Previous studies show that TPA and EGF induce Mcl-1 (Kozopas et al., 1993; Leu et al., 2000; Booy et al., 2011), and results in Fig. 1B confirm that both mitogens induce a time-dependent increase in Mcl-1 levels that are maximal after 2-6 hours and then decrease at longer time points (9-12 hours); Fig. 1C illustrates the dose-dependent induction of Mcl-1 by EGF and TPA at the 3-hour time point. The role of Mcl-1 in TPA and EGF-mediated transformation was determined by RNA interference (RNAi) in the anchorage-independent assay in JB6 cells transfected with control oligonucleotide and a small inhibitory RNA for Mcl-1 (siMcl-1). The results show that knockdown of Mcl-1 significantly inhibited TPA- and EGF-induced JB6 cell transformation (Fig. 1D), and these data demonstrate the critical role of Mcl-1 in mitogen-induced transformation using JB6 cells as a model.

Analysis of normal oral mucosa (NOM) and OSCC tissues (14 and 25, respectively) shows that Mcl-1 expression is significantly higher in tumor versus non-tumor tissues and high Mcl-1 levels were also observed in several oral cancer cell lines (Fig. 2A). Previous studies showed that Mith, a drug that blocks expression of Sp1 and other Sp transcription factors inhibits Mcl-1 expression in uroepidermoid carcinoma cells (Choi et al., 2011b), and results in Fig. 2, B and C, show that Mith also decreased expression of Mcl-1 protein and mRNA levels in HN22 and HSC4 cells. Moreover, Mith also co-ordinately decreased Mcl-1 and induced PARP cleavage at similar time points in both cell lines after treatment of 24–48 hours, whereas minimal effects were observed at earlier time points (Fig. 2D). We also confirmed that Mith inhibited TPA-induced neotransformation of JB6 cells (Fig. 2E) and this correlated with the observed interactions of TPA and Mcl-1 knockdown in these cells (Fig. 1, B and D). Mith also inhibited anchorage-independent in HSC2 and HSC4 oral cancer cells (Fig. 2F).

Because Mcl-1 is an important survival gene (Akgul, 2009; Mandelin and Pope, 2007; Yang-Yen, 2006), we further investigated the effects of Mith on HN22 and HSC4 cell growth and activation of caspases, which are markers of apoptosis. Fig. 3A illustrates that treatment with 15–60 nM Mith significantly inhibited growth of the OSCC cell lines and Mith also induced cleaved caspases-3 and -9 and PARP cleavage in these cell lines after treatment of 48 hours (Fig. 3B). Mith-induced apoptosis in HN22 and HSC4 cells was also confirmed by DAPI staining that showed a dose-dependent increase in apoptotic cells (Fig. 3C). The linkage between Mith-mediated downregulation of Mcl-1 and induction of apoptosis was confirmed by RNAi using siMcl-1 oligonucleotide. Knockdown of Mcl-1 in HN22 and HSC4 cells induced activation (cleavage) of caspase-3 and caspase-9 and this was accompanied by increased PARP cleavage (Fig. 3D) and increased DAPI staining for apoptotic cells (Fig. 3E). These results demonstrate that the proapoptotic activity of Mith is due to downregulation of Mcl-1.

Previous studies have demonstrated that the proapoptotic activity of Mcl-1 downregulation is also linked to induction of Bax (Cheng et al., 2010), and results in Fig. 4A show that Mith induces Bax [total protein and activated Bax (6A7)] expression in HN22 and HSC4 cells. Moreover, we also observed that Mith increased mitochondrial levels of Bax (Fig. 4B) and Bax oligomerization (Fig. 4C). The role of Mcl-1 knockdown in mediating Bax expression was investigated by RNAi (siMcl-1) and loss of Mcl-1 significantly increased total Bax protein in HSC4, but not in HN22 cells (Fig. 4D). However, knockdown of Mcl-1 significantly induced Bax (6A7) proteins in both HN22 and HSC4 cells, and this is consistent with a previous
reports showing that quercetin downregulates Mcl-1 and induces Bax in U937 cells (Cheng et al., 2010) (Fig. 4E). Moreover, siMcl-1 increased levels of mitochondrial Bax protein whereas it did not decrease total or cytosolic Bax protein expression, suggesting that the loss of Mcl-1 in HN22 and HSC4 cells primarily effects the subcellular distribution of Bax that is increased in mitochondria (Fig. 4F).

We also investigated the in vivo anti-tumorigenic activity of Mith (0.2 mg/kg/day) in an athymic nude mouse xenograft model using HN22 cells and observed inhibition of tumor volume and weight (Fig. 5A), and this was not accompanied by changes in body weights (Fig. 5B). Analysis of tumor lysates by Western blots showed decreased expression of Mcl-1 and no significant changes in Bax protein levels in tumors from animals treated with Mith compared with controls (Fig. 5C). However, Mith clearly increased cleaved caspase-3 and active Bax (6A7), which is consistent with in vitro data from HN22 cells (Figs. 4, D and E, and 5C). Mith also increased TUNEL-positive cells, indicating apoptotic cell death (Fig. 5D). We also found that Mith did not affect organ weights or levels of Mcl-1 protein in those organs obtained from Mith- and control-treated mice (Fig. 5E). Moreover, histopathological findings showed that there were no differences of organs from the control- and Mith-treated group, indicating no systemic toxicity at the dose of Mith used in this study (Fig. 5F).

**Discussion**

Numerous human cancers are typically resistant to apoptosis, and aberrant expression of the prosurvival Mcl-1...
Fig. 2. Mith modulates Mcl-1 expression and induces apoptosis in OSCC cells overexpressing Mcl-1. (A) NOM tissues and OSCC tissues were immunostained using antibodies against Mcl-1. Levels of Mcl-1 were analyzed by immunostaining of 14 NOM tissues samples and 25 OSCC tissues samples. *P < 0.05, Mcl-1 expression between NOM and OSCC tissues compared with NOM tissue samples. Mcl-1 expression in various oral cancer cell lines was determined by Western blot analysis. (B) Mcl-1 expression in HN22 and HSC4 cells treated with DMSO or various concentrations (15, 30, 60 nM) of Mith was determined by Western blots. Results are expressed as means ± S.D. for triplicate experiments. *P < 0.05 compared with DMSO-treated cells. (C) Mcl-1 mRNA levels were confirmed by RT-PCR in both cells and normalized to β-actin. Results are expressed as means ± S.D. for triplicate experiments. *P < 0.05 compared with DMSO-treated results. (D) HN22 and HSC4 cells were treated with DMSO or 60 nM Mith for 6, 12, 24, and 48 hours, and whole cell lysates were analyzed by Western blots. (E) for the soft agar assay, JB6 cells treated with TPA (20 ng/ml) or Mith incubated at 37°C in a 5% CO₂ incubator for 10 days, and colonies were counted as described in Materials and Methods. HSC4 and HSC2 cells were treated with Mith incubated for 20 days, and then colonies were counted as described above. *P < 0.05, Mith-induced effects. Results are expressed as means ± S.D. for triplicate experiments.
protein has been identified in many tumors (Krajewska et al., 1996; Erovic et al., 2005; Song et al., 2005; Fleischer et al., 2006). Immortalization of hematopoietic cells and high incidence of lymphomas in transgenic mice overexpressing Mcl-1 supports a role for this protein in tumorigenesis and cancer cell survival (Zhou et al., 1998, 2001; Craig, 2002). Down-regulation of Mcl-1 also enhances the sensitivity to diverse apoptotic stimuli in various cancer cells (Taniai et al., 2004; Rahmani et al., 2005; Song et al., 2005). In the present study, we showed that Mcl-1 protein is highly expressed in OSCC tissues compared with normal oral mucosa and inhibition of Mcl-1 induced caspase-dependent apoptosis. We also found that Mcl-1 was induced along with neoplastic cell transformation in JB6 cells treated with TPA or EGF and knockdown of Mcl-1 by RNAi inhibited JB6 cell transformation (Fig. 1). The linkage between Mcl-1 and cell transformation was also observed in HN22 and HSC4 OSCC cells; knockdown of Mcl-1 by RNAi decreased cell transformation and they was accompanied by induction of apoptosis.

Mith is an aureolic acid-type polyketide identified from a variety of strains of the bacterium Streptomyces and has been used to treat Paget’s disease (Koller and Miller, 1986).
Mith suppresses the expression of the human melanoma-associated gene ABCB8 and has potential therapeutic applications for treating melanoma patients (Sachrajda and Ratajewski, 2011). Mith also decreased VEGF and XIAP to induce apoptosis and inhibit tumor cell migration in glioma cells (Seznec et al., 2011). Analogs of Mith also possess anticancer properties and this is due, in part, to modulate the activity of Sp transcription factors as a new strategy for treatment of metastatic prostate cancer (Malek et al., 2012). In addition, treatment with a combination of Mith and other compounds downregulates Sp1 protein expression and exhibits synergistic antitumor activity in pancreatic cancer (Gao et al., 2011). We also investigated the anti-proliferative effects of Mith on OSCC and showed that Mith decreased all growth; in addition Mith also decreased expression of Mcl-1 protein in a concentration- and time-dependent manner and this was accompanied by inhibition of neoplastic cell transformation and induction of apoptosis. These results suggested that Mith may also be a potential drug for targeting Mcl-1 and treatment of OSCC. We also investigated the effects of Mith on other Bcl-2 family members to rule out the involvement of other Bcl-2 family members. The results showed that only Bcl-xL was affected by Mith in both cell lines indicating that Mith-mediated downregulation of Bcl-xL may also play a role in the induction of apoptosis (Supplemental Figure). Future studies will investigate the molecular pathways related to a role for Bcl-xL in Mith-induced apoptosis in oral cancer. Previously, we reported that tolfenamic acid decreased Mcl-1 mRNA, promoter activity, and protein levels showing effects on transcription (Choi et al., 2011a), whereas downregulation of Mcl-1 by sorafenib was due to inhibition of translation (Rahmani et al., 2005). In this study we show that Mith clearly decreased levels of Mcl-1 mRNA and protein (Figs. 2, B and C), indicating that Mith also acts as the transcriptional level.

Previous studies show that Mcl-1 interacts with Bak in adenovirus-infected cells and HeLa cells (Cuconati et al., 2003; Willis et al., 2005). Mcl-1 decreases Bak expression and inhibits its function by inhibiting conformational changes in several cancer cells. Thus, we hypothesized that knockdown of Mcl-1 may also increase Bak protein levels in OSCC, and the results showed that downregulation of Mcl-1 by Mith either did not affect or slightly decreased Bak protein expression (data not shown). Although inhibition of Bak by Mcl-1 has been suggested to play a critical role in its anti-apoptotic characteristic, the relationship between Mcl-1 and Bak is less well understood. Higher expression of Mcl-1 has previously been known to inhibit Bak activation (Antonsson et al., 2001). Bak is a cytosolic monomer and activation induces conformational changes (becoming 6A7 positive) and translocation into the outer mitochondria membrane (Antonsson et al., 2001; Leber et al., 2007). Recently, it was reported that the prosurvival activity of Mcl-1 is due to inhibition of Bak function.

Fig. 4. Downregulation of Mcl-1 by Mith and RNAi regulates Bak induce apoptosis in OSCC cells. (A) total Bax and activated Bax (6A7) were detected by Western blot analysis in HN22 and HSC4 cells. (B) mitochondrial fractions were prepared and analyzed by Western blots. Results are expressed as means ± S.D. for three replicate experiments, and significance (P < 0.05) compared with DMSO is indicated. Cox4 was used to normalize the mitochondrial fraction. (C) Bak oligomerization was detected in cell lysates after treatment with 0.2 mM BMH, and Western blot analysis as described in Materials and Methods. (D and E) total Bax and activated Bax (6A7) were detected by Western blot analysis in HN22 and HSC4 cells transfected with siMcl-1. (F) cytosolic and mitochondrial fractions from HN22 and HSC4 cells were analyzed by Western blots. Cox4 was used to normalize protein in mitochondrial fractions.
at mitochondria, and the anticancer drug quercetin blocks this response in leukemia cells (Germain et al., 2008; Cheng et al., 2010). Our results also showed that knockdown of Mcl-1 by RNAi or treatment with Mith activated Bax protein (6A7) and induced Bax translocation into mitochondria (Fig. 4). We also observed that siMcl-1 and Mith induced Bax oligomerization, which can induce the release of cytochrome c from the outer mitochondria membrane.

Previous reports show that Mith suppresses pancreatic tumor growth in a xenograft model (Yuan et al., 2007). We also observed that Mith inhibited tumor volume and weight in mice bearing HN22 cells as a xenograft (Figs. 5, A and B). Mith also decreased Mcl-1 expression, activated caspase-3 and increased TUNEL-positive cells, and activated Bax protein in tumors (Figs. 5, C and D). These results were consistent with in vitro data. A previous study reported 0.4 and 1.5 mg/kg doses of...
Mithramycin A Inhibits Mcl-1 and Induces Apoptosis

Mith had significant systemic side effects as evidenced by a loss of body weight, whereas a 0.05 mg/kg dose did not cause side effects (Jia et al., 2010). Another report showed that 0.25 mg/kg Mith did not show toxicity in a pancreatic tumor model (Yuan et al., 2007), and on the basis of these results, we selected a 0.2 mg/kg dose for this study and observed no detectable cytotoxic effects on the basis of histopathology and changes in body and organ weights. We also observed that Mith did not alter Mcl-1 protein expression in five organs/tissues obtained from Mith-treated mice compared with controls.

In summary, our results show that Mcl-1 is overexpressed in oral tumors and cancer cells and results of RNAi experiments and treatment with Mith demonstrate that Mith1 exhibits pro-oncogenic activity in oral cancer cells and tumors. Mcl-1 plays a critical role in suppressing activation of Bax and Bax-induced apoptotic responses and Mith effectively down-regulates Mcl-1, resulting in inhibition of oral cancer cell growth and tumor formation. These results suggest that Mith may have important clinical potential as an anticancer drug for treating OSCC.

Authorship Contributions
Participated in research design: Cho. Conducted experiments: Shin, Jung, Ryu. Performed data analysis: Safe, Cho. Wrote or contributed to the writing of the manuscript: Safe, Cho.

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Ji-Ae Shin, Ji-Youn Jung, Mi Heon Ryu, Stephen Safe, and Sung-Dae Cho

Supplement Figure. Other Bcl-2 family member protein expression (Bcl-xL, Bcl-2, Bad, and Bim) in HN22 and HSC4 cells treated with DMSO or various concentrations (15, 30, 60 nM) of Mith was determined by Western blots. Positive control is a cell lysate from KB human cervical cancer cells.