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ORIGINAL ARTICLE

Inhibition of myeloid cell leukemia-1 by tolfenamic acid induces apoptosis in mucoepidermoid carcinoma

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OBJECTIVES: The aim of this study was to evaluate the role of tolfenamic acid (Tol) and ampiroxicam (Amp) in the apoptotic regulation of YD-15 salivary mucoepidermoid carcinoma (MEC).

MATERIALS AND METHODS: The effect of Tol on apoptosis and its mechanism were examined using a 3-(4,5-dimethylthiazol-2-yl)-5-(2,4-disulfophenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay, Sub-G_1 population, Western blot analysis, 4′-6-Diamidino-2-phenylindole staining, reverse transcriptase polymerase chain reaction, immunostaining and small interfering RNA transfection.

RESULTS: Tol inhibited cell growth of YD-15 cells but Amp did not. Tol induces apoptosis in YD-15 cells as evidenced by nuclear fragmentation, accumulation of the sub-G1 phase and the activation of caspase 3. Tol inhibited myeloid cell leukemia-1 (MCL-1) at the protein and mRNA levels. The treatment of MCL-1 siRNA to YD-15 cells resulted in the activation of caspase 3 and the inhibition of cell growth. Moreover, MCL-1 was regulated by specificity protein 1, but not by mitogen-activated protein kinases.

CONCLUSION: These results suggest that Tol could be a potent anti-cancer drug for YD-15 MEC cells that acts by regulating the MCL-1 protein.
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Keywords: salivary mucoepidermoid carcinoma; tolfenamic acid; myeloid cell leukemia-1; apoptosis

Introduction

Mucoepidermoid carcinoma (MEC) is the most common malignant salivary tumor corresponding to 34% of all salivary malignancies (Spiro, 1986), and is a unique epithelial neoplasm composed of epidermoid, mucous and intermediate cells in variable proportions. Its clinical behavior is highly variable and ranges from being slow-growing to being locally aggressive and highly metastatic. Although there are many case reports related to MEC (Takeda and Kurose, 2006; Leong *et al*, 2007; Das and Kalyani, 2008; Tucci *et al*, 2009), the molecular risk factors for MEC have not yet been completely understood.

Non-steroidal anti-inflammatory drugs (NSAIDs) induce apoptosis in many cell types (Menzel *et al*, 2002; Abdelrahim *et al*, 2007; Ye *et al*, 2010; Zhang *et al*, 2010). In studies published recently, NSAIDs, such as celecoxib and aspirin, were shown to have anti-cancer effects by inducing apoptosis in oral squamous carcinoma cells (Kim *et al*, 2010). However, it has not been established whether other NSAIDs, such as tolfenamic acid (Tol) and ampiroxicam (Amp), have similar inhibiting effect on the proliferation of human oral cancer cells, especially in respect of human MEC cells; the molecular pathways involved in NSAID-induced apoptosis have not been well explored.

Although NSAIDs can inhibit cancer growth by regulating the activity of cyclooxygenase (COX), there is increasing evidence that the apoptotic action of NSAIDs involves COX-independent pathways (Zhang *et al*, 1999). Recently, several studies published that Tol has therapeutic potential that exhibits anti-cancer activity in pancreatic and colorectal cancer models irrespective of COX-2 activity (Ding *et al*, 2008; Konduri *et al*, 2009). Myeloid cell leukemia-1 (MCL-1), a member of the B-cell lymphoma-2 (Bcl-2) family was discovered based on its increased expression during cell commitment to differentiation in a human myeloid leukemia cell line. MCL-1 was, in studies published recently, found to be a critical factor for the survival of hematopoietic cells such as human neutrophils, leukemic large granular

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lymphocytes, and multiple myeloma cell lines (Zhou *et al*, 1997; Craig, 2002; Gojo *et al*, 2002; Kaufmann *et al*, 2005). Therefore, the down-regulation of MCL-1 in cancer cells can abrogate its pro-survival function because it is often over-expressed in cancer where it plays a pro-survival role.

Therefore, this study examined the effects of Tol on the growth of YD-15 salivary MEC cells as well as the mechanism of the anti-cancer action of this compound as related to MCL-1.

Materials and methods

Materials

Tolfenamic acid [N-(3-Chloro-2-methylphenyl)-anthranilic acid, Tol] was obtained from Tokyo Chemical Industry Co. (Tokyo, Japan) and Amp was purchased from LKT Laboratories (St Paul, MN, USA). Antibodies to cleaved caspase-3 (Asp175), MCL-1, phospho-extracellular signal-regulated kinases (ERK)1/2, total ERK1/2, phospho-p38 (Thr180/Tyr182), total p38, phospho-Jun N-terminal Kinase (JNK) (Thr183/Tyr185), and total JNK were obtained from Cell Signaling Technology (Denver, MA, USA). The actin and specificity protein1 (Sp1) antibodies was supplied by Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). SB203580, mithramycin A, 4′-6-Diamidino-2-phenylindole (DAPI) and propidium iodide (PI) was acquired from Sigma-Aldrich Chemical Co. (St Louis, MO, USA).

Cell culture and drug treatment

YD-15 mucoepidermoid carcinoma cells were obtained from the College of Dentistry, Yonsei University (Seoul, Korea). The cells were cultured at 37°C in RPMI1640 containing 10% fetal bovine serum (FBS) and 100 U/ml each of penicillin and streptomycin in an atmosphere containing 5% CO_2. The cells were treated with Tol or Amp (20, 40, and 60 μM) diluted in DMSO [vehicle control; 0.1% (v/v DMSO/RPMI1640 containing 10% FBS)] for 6, 12, 24, 48 and 72 h depending on the experimental designs.

3-(4,5-dimethylthiazol-2-yl)-5-(2,4-disulfophenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay.

A CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit (Promega, Madison, WI, USA) was used to examine the effects of 20, 40 and 60 μM of Tol and Amp on the cell viability in YD-15 cells. Briefly, the cells were treated with different concentrations of Tol and Amp. The viability was determined using a CellTiter 96 Aqueous One Solution detection kit and quantified using a microplated reader (BioTeck Instruments Inc., Winooski, VT, USA) at 490 and 690 nm (background).

Fluorescence-activated cell sorter analysis

After treatment with Tol and Amp, the detached cells (floaters) were collected by centrifugation and combined with the adherent cells. The cells were then fixed with ice-cold solution of 70% ethanol overnight at −20°C and treated with 0.02 mg/ml PI and 150 μg/ml RNase A. To detect nuclear fragmentation, sub-G_1 peak on DNA content was analysed using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Western blotting

The protein samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to membranes and blocked with 5% skim milk followed by hybridization with the indicated antibodies. The protein bands with the horseradish peroxidase-conjugated secondary antibody were observed using a chemiluminescence detection kit.

DAPI staining

The cells showing nuclear condensation and fragmentation were observed. The YD-15 cells treated with Tol and Amp were harvested by trypsinization and fixed in 100% methanol at RT for 20 min. The cells were deposited on slides, stained with a DAPI solution and viewed under a fluorescence microscope.

Reverse transcription-polymerase chain reaction

Total RNA was isolated from the cells using an Easy-blue Total RNA Extraction kit (iNtRON Biotechnology, Sung-Nam city, Korea). cDNA was synthesized from the total RNA using ImProm-II Reverse Transcription System (Promega) according to the manufacturer's instructions. cDNA was obtained by PCR amplification using glyceraldehyde 3-phosphate dehydrogenase (GAPDH)- and MCL-1-specific primers as described below under the following PCR conditions (25 or 30 cycles: 1 min at 95°C, 1 min at 60°C, and 1 min 30 at 72°C). GAPDH primers used were, forward: CGG AGT CAA CGG ATT TGG TCG TAT and reverse: AGC CTT CTC CAT GGT GGT GAA GAC. MCL-1 primers used were, forward: TGC TGG AGT TGG TCG GGG AA and reverse: TCG TAA GGT CTC CAG CGC CT. PCR products were analysed by 2% agarose gel electrophoresis.

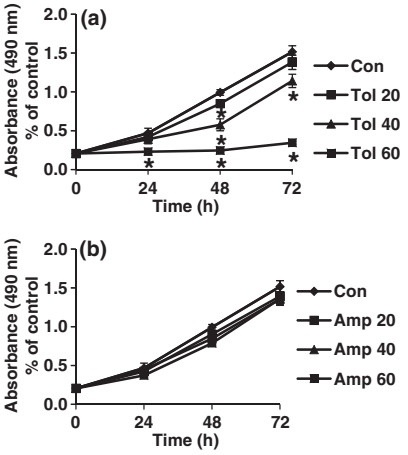

Figure 1 The effect of Tol and Amp on cell proliferation in YD-15 salivary MEC cells. YD-15 cells were treated with DMSO or 20, 40 or 60 μM of Tol **(a)** or Amp **(b)** for 24, 48 and 72 h. Cell proliferation was determined using a MTS assay. The points are the mean ± s.d. of three independent experiments. $^*P < 0.05$ as compared with the control group

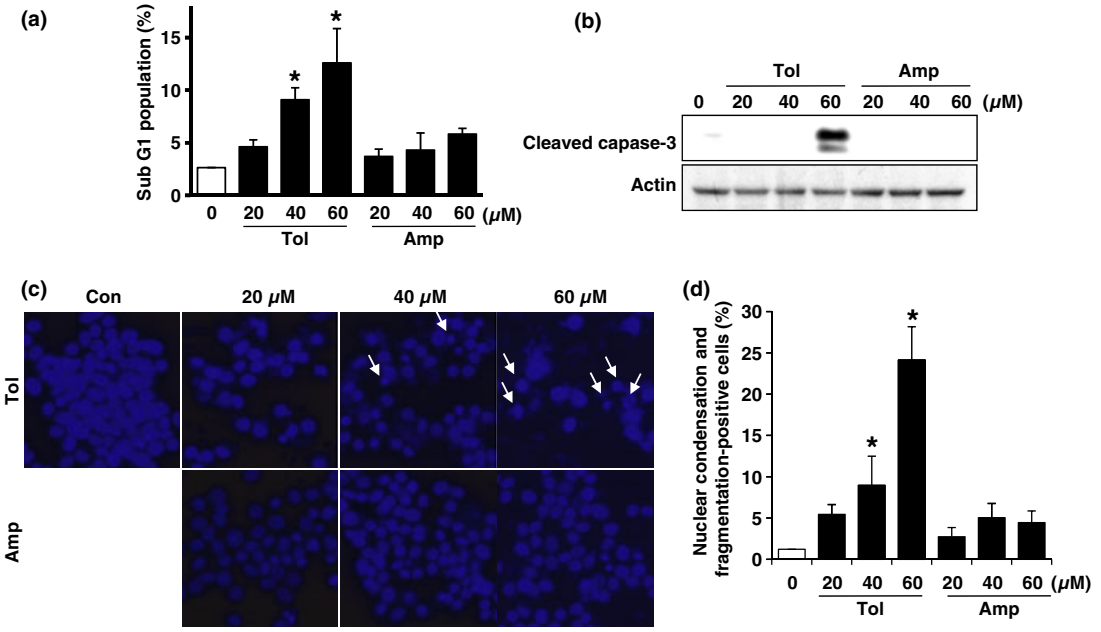

Figure 2 The effect of Tol and Amp on the apoptosis of YD-15 salivary MEC cells. YD-15 cells were treated with DMSO or 20, 40 or 60 μM of Tol or Amp for 72 h. **(a)** The sub-G_1 fraction was assessed by PI staining and flow cytometry analysis. The accumulation of sub-G_1 population (fold induction of control) was quantified and the results of triplicate experiments are expressed as the mean $\pm$ s.d. $^*P < 0.05$ as compared with the DMSO treatment group; **(b)** Immunoblot detection of the cleaved caspase 3 in whole cell lysates. Actin was used to normalize the protein loading from each treatment; **(c)** Fluorescence microscopy images of the DAPI-stained YD-15 cells showing the concentration-dependent appearance of an apoptotic morphology in the Tol-treated YD-15 cells; **(d)** The number of cells with nuclear fragmentation and condensation was quantified and the results of triplicate experiments are expressed as the mean $\pm$ s.d. $^*P < 0.05$ as compared with the DMSO treatment group

Immunocytochemistry

After specific treatments, the slide cultures were washed twice with cold phosphate-buffered saline, fixed with a Fixation Solution and permeabilized with Permeabilization Solution (BD Biosciences) for 1 h. After three more washes with phosphate buffered saline with tween 20, the cells were blocked with 1% bovine serum albumin and incubated with the indicated antibodies overnight at 4°C. Subsequently, the cells were incubated with the fluorescein isothiocyanate (FITC)-conjugated secondary antibodies for 2 h at room temperature, and observed by a fluorescence microscopy (scope A1, AX10; Carl Zeiss, Blauvelt, NY, USA).

Transfection of siRNA

The small interfering RNA (siRNA) mediating the down-regulation of human MCL-1 was transfected by SMART pool-specific MCL-1 (siMCL-1) or nonspecific

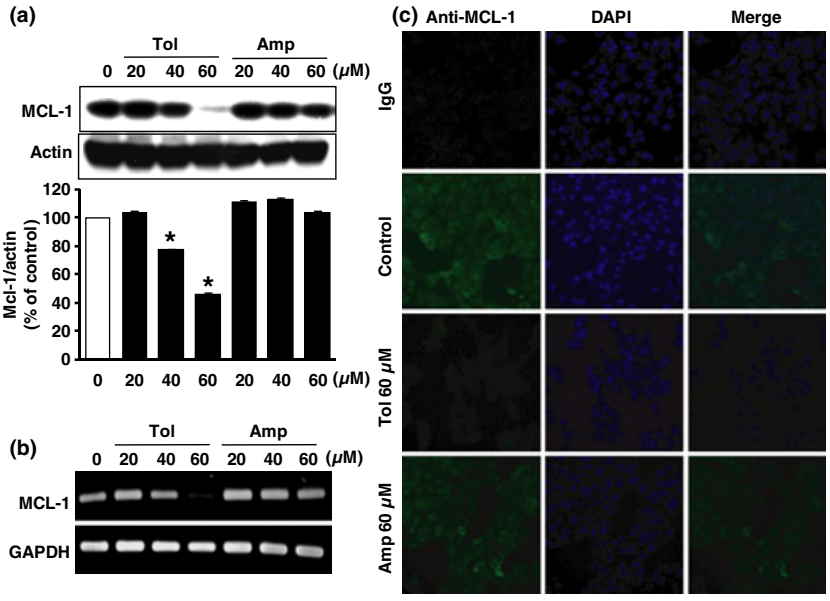

Figure 3 Effects of Tol and Amp on MCL-1 protein and mRNA expression in YD-15 salivary MEC cells. **(a)** The effects of 20, 40 or 60 μM of Tol or Amp for 72 h on the expressions of MCL-1 protein were determined by Western blot analysis; **(b)** The effects of DMSO, Tol or Amp for 72 h on MCL-1 mRNA expression were determined by RT-PCR as described in Materials and methods; **(c)** Immunostaining for MCL-1. YD-15 cells were treated with DMSO or 60 μM of Tol or Amp for 48 h and immunostained with IgG or MCL-1 antibodies

control (siCon) pool double-stranded RNA oligonucleotides using DharmaFECT2 transfection reagent (Dharmacon Inc., Lafayette, CO, USA) according to the manufacturer's protocol. Briefly, the cells were seeded in six-well plates and transfected transiently with 50 nM of siMCL-1 or siCon. After transfection, the cell viability was determined by MTS assays and the MCL-1 and caspase-3 protein levels were analysed by Western blotting.

Statistical analysis

The values are expressed as the mean ± s.d. The significance of the difference from the respective controls for each experimental test condition was assayed by using a *t*-test of the data obtained from triplicate experiments. A *P*-value of <0.05 was regarded as indicating a significant difference.

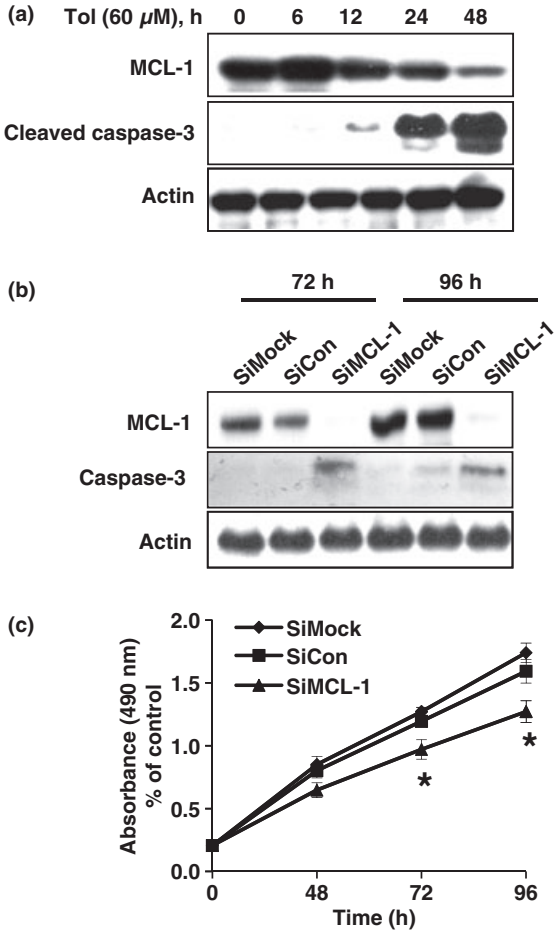

Figure 4 The function of the MCL-1 protein for the proliferation of YD-15 cells. **(a)** Time-dependent effect of Tol on MCL-1 and cleaved caspase 3 was performed in YD-15 cells treated with 60 μM of Tol for 0, 6, 12, 24 and 48 h; **(b)** RNA interference with siMCL-1. YD-15 cells were transfected with siCon (non-specific) or siMCL-1 for 72 or 96 h and the whole cell lysates were analysed by Western blot analysis for MCL-1, cleaved caspase 3 and actin. The values measured by Western blot analysis are representatives of two independent experiments; **(c)** Cell viability in siCon- or siMCL1-transfected YD-15 cells was detected using a MTS assay. The points are the mean ± s.d. of three independent experiments. $*P < 0.05$ as compared with the control group

Results

Tol, but not Amp inhibits the growth of YD-15 salivary MEC cells

At first, we examined the anti-proliferative activities of Tol and Amp in YD-15 salivary MEC cells. The cells were treated with Tol or Amp (0–60 μM) for 24, 48 and 72 h and then cell viability was determined using a MTS assay. Tol significantly decreased the cell viability in a concentration- and time-dependent manner, but Amp did not (Figure 1).

Tolfenamic acid, but not Ampiroxicam inhibits apoptosis in YD-15 salivary mucoepidermoid carcinoma cells

Next, the effects of Tol or Amp on the apoptosis of YD-15 cells were investigated. Sub-G_1 population analysis, western blot analysis using antibody against caspase 3 and DAPI staining were performed to determine if Tol or Amp increases apoptosis. Only Tol increased the Sub-G_1 population in a concentration-dependent manner, activated caspase 3 and caused nuclear condensation and fragmentation (Figure 2). These results suggest that the apoptotic cell death event contributes to the growth-inhibitory effect of Tol in YD-15 cells.

MCL-1 is attenuated by Tol to inhibit cell growth and induce apoptosis, but not Amp

In order to find out the mechanism underlying Tol-induced apoptosis, it was examined whether Tol affects the expression of MCL-1 protein and mRNA in YD-15 cell lines. The results showed that only Tol significantly decreased MCL-1 protein and mRNA in YD-15 cells (Figure 3a,b). Confirmation that Tol decreased MCL-1 protein expression in YD-15 cells was determined by immunocytochemical analysis (Figure 3c). MCL-1 immunostaining was observed in YD-15 cells treated with vehicle control (DMSO) or Amp, whereas Tol decreased MCL-1 staining. A time-course study revealed a decrease in the MCL-1 protein 12–48 h after the treatment of Tol and caspase 3 was activated simultaneously (Figure 4a). It was hypothesized that the attenuation of MCL-1 might induce apoptosis and inhibit cell growth. Therefore, the effect of siMCL-1 on the apoptosis of YD-15 cells was investigated. Figure 4b shows the activation of caspase 3 in YD-15 cells transfected with siMCL-1. siMCL-1 also decreased the viability of YD-15 cells significantly (Figure 4c).

MCL-1 is clearly regulated by Sp1, but not mitogen-activated protein kinases

To assess the role of Sp1 on Tol-induced apoptosis, the effect of Tol on the Sp1 protein in YD-15 cells was first analysed. The Tol treatment for 72 h decreased Sp1 protein concentration-dependently (Figure 5a). Furthermore, the effect of mithramycin A (a specific Sp1 DNA binding inhibitor) on MCL-1 protein and cell viability was determined by western blot analysis and an MTS assay. Figure 5b,c showed that the down-regulation of Sp1 by mithramycin A decreased the cell viability and

affected the MCL-1 protein. The effect of Tol on the phosphorylation status of mitogen-activated protein kinases (MAPKs) in YD-15 cells was also evaluated. Tol increased the phosphorylated forms of p38 MAPK and ERK1/2 without modifying JNK (Figure 5d). There was no change in the total form of MAPKs, as measured by Western blotting. Furthermore, the cells were pretreated with SB203580, a selective p38 MAPK inhibitor for 1 h, and treated with 60 μM of Tol for 72 h. The cells were harvested and cell viability was analysed. As shown in Figure 5e, SB203580 did not protect YD-15 cells from the decrease in cell viability induced by Tol.

Discussion

There is increasing epidemiological and experimental evidence that traditional non-prescription analgesics NSAIDs have a protective effect in various cancers (Agrawal and Fentiman, 2008) suggesting that NSAIDs are promising anti-cancer drug candidates. In studies published recently, several research groups reported that NSAIDs have anti-cancer effects of oral cancer cell lines (Hao *et al*, 2009; Kim *et al*, 2010; Park *et al*, 2010). An NSAID, Tol, has been demonstrated to exhibit anti-cancer activity in relation to pancreatic and colorectal cancer cells (Ding *et al*, 2008; Konduri *et al*, 2009). For examining whether Tol is capable of regulating the proliferation of oral cancer cells, we used a recently established and characterized **YD-15 MEC** cell-line, which is a very important set of tumor cells in the field of oral cancer biology (Lee *et al*, 2005). This study, first, demonstrated that Tol shows the inhibitory effect on the cell growth of YD-15 cell lines through the induction of apoptosis, whereas Amp was not effective in this case. MCL-1 is an anti-apoptotic member of the Bcl-2 family that was originally cloned as an early induction gene during differentiation of the myeloid cell line, ML-1 (Kozopas *et al*, 1993). Although MCL-1 is an essential anti-apoptotic factor in the development and differentiation of normal cells, the deregulation of MCL-1 expression often results in its over-expression, which contributes to several human diseases. Recently, there have been many reports suggesting the relationship between MCL-1 and carcinogenesis. In particular, several reports suggested that the down-regulation of MCL-1 induces apoptosis in oral cancer cells (Nagata *et al*, 2009; Park *et al*, 2010). In addition, many synthetic chemicals have been examined for their anti-cancer activities in a wide range of cancer cell lines and the down-regulation of MCL-1 expression has been reported to be one of the critical mechanisms for their anti-cancer activities (Tang *et al*, 2006; Ding *et al*, 2008; Kuroda *et al*, 2008). Therefore, this study examined the ability of Tol to down-regulate MCL-1 in YD-15 cells at the protein and mRNA levels in YD-15 cells. The results showed that Tol decreases MCL-1 significantly in a concentration-dependent manner, but Amp did not

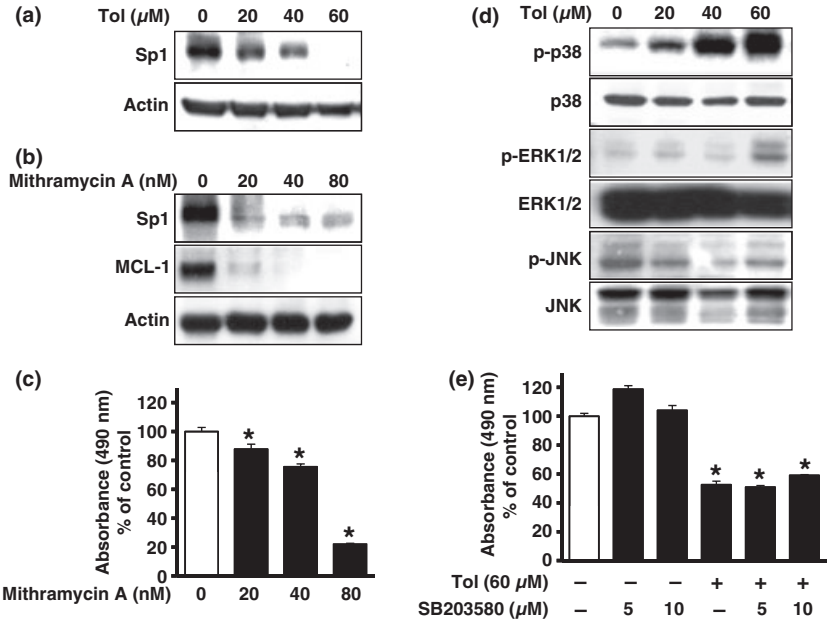

Figure 5 The effect of Tol on the upstream target proteins of MCL-1. (a) Effects of DMSO or Tol for 72 h on Sp1 protein expression were determined by Western blot analysis. The values measured by Western blot are representatives of two independent experiments; (b) Inhibition of the Sp1 protein by mithramycin A. YD-15 cells were treated with 20, 40 or 80 nM of mithramycin for 72 h and the whole cell lysates were analysed by western blot analysis for Sp1, and MCL-1. The values measured by Western blot are representatives of two independent experiments; (c) Cell viability in DMSO- or mithramycin A (20, 40 or 80 nM)-treated KB cells was detected using a MTS assay and the bars are the mean ± s.d. of three independent experiments. $*P < 0.05$ as compared with DMSO treatment group. (d) Effects of DMSO or Tol for 72 h on the expression of MAPKs and phosphor-MAPKs were determined by Western blot analysis. The values measured by Western blot are representatives of two independent experiments; (e) The effect of p38 MAPK inhibition on Tol-induced growth inhibition in YD-15 cells. The inhibition of p38 MAPK (5 or 10 μM) does not return from Tol-induced growth inhibition

cause any change. It suggests that only Tol down-regulating MCL-1 could induce apoptotic cell death. The time-course study also demonstrated the parallel induction of MCL-1 and cleaved caspase 3 indicating that the down-regulation of MCL-1 by Tol is associated with apoptosis. Next, the role of MCL-1 on the potential target for tumor therapy of MEC cells using siRNA MCL-1 was examined. In the present study, MCL-1 siRNA inhibited the cell growth of cultured YD-15 salivary MEC cells established from human tongue carcinomas. Furthermore, the inhibition of cell growth by MCL-1 siRNA was attributable to the activation of caspase 3. This strongly suggests that the down-regulation of MCL-1 induces apoptosis in salivary MEC cells.

The MCL-1 is strongly regulated by MAPKs, because it possesses many phosphorylation sites. It was reported that an interruption of the ERKs pathway by pharmacologic MEK inhibitors reduces the MCL-1 basal levels and the JNK pathway also transcriptionally up-regulates the expression of MCL-1 (Selzer *et al*, 2002; Park *et al*, 2010). These reports suggest that MCL-1 may be mediated in part by MAPKs. Therefore, we have shown the levels of p-ERK1/2, p-p38 and p-JNK in YD-15 cells and only p-p38 and p-ERK1/2 were increased significantly by the treatment of Tol to YD-15 cells. Because ERK1/2 pathway usually regulates MCL-1 positively based on data reported in the literatures, Tol appears to activate ERK1/2 pathway in order to response to its apoptotic action irrespective of the expression level of MCL-1 in this study. Inhibition of p38 MAPK with a pretreatment with a chemical inhibitor (SB203580) did not reverse Tol-inhibited cell growth. These results indicate that the Tol-induced apoptosis of YD-15 cells is not mediated by MAPKs.

Sp1 was reported to be associated with tumor growth and strongly expressed in tumor tissues, and that Tol attenuates the expression of the Sp1 protein to inhibit tumor cell growth in pancreatic and esophageal cancer cells (Abdelrahim *et al*, 2007; Papineni *et al*, 2009). In studies published recently, a chromatin immunoprecipitation assay revealed Sp1/Sp3 binding sites on the MCL-1 promoter indicating that Sp1 can regulate MCL-1 directly (Saxena *et al*, 2007). This study showed that Tol decreased the expression of the Sp1 protein. Sp1 DNA binding inhibitor (mithramycin A) was used to show direct evidence of the Sp1 protein in modulating the MCL-1 protein. These results confirmed that the attenuation of Sp1 protein expression down-regulates MCL-1 protein and inhibits the growth of YD-15 cells.

In summary, this study showed that Tol inhibits the proliferation of MEC cells, YD-15 cells and modulates the expression of MCL-1. This expression is totally dependent on the Sp1 protein and not MAPKs. To the best of our knowledge, this is the first report that the apoptotic mechanism of Tol in salivary MEC cells might involve the regulation of the MCL-1 protein. Therefore, the development of Tol and its related compounds targeting the MCL-1 protein may be a novel class of drugs for the treatment of salivary MEC.

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Author contributions

Kyeong-Hee Choi, Jung-Hyun Shim and Le Diem Huong performed the experiments and laboratory analyses. Nam-Pyo Cho performed the data analyses. Sung-Dae Cho designed the research plan and prepared the manuscript. All authors participated in the editing and final preparation of this manuscript.

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