치의과학박사학위논문

Apoptotic and chemosensitizing effects of TW-37 in human oral cancer cell lines by targeting STAT3-Mcl-1 signaling

인간 구강암 세포주에서 STAT3–Mcl-1 신호기전을 표적하는 TW-37의 세포자멸 및 상승 작용에 관한 연구

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ABSTRACT

Apoptotic and chemosensitizing effects of TW-37 in human oral cancer cell lines by targeting STAT3-Mcl-1 signaling

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(Directed by Professor **Sung-Dae Cho**, D.V.M., Ph.D.)

Background: Despite being one of the leading cancer types in the world, the diagnosis of oral cancer and its suitable therapeutic options remain limited. This study aims to investigate the single and chemosensitizing effects of TW-37, a BH3 mimetic in oral cancer, on human oral cancer cell lines.

Methods: The single and chemosensitizing effects of TW-37 was assessed *in vitro* using trypan blue exclusion assay, Western blotting, DAPI staining, Annexin V–FITC/PI double staining, and quantitative real-time PCR. Myeloid cell leukemia-1 (Mcl-1) overexpression models were established by transforming vector and transient transfection was performed to test for apoptosis.

Results: TW-37 enhanced the cytotoxicity of human oral cancer cell lines by inducing caspase-dependent apoptosis, which correlates with the reduction of the Mcl-1 expression via transcriptional and post-translational regulation. The ectopic expression of Mcl-1 partially attenuated the apoptosis-inducing capacity of TW-37 in human oral cancer cell lines. Besides, TW-37 decreased the phosphorylation of signal transducer and activator of transcription 3 (STAT3) at Tyr705 and nuclear translocation in human oral cancer cell lines at the early time points. Furthermore, TW-37 potentiated chemo-susceptibility of cryptotanshinone in human oral cancer cell lines by suppressing STAT3–Mcl-1 signaling compared with either TW-37 or cryptotanshinone alone, resulting in potent apoptosis.

Conclusions: This study not only unravels the single and chemosensitizing effects of TW-37 for treatment of human oral cancer but also highlights the likelihood of TW-37 as a good therapeutic strategy to enhance the prognosis of patients with oral cancer in the future.

Keywords: TW-37, Cryptotanshinone, Chemosensitivity, STAT3, Mcl-1, Oral cancer, Apoptosis

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TABLE OF CONTENTS

1. Review of literature

B-cell lymphoma-2 family and BH mimetics

B cell lymphoma-2 (BCL-2) family proteins are important cell-survival regulators classified by three functions: antiapoptotic, proapoptotic, and Bcl-2 homology 3 (BH3)-only protein [34] : 1) Antiapoptotic proteins: MCL-1, BCL-2, BCL-W, BCL-XL, BCL-B, and BFL-1/A1; 2) Proapoptotic proteins: BAK and BAX; 3) BH3-only proteins: BIM, BAD, BID, NOXA, and PUMA. The fate of cells is dependent on the interactions among these three groups of proteins. The BCL-2-regulated apoptotic pathway, namely 'intrinsic', 'stress' or 'mitochondrial' pathway is progressively conserved and found in animals with homologues of critical genes as related as humans and worms. [35-38] In healthy cells, the pro-survival BCL-2 proteins bind and prohibit BAX and BAK once activated partially, diminishing the capability of BAX/BAK to oligomerize and form pores to induce mitochondrial outer membrane permeabilization.[35] BH3-only proteins that target all pro-survival BCL-2 family members, can trigger apoptosis.[39] BH3 mimetics, synthetic small-molecule inhibitors, namely, offer an effective

strategy to revive cell death signaling in cancer cells [34]. Their mechanism of action is simple basically. BH3 mimetics imitate short sequences stemmed from the BH3 domain of the canonical binding partners of pro-survival BCL2 proteins which is the proapoptotic BH3-only proteins, hence preventing BH3-only/pro-survival BCL2 protein family interaction [34]. TW-37, one of BH3 mimetics, is a second-generation benzenesulphonyl offshoot of gossypol insulating from cotton seeds and roots. [40,41]. It is a reasonably appointed small molecule targeting the BH3-binding groove in BCL-2 where proapoptotic BCL-2 proteins bind and has a higher similarity and selectivity for MCL-1 and BCL-2 over BCL-XL [42]. TW-37 exploits strong antiproliferative effect in de novo chemoresistant lymphoma cells without any significant influence on normal peripheral blood lymphocytes. Recently, it was reported that the *in vitro* anti-tumor effects of TW-37 in human oral cancer cell lines [43] and the combination of TW-37 with AT-101 prolongs the time considerably to the failure of human oral squamous cell carcinoma OSCC-3 tumors in severe combined immunodeficiency disease mice as compared with monotherapy [44]. These suggest that TW-37 can be prospective drug candidates against human cancer including oral cancer.

Signal transducer and activator of transcription 3

Signal transducer and activator of transcription 3 (STAT3) is one of STAT family, which consists of seven structurally similar and highly conserved members and a cytoplasmic transcription factor that regulates cell proliferation, differentiation, apoptosis, angiogenesis, inflammation and immune responses [45]. Since STAT3 was discovered in 1994, STAT3 participates in cellular proliferation and survival [46-48]. In accordance with the role in cellular proliferation, accumulating data show that STAT3 signaling pathway suppresses apoptosis in cancer cells. Activated STAT3 can upregulate anti-apoptotic protein such as Bcl-2, Bcl-XL, and Mcl-1 to prevent apoptosis of tumor cells in multiple myeloma [49,50]. In addition, Aberrant STAT3 activation targets the progression of tumor via oncogenic gene expression in lots of human cancers [45] and persistent activation of STAT3 can cause the up-regulated expression of cycln D1, c-Myc and survivin to accelerate cell cycle progression in renal and colon cancers [46-48]. Inhibition of STAT3 signaling resulted in decreased cell proliferation and promote apoptosis in various cancers such [51-53] suggesting that STAT3 is a key regulator of cancer cell proliferation and survival.

Myeloid cell leukemia-1

Myeloid cell leukemia-1 (Mcl-1) is generally located in in the mitochondria, in which it inserts into the mitochondrial membrane through a hydrophobic tail [54-57]. The prototypical Mcl-1 protein, namely, Mcl-1L consists of 350 amino-acid residues and includes regions of similarity to other Bcl-2 family proteins, termed BH domains [58]. Mcl-1 is an antiapoptotic member of Bcl-2 family proteins [59], so it is essential to cell survival and homeostasis. It was reported the amplification and overexpression of Mcl-1 have been founded in various human hematological malignancies and solid tumors [60-64,42]. Mcl-1 also blocks apoptosis through binding to BH3 only protein and dissociating them from Bax and Bak which can form mitochondrial permeability transition pores in the mitochondrial membrane to induce cytochrome c release into the cytoplasm, electron transport change [30,31]. The overexpression of Mcl-1 protein in Oral squamous cell carcinoma (OSCC) cell lines was also sufficient to block the induction of apoptosis [32,33]. These suggest that Mcl-1 may be associated with cancer development and progression.

2. Introduction

Globally, head and neck squamous cell carcinoma (HNSCC) is one of the leading cancer types, of which oral squamous cell carcinoma constitutes > 90% of cases [1]. Despite witnessing a decline in the incidence of some cancer types, the incidence and mortality of oral cancer have steadily increased in both males and females [2]. The standard therapeutic options for managing patients with oral cancer include surgery, radiotherapy, and chemotherapy, which depend on the type, location, and stage of cancer at diagnosis. Despite significant advancements in the approaches of oral cancer treatment over the past decades, effective diagnosis and suitable therapeutic options of oral cancer remain limited, thereby triggering low prognosis of patients [3]. Hence, finding suitable therapeutic options for the treatment of patients with oral cancer is of utmost significance to enhance the therapeutic outcome.

In cancer therapy, targeting anti-apoptotic Bcl-2 family proteins is an attractive approach because of their oncogenic potential. The “BH3 mimetics” were designed to mimic the BH3 domain of pro-apoptotic

Bcl-2 proteins and to antagonize anti-apoptotic Bcl-2 family proteins, resulting in apoptosis [4]. Of these, TW-37 is an analog of gossypol, a plant-derived polyphenolic aldehyde, which can bind to Bcl-2, Bcl-xL, and myeloid cell leukemia-1 (Mcl-1) in the nanomolar range [5, 6]. A growing number of studies have demonstrated the therapeutic potential of TW-37 against various types of cancer by inducing S-phase cell cycle arrest or apoptosis *in vitro* and *in vivo* [7,8,9]. In HNSCC, TW-37 has also been reported to suppress tumor angiogenesis and sensitize the antitumor effect of cisplatin [10]. Recently, TW-37 functions as a potential apoptosis-inducing agent for the treatment of oral cancer by reducing heme oxygenase-1 and Bcl-2 [11, 12]. Furthermore, in combination with conventional chemotherapeutic drugs like cisplatin and 5-fluorouracil, TW-37 was sufficient in potentiating the chemosensitivity of tumors by inducing apoptosis [13, 14]. Hence, therapeutic approaches by TW-37 alone or combined with chemotherapeutic drugs are crucial to augment the survival of patients with oral cancer.

Based on the related literature [15], this study hypothesizes that the transcriptional regulation of Mcl-1 in human oral cancer cell lines upon

TW-37 treatment could correlate with the signal transducer and activator of transcription (STAT) family members, including STAT3 (H1). In addition, the combination of TW-37 and cryptotanshinone at low concentration ranges could potently suppress the Mcl-1 expression by inhibiting the STAT3 activation (H2). Hence, this study aims to explore whether TW-37 could be a valuable therapeutic option as monotherapy in human oral cancer, as well as combination therapy with cryptotanshinone, by targeting STAT3–Mcl-1 signaling.

3. Materials and Methods

3.1 Cell culture and chemical preparation

HSC-3, Ca9.22, and HSC-4 cell lines are obtained from Hokkaido University (Hokkaido, Japan), and the HN22 cell line was provided by Dankook University (Cheonan, Republic of Korea). All cell lines were maintained in DMEM/F12 medium (WELGENE, Gyeongsan, Republic of Korea) supplemented with 10% fetal bovine serum and 1% antibiotics (penicillin/streptomycin) at 37 °C in a 5% CO2 incubator. In addition, TW-37 was purchased from ApexBio (Houston, TX, USA), whereas cryptotanshinone and cycloheximide (CHX) were purchased from Sigma-Aldrich (St Louis, MO, USA). ABT-737 and Stattic were supplied from Selleckchem (Houston, TX, USA), Z-VAD-FMK was purchased from R&D Systems (Minneapolis, MN, USA), and MG132 was provided from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All chemicals were dissolved in dimethyl sulfoxide and stored at −20 °C.

3.2 Trypan blue exclusion assay

All cell lines were seeded in six-well plates and treated those with TW-37. Figure 1a shows the structure of TW-37. Then, 48 h after the TW-37

treatment, all cell lines were stained with 0.4% trypan blue solution (Gibco, Paisley, UK), and viable cell lines were counted using a hemocytometer.

3.3 Western blotting

All cell lines were extracted with 1X RIPA lysis buffer (EMD Millipore, Billerica, CA, USA) using phosphatase inhibitor tablets (Thermo Scientific Inc., Rockford, IL, USA) and protease inhibitor cocktails (Roche, Mannheim, Germany). The protein was quantified using a DC protein assay kit (Bio-Rad Laboratories, Madison, WI, USA). After normalization, protein lysates containing nearly 20–50 μg of protein were boiled with a protein sample buffer at 95 °C for 5 min and separated on SDS–PAGE. Next, the proteins were transferred to Immun-Blot PVDF membranes after the electrophoresis and blocked with 5% skimmed milk for 1 h at room temperature (RT). The membranes were incubated with the indicated primary antibodies overnight at 4 °C and were maintained with corresponding horseradish peroxidase (HRP)–conjugated secondary antibodies for 2 h at RT. The antibodies for all the target proteins have been listed in Table 1. The bands were then immune-reactivated with an ECL solution (Santa Cruz Biotechnology) and

visualized using ImageQuant LAS 500 (GE Healthcare Life Sciences, Piscataway, NJ, USA) or an X-ray film.

3.4 DAPI staining

After the TW-37 treatment, all cell lines were fixed overnight with 100% ethanol at −20 °C and 100% methanol at RT for 10 min. The fixed cell lines were then deposited on slides and stained with DAPI solution (5 μg/mL). Furthermore, morphological changes of nuclei in apoptotic cell lines were observed using a fluorescence microscope (Leica DMi8; Leica Microsystems GmbH, Wetzlar, Germany).

3.5 Annexin V–FITC/PI double staining

The induction of apoptosis was measured using the FITC Annexin V Apoptosis Detection Kit (BD Pharmingen, San Jose, CA, USA) according to the manufacturer's protocol. Briefly, all floating and adherent cell lines were collected, washed two times with PBS, and pelleted by centrifugation. After that, cell lines were resuspended in annexin V binding buffer containing 3-μL annexin V–FITC and 1-μL PI and incubated for 15 min at RT in the dark. Subsequently, cell lines were

transferred to a FACS tube and analyzed by flow cytometry using a FACSCalibur and counted 10,000 events per sample.

3.6 Quantitative real-time PCR

Using TRIzol Reagent (Life Technologies, Carlsbad, CA, USA), the total RNA was extracted. Afterward, 1 μg of RNA was reverse transcribed using an AMPIGENE cDNA Synthesis Kit (Enzo Life Sciences, Inc., NY, USA), and the resultant cDNA was subjected to PCR using AMPIGENE qPCR Green Mix Hi-Rox (Enzo Life Sciences). For quantitative real-time PCR, StepOnePlus Real-Time PCR System (Applied Biosystems, CA, USA) was used. In this study, the PCR conditions for all genes were as follows: 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. The relative amount of Mcl-1 gene was normalized to the amount of GAPDH and was evaluated using the $2^{-\Delta\Delta Ct}$ method. The qPCR primers were as follows: Mcl-1 sense, 5´-GTA TCA CAG ACG TTC TCG TAA GG-3´; Mcl-1 antisense, 5´-CCA CCT TCT AGG TCC TCT ACA T-3´; GAPDH sense, 5´-GTG GTC TCC TCT GAC TTC AAC-3´; and GAPDH antisense, 5´-CCT GTT GCT GTA GCC AAA TTC-3´.

3.7 Plasmid construction and transient transfection

The open reading frame of human Mcl-1 (NM_021960) gene from cDNA that was synthesized in HSC-3 cell lines was amplified using gene-specific primers (primer sequence: Mcl-1 sense, 5´-GAA TTC ATG TTT GGC CTC AAA AGA-3´, with an included EcoRI site; Mcl-1 antisense, 5´-GAA TTC CTA TCT TAT TAG ATA TGC-3´, with an included EcoRI site), followed by cloning into a pGEM® T-easy vector (Promega, Madison, WI, USA). The Mcl-1 was then established by sequence analysis. Finally, the Mcl-1 gene was cloned into the multi-cloning site of the pcDNA3.1 (+) vector (Invitrogen, San Diego, CA, USA). For the overexpression vector transfection, cell lines were transfected with vector constructs (pcDNA3.1 or pcDNA3.1-Mcl-1) using Lipofectamine 3000 transfection reagent (Life Technologies) according to the manufacturer's protocol.

3.8 Statistical analysis

The control and the treated groups were compared through Student's t-test (two tailed) using SPSS 22 (SPSS, Chicago, IL, USA). In this study, $P < 0.05$ was considered statistically significant.

3. Results

3.1 TW-37 represses cell viability and induces apoptosis in human oral cancer cell lines

A concentration–response test using the trypan blue exclusion assay was first performed to assess the potential cytotoxic effect of TW-37 on human oral cancer cell lines. TW-37 exhibited a concentration-dependent cytotoxic effect on HSC-3 cell lines (Fig. 1b). In addition, similar cytotoxicity was observed in the three human oral cancer cell lines, including Ca9.22, HSC-4, and HN22 (Fig. 6a). Furthermore, the levels of cleaved caspase-3 and cleaved PARP using Western blotting was analyzed to determine whether the cytotoxic effect of TW-37 was because of the induction of apoptosis. TW-37 induced apoptosis in four human oral cancer cell lines (Fig. 1c, Fig. 6b). Likewise, morphological changes in nuclei by fluorescence-based analysis exhibited TW-37-induced apoptosis in four human oral cancer cell lines, as evidenced by chromatin condensation or DNA fragmentation (Fig. 1d, Fig. 6c). Next, the apoptotic effect of TW-37 was examined using flow cytometric analysis. The rate of annexin V-positive cell lines was increased from 11.29% in the vehicle control group to 22.78 or 42.15% in the TW-37

treatment group (Fig. 1e). Moreover, TW-37-induced apoptosis relied on caspase activation in human oral cancer cell lines (Fig. 7). The findings provided above demonstrated that TW-37 elicits cytotoxicity in human oral cancer cell lines because of apoptosis induced by the caspase-dependent mechanism.

3.2 TW-37 augments apoptosis in human oral cancer cell lines by inhibiting the Mcl-1 expression

As TW-37 is a potent inhibitor of anti-apoptotic Bcl-2 family proteins, such as Bcl-2, Bcl-xL, and Mcl-1, I assessed whether TW-37-induced apoptosis in human oral cancer cell lines could regulate these proteins. The findings revealed that the Mcl-1 expression notably decreased after the TW-37 treatment, whereas the Bcl-2 and Bcl-xL expressions were inconsistent (Fig. 2a). I observed a decline in the Mcl-1 expression after 12 h TW-37 treatment, and PARP cleavage was coordinately enhanced at similar time points (Fig. 2b). In addition, real-time PCR was performed in a time-dependent manner to determine whether TW-37 could affect the levels of Mcl-1 mRNA. TW-37 markedly decreased the Mcl-1 mRNA levels up to 6 h, alleviated after 12 h, and then weakly decreased after 24 or 48 h (Fig. 2c, Fig. 8a). Thus, I next investigated

whether TW-37 treatment for 24 or 48 h could regulate the protein stability of Mcl-1 in human oral cancer cell lines. After treatment with CHX to stop new protein synthesis, TW-37 strongly degraded Mcl-1 protein (Fig. 2d). Consistently, the degradation of Mcl-1 protein in cells treated with TW-37 for 24 and 48 h was restored by MG132, a proteasome inhibitor (Fig. 2e). These findings suggested that the suppression of the Mcl-1 expression in a transcriptional and post-translational manner could contribute to TW-37-induced apoptosis in human oral cancer cell lines.

3.3 Ectopic expression of Mcl-1 abolishes TW-37-induced apoptosis in human oral cancer cell lines

In this study, cell lines were transiently transfected with pcDNA3.1 (a control vector) and pcDNA3.1-Mcl-1 (to overexpress Mcl-1) to elucidate the biological role of Mcl-1 protein in TW-37-induced apoptosis. The ectopic expression of Mcl-1 modestly attenuated the expression of cleaved PARP after the TW-37 treatment (Fig. 3a). I further established the suppressive effect of Mcl-1 in TW-37-induced apoptosis using flow cytometric analysis. The rate of annexin V-positive cell lines after the TW-37 treatment (52.15%) was partially diminished by the ectopic

expression of Mcl-1 (43.02%; Fig. 3b. These findings indicated that the Mcl-1 suppression could be partly needed for TW-37-induced apoptosis in human oral cancer cell lines.

3.4 TW-37 decreases the STAT3 phosphorylation at Tyr705 and nuclear translocation in human oral cancer cell lines

STAT signaling pathway plays a vital role in oncogenesis through the modulation of genes involved in anti-apoptotic Bcl-2 proteins such as Mcl-1 [16]. In this study, I explored the phosphorylation of STAT3 and STAT5 at early time points after the TW-37 treatment to determine whether TW-37 could regulate STAT family proteins. TW-37 markedly suppressed the STAT3 phosphorylation at Tyr705 in a time-dependent manner but not the STAT3 phosphorylation at Ser727 and STAT5 phosphorylation at Tyr694 (Fig. 4a, b). Consistently, a reduction of the STAT3 phosphorylation at Tyr705 was observed in a concentration-dependent manner in other human oral cancer cell lines, including Ca9.22, HSC-4, and HN22 (Fig. 6d). As the STAT3 phosphorylation exhibits translocation into the nucleus, I examined the phosphorylation status of STAT3 in the nucleus after the TW-37 treatment. Notably, TW-37 inhibited the nuclear translocation of p-STAT3Tyr705 (Fig. 4c). These

results suggested that TW-37 triggers the suppression of p-STAT3Tyr705 and nuclear translocation in human oral cancer cell lines.

3.5 TW-37 enhances the chemosensitivity of cryptotanshinone by modulating the STAT3–Mcl-1 axis in human oral cancer cell lines

I optimized the concentrations of TW-37 and cryptotanshinone to exclude their direct cytotoxic efficiency on human oral cancer cell lines to investigate the synergistic effect of TW-37 with cryptotanshinone as a potent STAT3 inhibitor. The combined treatment of TW-37 and cryptotanshinone potently decreased the growth of HSC-3 cell lines compared with either TW-37 or cryptotanshinone alone (Fig. 5a). Moreover, the rate of annexin V-positive cell lines was enhanced after the combined treatment of TW-37 and cryptotanshinone (Fig. 5b). Furthermore, the levels of p-STAT3 Tyr705 and Mcl-1 were confirmed by Western blotting to elucidate whether the synergistic effect of TW-37 with cryptotanshinone correlated with STAT3–Mcl-1 signaling. The combined treatment of TW-37 and cryptotanshinone remarkably abolished the expression of p-STAT3 Tyr705 and Mcl-1 compared with either TW-37 or cryptotanshinone alone, resulting in a potent induction of apoptosis (Fig. 5c). These findings suggested that TW-37 can

synergistically induce the chemosensitivity of cryptotanshinone by inducing apoptosis through suppressing STAT3–Mcl-1 signaling in human oral cancer cell lines.

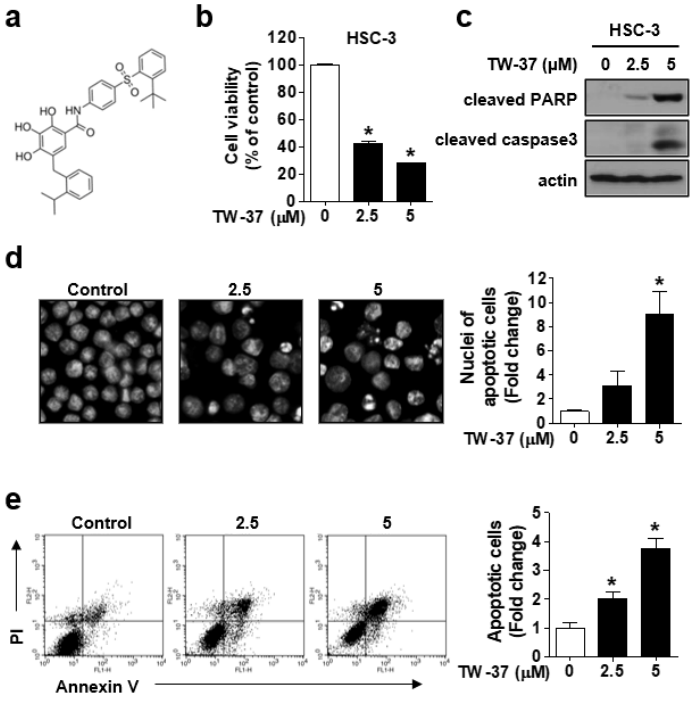

Fig. 1 TW-37 augments the cytotoxicity of human oral cancer cell lines by triggering apoptosis. **a** The chemical structure of TW-37. **b** HSC-3 cell lines were treated with 2.5- and 5-μM TW-37 for 48 h, following which the cell viability was measured using the trypan blue exclusion assay. **c** Cleavage of both PARP and caspase-3 was detected by Western blotting. Actin was used as a loading control. **d** HSC-3 cell lines were stained with DAPI and visualized by fluorescence microscopy (magnification, ×400). **e** FACS analysis of annexin V/PI staining. All bar graphs represent mean ± SD of the three independent experiments. *$P < 0.05$.

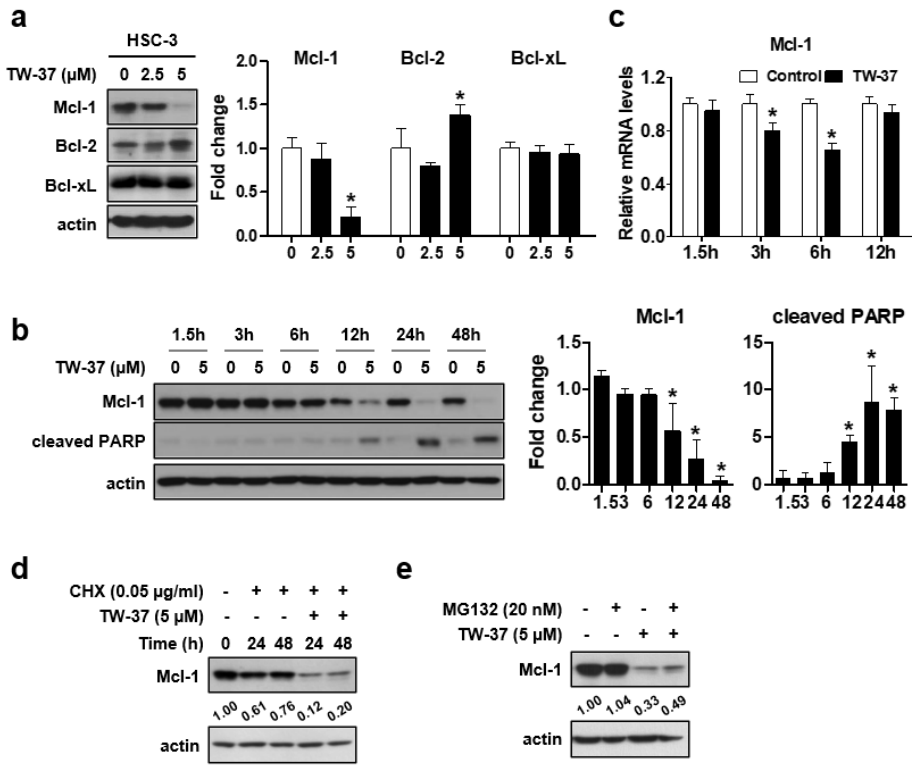

Fig. 2 TW-37 induces apoptosis in human oral cancer cell lines by suppressing the Mcl-1 expression. **a** The expressions of myeloid cell leukemia-1 (Mcl-1), Bcl-2, and Bcl-xL were detected using Western blotting. Actin was used as a loading control. **b** The expression levels of Mcl-1 and cleaved PARP were detected using Western blotting. Actin was used as a loading control. **c** Relative mRNA levels of Mcl-1 were measured by qPCR and normalized to GAPDH. All bar graphs represent mean ± SD of three independent experiments. *P < 0.05. **d** HSC-3 cell lines were pretreated with CHX for 1 h, followed by

treatment with 5-μM TW-37 for 48 h. The expression of Mcl-1 was analyzed using Western blotting. **e** After treatment with MG132 for 1 h, the HSC-3 cell lines were treated with 5-μM TW-37 for the indicated time points. The protein levels of Mcl-1 were detected with Western blotting. Actin was used as a loading control. Data represent the mean of the two independent experiments.

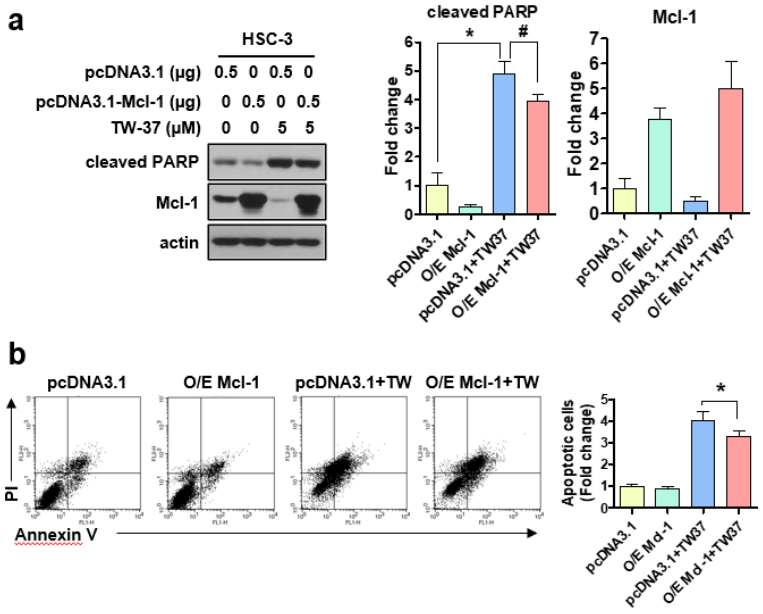

Fig. 3 Ectopic expression of Mcl-1 attenuates TW-37-induced apoptosis. HSC-3 cell lines were treated with DMSO or 5-μM TW-37 for 48 h after transiently transfected with 0.5-μg pcDNA3.1 or pcDNA3.1-Mcl-1 for 6 h. **a** Protein levels of myeloid cell leukemia-1 (Mcl-1) and cleaved PARP were determined using Western blotting. Actin was used as a loading control. **b** FACS analysis of annexin V/PI staining. All bar graphs represent mean ± SD of three independent experiments. $^{*}P < 0.05$; $^{\#}P < 0.05$.

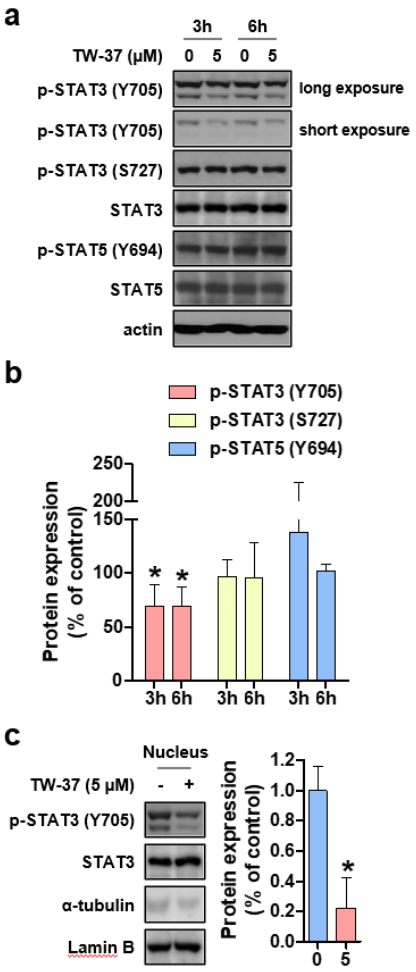

Fig. 4 TW-37 inhibits the STAT3 phosphorylation at Tyr^{705} and nuclear translocation. HSC-3 cell lines were treated with DMSO or 5-μM of TW-37 for 3 or 6 h. **a** Protein levels of p-STAT3 (Y705), p-STAT3 (S727), STAT3, p-STAT5 (Y694), and STAT5 were analyzed using Western blotting. Actin was used as a loading control. **b** The bar graphs represent mean ± SD of three

independent experiments. *$P < 0.05$. **c** The expression of nuclear p-STAT3 (Y705) and signal transducer and activator of transcription 3 (STAT3) was detected using Western blotting. In addition, α-tubulin and Lamin B were used as specific markers for cytosolic or nuclear fraction, respectively. The bar graphs represent mean ± SD of three independent experiments. *$P < 0.05$.

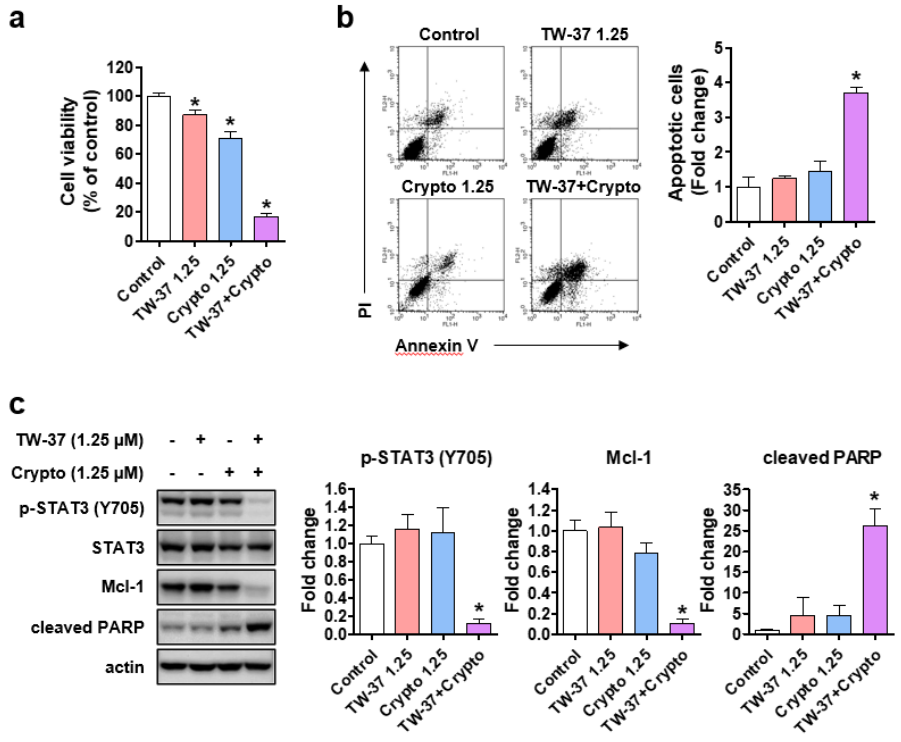

Fig. 5 TW-37 augments the chemosensitivity of cryptotanshinone in human oral cancer cell lines by modulating STAT3–Mcl-1 signaling. **a** The cell viability was measured using the trypan blue exclusion assay. **b** FACS analysis of annexin V/PI staining. All bar graphs represent mean ± SD of three independent experiments. **c** Protein levels of p-STAT3 (Y705), signal transducer and activator of transcription 3 (STAT3), and myeloid cell leukemia-1 (Mcl-1) were determined using Western blotting. Actin was used as a loading control. The bar graphs represent mean ± SD of three independent experiments. *$P < 0.05$.

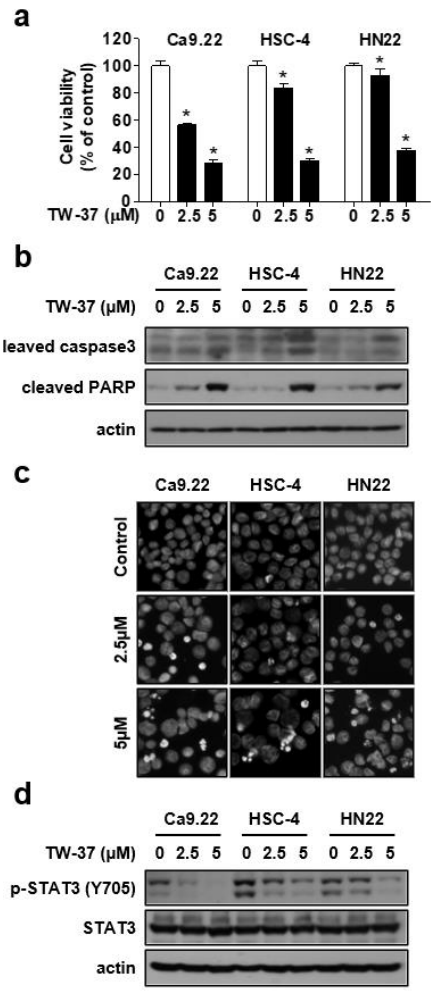

Fig. 6. TW-37 induces apoptosis in human oral cancer cell lines by suppressing p-STAT3^{Tyr705}. Ca9.22, HSC-4, and HN22 cell lines were treated with 2.5- and 5-μM TW-37 for 48 h. **a** The cell viability was measured using the trypan blue exclusion assay. Bar graphs represent the mean ± SD values of three independent experiments. $^{*}P < 0.05$. **b** Protein levels of cleaved caspase-3 and

cleaved PARP were analyzed using Western blotting. Actin was used as a loading control. **c** All cell lines stained with DAPI were visualized by fluorescence microscopy (magnification, ×400). **d** The expressions of p-STAT3 (Y705) and STAT3 were detected using Western blotting. Actin was used as a loading control.

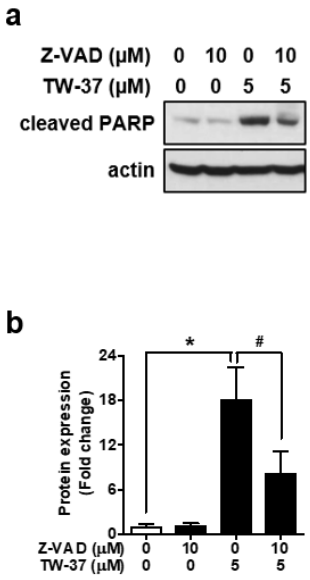

Fig. 7. TW-37-induced apoptosis depending on caspase activation in human oral cancer cell lines. HSC-3 cell lines were pretreated with 10-μM Z-VAD for 2 h with/without 5-μM TW-37 for 48 h. **a** Protein levels of cleaved PARP were analyzed using Western blotting analysis. Actin was used as a loading control. **b** Bar graphs represent the mean ± SD values of three independent experiments. $^{*}P < 0.05$; $^{\#}P < 0.05$.

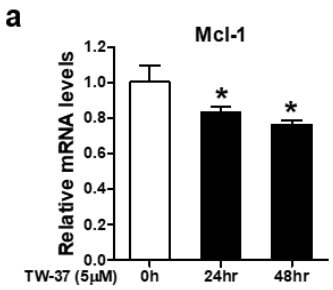

Fig. 8. a TW-37 slightly decreases Mcl-1 mRNA levels at 24 and 48 h. The HSC-3 cell lines were treated with 5-μM of TW-37 for 24 or 48 h. Relative mRNA levels of Mcl-1 were measured with qPCR and normalized to GAPDH. Bar graphs represent the mean ± SD values of triplicate experiments. $^{*}P < 0.05$.

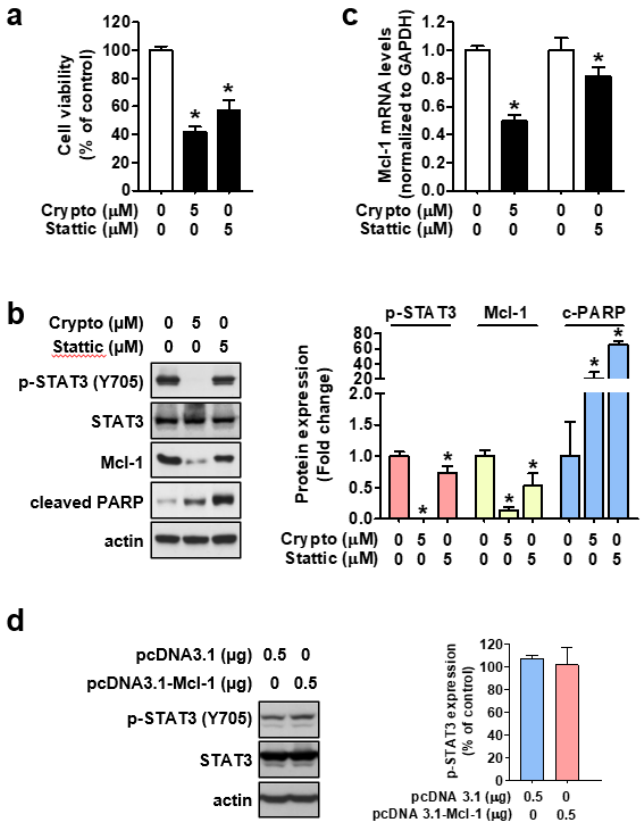

Fig. 9. STAT3 inactivation is sufficient to induce apoptosis via the inhibition of Mcl-1 expression. The HSC-3 cell lines were treated with 5-μM of cryptotanshinone or stattic for 24 h. **a** The cell viability was measured using the trypan blue exclusion assay. **b** Protein levels of p-STAT3 (Y705), STAT3, Mcl-1, and cleaved PARP were analyzed using Western blotting analysis. Actin was used as a loading control. **c** Relative mRNA levels of Mcl-1 were measured with qPCR and normalized to GAPDH. **d** The HSC-3 cell lines were transiently

transfected with 0.5-μg pcDNA3.1 or pcDNA3.1-Mcl-1 for 6 h. Protein levels of p-STAT3 (Y705) and STAT3 were determined using Western blotting analysis. Actin was used as a loading control. All bar graphs represent the mean ± SD values of three independent experiments. $^{*}P < 0.05$.

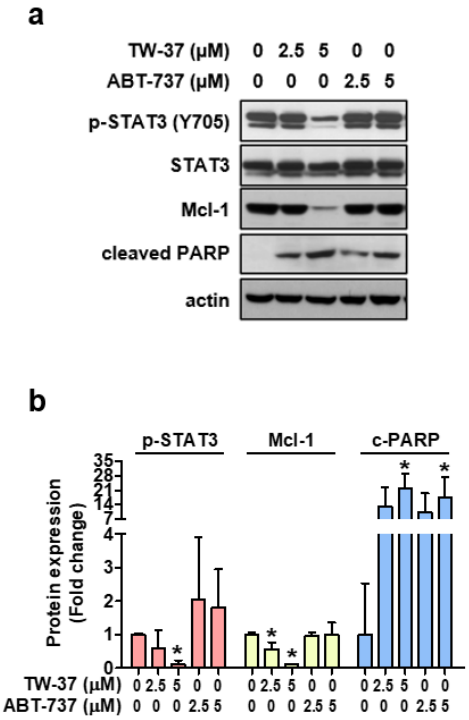

Fig. 10. TW-37-induced apoptosis is mediated by the suppression of STAT3-Mcl-1 signaling unlike ABT-737. The HSC-3 cell lines were treated with the indicated concentrations of TW-37 or ABT-737 for 48 h. **a** Protein levels of p-STAT3 (Y705), STAT3, Mcl-1, and cleaved PARP were analyzed using Western blotting analysis. Actin was used as a loading control. **b** Bar graphs represent the mean ± SD values of three independent experiments. $^{*}P < 0.05$.

5. Discussion

Although targeting anti-apoptotic Bcl-2 family proteins is a promising and well-established therapeutic strategy for the treatment of various tumors, developing Bcl-2 inhibitors and exploring their efficiency because of the oncogenic potential of anti-apoptotic Bcl-2 family proteins are still worthwhile. This study reports the single and chemosensitizing effects of TW-37, a potent inhibitor of anti-apoptotic Bcl-2 family proteins, for the treatment of oral cancer. The analysis of the effect of TW-37 on anti-apoptotic Bcl-2 family proteins in human oral cancer cell lines revealed that TW-37 suppressed the Mcl-1 expression at the transcriptional and post-translational levels, resulting in apoptosis (Fig. 2). Mcl-1, an anti-apoptotic member of Bcl-2 family proteins, is regulated at the transcriptional, post-transcriptional, and post-translational levels upon several factors such as growth factors [17]. In addition, Mcl-1 is overexpressed in various types of cancer, which is related to the evasion of cell death and acquisition of resistance to chemotherapeutic drugs [18]. Specifically, it was previously established that the Mcl-1 expression was higher in human oral tumors compared with healthy oral mucosa and was accountable for neoplastic cell

transformation under 12-O-tetradecanoylphorbol-13-acetate or epidermal growth factor [19]. This study reported that the ectopic expression of Mcl-1 partly declined the apoptotic activity of TW-37 in human oral cancer cell lines, suggesting that regulation of the Mcl-1 expression is needed for TW-37-induced apoptosis in human oral cancer cell lines (Fig. 3). Hence, seeking a Mcl-1-targeting inhibitor could provide a potential therapeutic benefit for oral cancer therapy.

STAT3, one of the STAT family members, is a transcription factor playing a vital role in pathological processes of cancer. Notably, the constitutive phosphorylation of STAT3 correlates with tumor initiation and progression by suppressing apoptosis and inducing proliferation, invasion, metastasis, and angiogenesis, which is accompanied by dimerization, translocation to the nucleus, and binding to promoters of target genes [20, 21]., it was previously illustrated that the phosphorylated STAT3 levels markedly elevated in tissues obtained from patients with oral cancer compared with tissues obtained from the healthy oral mucosa, suggesting that p-STAT3 is a good prognostic indicator of oral cancer [22]. In addition, Zhou et al. demonstrated that the deletion of putative STAT3 binding site within the promoter of Mcl-1 abolished the promoter activity of Mcl-1 in lung cancer cell lines,

suggesting the transcriptional regulation of Mcl-1 by STAT3 [15]. This study established that the TW-37 treatment markedly abrogated the p-STAT3Tyr705 levels in human oral cancer cell lines, thereby inhibiting the nuclear translocation of p-STAT3Tyr705 (Fig. 4). Using STAT3 inhibitors such as cryptotanshinone and stattic, it was also investigated whether STAT3 signaling affects the Mcl-1 level; this was performed to support the evidence that p-STAT3 is the upstream signal of Mcl-1. The results showed that these STAT3 inhibitors significantly decreased the cell viability and induced apoptosis in human oral cancer cell lines via the downregulation of Mcl-1 (Fig. 9a–c). In contrast, the ectopic expression of Mcl-1 had no effect on the activation of STAT3 (Fig. 9d). Moreover, the effects of TW-37 with those of ABT-737 on p-STAT3-Mcl-1 signaling were compared; TW-37 significantly inhibited p-STAT3-Mcl-1 signaling; however, ABT-737 did not affect it although both inhibitors showed similar apoptotic efficiency (Fig. 10). Thus, these findings indicate that the decreased expression of Mcl-1 protein induced by TW-37 in human oral cancer cell lines could arise from the inhibition of STAT3 activation and nuclear translocation, thereby validating H1 of this study. Nevertheless, further studies are required to demonstrate the

role of STAT3 on reduced Mcl-1 promoter activity upon TW-37 treatment.

To date, numerous STAT3-targeted therapies inhibiting the SH2 domain, upstream tyrosine kinase, and DNA-binding domain have been considered potential therapeutic approaches for clinical applications because targeting STAT3 could be a reasonable strategy for the development of chemotherapeutic drugs against cancer [23]. Cryptotanshinone, one of the major active ingredients isolated from the traditional Chinese medicinal herb Salvia miltiorrhiza Bunge (red sage), is a STAT3 inhibitor that binds to the SH2 domain of STAT3 [24]. In addition, cryptotanshinone reportedly suppressed the cell proliferation and induced cell cycle arrest and apoptosis in various cancer types by suppressing JAK2–STAT3 signaling, as well as PI3K–Akt–NF-κB signaling [25-27]. Reportedly, cryptotanshinone enhances the chemosensitivity of conventional chemotherapeutic drugs, such as doxorubicin and paclitaxel, in cancer cell lines by inhibiting the STAT3 signaling pathway [28, 29]. Accordingly, I optimized the concentrations of TW-37 and cryptotanshinone to exclude their direct cytotoxic efficiency on human oral cancer cell lines. The findings revealed that the combination of TW-37 and cryptotanshinone further enhanced the

chemosensitivity of human oral cancer cell lines than a single drug alone, which was, perhaps, caused by the inhibition of STAT3–Mcl-1 signaling (Fig. 5), thereby validating H2 of this study. Hence, this study suggests that TW-37 potentiates the apoptotic effect of cryptotanshinone in human oral cancer cell lines by suppressing STAT3–Mcl-1 signaling.

7. Conclusion

This study demonstrates that TW-37 alone and in combination with cryptotanshinone exerts a potent apoptotic effect on human oral cancer cell lines by inhibiting STAT3–Mcl-1 signaling. Overall, this study highlights that targeting STAT3 using TW-37 could be an attractive approach in enhancing the prognosis of patients with oral cancer in the future.

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국 문 초 록

인간 구강암 세포주에서 STAT3–Mcl-1 신호기전을 표적하는 TW-37의 세포자멸 및 상승 작용에 관한 연구

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연구개요 및 목적: 두경부편평세포암종 (HNSCC)은 전세계적으로 주요한 암 중에 하나이며 그 중 구강편평세포암종이 90 % 이상으로, 가장 흔하게 발생한다. 몇몇 다른 암의 발병률이 감소했음에도 불구하고, 구강암의 발병률 및 사망률은 남성과 여성 모두에서 꾸준히 증가하고 있는 실정이다. 지난 수십 년간 구강암 치료를 위한 수술법 및 방사선·화학 요법이 크게 발전 했음에도 불구하고 구강암의 효과적인 진단과 적절한 치료법은 여전히 제한적이기 때문에 환자의 예후가 불량한

편이다. 따라서 구강암 환자를 위한 진보된 치료전략을 위해 꾸준히 연구할 필요가 있다. 발암의 잠재력을 가지고 있는 anti-apoptotic 단백질인 Bcl-2 family을 표적으로 하는 것은 하나의 좋은 암 치료법이 될 수 있을 것이다. 'BH3 mimetics'는 pro-apoptotic Bcl-2 단백질의 BH3 도메인을 모방하고 anti-apoptotic Bcl-2 계열 단백질을 길항하여 세포자멸사를 유발하도록 설계되었다. TW-37은 식물 유래 polyphenolic aldehyde 인 gossypol의 유사체로, 많은 *in vitro* 및 *in vivo* 연구에서 암세포의 세포주기를 S기에 정지시키거나 세포자멸사를 유도함으로써 다양한 유형의 암에 대한 치료 가능성이 보고되어져 왔다. 본 연구에서는 구강암에서 BH3 mimetic 기능을 하는 TW-37이 인간 구강암 세포주에 미치는 단일효과 및 항암제감수성 증가 효과를 규명하는 것을 목표로 한다.

연구방법: TW-37의 단일효과 및 항암제감수성 증가 효과는 Trypan blue exclusion assay, Western Blotting, DAPI staining, Annexin V-FITC/PI double staining, 그리고 Quantitative real-time PCR 등을 사용하여 세포수준에서 평가되었다. Mcl-1 과발현 모델은 형질전환벡터를 이용하여 제작하였으며, 세포자멸사를 평가하기 위해 일시적인 형질주입법을 사용하였다.

연구결과: TW-37은 전사(transcriptional) 및 번역후(post-translational) 조절을 통한 myeloid cell leukemia-1 (MCL-1) 발현 감소와 상관관계가 있는 caspase 의존적 세포자멸사를 유도하여 인간 구강암 세포주의 증식을 억제하였다. Mcl-1의 이소성 발현은 인간 구강암 세포주에서 TW-37의 세포자멸사 유도 능력을 부분적으로 약화시켰다. 또한 TW-37은 signal transducer and activator of transcription 3 (STAT3)의 Tyr705 인산화 및 인간 구강암 세포주에서 초기시점의 핵내 이동 (nuclear translocation)을 감소시켰다. 특히, TW-37과 cryptotanshinone을 병용하는 것은 인간 구강암 세포주의 STAT3-Mcl-1 신호를 억제함으로써 TW-37 또는 cryptotanshinone 둘 중 하나의 단독 효과와 비교하여 cryptotanshinone의 항암제감수성을 크게 증가시켰으며 그 결과 현저한 세포사멸을 유도하였다.

결론: 본 연구는 인간 구강암 치료를 위한 TW-37의 단일효과 및 항암제감수성 증가 효과를 밝혀낼 뿐만 아니라, TW-37이 향후 구강암 환자의 예후를 향상시키기 위한 좋은 치료 전략이 될 수 있음을 제시한다.

주요어 : TW-37, Cryptotanshinone, 항암제민감성, STAT3, Mcl-1, 구강암, 세포자멸사

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