석 사 학 위 논 문

Tubeimoside-1 Sensitizes TRAIL-induced Apoptosis via OTUD4-STAMBPL1 -mediated c-FLIP Downregulation

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이 논문을 석사학위 논문으로 제출함

2023년 2월

계 명 대 학 교 대 학 원

의학과 면역학 전공

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송소래의 석사학위 논문을 인준함

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또한 학위준비 기간 동안 많은 시간을 함께 해주시고 완성도 있는 논문을 위해 아낌없이 조언 해주신 박종욱 교수님, 김신 교수님께도 감사의 마음을 전합니다. 감사드립니다.

제가 석사학생으로서 성장할 수 있도록 곁에서 가르침을 주신 박사님들과 연구실에 상주하며 동고동락한 연구원선생님 및 동료학생들에게도 감사의 마음을 전합니다. 감사드립니다.

이상 석사학생을 위해 귀한 시간과 지대한 관심을 아낌없이 내어주신 면역학 교실의 모든 교수님 및 박사님, 연구원 선생님들께 다시 한번 감사의 마음을 표합니다.

마지막으로 석사학위를 마무리하기까지 제게 가장 큰 힘이 되어주고 묵묵히 앞을 보며 걸을 수 있도록 응원해주신 사랑하는 저희 가족에게 감사의 마음을 전달합니다.

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송소래

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1. Introduction

Tubeimoside-1 (TBMS-1), triterpenoid saponins, has been identified in Chinese medicinal herb *Bolbostemma paniculatum* (Maxim) Franquet (Cucurbitaceae) (1). Multiple studies demonstrated that TBMS-1 exerts biological actions such as anti-inflammation, anti-angiogenesis and anti-cancer effects (2-4). Recently, Jiang *et al.* reported that TBMS-1 induces cytoprotective autophagy through Akt-mTOR-eEF-2K pathway (5). TBMS-1 induces accumulation of impaired lysosomal cathepsin activity and blocks autophagic flux, which could be aggravated the cytotoxic activity of TBMS-1 in cervical cancer cells (6). In consistence with previous studies, TBMS-1 triggers autophagy by ROS-induced AMPK activation and blocks autophagic flux by inhibiting lysosomal enzymes, which contributes to TBMS-1-induced anti-cancer activity (7). Besides, TBMS-1 exerts dual anti-cancer effects that involves the induction of mitochondrial fission and lysosomal membrane permeability, resulting in the induction of apoptotic cell death in cancer (8).

The tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) reveals a promising therapeutic potential in cancer therapy as it exclusively induces apoptosis in multiple cancer cells through binding to the death receptors (DRs), followed by the increment of caspase-dependent apoptosis except normal cells (9). However, many malignant cancer cells display TRAIL resistance through downregulation of the DRs and upregulation of anti-apoptotic proteins (10). Therefore, many studies have investigated that combined treatment with novel agents and TRAIL could be overcome TRAIL resistance and enhance TRAIL sensitivity in malignant cancer cells (11).

In this study, I found the effect of TBMS-1 on the

TRAIL-sensitization of cancer cells, and the underlying mechanism in cancer cells. TBMS-1 induced downregulation of c-FLIP through downregulation of STAMBPL1 deubiquitin enzyme, resulted in the enhancement to TRAIL-mediated apoptotic cell death in cancer.

2. Materials and Methods

2.1. Cell Lines and Cell Culture:

All human cancer cells Caki (renal carcinoma), A549 (lung cancer), HeLa (cervical cancer) and human normal umbilical vein cells (EA.hy926) were obtained for the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in DMEM (Dulbecco's modified Eagle's) medium supplemented with 10% fetal bovine serum (Welgene, Gyeongsan, Korea) and antibiotics (1% penicillin-streptomycin and 100 μg/mL gentamycin). All the cell lines were incubated at 37 °C, 5% CO_2 in humidified air. They were cultured by short tandem repeats profiling before use and were also routinely tested for bacteria infection during this study.

2.2. Antibodies and Reagents:

Tubeimoside I (TBMS-1, purity ≥95.19%) was purchased from Selleckchem (Houston, TX, USA) and dissolved in dimethyl sulfoxide (DMSO). Human recombinant TRAIL and z-VAD-fmk were purchased from R&D system (Minneapolis, MN, USA). MG132 was purchased from Calbiochem (San Diego, CA, USA). Cycloheximide were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The primary antibody anti-cFLIP was obtained from Enzo Life Sciences (San Diego, CA, USA). Anti-STAMBPL1 and anti-Ub were obtained from Biotechnology (St. Louis, MO, USA). Anti-PARP and anti-survivin were obtained from Cell Signaling Technology (Beverly, MA, USA) and R&D System

(Minneapolis, MN, USA), respectively. Anti-OTUD4 was obtained from Abcam (Cambridge, MA, USA).

2.3. Flow Cytometry Assay:

To analyze apoptosis, harvested cells were resuspended in 100 uL of phosphate-buffered saline and fixed in 200 μL of 95% ethanol at 4 °C. After, cells were incubated in 1.12% sodium citrate buffer including RNase at 37 °C for 30 min, then stained with propidium iodide (PI). The level of apoptosis was analysed by BD Accuri™ C6 flow cytometer (BD Biosciences, San Jose, CA, USA).

2.4. DAPI Staining and DNA Fragmentation Assay:

Caki cells treated with each alone of TBMS-1 and TRAIL or combinations of both. To detect the change of cellular nuclei, cells were fixed with 1% paraformaldehyde. The cells washed with PBS and stained with 4′,6′-diamidino-2-phenylindole solution (Roche, Mannheim, Germany) for 5 min. The nucleus condensation was examined by fluorescence microscope (Carl Zeiss, Jena, Germany). For detection of apoptosis, DNA fragmentation was measured by Cell Death Detection ELISA PLUS kit. The samples were measured at 405 and 490 nm with spectrophotometry (BMG Labtech, Ortenberg, Germany).

2.5. Western Blotting Analysis:

Total cell lysed were lysed using ERK lysis buffer containing protease inhibitors. The protein sample separated by 10% SDS-PAGE

and transferred to nitrocellulose membrane (GE Healthcare Life Sciences, Pittsburgh, PA, USA). The membranes were blocked with 5% nonfat dry milk and incubated overnight with the indicated primary antibody (1:700 dilution) at room temperature. Immunoreactive bands were detected with chemiluminescence kit (EMD Millipore, Darmstadt, Germany).

2.6. Reverse Transcription PCR (RT-PCR) and Quantitative Real-Time PCR (qPCR):

Total RNA was extracted from cells using the TRIzol Reagent (Life Technologies, Gaithersburg, MD, USA). The quality and concentration of the RNA were determined by spectrophotometry. Prepared cDNA using M-MLV reverse transcriptase (Gibco-BRL, Gaithersburg, MD, USA). For RT-PCR, used Blend Taq DNA polymerase (Toyobo, Osaka, Japan) with primers targeting genes. And qPCR performed using a Thermal Cycler Dice® Real Time System III (Takara Bio Inc., Shiga, Japan) with SYBR Fast qPCR Mix (Takara Bio Inc., Shiga, Japan). I used c-FLIP and STAMBPL1 primers for qPCR: c-FLIP (sense) 5′-CGC TCA ACA AGA ACC AGT G-3′ and (antisense) 5′-AGG GAA GTG AAG GTG TCT C-3′, and STAMBPL1 (sense) 5′-GGG ACC ATC GCA GTG ACA AT-3′ and (antisense) 5′-CCG ACA GAT GGA GCT TTG CT-3′. I indicated the threshold cycle number (Ct) of each gene with actin as the reference gene, and I provided the delta-delta Ct values of the genes.

2.7. Transfection:

For siRNA transfection, human OTUD4 was knocked down using siRNA obtained from Bioneer (Daejeon, Korea) and used with Lipofectamine RNAiMAX Reagent (Invitrogen, Calshad, CA, USA). For plasmid transfection, pcDNA 3.1 vector was acquired from Invitrogen (Carlsbad, CA, USA). Flag-HA-OTUD4 plasmid was acquired from life science market (Dundas St, Mong Kok, HK) and used with Lipidofect-P (Lipidomia, Seongnam, Korea).

2.8. Deubiquitination (DUB) and Immunoprecipitation Assay:

For in vitro DUB assay, Caki cells were transfected with HA-ubiquitin plasmid, and treated with TBMS-1 and proteasome inhibitor (MG132). Harvested cells were washed with PBS containing 10 mM N-Ethylmaleimide (NEM), boiled for samples containing 100 µL PBS/NEM plus 1% SDS for 10 min at 95 °C. Total protein was extracted using RIPA lysis buffer containing proteasome inhibitors and 5 mM NEM, lysed using syringe for 5-7 times and centrifuged. The supernatants were incubated with the indicated antibody overnight and reacted by attaching protein G agarose bead (Santa Cruz Biotechnology, St. Louis, MO, USA) for 2 h. Then, the supernatants washed with RIPA containing 5 mM NEM and boiled using 2X sample buffer. Protein samples were separated on SDS-PAGE and then transferred onto nitrocellulose membranes. The membrane was exposed denaturation condition overnight and blocked with 5% Bovine serum albumin (BSA) at least 3 h. Subsequently, the membrane was exposed using HRP-conjugated anti-Ub antibody. For immunoprecipitation, the cells were lysed with RIPA lysis buffer containing 10 mM NEM, 1 mM PMSF and sonicated all the samples. After then, centrifuged lysate's

supernatants were incubated with the indicated antibody overnight at 4 °C, and added Protein G agarose bead at 4 °C on rotator. Eluted immunoprecipitates were resolved on SDS-PAGE and inspected for association of proteins of interest using specific antibodies.

2.9. Statistical Analysis:

The data were analysed by a one-way ANOVA and post hoc comparisons (Student-Newman-Keuls) using the Statistical Package for Social Sciences 27.0 software supplied from SPSS Inc. (Chicago, IL, USA). A p-value < 0.05 was considered significant.

3. Results

3.1. TBMS-1 Enhances TRAIL Sensitization:

High concentration (20 μM) of TBMS-1 exerts its anticancer effects against various cancer cell lines including lung cancer cells (12,13). I investigated whether TBMS-1 enhances TRAIL-induced apoptosis in human renal carcinoma cells. As shown in Figure 1A, individual TBMS-1 (5 μM) and TRAIL (50 ng/mL) had no effect on apoptosis in human renal carcinoma Caki, lung carcinoma A549 and cervical carcinoma HeLa cells, but combined treatment with TBMS-1 plus TRAIL remarkably increased the sub-G1 population and cleavage of PARP. However, TBMS-1 plus TRAIL treatment had no effect on morphological apoptotic bodies and sub-G1 populations in normal human EA.hy926 cells (Figure 1B). Combined treatment with TBMS-1 plus TRAIL showed exemplary chromatin damage in the nuclei and increased DNA fragmentation (Figure 1C). The pretreatment of pan-caspase inhibitor, z-VAD-fmk (z-VAD), markedly inhibited combined treatment-mediated increase of sub-G1 population and cleavage of caspase-3 and PARP (Figure 1D). These data indicate that combined treatment with TBMS-1 and TRAIL might induce apoptosis dependent on caspase in cancer cells, but not normal cells.

3.2. Decrease of c-FLIP by TBMS-1 is Involved in TRAIL Sensitization:

I investigated the expression levels of apoptosis-related proteins in

TBMS-1-treated Caki cells. TBMS-1 significantly downregulated survivin and c-FLIP expression, while other apoptosis-related proteins (Bcl-xL, Mcl-1, Bcl-2, Bim, Bax, cIAP1, cIAP2, and XIAP) were not affected by TBMS-1 (Figure 2A). To investigate the functional role of survivin and c-FLIP in combined treatment-induced apoptosis, I used survivin- or c-FLIP-overexpressed stable cells. Ectopic expression of c-FLIP inhibited sub-G1 population and PARP cleavage by combined treatment, but not in survivin-overexpressed cells (Figure 2B, C). These results indicate an essential role of c-FLIP downregulation in process of TBMS-1-induced TRAIL sensitization.

3.3. TBMS-1 Inhibits the c-FLIP Expression via Ubiquitin -Proteasome System:

To explore the TBMS-1-mediated c-FLIP downregulation at the transcriptional level, I checked c-FLIP mRNA level. TBMS-1 did not alter cFLIP mRNA (Figure 3A). Next, I investigated the effect of TBMS-1 on c-FLIP protein stability using the cycloheximide (CHX). Combined treatment with CHX and TBMS-1 more reduced c-FLIP protein level compared to CHX alone (Figure 3B). Pretreatment of proteasome inhibitor (MG132) prohibited TBMS-1-mediated c-FLIP downregulation (Figure 3C). In addition, I examined c-FLIP ubiquitination by TBMS-1 and found that TBMS-1 enhances ubiquitination of c-FLIP protein (Figure 3D). Collectively, these findings indicate that TBMS-1-induced c-FLIP downregulation is modulated by ubiquitin-proteasome pathway.

3.4. TBMS-1 Downregulates STAMBPL1 Expression at the Post-Translational Level:

The E3 ligases and deubiquitinases (DUBs) play a critical role in c-FLIP protein stabilization through ubiquitin-proteasome system (UPS). To investigate which E3 ligases and DUBs involved in TBMS-1-induced c-FLIP downregulation, I examined expression levels of various E3 ligases and DUBs for c-FLIP. Itch E3 ligase expression was not changed, while the expression levels of Cbl were slightly decreased by TBMS-1 treatment (Figure 4A). I did not detect upregulation of the other E3 ligases. However, TBMS-1 significantly downregulated STAMBPL1 expression, while other DUBs (USP2, USP8, and USP9x) were not affected by TBMS-1 (Figure 4A). I also found similar effect of TBMS-1-induced downregulation of c-FLIP and STAMBPL1 in HeLa and A549 cells (Figure 4B). However, STAMBPL1 mRNA expression was not changed by TBMS-1 (Figure 4C). Taken together, these data suggested that STAMBPL1 is involved in TBMS-1-induced c-FLIP stabilization.

3.5. STAMBPL1-Dependent c-FLIP Degradation is Important to the Sensitization Effect of TBMS-1 on TRAIL-Induced Apoptosis:

I further examined whether TBMS-1 can regulate STAMBPL1 stabilization. As shown in Figure 5A, TBMS-1 markedly inhibited STAMBPL1 stability compared with CHX alone. To rule out off-target effect of TBMS-1, I examined c-FLIP expression in STAMBPL1 siRNA transfected cells. As shown in Figure 5B, knockdown of STAMBPL1

markedly downregulated c-FLIP protein levels. To further confirm the involvement of STAMBPL1 on c-FLIP downregulation by TBMS-1, I transiently overexpressed STAMBPL1. Overexpression of STAMBPL1 markedly inhibited TBMS-1-induced c-FLIP downregulation, sub-G1 population and PARP cleavage by combined treatment (Figure 5C). Contrary to overexpression system, siRNA-mediated STAMBPL1 knockdown decreased c-FLIP protein expression and increased sensitivity to TRAIL (Figure 5D). Taken together, our results suggest that TBMS-1 can degrade c-FLIP expression via downregulation of STAMBPL1, and downregulation of c-FLIP contributes to TBMS-1-induced TRAIL sensitization.

3.6. TBMS-1 Regulates OTUD4 Expression at Transcriptional Level:

Because TBMS-1 downregulated STAMBPL1 expression at the post-translational regulation, I explored the various DUBs mRNA expression by TBMS-1 treatment. Only OTUD4 mRNA expression was decreased by TBMS-1, and knockdown of OTUD4 using siRNA inhibited STAMBPL1 protein expression (Figure 6A,B). Also, TBMS-1 induced OTUD4 downregulation in many cancer cell lines (Figure 6C). OTUD4 silencing also resulted in STAMBPL1 and c-FLIP downregulation (Figure 6D). These data showed that TBMS-1 decreases OTUD4 mRNA expression, and OTUD4 is involved in downregulation of STAMBPL1 and c-FLIP expression by TBMS-1.

3.7. OTUD4 is Essential for STAMBPL1-mediated c-FLIP Deubiquitination by TBMS-1:

I found the interaction with OTUD4-STAMBPL1-c-FLIP (Figure 7A). Knockdown of OTUD4 more decreased STAMBPL1 and c-FLIP by CHX alone, and proteasome inhibitors (MG132 and lactacystin) were reversed by OTUD4 depletion-induced STAMBPL1 and c-FLIP downregulation (Figure 7B,C). I examined the involvement of OTUD4 in TBMS-1-mediated TRAIL sensitization and found the increase of apoptosis by knockdown of OTUD4 in TRAIL-treated cells (Figure 7D). Because STAMBPL1 interacted with both OTUD4 and c-FLIP, I investigated the STAMPBL1 and c-FLIP ubiquitination by OTUD4. Overexpression of OTUD4 decreased STAMBPL1 ubiquitination, whereas ubiquitination of c-FLIP was not inhibited (Figure 7E,F). Altogether, the above results suggested that OTUD4 is essential for STAMBPL1-mediated c-FLIP deubiquitination and degradation by TBMS-1.

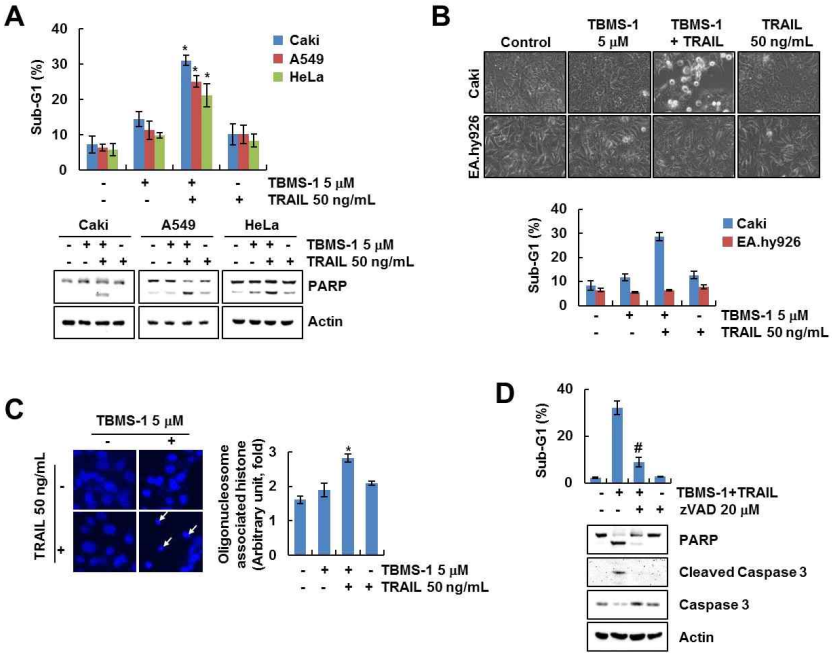

Figure 1. TBMS-1 enhances TRAIL-mediated apoptosis. (A-D) Cells were treated TBMS-1 (5 μM) plus TRAIL (50 ng/mL) for 24 h. (A) The sub-G1 population was determined by flow cytometry. The protein were detected by western blotting. (B) Cell morphology was determined by interference light microscopy. The levels of apoptosis were determined by flow cytometer. (C) DAPI staining was detected condensation and fragmentation of nuclei. Measurement of the cytoplasmic histone-associated DNA fragments using a DNA fragmentation detection kit. (D) Caki cells were pretreated with zVAD (20 μM). The apoptosis was analyzed by flow cytometry. The protein expression were detected by western blotting. The values in graph indicate the mean ± SD of three independent samples. * $p < 0.05$ compared to the control. # $p < 0.05$ compared to the treatment of combinations of TBMS-1 and TRAIL.

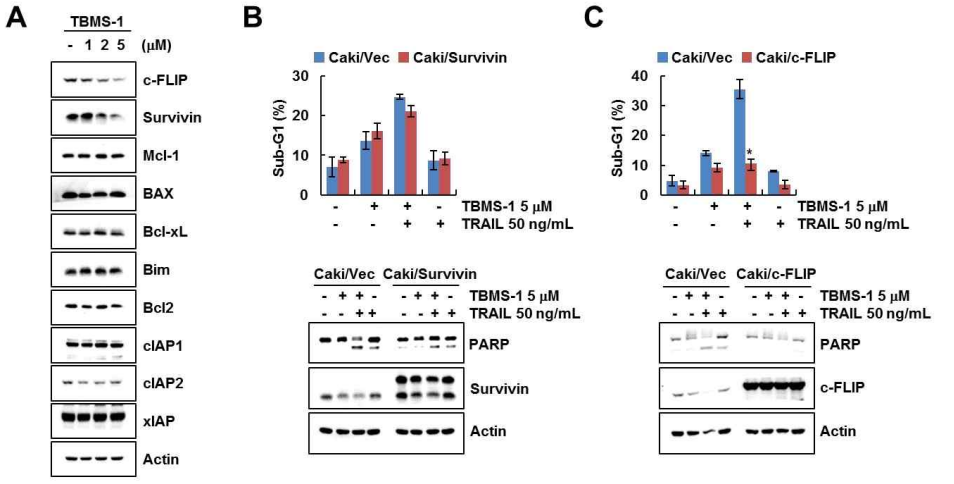

Figure 2. TBMS-1 induces downregulation of c-FLIP expression. (A) Caki cells were treated with 1, 2, and 5 μM TBMS-1 for 24 h. (B,C) Caki and survivin- or c-FLIP-overexpressed Caki cells (B) Caki/survivin or (C) Caki/c-FLIP were treated with 5 μM TBMS-1 or/and 50 ng/mL TRAIL for 24 h. (A-C) The protein expression and (B,C) sub-G1 population were measured by Western blotting and flow cytometry, respectively. The values in graph indicate the mean ± SD of three independent samples. * $p < 0.05$ compared to the control.

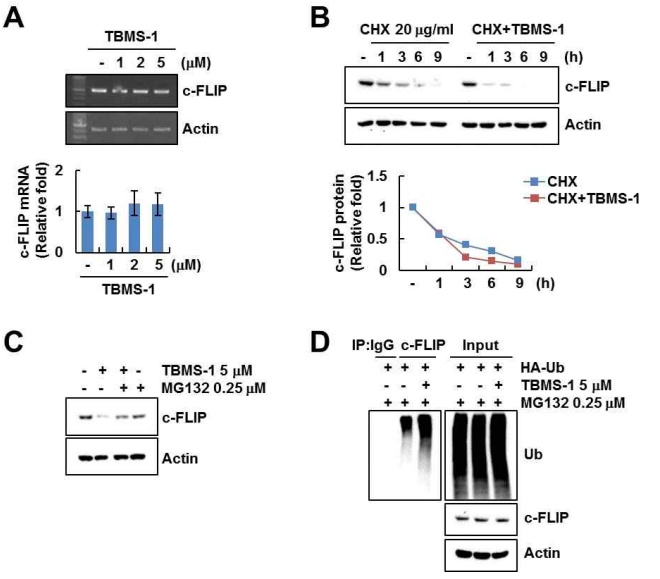

Figure 3. TBMS-1 inhibits the c-FLIP expression via ubiquitin-proteasome system. (A) Caki cells were treated with 1-5 μM TBMS-1 for 24 h. The mRNA levels of c-FLIP and actin were examined by RT-PCR and qPCR. (B) Caki cells were pretreated with 20 μg/mL cycloheximide (CHX) for 30 min, then treated with 5 μM TBMS-1 for the indicated time periods. The band intensity was measured using Image J. (C) Caki cells were pretreated with 0.25 μM MG132 for 30 min and then treated with 5 μM TBMS-1 for 24 h. The protein expression was measured by Western blotting. (D) Caki cells were transfected with HA-ubiquitin plasmid for 24 h. Then cells were treated with 0.25 μM MG132 and 5 μM TBMS-1 for 24 h. c-FLIP ubiquitination was detected by Western blotting using HRP-conjugated anti-Ub antibody. The values in graph indicate the mean ± SD of three independent samples.

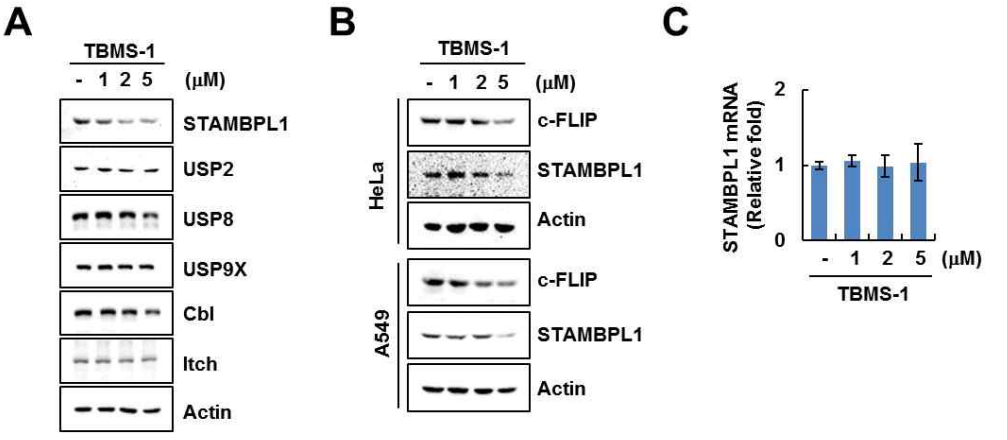

Figure 4. TBMS-1 induces down-regulation of STAMBPL1 expression. (A) Caki cells, (B) A549 cells and Hela cells were treated with 1-5 μM TBMS-1 for 24 h. (A,B) The protein expression was measured by Western blotting. (C) Caki cells were treated with each dose of TBMS-1 for 24 h. The mRNA levels of STAMBPL1 and actin were examined by qPCR. The values in graph indicate the mean ± SD of three independent samples.

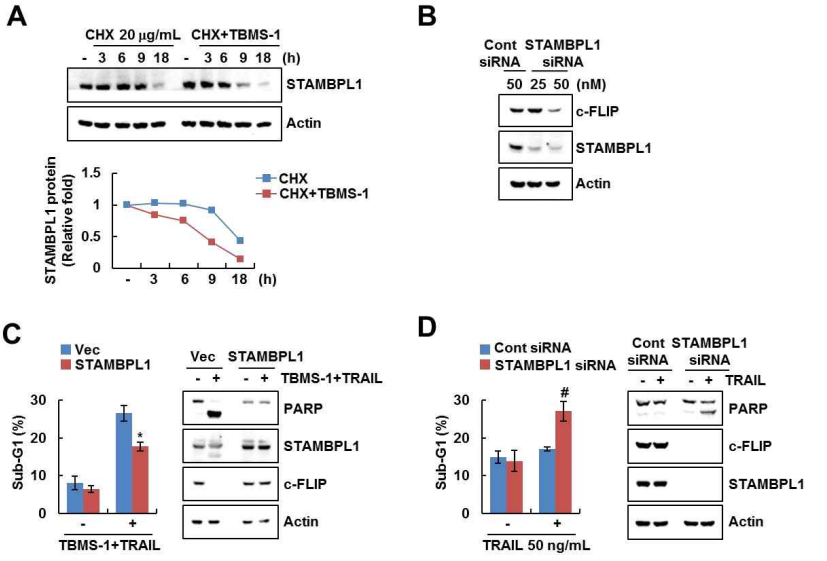

Figure 5. STAMBPL1 regulates c-FLIP stability and enhances TRAIL-mediated apoptosis. (A) Caki cells were pretreated with 20 μg/mL cycloheximide (CHX) for 30 min, then treated with 5 μM TBMS-1 for the indicated time periods. The band intensity was measured using Image J. (B,D) Caki cells were transfected by control siRNA (siCont) or STAMBPL1 siRNA (siSTAMBPL1). (C) Caki cells were transfected with pcDNA3.1(+) (Vec) or pcDNA3.1(+)/STAMBPL1 plasmids, then treated with combinations of 5 μM TBMS-1 with 50 ng/ml TRAIL for 24 h. (D) Treated with 50 ng/mL TRAIL for 24 h; (A-D) The protein expression levels and (C,D) sub-G1 population were examined by Western blotting and flow cytometry, respectively. The values in graph indicate the mean ± SD of three independent samples. * $p < 0.05$ contrasted with the combinations of TBMS-1 and TRAIL in Vec-transfected cells. # $p < 0.05$ contrasted with TRAIL in Cont siRNA-transfected cells.

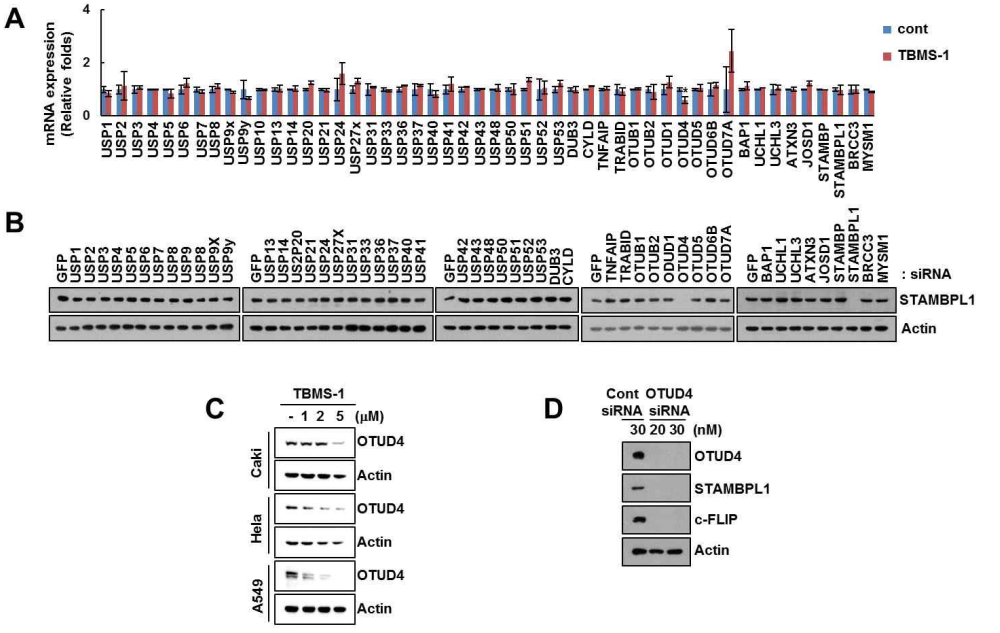

Figure 6. TBMS-1 regulates OTUD4 expression at transcriptional level. (A) Caki cells were treated with 5 μM TBMS-1 for 24 h. The mRNA levels of 50 DUBs were examined by qPCR. (B) Caki cells were transfected by control siRNA or each DUB siRNA. (C) Caki cells, HeLa cells and A549 cells were treated with 1-5 μM TBMS-1 for 24 h. (D) Caki cells were transfected by control siRNA (siCont) or OTUD4 siRNA (siOTUD4) for 48 h. (B-D) Protein expression level was determined using Western blotting. The values in graph indicate the mean ± SD of three independent samples. * $p < 0.05$ compared to the control.

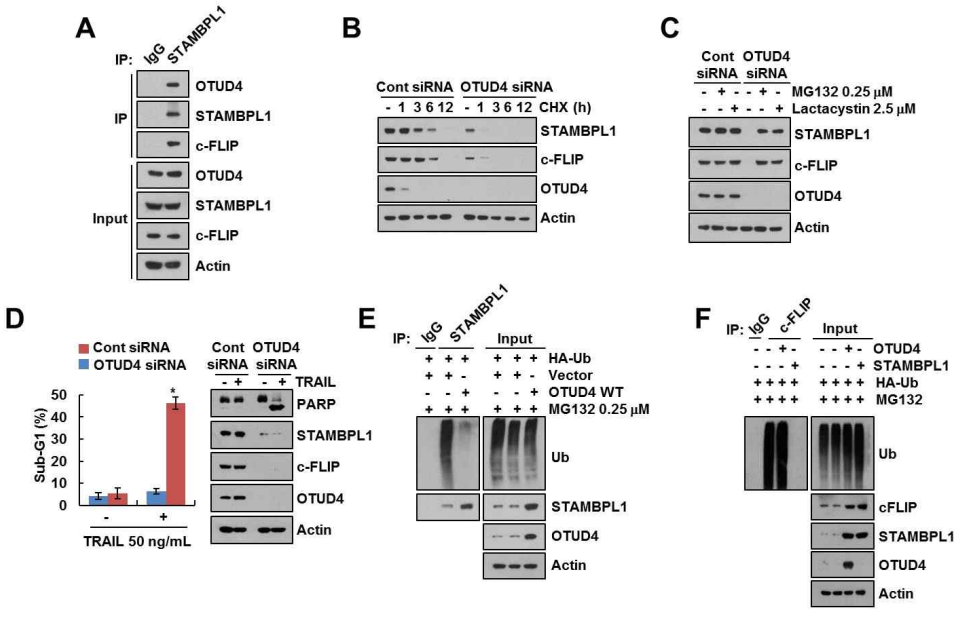

Figure 7. OTUD4 interacts with STAMBPL1 and regulates STAMBPL1 stability. (A) Endogenous protein-protein interaction was demonstrated by IP using anti-STAMBPL1 (B-D) Caki cells were transfected by control siRNA or OTUD4 siRNA; and treated with (B) 20 μg/mL cycloheximide (CHX) for the indicated time periods. (C) with 0.25 μM MG132 or 2.5 μM Lactacystin for 24 h. (D) Treated with 50 ng/mL TRAIL for 24 h; sub-G1 population were measured by flow cytometry. (E,F) Caki-1 cells were co-transfected by the vector, WT OTUD4, WT STAMBPL1 and HA-ubiquitin (HA-Ub), and treated with 0.5 μM MG132 for 12 h to analyze (E) STAMBPL1, (F) c-FLIP ubiquitination. Immunoprecipitation was performed using an (E) anti-STAMBPL1 antibody and (F) anti-c-FLIP antibody. (A-F) Protein expression level was determined using Western blotting, respectively. The values in graph indicate the mean ± SD of three independent samples. * $p < 0.05$ compared to the control.

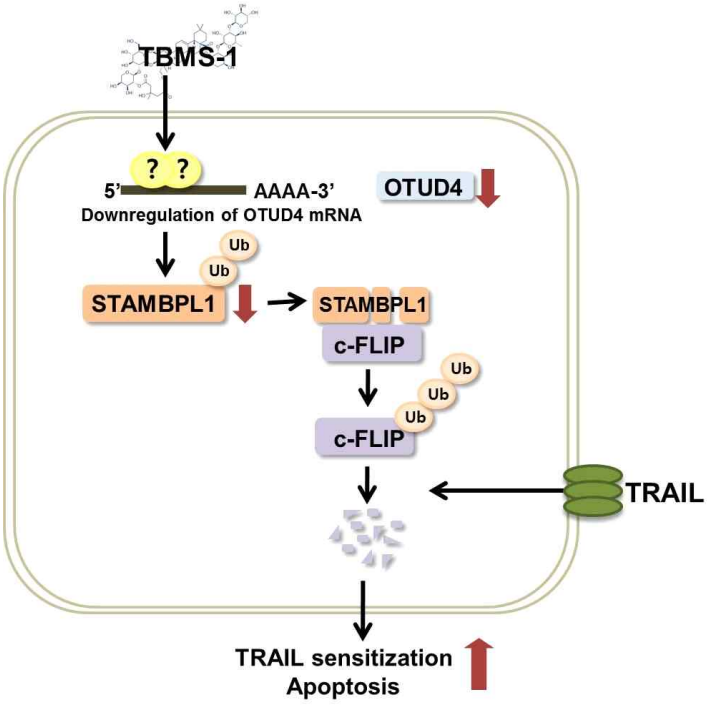

Figure 8. The scheme of the anti-cancer mechanism of TBMS-1. TBMS-1 decreases OTUD4 expression at the transcription level, stabilizes STAMBPL1 and decreases STAMBPL1 expression. STAMBPL1 interacts with c-FLIP and induces deubiquitination of c-FLIP. Eventually, sensitivity of TRAIL is increased in TBMS-1 treatment.

4. Discussion

In this study, I found that TBMS-1 enhanced TRAIL-induced apoptosis in cancer cells, but not in normal cells. I found that TBMS-1-induced c-FLIP downregulation contributes to TBMS-1-induced TRAIL sensitization. Downregulation of STAMBPL1 is associated with c-FLIP destabilization in TBMS-1-treated cells. Therefore, I suggested that TBMS-1 induces STAMBPL1-dependent c-FLIP degradation, resulting in the augment of TRAIL-induced apoptosis.

Previous research demonstrated that TBMS-1 revealed a time- and dose-dependent inhibitory effect on the growth and proliferation of various tumor cell lines with IC50 values being at 10-50 μM concentrations (4). In our study, a mechanism for TRAIL-mediated apoptosis enhancement by low concentration (5 μM) of TBMS-1 was performed. As shown in Figure 2A, c-FLIP was downregulated by TBMS-1, whereas expression of other apoptosis-related protein (Bcl-xL, Mcl-1, Bcl-2, Bim, Bax, cIAP1, cIAP2, and XIAP) was not changed. Interestingly, although the expression of survivin was decreased by TBMS-1 treatment, the apoptosis inhibitory effect by the combined treatment with TBMS-1 plus TRAIL could not be confirmed by overexpression of survivin. This is presumed to be that survivin downregulation does not play a significant role in apoptosis by the combined treatment.

It has been well documented that c-FLIP play a pivotal role in the resistance of cancer cells to death receptor-induced cell death. Previously, Zhao *et al.* reported that mTOR complex 2 is involved in modulation of Cbl-dependent c-FLIP stability and sensitivity of

TRAIL-induced apoptosis (14). According to our results (Figure 4A), Cbl expression decreased, not increased, by TBMS-1, suggesting that Cbl is not related to the regulation of c-FLIP stability by TBMS-1. Therefore, I focused protein expression levels of DUBs in TBMS-1 treated cells. Jeong *et al.* reported that USP8 directly deubiquitylates and stabilizes c-FLIP. Depletion of USP8 induces c-FLIP downregulation, promoting TRAIL-induced apoptosis (15). USP8 was slightly decreased by TBMS-1 treatment in Caki cells (Figure 4A). It need to further studies to identify USP8 involved in TBMS-1-mediated c-FLIP downregulation. Interestingly, TBMS-1 dramatically reduces STAMBPL1 expression. Ectopic expression of STAMBPL1 inhibits TBMB-1-induced c-FLIP downregulation and combined treatment with TBMS-1 plus TRAIL-induced apoptosis. In previous studies, I also found that honokiol enhances TRAIL-induced apoptosis through STAMBPL1-mediated survivin and c-FLIP degradation (16). Overexpression of STAMBPL1 reduced ubiquitination of endogenous c-FLIP and physical interaction between STAMBPL1 and c-FLIP was detected in immunoprecipitation assay (16). Knockdown of STAMBPL1 by shRNA triggered caspase-dependent apoptosis through XIAP degradation in prostate cancer cells (17). However, in our study, XIAP was not downregulated by TBMS-1 (Figure 2A). This contradiction is believed to be due to the difference between the cell line used and the downregulation of STAMBPL1. I have not completely ruled out the involvement of other regulation pathway of XIAP in TBMS-1 treated cells. The zinc metalloprotease STAMBPL1 has multiple functions, but the role of STAMBPL1 is unclear in cancer cells. I found that downregulation of STAMBPL1 might be involved in TBMS-1-induced downregulation of c-FLIP (Figure 5C).

Moreover, TBMS-1 decreased OTUD4 mRNA expression and

ubiquitination of STAMBPL1 (Figure 6A and 7E). To verity the involvement of OTUD4-dependent STAMBPL1 in TBMS-1-induced c-FLIP degradation, I examined STAMBPL1 and c-FLIIP ubiquitination by OTUD4 overexpression. Overexpression of OTUD4 only inhibited ubiquitination of STAMBPL1, but not c-FLIP (Figure 7F). Therefore, these results that STAMBPL1 is essential for OTUD4-mediated c-FLIP downregulation by TBMS-1.

Taken all together, I showed that TBMS-1 degrades c-FLIP protein expression through downregulation of STAMBPL1 deubiquitin enzyme. Moreover, decrease of OTUD4 by TBMS-1 was involved in STAMBPL1-mediated c-FLIP downregulation. Thus, combined treatment with TBMS-1 plus TRAIL might be a potential therapeutic strategy in TRAIL-resistant cancer.

5. Summary

The tubeimoside-1 (TBMS-1) from traditional Chinese medicine herb is frequently employed as an anti-cancer drug. Here, I examine to effect of TBMS-1 on TRAIL sensitization and elucidate the cell death mechanism of TBMS-1 in TRAIL-mediated cell death in the cancer cell. Sub-toxic doses of TBMS-1 or TRAIL alone does not induce apoptosis However, the combined treatment with TBMS-1 and TRAIL induces cell apoptosis in various cancer cells. TBMS-1 downregulates STAMBPL1 deubiquitinating enzyme results in destabilization of c-FLIP expression. Furthermore, TBMS-1 induces downregulation of OTUD4 mRNA expression. The OTUD4 interacts STAMBPL1 and regulate stability of STAMBPL1. These results suggested that TBMS-1 induces apoptosis through OTUD4-STAMBPL1 mediated c-FLIP downregulation and enhances sensitization with TRAIL.

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Tubeimoside-1 Sensitizes TRAIL-induced Apoptosis via OTUD4-STAMBPL1-mediated c-FLIP downregulation

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(Abstract)

Tubeimoside-1 (TBMS-1), the traditional Chinese medicinal herb, is commonly used as anti-cancer agent. Here, I investigated the effect of TBMS-1 in sensitization to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in cancer cells. Sub-lethal concentration of TBMS-1 alone and TRAIL alone did not affect cancer cell death, whereas combined treatment with TBMS-1 and TRAIL increase apoptotic cell death. Mechanistically, TBMS-1 destabilized c-FLIP expression through decrease of STAMBPL1, a deubiquitinating enzyme. STAMBPL1 interacted and deubiquitinased c-FLIP. Moreover, knockdown of STAMBPL1 markedly increased sensitivity to TRAIL, and overexpression of STAMBPL1 prevented TBMS-1 and TRAIL-induced apoptosis by regulating c-FLIP stabilization. Also, TBMS-1

downregulated OTUD4 mRNA expression. Decreased OTUD4 regulates STAMBPL1 expression and stability. These results provided that TBMS-1 increases TRAIL sensitization and induces apoptosis via OTUD4-STAMBPL1 mediated c-FLIP downregulation.

Tubeimoside-1에 의한 TRAIL 매개 세포사멸 증대 기전 연구

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(초록)

천연 약재로써 항암효과가 있는 Tubeimoside-1 (TBMS-1)와 TRAIL을 병합 처리하여 암세포에 대한 항암 효과를 조사하였다. 낮은 독성 농도의 TBMS-1와 TRAIL의 병합처리는 암세포에서 세포사멸을 증진시켰으나 정상세포에서는 효과가 없었다. TBMS-1은 탈유비퀴틴화 효소인 STAMBPL1의 감소를 통해 c-FLIP을 탈유비퀴틴화 시켜 c-FLIP 발현을 불안정화를 유도하였다. STAMBPL1의 발현억제는 TRAIL에 대한 민감성을 현저하게 증가시켰고, STAMBPL1의 과발현은 c-FLIP 안정화를 조절함으로써 TBMS-1 및 TRAIL-유도 세포 사멸을 억제하였다. TBMS-1은 OTUD4 mRNA 발현 감소를 유도하였다. TBMS-1에 의한 OTUD4 단백질의 발현 감소는 STAMBPL1의 단백질 발현 감소를 유도하였다. 이 결과는 TRAIL 매개의 세포 사멸 증진을 위한 민감제로 TBMS-1 가능성을 제시한다.