Thesis for Master's Degree

The BMI-1 Inhibitor, PTC596, Induces Apoptosis through Down-regulation of Mcl-1 Expression in Cancer Cells

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1. Introduction

Millions of people in the world die of cancers every day, therefore there are many treatment options for various cancers. However, few can be completely cured. Undoubtedly, effective treatment of cancer has become an urgent issue (1). Additionally, human renal cell carcinoma is one of the common diseases and cause of death in the whole world (2). As known, cell death includes programmed cell death and non-programmed cell death, among them, apoptosis is a form of programmed cell death, which exhibits specific morphological changes and shows important role in cancer therapy (3). Chemotherapy is a kind of effective strategy to kill cancer cells, although most of patients suffering from cancers behave chemotherapeutic resistance, development of potential anti-cancer drugs still one of the main methods of cancer therapy (4).

Cancer stem cells (CSCs) can generate tumors through the stem cell processes by self-renewing and differentiation into various cell types resulting in the sustenance of tumorigenesis (5,6). Accordingly, chemicals related to cancer stem cells could be the new candidates for cancer therapy (7). BMI-1 belongs to the polycomb family and directly participates in the regulation of cell proliferation, cell invasion, cell apoptosis and plays an essential role in the self-renewal of adult stem cells and leukemia stem cells (8-12). Taken together, BMI-1 is expected to become an effective target for cancer therapy (11). Previous studies have showed that up-regulated BMI-1 is associated in development of various human tumors, including leukemia, breast, gastric, lung, prostate, colorectal and ovarian cancers (9,13-19).

As BMI-1 is highly expressed in various cancers and relates to the

cancer stem cells, inhibition of BMI-1 significantly could be an efficient method to the increase of sensitization of anti-cancer drugs (20). Furthermore, PTC596, a novel inhibitor of BMI-1, classically induced apoptosis through down-regulation of Mcl-1 expression (21). Even though BMI-1 inhibitor with the anti-cancer effect has been identified in several cancer cells, the deeper molecular mechanisms has not been completely confirmed.

Post-translational modification is an important mechanism for apoptosis, which involves three pathways such as phosphorylation, acetylation and ubiquitination (22). Among them, ubiquitination plays a crucial role in cell apoptosis caused by a lot of chemicals (23). The ubiquitin proteasome system (UPS) can be regulated by E3 ubiquitin ligases and deubiquitinases, and is a crucial pathway for the protein degradation (24,25).

Consequently, I investigated that inhibition of BMI-1 by siRNA and specific inhibitor induce cancer cell apoptosis and the decrease of cancer cell stem-like cell population. Furthermore, the BMI-1 inhibitor PTC596 markedly down-regulated Mcl-1 expression by reducing a deubiquitinase DUB3 expression at a post-translational level in cancer cells. Herein, these results demonstrated the induction of apoptosis by PTC596 is involved in DUB3-mediated Mcl-1 down-regulation.

2. Materials and Methods

2.1. Cell Lines, Cell Culture Conditions and Chemicals:

Human renal cancer cell carcinoma (Caki), human breast cancer cell carcinoma (MDA-MB-231), human cervical cancer cell carcinoma (HeLa), human colorectal cancer cell carcinoma (HCT116), human lung cancer cell carcinoma (A549), human prostate cancer cell carcinoma (DU145), human hepatocellular cancer cell carcinoma (SK-Hep1) and kidney normal cells (TCMK-1) were offered from American Type Culture Collection (Manassas, VA, USA). Human mesangial cells were obtained from Lonza (Basel, Switzerland). Caki, MDA-MB-231, HCT116, DU145 and SK-Hep1 cells were grown in an appropriate Dulbecco's Modified Eagle Media medium, A549 and HeLa cells were grown in Roswell Park Memorial Institute Medium. Both two mediums supplemented with 10% fetal bovine serum (Welgene, Gyeongsan, Korea), 1% penicillin - streptomycin and 100 μg/mL gentamycin (Thermo Fisher Scientific, Waltham, MA, USA). PCR primers were provided by Macrogen (Seoul, Korea). PTC596 was purchased from Selleck Chemicals (Houston, TX, USA). Sigma Chemical Co. supplied cycloheximide, MG132 and anti-actin (St. Louis, MO, USA), z-VAD-fmk and anti-survivin (Minneapolis, MN, USA) were offered from R&D Systems. Cell Signaling Technology (Beverly, MA, USA) furnished anti-PARP, anti-Bcl-xL, anti-Mcl-1, anti-BMI-1 and anti-cleaved caspase 3 antibodies. Lactacystin and anti-caspase 3 antibody were supplied by Enzo Life Sciences (San Diego, CA, USA). BD Biosciences (San Jose, CA, USA) offered anti-XIAP, anti-Bax and anti-Bim

antibodies. Anti-Mcl-1, anti-cIAP1, anti-cIAP2, anti-Bcl-2 and anti-β-TrCP were obtained from Santa Cruz Biotechnology (St. Louis, MO, USA). Abnova (Taipei City, Taiwan) supplied the anti-USP9X. Anti-USP1 and anti-FBW7 were purchased from Bethyl Laboratories (Montgomery, TX, USA). Novus Biologicals (Centennial, CO, USA) offered the anti-DUB3 antibody. Anti-OTUD1 was supplied by MERCK (Kenilworth, NJ, USA).

2.2. Cancer Cell Stem-like Cell Population Analysis:

To analyze the population of cancer stem cells, cancer cells were treated with PTC596 for 12 h, ALDEFLUOR™ Kit for ALDH assay (STEMCELL™ TECHNOLOGIES) (Cambridge, MA, USA) was used to measure stem cells, then analyzed by flow cytometry (BD Biosciences, San Jose, CA, USA).

2.3. DNA Fragmentation and DAPI Staining:

To measure DNA fragmentation, cell death detection ELISA plus kit (Boehringer Mannheim, Indianapolis, IN, USA) was used. To identify nuclei condensation, cells were stained with 300 nM 4′,6′-diamidino-2-phenylindole solution (Roche, Mannheim, Germany), then analyzed by fluorescence microscope.

2.4. Quantitative Polymerase Chain Reaction (qPCR) and Reverse Transcription Polymerase Chain Reaction (RT-PCR):

RNA was isolated by using the TriZol reagent (Life Technologies, Gaithersburg, MD, USA). cDNA was obtained using M-MLV reverse transcriptase (Gibco-BRL, Gaithersburg, MD, USA). Blend Taq DNA polymerase (Toyobo, Osaka, Japan) with primers were used for targeting Mcl-1 and actin. The amplified products were separated by electrophoresis on a 2% agarose gel and detected under ultraviolet light. For real-time PCR, SYBR Fast qPCR Mix (Takara Bio Inc., Shiga, Japan) was used, and reactions were analyzed on Thermal Cycler Dice® Real Time System III (Takara Bio Inc., Shiga, Japan).

2.5. Asp-Glu-Val-Asp-ase (DEVDase) Activity Assay:

To detect caspase activity, Caki cells were treated with PTC596 for 24 h, then harvested and incubated with reaction buffer containing substrate [acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA)] (26).

2.6. Flow Cytometry and Western Blotting Analysis:

To calculate sub-G1 population, cells were mixed with 100% ethanol, then incubated in 1.12% sodium citrate buffer containing RNase at 37 °C for 30 min, next added to 50 μg/mL propidium iodide, finally analyzed by Guava® easyCyte™ Flow cytometer. The total lysates were obtained in RIPA lysis buffer (20 mM HEPES and 0.5% Triton X-100, pH 7.6), then proteins were separated by SDS-PAGE, next transferred to nitrocellulose membranes (GE Healthcare Life Science, Pittsburgh, PO, USA). After blocking using nonfat milk for 30 min, overnight with primary antibody. Next day incubated with secondary antibody, finally

checked by iBright™ Imager (Thermo Fisher Scientific, Waltham, MA, USA).

2.7. siRNA Transfection:

BMI-1 siRNA was bought from Santa Cruz Biotechnology (St. Louis, MO, USA). USP1 siRNA and DUB3 siRNA were purchased from Bioneer (Daejeon, Korea). For knockdown by siRNA, Caki cells were transfected using Lipofectamine® RNAiMAX Reagent (Invirtrogen, Calshad, CA, USA).

2.8. Plasmid Transfection:

To construct stable cell lines, Caki cells were transfected with the pcDNA3.1(+)/Vector or pcDNA3.1(+)/Mcl-1 plasmids using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) for 24 h, and cells were selected by 700 μg/mL G418 (Invitrogen). DUB3 plasmid was purchased from Addgene and transfected using Lipofectamine™ 2000 (Invitrogen) in Caki cells.

2.9. Densitometry:

The band intensities were performed using the gel analysis plugin for the open source software ImageJ 1.46 (Imaging Processing and Analysis in Java: http://rsb.info.nih.gov/ij).

2.10. Statistical Analysis:

The data were analyzed by a one-way ANOVA and post-hoc comparisons (Student-Newman-Keuls) using the Statistical Package for Social Sciences 25.0 software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Inhibition of BMI-1 Decreases Cancer Cells Stem-like Cell Population:

Previous study has demonstrated that BMI-1 is associated with self-renewal of cancer stem cells (9). To determine whether inhibition of BMI-1 can reduce stem-like cell population, I used siRNA and pharmacological inhibitor. PTC596, a novel BMI-1 inhibitor, decreased cancer cell stem-like cell population in Caki and HeLa cells (Figure 1A). Also, knockdown of BMI-1 by siRNA inhibited cancer cell stem-like cell population in Caki and A549 cells (Figure 1B&C). Initially, these results showed that inhibition of BMI-1 induces decrease of cancer cell stem-like cell population. Furthermore, the BMI-1 inhibitor, PTC596, could be a potential drug against cancer stem cells.

3.2. Effects of BMI-1 Inhibitors on Apoptosis in Various Cancer Cells:

To select the most effective drug for research, two BMI-1 inhibitors, PTC209 and PTC596 were treated to three cancer cell lines [human renal cancer cell carcinoma (Caki), human cervical cancer cell carcinoma (HeLa) and human colorectal cancer cell carcinoma (HCT116)]. Interestingly, PTC596 more effectively induced apoptosis compared to PTC209 in all cancer cell lines (Figure 2A-C). According to these data, I selected PTC596 as BMI-1 inhibitor for further study.

3.3. PTC596 Induces Apoptosis through a Caspase-dependent Manner:

Furthermore, we evaluated whether PTC596 induces apoptosis through the caspase-dependent pathway. As shown in Figure 3A, PTC596 treatment induced typical apoptotic morphology and chromatin condensation in Caki cells. Additionally, PTC596 treatment induced both sub-G1 population and PARP cleavage in a dose-dependent manner (Figure 3B). As expected, PTC596 markedly induced DNA fragmentation (Figure 3C). In Figure 3D, PTC596 increased DEVDase (caspase-3) activity. Furthermore, to explore the involvement of caspase activation in PTC596-induced apoptosis, I treated a pan-caspase inhibitor, z-VAD-fmk (z-VAD). z-VAD inhibited the increase of sub-G1 population, the expression of cleaved PARP and cleaved caspase 3 in PTC596-treated cells (Figure 3E). According to these results, PTC596 induced apoptosis in a caspase-dependent manner.

3.4. Inhibition of BMI-1 Induces Down-regulation of Mcl-1 Expression in Various Cancer Cells:

To speculate the detail mechanism of PTC596-induced apoptosis, apoptosis-related proteins were detected by Western blotting in Caki cells. The data showed that PTC596 down-regulated Mcl-1 expression, whereas the expression levels of other proteins (cIAP1, cIAP2, XIAP, survivin, Bcl-XL, Bcl-2, Bim and Bax) were not changed (Figure 4A). In addition, inhibition of BMI-1 by siRNA also exhibited similar results compared with PTC596 (Figure 4B). Moreover, both PTC596 and BMI-1 siRNA significantly decreased Mcl-1 expression in MDA-MB-231 and

HeLa cells (Figure 4C&D). All these experiments demonstrated that inhibition of BMI-1 down-regulated Mcl-1 expression.

3.5. Down-regulation of Mcl-1 is Involved in PTC596-induced Apoptosis:

As expected, overexpression of Mcl-1 blocked PTC596-induced apoptosis (Figure 5A). Since Mcl-1 plays a critical role in PTC596-induced apoptosis of cancer cells, I examined the underlying mechanism of PTC596-mediated Mcl-1 down-regulation. The mRNA level of Mcl-1 was not changed by PTC596 and BMI-1 siRNA treatment (Figure 5B). To identify the protein stability of Mcl-1, a protein biosynthesis inhibitor, cycloheximide (CHX) was used. Combined treatment with CHX and PTC596 rapidly induced degradation of Mcl-1 expression compared to treatment of CHX alone (Figure 5C). Additionally, to investigate the regulation of UPS-mediated post-translational modification of Mcl-1, two proteasome inhibitors (MG132 and lactacystin) were treated. As shown in Figure 5D, MG132 and lactacystin prevented the degradation of Mcl-1 expression by PTC596. These data revealed that PTC596-induced down-regulation of Mcl-1 expression is regulated in a post-translational level.

3.6. Inhibition of DUB3 is Associated with PTC596-induced Mcl-1 Down-regulation and Apoptosis:

There are a lot of studies have assessed that ubiquitination is related to the regulation of Mcl-1 expression. Especially, E3 ubiquitin ligases

and deubiquitinases play an important role in UPS (27-31). I examined whether expression of E3 ligases and deubiquitinases are involved in Mcl-1 degradation. There was no obvious change in E3 ligases, while expression levels of USP1 and DUB3 deubiquitinases decreased by PTC596 treatment (Figure 6A&B). Additionally, knockdown of USP1 and DUB3 reduced the Mcl-1 protein levels (Figure 6C). mFurthermore, overexpression of DUB3 slightly blocked PTC596-induced sub-G1 population and Mcl-1 degradation (Figure 6D). Collectively, these results indicated that inhibition of DUB3 is associated with PTC596-induced Mcl-1 down-regulation and apoptosis.

3.7. Apoptotic Effect of PTC596 in Various Cell Lines:

Consequently, the effect of PTC596 was evaluated in various cell lines. To verify the apoptotic effect of PTC596 against cancer cell lines, the results explored that PTC596 increased the population of apoptotic cells and cleaved PARP in human hepatocellular cancer cell carcinoma (SK-Hep1) and human prostate cancer cell carcinoma (DU145). (Figure 7A). In contrast, PTC596 did not affect cell morphology and sub-G1 population in two normal cell lines, such as MC and TCMK-1 cells (Figure 7B). Taken together, PTC596 has a selective apoptotic effect on cancer cells.

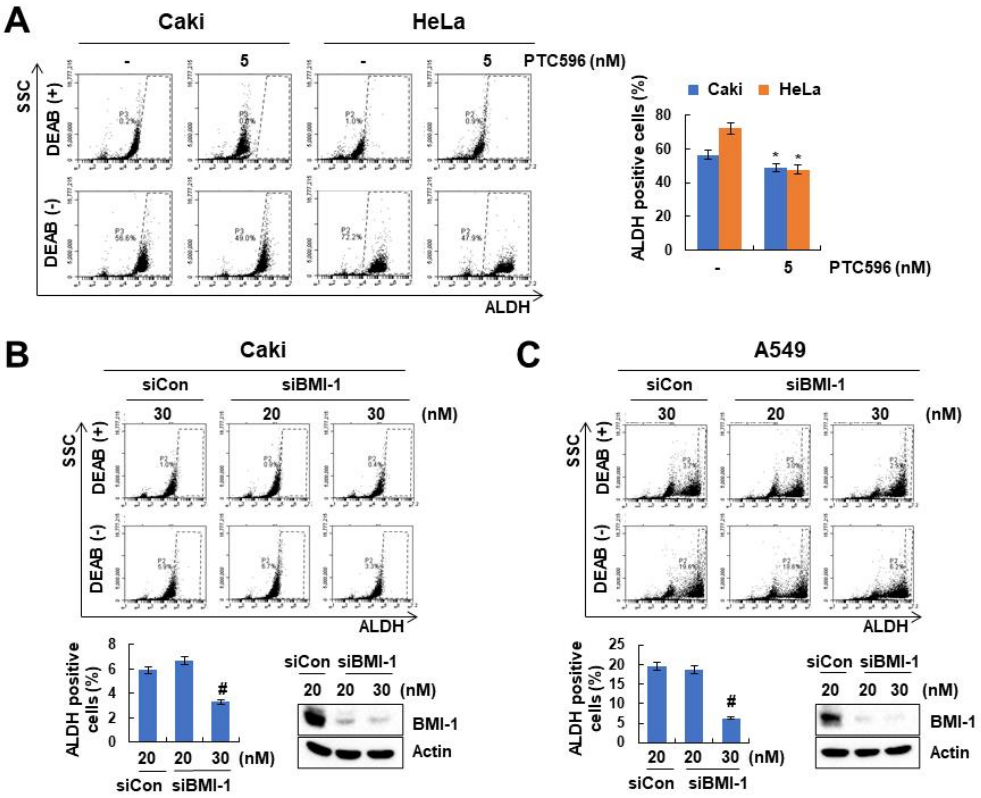

Figure 1. Inhibition of BMI-1 decreases cancer cell stem-like cell population. (A) Caki and HeLa cells were treated with BMI-1 inhibitor PTC596 (1, 2, 5 nM) for 12 h. (B,C) Caki and A549 cells were transfected with siCon or siBMI-1 siRNA for 24 h. The population of stem-like cells were detected by flow cytometry and the protein expression was quantified by Western blotting (A,B). $p^{*} < 0.01$ compared to the control. $p^{\#} < 0.01$ compared to the siCon.

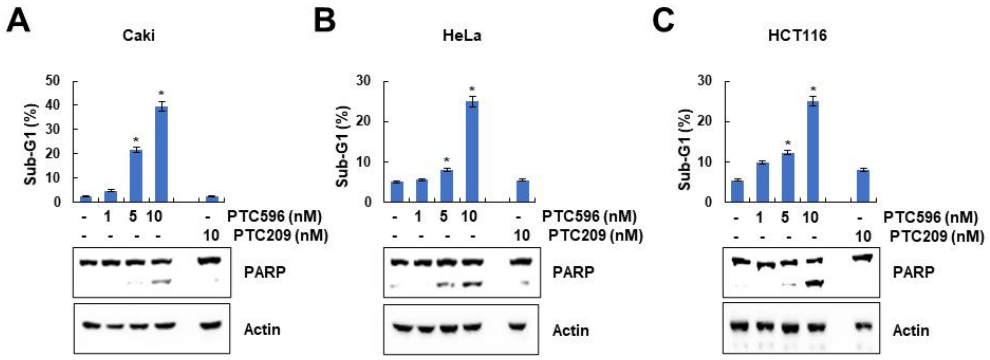

Figure 2. Effects of BMI-1 inhibitors on apoptosis in various cancer cells. Caki (A), HeLa (B) and HCT116 (C) cells were treated with PTC596 (1, 5, 10 nM) or PTC209 (10 nM). The sub-G1 population and protein expression were measured by flow cytometry and Western blotting, respectively. The values in graph (A-C) represent the mean ± SD of three independent samples. $p^* < 0.01$ compared to the control.

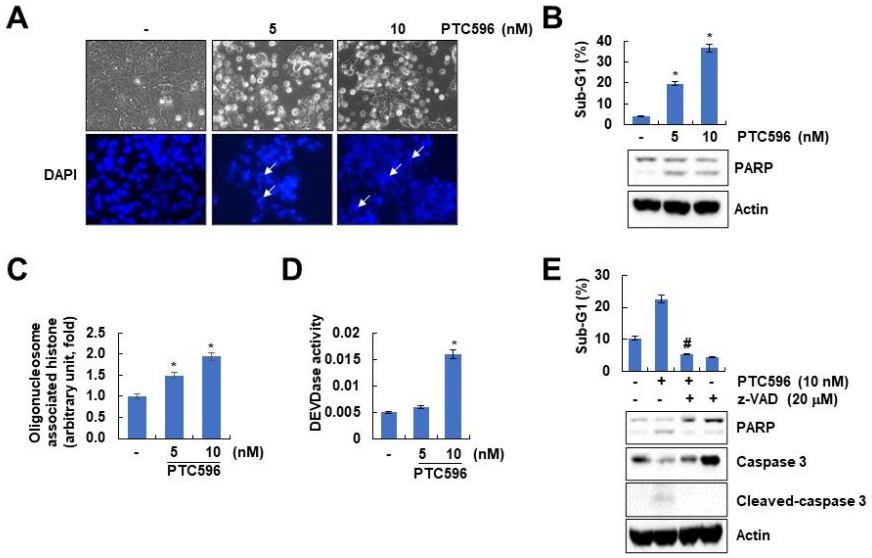

Figure 3. PTC596 induces apoptosis through a caspase-dependent manner. (A-D) Caki cells were treated with PTC596 (5, 10 nM) for 24 h. The cell morphology and nuclear condensation were examined by using microscope and by 4′, 6′-diamidino-2-phenylindole (DAPI) staining, respectively (A). The sub-G1 population and protein expression were measured by flow cytometry and Western blotting, respectively (B). The fragmentation of the nuclei was examined by using a DNA fragmentation assay kit (C). The caspase-3 activity was detected by DEVDase assay (D). (E) Caki cells were pre-treated with 20 μM z-VAD-fmk (zVAD) for 30 min then treated with 10 nM PTC596 for 24 h. The sub-G1 population and protein expression were measured by flow cytometry and Western blotting, respectively. The values in graph (E) represent the mean ± SD of three independent samples. $p^{*} < 0.01$ compared to the control. $p^{\#} < 0.01$ compared to the treatment of PTC596.

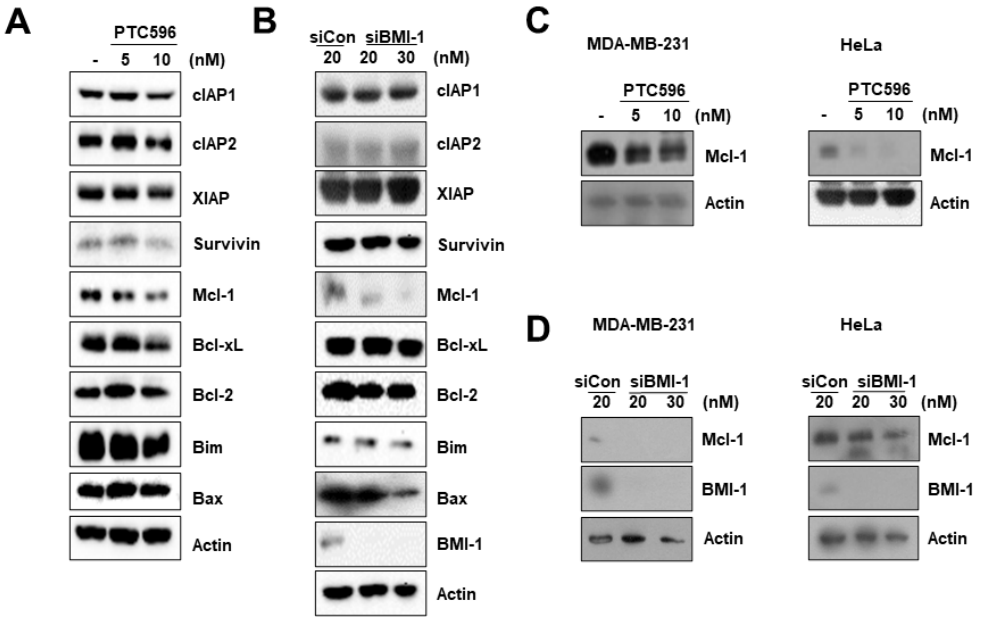

Figure 4. Inhibition of BMI-1 induces down-regulation of Mcl-1 expression in various cancer cells. (A,B) Caki were treated with PTC596 (5, 10 nM) (A) for 24 h or transfected with siCon or siBMI-1 siRNA (B) for 48 h. (C,D) MDA-MB-231 and HeLa cells were treated with PTC596 (5, 10 nM) (C) for 24 h or transfected with siCon or siBMI-1 siRNA (D) for 48 h. The expression levels of apoptosis related proteins were measured by Western blotting (A-D).

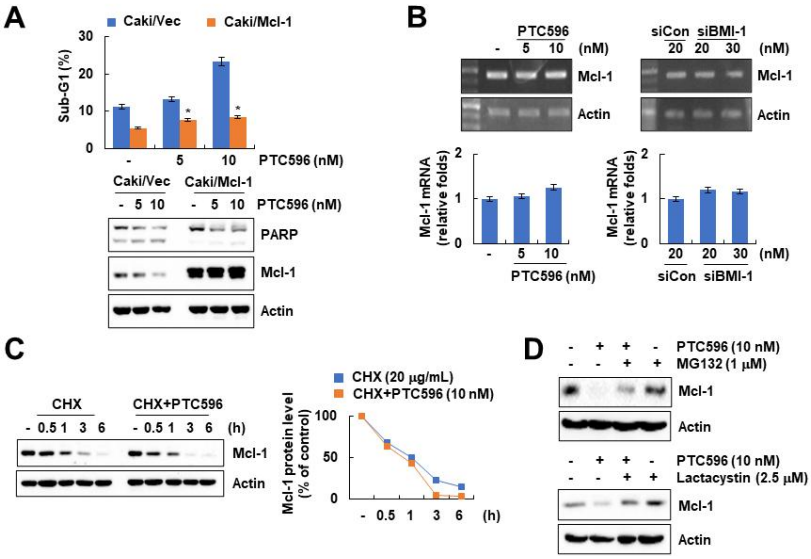

Figure 5. Down-regulation of Mcl-1 is involved in PTC596-induced apoptosis. (A) Vector cells (Caki/Vec) and Mcl-1 overexpressed cells (Caki/Mcl-1) were treated with PTC596 (5, 10 nM) for 18 h. (B) Caki cells were treated with PTC596 (5, 10 nM) for 24 h or transfected with siCon or siBMI-1 siRNA for 48 h. (C) Caki cells were treated with or without PTC596 (10 nM) in the presence of cycloheximide (20 μg/mL) for the indicated time periods. (D) Caki cells were pretreated with MG132 (1 μM) and lactacystin (2.5 μM) for 30 min, then treated with PTC596 (10 nM) for 24 h. The sub-G1 population and protein expression were measured by flow cytometry and Western blotting, respectively (A,C,D). The mRNA levels of Mcl-1 and actin were quantified by reverse transcription polymerase chain reaction and real-time PCR (B). The band intensity was examined by using Image J (C). The values in graph (D) represent the mean ± SD of three independent samples. $p^* < 0.01$ compared to the control in Caki/Vec cells.

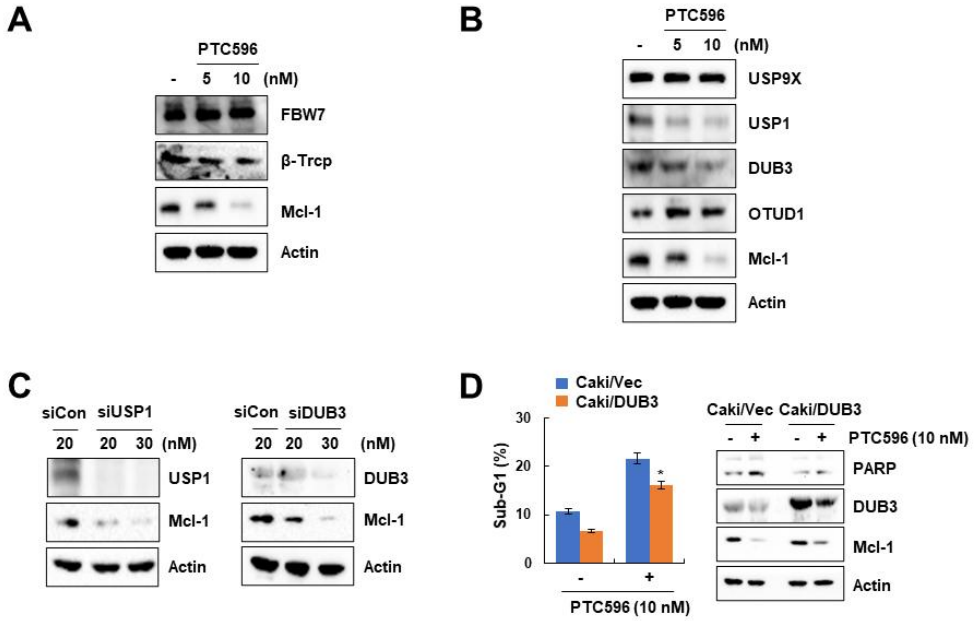

Figure 6. Inhibition of DUB3 is associated with PTC596-induced Mcl-1 down-regulation and apoptosis. (A,B) Caki cells were treated with PTC596 (5, 10 nM) for 24 h. (C) Caki cells were transfected with siCon, siUSP1 or siDUB3 siRNA for 48 h. (D) Caki cells were transfected with Caki/Vec or Caki/DUB3 plasmids for 24 h, and then treated with PTC596 (10 nM) for 24 h. The sub-G1 population and protein expression were measured by flow cytometry and Western blotting, respectively (A-D). $p^{*} < 0.01$ compared to the PTC596 treatment in Caki/Vec cells.

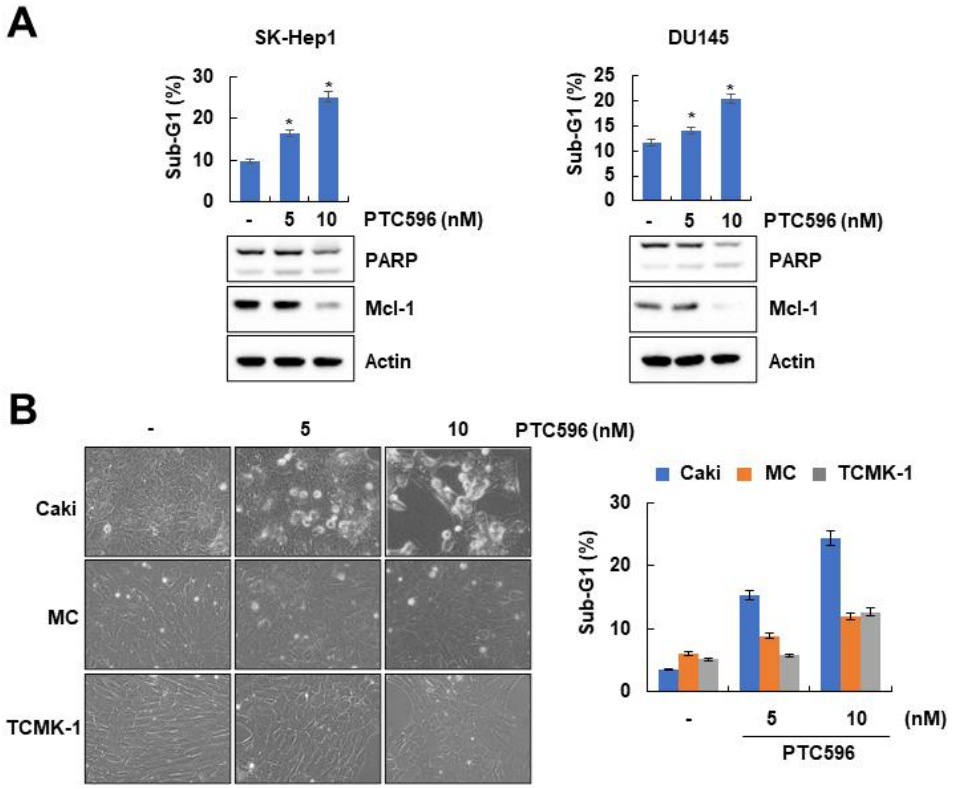

Figure 7. Apoptotic effect of PTC596 in various cell lines. (A,B) SK-Hep1, DU145 cells (A) and normal cells (MC and TCMK-1 cells) (B) were treated with PTC596 (5, 10 nM) for 24 h. The sub-G1 population and protein expression were measured by flow cytometry and Western blotting, respectively (A,B). Cell morphology was measured by interference light microscope (B). $p^{*} < 0.01$ compared to the control.

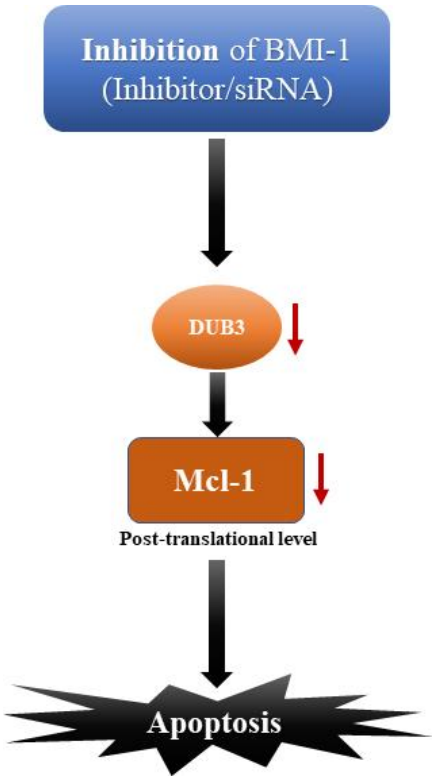

Figure 8. Schematic diagram revealing the molecular mechanism of PTC596-induced apoptosis. Inhibition of BMI-1 by PTC596/siRNA induces cancer cell death through DUB3-mediated Mcl-1 down-regulation.

4. Discussion

Previous studies have identified that BMI-1 is involved in cancer stem cells and participated in various cancer cell death (16,32-34). However, the molecular mechanism for the therapeutic effect of inhibition of BMI-1 remains unclear.

I found that inhibition of BMI-1 by siRNA or inhibitor could effectively induce cancer cell apoptosis and decrease cancer cell stem-like cell population. Li *et al.* identified that BMI-1 regulated stem cell-like properties by miR-27a and miR-155 in gastric cancer cells (35). Similarly, Koh *et al.* also showed miR-128 is involved in suppression of lung cancer stem cells by inhibiting BMI-1 (36). As shown in Figure 1, PTC596 and knockdown of BMI-1 reduced the cancer cell stem-like cell population in multiple cancer cell lines (Caki, HeLa and A549). Multiple BMI-1 inhibitors such as QW24, PTC209 and PTC596 were identified (9,37,38). Treatment of PTC596 increased the sub-G1 population and PARP cleavage at lower concentration (5 nM) rather than other BMI-1 inhibitors (<50 nM) (Figure 2). Therefore, PTC596 was used for investigating the anticancer mechanism in the present study.

Notably, resent studies have identified that PTC596 induces apoptosis and down-regulation of Mcl-1 expression (21,39,40). However, how to regulate Mcl-1 expression by PTC596 have not been investigated. I found that PTC596 and BMI-1 siRNA suppressed Mcl-1 protein level, but not Mcl-1 mRNA level (Figure 4&5B). Moreover, proteasome inhibitors attenuated PTC596-induced down-regulation of Mcl-1 expression (Figure 5D). Therefore, inhibition of BMI-1 decreased Mcl-1 expression at the post-translational level. In UPS, E3 ubiquitin ligases and deubiquitinases play crucial roles in protein degradation. Previous

studies suggested that several E3 ligases (FBW7 and β-TrCP) and deubiquitinases (USP1, USP9X, DUB3 and OTUD1) are involved in Mcl-1 down-regulation (27-31,41). Chen *et al.* explored that knockdown of FBW7 markedly increased Mcl-1 expression resulting the reduction of cell death in U87 cells (27). USP1 and DUB3 can regulate Mcl-1 protein degradation in colorectal and ovarian cancer, respectively (29,31). As shown in Figure 6, E3 ligases (FBW7 and β-TrCP) of Mcl-1 were not altered by PTC596, whereas USP1 and DUB3 were decreased. Furthermore, knockdown USP1 and DUB3 reduced Mcl-1 expression (Figure 6C). Ectopic expression of DUB3 blocked PTC596-induced apoptosis and Mcl-1 down-regulation (Figure 6D).

Previous studies demonstrated that BMI-1 depletion induced PH domain, leucine-rich repeat protein phosphatase 1/2 expression (PHLPP1 and PHLPP2) and dephosphorylation of Akt in endometrial cancer and leukaemia stem cells (42,43). In my study, I checked AKT signaling pathway in PTC596-treated MDA-MB-231 cells and found that PTC596 decreased phosphorylation of AKT. According to that the mechanism between PTC596-induced apoptosis and AKT signaling pathway need to further study.

Collectively, this study illustrated that BMI-1 inhibitor PTC596 and siRNA induce apoptosis through DUB3-mediated Mcl-1 degradation. Therefore, PTC596 may be a potential drug for cancer therapy in the future.

5. Summary

BMI-1 is a polycomb oncogene, which highly expressed in various types of cancer cells. Expression of BMI-1 is related to cancer cell invasion, proliferation, distant metastasis, apoptosis. However, the function of BMI-1 in cancer cells is not fully understood. This study demonstrated that inhibition of BMI-1 by siRNA or inhibitor PTC596 induces apoptosis in many cancer cells, but not in normal cells. Moreover, inhibition of BMI-1 down-regulated Mcl-1 expression by reducing the deubiquitinase DUB3 expression. Furthermore, overexpression of Mcl-1 and DUB3 prevented PTC596-induced apoptosis. These results provided strong evidences for inhibiting BMI-1 induces apoptosis through DUB3-dependent Mcl-1 degradation in various cancer cell lines.

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The BMI-1 Inhibitor, PTC596, Induces Apoptosis through Down-regulation of Mcl-1 Expression in Cancer Cells

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(Abstract)

BMI-1, which is known as a polycomb ring finger oncogene, has been found highly expressed in multiple cancer cells, which is also involved in cancer cell proliferation, invasion and apoptosis. More importantly, the regulation of self-renewal of stem cells are associated with the expression of BMI-1. In this study, siRNA-mediated knockdown of BMI-1 expression and a novel molecular inhibitor PTC596 enhanced cancer cells apoptosis and decreased cancer stem-like cell population. PTC596 and BMI-1 siRNA decrease Mcl-1 protein expression through the down-regulation of DUB3 expression. Moreover, ectopic expression of Mcl-1 and DUB3 inhibited PTC596-induced apoptosis. In summary, inhibition of BMI-1 may be a potential therapeutic strategy against cancer.

BMI-1 저해제 PTC596에 의한 암세포사멸 유도 기전 규명

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(초록)

종양 및 혈액세포에서 발견되는 암줄기세포는 일반 줄기세포와 같이 자기재생 능력, 암 형성 능력 및 약물 저항성을 지니기 때문에 암세포가 항암제에 대한 저항성을 가지게 되어 종양 재형성에 관여한다. Polycomb ring finger 종양 유전자로 알려진 BMI-1은 종양세포에서 발현이 높으며, 암줄기세포의 재생 능력 조절을 통해 암세포 증식 및 전이 유도에 관여한다. 따라서 BMI-1 발현 억제는 암줄기세포의 증식 및 자가 재생능력을 억제함에 따라 종양 형성 및 암세포사멸을 유도할 수 있다. 본 연구에서는 BMI-1 발현 및 활성 억제에 따른 다양한 암세포사멸의 유도 기전에 대해 조사하였다. BMI-1 활성 저해제인 PTC596과 siRNA를 이용한 BMI-1 발현 억제는 다양한 암세포에서 사멸을 유도하며, Bcl-2 부류 단백질 중 항세포사멸 기능을 가진 Mcl-1이 프로테아좀 의존적인 경로를 통하여 억제된다. 흥미롭게도 유비퀴틴-프로테아좀 시스템에 중요하게 관여하는 탈유비퀴틴화 효소인 DUB3에 의해 Mcl-1이 분해되며, Mcl-1 및 DUB3 과발현은 PTC596

에 의한 암세포사멸을 억제한다. 이상의 연구 결과를 통하여 BMI-1 활성 및 발현 억제는 DUB3 매개 Mcl-1의 단백질 분해를 통하여 암세포사멸을 증진시키며 이는 BMI-1이 암 치료에 있어 중요한 표적임을 제시한다.