YM155 induces apoptosis through downregulation of specificity protein 1 and myeloid cell leukemia-1 in human oral cancer cell lines

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BACKGROUND: YM155 is a small-molecule pro-apoptotic agent which has shown to inhibit survivin expression and induce apoptosis in various cancer cells. In this study, we investigated the function and molecular mechanism of YM155 in human oral cancer cells.

METHODS: The apoptotic effects and related signaling pathways of YM155 were evaluated using trypan blue exclusion assay, 4',6-diamidino-2-phenylindole staining, Western blotting, RT-PCR, and siRNA.

RESULTS: YM155 inhibited the growth and caused caspase-dependent apoptosis in MC3 and HN22 cells. YM155 significantly suppressed the level of survivin protein expression through proteasome-dependent protein degradation to confirm its survivin-inhibiting function. YM155 reduced myeloid cell leukemia-1 (Mcl-1) protein, but it did not alter Mcl-1 mRNA. It was associated with the facilitation of lysosome-dependent protein degradation. The modifications of Mcl-1 and survivin by YM155 were caspase-independent manner. Treatment of MC-3 and HN22 cells with YM155 inhibited specificity protein 1 (Sp1) and the knockdown of Sp1 by siRNA demonstrated that Mcl-1 was regulated by Sp1 protein.

CONCLUSIONS: We demonstrated the novel mechanism that YM155 causes apoptosis of human oral cancer cell lines through downregulation of Sp1 and Mcl-1. Therefore, it may be a potential anticancer drug candidate for the treatment of oral cancer.

Keywords: apoptosis; myeloid cell leukemia-1; oral cancer; specificity protein 1; YM155

Introduction

Various prevention and treatment modalities are reestablished for cancer treatment; however, the number of new cases is still increasing annually. Oral cancer is one of the most frequent cancers worldwide, the sixth most common, and incidence rates are higher in men than women (1, 2). Recently, early detection and screening programs have decreased the mortality rates, and it can be potentiated by chemotherapy. Thus, discovering new treatment to control oral cancer has been pursued by many researchers.

YM155, also called as a sepantronium bromide, is an imidazolium derivative that represses survivin expression and induces apoptosis in a variety of other tumor cell lines such as prostate, colon, and lung cancer (3, 4). Although survivin is a smallest member of inhibitor of apoptosis (IAP) gene family, it is expressed during embryonic development and is truant in most normal tissues. In a context for decreasing cancer cells selectively while normal cells are not disturbing, survivin becomes an ideal target for cancer therapy. An attention has been growing due to its overexpression in human tumors universally, its prominent role in networks of cellular division, intracellular signaling, and anti-apoptosis. (5–7).

Specificity protein 1 (Sp1) is a zinc-finger-type DNA-binding domain that binds to guanine–cytosine (GC)-rich motifs and is involved in many cellular processes such as tumor cell growth, survival, angiogenesis, and apoptosis (8–11). Also, the expression of Sp1 is highly dominated in many tumor and cancer cell lines. Previously, our group had identified that decreasing Sp1 level regulates the expression of anti-apoptotic proteins, such as survivin and myeloid cell leukemia-1 (Mcl-1) in prostate cancer cell lines (12). It has been recommended that targeting Sp1 and its downstream target protein may have a potential for oral cancer therapy.
In this study, we evaluated the apoptotic effect of YM155 on oral cancer cells and examined the effect of YM155 on expression of various pro and anti-apoptotic proteins. We provided the evidence that YM155 downregulates survivin and Sp1 protein expression in oral cancer cells. Taken together, these findings provide a framework for understanding the mechanism of action about YM155 against the human oral cancer cells.

**Material and methods**

**Reagents and antibodies**

YM155 was purchased from Selleck chemicals (Houston, TX, USA). 4′-6-Diamidino-2-phenylindole (DAPI), chloroquine (CQ), and cycloheximide (CHX) were obtained from Sigma–Aldrich chemical Co. (St Louis, MO, USA). Antibody against Cytochrome-C was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against cleaved caspase-3, cleaved PARP, surviving, Mcl-1, Bcl-xl, Bak, Bcl-2, Bad, p-STAT3, STAT3, p-ERK, and ERK were supplied by Cell signaling Technology, Inc. (Charlottesville, VA, USA). The pan-caspase inhibitor, z-VAD, was purchased from R&D systems (Minneapolis, MN, USA).

**Cell culture and drug treatment**

MC3 human oral cancer cell line was obtained from College of Stomatology, Fourth Military Medical University (Xi’an, China), and HN22 human oral cancer cell line was kindly gifted from Dankook University (Cheonan, Korea). Both cells were cultured in Dulbecco’s Modified Eagle Medium supplement with 10% fetal bovine serum (FBS) and 100 U/ml each penicillin at 37°C under 5% CO₂ in a humidified atmosphere. Exponentially growing cells were exposed to various concentrations of drugs for indicated time periods.

**Trypan Blue exclusion assay**

To examine the cell viability effect of YM155 on the cancer cells, we performed the trypan blue exclusion assay with trypan blue (0.04%). After YM155 treatments for 24 h, cells were counted using a hemocytometer and data were expressed as the percentage of cell viability compared to that of the control. Each experiment was carried out in triplicate, and the results were expressed as the mean ± SD for each treatment group.

**DAPI staining**

The apoptotic cells with chromatin condensation and fragmentation were visualized using fluorescence nuclear dye, DAPI. Both MC3 and HN22 cells were treated with various dose of YM155, harvested by trypsinization, and suspended in PBS. The cells were fixed in 100% methanol at room temperature for 10 min, deposited slides, and then stained with DAPI solution (2 μg/ml). Apoptotic cells were assessed by a fluorescence microscope and pictured.

**Western blot analysis**

Whole cell lysates were extracted with lysis buffer, and protein concentrations were measured using a DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Equivalent samples (20 μg of protein) were subjected to SDS-PAGE, and the proteins were transferred to polyvinylidene fluoride (PVDF) membrane. After blocking with 5% skim milk in tris-buffered saline Tween-20 (TBST) for 2 h at room temperature, the membrane was probed with the specific primary antibody for overnight at 4°C. After washing the membrane with TBST buffer, they were incubated with horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. Protein of interest was detected with ECL reagents (Santa Cruz, CA, USA).

**Preparation of cytosolic and mitochondrial fractions**

After the treatment of MC3 cells with YM155, cytosolic and mitochondrial fractions were separated and extracted from digitonin or triton X-100 permeabilization according to the protocol of Zong et al. (13). MC3 cells were briefly washed with PBS, and the pellets were resuspended in plasma membrane extraction buffer (a 250 mM sucrose solution containing 10 mM HEPES [pH 7.5], 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, and 1 μg/ml aprotinin) containing 0.05% digitonin at room temperature for 1 min. The supernatant was separated from pellet by the centrifugation for 5 min at 13 000 g rpm. Then, the supernatant-containing cytosolic proteins were isolated, and the mitochondrial pellet was collected. The mitochondrial protein was resuspended by plasma membrane extraction buffer containing 0.5% triton X-100. The mitochondrial proteins were isolated from the last centrifugation, and result was analyzed using Western blotting with appropriate mitochondrial and cytosolic marker, respectively.

**Reverse transcription–polymerase chain reaction (RT-PCR)**

Total RNA from MC3 cells treated with various dose of YM155 for 24 h was isolated using easy-BLUE total RNA extraction kit (iNTRON, Daejeon, Korea). Then, the cDNA was synthesized from total RNA using the ImProm-II reverse transcription system (Promega, Madison, WI, USA). Survivin, Mcl-1, and β-actin were amplified by PCR using specific primers; Survivin: 5′-ATG GCC GAG GCT GGC TTC ATC-3′ (S), 5′-ACG GC CAC TTT CTT CGC AGT T-3′ (AS), Mcl-1: 5′-TGC TGG AGT TGG TCG GGG AA-3′ (S), 5′-TGC TAA GGT CTC CGC CT-3′ (AS), β-actin: 5′-GTG GGC CCC AGG CAC CA-3′ (S), 5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′ (AS), respectively. Survivin amplification was performed in 30 cycles, Mcl-1 amplification was performed for 28 cycles (1 min at 95°C, 1 min at 60°C, and 1 min 30 s at 72°C), and β-actin amplification was performed for 25 cycles (1 min at 95°C, 1 min at 60°C, and 1 min 30 s at 72°C). PCR products were analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining with UV detector.

**Small interfering RNA (siRNA) transfection**

Cells were grown in a 6-well plate, and siRNA transfection was performed according to the manufacturer’s instruction. On TARGET plus, SMART pool small interfering RNA (siRNA) sequences targeting Sp1 (5′-GGUAGCUCUAA GUUUGAATT-3′) (14) and non-targeting negative control were purchased from Dharmacon Research (Lafayette, CO, USA).
CO, USA). MC3 cell was transfected with 50 nM siRNA for 48 h, and then, the subsequent protein expressions were analyzed by Western blotting.

**Statistical analysis**

Each protein expression level was quantified using the TINA software and expressed as mean ± SD. After the data collection, it was compared with the Student’s t-test to determine the differences between the control and treated groups; P value of <0.05 was considered statistically significant.

**Results**

**YM155 exhibits potent antiproliferative activity and induces apoptosis against human oral cancer cells**

The antiproliferative activity of YM155 was examined using trypan blue exclusion assay upon the treatment with DMSO or various doses of YM155 (2.5, 5, 10 nM). The result showed that YM155 inhibited the cell growth of MC3 in a concentration-dependent manner (Fig. 1A). Then, the apoptotic effect of YM155 was further determined by DAPI staining and subsequent Western blot analysis. Nuclear condensation and fragmentation was clearly observed in a concentration-dependent manner when the YM155 exposed cells by visualizing with DAPI staining (Fig. 1B). When we checked the expression of cleaved PARP and caspase-3 activation, YM155 induced spontaneous apoptosis in oral cancer cell (Fig. 1C). To verify the involvement of caspase-3 in YM155-induced apoptosis, we utilized the pan-caspase inhibitor; z-VAD. The result suggested that YM155 induce caspase-dependent apoptosis as revealed by the inhibition of PARP cleavage, caspase-3 activation when the presence of z-VAD (Fig. 1D). Then, we investigated the release of Cytochrome-C from the mitochondria to cytosol upon YM155 treatment. As compared each fractionation marker expression (Cox-4 and α-tubulin, respectively), YM155 showed the release of Cytochrome-C from mitochondria to the cytosolic fraction (Fig. 1E). Collectively, these results support that YM155 decreased cell viability and induced apoptosis in MC3 human oral cancer cells.

**YM155 inhibits survivin protein expression and induces its proteasomal degradation**

When we examined the effect of YM155 on survivin expression, YM155 (2.5, 5, 10 nM) exposed MC3 cell for 24 h inhibited survivin expression in a concentration-dependent manner (Fig. 2A). However, as shown in Fig. 2B, the survivin mRNA expression was barely changed by YM155 treatment. To further investigate the regulation of survivin protein by YM155 treatment, we assessed the effect of YM155 on protein degradation using the protein synthesis inhibitor, cycloheximide (CHX) or proteasome inhibitor, MG132. The survivin protein expression was significantly decreased in the presence of CHX with YM155 compared with the third lane in Fig. 2A which means YM155 reduces the de novo synthesis of surviving (Fig. 2C). We also analyzed notably recovered survivin expression inhibited by YM155 when we inhibited proteasomal degradation by MG132 (Fig. 2D).
together, findings suggest that YM155 may regulate survivin protein through proteasome-dependent protein’s degradation pathway.

**YM155 downregulates survivin inhibitor, Mcl-1 protein, in MC3 cells via lysosomal-dependent degradation**

It has been reported that myeloid cell leukemia (Mcl-1) is an important survival factor for malignant tissues (15). Thus, we also expanded our investigation to dissect the effect of YM155 on Mcl-1 expression in MC3 cells. Only Mcl-1 protein levels decreased significantly when the YM155 was applied dose dependently (Fig. 3A), but Mcl-1 mRNA level was not altered (Fig. 3B). Thus, Mcl-1 protein turnover rate in MC3 cell right after protein synthesis might have been abrogated by CHX. As shown in Fig. 3C, Mcl-1 degradation was greatly increased in the presence of CHX with YM155. Further, to investigate whether YM155 effects on Mcl-1 degradation was proteasome or lysosome dependent, we performed Western blot analysis in MC3 cells after treating with proteasome inhibitor MG132 and lysosome inhibitor chloroquine (CQ). The result showed that addition of MG132 to YM155-treated MC3 cells was ineffective. However, Mcl-1 expression was restored when lysosomal degradation was inhibited by CQ (Fig. 3D). We further investigated the effect of YM155 on the various expressions of anti-apoptotic and pro-apoptotic proteins in MC3 cell. There was no change in Bcl-xl, Bak, Bcl-2, Bax and Bad (Fig. 3E). Further, pre-treatment of MC3 with z-VAD, pan-caspase inhibitor with subsequent 1 h treatment of YM155, showed no alteration in the expression of Mcl-1 level. In contrast, survivin level was decreased (Fig. 3F). Therefore, reduction of the survivin protein expression by YM155 was not caspase-dependent apoptosis but lysosomal-dependent degradation.

**Downregulation of Sp1 by YM155 and Sp1 siRNA regulates Mcl-1 in MC3 cells**

Development of various types of cancers and other diseases is the result of the activation and abnormal expression of Sp1 (16). Thus, we further examined the effect of YM155 on Sp1 expression in MC3 cells. The result supported that YM155 dose-dependently decreased Sp1 expression (Fig. 4A). Then, we further conducted Sp1 siRNA transfection for 48 h. Marked Sp1 knockdown was noted with decreased in Mcl-1 expression in 48 h after transfection. No decrease in the expression of survivin was noted in response to Sp1 siRNA transfection (Fig. 4B). Exposure of MC3 cells to YM155 for 24 h did not affect the protein kinases expressions including p-STAT3, STAT3, p-ERK, and ERK (Fig. 4C) which indicated that YM155 specifically inhibits Sp1 expressions.

**YM155 inhibits the growth of other cancer cell and induces apoptosis**

To determine induction of universal cell death by YM155 in other oral cancer cell, HN22 cells were treated with YM155 for 24 h. Likewise in MC3 cells, cell viability was assessed with trypan blue exclusion assay and the triggering of apoptosis with DAPI and subsequent Western blot analysis. YM155 at concentration of 15, 30, 60, and 120 nM significantly decreased the viability of HN22 cells in a concentration-dependent manner (Fig. 5A). When the HN22 cells were exposed to YM155, the hallmark for apoptosis which condensation and fragmentation of nuclei and PARP cleavage and caspase-3 activation was clearly increased (Fig. 5B,C). As shown in Fig. 5D, the expression levels of survivin, Mcl-1, and Sp1 were significantly decreased in HN22 cells after the exposure with YM155. These results suggest that YM155 induces apoptosis of other human oral cancer cells.
Discussion
As we all know, the aberrant cell proliferation and apoptosis resistance are one of the hall phenotypes of cancer cells (17), survivin becomes a novel target for anticancer therapy because it is highly expressed in a broad range of tumors tissues (18–20). Increased expression of survivin in cancer leads to decreased overall survival in several malignancies, including non-small cell lung (21–23), gastric (24–27), colorectal (28–30), breast carcinoma (31), neuroblastoma (32), prostate cancer (33), pancreatic cancer (34), hepatocellular carcinoma (35), and hematologic malignancies (36, 37). Recently, several groups reported phase I and phase II trials of YM155 as a small-molecule suppressor of survivin in patients with advanced solid tumors showing the possibility of its clinical application (38, 39). Although YM155 may have potential benefits for the treatment of various cancers, the anticancer activity of YM155 against oral cancer cells has not yet been established. In the present study, we have examined that whether the novel agent, YM155 may inhibit the expression of survivin in human oral cancer cells. We found that YM155 potently inhibited
the cell growth of MC3 and HN22 human oral cancer cells. As well as, our data indicate that YM155 exhibits cytotoxic activity through downregulation of survivin and induces apoptosis by chromatin condensation and nuclear fragmentation and cleavage of PARP protein. Taken together, these findings suggest that YM155 may be a hopeful candidate for oral therapy as a novel apoptosis inducer.

Irregular expression of Mcl-1, a member of the B-cell lymphoma-2 (Bcl-2) family protein, has been recognized as one of causative proteins in many tumors. (40–42). Downregulation of Mcl-1 might reinforce to increase the apoptotic stimuli in various cancers cells (43). In this study, when we investigated the effect of YM155 on Mcl-1 expression, we found that YM155 concentration dependently decreased Mcl-1 expression in oral cancer cells. However, the Mcl-1 mRNA level was barely changed when we further evaluated. It has been reported that the regulation of Mcl-1 protein can be degraded by the proteasome system. (44, 45). Undeviating with these findings, YM155 treatment shows inhibition of protein synthesis by cycloheximide. In addition, the pre-treatment of chloroquine to MC3 cells blunted YM155 provoked the decrease in Mcl-1 level, whereas cotreatment with MG132 and CQ were ineffective which means enhanced lysosomal degradation may be dominant (46). It is also supported that YM155 decreased the interaction between survivin and XIAP and then eventually induced lysosomal degradation. Corresponding with previous findings, our result also confirmed that YM155 may modulate Mcl-1 protein through lysosomal-dependent degradation pathway.

Previously, many reports have shown that Sp1 is related with tumor growth and is overexpressed in innumerable cancer cells (47, 48). Aberrant expression of Sp1 contributes to the development of various types of cancers and other diseases (16). When we explored to determine the effect of YM155 on Sp1 in MC3 and HN22 cell line whether Sp1 is involved in oral cancer development, we found that YM155 decreases Sp1 expression concentration dependently in both MC3 and HN22 cell lines. YM155 modulates the Sp1 protein expression; it becomes necessary to determine the response on key candidates and its downstream signaling pathway. Therefore, the effects of Sp1 by siRNA transfection were examined for checking Mcl-1 and survivin proteins expression. We found that siSp1 induced downregulation of Mcl-1 but not that of survivin. RNA interference experiments using Sp1 siRNA are indicating that Sp1 regulates only Mcl-1 protein but did not diminish the surviving expression. It is also suggesting that survivin may not be a downstream target of Sp1. Furthermore, we examined the effects of YM155 on other kinase proteins, and the result showed that there was no significant decrease in expression of other kinase proteins.

Collectively as we illustrated in summary Fig. 6, the present study demonstrates that YM155 treatment resulted in apoptosis and inhibition of cell proliferation through the downregulation of survivin, Sp1 and Mcl-1 in MC3 and HN22 cells. Moreover, Mcl-1 was identified as a key downstream molecule of Sp1. Our data suggest that Mcl-1 may play an important role as a down-stream regulator for Sp1. Therefore, we suggest that YM155 is a promising anticancer drug candidate for the treatment of oral cancer.

References


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