Proteasome inhibitor MG132 potentiates TRAIL-induced apoptosis in gallbladder carcinoma GBC-SD cells via DR5-dependent pathway

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Abstract. TRAIL is a tumor-selective apoptosis-inducing cytokine playing a vital role in the surveillance and elimination of some tumor cells. However, some tumors are resistant to TRAIL treatment. Proteasome inhibitor MG132 exhibits anti-proliferative and pro-apoptotic properties in many tumors. In this study, we demonstrated that proteasome inhibitor MG132 *in vitro* and *in vivo* potentiates TRAIL-induced apoptosis in gallbladder carcinoma GBC-SD cells. MG132 was able to inhibit the proliferation of GBC-SD cells and induce apoptosis in a dose-dependent manner. The induction of apoptosis by proteasome inhibitor MG132 was mainly through the extrinsic apoptotic pathways of caspase activation such as caspase-8, caspase-3 and PARP cleavage. In addition, this process was also dependent on the upregulation of death receptor 5 (DR5), which promoted TRAIL-induced apoptosis in GBC-SD cells. Taken together, these findings indicate that MG132 possesses anti-gallbladder cancer potential that correlate with regulation of DR5-dependent pathway, and suggest that MG132 may be a promising agent for sensitizing GBC-SD cells to TRAIL-induced apoptosis.

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Abbreviations: GBC, gallbladder cancer; TNF, tumor necrosis factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; DISC, death inducing signaling complex; UPS, ubiquitin-proteasome system

Key words: gallbladder cancer, proteasome inhibitor, TRAIL, DR5, apoptosis

Introduction

Gallbladder cancer (GBC) is the most common, aggressive malignancy of the bile duct worldwide, showing an increasing tendency in its incidence and mortality, and associated with extremely poor prognosis (1,2). The 5-year survival rate has improved due to recent advances in diagnostic and therapeutic approaches, but the prognosis is still poor for the patients with an advanced stage, high local recurrence and distant metastasis. Because of low surgical resection rate and serious side effects caused by ineffective chemotherapy and radiation therapy, the majority of patients now have unsatisfactory results (3,4). Therefore, new effective strategies for this lethal neoplasm is urgently needed.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of the tumor necrosis factor (TNF) family, has the ability to induce apoptosis in transformed, but not normal cells, via extrinsic signaling pathways (5,6). TRAIL initiates cell death upon binding to death receptor DR4 (TRAIL-R1) and/or DR5 (TRAIL-R2) on the surface of cancer cells, which subsequently causes the formation of DISC (death inducing signaling complex) and caspase cascades (7). Caspase-8 is activated in the death receptor signaling pathway due to its death domain (8). The active caspase-8 cleaves and directly activates downstream effector caspase-3 and PARP, and finally initiates caspase-dependent apoptosis (9). TRAIL therapy has emerged as a promising cancer therapeutic strategy. However, some tumor cells can downregulate the expression of TRAIL-receptors and acquire resistance to TRAIL-induced apoptosis (10,11). As such, TRAIL combination treatments that can upregulate TRAIL-receptor expression or inhibit downregulation were proved to be of therapeutic benefit (12-14).

The ubiquitin-proteasome system (UPS) plays a vital role in the regulation of protein levels in eukaryotic cells, and related to cell proliferation, survival, differentiation and programmed cell death (15,16). The activity of UPS is closely associated with the incidence and progression of many human

malignant tumors. Now, targeting the proteasome is evidenced to be one of the most effective strategies for cancer treatment (17). MG132, a reversible peptide-aldehyde of specific proteasome inhibitor (Fig. 1A), can effectively inhibit the activity of 26S proteasome and cause accumulation of certain proteins harmful to the survival of tumor cells, inducing cell cycle arrest and cell apoptosis (18-20). Evidence has shown that MG132 could inhibit the proliferation of tumor cells in a dose-dependent manner in cell cycle transformation, and with the cell cycle protein Cyclin A, Cyclin B and P27 accumulation, and cell cycle arrests in G2/M phase (21). Likewise, it has been reported that MG132 induced apoptosis of tumor cells by caspase-related signal transduction pathway mediated by death receptor (18). To date, the effect of proteasome inhibitor MG132 on GBC still remains unclear, and also the effect on TRAIL-induced apoptosis in GBC remains unknown. Therefore, it may be more promising to uncover the mechanism that links proteasome dysfunction caused by MG132 and TRAIL-induced apoptosis to the treatment of GBC.

In this study, we investigated the effect of the representative proteasome inhibitor MG132 on TRAIL-induced apoptosis in GBC-SD cells *in vitro* and *in vivo*, and further elucidated the mechanism of the cell apoptosis induction. Our data evidenced that MG132 can inhibit the proliferation of GBC-SD cells and induce apoptosis. The induction of apoptosis by MG132 was mainly through the upregulation of DR5 and subsequent caspase activation, which could promote TRAIL-induced apoptosis in GBC-SD cells. Therefore, the combinatorial treatment of MG132 with TRAIL could be a new and effective strategy for TRAIL-resistant GBC.

Materials and methods

Reagents. The proteasome inhibitor MG132 (Z-Leu-Leu-Leucinal, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) as a 40-μM stock solution and stored at -20˚C. DMSO was purchased from the Beyotime Institute of Biotechnology (Jiangsu, China). Antibodies against caspase-3, PARP, caspase-8, DR5 and DR4 were obtained from Sigma-Aldrich. DMEM cell culture medium and fetal bovine serum (FBS) were purchased from Gibco Co. Ltd. (USA).

Cell line and mice. The human gallbladder cancer cell line (GBC-SD) was purchased from the Committee on Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Female BALB/c mice, 7 weeks of age and weighing ~25 g, were obtained from the Science Department of Experimental Animals of Fudan University in China. All mice were housed in a specific pathogen-Free (SPF) level B animal facility. The study was approved by the Review Board of Fudan University Shanghai Cancer Center and Fudan Medical College.

Cell culture and experimental conditions. GBC-SD cells were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin in a humidified incubator containing 5% CO_2 at 37˚C. Then, the cells were seeded into 96-well plates at a density of 2,000 cells per well, incubated for 12 h, and further treated with different concentrations of MG132 (2.5, 5, 10, 20, 40, 80 and 160 μM) for 24, 48 and 72 h. Then, 10 μl of Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) reagent was added to each well of the plates and the plates were incubated for 1 h at 37˚C. After removal of the supernatant, 150 μl of DMSO was added to each well. The optical density (OD) was measured at 450 nm using a spectrophotometric plate reader (Bio-Rad Co., Hercules, CA, USA) and the cell survival rate was determined according to the absorbance relative to that of untreated controls. Data are reported as the mean ± standard deviation of OD values obtained in each group. The concentrations of MG132 in this study were previously confirmed as being the most favorable for the following experiment.

Flow cytometric analysis of apoptosis. Apoptosis was performed with the Annexin V/PI apoptosis kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). GBC-SD cells were treated with MG132 (10 μM), TRAIL (100 ng/ml), and the combination of MG132 and TRAIL (10 μM + 100 ng/ml) for 48 h. After washing twice with cold PBS, the cells were resuspended in 100 μl binding buffer at a density of x10^6 cells/ml. Then, 2.5 μl of Annexin V-FITC and 1 μl of PI working solution (100 μg/ml) were added to these cells and incubated for 30 min in the dark. The samples were analyzed by a flow cytometer (BD Biosciences, San Diego, CA, USA).

Western blot assay. GBC-SD cells were harvested, washed twice with cold PBS, and lysed in RIPA buffer (Beyotime, Shanghai, China) supplemented with protease inhibitor (Roche Applied Science, Indianapolis, IN, USA) at 4˚C for 5 min. Total protein of the supernatant was determined by the bicinchoninic acid (BCA) assay kit (Beyotime) with BSA as a standard. Equal amounts of protein (40 μg/lane) from each group were separated by SDS-PAGE and transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% skim milk, and incubated with the primary antibodies against caspase-3, PARP, caspase-8, DR5, DR4, and GAPDH at 4˚C overnight. Then, the membranes were incubated with a horseradish peroxidase-conjugated goat anti-rabbit/anti-mouse secondary antibody (1:5,000; Abcam, Cambridge, UK) for 1 h at room temperature, and visualized by a Gel Doc 2000 (Bio-Rad).

Real-time PCR. Primers were synthesized and purchased from Beijing Sunbiotech Co., Ltd. (Table I). Total RNA of GBC-SD cells was extracted after 48 h of incubation using the Purelink™ Micro-to-Midi purification system (Invitrogen Co.). Then, the cDNA was synthesized using 1 μl of total RNA with 0.5 μl AMV reverse transcriptase. PCR was carried out using 2.5 μl cDNA, 0.1 μl Ex Taq HS, 0.1 μl forward primer and 0.1 μl reverse primer. The PCR program consisted of an initial 2-min step at 94˚C, and 35 cycles of 15 sec at 94˚C, 40 sec at 60˚C, and 1 min cycles at 72˚C, followed by 72˚C for 5 min. The results were determined using a UV gel imaging system and analyzed using Quantity One software (Bio-Rad Inc.), and presented as the ratio of target genes to internal control GAPDH.

Xenograft tumor experiments. Subcutaneous injection of MG132 (10 μM), TRAIL (100 ng/ml), and their combination were performed to evaluate the growth of GBC-SD xenografts in athymic nude mice. All mice were housed in the SPF

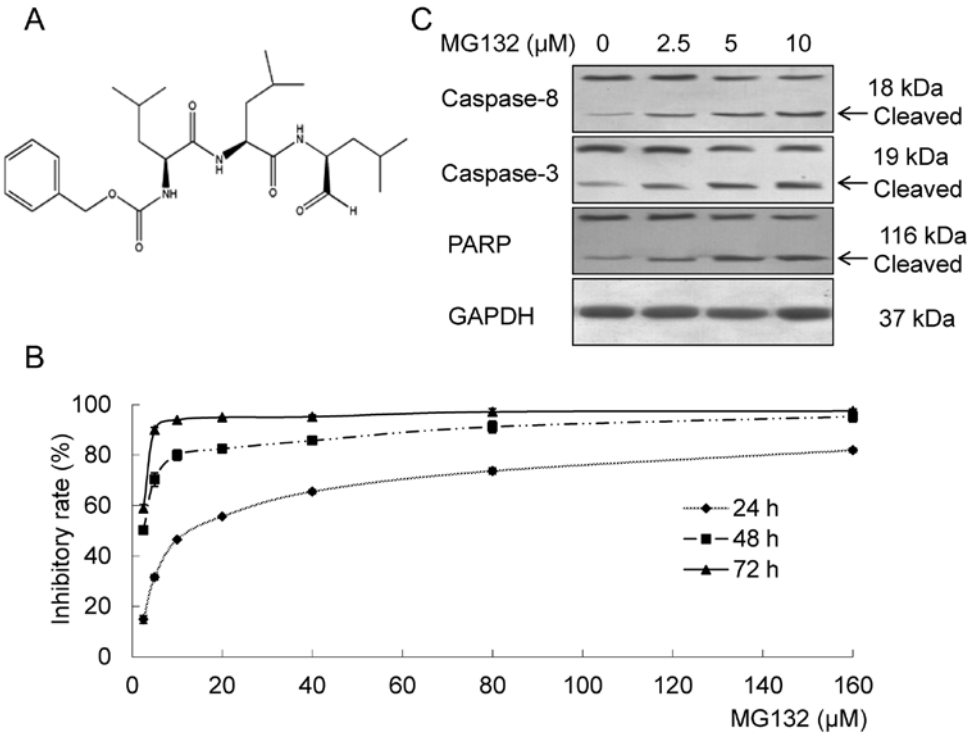

Figure 1. MG132 (A) effectively inhibits the proliferation of gallbladder cancer cells (B) and induces apoptosis (C). (A) The structure of MG132. (B) The GBC-SD cells were treated with various concentrations of MG132 for 24, 48 and 72 h. Effects of MG132 on cell proliferation were determined by CCK-8 assay. Data are expressed as the mean ± SD from three independent experiments. (C) The indicated cancer cells were treated with the given concentrations of MG132 for 48 h, and then the whole-cell proteins were extracted for western blotting (C). Data are expressed as the mean ± SD from three independent experiments.

Table I. Primer sequences for detection of mRNA expression.

Gene name	Sequence	Amplicons (bp)
Caspase-8	F: 5'-CGACCTTTGGTAGGCCAATC-3'	356
	R: 5'-GCCAATTTGTATTGCCCAACTAT-3'	
Caspase-3	F: 5'-GGTTCATCCAGTCCCTTTGC-3'	278
	R: 5'-GCGAGTGAGAATGTGCATAAATTC-3'	
PARP	F: 5'-ACGCACAATGCCTATGAC-3'	442
	R: 5'-CCAGCGGAACCTCTACAC-3'	
DR4	F: 5'-CTGAGCAACGCAGACTCGCTGTCCAC-3'	506
	R: 5'-TCCAAGGACACGGCAGAGCCTGTGCCAT-3'	
DR5	F: 5'-GCCTCATGGACAATGAGATAAAGGTGGCT-3'	502
	R: 5'-CCAAATCTCAAAGTACGCACAAACGG-3'	
GAPDH	F: 5'-TGTGTCCGTCGTGGATCTGA-3'	346
	R: 5'-CCTGCTTCACCACCTTCTTGA-3'	

level B animal facility. GBC-SD cells in log-phase growth were subcutaneously injected into the right flank of the mice. On day 10, the mice were randomly divided into four groups (6 mice/group). The control group received an injection of vehicle (10% DMSO and 90% PBS) intraperitoneally (i.p.) each day. The other three groups were administered an i.p. injection of MG132 at 10 mg/kg, TRAIL at 80 μg/kg and the combination of MG132 and TRAIL, respectively, at 10 mg/kg and 80 μg/kg every day. Visible subcutaneous tumor volumes were measured every 3 days with calipers, and then calculated by the formula: volume = (length x width^2) / 2, where length and width represent the length and width of the tumor, respectively. After 3 weeks of the treatment, all nude mice were sacrificed, and the tumor tissue was removed and weighed.

Statistical analysis. All data are presented as mean ± SD. Statistical analysis was conducted using SPSS 11.0 (SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) was used to evaluate within group data and one-way ANOVA was used to evaluate between group data. Least squares difference was

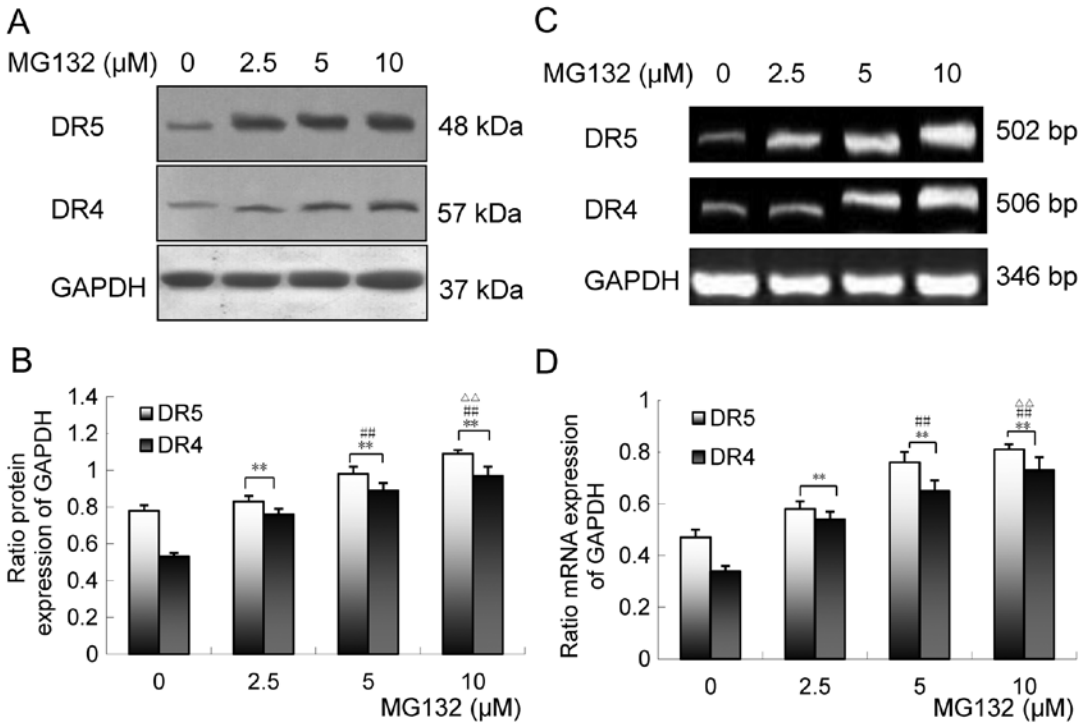

Figure 2. MG132 induces apoptosis by upregulating DR5 and DR4 expression in GBC-SD cells. The GBC-SD cells were treated with the given concentrations of MG132 for 48 h. The expression levels of DR5 and DR4 were detected by western blotting (A) and RT-PCR analysis (C), and GAPDH was used as a loading control. The data of DR5 and DR4 proteins (B) and genes (D) expression are expressed as the mean ± SD (n=3). **P<0.01 compared with the 0 μM group; $^{\#\#}$P<0.01 compared with the 2.5 μM group; $^{\Delta\Delta}$P<0.01 compared with the 5 μM group.

used for pairwise comparisons between groups. The statistical significance was defined as P<0.05.

Results

MG132 inhibits the proliferation of GBC-SD cells and induces apoptosis. To investigate the effect of MG132 on the proliferation of cells, GBC-SD cells were treated with increasing concentrations of MG132 (2.5, 5, 10, 20, 40, 80 and 160 μM) for 24, 48 and 72 h. We found that MG132 played a potent cytotoxic role on GBC-SD cells in a time- and dose-dependent manner (P<0.01) (Fig. 1B). Moreover, this inhibitory rate in GBC-SD cells increased rapidly when the increasing concentrations of MG132 were between 0 and 10 μM, when the increasing inhibitory rate slowed down the concentrations of MG132 were between 10 and 160 μM. Hence, MG132 has a more sensitive killing effect on GBC-SD cells at the concentrations of 0-10 μM than at 10-160 μM, and we chose the low concentrations of MG132 (2.5, 5 and 10 μM) at 48 h for the following treatment. We further detected the hallmarks of apoptosis in GBC-SD cells treated with MG132, and found that the protein levels of cleavage of caspase-8, caspase-3 and PARP obviously increased in a dose-dependent manner, which indicated that MG132 activated these caspases in the above cells (Fig. 1C). Therefore, MG132 clearly induces apoptosis in GBC-SD cells, and has an important mechanism for its tumor-killing effect.

MG132 induces apoptosis by upregulating the levels of DR5 and DR4 in extrinsic apoptotic pathway in GBC-SD cells. The data in a previous study **(?)** clearly exhibited that MG132 induced caspase-dependent apoptosis, especially activiated caspase-8 which is an initiator caspase in the extrinsic apoptotic pathway. To further determine the mechanism on the activation of extrinsic apoptosis invovled in this experiment, we detected the expression of DR5 and DR4 in the experiment, which are well-known as TRAIL death receptors and can initiate extrinsic apoptotic pathway. During the given concentration range of 2.5-10 μM, MG132 obviously upregulated the protein levels of DR5 and DR4 in a dose-dependent manner in GBC-SD cells, and the expression of DR5 was more obvious than that of DR4 (Fig. 2A and B). Consistent with the protein expression of DR5 and DR4 in MG132-treated cells, the mRNA levels of DR5 and DR4 were also substantially increased when treated with MG132 in GBC-SD cells (Fig. 2C and D). These results indicate that MG132 induces DR5 and DR4-dependent apoptosis through extrinsic apoptotic pathway in GBC-SD cells.

MG132 potentiates TRAIL-induced apoptosis through caspase-dependent pathway in GBC-SD cells. In the above experiments, we confirmed that MG132 can enhance the expression of DR5 and DR4 in GBC-SD cells, which have been proved to be major death receptors for the death ligand TRAIL. Therefore, we hypothesized that MG132 could enhance the apoptosis induced by TRAIL. To confirm this hypothesis, we compared the cytotoxic effect of MG132 alone, TRAIL alone, and their combination on GBC-SD cells. We found that the given concentration of MG132 or TRAIL could effectively inhibit the survival of GBC-SD cells, while the combination could play a more effective role in the killing of GBC-SD cells (Fig. 3A). Consistently, the apoptosis induced by the combination of MG132 and TRAIL was more potent than each single agent (Fig. 3B and C). Moreover, we determined whether the

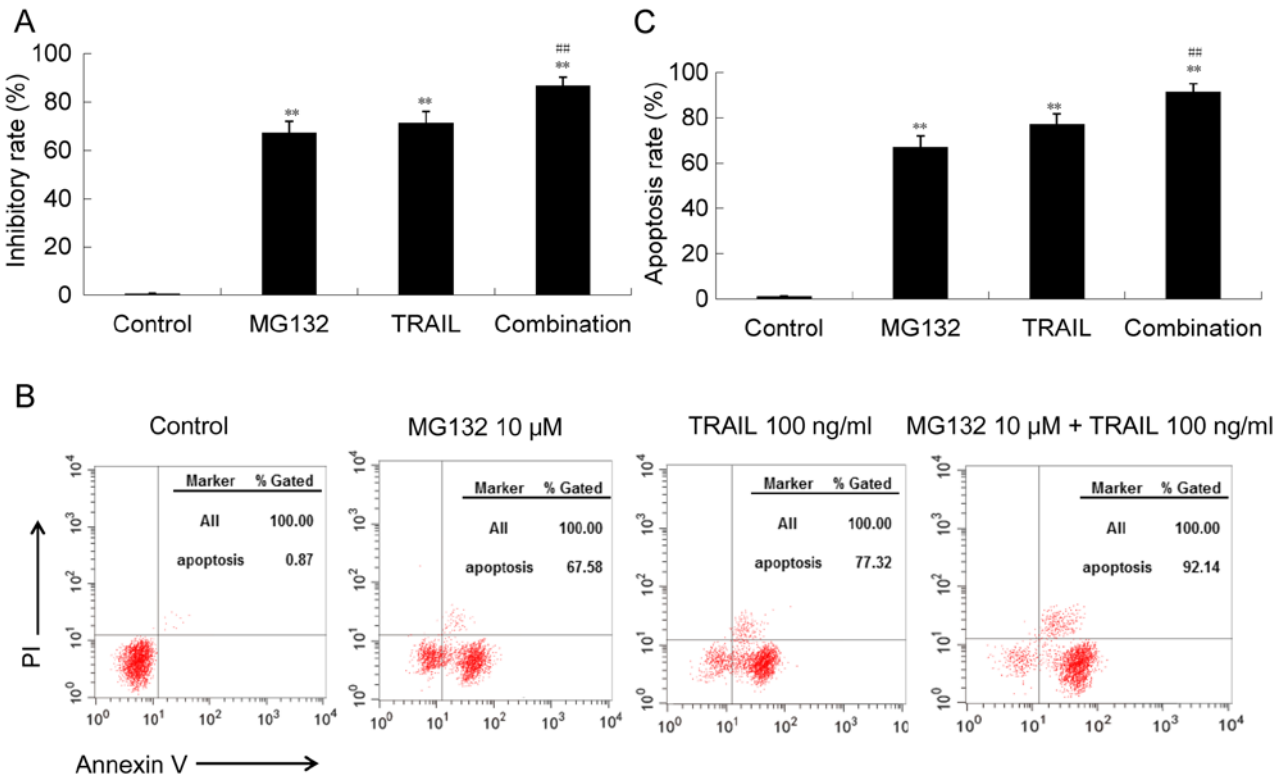

Figure 3. MG132 enhances the killing effect of TRAIL on GBC-SD cells. (A) The GBC-SD cells were treated with different concentrations of MG132, TRAIL, and the combination of MG132 and TRAIL for 48 h, and then determined by CCK-8 assay. Data are expressed as the mean ± SD from three independent experiments. (B) The GBC-SD cells were treated with MG132 alone, TRAIL alone and their combination for 48 h, and then stained with Annexin V/PI double staining. The lower left quadrant (Annexin V^-/PI^-), lower right quadrant (Annexin V^+/PI^-) and upper right quadrant (Annexin V^+/PI^+) represent the percentage of non-apoptotic cells, early apoptotic cells and late apoptotic cells, respectively. (C) The ration of apoptotic cells is presented as the mean ± SD (n=3). $^{**}P<0.01$ compared with the control group; $^{\#\#}P<0.01$ compared with the MG132 group or TRAIL group.

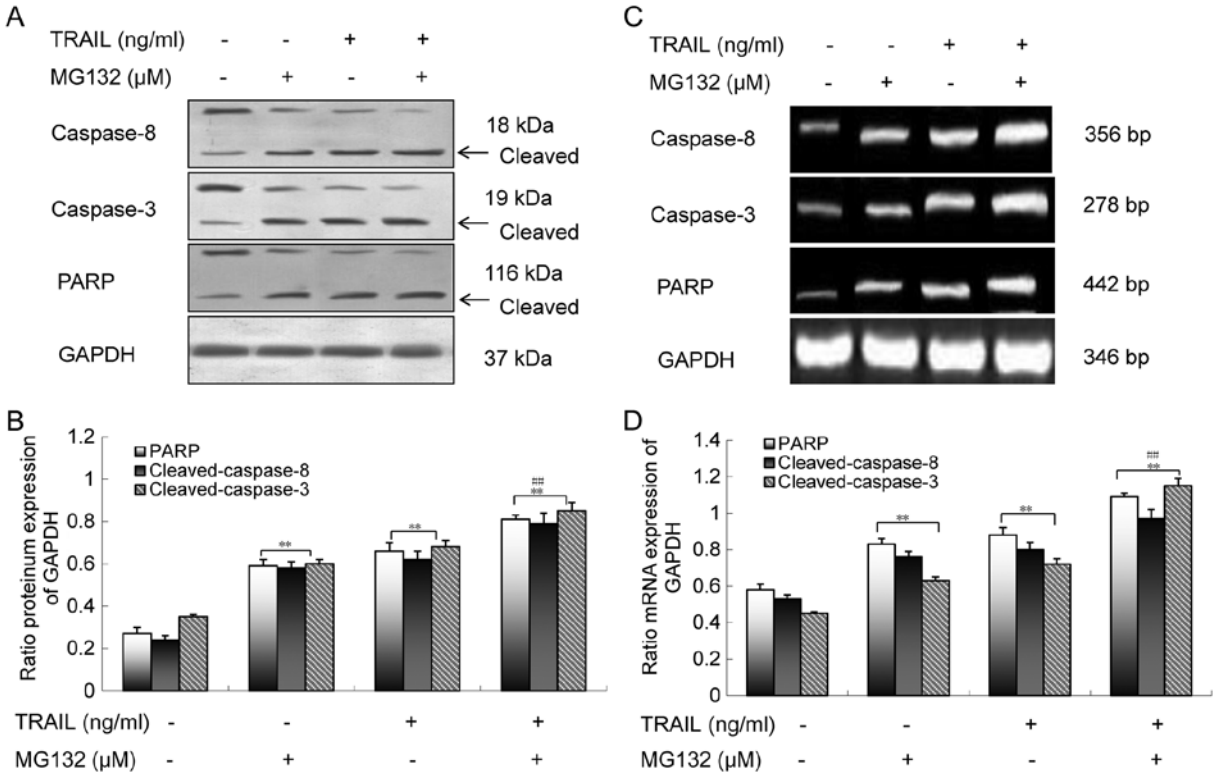

Figure 4. MG132 potentiates TRAIL-induced apoptosis in GBC-SD cells by activating caspase-dependent pathway. The GBC-SD cells were treated with the given concentrations of MG132, TRAIL, and their combination for 48 h, and then the whole-cell proteins were extracted for western blotting (A) or RT-PCR analysis (C). The data of apoptosis-related proteins (B) and genes (D) activities are expressed as the mean ± SD (n=3). $^{**}P<0.01$ compared with the control group; $^{\#\#}P<0.01$ compared with the MG132 group or TRAIL group.

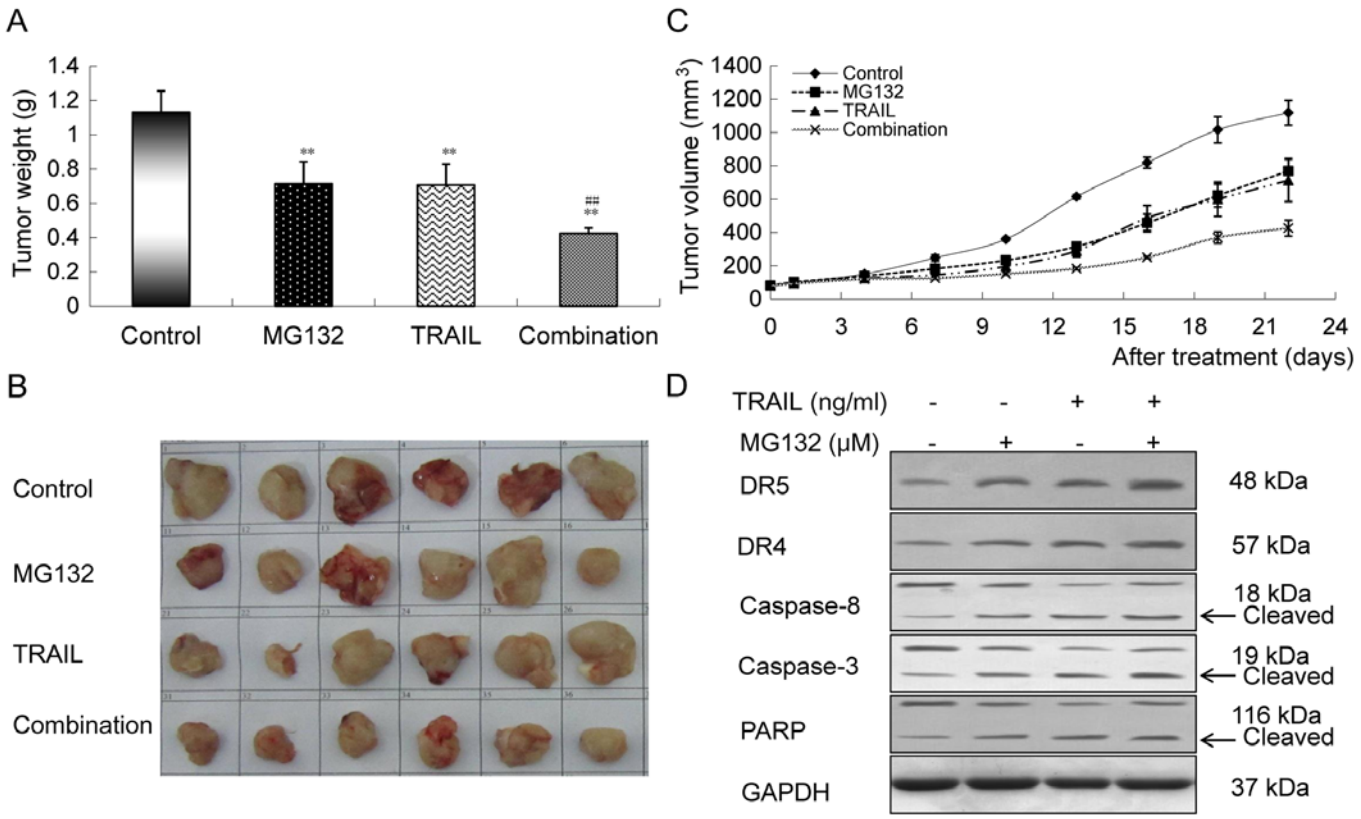

Figure 5. MG132 *in vivo* enhances the killing effect of TRAIL (A-C) on GBC-SD cells by activating the extrinsic apoptotic pathway (D). GBC-SD cells were subcutaneously injected into the right flank of the nude mice, and then the mice were treated with MG132 alone, TRAIL alone, their combination or vehicle (10% DMSO and 90% PBS) intraperitoneally every day for 3 weeks. (A and B) Tumors were excised from the animals and weighed. (C) Tumor dimensions were periodically measured with calipers. The data are illustrated as the mean ± SD (n=6). (D) The apoptosis-related proteins of tumor tissues were extracted for western blotting. Data are expressed as the mean ± SD from three independent experiments. **P<0.01 compared with the control group; $^{\#\#}$P<0.01 compared with the MG132 group or TRAIL group.

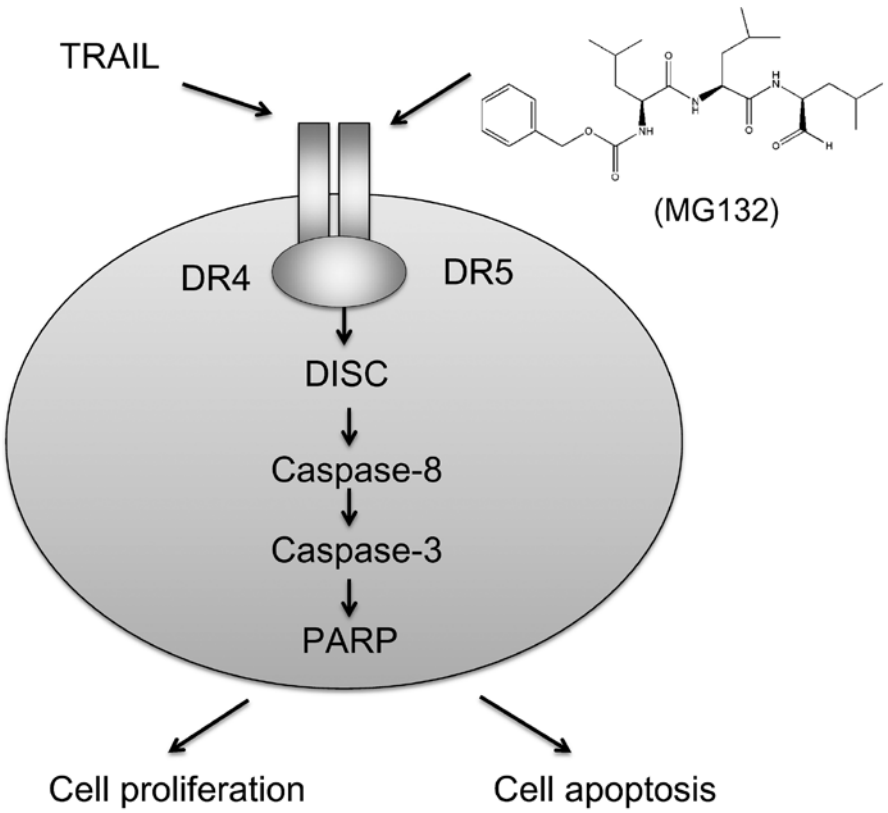

Figure 6. Schematic diagram of the signaling pathway induced by the combined treatment with MG132 and TRAIL.

combination of MG132 and TRAIL induces apoptosis by activation of the caspase-dependent pathway. We compared the effects of MG132 alone, TRAIL alone, and their combination in inducing caspase-8, caspase-3 and PARP on GBC-SD cells, and found that the tested protein and mRNA levels induced by the combination were higher than those treated with either MG132 or TRAIL alone (Fig. 4). Hence, these data further support that MG132 enhances TRAIL-induced apoptosis through caspase-dependent pathway in GBC-SD cells.

The combination of MG132 and TRAIL augments the killing effect of gallbladder cancer cells in vivo. To observe whether MG132 could enhance the killing effect of TRAIL on GBC-SD cells *in vivo*, we measured the weight and volume of GBC-SD xenografts in this experiment (Fig. 5A and C). Tumors excised from the mice are shown in Fig. 5B, and the tumor weights after 3 weeks of treatment showed an decreasing tendency from control to MG132 to TRAIL to combination groups (Fig. 5A). The differences between the groups were statistically significant ($P<0.01$). In addition, a significant reduction was also detected in tumor volumes treated with the combination compared to those treated with either MG132 or TRAIL alone (Fig. 5C). To examine whether the impact of MG132 and TRAIL on tumor growth inhibition was related to extrinsic apoptotic pathway, we detected the protein levels of DR5, DR4, caspase-8, caspase-3 and PARP in the gallbladder tumor tissues by western blot analysis, and found that the upregulation of DR5, DR4, cleaved caspase-3, caspase-8, and cleaved PARP induced by the combination were more obvious than those treated with either MG132 or TRAIL alone, which was in agreement with the results of the *in vitro* experiment (Fig. 5D).

Discussion

In this study, we demonstrated that the proteasome inhibitor MG132 can effectively inhibit the proliferation of GBC-SD cells and induce cell apoptosis *in vitro* and *in vivo*. Apoptosis of GBC-SD cells induced by MG132 were mainly through the activation of extrinsic apoptosis pathway. In addition, we evidenced that MG132 can enhance TRAIL-induced apoptosis in GBC-SD cells *in vivo* and *in vitro*. As far as we know, this is the first study to indicate that MG132 induces apoptosis in GBC-SD cells by activating the extrinsic apoptotic pathway.

Death receptor DR5 and DR4 are pro-apoptotic receptors of TRAIL ligand, which can trigger the extrinsic apoptotic signaling when interacting with TRAIL (7). To date, TRAIL-receptors, especially DR5 and DR4, have emerged as key mediators of cell apoptosis (12,22). In this study, we found that MG132 can upregulate the expression of DR5 and DR4 protein and mRNA in GBC-SD cells in a dose-dependent manner, and the expression level of DR5 was significantly stronger than that of DR4. Then it was investigated whether upregulation of DR5 caused by MG132 could potentiate TRAIL-induced apoptosis in GBC-SD cells.

The ubiquitin-proteasome system (UPS) is a key regulator of protein degradation in cellular biological processes including apoptosis (23). It has been confirmed that death receptor-mediated apoptotic pathway is regulated by UPS (24). In this study, we found that proteasome inhibitor MG132 can increase the expression level of DR5 protein in a dose-dependent manner. Thus, we speculate that the upregulation of DR5 protein in tumor cells may be attributed to the dysfunction of proteasome caused by MG132. Furthermore, the expression of DR5 also increased at the transcriptional level. In this study, we observed that MG132 can enhance the expression level of DR5 mRNA in a dose-dependent manner. Hence, the transcriptional regulation of DR5 expression is another mechanism for the upregulation of DR5 expression. In conclusion, the upregulation of DR5 expression caused by MG132 may be due to enhanced protein stability and gene transcription, which is consistent with previous studies (25-27).

At present, TRAIL has been proven to have significant potential in cancer therapy for its good tumor specificity and favorable safety (28). TRAIL-based therapies are being increasingly explored also in clinical trials (29,30). Unfortunately, many tumor cells are resistant to TRAIL-induced apoptosis. Combination therapy has been evidenced to be an effective way to overcome tumor resistance. Some studies have demonstrated that treatment with proteasome inhibitors can upregulate TRAIL-receptors expression on tumor cell surface, which promoted sensitization to TRAIL-induced apoptosis (31,32). In this study, we found that MG132 *in vitro* and *in vivo* can enhance TRAIL-mediated apoptosis in GBC-SD cells, and this process involves upregulation of DR5 and caspase-dependent pathways (Fig. 6). Thus, we speculate that MG132 can enhance TRAIL-induced apoptosis via upregulating DR5 expression in GBC-SD cells, which may overcome TRAIL resistance in cancer therapy.

In conclusion, we have established that proteasome inhibitor MG132 can potentiate TRAIL-induced apoptosis both *in vitro* and *in vivo*, and uncovered that MG132 induces apoptosis by its effect on the upregulation of DR5. In this study, we have provided experimental evidence demonstrating a possible tumor suppressor role of MG132 in GBC-SD cells, and its increasing effect on TRAIL-mediated activities.

Acknowledgements

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References

1. Bizama C, García P, Espinoza JA, Weber H, Leal P, Nervi B and Roa JC: Targeting specific molecular pathways holds promise for advanced gallbladder cancer therapy. Cancer Treat Rev 41: 222-234, 2015.
2. Misra S, Chaturvedi A, Misra NC and Sharma ID: Carcinoma of the gallbladder. Lancet Oncol 4: 167-176, 2003.
3. Henley SJ, Weir HK, Jim MA, Watson M and Richardson LC: Gallbladder cancer incidence and mortality, United States 1999-2011. Cancer Epidemiol Biomarkers Prev 24: 1319-1326, 2015.
4. Chan E and Berlin J: Biliary tract cancers: Understudied and poorly understood. J Clin Oncol 33: 1845-1848, 2015.

5. Walczak H, Miller RE, Ariail K, Gliniak B, Griffith TS, Kubin M, Chin W, Jones J, Woodward A, Le T, *et al*: Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. Nat Med 5: 157-163, 1999.
6. Nesterov A, Nikrad M, Johnson T and Kraft AS: Oncogenic Ras sensitizes normal human cells to tumor necrosis factor-alpha-related apoptosis-inducing ligand-induced apoptosis. Cancer Res 64: 3922-3927, 2004.
7. Pan G, O'Rourke K, Chinnaiyan AM, Gentz R, Ebner R, Ni J and Dixit VM: The receptor for the cytotoxic ligand TRAIL. Science 276: 111-113, 1997.
8. Bellail AC, Tse MC, Song JH, Phuphanich S, Olson JJ, Sun SY and Hao C: DR5-mediated DISC controls caspase-8 cleavage and initiation of apoptosis in human glioblastomas. J Cell Mol Med 14A: 1303-1317, 2010.
9. Fernald K and Kurokawa M: Evading apoptosis in cancer. Trends Cell Biol 23: 620-633, 2013.
10. Shankar S and Srivastava RK: Enhancement of therapeutic potential of TRAIL by cancer chemotherapy and irradiation: mechanisms and clinical implications. Drug Resist Updat 7: 139-156, 2004.
11. Shin EA, Sohn EJ, Won G, Choi JU, Jeong M, Kim B, Kim MJ and Kim SH: Upregulation of microRNA135a-3p and death receptor 5 plays a critical role in Tanshinone I sensitized prostate cancer cells to TRAIL induced apoptosis. Oncotarget 5: 5624-5636, 2014.
12. Stuckey DW and Shah K: TRAIL on trial: Preclinical advances in cancer therapy. Trends Mol Med 19: 685-694, 2013.
13. Sarhan D, Wennerberg E, D'Arcy P, Gurajada D, Linder S and Lundqvist A: A novel inhibitor of proteasome deubiquitinating activity renders tumor cells sensitive to TRAIL-mediated apoptosis by natural killer cells and T cells. Cancer Immunol Immunother 62: 1359-1368, 2013.
14. Dokouhaki P, Schuh NW, Joe B, Allen CA, Der SD, Tsao MS and Zhang L: NKG2D regulates production of soluble TRAIL by ex vivo expanded human γδ T cells. Eur J Immunol 43: 3175-3182, 2013.
15. Nalepa G, Rolfe M and Harper JW: Drug discovery in the ubiquitin-proteasome system. Nat Rev Drug Discov 5: 596-613, 2006.
16. Adams J: The proteasome: A suitable antineoplastic target. Nat Rev Cancer 4: 349-360, 2004.
17. Johnson DE: The ubiquitin-proteasome system: Opportunities for therapeutic intervention in solid tumors. Endocr Relat Cancer 22: T1-T17, 2015.
18. He Q, Huang Y and Sheikh MS: Proteasome inhibitor MG132 upregulates death receptor 5 and cooperates with Apo2L/TRAIL to induce apoptosis in Bax-proficient and -deficient cells. Oncogene 23: 2554-2558, 2004.
19. Pandit B and Gartel AL: Proteasome inhibitors induce p53-independent apoptosis in human cancer cells. Am J Pathol 178: 355-360, 2011.
20. Ustundag Y, Bronk SF and Gores GJ: Proteasome inhibition-induces endoplasmic reticulum dysfunction and cell death of human cholangiocarcinoma cells. World J Gastroenterol 13: 851-857, 2007.
21. Kim OH, Lim JH, Woo KJ, Kim YH, Jin IN, Han ST, Park JW and Kwon TK: Influence of p53 and p21Waf1 expression on G2/M phase arrest of colorectal carcinoma HCT116 cells to proteasome inhibitors. Int J Oncol 24: 935-941, 2004.
22. Watts TH: TNF/TNFR family members in costimulation of T cell responses. Annu Rev Immunol 23: 23-68, 2005.
23. Vucic D, Dixit VM and Wertz IE: Ubiquitylation in apoptosis: A post-translational modification at the edge of life and death. Nat Rev Mol Cell Biol 12: 439-452, 2011.
24. Song JJ, Szczepanski MJ, Kim SY, Kim JH, An JY, Kwon YT, Alcala MA Jr, Bartlett DL and Lee YJ: c-Cbl-mediated degradation of TRAIL receptors is responsible for the development of the early phase of TRAIL resistance. Cell Signal 22: 553-563, 2010.
25. Li X, Huang T, Jiang G, Gong W, Qian H and Zou C: Proteasome inhibitor MG132 enhances TRAIL-induced apoptosis and inhibits invasion of human osteosarcoma OS732 cells. Biochem Biophys Res Commun 439: 179-186, 2013.
26. Han B, Yao W, Oh YT, Tong JS, Li S, Deng J, Yue P, Khuri FR and Sun SY: The novel proteasome inhibitor carfilzomib activates and enhances extrinsic apoptosis involving stabilization of death receptor 5. Oncotarget 6: 17532-17542, 2015.
27. Ko H, Jeong MH, Jeon H, Sung GJ, So Y, Kim I, Son J, Lee SW, Yoon HG and Choi KC: Delphinidin sensitizes prostate cancer cells to TRAIL-induced apoptosis, by inducing DR5 and causing caspase-mediated HDAC3 cleavage. Oncotarget 6: 9970-9984, 2015.
28. Kelley SK and Ashkenazi A: Targeting death receptors in cancer with Apo2L/TRAIL. Curr Opin Pharmacol 4: 333-339, 2004.
29. Merchant MS, Geller JI, Baird K, Chou AJ, Galli S, Charles A, Amaoko M, Rhee EH, Price A, Wexler LH, *et al*: Phase I trial and pharmacokinetic study of lexatumumab in pediatric patients with solid tumors. J Clin Oncol 30: 4141-4147, 2012.
30. Jazirehi AR, Kurdistani SK and Economou JS: Histone deacetylase inhibitor sensitizes apoptosis-resistant melanomas to cytotoxic human T lymphocytes through regulation of TRAIL/DR5 pathway. J Immunol 192: 3981-3989, 2014.
31. Lee YJ, Seol JW, Jeong JK, Moon MH and Park SY: Inhibition of the ubiquitin-proteasome system sensitizes TRAIL-resistant prostate cancer cells by up-regulation of death receptor 5. Mol Med Rep 4: 1255-1259, 2011.
32. de Wilt LH, Kroon J, Jansen G, de Jong S, Peters GJ and Kruyt FA: Bortezomib and TRAIL: A perfect match for apoptotic elimination of tumour cells? Crit Rev Oncol Hematol 85: 363-372, 2013.