Corilagin induces human glioblastoma U251 cell apoptosis by impeding activity of (immuno)proteasome

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Abstract. Glioma is a type of common primary intracranial tumor, which is difficult to treat. It has been confirmed by research that corilagin (the primary active constituent of the matsumura leafflower herb) has significant antitumor effect. In particular, our previous research demonstrated that corilagin effectively promotes apoptosis of glioma U251 cells and has a synergistic effect when used with temozolomide. However, the mechanism by which corilagin causes apoptosis in U251 cells has yet to be investigated. Proteasomes are catalytic centers of the ubiquitin-proteasome system, which is the major protein degradation pathway in eukaryotic cells; they are primarily responsible for the degradation of signal molecules, tumor suppressors, cyclins and apoptosis inhibitors and serve an important role in tumor cell proliferation and apoptosis. The present study investigated the pro-apoptotic effect of corilagin on glioma U251 cells and confirmed that decreased proteasome activity and expression levels serve an important role in corilagin-induced U251 cell apoptosis.

Introduction

Glioma the most common type of primary brain tumor, accounts for >70% of intracranial malignant tumors. According to histopathological characteristics, World Health Organization classifies glioma into four grades (1). Glioblastoma multiforme (GBM) belongs to grade IV and is the most malignant glioma. The prognosis of GBM is poor: In Switzerland and the USA, the 5-year survival rate of patients with glioblastoma is only 2.9% (2). GBM is known for its resistance to treatments such as temozolomide (TMZ), the most commonly used chemotherapy drug to treat glioma (3,4). This makes the development of novel drugs for treating glioma essential.

Corilagin is the primary active constituent of the matsumura leafflower herb (a *Phyllanthus* plant from the Euphorbiaceae family), which has potential for various biological activities, including antitumor, anti-inflammatory, and hepatoprotective activity (5). Corilagin inhibits malignant tumor cell proliferation and promotes tumor cell apoptosis (6). Both *in vitro* and *in vivo* tests have demonstrated that corilagin exerts a significant inhibitory effect on ovarian and colon cancer, hepatocellular (7) and esophageal squamous cell carcinoma (8) and other types of tumor cell. Corilagin inhibits breast cancer growth via reactive oxygen species (ROS)-dependent apoptosis and autophagy (9). Moreover, it induces apoptosis, autophagy and ROS generation in gastric cancer cells *in vitro*. (10). Our previous research (11) demonstrated that corilagin effectively inhibits the proliferation of U251 and stem-like cells. It is reported that corilagin also affects TMZ-resistant T98G glioma cells (12). Taken together, these findings suggest the potential use of corilagin in glioma treatment. However, the precise mechanism by which corilagin promotes U251 cell apoptosis warrants further research.

A key feature of glioma, similar to that of other advanced tumors, is increased protein synthesis and degradation (13). This increase is due to the role of proteasomes in the degradation of signaling molecules, tumor suppressors, cyclin and apoptosis inhibitors; proteasomes are thus associated with glioma occurrence and development. The 26S proteasome is the primary proteolytic mechanism that regulates protein degradation in eukaryotic cells. The 26S proteasome comprises 20S catalytic and 19S regulatory particles. The 20S proteasome is a cylindrical structure comprising α and β subunits arranged in four stacked heteroheptamer rings (14). The two β-rings face three active sites of the β1, β2, and β5 subunits, which face the core particle (15). When stimulated by inflammatory cytokines, such as IFN-γ, immune cells,

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express large multifunctional peptidase (LMP)2 (β1i), multicatalytic endopeptidase complex-like-1 (MECL-1; β2i) and LMP7 (β5i). These three inducible subunits, β1i, β2i,and β5i, replace the constitutive subunits β1, β2, and β5 to form immunoproteasomes (16). Moreover, β1/β1i, β2/β2i, and β5/β5i are responsible for caspase-, trypsin- and chymotrypsin-like activity, respectively (17). Studies (18-20) have shown that β1i also exhibits a certain chymotrypsin-like activity. However, the inhibitory effect of corilagin-induced U251 cell apoptosis mediated by proteasome has yet to be investigated. The present study investigated the role of proteasomes in corilagin-induced U251 cell apoptosis.

Materials and methods

Chemicals and reagents. Corilagin analytical standard (purity, >99%) was purchased from Sigma-Aldrich (Merck KGaA) and TMZ from Selleck Chemicals. Anti-β1, anti-β2, anti-β5, anti-β1i, anti-β2i and anti-β5i antibodies were procured from Abcam; anti-β-actin and horseradish peroxidase (HRP)-conjugated goat anti-mouse and anti-rabbit IgG secondary antibodies were acquired from OriGene Technologies, Inc.; MG-132, bortezomib, carfizomib and PR-957 were procured from Selleck Chemicals. DMEM, streptomycin penicillin, and fetal calf serum were obtained from HyClone (GE Healthcare Life Sciences); trypsin was obtained from Gibco (Thermo Fisher Scientific, Inc.). BCA protein assay kit was purchased from Beyotime Institute of Biotechnology, an Annexin V/PI apoptosis detection kit from BD Biosciences and proteasome activity detection kit from Promega Corporation.

U251 cell line culture. U251 cells ware cultured at 37˚C in 5% CO_2 in high glucose DMEM and 10% fetal bovine serum supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). In order to evaluate proteasome involvement in corilagin-induced cell differentiation, cells were treated with proteasome inhibitors MG-132 (0, 200 nM, 1, 5 μM) and bortezomib (0, 5, 40, 100 nM) for 72 h and carfizomib (0, 100, 200 nM, 1 μM) and PR-957 (0, 100 nM, 1, 10 μM) for 24 h at 37˚C. In order to prove whether corilagin increases TMZ-induced apoptosis of U251 cells, flow cytometry was performed using U251 cells treated with increasing concentrations of corilagin (0, 25, 50, 100 and 200 μg/ml) and/or TMZ (0, 25, 50, 100 and 200 μg/ml) for 72 h at 37˚C. In order to demonstrate that proteasome inhibition mediated corilagin-induced apoptosis, flow cytometry was performed using U251 cells treated with corilagin + MG-132 (200 nM) and corilagin + bortezomib (5 nM) for 72 h at 37˚C.

Cell Counting Kit (CCK)-8 assay. A total of 100 μl cell suspension (5,000 cells/well) was inoculated in a 96-well plate, which was placed in a 37˚C cell incubator for 24 h. Next, 10 μl corilagin at various concentrations (0, 25, 50 and 100 μg/ml) was added to the wells. After 24, 48 and 72 h of incubation at 37˚C, 10 μl CCK-8 reagent (Beyotime Institute of Biotechnology) was added to each well, followed by incubation at 37˚C for 2 h. The developed color was measured by determining the absorbance at 450 nm wavelength (A). Finally, the cell survival rate (%) was calculated as $[(A_{\text{experimental well}}-A_{\text{blank well}})/(A_{\text{control well}}-A_{\text{blank well}})] \times 100\%$.

Clonogenic survival assay. U251 cells were treated with corilagin (0, 25, 50 and 100 μg/ml) for 48 h at 37˚C. The cells in the logarithmic growth phase were inoculated at a density of 500-1,000 cells/well in a 6-well plate in 10% fetal bovine serum complete DMEM and incubated for 7-14 days at 37˚C. For each concentration, three auxiliary wells were used. Every 3 days, cell growth was monitored and the medium was changed. When a single clone grew to a visible size, the medium was removed and cells were washed twice with phosphate-buffered saline and fixed with methanol for 20 min at room temperature. After the methanol was removed, the cells were stained with 0.2% crystal violet for 30 min at room temperature and images were captured with an inverted light microscope (Olympus IX71) at x100 magnification.

Apoptosis by flow cytometry. U251 cells were incubated with different concentrations (0, 25, 50, 100 and 200 μg/ml) of corilagin for 24, 48, 72 and 96 h at 37˚C. cells were collected and washed with phosphate-buffered saline three times. For the detection of apoptosis, cells were stained using a Annexin V/PI Apoptosis Detection kit according to the manufacturer's instructions. Samples were analyzed by flow cytometry (BD FACSAria; BD Biosciences). The data were analyzed by FlowJo 7.6.1 software (FlowJo LLC).

Western blot analysis. Corilagin (0, 25, 50 and 100 μg/ml) was added to U251 cells (30-40% density) at 37˚C which were inoculated in 6-well plates. After 48 h, proteins were extracted. Protein concentration was determined using BCA protein assay kit. U251 cells were collected and lysed using RIPA cell lysis buffer (Invitrogen; Thermo Fisher Scientific, Inc.). Following two washes with PBS, cells were lysed with lysis buffer [20 mM Tris-HCl (pH, 7.5), 150 mM NaCl, 1 mM EDTA, 1% NP-40] containing Complete Protease Inhibitor Cocktail (Roche Applied Science). The cell lysates (40 μg) were separated by 12% SDS-PAGE and transferred onto a PVDF membrane. Membranes were blocked with 5% skimmed milk for 1 h at room temperature and incubated with primary antibodies at a dilution of 1:1,000 overnight at 4˚C. Antibodies against the following proteins were used: β1, β2, β5, β1i, β2i, β5i and β-actin. Luminata Forte Western HRP Substrate (EMD Millipore) was used for the development of positive signals. The relative protein expression of genes was quantified by ImageJ v.1.46r software (National Institutes of Health) using β-actin as a control.

Assay for proteasome activity. The 26S proteasome activity was analyzed based on a previously published method of Promega Corporation proteasome activity assay kit (21). Cells were harvested and 10,000 cells in high glucose complete DMEM were incubated with detection reagents containing fluorogenic substrates of caspase-, trypsin- and chymotrypsin-like activity at 37˚C for 1 h. The caspase-like activity was determined with Z-LLEC (45 μM), trypsin-like activity with Ac-RLR-AMC (40 μM) and chymotrypsin-like activity with Suc-LLVY-AMC (18 μM) at 37˚C for 1 h. The fluorescence intensity was measured at the following wavelengths: Excitation, 380 and emission, 460 nm.

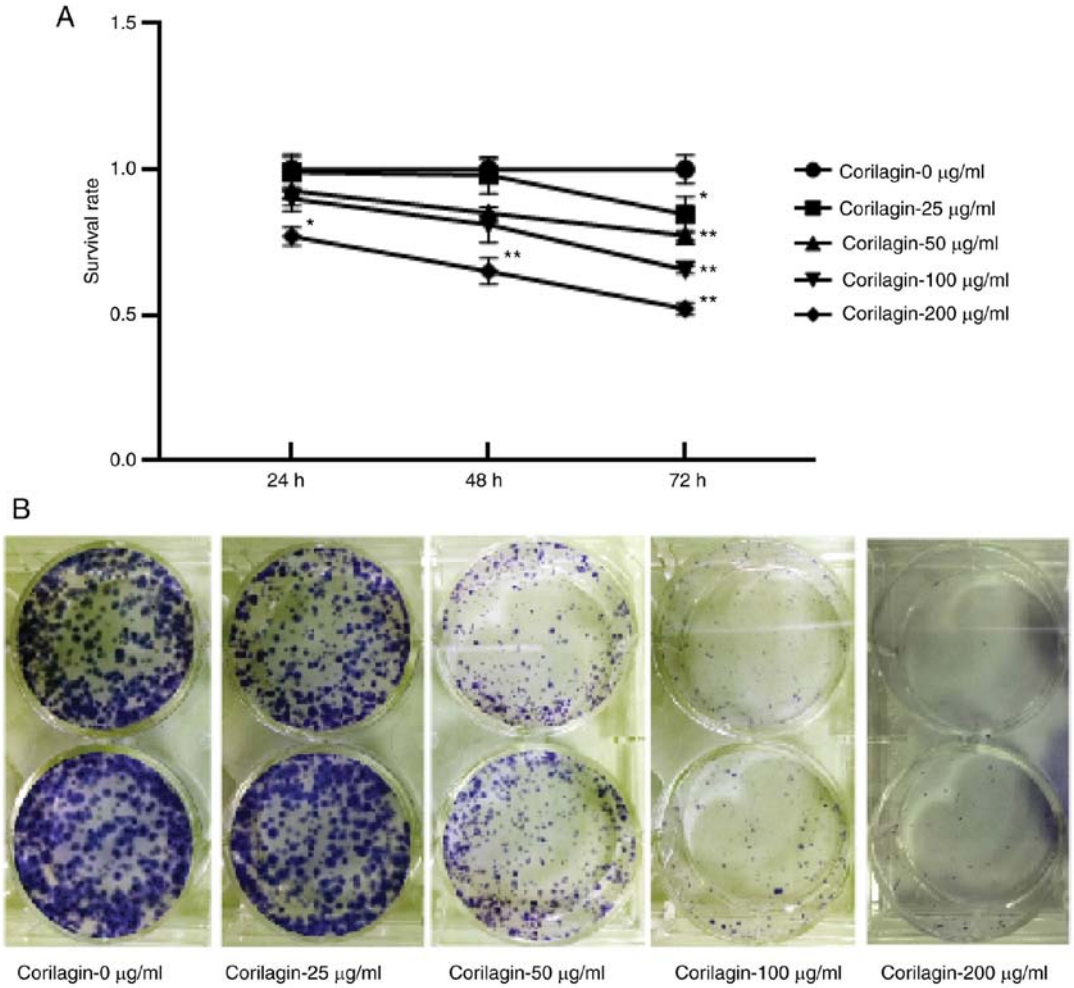

Figure 1. Corilagin deceases U251 proliferation and clonal formation. (A) Cell proliferation and toxicity were analyzed by Cell Counting Kit-8 assay. (B) U251 cells were treated with corilagin. Data are presented as the mean ± SEM (n=5/group). *P<0.05, **P<0.01 vs. corilagin-0 μg/ml.

Statistical analysis. Data were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test by GraphPad Prism 8.0.2 (GraphPad Software, Inc.). Data are presented as the mean ± SEM (≥3). P<0.05 was considered to indicate a statistically significant difference.

Results

Corilagin inhibits U251 proliferation. In order to determine the role of corilagin in regulating U251 growth, CCK-8 assay was performed to detect U251 cell survival following exposure to increasing corilagin concentrations (0, 25, 50, 100 and 200 μg/ml) for 24, 48 and 72 h. The results indicated that all concentrations of corilagin significantly decreased survival over 72 h, and 200 μg/ml corilagin at 24 and 48 h also significantly decreased the survival (Fig. 1A). Clone formation experiments on U251 cells treated with varying concentrations of corilagin for 48 h also demonstrated that the number of cell clones decreased in a dose-dependent manner (0, 25, 50, 100 and 200 μg/ml; Fig. 1B). Taken together, these data suggested that corilagin inhibited U251 cell proliferation.

Corilagin promotes U251 apoptosis. In order to determine the role of corilagin in regulating U251 apoptosis, apoptosis of U251 cells was assessed using an Annexin V/PI staining kit. The results revealed that U251 cell apoptosis rates significantly increased following corilagin treatment (0, 25, 50, 100 and 200 μg/ml) for 24, 48 and 72 h (Fig. 2A). Following treatment with 200 μg/ml corilagin treatment for 24, 48, and 72 h, the ratio of apoptotic cells significantly increased by 1.8-, 2.7- and 4.4-fold, respectively (Fig. 2B-D). Additionally, 50 μg/ml corilagin at 24 h and 100 μg/ml at all three time points significantly increased the ratio of apoptotic cells. Taken together, these results suggested that corilagin effectively promoted U251 apoptosis.

Corilagin combined with TMZ increases apoptosis of U251 cells. In order to determine the role of corilagin + TMZ in regulating U251 apoptosis, flow cytometry was performed. Images of Annexin V/PI double staining of U251 cells treated with increasing concentrations (0, 25, 50, 100 and 200 μg/ml) of corilagin, TMZ and corilagin + TMZ for 72 h are shown in Fig. 3A-C. The percentage of apoptotic cells (Annexin V^+/PI^+ and Annexin V^+/PI^-) treated with corilagin, TMZ and corilagin + TMZ was calculated (Fig. 3D-F). At 100 μg/ml corilagin + TMZ increased the apoptosis rate from 58% in the TMZ-alone group to 94% Furthermore, 200 μg/ml corilagin + TMZ increased the apoptosis rate from 62% in

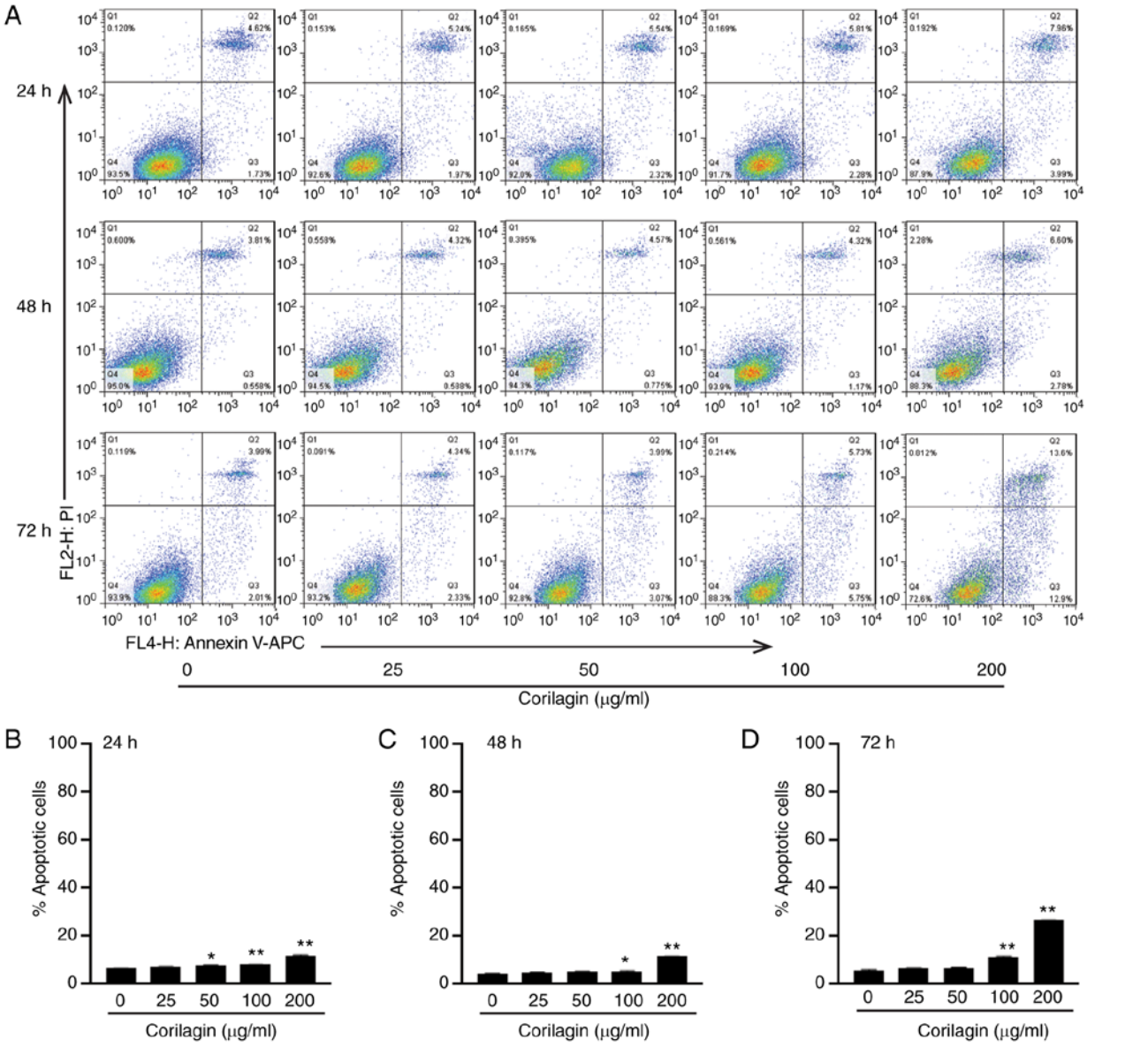

Figure 2. Images of Annexin V/PI doubling staining of U251 cells. Cells were treated with increasing concentrations of corilagin (0, 25, 50, 100 and 200 μg/ml) for (A) 24, 48 and 72 h and subjected to flow cytometry. The ratios of apoptotic cells (Annexin V^+/PI^+ and Annexin V^+/PI^-) after (B) 24, (C) 48 and (D) 72 h were calculated. Data are presented as the mean ± SEM (n=5/group). $^*P<0.05$, $^{**}P<0.01$ vs. 0 μg/ml corilagin.

the TMZ-alone group to 90% and 50 μg/ml corilagin + TMZ increased the apoptosis rate from 17 to 40%. However, 50 μg/ml corilagin group did not significantly increase the apoptosis rate compared with 0 μg/ml corilagin (Fig. 3E and F). Taken together, these results suggested that corilagin + TMZ increased apoptosis of U251 cells.

Corilagin decreases proteasome activity. In order to determine the role of proteasome in corilagin-induced U251 cell apoptosis, the effect of corilagin on proteasome activity was investigated. U251 cells were treated with corilagin at different concentrations (0, 25, 50 and 100 μg/ml) and durations (4, 8, 24 and 48 h). The proteasome activity, including caspase-, trypsin- and chymotrypsin-like activity, was measured using fluorescent-labeled peptides. At 4 h, corilagin treatment significantly increased caspase-, trypsin- and chymotrypsin-like activity in a dose-dependent manner (Fig. 4A). At 8 h, chymotrypsin-like activity was significantly increased by 100 μg/ml corilagin (Fig. 4B). At 24 and 48 h, caspase-, trypsin- and chymotrypsin-like activities significantly decreased in a dose-dependent manner (Fig. 4C and D). These results suggested that corilagin-induced apoptosis was associated with proteasome activity.

Corilagin decreases expression levels of proteasome subunits. In order to determine the effect of corilagin on proteasome catalytic subunit expression levels, U251 cells were treated with corilagin (100 μg/ml) for different durations (4, 8 and 24 h). The protein expression levels of the constitutive (i.e., β1, β2, and β5; Fig. 5A) and inducible proteasome catalytic subunits (i.e., β1i, β2i and β5i; Fig. 5B) were detected by western blotting. Corilagin treatment significantly decreased the protein levels of constitutive subunits (β1 and β2) and immunosubunits (β1i, β2i and β5i) but increased those of

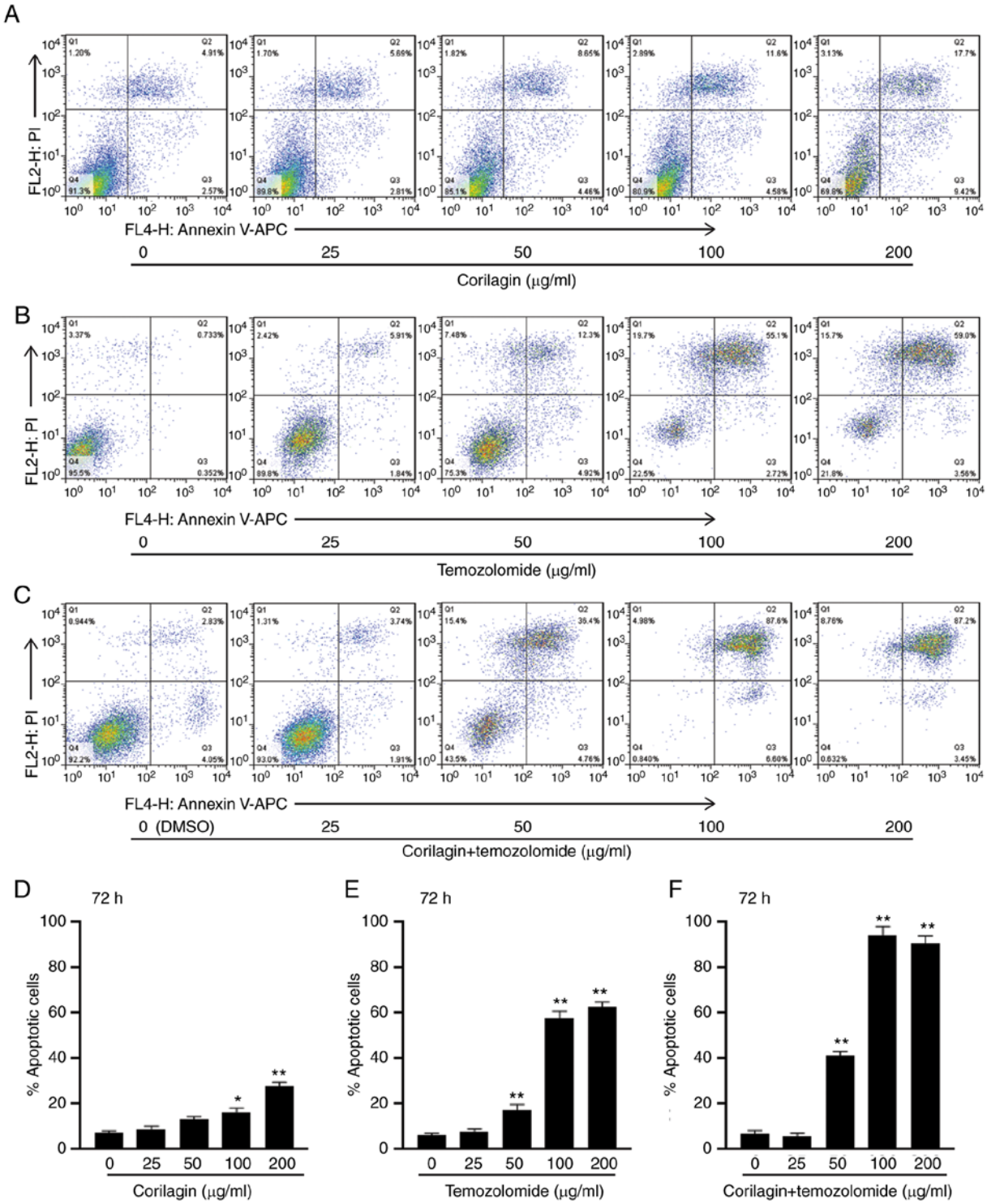

Figure 3. Corilagin combined with TMZ increases apoptosis of U251 cells. Cells were treated with increasing concentrations (0, 25, 50, 100 and 200 μg/ml) of (A) corilagin, (B) temozolomide and (C) corilagin + temozolomide for 72 h and subjected to flow cytometry. Percentage of apoptotic cells (Annexin V^+/PI^+ and Annexin V^+/PI^-) cells treated with (D) corilagin, (E) temozolomide and (F) corilagin + temozolomide was calculated. Data are presented as the mean ± SEM (n=5/group). $^*P<0.05$, $^{**}P<0.01$ vs. 0 μg/ml.

the constitutive subunit β5 (Fig. 5C and D), indicating that the corilagin-induced decrease in proteasome activity in U251 cells was primarily mediated by decreased expression levels of proteasome subunits β1/β1i, β2i and β5i.

Proteasome inhibitors promote U251 apoptosis. In order to determine the role of proteasome in corilagin-mediated regulation of U251 apoptosis, the apoptotic rate of U251 cells treated with proteasome inhibitors MG-132 (0, 200 nM, 1, 5 μM; Fig. 6B), bortezomib (0, 5, 40, 100 nM; Fig. 6E) for 72 h and carfizomib (0, 100, 200 nM, 1 μM; Fig. 6H) and PR-957 (0, 100, 1, 10 μM; Fig. 6K) for 24 h was assessed using an Annexin V/PI staining kit. Before detecting apoptosis, the effects of proteasome inhibitors on the activity of three

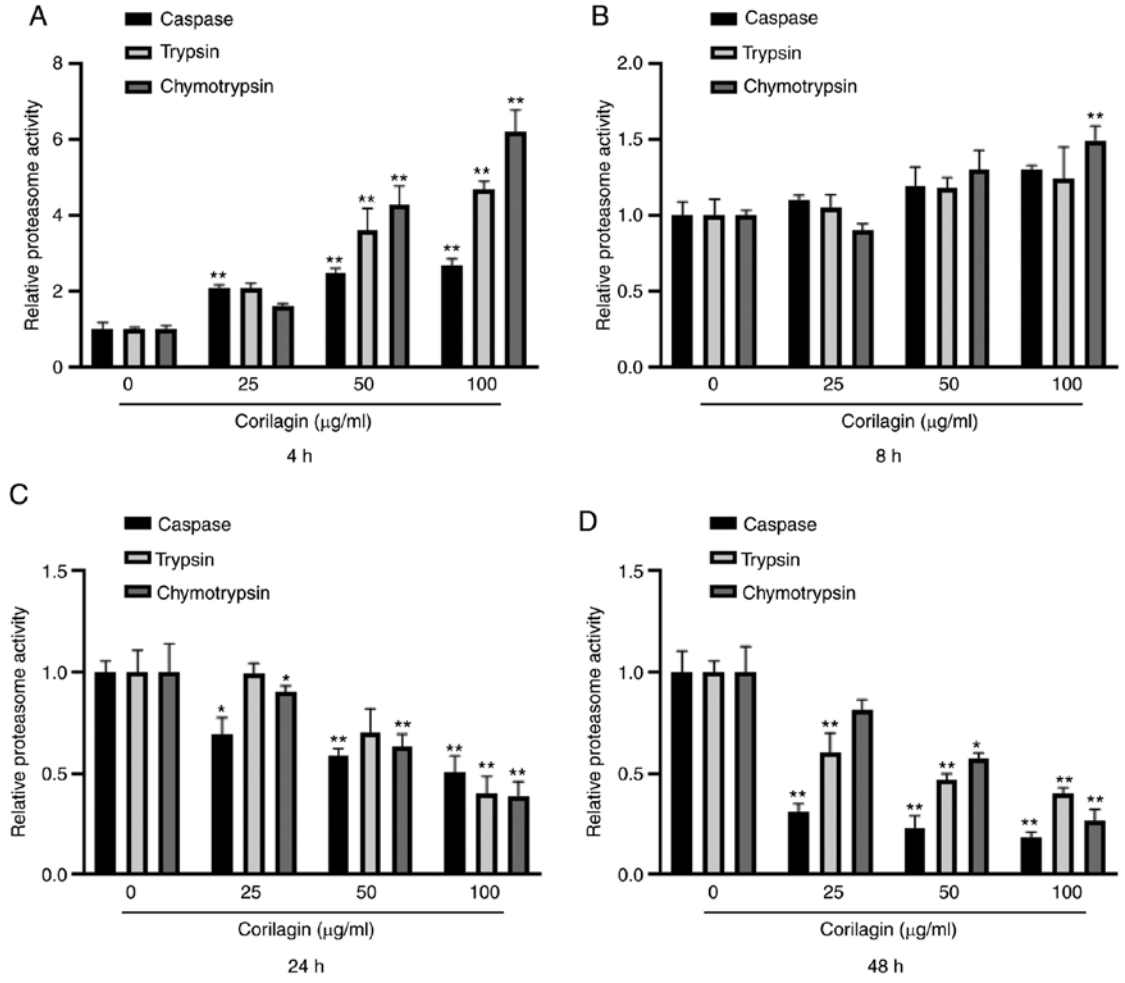

Figure 4. Corilagin decreases proteasome activity. (A) U251 cells were treated with corilagin (25, 50 and 100 μg/ml) for (A) 4, (B) 8, (C) 24 and (D) 48 h. Caspase-like, trypsin-like and chymotrypsin-like proteasome activity were measured by fluorogenic substrates. Data are presented as the mean ± SEM (n=5/group). *P<0.05, **P<0.01 vs. 0 μg/ml.

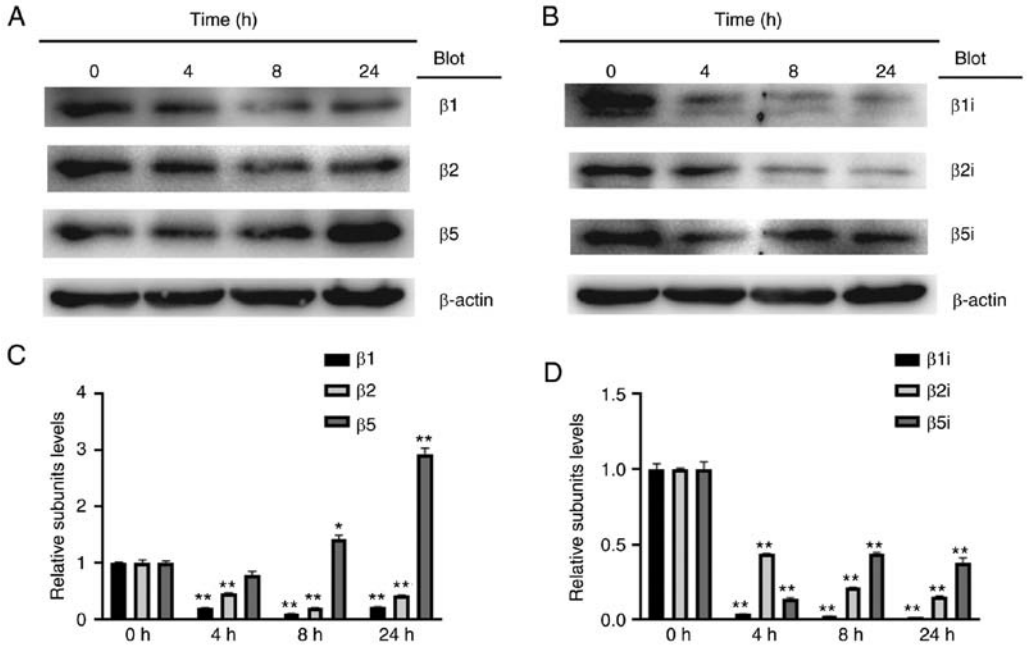

Figure 5. Corilagin decreases expression levels of proteasome catalytic subunits. U251 cells were treated with 100 μg/ml corilagin for different durations (0, 4, 8 and 24 h). The protein levels of (A) constitutive catalytic subunits (β1, β2 and β5) and (B) immuno-subunits (β1i, β2i and β5i) were detected by western blot analysis. Quantification of the protein levels of (C) constitutive catalytic subunits and (D) immuno-subunits was performed. Data are presented as the mean ± SEM (n=5/group). *P<0.05, **P<0.01 vs. 0 h.

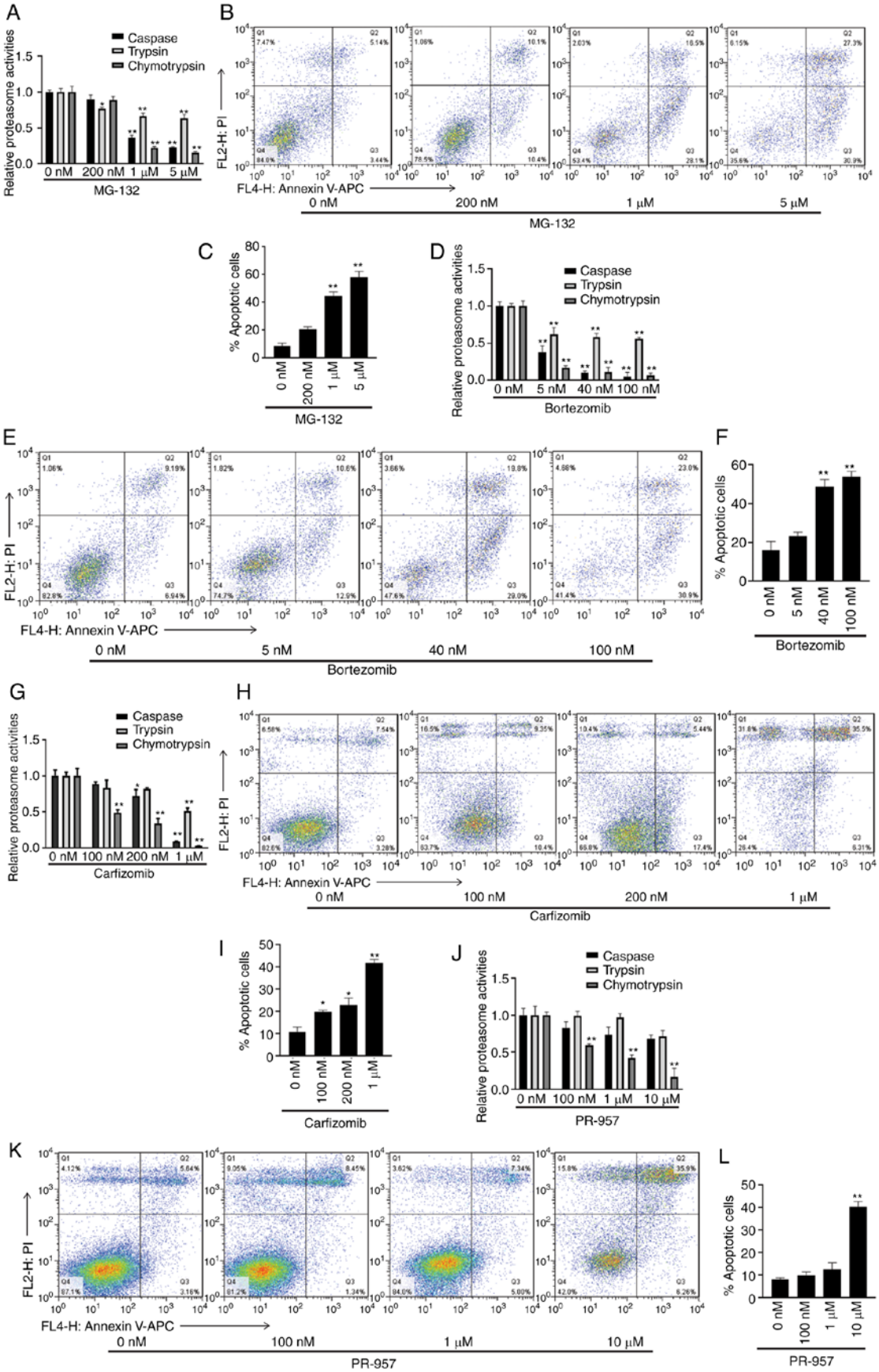

Figure 6. MG-132, Bortezomib, carfizomib and PR-957 induce U251 apoptosis. (A) Caspase-, trypsin- and chymotrypsin-like proteasome activity of U251 cells treated with MG-132 (100, 500 nM, 1 and 5 μM) for 72 h were measured. (B) Flow cytometry analysis of U251 cells treated with increasing concentrations of MG-132 for 72 h. (C) Ratio of apoptotic (Annexin V^+/PI^+ and Annexin V^+/PI^-) of MG-132-treated U251 cells was calculated. (D) Proteasome activity, (E) flow cytometry and (F) ratio of apoptotic U251 treated with bortezomib (0, 5, 40 and 100 nM) for 72 h. (G) Proteasome activity, (H) flow cytometry and (I) ratio of apoptotic cells of U251 treated with carfizomib (0, 100, 200 nM, 1 μM) for 24 h. (J) Proteasome activity, (K) flow cytometry and (L) ratio of apoptotic U251 cells treated with PR-957 (0, 100 nM, 1, 10 μM) for 24 h. Data are presented as the mean ± SEM (n=5/group). *P<0.05, **P<0.01) vs. 0 nM.

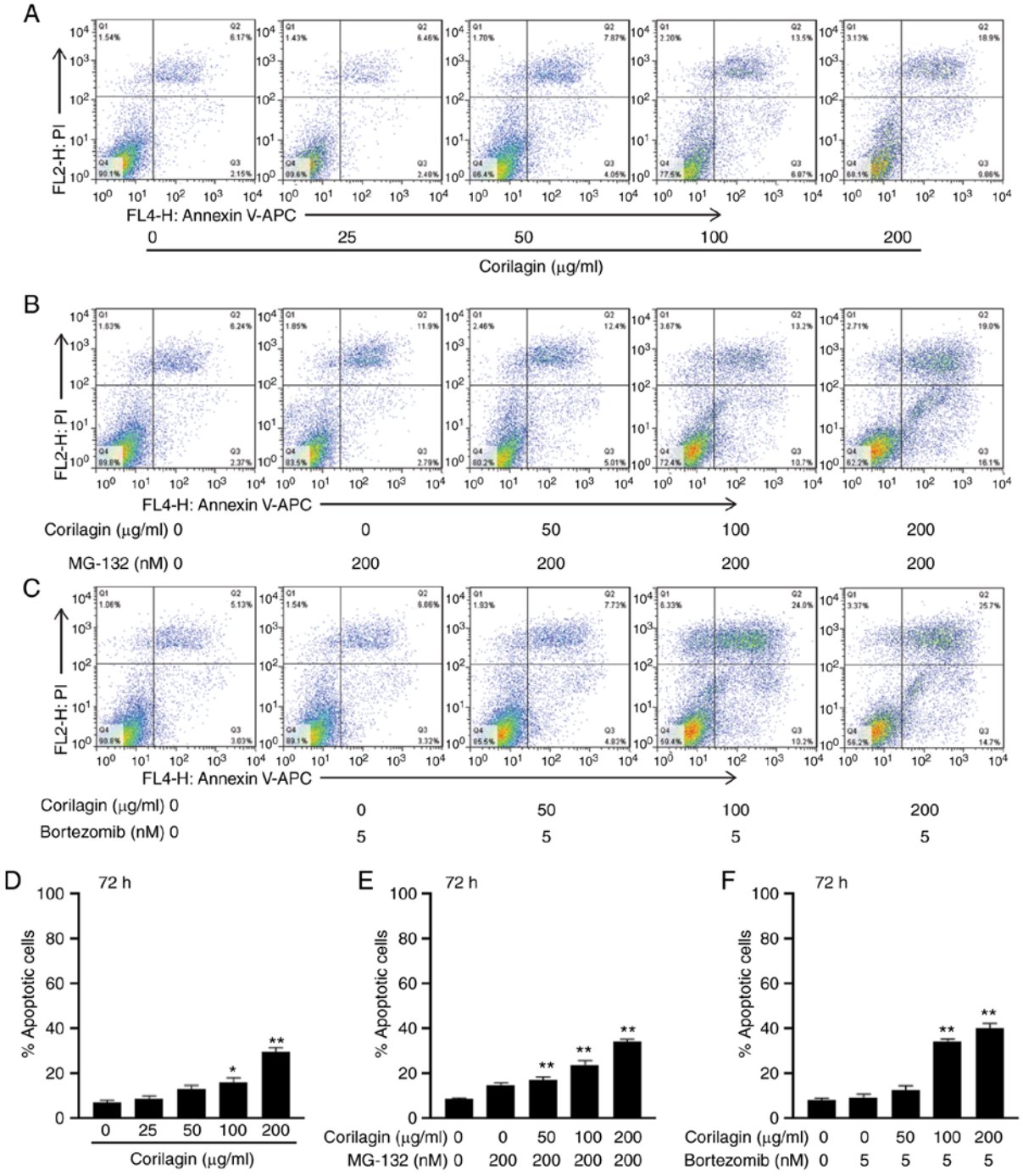

Figure 7. Proteasome inhibitors increase corilagin-induced apoptosis of U251 cells. Annexin V/PI doubling staining of U251 cells treated with (A) increasing concentrations of corilagin (0, 25, 50, 100 and 200 μg/ml), (B) corilagin + MG-132 (200 nM) and (C) corilagin + bortezomib (5 nM) for 72 h was assessed by flow cytometry assay. Percentages of apoptotic cells (Annexin V^+/PI^+ and Annexin V^+/PI^-) treated with (D) corilagin, (E) corilagin + MG-132 and (F) corilagin + bortezomib were calculated. Data are presented as the mean ± SEM (n=5/group). $^*P<0.05$, $^{**}P<0.01$ vs. untreated control.

proteasome enzymes was assessed. MG-132 and bortezomib significantly inhibited caspase- and chymotrypsin-like activity; lower concentrations of bortezomib were needed to achieve this inhibitory effect (Fig. 6A and D). Carfilzomib and PR-957 significantly inhibited chymotrypsin-like enzyme activity and carfilzomib also significantly inhibited caspase-like enzyme activity at a concentration of 1 μM (Fig. 6G and J). The apoptosis assay results showed that all inhibitors increased the percentage of apoptotic cells in a dose-dependent manner (Fig. 6C, F, I and L).

Proteasome inhibitors increase corilagin-induced apoptosis of U251 cells. In order to demonstrate that proteasome inhibition mediated corilagin-induced apoptosis, flow cytometry was performed using U251 cells treated with corilagin + MG-132 (200 nM) and corilagin + bortezomib (5 nM). Images of Annexin V/PI double staining of U251 cells treated with increasing concentrations (0, 25, 50, 100 and 200 μg/ml) of corilagin, corilagin + MG-132 (200 nM), and corilagin + bortezomib (5 nM) for 72 h are shown in Fig. 7A-C. The percentages of apoptotic cells

(Annexin V^+/PI^+ and Annexin V^+/PI^-) of cells treated with corilagin, corilagin + MG-132 and corilagin + bortezomib were calculated (Fig. 7D-F). The results showed that corilagin + MG-132 (200 nM) increased the percentage of apoptotic cells compared with corilagin-alone (50, 100 and 200 ug/ml) from 13, 16 and 27 to 17, 23 and 34%, respectively (Fig. 7E). Corilagin + bortezomib (5 nM) increased the percentage of apoptotic cells to 12, 34 and 40% (Fig. 7F). These results indicated that proteasome inhibitors increased corilagin-induced apoptosis.

Discussion

The present study investigated the role of proteasome activation in corilagin-induced U251 cell apoptosis. Corilagin primarily stimulated U251 cell apoptosis and decreased the activity and expression levels of proteasome subunits, including immunosubunits. U251 cells were treated with proteasome inhibitors, which were shown to promote apoptosis in these cells. In order to demonstrate that proteasome inhibition mediates corilagin-induced apoptosis, U251 cells were treated with corilagin in combination with the proteasome inhibitors MG-132 and bortezomib. The results of Annexin V/PI staining analysis for corilagin, corilagin + MG-132 and corilagin + bortezomib indicated that proteasome inhibitors increased corilagin-induced apoptosis. The present study therefore identified a novel potential mechanism for proteasomes in corilagin-induced U251 cell apoptosis.

The present study demonstrated that corilagin effectively promoted the apoptosis of glioma U251 cells. Research has indicated that corilagin inhibits the growth of U251 and TMZ-resistant T98G glioma cells (12). Qiu *et al* (8) determined that corilagin downregulates the E3 ubiquitin ligase RING finger protein 8 in the ubiquitin-proteasome pathway, which disrupts the DNA damage repair response and promotes cell death in esophageal squamous cell cancer cells.

During corilagin-induced U251 cell apoptosis, the proteasome catalytic subunit expression levels were all downregulated in the present study, except for those of β5. However, proteasome activity first increased and then decreased, particularly at 4 h, at which time proteasome activity was notably increased. This appears to contradict the decreased expression levels. The proteasome is a biological complex composed of multiple molecules, the assembly of which is complex and strictly regulated by various mechanisms, such as the assembly of proteasome activator 200 (22) and Adc17, the absence of which aggravates proteasome defects (23). The levels of proteasome are affected by proteasome subunits and assembly chaperones (17). Proteasome activity is also regulated by phosphorylation of 26S subunits (24) and by protein kinases that have been reported to stimulate proteasomal activity (25). For example, increased cAMP levels and protein kinase A activation cause phosphorylation of the 19S subunit Rpn6 (26), which increases peptide, ATP and Ubiquitin conjugate hydrolysis rates (27). The inconsistency between changes in proteasome activity and catalytic subunit expression levels require further analysis. It was hypothesized that in the early stage (4 h) of corilagin intervention in cells, proteasome subunit expression levels were affected and proteasome assembly was altered, but the proteasome activity was stimulated in response to external stress. After 8 h, proteasome function became increasingly damaged, causing proteasome activity to continue to decline, resulting in decreased physiological cell activity, finally leading to apoptosis.

In glioma cells, the expression levels of immunosubunits are high (28), but their mechanism of involvement in glioma occurrence and development remains unclear. Studies have revealed that immunoproteasomes regulate T helper cell differentiation (29,30). Several factors, including IFN-γ and TNF-α, promote catalytic subunit expression to produce immunoproteasomes and thereby affect the enzymatic activity of their substrates (17) and alter cell function (31). Notably, in the present study, β5 expression was upregulated and β5i expression was downregulated during corilagin intervention. It was hypothesized that corilagin changed the proteasome structure. The assembly of proteasome subunits is complex and strictly regulated; it is achieved by the controlled expression of proteasome subunits by a common transcription factor (such as Rpn4) in yeast (32). Cells adjust proteasome-mediated degradation by regulating proteasome levels via coordinated expression of proteasome subunits and assembly chaperones (17).

In order to demonstrate that corilagin promoted apoptosis by interfering with proteasome activity, U251 cells were treated with four proteasome inhibitors. Proteasome inhibitors inhibited proteasome activity and promoted apoptosis of U251 cells. Proteasome inhibitors are widely used to study the function of proteasomes and are employed as anticancer drugs to treat various types of cancer (18,33,34). The following proteasome inhibitors were used in the present study: MG-132, a reversible inhibitor of β1, β2, and β5; bortezomib, a reversible inhibitor of β5, β5i and β1i; carfilzomib, an irreversible inhibitor of β5 and β5i and PR-957, a specific irreversible inhibitor of β5i (35,36). Yoo *et al* (37) reported that glioma stem cells are sensitive to proteasome inhibitors. MG-132 has also been used in glioma research (38,39). Both bortezomib and carfilzomib have been approved for the treatment of multiple myeloma (40) and their use has also been reported in glioma research (40,41). The International Journal of Radiation Oncology, Biology, Physics published a phase-II clinical trial in 2018 (42) that evaluated the efficacy and safety of bortezomib in combination with TMZ and local radiotherapy for GBM. Bortezomib inhibits cell adhesion, angiogenesis and cytokine-mediated intercellular communication, thereby affecting the tumor microenvironment (33). Bortezomib and TMZ in combination with local radiotherapy is particularly beneficial for people with O-6-methylguanine-DNA methyltransferase methylation and its side effects (such as lymphopenia, neutropenia and thrombocytopenia) are generally mild (42). PR-957, which specifically inhibits β5i activity (35), is used to study autoimmune (43) and inflammatory disease (36). To the best of our knowledge, however, it has not been used in the study of glioma treatment. The present study demonstrated that PR-957 effectively promoted U251 cell apoptosis and its mechanism of action warrants further investigation. Given the inhibitory effect of proteasome inhibitors on glioma, future studies should investigate whether corilagin can be combined with proteasome inhibitors to improve its therapeutic effects on glioma.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XQ and FJ designed the experiments. JL and WM performed western blot analysis, activity assays and cell experiments. PC performed flow cytometry. XQ and DP analyzed data and wrote the manuscript. XQ and FJ authenticated all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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