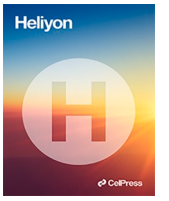
Heliyon 10 (2024) e33935

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Ginsenoside Rg1 inhibits multiple myeloma and overcomes bortezomib resistance through AMPK-mTOR pathway

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ARTICLE INFO

Keywords:
Ginsenoside Rg1
Multiple myeloma
Autophagy
Bortezomib
Drug resistance

ABSTRACT

Background: The resistance of multiple myeloma (MM) to bortezomib (BTZ) has brought multiple challenges to its clinical use. Numerous ginsenosides have potential anti-tumor effects, however, the research on the role of Rg1 in MM has not been reported.

Objective: To examine the inhibitory impact of Rg1 on the growth of MM and reduce the drug resistance of MM to BTZ through *in vivo* and *in vitro* experiments, and to explore their potential mechanism.

Methods: BTZ drug-resistant cell line RPMI8226R was constructed. Mouse tumor-bearing model was developed by abdominal subcutaneous injection of MM cells. MM cells were treated with AMPK inhibitor Compound C or autophagy inhibitor Chloroquine together with Rg1. RPMI8226R cells were treated with BTZ and Rg1. Cell multiplication was detected using Methylthiazolyldiphenyl-tetrazolium bromide assay. Apoptosis was assessed using flow cytometry. Immunofluorescence assay was employed to assess the autophagy markers LC3. Western blot was utilized to assess the protein expression. Immunohistochemistry was used to detect cell proliferation and apoptosis in tumor tissues.

Results: In vitro experiments demonstrated that Rg1 could hinder the proliferation of MM cells, promote apoptosis and enhance autophagy. Rg1 could also increase the sensitivity of RPMI8226R to BTZ. In vivo experiments illustrated that Rg1 could hinder the development of MM cells in mice, weaken the proliferation of tumor cells and enhance their apoptosis. Further study found that the anti-MM impact of Rg1 was linked to AMPK-mTOR pathway, the autophagy degree of RPMI8226R was higher than that of RPMI8226, and that Rg1 could inhibit MM and overcome drug resistance through autophagy induced by AMPK-mTOR pathway.

Conclusion: Rg1 has significant anti-MM effect and can overcome BTZ resistance, and its potential mechanism is related to the regulation of autophagy induced by AMPK-mTOR pathway. Rg1 is a promising adjuvant drug for the treatment of MM.

1. Introduction

Multiple myeloma (MM) is a malignant tumor that originates from B cell line and accounts for 10 % of hematological tumors [1]. In recent years, the incidence of drug resistance in MM patients has gradually gone up [2]. Bortezomib (BTZ) is the main drug in clinical treatment of MM, however, the emergence of drug resistance greatly reduces the therapeutic effect [3]. Therefore, it is crucial to

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https://doi.org/10.1016/j.heliyon.2024.e33935
Received 16 April 2024; Received in revised form 28 June 2024; Accepted 1 July 2024
Available online 1 July 2024

undertake research in order to identify the best combination of BTZ and formulate a novel combined medication scheme to achieve better curative effect.

Ginsenoside Rg1 is a tetracyclic triterpenoid derivative derived from the root of *Panax ginseng C.A.Mey of Araliaceae*. It has been proved to have auxiliary anti-tumor effect [4] and hinder tumor cell mitosis as well as cancer cell proliferation [5]. Several studies have demonstrated that Rg1 has the ability to enhance the reactivity of triple negative breast cancer cell lines to chemotherapeutic drugs [6]. However, there are few reports on its function and application in malignant hematological diseases. In this study, the inhibitory impact of ginsenoside Rg1 on MM and the sensitizing effect on drug-resistant strains were observed *in vivo* and *in vitro*, and its potential mechanism discussed.

2. Materials and methods

2.1. Cell culture and drug treatment

RPMI8226 cells (Shanghai ATCC cell bank) were cultured in RPMI 1640 medium (2591986, Gibco) containing 1 % penicillin-streptomycin (P7630, Solarbio) and 10 % FBS (22323002, Corning®) at 37 °C, 5 % CO_2 and saturated humidity. Rg1 was co-treated with Compound C(CC) (CAS: 866405-64-3, Sigma), an inhibitor of AMPK, in RPMI8226 cells at a final concentration of 5 μmol/L. Rg1 was co-treated with an autophagy inhibitor Chloroquine (CQ) (CAS: 50-63-5, Sigma) at a final dosage of 1 μM in RPMI8226 cells. Meanwhile, RPMI8226 was treated with PBS as the Control group.

2.2. Drug-resistant strain construction and drug treatment

1×10^5/mL of RPMI8226 cell line in logarithmic growth period was inoculated into a 6-well plate and treated with 0.01 μM BTZ (179324-69-7, MCE). After the cell growth returned to normal, it was treated with 0.02 μM BTZ. The drug concentration was gradually increased to 0.2 μM to obtain a drug-resistant cell line (RPMI8226R). After preincubation with autophagy inhibitor Chloroquine (CQ) (CAS: 50-63-5, Sigma) for 1 h (1 μM), RPMI8226R cells were fixed with Rg1 for 24 h. Meanwhile, RPMI8226R was fixed with PBS as the Control group.

2.3. Construction of tumor-bearing mice

Four-week-old female BALB/c nude mice were obtained from Zhejiang Center of Laboratory Animals (SCXK (Zhejiang) 2019-0002) and were reared in an independent ventilated cage with stable temperature of 20–26 °C and consistent humidity of 50%–56 % for one week. Thereafter, 1×10^7/0.2 mL RPMI8226/RPMI8226R cells were subcutaneously inoculated under the abdomen of the nude mice. All animal experiments procedures strictly followed the guidelines established by the National Institutes of Health for the use of experimental animals. The current study was approved by the Institutional Animal Care and Use Committee of Zhejiang Center of Laboratory Animals (approval number: ZJCLA-IACUC-20020112).

2.4. Mouse grouping and administration treatment

After the tumor grew to about 100 mm^3, 12 RPMI8226 tumor-bearing mice were randomly categorized into two groups: Control group and Rg1 group. Mice in Control group were given PBS, while mice in Rg1 group were given Rg1 (50 mg/kg). The mice were administered intraperitoneal injection every 3 days for 15 days. Similarly, 24 RPMI8226R tumor-bearing mice were randomly categorized into four groups: Control group (PBS), BTZ group (0.25 mg/kg), Rg1 group (50 mg/kg) and BTZ + Rg1 group (0.25 mg/kg BTZ+50 mg/kg Rg1).

2.5. Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay

Cells were seeded in 96-well plates at a density of 5×10^3/100 μL. 10 μL of 0.5 mg/mL MTT solution (E606334, Sangon Biotechnology) was added to each well and incubated for 4 h. After centrifugation, the medium was carefully removed, and then 100 μL of Formazan Solubilization Solution was added to each well. After shaking gently for 10 min, the absorbance at 570 nm was assessed using enzyme-labeled instrument (MK-3, Thermo Fisher Scientific Shier Technology).

2.6. Flow cytometry

Once the cells were collected, 195 μL Binding Buffer was added and then resuspended in the flow tube to attain the cell density of 5×10^5. 5 μL Annexin V-FITC reagent (E606336, Sangon Biotechnology) was then added and incubated in the dark for 15 min. 200 μL Binding Buffer was employed to rinse the cells, 190 μL Binding Buffer was added to resuspend the cells, 10 μL of PI reagent was added, and the cells were assessed using flow cytometry.

2.7. Western blot

Protein was isolated from MM cells by centrifugation (4 °C, 5000 rpm, 20 min) in Eppendorf high-speed freezing centrifuge

(Centrifuge 5804 R). The cell lysate was prepared with RIPA Lysis Buffer (C500005, Sangon Biotechnology) and Phenyl Methyl Sulfonyl Fluoride (CAS: 329-98-6, Sangon Biotechnology) at a ratio of 100:1. The BCA method protein concentration determination kit (C503021, Sangon Biotechnology) and microplate analyzer (MK-3, Thermo Fisher Scientific) were employed to assess the protein concentration. Protein samples were separated using SDS-PAGE electrophoresis (Bio-Rad, USA). Thereafter, the protein was moved to PVDF membrane (Millipore, USA), and subjected to overnight incubation at 4 °C with primary antibodies. The antibodies *p*-AMPKα (#50081, 1/1000) and AMPKα (#5832, 1/1000) were purchased from Cell Signaling Technology, Inc. The antibodies mTOR (ab134903, 1/10000), *p*-mTOR (ab109268, 1/2000), Bax (ab32503, 1/1000), Bcl-2 (ab182858, 1/2000), Cleaved-Caspase3 (ab214430, 1/5000), GAPDH (ab9485, 1/2500), LC3B (ab192890, 1/2000), Beclin 1 (ab210498, 1/1000), p62 (ab109012, 1/10000) and Goat anti-rabbit IgG H&L (HRP) (ab6721, 1/2000) were obtained from Abcam (Shanghai) Trading Co., Ltd. The second antibody was incubated for 2 h. The protein bands were exposed using ECL luminescent reagent (Sangon Biotechnology, C500044) and chemiluminescence gel imaging system (FluorChem FC3, USA). ImageJ software was employed to evaluate the gray value.

2.8. Immunofluorescence (IF)

Cells were collected and treated with 4 % Paraformaldehyde (Solarbio, P1110) for 30 min. Subsequently, 100 μL of primary antibody (LC3B antibody, ab192890, 1 μg/mL was incubated overnight at 4 °C in the dark. The second antibody (ab150081, 1/500) was incubated in the refrigerator at 4 °C for 2 h in the dark. Nuclear DNA was labeled with DAPI (E607303, Sangon Biotechnology) and stored in the dark. The images were examined and captured using a fluorescent inverted microscope (Zeiss, Axio Observer. A1).

2.9. Immunohistochemical (IHC) staining

After the tumor tissue was treated with 4 % paraformaldehyde at room temperature for 24 h, it was made into paraffin sections (3~5 μm) with a dehydrator (Excelsior AS, Thermo Fisher Scientific), an embedding machine (HistoStar, Thermo Fisher Scientific) and a slicer (RM2245, Leica). KI67 Cell Proliferation Kit (IHC, E607235, Sangon Biotechnology) was employed to detect tumor tissue, and the cell proliferation was observed under the microscope. Three low power fields (× 100) were randomly chosen under the microscope. The intensity of positive staining was divided into +++, ++, + and –, with their corresponding scores of 3, 2, 1 and 0 respectively. The proportion of stained cells was categorized into 76~100 %, 51~75 %, 26~50 % and 0~25 %, with the corresponding scores of 4, 3, 2 and 1. The staining results were statistically analyzed using semi-quantitative counting method based on the staining intensity and the distribution of positive cells. Colorimetric tuned apoptosis assay kit (C1098, Beyotime) and fluorescence

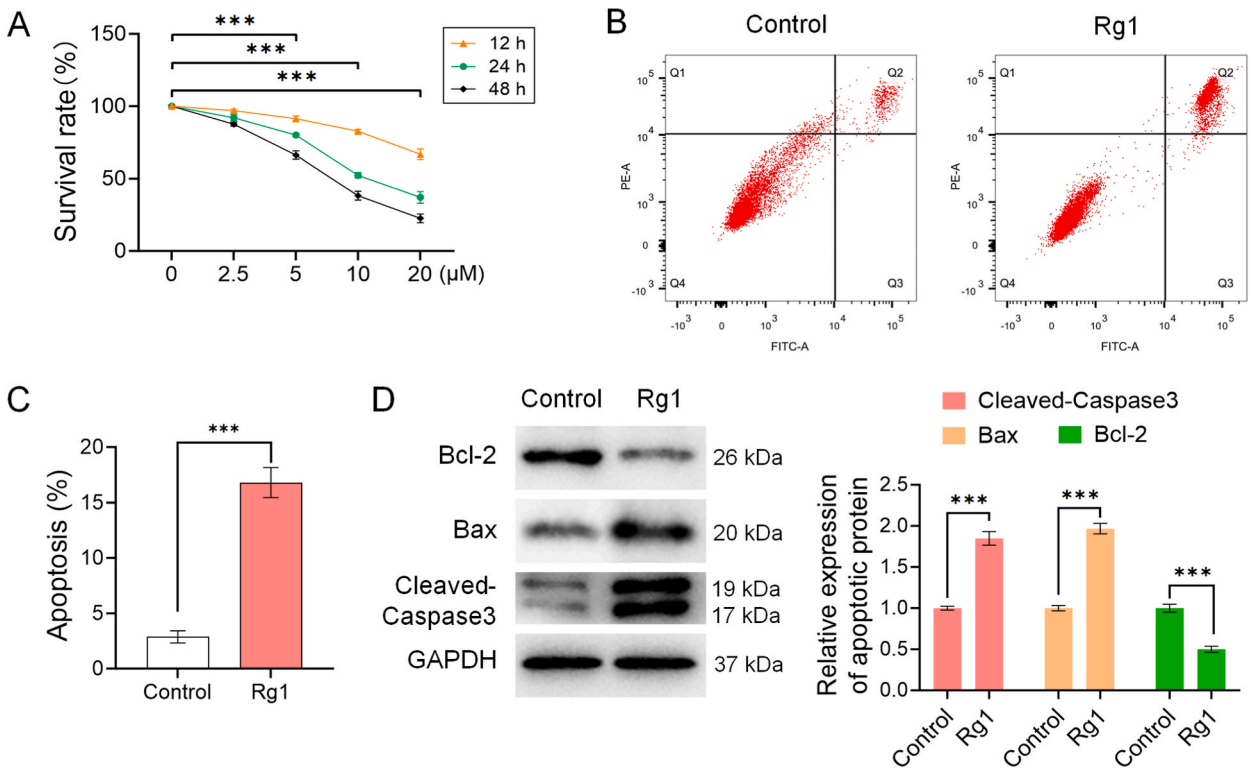

Fig. 1. Rg1 inhibits the proliferation of RPMI8226 cells and promotes apoptosis. A: MTT assay. B–C: Apoptosis was examined using flow cytometry. D: Western blot detection of apoptosis-related proteins. ***P < 0.001.

microscope were used to observe the apoptosis of tumor tissue. The fluorescence intensity was evaluated using ImageJ software.

2.10. Data analysis

The data were all expressed as mean ± standard deviation ($\bar{x}$ ±S) and were evaluated using SPSS 26.0 software. T test was utilized for pairwise comparison, while One-way analysis of variance was employed for comparison among groups, with $P < 0.05$ considered as statistically significant.

3. Results

3.1. Rg1 inhibits MM cell proliferation and promotes apoptosis

In order to clarify the anti-MM effect of Rg1, this study observed the impact of Rg1 on the proliferation and apoptosis of RPMI8226 cells. The findings illustrated that, in comparison to the 0 μM group, Rg1 with 5~20 μM had a considerable inhibitory impact on the proliferation of RPMI8226 cells for 12, 24 or 48 h ($P < 0.001$). In addition, the inhibitory rate of Rg1 with 10 μM for 24 h was about 50 % (Fig. 1A). In comparison to the Control group, the apoptosis rate of RPMI8226 cells in Rg1 group increased substantially after being treated with Rg1(10 μM) for 24 h ($P < 0.001$, Fig. 1B–C). Furthermore, the expression levels of apoptosis-related proteins Cleaved-Caspase3 and Bax was dramatically elevated, while Bcl-2 was notably decreased ($P < 0.001$, Fig. 1D). These findings demonstrated that 10 μM Rg1 had a good inhibitory impact on RPMI8226 cells.

3.2. Rg1 inhibits the growth of MM tumor

In vitro experiments showed that 50 mg/kg Rg1 can not only substantially suppress the growth of tumor (RPMI8226 cells) ($P < 0.001$, Fig. 2A), but also inhibit the proliferation of tumor cells (Fig. 2B) and promote apoptosis (Fig. 2C). Compared with the Control

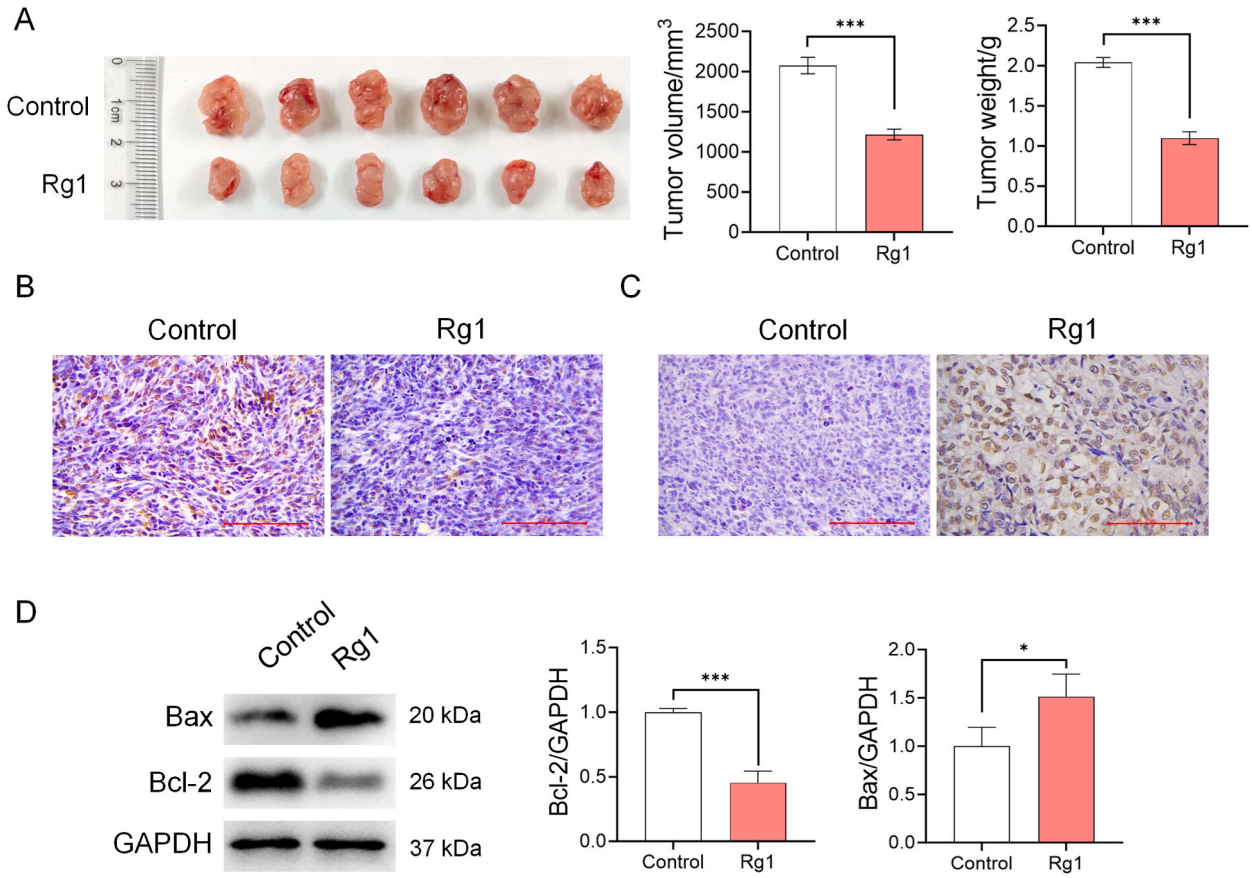

Fig. 2. Rg1 inhibits the growth of MM tumor. A: tumor volume and weight. B: Ki-67 experiment was employed to examine cell proliferation. C: TUNEL experiment was employed to evaluate apoptosis. D: Western blot was employed to examine apoptosis-related protein expression. ***P < 0.001.

group, the expression of Bax in tumor tissues of Rg1 group increased significantly ($P < 0.05$, Fig. 2D), while the expression of Bcl-2 reduced significantly ($P < 0.001$, Fig. 2D).

3.3. Rg1 induces autophagy of MM cells through AMPK-mTOR pathway

To examine the effect and mechanism of Rg1 on autophagy of RPMI8226 cells, our study employed CC to interfere with AMPK pathway. The results demonstrated that Rg1 could obviously increase the autophagy activity of RPMI8226 cells ($P < 0.001$, Fig. 3A–B). In comparison to the Rg1 group, the autophagy degree of Rg1+CC group was considerably reduced ($P < 0.01$, Fig. 3A), whereas the levels of LC3B-II/LC3B-I, Beclin1 and *p*-AMPK were significantly reduced, the *p*-mTOR and p62 were substantially increased ($P <$

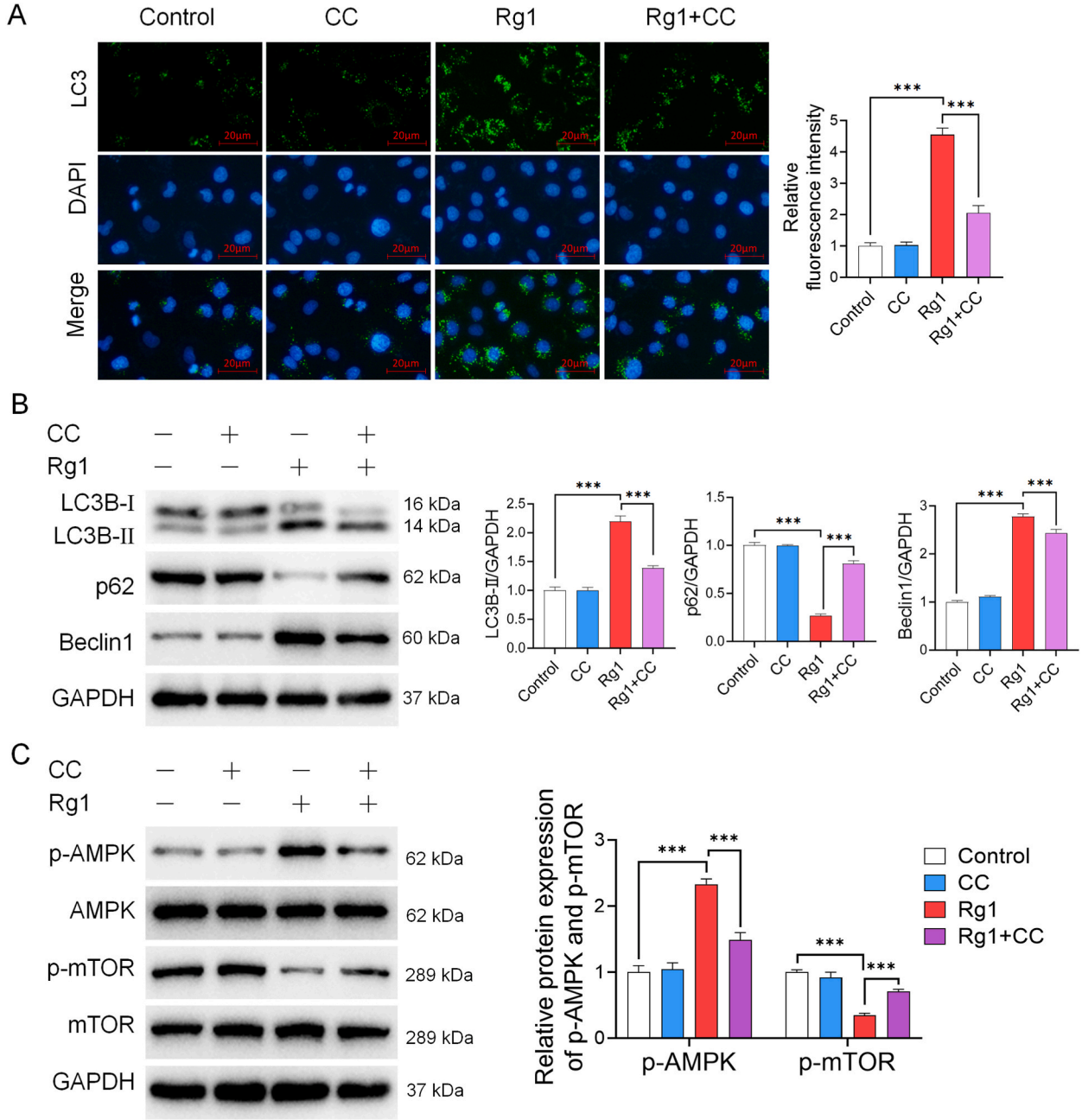

Fig. 3. Autophagy of RPMI8226 cells stimulated by Rg1 through AMPK-mTOR pathway. A: IF detects autophagy flow. B: Western blot was employed to examine the expression of autophagy-related proteins. C: Western blot was employed to examine the expression of AMPK-mTOR pathway related proteins. ***$P < 0.001$.

0.001, Fig. 3B–C). These findings illustrated that Rg1 partly increased autophagy of RPMI8226 cells through AMPK-mTOR pathway.

3.4. Rg1 inhibits MM cell proliferation and promotes apoptosis through autophagy

After interfering with Rg1-treated RPMI8226 cells with autophagy inhibitor Chloroquine (CQ), the proliferation and apoptosis of RPMI8226 cells were observed. The findings illustrated that compared with Rg1 group, Rg1+CQ group significantly increased the cell survival rate ($P < 0.001$, Fig. 4A), the apoptosis rate ($P < 0.01$, Fig. 4B–C), the expression of apoptotic proteins Cleaved-Caspase3. Bax was significantly reduced whereas Bcl-2 significantly increased ($P < 0.05$, Fig. 4D). These findings illustrated that Rg1 partially suppresses RPMI8226 cell proliferation and induces apoptosis through autophagy.

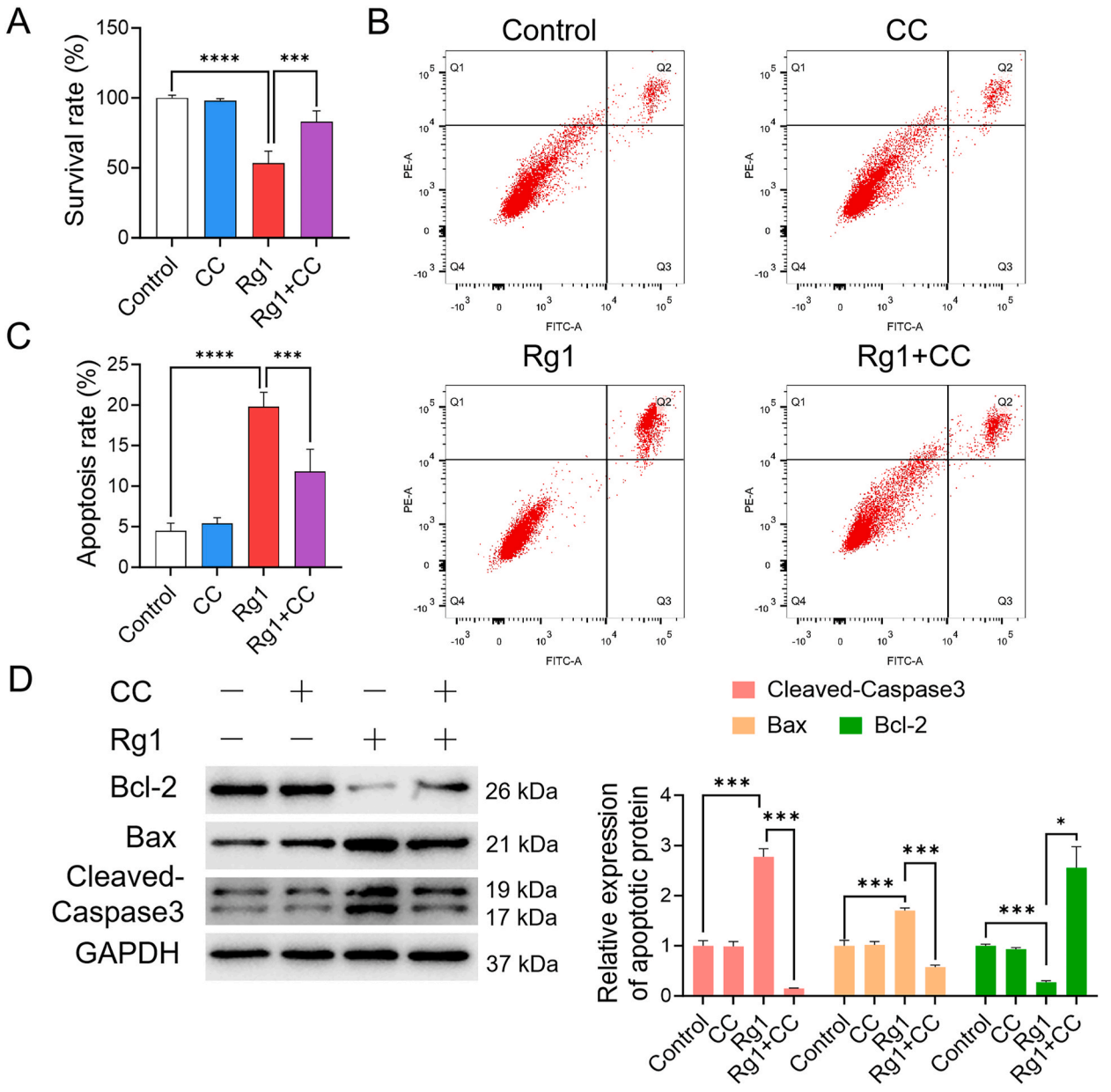

Fig. 4. Rg1 inhibits RPMI8226 cell proliferation and induces apoptosis through autophagy. A: MTT assay was utilized to evaluate cell proliferation. B–C: Apoptosis was evaluated using flow cytometry. D: Western blot detection of apoptosis-related protein expression. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

3.5. Rg1 can enhance the sensitivity of drug-resistant strain RPMI8226R to BTZ

To examine the effect of Rg1 on the drug resistance of RPMI8226 cells to BTZ, we constructed a BTZ-resistant strain RPMI8226R. Fig. 5A illustrates the survival rate of the common strain RPMI8226 at 0.01 μM is about 50 %, while the survival rate of RPMI8226R at 0.2 μM is equally about 50 %, and its sensitivity is weakened by nearly 20 times. However, after co-treatment with Rg1, the survival rate of RPMI8226R at 0.2 μM was substantially improved ($P < 0.001$, Fig. 5B). In comparison to the Control group, the apoptosis rate, expression of apoptotic proteins Cleaved-Caspase3 and Bax in Rg1 group increased significantly, while Bcl-2 decreased considerably ($P < 0.001$, Fig. 5C–D). The combination of Rg1 and BTZ (0.1 μM) increased the sensitivity of RPMI8226R to BTZ, and the effect was greater than that of Rg1 alone.

3.6. Rg1 overcomes BTZ resistance in MM

To verify the effect of Rg1 on BTZ resistance of RPMI8226R cells, we conducted *in vivo* experiments. Fig. 6 illustrates that BTZ (0.25 mg/kg) has a very weak inhibitory effect on drug-resistant tumors, while Rg1 can substantially enhance the inhibitory impact of BTZ on tumors, and the effect is greater than that of Rg1 alone (Fig. 6A). In comparison to the Rg1 group and BTZ group, the cell proliferation rate of Rg1+BTZ group decreased considerably (Fig. 6B), along with increased apoptosis rate (Fig. 6C), increased Bax protein expression ($P < 0.05$, Fig. 6D), and reduced Bcl-2 protein expression ($P < 0.05$, Fig. 6D).

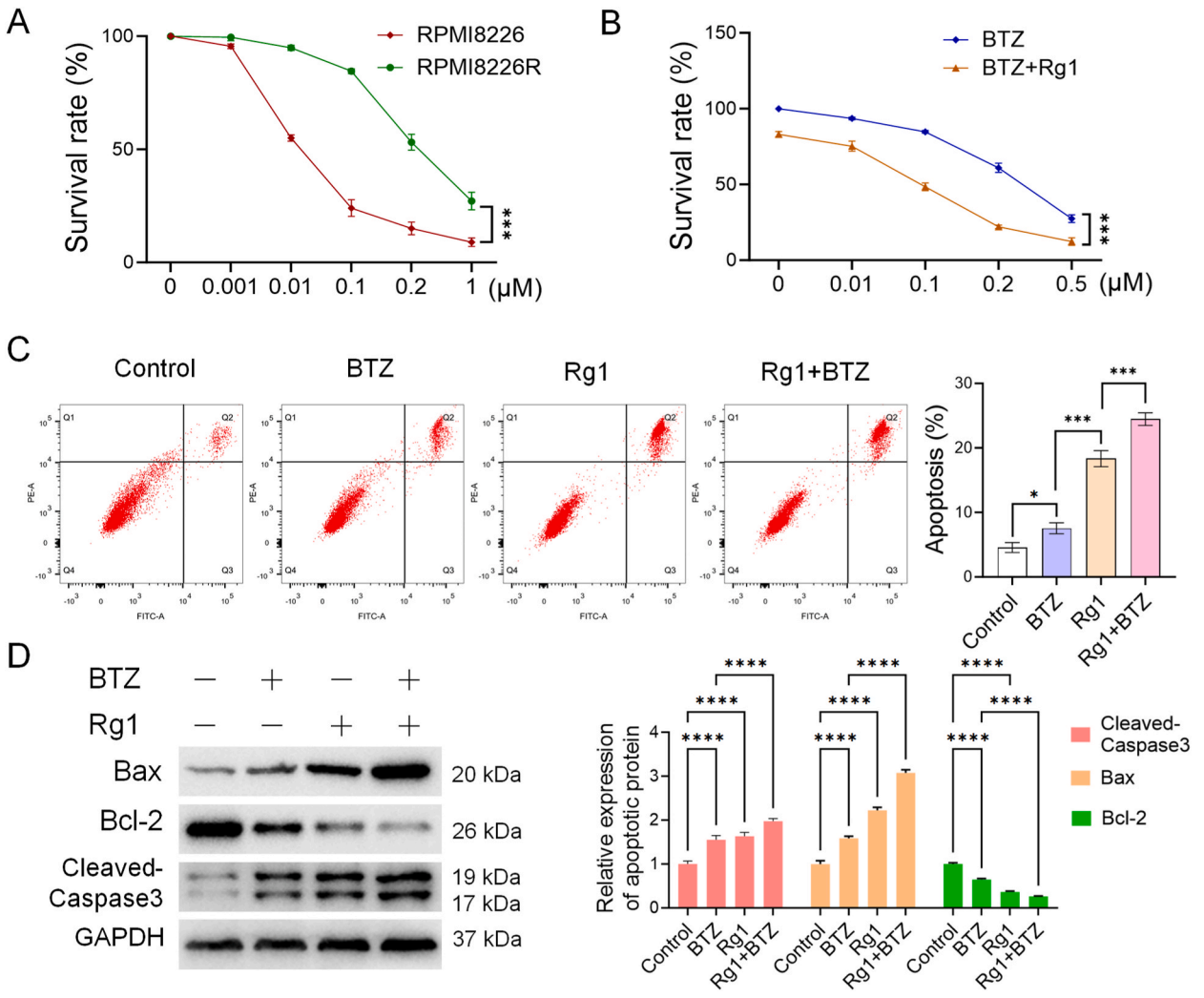

Fig. 5. Rg1 enhances the sensitivity of RPMI8226R to BTZ. A: MTT assay was utilized to examine cell viability. B: MTT assay was employed to examine cell proliferation. Comparison between two groups at the same concentration. C: Apoptosis was examined using flow cytometry. D: Western blot was utilized to examine apoptosis-related protein expression. *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$.

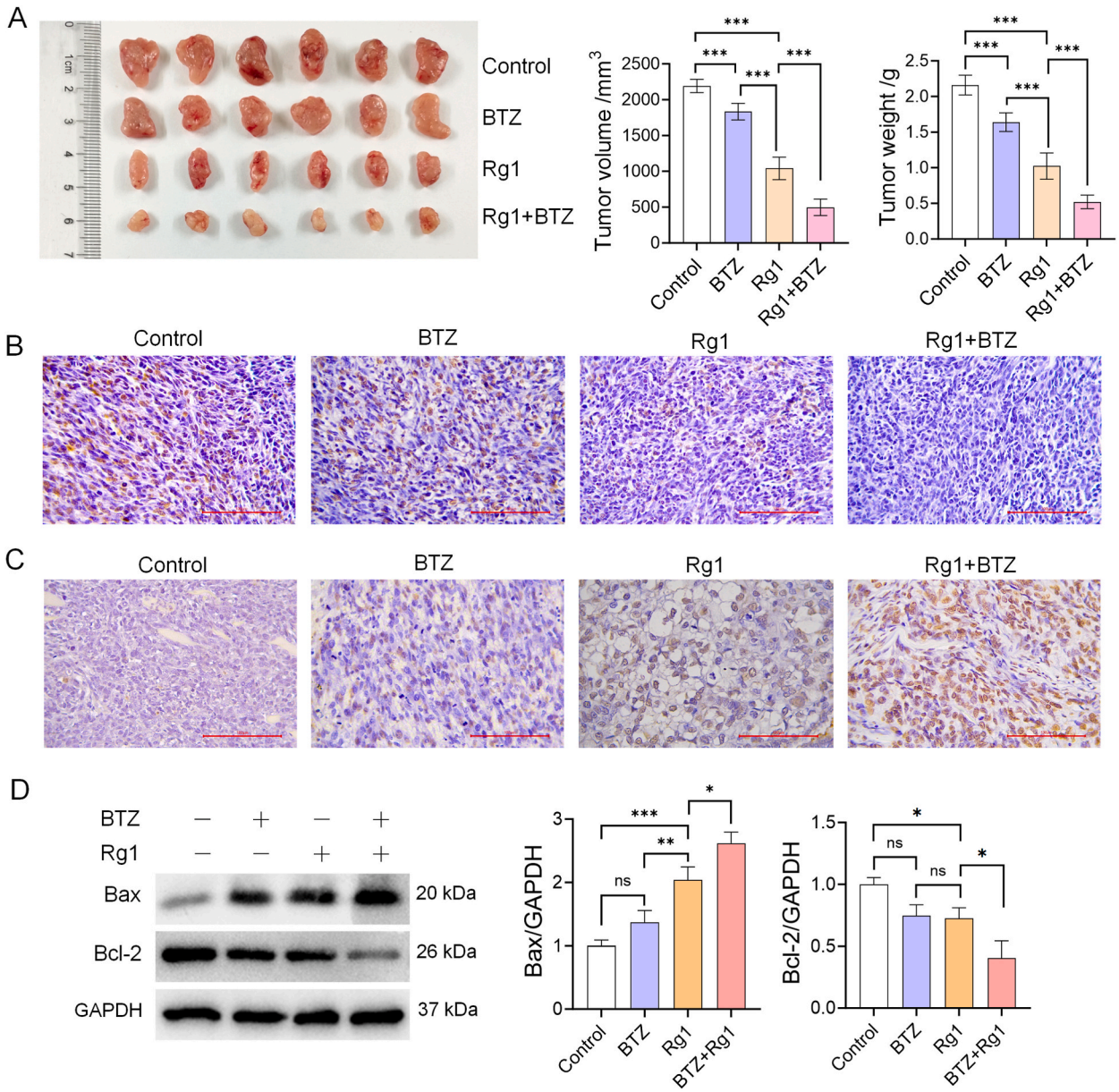

Fig. 6. Rg1 overcomes BTZ resistance. A: Tumor volume and weight. B: Ki-67 experiment was employed to examine cell proliferation. C: TUNEL experiment was employed to examine apoptosis. D: Western blot was employed to evaluate apoptosis-related protein expression. Ns $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

3.7. The autophagy level of RPMI8226R drug-resistant strain increased

To clarify the role of autophagy in the occurrence of drug resistance in BTZ, our study compared the autophagy between RPMI8226 and RPMI8226R. Our findings illustrated that compared with RPMI8226, RPMI8226R significantly increased the autophagy activity ($P < 0.001$, Fig. 7A). Additionally, RPMI8226R showed a considerable increase in the autophagy markers LC3B-II and Beclin1 expression, while causing a notable decrease in p62 ($P < 0.001$, Fig. 7B).

3.8. Rg1 overcomes BTZ resistance through autophagy induced by AMPK-mTOR pathway

In order to examine the function and possible mechanism of autophagy in Rg1 overcoming BTZ resistance, our study intervened with autophagy inhibitor CQ. The findings demonstrated that Rg1 treatment could substantially inhibit the cellular activity of RPMI8226R ($P < 0.001$, Fig. 8A), promote apoptosis ($P < 0.0001$, Fig. 8B) and autophagy ($P < 0.001$, Fig. 8D). At the same time, Rg1

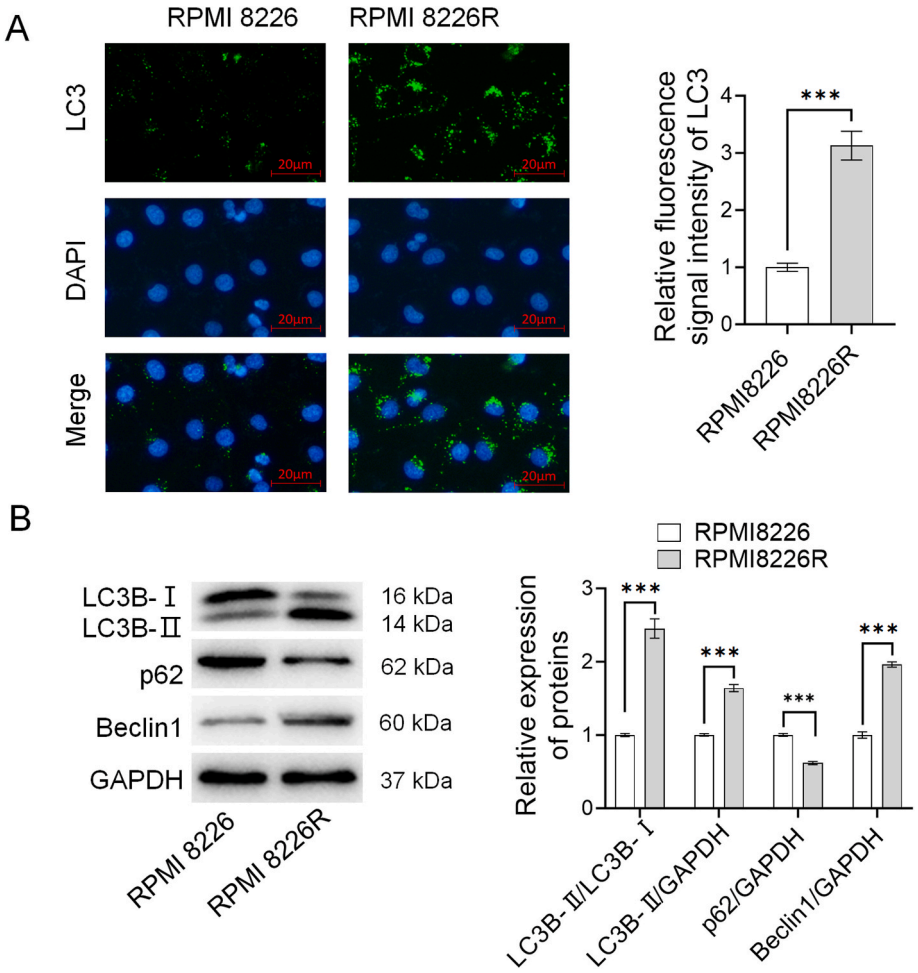

Fig. 7. The autophagy level of drug-resistant strain RPMI8226R increased. A: IF to detect autophagy flow. B: Western blot was employed to examine the expression of autophagy-associated proteins. ***P < 0.001.

treatment was observed to significantly increase *p*-AMPK and decrease *p*-mTOR and Cleaved-Caspase3 (P < 0.01, Fig. 8C–D). Additionally, CQ pretreatment weakened the effect of Rg1 on RPMI8226R cells.

4. Discussion

Multiple myeloma (MM) is the second most prevalent hematological tumor, and it is incurable [7]. Bortezomib (BTZ) functions as a reversible 26S proteasome inhibitor. Currently, numerous combined medication schemes for clinical treatment of MM are based on BTZ [8]. However, it has been found that some patients with MM are resistant to BTZ [9]. Numerous natural ingredients have anti-tumor effects, while some have the potential to overcome drug resistance [10–12]. This provides a novel idea for potential clinical treatment of MM and the exploration for new auxiliary anti-tumor drugs.

Autophagy is a complex cell killing mechanism, which has cell protection and cytotoxicity, and can induce chemical sensitization and chemoresistance of cancer cells [13]. It has been proved that autophagy is a survival strategy for MM drug resistance [14]. Total steroidal saponins from *Solanum nigrum* L. activated autophagy through mTOR signaling pathway to combat multiple drug resistance, thus promoting the death of adriamycin-resistant K562 cells [15]. Solanine can play an anti-MM role by partially activating autophagy, and it has a synergistic effect with BTZ [13]. Therefore, targeted autophagy is an effective intervention strategy for cancer treatment and an important mechanism for overcoming drug resistance in cancer treatment [16,17].

The mTOR is closely linked to the cell survival, proliferation, apoptosis and autophagy. mTORC1 is one of its main active components and serves a crucial part in sustaining metabolic balance, mitochondrial biogenesis and lysosomal biogenesis [18]. AMPK is the key molecule of energy metabolism regulation, and it is also the upstream kinase of mTOR, which can inhibit the activity of the latter. Sorafenib and amygdalin can induce apoptosis of HepG2 cells by targeting AMPK/mTOR pathway [19]. When nutrition is deficient, the increase of AMPK activity reduces the activity of downstream mTORC1, which leads to phosphorylation of the target in the complex before autophagy initiation, thus inducing autophagy [20]. The PFAP treatment has been found to regulate the

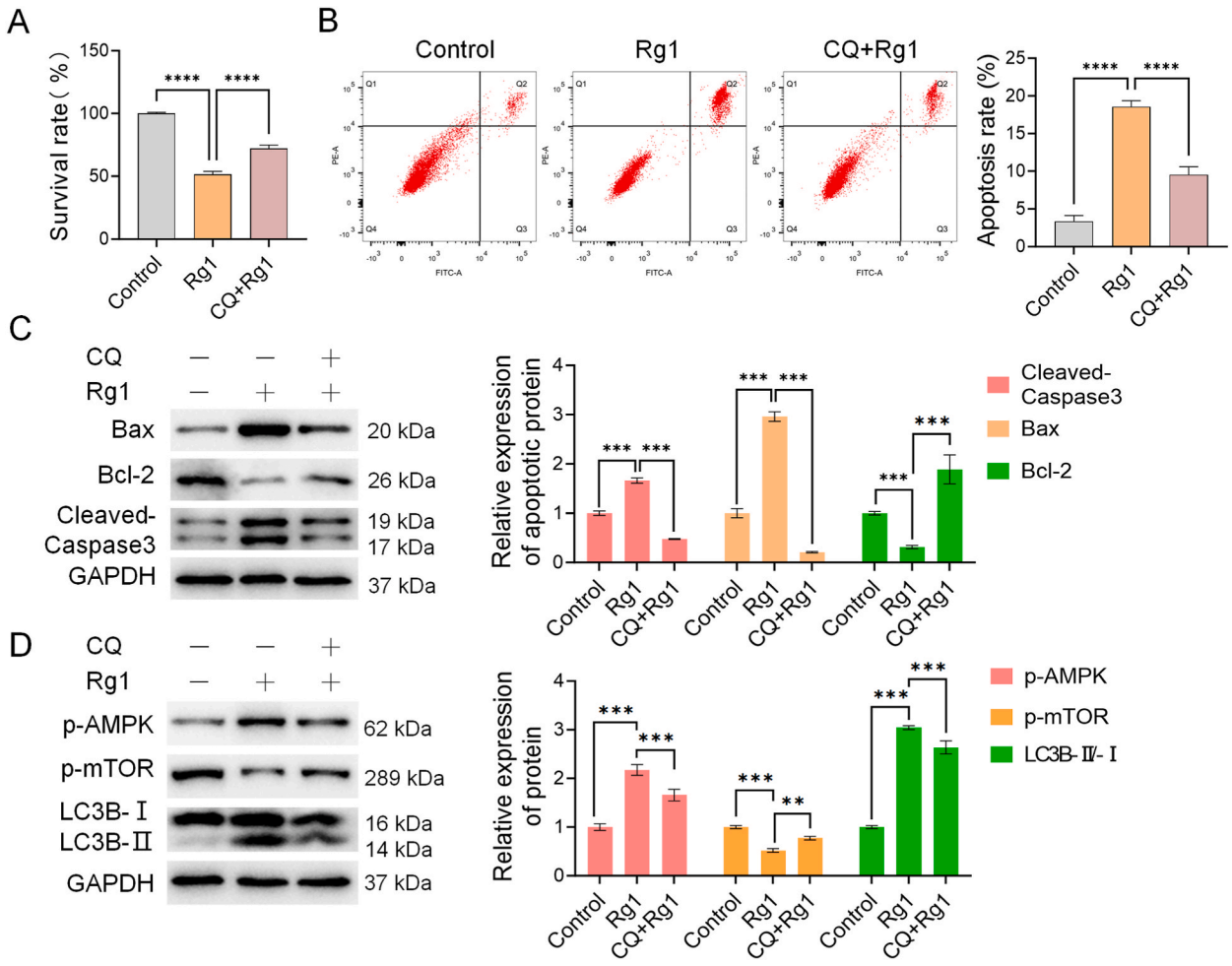

Fig. 8. Rg1 overcomes drug resistance through autophagy induced by AMPK-mTOR pathway. A: MTT assay was employed to assess RPMI8226R cell proliferation. B: Apoptosis was detected using flow cytometry. C: Western blot was employed to assess the expression of apoptosis-related proteins. D: Western blot was employed to assess autophagy and AMPK-mTOR pathway associated protein expression. **P < 0.01, ***P < 0.001, ****P < 0.0001.

AMPK-mTOR pathway to activate autophagy in A549 cells [21].

Rg1 is a potential anti-tumor drug [4,22], which can delay the mitosis of cancer cells and suppress the proliferation of cancer cells [5]. In nasopharyngeal carcinoma cells, Rg1 has been found to decrease the cell activity, promote apoptosis and increase the expression of LC3II protein [23]. In triple-negative breast cancer cell line, Rg1 can inhibit cell proliferation, reduce tumor occurrence in rats, and show chemical sensitization characteristics [6,24]. However, its research in MM is rarely reported. The findings of this investigation show that Rg1 can inhibit the development of MM *in vivo* and *in vitro*, and promote apoptosis. *In vitro*, Rg1 induces excessive autophagy of MM cells through AMPK-mTOR pathway, thus hindering the proliferation of MM cells and promoting apoptosis. We constructed a BTZ-resistant strain RPMI8226R. Through in vivo and in vitro experiments, it was found that Rg1 can increase the sensitivity of RPMI8226R to BTZ and overcome drug resistance. Additionally, this study found that compared with RPMI8226, the autophagy level of RPMI8226R increased, which suggested that autophagy played a role in the formation of BTZ drug resistance. Further study showed that Rg1 could increase the sensitivity of RPMI8226R to BTZ by enhancing autophagy. This process is believed to be linked with the regulation of AMPK-mTOR pathway. The above results prove that Rg1 can resist MM and improve the sensitivity of BTZ-resistant strains, and reveal some of its mechanisms, providing a potential auxiliary anti-tumor drug for the clinical treatment of MM.

5. Conclusion

This study proved the anti-tumor effect of Rg1 on MM. Rg1, either alone or in conjunction with BTZ, offers a promising supplemental anti-tumor medication for clinical use, allowing for the overcoming of BTZ resistance in MM. The limitation of this investigation is that only one cell line was utilized. Additional research is required to further clarify the molecular mechanism of Rg1 against MM in different cell lines or through alternative approaches.

Funding

This work was supported by The Natural Science Foundation of Ningbo Municipality (Grant no. 2021J018).

Ethics approval

This study was reviewed and approved by the Institutional Animal Care and Use Committee of Zhejiang Center of Laboratory Animals with the approval number: ZJCLA-IACUC-20020112, dated June 23, 2022.

Data availability statement

Data associated with the study has not been deposited into a publicly available repository. Data are available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Li Lin: Writing – original draft, Supervision, Resources, Project administration, Methodology, Formal analysis, Conceptualization. **Dong Chen:** Writing – review & editing, Validation, Formal analysis, Data curation. **Shuangyue Li:** Writing – review & editing, Validation, Software, Investigation, Formal analysis. **Tiantian Wang:** Writing – review & editing, Visualization, Validation, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33935.

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