Histol Histopathol (2023) 38: 1069-1077

Histology and Histopathology
From Cell Biology to Tissue Engineering

ORIGINAL ARTICLE Open Access

Apoptotic effect of selenium mushroom extract from Qinba on multiple myeloma cells

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Summary. Qinba selenium mushroom is a mushroom belonging to the Basidiomycetes family, which is believed to have anti- oxidant, anti-tumoral and anti-mutagenic activities. However, the efficacy of Qinba selenium mushroom against multiple myeloma has not been confirmed. The present study aimed to investigate the apoptotic effect of FA-2-b-β, the selenium mushroom extract from Qinba on multiple myeloma (MM) cells. The MM RPMI-8226 cells were treated with FA-2-b-β at different concentrations and time points. MM RPMI-8226 cell proliferation and apoptosis were detected by the Cell Counting Kit-8 (CCK-8) assay and Annexin V/propidium iodide (PI) assay, RT-QPCR and western blotting analyses were performed to determine the proteins and pathways involved. The results of the present study demonstrated that FA-2-b-β has high anti-proliferative activities and strong pro-apoptotic effects on MM RPMI-8226 cells, and its pharmacological effects on proliferation changes occurred in a dose- and time-dependent manner. In addition, we found that FA-2-b-β was able to induce cell apoptosis and promote cell cycle arrest at G0/G1 phase. In summary, the results illustrate the involvement of FA-2-b-β in mediating G0/G1 cell cycle arrest and apoptosis in MM RPMI-8226 cells, which suggested that FA-2-b-β might have therapeutic potential against multiple myeloma as an effective compound, and may provide useful information for the development of a novel therapeutic target in this area.

Key words: FA-2-b-β, Qinba selenium mushroom, Cell cycle, Apoptosis

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www.hh.um.es. DOI: 10.14670/HH-18-571

Introduction

Multiple myeloma (MM) is a mature B-cell tumor, which is mainly characterized by excessive proliferation of malignant clonal plasma cells and production of monoclonal immunoglobulin, causing bone destruction, bone pain or fracture, anemia, renal insufficiency and other symptoms (Gerecke et al., 2016; Swerdlow et al., 2016). Due to the inhibition of the production of normal immunoglobulin, it is prone to various bacterial infections. At present, MM is the second most common hematological malignancy in the United States, accounting for 13% of blood cancers and 1% of all cancers (Raab et al., 2016). MM mostly occurs in middle-aged and elderly people, and women are more likely to suffer from this disease than men (Raab et al., 2016). As population ages, the number of patients suffering from this disease is increasing. Although bortezomib, as a representative of proteasome inhibitor, has made great progress in clinical treatment, the problems of refractory, relapse and drug resistance have not been solved. MM is still an incurable malignant blood disease, which seriously threatens the lives of patients. Therefore, the research and development of new drugs for the disease is of great significance.

Qinba selenium mushroom, also named Agaricus blazei murill (ABM) is rich in selenium polysaccharide, glycoprotein, nucleic acid, fat, saponins, tanni, sterol, minerals, trace elements and essential amino acids required by the human body (Rathore et al., 2017). Since 1960, ABM has been widely used in alternative medicine in Brazil (Li et al., 2019). It has been reported that ABM may be active in alleviating atopic dermatitis and reducing diabetes risk (Khursheed et al., 2020), but the molecular mechanism of the anti-cancer activity of ABM is unknown. In recent years, many experts have isolated and purified steroids, polysaccharides, glycoprotein complexes and nucleic acids from ABM. The active components and pharmacological effects of the drug have been studied. ABM has been found to have an anti-tumor function (Xie et al., 2018; Kumar et

al., 2020; Murad et al., 2021), improve immunity (Cheng et al., 2017; Ryan et al., 2018), and decrease the percentage of body fat (Lin et al., 2019), and has a bactericidal effect (Hetland et al., 2020). With the deepening of research on ABM, the β-1,6-glucan isolated from ABM polysaccharide can inhibit the proliferation of tumor cells. It can regulate the growth and senescence of tumor cells and improve the immunity of the body, and it may play an anti-tumor effect by inducing apoptosis of tumor cells (Matsushita et al., 2018; Taofiq et al., 2019). Tangen et al. (2017) reported that polysaccharide from ABM was able to induce apoptosis of leukemia cells; Matsushita et al. (2018) showed that the water extract of ABM inhibited the proliferation of pancreatic cancer cells and induce their apoptosis; In addition to the anti-tumor effect, the water extract of ABM combined with chemotherapy drug (azide) has a good anti-tumor sensitization effect on gastric cancer model mice. The proteoglycan extracted from ABM, namely acid RNA protein complex FA-2-b-β is an anti-cancer compound, which plays a role in regulating the activity of natural killer cells and macrophages (Lin et al., 2019). It is suggested that the anti-tumor effect of FA-2-b-β is closely related to the enhancement of body immunity (Cheng et al., 2021). This natural compound, which has few or no side effects, is more effective in treating tumors than existing natural products. Studies have reported that FA-2-b-β has inhibition rates of 85.8% and 74.5% in sarcoma 180/ICR-JCL mice after intravenous injection and oral administration for 3 weeks, and complete tumor regression rates of 33.3% and 35.2% after 6 weeks of administration, respectively (Zhang et al., 2016).

In the present study, we examined the anti-cancer activity of FA-2-b-β in RPMI8226 cells, and RPMI8226 cell proliferation was potently inhibited by FA-2-b-β in a time- and dose-dependent manner. FA-2-b-β was able to induce cell apoptosis *in vitro* and elevated Bax expression, while it decreased the level of Bcl-2. Because our studies demonstrated that the treatment of RPMI8226 cells with FA-2-b-β resulted in cell cycle arrest at G0/G1 phase, following FA-2-b-β treatment, the expression of CDK2 and CDK4 decreased. The decreased level of cyclin CDK2 and CDK4 made evident FA-2-b-β induced cell cycle arrest at G0/G1 phase in RPMI8226 cells. Thus, the results of the present study suggest that the anti-tumor activity of FA-2-b-β may be due to decreased cellular proliferation, but also the induction of cell apoptosis. Therefore, FA-2-b-β has potential for future treatment approaches for MM. The present study presents a number of limitations and future studies should include experiments *in vivo* in order to determine the direct effect of FA-2-b-β in MM. The purpose of this study was to utilize the anti-cancer compound FA-2-b-β extracted from ABM to treat myeloma cell lines and to detect its effect on inducing apoptosis, so as to explore its anti-tumor mechanism and provide experimental data for the development of new anti-tumor drugs.

Materials and methods

Drugs and reagents

ABM was provided by Shaanxi Ziyang Co., Ltd., and its RNA protein complex FA-2-b-β was extracted and identified by Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, as previously described (Akiyama et al., 2011). The FA-2-b-β was dissolved in PBS and stored at 4°C for subsequent experimentation.

Cell culture and culture

Multiple myeloma cells (RPMI-8226) were purchased from ATCC and primary Multiple myeloma cells were maintained in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) media with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc) and 1% penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in an atmosphere with 5% CO_2. During the expansion phase, RPMI8226 cells were maintained at a concentration of 0.5-1×10^6 cells/mL in culture medium. According to different experimental purposes, the appropriate number of cells for use in the experiments was determined and treated with 0.5 mg/ml, 1mg/ml, 1.5mg/ml, 2 mg/ml or 2.5 mg/ml FA-2-b-β for 24, 48 and 72h.

Proliferation assay

A CCK-8 assay was used to detect cell proliferation. MM RPMI8226 cells were seeded onto 96-well plates (KeyGene, Nanjing, China) at a density of 5×10^4 cells/well in 100 μL of culture. Then they were exposed to various concentrations of FA-2-b-β(0.5-2.5 mg/ml) for 24, 48, and 72 h in 96-well plates. The experiments were performed in triplicate. After incubation with FA-2-b-β, 10 μL CCK-8 solution (Dongjido, Japan) was added to each well and incubated for 1h at 37°C according to the manufacturer's instructions. The absorbance was measured at 450 nm using an ELX800 absorbance microplate reader (Biotech Instruments, NY, USA).

Assessment of apoptosis using flow cytometry

MM RPMI8226 cells were seeded in a culture flask at a density of 1×10^6 cells and treated with FA-2-b-β for 24, 48, 72h. Then, the cells were detected using the Annexin VFITC Apoptosis Detection Kit (BDBiosciences). After staining and incubating, according to the manufacturer's protocol, apoptosis was measured by flow cytometry (BDBiosciences, USA).

Cell cycle distribution analysis

The distribution of MM RPMI8226 cells which stained with propidium iodide (PI) in different phases of the cell cycle were estimated by flow cytometry (BDBiosciences, USA). MM RPMI8226 cells treated

with or without FA-2-b-β were fixed in 75% ethanol, and incubated at 4°C for at least 2h. Subsequently, fixed cells were washed in PBS buffer, stained in a PI-containing solution (50 μg propidium iodide/ml, 50 μg RNAse A/ml, 0.1M EDTA in PBS, pH 7.4) and analyzed on flow cytometry (BD Biosciences).

Reverse transcription-quantitative (RT-q) PCR

Total RNA from MM RPMI8226 cells was extracted by using TRIzol (Invitrogen, USA).

The purity and concentration of RNAs were verified by absorbance on NanoDrop2000 (Thermo, USA) at 260 nm and 280 nm. The samples with ratios from 1.8 to 2.0 were qualified for next reverse transcription reaction. iScriptcDNA Synthesis Kit (Thermo, USA) was used to synthesize cDNAs according to the manufacturer's instructions. Each real-time PCR was prepared in a 20 μL reaction mixture and performed on Applied Biosystems 7500 Real-Time PCR System (Thermo, USA). The Real Time PCR reaction was performed with SYBRGreen (Biored, USA) the thermocycling conditions according to the manufacturer's instructions. PCR primer (Invitrogen, USA) sequences for gene expression analyses are presented in Table 1. Target Gene relative mRNA levels were calculated with normalization to β-actin values using the $2^{-\Delta\Delta ct}$ method.

Western blot analysis

MM RPMI8226 cells were harvested and rinsed in PBS and lysed with SDS lysis buffer (Solarbio, China). The samples were boiled for 10 min, loaded on 8-12% SDS polyacrylamide gels and run at 100V, samples were trans blotted onto nitrocellulose membranes for 1h at 100V. Nitrocellulose membranes were blocked for at least 1h in 5% skimmed dried milk at room temperature, washed with 0.05% Tween-20 in TBS, pH 7.4 and incubated with primary antibody (diluted in 1% bovine serum albumin) overnight (anti-CDK2, anti-CDK4, anti-Bcl-2, anti-Bax, Abcame, USA) at 4°C. Membranes were washed (three times in TBS) and exposed to secondary antibody (diluted 1:1000 in 1% bovine serum albumin in TBS) for 1 hour (anti-Mouse IgG, E030110-01, Earthox; anti-Rabbit IgG, E030120-01, Earthox; anti-Goat IgG, E030130-01, Earthox;) then rinsed three times in TBS. Protein bands were visualized by ECL (ECL kit; Molecular Biotech) according to the manufacturers protocol. The band intensity was measured and quantified with a chemiluminescence detection system (ChemiDoc XRS, Bio-Rad, USA).

Statistical analysis

Analyses of significant differences were performed by using one-way ANOVA and the T-test. Data shown are representative of three independent experiments with cells. Data are presented as mean ± SD. A P-value less than 0.05 was considered to indicate statistically significant differences. Statistics were performed using GraphPad Prism 6 Software.

Results

Effects of FA-2-b-β on the proliferation of RPMI-8226 cell

The CCK-8 assay was employed in order to determine the inhibitory effect of FA-2-b-β on MM cell proliferation. Figure 1 shows that FA-2-b-β had a concentration and time dependent anti-proliferative effect on RPMI-8226 cells. The number of viable cells decreased sharply by 48 and 72h in the 1.5 mg/ml of FA-2-b-β-treated group, indicating that FA-2-b-β had significant effects on cell proliferation at the late stage (48 and 72h) but not in the early stage (24h).

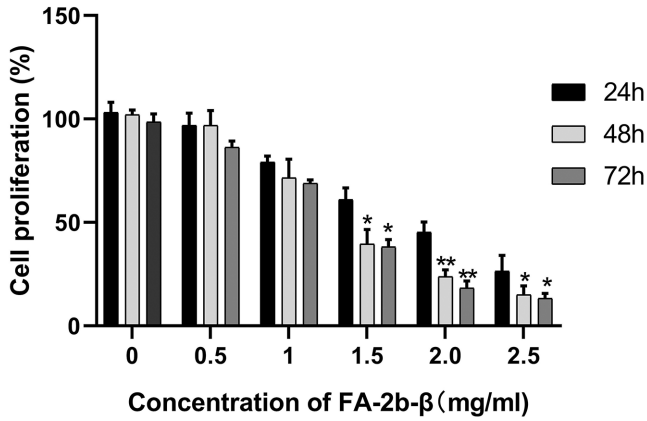

Fig. 1. FA-2-b-β inhibits the cell proliferation of RPMI-8226 cell. CCK8 assay of RPMI-8226 cells treated with different doses of FA-2-b-β for 24h, 48h and 72h (n=5). The plots represent the mean ± SD of five replicates. Asterisks indicate statistically significant differences as analyzed by one-way ANOVA (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

Table 1. PCR primer sequences.

Gene	Forward (5'-3')	Reverse (5'-3')
β-actin	TGGCACAAAGCACAATGAA	CTAAGTCATAGTCCGCCTAGAAGC
Bcl-2	GGATTGTGGCCTTCTTTGAG	TACCCAGCCTCCGTTATCCT
Bax	CCGATTCATCTACCCTGCTG	TGAGCCAATTCCAGAGGCAGT

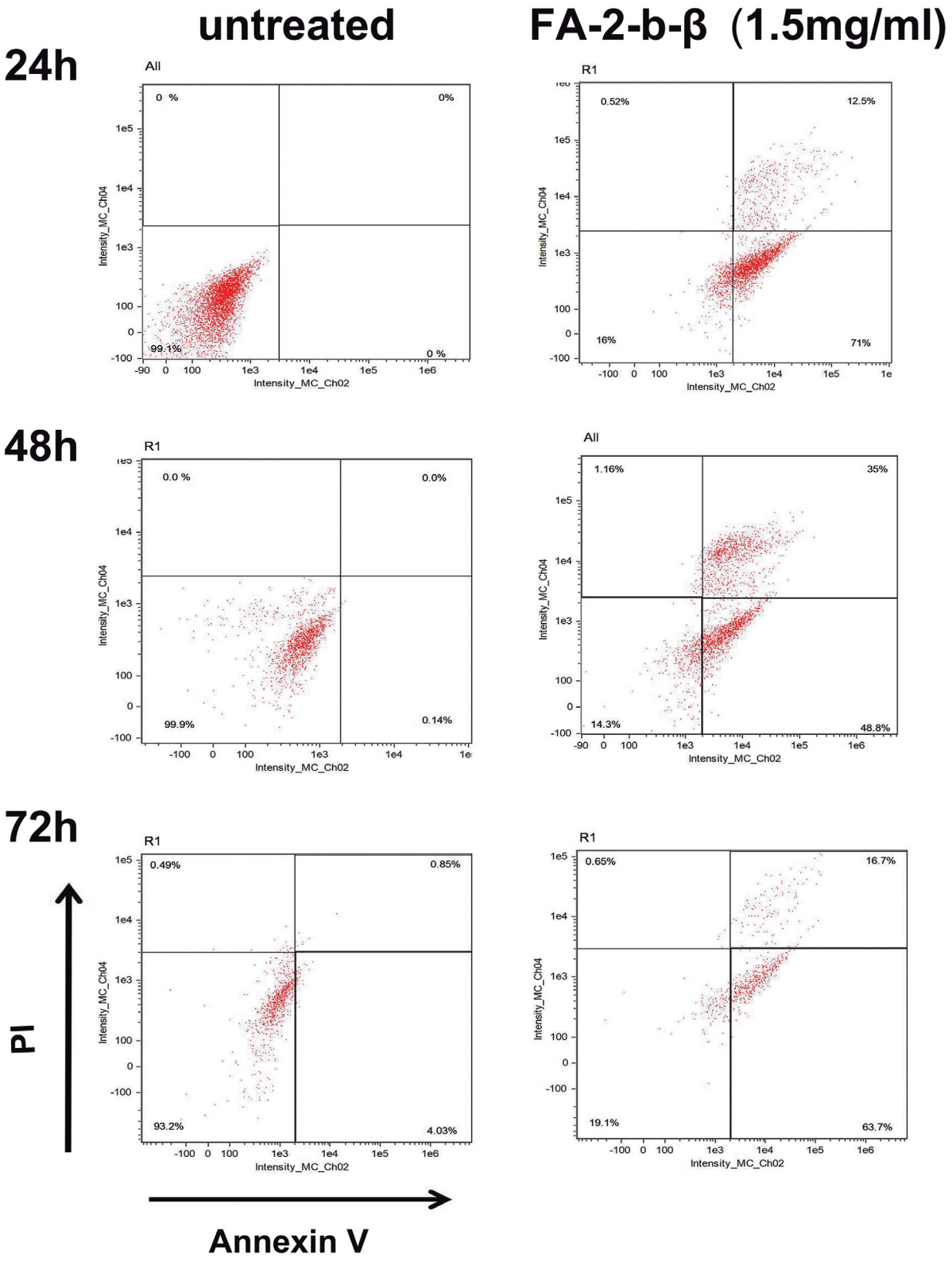

Fig. 2. FA-2-b-β induces apoptosis in RPMI-8226 cells. RPMI-8226 cells were treated with or without FA-2-b-β (1.5 mg/ml) for 24h, 48h and 72h, stained with Annexin V-FITC/propidium iodide, and analyzed for apoptosis by flow cytometry. Data are representative of 3 independent experiments. Staining for FITC-conjugated Annexin V was used as a marker for early apoptosis whereas propidium iodide (PI) staining was used as a marker for cell death.

FA-2-b-β induced apoptosis in RPMI8226 cells

We next determined the effects of FA-2-b-β on cellular apoptosis in RPMI8226 cells.

In the right quadrants of flow cytometry graphs, FA-2-b-β significantly increased the number of apoptotic cells. In RPMI8226 cells, the total percentage of apoptotic cells (early + late apoptosis) was increased from 0% at basal level (zero time) to 83.5%, 83.8% and 80.4% when treated with FA-2-b-β at 1.5 mg/ml for 24h, 48h, 72h, respectively (Figs. 2, 3). In aggregate, FA-2-b-β remarkably induces apoptotic cell death in RPMI8226 cells. To further investigate the molecular mechanism underlying FA-2-b-β induced apoptosis in RPMI8226 cells, the RT-PCR analysis that was performed focused on apoptosis-related genes, including Bax and BCL-2. As illustrated in Figure 4A, after the RPMI8226 cells were treated with FA-2-b-β at 1.5 mg/ml, the anti-apoptosis gene Bcl-2 mRNA expression was significantly impaired in the RPMI8226 cells, while the expression of pro-apoptosis gene Bax mRNA was increased (Fig. 4B). Furthermore, western blotting was performed to evaluate the expression of Bax and BCL-2, as presented in Fig. 4C, FA-2-b-β increased the expression of Bax and inhibited the expression of Bcl-2, which further confirmed that FA-2-b-β promoted apoptosis of RPMI8226 cells. These results suggested that FA-2-b-β induced apoptosis in RPMI8226 cells, which may be related to the inhibition of Bcl-2 expression.

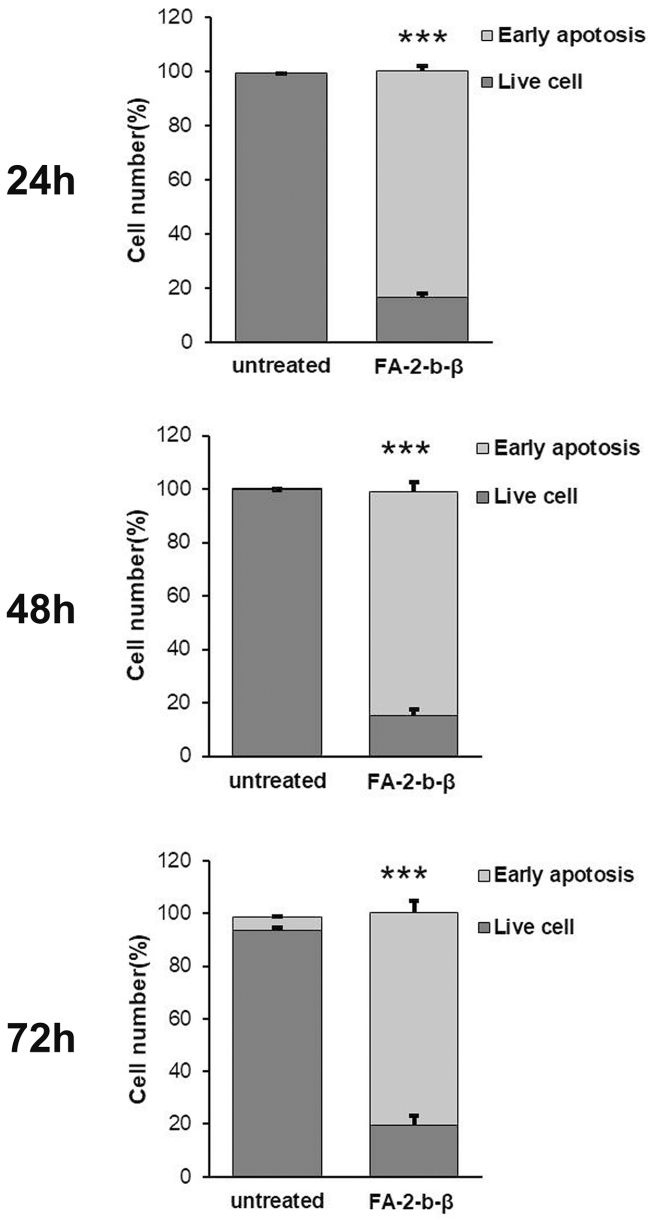

Fig. 3. FA-2-b-β induces apoptosis in RPMI-8226 cells. RPMI-8226 cells were treated with or without FA-2-b-β (1.5 mg/ml) for 24h, 48h and 72h, stained with Annexin V-FITC/propidium iodide, quantification of live and dead cells, data are shown as mean ± SEM. *P< 0.05. **P<0.01.

FA-2-b-β induced RPMI8226 cell-cycle arrest

To determine the function of FA-2-b-β in the regulation of the cell cycle, the effect of FA-2-b-β on cell-cycle progression in RPMI8226 cells was measured by flow cytometry. We evaluated the distribution of cell cycle in RPMI8226 cells treated with FA-2-b-β at the concentration 1.5 mg/ml for 48h. As shown in Figure 5, a significant change in the population of cell cycle distribution was observed after treatment with 1.5 mg/ml for 48 hours. Compared with control groups, the G0/G1 fraction of RPMI8226 cells treated with 1.5 mg/ml FA-2-b-β for 48 h increased by 11.4% ($P<0.05$, Fig. 5A,B). The percentages of S phase cells were decreased correspondingly (34.1% in the control vs. 19.7% in FA-2-b-β 1.5 mg/ml, $p<0.01$). These data indicated that FA-2-b-β blocked or delayed the progression of cells from G0/G1 phase into S phase, and induced cells toward apoptosis. Western blot analysis also confirmed that after treatment for 48h with FA-2-b-β at 1.5 mg/ml, the protein expression of Cyclin-dependent kinase (CDK) 2 and CDK4 were suppressed significantly (Fig. 6). CDK2 and CDK4 protein are core factors and play key roles in the cell cycle G1/S phase regulation network. These results suggested that FA-2-b-β can induce cell cycle arrest at the G0/G1 phase by reducing the expression of CDK2 and CDK4 in RPMI8226 cells, thus inhibiting the proliferation of RPMI8226 cells.

Discussion

MM is a hematological malignancy characterized by the proliferation of clonal plasma cells in the bone marrow (BM) accompanied by secretion of monoclonal immunoglobulin (Kumar et al., 2017). MM is one of the most common malignant cancers worldwide and mostly occurs in middle-aged and elderly people (Raab et al., 2016). As standard chemotherapy and hematopoietic stem-cell transplantation still do not offer a cure for

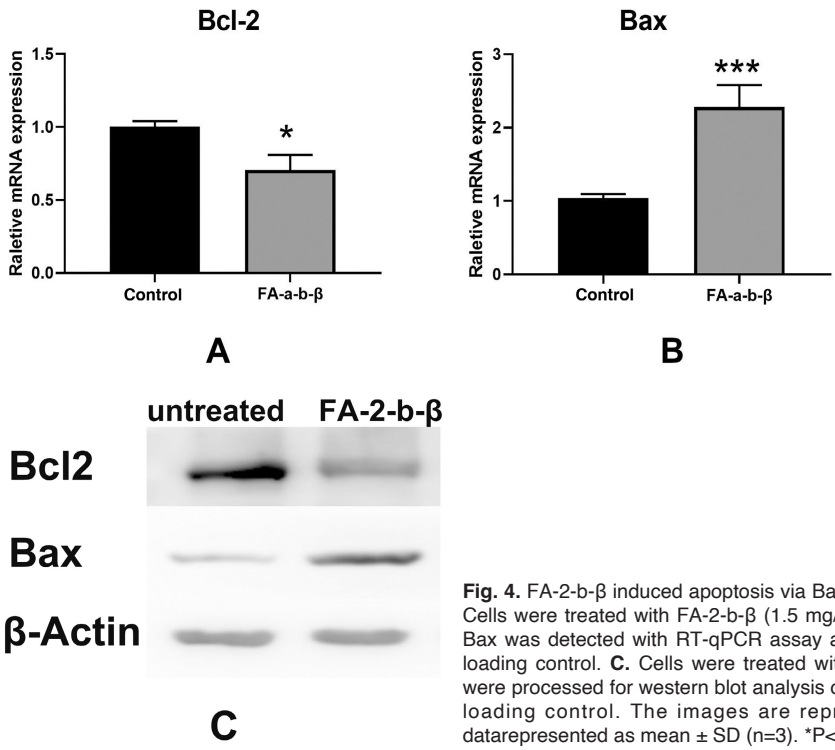

Fig. 4. FA-2-b-β induced apoptosis via Bax expression and Bcl-2 repression in RPMI-8226 cells. **A.** Cells were treated with FA-2-b-β (1.5 mg/ml) for 48h, then the mRNA expression of Bcl-2 **(A)** and Bax was detected with RT-qPCR assay as described in methods **(B)**, and β-actin was used as a loading control. **C.** Cells were treated with FA-2-b-β (1.5 mg/ml) for 48h as indicated, then cells were processed for western blot analysis of Bax and Bcl-2 protein expression. β-actin served as the loading control. The images are representative of three independent experiments and datarepresented as mean ± SD (n=3). *P<0.05. **P<0.01.

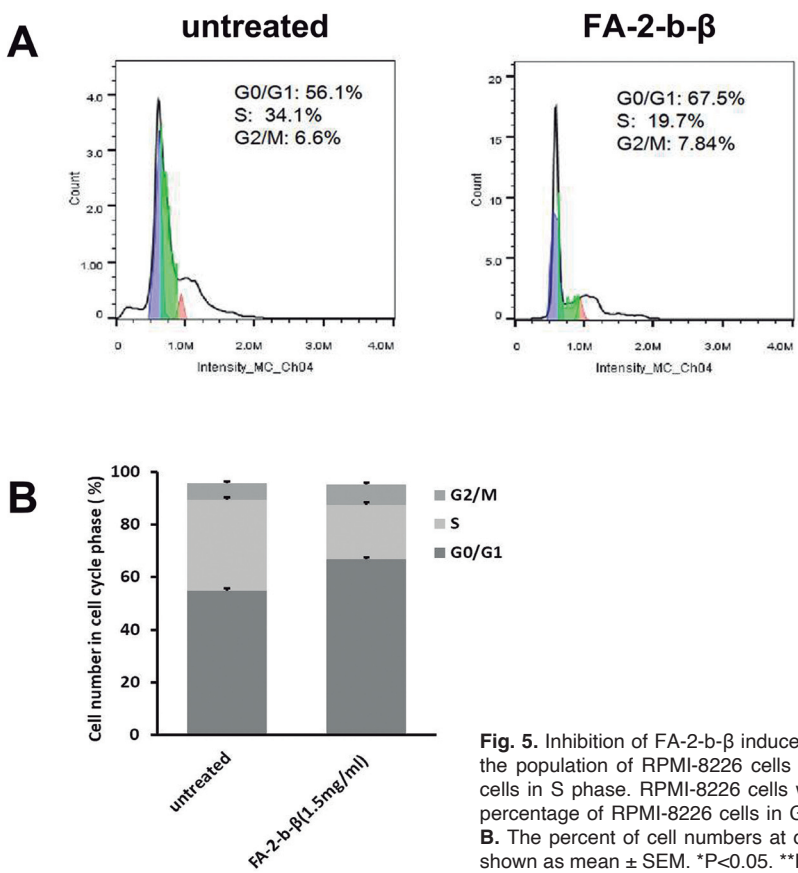

Fig. 5. Inhibition of FA-2-b-β induced cell cycle arrest of RPMI-8226 cells. **A.** FA-2-b-β increased the population of RPMI-8226 cells in G0/G1phase but decreased the population of RPMI-8226 cells in S phase. RPMI-8226 cells were treated with or without 1.5mg/ml FA-2-b-β for 48h. The percentage of RPMI-8226 cells in G0/G1, S, and G2/M phases are indicated in each histogram. **B.** The percent of cell numbers at different phases of cell cycle (G0/G1, S and G2/M). Data are shown as mean ± SEM. *P<0.05. **P<0.01.

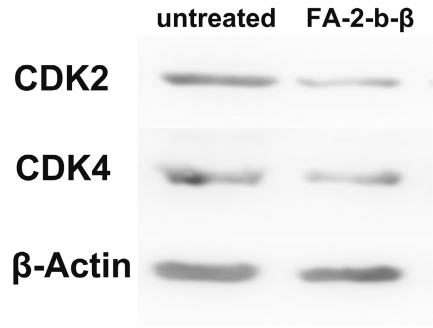

Fig. 6. Effects of FA-2-b-β on cell cycle regulatory proteins in RPMI-8226 cells. (A) Cell-cycle regulatory proteins CDK2, CDK4 were examined using Western blot analyses. Cells were pretreated with or without FA-2-b-β (1.5mg/ml) for 48h. β-actin as a loading control. Relative changes in the protein bands were measured using commercially available quantitative software.

patients, alternative strategies are required for therapeutic intervention (Kumar et al., 2017). ABM has been used in the treatment of a variety of diseases, including diabetes, atherosclerosis, hepatitis, hypercholesterolemia and heart disease (Hetland et al., 2021). ABM has been shown to possess immune-potentiating, anti-oxidant, anti-tumoral activities (Bahrami et al., 2019). It has been proved that the β-1,6-glucan isolated from ABM polysaccharide can inhibit the viability of colon cancer cells *in vitro* and *in vivo* (Cheng et al., 2020). It has been reported that the compound derived from AbM for anti-cancer activity is a proteoglucan, acid RNA protein complex, FA-2-b-β, which functions in regulating natural killer cell and macrophage activity (Lin et al., 2019). However, the role of FA-2-b-β in patients with MM requires further investigation. Studies have shown that ABM induces cancer cell apoptosis through Bax and caspase dependent pathways (Shimizu et al., 2016). However, the role of FA2bβ in the treatment of MM is not well understood. Based on these results, we explored the inhibition effects of FA2bβ on RPMI8226 cells for further study. Our study demonstrated that decreased proliferation of RPMI8226 cells occurs in response to treatment with FA-2-b-β, and its pharmacological effects on proliferation changes occurred in a dose- and time-dependent manner. In addition, we found that FA2bβ could induce cell apoptosis and promote cell cycle arrest at G0/G1 phase. The antitumor mechanism of FA-2-b-β in the RPMI8226 cells was the induction of G0/G1 cell cycle arrest and promotion of cell apoptosis. This study is the first to elucidate the function of FA-2-b-β in MM.

Apoptosis is controlled by genes. There are many genes involved in the process of apoptosis, among which Bcl-2, p53, caspase-3 and c-myc play important regulatory roles. Imbalances in expression levels of the Bcl-2 family members result in defects in programmed cell death associated with chemoresistance, malignancy, and aggressiveness of tumors (Vasco-Mogorrón et al., 2021). Thus, regulation of anti-apoptotic proteins may represent an important strategy for sensitizing MM cells to various therapeutic agents. There is higher expression of the anti-apoptotic Bcl-2 and there is a lower level of expression of Bax in MM (Touzeau et al., 2018). It has been reported that FA2bβ induces cell apoptosis through the Wnt/βcatenin pathway in Chronic myeloid leukemia (Sun et al., 2020). And β-catenin can decrease cell apoptosis via Bcl-2 or Bax targeting (Yamaguchi et al., 2019). Other studies demonstrated that anti-cancer agents regulate Bcl-2 family members (including Bax or Bcl-2) (Liang et al., 2018; Yamaguchi et al., 2019; Tajadura et al., 2020). This evidence strongly supports the results of the present study, which demonstrated that FA-2-b-β induced RPMI8226 cell apoptosis *in vitro*, accompanied by elevated Bax expression and decreased level of Bcl-2.

The cell cycle determines cell proliferation and regulates complex processes that determine cell growth and division. All signaling pathways involved in the cell cycle must be precisely regulated in order to determine the fate of the cell. To date, numerous anticancer drugs have been proved to arrest the cell cycle at a certain phase (Bonelli et al., 2019; El Gaafary et al., 2019; Hoffman and Yano, 2019; Kawami et al., 2019; Lopez Perez et al., 2019; Yamashita et al., 2020; Tang et al., 2020). Cell cycle disorder is an important feature of tumor cells, so cyclin is an important target for the anti-cancer effect of FA2bβ. Among them, CDK2 and CDK4 play a crucial role in the regulation of G1-S transition and modulation of G2 progression (Chen et al., 2020; Hume et al., 2020; Liu et al., 2020). Following FA2bβ treatment, the expression of CDK2 and CDK4 decreased, confirming that G0/G1 phase arrest occurred after FA2bβ treatment in RPMI8226 cells. It has been reported that FA2bβ treatment contributed to cell cycle arrest during the G1 phase and this observation is supported by a previous study (Sun et al., 2020). The results suggest that the anti-tumor effect of FA2bβ on the RPMI8226 cells was mainly attributed to the induction of G0/G1 cell cycle arrest by targeting CDK2 and CDK4.

Thus, the results of the present study suggest that the anti-tumor activity of FA2bβ may be due to decreased cellular proliferation, but also the induction of cell apoptosis. Therefore, FA2bβ has potential for future treatment approaches for MM. The present study presents a number of limitations and future studies should include an experiment *in vivo* in order to determine the direct effect of FA2bβ in MM.

Acknowledgements. This study was supported by Gansu Youth Science and Technology Fund (No. 20JR5RA190).

Declaration of Interest Statement. The authors declare no conflict of interests.

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Accepted December 9, 2022