Agaricus blazei extract (FA-2-b-β) induces apoptosis in chronic myeloid leukemia cells

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Abstract. *Agaricus blazei Murill* (AbM) is a mushroom belonging to the Basidiomycetes family, which is believed to have antitumor and antioxidative activities. Proteoglycans and ergosterol are considered the key compounds of AbM for antitumor properties and so are used in complementary and alternative medicine as an anticancer drug. AbM is used to avoid serious side effects that would inevitably affect patients. Currently, the efficacy of AbM against chronic myeloid leukemia (CML) has not been established. The present study aimed to investigate the antitumor activities of the acidic RNA protein complex, FA-2-b-β, extracted from wild edible AbM. The CML K562 cells or primary CML bone marrow (BM) cells were treated with FA-2-b-β at different concentrations and time points. CML cell line proliferation and apoptosis were determined using the CCK-8 assay or Annexin V/propidium iodide (PI) labeling, RT-qPCR and western blotting was performed to determine the involvement of the Wnt/β-catenin-associated apoptotic pathway. The results of the present study demonstrated that FA-2-b-β has a high anti-proliferative potency and strong pro-apoptotic effects. Thus, daily intake of mushrooms containing FA-2-b-β may be an adequate source as an alternative medicine in the management of CML, and may provide useful information for the development of a novel therapeutic target in this area.

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Abbreviations: AbM, *Agaricus blazei* Murill; CML, chronic myeloid leukemia; BM, bone marrow; PB, peripheral blood; ABL1, abelson; BCR, breakpoint cluster region; TK, tyrosine kinase

Key words: CML, AbM, Wnt/β-catenin signaling pathway, apoptosis cycle

Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative disorder that stems from myeloid CD34$^+$/CD38$^-$/CD44$^+$ progenitors in the bone marrow (BM) (1). The incomplete differentiation of hematopoietic stem cells in CML results in immature leucocytes in both the BM and peripheral blood (PB) (2). Translocation of the abelson (ABL1) gene at chromosome 9 produces the so called 'Philadelphia chromosome,' which is considered the main cause of CML (3). This mutation activates the BCR-ABL1 fusion oncoprotein, which modulates a number of signaling pathways and enables stem cells to avoid cell death. Furthermore, this potent tyrosine kinase (TK) activates different signaling pathways, including the Wnt/β-catenin, Bcl-2 and JAK/STAT MAPK/JNK signaling pathways, that are responsible for the pathogenesis of CML (3-5). Thus, BCR-ABL1 is considered a therapeutic chemotherapy target for CML, using the imatinib class of drugs. However, the main drawback of therapeutic TK inhibitors is the severe side effects exhibited in patients with CML (6), including cardiovascular, pulmonary, gastrointestinal and endocrine toxicities (7), as well as development of resistance (8). Thus, it is essential to investigate alternative agents to minimize the side effects.

Complementary and alternative medicine, extensively used for the treatment of disease (9), may be an alternative approach in decreasing the side effects of patients with cancer undergoing chemotherapy, which compromises patients' quality of life (10). Natural compounds are well recognized for the management of several diseases, including CML (11). Mushroom-derived compounds have the ability to act as anti-leukemia, antiviral, antioxidant and anti-inflammatory agents, and exert tumor apoptosis-inducing properties (12). Additionally, it has been reported that chemical substances derived from microorganisms, plants and animals possess anticancer potential (13). Thus, it is of great interest to investigate whether there is anti-CML potential associated with different mushrooms and determine the underlying molecular mechanism of action.

Agaricus blazei Murill (AbM) has been extensively applied in alternative medicine in Brazil since 1960 (11). It has been reported that the putative active functions of AbM lie in relieving atopic dermatitis and decreasing glycosuria (9).

Although several polysaccharides contain AbM, including dextran, mannan and heteropolysaccharides (14,15), the molecular mechanism underlying such agents in anticancer activity is yet to be investigated. The most useful compound derived from AbM for anticancer activity is a proteoglucan, acid RNA protein complex, FA-2-b-β, which functions in regulating natural killer cell and macrophage activity (16). In addition, AbM-derived linoleic acid is a bactericidal agent that regulates host immunity, enhancing helper T cell expansion and natural killer cell activation, and helping to decrease the percentage of body fat and visceral fat, as well as blood cholesterol and glucose levels in human volunteers of clinical research (14,17). This evidence strongly supports the use of AbM as a health-promoting supplement for immunomodulation (14,17). Steroids have also been identified as an effective component in AbM against acute monocytic leukemia (18).

The Wnt/β-catenin signaling pathway is involved in embryogenesis, cell development, proliferation, differentiation and self-renewal, and homeostasis of multiple organ systems (19). Thus, dysregulation of the Wnt/β-catenin signaling pathway is closely associated with tumorigenesis (20). For example, upregulation of β-catenin is observed in CML, with the expansion of the BCR/ABL gene (21). Conversely, inhibition of β-catenin expression decreases drug resistance in CML, with delayed entry into the blast phase (22).

However, the ability of AbM to inhibit human leukemia *in vitro* remains unknown. Thus, the present study aimed to investigate the effect of FA-2-b-β, derived from AbM, in the induction of apoptosis in human leukemia cells *in vitro*, and considered whether such a potential effect targeted the Wnt/β-catenin signaling pathway. The results of the present study provide insight on naturally-derived compounds in the development of a novel therapeutic target in the management of CML.

Materials and methods

Cell culture and reagents. The CML K562 cells and primary CML BM cells from four female patients and one male patient with CML (mean age, 48 years; age range, 40-56 years) were collected. CML K562 cells were purchased from Lanzhou University Medical School (Lanzhou, China), and primary CML BM cells were purchased from Gansu Provincial People's Hospital (Lanzhou, China). The BM samples from patients with chronic leukemia were extracted and isolated by density gradient centrifugation at 1,000 x g (1,000 r/min) at 4˚C for 3 h, using a sterile operating table, after obtaining written informed consent from the patients. The cell density was adjusted to $5x10^6$/ml. Either the K562 or the primary CML BM cells were maintained in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc), at 37˚C in an atmosphere with 5% CO_2. Sub-culturing of the cells was performed when 80% confluence was reached.

Details of the primary antibodies are as follows: Anti-c-myc antibody (1:1,000; cat. no. 5605; Cell Signaling Technology, Inc.), anti-cyclin D1 antibody (1:1,000; cat. no. 2978; Cell Signaling Technology, Inc.), anti-CD44 antibody (1:1,000; cat. no. 3570; Cell Signaling Technology, Inc.), anti-c-Jun antibody (1:1,000; cat. no. 9165; Cell Signaling Technology, Inc.), anti-MMP antibody (1:1,000; cat. no. 3801; Cell Signaling Technology, Inc.), anti-Lef/Tcf antibody (1:1,000; cat. no. 2203; Cell Signaling Technology, Inc.), anti-Bcl-2 antibody (1:1,000; cat. no. 12789-1-AP; ProteinTech Group, Inc.), anti-Bax antibody (1:1,000; cat. no. ab32503; Abcam), anti-β-actin antibody (1:1,000; cat. no. GB-12001; Beijing Biosynthesis Biotechnology Co., Ltd.), anti-β-catenin antibody (1:1,000; cat. no. ab22656; Abcam). The HRP-linked goat anti-rabbit IgG (1:4,000; cat. no. 7074) or goat anti-mouse IgG (1:1,000; cat. no. 7076) were purchased from Cell Signaling Technology, Inc.

AbM was supplied by the Edible Mushroom Center of Shanxi Agricultural University (Shanxi, China) (23). The RNA-protein complex, FA-2-b-β, was extracted in the School of Chemistry and Chemical Engineering, Lanzhou University (Lanzhou, China), as previously described (24). The purified compounds were obtained from Lanzhou University using ethanol precipitation, gel filtration, DEAE-cellulose and Sephadex G-200 column chromatography (15). The fraction FA-2-b-β was dissolved in distilled water for subsequent experimentation.

Cell proliferation assay. K562 cells or primary CML BM cells (10,000) were seeded into 96-well plates in a total of 100 μl and cultured with FA-2-b-β. FA-2-b-β at the concentrations of 1.2, 1.5, 1.8, 2.1 and 2.4 mg/ml or vehicle only (RPMI-1640) were cultured at 37˚C for 24, 48 and 72 h. To prevent medium evaporation, 100 μl PBS was added into each well. Cell proliferation was assessed via the Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc.) according to the manufacturer's protocol (25). The solution (10 μl/ml) was added and incubated for 1 h at 37˚C. The solution in the wells was aspirated gently following centrifugation at 500 x g for 5 min at 4˚C. Following mixing for 10 min, the absorbance was measured at 450 nm using an ELX800 absorbance microplate reader (BioTek Instruments, Inc.). Each assay was repeated five times.

Apoptosis and cell cycle assay. For the apoptosis assay, K562 cells or primary CML BM cells (100,000) were seeded into 6-well plates with different concentrations of FA-2-b-β, at a dose range of 0, 1.2, 1.5, 1.8 and 2.4 mg/ml and time periods of 24 and 48 h. These cells were harvested after 24 or 48 h of treatment and permeabilized using Triton-X, washed and resuspended in binding buffer (50 mM HEPES, 700 mM NaCl, 12.5 mM $CaCl_2$ pH 7.4 in 100 μl) with the addition of 2.5 μl Annexin V (cat. no. R8021) and 5 μl PI (cat. no. EZ2811E231) from an Annexin V-FITC Apoptosis Detection kit (BD Biosciences). Briefly, 100,000 cells were washed with ice-cold PBS, resuspended in 195 μl PBS and stained for 15 min at room temperature with 5 μl FITC conjugated anti-Annexin V antibody. Unbound Annexin V antibody was removed by washing with binding buffer. The percentage of apoptotic K562 cells and primary CML BM cells (Annexin V-positive) was determined by flow cytometry analysis. Apoptotic cells were pre-treated at 37˚C in the presence or absence of 3 mmol/l N-acetylcysteine (NAC; BD Biosciences) for cell cycle analysis for 2 h, followed by treatment with different concentrations of FA-2-b-β for 24 or 48 h at 37˚C. Subsequently, the supernatant was removed, and the treated cells were washed three times with PBS and fixed using 70% ethanol overnight at 4˚C. The cells were washed with PBS buffer, incubated with 5 μl RNase (0.25 mg/ml; BD Biosciences) for 30 min at 37˚C. The pelleted

Table I. PCR primer sequences.

Gene	Forward (5'-3')	Reverse (5'-3')
Actin	TGGCACAAAGCACAATGAA	CTAAGTCATAGTCCGCCTAGAAGC
β-catenin	GTGCATCTACACCGACAACTCC	GTTCCACTTGAGCTTGTTCACC
Bcl-2	GGATTGTGGCCTTCTTTGAG	TACCCAGCCTCCGTTATCCT
Bax	CCGATTCATCTACCCTGCTG	TGAGCCAATTCCAGAGGCAGT

cells were resuspended in PBS-diluted (1:100) Annexin V-FITC Apoptosis reagent (50 μg/ml) and incubated for 15 min at room temperature in the dark for flow cytometry (BD FACSuite v1.0.538.41; BD Biosciences).

Reverse transcription-quantitative (RT-q) PCR. Total RNA from K562 and primary CML BM cells was extracted using TRIzol® reagent (Takara Bio, Inc). RNase-free DNase I (Promega Corporation) was used to remove the genomic DNA. RT was performed at 70°C for 5 min using the PrimeScript RT MasterMix Reverse transcriptase cDNA synthesis kit (cat. no. SD2193; Takara Bio, Inc.). The Real Time PCR reaction was performed with SYBRGreen (cat. no. SD3034; Takara Bio, Inc.) using the qPCR Applied Biosystems 7500 Real-time System StepOne PLUS with the following thermocycling conditions: Initial denaturation at 95°C for 4 min, then 40 cycles at 95°C for 15 sec and 60°C for 45 sec. β-actin was used as the internal control for AbM inhibition of apoptosis determining Bcl-2, Bax and β-catenin expression. Primers were designed by Takara Bio, Inc, and are presented in Table I. The expression levels of the aforementioned genes were determined using the $2^{-\Delta\Delta Cq}$ method (26).

Western blot analysis. K562 and primary CML BM cells were seeded into a 6-well plate and incubated with FA-2-b-β for 24/48 h at 37°C. Cells were lysed with SDS lysis buffer (cat. no. P1200; Beijing Solarbio Science & Technology Co., Ltd.) and total protein was obtained. Protein concentration was measured using a bicinchoninic acid protein assay. After protein concentration was measured, equal amounts of protein (10 μg/lane) were separated via 8-12% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (EMD Millipore). Membranes were blocked with 5% skimmed dried milk for 1 h at room temperature, and then incubated with the appropriate primary antibodies overnight at 4°C. After primary antibody incubation, the membranes were washed three times with TBS-Tween (0.05% Tween-20; Boster Biological Technology), and then the corresponding HRP-linked secondary antibody was added for 2 h at room temperature. Apoptosis protein bands were detected using enhanced chemiluminescence (ECL kit; cat. no. P10010; Molecular Biotech Co., Ltd.) according to the manufacturer's protocol and analyzed using Image Lab™ software v6.0 (Bio-Rad Laboratories, Inc.).

Statistical analysis. Statistical analysis was performed using SPSS software (version 18; SPSS, Inc.). All quantitative data were analyzed using one-way ANOVA, followed by Dunnett's post hoc multiple comparison test to compare all columns with the control column. Data shown are representative of
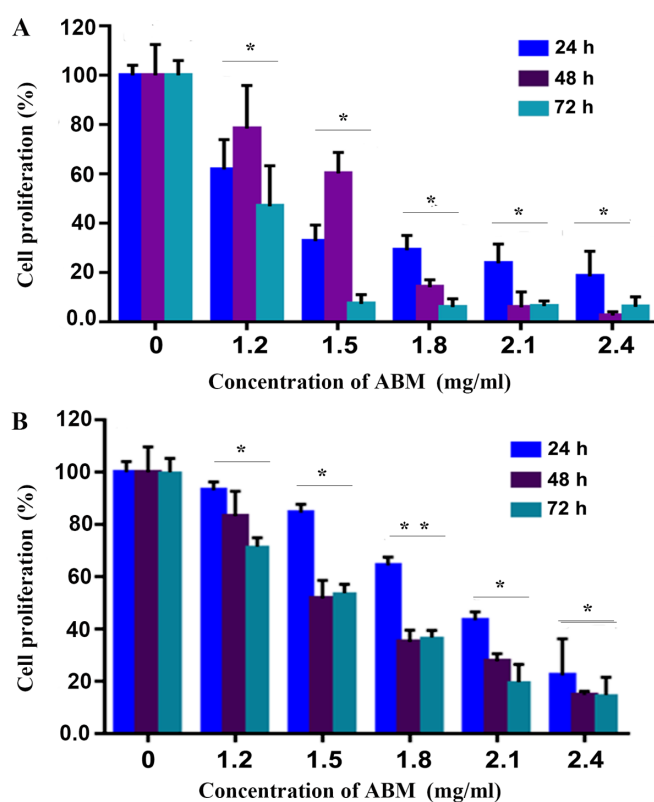

Figure 1. AbM inhibits the cell proliferation of K562 or primary CML BM cells. Cell proliferation was inhibited in (A) K562 or (B) primary CML BM cells at the indicated time points according to the CCK-8 assay. The plots represent the mean ± SD of five replicates (after 24, 48 and 72 h).

three independent experiments with cells. Data are presented as the mean ± SD. P<0.05 and P<0.001 were considered to indicate statistically significant differences.

Results

FA-2-b-β inhibits CML cell proliferation. The CCK-8 assay was employed in order to determine if FA-2-b-β was able to inhibit the cell proliferation of CML. FA-2-b-β significantly inhibited proliferation of K562 cells or primary CML BM cells (P<0.05), in a concentration dependent manner (Fig. 1A and B). This effect was enhanced in 1.2 mg/ml of FA-2-b-β over the entire culture period.

FA-2-b-β induces CML apoptosis. Some of the K562 or primary CML BM cells were completely damaged or inhibited by FA-2-b-β at the dosage used (Fig. 1A and B), but it was unclear if other residual cells were still viable. The apoptosis of
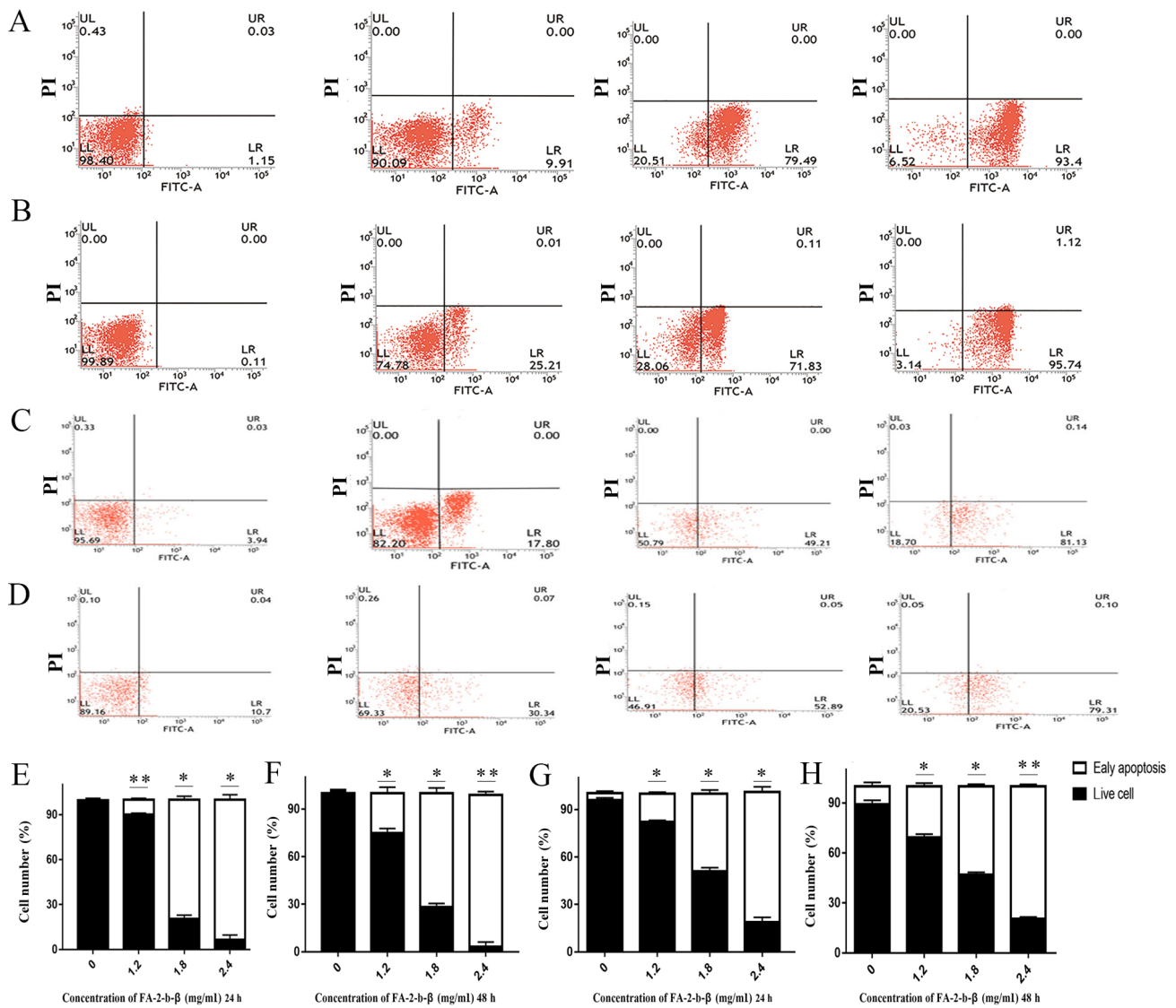
Figure 2. Flow cytometry analysis reveals AbM increased the apoptotic K562 cells and primary CML BM cells percentages in a concentration-dependent manner. K562 cells and primary CML BM cells were treated with different concentrations of AbM (0, 1.2, 1,8 and 2.4 mg/ml). K562 cells treated with different concentrations of AbM for (A) 24 and (B) 48 h. Primary CML BM cells treated with different concentrations of AbM for (C) 24 and (D) 48 h. Quantification of K562 cells treated with different concentrations of AbM for (E) 24 and (F) 48 h. Quantification of primary CML BM cells treated with different concentrations of AbM for (G) 24 and (H) 48 h. Data shown are representative of three independent experiments with K562 cells and primary CML BM cells. Data are presented as the mean ± SD. *P<0.05; **P<0.01 vs. 0 mg/ml.

the FA-2-b-β treated K562 cells was further determined using flow cytometry. Annexin V-FITC/PI double staining assay was used in order to determine the percentage of cells at different survival status in the entire culture (Fig. 2A-H). The proportion of apoptotic cells increased following treatment with FA-2-b-β. The total apoptotic K562 cells percentages of the control group were 1.15 and 0.11% at 24 and 48 h, respectively. This apoptotic rate was increased to 9.91, 79.49 and 93.40% (P<0.05; Fig. 2E), and 25.21, 71.83 and 95.74% (Fig. 2B) following treatment with FA-2-b-β (at 1.2, 1.8 and 2.4 mg/ml, respectively) for 24 and 48 h, respectively (Fig. 2E-F). The total apoptotic primary CML BM cell percentages of the control group were 3.94 and 10.70% at 24 and 48 h mock treatment, respectively; whereas apoptosis in the primary cells increased to 17.80 and 30.34%, 49.21 and 52.89%, and 81.13 and 79.31% following treatment with FA-2-b-β (at 1.2, 1.8 and 2.4 mg/ml, respectively) for 24 and 48 h, respectively (P<0.05; Fig. 2G and H).

FA-2-b-β induced CML cells apoptosis through inhibited G1 phase. Cell cycle distribution of the K562 cells or primary CML BM cells was evaluated using flow cytometry, in order to determine the mechanisms underlying the cytotoxic activity of FA-2-b-β on the aforementioned cell types. The association between the DNA content and the intensity of the PI was calculated following treatment with FA-2-b-β at 0, 1.2, 1.8 and 2.4 mg/ml, for 24 and 48 h (Fig. 3A-H). There was an increase in the G_1 phase, but a partial decrease in the S phase in a dose-dependent manner. The proportion of K562 cells at G_1 or S phases was 33.73 and 33.73%, and 46.13 and 46.13%, respectively, with mock treatment for 24 and 48 h, respectively. The proportion of cells in G_1 phase increased to 34.94, 37.35 and 41.33% using 1.2, 1.8 and 2.4 mg/ml of FA-2-b-β for 24 h (Fig. 3A), and to 37.01, 56.95 and 58.59% using 1.2, 1.8 and 2.4 mg/ml of FA-2-b-β for 48 h (Fig. 3C); whereas the proportion of cells in S phase partially decreased to 54.05, 58.91 and
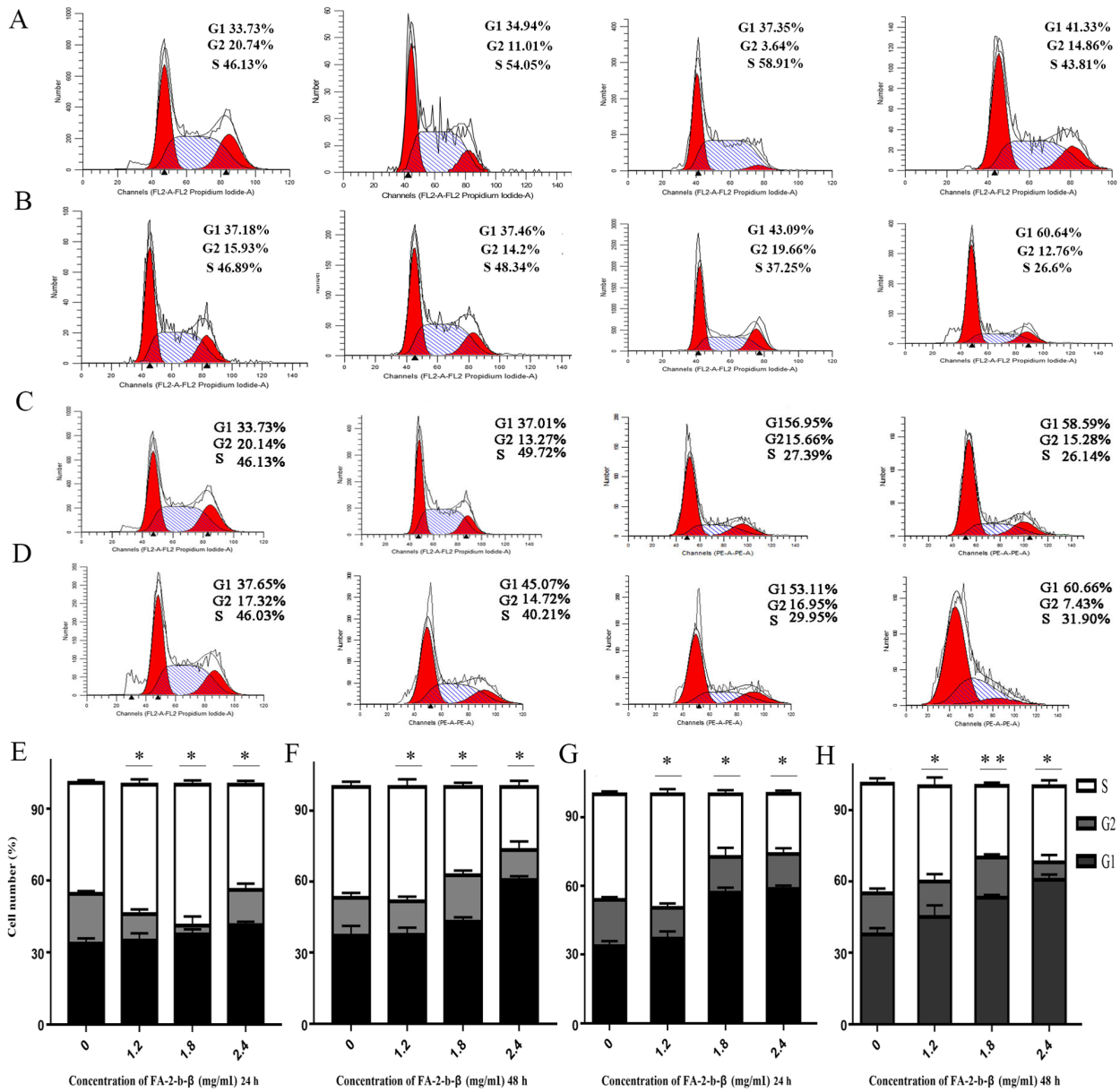
Figure 3. AbM promotes K562 cells and primary CML BM cells cycle. AbM induced cell cycle G1 phase arrest. K562 cells and primary CML BM cells were treated with different concentrations of AbM (0, 1.2, 1,8 and 2.4 mg/ml). Representative flow-cytograms are shown. Bar graphs indicate the mean percentages. (A) K562 cells and (B) primary CML BM cells treated with different concentrations of AbM for 24 h. (C) K562 cells and (D) primary CML BM cells treated with different concentrations of AbM for 48 h. Quantification of K562 cells treated with different concentrations of AbM for (E) 24 and (F) 48 h. Quantification of primary CML BM cells treated with different concentrations of AbM for (G) 24 and (H) 48 h. Data shown are representative of three independent experiments. K562 and primary CML BM cells data analysis revealed the proportion of cells in each phase of the cell cycle. Data are presented as the mean ± SD. *P<0.05, **P<0.01 vs. 0 mg/ml.

43.81% at 24 h (Fig. 3A), and 49.72, 27.39 and 26.14% at 48 h (Fig. 3C). In the primary CML BM cells, the proportion of cells in G_1 and S phases were 37.18 and 37.65%, and 46.89 and 46.03%, respectively, with mock treatment for 24 and 48 h, respectively. The proportion of cells in G_1 phase increased to 37.46, 43.09 and 60.64% using 1.2, 1.8 and 2.4 mg/ml of FA-2-b-β for 24 h (Fig. 3B), and to 45.07, 53.11 and 60.66% using 1.2, 1.8 and 2.4 mg/ml of FA-2-b-β for 48 h (Fig. 3D). The proportion of cells in S phase decreased to 48.34, 37.25 and 26.6% at 24 h (Fig. 3B), whereas the proportion of cells in S phase partially decreased to 40.21, 29.95 and 31.90% at 48 h (Fig. 3D) in the presence of 1.2, 1.8 and 2.4 mg/ml FA-2-b-β, respectively (Fig. 3A-H).

Cyclin D1 is a key cell cycle regulator that is essential for the G_1 phase, whereby its expression is closely associated with the development and prognosis of numerous types of cancer. Following FA-2-b-β treatment, the expression of cyclin D1 decreased in a concentration-dependent manner, confirming that G_1 phase arrest occurred after FA-2-b-β treatment in K562 cells or primary CML BM cells (Fig. 4A and B or Fig. 5A and B). Thus, FA-2-b-β induced both cell cycle arrest and apoptosis in K562 cells and primary CML BM cells, demonstrating similar findings to those that occur in the patients undergoing chemotherapy (27).

The present study further tested the hypothesis that FA-2-b-β may exert its effect on CML cells through negative
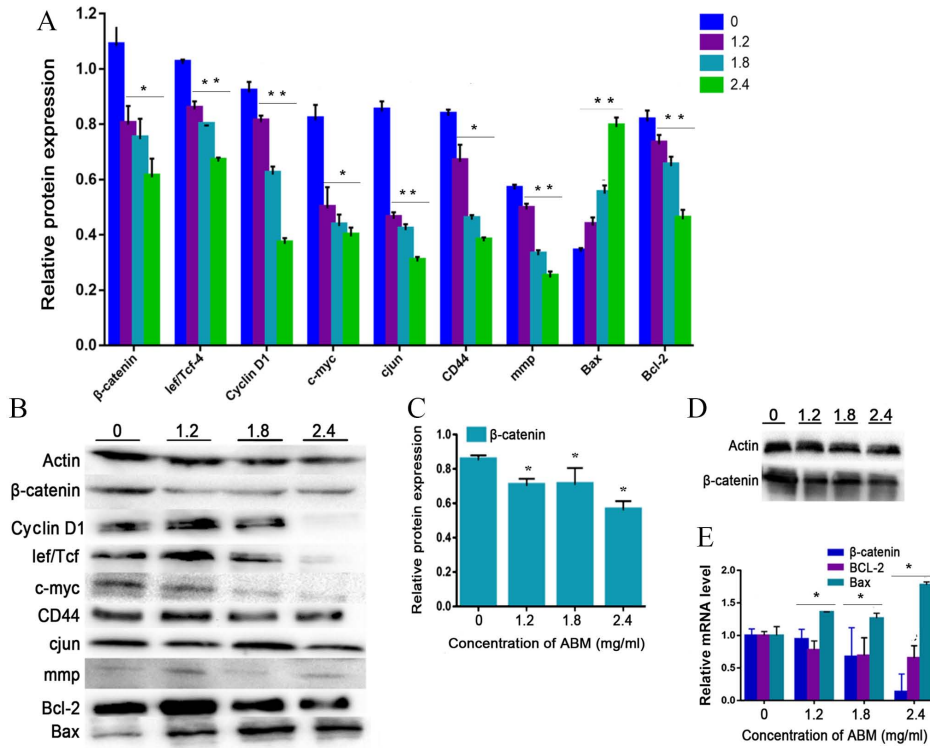
Figure 4. AbM induces apoptosis of K562 cells. K562 cells were treated with different concentrations of AbM (0, 1.2, 1.8 and 2.4 mg/ml) for 48 h and the expression of apoptosis-associated genes and proteins was determined using western blot analysis for (A and B) 24 h (*P<0.05 and **P<0.001) or (C and D) 48 h and (E) reverse transcription-quantitative PCR. The relative expression levels of β catenin, Bax and Bcl-2 genes in K562 cells were quantified according to the gene expression levels of actin. The relative expression levels of cyclin D1, Lef/Tcf, c-myc, β catenin, CD44, c-Jun, mmp, Bax and Bcl-2 proteins in K562 cells were quantified according to the protein expression levels of actin. Data are representative of three independent experiments and are expressed as the mean ± SD. *P<0.05 vs. 0 mg/ml.
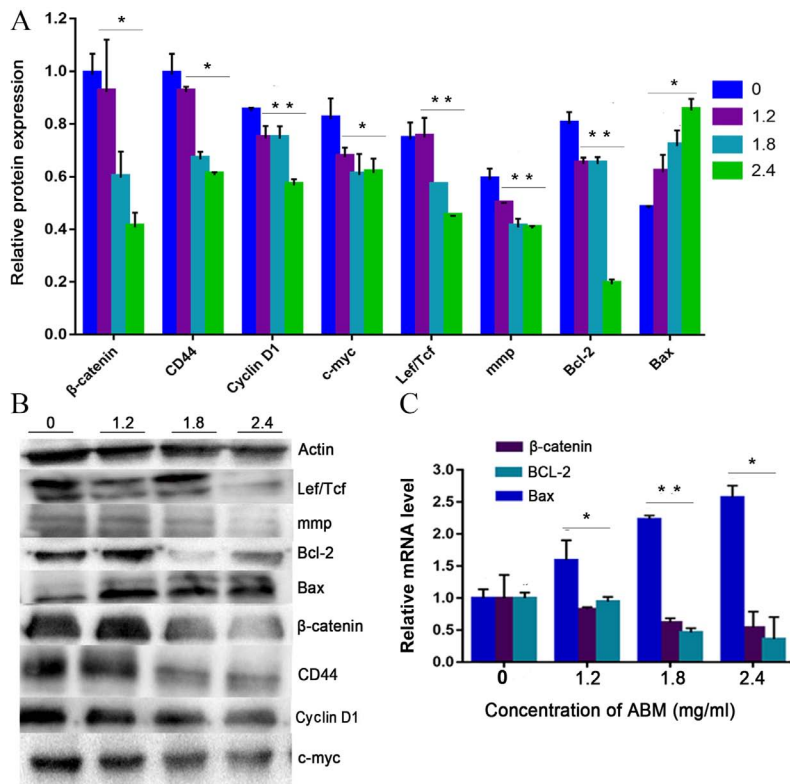

Figure 5. AbM induces apoptosis of CML cells. Primary CML BM cells were treated with different concentrations of AbM (0, 1.2, 1.8 and 2.4 mg/ml) for 24 h and the expression of apoptosis-related genes and proteins was determined using (A and B) western-blot analysis (*P<0.05 and **P<0.001) and (C) reverse transcription-quantitative PCR. The relative expression levels of β catenin, Bax and Bcl-2 genes in primary CML BM cells were quantified according to the expression levels of actin. The relative expression levels of cyclin D1, Lef/Tcf, c-myc, β catenin, CD44, c-Jun, mmp, Bax and Bcl-2 proteins in primary CML BM cells were quantified according to the expression levels of Actin. Data are representative of three independent experiments and are expressed as the mean ± SD. *P<0.05, **P<0.001.

modulation of Wnt/β-catenin signaling. The K562 cells or primary CML BM cells were dose dependently incubated with FA-2-b-β, following which RT-qPCR and western blot analysis were performed to assess the level of Wnt/β-catenin-related apoptosis gene and protein expression at 24 h. The data are presented as the mean of three independent experiments (P<0.05). β-catenin-associated apoptosis protein and gene expression in K562 cells or primary CML BM cells, exposed to FA-2-b-β at 1.2, 1.8 and 2.4 mg/ml for 24 h (Fig. 4A, B and E, and Fig. 5) or 48 h (Fig. 4C and D), was determined. β-catenin expression decreased at 24/48 h in a dose-dependent manner. Comparison was performed in the protein extracts between the drug-treated cells and controls in order to determine if there was an influence of spontaneous fluctuations in the associated apoptosis genes and proteins at each concentration. Actin was also evaluated to allow normalized protein and gene loadings. Bcl-2 and Bax protein and gene expression has been compared with the control cells. Bcl-2 expression decreased whilst Bax expression increased, in a dose-dependent manner. Based on our current study, it was observed that FA-2-b-β decreases cyclin D1 protein expression for binding to the promoter region and decreases β-catenin protein and gene expression for nuclear localization. The present study revealed ~50 or 25% of downregulated β-catenin mRNA in CML cells treated with FA-2-b-β 1.8 or 2.4 mg/ml for 24 h (Figs. 4E and 5C). Thus, FA-2-b-β may stimulate β-catenin via Lef/Tcf trans-inhibition activity, subsequently downregulating cyclin D1 in K562 cells as well as in the primary cells (P<0.05; Figs. 4A and 5A).

Discussion

Chemo-resistance remains a major challenge for clinicians in cancer therapy. Anti-cancer therapy is usually utilized to induce apoptosis or repair defects in cancer cells. Chemo-resistance is partially due to cancer-induced mutations in those genes responsible for apoptotic pathways (28). Thus, inhibition of cell proliferation and/or promotion of cell death are strategies in the management of cancer (29). It is necessary to develop novel therapeutic strategies for the treatment of CML. A significant advantage of drug repositioning over the conventional drug discovery and development process is the rapid implementation of anti-leukemia activity. As natural compounds derived from plants are easily accessible, such materials are routinely used for anti-cancer treatment in China, Japan, Brazil and the United States (30).

AbM has been used for the treatment of numerous types of diseases, including; Diabetes, atherosclerosis, hepatitis, hyper-cholesterolemia and heart disease (31). Immune-enhancing effects have been demonstrated to occur with AbM, including anti-oxidant, anti-mutagenic and anti-tumor effects (32). The positive mechanism of action of various medicinal plants in acute myeloid leukemia occurs via polysaccharides, which is primarily associated with; inhibition of cell proliferation, invasion and further induction of cancer cell apoptosis (33). Apoptosis is a form of programmed cell death without the accompanied inflammation, a phenomenon that has been a popular topic in anti-cancer research (34). The development of disrupted apoptotic pathways has been demonstrated to be associated with chemo-resistance of leukemia (35). This is consistent with the results of the present study which demon-

strated that decreased viabilities of K562 cells or primary CML BM cells occurs in response to treatment with FA-2-b-β. Additionally, the present study observed that FA-2-b-β-treated K562 cells or primary CML BM cells presented morpho-logical characteristics of apoptosis that are associated with downregulation of cyclin Dl and CD44. Cyclin Dl is a target of the Wnt/β-catenin pathway, which is associated with a number of steps of CML development (36). Conversely, cyclin Dl and CD44 are believed to be key genes of the Wnt/β-catenin pathway (36,37). Thus, the results of the present study suggest that FA-2-b-β-induced apoptosis in CML cells utilizes the Wnt/β-catenin pathway. β-catenin is another key mediator in cancer progression, which is involved in the regulation of cell cycle transition and cell apoptosis (38). This is consistent with the results of a previous study which demonstrated that AbM extracts induce apoptotic effects on leukemia cells (39).

c-Jun, c-myc, cyclin D1, CD44 and mmp all contribute to cancer cell proliferation and invasion (40). c-Jun activates β-catenin/Tcf transcription activity via the Wnt/β-catenin pathway, which is reported to be induced in CML (41). Furthermore, β-catenin interacts directly with c-Jun to acti-vate the cyclin D1 and c-myc genes. Currently, a number of β-catenin signaling pathway inhibitors are under investigation, with the aim to disrupt β-catenin activity and its interaction with the transcription factors (42). The present study observed that the effect of FA-2-b-β for anti-cancer activity is via targeting c-myc (Wnt genes). FA-2-b-β decreased β-catenin and c-Jun production, particularly at the G_1-S transition which resulted in cell cycle arrest and cell apoptosis at the G_0/G_1 phase. The results of the present study are supported by a previous study (43), which demonstrated that accumulated β-catenin activates the Wnt signaling pathway. Furthermore, knockout of Wnt signaling decreases β-catenin via the degra-dation of cytoplasmic β-catenin (44).

Kim *et al* (45), demonstrated that inhibition of β-catenin reverses the transformed properties in cancer, suggesting that β-catenin plays a key role in oncogenesis. These results may shed light on the development of pharmacological therapeutic targets against tumors. The results of the present study are consistent with others in demonstrating that β-catenin is impor-tant in the pathogenesis and progression of CML (46). β-catenin knockout decreases the risk of development of CML (47). Conversely, enhanced Wnt/β-catenin activity is associated with poor clinical outcomes with chemotherapy during 'blast crisis' in CML (48), and a meta-analysis revealed that high-dose imatinib achieved a major molecular response at 12 months of therapy (49-51). The present study demonstrated that FA-2-b-β inhibited the proliferation of K562 cells or primary CML BM cells by inducing apoptosis and G_1 arrest. Furthermore, the present study revealed that FA-2-b-β suppressed the expression of β-catenin as well as the protein products of Wnt target genes; c-myc, c-Jun and cyclin D1 in the G_1 transition. Subsequently, FA-2-b-β treatment contributed to cell cycle arrest during the G_1 phase and this observation is supported by a previous study (52). In the absence of Wnt signaling, β-catenin remains low through the degradation of cytoplasmic β-catenin, which prevents induction of the transcription of a number of proliferation-associated genes, including; cyclin D1, c-myc and fibronectin. A decrease in transcription, which would normally cause malignant transformation (53), was detected following

downregulation of the protein, β-catenin after treatment with FA-2-b-β. This ultimately inhibits the Wnt signaling pathway, accompanied by decreased cyclin D1, c-myc and CD44 in K562 cells or primary CML BM cells, in a dose-dependent manner. On the other hand, β-catenin is also capable of decreasing cell apoptosis via Bcl-2 or Bax targeting (36). Thus, the results of the present study suggest that the anti-tumor activity of AbM may be due to decreased cellular proliferation, but also the induction of cell apoptosis (54). This is supported by other studies that demonstrate that anti-cancer agents regulate Bcl-2 family members (including Bax or Bcl-2) (55-57). This evidence strongly supports the results of the present study, which demonstrated that FA-2-b-β induced leukemia cell apoptosis *in vitro*, accompanied by elevated Bax expression and decreased level of Bcl-2. These results suggest that cyclin D1, c-myc, c-Jun and CD44 can drive proliferation in CML as a consequence of β-catenin activity. These data further confirm that the role of β-catenin is indispensable for self-renewal of CML line cells.

The present study presents a number of limitations. First, it would provide more insight to perform the experiment evaluating TK inhibitor-resistant cells and normal BM cells. Secondly, future studies should include the experiment *in vivo* in order to determine the direct effect of AbM in CML.

FA-2-b-β exhibits anti-cancer potential through modulating different nodes of Wnt/β-catenin signaling pathways, which subsequently affects apoptosis, as well as cell cycling. Therefore, FA-2-b-β has potential for future treatment approaches for CML. The mushroom extract has minimal toxicity in animal models (58) and, to the best of our knowledge is considered safe for human consumption. As *Agaricus blazei extract* contains natural compounds derived from plants, which can be obtained at relatively low costs for the patients, it is routinely used in a number of clinics throughout China as an anti-cancer therapy.

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Availability of data and materials

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

Authors' contributions

MC and YS managed the design and coordination of the present study. YS provided professional writing. YS, QZ and KY carried out acid RNA protein complex (FA-2-b-β) extraction from Agaricus blazei Murill. LD, ZM, SY and KB performed the in vitro experiments. PY and XZ analyzed the results. All authors read and approved the final published version of this article.

Ethics approval and consent to participate

The present study was approved by the Human Ethics Committee of Gansu Provincial Hospital (approval no. 2019-157). All patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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