A β-1,3-1,6-glucan from *Aureobasidium pullulans* enhances apoptotic and autophagic pathways in colorectal cancer cells

JI-WON PARK[1], GYEONG MIM MUN[1], TAE MIN JO[1], JI A SHIN[2],
TAKAO KUGE[3], HISASHI KIMOTO[4] and JIN-KYUNG KIM[1]

[1]Department of Biomedical Science, Daegu Catholic University, Gyeongsan, Gyeongsangbuk 38430, Republic of Korea; [2]Department of Pharmaceutical Engineering, Daegu Catholic University, Gyeongsan, Gyeongsangbuk 38430, Republic of Korea; [3]ADEKA Corporation, R&D Division, Tokyo 116-8553, Japan; [4]Graduate School of Bioscience and Biotechnology, Fukui Prefectural University, Awara, Fukui 910-4103, Japan

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Abstract. Although β-glucan exhibits various biological activities, the anticancer mechanisms of a low-molecular-weight β-glucan isolated from *Aureobasidium pullulans* (LMW-AP-FBG) in colorectal cancer remain unclear. Therefore, this study investigated the anticancer potential of LMW-AP-FBG in human colon cancer cells. Treatment of SW480 cells with LMW-AP-FBG resulted in a significant reduction in cell viability in a concentration- and time-dependent manner, with half-maximal inhibitory concentrations (IC_{50}) of 557.2, 497.4 and 300.6 μg/ml at 24, 48 and 72 h, respectively. Apoptotic cell death was evidenced by increased caspase-3/7 enzymatic activity and proteolytic cleavage of caspase-3, -7, -8, -9 and poly (ADP-ribose) polymerase. Notably, pharmacological inhibition of caspases using the pan-caspase inhibitor Z-VAD-FMK only partially restored cell viability, suggesting the involvement of caspase-independent cell death mechanisms. Mitochondrial dysfunction was further indicated by dissipation of mitochondrial membrane potential, cytosolic accumulation of cytochrome c, and downregulation of the anti-apoptotic protein Bcl-2, collectively supporting activation of the intrinsic apoptotic pathway. In parallel, the expression of autophagy-related proteins was markedly increased, implying concomitant induction of autophagic processes. Mechanistically, LMW-AP-FBG suppressed the PI3K/AKT/mTOR signaling axis, a central regulator of cell survival and autophagy. Collectively, these findings demonstrate that LMW-AP-FBG exerts anticancer effects in SW480 colon cancer cells through coordinated activation of apoptotic and autophagy-associated pathways, highlighting its potential as a bioactive anticancer agent and warranting further *in vivo* validation.

Introduction

Colorectal cancer (CRC) remains one of the most prevalent malignancies worldwide and continues to represent a major contributor to cancer-related mortality (1). Recent data indicate that, as of 2022, CRC ranks as the third most common cancer globally and is the second leading cause of cancer mortality following lung cancer (2). In Korea, statistics show that as of 2022, CRC is the second most frequently diagnosed cancer after thyroid cancer, and by 2023, it has become the third-leading cause of cancer-related death following lung and liver cancer (3). A particular concern in Korea is the notably high incidence of CRC among younger individuals, the highest rate reported globally (4). Although advances have been achieved in surgical procedures, chemotherapy protocols, and targeted therapies, the prognosis for patients with advanced CRC remains poor, primarily due to therapeutic resistance, the development of metastases, and adverse treatment-related effects (5,6). These ongoing obstacles underscore the urgent need for innovative therapeutic approaches capable of effectively inhibiting tumor progression with reduced toxicity. Within this framework, natural compounds, including phytochemicals and dietary constituents, have emerged as promising candidates for the development of new therapeutic modalities for CRC.

β-glucans are naturally occurring polysaccharides that serve as key structural components within the cell walls of yeast, fungi, and cereal grains. In recent years, extensive research has demonstrated their wide-ranging physiological activities, such as modulation of immune responses (7,8), regulation of metabolic processes (9-11), and potential anticancer properties (12-14). These advances support a growing interest in β-glucans as bioactive agents suitable for use both as functional food ingredients and as adjunctive therapies. Notably, β-(1→3),(1→6)-D-glucan, derived from an edible cultured fungus through fermentation with the strain *Aureobasidium pullulans* (AP-FBG), has gained attention for its increased solubility and enhanced bioavailability, attributes

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Correspondence to: Professor Jin-Kyung Kim, Department of Biomedical Science, Daegu Catholic University, 13-13 Hayang-ro, Hayang-eup, Gyeongsan, Gyeongsangbuk 38430, Republic of Korea
E-mail: toto0818@cu.ac.kr

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that may improve its therapeutic efficacy (15-19). Furthermore, emerging data suggest that derivatives of AP-FBG with low molecular weight (LMW-AP-FBG) exhibit considerable promise for therapeutic development (17,20-22). In parallel, low-molar-mass oat β-glucan has been reported to influence autophagy- and apoptosis-associated processes during the early stages of chemically induced colorectal carcinogenesis in animal models (23). Nevertheless, such *in vivo* observations provide limited insight into the direct cellular responses and molecular signaling events elicited by β-glucans in human colorectal cancer cells.

Programmed cell death pathways, including apoptosis and autophagy, are essential for maintaining cellular homeostasis and have a significant impact on cancer development (24,25). Apoptosis (type I cell death) is characterized by caspase activation, DNA fragmentation, and notable morphological changes, while autophagy (type II cell death) involves the lysosomal degradation of intracellular components (24-26). Dysregulation of these mechanisms is strongly associated with tumorigenesis and the development of resistance to therapy, which underscores their relevance as potential targets for cancer treatment (24-26). Recent studies suggest that β-glucans, in addition to their recognized immunomodulatory effects, might influence programmed cell death pathways, particularly apoptosis and autophagy, across diverse cancer types (20,23,27,28). Nevertheless, the detailed molecular mechanisms underlying these effects in CRC remain largely unknown. Considering the pivotal function of apoptosis-autophagy crosstalk in the regulation of tumor progression and therapeutic response, investigating the modulatory effects of β-glucans on these pathways in CRC is critical for advancing their potential therapeutic applications and designing new intervention strategies. In this study, we sought to investigate the effects of LMW-AP-FBG on apoptosis and autophagy in colorectal cancer cell lines. Understanding the influence of this agent on cell death pathways could support its further development as a promising therapeutic option for CRC.

Materials and methods

Materials. RPMI-1640 medium and Dulbecco's modified Eagle's medium (DMEM) were purchased from WELGENE (Gyeongsan, Korea). Penicillin/streptomycin was obtained from Cytiva (Marlborough, MA, USA). Fetal bovine serum (FBS) was purchased from Atlas (Fort Collins, USA). Z-VAD-FMK, a pan-caspase inhibitor, was procured from Enzo Life Sciences (New York, USA). WestGlow™ PICO PLUS ECL Chemiluminescent substrate was acquired from BioMax (Guri, Korea). Pierce™ BCA protein assay Kit was sourced from Pierce (Rockford, IL, USA). PRO-PREP™ solution was provided by iNtRON Biotechnology (Seongnam, Korea). Primary antibodies targeting caspase-3 (#9662), cleaved caspase-3 (#9664), caspase-7 (#9492), cleaved caspase-7 (#9491), caspase-8 (#4790), cleaved caspase-8 (#9496), caspase-9 (#9502), cleaved caspase-9 (#9501), Poly (ADP-ribose) polymerase (PARP, #9542), Bcl-2 (#3498), Bax (#2772), autophagy-related 3 (Atg3, #3415), Atg5 (#12994), Atg7 (#8558), microtubule-associated proteins 1A/1B light chain 3 (LC3, #12741), p62 (#5114), phosphatidylinositol 3-kinase (PI3K, #4249), phospho-PI3K (p-PI3K, #4228), protein kinase B (AKT, #9272), phospho-AKT (p-AKT, #9271), mammalian target of rapamycin (mTOR, #2972), and phospho-mTOR (p-mTOR, #2971) were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies for apoptosis-inducing factor (AIF, sc-13116), cytochrome C (sc-7159) and beclin-1 (BCEN-1, sc-12427) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-rabbit (GTX213110) and goat anti-mouse IgG (GTX213111) were supplied by Genetex (Irvine, CA, USA). GAPDH loading control monoclonal antibody as well as other unspecified reagents were purchased from Sigma (St. Louis, MO, USA).

Preparation of LMW-AP-FBG. LMW-AP-FBG, kindly provided by Professor Hisashi Kimoto (Fukui Prefectural University, Fukui, Japan), was dissolved in RPMI-1640 medium at a concentration of 10 mg/ml and stored at 4˚C until use. To briefly outline the manufacturing process of LMW-AP-FBG, AP-FBG was suspended in distilled water to reach a final concentration of 1% (w/v), and sodium hydroxide was then added to attain an ultimate alkali concentration of 2% (w/v). The resulting mixture underwent heat treatment in a boiling water bath for 1 h to achieve alkaline hydrolysis. The alkaline hydrolysate was neutralized with hydrochloric acid and desalted by dialysis against ultrapure water using a dialysis membrane. Size-exclusion high-performance liquid chromatography (SE-HPLC, Tosoh Corporation, Tokyo, Japan) with a KW 802.5 Shodex column (30 cm x 8 mm i.d., Resonac Corporation, Tokyo, Japan) using a phosphate buffer (0.05 M) containing NaCl (0.3 M, pH 7.0) as the mobile phase was employed to evaluate the degradation of the polymer. Chromatographic separation was conducted at 30˚C with a flow rate of 1.0 ml/min, and detection was accomplished by a refractive index detector. According to SE-HPLC analysis, the average molecular weight of native AP-FBG was 183,683 Da, which decreased to 2,675 Da after alkaline hydrolysis.

Cell Culture. Human colorectal cancer cell lines (SW480, HCT116, and Caco-2), human pancreatic cancer cell lines (AsPC-1, PANC-1, and MIA-PaCa-2), a human lung cancer cell line (A549), an ovarian cancer cell line (SKOV3), a mouse colon cancer cell line (CT26), and a human normal colon fibroblast cell line (CCD-18Co) were obtained from the Korea Cell Bank (Seoul, Korea). All experiments were performed using low-passage cells (fewer than 10 passages) in accordance with the supplier's guidelines. These cell lines were maintained in either RPMI-1640 or DMEM, each supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin solution at 37˚C in a 5% CO_2 atmosphere.

Cell viability assay. Cell viability was evaluated using the CellTiter-Glo® 2.0 Cell Viability Assay (Promega, Wisconsin, USA). In brief, SW480 cells were plated ($5x10^3$ cells) in 96-well plates. Following exposure to different concentrations of LMW-AP-FBG (prepared in complete medium) for 24, 48, or 72 h, the assay reagents were added and the plates were placed on an orbital shaker for 2 min. Luminescence was measured using the GloMax 96 Microplate Luminometer (Promega, Wisconsin, USA). The IC_{50} values were determined using GraphPad PRISM software 4.0 (GraphPad Software Inc., San Diego, CA, USA).

In vitro wound healing assay. SW480 cells were seeded ($2x10^4$ cells) in 24-well plates and cultured for 24 h to reach confluence. A linear scratch was created using a sterile 200-μl pipette tip, followed by gentle washing to remove detached cells. The cells were then treated with the indicated concentrations of LMW-AP-FBG and incubated for an additional 24 h. Cells that migrated into the wound region were visualized using a DM-IL-LED inverted microscope equipped with an EC3 camera (Leica Microsystems, Heerbrugg, Switzerland). Wound closure was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA) by calculating the percentage of wound closure as $[(A_0-A_{24})/A0]$ x100, where A_0 and A_{24} represent the wound area at 0 and 24 h, respectively. Three random fields per well were analyzed, and experiments were performed in triplicate.

Measurement of apoptotic cells. The proportion of apoptotic cells was determined with the Muse™ Annexin V & Dead Cell Kit (Luminex, Austin, TX, USA), following the manufacturer's protocols. SW480 cells were cultured in 24-well plates at a density of $5x10^4$ cells/well and treated with increasing doses (0.25, 0.5, and 1 mg/ml) of LMW-AP-FBG for 48 h. Cells were washed with PBS, collected, and incubated with Annexin V and 7-Aminoactinomycin D-dead cell marker (7-AAD) for 20 min at room temperature in the dark. The percentages of cells undergoing early and late apoptosis were analyzed using the Muse™ Cell Analyzer (Luminex, Austin, TX, USA).

Western Blot analysis. For whole cell lysate preparation, SW480 cells were treated with various concentrations of LMW-AP-FBG for 48 h, washed with ice-cold PBS, and lysed with 50 μl of PRO-PREP™ protein extraction solution containing a freshly prepared mixture of complete protease inhibitors (Roche, Switzerland). A total of 20 μg of protein was separated by 12% or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto a PVDF membrane (Millipore, Massachusetts, USA). After blocking the membranes with 5% non-fat milk in 0.1% Tween 20 Tris Buffered Saline (TBS-T) for 2 h, membranes were incubated overnight at 4˚C in the presence of primary antibodies. The membranes were subsequently treated for 3 h at 4˚C with secondary antibodies. Chemiluminescent substrate was then applied, and signals were visualized using the DAVINCH-Chemi CAS-400 SM (Davinch-k, Seoul, Korea) and quantified by Total Lab analysis software (Nonlinear Dynamics, Dunham, NC, USA).

Measurement of the Mitochondrial Membrane Potential. Mitochondrial membrane potential (MMP) levels were assessed using the JC-1 Mitochondrial Membrane Potential Detection Kit (Biotium, Fremont, CA, USA) following the manufacturer's protocol. In brief, SW480 cells cultured in 24-well plates ($0.5x10^5$ cells/well) were exposed to the indicated concentrations of LMW-AP-FBG for 48 h. Following treatment, the cells were washed, collected using trypsin-EDTA, and incubated with JC-1 Dye (Biotium) for 15 min in a 37˚C in a CO_2 incubator. The proportion of depolarized cells was determined by flow cytometry (BD Bioscience, Becton Dickinson, USA) and results were analyzed with FlowJo Software (Tree Star, Inc, Ashland, OR, USA).

Statistical analysis. All data are presented as mean ± SEM. Statistical significance was determined using a two-tailed Student's t test or a one-way ANOVA followed by Bonferroni's post-hoc test in GraphPad PRISM software 4.0. A value of $P<0.05$ was considered statistically significant.

Results

LMW-AP-FBG treatment reduced the cell viability of SW480 cells. To assess the inhibitory effects of LMW-AP-FBG on the viability of various cancer cell lines, an initial screening was performed using the CellTiter-Glo 2.0 Cell Viability Assay. LMW-AP-FBG treatment at 0.5 mg/ml markedly reduced cell viability across multiple cancer cell types, including colon, pancreatic, ovarian, and lung cancer cells, with PANC-1 cells showing the most pronounced sensitivity (Fig. S1). These results indicate that LMW-AP-FBG exerts broad antiproliferative effects against diverse cancer cell lines.

Based on this preliminary screening, SW480 cells were selected for subsequent mechanistic studies owing to their well-characterized genetic alterations relevant to colorectal cancer and their suitability for *in vitro* anticancer evaluations. SW480 cells were then treated with increasing concentrations of LMW-AP-FBG for 24, 48, and 72 h, resulting in a clear concentration- and time-dependent decrease in cell viability (Fig. 1A). The calculated half-maximal inhibitory concentrations (IC_{50}) were 557.2 μg/ml at 24 h, 497.4 μg/ml at 48 h, and 300.6 μg/ml at 72 h.

To evaluate the selectivity of LMW-AP-FBG toward cancer cells, its cytotoxic effects were examined in normal human colorectal CCD-18Co cells under identical experimental conditions. No significant reduction in cell viability was observed at any tested concentration over 24, 48, and 72 h (Fig. 1B), indicating minimal cytotoxicity in normal cells.

Given that enhanced cell migration is a key characteristic of malignant progression, we next assessed the effect of LMW-AP-FBG on the migratory behavior of SW480 cells using a wound healing assay. Notably, LMW-AP-FBG significantly suppressed migratory activity at concentrations of 0.5 and 1 mg/ml (Fig. 1C and D), suggesting a potential role in suppressing cancer cell motility.

LMW-AP-FBG enhances apoptosis in SW480 cells. Given that LMW-AP-FBG reduced SW480 cell viability, we subsequently assessed whether LMW-AP-FBG triggers apoptosis using the Muse Annexin V assay kit. Early and late apoptotic populations were quantified following 48 h of LMW-AP-FBG exposure. LMW-AP-FBG led to an approximate 2- to 3-fold increase in early apoptotic cells compared to controls. Specifically, the proportions of early apoptotic cells at concentrations of 0.25, 0.5, and 1 mg/ml were 28.4±4.2%, 33.0±3.1%, and 29.0±1.6%, respectively, while the control was 11.0±2.9% (Fig. 2A and B). These data suggest that LMW-AP-FBG promotes apoptosis in SW480 cells.

Furthermore, to investigate the involvement of caspases in LMW-AP-FBG-induced apoptosis in SW480 cells, caspase-3 and -7 activities were quantitatively assessed using the Caspase Glo 3/7 luminescence assay. The activity levels of caspase-3 and -7 were markedly increased at both 0.5 and 1 mg/ml concentrations of LMW-AP-FBG, as demonstrated

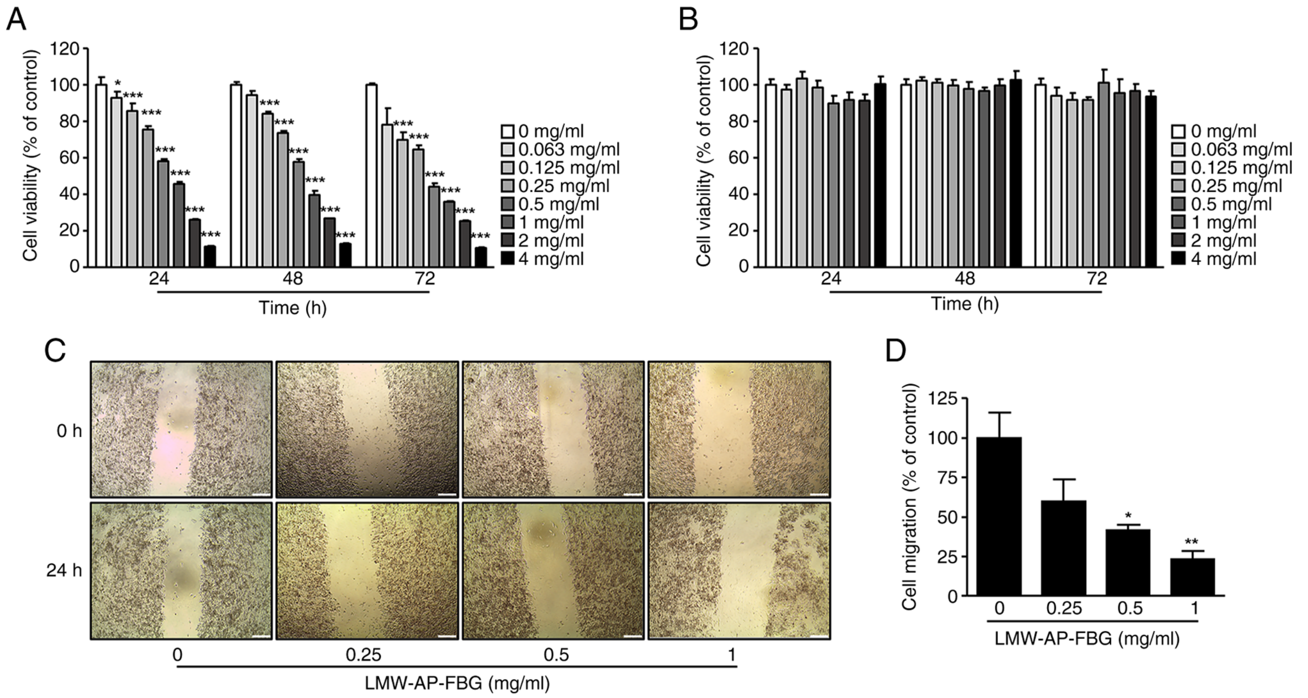

Figure 1. LMW-AP-FBG reduces viability and migration in SW480 cells. (A) Dose- and time-dependent decrease in SW480 cell viability after exposure to serial two-fold dilutions of LMW-AP-FBG (0.0634.0000 mg/ml) for 24, 48 and 72 h. (B) Viability of CCD-18Co cells under the same treatment conditions. (C) Representative phase-contrast images of wound closure in SW480 cells treated with the indicated concentrations of LMW-AP-FBG at 0 and 24 h (scale bars, 100 μm). (D) Quantitative analysis of wound closure expressed as the percentage of wound closure relative to initial wound area. Data represent mean ± SEM from three independent experiments (n=3). *P<0.05, **P<0.01 and ***P<0.001 compared to control (0 mg/ml). LMW-AP-FBG, low-molecular-weight β-glucan isolated from *Aureobasidium pullulans*.

in Fig. 2C. Given that caspases function as crucial proteases in apoptosis by cleaving numerous substrate proteins (23,29), we next examined caspase activation in SW480 cells following LMW-AP-FBG treatment. Western blot analysis showed that the total protein levels of caspase-3, -7, -8, and -9 remained largely unchanged across the tested concentrations (Fig. 2D and E). In contrast, their cleaved forms were markedly elevated in a concentration-dependent manner, as confirmed by densitometric analysis (Fig. 2D and E). In parallel, PARP cleavage was prominently induced, as reflected by the accumulation of cleaved PARP alongside a concomitant decrease in full-length PARP. Quantitative analysis further substantiated these findings, demonstrating significant upregulation of cleaved caspases and PARP relative to the control group. Collectively, these findings indicate that LMW-AP-FBG triggers apoptosis by activating caspases involved in both extrinsic and intrinsic apoptotic processes.

Apoptosis induced by LMW-AP-FBG in SW480 cells is partially caspase-dependent. To further clarify whether LMW-AP-FBG-induced cell death in SW480 cells is caspase-dependent, experiments employing the pan-caspase inhibitor Z-VAD-FMK were conducted. Specifically, SW480 cells were co-incubated with Z-VAD-FMK (20 μM) and various concentrations of LMW-AP-FBG for 48 h, followed by assessment of cell viability using the CellTiter-Glo 2.0 Cell Viability Assay. As illustrated in Fig. 3A, treatment with 0.25 mg/ml LMW-AP-FBG alone resulted in 59.8% cell viability, while co-treatment with Z-VAD-FMK restored viability to 74.3%. Furthermore, at 1 mg/ml LMW-AP-FBG, the presence of Z-VAD-FMK led to an approximately 13% increase in viability compared to LMW-AP-FBG alone (Fig. 3A).

Under equivalent conditions, apoptosis measurements revealed that the proportion of total apoptotic cells was reduced in all Z-VAD-FMK co-treated groups in comparison to those receiving only LMW-AP-FBG (Fig. 3B and C). More precisely, Z-VAD-FMK co-treatment reduced LMW-AP-FBG-induced apoptosis from 20.5±2.6% to 11.6±1.97% at 0.25 mg/ml, and from 25.1±2.1% to 16.2±3.1% at 0.5 mg/ml (Fig. 3C). Collectively, these findings support the notion that the reduction in SW480 cell viability induced by LMW-AP-FBG is at least partially mediated through caspase-dependent apoptotic pathways.

LMW-AP-FBG induces mitochondria-mediated apoptosis in SW480 cells. Mitochondria play a pivotal role in apoptosis. During this process, mitochondrial outer membrane permeabilization (MOMP) markedly increases, facilitating cytochrome c release via mitochondrial pores, which subsequently activates caspases and triggers cell death. To evaluate whether LMW-AP-FBG promotes an increase in MOMP in SW480 cells, we assessed MMP using JC-1 staining. When MMP decreases, JC-1 aggregates (red fluorescence) dissociate into monomers, resulting in enhanced green fluorescence. Upon treatment with LMW-AP-FBG concentrations of 0.5 and 1 mg/ml, the proportion of JC-1 aggregates decreased from 89.58±0.58% of the control to 71.58±5.64% and 56.05±2.84%, respectively; conversely, JC-1 monomers increased from 8.49±0.87% of the control to 22.28±4.60% and 34.95±2.77%, respectively (Fig. 4A and B).

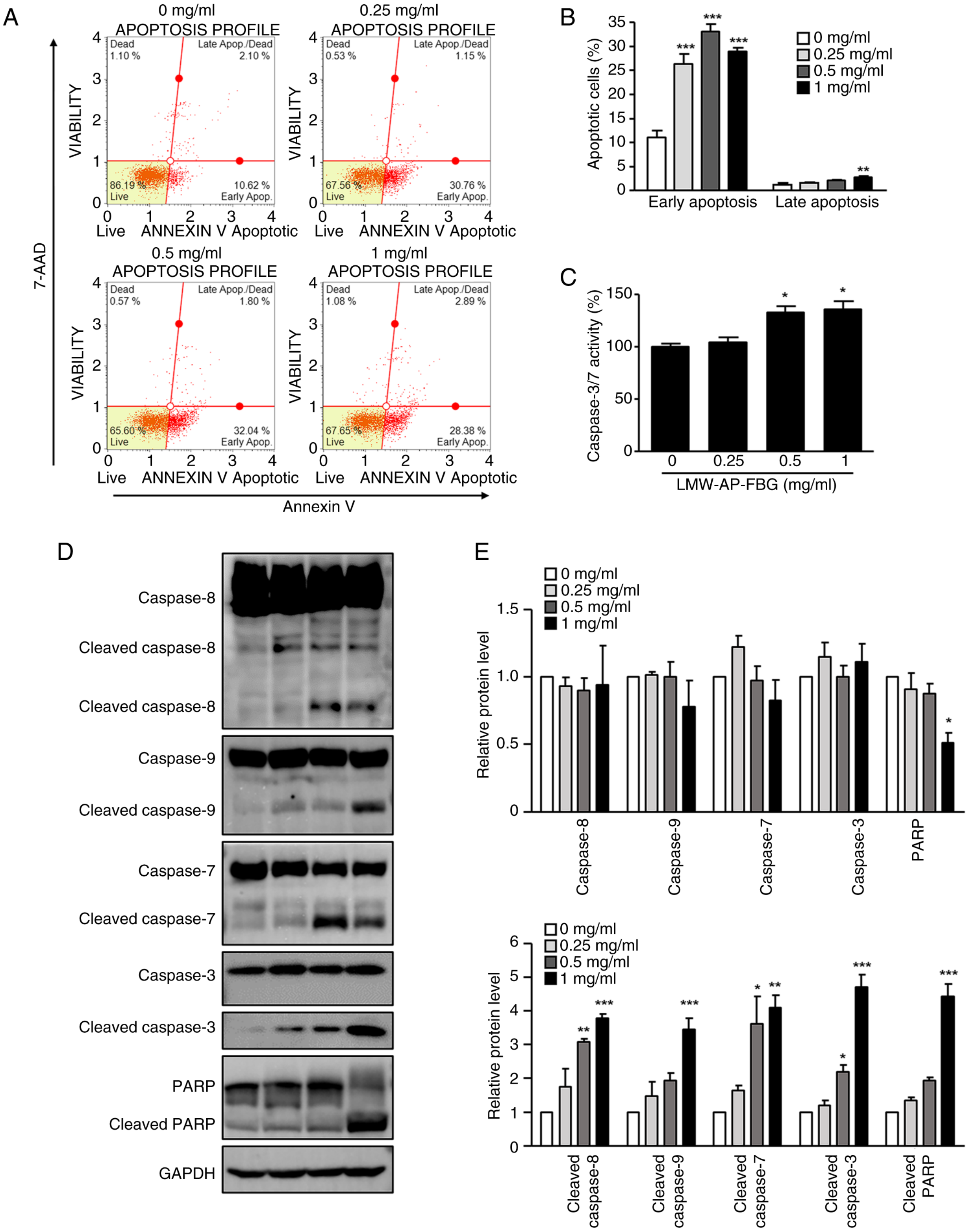

Figure 2. LMW-AP-FBG promotes apoptosis in SW480 cells. SW480 cells were exposed to the indicated concentrations of LMW-AP-FBG for 48 h, and apoptosis was evaluated by Annexin V/7-AAD staining. (A) Representative flow cytometry dot plots are shown. (B) Quantification of early and late apoptotic cells is provided. (C) Caspase-3/7 activity was determined using the Caspase-Glo 3/7 Assay. (D) Representative Western blot images showing both full-length (total) and cleaved forms of caspases and PARP in SW480 cells. (E) Densitometric analysis of full-length and cleaved caspases and PARP. Data are presented as mean ± SEM from three independent experiments (n=3). *P<0.05, **P<0.01 and ***P<0.001 vs. control (0 mg/ml). 7-AAD, 7-aminoactinomycin D; LMW-AP-FBG, low-molecular-weight β-glucan isolated from *Aureobasidium pullulans*; PARP, poly (ADP-ribose) polymerase.

In line with the observed decrease in MMP, treatment of SW480 cells with LMW-AP-FBG resulted in reduced Bcl-2 protein levels and elevated cytosolic cytochrome c levels. Notably, cytochrome c increased approximately 6-8-fold compared to the control group after 48 h of LMW-AP-FBG exposure, while Bcl-2 expression was significantly diminished

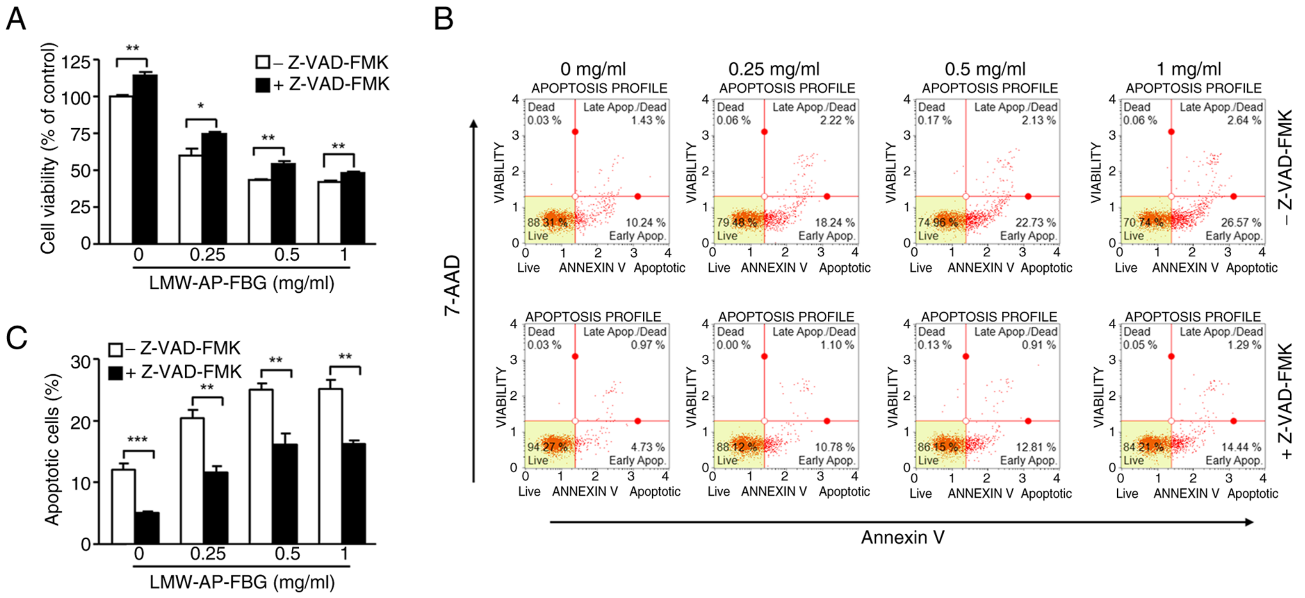

Figure 3. Apoptosis induced by LMW-AP-FBG in SW480 cells is partially caspase-dependent. (A) Cell viability in SW480 cells was determined after 48 h incubation with LMW-AP-FBG alone or together with Z-VAD-FMK using the Titer Glo luminescence assay. (B) Apoptosis was evaluated by Annexin V/7-AAD staining after treatment for 48 h with LMW-AP-FBG, either with or without 20 μM Z-VAD-FMK. (C) Bar graphs summarize the proportion of apoptotic cells after exposure to LMW-AP-FBG alone or in combination with Z-VAD-FMK. Data are reported as mean ± SEM from three independent experiments (n=3). $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$. 7-AAD, 7-aminoactinomycin D; LMW-AP-FBG, low-molecular-weight β-glucan isolated from *Aureobasidium pullulans*.

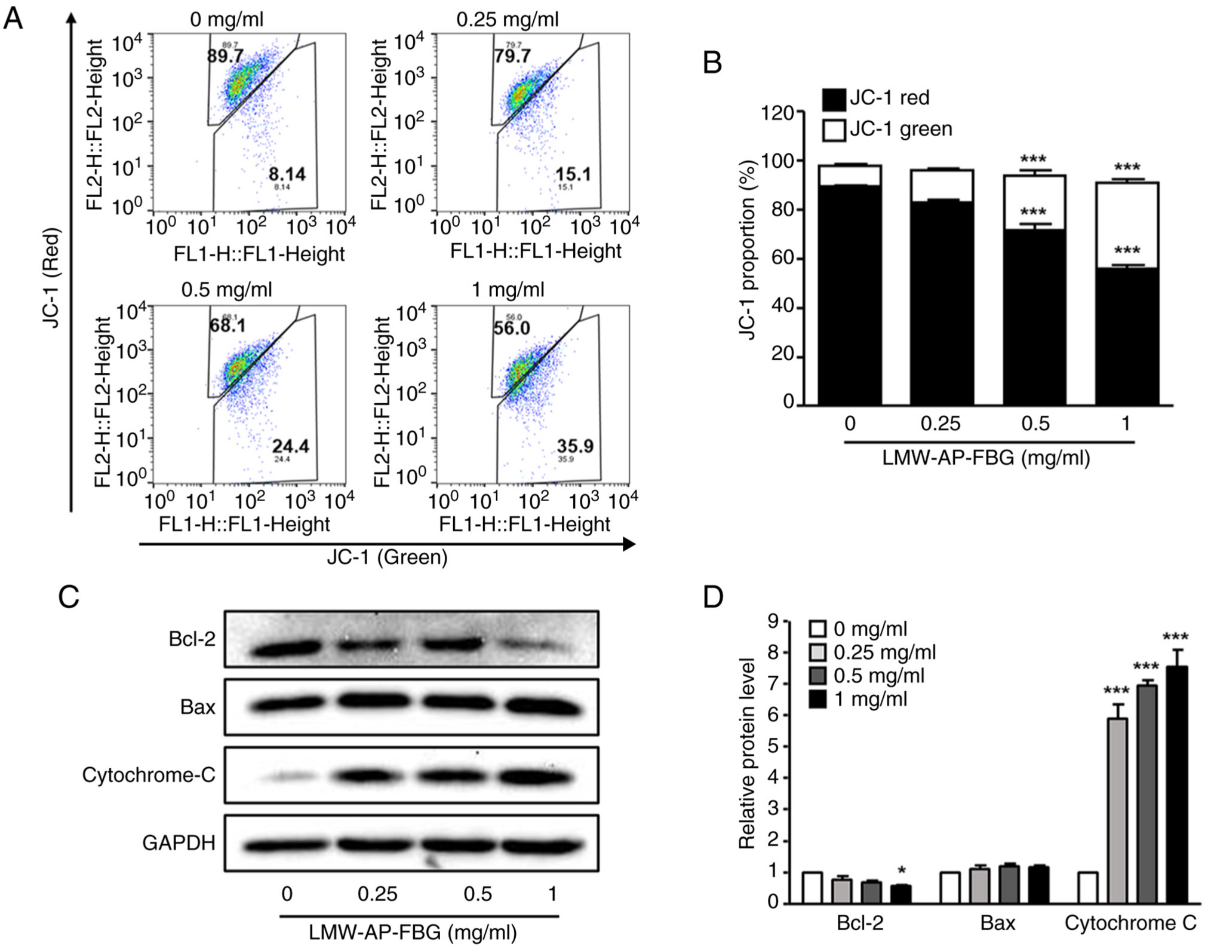

Figure 4. LMW-AP-FBG initiates mitochondrial-dependent apoptosis in SW480 cells. (A) Mitochondrial membrane potential was assessed 48 h after LMW-AP-FBG exposure using JC-1 dye and flow cytometry. (B) Quantitative analysis of the JC-1 monomer to aggregate ratio is shown in the bar graph. (C) The protein expression of Bcl-2, Bax and Cytochrome C after 48 h of treatment was evaluated by Western blot; GAPDH served as an internal loading control. (D) Bar graphs present the relative expression levels of Bcl-2, Bax and Cytochrome C. Results are expressed as mean ± SEM (n=4). $^{*}P<0.05$ and $^{***}P<0.001$ vs. control (0 mg/ml). LMW-AP-FBG, low-molecular-weight β-glucan isolated from *Aureobasidium pullulans*.

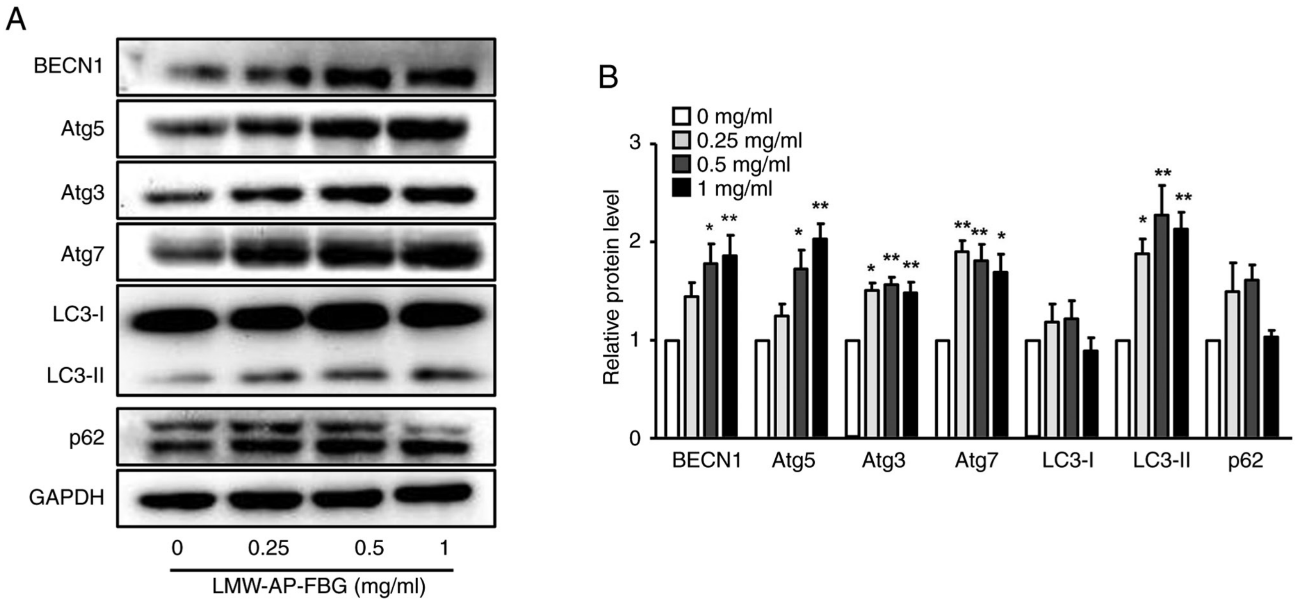

Figure 5. Effects of LMW-AP-FBG on autophagy in SW480 cells. (A) Representative Western blots display levels of autophagy-associated proteins in SW480 cells after 48 h treatment with various concentrations of LMW-AP-FBG. Western blotting was performed on equal amounts of protein extracts for the indicated targets. (B) Quantitative analysis of the relative protein levels is presented as a bar graph. Results are expressed as mean ± SEM (n=4). *P<0.05 and **P<0.01 vs. control (0 mg/ml). LMW-AP-FBG, low-molecular-weight β-glucan isolated from *Aureobasidium pullulans*; BECN1, beclin-1; ATG, autophagy-related; LC3, microtubule-associated proteins 1A/1B light chain 3.

at 1 mg/ml of LMW-AP-FBG (Fig. 4C and D). Collectively, these findings indicate that LMW-AP-FBG induces apoptosis via mitochondrial pathways regulated by Bcl-2 family proteins.

To further determine whether LMW-AP-FBG induces AIF-mediated caspase-independent apoptosis, AIF localization was examined. However, LMW-AP-FBG treatment did not promote AIF translocation compared with the control group (Fig. S2) and there was no evidence to suggest that LMW-AP-FBG promoted AIF translocation.

LMW-AP-FBG induces autophagy-associated molecular changes in SW480 cells. Since the extent of apoptosis induced by LMW-AP-FBG in SW480 cells was less than the observed inhibition of cell viability, we postulated that LMW-AP-FBG may trigger other forms of cell death in addition to apoptosis. To investigate this possibility, we quantified levels of autophagy-associated proteins using Western blot analysis. At concentrations of 0.5 and 1 mg/ml, expression levels of BECN1, Atg5, Atg3, and Atg7 were 1.5-fold greater compared with the control group (Fig. 5A and B). Furthermore, while LC3-I levels remained unchanged, LC3-II expression was significantly upregulated in a dose-dependent manner with LMW-AP-FBG treatment (Fig. 5A and B). For p62, no significant alteration was observed upon LMW-AP-FBG administration, suggesting that although autophagy-related machinery was activated, enhanced autophagic flux was not clearly evident under the experimental conditions.

LMW-AP-FBG reduces PI3K/AKT/mTOR signal transduction pathway. Given that our results demonstrated that LMW-AP-FBG could induce apoptosis and activate autophagy-related pathways, we subsequently investigated the PI3K/AKT/mTOR signaling pathway, which is known to play a central role in modulating the balance between these two processes (30,31). As depicted in Fig. 6, treatment with LMW-AP-FBG resulted in a marked reduction in the phosphorylation of PI3K, AKT, and mTOR compared to their respective total protein levels. These outcomes imply that LMW-AP-FBG substantially suppresses the activation of the PI3K/AKT/mTOR pathway, thereby inhibiting downstream signaling events that govern cellular proliferation and survival. Consequently, the inhibition of this pathway is likely to contribute to the observed induction of apoptotic and autophagy-related responses in SW480 cells.

Discussion

β-glucans are well recognized for a wide array of physicochemical and biological activities; however, certain intrinsic limitations-including challenges in purification, variability in structural conformations, and inadequate aqueous solubility-have consistently impeded detailed clarification of their structure-activity relationships (32,33). To address these issues, we adopted a sodium hydroxide-mediated hydrolysis approach aimed at reducing both molecular weight and viscosity, thereby enhancing both the consistency and bioavailability of β-glucan samples. In particular, we subjected crude AP-FBG to hydrolysis and then filtration, obtaining LMW-AP-FBG with a mean molecular weight below 3,000 Da. In subsequent functional assays, this LMW-AP-FBG demonstrated significant growth-inhibitory effects on SW480 CRC cells at concentrations as low as 0.25 mg/ml. Notably, this active concentration is approximately one-tenth of concentrations typically reported in the literature (20,21). For example, earlier studies in which LMW-AP-FBG was generated through mechanochemical ball milling instead of chemical hydrolysis using sodium hydroxide, showed that anticancer effects were seen in CT-26 cells only at concentrations of 2.5 mg/ml (20). These

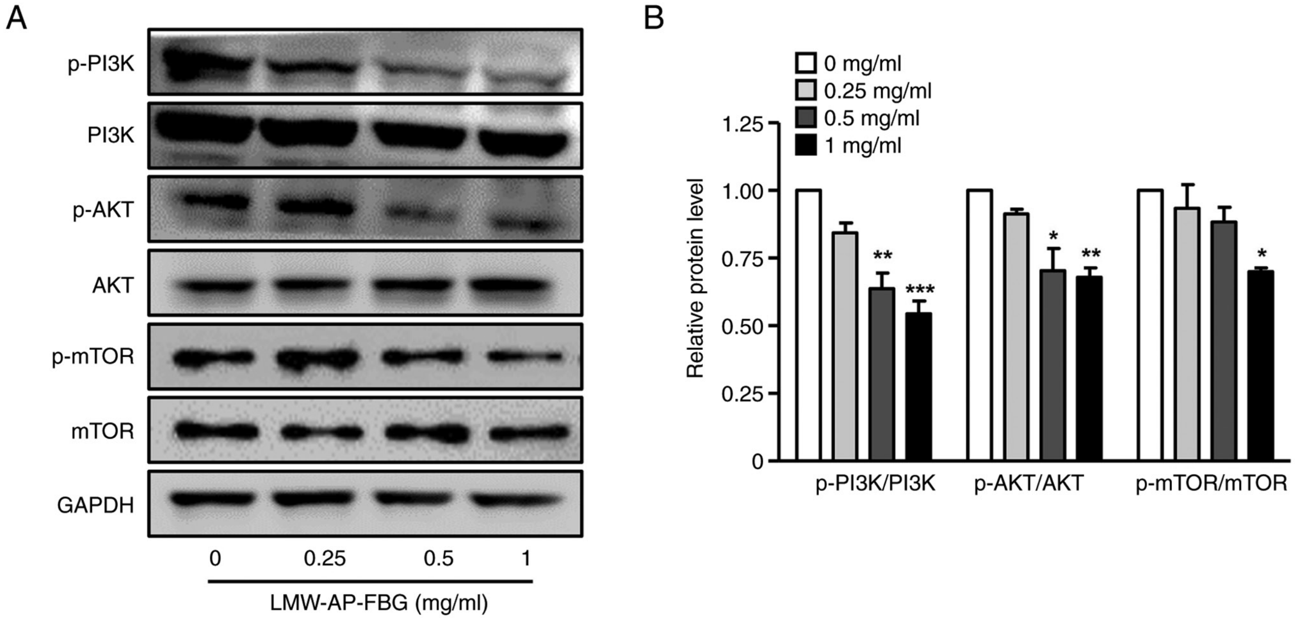

Figure 6. Effects of LMW-AP-FBG on the PI3K/AKT/mTOR signaling pathway in SW480 cells. (A) Representative Western blot images showing the expression levels of proteins involved in the PI3K/AKT/mTOR signaling pathway in SW480 cells treated with various concentrations of LMW-AP-FBG for 48 h. Equal amounts of total protein were subjected to Western blot analysis using the indicated antibodies. (B) Quantitative densitometric analysis of relative protein expression levels. Results are expressed as mean ± SEM (n=4). $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared with the control (0 mg/ml). LMW-AP-FBG, low-molecular-weight β-glucan isolated from *Aureobasidium pullulans*; p-PI3K, phospho-phosphatidylinositol 3-kinase; PI3K, phosphatidylinositol 3-kinase; p-AKT, phospho-protein kinase B; AKT, protein kinase B; p-mTOR, phospho-mammalian target of rapamycin; mTOR, mammalian target of rapamycin.

data underscore the methodological strengths of the sodium hydroxide-mediated hydrolysis approach, which enables efficient production of LMW-AP-FBG with greater bioactivity. This further supports the importance of processing methods in determining both the structural attributes and therapeutic efficacy of β-glucans, offering critical guidance for the development of β-glucan-based therapeutics.

Our results demonstrate that LMW-AP-FBG suppresses the proliferation of SW480 cells primarily by inducing apoptotic cell death. This conclusion is substantiated by cell viability assays and Annexin V/7-AAD staining, which revealed a significant increase in the proportion of cells undergoing early apoptosis, while progression to late apoptosis was comparatively limited. Importantly, administration of the pan-caspase inhibitor Z-VAD-FMK inhibited apoptosis induction but resulted in only partial restoration of cell viability. Collectively, these observations indicate that, although apoptosis is a key mediator of the antiproliferative effect, other pathways likely contribute. The partial recovery of proliferation upon caspase inhibition suggests involvement of additional caspase-independent cell death pathways, such as AIF-mediated apoptosis or autophagy. Under physiological conditions, the anti-apoptotic protein Bcl-2 inhibits mitochondrial apoptosis by binding and sequestering the pro-apoptotic protein Bax, thereby preventing Bax oligomerization and pore formation in the mitochondrial outer membrane (34). In this study, exposure to LMW-AP-FBG resulted in a pronounced decrease in Bcl-2 expression in SW480 cells and was accompanied by a simultaneous increase in cytosolic cytochrome c. This observation indicates that LMW-AP-FBG compromises mitochondrial membrane integrity, promoting cytochrome c release and subsequent activation of the intrinsic apoptotic pathway. In addition to caspase-dependent signaling, the intrinsic pathway includes caspase-independent mechanisms involving mitochondrial proteins such as AIF and Endo G, which may translocate to the nucleus (35,36). However, our findings suggest that AIF-dependent signaling is not a major contributor to LMW-AP-FBG-induced apoptosis. -Taken together, these findings indicate that LMW-AP-FBG induces apoptosis in SW480 cells primarily through mechanisms associated with Bcl-2 downregulation and cytochrome c release rather than through AIF-mediated caspase-independent pathways.

Of note, the extent of apoptosis observed did not entirely explain the decrease in cell viability, implying that other cell death modalities may also be operative. Supporting this notion, Western blot analyses revealed elevated levels of autophagy-related initiation and elongation proteins, including BECN1, Atg5, Atg3, and Atg7, together with a dose-dependent increase in LC3-II accumulation. The absence of significant alteration in p62 expression indicates that, although LMW-AP-FBG triggers autophagy, it may not fully promote autophagic flux. Collectively, these findings indicate that autophagy, in parallel with apoptosis, may be involved in the cellular response to LMW-AP-FBG treatment and potentially contribute to its cytotoxic effects in SW480 cells. Further studies employing lysosomal inhibitors or LC3 turnover assays will be required to determine whether this autophagic response primarily serves cytoprotective or cytotoxic functions.

Our findings are consistent with a growing body of evidence indicating that natural polysaccharides, such as β-glucans and fucoidans, exert antitumor effects through modulation of mitochondrial function, Bcl-2 family proteins, and autophagy-related signaling pathways (37-39).

Significantly, concurrent engagement of apoptotic and autophagy-related pathways by LMW-AP-FBG highlights its potential as a multi-targeted anticancer candidate. The participation of multiple cell death pathways in cancer cells is now widely acknowledged as a key determinant of anticancer treatment efficacy (40,41). Our results support the hypothesis that LMW-AP-FBG may leverage these complementary mechanisms to augment its anticancer properties.

The PI3K/AKT/mTOR pathway orchestrates essential cellular functions, such as proliferation, energy metabolism, and survival (30,31). Persistent activation of this signaling axis has been associated with colorectal cancer development and resistance to therapies (31,42). Notably, inhibition of this pathway enhances apoptosis mediated by mitochondria and increases autophagic processes across a range of cancer cell systems (43). As such, investigating this network in our study was crucial to elucidate the molecular basis of the anticancer actions of LMW-AP-FBG in SW480 colon cancer cells. We found that LMW-AP-FBG treatment caused significant suppression of PI3K/AKT/mTOR signaling, accompanied by enhanced apoptotic and autophagy-related responses. These observations indicate that LMW-AP-FBG may modulate two major cell death pathways, at least in part, through disruption of a central signaling cascade. Collectively, these data broaden the perspective on its molecular mechanisms and emphasize its potential as a candidate for therapeutic intervention in CRC.

The findings of this study suggest that LMW-AP-FBG may have potential clinical applications as an adjunct therapeutic agent in colorectal cancer treatment. In particular, β-glucans are known to modulate immune responses and enhance the efficacy of conventional anticancer therapies. Therefore, LMW-AP-FBG may be considered as a promising candidate for combination therapy with existing chemotherapeutic agents or targeted therapies. However, further *in vivo* and clinical studies are required to validate its therapeutic efficacy and safety.

There are several limitations to this study. It should be noted that the present findings were obtained using an *in vitro* cell culture system. Although these results provide important mechanistic insights into the anticancer effects of LMW-AP-FBG, further studies using animal models and clinical samples are required to confirm its therapeutic potential *in vivo*. First, as our data are derived from *in vitro* assays using a single colon cancer cell line, the results may not accurately represent *in vivo* biology. Second, even though both apoptosis and autophagy were observed, the distinct roles each process plays in LMW-AP-FBG-induced cell death remain to be clarified. Thus, subsequent investigations should focus on defining the contribution of autophagy by employing pharmacological inhibitors or genetic silencing approaches targeting autophagy-related genes. Evaluation using *in vivo* xenograft models will be required to further assess both the therapeutic efficacy and safety profile of LMW-AP-FBG. In addition, a detailed analysis of the crosstalk between Bcl-2 family members and autophagy regulators could yield a deeper understanding of the complex anticancer mechanisms underlying LMW-AP-FBG action.

Collectively, our results indicate that LMW-AP-FBG exerts anticancer effects against SW480 cells through induction of mitochondria-mediated apoptosis accompanied by engagement of autophagy-related processes, a mechanism that is associated, at least in part, with inhibition of the PI3K/AKT/mTOR pathway. These findings underscore the potential of LMW-AP-FBG as a promising natural candidate for anticancer applications and justify further mechanistic and translational research.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

JWP performed the experiments and prepared the original draft of the manuscript. GMM, TMJ, JAS and TK performed experiments and contributed to the methodology. HK contributed to the conceptualization and validation of the study and critically reviewed and edited the manuscript. JKK conceived and designed the study, acquired funding, administered the project, and supervised the review and editing of the manuscript. JWP, TMJ and JKK confirm the authenticity of all the raw data. All authors have read and approved the final version of the 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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