A polysaccharide from the fruiting bodies of *Agaricus blazei* Murill induces caspase-dependent apoptosis in human leukemia HL-60 cells

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**Abstract** Polysaccharides are the major active ingredients of fungus *Agaricus blazei* for treating and preventing cancer. However, there are no reports showing anti-tumor activity of *A. blazei* polysaccharides (ABP) on human leukemia (HL)-60 cells in vitro and in vivo. In this study, we demonstrated that ABP efficiently inhibited proliferation of cultured HL-60 cells, which was associated with the induction of apoptosis. The increase in ABP-induced apoptosis was accompanied by loss of mitochondrial membrane potential (ΔΨm), cytochrome c release from the mitochondria, activation of caspase-3, degradation of poly(ADP-ribose) polymerase (PARP), and the elevated ratio of Bcl-2-associated X (Bax)/B-cell lymphoma 2 (Bcl-2). Moreover, z-DEVD-fmk, a caspase-3 inhibitor, reversed the cytotoxic effects and apoptotic characteristics induced by ABP in HL-60 cells. Furthermore, we confirmed that ABP could obviously inhibit the solid cancer growth of leukemia HL-60 in tumor xenograft model. These data demonstrated that ABP effectively induced the apoptosis of HL-60 cells via a signaling cascade of mitochondrial caspase-3-dependent pathway.

**Keywords** *Agaricus blazei* · Polysaccharides · HL-60 cells · Apoptosis · Caspase-3 · Mitochondria

**Introduction**

Human leukemia is the most common hematological malignancy and the major leading cause of human death, even though various treatment strategies were developed [1]. Recently, several lines of evidence indicated that traditional Chinese herbal medicines have gained wide attention as alternative clinical options for the treatment of various malignant diseases, including leukemia, due to their anti-viral, antioxidant, anti-inflammatory, and tumor apoptosis-inducing properties [2, 3]. Thus, the characterization and development of chemical compounds from these herbal medicines with anti-tumor effects have become a very important topic.

Recently, it is documented that many polysaccharides from fungi, such as lentinian, schizophyllan, polysaccharide-K (PSK), and polysaccharide-P (PSP), could effectively inhibit the growth of various transplantable tumors in experimental animals and increase the survival rate [4-6]. An interesting example is *Agaricus blazei* Murill, which is a mushroom native to Brazil, and is widely used among cancer patients as complementary and alternative medicine [7]. Growing pieces of evidence had proved that some polysaccharides and polysaccharide-protein complexes isolated from *A. blazei* were bioactive principles responsible for treating and preventing cancer [8]. However, up to now, anti-tumor effect and the exact mechanism of *A. blazei* polysaccharides in models of human
leukemia in vitro and in an in vivo xenograft mouse model have not been addressed in any of the foregoing studies. Bearing this in mind, we therefore investigated the effects of a polysaccharide from *A. blazei* on proliferation and apoptosis in HL-60 human promyelocytic leukemia cells in vitro and further examined the anti-tumor effect in vivo. The molecular mechanisms involved in these processes were also elucidated using the HL-60 cell line. Our results clearly demonstrated that this polysaccharide could induce apoptosis in HL-60 cells through the caspases-3-dependent mitochondrial pathway.

**Materials and methods**

**Materials**

The dried fruiting bodies of *A. blazei* were purchased from the local marked in Xi’an, China. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Sigma Co. (St. Louis, MO, USA). DEAE-Sepharose Fast Flow and Sephadex G-100 were from Pharmacia Co. (Uppsala, Sweden). Anti-PARP antibody was from BD Pharmingen (Franklin Lakes, NJ, USA). Antibodies against Bcl-2-associated X (Bax) and B-cell lymphoma 2 (Bcl-2) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit was from KeyGen Biotech Co. (Nanjing, China). Caspase-3 colorimetric assay kit was from R&D Systems, Inc. (Minneapolis, MN, USA). Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Gibco Invitrogen Co. (Grand Island, NY, USA). All other reagents of analytical grade were purchased from Shanghai Chemical Co. (Shanghai, China).

**Polysaccharide purification**

The dried fruiting bodies of *A. blazei* (500 g) was sufficiently extracted with toluene–ethanol (1:1, v/v) by refluxing in a Soxhlet apparatus for 6 h to remove fats and waxes, and then decocted with distilled water at 90 °C for three times and each time for 3 h. The extract solution was combined by centrifugation, concentrated to 20 % of the original volume under a reduced pressure, and associated proteins in the extracts were removed with the Sevag method [9], followed by dialyzing against distilled water. The retentate was concentrated and added with 3 volumes of 95 % EtOH to precipitate crude polysaccharides (named CABP, 42.4 g, recovery 8.48 %).

The crude polysaccharides were redissolved in distilled water, forced through a filter (0.45 μm), and then applied to a DEAE-Sepharose Fast Flow column (2.6 cm×30 cm) equilibrated with 0.02 mol/L PBS (pH 7.2). After loading with sample, the column was eluted first with 0.02 mol/L PBS and followed by a linear gradient of 0→2 M NaCl in distilled water at a flow rate of 1.0 mL/min. The collected samples eluted by PBS were further applied to a Sephadex G-100 column (2.6 cm×100 cm) and eluted with 0.02 mol/L PBS at a flow rate of 1 mL/min. One polysaccharide fraction obtained was named as *A. blazei* polysaccharide (ABP). All the collection in each tube was monitored by the phenol–sulfuric acid assay at 490 nm [10].

**Cell culture**

HL-60 (human leukemia), HepG-2 (human hepatocellular carcinoma), HeLa (human cervix adenocarcinoma), A549 (human lung adenoma), and HT-29 (human colon carcinoma) cell lines were obtained from the Cell Bank of Chinese Academy of Science and cultured in RPMI 1640 media supplemented with 10 % heat-inactivated FBS and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin) in a 5 % CO2 humidified incubator at 37 °C.

**Quantification of cytotoxicity**

Cell viability was assessed by measuring their ability to metabolize MTT as described elsewhere [11]. Briefly, the cells (2×10^5 cells/mL) were seeded in a 96-well plate and cultivated for 24 h, followed by the addition of 0, 25, 50, 100, 200, 400, and 800 μg/mL of ABP. After 48 h, 20 μL of MTT (5 mg/mL in PBS) solution was added to each well, and the plates were incubated for an additional 4 h. The medium was discarded and the purple-blue MTT formazan precipitate was dissolved with 100 μL of dimethyl sulfoxide (DMSO). The optical density was measured at 570 nm by a Benchmark microtiter plate reader (Bio-Rad Laboratories, CA, USA). Cell growth inhibition rate (%) was calculated using the following equation: inhibitory rate (%)=(1−(A570treatment /A570control))×100 %. Half maximal inhibitory concentration (IC50) value was obtained by the Logit method and was determined from the dose–response curve. The experiments were performed in triplicate and repeated at least three times.

**Flow cytometric analysis of apoptosis**

To detect apoptotic cells (annexin V+/PI−) and necrotic cells (annexin V+/PI+), HL-60 cells were stained with FITC-conjugated annexin V and PI using a commercially available Annexin V-FITC/PI apoptosis detection kit according to the manufacturer’s instructions. After ABP treatment, the cells were collected and washed twice in ice-cold PBS and resuspended in 300 μL of binding buffer at 2×10^5 cells/mL. To discriminate between early apoptosis and necrosis, the cells were simultaneously stained with annexin V and PI before analysis. At least 10,000 events were analyzed for each
sample by flow cytometry (FACSCalibur, BD Biosciences) using CellQuest software.

Evaluation of mitochondrial membrane potential (ΔΨm)

The uptake of the cationic fluorescent dye rhodamine 123 has been used for the estimation of mitochondrial membrane potential [12]. Rh123 selectively enters mitochondria with an intact membrane potential and is retained in the mitochondria. Once the ΔΨm is lost, Rh123 is subsequently washed out of the cells. The cells were seeded at 1 × 10^5 cells/well into 12-well plates. After 24-h incubation, the cells were treated with serial dilutions of ABP (50, 100, and 200 μg/mL) for 48 h, followed by the addition of R123 (10 μM) to the samples for 20 min at 37 °C. Thereafter, harvested cells were washed twice with PBS, added with PI (50-μM final concentrations), and analyzed by means of flow cytometry.

Caspase-3 assays

Caspase-3 activity was measured with the use of the caspase-3 colorimetric assay kit according to the recommended protocol, which detects enzyme activity based on the cleavage of Asp-Glu-Val-Asp (DEVD)-pNA. Briefly, HL-60 cells (2 × 10^5 cells/mL) in RPMI 1640 medium were treated with ABP at the indicated concentration for 48 h at 37 °C. The cell pellets were resuspended in lysis buffer and left on ice for 30 min. The lysates were centrifuged at 10,000×g for 10 min, and the enzyme activity was measured on 96-well flat-bottom microplates with equivalent of 100 μg of protein from the cell lysate in reaction buffer containing DEVD-pNA, a specific substrate of caspase-3. Next, the cells were incubated for 1 h at 37 °C, and caspase-3 activity was measured at 405 nm with the use of a microtiter plate reader. The increase in caspase-3 activity was calculated relative to the absorbance value of the negative control.

Preparation of cytosolic and mitochondrial fractionations and Western blot analysis

Treated and untreated HL-60 cells were centrifuged at 400×g at 4 °C, washed in PBS, and lysed in lysis buffer [50 mM Tris-HCl (pH 7.4), 1 % Nonidet-P40, 0.25 % sodium deoxycholate, and 150 mM NaCl] at 4 °C for 30 min. Protein concentration was determined using the Bradford method [13]. Cytosolic, mitochondrial, and whole cell lysates were prepared using the method of Liu et al. [14]. Equal amounts of proteins (30 μg) were subjected to electrophoresis on 12 % SDS-polyacrylamide gels (SDS-PAGE) and transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA). The membranes were then blocked in 5 % powdered nonfat milk in a Tris/Tween solution for 2 h and then incubated with specific primary antibodies (1:200 for cytochrome c; 1:1,000 for β-actin, Bax, Bcl-2, and PARP) in blocking solution overnight at 4 °C. The immunobots were developed with an HRP-conjugated secondary IgG antibody (goat anti-mouse for cytochrome c; goat anti-rabbit for β-actin, Bax, Bcl-2, and PARP) at a 1:2,500 dilution and visualized by an enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Piscataway, NJ, USA). β-Actin was used as internal control.

Animals and experimental procedures

Male athymic nude mice, approximately 6 weeks of age, were purchased from the Animal Center of the Fourth Military Medical University and housed in plastic cages under standard conditions and had access to rodent chow and water ad libitum. All animal studies were conducted according to institutional guidelines approved by the Animal Care and Use Committee of the Fourth Military Medical University (Xi’an, China).

After housing for a week, the mice were inoculated with 2 × 10^6 HL-60 cells in 0.2 mL of PBS in the right flank of

Table 1: Cytotoxic activity of ABP on cancer cell growth in vitro

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Tumor type</th>
<th>IC50 (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL60</td>
<td>Human leukemia</td>
<td>96.6</td>
</tr>
<tr>
<td>A549</td>
<td>Human lung adenoma</td>
<td>136.5</td>
</tr>
<tr>
<td>HT-29</td>
<td>Human colon carcinoma</td>
<td>235.5</td>
</tr>
<tr>
<td>HepG-2</td>
<td>Human hepatocellular carcinoma</td>
<td>426.3</td>
</tr>
<tr>
<td>HeLa</td>
<td>Human cervix adenocarcinoma</td>
<td>152.2</td>
</tr>
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</table>

IC50 is defined as the extract concentration causing a 50 % decrease in the survival curve compared to that of the control. The values were represented as mean ± S.D. from three independent experiments.

Cytotoxicity was measured with the MTT assay.

Fig. 1 Annexin V-FITC/PI staining of HL-60 cells exposed to different concentrations (50, 100, and 200 μg/mL) of ABP. Results are presented as means ± S.D. (n=3). *P<0.05 or **P<0.01 as compared with the control group.
unanesthetized mice. After 7 days, palpable tumors of volume 100–200 mm$^3$ were developed, and the xenograft tumor-bearing mice were randomly divided into five groups (with five mice per group): one negative control group, three doses of ABP-treated groups, and one positive control group. The tumor-bearing mice were treated with ABP at the dosage of 25, 50, and 100 mg/kg. The negative control group received 0.9 % normal saline. The positive group was treated with 30 mg/kg cyclophosphamide (CTX) only. All of these drugs were injected intraperitoneally every 3 days up to 20 days. At the end of the experiment, the animals were euthanized by cervical dislocation, and the solid tumors were picked up and weighed. The in vivo tumor inhibition (RI) ratio was calculated according to the formula: RI=[(A−B)/A]×100 %, where A is the mean tumor weight of the negative control group and B is the average tumor weight of treatment group.

Data analysis

Data presented are the means ± S.D. of at least three independent determinations. All experiments were done at least three times, with three or more independent observations each time. Statistical analysis was performed using Student’s t test.

Results and discussion

The cytotoxicity of ABP on various cancer cells

The growth inhibitory effect of polysaccharide ABP on five different cancer cell lines was tested in vitro using MTT assay following exposure of cancer cells to ABP (0 to 800 μg/mL) for 48 h. The results in Table 1 indicated that ABP treatment exhibited different degrees of cytotoxicity on these cells based on IC$_{50}$ values, ranging from 96.6 to 426.3 μg/mL. In particular, HL-60 human leukemia cell line was more sensitive to ABP treatment. The data demonstrated that ABP had a specific suppressing effect on the growth of HL-60 cells in vitro.

ABP induces apoptosis of HL-60 cells

ABP markedly depressed the proliferation of HL-60 cell as demonstrated by the MTT results. Therefore, it was speculated that ABP induced apoptosis of HL-60 cells. In an attempt to elucidate whether the loss of cell viability occurred as a consequence of apoptosis, the HL-60 cells were incubated with different concentrations of ABP for 24 h, and then, the occurrence of apoptotic cells was quantified by flow cytometry using annexin V/PI double-staining assay. As shown in Fig. 1, the percentage of...
Annexin V-FITC-positive cells increased with the concentration of ABP applied, which indicated that ABP-induced growth suppression of HL-60 cells involves the induction of apoptosis.

ABP induces loss of \( \Delta \Psi_m \) and release of cytochrome c

Disruption of \( \Delta \Psi_m \), as markers of mitochondrial function, is one of the earliest intracellular events that occur following the induction of apoptosis [15]. In this study, we investigated the effect of ABP on the change of \( \Delta \Psi_m \) using a cationic dye rhodamine 123, which can cross over the mitochondrial membrane and accumulate in the matrix, reflecting the change of \( \Delta \Psi_m \) [16]. The results in Fig. 2a demonstrated that treating HL-60 cells with ABP for 48 h resulted in a dose-dependent loss of the mitochondrial membrane potential.

In line with the dissipation of \( \Delta \Psi_m \), efflux of some mitochondrial inner membrane proteins, such as cytochrome c, is also involved in the initiation of the mitochondria-dependent apoptosis [17]. As indicated in Fig. 2b, ABP induced cytochrome c release from the mitochondria into the cytosol in a concentration-dependent manner. Altogether, these findings indicated that ABP-induced apoptosis occurred through triggering the loss of \( \Delta \Psi_m \) with a subsequent release of cytochrome c from mitochondria into the cytoplasm.

ABP activates caspase-3 activity and induces cleavage of PARP

Caspase, a family of cysteine proteases, is known to form integral parts of the apoptotic pathway [18]. Therefore, we investigated the protein level and activity of caspase-3. Results showed that caspase-3 activity significantly increased as the concentration of ABP raised (Fig. 3a). Moreover, ABP treatment caused cleavage of PARP, 116 into 89-kDa fragment (Fig. 3b), which corresponded with the activation of caspase-3. In addition, pre-treatment of cells for 2 h with 25-\( \mu \)M z-DEVD-fmk, a caspase-3 inhibitor, blocked ABP-induced apoptosis (data not shown), confirming the involvement of caspase-3 in the apoptotic events in ABP-treated cells.

ABP increases Bax protein expression but decreases Bcl-2 protein expression

Bcl-2 family proteins such as anti-apoptotic Bcl-2 protein and pro-apoptotic Bax protein play a critical role in the regulation of apoptosis [19, 20]. We further examined the effect of ABP on the expression of Bcl-2 and Bax proteins. As shown in Fig. 4, the protein level of the pro-apoptotic protein, Bax, increased as the concentration of ABP raised. Conversely, the protein level of the anti-apoptotic Bcl-2 protein did not significantly change in HL-60 cells. These results indicated that ABP may disarrange the pro-apoptotic/anti-apoptotic (Bax/Bcl-2) ratio, thus leading to apoptosis in HL-60 cells.

ABP inhibits the growth of solid tumor implanted in mice

Based on all the results from in vitro studies, we find that ABP induced apoptosis in human leukemia HL-60 cells through mitochondrial- and caspases-dependent pathways. In this context, we investigated whether or not ABP can affect HL-60 cells in vivo by injecting HL-60 cells by s.c. into the mice for generating leukemia tumor xenograft model. Table 2 showed the tumor weight and inhibition ratio for ABP treatments in athymic nude mice. ABP treatment did not alter body weight, but significantly decreased the tumor weight compared to controls, and the percentages of tumor inhibition were 23.72 for 25 mg/kg of ABP, 47.44 for 50 mg/kg of ABP, and 54.49 for 100 mg/kg of ABP, respectively. The results demonstrated that ABP could suppress tumor growth in vivo.

### Conclusions

In conclusion, this study demonstrated that ABP could inhibit the proliferation of HL-60 cells by induction of apoptosis through activation of the mitochondrial-mediated intrinsic caspase pathway. It was found that the apoptotic effects of ABP...
ABP are mediated through the dissipation of ΔΨm, release of cytochrome c from the mitochondria into the cytosol, activation of caspase-3, cleavage of PARP, and elevated the ratio of Bax/Bcl-2. In addition, ABP administration significantly inhibited tumor growth in vivo. Thus, the present results suggested that ABP could be considered as a promising source for developing novel therapeutics for the treatment of human leukemia.

Conflicts of interest None

References
