

Polysaccharide peptide isolated from grass-cultured *Ganoderma lucidum* induces anti-proliferative and pro-apoptotic effects in the human U251 glioma cell line

CHUNHUA WANG^{1,2}, DONGMEI LIN³, QUAN CHEN¹,
SHUQIAN LIN^{3,4}, SONGSHENG SHI^{1,2} and CHUNMEI CHEN^{1,2}

¹Department of Neurosurgery, The Affiliated Union Hospital of Fujian Medical University; ²Fujian Neurosurgical Institute, Fuzhou, Fujian 350001; ³National Engineering Research Center of JUNCAO Technology, Fujian Agricultural and Forestry University; ⁴Fuzhou Institute of Green Valley Bio-Pharm Technology, Fuzhou, Fujian 350002, P.R. China

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Abstract. The *Ganoderma lucidum* (*G. lucidum*) mushroom is one of the most extensively studied functional foods, known for its numerous health benefits, including the inhibition of tumor cell growth. The present study assessed the anti-proliferative and pro-apoptotic activity of a novel *G. lucidum* polysaccharide peptide (GL-PP) in human glioma U251 cells, which was purified from grass-cultured *G. lucidum*. GL-PP is a glycopeptide with an average molecular weight of 42,635 Da and a polysaccharide-to-peptide ratio of 88.70:11.30. The polysaccharides were composed of l-arabinose, d-mannose and d-glucose at a molar ratio of 1.329:0.372:2.953 and a total of 17 amino acids were detected. The results of the current study demonstrated that GL-PP significantly inhibited U251 cellular proliferation. The proportion of G₀/G₁ phase cells and sub-G₁ phase cells significantly increased as the concentration of GL-PP increased, as did the activity of caspase-3. These results indicate that GL-PP directly inhibited human glioma U251 proliferation by inducing cell cycle arrest and promoting apoptosis.

Introduction

Malignant gliomas are the most common type of brain tumor, with high rates of recurrence and mortality (1). Despite the aggressive use of surgery, radiotherapy and chemotherapy, the average life expectancy of patients with high-grade glioma is 14 months after diagnosis (2). Novel treatments

are therefore required to improve this figure. In recent years, natural products isolated from herbs and plants have received substantial attention for their potential as novel anticancer drugs (3).

Ganoderma lucidum is a mushroom that has been used for medicinal purposes for centuries in China and other oriental countries. *G. lucidum* polysaccharides (GL-PS) are primary bioactive components of the fungus that can exert various pharmacological effects, including immunomodulation (4-10), antitumor (11-16), anti-angiogenesis (17,18), anti-oxidation (19-22), anti-inflammatory (23) and hepatoprotective effects (24,25).

The potential antitumor effects of GL-PS have received substantial attention and have been the subject of extensive investigation. It has been demonstrated that GL-PS is effective at inhibiting tumor growth *in vivo* via multiple mechanisms, particularly via the activation of immune effector cells such as lymphocytes (9,26-29), macrophages (9) and natural killer cells (29,30). GL-PS has also been revealed to inhibit tumor-induced neovascularization processes. However, GL-PS is considered to possess weak or no anti-tumor activity *in vitro* (17,28,31-34). Previous studies have demonstrated that GL-PS may directly inhibit the *in vitro* proliferation of certain types of cancer cell, including breast cancer (14), ovarian cancer (15), colorectal cancer (23) and hepatoma cells (11). However, whether GL-PS elicits anti-tumor effects on gliomas remains unknown. In the present study, we isolated and identified a 42,635 Da *G. lucidum* polysaccharide peptide (GL-PP) from *G. lucidum* cultivated with JUNCAO grasses and determine its antitumor effects on gliomas for the first time. It was revealed that this GL-PP exhibited anti-proliferative and apoptosis-inducing effects in human U251 glioma cells.

Materials and methods

Preparation of GL-PP. GL-PP was isolated from the boiled extract of *G. lucidum* cultivated with JUNCAO grasses (National Engineering Research Center of Juncao Technology, Fujian, China). An ethanol precipitation was then performed,

Correspondence to: Dr Chunmei Chen, Department of Neurosurgery, The Affiliated Union Hospital of Fujian Medical University, 29 Xinquan Road, Fuzhou, Fujian 350001, P.R. China
E-mail: chenm_mail@126.com

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followed by dialysis and de-proteination according to the Sevag method, as previously described (35).

The homogeneity and molecular weight of GL-PP was identified using high-performance gel permeation chromatography (HPGPC) with a Waters 2695 HPLC apparatus (Waters, Milford, MA, USA), equipped with a Waters 2515 HPLC pump (Waters), a gel permeation column TSK4000PW (21.5x300 mm, 10 μ m; Tosoh, Tokyo, Japan) and a Waters 2414 refractive index detector (Waters). Water (HPLC grade) was used as the mobile phase with a gradient elution (flow rate of 0.8 ml/min) at 35°C. Dextran standards were obtained from the National Institutes for Food and Drug Control and molecular weights ranging from 2,500 to 84,400 Da were used to generate a calibration curve (36). The molecular weight of GL-PP was estimated using Waters Empower software (version 5.0; Waters).

Monosaccharides were determined using hydrophilic interaction liquid interface chromatography and an evaporative light scattering detector (HILIC-ELSD) via a Waters Alliance 2695 HPLC system and 2424 ELSD (both from Waters). Chromatographic separation was performed on a Sugar-D column (4.6x250 mm; 5 μ m; Nacalai Tesque, Inc., Kyoto, Japan). The column was run at 35°C and the mobile phase consisted of 75% acetonitrile and 25% water, at a flow rate of 0.8 ml/min. The drift tube temperature and air carrier gas pressure of the ELSD was set to 55°C and 45 psi, respectively. The injection volume was 10 μ l. The identity of sample monosaccharides were determined by comparing the retention time of peaks with those of known standards including, rhamnose, fructose, xylose, arabinose, glucose, galactose and mannose.

The amino acid composition of GL-PP was determined using the Hitachi-L8800 amino acid analyzer (Hitachi High-Technologies Co., Tokyo, Japan) according to the Chinese National Standard (37).

GL-PP was dissolved in serum-free Dulbecco's modified Eagle's medium (DMEM; HyClone; GE Healthcare Life Sciences, Chicago, IL, USA), then filtered through a 0.22- μ m filter and stored at 4°C. This medium was further diluted to the indicated concentration prior to each assay.

Endotoxin test. The presence of an endotoxin in GL-PP was detected using Tachypleus Amebocyte Lysate (TAL; Fuzhou Xin Bei Biochemical Industry Co., Ltd., Fuzhou, China) according to the manufacturer's instructions. A total of 100 μ l TAL reagent was added to 100 μ l of 800 μ g/ml GL-PP, 100 μ l endotoxin standard (Fuzhou Xin Bei Biochemical Industry Co., Ltd.) and 100 μ l endotoxin-free water. The mixture was gently agitated, covered with foil and incubated at 37°C for 1 h. Since endotoxin contamination results in the formation of a hard gel substance, samples were observed for the formation of this gel by performing a gentle 180° tube inversion following the incubation period.

Cell culture. The human glioma U251 cell line was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) where it was routinely maintained in DMEM containing 10% inactivated fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences) and 100 U/ml penicillin/streptomycin. Cells were grown at 37°C in an atmosphere of 5% CO₂.

Cell proliferation assay. Cell proliferation and viability were analyzed using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). U251 cells were seeded onto 96-well culture plates at a density of 2,000 cells/well and cultured for 24 h prior to treatment. GL-PP was added to the culture at a final concentration of 50, 100, 200, 400 or 800 μ g/ml and cells were incubated for a further 24, 48 or 72 h. Wells that were not treated with GL-PP were used as negative controls. The CCK-8 reagent was then added to the cultures according to manufacturer's instructions and absorbance was measured at 450 nm using an ELISA MK3 microplate reader (Thermo Fisher Scientific Inc., Waltham, MA, USA). All measurements were performed in triplicate.

Cell cycle assay. U251 cells (2.5x10⁴ cells/ml) were plated onto a 6-well plate and harvested at 48 h following treatment. Cells were fixed in 70% ice-cold ethanol, washed with ice cold PBS and stained with 50 mg/ml propidium iodide in the presence of 50 mg/ml RNase A for 30 min. Staining procedures were performed under low light at room temperature. Analyses were performed using a flow cytometer (Cytomics FC 500; Beckman Coulter Inc., Brea, CA, USA). Cell proportions in sub-G₁, G₀/G₁, S and G₂/M phases were analyzed using ModFit LT software (version 2.0; Verity Software House, Topsham, ME, USA). Tests were performed in triplicate for each sample.

Western blotting. A 6-well cell culture plate was prepared as aforementioned. Total protein was purified from cells using a cell lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) containing 1 mM phenylmethylsulfonyl fluoride. Protein concentrations were subsequently measured using a BCA kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Samples (150 μ g protein/lane) were then separated on 12% SDS-PAGE gel and transferred onto a nitrocellulose membrane. β -actin (Cell Signaling Technology Inc., Danvers, MA, USA) was used as a loading control. The membrane was blocked with 5% bovine serum albumin (Sangon Biotech, Shanghai, China) at room temperature for 2 h and incubated overnight at 4°C with a rabbit polyclonal anti-active caspase-3 antibody (1:1,000, cat. no. ab2302; Abcam, Cambridge, UK). The membrane was then incubated with a horseradish peroxidase conjugated goat anti-rabbit immunoglobulin G (1:2,000, cat. no. sc-2004; Santa Cruz Biotechnology Inc., Dallas, TX, USA) at room temperature for 2 h. Protein expression was detected using a Chemiluminescent HRP Substrate (EMD Millipore, Billerica, MA, USA) and exposed on X-ray film. The optical density of each band was determined using Quantity One software (version 4.6.1; Bio-Rad Laboratories Inc., Hercules, CA, USA). The expression of cleaved caspase-3 was expressed as a ratio to β -actin used as an internal control. Tests were performed in triplicate for each sample.

Caspase-3 activity assay. The activity of caspase-3 was determined using a Caspase 3 Activity Assay kit (Beyotime Institute of Biotechnology). To evaluate the activity of caspase-3, cell lysates were prepared following cell treatment with GL-PP for 48 h. Each 10 μ l cell lysate was incubated with 80 μ l reaction buffer [1% NP-40, 20 mmol/l Tris-HCl (pH 7.5), 137 mmol/l NaCl, 10% glycerol] containing 10 μ l caspase-3

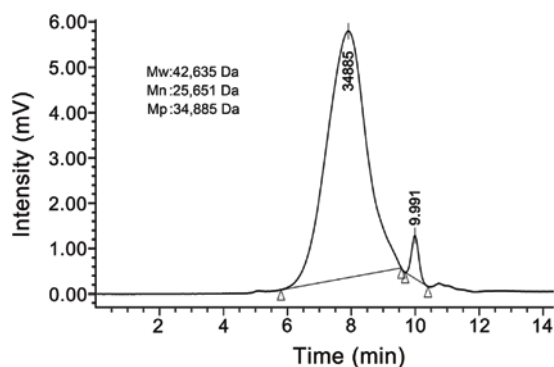


Figure 1. Typical gel-permeation chromatogram of GL-PP samples. Chromatograms of the average molecular weight and homogeneity of GL-PP as determined by high-performance gel permeation chromatography (Mw: mean molecular weight; Mn: Number-average molecular weight; Mp: Peak molecular weight) GL-PP, *Ganoderma lucidum* polysaccharide peptide.

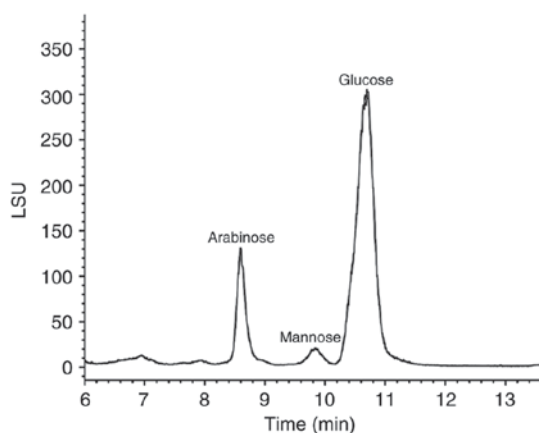


Figure 2. Identification and determination of major monosaccharides in the *Ganoderma lucidum* polysaccharide peptide using hydrophilic interaction liquid interface chromatography and evaporative light scattering detector analysis. LSU, light scattering unit.

substrate [2 mmol/l acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA)] at 37°C for 2 h. Caspase-3 can catalyze the substrate Ac-DEVD-pNA to produce pNA. Thereafter, absorbance of pNA at 405 nm was measured using an ELISA plate reader (MK3; Thermo Fisher Scientific Inc.). The analysis procedure followed the manufacturer's instructions. All experiments were performed in triplicate.

Statistical analysis. The data were expressed as mean \pm standard deviation. Multigroup comparisons of the means were carried out by one-way analysis of variance test with post hoc Student-Newman-Keuls test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Characterization of GL-PP. GL-PP was determined to be a polysaccharide peptide with a mean molecular weight (Mw) of 42,635 Da (Fig. 1), a purity of 96.89% and a polysaccharide to peptide ratio of 88.70:11.30%. HILIC-ELSD data indicated that polysaccharides were primarily composed of l-arabinose, d-mannose and d-glucose at a molar ratio of 1.329:0.372:2.953 (Fig. 2).

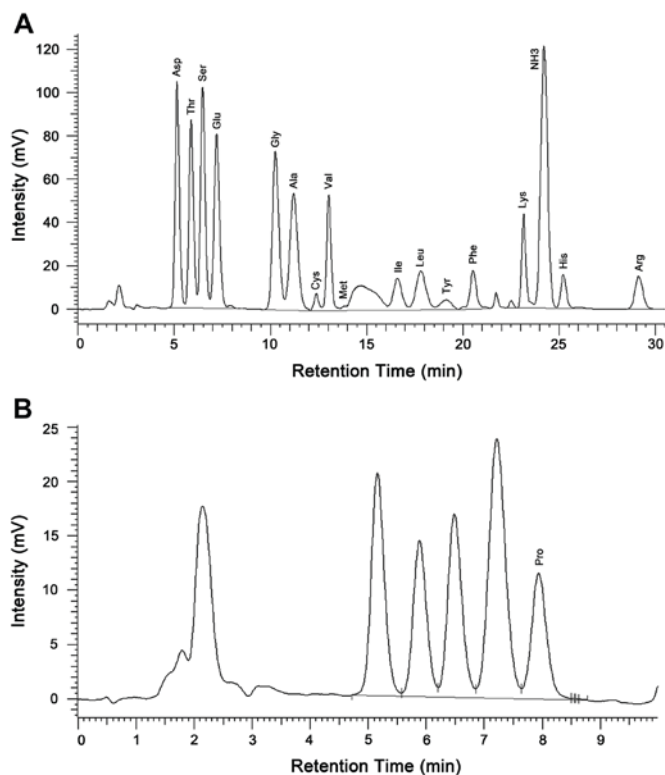


Figure 3. The fingerprint amino acid composition spectrum of GL-PP. (A) Amino acid composition spectrum in channel 1. (B) Amino acid composition spectrum in channel 2.

The amino acid automatic analyzer indicated that the peptide contained 17 amino acids, including Asp, Thr, Ser, Glu, Gly, Ala, Cys, Val, Met, Ile, Leu, Tyr, Phe, Lys, His, Arg and Pro, with a respective mass ratio of: 1.45:1.08:1.12:1.45:0.84:0.87:0.19:0.64:0.01:0.38:0.56:0.23:0.47:0.58:0.30:0.53:0.60 (Table I and Fig. 3). No detectable level of endotoxin (≤ 0.10 EU/ml) was identified in the GL-PP samples.

GL-PP inhibits the proliferation of U251 cells. Cells were incubated with varying concentrations of GL-PP (50, 100, 200, 400 and 800 $\mu\text{g/ml}$) for 24, 48 or 72 h. GL-PP markedly inhibited the proliferation of U251 cells in a dose-dependent manner compared with untreated controls. The maximum inhibition observed at 48 h among all treatment groups except 400 $\mu\text{g/ml}$ group (Fig. 4). The half-maximal inhibitory concentration of GL-PP at 48 h was 274.1 $\mu\text{g/ml}$.

GL-PP blocks cell cycle progression at the G_0/G_1 phase and induces apoptosis. To examine how GL-PP inhibited cell proliferation and viability, flow cytometry was utilized to determine the effect of GL-PP on cell cycle progression. Results revealed that the presence of G_0/G_1 phase cells was increased from 62.9 to 74.94% at 48 h with increasing concentrations of GL-PP among all groups. This increase was accompanied by a significant decrease in the percentage of S phase cells, whereas the fraction of G_2/M phase cells was primarily unchanged. The sub- G_1 phase cell population, which indicate the number of late-stage apoptotic cells, also increased significantly in GL-PP treated cells in a dose-dependent manner (Fig. 5). This result demonstrated that GL-PP induced G_0/G_1 phase arrest and apoptosis induction.

Table I. Amino acid comparisons of *Ganoderma lucidum* polysaccharide peptide.

Amino acid	Category	Contents (%)
Asparagine	N	1.45
Threonine	N	1.08
Serine	N	1.12
Glutamic acid	N	1.45
Glycine	N	0.84
Alanine	N	0.87
Cystine	N	0.19
Valine	E	0.64
Methionine	E	0.01
Isoleucine	E	0.38
Leucine	E	0.56
Tyrosine	N	0.23
Phenylalanine	E	0.47
Lysine	E	0.58
Histidine	N	0.30
Arginine	N	0.53
Proline	N	0.60
Total	-	11.3

E, essential; N, non-essential.

GL-PP induces apoptosis specific caspase-3 activity in U251 cells. To examine the induction of apoptosis by GL-PP, the activation of caspase-3, a primary enzyme in cell apoptosis, was assessed. The expression of active caspase-3 in GL-PP-treated U251 cells was determined using western blotting. The active caspase-3 level was increased in a dose-dependent manner following treatment with 50, 100, 200, 400 or 800 $\mu\text{g/ml}$ GL-PP for 48 h (Fig. 6). In addition, the activation of caspase-3 was analyzed by measuring the catalytic capability to the substrate Ac-DEVD-pNA. The result showed the activation of caspase-3 significantly increased in GL-PP treated groups in a dose-dependent manner (Fig. 7). This agrees with the result obtained by western blot analysis (Fig. 6).

Discussion

GL-PS and *G. lucidum* glycopeptides have been identified as potential natural sources of anticancer compounds (38). The present study identified, purified and tested the anti-glioma activity of a novel glycopeptide from grass-cultured *G. lucidum*, GL-PP, *in vitro*. GL-PP strongly inhibited human U251 glioma cell proliferation in a dose-dependent manner through cell cycle arrest and induction of apoptosis.

The sustenance of proliferative signaling and the resistance to apoptosis are two hallmarks of cancer (39). In the present study, human U251 glioma cells treated with GL-PP, exhibited G_0/G_1 cell cycle arrest and inhibited proliferation. Furthermore, the proportion of hypoploid cells (sub- G_1) and the activity of caspase-3 markedly increased, indicating that GL-PP induced U251 glioma cell apoptosis.

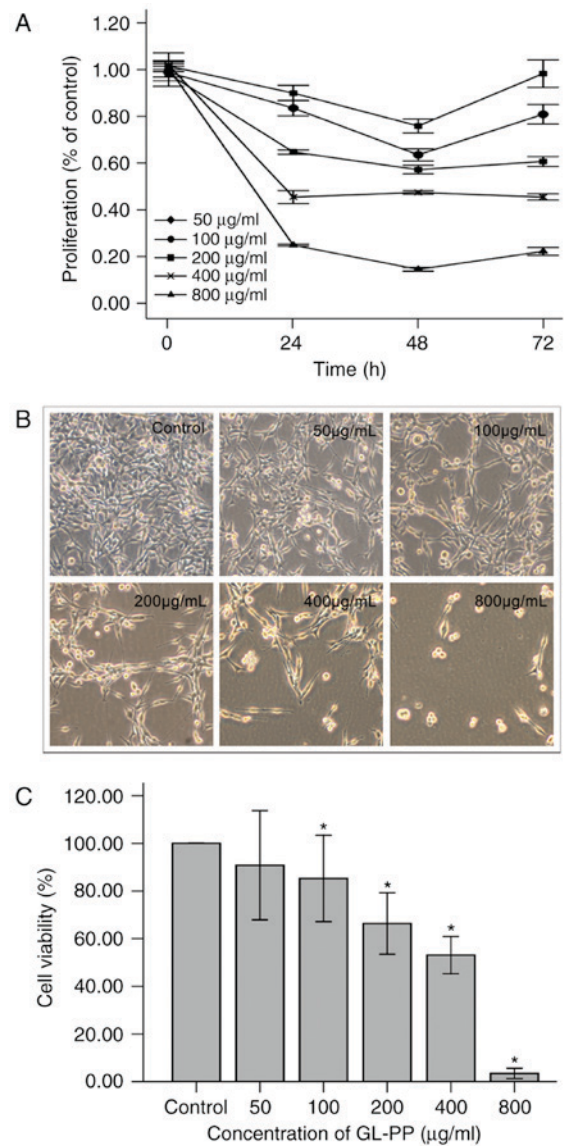


Figure 4. Effect of GL-PP on the proliferation and viability of U251 glioma cells. (A) The proliferation of U251 cells was markedly inhibited by GL-PP in a dose-dependent manner compared with controls. (B) U251 cells in culture under light microscope (magnification, $\times 100$) following treatment with GL-PP for 48 h. (C) Cell viability was examined 48 h after treatment with GL-PP using a Cell Counting Kit-8 assay. Except the 50 $\mu\text{g/ml}$ GL-PP group, the ratio of cell viability in all GL-PP treated groups was significantly lower than control group. Data are presented as the mean \pm standard deviation, * $P < 0.05$ vs. control. GL-PP, *Ganoderma lucidum* polysaccharide peptide.

Although the antitumor activity of GL-PS/GL-PP has been demonstrated in previous studies (8,10,14,38,40,41), whether GL-PS/GL-PP directly inhibits cancer cell proliferation or induces cellular apoptosis remains unknown. A study by Cao *et al* (31) indicated that GL-PP inhibited human lung carcinoma cell migration in a dose-dependent manner by inhibiting the expression and activity of matrix metalloproteinase-9. However, no inhibitory effect on cellular proliferation was identified. Li *et al* (42) also demonstrated that GL-PS had no cytotoxic effect on human prostate carcinoma PC-3M cells. In addition, a study by Oliveira *et al* (32) revealed that polysaccharide extracts from *G. lucidum* exhibited no cytotoxic activity, as all tumor cell lines (AGS, MCF-7, NCI-H460 and HCT-15) exhibited a concentration causing a 50% reduction in proliferation $> 400 \mu\text{g/ml}$.

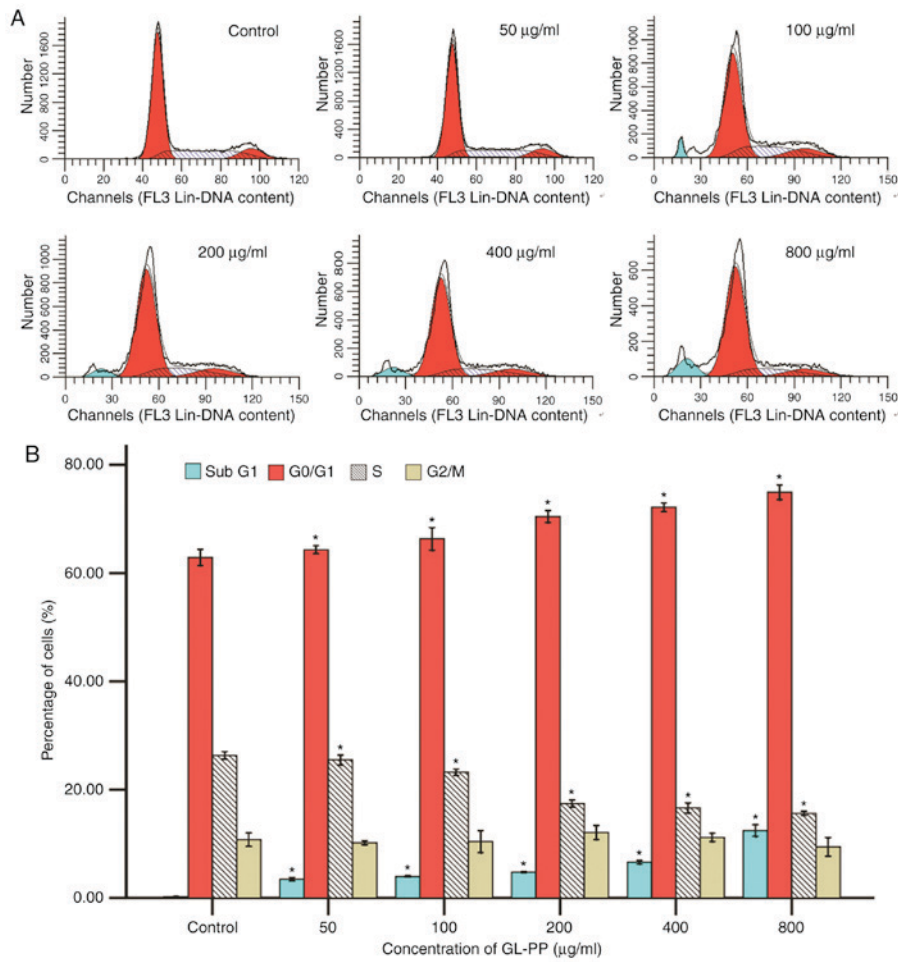


Figure 5. Cell cycle distribution of U251 cells exposed to different concentrations of GL-PP. (A) Cells were incubated with GL-PP for 48 h and the percentage of cells in each cell cycle phase was determined using flow cytometry. (B) Cell cycle distribution of different treatment groups. The population of cells in the sub-G₁ phase consisted of cellular fragments resulting from apoptosis. Data are presented as the mean ± standard deviation. *P<0.05 vs. control. GL-PP, *Ganoderma lucidum* polysaccharide peptide.

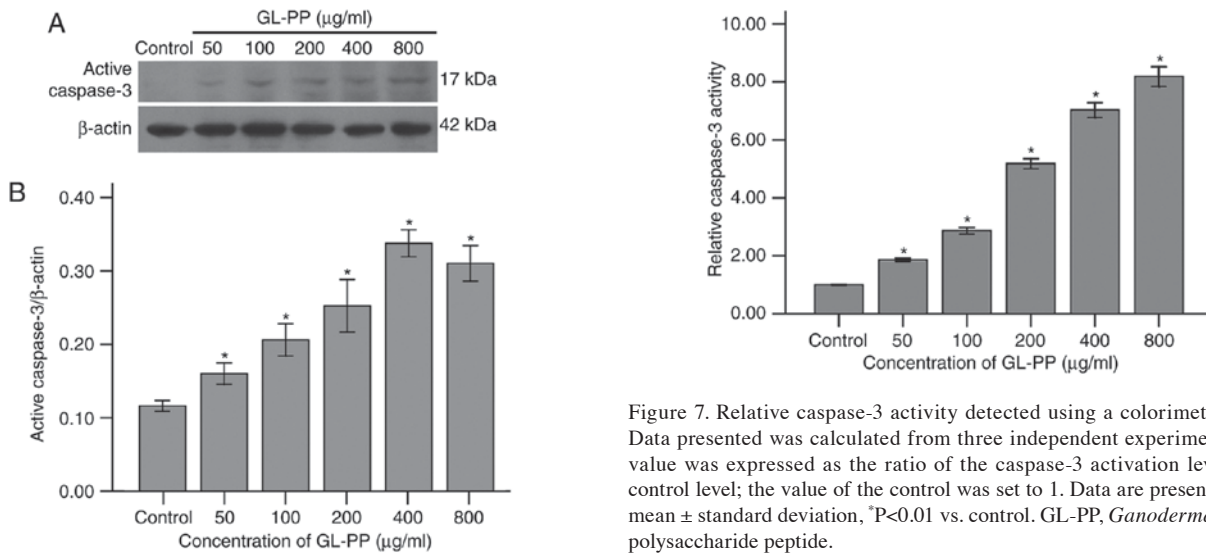


Figure 6. Effect of GL-PP on the expression of active caspase-3 in U251 cells. (A) Caspase-3 expression in U251 cells was detected by western blotting 48 h after treatment with the indicated concentrations of GL-PP. (B) The expression of active caspase-3 presented was calculated from three independent experiments. Each value was expressed as the ratio of active caspase-3 to β-actin. Data are presented as the mean ± standard deviation, *P<0.01 vs. control. GL-PP, *Ganoderma lucidum* polysaccharide peptide.

Figure 7. Relative caspase-3 activity detected using a colorimetric assay. Data presented was calculated from three independent experiments. Each value was expressed as the ratio of the caspase-3 activation level to the control level; the value of the control was set to 1. Data are presented as the mean ± standard deviation, *P<0.01 vs. control. GL-PP, *Ganoderma lucidum* polysaccharide peptide.

However, other studies have demonstrated that GL-PS/GL-PP significantly inhibited the proliferation of certain types of cancer cell *in vitro*, including breast cancer (14), ovarian cancer (15), hepatoma (11) and colorectal adenocarcinoma cells (11). The

findings of the present study are similar to these results, but concern glioma cells.

The apparent discrepancies of previous studies on the anti-proliferative effects of GL-PS/GL-PP may be due to the variability in the chemical constituents of GL-PS/GL-PP and the different origins of the cancer cell lines utilized. It has been determined that the biological activities of polysaccharides are associated with their chemical conformation, molecular weight, chemical modification and content of bound protein (43-46). For example, the native triple-helix conformation with β -(1-3) linkages in the primary glucan chain and additional β -(1-6) branch points of polysaccharides are considered to be necessary for antitumor activity (47). Furthermore, analysis performed by Yeh *et al* (9) compared de-proteinized polysaccharides with ling zhi-8 (LZ-8) and determined that LZ-8 activates murine macrophages and T lymphocytes, but a de-proteinized polysaccharide only acts as a macrophage activator, indicating that retaining proteins and the natural complex of polysaccharides may serve a vital role in the biological activities that they exhibit.

The GL-PP used in the present study was isolated from *G. lucidum* and cultivated with JUNCAO grasses. According to a previous study (48), the glycopeptides obtained from grass-cultured *G. lucidum* (GLPG) have a similar molecular weight, monosaccharide constituent and amino acid composition to those obtained from wood-log-cultured *G. lucidum* (GLPW), which are each primarily attached by β -glycosidic linkages. GLPG has a higher purification rate (2.8 times that of GLPW) and is more environmentally friendly than GLPW, which relies on a large consumption of forest resources. Compared with previous studies (15,17,26), the GL-PP extract utilized in the present study has a smaller molecular weight (42,635 kDa), contained fewer monosaccharides and exhibited a higher proportion of peptide (11.3%). Only glucose, mannose and arabinose were detected in the GL-PP isolated in the current study. The other monosaccharides often present in GL-PS/GL-PP extracts, including galactose, rhamnose, fucose, xylose (15,17,26), were not detected. In addition, 17 different amino acids were detected in the GL-PP extract utilized in the present study, including tyrosine, which has not been detected in GL-PS/GL-PP in previous studies. These differences may be partly due to the different isolation procedures and detection methods used, and may ultimately serve a role in the unique biological activities identified in the present study.

Although the present study determined the molecular weight, and monosaccharide and amino acid composition of GL-PP, to clearly ascribe the anti-proliferative effects to its chemical structure, a more thorough characterization is required. Elucidation of the molecular mechanisms and signaling pathways involved in GL-PP anti-glioma activity should also be performed in future studies. Additionally, although the direct anti-glioma activity of GL-PP has been observed *in vitro*, it may be limited by a number of elements *in vivo*, including biotransformation and the blood-brain barrier. Addressing these limitations may clarify the immunomodulatory activities of GL-PP and its inhibitory role in glioma growth *in vivo*.

In conclusion, the present study demonstrated that the GL-PP isolated from the *G. lucidum* cultivated with Juncao grasses demonstrates a potent inhibitive capacity on human

U251 glioma cell lines. The demonstrated *in vitro* anti-glioma activity of GL-PP was determined to be dose-dependent, and mediated by cell cycle arrest and induction of apoptosis. Therefore, GL-PP may represent a natural addition to the treatment regimen of patients with glioma.

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