

MAP30 promotes apoptosis of U251 and U87 cells by suppressing the LGR5 and Wnt/ β -catenin signaling pathway, and enhancing Smac expression

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Received December 19, 2016; Accepted November 16, 2017

DOI: 10.3892/ol.2018.8073

Abstract. Significant antitumor activity of *Momordica* anti-human immunodeficiency virus protein of 30 kDa (MAP30) purified from *Momordica charantia* has been the subject of previous research. However, the effective mechanism of MAP30 on malignant glioma cells has not yet been clarified. The aim of the present study was to investigate the effects and mechanism of MAP30 on U87 and U251 cell lines. A Cell Counting Kit-8 assay, wound healing assay and Transwell assay were used to detect the effects on U87 and U251 cells treated with different concentrations of MAP30 (0.5, 1, 2, 4, 8 and 16 μ M) over different periods of time. Proliferation, migration and invasion of each cell line were markedly inhibited by MAP30 in a dose- and time-dependent manner. Flow cytometry and fluorescence staining demonstrated that apoptosis increased and the cell cycle was arrested in S-phase in the two investigated cell lines following MAP30 treatment. Western blot analysis demonstrated that leucine-rich-repeat-containing G-protein-coupled receptor 5 (LGR5) expression and key proteins in the Wnt/ β -catenin signaling pathway were apparently decreased, whereas second mitochondria-derived activator of caspase (Smac) protein expression significantly increased with MAP30 treatment in the same manner. These results suggest that MAP30 markedly induces apoptosis in U87 and U251 cell lines by suppressing LGR5 and the Wnt/ β -catenin signaling pathway, and enhancing Smac expression in a dose- and time-dependent manner.

Introduction

Gliomas are the most common primary brain tumor in adults and are categorized into four World Health Organization grades on the basis of histopathological characteristics (1). Despite current multidisciplinary treatments including surgery, radiotherapy and chemotherapy, no significant prognostic improvement has been obtained in patients with gliomas over the last decade, because of the aggressive nature of high-grade gliomas, the survival rates of patients with high-grade gliomas are <10% at 5 years (2). Previously, efforts have been made to identify new effective therapeutic agents.

Momordica anti-human immunodeficiency virus protein of 30 kDa (MAP30), first extracted and purified from *Momordica charantia* in 1990, is a type I ribosome-inactivating protein (RIP) (3). Previous studies indicated that MAP30 exhibits a variety of anti-infection, anti-diabetic, antiviral and antitumor bioactive effects (4-10). The antitumor ability of MAP30 has been the subject of previous studies (11-15). However, certain researchers have already noted the effects of MAP30 on the inhibition of U87 cells in screening for anti-cancer effects (3,7,16,17). However, the mechanism of MAP30 on glioma cells has not been elucidated in detail. The aim of the present study was to investigate the effects and mechanism of MAP30 on U87 and U251 glioblastoma cell lines *in vitro*. Although some researchers identified that the DNA profile of U87MG was different from that of the original cells in 2016, it is likely to be a bona fide human glioblastoma cell line of unknown origin; therefore, in the present study the U87 and U251 cell lines were used.

Materials and methods

Materials. High-glucose Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin G and streptomycin were all purchased from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). The U87 and U251 human malignant glioblastoma cell lines were purchased from China Infrastructure of Cell Line Resources (Beijing, China). MAP30 was kindly provided by Professor Y.F. Meng (College of Life Sciences, Sichuan University, Chengdu, China) (18).

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Key words: *Momordica charantia* anti-human immunodeficiency virus protein 30, glioma, apoptosis, leucine-rich-repeat-containing G-protein-coupled receptor 5, Wnt/ β -catenin signaling, second mitochondria-derived activator of caspase

The primary antibodies against leucine-rich-repeat-containing G-protein-coupled receptor 5 (LGR5; cat. no. AB75850), β -catenin (cat. no. AB32572), glycogen synthase kinase 3 β phosphorylated at Ser⁹ (pGSK-3 β ^{Ser9}; cat. no. AB75814), c-Myc (cat. no. AB32072), cyclin D1 (cat. no. AB134175) and second mitochondria-derived activator of caspase (Smac; cat. no. AB32023) used for western blot analysis were purchased from Abcam (Cambridge, UK).

Cell culture. U87 and U251 cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin at 37°C in a humidified atmosphere of 5% CO₂ in air. The medium was changed every 3–4 days, and cultures were split using 0.25% trypsin. All experiments were carried out on cells with viability >95%.

Cell viability assay. When U87 and U251 cells reached the exponential growth phase, they were treated with MAP30 (2.7 μ M) or PBS (0.01 M, pH 7.2–7.4; Gibco; Thermo Fisher Scientific, Inc.), and images were captured under an inverted light microscope (x400 magnification; Olympus CKX41; Olympus Corporation, Tokyo, Japan) at 0 and 24 h.

A Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) assay was used to evaluate cell viability. U87 and U251 cells were resuspended in DMEM with 10% FBS at a density of 5x10³ cells/well and cultured in 96-well plates. The cells were treated with various concentrations (0.5, 1, 2, 4, 8 and 16 μ M) of MAP30 for 24, 48 and 72 h, prior to medium being replaced with 10 μ l CCK-8 and 100 μ l FBS-free DMEM. Cells were incubated for 1 h at 37°C and the absorbance of each well was measured at 450 nm using a microplate reader.

Cell migration and invasion assays. The effect of MAP30 on glioma cell migration was assessed using wound-healing assays. U87 and U251 cells were seeded in multiwell plates and cultured until reaching confluence. A 10- μ l pipette tip was used to make a straight scratch. The cells were washed with PBS, and treated for 24 h with MAP30 (2.7 μ M) or PBS (0.01 M, pH 7.2–7.4) as a control. Images of the wounds were captured under an inverted light microscope (x400 magnification) at 0 and 24 h, and measured.

A cell invasion assay was performed using 24-well Transwell chambers with an 8 μ m aperture (Costar; Corning Incorporated, Corning, New York, NY, USA). The inserts were coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). U87 and U251 cells were trypsinized and transferred to the upper Matrigel chamber with FBS-free DMEM at a density of 1x10⁵ cells/well. The lower chamber was filled with DMEM containing 20% FBS as a chemoattractant. After 24 h of incubation at 37°C, the non-invasive cells on the upper membrane surface were gently removed with a cotton tip, and the cells that passed through the filter were stained with hematoxylin at room temperature for 1 h and counted under an inverted light microscope (x400 magnification).

Plate colony formation assays. Colony formation ability of the glioblastoma cell lines was determined using plate colony formation assays. Briefly, U87 and U251 cells were cultured in DMEM containing 10% FBS in 6-well plates at

a concentration of 1,000 cells/well. The cells were treated with MAP30 0.51 and 0.60 μ M at 1/4 of half-maximal inhibitory concentration (IC₅₀) for U87 and U251 cells at 48 h. or PBS. After 7 days, the colonies were washed gently with PBS, fixed with 4% paraformaldehyde at room temperature for 5 min and stained with Giemsa (Sigma; Merck KGaA, Darmstadt, Germany) at room temperature for 30 min; images were captured under an inverted light microscope (x400 magnification) and quantified.

Hoechst 33342/propidium iodide (PI) staining. U87 cells were treated for 24 h with MAP30 (1.4 and 2.7 μ M) or PBS (0.01 M, pH 7.2–7.4), fixed with ice-cold 70% ethanol (4°C), washed three times with PBS, stained with Hoechst 33342/PI solution (50 μ g/ml, Sigma-Aldrich; Merck KGaA) for 10 min and washed three times with PBS. The nuclear morphological changes were observed and images were captured using fluorescence microscopy (x400 magnification; EVOS-FL; Thermo Fisher Scientific, Inc.).

Cell cycle and apoptosis assays. U87 cells were cultured in 25 ml flasks for 24 h with MAP30 (1.4 and 2.7 μ M) or PBS (0.01 M, pH 7.2–7.4). For cell cycle analysis, the cells were harvested, washed, fixed with ice-cold 70% ethanol at -20°C overnight, prior to washing with PBS and suspended in 300 μ l staining buffer (BD Biosciences) containing 10 μ l propidium iodide (PI) and 5 μ l RNase (BD Biosciences) for 15 min at room temperature in the dark.

An annexin V-fluorescein isothiocyanate (FITC)/PI Apoptosis Detection kit (BD Biosciences) was used to determine the effects of MAP30 on apoptosis of U87 cells. The cells were harvested, washed twice with ice-cold PBS, and resuspended in binding buffer at a concentration of 1x10⁶ cells/ml. A 500 μ l volume of the solution was transferred into a 5-ml culture tube, and 5 μ l annexin V-FITC and 5 μ l PI were added to the solution with slight shaking, prior to incubation for 15 min at room temperature in the dark.

Samples prepared using the aforementioned methods were analyzed using flow cytometry (FC500 instrument; Beckman Coulter, Inc., Brea, CA, USA) within 1 h of staining.

Western blotting. U87 cells were incubated with MAP30 at 1.4 and 2.7 μ M for 24 and 48 h, and total cell proteins were extracted with ice-cold lysis buffer (1 mM EDTA, pH 8.0, 50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS and 0.5% sodium deoxycholate, pH 7.4). After 30 min on ice, cell debris was removed by centrifugation at 14,000 x g for 20 min at 4°C. A basic protein quantification kit (BioVision, Inc., CA, USA) was used to determine total protein concentrations. Equal amounts (20 μ g) of whole cell protein extracts from each sample were size-fractionated by 8% SDS-PAGE and transferred onto a polyvinyl difluoride membrane (BioVision, Inc., Milpitas, CA, USA). Membranes were first blocked in 5% non-fat milk powder (suspended in Tris-buffered saline with Tween-20; cat. no. AB64204) at room temperature for 2 h, and incubated with a specific primary antibody (using GAPDH as a control; cat. no. AB181602; dilution 1:2,000; Abcam) overnight at 4°C. Following washing with PBS containing Tween-20, membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (cat. no. AB6721;

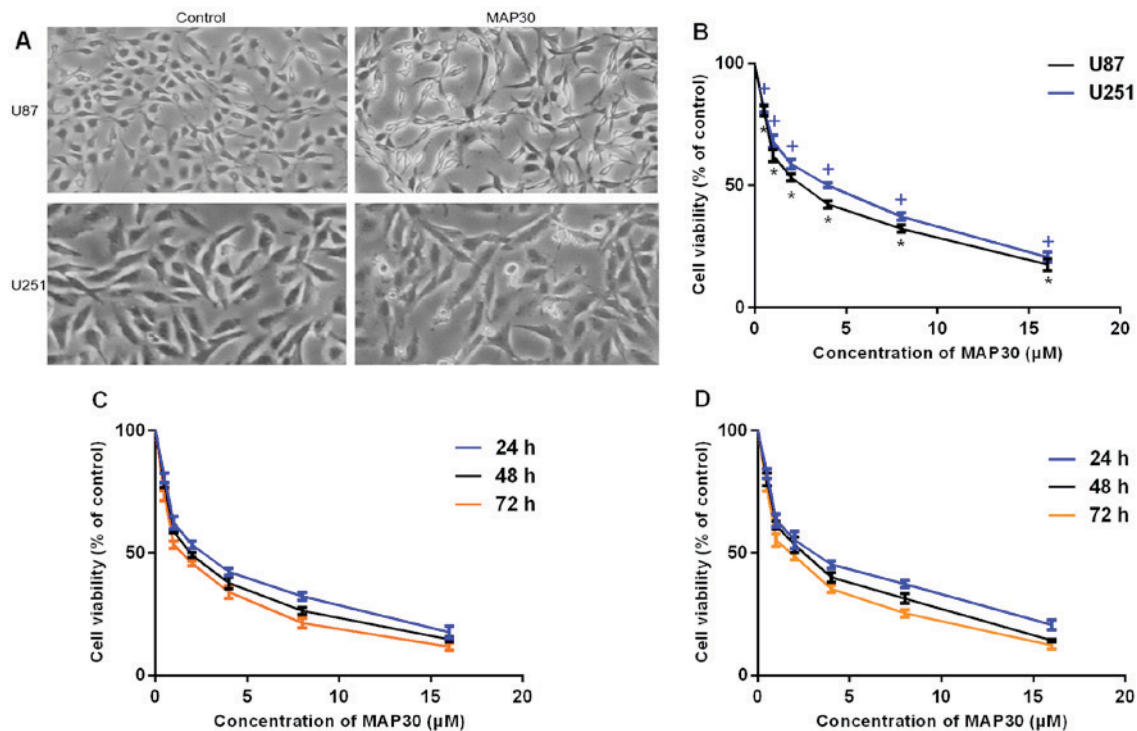


Figure 1. MAP30 inhibits the viability of glioblastoma cells in a dose- and time-dependent manner. (A) U87 and U251 cells were treated with MAP30 (2.7 μ M) or PBS for 24 h. Images were captured of the morphological changes in these cells under microscopy (x400 magnification). Images are representative of three independent experiments. (B) U87 and U251 cell lines were treated with different concentrations of MAP30 (0–16 μ M) for 24 h, and cell viability relative to the control (PBS-treated cells) was assessed. (C) U87 cells and (D) U251 cells were treated with different concentrations of MAP30 (0–16 μ M) for 24, 48 or 72 h, and cell viability relative to the control (PBS-treated cells) was assessed. Results are presented as the mean \pm standard deviation of three independent experiments. * P <0.05 vs. control. MAP30, *Momordica* anti-human immunodeficiency virus protein of 30 kDa.

Abcam; dilution, 1:3,000) at room temperature for 2 h, and protein bands were developed with Amersham ECL Western Blotting Detection reagent (GE Healthcare Life Sciences, Little Chalfont, UK) and visualized using a gel imaging analysis system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Results are expressed as the mean \pm standard deviation of multiple independent experiments. Statistical analyses were performed using two-way analysis of variance with Student-Newman-Keuls post hoc test or Student's *t*-test using SPSS software (version 20.0; IBM Corp., Armonk, NY, USA). The IC_{50} value was estimated from CCK-8 dose-response data via linear regression and log transformation. P <0.05 was considered to indicate a statistically significant difference.

Results

MAP30 affects the viability of glioblastoma cells. After treatment with MAP30 at IC_{50} concentration for 24 h, the morphological changes in the glioma cells were observed (Fig. 1A). Following treatment of U87 and U251 cells with different concentrations of MAP30 (0.5, 1, 2, 4, 8 and 16 μ M) for 24–72 h, cell viability was measured by CCK-8. It was identified that MAP30 significantly inhibited the viability of U87 and U251 cells (Fig. 1B). The IC_{50} values for MAP30 were identified to be 2.7 ± 0.22 and 3.9 ± 0.18 μ M at 24 h, 2.03 ± 0.03 and 2.4 ± 0.03 μ M at 48 h, and 1.6 ± 0.15 and 1.9 ± 0.16 μ M at 72 h for U87 and U251 cells, respectively (Fig. 1C and D). These results reveal a time- and dose-dependent effect of the action of MAP30.

Effects of MAP30 on cell migration and invasion. Wound healing assays and Transwell assays were used to determine the effects of MAP30 on the migration and invasion of glioblastoma cells. Fig. 2A presents the wound healing of U87 cells treated with MAP30 in comparison with the control. The wound healing rate was 37.3% for MAP30-treated U87 cells compared with 60% for the control cells, representing a significant decrease (P <0.05; Fig. 2B). Fig. 2C presents the wound healing of U251 cells treated with MAP30 in comparison with the control. The wound healing rate was 43.5% for MAP30-treated U251 cells compared with 70.5% for the control cells, representing a significant decrease (P <0.05; Fig. 2D). Fig. 2E presents images of the invading control and MAP30-treated U87 and U251 cells on the bottom of the membrane. The proportions of invading MAP30-treated U87 and U251 cells were respectively decreased to 34.7 and 41.3% compared with their control counterparts (P <0.05; Fig. 2F).

Plate colony formation assays. Compared with cells treated with PBS, application of MAP30 at 0.51 and 0.60 μ M (1/4 of the IC_{50} for U87 and U251 cells, respectively, at 48 h) significantly suppressed colony formation of U87 and U251 cells. The number of colonies formed was 5 and 12 for MAP30-treated U87 and U251 cells, respectively, compared with 78 and 113 for control U87 and U251 cells, respectively, representing a significant decrease (P <0.05; Fig. 2G).

Cell apoptosis and cycle assays. Following Hoechst 33342/PI double staining, U87 and U251 cells treated with MAP30

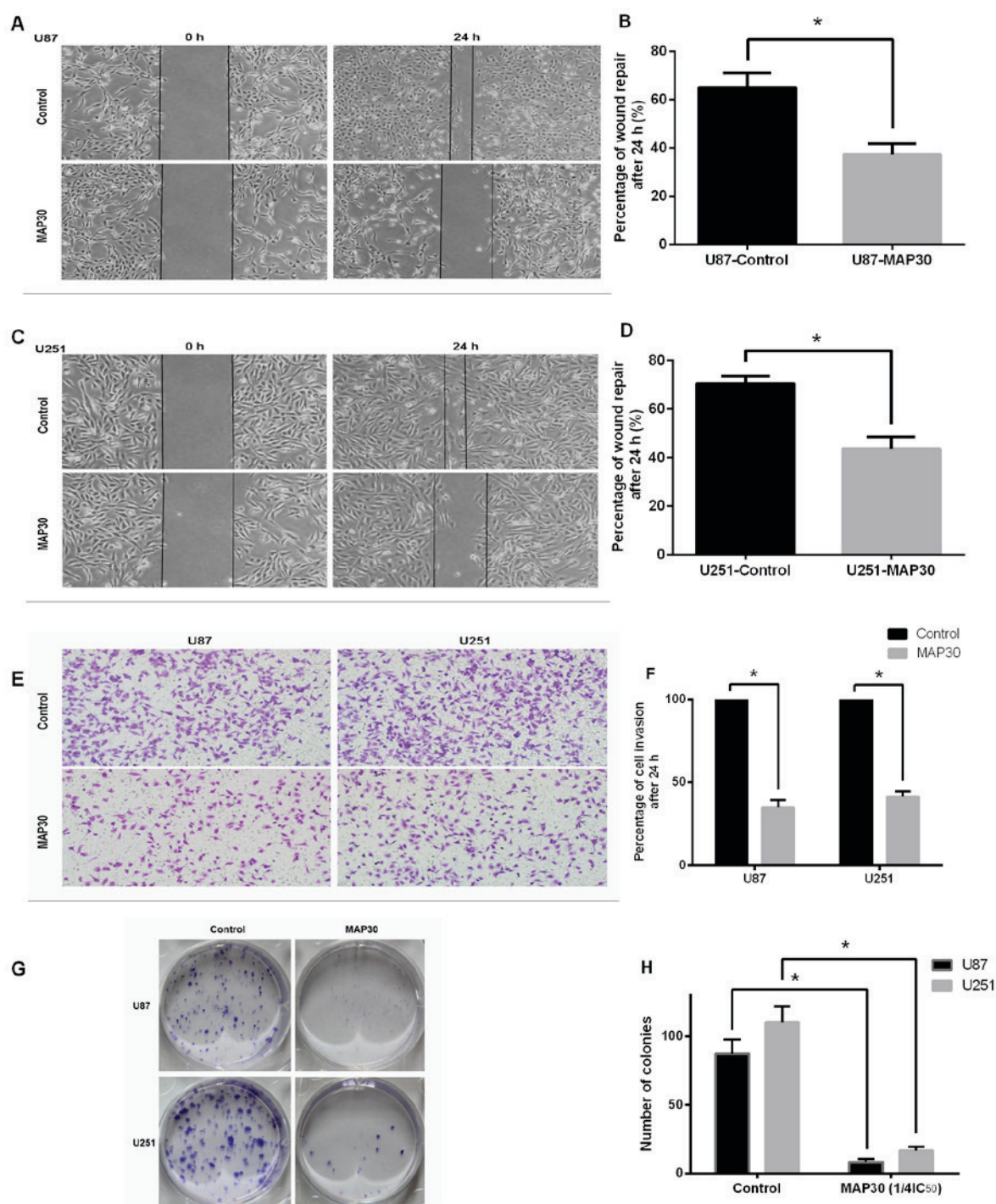


Figure 2. MAP30 inhibits U251 and U87 cell migration, invasion and colony formation *in vitro*. (A) Representative images of U87 cell wound healing at 0 and 24 h under microscopy (x400 magnification). (B) Quantification of the wound healing assays following treatment of U87 cells with MAP30 (2.7 μ M) or PBS for 0 and 24 h. (C) Representative images of U251 cell wound healing at 0 and 24 h following treatment of U87 cells with MAP30 (2 μ M) or PBS for 0 and 24 h. (D) Quantification of the wound healing assays. (E) Images of Transwell assay results of the two cell lines were captured at 0 and 24 h after treatment with MAP30 (2.7 μ M) or PBS under microscopy (x400 magnification). (F) Quantification of the Transwell assay results. (G) MAP30 significantly suppressed colony formation. (H) U87 and U251 cells were treated with MAP30 (1/4 of the IC₅₀ for U87 and U251 at 48 h) or PBS; the number of colonies was significantly decreased compared with the control cells. * $P < 0.05$ vs. control. MAP30, *Momordica* anti-human immunodeficiency virus protein of 30 kDa.

exhibited typical apoptotic morphological changes including chromatic agglutination, karyopyknosis and nuclear fragmentation (Fig. 3A and B).

After 24 h of treatment with MAP30 at a dose of 1.4 μ M, early apoptosis was increased to 13.87 and 16.77% in the U87 and U251 cell lines, respectively. The early apoptosis ratio increased to 49.61 and 51.17% in these two cell lines

when the concentration of MAP30 was increased to 2.7 μ M (Fig. 3C and D). The baseline early apoptotic proportions were 0.08 and 0.25%, respectively, for U87 and U251 cells treated with PBS ($P < 0.05$; Fig. 3F).

Cell cycle assays indicated that MAP30 induced S-phase arrest in U87 cells. The proportions of S-phase U87 and U251 cells treated with MAP30 (1.4 and 2.7 μ M) for 24 h

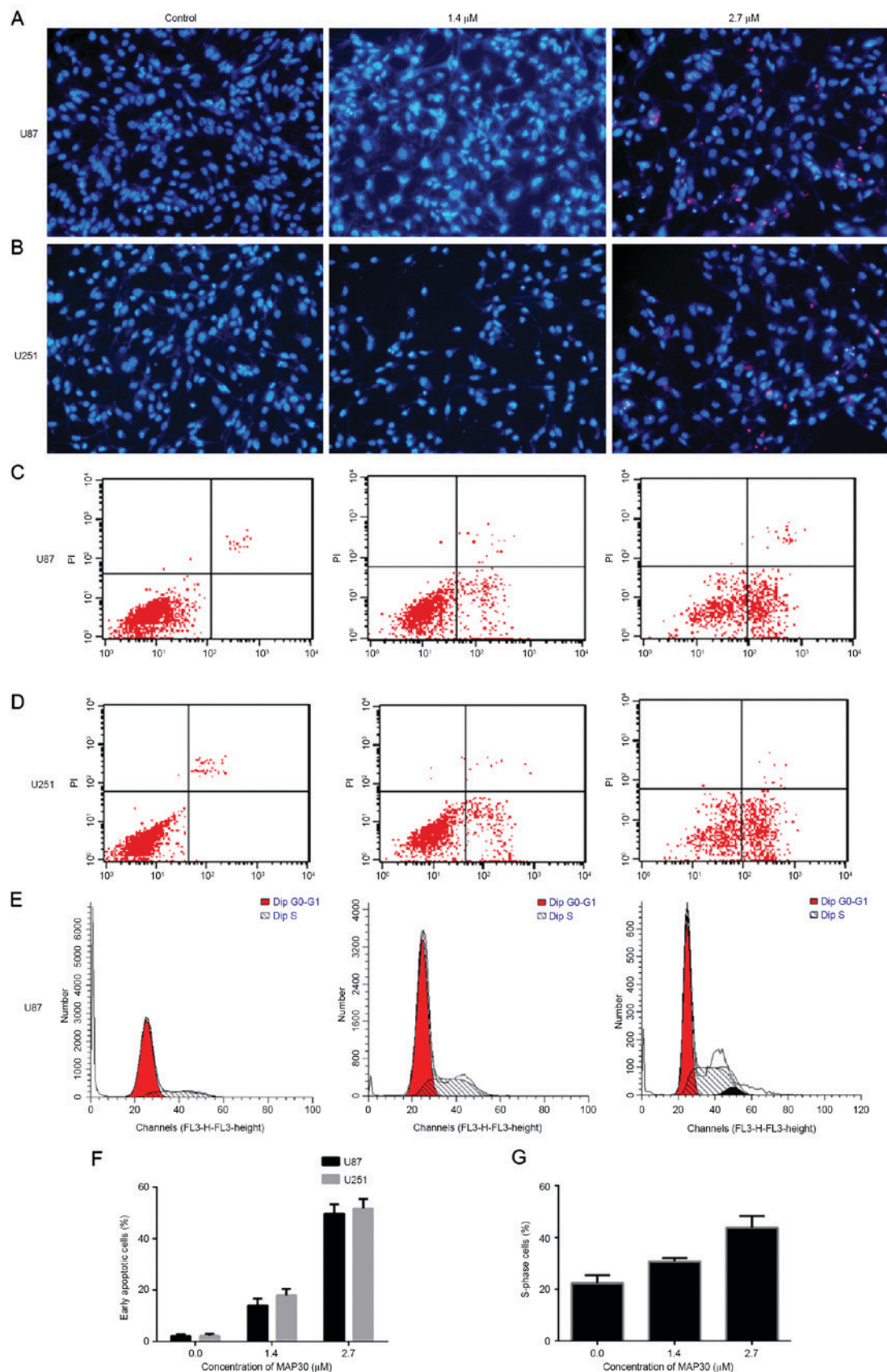


Figure 3. MAP30 induces apoptosis and arrests cell cycle at S-phase in glioblastoma cells. (A) U87 and (B) U251 cells were treated with MAP30 (1.4 and 2.7 μ M) or PBS for 24 h, and alterations in cell morphology were determined following Hoechst 33342/PI staining and fluorescence microscopy (x400 magnification). (C) U87 and (D) U251 cells, following treatment with MAP30 (1.4 and 2.7 μ M) or PBS for 24 h, underwent annexin V-PI double staining and were analyzed by flow cytometry. (E) U87 cells following treatment with MAP30 (1.4 and 2.7 μ M) or PBS were stained with PI and analyzed using flow cytometry. MAP30 was identified to arrest the cell cycle at S-phase. (F) The annexin V-PI double staining results indicated that MAP30 promotes apoptosis of U87 and U251 cells in a dose-dependent manner. (G) The PI staining results indicated that MAP30 arrests the cell cycle at S-phase in a dose-dependent manner. All experiments were repeated three times, and images are representative. MAP30, *Momordica* anti-human immunodeficiency virus protein of 30 kDa; PI, propidium iodide.

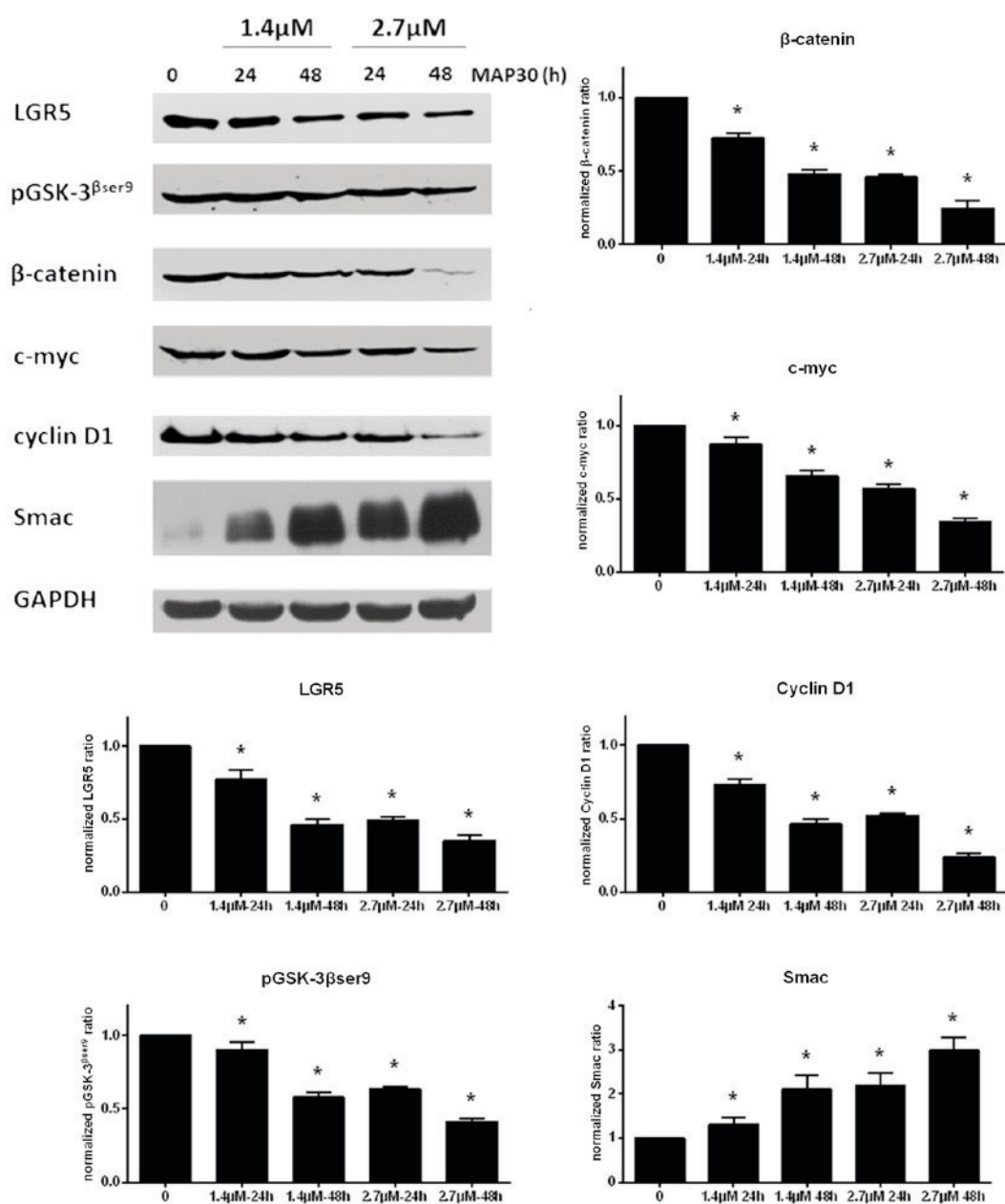


Figure 4. Western blot results indicate that MAP30 inhibits expression of LGR5 and the key proteins of the Wnt/ β -catenin signaling pathway, and upregulate Smac expression in glioma. U87 cells were treated with different concentrations of MAP30 (1.4 and 2.7 μ M) or PBS for 24 or 48 h. Quantitative analysis of protein expression from three independent experiments. * $P < 0.05$ vs. control. MAP30, *Momordica* anti-human immunodeficiency virus protein of 30 kDa; LGR5, leucine-rich-repeat-containing G-protein-coupled receptor 5; Smac, second mitochondria-derived activator of caspase; pGSK-3 β Ser⁹, glycogen synthase kinase 3 β phosphorylated at Ser⁹.

significantly increased from 21.73% (PBS-treated) to 30.79 and 41.33%, respectively ($P < 0.05$; Fig. 3E and G).

Western blot analysis. The western blotting results demonstrated that the expression of LGR5, pGSK-3 β Ser⁹, β -catenin, c-Myc and cyclin D1 decreased, and the expression of Smac markedly increased, as the dose (1.4–2.7 μ M) and treated time (24–48 h) of MAP30 increased (Fig. 4; $P < 0.05$).

Discussion

Previously, a promising anti-angiogenic therapy (bevacizumab) failed to improve overall survival rates in two independent

Phase III trials (AVAglio and RTOG 0825), suggesting that gliomas are refractory to treatment (19,20). Therefore, development of novel and effective therapeutic agents is highly desirable. Although previous studies have identified that MAP30 possesses antitumor effects in various tumor cells, including glioblastoma cells, the underlying molecular mechanism of MAP30 on glioblastoma cells has not yet been elucidated (4,6,12–14,16,21,22). The results of the present study demonstrated that MAP30 displayed significant inhibition of proliferation, invasion and migration on U87 and U251 cell lines in a dose- and time-dependent manner. The IC₅₀ values for U87 cells were lower compared with those for U251 cells, indicating that U87 cells might be more sensitive to MAP30.

Therefore, the U87 cell line was selected for additional experiments. The U87 cell line is the most representative glioma cell line. Although it was identified that the DNA profile of U87MG cells was different from that of the original cells in 2016, it is likely to be a bona fide human glioblastoma cell line of unknown origin (23). These cells are thought to exhibit the characteristics of human glioblastoma (24-27), including proliferation, invasion and migration. The U87 cell line remains one of the most representative glioma cell lines. In addition, the U251 cell line was investigated to support the results from the U87 cells.

The flow cytometric and fluorescence staining results demonstrated that MAP30 induced apoptosis and arrested cell cycle in S-phase. The apoptosis-promoting and cell cycle-arresting abilities of MAP30 were identified in previous studies (14,28-30). MAP30 was considered to cleave adenine-ribose glycosidic bonds by targeting the conserved host protein synthesis machinery, and prevent the elongation of DNA/RNA, resulting in DNA replication arrest in S-phase (3,16,17).

The apoptotic mechanism of MAP30 in glioma cells has not been clarified. LGR5, a downstream target gene of the Wnt signaling pathway, serves a key function in tumorigenesis, and the Wnt signaling pathway is vital in proliferation, differentiation and morphogenesis of gliomas (31-36). Our previous studies revealed that LGR5 expression increased with the malignant degree of human gliomas, and knockdown of LGR5 was identified to lead to significant inhibition of the proliferation of glioma cells *in vitro* and *in vivo* (37). Additionally, Hsu *et al* (38) demonstrated that depletion of LGR5 induced apoptosis through the loss of mitochondrial membrane potential, and inhibited the activity of Wnt/ β -catenin signaling by suppressing the expression of c-Myc and cyclin D in CRC cells (38). The western blotting results of the present study indicated that the expression of LGR5, pGSK-3 β ^{Ser9}, β -catenin, c-Myc and cyclin D1 were markedly decreased in U87 cells following MAP30 treatment in a dose- and time-dependent manner. Thus, we hypothesize that suppression of expression of LGR5 and the Wnt/ β -catenin signaling pathway may be part of the mechanism by which MAP30 inhibits proliferation and induces apoptosis in glioma cells.

On the other hand, our results indicate that the expression of Smac is significantly enhanced in U87 cells in a dose- and time-dependent manner. Evasion of programmed cell death is a hallmark of human cancer (39). Smac is considered to be an important anticancer factor in the mitochondrial apoptosis pathway, which binds directly to and neutralizes inhibitor of apoptosis (IAP) proteins, thus leading to activation of the intrinsic pathway of apoptosis (39-42). In addition, Smac mimetic-mediated depletion of IAP proteins may lead to ubiquitination, proteasomal degradation of substrates and activation of nuclear factor κ B (NF- κ B), a transcription factor with important functions in cell death and survival signaling, and all the functions of Smac promote apoptosis (39,41,43).

In addition, previous studies fused MAP30 with human-derived cell penetrating peptide or poly (ethylene glycol) to increase the uptake efficiency, cytotoxic activity and *in vivo* half-life and decrease immunogenicity of MAP30 (12,44). Even more importantly, RIPs may exhibit

little or no detectable adverse effects on normal cells as they may recognize features on membranes dominant to tumor cells (4,17,45-47). All these characteristics would broaden the number of applications of MAP30.

On the basis of the results of the present study, it is concluded that the mechanisms of MAP30 against malignant glioma cell lines may be associated with potent suppression of LGR5 and the Wnt/ β -catenin signaling pathway, and enhancement of Smac expression in a dose- and time-dependent manner, indicating that MAP30 may be a novel and effective therapeutic natural plant agent for the treatment of gliomas. However, further study using *in vivo* models and elucidation of the underlying molecular mechanism of action of Smac are required to confirm these results.

Acknowledgements

The present study was supported by the Peking University People's Hospital Research and Development Funds (grant nos. 2104000004, RDB2011-14 and RDC2015-05) and by the National Natural Science Foundation of China (grant no. 81001009). The authors would like to thank Professor Meng (College of Life Sciences, Sichuan University, Chengdu, China) for providing the highly purified MAP30. The authors also thank Professor Xiangjun He, Dr Junjie Miao, Dr Dongliang Wang (Peking University People's Hospital, Beijing, China) and Dr Jiangfeng Liang (Peking University International Hospital, Beijing, China) for providing the laboratory and technical advice.

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