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Study on the In Vivo and In Vitro Anticancer Activity of Gloydius Brevicaudus Venom Against Lung Cancer

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Abstract

The objective of this study was to evaluate the anticancer effects of Gloydius brevicaudus venom (GBV), a natural bioactive substance, on lung cancer through in vivo and in vitro experiments, and to explore its mechanism of action. Methods included measuring the inhibition rate with the CCK-8 assay, detecting cell apoptosis via flow cytometry, and analyzing the expression of apoptosis-related proteins by Western blot. A nude mouse xenograft model was established, with intratumoral injection as the administration route, administered every two days. The therapeutic effect of GBV on the xenograft model was observed, and immunohistochemical analysis was performed to detect the expression of tumor proliferation marker Ki-67 in the nude mice. The results from in vitro experiments showed that GBV exhibited cytotoxicity against tumor cells and induced apoptosis. GBV significantly upregulated the expression of proteins Bax, Cleaved-Caspase-3, Cleaved-Caspase-8, and Cleaved-Caspase-9 ($P<0.05$), while downregulating the expression of protein Bcl-2 ($P<0.05$). In vivo results demonstrated that medium and high concentrations of GBV significantly inhibited the growth of A549 tumor xenografts in nude mice. Immunohistochemical analysis showed that GBV at medium and high concentrations significantly reduced the expression level of Ki-67 in vivo. In conclusion, GBV can inhibit proliferation and induce apoptosis in A549 cells in vitro through both the mitochondrial pathway and death receptor pathway. In vivo, GBV significantly inhibits the growth of A549 xenografts and regulates tumor cell cycle and proliferation.

Keywords: Non-small cell lung cancer; GBV; Proliferation; Apoptosis

Lung cancer has gradually evolved into a global health issue, with both its incidence and mortality rates continuously rising, with non-small cell lung cancer (NSCLC) being the most common subtype [1, 2]. Currently, chemotherapy remains the primary treatment for lung cancer, with platinum-based chemotherapeutic agents being the first-line drugs [3]. However, chemotherapy often fails to achieve ideal clinical efficacy due to the development of secondary resistance [4], making it necessary to search for novel anti-tumor drugs. In recent years, numerous studies both domestically and internationally have indicated that snake venom holds great potential in cancer treatment [5-8]. Although some snake venom-derived drugs have successfully entered clinical trials for

the treatment of cardiovascular diseases [9], such as angiotensin-converting enzyme inhibitors like captopril [10], their application in cancer research remains at a relatively early stage [11]. Notably, research on the anti-tumor effects of Jiangsu-Zhejiang short-tailed pit viper venom is relatively scarce. Therefore, the aim of this study is to observe the effects of GBV on the proliferation, migration, and apoptosis of non-small cell lung cancer A549 cells in vitro, providing a feasible experimental basis for its clinical application in lung cancer treatment.

Materials and Methods

1.1 Reagents

GBV was provided by Jiaxing Changsheng Agricultural Development Co., Ltd.; cisplatin was purchased from Solebio Biotechnology Co., Ltd.; fetal bovine serum from Procell; DMEM/F-12 medium from Procell; RIPA lysis buffer, protein concentration assay kits, and Matrix-Gel matrix gel from Shanghai Biotain Biotechnology Co., Ltd.; CCK-8 assay kit from Biosharp; Transwell chambers from Yisheng Biotechnology Co., Ltd.; cell apoptosis detection kit from Wuhan Pusano Co., Ltd.; Bcl-2, Bax, Caspase-3, Cleaved-Caspase-3, GAPDH, Cleaved-Caspase-8, Cleaved-Caspase-9 from Proteintech, USA; immunohistochemistry kit and centrifuge from Shanghai Biotain Biotechnology Co., Ltd., Japan HITACHI; incubators and ultra-low temperature freezers from Thermo Fisher Scientific.

1.2 Cell Line

A549 cells were purchased from Oricell.

1.3 Animals

Thirty male Balb/c nude mice, aged 4-5 weeks with a body weight of 16-20 g, were purchased from the Zhejiang Provincial Academy of Medical Sciences. All nude mice were housed in the SPF-grade animal facility of the Zhejiang Provincial Academy of Medical Sciences. All animal experiments were approved by the Animal Experimental Center and adhered to the National Institutes of Health's "Guide for the Care and Use of Laboratory Animals."

1.4 Methods

1.4.1 Experimental Groups

Cisplatin group (treated with cisplatin working solution at concentrations of 2, 4, 6, 8, and 10 μg/mL), GBV group (treated with Jiangsu-Zhejiang short-tailed pit viper venom working solution at concentrations of 2, 4, 6, 8, and 10 μg/mL), and NS control group.

1.4.2 Solution Preparation

GBV working solution: Dissolve 1 mg of lyophilized GBV powder in 1 mL complete medium to prepare a 1 mg/mL GBV working solution, then dilute to the desired concentration before use.

Cisplatin working solution: Dissolve 20 mg of cisplatin powder in 20 mL complete medium to prepare a 1 mg/mL cisplatin working solution, then dilute to the desired concentration before use.

1.4.3 Cell Culture

Cells were cultured in DMEM/F-12 medium containing 10% fetal bovine serum and placed in a 37°C, 5% CO2 incubator. Cells were passaged or used for experiments when they reached 80%-90% confluence.

1.4.4 CCK-8 Assay to Detect A549 Cell Proliferation Inhibition Rate

A549 cells were seeded in 96-well plates at 100 μL per well, approximately 5000 cells per well, with six replicates per concentration. After 24 hours of incubation, when the cells had fully adhered, the original medium was discarded and replaced with various concentrations (0, 2, 4, 6, 8, 10 μg/mL) of GBV or cisplatin, and cells were incubated for 24 hours. Wells treated with 0 μg/mL served as controls, and an additional blank control group was included. After 24 hours, 10 μL CCK-8 solution was added to each well and incubated for 1 hour. Absorbance at 450 nm was measured using a microplate reader. The cell proliferation inhibition rate was calculated using the formula: (Control group OD - Experimental group OD) / (Control group OD - Blank group OD) × 100%.

1.4.5 Flow Cytometry to Detect A549 Cell Apoptosis Rate

A549 cells were seeded in 6-well plates at a density of 2×10^5/mL. After 24 hours of incubation, the medium was discarded, and cells were treated with 8 μg/mL of the appropriate drug for 24 hours. After treatment, cells were washed twice with pre-cooled PBS, centrifuged at 1000 rpm for 5 minutes, and the supernatant was discarded. The cells were resuspended in 500 μL Binding Buffer, and 5 μL of Annexin V-FITC and 5 μL PI staining solution were added. Cells were mixed gently and incubated in the dark at room temperature for 10-15 minutes. A flow cytometer was used to collect data within 1 hour, and FlowJo software was used for data analysis. The apoptosis rate was calculated as the sum of early and late apoptosis.

1.4.6 Western Blot to Detect Apoptosis-related Protein Expression

Cells from each group were collected, and proteins were extracted using RIPA lysis buffer on ice. After low-speed centrifugation, the supernatant was collected, and protein concentration was measured using a BCA protein assay kit. A total of 20 μg protein was subjected to SDS-PAGE electrophoresis, followed by membrane transfer, blocking, primary antibody incubation, secondary antibody incubation, and ECL detection. The bands were analyzed using ImageJ software, and the relative expression of the target proteins was calculated by comparing the grayscale value of the target protein to that of the internal control.

1.4.7 A549 Tumor-bearing Nude Mouse Model

Nude mice were acclimatized in the animal room for 1 week. Approximately 3 million A549 cells were injected into the axillary region of each mouse. Tumor formation was considered successful when a firm, millet-sized nodule could be felt at the injection site 5-7 days after inoculation.

1.4.8 Animal Experiment Grouping and Administration

The animals were divided into the NS control group, GBV low-dose group (0.5 mg/kg), GBV medium-dose group (1.0 mg/kg), GBV high-dose group (2.0 mg/kg), and cisplatin group (5 mg/kg), with 6 mice per group, totaling 30 mice. Intratumoral injections were performed using a 1 mL insulin syringe, with injections given every two days for a total of 6 injections.

1.4.9 Observation and Measurement

After tumor formation, the injection sites were observed for ulceration or exudation, and tumor volume was measured using calipers. Tumor volume was calculated using the formula: $V = 0.5 \times x \times y^2$ (x: longest tumor diameter, y: largest perpendicular tumor diameter). The physical condition, food intake, defecation, and body weight of the mice were monitored. When the tumor reached 80-120 mm^3, treatment began. After the experiment, all mice were euthanized, tumors were excised, photographed, and tumor volume and weight were measured. Tumor growth inhibition rate was calculated as: Tumor growth inhibition rate = (Control group average tumor weight - Treatment group average tumor weight) / Control group average tumor weight × 100%.

1.4.10 Immunohistochemical Ki-67 Staining

Tumor tissue sections were prepared, treated with 3% H_2O_2 solution, and incubated in the dark for 10 minutes. The sections were washed three times with PBS for 5 minutes each, followed by blocking with 5% BSA for 30 minutes at room temperature. After removing the blocking solution, primary antibody was applied and incubated overnight at 4°C. The sections were then incubated with secondary antibody at room temperature for 1 hour. After washing with PBS, DAB staining solution was applied for 3-5 minutes, followed by counterstaining with hematoxylin. Sections were dehydrated in gradient alcohol and xylene, mounted, and examined under a microscope.

1.5 Statistical Analysis

Data were analyzed using GraphPad Prism software version 9.0. Quantitative data are expressed as mean ± standard deviation ($x \pm s$). One-way ANOVA was used for comparisons between groups, and a p-value of <0.05 was considered statistically significant.

2 Results

2.1. Effect of GBV on the Proliferation of A549 Cells

The results of the CCK-8 assay are shown in Figure 1. Compared to the NS control group (0 μg/mL), both GBV and cisplatin at various concentrations significantly inhibited the proliferation of A549 cells, with statistically significant differences ($P<0.001$), as shown in Figure 1. At concentrations of 2, 4, and 6 μg/mL, GBV showed less inhibition of A549 cell proliferation than cisplatin. However, at concentrations of 8 and 10 μg/mL, GBV exhibited superior inhibition compared to cisplatin. Based on these results, a concentration of 8 μg/mL of GBV was selected for subsequent experiments.

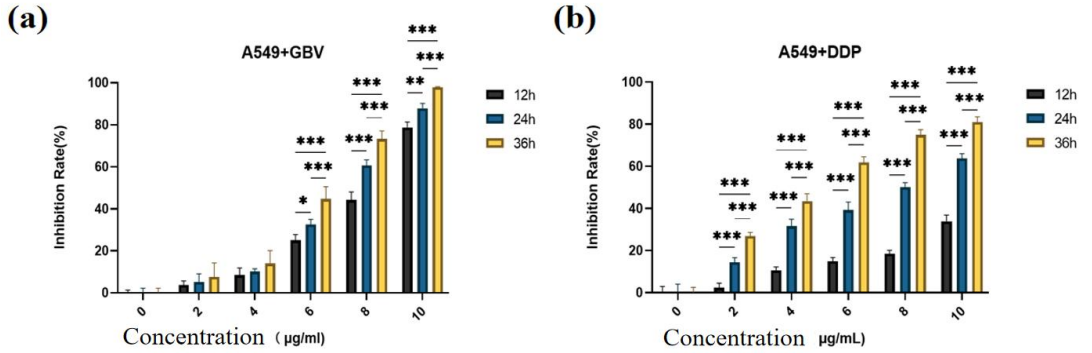

Figure 1 Effect of different concentrations of GBV on the proliferation of A549 cells (x±s, n=3)

2.2. Effect of GBV on the Apoptosis Rate of A549 Cells

The results of flow cytometry are shown in Figure 2. The total apoptosis rate in the NS control group was (5.52±1.09)%, in the GBV group it was (65.13±1.50)%, and in the cisplatin group it was (54.06±2.66)%. Compared to the NS control group, both GBV and cisplatin significantly increased the apoptosis rate, with statistically significant differences ($P<0.001$). Furthermore, the apoptosis rate was significantly higher in the GBV group than in the cisplatin group ($P<0.001$).

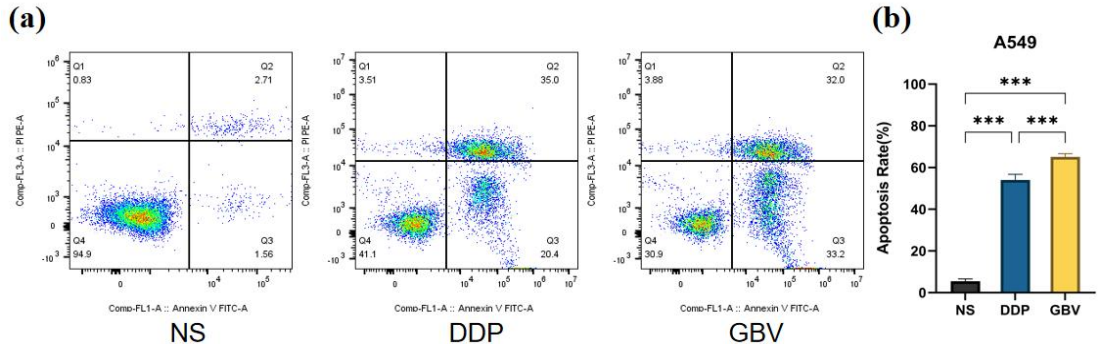

Figure 2 Apoptosis rate of A549 cells after 24 hours of treatment with GBV

2.3. Effect of GBV on the Expression of Apoptosis-Related Proteins in A549 Cells

Western blot analysis was used to assess the expression levels of apoptosis-related proteins Bcl-2, Bax, Caspase-3, Cleaved-Caspase-3, Cleaved-Caspase-8, and Cleaved-Caspase-9. The experimental results are shown in Figure 3 (a), (b), and (c). Compared to the NS control group, the GBV and cisplatin groups showed a significant increase in the expression of Bax and Cleaved-Caspase-3, -8, and -9 ($P<0.05$ or $P<0.001$), and a significant decrease in the expression of Bcl-2 and Caspase-3 ($P<0.05$ or $P<0.001$). The Bax/Bcl-2 ratio was significantly higher than that in the control group ($P<0.001$). Compared to the cisplatin group, the GBV group showed further increases in Cleaved-Caspase-3, -9 expression and the Bax/Bcl-2 ratio ($P<0.05$ or $P<0.01$). There were no significant differences in Caspase-3 and Cleaved-Caspase-8 expression between the GBV and cisplatin groups ($P>0.05$).

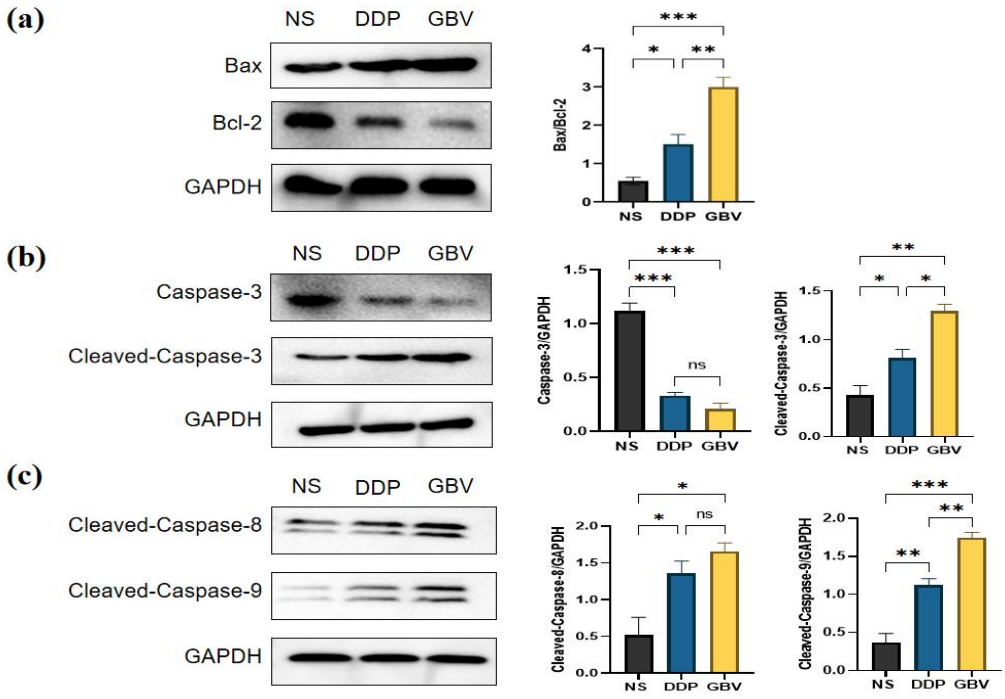

Figure 3 Effect of GBV on the expression of apoptosis-related proteins

2.4. Effect of GBV on Tumor Growth in A549 Tumor-Bearing Nude Mice

The tumors from the NS group, GBV low, medium, and high concentration groups, and the cisplatin group were extracted for comparison, as shown in Figure 4 (a). It was found that the medium and high concentrations of GBV, as well as cisplatin, significantly inhibited the growth of the implanted tumors, with the 2.0 mg/kg GBV group showing

slightly stronger anti-tumor effects than the 5.0 mg/kg cisplatin group. The weights of the tumor masses from each group are shown in Figure 4 (b). The tumor weights in the NS control group were 0.277±0.04 g, in the GBV low concentration group were 0.281±0.07 g, in the GBV medium concentration group were 0.190±0.07 g, in the GBV high concentration group were 0.085±0.02 g, and in the cisplatin group were 0.100±0.04 g. Tumor weights in the GBV medium and high concentration groups, as well as the cisplatin group, were significantly lower than in the NS control group ($P<0.05$ or $P<0.001$), while no significant difference was observed between the GBV low concentration group and the NS control group ($P>0.05$). The tumor inhibition rates for the cisplatin, GBV high concentration, and GBV medium concentration groups were 63.85%, 69.87%, and 31.65%, respectively. During the administration period, tumor mass diameters were measured, and volumes were estimated. The tumor growth curves are shown in Figure 4 (c). Starting from the first administration, the tumor volumes in the NS control group and the GBV low concentration group increased significantly, while the tumor volumes in the GBV medium concentration group showed a slight increase. The tumor volumes in the GBV high concentration and cisplatin groups showed almost no growth, and even slightly decreased. Tumor volumes in the GBV medium and high concentration groups, as well as the cisplatin group, were significantly smaller than in the NS control group ($P<0.001$ or $P<0.05$), while no significant difference was found between the GBV low concentration group and the NS control group ($P>0.05$).

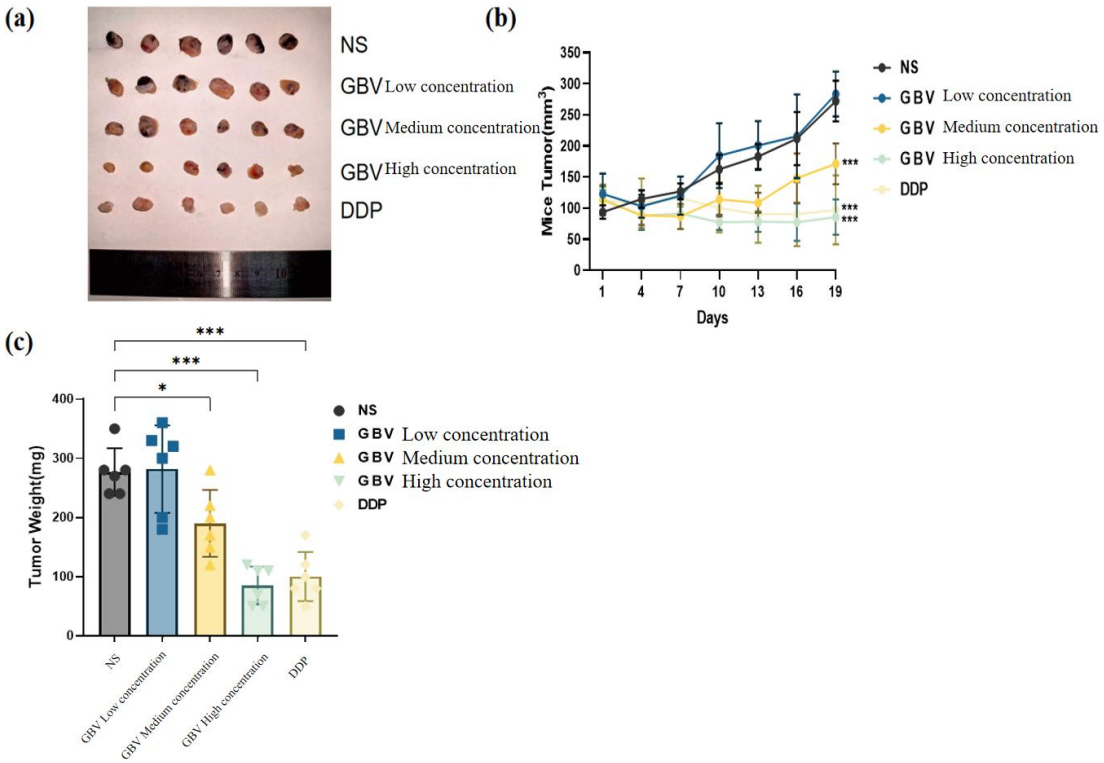

Figure 4 Effect of GBV on tumor growth in A549 tumor-bearing nude mice

2.5. Immunohistochemical Ki-67 Analysis

Immunohistochemical staining was performed to detect the expression level of Ki-67 in tumor tissue. The experimental results are shown in Figure 5 (a). In the NS control and GBV low concentration groups, the tumor cells showed evident proliferation, with a large amount of brown staining visible, indicating a high level of Ki-67 expression. In the GBV high concentration and cisplatin groups, the tumor cells showed less proliferation, with less visible brown staining, indicating lower levels of Ki-67 positive expression. Quantitative analysis of the positive area of tumor tissue sections is shown in Figure 5 (b). The positive areas in the GBV medium and high concentration groups, as well as the cisplatin group, were significantly lower than in the NS control group ($P<0.001$ or $P<0.01$), while no significant difference was observed between the GBV low concentration group and the NS control group ($P>0.05$).

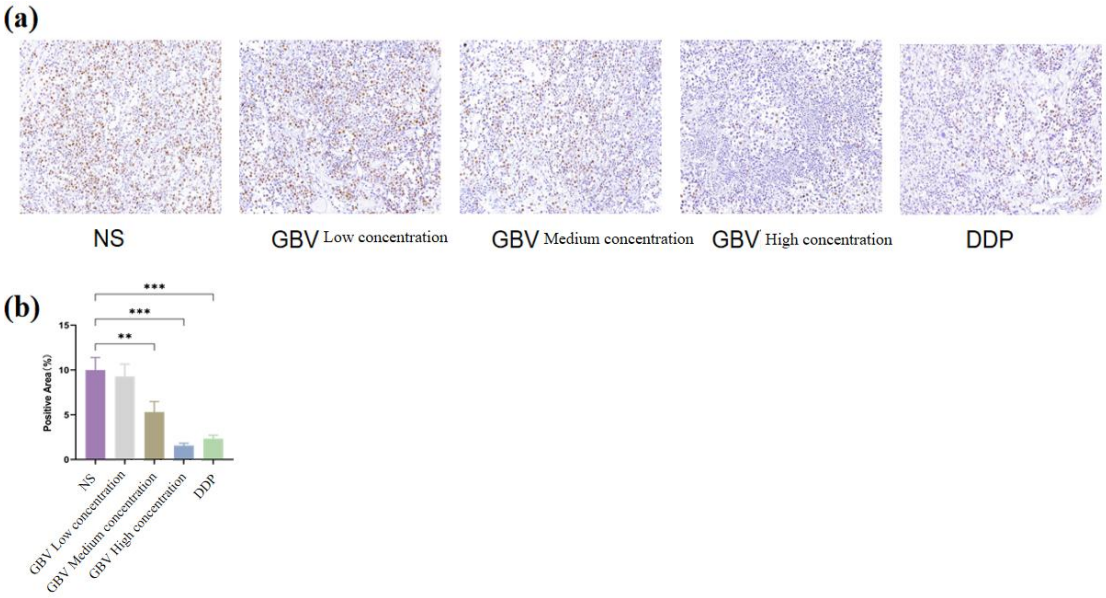

Figure 5 Immunohistochemical Ki-67 staining results in tumor tissue from nude mice

3 Discussion

Currently, the treatment of non-small cell lung cancer (NSCLC), particularly in advanced stages, is often hampered by severe side effects [12] and the development of resistance, leading to poor prognosis. Snake venom is a natural compound containing various enzymes, and compared to chemically synthesized drugs, natural products like snake venom have complex structures and diverse biological activities, which are more easily absorbed and metabolized by the human body [13-14]. The results of this study demonstrate that GBV can inhibit the proliferation of A549 cells in a dose-dependent manner, and at higher concentrations, its anti-tumor effects are stronger than the commonly used clinical chemotherapeutic drug, cisplatin. Further in vitro experiments

confirmed that GBV not only significantly impaired the migration ability of A549 cells but also promoted A549 cell apoptosis.

In recent decades, cancer research has mainly focused on developing improved drugs and radiotherapies aimed at inducing maximal tumor cell death, thereby leading to tumor shrinkage and blocking invasion [15]. Programmed cell death includes apoptosis, necroptosis, and pyroptosis, with apoptosis being a finely regulated, self-induced death process controlled by signaling pathways and proteins [16]. Apoptosis can be triggered by either extrinsic or intrinsic pathways. The extrinsic apoptotic pathway is initiated by the activation of death receptors on the cell membrane, leading to the intracellular activation of caspase-8, which then triggers the downstream apoptotic cascade. The intrinsic pathway is initiated by various stress signals within the cell, ultimately causing an increase in mitochondrial outer membrane permeability, the release of cytochrome c into the cytoplasm, and the activation of the initiator caspase-9 [17], which in turn activates Caspase-3, inducing apoptosis. In this study, flow cytometry revealed that treatment with GBV for 24 hours significantly increased the apoptosis rate of A549 cells, with the total apoptosis rate in the GBV group being higher than that in the cisplatin group, suggesting that GBV may exert its anti-tumor effects by inducing A549 cell apoptosis.

To further explore the mechanisms underlying GBV-induced apoptosis, Western blotting was used to assess the expression of Bcl-2 family proteins and Caspase family proteins. The Western blot results showed that GBV significantly increased the expression of Bax and Cleaved-Caspase-3, -8, and -9, while decreasing the expression of Bcl-2 and Caspase-3, leading to a significant increase in the Bax/Bcl-2 ratio. These results suggest that GBV may induce apoptosis in A549 cells by upregulating the expression of Bax and Cleaved-Caspase-3, while downregulating Bcl-2 expression, thus activating the mitochondrial apoptosis pathway. Furthermore, the activation of Caspase-8 suggests that the extrinsic death receptor pathway may also contribute to the apoptotic signaling. Therefore, it can be inferred that GBV induces programmed cell death in A549 cells by synergistically activating both the mitochondrial apoptosis pathway and the extrinsic death receptor pathway.

In vivo evaluation models play a crucial role in the development of candidate anti-tumor drugs. In this study, we established a nude mouse model with human lung cancer A549 cell transplantation to evaluate the in vivo anti-tumor activity of GBV. The experimental results showed that the high-concentration GBV group and the cisplatin group significantly inhibited tumor growth, with inhibition rates of 69.87% and 63.85%, respectively. The GBV medium concentration group also showed some inhibitory effects, with an inhibition rate of 31.65%. Next, we used immunohistochemical methods to detect the expression of Ki-67 in A549 tumors to explore the impact of GBV on tumor cell proliferation. Ki-67 is a widely used marker for assessing cell proliferation activity, and its expression level correlates positively with cell proliferation. High expression of Ki-67 is observed in various tumor tissues, such as breast cancer [18], lung cancer [19], and pancreatic cancer [20]. Ki-67 is active in both interphase and mitotic cells, beginning to appear in late G1 phase, gradually increasing during S and G2 phases, reaching its peak

in M phase, and not being expressed in early G0 and G1 phases [21]. Immunohistochemical results showed that the Ki-67 expression level was high in the NS control group, while it was significantly reduced in the GBV high-concentration group compared to the NS control group, indicating that high-concentration GBV effectively inhibits tumor cell proliferation, demonstrating a favorable therapeutic effect, which is concentration-dependent. These experimental results suggest that GBV may regulate the cell cycle process to affect tumor cell proliferation.

In conclusion, GBV demonstrates significant anti-tumor activity both in vitro and in vivo and holds promise as a novel anti-lung cancer drug.

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