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Full paper

# Plumbagin inhibits growth of gliomas *in vivo* via suppression of FOXM1 expression

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A B S T R A C T

Plumbagin is a natural compound that is isolated from the root of the medicinal plant *Plumbago zeylanica* L. Based on a previous *in vitro* study performed by our group, which demonstrated the effectiveness of plumbagin against glioma cells, we further ascertained whether plumbagin exhibits the same effectiveness against glioma cell xenografts in nude mice. Our results revealed that tumor volume was reduced by 54.48% in the plumbagin-treated group compared with the controls. Furthermore, there were no obvious signs of toxicity as assessed by the organ sizes and cell morphologies of the mice that were treated with plumbagin. Immunofluorescence assays further revealed that plumbagin significantly inhibited glioma cell proliferation and induced cell apoptosis. Importantly, we also determined that the expressions of FOXM1 and its downstream target effectors, including cyclin D1 and Cdc25B, were downregulated in the treated group, while the expressions of p21 and p27 were increased; the latter findings corroborate the results of our previous *in vitro* study. Taken together, these findings indicate that plumbagin may be a natural downregulator of FOXM1 with potential therapeutic effectiveness for the treatment of gliomas.

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## 1. Introduction

Glioblastoma multiforme (GBM) is the most common primary brain tumor and is also the most deadly glioma (1). Currently, the standard treatment for malignant glioma is surgery followed by external radiation and chemotherapy. However, these treatments are not efficacious; the median survival is still 15–18 months from diagnosis (2). Temozolomide (TMZ) is an alkylating agent that is often used in the management of GBM patients in combination with radiotherapy. However, the efficacy of TMZ is limited due to the occurrence of chemoresistance and the inability of TMZ to induce tumor cell death (3–5). Therefore, the development of more effective therapeutic agents is critical for the treatment of high-grade gliomas in neurooncology.

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) is an active constituent of the roots of the medicinal plant *Plumbago zeylanica* L that has been demonstrated to exhibit antimicrobial (6), antiatherosclerotic (7), and anticarcinogenic effects (8). A large number of published studies have suggested that plumbagin exhibits anti-proliferative and pro-apoptotic properties in a variety of tumor cell lines and animal tumor models (9–12). Plumbagin has been reported to induce the generation of reactive oxygen species (ROS), which results in apoptosis and cell cycle arrest (13, 14). Some studies have also shown that plumbagin inhibits the Akt/mTOR and NF-κB pathways in breast cancer (15, 16). Additionally, plumbagin has been reported to inhibit tumor angiogenesis and tumor growth in endothelial cells (17). Furthermore, some *in vivo* studies of the effects of plumbagin on tumorigenesis have revealed that plumbagin can significantly inhibit tumor cell growth in various animal

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tumor models, including models of ovarian cancer (11), prostate cancer (18), breast cancer (15) and leukemia (19). A previous *in vitro* study performed by our group demonstrated that plumbagin inhibits glioma cell growth and induces apoptosis (20). However, the efficacy of plumbagin in the treatment of gliomas *in vivo* has yet to be investigated.

Forkhead box M1 (FOXM1) is a transcription factor that induces the expression of genes involved in cell cycle progression and apoptosis (21, 22). Because the proliferation-specific oncogenic gene *FOXM1* is strongly up-regulated in a variety of human tumors, including GBMs, it is considered an attractive target for the prevention of and/or therapeutic interventions for cancer (23–26). However, little information regarding FOXM1 inhibitors is currently available. Recent research has demonstrated that FOXM1 knockdown sensitizes recurrent GBM cells to TMZ cytotoxicity (27). In our previous study, we found that plumbagin exerts its anti-tumor activities partially by inhibiting FOXM1 activity in gliomas (20). However, *in vivo* studies are also needed to further ascertain anti-tumor activity of plumbagin via the inactivation of FOXM1.

In this study, we report that plumbagin inhibited glioma cell proliferation and promoted apoptosis in a nude mouse model. We also observed that the plumbagin-mediated inhibition of the cell growth was accompanied by inhibitions of the expressions of FOXM1, downstream target genes of FOXM1 (*i.e.*, cyclin D1 and Cdc25B), and proliferative markers (Ki67) and induction of the expressions of p21, p27 and cleaved caspase-3.

## 2. Materials and methods

### 2.1. Cell lines and culture conditions

All of the human glioma cell lines used in this study (*i.e.*, U87, A172, SHG44, and U251) were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences (CAS). These cells were cultured in DMEM/F-12 media supplemented with 10% fetal bovine serum (FBS, Sijiqing Bioengineering Material Co., China) and grown in a humidified incubator with 5% $CO_2$ at 37 °C.

### 2.2. Antibodies and reagents

Antibodies against cleaved caspase-3, cyclin D1, Cdc25B, p21, p27, and β-actin were obtained from Cell Signaling Technology. The anti-FOXM1 antibody was acquired from Santa Cruz, and Ki67 was from Thermo. Leibovitz's L-15 was purchased from Gibco. Plumbagin and all other chemicals were purchased from Sigma–Aldrich.

### 2.3. Cell viability assay

Cell viability was measured with the CCK-8 assay. In brief, glioma cells were seeded in 96-well plates and incubated overnight. Then, the cells were treated with plumbagin at different concentrations (0.625, 1.25, 2.5, 5, 10 and 20 μM) for 48 h. CCK-8 reagent (10 μL) was added to the cells, and the plates were then read at 450 nm using a spectrophotometer.

### 2.4. Glioma xenografts in nude mice

The study was approved by the Ethics Committee of the Xuzhou Medical College. Female athymic nude mice aged 4–6 weeks were purchased from the Experimental Animal Center of Xuzhou Medical College and maintained in pathogen-free conditions. U87 cells ($1 \times 10^6$ cells) in 100 μL of Leibovitz's L-15 were transplanted subcutaneously into the right flanks of the mice. When the tumors reached 100–150 $mm^3$, treatment was initiated. The tumor-bearing mice were randomly divided into two groups ($n = 5$ per group). Group 1 was treated with vehicle (0.9% sodium chloride) alone, and Group 2 was treated with plumbagin in vehicle at a dose of 2 mg/kg/day via intraperitoneal injections that were performed 5 times per week. All of the mice were sacrificed after 24 days of treatment. The tumors and vital organs of the control and treated mice were harvested and prepared for histological study.

### 2.5. Histopathology

The tumors and liver, kidney and lung tissues were fixed in 4% paraformaldehyde and then dehydrated in 20% followed by 30% sucrose at 4 °C until they sank. The fresh-frozen tissues were continuously sectioned at a thickness of 12 μm and then stained with hematoxylin–eosin (H–E) for microscopic examination. The tumor sizes were measured using calipers, and the tumor volumes were calculated according to the following formula: $V (mm^3) = a \times b^2/2$, where a is the longest tumor axis, and b is the shortest tumor axis.

### 2.6. Immunofluorescence staining

The sections containing the tumor were incubated with 0.3% triton X-100 followed by 10% goat serum and were then incubated overnight with Ki67 or cleaved caspase-3 primary antibody at 4 °C. To visualize the Ki67- or cleaved caspase-3-positive cells, the sections were incubated with Alexa-594-conjugated secondary antibody for 1 h at room temperature in the dark. DAPI was used to stain the cell nuclei. All sections were examined and photographed with a microscope with an attached fluorescence detector (IX71 Olympus).

### 2.7. Western blot analysis

Parts of the excised tumor tissues of the gliomas from each group of mice were used to prepare whole tissue lysates. Total protein extracts from the tumor tissue lysates were subjected to Western blot analyses as described previously (28). The expressions of FOXM1, p21, p27, cyclin D1 and Cdc25B were examined with specific primary antibodies, and β-actin was used as the loading control.

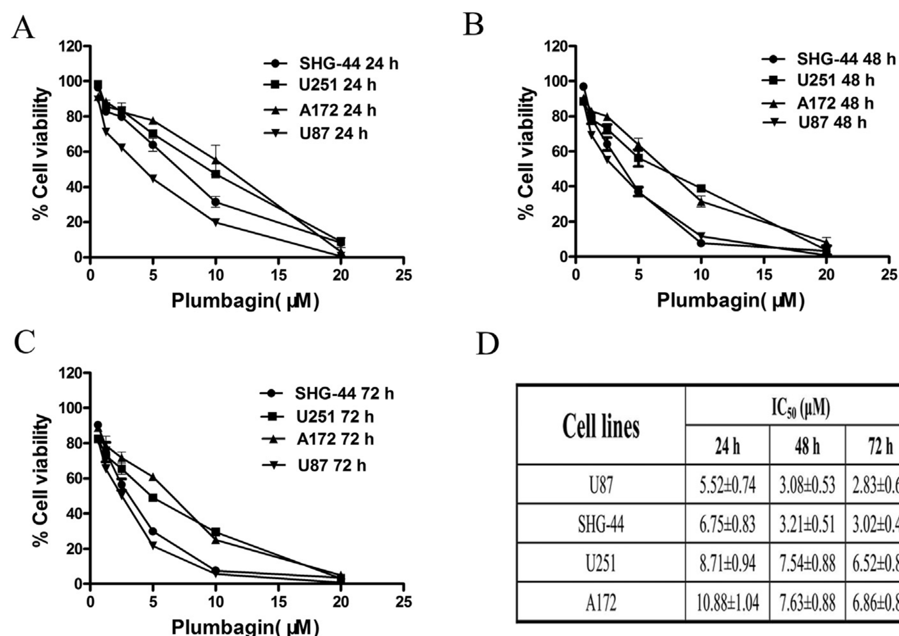
### 2.8. Statistical analysis

The data are presented as the means and the standard deviations of three to five independent experiments. Significant differences between the control and plumbagin-treated groups were determined with Student's *t* tests. Values of $P < 0.05$ were considered statistically significant.

## 3. Results

### 3.1. Plumbagin inhibits glioma cell growth in vitro

To assess the efficacy of plumbagin against glioma cells *in vitro*, we first examined the effects of plumbagin on glioma cell viability using the CCK-8 assay. As shown in Fig. 1, all of the glioma cells from all four of the glioma cell lines that were treated with plumbagin exhibited inhibitions in growth that were concentration- and time-dependent. The $IC_{50}$ values of plumbagin for the inhibition of the glioma cell lines are summarized in Fig. 1D. The greatest activity was observed against the U87 cells, and the $IC_{50}$ value obtained for this cell line was 2.83 μM at 72 h of incubation. These data indicate that plumbagin significantly inhibited glioma cell growth *in vitro*.




| Cell lines | $IC_{50}$ (μM) | | |
|---|---|---|---|
| | 24 h | 48 h | 72 h |
| U87 | 5.52±0.74 | 3.08±0.53 | 2.83±0.65 |
| SHG-44 | 6.75±0.83 | 3.21±0.51 | 3.02±0.48 |
| U251 | 8.71±0.94 | 7.54±0.88 | 6.52±0.83 |
| A172 | 10.88±1.04 | 7.63±0.88 | 6.86±0.84 |

**Fig. 1.** Effect of plumbagin on the viability of glioma cells *in vitro*. Cells were treated with vehicle (0.1% DMSO) or different concentrations of plumbagin (0.625, 1.25, 2.5, 5, 10 and 20 μM) for 24 (A), 48 (B) and 72 h (C). Cell viability was measured with CCK-8 assays. (D) The $IC_{50}$ values of for the SHG-44, U251, U87 and A172 cell lines at 24, 48 and 72 h.

### 3.2. Effects of plumbagin on the growth of glioma cells in vivo

A large number of studies of other tumors types have shown that plumbagin inhibits tumorgenesis in mouse tumor models (15, 18, 29). After demonstrating the effects of plumbagin on glioma cell growth *in vitro*, we tested the ability of plumbagin to inhibit glioma growth *in vivo*. We injected U87 cells subcutaneously into right flanks of nude mice. The treatments were continued for 24 days for the mice that were injected with U87 tumor cells. All of the control mice were treated with an equal volume of 0.9% sodium chloride. As shown in Fig. 2A and B, the inhibition of tumor growth was most evident in the mice that were treated with plumbagin at 2 mg/kg/d; an approximately 54.48% reduction in tumor size was observed in this group. The average tumor volumes were 2393.72 $mm^3$ in the control group and 1029.73 $mm^3$ in the plumbagin-treated group. We also observed an obvious difference in tumor weight between the treated and control groups. Compared with the control group, the tumor weight was reduced by 44.54% in the plumbagin-treated group (Fig. 2D). These results suggest that plumbagin inhibited glioma cell growth *in vivo*.

Next, we examined the toxicity of plumbagin *in vivo* via parallel monitoring of the body weights and examinations of the H&E stained tissue sections of the livers, kidneys and lungs of the plumbagin-treated mice. As shown in Fig. 2C, gross examinations of the vital organs, *i.e.*, the lungs, livers and kidneys, of the plumbagin-treated and control groups revealed similar sizes and cell morphologies.

### 3.3. Effects of plumbagin on cell proliferation and apoptosis in vivo

To further study the effects of plumbagin on tumor growth *in vivo*, Ki67 and cleaved caspase-3 were used to evaluate cell proliferation and apoptosis, respectively. The percentage of Ki67-positive cells was decreased by 51.65% in the plumbagin-treated group (Fig. 3A and B). In contrast, the percentage of apoptotic cells was significantly increased by 51.28% in the plumbagin-treated group (Fig. 3C and D). Taken together, these data reveal that plumbagin can inhibit tumor cell proliferation and induce cell apoptosis *in vivo*.

### 3.4. Effects of plumbagin on the expressions of FOXM1 and its downstream target genes in vivo

A previous *in vitro* study performed by group demonstrated that plumbagin inhibits glioma cell growth by down-regulating the expression of FOXM1 (20). To further ascertain whether plumbagin exerts the same effect on FOXM1 expression *in vivo*, we extracted the total proteins from the tumor tissues to assess the levels of FOXM1 and its downstream effectors in the control and plumbagin-treated groups. As shown in Fig. 4, the FOXM1, cyclin D1 and Cdc25B levels were decreased in the tumors from the plumbagin-treated group compared with the tumors from the control group. In contrast, the p21 and p27 levels were increased in the plumbagin-treated group. Together with the data from our previous study, these data demonstrate that plumbagin-mediated antitumor activity occurs via the inactivation of FOXM1.

## 4. Discussion

Malignant glioma has long resulted in high mortality rates worldwide. Unfortunately, there is no effective therapy for this malignant disease due to its rapid proliferation, high invasiveness and resistance to radiotherapy and chemotherapy (30, 31). Therefore, it is necessary to develop highly effective and safe drugs for the treatment of gliomas. The previous study performed by our group demonstrated that plumbagin effectively inhibits glioma cell growth, migration and invasion *in vitro* (20). In the present study, we generated gliomas in nude mice via the implantation of U87 cells to further evaluate the effect of plumbagin *in vivo*. Our results indicated that plumbagin significantly inhibited gliomagenesis and tumor growth in this nude mice model. Additionally, we also found that the plumbagin-mediated antitumor activity was accompanied by the inhibition of the expression of FOXM1 *in vivo*, which corroborates the results of our previous *in vitro* study (20).

A number of studies have demonstrated the potential usefulness of plumbagin as an anti-cancer treatment (14, 29, 32). Plumbagin has been demonstrated to significantly inhibit tumorigenesis and tumor cell growth *in vivo* in breast cancer (11, 15), lung cancer (29),

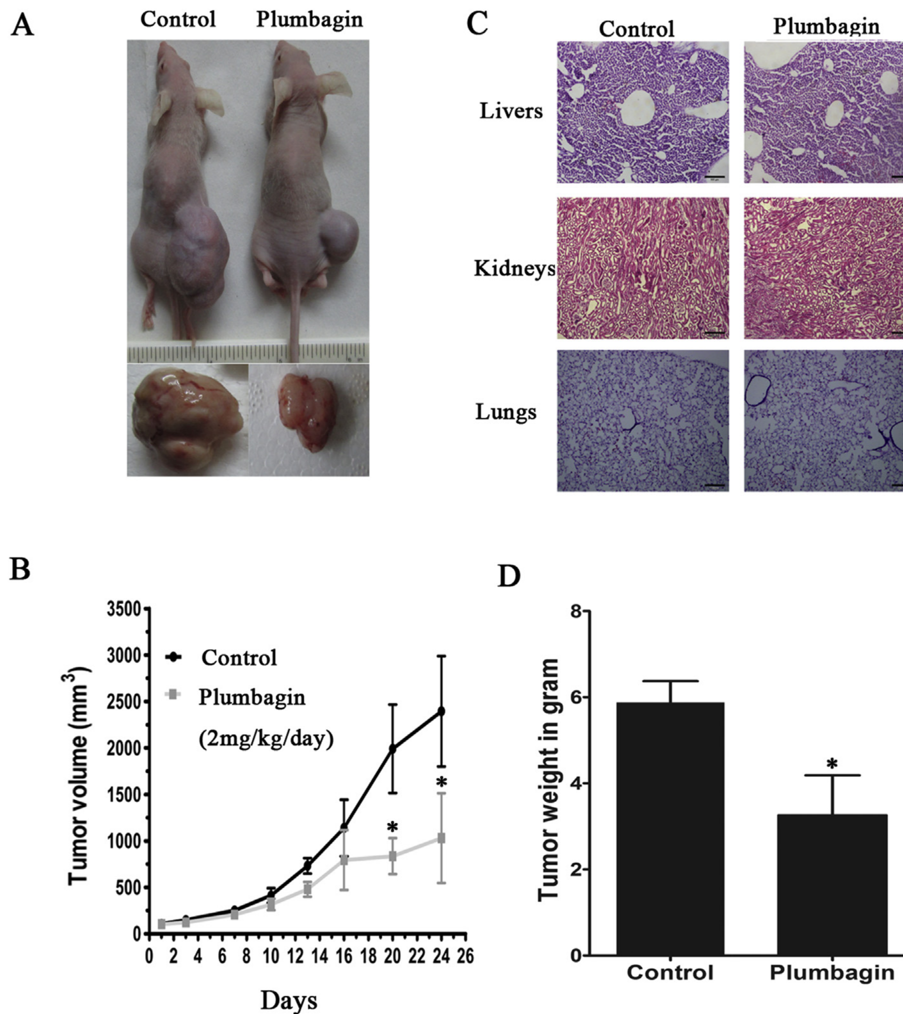



**Fig. 2.** Plumbagin inhibits glioma cell growth *in vivo*. (A) Representative tumor-bearing nude mice and tumors isolated from the control and plumbagin-treated groups. (B) The mean tumor volumes were assessed at the indicated numbers of days after plumbagin treatment. (C) Tissue sections of the livers, kidneys and lungs stained with H&E from the control and plumbagin-treated mice were observed; scale bar = 100 μm. (D) The mean tumor weights were measured. The data obtained from three independent experiments are represented as the means ± the SEMs. $^{*}P < 0.05$, plumbagin vs. vehicle-treated mice.

and prostate cancer (18). Consistently, we found that treatment with plumbagin resulted in significant glioma growth inhibition in mice compared to the untreated group. No signs of toxicity were observed in the plumbagin-treated group as assessed with H&E staining of the vital organs, which is consistent with previous studies that have observed no toxic influences of plumbagin on vital organs, including the liver, kidneys and lungs, when used against breast cancer (15) and leukemia (19). The Ki67 and cleaved caspase-3 proteins are cellular markers of proliferation and apoptosis, respectively (33, 34). The significant reduction in Ki67-positive cells and significant increase in cleaved caspase-3 expression further indicate that plumbagin effectively inhibited gliomagenesis and glioma cell growth *in vivo*, and these results corroborate those of our previous *in vitro* study (20).

FOXM1 is an oncogenic transcription factor that is overexpressed in various human malignancies, including GBM, and is closely associated with tumor cell proliferation and apoptosis (26, 35). In addition to our previous investigation, many other studies have found that the down-regulation of FOXM1 inhibits glioma cell growth (26, 36, 37). Thus, the development of agents targeting FOXM1 could have significant therapeutic implications for the treatment of gliomas (38). We have previously reported that plumbagin inhibits glioma cell growth by down-regulating the expression and activity of FOXM1 *in vitro* (20). Consistently, we also found that FOXM1 was down-regulated in plumbagin-treated mice compared with control mice. Furthermore, the expressions of downstream target effectors of FOXM1 were also affected by plumbagin treatment. These results further support the conclusion that the down-regulation of FOXM1 mediates the plumbagin-induced inhibition of glioma cell growth *in vivo*. However, in-depth studies of the mechanism by which plumbagin regulates FOXM1 should be performed in the future.

In conclusion, our present findings support those of our previous *in vitro* study and suggest that plumbagin inhibits gliomagenesis and tumor cell growth via the inactivation of FOXM1. Moreover, we strongly believe that plumbagin may function as a potential downregulator of FOXM1 and could be useful in the treatment of malignant gliomas.

## Ethical statement

All procedures performed in the studies involving animals were in accordance with the ethical standards of the Xuzhou Medical College.

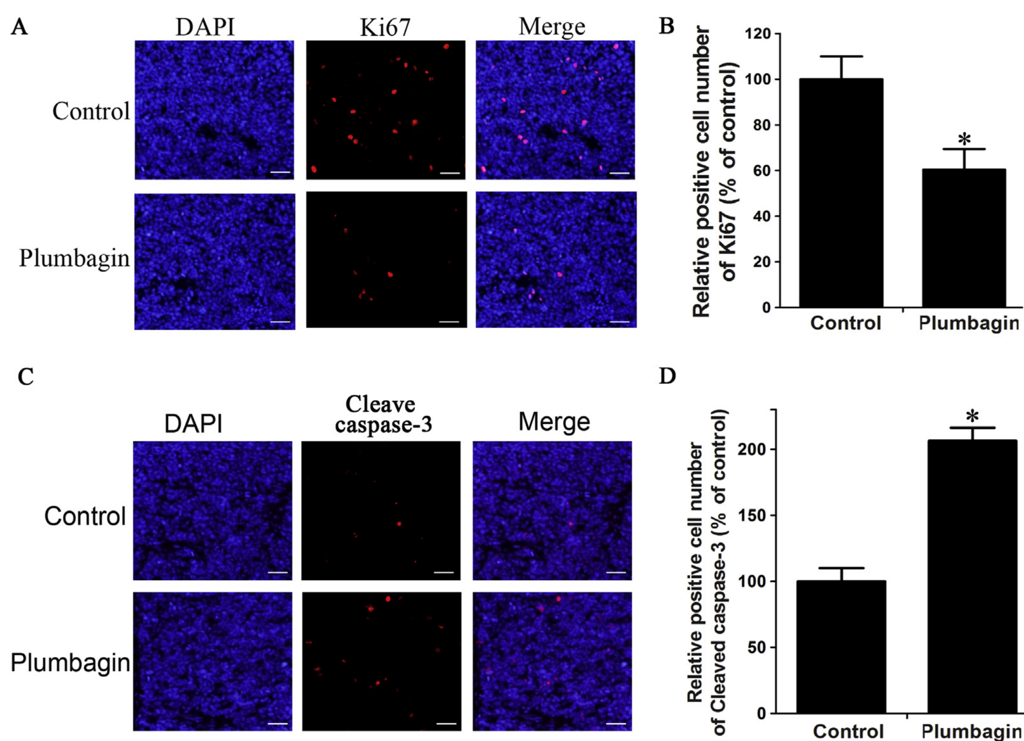



**Fig. 3.** The effects of plumbagin on cell proliferation and apoptosis *in vivo*. Tumor cell proliferation and apoptosis were assessed with anti-Ki67 (A) and anti-cleaved caspase-3 immunostaining (C). (B) and (D) Quantitative analyses of the percentages of positive cells in the plumbagin-treated group normalized to the control group. These results are presented as the means ± the SEMs of three independent experiments. $^{*}P < 0.05$, scale bar = 50 μm.

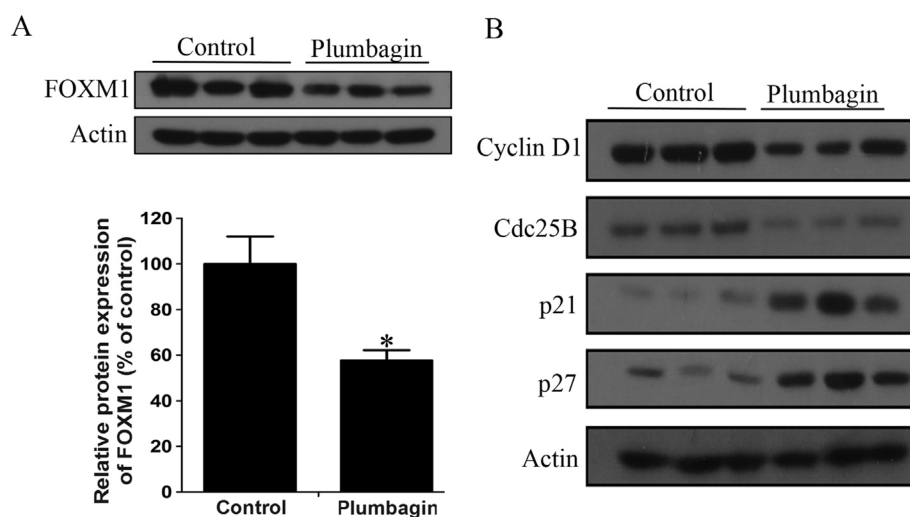



**Fig. 4.** The effects of plumbagin on the expression of FOXM1 and its downstream effectors *in vivo*. (A) Plumbagin inhibits the expression of FOXM1 in U87 xenografts. (B) Modulation of the protein expressions of the downstream target genes of FOXM1 by plumbagin in U87 xenografts.

## Conflict of interest

The authors declare that they have no conflicts of interest.

## Acknowledgments

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