Aplysin induces apoptosis in glioma cells through HSP90/AKT pathway

An-jing Gong¹, Li-li Gong³, Wei-cheng Yao¹, Na Ge², Lu-xiang Lu¹ and Hui Liang²

¹Department of Neurosurgery, The Affiliated Hospital of Qingdao University, Qingdao 266003, China; ²The Institute of Human Nutrition, Medical College of Qingdao University, Qingdao 266021, China; ³Department of Rehabilitation, The Affiliated Hospital of Qingdao University, Qingdao 266003, China

Corresponding author: Hui Liang. Email: ajgqdfy@163.com

Abstract

Glioma is one of the most common malignancies in the world. However, an effective regimen is lacking. Increasing evidence indicated that PI3K/AKT signaling is critical for the survival of glioma. In this study, we aimed to study the effect of aplysin on the survival and proliferation of GL26 glioma cells and the involved mechanisms. The data showed that aplysin suppressed the viability of glioma cells in both dose- and time-dependent manners. It also induced G₀/G₁ arrest and apoptosis in glioma cells. Western blot assays revealed that aplysin treatment changed p-AKT expression by impairing the formation of Heat shock protein 90/AKT complex. Aplysin significantly increased the survival time of mice-bearing glioma and reduced the weights of the established gliomas. Collectively, aplysin can inhibit the proliferation of GL26 glioma cells and induce apoptosis *in vitro*, perhaps through suppressing PI3K/AKT pathway. It can also inhibit glioma growth *in vivo* and prolong the survival of mice. Thus, aplysin may be a novel therapeutic drug for glioma.

Keywords: Aplysin, glioma, HSP90, AKT pathway

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Introduction

Glioma is a type of malignant cerebral tumor with high morbidity and poor prognosis. Surgery usually fails to completely eliminate glioma. Generally, glioma is insensitive to radiotherapy or chemotherapy, and hence, has a high incidence of relapse and mortality. Thus, it is of great necessity to find new strategies to improve the survival of patients.¹

Increasing evidence indicates that the aberrant activation of specific molecular pathway plays an important role in the progression, recurrence, and invasion of glioma. Several putative molecular signaling essential for glioma includes PI3K/AKT, mitogen-activated protein kinase (MAPK), and Wntless (WNT)/ β -catenin pathways.² PI3K/AKT pathway has been well documented to be activated in glioma.³ The activation of this signaling is required for the survival, proliferation, invasion, and tumorigenesis of glioma cells.³ PTEN (phosphatase and tensin homology deleted on chromosome 10) deletion or mutation, a lesion widely reported in a wide range of cancers, results in the activation of PI3K/AKT pathway.³ Targeting PI3K/AKT pathway has also been shown to be an effective way to treat glioma.³ Therefore, the identification of new compounds that can suppress the activation of PI3K/AKT pathway is always of interest.

Aplysin (C₁₅H₁₉OBr) is a seaweed bromo sesquiterpene compound from *Laurencia tristicha* with a molecular weight of 295. Our previous studies verified that aplysin can reduce ethanol-induced hepatic injury in mice.⁴ More intriguingly, this compound also exerts antitumor activity on sarcoma,⁵ human breast cancer,⁶ and human gastric cancer⁷ by inducing apoptosis.^{8,9} However, the mechanism by which aplysin triggers apoptosis in cancer cells is still unknown.

In this study, we provided evidence that aplysin can suppress the growth of glioma cells. Furthermore, we investigated the mechanisms underlying antitumor effects of aplysin on glioma cells *in vitro* and *in vivo*. We found that aplysin deactivated the PI3K/AKT pathway in glioma cells and destroyed Heat shock protein 90 (Hsp90)/AKT complex, to induce the apoptosis in glioma cells.

Methods

Preparation of aplysin

Aplysin was prepared following the methods described by Sun et al.⁹ Three *L. tristicha* were provided and validated by the Institute of Oceanology, Chinese Academy of Sciences. The dried sample (5 kg) was soaked in 95% ethanol for three days and extracted for three times to obtain 325 g extraction.

The extraction was extracted with ethyl acetate, and a final 105 g extraction was obtained. Then the extraction was separated using silica gel column chromatography, in which petroleum ether-acetone was used for gradient washing. The washed solution was purified with repeat silica, bio-beads, Sephadex LH-20 column chromatography, and reversed-phase High Performance Liquid Chromatography (HPLC). The obtained white compound was verified as aplysin ($C_{15}H_{19}OBr$) with a molecular weight of 295.

Cell culture

GL26 glioma cell line was purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. Mouse GL26 glioma cells were cultured in 10% Fetal bovine serum (FBS)-containing Dulbeccos Modified Eagle Medium (DMEM) (penicillin 100 U/mL, streptomycin 100 μ g/mL) at 37°C in a 5% CO_2 condition.

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium (MTT) assays

Cells were plated in each well of 96-well plates at the concentration of 1×10^6 mL, and treated with different concentrations of aplysin (0, 20, 40, and 80 μ g/mL) overnight. Each group had six replicates. At the indicated time points (24, 48, or 72 h), 50 μ L MTT (2 mg/mL) was added to each well and incubated for another 4 h. The supernatant was then removed and 150 μ L Dimethyl sulfoxide (DMSO) was added and mixed by shaking. Microplate reader was used to detect the absorbance at 550 nm, which was used to plot the cell proliferation curve.

Cell cycle analysis by flow cytometry

GL26 cells were diluted to 1×10^6 mL by Phosphate Buffered Saline (PBS), transferred to 6-well plates, and treated with aplysin for 48 h. Pan caspase inhibitor, Z-VAD-FMK (50 μ M), was added to prevent the onset of apoptosis at the same time. After harvesting and centrifugation, the supernatant was removed and the cell pellet was washed twice with PBS. One microliter of 70% ethanol was added and incubated overnight at -20°C. The cells were centrifuged and the supernatant was discarded. Pellets were then washed twice with PBS, digested by Ribonuclease (RNase) for 30 min, and dyed with propidium iodide (PI) (50 mg/L). The cells were put in dark at room temperature for 10 min, followed by being tested with flow cytometry (Aria II, BD Biosciences).

Cell apoptosis detection

GL26 cells were treated with aplysin for 48 h. Then, cells were centrifuged and washed twice with PBS. The supernatant was discarded. Then, the cells were processed with Annexin/PI staining apoptosis detection kit (Aria II, BD Biosciences) following the manufacturer's protocols, and loaded on flow cytometer for apoptosis detection (Aria II, BD Biosciences).

Western blot assay

Western blot assay was used to detect the protein expression of Hsp90, β -actin, AKT and p-AKT in GL26 cells according to the routine procedures. To make the statistical

analysis easier, the ratio of the densitometry of each blot to β -actin was calculated first, so that the relative value could be expressed as the percentage of this ratio of the aplysin-treated groups to the ratio of the control group. The involved antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

Co-immunoprecipitation assay

After aplysin treatment, total proteins were extracted with lysis solution. The protein concentration was measured using the Bio-Rad assay. Equal amount of cell lysate from each group was incubated with 2 μ g primary antibody (anti-AKT) for 2 h. Cell lysate was centrifuged at $1,000 \times g$ for 30 s to obtain cell pellets. The pellets were washed with Radio Immunoprecipitation Assay (RIPA) buffer for five times. Bound proteins were eluted in sample buffer, separated on 10% SDS-PAGE, and blotted with anti-AKT and anti-Hsp90 antibodies. Protein bands were detected using the Amersham ECL system and scanned by Image-Quant 5.2 software (Amersham).

Animal studies

C57BL/6 mice were randomly divided into four groups ($n = 10$). The mice were anesthetized by intraperitoneal (i.p.) injection of xylazine 12 mg/kg and ketamine 30 mg/kg, and were placed in a stereotactic frame with ear bars. Then, 1×10^5 GL26 cells were stereotactically injected at the bregma 2 mm to the right of the sagittal suture and 3 mm below the surface of the skull of anesthetized C57BL/6 mice with a sterile Hamilton syringe fitted with a 26 gauge needle. Seven days later, Groups A, B, and C were intratumorally injected with 20, 40, and 80 mg/kg-bw aplysin at the same site with Hamilton syringes (low dose, medium dose, and high dose), respectively, while group D was selected as control. The survival curve of mice was plotted. The mice were euthanized 40 days later, and glioma was harvested and weighted.

Statistical analysis

All data were expressed as means $\pm$ SD. Student's *t*-test was used to compare intergroup differences and $P < 0.05$ was regarded as significant. SPSS19.0 software was used for statistical analysis.

Results

Effects of aplysin on cell proliferation and apoptosis

Aplysin is a natural product from marine organisms (Figure 1). MTT assays showed that aplysin of different concentrations significantly inhibited the proliferation of GL26 glioma cells after 24, 48, or 72 h incubation. The effect was dependent on the dose and time (Figure 2(a) and 2(b)). After 48-h treatment with aplysin, the number of cells in G_0/G_1 phase was significantly increased, compared with the control, whereas the numbers of cells in S and G_2/M phases were reduced (Figure 3). The results suggested that aplysin inhibited cell proliferation by arresting cell cycle progression in G_0/G_1 phase. After 48-h treatment with aplysin, the number of apoptotic cells was significantly increased (Figure 4).

Figure 1 The structure of aplysin

Figure 2 Aplysin reduced the viability of glioma cells. (a) GL26 glioma cells were treated without or with aplysin (20, 40, and 80 $\mu\text{g/ml}$) for 48 h, followed by MTT assay-based determination of cell viability. The bars represent the Mean $\pm$ SD of three independent experiments. (b) Aplysin (80 $\mu\text{g/ml}$) was added to the culture of GL26 glioma cells. The survival rates were evaluated at three time points (24, 48, and 72 h). The bars represent the Mean $\pm$ SD of three independent experiments

Effects of aplysin on the expression of Hsp90, AKT, and p-AKT

The protein expression of AKT and p-AKT in aplysin-treated and control cells were shown in Figure 5(a). The expression of phosphorylated AKT was reduced in GL26 glioma, while total AKT level remained unchanged, suggesting that aplysin may inhibit PI3K/AKT signaling pathway (Figure 5(b)). The level of AKT-binding Hsp90 was found

to be decreased in the dose-dependent manner, while the total Hsp90 remained unchanged, suggesting that aplysin inhibited the formation of the Hsp90/AKT complex (Figures 6(a), (b), 7(a) and (b)).

Effects of aplysin on the survival of glioma-bearing mice

In an animal model, aplysin was shown to reduce the growth of glioma in mice, evidenced by the prolonged survival of mice suffering from the established glioma xenografts and the decreased weight of the tumors due to aplysin treatment (Figures 8, 9, and Table 1).

Discussion

In the present study, aplysin significantly inhibited the proliferation of GL26 glioma cells and induced apoptosis in both dose- and time-dependent manners. Further study showed that aplysin inhibited the p-AKT expression and the formation of Hsp90/AKT complex, suggesting that aplysin may inhibit the glioma via PI3K/AKT signaling pathway. *In vivo* results also demonstrated that aplysin can inhibit glioma and prolong survival of mice-bearing glioma.

Aplysin is a natural compound extracted from *L. tristichia*. Our previous study has reported its protective effect on ethanol-induced liver injury,⁴ and its antitumor effects on some types of tumors.^{5,7} One mechanism of antitumor medications is to induce apoptosis. Our result also demonstrated that aplysin effectively induced apoptosis in GL26 cell and this effect was increased along with aplysin concentrations. In addition, glioma cell cycle progression were arrested at G₀/G₁ phase by aplysin, resulting in slower growth and proliferation, suggesting cell cycle arrest also mediated the effect of aplysin on glioma. It should be mentioned that, pan caspase inhibitor Z-VAD-FMK was used to prevent apoptosis to maximize the change in the distribution of G₀/G₁, S, and G₂/M phases in glioma cells treated with aplysin. So no sub G₀/G₁ population representing apoptotic cells were observed in our study. Aplysin at 40 and 80 $\mu\text{g/ml}$ also significantly reduced the growth of glioma xenografts and prolonged the survival of mice, supporting its antitumor effect.

Hsp90 is a protein chaperon that maintains the stability and function of various signaling proteins involved in cell proliferation, growth, and survival. The serine-threonine kinase (Akt), also referred to as protein kinase B, is closely associated with growth factor-induced signaling pathway. By stimulating growth factors and cytokines, Akt is recruited from cytosol to plasma membrane and then phosphorylated at two key regulatory sites, Thr308 and Ser473, by 3-phosphoinositide-dependent protein kinase-1 (PDK1).^{10,11} Akt is a well-known binding protein of HSP90. HSP90 is essential for the proper function of Akt, because they form a chaperone-substrate protein complex, and the reduction in Hsp90/Akt binding results in Akt inactivation.¹²

Our results suggested that aplysin inhibited the glioma by suppressing AKT binding of Hsp90. Hsp90 is a chaperone protein responsible for correct folding, stability, and activation of its client proteins. It plays a fundamental role

Figure 3 Aplysin arrested glioma cells at G_0/G_1 phase. GL26 glioma cells were treated without or with aplysin (20, 40, and 80 $\mu\text{g/ml}$) for 48 h. Then, cytometrical analysis quantified the distributions of glioma cells in G_0/G_1 , S, and G_2/M phases. The plots are shown in the figure

Figure 4 Aplysin triggered apoptosis in glioma cells. Annexin V and PI double staining were performed on GL26 cells after the treatment of aplysin for 48 h. Annexin V⁺/PI⁻ cells stand for the early apoptotic population

Figure 5 Aplysin reduced the activation of AKT pathway. (a) The expression of phosphorylated and total AKT was detected by western blot assays in GL26 glioma cells treated with aplysin of indicated concentrations. (b) The expression was quantified with ImageJ software

Figure 7 Aplysin has no influence on total HSP90 levels. (a) GL26 cells were incubated with aplysin of 0, 20, 40, and 80 μg/mL for 48 h, followed by immunoblot analysis of total HSP90 expression. (b) The expression was quantified with ImageJ software

Figure 6 The binding of HSP90 to AKT was compromised by aplysin. (a) GL26 cells were incubated with aplysin of 0, 20, 40, and 80 μg/mL for 48 h, followed by co-immunoprecipitation analysis of AKT-binding HSP90 expression. AKT expression was also detected to test IP efficiency. (b) The expression was quantified with ImageJ software

Figure 8 Aplysin prolonged the survival of mice-bearing glioma. GL26 glioma cells were intracranially injected into mice to establish glioma xenograft model. After the administration of aplysin, the survival of mice in each group was recorded and plotted. Groups A, B, and C were injected with 20, 40, and 80 mg/kg-bw aplysin (low dose, medium dose and high dose), respectively, while group D was selected as control

in normal and stress conditions, as well as in pathological states such as cancer.^{13,14} It has been shown that AKT associates with molecular chaperone HSP90, and the stability of AKT protein depends on the HSP90/AKT complex.¹⁵

HSP90 forms a multimolecular complex with these signaling molecules to ensure their functional stability, and to facilitate the phosphorylation-dependent activation of AKT.^{16,17}

Figure 9 The weights of glioma treated with aplysin were reduced in a dose-dependent manner. Forty days after the administration of aplysin, the mice were sacrificed and the tumors were obtained for weighting. The weights of the glioma were shown here. Means $\pm$ SD. The bars represent the Mean $\pm$ SD of tumor weights in each group

Table 1 Tumor weight after treatment with aplysin of different concentrations

Group	Tumor weight (g)	P value
Low dose	0.823 $\pm$ 0.213*	P < 0.05
Medium dose	0.752 $\pm$ 0.453*	P < 0.05
High dose	0.543 $\pm$ 0.265*	P < 0.05
Control	1.051 $\pm$ 0.326	

*Significantly different from control.

Akt pathway has been well known to promote the survival of glioma cells. Robinson et al.¹⁸ showed that PI3K/AKT signaling is indispensable for the growth of glioma in mice and increases the resistance of glioma cells to apoptosis induced by various stimuli.¹⁸ Therefore, the suppression of PI3K/AKT signaling may be associated with the effect of aplysin on glioma cells. Although we have provided evidence that aplysin can suppress the activation of Akt by reducing the binding between Akt and HSP90, the possibility that aplysin can affect AKT signaling by other mechanisms cannot be excluded. PI3K and mTOR are both believed to be effective targets for suppressing AKT signaling. For instances, oleanolic acid has been recently shown to suppress the activation of mTOR in cancer cells in an adenosine 5'-monophosphate-activated protein kinase-dependent mechanism.^{19–21} Therefore, it is worth further studying if aplysin also suppresses the activation of PI3K/AKT signaling by targeting the components other than Akt.

In conclusion, our study demonstrated that aplysin can effectively inhibit the glioma growth and induce apoptosis probably through the PI3K/AKT signaling pathway. Further studies on the antitumor efficiency of aplysin and the underlying mechanisms are needed before it can be developed as a novel anticancer drug.

Author contribution: A.G. and H.L. designed the research. A.G. and N.G. performed the research. A.G., W.Y., L.L., and H.L. analyzed the data. A.G. and H.L. wrote the manuscript.

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