Int J Clin Exp Med 2016;9(5):8062-8068
www.ijcem.com /ISSN:1940-5901/IJCEM0017697

Original Article

Aplysin suppresses the invasion of glioma cells by targeting Akt pathway

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Received October 12, 2015; Accepted March 21, 2016; Epub May 15, 2016; Published May 30, 2016

Abstract: Malignant glioma is featured with high invasiveness. However, current drugs have limited outcomes to impair glioma invasion. The activation of Akt pathway is one of major mechanisms of glioma invasion. In this study, we investigated if aplysin, a natural compound from seaweed, has an inhibitory effect on the invasiveness of glioma cells. Aplysin was found to reduce the number of invasive U-87 MG and U-251 MG glioma cells, as well as primary glioma cells, in a dose-dependent manner. Invasion-associated proteins were found to be underexpressed in aplysin-treated glioma cells. Akt pathway was inactivated by aplysin and reactivating Akt pathway rescued the inhibitory effect of aplysin on invasion-associated proteins and the invasiveness of U-87 cells. Aplysin has no cytotoxicity to normal cells. Collectively, aplysin can suppress the invasion of glioma cells through suppressing Akt pathway.

Keywords: Akt, aplysin, glioma, invasion

Introduction

Malignant glioma is the most common brain cancer with high invasiveness and poor prognosis. In addition, glioma frequently recurs in the patients after surgery, because surgery is difficult to eliminate the remaining glioma cells due to their invasion. The current therapeutic regimens have limit outcomes to inhibit glioma invasion. Thus, it is high required to develop new strategy to minimize the invasiveness of glioma cells.

Accumulated evidences have demonstrated that some specific molecular pathways are important for the invasive property of malignant glioma. 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 deletion or mutation, a lesion widely reported in a wide range of cancers, result 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 suppress the activation of PI3K/AKT pathway is always of interest.

Aplysin ($C_{15}H_{19}OBr$) 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]. Furthermore, this compound also exerts antitumor activity on sarcoma [5], human breast cancer [6] and human gastric cancer [7] by inducing apoptosis [9]. However, it is still unknown if aplysin can suppress the invasiveness of glioma cells.

In the present study, we found that aplysin was able to suppress the invasion of glioma cells. Furthermore, we investigated the mechanisms of anti-invasion activity of aplysin on glioma cells *in vitro*.

Materials and methods

Preparation of aplysin

Aplysin solutions were prepared with the method of Sun [9]. Three laurencia 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 3 days and extracted 3 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. Washed solution was purified with repeat silica, bio-beads, Sephadex LH-20 column chromatography and reversed-phase PHLC. The obtained white compound was verified as aplysin ($C_{15}H_{19}OBr$) with a molecular weight of 295.

Cell culture and chemicals

U-87 MG and U-251 MG cells were purchase from American Tissue Culture Collection (ATCC, USA), and were cultured in 10% FBS-containing DMEM (100 U penicillin ml^{-1}, 100 streptomycin $\mu g\ ml^{-1}$) under 37°C and 5% CO_2. An Akt activator SC79 was purchased from Millipore (Billercia, MA, USA).

Transwell assay

U-87 MG and U-251 MG cells were re-suspended in 200 mL serum-free media, followed by being plated into the upper chamber of 24-well hanging cell culture insert (Millipore, Billercia, MA, USA) pre-coated with Matrigel (BD Biosciences, Sparks, MD, USA). 900 mL DMEM media containing 20% FBS was added to lower chamber of each well. 48 h later, cells were fixed by 4% paraformaldehyde and then dyed with crystal violet.

Wound healing assay

3×10^5 U-87 MG and U-251 MG cells were seeded in a 6-well plate. Then, the cells were cultured in starvation media overnight. A scratch on cell monolayer was made with help of a pipette tip, and then washed with starvation media to remove the nonadherent cells. Aplysin was added to the meida, and 24 h later, photographs were captured at the indicated time points.

Reverse transcription PCR assays

U-87 MG cells were treated with aplysin. 24 h later, total mRNA was isolated with Trizol solution (Invitrogen) according to the instructions of manufacturer, followed by reverse transcription reaction performed with Reverse Transcription Kit (Toyobo, Japan) for the generation of cDNA. cDNA was amplified by PCR assays, followed by agarose gel electrophoresis.

Western blot

After cells were lysed and the proteins were extracted, western blot was used to detect the protein expression according to the routine procedures. The involved antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

ELISA assays

ELISA assay were performed according to the routine procedures. Briefly, aplysin was added to cell media. 48 h later, the two-antibody sandwich ELISA was developed for detection of indicated proteins. The absorbance was read at a 450 nm wavelength. The concentration of proteins was evaluated based on standard curves.

Luciferase assay

The activation of Akt signaling was examined by dual luciferase assay. 5×10^4 cells were seeded in 24-well tissue culture plates and transfected with a luciferase plasmid containing FOXO-responsive promoter (Millipore, Billercia, MA, USA) and control renilla luciferase reporter vector (pRL-TK) (40:1) (Promega, Madison, WI, USA), using Lipofectamine 2000 (Invitrogen). Overnight, cells were treated with aplysin. 24 h later, cells were collected and suspended in lysis buffer (Promega) and luciferase activities were monitored for luciferase and renilla activity using reagents in dual-luciferase reporter assay kit and a luminometer (Promega), as recommended by the manufacturer.

Cell viability assay

1×10^4 cells were plated on 96-well plates. Overnight, aplysin was added to the cell culture at the indicated concentrations. 48 h later, cells was treated with the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, 1 mg ml^{-1}) followed by absorption determination on a microplate reader (Tecan, Switzerland).

Statistical analysis

All data were expressed as mean ± SD. T test was used to compare inter-group differences and $P<0.05$ and $P<0.01$ were regarded as significant and very significant, respectively. SPSS19.0 software was used for statistical analysis.

Results

Aplysin reduced the invasiveness of glioma cells in a dose-dependent manner

The structure of aplysin was presented in **Figure 1A** and **1B**. First of all, we employed two

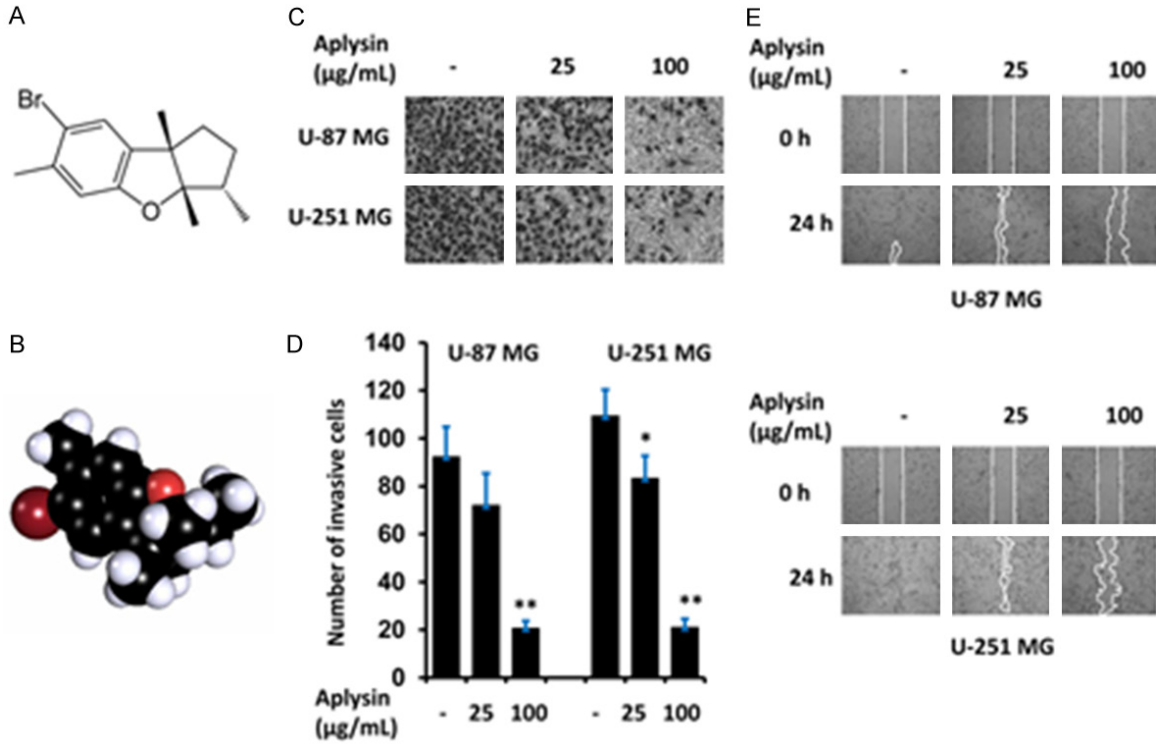

Figure 1. Aplysin reduces the invasiveness of glioma cells. A. Aplysin structure was shown here. B. 3D structures of aplysin was illustrated here. Black ball, carbon; white ball, hydrogen; pink ball, oxygen; red ball, bromine. C. U-87 MG and U-251 MG cells were treated with aplysin (25 μg ml^{-1} and 100 μg ml^{-1}) for 24 h. Transwell assays were performed to determine the number of invasive cells. (400 folds) D. The bars represented means ± SD of three independent experiments. E. Would healing assays were used to determine the motility of U-87 MG and U-251 MG glioma cells after the above treatments. The while lines defined the area not occupied by cells (200 folds).

glioma cell lines, U-87 MG and U-251 MG, as well as a primary glioma cells from patients, to study the effect of aplysin on the invasiveness of cancer cells. After the treatment of aplysin (25 and 100 μg ml^{-1}), the number of invasive cells in U-87 MG and U-251 MG cell lines were found to be reduced in a dose-dependent manner (**Figure 1C** and **1D**). Furthermore, we used wound healing assays to confirm the effect of aplysin on motility of glioma cells. The results indicated that aplysin was able to suppress the motility of U-87 MG and U-251 MG cells, evidenced by larger size of non-cell surfaces under aplysin treatment (**Figure 1E**).

Aplysin suppressed the expression of invasion-associated gene expression in glioma cells

Many proteins have been well documented to be closely associated with enhanced invasiveness of glioma cells. Subsequently, we aimed to verify if these genes were affected by the treatment of aplysin. Reverse transcription PCR assays revealed that mRNA abundances of VEGF, MMP2, MMP9, TIMP1 and TIMP4 were all decreased in U-87 MG cells stimulated with aplysin (**Figure 2A**). Consistently, immunoblot analysis also indicated that aplysin reduced the expression of the above invasion-promoting proteins (**Figure 2B**). ELISA assays further confirmed that secretion of these proteins were suppressed by aplysin (**Figure 2C**). The inhibitory effect of aplysin on these proteins appeared to possess a dose-dependent fashion.

Aplysin suppressed the activation of Akt/mTOR pathway in glioma cells

Next, we studied the molecular mechanism by which aplysin exerted its anti-invasion activity on glioma cells. Immunoblot assays revealed that the activation of Akt/mTOR pathway was suppressed by aplysin treatment, evidenced by the decreased phosphorylation of Akt and mTOR proteins (**Figure 2D**). Also, the expression levels of luciferase under the regulation of FOXO-responsive promoter were found to be

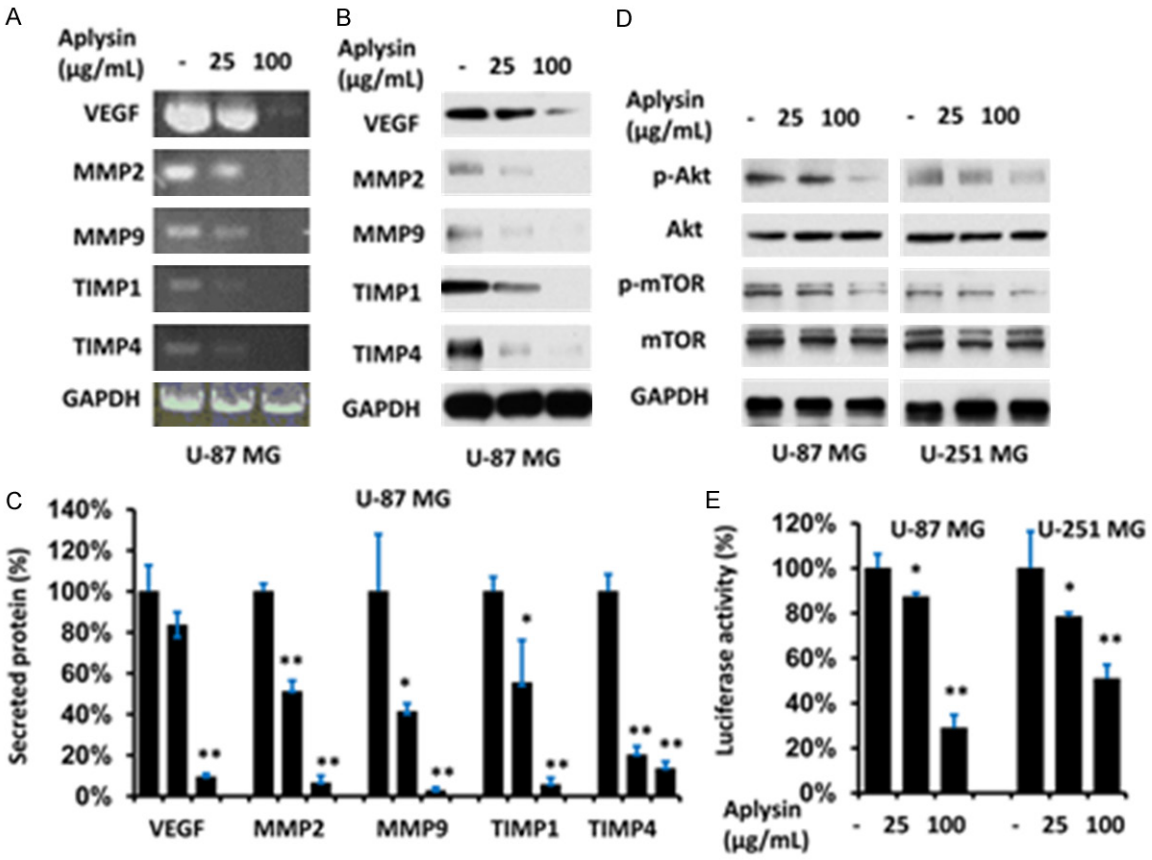

Figure 2. Aplysin suppressed the expression of invasion-promoting genes and the activation of Akt/mTOR pathway. A. U-87 MG cells were treated with aplysin (25 μg ml^{-1} and 100 μg ml^{-1}) for 24 h. Reverse transcription PCR assays were employed to determine the abundance of VEGF, MMP2, MMP9, TIMP1 and TIMP4 mRNA. GAPDH was used as endogenous reference. B. Immunoblot assays were employed to determine the expression levels of the above proteins. GAPDH was used as endogenous reference. C. ELISA assays were used to detect the concentrations of these invasion-associated proteins secreted into the cell media. The bars represented means ± SD of three independent experiments. D. U-87 MG and U-251 MG cells were treated with aplysin (25 μg ml^{-1} and 100 μg ml^{-1}) for 24 h. Immunoblot assays were employed to determine the expression levels of phosphorylated and total Akt as well as mTOR. GAPDH was used as endogenous reference. E. Under the same treatment, luciferase assays were used to evaluate the expression of luciferase driven by FOXO-responsive promoters. The bars represented means ± SD of three independent experiments.

reduced by aplysin (**Figure 2E**), suggesting that Akt/mTOR pathway was suppressed under this treatment.

Suppression of Akt/mTOR is required for the reduced expression of invasion-associated proteins

Subsequently, we were interested in the role of Akt/mTOR in the inhibitory effect of aplysin on the expression of VEGF, MMP2, MMP9, TIMP1 and TIMP4. We employed SC79, an activator specific for Akt, to reactivate Akt pathway in glioma cells treated with aplysin. Immunoblot assays confirmed that this strategy was able to reactivate this signaling in glioma cells (**Figure 3A**). Then, the expression levels of invasion-associated genes were detected by immunoblot assays in U-87 MG cells co-treated with aplysin and SC79. The results revealed that Akt reactivation abolished the inhibitory of aplysin on the expression of the above proteins (**Figure 3B**).

Reactivation of Akt/mTOR pathway rescues the effect of aplysin on invasiveness of glioma cells

To confirm the role of Akt/mTOR pathway in aplysin suppression of invasion of glioma cells, we reactivated this pathway by treating an activator SC79. Transwell assay revealed that the

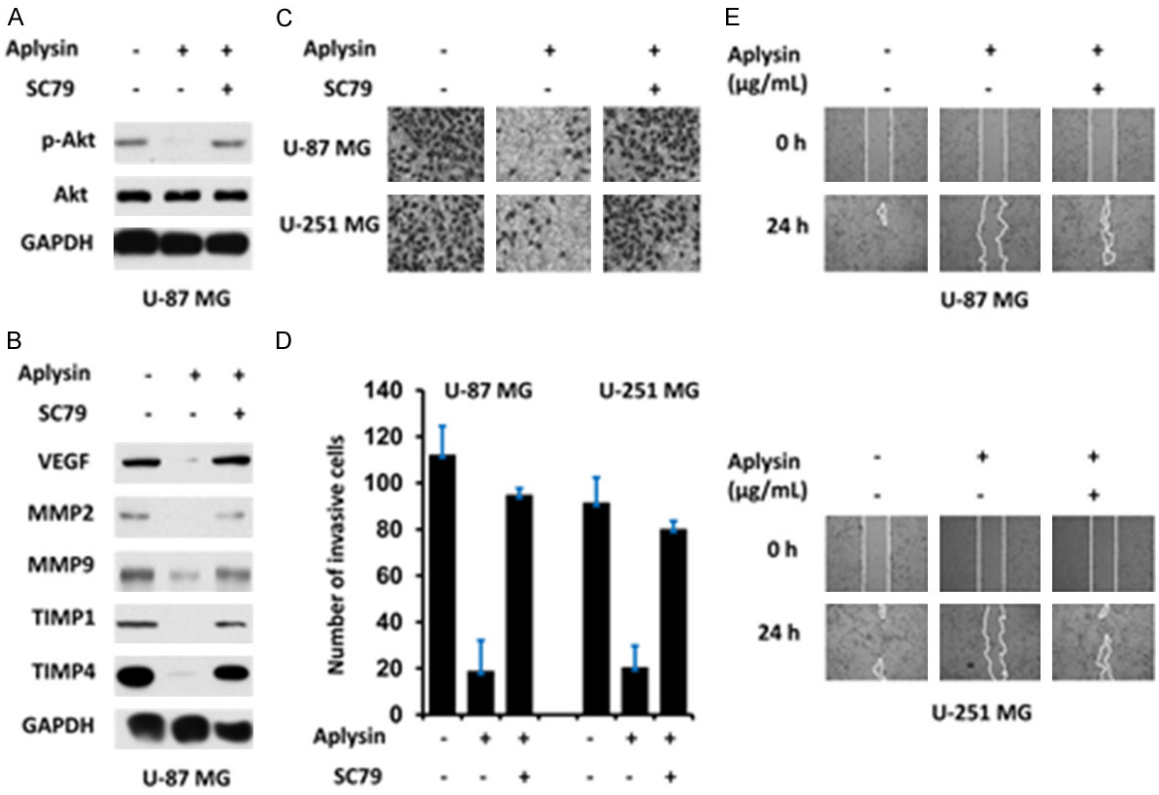

Figure 3. Akt reactivation abolished the inhibitory effect of aplysin on invasion-promoting gene expression and glioma invasiveness. A. U-87 MG cells were treated with aplysin (100 μg ml^{-1}) in the presence or absence of SC79 (4 μg ml^{-1}) for 24 h. Immunoblot assays were employed to determine the levels of phosphorylated and total Akt. GAPDH was used as endogenous reference. B. The expression levels of VEGF, MMP2, MMP9, TIMP1 and TIMP4 proteins were also detected by immunoblot assays. C. U-87 MG and U-251 MG cells were treated with aplysin (100 μg ml^{-1}) in the presence or absence of SC79 (4 μg ml^{-1}) for 24 h. Transwell assays were performed to determine the number of invasive cells (400 folds). D. The bars represented means ± SD of three independent experiments. E. Would healing assays were used to determine the motility of U-87 MG and U-251 MG glioma cells after the above treatments. The while lines defined the area not occupied by cells (200 folds).

number of invasive cells is much higher in both U-87 MG and U-251 MG cells co-treated with aplysin and SC79, than that treated with aplysin alone (**Figure 3C** and **3D**). Furthermore, wound healing assays also confirmed that SC79 abolished the inhibitory effect of aplysin on invasiveness of glioma cells (**Figure 3E**).

Aplysin has no cytotoxicity to normal cells

To determine if the application of aplysin was able to induce cytotoxicity to normal cells, we employed normal neuronal cell line, HCN2, normal liver cell line, L-02, normal endothelial cell line, HUV-EC-C, and normal lung fibroblast cell line, MRC-5, to detect their viability under the treatment of aplysin of different concentrations. The data indicated that aplysin has no significant influence on the survival rates of the tested cell lines (Supplementary Figure 1).

Discussion

Aplysin is a bromine compound originating from marine organisms. Its bioactivities include hepatoprotection and tumor suppression. The antitumor activity of aplysin is mainly associated with its ability to induce apoptosis in cancer cells [8, 9]. It is interesting if aplysin exerts its antitumor activity through more mechaisms other than apoptosis induction. In the present study, we provided evidence that aplysin was able to affect malignant phenotypes of glioma by suppressing its invasiveness. To our knowledge, this is the first time to show that aplysin can affect the invasion of cancer cells. This finding suggested that this marine natural compound can suppress cancer progression by multiple ways.

Although the antitumor function of aplysin has been verified for a long time, the molecular

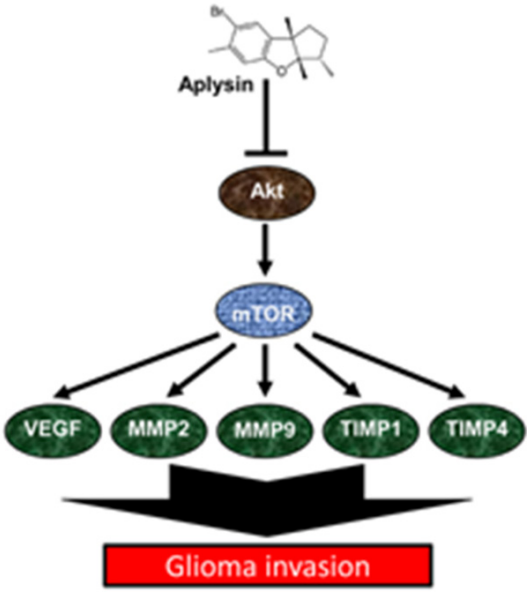

Figure 4. The illustration of the molecular mechanism by which aplysin suppresses glioma invasion. Aplysin inhibited the activation of Akt/mTOR pathway. In turn, invasion promoting proteins, such as VEGF, MMP2, MMP9, TIMP1 and TIMP4 were underexpressed. Finally, the invasion of glioma cells was suppressed by this stimulation.

pathways that are affected by this small molecule have not been identified yet. In this study, we found that Akt pathway, which is constantly activated in glioma cells [10], is suppressed by aplysin treatment. Given that Akt pathway plays a critical role in the invasion of glioma cells [11], its suppression resulting from aplysin treatment can account for this natural compound's anti-invasion effect. This is the first time to identify the molecular pathway affected by the stimulation of aplysin.

Akt pathway has been believed to play multiple important roles in the biology of a wide range of cancers, not limited to glioma [12]. Numerous evidences also imply Akt pathway as a promising target for cancer therapy [13]. In fact, many compounds that suppress the activation of Akt pathway have been confirmed to suppress the invasiveness of glioma in previous studies [14]. For instances, Cannabidiol has been found to suppress the invasion of U-87 MG and T98G glioma cells, and suppressed the activation of Akt pathway in a dose-dependent manner [15]. Eckol can reduce the invasiveness of glioblastoma stem-like cells, perhaps through targeting Akt signaling [16]. However, it is still unknown if these compounds induce cytotoxicity to normal cells. Here, we detect the viability of normal cells treated with aplysin, and the results revealed aplysin has no significant cytotoxicity to the cell lines derived from brain, lung and liver. This is the first anti-glioma-invasion compound that has been experimentally verified not to be toxic to normal tissue, to our knowledge.

Collectively, we demonstrated that, in addition to its low cytotoxicity to normal tissues, aplysin can suppress Akt pathway in glioma cells, and reduce their invasiveness (**Figure 4**). These properties may enable aplysin to be a promising lead compound for the development of anti-invasion drugs.

Acknowledgements

This study was supported by the grant from Qingdao basic research project (10-3-4-3-11-jch).

Disclosure of conflict of interest

None.

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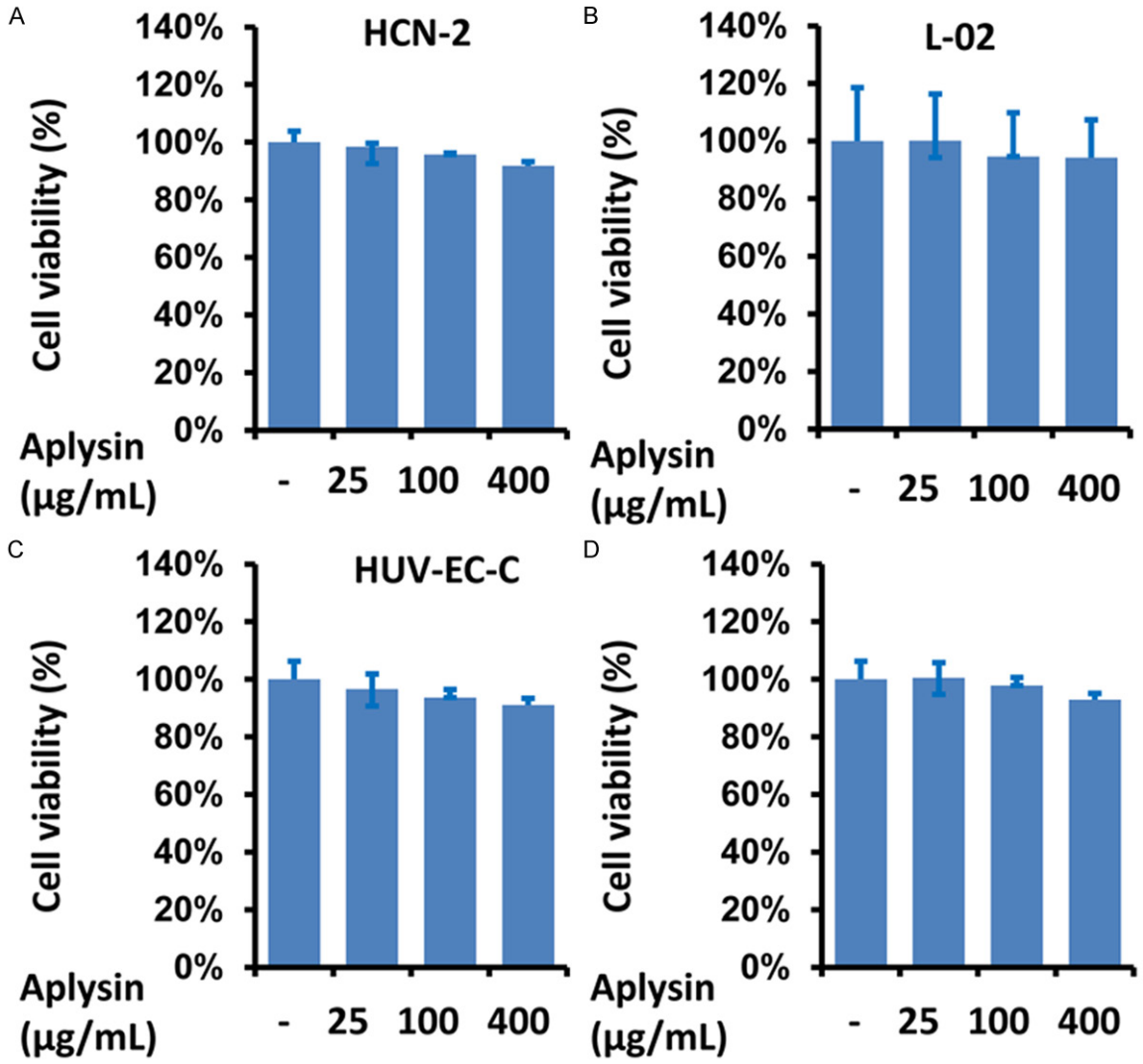

Supplementary Figure 1. Aplysin had no significant cytotoxicity to normal cells. (A) HCN-2 cells were treated with aplysin (25 μg ml^{-1}, 100 μg ml^{-1} and 400 μg ml^{-1}) for 48 h. MTT assays were used to examine the viability. The bars represented means ± SD of three independent experiments. L-02 (B), HUV-EC-C (C) and MRC-5 (D) cells were subjected to the above experiment.