Study on the mechanism of multi-AGC kinase AT13148 on Notch signaling pathway in glioblastoma

Type
Research paper

Keywords
glioblastoma, small molecule drug, AT13148, Notch, ITGB1, NOTCH1

Abstract
Introduction
Glioblastoma is the most malignant astrocytoma, and its therapeutic effect is not ideal. Notch signaling pathway plays an important role in tumor proliferation and invasion. Whether small molecule drug AT13148 can affect glioblastoma by regulating Notch signaling pathway is the focus of this study.

Material and methods
In vitro, glioblastoma U87 cell line transfected with sh-ITGB1 (U87sh-ITGB1), U87 cell line transfected with oe-ITGB1 (U87oe-ITGB1) and control group were treated with a small molecular drug AT13148. RT-qPCR, western-blot and clone formation ability assays were used to detect the mRNA and protein expression of the ITGB1 and the key gene NOTCH1, as well as the proliferation of cancer cells. Therapeutic effects of AT13148 were examined in vivo using a nude mice model of U87 cells. After treatment with AT13148, volume of tumors were calculated, and RT-qPCR and western-blot were used to evaluate the mRNA and protein expression of the ITGB1 and NOTCH1.

Results
AT13148 inhibits the activity of U87 cells. Lentiviral transfection of sh-ITGB1 and oe-ITGB1 can interfere with the expression of ITGB1 in U87 cells. AT13148 could down-regulate both the expression of ITGB1 and NOTCH1. Moreover, AT13148 affects the cloning ability of U87 cells. AT13148 can also inhibit the proliferation of U87 cells. Furthermore, AT13148 inhibited the proliferation and invasion of transplanted tumors in vivo.

Conclusions
This study indicated that AT13148 could affect the expression of ITGB1 and NOTCH1, which also could be a potential potential anti-glioblastoma small molecule drug candidate in clinic medicine.
Study on the mechanism of multi-AGC kinase AT13148 on Notch signaling pathway in glioblastoma

Running title: Mechanism of AT13148 on glioblastoma

Yanan Li¹#, Guosheng Han¹#, Weijie Min¹#, Mengmeng Li², Maomao Wang¹, Chao Chen¹, Yi Chen¹, Laixing Wang¹*, Zhijian Yue¹*

¹Department of Neurosurgery, Changhai Hospital, Second Military Medical University, Shanghai 200433, China

²Department of Rheumatology, Changzheng Hospital, Navy Medical University, Shanghai 200003, China

#Contributed equally to this work.

*Corresponding authors: Zhijian Yue and Laixing Wang

Department of Neurosurgery, Changhai Hospital, Second Military Medical University, No. 168 Changhai Road, Shanghai 200433, China

Tel: +86-021-31161765

Fax: +86-021-31161765

E-mail: ypv8droe@sina.com
Abstract

Introduction: Glioblastoma is the most malignant astrocytoma, and its therapeutic effect is not ideal. Notch signaling pathway plays an important role in tumor proliferation and invasion. Whether small molecule drug AT13148 can affect glioblastoma by regulating Notch signaling pathway is the focus of this study.

Material and methods: In vitro, glioblastoma U87 cell line transfected with sh-ITGB1 (U87sh-ITGB1), U87 cell line transfected with oe-ITGB1 (U87oe-ITGB1) and control group were treated with a small molecular drug AT13148. RT-qPCR, western-blot and clone formation ability assays were used to detect the mRNA and protein expression of the ITGB1 and the key gene NOTCH1, as well as the proliferation of cancer cells. Therapeutic effects of AT13148 were examined in vivo using a nude mice model of U87 cells. After treatment with AT13148, volume of tumors were calculated, and RT-qPCR and western-blot were used to evaluate the mRNA and protein expression of the ITGB1 and NOTCH1.

Results: AT13148 inhibits the activity of U87 cells. Lentiviral transfection of sh-ITGB1 and oe-ITGB1 can interfere with the expression of ITGB1 in U87 cells. AT13148 could down-regulate both the expression of ITGB1 and NOTCH1. Moreover, AT13148 affects the cloning ability of U87 cells. AT13148 can also inhibit the proliferation of U87 cells. Furthermore, AT13148 inhibited the proliferation and invasion of transplanted tumors in vivo.

Conclusion: This study indicated that AT13148 could affect the expression of ITGB1 and NOTCH1, which also could be a potential potential anti-glioblastoma small molecule drug candidate in clinic medicine.

Keywords: glioblastoma; small molecule drug; AT13148; Notch; ITGB1; NOTCH1
Introduction

Glioma originate from neuroepithelial tissues, most of which are malignant [1]. Glioma includes astrocytoma, oligodendroglioma, ependymoma, mixed gliomas, and neuroepithelial tumors [2]. According to the malignant degree of the tumors, WHO classifies gliomas into grade I (low proliferative capacity, low invasive astrocytoma and oligodendroglioma) to grade IV (highly invasive, mitotic active, necrotizing glioblastoma) [3]. For malignant glioma, the preferred treatment is still surgical treatment, which can achieve the total removal of tumors within a controlled range [4]. When tumors invade functional areas or conduction tracts, they need to be operated with the help of neuronavigation, functional magnetic resonance imaging, or even wake-up during operation for functional protection [5]. Regular radiotherapy and chemotherapy were given after operation. At present, the most commonly used chemotherapeutic drug is temozolomide. The dosage of temozolomide is related to the patient's body surface area, and there are differences between the period of radiotherapy and after radiotherapy [6]. Although many new techniques have been developed to improve the resection of glioblastoma, the bioavailability and blood brain concentration of temozolomide can reach a good state after operation, but for the overall prognosis of glioblastoma, there is no significant improvement. The use of chemoradiation, immunotherapy, and radio sensitizers as an adjuvant therapy cannot reduce the high rates of recurrence within a few months after treatment [7,8]. After glioblastoma diagnosis, in patients without treatment overall survival is typically 3 months [9]. Study has reported that the 10-year survival rate of glioblastoma was only 0.71% [10]. In addition to actively improving surgical techniques, scientists have also explored new treatment methods from various aspects, such as small molecule drugs, immunotherapy, photodynamic therapy and so on, but the effect is not satisfactory...
Notch signaling pathway is widely existing in vertebrates and invertebrates and a highly conserved signaling pathway. It plays an crucial role in cell proliferation and apoptosis [14]. AS-IVAstragaloside IV effectively reversed hypoxia-induced pulmonary vascular remodeling and pulmonary artery smooth muscle cell proliferation via the Notch signaling pathway [15]. It has been studied that Notch signaling pathway can not only regulate cell proliferation and differentiation, but also interact with other signaling pathways and growth factors [16]. The crosstalk between Notch and mitogen-activated protein kinase (MAPK) pathway plays a role in MEK inhibitor resistance in BRAFV600E metastatic melanoma and promotes migration in GNAQQ209L uveal melanoma (UM) cells [17]. Study has confirmed that miR-223 can inhibit the expression of F-box and WD repeat domain-containing7 (FBXW7), and ultimately increase the proliferation and apoptosis of colorectal cancer cells through Notch and Atk/mTOR signaling pathways [18]. The abnormal expression of Notch signaling pathway can lead to vascular malformation in mice [19]. Notch signaling pathway has become a very important target in cancer therapy. It has been found activation of Notch signaling pathway is associated with the development of glioblastoma [20]. The proliferation and cloning ability of glioma stem cells can be significantly enhanced by over-expression of Notch1 intracellular domain (NICD) [21]. The most influential gene integrin β1 (ITGB1) was previously identified by screening for genes that changed after Notch signaling pathway was activated in glioma [22]. ITGB1 is a member of the integrin family, which is formed by 18α and 8β transmembrane subunits. ITGB1 is a transmembrane receptor that mediates the connection between cells and their external environment [23]. It can activate PI3K/Akt pathway and p130cas/JNK signal transduction pathway, and regulate cell
proliferation, invasion and migration [24].

AT13148 is a new oral competitive multi-target kinase inhibitor of adenosine triphosphate (ATP). It was found that AT13148 affected the phosphorylation of rho-associated kinase (ROCK), protein kinase B (PKB), p70S6K and protein kinase (PKA) substrates, thus inducing apoptosis of cancer cells [25]. Inhibition of recombinant glutamate cysteine ligase modifier (GCLM) by AT13148 can improve the prognosis of chemotherapy in glioma patients. In previous work, we found that AT13148 was closely associated with seven genes in Notch signaling pathway which including ITGB1 [22]. Previous study showed that Mega-Itgb1 modulated angiogenesis upstream of Notch signaling [26]. Whether AT13148 can inhibit ITGB1 and further regulate Notch signaling pathway, thus affecting the proliferation and invasion of glioblastoma, needs more research. In this research, we aimed to explore the regulation of AT13148 on ITGB1 and its effect on glioblastoma and provide some ideas for clinical treatment of glioblastoma.

**Materials and Methods**

**Cell line and culture**

U87 human glioma cells were obtained from the First Affiliated Hospital of Naval Military Medical University (Shanghai, China) and routinely cultured in DMEM (Gibco, US) included with 10% fetal calf serum (Gibco, USA) and at 37 °C in incubator (Jingsheng Instrument, China) with humidified atmosphere and 5% CO2.

The culture medium was removed and PBS was used to wash the cells for subculture. After added 0.25% trypsin, the cells were then placed in an incubator (Jingsheng Instrument, China) at 25 °C for 1 min. During the period of continuous observation, when the sample changed like pinholes, cell suspension was moved to a centrifugal tube (AxyGen PCR-02-C, USA), and centrifuged for 3 min at 500 rpm
Samples with fresh culture medium were resuspended and counted, and then inoculated in the petri dish.

**The optimum concentration of AT13148 assay**

U87 cell suspension (10 000 cells/100 μL/well) was inoculated into 96-well plate. The culture plate was placed in the incubator (Thermo Forma, USA) for 24 h (37 °C, 5% CO₂). Different concentrations of AT13148 (50 μM, 20 μM, 10 μM, 5 μM, 2 μM, 1 μM, 500 nM, 300 nM, 50 nM, 20 nM, 10 nM, control, Selleck, China) were put in the 96-well plate with U87 cell suspension for 24 h. Then cells were incubated with 10 μL CCK-8 (Yeasen, China) in incubator (Jingsheng Instrument, China) for 4 h. Lastly, the absorbance value (OD) at 450 nm wavelength was measured by an enzyme-labeled instrument (Bio-tek, USA). Cell viability was quantified with the following formula: 

\[
\frac{(OD_{exp}-OD_{blank})}{(OD_{nc}-OD_{blank})} \times 100%.
\]

Each sample was measured three times and each sample had three biological replicates. AT13148 (water insoluble, 313.78, C₁₇H₁₆ClN₃O) was purchased from MedChemExpress (USA) with purity of 99.54%.

**Lentivirus packaging and transfection**

The DNA-lipofectamine 2000 complex were prepared according to the lipofectamine 2000 protocol [27]. The medium of U87 cell suspension was removed, and added 2 mL lentivirus medium with DMEM and 10% thermal inactivation fatal bovine serum (HI-BS) for 48 h. After that operation, the U87 cells were transferred to medium with DMEM, 10% HI-FBS, 1% penicillin/streptomycin (P/S) and 0.5 mg/Ml. U87 was cultured in the medium without antibiotics until the density reached 30%. The 293T cell were transfected by overexpression plasmid (oe-ITGB1) and interference expression plasmid (sh-ITGB1) respectively. Inclusion of the green fluorescent protein (GFP) gene in the vector smooths the tracking of any transduction in subsequent experiments (Supplementary Fig.1). Viruses made from 293T cell were
collected, and filtered by 0.5 μm microporous membrane. One mL lentivirus medium and 1 mL viruses solution were added to U87 cells in the presence of 4 μg/mL polybrene. Then the cells were transferred to the objective cell culture medium containing DMEM and 10% FBS without antibiotic. The expression of GFP was detected by fluorescence microscope (CKX41; Olympus, Tokyo, Japan) after transfection of oe-ITGB1 empty vector, oe-ITGB1 vector, sh-ITGB1 empty vector and sh-ITGB1 vector for 72 hours inorder to determine the transfection efficiency.

**RNA extraction and qRT-PCR**

RNA was isolated from cells or tumor tissues using Trizol (invitrogen, USA) following manufacturer’s protocol. The isolated RNA was transcribed into cDNA using PrimeScript RT reagent Kit with gDNA Eraser (Takara, Japan). The 20 μL reaction system consisted of 10 μL genomic DNA removal solution, 1 μL primeScript RT Enzyme Mix I, 1 μL RT Primer Mix, 4 μL 5× PrimeScript Buffer 2, and 4 μl 1 RNase Free dH2O. The reverse transcription conditions performed on the Eppendorf Mastercycler X50a (Eppendorf, Germany) were as follows: 37 °C for 15 min, 85 °C for 5 s, and 4 °C for 15 min. All primers used in this study are listed at Table 1. In this study, all the primers were synthesized by Sangon Biotech, Shanghai, China. qRT-PCR was performed using SYBR Primer Ex Taq™ II (Takara, Japan) on ViiA™ 7 system (Applied Biosystems, USA) and operation process was carried out according to the manufacturer’s protocol. The 20 μL reaction system consisted of 10 μL SYBR Premix Ex Taq, 0.4 μL ROX Reference Dye II, 0.8 μL forward primer and reverse primer, 4 μL cDNA, and 4 μL ddH2O. The reaction consisted 40 cycles and the procedure was as follows: 95 °C for 30 s, 95 °C for 5 min, 60 °C for 34 s, 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. Each group consisted of three biological replicates with three technical replicates per sample. GAPDH was used for
normalizing different samples as internal control. The calculation formula is as follows: \( \Delta \Delta CT = \Delta CT(\text{target gene}) - \Delta CT(\text{reference gene}) \).

**Western-blot assay**

Cell lysates were prepared using RIPA buffer (Santa Cruz Biotechnology). After centrifugation at 12,000 rpm at 4 °C for 20 min, supernatants were collected and protein concentrations were determined by the DC Protein Assay Kit (Bio-Rad Laboratories). Cell protein lysates were separated for 70 min by 15% 10% and 5% SDS-PAGE with 80 V, and then transferred onto the polyvinylidene fluoride (PVDF) membranes (Immobilon; Millipore, Bedford, MA, USA) using a semidry system for 1 h at 300 mA. Polyvinylidene difluoride membranes were blocked with 5% skim milk (Wako Pure Chemical Industries, Osaka, Japan) in phosphate buffer saline (PBS) at 25 °C for 1 h. Then they were incubated with the primary antibodies, diluted in 5% skim milk at 37 °C for 2 h. The membrane was washed and incubated with the following primary antibodies: NOTCH 1 (Affinity, AF5307, 1:500), ITGB1 (Affinity, BF0036, 1:500), GAPDH (Abcam, ab8245, 1:1000). Secondary antibody used were Goat anti Rabbit IgG (H+L) HRP (S0001, 1:10000) and Goat Anti-Mouse IgG (H+L) CY3-conjugated (S0012, 1:100). After the washing operation, enhanced chemiluminescence assays (NEN™ Life Science Products, Boston, MA, USA) were performed, and all the positive bands were identified on Fuji Medical X-ray film.

**Colonial formation assay**

The cells suspended in complete growth medium with 0.3% low-melting agarose, then cells were transferred into solidified 0.6% agarose in six-well culture plates (2 × 10^4 cells). The medium was changed every three days. Two weeks after incubation, using Omnicon 3600 image analysis system the numbers of colonies was counted. The colonies was stained using a 0.04% crystal violet solution for 2 h and visualized.
Each group consisted of three biological replicates.

**Xenograft tumor in nude mice**

All the experiments performed in this study were approved by the Experimental Animal Ethics Committee at the Second Military Medical University (Shanghai, China). Pathogen-free, female, 4 week-old BALB/c-nu (nude) mice were purchased from Charles River Laboratories (Wilmington, MA, USA). To analyze the consequence of AT13148 on tumor cells, U87 cell line ($6 \times 10^6$) were injected into the mice. After 3 weeks of feeding, the mice with tumor were randomly divided into control group and experimental group with 6 mice in each group. The mice in the experimental group were given 45 mg/kg/day AT13148, while the mice in the control group were given 45 mg/kg/day physiological saline. After 12 days, animals were sacrificed as per institutional guidelines. Body weight and tumor volume were measured over the whole time of the experiment every 3 days. Volumes of the tumor were measured with an equation: volume = (length $\times$ width $\times$ height/2).

**Immunohistochemistry assay**

For immunohistochemistry staining, slides were created from paraffin-embedded murine specimens. Each group contained six slices. The slices were immersed in xylene for 15 min, then dehydrated in ethanol and washed with PBS lately. Antigen repair was carried out according to the instructions of the primary antibody. The primary antibody of NOTCH 1 (Affinity, AF5307, 1:500) and ITGB1 (Affinity, BF0036, 1:500) were used to incubate slides, for 12 h at 4 ℃ and 1 h at 25 ℃. Following a washing step, all slides were incubated with secondary antibodies in blocking solution for 1 hour at room temperature. After PBS was removed, DAB was added to the slices and incubated for 10 minutes. Distilled water was used to wash the slices, and then hematoxylin was used for re-staining. The slides were assessed under...
a microscope (BX51M, OLYMPUS, Tokyo, Japan).

**Statistical analysis**

All data in this study were analyzed by SPSS standard version 20.0. Two-tailed Student’s t-test was used to do the significant analysis. Error bars represent the error of the mean (± SD). For whole analyses, value of $P < 0.05$ was deemed as significant.

**Results**

**AT13148 inhibits the activity of U87 cells**

We used CCK-8 kit to measure proliferation of U87 cells treated with different concentration AT13148. According to the concentration curve (Figure 1A), the IC$_{50}$ concentration of AT13148 was 3.4 μM. We treated U87 cells with 3.4 μM AT13148, and found that compared with the control group, the relative viability of group AT13148 was inhibited by 54% ($P < 0.001$, Figure 1B).

**Lentivirus transfection of sh-ITGB1 and oe-ITGB1 can interfere with the expression of ITGB1 in cell line U87**

Transfection efficiency was determined by fluorescence microscopy, and the result indicated that, the transfection efficency of sh-ITGB1 and oe-ITGB1 viruses were high, and the viruses can be used in the further experiments (Figure 2A). As shown in Figure 2B, the relative expression level of ITGB1 in sh-ITGB1 group was significantly lower than that in control group ($P < 0.05$), while higher in oe-ITGB1 group ($P < 0.05$). After transfection of U87 cells with lentivirus, the expression of ITGB1 protein in experimental group and control group changed (Figure 2C). Therefore, lentiviral transfection of sh-ITGB1 and oe-ITGB1 can interfere with the expression of ITGB1 in U87 cells.

**AT13148 regulates both ITGB1 and NOTCH1**

U87, U87$^{oe-ITGB1}$, and U87$^{sh-ITGB1}$ cells were treated with AT13148 with the
concentration IC50. The relative expression level of ITGB1 in U87oe-ITGB1, U87sh-ITGB1 and U87 group treated with AT13148 were significantly lower than that in control group (P < 0.05) after 48 h culture (Figure 3A). The expression of gene ITGB1 in U87sh-ITGB1 cell treated with AT13148 was the lowest. From the results of western blot, we can see that the protein expression of ITGB1 in each experimental group decreased in varying degrees compared with that in the control group (Figure 3C). All the results showed that AT13148 had a regulatory effect on ITGB1.

The same research method was used to study the effect of AT13148 on NOTCH1. As shown in Figure 3B, after treatment with AT13148, the expression of NOTCH1 was significantly decreased in the U87sh-ITGB1 cell and U87 cell compared with that in the control group (P < 0.05). Figure 3D showed that AT13148 also affects the protein expression of NOTCH1.

**AT13148 inhibits the clone formation ability of U87 cells**

To test whether AT13148 had an effect on the proliferation of glioblastoma cells, colony formation assay was carried out (Figure 4) in the U87 cells. After treatment with AT13148, the number of clones in the U87 group was significantly lower than that in the control group without AT13148 (P < 0.05). There was no significant difference in the number of clones between the U87oe-ITGB1 after treated with AT13148 and the control group (P > 0.05), while the number of clones in the group of U87sh-ITGB1 after treated with AT13148 was significantly lower than that in the control group (P < 0.05).

**AT13148 affects the volume of tumors in nude mice**

In this study, the nude mice model of glioblastoma U87 was established by subcutaneous injection. Twelve mice were successfully modeled (Figure 5A, 5B). During AT13148 treatment, there was no significant change in the body weight of
nude mice between the experiment group and the control ($P > 0.05$), and its fluctuation ranged from 19 to 22 g (Figure 5C). During the whole period of treatment, the tumor volume of mice in the AT13148 group was significant lower than that in the control group at 6th, 9th, and 12th day ($P < 0.05$) (Figure 5D).

**AT13148 affects the expression of ITGB1 and Notch1 in tumor tissues**

We extracted the RNA and protein of tumor tissue to verify whether AT13148 can affect the expression of ITGB1 and Notch1 *in vivo*. The results of qRT-PCR indicated that, compared with that in the control group, the expression of gene ITGB1 and NOTCH1 in transplanted tumors decreased 90% and 74%, respectively ($P < 0.05$) (Figure 6A. The protein expressions of ITGB1 and NOTCH1 were also inhibited (Figure 6B). Immunohistochemical result suggested that ITGB1 and NOTCH1 was mainly expressed in the control group (Figure 6C, 6D).

**Discussion**

Glioblastoma is a primary malignant glioma, which seriously threatens the life of patients. This study revealed that AT13148 could not only regulate the expression of ITGB1 and NOTCH1 but also inhibit the growth of glioblastoma.

It is recently shown that *ITGB1* is involved in lots of processes including migration [28], cell proliferation [29], and invasion [28]. ITGB1 involved in urokinase plasminogen activator-mediated angiogenesis through urokinase plasminogen activator receptor (PLAUR) [30,31]. Study previously reported that ITGB1 can promote proliferation of glioma cells via feedback regulation of the Notch signaling pathway [32]. This study revealed that AT13148 could downregulate the expression of ITGB1 and inhibit the

AT13148 belongs to multi-AGC kinase and ATP-competitive inhibitor. The AGC kinase family is important cell proliferation and survival. AGC activity was
significantly reduced in HGC27 tumors treated with AT13148. AT13148 as the target of AGC kinase showed good anti-gastric cancer activity in vitro and in vivo [33]. AGC kinase may play a similar role in this study. Study has demonstrated that AT13148 has an antiproliferative activity in a series of in vitro models harboring applicable genetic abnormalities, that incorporating gene of phosphate and tension homology deleted on chromosome ten (PTEN), kirsten rat sarcoma viral oncogene (KRAS), Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (PIK3CA) and Human Epidermal GrowthFactor Receptor 2 (HER2) aberrations. AT13148 caused substantial blockade of protein kinase B (PKB), p70S6K, protein kinase A (PKA), ROCK and SGK substrate phosphorylation. AT13148 also caused the apoptosis in a time-dependent and concentration manners in tumor cells with clinically related genetic deficiency [34]. BT474 and PC3 cells treated with AT13148 affect the metabolites of gene proteins related to nitric oxide synthase (NOS) [35]. And activated Notch tumorigenesis was fueled by hampering the immune response or by NOS overexpression to mimic a protumorigenic environment [36]. This suggests that AT13148 may affect the Notch signaling pathway by affecting NOS, which is also our future lines of inquiry.

Due to the existence of blood-brain barrier (BBB) and blood-brain tumor barrier (BBTB), most chemotherapy drugs can not directly reach the tumor site, which reduces the therapeutic effect of glioblastoma [37]. In this study, although the optimal concentration of AT13148 on U87 cells was determined according to CCK-8 assay, the in vivo experiment must be considered in the future. It is an important project to improve the permeability of AT13148 to BBB/BBTB for future in vivo research. Study has suggested that the active efflux of BBB/BBTB can be overcome by modifying the structure of candidate drugs and inhibiting the active efflux transport
receptors. For instance, small drug paclitaxel acquires increased BBB/BBTB permeability by covalent conjugation of N-docosahexaenoic acid [38]. Another perspective mainly includes a series of technologies to open or block the BBT/BBTB, such as focused ultrasound, radio-frequency microwaves, and laser interstitial thermotherapy [37].

**Conclusion**

In summary, the small molecule drug AT13148 regulates the expression of ITGB1 and NOTCH1 in the Notch pathway. *In vivo* experiments prove that it can affect the volume of tumors in nude mice. Our data suggested that AT13148 may serve as a potential anti-glioblastoma small molecule drug candidate in clinic medicine.
Declarations

Ethics Approval
All the experiments performed in this study were approved by the Experimental Animal Ethics Committee at the Second Military Medical University.

Funding
This work was supported by Natural Science Foundation of Shanghai (grant number 17ZR1438300).

Conflicts of interest
The authors declare that they have no conflicts of interest.

Availability of data and material
The data used to support the findings of this study are available from the corresponding author upon request.

Authors' contributions
Conception and design of the research: ZY and LW; acquisition of data: ML, MW, CC and YC; analysis and interpretation of data: ML, MW, CC and YC; statistical analysis: ML, MW, CC and YC; obtaining funding: ZY; drafting the manuscript: YL, GH and WM; revision of manuscript for important intellectual content: ZY and LW. All authors read and approved the final manuscript.

Acknowledgements
None.
Referencias

1. Moots PL, Johnson MD, Jennings MT, Cmelak AT (2012) Glioma and Other Neuroepithelial Neoplasms. John Wiley & Sons, Ltd,
Notch and Akt/mTOR pathways. Molecular medicine reports 2021; 23:154-163.


34. Yap TA, Walton MI, Grimshaw KM et al. Abstract 928: The novel clinical candidate AT13148 is an oral multi-AGC kinase inhibitor with potent


Figure legends

**Figure 1.** Relative viability of U87 cells. (A) Relationship between AT13148 concentration and U87 cell viability. (B) Relative viability of U87 cells in control group and AT13148 treatment group. *** $P < 0.001$.

**Figure 2.** Transfection of the U87 cells with lentivirus for overexpressed (oe-ITGB1) and interference expressed ITGB1 (sh-ITGB1). (A), The transfection efficiency of lentivirus in the oe-ITGB1 with empty vector, oe-ITGB1, sh-ITGB1 with empty vector, and sh-ITGB1 was detected by fluorescence microscope. (B), Relative expression level of ITGB1 in the control, sh-ITGB1, and oe-ITGB1 group. (C), Relative protein expression level of ITGB1 in the control, sh-ITGB1, and oe-ITGB1 group.

**Figure 3.** Relative expression of ITGB1 and NOTCH1. (A, B), Relative expression level of ITGB1 and NOTCH1 in the control, U87oe-ITGB1+AT13148, U87sh-ITGB1+AT13148, and U87+AT13148 group. (C, D), Relative protein expression level of ITGB1 and NOTCH1 in the control, U87oe-ITGB1+AT13148, U87sh-ITGB1+AT13148, and U87+AT13148 group. * $P < 0.05$.

**Figure 4.** Number of colonies in the control, U87oe-ITGB1+AT13148, U87sh-ITGB1+AT13148, and U87+AT13148 group. * $P < 0.05$.

**Figure 5.** AT13148 affects the volume of tumors in nude mice. (A, B), Nude mice with U87 xenograft tumor. The up row was control group mice and the down row was experimental group mice. (C) The weight of mice. (D) The tumor volume.

**Figure 6.** AT13148 regulates the expression of ITGB1 and NOTCH1 in the tumor. (A) Relative expression level of ITGB1 and NOTCH1 in different group. (B) Relative protein expression level of ITGB1 and NOTCH1 in different group. (C, D), Immunohistochemical detection of ITGB1 and NOTCH1 in different group. * $P <$
Supplementary Figure 1. The diagram of vector structure.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5(ve)’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITGB1</td>
<td>CCTTGGATGACTTGATTG</td>
<td>CCTTTGCTACGGTTGGTTAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AT</td>
</tr>
<tr>
<td>NOTCH1</td>
<td>GAGGCGTGCGACGACTATGC</td>
<td>CTTGACTCCGTCAGCGTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GGAGCGAGATCCCTCCAAA</td>
<td>GGCTGTTGTCATACTTCTCA</td>
</tr>
<tr>
<td></td>
<td>AT</td>
<td>TG</td>
</tr>
</tbody>
</table>
IC50 = 3.4 μM
Revised figure 4
Revised figure 5
Revised figure 6

**Figure 6**

**A**

[Graph showing the relative expression level of ITGB1 and NOTCH1 under control and AT13148 conditions.]

**B**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>AT13148</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITGB1</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
</tr>
<tr>
<td>NOTCH1</td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
</tr>
<tr>
<td>ACTIN</td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
</tr>
</tbody>
</table>

**C**

![Image](image7)

**D**

![Image](image8)