

Research Article

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Growth suppression of glioma cells using HDAC6 inhibitor, tubacin

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Abstract: In cancer research, autophagy has been revealed as one of the major ways to maintain the metabolism of cancer cells, including glioma cells, through protein degradation. Meanwhile, autophagy is also regarded as a kind of mechanism to protect glioma cells from a harmful stimulus, such as chemical and radiation treatment. So, the inhibition of autophagy may be very helpful in curing glioma. This study aimed to determine the effect of autophagic inhibition on glioma cells using tubacin, a specific inhibitor of histone deacetylase 6 (HDAC6). According to the results, tubacin inhibited the growth of both U251 and LN229 cells, which was accompanied by lower HDAC6 activity and accumulated autophagosome. The inhibition of HDCA6 also led to accumulation of autophagosome and death of glioma cells. Moreover, the combined treatment of tubacin and temozolomide, an alkylating agent used to treat glioblastoma, induced more severe glioma cell death. Thus, it can be concluded that inhibition of HDAC6 suppressed growth and drug resistance of glioma cells in-vitro through autophagic suppression and blocking of fusion of autophagosome and lysosome.

Keywords: HDAC6; Glioma; Drug resistant; Autophage

1 Introduction

Among the primary central nervous system (CNS) tumors, 40% are glial neoplasms. According to the histopathological and clinical features identified by the World Health

Organization (WHO), these neoplasms are classified into four grades [1]. High-grade gliomas, including glioblastoma multiforme (GBM) and anaplastic astrocytoma, threaten health most. Histological features of glioblastoma are marked by vascular proliferation, high cell density with mitotic activity and aggressive invasion into normal brain tissue [2]. Surgical resection followed by radiation and temozolomide treatment is the most frequent strategy used clinically to cure patients with glioma. However, radiation and temozolomide treatment can also induce protective autophagy to prevent excessive death of glioma cells [3].

Autophagy has been reported as one of the main pathways for degradation of proteins, organelles and recycling materials in cells. This process is regulated by more than 30 autophagy-related proteins, such as Beclin1, ULK1, Atg5 and LC3B [4]. Responding to cellular stress, activated ULK1 and Beclin1 promote the initiation of autophagy, and then two ubiquitin-like (Atg12 and Atg8/LC3) conjugation systems mediate the autophagosome formation [5]. Finally, autophagosomes will fuse with lysosomes and degrade the contents engulfed in autophagosome [6]. Previous reports indicated that sustained autophagy could induce death of tumor cells [7,8]. Moreover, blocking fusion of autophagosome and lysosome by CQ could enhance the anti-cancer effect of nutrient deprivation [9]. Thus, inhibition of late autophagy may be a potential strategy to antagonize tumor.

Recent research has demonstrated that many histone deacetylase (HDAC) inhibitors like vorinostat and depsipeptide participate in regulating the process of autophagy [10]. For example, HDAC6 has been reported to promote late autophagy. However, the mechanism of HDAC6-mediated autophagy is still unclear. Meanwhile, much less is known about the role of HDAC6 in glioma. In the current study, high HDAC6 expression in glioma tissue was detected, and it was found that tubacin, a specific small-molecule inhibitor of HDAC6, could suppress the growth and drug resistance of glioma cells invitro [11]. Moreover, it also demonstrated that tubacin suppressed late autophagy by influencing fusion of autophagosome and lysosome.

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2 Materials and methods

2.1 Patients

The surgical specimens from 5 patients with glioma from 2015 to 2016 in our hospital's department of neurosurgery were selected. Three specimens were collected from each case: tumor tissue, critical tissue and normal tissue, respectively. All tissues were frozen in liquid nitrogen tanks for later polymerase chain reaction (PCR) testing experiments. All of the cases had complete clinicopathological data. This study was approved by the hospital ethics committee and received the consent of the patients and their families.

2.2 Antibodies and reagents

Temozolomide, tubacin, Hoechst33342 and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), antibiotics and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). Polyvinylidene difluoride (PVDF) membranes (Hybond-P) and enhanced chemiluminescence (ECL) kit were purchased from GE Healthcare Life Sciences (Piscataway, NJ, USA). Antibodies against acetyl- α -tubulin, α -tubulin, LC3B, p62/SQSTM1, and HRP-conjugated sheep anti-rabbit IgG were all obtained from Cell Signaling Technology (Danvers, MA, USA). LAMP2 mouse monoclonal IgG was purchased from Santa Cruz.

2.3 Cell culture and immunoblotting

U251 and LN299 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM supplemented with 10% FBS (100 μ g/ml) penicillin and 100 μ g/ml streptomycin at 37°C in a humidified incubator with 5% CO₂. U251 cells were seeded in 6-well plates for 24 hrs. Then cells were treated with or without tubacin. After indicated times, cells were washed thoroughly with ice-cold PBS and lysed with radioimmunoprecipitation assay (RIPA) buffer (Beyotime). Samples were then boiled and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes. After incubation in blocking buffer (50 mM Tris-buffered saline (pH7.4) containing 5% non-fat milk and 0.1% Tween-20), the membranes were probed with indicated antibodies, fol-

lowed by a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG. Bands were revealed with the LumiGlo Chemiluminescent Substrate System (KPL).

2.4 Immunofluorescence staining

U251 cells were fixed with 4% paraformaldehyde and permeabilized with 100% methanol. 10% normal goat serum was used to block the nonspecific binding with primary antibodies. Then cells were incubated with appropriate primary antibodies at 4°C overnight. After PBS wash, cells were incubated with CF568-conjugated goat-anti-mouse IgG or CF488-conjugated goat-anti-rabbit IgG (Biotium, Hayward, CA, USA) at room temperature for 1 hr. Nuclei were revealed by Hoechst33342 staining. Fluorescence images were collected under a Leica DMIRB fluorescent microscope (Leica Microsystems, Wetzlar, Germany) armed with a spinning disk confocal microscopy system (UltraView cooled CCD; Perkin Elmer, Waltham, MA, USA).

2.5 Cell proliferation assay

The same amount of U251 cells were seeded in 6-well plates in triplicate. After indicated times and treatment, cells were fixed with 10% methanol and stained with 0.1% crystal violet (dissolved in 10% methanol). After staining, wells were washed three times with PBS and de-stained with acetic acid. The absorbance of the crystal violet solution was measured at 590nm.

2.6 Real-time PCR

Total mRNA from tissues of patients with GBM was extracted using trizol[®] (Invitrogen) while Omniscript RT Kit[®] (Qiagen) was used to produce cDNA. RT-PCR was performed using LC480 Real-Time PCR System (Roche) and SYBR Green PCR Master Mix (BioRad). Primers for qRT-PCR included *HDAC6* sense (5'-AGTCTTATGGATGGCTATTGCATG-3'), *HDAC6* antisense (5'-TGGACCAGTTAGAGGCCTCAGG-3'). β -actin sense (5'-TCTACAATGAG-CTGCGTGTG-3'), β -actin antisense (5'-GGTCAGGATCTTCATGAGGT-3').

2.7 Statistical analysis

Statistical analysis was performed with SPSS 10.0 software. Data were presented as mean \pm standard deviation (SD). The difference between the two groups was evalu-

ated by an un-paired t test. A value of $p < 0.05$ was considered as statistically significant.

3 Results

3.1 HDAC6 is highly expressed in tumor tissue of patients with GBM

To check the expression of HDAC6 in glioma tissue, the mRNA level of HDAC6 was detected by quantitative real-time PCR. The results showed that HDAC6 expression in tumor tissue from these patients was higher than in the control, whereas the average level of HDAC6 mRNA in tumor tissues was high compared with adjacent tissue of tumor from single patients as shown in Fig. 1A and B. In the samples from the same patients, the mRNA levels of HDAC6 in tumor tissues were also higher than in tissue adjacent to the tumor.

3.2 Tubacin growth inhibitory and temozolomide (TMZ) resistance effects on glioma cells

When treated with tubacin, the growth of U251 and LN299 cells was inhibited in a concentration-dependent way, as shown in Fig. 2A. Like chloroquine, tubacin also promotes

accumulation of autophagosome, so it was possible to determine whether tubacin can influence the TMZ resistance of glioma cells. As the results in Fig. 2B show, tubacin can reinforce TMZ toxicity on U251 cells and LN229 cells. Therefore, HDAC6 inhibition by tubacin can suppress the proliferation and TMZ resistance of glioma cells.

3.3 Autophagy suppression by HDAC6 inhibitor

We found that tubacin could inhibit the activity of HDAC6 and suppress autophagy. The lys-40 site of α -tubulin was the target of the catalytic activity of HDAC6 [12]. So, we used the acetylated level of lys-40 site in α -tubulin as the indicator of HDAC6 activity. The switch from LC3B-I to LC3B-II was considered as a marker of increased autophagic flux. After treatment with tubacin, the levels of LC3B-I and LC3B-II in U251 cells both increased, which meant a blockade of autophagic flux. Additionally, p62 has been reported as a marker for indicating the activity of late autophagy [13]. Tubacin treatment also induced p62 accumulation in U251 cells, which meant an impairment of autophagy as shown in Fig. 3A. Also, we found that tubacin treatment increased autophagosome formation, as shown in Fig. 3B. These results indicated that tubacin suppressed autophagy.

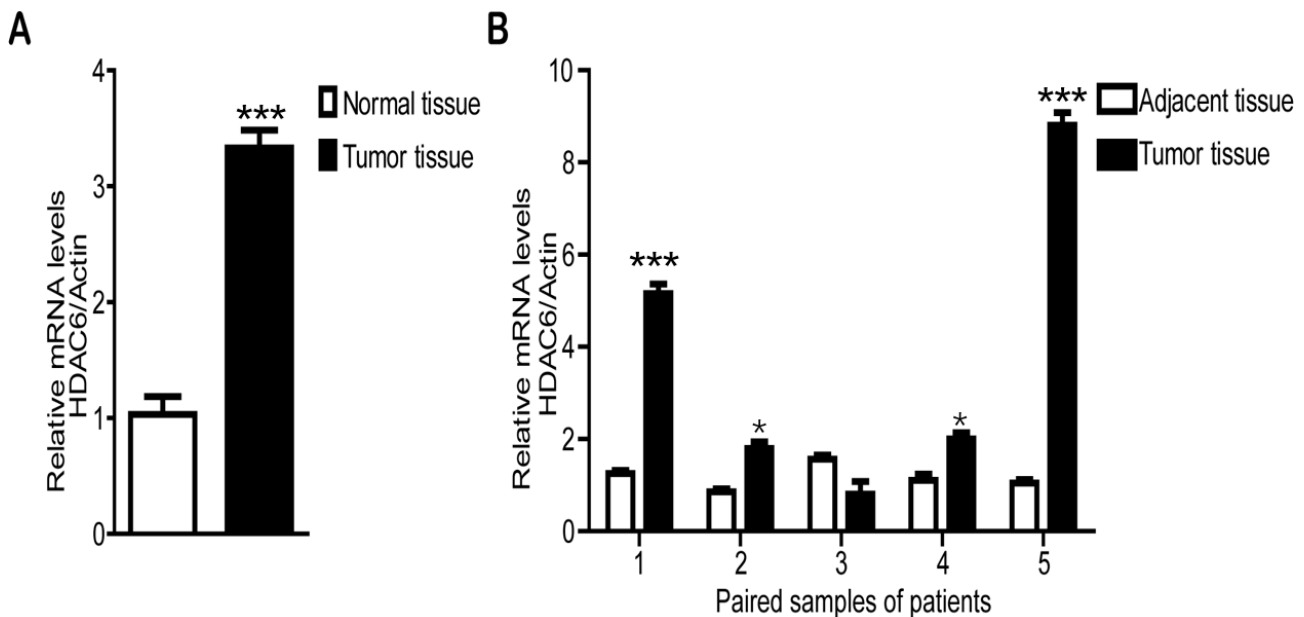


Figure 1: (a) HDAC6 mRNA in tissues from patients with GBM (b) HDAC6 mRNA in tumor tissues compared with adjacent tissue of tumor from single patients. The data shown in the figure was derived from 3 independent experiments. * $P < 0.05$, *** $P < 0.001$ versus normal tissue in panel A and versus adjacent tissue in panel B.

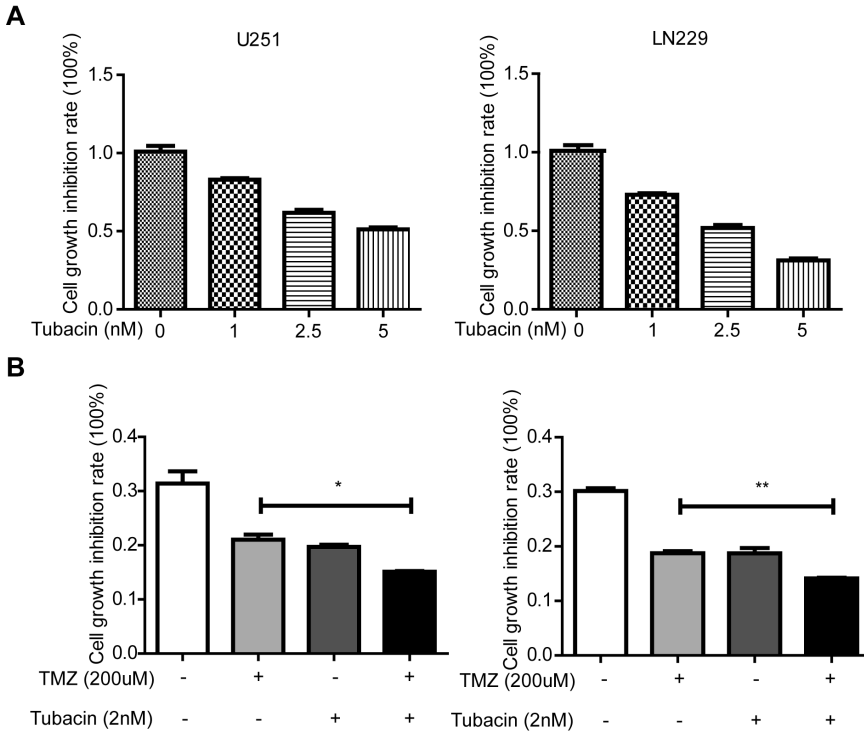


Figure 2: Tubacin inhibited the growth of glioma cells and enhanced the toxicity of temozolomide. (a) Human glioma cell lines LN229 and U251 were plated in 96-well plates (5×10^3 /well) and cultured at 37°C for 24 hours. Then cells were treated with or without various concentrations of tubacin for 8 hours. Cell growth was monitored by crystal violet staining. (b) LN229 and U251 cells were plated in 96-well plates. After 24 hours, cells were treated with or without TMZ ($200\mu\text{M}$) in the presence or absence of tubacin (2nM) at 37°C for 8 hours. Cell viability was measured by crystal violet staining.

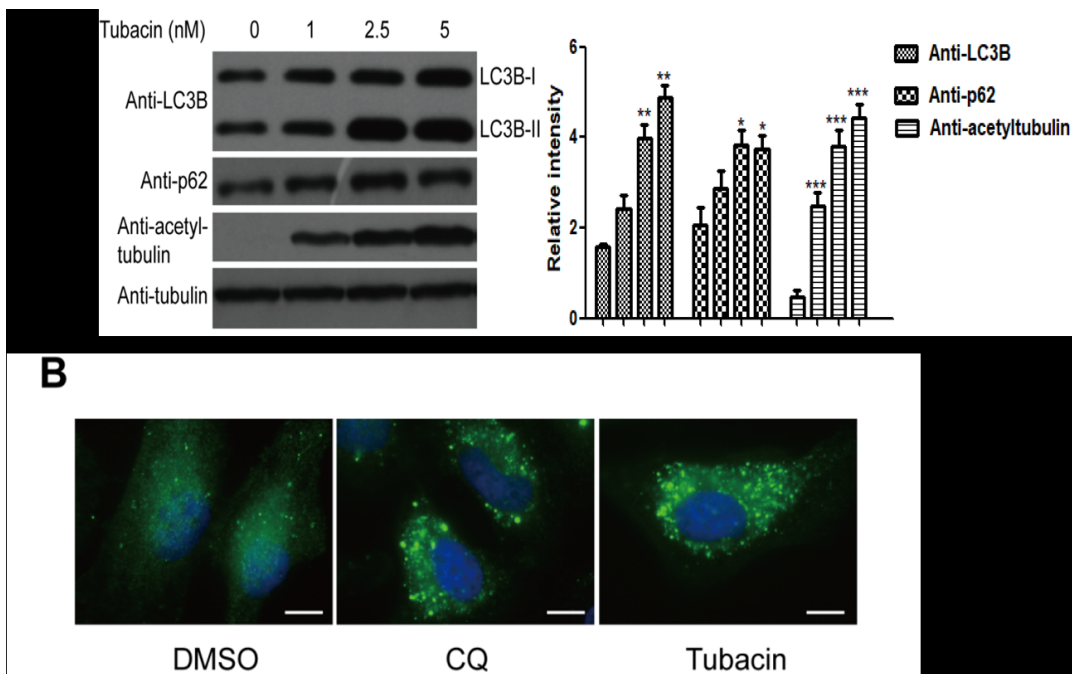


Figure 3: Inhibition of HDAC6 by tubacin suppressed autophagic degradation. (a) U251 cells were treated with the indicated concentration of tubacin. After 8 hours, cells were lysed and used for immunoblotting detection with antibodies. (b) U251 cells were plated and treated with CQ ($25\mu\text{M}$) or tubacin (2nM) for 8 hours. Then cells were fixed and used to detect autophagic puncta by immunofluorescence with LC3B antibodies. Scale bars, $10\mu\text{M}$. The data shown was derived from 3 independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus no tubacin group.

3.4 Blocking of fusion of autophagosome and lysosome by tubacin

To further investigate the mechanism by which tubacin suppresses autophagic degradation, we used double-staining immunofluorescence to detect the fusion of autophagosome and lysosome. LC3B antibody was used to label the autophagosome, and LAMP2 antibody was used to label the lysosome [14,15]. In our results, tubacin treatment strongly blocked the fusion of autophagosome and lysosome (Fig. 4A). According to the statistical analysis of the fused autolysosome in U251 cells, we found suppression of autophagosome and lysosome fusion (Fig. 4B). So, we concluded that tubacin suppressed late autophagy by blocking the formation of the autophagosome-lysosome complex.

4 Discussion

Currently, most therapeutic strategies used to cure tumor focus on apoptosis of tumor cells. However, apoptosis evasion is one of the major hallmarks of various types of cancer, including glioblastoma [16]. So, it is necessary to develop new methods that target non-apoptotic death pathways. Previously, there was some controversy about the role of autophagy in cancer. Some researchers thought the process of autophagy was a kind of protective pathway to antagonize stress or nutrition deficiency [17]. However, drugs that cannot induce apoptosis also promote cell death in many GBM cell lines. This may be due to disposal of organelles by autophagic degradation and accumulation of autophagosome [18].

In this study, we examined the role of HDAC6 in the autophagy process and found that HDAC6 contributed to tumor development. Many small molecules are used to inhibit autophagy, such as chloroquine, 3-MA or LY294002. But the main challenge in current research on autophagy is the lack of specific inhibitors targeting the autophagic process. Previously, we found that tubacin, a specific small-molecule inhibitor of HDAC6, could inhibit the activity of HDAC6 and suppress autophagy. In autophagy, cargos are recognized by an autophagic cargo receptor, such as p62. Then these cargos are transferred to an autophagosome. Finally, the autophagosome fuses with lysosome and degrades the contents, with the help of proteolysis enzymes in lysosome. Like chloroquine, HDAC6 inhibitor-induced accumulation of autophagosome has been considered one of the main factors that causes autophagic cell death [9]. But, unlike chloroquine, HDAC6 inhibitor does not influence the formation of lysosome.

In our study, tubacin inhibited the growth of glioma cells and enhanced the toxicity of temozolomide in human glioma cell lines LN229 and U251 when treated with tubacin for 8 hours, which means HDAC6 inhibition by tubacin can suppress the proliferation and TMZ resistance of glioma cells.

HDAC6 is a special enzyme in the histone deacetylase family. Unlike other histone deacetylase, HDAC6 does not interact with histones. Besides deacetylating α -tubulin, HDAC6 also deacetylates cortactin, Hsp90. Moreover, HDAC6 is involved in many processes that are linked to cancer, including cell survival, motility, and metastasis, which makes it an attractive therapeutic target [19]. In our results, HDAC6 was highly expressed in GBM tumors com-

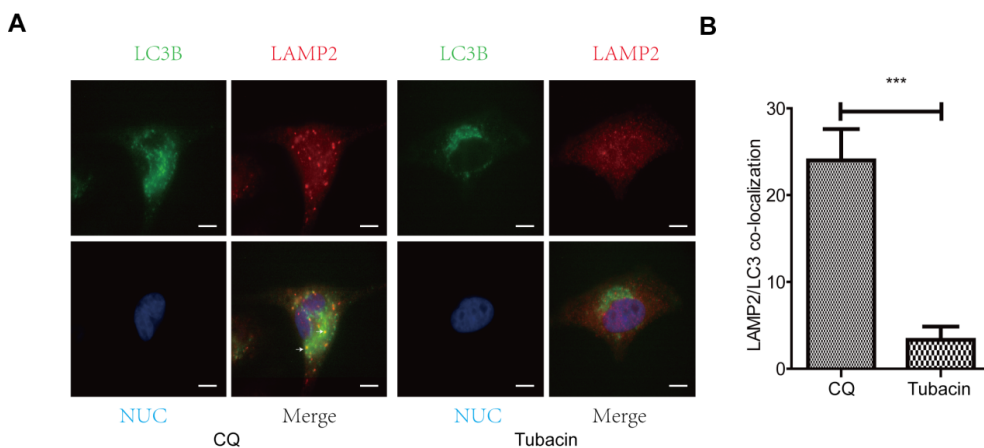


Figure 4: Tubacin treatment blocked the fusion of autophagosome and lysosome. (a) U251 cells were treated with CQ (25 μM) or tubacin (2 nM) for 8 hours followed by immunofluorescence. Autophagosome was detected by LC3B antibody combined with CF488-conjugated goat-anti-rabbit antibody (green). Lysosome was detected by LAMP2 antibody combined with CF568-conjugated goat-anti-mouse antibody (rabbit). Scale bars, 10 μm. (b) Fused puncta, which indicated fused autolysosome, in 30 cells were obtained for statistical analysis.

pared with normal brain tissue, indicating that HDAC6 might promote tumor progression in GBMs. In summary, inhibition of HDAC6 is a potential choice to suppress growth and drug resistance of glioma. In conclusion, inhibition of HDAC6 can suppress both growth and drug resistance of glioma cells in vitro through autophagy suppression and blocking of fusion of autophagosome and lysosome.

Study limitations: In this article, we mainly compared the tumor tissue, the critical (scar) tissue and the normal tissue samples of the patients and carried out the related cell experiments. In this study, the number of patients was somewhat small, and statistical deviation was prone to occur. In addition, we did not use the expression of HDAC6 gene in our experiment to observe its effect on the growth of tumor cells. Therefore, it is necessary to increase the number of samples of tumor tissue in the future, use the over-expression experiment and study the related mechanism.

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