Original Article

Inhibition of histone deacetylase 6 by tubastatin A as an experimental therapeutic strategy against glioblastoma

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Abstract

Background and Aim: Glioblastoma is the most lethal brain tumor. No effective curative treatment is available yet, and it is treated by surgery, temozolomide (TMZ), and radiotherapy, with an average overall survival of around 15 months. Inhibitors of histone deacetylases (HDACs) are being explored against a variety of tumors, including glioblastoma. Specific inhibitors of HDAC6, such as tubastatin A (Tub A), may potentially be beneficial as HDAC6 has been demonstrated to be the most expressed HDACs in glioblastoma. Our aim was to test whether Tub A could reverse the malignant phenotype of U87MG cells via the inhibition of HDAC6. **Materials and Methods:** U87MG cells were treated with cyclopamine (Cyp), TMZ, and Tub A. Two double treatments were performed as well (Cyp + Tub A and TMZ + Tub A). Colony formation, wound healing, Caspase-Glo 3/7, quantitative reverse transcription–polymerase chain reaction, luciferase assay, and Western blot assays were conducted to determine clonogenic and migration capacity, apoptosis, activation of the Sonic Hedgehog pathway, acetylation of α-tubulin and epithelial-to-mesenchymal transition, and autophagic flux of U87MG glioblastoma cells, respectively. **Results:** Tub A treatment caused a reversal of the U87MG malignant phenotype by reducing its clonogenic and migratory cellular potential, and inducing apoptosis. Sonic Hedgehog pathway inhibition, together with reversal of epithelial-to-mesenchymal transition and reduced autophagic flux, was also induced by the effect of Tub A. **Conclusions:** HDAC6 might be a good target for glioblastoma treatment.

Keywords: Autophagy, epithelial-to-mesenchymal transition, glioblastoma, histone deacetylase 6, Sonic Hedgehog pathway, tubastatin A

Introduction

Glioblastoma multiforme (GBM) or Grade IV astrocytoma is the most aggressive and most common malignant brain tumor.[1] Current treatment of GBM consists of maximal resection followed by radiotherapy and chemotherapy,[2] and has remained unchanged since 2005.

Histone deacetylases (HDACs) regulate cellular processes such as cell cycle, proliferation, cell survival, and angiogenesis, which make them an interesting therapeutic target for the treatment of cancer.[3] An aberrant expression or dysfunction of these proteins is related to different types of tumors, such as colon cancer,[4] gastric cancer,[5] and GBM.[6] Accordingly, some HDAC inhibitors have been developed to treat cancer. These drugs can revert the acetylation status of proteins found in different kinds of tumors, and reactivate the expression of tumor suppressor genes. Moreover, these inhibitors are tumor specific, and spare healthy cells.[7]

HDAC6 is the most overexpressed isoform of HDAC in GBM.[6] This isoform is the only one which contains two

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functional deacetylation domains.[8] Although almost all HDACs are located in the nucleus, HDAC6 is predominantly present in the cytoplasm.[9,10] HDAC6 can perform the deacetylation of nonhistone substrates, including cortactin, HSP90, and α-tubulin, which are its major targets. HDAC6 has been shown to be deregulated in other cancer types, such as hepatocellular carcinoma[11,12] and ovarian cancer.[13]

Tubastatin A (Tub A) is a hydroxamic acid-derived compound, which specifically inhibits HDAC6.[14] Treatment with Tub A showed a significant effect on tumor growth and primary cilia restoration in a model of cholangiocarcinoma, both in cell lines and an animal model.[15] Tub A also reduced proliferation and motility in an esophageal squamous cell carcinoma cell line.[16]

The aim of this study was to investigate whether HDAC6 could be a good therapeutic target for GBM treatment. We examined the effects of the HDAC6 inhibitor, Tub A, on a GBM cell line, U87MG, in order to clarify whether the selective inhibition of HDAC6 by Tub A could reverse the malignant phenotype of U87MG cells.

Materials and Methods

Cell culture

The glioblastoma-derived cell line, U87MG (ATCC® HTB-14™), was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA, ATCC® HTB-14™). U87MG cell line was cultured in DMEM GlutaMAX™ (Dulbecco's Modified Eagle Medium, ref. 61965-026, Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 4% nonessential amino acids (MEM NEAA, ref. 11140-035, Thermo Fisher Scientific) in an incubator at 37°C, 5% CO_2, and 95% relative humidity.

Drug treatment

U87MG cells were treated with cyclopamine (Cyp; an inhibitor of Smo, which is an activator protein of the Sonic Hedgehog pathway; C-8700, LC Laboratories, Woburn, MA, USA), Tub A (an inhibitor of HDAC6; S8049, Selleckchem, Houston, TX, USA), and temozolomide (TMZ; alkylating agent against glioblastoma; T2577, Sigma, St. Louis, MO, USA) in a final concentration of 20, 20, and 500 μM, respectively. In addition, two combined treatments were performed (Cyp + Tub A and TMZ + Tub A). These concentrations were calculated using the MTT assay (1117140010, Sigma), and every concentration contained 20 μM dimethyl sulfoxide (DMSO), also used as a control of treatment.

Colony formation assay

Colony formation assay was conducted to determine the clonogenic capacity of the cell line under normal conditions (control group) or after treatment with a drug. A total of 300 cells previously treated with the different individual drugs and combinations for 72 h were seeded in 6-well plates and incubated for 10 days. Subsequently, the cells were washed with phosphate-buffered saline and fixed with 4% paraformaldehyde for 30 min. Once the cells were fixed, they were stained with crystal violet solution (Sigma) for 15 min. Finally, the colonies were counted using the Suntex 560 Colony Counter (Gemini, Apeldoorn, The Netherlands).

Wound healing assay

For this assay, 100,000 cells, which were previously treated with the different individual drugs and combinations for 72 h, were seeded in 24-well plates and incubated for 24 h until ~100% confluence was reached. The following day, a scratch was made using a 200 μL pipette tip, and the medium was replaced by one containing 2.5% fetal bovine serum. Photographs were taken using SMZ18 stereomicroscope equipped with a high-definition DS-Fi2 camera (Nikon Instruments Inc., Tokyo, Japan) at different time points to visualize the scratch healing, and the percentage of the area showing cell migration was monitored using the ImageJ Software 1.52a (NIH, Bethesda, MD, USA).

Caspase-Glo 3/7 assay

A total of 5000 cells were seeded in 96-well plates. The day after seeding the cells in the wells, cells were treated with the different individual drugs and combinations, and incubated for 24, 48, and 72 h. Afterward, the Caspase-Glo 3/7 reagent (Promega, Madison, WI, USA) was added following the manufacturer's instructions, and luminescence was measured by a Luminoskan Ascent (Thermo Electron Corporation, Rockford, IL, USA).

Quantitative reverse transcription–polymerase chain reaction

300,000 cells were seeded in 6-well plates, followed by incubation for 24 h. Then, cells were treated with 20 μM Tub A or DMSO for 72 h, and RNA was extracted by following the TRIzol Reagent T9424 protocol (Sigma, St. Louis, MO, USA).

RNA was retrotranscribed into cDNA using SuperScript™ II Reverse Transcriptase (Cat. No. 18064, Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocol. Then, quantitative reverse transcription–polymerase chain reaction (RT-qPCR) was performed to analyze the mRNA expression of *Gli1*, using the following primer sequences *Gli1* forward 5'-AAGCGTGAGCCTGAATCTGT-3', *Gli1* reverse 5'-AGCATGTACTGGGCTTTGA-3'. Data were analyzed using the $2^{-\Delta\Delta CT}$ method.[17]

Luciferase assay

Sonic Hedgehog activity was analyzed by luciferase assay. For this assay, 50,000 cells were seeded in 24-well plates and incubated until the next day. Thereafter, cells were transfected with a plasmid with mutated 8× Gli1 promoter (negative control), or a plasmid containing *Renilla* luciferase (wild-type or control group), and a control plasmid in a ratio of 4:1:5 in a total amount of 0.5 μg by following the Lipofectamine 2000 protocol. All plasmids were a kind gift from Dr. Riobo-Del Galdo (University of

Leeds). A third group was created, by adding 20 μM Tub A to cells in the control group. One day after transfection, the culture medium was replaced by one without fetal bovine serum, and cells were incubated for 24 h. Then, luciferase was measured using Dual-Glo® Luciferase Assay System (E2920, Promega, Madison, WI, USA) in a GloMax 20/20 Luminometer (Promega, Madison, WI, USA) following the manufacturer's protocol.

Western blot assay

A total of 300,000 cells were seeded in a 6-well plate and incubated for 24 h. The cells were then treated with Tub A, Cyp, and TMZ or DMSO for 72 h, and combinations of Cyp + Tub A, and TMZ + Tub A, and proteins were extracted with radio-immunoprecipitation assay buffer. Proteins were quantified, and the same amount of protein was loaded per lane in sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotted. After blocking with 3% bovine serum albumin solution, the blots were incubated overnight at 4°C with rabbit anti-Slug (Cat# 9585, dilution 1:1000, Cell Signaling, Danvers, MA, USA), rabbit anti-LC3B (Cat# 3868, dilution 1:1000, Cell Signaling), mouse anti-acetylated α-tubulin (Cat# T6793, dilution 1:10,000, Sigma), mouse anti-α-tubulin (Cat# T6074, dilution 1:10,000, Sigma), and mouse anti-β-actin (Cat# A5441, dilution 1:10,000, Sigma). After primary antibody incubation, membranes were washed and incubated for 1 h at room temperature with anti-mouse (Cat# 10794347, dilution 1:20,000, Fisher Scientific) or anti-rabbit secondary antibodies (Cat# 10196124, dilution 1:20,000, Fisher Scientific, Madrid, Spain) conjugated with horseradish peroxidase (HRP) (1:1000, Bio-Rad Laboratories, Hercules, CA, USA). Lumi-Light PLUS (#12015196, Roche Diagnostics Deutschland GmbH, Mannheim, Germany) was used as the HRP substrate for immunodetection.

For the determination of autophagy by Western blot, we seeded the U87MG cells in 6-well plates for 24 h. Then, we treated cells with 20 μM DMSO or 20 μM Tub A for 72 h. After this time, the culture medium was changed for new medium, which also contained DMSO or Tub A, but, in addition, bafilomycin A1 (Baf A1) was added for 4 h to inhibit the degradation of autophagosomes, creating four experimental groups: (1) DMSO, (2) Baf A1, (3) Tub A, and (4) Tub A + Baf A1. After 4 h of treatment with Baf A1, protein extraction was performed. The protein extracts were quantified and a Western blot was performed to study the expression of the autophagy marker LC3B, with the use of the anti-LC3B antibody (AB48394, Abcam, Cambridge, UK). This antibody marks both the cytoplasmic form (LC3B-I) and the lipidated form found in the membrane of autophagosomes (LC3B-II).

Statistical analysis

Data are presented as means ± standard deviations. GraphPad 8.0 Software (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analysis. Statistical tests used were the Student's *t*-test and one-way analysis of variance followed by Tukey's *post hoc* test. $P < 0.05$ was considered statistically significant.

Results

Tubastatin A decreases clonogenicity of U87MG glioblastoma cells

In order to analyze the effect of Tub A on the clonogenic capacity of U87MG cells, a colony formation assay was performed in adherence-dependent conditions. Tub A was able to induce a significant decrease ($P < 0.0001$) in the number of colonies formed in comparison to the control group [Figure 1]. TMZ alone also reduced the clonogenic ability of U87MG cells and the number of colonies. This effect was enhanced when Tub A was administered together with the chemotherapeutic agent TMZ, which is used in the current treatment regimen against GBM. This combination gave the best result among all the drugs and combinations examined in this experiment.

Tubastatin A inhibits migration of U87MG glioblastoma cells

Wound healing assay was performed to study the effect of Tub A on the migration capability of U87MG cells. Cyp treatment did not have any noticeable impact on wound closure [Figure 2]. However, cells treated with TMZ or Tub A were not able to close the wound, indicating that these individual treatments had an inhibitory effect on cell migration. Similar to the colony formation assay, the combination of TMZ and Tub A showed the best result with increased inhibition of migration. These results, together with those from the colony formation assay, indicate that Tub A might have a synergistic effect with TMZ.

Tubastatin A induces apoptosis of U87MG glioblastoma cells

Once we identified that Tub A has an anticancer effect on U87MG cells, we aimed to investigate if it could also induce

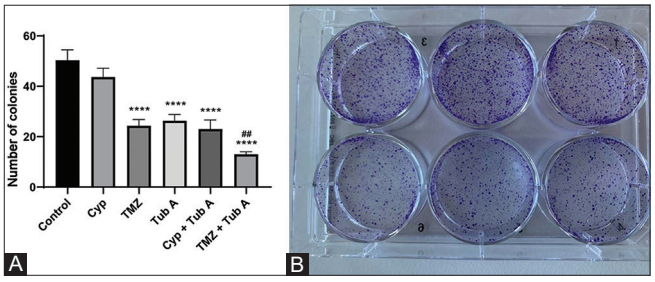

Figure 1: Tubastatin A reduces the clonogenic potential of U87MG glioblastoma cells. (A) Analysis of the colony formation assay. Data are presented as the mean ± SD from $n = 3$ independent samples assayed in triplicate. ****$P < 0.0001$, *vs*. control; ##$P < 0.01$, *vs*. TMZ (one-way analysis of variance followed by Tukey's *post hoc* test). (B) Representative image of the colony formation assay. The top and bottom three wells correspond to the control and Tub A groups, respectively. Cyp: Cyclopamine, SD: Standard deviation, TMZ: Temozolomide, Tub A: Tubastatin A

apoptosis. Accordingly, an apoptosis assay was performed using Caspase-Glo 3/7. Tub A induced an apoptotic response within the first 24 h of treatment. This effect decreased in the next 2 days [Figure 3]. However, TMZ showed the opposite effect. The apoptosis induction was lower in the first 24 h than at 72 h. The most remarkable result of this experiment

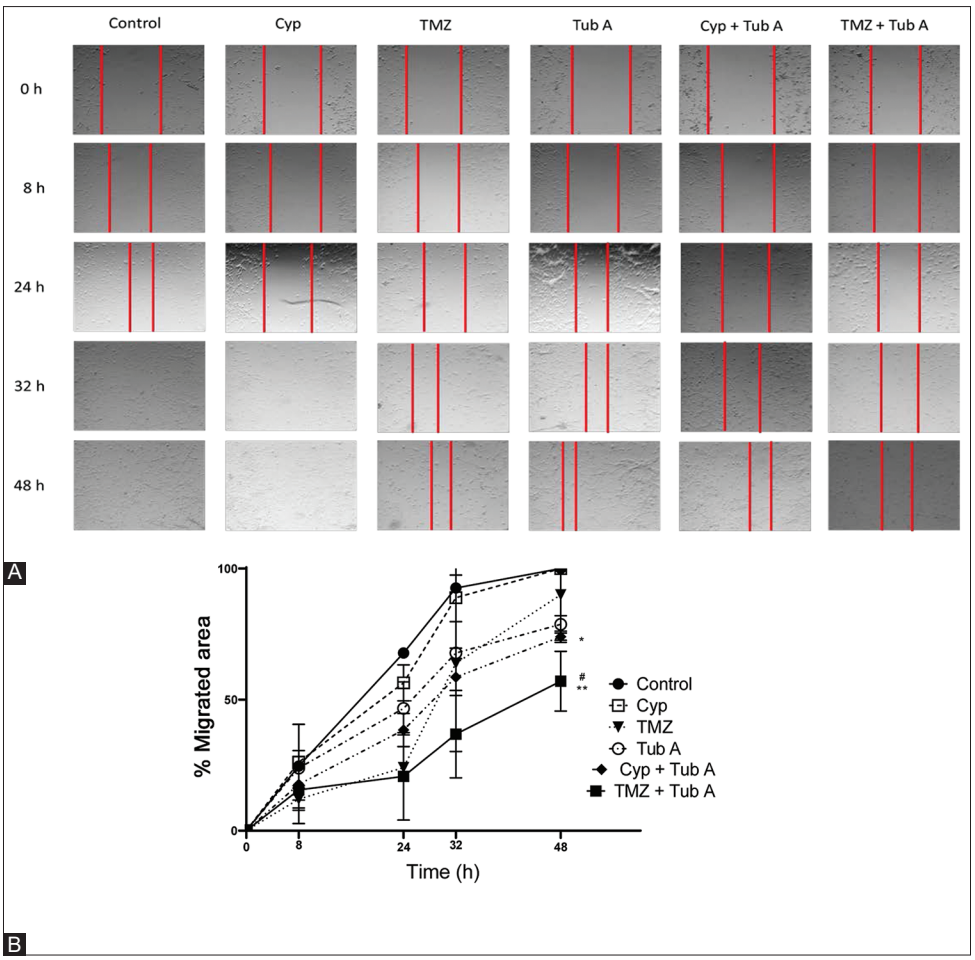

Figure 2: Tubastatin A reduces the migratory potential of U87MG glioblastoma cells and demonstrates a synergistic effect with TMZ as a combination treatment (wound healing assay). (A) Images of the wound healing assay taken at different time points. Red bars show the migration front. (B) Analysis of the migrated area in each condition at different time points of the experiment. Data are presented as the mean ± SD from $n = 3$ independent samples assayed in triplicate. *$P < 0.05$, **$P < 0.01$, *vs*. control; #$P < 0.05$, *vs.* TMZ (one-way analysis of variance followed by Tukey's *post hoc* test). The experiment was repeated three times. Cyp: Cyclopamine, SD: Standard deviation, TMZ: Temozolomide, Tub A: Tubastatin A

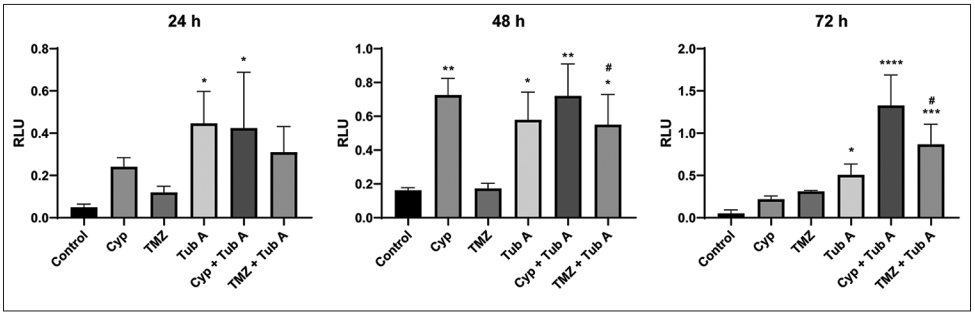

Figure 3: Tubastatin A induces apoptosis in U87MG glioblastoma cells and shows a synergistic effect with TMZ as a combination treatment (Caspase-Glo 3/7 assay at 24, 48, and 72 h). Data are presented as the mean ± SD from $n = 4$ independent samples assayed in triplicate. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, *vs*. control; #$P < 0.05$, *vs*. TMZ (one-way analysis of variance followed by Tukey's *post hoc* test). A405: Absorbance at 405 nm, A492: Absorbance at 492 nm, Cyp: Cyclopamine, RLU: Relative light units, SD: Standard deviation, TMZ: Temozolomide, Tub A: Tubastatin A

was the synergistic effect demonstrated by a combination of TMZ and Tub A, which induced the highest apoptosis at 72 h, suggesting that this combination might be a good candidate for GBM treatment.

Tubastatin A decreases the activation of the Sonic Hedgehog pathway in U87MG glioblastoma cells

To evaluate if Tub A could decrease the activation of the Sonic Hedgehog pathway, RT-qPCR of *Gli1* gene was first performed after treatment with Tub A or DMSO as a vehicle control. The levels of *Gli1* mRNA were lower in the cells, which were previously treated with Tub A [Figure 4A]. Subsequently, a luciferase assay was conducted to assess the activation status of this pathway under control conditions and after treatment with Tub A. In baseline conditions, the Sonic Hedgehog pathway was activated because the signal in the control group was higher than the signal in the negative control group. Treatment with Tub A decreased this activation, which correlates with the RT-qPCR result [Figure 4B]. Thus, the results confirmed that Tub A inhibits the Sonic Hedgehog pathway in U87MG cells.

Tubastatin A increases acetylation of α-tubulin and reverses epithelial–mesenchymal transition in U87MG glioblastoma cells

Western blot analysis of acetylated α-tubulin and total α-tubulin was performed to examine if Tub A was an inhibitor of HDAC6, the main deacetylase of α-tubulin. To do so, cells were treated with the individual treatments and combinations for 72 h and proteins were extracted. On performing Western blot, the levels of acetylated α-tubulin increased in the presence of Tub A, confirming HDAC6 inhibition by Tub A [Figure 5]. Furthermore, we studied the protein levels of Slug, a mesenchymal marker. Tub A and its combinations reverted the mesenchymal phenotype, and transformed the phenotype of the U87MG cells into a more epithelial one. Thus, the results showed that the inhibition of HDAC6 by Tub A reverses epithelial–mesenchymal transition in U87MG cells.

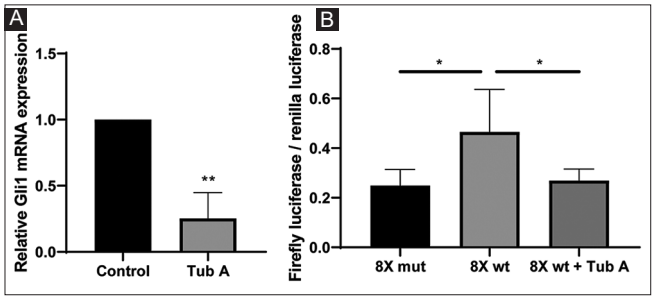

Figure 4: Tubastatin A inhibits Sonic Hedgehog activation in U87MG glioblastoma cells. (A) Analysis of *Gli1* expression by quantitative reverse transcription–polymerase chain reaction after tubastatin A treatment. Data are presented as means ± standard deviation from $n = 3$ independent samples assayed in triplicate. $^{**}P < 0.01$, *vs*. control (Student's *t*-test). (B) Analysis of Sonic Hedgehog activity by luciferase assay after tubastatin A treatment. Data are presented as means ± standard deviation from $n = 4$ independent samples assayed in triplicate. $^{*}P < 0.05$ (one-way analysis of variance followed by Tukey's *post hoc* test)

Tubastatin A reduces the autophagic flux in U87MG glioblastoma cells

Western blot was performed for LC3B protein, whose lipidated version, LC3BII, is an autophagosome marker. Under normal conditions, treatment with Baf A1 increased the expression of LC3BII due to the inhibition of autophagosome degradation [Figure 6]. Nevertheless, bafilomycin treatment of Tub A-treated cells did not result in an increased expression of LC3BII, supporting the hypothesis that HDAC6 is a relevant protein in the autophagic process.

Discussion

GBM is one of the most common and malignant brain tumors.[1] Current treatment of GBM comprises maximal resection followed by radiotherapy and chemotherapy.[2] As patient prognosis continues to be poor, identification of a new therapeutic target against this tumor is of paramount importance.

Panobinostat, a pan-inhibitor of HDACs, has been tested with limited success against glioblastoma. The results from this study demonstrated that it potentiates the effects of TMZ and reverses epithelial–mesenchymal transition in glioblastoma cells.[18] Furthermore, panobinostat acts synergistically with several other compounds, including DZNep, TMZ, and APR-246 to reduce clonogenicity in addition to inducing apoptosis in glioblastoma cells.[19-22]

HDAC6 has been proven to be the most expressed HDAC in GBM, based on the analysis of protein expression of HDAC1 to HDAC11 in GBM tumor samples.[6] Therefore, new inhibitors specifically directed against HDAC6, and not only pan-inhibitors of HDACs such as panobinostat, should be tested against GBM. One of these inhibitors is Tub A, which has been shown to enhance TMZ-induced apoptosis

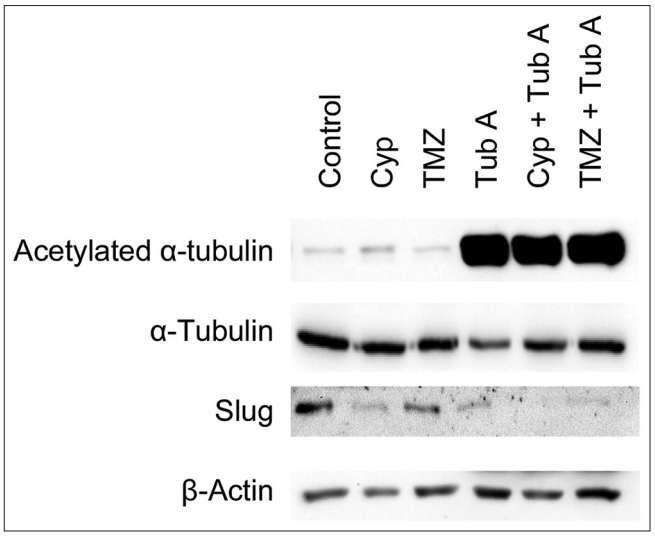

Figure 5: Tubastatin A reverses epithelial-to-mesenchymal transition in U87MG glioblastoma cells. Western blot image of acetylated α-tubulin, total α-tubulin, Slug. Every treatment was added for 72 h. Cyp: Cyclopamine, TMZ: Temozolomide, Tub A: Tubastatin A

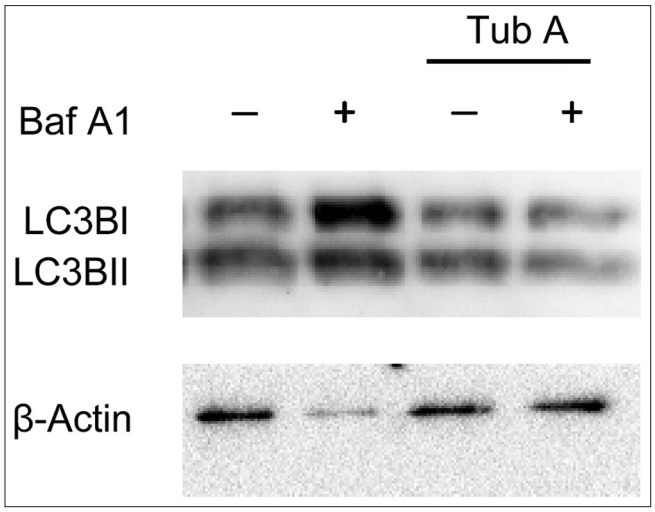

Figure 6: Tubastatin A reduces the autophagic flux in U87MG glioblastoma cells. Western blot image of LC3B and β-actin. Cells were treated with 20 nM tubastatin A for 72 h, and treated with 100 nM Baf A1 6 h before protein extractions. Baf A1: Bafilomycin A1, Tub A: Tubastatin A

and reverse the malignant phenotype of glioblastoma cell lines LN405 and T98G.[23] LN405 is a *p53*-mutated, *PTEN*-mutated, and MGMT-negative cell line, while T98G presents *p53* mutations, *PTEN* mutations, and MGMT expression. Silencing of *HDAC6* has also been shown to have the beneficial effect of inhibiting GBM growth, as HDAC6 promotes cell proliferation and confers TMZ resistance in GBM.[24]

In the present study, the effects of Tub A in the U87 glioblastoma cell line (wild-type *p53*, mutated *PTEN*, and negative MGMT) were examined to determine its impact on clonogenicity, migration, and apoptosis as well as on the Sonic Hedgehog pathway status, epithelial–mesenchymal transition, and autophagic flux, with the overall aim of inhibiting the malignant phenotype of GBM cells.

Treatment with Tub A decreased the clonogenic ability of U87MG cells, concurrent with the results obtained from a model of cholangiocarcinoma.[15] The greatest inhibition of colony formation observed in this study was with a combination of Tub A and TMZ, which indicates that HDAC6 inhibition may sensitize the tumor cells to the chemotherapeutic agent.

The same effect was detected in the wound healing assay. Inhibition of HDAC6 with Tub A was able to reduce the migration ability of U87MG cells, along with the formation of colonies. HDAC6 inhibition also reduced migration in pancreatic[25] and prostate cancer.[26] Again, the best condition to reduce migration was a combination of Tub A with TMZ, strengthening the hypothesis of the synergistic effect of these drugs.

Furthermore, we investigated if Tub A could induce apoptosis in the GBM-derived cell line. The HDAC6 inhibitor induced apoptosis at 24 h compared to the control group, demonstrating a decreasing effect afterwards. In contrast, TMZ exhibited an increasing effect. The greatest effect was observed with a combination of Tub A and TMZ at 72 h after treatment.

Next, we examined if Tub A could inhibit the Sonic Hedgehog pathway. U87MG cells treated with Tub A exhibited reduced levels of *Gli1* mRNA. This result obtained from RT-qPCR was translated into a decreasing activity in the luciferase assay, confirming that the inhibition of HDAC6 by Tub A inhibits the Sonic Hedgehog pathway. This effect has also been observed in GBM-derived cell lines treated with another HDAC6 inhibitor, Tubacin,[27] confirming our results.

In this study, Western blot assay was performed to study epithelial–mesenchymal transition in U87MG cells after treatment with Tub A. We observed that in the presence of Tub A, the levels of acetylated α-tubulin were upregulated due to HDAC6 inhibition. These elevated levels of acetylated α-tubulin were correlated with decreased levels of the mesenchymal marker Slug. As shown by Gu *et al*.,[28] this increase in acetylated α-tubulin could be used as a marker of the epithelial cell phenotype. Taken together, these results reveal that inhibition of HDAC6 may reverse epithelial–mesenchymal transition.

Finally, the present study aimed to evaluate if Tub A could have an effect on autophagy. Tub A-treated cells demonstrated a decrease in autophagic flux compared to the control. This has been previously reported in a cholangiocarcinoma model after treatment with another HDAC6 inhibitor, ACY-1215.[29] Thus, our findings confirmed that inhibition of HDAC6 inhibits the autophagic process in U87MG cells.

Limitations

Our experiments have been done in one glioblastoma cell line, which obviously does not cover the heterogenic phenotype of this tumor. The inhibitory mechanism by which Tub A acts against glioblastoma is not deciphered yet. Comparisons with another HDAC6 inhibitor like ACY-1215 and *in vivo* experiments would be highly recommended.

Conclusions

HDAC6 seems to have potential as an effective therapeutic target in GBM treatment. The treatment strategy of this tumor has remained unchanged in the last 15 years, and it is still not satisfactory. This study analyzed Tub A, which has some efficacy against glioblastoma, and seems to demonstrate a synergistic effect with TMZ, which is currently used to treat this brain tumor.

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Institutional review board statement

The ethical approval is waived because the cell lines are available commercially.

Conflicts of interest

There are no conflicts of interest.

Editor note: JSC is an Editorial Board member of *Glioma*. The article was subjected to the journal's standard procedures, with peer review handled independently of this Editorial Board member and his research group.

References

1. Ostrom QT, Cioffi G, Gittleman H, Patil N, Waite K, Kruchko C, *et al.* CBTRUS statistical report: Primary brain and other central nervous system tumors diagnosed in the United States in 2012-2016. Neuro Oncol 2019;21:v1-100.
2. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, *et al.* Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 2005;352:987-96.
3. Verza FA, Das U, Fachin AL, Dimmock JR, Marins M. Roles of histone deacetylases and inhibitors in anticancer therapy. Cancers (Basel) 2020;12:1664.
4. Wilson AJ, Byun DS, Popova N, Murray LB, L'Italien K, Sowa Y, *et al.* Histone deacetylase 3 (HDAC3) and other class I HDACs regulate colon cell maturation and p21 expression and are deregulated in human colon cancer. J Biol Chem 2006;281:13548-58.
5. Deng R, Zhang P, Liu W, Zeng X, Ma X, Shi L, *et al.* HDAC is indispensable for IFN-γ-induced B7-H1 expression in gastric cancer. Clin Epigenetics 2018;10:153.
6. Li S, Liu X, Chen X, Zhang L, Wang X. Histone deacetylase 6 promotes growth of glioblastoma through inhibition of SMAD2 signaling. Tumour Biol 2015;36:9661-5.
7. Sanchez GJ, Richmond PA, Bunker EN, Karman SS, Azofeifa J, Garnett AT, *et al.* Genome-wide dose-dependent inhibition of histone deacetylases studies reveal their roles in enhancer remodeling and suppression of oncogenic super-enhancers. Nucleic Acids Res 2018;46:1756-76.
8. Guardiola AR, Yao TP. Molecular cloning and characterization of a novel histone deacetylase HDAC10. J Biol Chem 2002;277:3350-6.
9. Verdel A, Curtet S, Brocard MP, Rousseaux S, Lemercier C, Yoshida M, *et al.* Active maintenance of mHDA2/mHDAC6 histone-deacetylase in the cytoplasm. Curr Biol 2000;10:747-9.
10. Bertos NR, Gilquin B, Chan GK, Yen TJ, Khochbin S, Yang XJ. Role of the tetradecapeptide repeat domain of human histone deacetylase 6 in cytoplasmic retention. J Biol Chem 2004;279:48246-54.
11. Ding G, Liu HD, Huang Q, Liang HX, Ding ZH, Liao ZJ, *et al.* HDAC6 promotes hepatocellular carcinoma progression by inhibiting P53 transcriptional activity. FEBS Lett 2013;587:880-6.
12. Kanno K, Kanno S, Nitta H, Uesugi N, Sugai T, Masuda T, *et al.* Overexpression of histone deacetylase 6 contributes to accelerated migration and invasion activity of hepatocellular carcinoma cells. Oncol Rep 2012;28:867-73.
13. Bazzaro M, Lin Z, Santillan A, Lee MK, Wang MC, Chan KC, *et al.* Ubiquitin proteasome system stress underlies synergistic killing of ovarian cancer cells by bortezomib and a novel HDAC6 inhibitor. Clin Cancer Res 2008;14:7340-7.
14. Butler KV, Kalin J, Brochier C, Vistoli G, Langley B, Kozikowski AP. Rational design and simple chemistry yield a superior, neuroprotective HDAC6 inhibitor, tubastatin A. J Am Chem Soc 2010;132:10842-6.
15. Gradilone SA, Radtke BN, Bogert PS, Huang BQ, Gajdos GB, LaRusso NF. HDAC6 inhibition restores ciliary expression and decreases tumor growth. Cancer Res 2013;73:2259-70.
16. Tao H, Chen YY, Sun ZW, Chen HL, Chen M. Silence of HDAC6 suppressed esophageal squamous cell carcinoma proliferation and migration by disrupting chaperone function of HSP90. J Cell Biochem 2018;119:6623-32.
17. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C (T) method. Nat Protoc 2008;3:1101-8.
18. Urdiciain A, Meléndez B, Rey JA, Idoate MA, Castresana JS. Panobinostat potentiates temozolomide effects and reverses epithelial – Mesenchymal transition in glioblastoma cells. Epigenomes 2018;2:5.
19. De La Rosa J, Urdiciain A, Zazpe I, Zelaya MV, Meléndez B, Rey JA, *et al.* The synergistic effect of DZ-NEP, panobinostat and temozolomide reduces clonogenicity and induces apoptosis in glioblastoma cells. Int J Oncol 2020;56:283-300.
20. De La Rosa J, Urdiciain A, Zelaya MV, Zazpe I, Meléndez B, Rey JA, *et al.* APR-246 combined with 3-deazaneplanocin A, panobinostat or temozolomide reduces clonogenicity and induces apoptosis in glioblastoma cells. Int J Oncol 2021;58:312-30.
21. de la Rosa J, Urdiciain A, Aznar-Morales JJ, Meléndez B, Rey JA, Idoate MA, *et al.* Panobinostat and its combination with 3-Dezaneplanocin-A induce apoptosis and inhibit *in vitro* tumorigenesis and metastasis in GOS-3 glioblastoma cell lines. Cancer Transl Med 2018;4:39-47.
22. de la Rosa J, Urdiciain A, Meléndez B, Rey JA, Idoate MA, Castresana JS. *In vitro* therapy against glioblastoma cells by 3-Dezaneplanocin-A, panobinostat and temozolomide. Glioma 2018;1:22-6.
23. Urdiciain A, Erausquin E, Meléndez B, Rey JA, Idoate MA, Castresana JS. Tubastatin A, an inhibitor of HDAC6, enhances temozolomide-induced apoptosis, and reverses the malignant phenotype of glioblastoma cells. Int J Oncol 2019;54:1797-808.
24. Wang Z, Hu P, Tang F, Lian H, Chen X, Zhang Y, *et al.* HDAC6 promotes cell proliferation and confers resistance to temozolomide in glioblastoma. Cancer Lett 2016;379:134-42.
25. Song H, Niu X, Quan J, Li Y, Yuan L, Wang J, *et al.* Discovery of specific HDAC6 inhibitor with anti-metastatic effects in pancreatic cancer cells through virtual screening and biological evaluation. Bioorg Chem 2020;97:103679.
26. Thakur N, Hamidi A, Song J, Itoh S, Bergh A, Heldin CH, *et al.* Smad7 enhances TGF-β-induced transcription of c-Jun and HDAC6 promoting invasion of prostate cancer Cells. iScience 2020;23:101470.
27. Yang W, Liu Y, Gao R, Yu H, Sun T. HDAC6 inhibition induces glioma stem cells differentiation and enhances cellular radiation sensitivity through the SHH/Gli1 signaling pathway. Cancer Lett 2018;415:164-76.
28. Gu S, Liu Y, Zhu B, Ding K, Yao TP, Chen F, *et al.* Loss of α-tubulin acetylation is associated with TGF-β-induced epithelial-mesenchymal transition. J Biol Chem 2016;291:5396-405.
29. Peixoto E, Jin S, Thelen K, Biswas A, Richard S, Morleo M, *et al.* HDAC6-dependent ciliophagy is involved in ciliary loss and cholangiocarcinoma growth in human cells and murine models. Am J Physiol Gastrointest Liver Physiol 2020;318:G1022-33.