Tigecycline Inhibits Glioma Growth by Regulating miRNA-199b-5p–HES1–AKT Pathway
Rui Yang1, Liang Yi1, Zhen Dong1, Qing Ouyang2, Ji Zhou2,3, Yi Pang1, Yanan Wu1, Lunshan Xu2, and Hongjuan Cui1

Abstract

Tigecycline is a broad-spectrum, first-in-class glycylcycline antibiotic currently used to treat complicated skin infections and community-acquired pneumonia. However, there is accumulating evidence showing that tigecycline has anticancer properties. In this study, we found tigecycline could inhibit cell proliferation by inducing cell-cycle arrest, but not apoptosis in glioma. To find the underlying mechanism of how tigecycline inhibits cell proliferation, the expression of miRNAs, which were related to regulating cell-cycle progression, was detected with miRNA assay. We found that miR-199b-5p expression was significantly increased after tigecycline treatment, and miR-199b-5p target gene HES1 was downregulated. In addition, the PI3K/AKT pathway was inhibited and p21 expression was increased. When treated with tigecycline and miR-199b-5p antagonist simultaneously in glioma cells, we found that miR-199b-5p antagonist could partly block the effects induced by tigecycline. Tigecycline effectively upregulated miR-199b-5p expression and inhibited tumor growth in the xenograft tumor model of U87 glioma cells. These results suggest that tigecycline may induce cell-cycle arrest and inhibit glioma growth by regulating miRNA-199b-5p–HES1–AKT pathway. Thus, tigecycline is a promising agent in the treatment of malignant gliomas.

Introduction

Malignant gliomas are the highly malignant primary central nervous system neoplasms associated with poor survival and are invariably fatal (1). They are considered the most aggressive form of human cancers, characterized largely by their rapid growth, extensive angiogenesis, and invariable resistance to all current therapies (2). Despite advances in the diagnosis and treatment, the current standard of care for patients with glioma provides only palliation with a median survival of about 15 months (3, 4). Therefore, exploration of a new approach is urgently needed.

Tigecycline is a first-in-class glycylcycline antibiotic, developed as a third-generation structural analogue of older tetracyclines (5, 6). As a new member of the broad-spectrum antibiotic drug, it displays potent activity against both gram-positive and gram-negative bacteria, including many multidrug-resistant pathogens (7). Tigecycline is currently approved in North America for the treatment of adults with intra-abdominal, skin or skin structure infections, or those with community-acquired pneumonia. The drug has also been used for the treatment of additional infections, including ventilator-associated pneumonia, nosocomial sepsis, and bacteremia (8). Beyond its role as an antimicrobial, there is accumulating evidence showing that tigecycline has anticancer properties. It could inhibit human acute myeloid leukemia and gastric cancer growth (9, 10). However, the function of tigecycline in glioma is still unknown.

miRNAs, a family of short endogenous noncoding RNAs, act as posttranscriptional gene regulators through binding their target mRNAs (11). Growing evidence has proved that miRNAs regulate major cellular processes involved in tumor biology, including cell proliferation, differentiation, apoptosis, and metastasis (12, 13). miR-199b-5p could regulate Notch pathway through its targeting of the transcription factor HES1 (14, 15). Overexpression of miR-199b-5p could inhibit AKT signaling pathway and decrease cyclin D1 expression and negatively regulated proliferation by inducing cell-cycle arrest at G1 phase in breast cancer cells (16). Downregulation of HES1 expression by miR-199b-5p negatively regulated the proliferation rate and anchorage-independent growth of medulloblastoma cells (14). This evidence demonstrated that miR-199b-5p plays important roles in the tumor progression.

HES1, a basic helix-loop-helix transcriptional repressor, is a downstream target of the Notch signaling pathway (17). The HES1 protein contains both DNA-binding and protein–protein interaction domains important for its function as transcriptional regulator (including negative regulation of its own transcription; refs. 18, 19). Notch-independent HES1 expression can also result from Hedgehog and c-Jun N-terminal kinase signaling, as well as from RAS/MAPK signaling (20). Regulation of HES1 expression and activity is dependent on the tissue, spatial, and temporal factors and the proteins with which it interacts (17, 20). Overexpression of HES1 is associated with a variety of human cancers, including T-cell acute lymphoblastic leukemia (ALL) and breast, cervical, prostate, ovarian, colon, and non–small cell lung cancer (NSCLC; refs. 21–25). Recently, HES1 was shown as a self-renewal
In this research, we investigated the effect of tigecycline on malignant glioma cells. We found that tigecycline could inhibit the proliferation of glioma cells, but it had no effect on normal cells. Tigecycline induced cell-cycle arrest but not apoptosis in glioma cells. In addition, we found that miR-199b-5p was upregulated and its target gene HES1 was downregulated after tigecycline treatment. By treating glioma cells with tigecycline and miR-199b-5p antagonist, we found that miR-199b-5p antagonist could block all the effects induced by tigecycline. Tigecycline effectively inhibited tumor growth in the xenograft tumor model of U87 glioma cells. Thus, tigecycline seems to be a promising agent for the treatment of malignant gliomas.

Materials and Methods

Cell culture

Primary astrocytes were prepared as described previously (26). In brief, cerebral cortices were dissected from postnatal (P0–P2) Sprague–Dawley rats and dissociated in 0.25% trypsin at 37°C for 10 minutes. After passage through a Pasteur pipette several times, the primary astrocytes were cultured in DMEM/F12 (Invitrogen), supplemented with 10% FBS (Sigma-Aldrich), 2 mmol/L l-glutamine (Invitrogen), 100 U/mL penicillin, 0.1 mg/mL streptomycin (Invitrogen), and 0.1 mmol/L nonessential amino acid (Invitrogen). The human glioma cell lines U118, U87, and U251, normal glia cell SVG/P12, and murine glioma cell line GL261 were purchased from ATCC in 2014 and were passaged in our laboratory for less than 6 months. Glioma cells were cultured in DMEM (Invitrogen), supplemented with 10% FBS, 2 mmol/L l-glutamine, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. The human normal glia cells SVG/P12 were cultured in MEM (Invitrogen), supplemented with 10% FBS, 2 mmol/L l-glutamine, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. All cells were maintained in a humidified atmosphere containing 5% CO2 at 37°C.

Reagents

Tigecycline (Wyeth) was dissolved in DMSO. Rabbit monoclonal anti-HES1, anti-cyclin D1, anti-CDK4, and anti-CDK6, mouse monoclonal anti–caspase-3, and anti-GAPDH were obtained from Santa Cruz Biotechnology. Rabbit monoclonal anti-AKT, anti-phospho-AKT, and anti-p21 were obtained from Santa Cruz Biotechnology. Rabbit monoclonal anti-HES1, anti-cyclin D1, anti-CDK4, and anti-CDK6, mouse monoclonal anti–caspase-3, and anti-GAPDH were obtained from Santa Cruz Biotechnology. Rabbit monoclonal anti-AKT, anti-phospho-AKT, and anti-p21 were obtained from Cell Signaling Technology. Mouse monoclonal anti-Ki67, Annexin V-FITC Apoptosis Detection Kit and propidium iodide (PI) were obtained from BD Biosciences. Synthetic antagonimir-199b-5p and anti-miRNA inhibitor negative control were obtained from RiboBio.

Cell viability assay

For cell viability assay, 5 × 10^3 glial cells or primary astrocytes or 2 × 10^5 glioma cells per well were seeded in 96-well plates and allowed to attach overnight at 37°C. Culture medium containing vehicle or drugs was added to the medium in each well, and cells were cultured at 37°C for indicated time points. 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, 298-23-1) cytotoxicity assay was used to measure cell viability according to the manufacturer’s protocol. At indicated time points, cells in 96-well plate were incubated with MTT (125 μg/mL) in growth medium for 4 hours at 37°C. Formazan crystals were solubilized with DMSO and quantified by measuring the absorbance at 560 nm on a plate reader. Data were presented as the percentage of survival relative to vehicle-treated control culture.

BrdUrd staining

For bromodeoxyuridine (BrdUrd) immunofluorescent staining, cells were grown on coverslips. Culture medium containing vehicle or drugs was added to the medium in each well, and cells were incubated at 37°C for 48 hours and then incubated with 10 μg/mL BrdUrd (Sigma) for 30 minutes, washed with PBS, and fixed in 4% paraformaldehyde for 20 minutes. Subsequently, cells were pretreated with 1 mol/L HCl and blocked with 10% goat serum for 1 hour, followed by a monoclonal rat primary antibody against BrdUrd (1:200, ab6326; Abcam) for 1 hour and Alexa Fluor 594 goat anti-rat IgG secondary antibody, (H+L; Invitrogen). DAPI (300 nmol/L) was used for nuclear staining; the percentage of BrdUrd was calculated from at least 10 microscopic fields (Nikon 80i; Nikon Corporation).

Soft agar assay

In total, 2 × 10^5 cells were mixed with 0.3% Noble agar in growth medium containing vehicle or drugs and plated into 6-well plates containing a solidified bottom layer (0.6% Noble agar in growth medium). The colonies were photographed after 14 to 21 days and recorded.

Flow cytometry

Cells were cultured with medium containing vehicle or drugs at 37°C for 24 hours. For apoptosis assay, cells were collected and washed twice with cold PBS buffer, resuspended in 100 μL of binding buffer, incubated with 5 μL Annexin V conjugated to FITC and 10 μL PI for 15 minutes, and analyzed by flow cytometry with CellQuest analysis software. For cell-cycle assay, 1 × 10^6 cells were harvested and washed twice with cold PBS, followed by fixation with ice-cold 70% ethanol overnight at 4°C. After washing twice with PBS, the cells were incubated with PI (BD Biosciences) and RNaseA for 30 minutes at room temperature. The cells were then analyzed using a FACS C6 (BD Biosciences) with CellQuest software.

Western blot analysis

Drug- or vehicle-treated cells were lysed in a lysis buffer containing 50 mmol/L Tris-HCL pH 7.5, 150 mmol/L NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, AND 0.1% SDS with complete protease inhibitor cocktail (Roche) and phosphatase inhibitors (Sigma-Aldrich). Cell lysates were separated by 10% SDS-PAGE and were transferred to a polyvinylidene difluoride membrane. SDS-PAGE gels were calibrated using MagicMark XP Western Standard (Invitrogen). Primary antibodies were used at a dilution of 1:1,000. Secondary antibodies (peroxidase-labeled anti-mouse and anti-rabbit antibodies) were used at a dilution of 1:5,000. Bound antibodies were visualized by chemiluminescence using the ECL Prime Western Blotting Detection System (GE Healthcare), and luminescent images were analyzed with a Lumino Image (LAS- 4000 Mini; FujiFilm Inc.).

miRNA assay

Cells were seeded and cultured with medium containing vehicle or drugs at 37°C for 24 hours. Total RNA was extracted from
cultured cells using QIAzol and the miRNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol. Then, the expressions of miRNA that are related to regulating cell cycle were detected with RiboArray miDETECT MicroRNA Assay (RiboBio). Cluster 3.0 was used to generate heatmap.

RNA extraction, reverse transcription, and qRT-PCR
Total RNA was extracted from cultured cells using QIAzol and the miRNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol. The purity and concentration of all RNA samples were quantified using NanoDrop 2000 (Thermo Fisher Scientific). The qRT-PCR method has been previously described (27). PCR was performed in 96-well plates using StepOnePlus (Applied Biosystems). All reactions were performed in triplicate. Hsa-miR-199b-5p and endogenous control RNO6B TaqMan microRNA assays were obtained from Applied Biosystems. SYBR Green qRT-PCR was performed for qRT-PCR of miRNA, and the β-actin housekeeping gene was used to normalize the variation in the cDNA levels.

Transient transfection
The miR-199b-5p antagomir (100 nmol/L) was transfected without Lipofectamine RNAiMAX Reagent according to the manufacturer’s protocol (RiboBio). We cultured glioma cells with medium containing tigecycline and miR-199b-5p antagomir simultaneously in 6-well plates.

Animal studies
Animal experiments were performed in compliance with the Guidelines of the Institute for Laboratory Animal Research, Southwest University (Chongqing, China). Five- to 6-week-old male nude mice were used in the experiments. Subcuticular injections were performed following a previous protocol with minor modifications (28). Briefly, U87 (1 x 10⁶ cells in 100 µL of PBS) were inoculated subcutaneously into the right flank of nude mice. Tumor volume was measured every 4 days. Tumor volume was calculated by \( V = (L \times W^2)/2 \), in which \( L \) is the length and \( W \) is the width in millimeters. When the tumors reached a mean volume of 50 to 70 mm³, 100 µL of tigecycline (100 mg/kg in DMSO) or vehicle (DMSO) were injected intraperitoneally once per day for 10 days.

Immunohistochemical staining
Paraffin-embedded tumor tissues were sectioned at 5 µm, deparaffinized, and rehydrated. For antigen retrieval, sections were treated for 20 minutes at 95°C in 10 mmol/L citrate buffer (pH 6.0) in a laboratory microwave oven and subsequently washed in PBS. For IHC, after quenching of endogenous peroxidase activity and blocking with normal goat serum, sections were incubated sequentially with K67 primary antibodies (1:100, clone 550609; BD Pharmingen), biotinylated goat anti-mouse IgG, and the ABC reagent (Vector Laboratories). The immunostaining was visualized with 3, 3’-diaminobenzidine (Sigma). Sections were then counterstained with hematoxylin before being examined using a light microscope.

Statistical analysis
All observations were confirmed by at least three independent experiments. Quantitative data are expressed as the mean ± SD. Two-tailed Student t test was performed for paired samples. \( P < 0.05 \) was considered statistically significant.

Results
Tigecycline inhibited cell growth and proliferation in glioma cells
To examine the effect of tigecycline on cell viability, glioma cells were treated with different concentrations of tigecycline. As shown in Fig. 1A, the proliferation rates of tested cell lines (GL261, U87, U251, and U118) were reduced in a dose-dependent manner. In addition, the cell viabilities were reduced with the increased time of incubation (Fig. 1B). The vehicle (DMSO) had no effect on the glioma cell viability (data not shown). Importantly, tigecycline alone did not affect cell viability of normal cells, such as human glia cell line SVGp12 and rat primary astrocytes (Fig. 1A and B). Above data were confirmed by BrdUrd incorporation in the SVGp12, U87, and U118 cell lines, where the tigecycline-treated glioma cells showed more than 25% reduction in DNA synthesis compared with control cells in glioma cell lines, but not glia cell line (Fig. 1C). To further assess the effect of tigecycline on colony formation, soft agar assay was employed in vitro. As shown in Fig. 1D, the colonies were smaller and lesser in tigecycline-treated cells compared with the controls. These results demonstrated that tigecycline could inhibit cell growth and proliferation in glioma cells.

Tigecycline induced cell-cycle arrest, but not apoptosis, in glioma cells
To determine whether the reduced cell viability was due to apoptosis, glioma cells were stained with Annexin V-FITC and PI, then analyzed by flow cytometry. As shown in Fig. 2A, there was no significant apoptosis after tigecycline treatment. In addition, it was noted that the expression of 17 kDa cleaved fragment of caspase-3 was not detected after treatment with tigecycline. These results also confirmed that tigecycline did not induce apoptosis in glioma cells (Fig. 2B). As cell-cycle progression is usually related to cell proliferation, the U87 and U118 cell cycles were analyzed by flow cytometry to examine whether tigecycline inhibits cell proliferation by inducing cell-cycle arrest. Representative histograms and the results were summarized in Fig. 2C, and the treatment with tigecycline resulted in a marked increase in the percentage of both U87 and U118 cells in the G0 phase. The results demonstrated that tigecycline could induce cell-cycle arrest at G0 phase in glioma cells. To confirm the results, we measured the expression of cyclin D1, CDK4, and CDK6, which could promote cells to pass the G1-S checkpoint. We found that the expressions of cyclin D1 and CDK4 were reduced after tigecycline treatment (Fig. 2D). These results suggested that tigecycline inhibited cell proliferation by inducing cell-cycle arrest, but not apoptosis in glioma.

Tigecycline treatment of glioma cells induced the dysregulation of miR-199b-5p and HES1
To investigate the unknown mechanisms of proliferation inhibition after the exposure of glioma cells to tigecycline, we focused on miRNAs that are related to regulating cell-cycle progression. Using RiboArray miDETECT MicroRNA Assay, we assessed the expression of miRNAs that are related to regulating cell-cycle progression after the exposure of U87 cells to 10 µmol/L tigecycline for up to 24 hours (Fig. 3A). We found that the levels of miR-199b-5p was significantly upregulated at 24 hours after tigecycline treatment compared with control cells (Fig. 3B). To confirm this result, we assessed miR-199b-5p expression after tigecycline treatment by employing qRT-PCR. The expression of miR-199b-5p was significantly increased at 12 and 24 hours after tigecycline
treatment compared with their controls in U87 and U118 cells (Fig. 3C). Then, we measured the expression of HES1, which is a target gene of miR-199b-5p. We found that the expression of HES1 was reduced after tigecycline treatment in glioma cells (Fig. 3D). Merino and Singla had reported that the overexpression of HES1 was related to AKT activation and cell proliferation (29). The expression of PTEN was significantly increased after tigecycline treatment (Fig. 3D). We assessed the phosphorylation of AKT and its downstream target p21 by using Western blotting and qRT-PCR. Figure 3D showed that AKT phosphorylation at position Ser473 was significantly reduced at 12 and 24 hours after the application of tigecycline. In addition, the expression of p21 was increased. All these data demonstrated that tigecycline suppressed HES1 expression and AKT phosphorylation by upregulating miR-199b-5p.

**MiR-199b-5p antagomir blocked the effects induced by tigecycline in glioma cells**

As tigecycline upregulated miR-199b-5p and decreased HES1 expression and AKT phosphorylation, we hypothesized that tigecycline might inhibit cell proliferation by regulating miR-199b-5p–HES1–AKT pathway in glioma. To prove the inference, we exposed glioma cells to tigecycline and miR-199b-5p antagomir simultaneously. qRT-PCR suggested that the expression of miR-199b-5p was reduced compared with the tigecycline-treated glioma cells (Fig. 4A). The results suggested that the upregulation of miR-199b-5p in tigecycline-treated cells was blocked. In addition, Western blot assay showed that miR-199b-5p antagomir increased the HES1 expression in tigecycline-treated cells (Fig. 4B). Then, we investigated the proliferation rate of glioma cells that were treated with tigecycline or tigecycline and miR-199b-5p antagomir simultaneously for up to 48 hours by using MTT assay. The results revealed that the downregulation of miR-199b-5p in tigecycline-treated cells could increase cell proliferation rate (Fig. 4C). Cell cycle was analyzed by flow cytometry; the results showed that the downregulation of miR-199b-5p promoted cell proliferation by blocking the cell-cycle arrest induced by tigecycline in glioma cells (Fig. 4D). Similarly, the downregulation of miR-199b-5p also increased cyclin D1 expression in tigecycline-treated glioma cells (Fig. 4E). Finally, we measured the activation of AKT. Western blot assay showed that miR-199b-5p antagomir increased the...
phosphorylation level of AKT compared with tigecycline-treated cells (Fig. 4E). Taken together, all these results revealed that miR-199b-5p antagomir blocked the effects induced by tigecycline and demonstrated that tigecycline inhibited cell proliferation by regulating miR-199b-5p–HES1–AKT pathway in glioma.

We determined whether tigecycline inhibits growth and upregulates miR-199b-5p in U87 cells in vivo. Nude mice were inoculated subcutaneously with 1 × 10^6 U87 cells. When tumors reached 50 to 70 mm^3 in volume, tigecycline (100 mg/kg in DMSO) or DMSO were administered intraperitoneally every 24 hours for 10 days, and tumor growth was observed for 10 days after the cessation of treatment. Ten days after the cessation of drug injection, tumor growth was significantly inhibited by tigecycline (Fig. 5A). At the termination of the experiment, tumors were excised, and the weight of wet tumors was lower after tigecycline treatment compared with their controls (Fig. 5B). To determine whether tigecycline suppressed the tumor progression...
of U87 cells by inhibiting cell proliferation, the expression of Ki67, a well-known cell proliferation marker, was examined in tumor xenograft tissues by immunohistochemical staining. As shown in Fig. 5C, the expression of Ki67 in tumor tissues treated with tigecycline was decreased compared with the controls. Western blot assay showed that the expression of cyclin D1 and CDK4 was significantly decreased (Fig. 5E). These results suggested that tigecycline might induce cell-cycle arrest and inhibit cell proliferation in tumor xenograft mice. To examine whether tigecycline upregulated miR-199b-5p in vivo, we examined the expression of miR-199b-5p by using qRT-PCR. We found that the expression of miR-199b-5p increased significantly in tumors treated with tigecycline (Fig. 5D). In addition, Western blot assay showed that the expression of HES1 and the phosphorylation level of AKT were decreased in tumors treated with tigecycline compared with those of DMSO-treated (Fig. 5E). These results demonstrated that tigecycline inhibited tumor growth by regulating miR-199b-5p–HES1–AKT pathway in glioma.

Discussion

Tigecycline is a broad-spectrum, first-in-class glycylcycline antibiotic currently used to treat complicated skin infections and community-acquired pneumonia (5). However, there is evidence shown that tigecycline has anticancer properties in human acute myeloid leukemia (9). In our previous study, we found that tigecycline inhibits proliferation and induces autophagy in gastric cancer cells by activating the AMPK pathway (10). In this study, we demonstrated that tigecycline inhibited cell proliferation in glioma cells in a concentration-dependent manner with IC50 of approximately 10 μmol/L. Tigecycline had no effect on cultured astrocytes, suggesting that the reduction of cell viability is restricted to neoplastic types. In addition, using soft agar assay, we found that tigecycline inhibited survival and self-renewal of glioma cells in vitro. Tumor xenograft experiments in nude mice indicated that tigecycline significantly inhibited tumor growth in vivo. Moreover, immunostaining assays revealed that the expression of Ki67 in tumor tissues treated with tigecycline was decreased compared with the controls, suggesting that tigecycline inhibited the tumor formation of glioma cells by reducing cell proliferation. Flow cytometry and Western blot assay showed that the reduction of cell viability after exposure to tigecycline was not due to apoptosis. In addition, we did not find any autophagy after the treatment with tigecycline in glioma cells. Instead, tigecycline induced cell-cycle arrest at G1 phase in glioma cells. Furthermore, Western blot analysis with expression of cyclin D1, CDK4, and p21 confirmed tigecycline-induced cell-cycle arrest in glioma cells. All of our findings together indicated that tigecycline inhibited cell proliferation and growth by inducing cell-cycle arrest, but not apoptosis, in glioma cells.

To understand the mechanism of reduction of cell proliferation that we observed after tigecycline exposure, our study focused on...
miRNAs. Studies have shown that miRNAs are involved in cancer pathogenesis by regulating cell proliferation and tumor growth (30, 31). Therefore, more and more researchers focus in their potential applications in cancer therapeutics (32). Previous research has discovered different expressions of miRNAs between the poor and good survival groups (33). miRNA array analysis showed that these miRNAs are involved in pathways regulating tumor cell proliferation, cell cycles, and cell apoptosis. Using Riboblock miDETECT MicroRNA Assay, we assessed the expression of miR-199b-5p after tigecycline treatment for 48 hours, and proliferation rates were assessed using MTT assay. D, U87 and U118 cells were treated with 10 μmol/L tigecycline, tigecycline and miR-199b-5p antagomir, or DMSO for 48 hours; cell cycle was analyzed by flow cytometry. E, Western blot assay was performed to assess the expression of p-AKT, p21, and cyclin D1 after tigecycline or tigecycline and miR-199b-5p antagomir treatment for 48 hours. All data are shown as the mean ± SD; **, P < 0.01. All P values are based on analysis control versus treatment.

Figure 4.
MiR-199b-5p antagomir (Antag) blocked the effects induced by tigecycline (TIG) in glioma cells. A, qRT-PCR was performed to assess the expression of miR-199b-5p after tigecycline or tigecycline and miR-199b-5p antagomir treatment for 48 hours. B, Western blot analyses of HES1 after treatment of U87 and U118 cells with tigecycline, tigecycline and antagomir, or control (DMSO). C, U87 and U118 cells were treated with tigecycline or tigecycline and miR-199b-5p antagomir for 48 hours, and proliferation rates were assessed using MTT assay. D, U87 and U118 cells were treated with 10 μmol/L tigecycline, tigecycline and miR-199b-5p antagomir, or DMSO for 48 hours; cell cycle was analyzed by flow cytometry. E, Western blot assay was performed to assess the expression of p-AKT, p21, and cyclin D1 after tigecycline or tigecycline and miR-199b-5p antagomir treatment for 48 hours. All data are shown as the mean ± SD; **, P < 0.01. All P values are based on analysis control versus treatment.
increased. These results demonstrated that tigecycline inhibited HES1 expression and the activation of AKT signaling.

MiR-199b-5p could regulate Notch pathway through its targeting of the transcription factor HES1 (14, 15). In this study, we investigated the function of miR-199b-5p for the regulation of HES1 after tigecycline exposure in glioma cells. We clearly demonstrated the upregulation of miR-199b-5p at 12 and 24 hours after exposure of glioma cells to tigecycline and demonstrated that this upregulation was accompanied by HES1 downregulation. Exposing glioma cells to both tigecycline and miR-199b-5p antagomir, we found that the upregulation of miR-199b-5p and the downregulation of HES1 induced by tigecycline were all blocked. miR-199b-5p antagomir increased the phosphorylation level of AKT compared with tigecycline-treated cells. In addition, the reduction of cell proliferation and cell-cycle arrest induced by tigecycline was also blocked after miR-199b-5p antagomir treatment. Finally, we measured the activation of AKT. Western blot assay showed that miR-199b-5p antagomir increased the phosphorylation level of AKT and decreased p21 expression compared with tigecycline-treated cells. These results suggested that tigecycline-induced miR-199b-5p upregulation might be a novel mechanism of HES1 downregulation after drug exposure.

In conclusion, we first demonstrated that tigecycline inhibited glioma cells proliferation and growth both in vitro and in vivo. Second, we identified the miR-199b-5p–HES1–AKT pathway as a novel mechanism underlying the antitumor effect of tigecycline. Therefore, we suggest the use of tigecycline as a new anticancer agent for malignant glioma because of its prominent effect and its new anticancer mechanism of regulating miR-199b-5p–HES1 pathway.

Figure 5.
An in vivo study of the enhanced antitumor effect of tigecycline. A, U87 cells (1 × 10^6) were injected subcutaneously into the right flank of male nude mice (n = 4 for each group). When the tumors reached 50 to 70 mm³ in volume, intraperitoneal injections of tigecycline (TIG; 100 mg/kg) were administered every 24 hours for 10 days, and tumor volume was measured for another 10 days after the cessation of treatment. B, size and weight of xenograft tumor were measured at the termination of the experiment. C, immunohistochemical staining for Ki67 in tumor tissues. DMSO, DMSO for control; TIG, tigecycline treatment. D, qRT-PCR was performed to assess the expression of miR-199b-5p in the tumor tissues treated with DMSO or tigecycline. E, Western blot analyses of HES1, p-AKT, p21, cyclin D1, and CDK4 in the tumor tissues treated with DMSO or tigecycline. All data are shown as the mean ± SD; **, P < 0.01. All P values are based on analysis control versus treatment.
Tigecycline Inhibits Glioma Growth

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: R. Yang, Z. Dong, L. Xu, H. Cui
Development of methodology: R. Yang, L. Yi, Z. Dong, J. Zhou, Y. Pang, Y. Wu, H. Cui
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Yang, L. Yi, Q. Ouyang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Yang, L. Yi, Z. Dong, Y. Wu, L. Xu, H. Cui
Writing, review, and/or revision of the manuscript: R. Yang, L. Yi, H. Cui
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Yi, Z. Dong, Q. Ouyang, J. Zhou, L. Xu, H. Cui
Study supervision: R. Yang, H. Cui

References
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