Biochemical and Biophysical Research Communications 446 (2014) 105–112

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Antibiotic drug tigecycline inhibited cell proliferation and induced autophagy in gastric cancer cells

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ARTICLE INFO

Article history:
Received 10 February 2014
Available online 28 February 2014

Keywords:
Tigecycline
Gastric cancer
Proliferation inhibition
Autophagy

ABSTRACT

Tigecycline acts as a glycylcycline class bacteriostatic agent, and actively resists a series of bacteria, specifically drug fast bacteria. However, accumulating evidence showed that tetracycline and their derivatives such as doxycycline and minocycline have anti-cancer properties, which are out of their broader antimicrobial activity. We found that tigecycline dramatically inhibited gastric cancer cell proliferation and provided an evidence that tigecycline induced autophagy but not apoptosis in human gastric cancer cells. Further experiments demonstrated that AMPK pathway was activated accompanied with the suppression of its downstream targets including mTOR and p70S6K, and ultimately induced cell autophagy and inhibited cell growth. So our data suggested that tigecycline might act as a candidate agent for pre-clinical evaluation in treatment of patients suffering from gastric cancer.

1. Introduction

Nowadays the gastric cancer is the second-leading cause of cancer-related mortality worldwide, and advanced gastric cancer has a poor prognosis [1]. Although the incidence of gastric cancer has declined over the past two decades, it still has a high death rate [2]. There have been some progresses in the treatment of gastrointestinal malignancy, including gastrectomy, chemotherapy and radiochemotherapy. Nevertheless, none of these has a good prognosis for advanced stage patients [1]. Therefore, it's necessary to identify some new agents that could effectively target gastric cancer.

As the first representative of the glycylcyclines, tigecycline is approved for clinical anti-bacterial treatment by US FDA in 2005, which is derived from minocycline by using a t-butylglycylamido group to take the place of the hydrogen at position nine [3]. Moreover, as a new member of the broad-spectrum antibiotic drug, tigecycline has a structurally and functionally similarity with tetracycline [4]. Accumulating evidence has shown that tetracycline and their derivatives such as doxycycline and minocycline have anti-cancer properties, which are out of their broader antimicrobial activity. Recent reports showed that tigecycline could block the protein synthesis of mitochondria in hematological malignancies [5]. However, whether the antibacterial tigecycline has an efficacy on tumor growth, especially on gastric cancer cells remains unknown.

Particularly, adenosine monophosphate-activated protein kinase (AMPK), a family of serine/threonine protein kinase, is one of the considerable stress-sensing enzymes, which could sensitize cellular stress and actively regulate metabolism and cell proliferation [6]. For instance, AMPK is activated by the increasing amount of AMP bound to AMPK with the ATP depletion by multiple mechanisms including the inhibition of mitochondrial ATP synthesis and the increasing of the concentration of AMP and ADP [7]. Furthermore, the mammalian target of rapamycin (mTOR) pathway is essential for translation initiation and protein synthesis, which could be inhibited by AMPK, resulting in inhibition cell proliferation, inducing autophagy and so on [8].

Autophagy is a celluar catabolic process with degrading and recycling intracellular components in response to cell stress and its initiation is essentially regulated by autophagy-related genes (ATGs) [9]. Under stress condition, AMPK/mTOR pathway regulates cell autophagy through activating autophagy-initiating kinase, resulting in cell death [10].

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http://dx.doi.org/10.1016/j.bbrc.2014.02.043
0006-291X/

In this study, we reported that tigecycline inhibited cell proliferation and induced autophagy in human gastric cancer cells. We also showed that tigecycline inhibited tumor growth in vivo. More importantly, our findings provided an initial evidence for the potential of therapeutic tigecycline using as a novel agent for gastric cancer treatment.

2. Materials and methods

2.1. Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Southwest University. Human primary gastric cancer specimens were harvested with patient written consent from the hospital in Southwest University. All procedures were done according to protocols approved by the Ethics Committees of the Southwest University.

2.2. Primary gastric cancer cell suspension preparation and cell line culture

Human primary gastric cancer specimens were harvested with patient written consent. Specimens were dissociated and digested into single cell suspension by collagenase, DNase cooktail and then grown in culture medium supplemented with 20 ng/l EGF and 20 ng/l b-FGF (Sigma), we temporarily named the primary gastric cancer cell GAM-016 according to the patient sex and case number. MKN-45 Cell line (purchased from Shanghai cell bank, Chinese Academy of Sciences, Shanghai, China) was grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS; Invitrogen), 1% penicillin–streptomycin. Both cells were cultured at 37 °C in 5% CO_2 humidified incubator, and subcultured at the split ratio of 1:3 every 3 days.

2.3. Drug treatment

Tigecycline (TIG; Wyeth, Canada) was dissolved in Dimethyl Sulfoxide (DMSO) as 100 mM stock solutions. Primary gastric cancer cells and cell line MKN-45 were treated with tigecycline (1 μM, 5 μM, 10 μM) or DMSO for 72 h. Micrographs of cell morphology were taken by an Olympus microscopy (Olympus, Japan). Cell survival was analyzed by Trypan blue exclusion assay.

2.4. Cell proliferation assay

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Cells were seeded in 96-well plate at 400 cells per well for overnight. After incubated with either tigecycline or DMSO for the indicated time period, 20 μl MTT (5 μg/ml MTT in PBS; Sigma) was added to each well and incubated at 37 °C for 2 h and then removed the supernatant. DMSO (Sigma, 200 μl per well) was used to dissolve the cell pellets. After shaking for 10 min, the absorbance was measured at a wavelength of 570 nm.

2.5. BrdU staining

Cells were grown on coverslips and treated with either DMSO or tigecycline. After treatment, The thymidine analog 5-bromo-2-deoxyuridine (BrdU; Sigma) stock solution (10 mg/ml) in saline was diluted by 1000×, and added into the culture medium, and incubated for 30 min. Cells were washed with PBS, fixed in 4% paraformaldehyde (PFA) for 15 min, and permeabilized with 0.3% Triton X-100 for 5 min. Cells were blocked with 10% goat serum for 1 h, incubated with a primary antibody for 1 h, and followed by the appropriate secondary antibody for 1 h, and counterstained with 300 nM DAPI (C1002, Beyotime) for nuclei. Primary antibody was rat anti-BrdU (Abcam, ab6326), and used at 1:200. Secondary antibody was Alexa Fluor 488 goat anti-rat IgG(H+L) (A11006, Invitrogen), and used at 1:600. Cells were mounted in fluorescence mounting medium (P0126, Beyotime) and examined by using a Nikon microscope with Image-Pro Plus software for image analysis.

2.6. Apoptosis assay

The apoptotic ratios of cells were performed by the Annexin V-FITC apoptosis detection kit (Sigma). Briefly, gastric cancer cells were exposed to 10 μM tigecycline or DMSO for 72 h, and then the 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 min, and analysed by flow cytometry with CellQuest analysis software.

2.7. MDC staining

Cells were grown on coverslips and treated with either DMSO or tigecycline. After treatment, cells were labeled with the autofluorescent marker monodansylcadaverine (MDC, 50 μM; Sigma, 30,432) at 37 °C for 15 min in dark room. Cells were washed and fixed by 4% PFA for 15 min. Cells were mounted in fluorescence mounting medium and examined by using a Nikon microscope with Image-Pro Plus software for image analysis.

2.8. Immunofluorescence staining

Cells were grown on coverslips and treated with either DMSO or tigecycline. After treatment, cells were fixed by 4% PFA for 15 min, blocked with 5% milk for 1 h, incubated with the primary antibody, and then followed the appropriate secondary antibody. Subsequently, cells were counterstained with 300 nM DAPI for nuclei. Primary antibodies for LC3B (#4108S, Cell Signaling Technology) and tubulin (AT819, Beyotime) were used at 1:200 and 1:500, respectively. Secondary immunofluorescence antibodies Cy3-labeled goat anti-mouse IgG (A0521, Beyotime) and FITC-labeled goat anti-rabbit IgG (A0562, Beyotime) were used at 1:500, respectively. Cells were mounted in fluorescence mounting medium and examined by using a Nikon microscope with Image-Pro Plus software for image analysis.

2.9. Western blot assay

Proteins were separated by SDS–PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocked with 5% nonfat milk in TBST for 1 h, the membrane was washed and incubated with primary antibody. Membrane was washed three times, and incubated with the horseradish peroxidase-conjugated secondary antibody. The signal was captured by the ECL reagent (Beyotime) and visualized by Western blotting detection instruments (Clinx Science). Primary antibodies were used: rabbit anti-LC3B (#4108S, 1:1000), rabbit anti-phospho-AMPK (#2535P, 1:1000), rabbit anti-phospho-mTOR (#5536P, 1:1000), rabbit anti-phospho-p70S6K (#9234P, 1:1000), rabbit anti-AMPK (#2603P, 1:1000), rabbit anti-mTOR (#2983P, 1:1000), rabbit anti-p70S6K (#2708P, 1:1000) were purchased from Cell Signaling Technology; mouse anti-α-tubulin (AT819, 1:1000) was purchased from Beyotime Biotech. Secondary antibodies were used: HRP-labeled goat anti-mouse IgG(H+L) (A0216, 1:5000), HRP-labeled goat anti- rabbit IgG(H+L) (A0208, 1:5000), HRP-labeled donkey anti-goat IgG(H+L) (A0181, 1:5000) were purchased from Beyotime.

2.10. Xenograft tumor model

One million cells were injected subcutaneously into right flank of 4 weeks old severe combined immune deficiency (SCID) mice. After the appearance of palpable tumor, the mice were treated with tigecycline once daily (100 mg/kg by i.p. injection) or vehicle control (DMSO) for 5 days. Each group had 6 mice. Tumor volumes were calculated daily based on caliper measurements of tumor length and width (volume = tumor length $\times$ width^2 $\times$ 0.5236). Twenty-one days after injection, mice were sacrificed by CO_2, tumors were excised and measured.

2.11. Statistical analysis

Assays were set up in triplicates and the results were presented as mean ± SD. Variance between the experimental groups was determined by two-tailed *t*-test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Tigecycline inhibited cell growth and proliferation in human gastric cancer cells

Cells proliferation assay was conducted after 72 h treatment, tigecycline led to cell proliferation inhibition in a dose-dependent manner in gastric cancer cells (Fig. 1A). We followed the course of cell growth for 7 days after the addition of tigecycline. Tigecycline at 5 μM or 10 μM led to a dramatically decrease in cell proliferation in a dose- and time-dependent manner (Fig. 1B). In addition, BrdU staining result also showed that 10 μM tigecycline treatment for 72 h resulted in a significant reduction in the percentage of BrdU-positive cell compared to DMSO-treated cells (Fig. 1C). All together, the result verified that tigecycline dramatically inhibited cell growth and proliferation in gastric cancer cells.

3.2. Tigecycline induced autophagy not apoptosis in human gastric cancer cells

As show in Fig. 2A, flow cytometry data showed that tigecycline didn't induce apoptosis in gastric cancer cells, but it did induce autophagy (Fig. 2B). Monodansylcadaverine (MDC), as a lysosomotropic agent, has recently been found to be a late-stage marker for autophagosomes by accumulating in acidic organelles and interacting with autophagic vacuole membrane lipids [11]; therefore, MDC staining was performed to measure the induction of autophagy. Accumulation of MDC in autophagic vacuoles was increased in tigecycline-treated cells in a time-dependent manner (Fig. 2B). Hallmarks of autophagosome formation include the conversion of LC3-I to the PE-conjugated form (LC3-II) during autophagosome closure [12], resulting in an increased level of LC3-II, which reflects the autophagy. Coincident with MDC staining, the LC3-II form was observed after exposure to tigecycline, and the conversion of LC3-I to LC3-II was increased in a time-dependent manner (Fig. 2C and D). All together, these observations determined that tigecycline induced autophagy in gastric cancer cells.

3.3. AMPK/mTOR/p70S6K pathway was activated after tigecycline treatment

AMPK phosphorylation at position Thr172 was determined by Western blotting analysis with an anti-phospho-AMPK antibody. AMPK phosphorylation was significantly increased after 10 μM tigecycline treatment either in MKN-45 or GAM-016 cells (Fig. 3A). The phosphorylated AMPK suppressed its downstream targets including mTOR or p70S6K, ultimate induced cell autophagy and inhibited cell growth [13–15]. Similarly, we assessed the phosphorylation of mTOR and p70S6K after tigecycline treatment. Compared with the control group, tigecycline significantly downregulated mTOR phosphorylation at position Ser2448 as well as p70S6K phosphorylation at position Thr389, but not in the total mTOR or p70S6K (Fig. 3B and C). These result clearly demonstrated that tigecycline activated of AMPK and suppressed mTOR and p70S6K.

3.4. Tigecycline inhibited tumor growth in xenograft model of human gastric cancer cells

Primary gastric cancer cells GAM-016 were transplanted subcutaneously into SCID mice, which were injected with 100 mg/kg tigecycline daily after 5 days. Tigecycline significantly inhibited tumor growth (Fig. 4A and B). Treatment with tigecycline did not alter the appearance or behavior of the mice. And the weight of mice were all around 21.5 ± 0.5 g during the experiment, with no significant difference. Moreover, we detected autophagy related protein expression in tumors dissected from the mice, and found that tigecycline still reduced relevant protein expression (Fig. 4C). These result suggested that tumor growth inhibition was accompanied or caused by tigecycline-induced autophagy.

4. Discussion

In this study, we found that the antimicrobial agent tigecycline had anti-tumor activity from in vivo and vitro experiments. Tigecycline acts as a glycylcycline class bacteriostatic agents and actively resists a series of bacteria [16]. Tigecycline could reversibly bind to a single, high-affinity intracellular site on the 30S ribosomal subunit in pathogens, obstructing amino-acyl transfer RNA molecules to enter into the ribosome's A-site, therefore blocking the incorporation of amino acid residues into elongating peptide chains resulting in protein synthesis suppression [17,18]. Particularly, tigecycline seems to act on the ribosomal more efficiently than tetracycline, and has an ability to get over ribosome-based tetracycline resistance in a manner distinct from that of tetracycline [19,20]. However, the biological effect of anti-tumor with this antimicrobial agent of tigecycline is sparsely studied. Here we provided detail evidence that tigecycline performed anti-tumor activity through distinct mechanisms in human gastric cancer cells. Our result showed that tigecycline inhibited cell proliferation and growth in human gastric cancer cells.

Autophagy can be induced in several circumstances including aging, nutrient deprivation, as well as chemical and radiation cancer therapy [21,22]. Autophagy is a process of catabolism for the degradation and recycling of macromolecules and organelles [23,24], which begins with the formation of double-membraned vesicles called autophagosomes, undergoes acidification after maturation and subsequently fuses with lysosomes to form autophagolysosomes [25]. Previous studies suggested that autophagy is also called autophagic cell death or type II cell death, considered as a particular mode of cell death which is resembled to apoptosis [26]. Our result showed that tigecycline induced autophagy in gastric cancer cells. It's obvious that acidic vesicular organelles formed after exposure to tigecycline, which was characterized through monodansylcadaverine (MDC) staining. Additionally, the features of increased LC3-II and a punctuated pattern of LC3 fluorescence also indicated that tigecycline markedly induced autophagy.

Serving as a key energy sensor in cellular stress-sensing pathways, AMPK is conserved and could control cellular proliferation by acting as a checkpoint of metabolism under the circumstance on which nutrients are sick [27,28]. Additionally, AMPK also plays

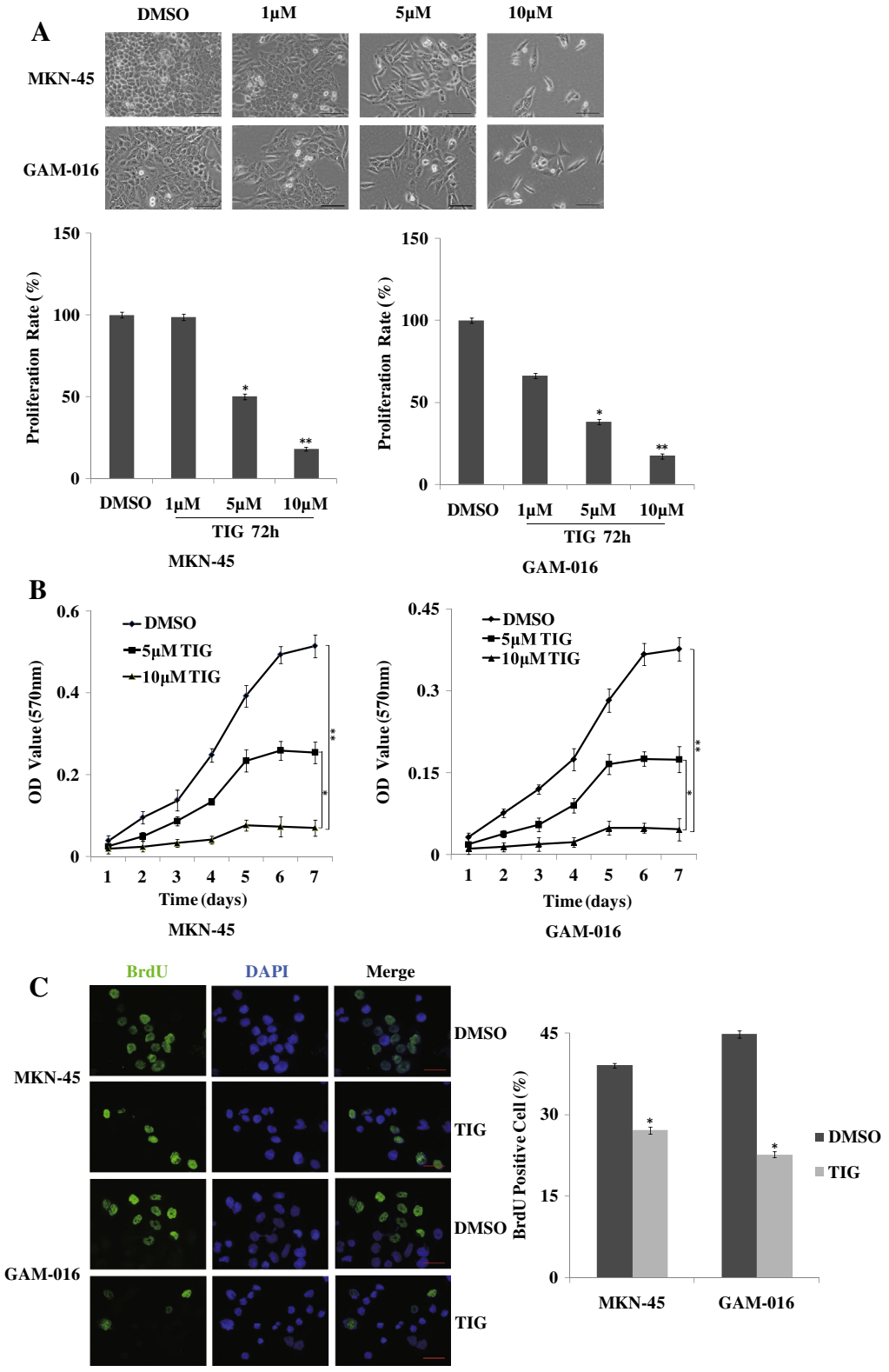

Fig. 1. Tigecycline inhibited cell proliferation in human gastric cancer cells. (A) Gastric cancer cell line MKN-45 or primary gastric cancer cell GAM-016 was seeded in 6-well plate. After tigecycline treatment for 72 h, cell morphology was observed and the amount of cell was determined by trypan blue assay, and the cell proliferation rate was calculated. Data was showed compared to DMSO control. (B) MKN-45 or GAM-016 cells were seeded at 400 cells per well in 96-well plate. After treated with tigecycline, a MTT assay was conducted daily from 1 to 7 days. The result was expressed as the absorbance from 5 independent experiments. (C) MKN-45 and GAM-016 cells were grown on coverslips and treated with either DMSO or 10 μM tigecycline for 72 h. After treatment, BrdU was added and incubated for 30 min. Cells were stained with an antibody against BrdU (green), counterstained with DAPI (blue). The immunofluorescent staining of BrdU in MKN-45 or GAM-016 was showed and the percentage of BrdU-positive cells was calculated. Cells were counted from at least 10 randomly selected fields. Each bar represented the average ± SD of three independent experiments. Statistical analysis was performed using the two-tailed Student's *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$.

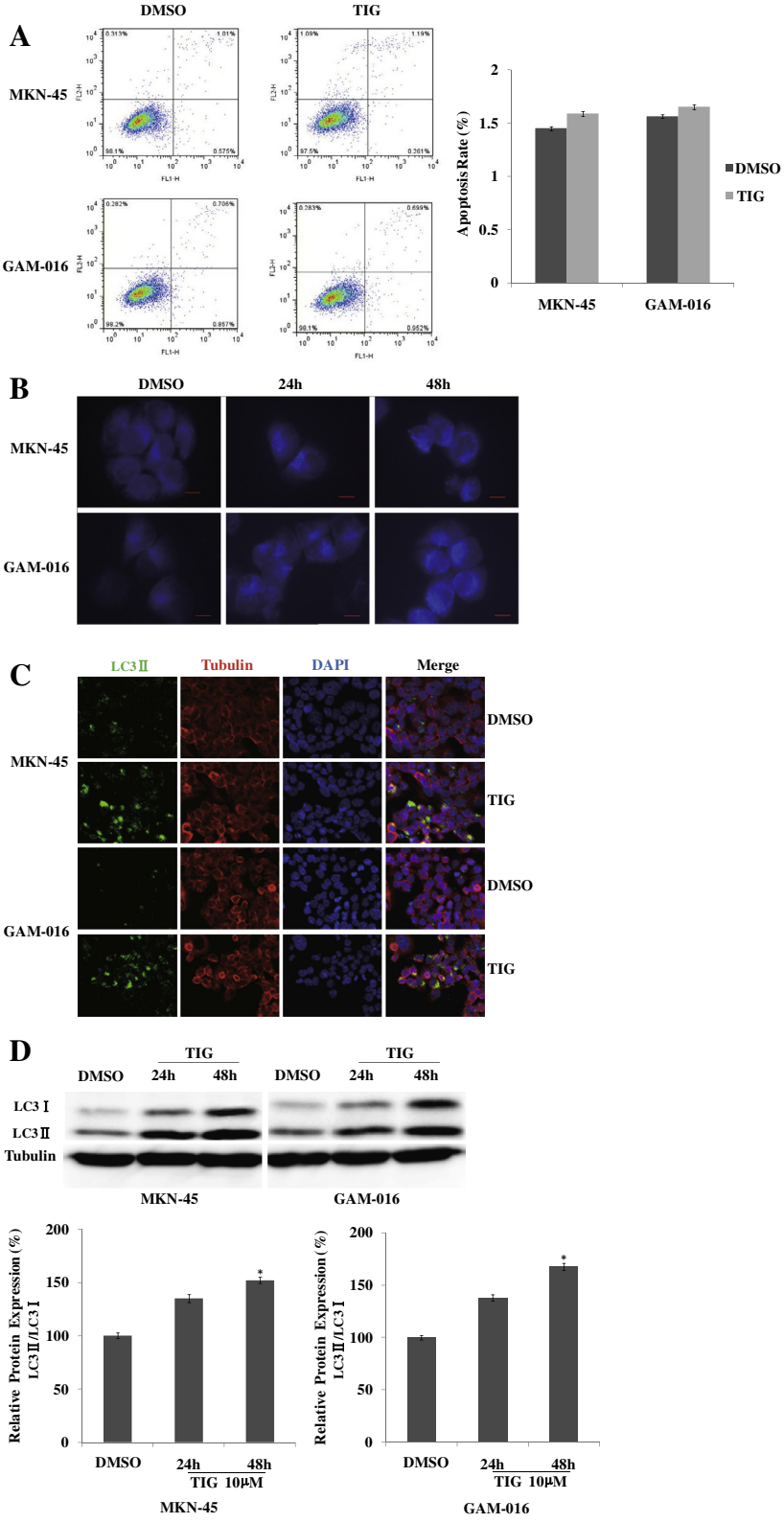

Fig. 2. Tigecycline induced autophagy but not apoptosis in human gastric cancer cells. (A) MKN-45 and GAM-016 cells were treated with either 10 μM tigecycline or DMSO for 72 h, and apoptosis was analyzed by Annexin V/PI double staining. (B) MKN-45 and GAM-016 cells were treated with either 10 μM tigecycline or DMSO for the indicated times and autophagosome formation was measured by MDC staining respectively. (C) Autophagic-related protein LC3-II was examined by immunofluorescent staining after tigecycline treatment in MKN-45 and GAM-016 cells. Cells were treated with 10 μM tigecycline or DMSO for 48 h and stained with antibodies against LC3-II (green) and tubulin (red), and counterstained with DAPI (blue). (D) Western blot assay was performed to assess the conversion of LC3-II to LC3-I after tigecycline treatment in MKN-45 and GAM-016 cells respectively. Cells were treated with 10 μM tigecycline for the indicated times. Protein expression level was normalized relative to tubulin. Data was showed compared to DMSO control. Each bar represented the average ± SD of three independent experiments. Statistical analysis was performed using the two-tailed Student's *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$.

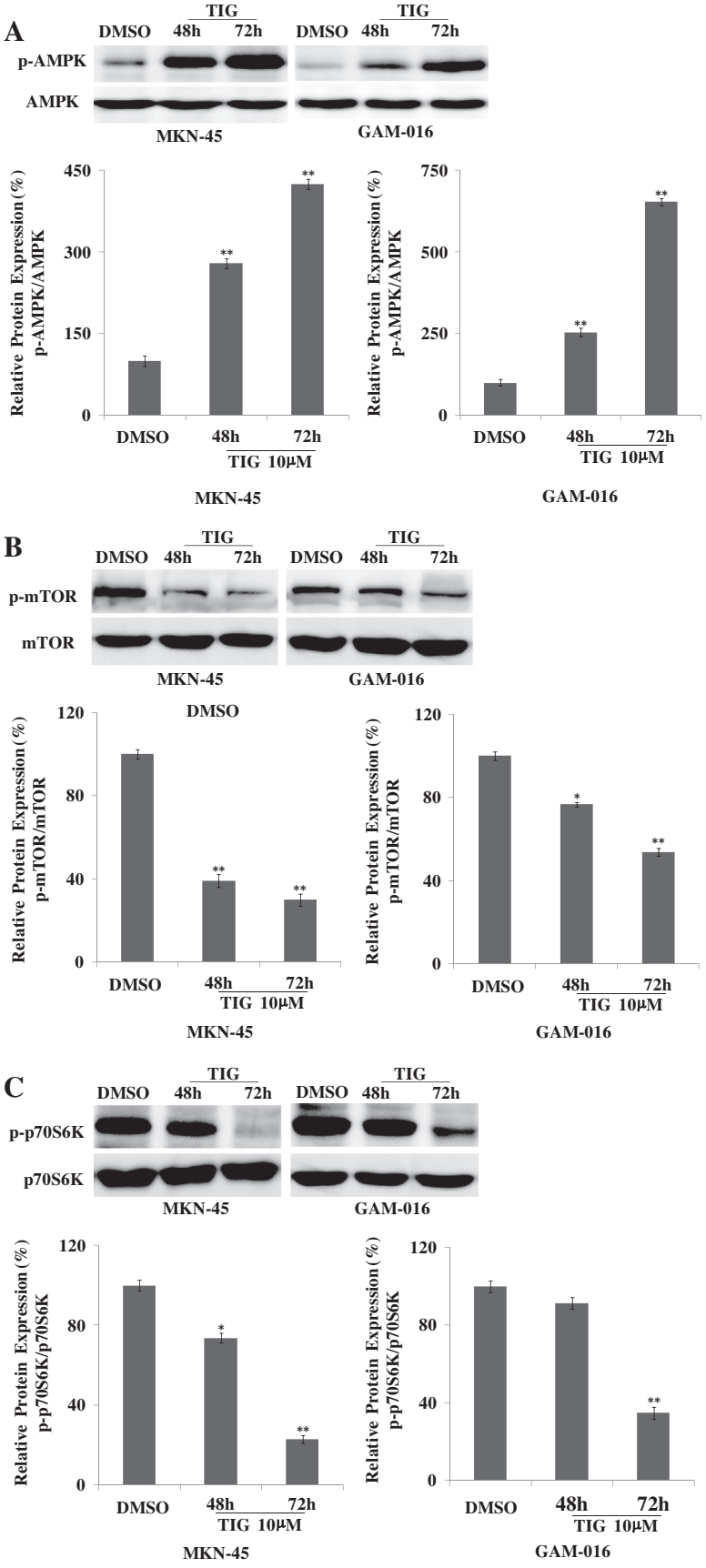

Fig. 3. AMPK/mTOR/p70S6K pathway was activated after tigecycline treatment. Western blot assay was performed to assess the level of phosphorylated AMPK (A), phosphorylated mTOR (B) and phosphorylated p70S6K (C) in MKN-45 and GAM-016 cells, respectively. Cells were treated with 10 μM tigecycline for the indicated times, and DMSO was used as control. Protein expression level was normalized relative to tubulin. Data was showed compared to DMSO control. Each bar represented the average ± SD of three independent experiments. Statistical analysis was performed using the two-tailed Student's *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$.

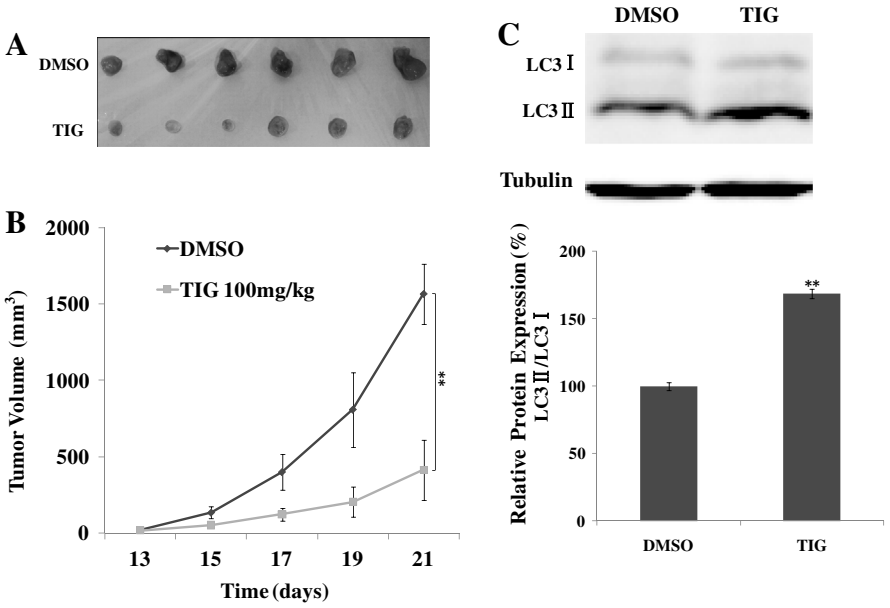

Fig. 4. Tigecycline inhibited tumor growth in xenograft model of human gastric cancer cells. (A) Photograph of tumors resected from SCID mice after treated with tigecycline (100 mg/kg once daily by i.p. injection) or DMSO control daily for 5 days. (B) Human primary gastric cancer cells GAM-016 were injected subcutaneously into the flank of SCID mice. When tumors were palpable, mice were treated with tigecycline or DMSO daily for 5 days, and tumor volume was measured ($n = 6$ per group). (C) Tumors from tigecycline-treated or DMSO control mice were excised, and total protein was extracted and analyzed by Western blot for LC3II/I and tubulin expression. Data was presented as mean ± SD. Statistical analysis was performed using the two-tailed Student's *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$.

an important role in regulating autophagy-related pathway, which could induce cells proceed autophagy process to ensure sufficient metabolites by decomposing cellular cytosolic components (microautophagy) and organelles (macroautophagy) once cancer cells faced a condition of shortage of nutrients [28]. The most described mechanism by which AMPK controled cell growth as well as regulated autophagy is through suppression of the mTOR pathway. When AMPK is activated and phosphorylated, it causes mTOR suppression, restrains cell growth and induces autophagy by regulating the phosphorylation of downstream effectors p70S6K [13]. To the best of our study, we originally showed that tigecycline treatment could activated and phosphorylated AMPK, further suppressed mTOR/p70S6K phosphorylation. So our data implied that AMPK might be, at least in part, one of the appreciated stress-sensing pathways which was involved in autophagy induced by tigecycline.

Although previous data have reported the mechanism of tigecycline acting as an antimicrobial agent by binding to ribosomal subunit and then blocking protein synthesis in prokaryote. There is no clear evidence to illustrate its role playing in eukaryote, especially in cancer cells. Recent reports showed that tigecycline could block mitochondrial protein synthesis through binding to its ribosomal subunit but not cytoplasmic ribosome subunit [5]. Mitochondrial dysfunction may cause cellular changes such as ATP decreasing. Under this condition, AMPK/mTOR pathway could be activated or inactivated by sensing cellular changes, which would trigger cell autophagy.

In summary, our result demonstrated that tigecycline inhibited cell proliferation as well as induced cell autophagy in human gastric cancer cells, which brought a fresh clue to evaluate tigecycline using as a potential anti-tumor drug for gastric cancer treatment.

Conflict of interest statement

The authors declare no potential conflicts of interest.

Acknowledgments

We are grateful for Jianhua Wang preparing primary human gastric cancer cell suspension. This study was supported by the National Basic Research Program of China (No. 2012cb114603), the National Natural Science Foundation of China (81201551), the Research Fund for the Doctoral Program of High Education of China (20130182110003), the Natural Science Foundation of Chongqing (cstc2013jcyjy0007), and the Fundamental Research Funds for the Central Universities (XDJK2013B020, SWU112033).

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