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# MTH1 Inhibitor TH287 Suppresses Gastric Cancer Development Through the Regulation of PI3K/AKT Signaling

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## Abstract

***Background:*** Cancer cells evade oxidative stress through the MutT homologue-1 (MTH1), a member of the Nudix family. MTH1 maintains genome integrity and the viability of tumor cells. A new class of MTH1 inhibitors have attracted interest as anticancer agents, but their mechanisms of action remain poorly characterized. In this study, the authors evaluated the anticancer effects of the MTH1 inhibitor TH287 on gastric cancer (GCa) cells.
***Materials and Methods:*** BGC-823 and SGC-7901 cells were treated with TH287 and CCK-8, and colony-forming assays were performed. Cell migration was assessed through Transwell and scratch assays. Apoptotic status was measured via flow cytometry and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) staining. Cell cycle status was assessed by propidium iodide (PI) staining. The expression of PI3K/AKT signaling-related proteins was verified by western blotting.
***Results:*** TH287 inhibited cell viability, reduced cell proliferation, inhibited apoptosis, induced G2/M arrest, and suppressed cell migration. A loss of mitochondrial membrane potential and reduced Bcl-2/Bax expression were also observed in TH287-treated cells. These effects were mediated through the inhibition of pro-oncogenic PI3K/AKT signaling.
***Conclusions:*** These findings indicate that the MTH1 inhibitor TH287 mediates an array of anticancer effects in GCa cells through its effects on mitochondrial function and PI3K/AKT signaling. Collectively, these data highlight the promise of TH287 as a novel therapeutic option for GCa cells.

**Keywords:** gastric cancer, TH287, MuT homologue-1



## Introduction

Reactive oxygen species (ROS) production is elevated in cancer cells, which leads to DNA damage.[1] Moreover, oxidative stress is associated with genomic instability and disease progression.[2] DNA is compromised during oxidative stress due to the integration of damaged bases from the oxidized deoxyribose nucleoside triphospate (dNTP) pools. The most common oxidative base, 8-oxo-G, is prone to mispairing with adenine, leading to errors during DNA replication, transcription, and protein translation. Previous studies indicate that 8-oxo-G is closely related to cancer progression through its ability to regulate telomerase activity.[3,4] 8-oxo-G lesions facilitate telomere lengthening under normoxic conditions, whereas oxidative stress promotes disordered or shorted telomeres and subsequent cell death.[5]

MutT homologous protein 1 (MTH1) is a Nudix hydrolase expressed in the cytoplasm and mitochondria of all cells. MTH1 hydrolyzes oxypurine triphosphate nucleotides, including 8-oxy-G, to prevent cell damage and subsequent death.[6] MTH1 also hydrolyzes methylated bases, preventing their insertion into DNA chains and preventing their interference with normal DNA replication and gene expression.[1,7–9] MTH1 is frequently overexpressed in a number of human cancers and is now recognized as a promising cellular target in cancer therapy. Altered MTH1 expression leads to cell damage and disease. A loss of MTH1 function through both pharmacological inhibition and genetic silencing leads to enhanced RNA mutagenesis that is mediated through 8-oxo-G.[10] Conversely, the overexpression of MTH1 is associated with ulcerative colitis-associated carcinogenesis and is considered pro-oncogenic in many cell types.[11] TH287 is first-in-class inhibitors of the Nudix hydrolase protein family and highly selective toward MTH1, with no relevant inhibition of other members of the Nudix

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protein family.[1] Moreover, TH287 has been shown to selectively kill a variety of cancer cell lines and regarded as a new antitumor drug.[12–14]

Gastric cancer (GCa) is a prevalent malignancy, representing the third highest cause of cancer-related mortality, with 1 million new cases diagnosed in 2018, 700,000 of which resulted in death.[15,16] Although GCa patients diagnosed during the early stages show high recovery rates, advanced gastric carcinoma frequently leads to recurrence, metastasis, and drug resistance. GCa is a malignant cancer that develops in the lining of the stomach; the influencing factors of which include age, diet, and stomach disease. The symptoms of GCa include indigestion and stomach discomfort or pain and its early diagnosis influences prognosis and future treatment options. Current treatments include surgery, endoscopic mucosal resection, chemotherapy, radiation therapy, chemoradiation, targeted therapy, and immunotherapy. New cellular targets have also emerged for GCa therapeutics. MTH1 inhibitors, such as TH588 and TH287, lead to cytotoxicity in cancer cells and have been touted as novel agents in the treatment of some forms of cancer in humans, including GCa.[1,11,17–19] In this study, the authors investigated the effects of the MTH1 inhibitor TH287 on GCa cells and investigated its underlying mechanism(s) of action.

## Materials and Methods

### *Cell lines and reagents*

BGC-823 and SGC-7901 cells were purchased from the Shanghai Institute of Biochemistry and Cell Biology. Cells were grown in RPMI 1640 (Hyclone) plus 10% fetal bovine serum (FBS) and 100 U/mL of pen/strep at 37°C in 5% $CO_2$. TH287 and the AKT activator were purchased from Selleck Chemicals. Antibodies were purchased from Proteintech.

### *Cell growth analysis*

Cells ($7\times10^3$ cells) were seeded into 96-well plates and treated with TH287 at 0, 4, 8, 16, 32, 64, and 128 $\mu$M and the relevant vehicle controls for 24, 48, and 72 h. CCK8 (10 $\mu$L) was added to each well for 2 h, and absorbances were measured at 450 nm. Cell viability(%) = (experimental group A450-background group A450)/(control group A450-background group A450).

### *Colony formation assays*

Cells ($\sim$2000) were plated in six-well dishes and treated with TH287 (4, 16, and 64 $\mu$M) for 2 weeks. Cells were then fixed with 4% Paraformaldehyde (PFA) for 10 min and stained with 1% crystal violet for 20 min. Representative images were obtained via microscopy.

### *Scratch assays*

Cells ($8\times10^5$ per well) were seeded into six-well plates and grown to confluence. A scratch was performed using a 10-$\mu$L pipette tip, and cells were treated with vehicle controls or TH287 (4, 16, and 64 $\mu$M). Wound widths were imaged after 24 h and measured.

### *Transwell assays*

Cells were seeded into the upper wells of Transwell filters (Costar, 8.0 $\mu$m, $5\times10^4$ per well) for 12 h in serum-free media. Complete RPMI 1640 medium was added to the bottom chambers. Cells were treated with TH287 (4, 16, and 64 $\mu$M) for 8 h and left for another 24 h. Cells that failed to migrate were removed with a cotton swab, whereas migrating cells were fixed in 4% PFA, stained with crystal violet, and imaged from five randomly selected fields (200×).

### *Western blotting*

Cells were lysed in RIPA buffer, and total proteins (40 $\mu$g per lane) were resolved on SDS-PAGE gels. Proteins were transferred onto polyvinylidene fluoride (PVDF) membranes and probed with anti-Bcl-2, anti-Bax, anti-PI3 Kinase, anti-p-Akt, anti-Akt, and anti-GAPDH primary antibodies (all obtained from Proteintech). Membranes were washed in Tris Buffered Saline Tween and labeled with the appropriate Horseradish Peroxidase (HRP) secondary antibodies for 2 h. Protein bands were visualized using the ECL system (Beyotime).

### *Flow cytometry*

Cells ($5\times10^5$ per well) were treated with TH287 (4, 16, and 64 $\mu$M) for 24 h, washed in ice-cold phosphate-buffered saline, and fixed in 70% ethanol for 1 h on ice. Cells were then stained with propidium iodide (PI) at 37°C for 30 min and assessed by flow cytometry (BD Biosciences) for cell cycle analysis.

### *Apoptosis assays*

Cells treated as above were resuspended in 200 $\mu$L of binding buffer and stained with annexin V-FITC and PI for 30 min in the dark. Cell apoptotic status was then assessed on a flow cytometer.

### *Mitochondrial membrane potential assessments*

A loss of mitochondrial membrane potential (MMP) ($\Psi$m) is an early indicator of apoptosis.[20] For MMP assessments, cells were treated with TH287 (4, 16, and 64 $\mu$M) or Mitochondrial oxidative phosphorylation uncoupler (CCCP) (10 $\mu$M) for 20 min and labeled with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) in the dark for 20 min. Cells were then washed in staining buffer, and fluorescence intensities were assessed by microscopy.

### *Statistical analyses*

SPSS 21 software was used for statistical analyses. Data shown are the mean ± standard deviation. Treatment groups were compared using a one-way analysis of variance. $p$-Values $\leq$0.05 were considered significant.

## Results

### *TH287 reduces the viability and proliferation of GCa cells*

Cell viability assays showed that TH287 was cytotoxic to the GCa cell lines assessed in both a concentration- and

time-dependent manner (Fig. 1A, B). More than 50% survival was observed at the highest concentration of 128 $\mu$M TH287 for 24 h. However, less than 50% cell survival was observed at 16 $\mu$M TH287 in BGC-823 cells and 64 $\mu$M TH287 in SGC-7901 after 48 h. Colony formation assays showed that cell cloning ability of the GCa cell lines was significantly inhibited following exposure to TH287 for 2 weeks (Fig. 1C, D). These data highlight the anti-GCa effects of TH287.

### *TH287 inhibits the migration of GCa cells*

Wound healing and Transwell assays were performed to assess GCa cell metastasis in response to TH287 treatment. After stimulation with a range of concentrations of TH287 (4, 16, and 64 $\mu$M) for 24 h, a loss of GCa cell migration was observed compared with the vehicle control groups (Fig. 2A, B, E, and F). TH287 also inhibited the migration of SGC-7901 cells (Fig. 2C, D, G, and H), confirming its antimetastatic effects in GCa cells.

### *TH287 induces apoptosis in GCa cells*

The authors next assessed the effects of TH287 on GCa apoptosis following annexin V-FITC/PI staining. Cells that were annexin V+/PI– or annexin V+/PI+ were deemed apoptotic. TH287 treatment (4, 16, and 64 $\mu$M) led to a significant rise in the number of apoptotic cells with the most potent effects observed at 64 $\mu$M (Fig. 3A–D). The induction of GCa cell apoptosis in response to TH287 was confirmed by western blotting of known apoptotic markers. TH287 treatment led to a loss of Bcl-2 expression (Fig. 4A, C) and a decreased Bcl-2/Bax ratio in GCa cells in response to TH287 treatment (Fig. 4B, D), suggesting that TH287 induces apoptosis via a mitochondrial-dependent pathway.

### *TH287 induces MMP collapse in GCa cells*

A loss of MMP occurs during apoptosis. In healthy mitochondria, JC-1 produces red fluorescence but stains mitochondria green when MMP is reduced. Cells were JC-1 stained following TH287 treatments to assess its effects on cellular MMP. Control cells retain red-stained mitochondria as expected suggestive of a normal $\Delta\psi$m, whereas cells treated with TH287 (4, 16, or 64 $\mu$M) for 24 h have green-stained mitochondria, indicative of a loss of MMP in GCa cells.

### *TH287 induces cell cycle arrest in GCa cells*

To further investigate the cytotoxic effects of TH287, GCa cells were stimulated with TH287 for 24 h and cell cycle status was assessed by flow cytometry. Figure 5A and C shows that TH287 markedly increased G2/M tetraploid cell populations with 4, 16, and 64 $\mu$M TH287 leading to 55.95%, 74.84%, and 81.12% cell arrest in the G2/M phase compared with 5.75% in control BGC-823 cells and 50.64%, 74.71%, and 77.49% cell arrest in the G2/M phase compared with 7.19% in SGC-7901 cells. These data suggest that TH287 induces G2/M phase arrest in GCa cells.

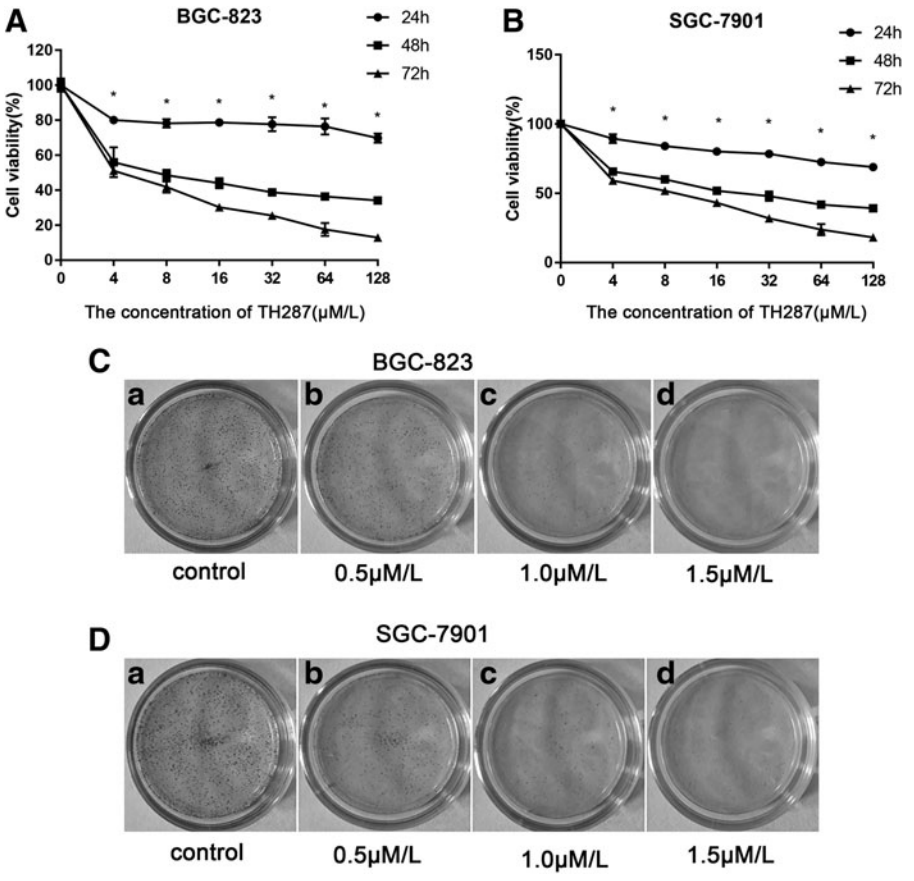


**FIG. 1.** TH287 reduces the viability and inhibits the proliferation of GCa cells. **(A, B)** MTT cell assays following TH287 treatment. **(C, D)** Colony formation assays in TH287-treated cells. Data are the mean ± SD ($n = 3$). *$p < 0.05$ versus control cells. GCa, gastric cancer; Cell Counting Kit-8 (CCK-8); SD, standard deviation.

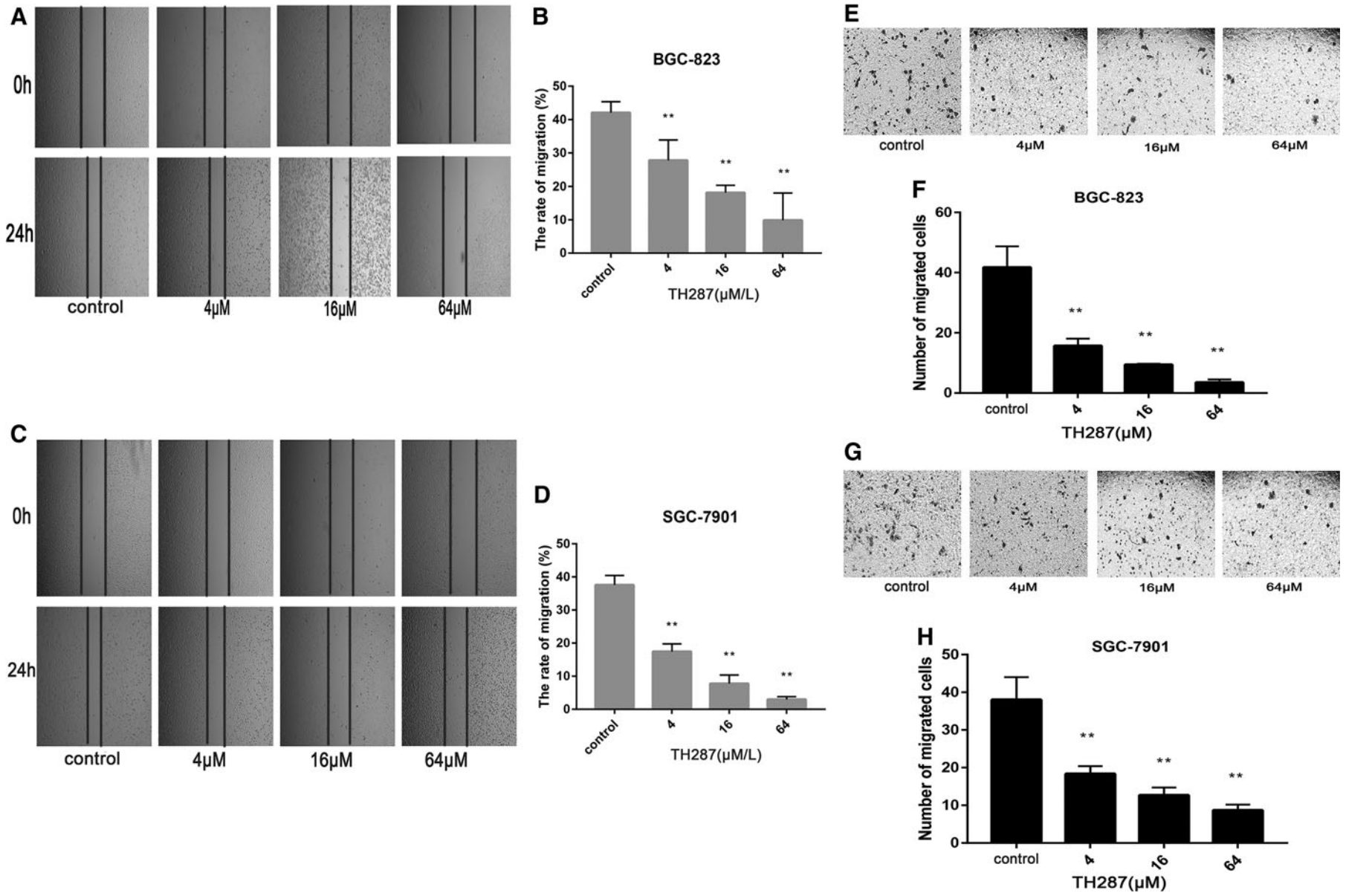


**FIG. 2.** TH287 inhibits the migration of GCa cells. **(A–C)** Wound healing assays following TH287 treatment. **(E, G)** Transwell assays in TH287-treated groups (*upper panels*). **(B, D, F, and H)** Migration assays in TH287-treated groups ($n = 3$ for all assays). $^*p < 0.05$ versus control cells.

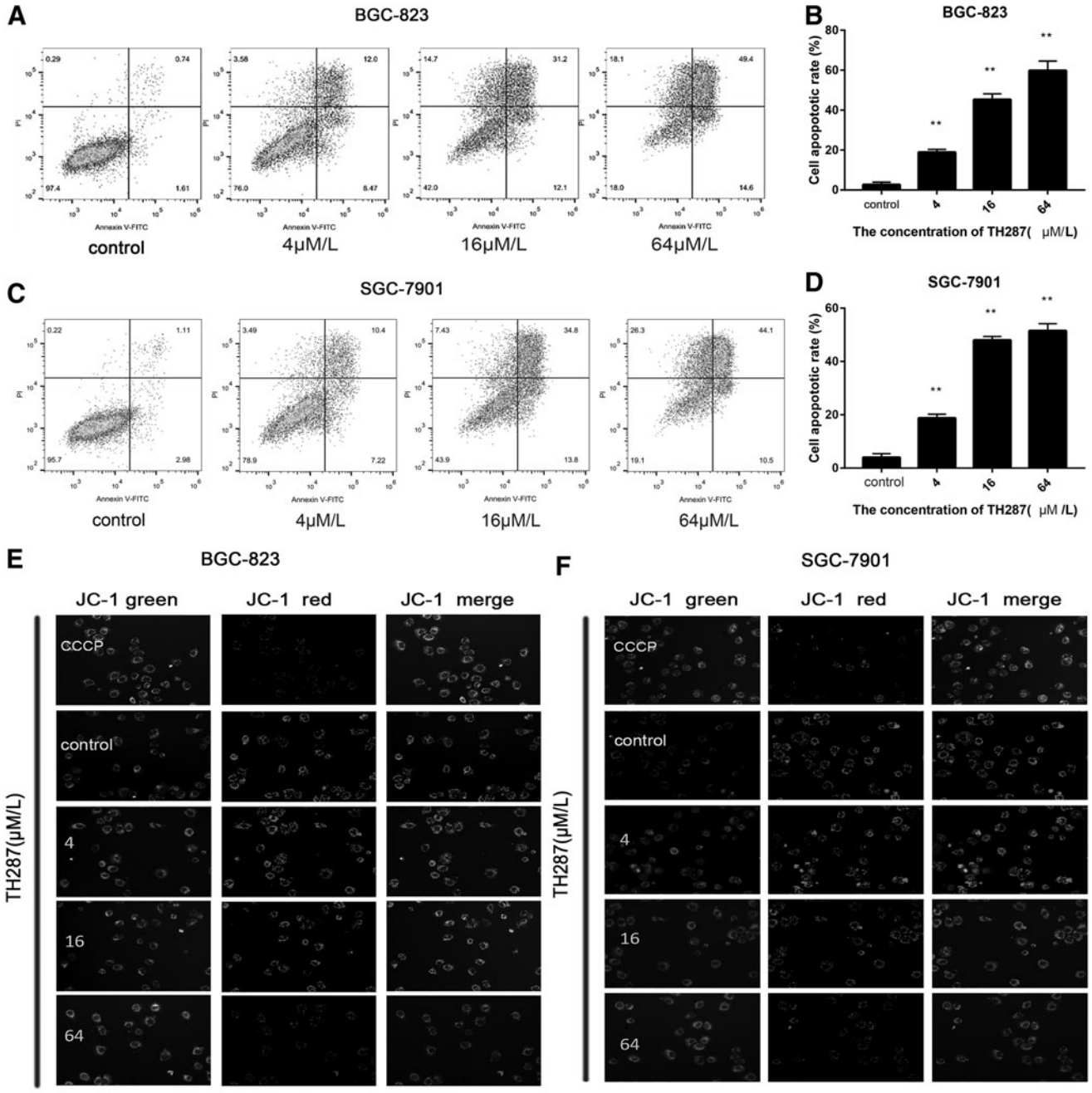


**FIG. 3.** TH287 induces apoptosis in GCa cells. **(A, C)** Annexin V-FITC/PI staining following 24 h treatment with 0.2% Dimethyl sulfoxide (DMSO) or TH287. **(B, D)** The number of apoptotic cells increased after stimulation with 0.2% DMSO and TH287. **(E, F)** MMP assessments in GCa cells through JC-1 staining in cells treated with 0.2% DMSO and TH287. $^{**}p < 0.01$ versus control group. DMSO; JC-1; MMP, mitochondrial membrane potential; PI, propidium iodide.

### *TH287 inhibits PI3K/Akt signaling*

To further investigate the effects of TH287 treatment on GCa cells, the authors assessed its effects on components of the PI3K/Akt signaling axis, a known driver of oncogenesis. In response to TH287 treatment, the levels of p-Akt and PI3K significantly declined as assessed by western blotting TH287-treated cells (Fig. 6A, B). To investigate whether the PI3K/Akt signaling axis mediated the anti-GCa effects of TH287 treatment, cells were treated with the Akt activator SC79 following TH287 induction and cell viability; apoptosis and metastatic properties were assessed. The authors found that TH287 reduced cell viability, increased cell apoptosis, and reduced the ratio of p-Akt/Akt and Bcl-2/Bax compared with the control group. Moreover, when cells were treated with TH287 in combination with SC79, the effect of TH287 on the GCa cell lines was partially reversed compared with TH287 treatment alone (Fig. 6C–F). These data strongly

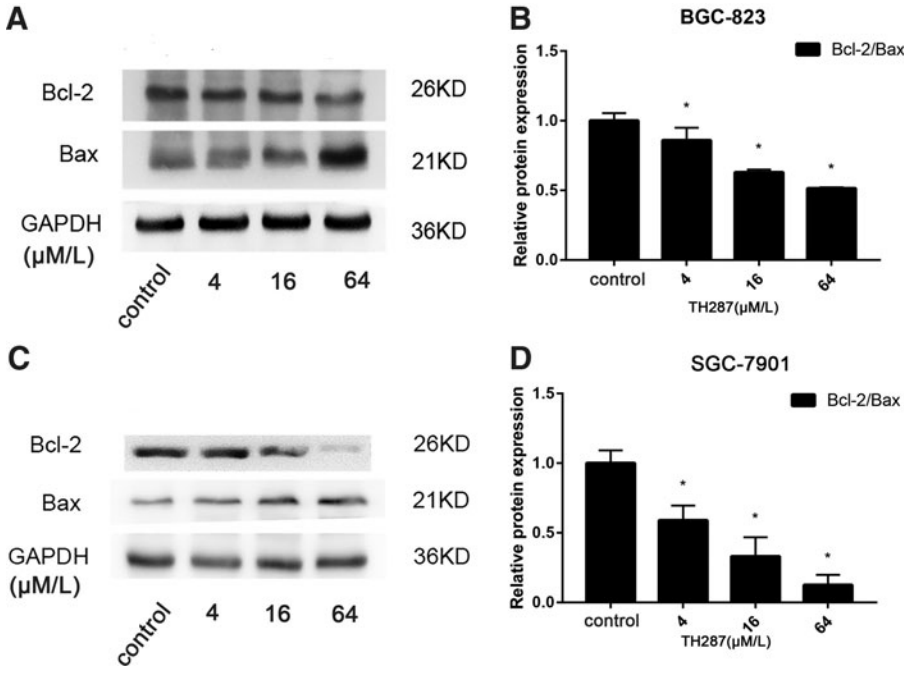


**FIG. 4.** Bcl-2/Bax expression following TH287 treatments in GCa cells (**A–D**). Data are the mean ± SD ($n = 3$). *$p < 0.05$ versus control group.



suggest that TH287 inhibits proliferation and stimulates apoptosis in GCa cells via PI3K/Akt signaling.

## Discussion

GCa remains a major threat to human health across the globe.[21] Although the early stages of GCa can be effectively treated through surgery, recurrence rates remain high, resulting in a poor prognosis.[22] Multidrug combined chemotherapy regimens are efficient treatment options in advanced cancer for recurrent and metastatic disease. However, these treatments are not satisfactory, as multidrug resistance frequently occurs in chemotherapy-treated patients.[23] New treatment options for GCa in the advanced stages are therefore urgently required. In recent years, MTH1 has been regarded as a new target in cancer therapy,[24] and small-molecule inhibitors of MTH1 have shown promise in *in vitro* cancer assays. However, to date, the mechanism(s) by which MTH1 exerts pro-cancerous effects and the efficacy of targeting this host cell factor in GCa remains poorly characterized. This was the first study to fully assess the role of MTH1 during GCa progression.

The authors show that the MTH1 inhibitor TH287 is cytotoxic to GCa cells. TH287 led to a loss of GCa cell proliferation, enhanced the rates of GCa cell apoptosis, and induced G2/M arrest in GCa cells. The authors further demonstrated that these effects of TH287 were mediated through the pro-oncogenic PI3K/AKT signaling axis as these effects could be reversed through AKT activation.

The authors found that the MTH1 inhibitor TH287 significantly reduced GCa cell viability and suppressed the proliferation of GCa cells *in vitro*. TH287 also inhibited the migration of GCa cells highlighting its anti-GCa effects. Cell cycle arrest is an effective means to control tumor growth and progression. Drugs can suppress cell proliferation via inducing cell cycle arrest.[25] TH287 induces DNA damage through its inhibitory effects on MTH1. DNA damage activates an array of genes that act to repair the damage,[26] which arrests the cell cycle status to prevent apoptosis.[27] The G2/M phase is essential for DNA repair. Previous studies have demonstrated that the recovery of the G2/M checkpoint is regulated by mTOR signaling.[28] In this study, TH287 treatment led to significant cell cycle arrest, confirming its ability to suppress GCa progression through the G2/M phase.

Apoptosis regulates embryogenesis, metamorphosis, differentiation, cell cycle progression, and cell homeostasis.[29] Mitochondria are important organelles that control cell fate.[30,31] The loss of MMP leads to apoptosis. The authors found that TH287 led to a collapse of MMP and induces apoptosis, evidenced by the enhanced levels of annexin V-FITC/PI staining in GCa cells in response to TH287 treatment. These results suggested that treatment with TH287 leads to higher apoptotic rates in GCa cells, highlighting its potential as a GCa therapeutic.

Previous studies have shown that the when the MMP of the outer mitochondrial membrane is altered, Cyt-c is released from mitochondrial membranes and stimulates apoptotic factors. During mitochondria-mediated cell death, Bax translocates from the cytoplasm to the outer mitochondrial membrane.[32] Bax and Bcl-2 play a crucial part in cell apoptosis and tumor occurrence.[33] The results of this study showed that TH287 reduced the Bcl-2/Bax ratio, which is consistent with its ability to induce apoptosis through the loss of Bcl2/Bax mitochondrial localization.[34] These results indicate that TH287 activates mitochondria-dependent pathways to trigger apoptosis in GCa cells.

Notably, the authors confirmed that TH287 mediates its anti-GCa effects through the PI3K/Akt pathway. PI3K/Akt/mTOR signaling is key to cell proliferation, apoptosis, autophagy, and cell cycle progression.[35–39] Targeting PI3K/Akt signaling represents a viable pathway for GCa treatment.[40,41] Moreover, the activation of PI3K/Akt signaling induces a range of pro-oncogenic signaling cascades.[42] Drugs

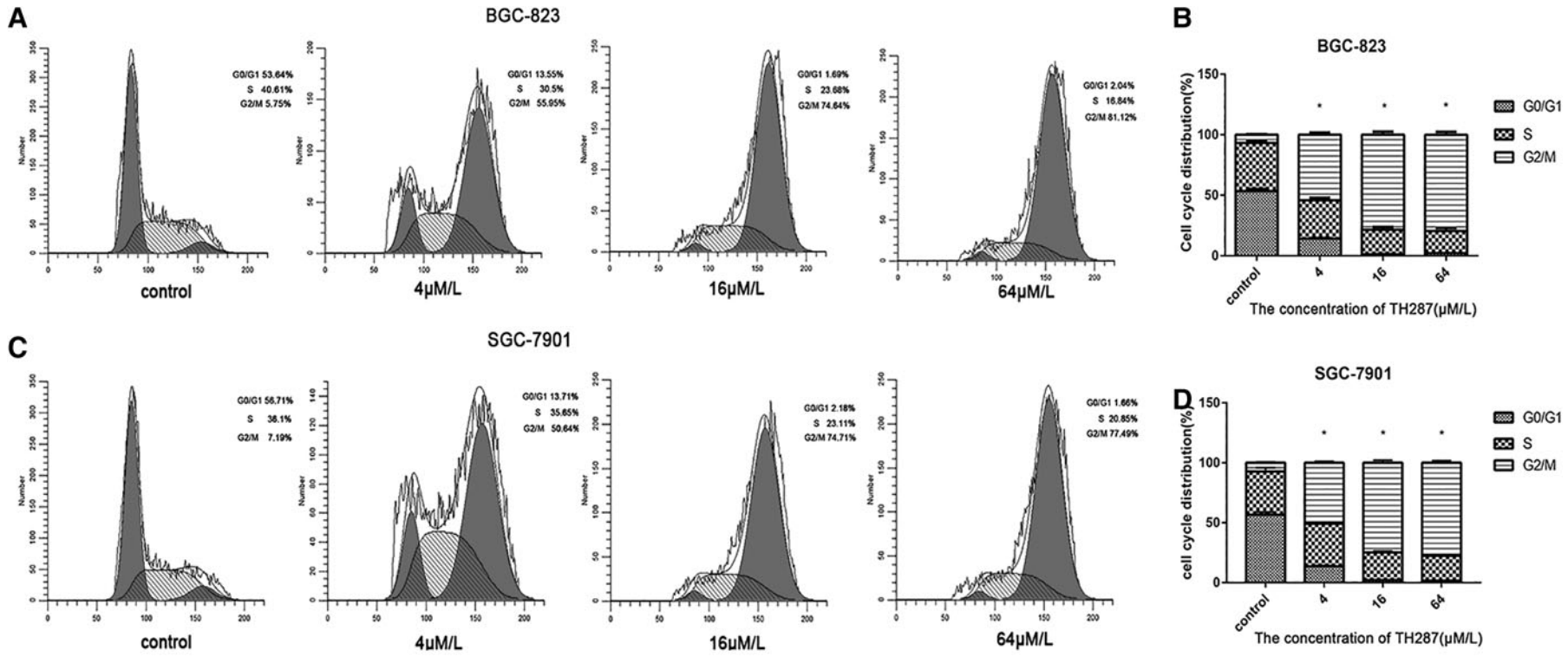


**FIG. 5.** TH287 induces G2/M phase arrest. **(A, C)** PI staining was performed to assess cell cycle progression. Cells were analyzed on a flow cytometer. **(B, D)** GCa cell cycle distribution. Data are the mean ± SD ($n = 3$). *$p < 0.05$ versus controls.

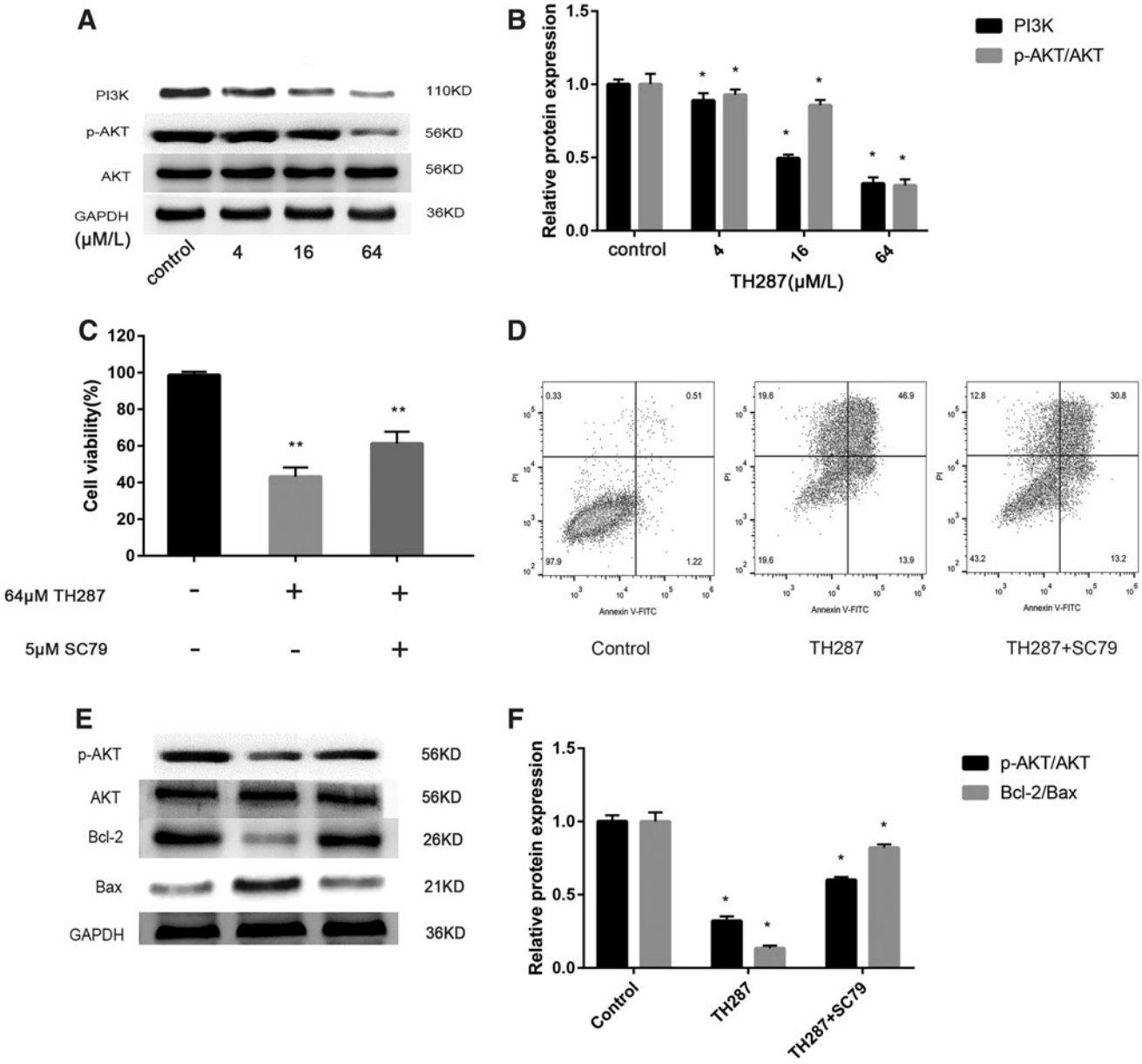


**FIG. 6.** TH287 inhibits the pro-oncogenic PI3K/Akt cascade. **(A)** Western blot analysis of the expression of PI3K/Akt signaling components in the indicated GCa cells. **(B)** TH287 treatment leads to a loss of PI3K and p-Akt/Akt. **(C–F)** The Akt activator SC79 alleviates the anti-GCa effects of TH287. $*p < 0.05$ versus controls.

that target PI3K/Akt can induce apoptosis in GCas. To fully define the mechanism(s) governing the effects of TH287 on GCa cell proliferation, the authors assessed PI3K/AKT signaling in GCa cells. The authors found that TH287 could effectively suppress PI3K/Akt signaling evidenced by the loss of p-Akt expression in the *in vitro* GCa cell lines investigated. The combination of TH287 and the Akt activator SC79[43,44] further supported the hypothesis that TH287 induces GCa cell apoptosis through its effects on the PI3K/Akt axis as SC79 alleviated the anti-GCa effects of TH287 treatment. Taken together, these data highlight a novel mechanism through which TH287 mediates its anti-GCa effects through its ability to suppress pro-oncogenic PI3K/Akt signaling in GCa cells lines that ultimately lead to the induction of apoptosis in GCa cells.

## Conclusions

In summary, the authors found that the MTH1 inhibitor TH287 significantly reduces GCa cell viability and suppresses the proliferation of GCa cells through its ability to induce G2/M arrest. TH287 also inhibited the migration of GCa cells. These effects were mediated through the suppression of PI3K/AKT signaling since AKT activation could alleviate the anti-GCa effects of TH287. Taken together, these data highlight the potential of TH287 as an anticancer therapeutic that can induce apoptosis in cancer cells and reduce the metastatic phenotypes of cancer cells. However, there are some limitations in the treatment of TH287. The compound TH287 has very low solubility and is insoluble in water. When it is necessary to increase the concentration of

the drug, Dimethyl sulfoxide (DMSO), tween 60, and other solvents may be used *in vivo* experiments. Further *in vivo* studies are now required to fully reveal the promise of this compound as a much needed anti-GCa therapeutic.

### Disclosure Statement

No competing financial interests exist.

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