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Original Article

Growth inhibition of human gastric adenocarcinoma cells *in vitro* by STO-609 is independent of calcium/calmodulin-dependent protein kinase kinase-beta and adenosine monophosphate-activated protein kinase

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Abstract: Adenosine monophosphate (AMP)-activated protein kinase is a recently identified downstream target of calcium/calmodulin-dependent protein kinase kinase-beta, and is involved in the regulation of cell metabolism and cell proliferation. STO-609 is a selective antagonist of calcium/calmodulin-dependent protein kinase kinase-beta. In the present study, we found that STO-609 suppressed AMP-activated protein kinase activity, reduced expression of Akt and ERK, and increased cell apoptosis in SNU-1 and N87 cells but not normal gastric epithelial cells (CCL-241). Interestingly, we found such effects of STO-609 on gastric cancer cells were not affected after the knock-down of CaMKK-β and AMPK. In conclusion, STO-609 is an effective cytotoxic agent for gastric adenocarcinoma *in vivo*.

Keywords: Adenocarcinoma, apoptosis, AMP-activated protein kinase, calcium/calmodulin-dependent protein kinase kinase-beta, gastric carcinoma, short hairpin RNA

Introduction

Gastric adenocarcinoma is a common malignancy and has a poor prognosis. In terms of recent advances in targeted therapy in oncology, there have been few biomarkers and cancer cell pathways identified for early detection or intervention in gastric cancer.

Calcium/calmodulin-dependent protein kinase kinase-beta (CaMKK-β) is a serine/threonine kinase that is activated by elevated intracellular calcium [1, 2]. CaMKK-β is believed to be an upstream kinase for calcium/calmodulin-dependent protein kinase type-1 (CaMK-I) and calcium/calmodulin-dependent protein kinase type-1V (CaMK-IV) [3, 4]. In 2015, CaMKK-β was found to be overexpressed in tissue biopsy samples of human gastric adenocarcinoma [5].

The adenosine monophosphate (AMP)-dependent protein kinase (AMPK) is a recently identified CaMKK-β downstream target [3, 4]. AMPK has a role in maintaining cellular metabolic homeostasis and is activated by decreasing ATP levels and an increasing cellular AMP: ATP ratio [1-3]. Recently, the AMPK inhibitor, compound C has been shown to be an AMPK-independent agent in cerebral glioma [6]. CaMKK-β can activate AMPK by phosphorylation of its alpha subunit, Thr172 [2]. The activation of AMPK by CaMK-β is independent of the AMP: ATP ratio [4]. AMPK has been shown to play a role in the cellular motility and cell invasion and has been proposed as a potential therapeutic target in the treatment of prostate cancer [7]. AMPK exerts its effects on biosynthetic pathways by inhibiting acetyl Co-A carboxylase (ACC). Recent studies have shown that agonists of AMPK, including 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) and metformin, can inhibit cancer cell proliferation [7-11].

Recently, an effective and selective antagonist of CaMKK-β, 1, 8-naphthoylene benzimidazole-3-carboxylic acid (STO-609), was synthesized

by Tokumitsu and colleagues [12]. STO-609 can permeate cell membranes to act as a competitive inhibitor of adenosine triphosphate (ATP). These investigators have demonstrated that STO-609 inhibits CaMKK-β activity *in vitro*, making it a valuable tool for assessing this kinase pathway [12]. Recent studies have demonstrated that STO-609 can inhibit AMPK phosphorylation, which is also a CaMKK-β downstream target [13].

This study was done to determine whether pharmacological inhibition of the CaMKK-β/AMPK pathway by STO-609 had any effects on cell growth and behavior in cell lines of human gastric adenocarcinoma.

Materials and methods

Cell culture

Human gastric cancer cell lines SNU-1, N87 and normal gastric epithelial cells CCL-241 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). All gastric cancer cells were cultured in RPMI-1640 (Life Technologies, Bethesda, Maryland, USA). CCL-241 cells were maintained in Hybri-Care Medium (ATCC) containing epidermal growth-factor (EGF) (30 ng/ml) (Sigma, St. Louis, MO, USA). All culture media were supplemented with 10% fetal bovine serum (FBS), penicillin (50 units/ml) and streptomycin (50 μg/ml).

Assessment of cell viability

Cell viability was assessed microscopically, and viable cell counting was performed following cell staining with Trypan blue.

Short hairpin RNA (shRNA) and lentivirus

The AMPK short hairpin RNA (shRNA) clone, the CaMKK-β shRNA clone and the non-target hairpin (negative control) were purchased from OriGene (Rockville, MD, USA). 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and were used to produce the shRNA lentivirus supernatant, using published methodology [14]. Briefly, the viral particles were used to infect gastric cancer cells for 12 h in the presence of polybrene (8 μg/ml). After 48 hours of infection, an antibiotic selection was initiated. Puromycin (2 μg/ml) was used to select stable clones and cell populations. Gene knockdown was confirmed by Western blot analysis.

Western blot analysis

The Western blots were performed on 70 μg of protein extract. The gastric adenocarcinoma cells, SNU-1 and N87, were lysed in lysis buffer (0.5% sodium deoxycholate, 50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS) containing 50 mM NaF, 5 mM EDTA, 1 mM dithiothreitol (DTT), and 10 μg/ml aprotinin. Cell lysates were resolved using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The samples were immunoblotted with primary antibodies including anti-phosphor-AMPK-Thr172, AMPK, phosphor-Akt-Ser473, phosphor-Akt-Thr308, Akt, phosphor-ERK, ERK, CaMKKβ and β-actin, followed by incubation with secondary antibodies. Antibodies were obtained from Cell Signaling Technology (Beverly, MA) and used at a dilution of 1:1000. Electrophoresis gel bands were visualized using an enzyme-linked chemiluminescence detection kit (ECL, Amersham Biosciences, Piscataway, NJ) as recommended by the manufacturer. Gel band density was quantified using Scion Image software (Scion Corp, Frederick, MD).

Analysis of cell apoptosis and necrosis

Gastric adenocarcinoma cells (3×10^5) were treated with STO-609 for 72 h, harvested with trypsin and then washed twice with phosphate-buffered saline (PBS). Measurement of cell apoptosis and necrosis was performed using immunofluorescence with the commercial Apoptosis and Necrosis Quantification Kit, obtained from Biotium Inc. (Hayward, CA, USA) as described previously [14]. Following cell staining, immunofluorescence in the fluorescein isothiocyanate (FITC) and propidium iodide (PI) channels were detected using flow cytometry acquisition (FACSCAN analyzer-BD). BD FACSDiva software was employed to analyze the data. Caspase-dependent cleavage of Poly ADP ribose polymerase (PARP) analysis was performed with Western analysis using the specific antibody.

Evaluation of cell autophagy

Cell autophagy was evaluated by the conversion of the microtubule-associated light chain 3

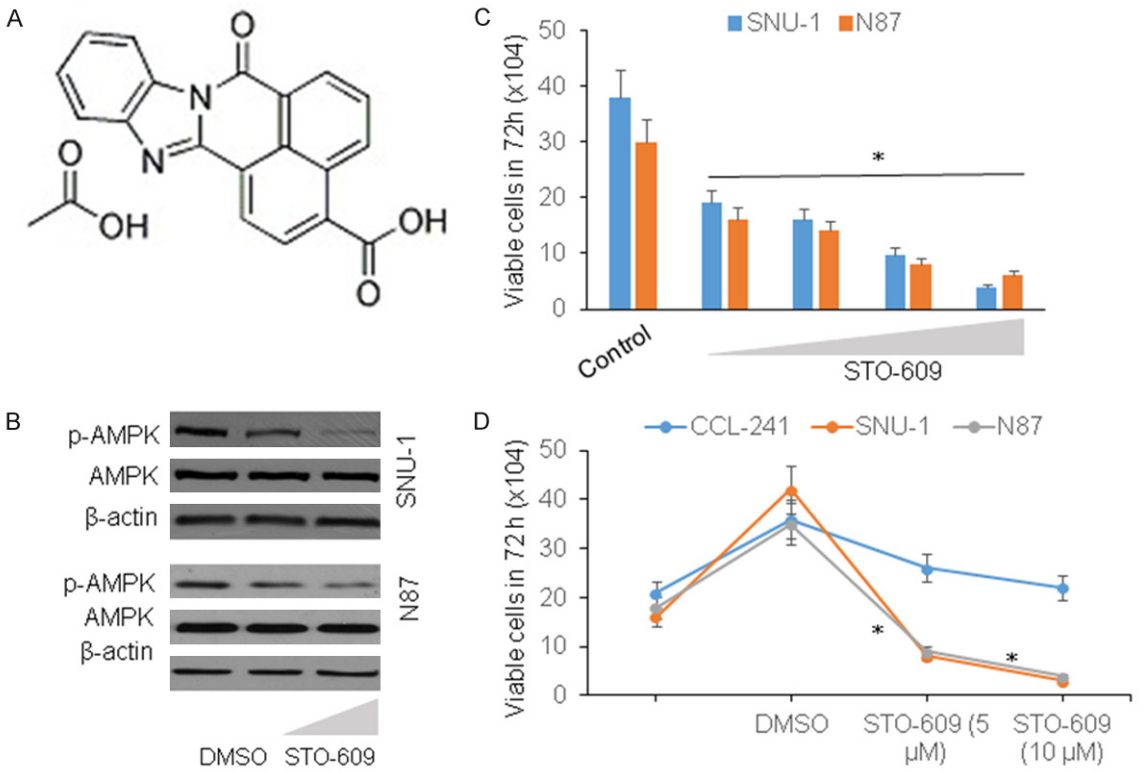

Figure 1. STO-609 is an effective growth inhibitor of gastric adenocarcinoma cell lines (SNU-1 and N87) *in vitro.* A. The chemical structure of STO-609. B. Immunoblots illustrating p-AMPK levels in gastric adenocarcinoma cells (SNU-1 and N87) treated with dimethyl sulfoxide (DMSO) (vehicle) or STO-609. C. Histogram illustrating the dose-dependent effect of STO-609 (2.5, 5, 10 and 20 μM) on the viability of the two gastric cancer cell lines (SNU-1 and N87). D. Proliferation assay results illustrating the anti-proliferative effect of STO-609 on gastric adenocarcinoma cells (SNU-1 and N87) versus normal gastric epithelial cells (CCL-241). *$P \leq 0.01$. Results indicated are representative of four independent experiments.

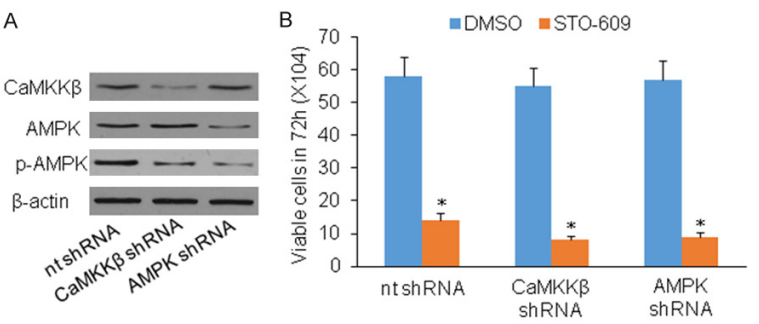

Figure 2. The anti-proliferative action of STO-609 is CaMKK-β/AMPK-independent. A. Western blotting analysis presenting the effects of 'silencing' CaMKK-β and AMPK showing decreasing AMPK activity. B. Proliferation assay shows the effects of STO-609 (10 μM) on normal gastric epithelial cells (CCL-241) and CaMKK-β and AMPK shRNA-expressing SNU-1 cells. *$P \leq 0.01$. Results indicated are representative of four independent experiments.

protein LC3A/B-I to LC3A/B-II with Western analysis using the specific antibody.

Trans-well invasion assay

An invasion assay was conducted with BD BioCoat Matrigel invasion chambers (BD Biosciences). Cells of the human gastric cancer cell line, SNU-1 were treated with increasing concentrations of STO-609 for 48 h. Cells were detached from culture plates with trypsin and washed three times with PBS. Cells were seeded (4×10^4 cells per well) in RPMI-1640 without serum in the upper chamber. As a chemo-attractant, RPMI-1640 medium containing 10% FBS was added to the lower compartment of the chamber. Following overnight incu-

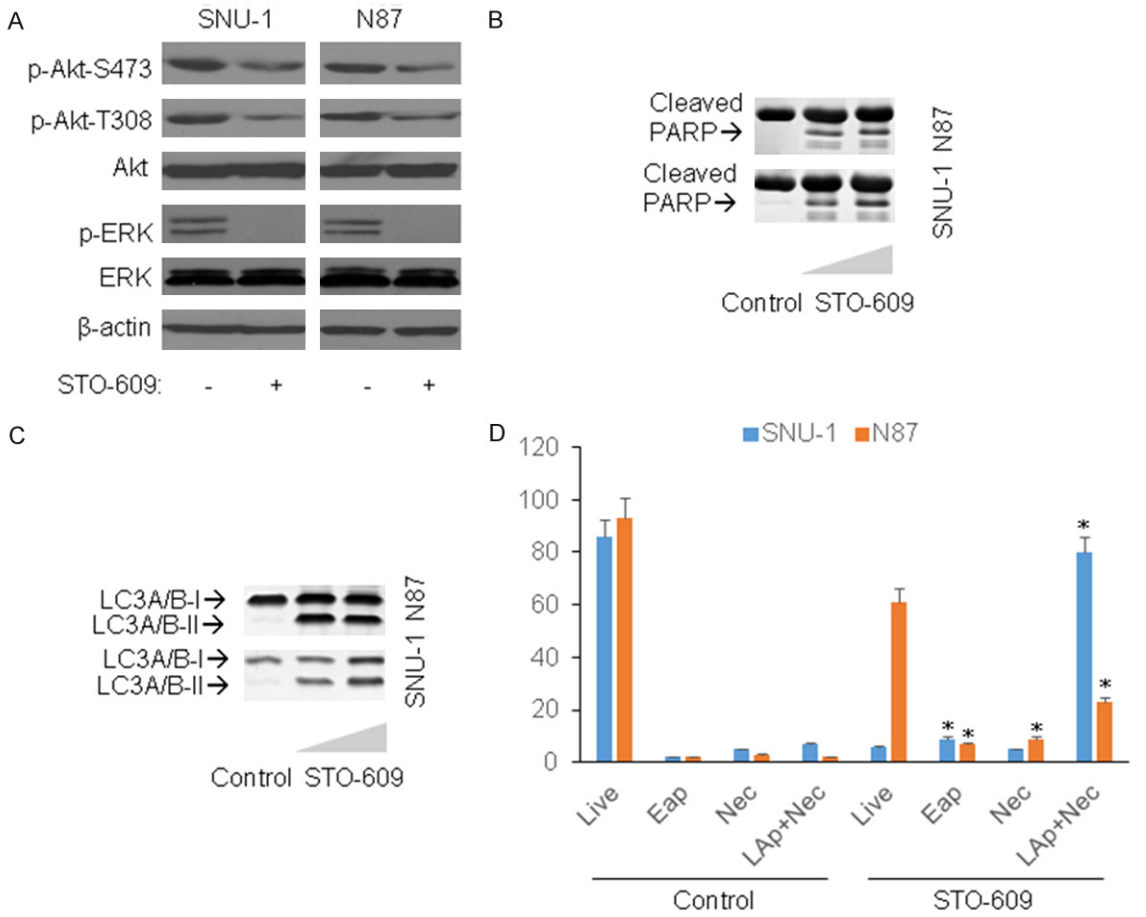

Figure 3. STO-609 inhibition affects several cellular survival pathways. (A-C) Western blotting results show the effects of STO-609 (5 and 10 μM) in gastric adenocarcinoma cells (SNU-1 and N87) on phosphorylation of Akt and ERK (A), apoptosis (B) and autophagy (C). Dimethyl sulfoxide (DMSO), a vehicle for STO-609, was used as control. (D) Apoptosis/necrosis analysis of gastric cancer cells treated with 10 μM STO-609. *$P \leq 0.01$ vs control. Results indicated are representative of three independent experiments.

bation, the non-invading cells were removed. After fixation with methanol and washing, the cell membranes were stained with Giemsa. Cell invasion was assessed by measurements using light microscopy. All experiments were performed in triplicate.

Statistical analysis

Quantitative data were expressed as mean ± SD. Student's t-test was used to compare two independent groups under a variety of conditions. $P < 0.05$ was considered statistically significant.

Results

STO-609 inhibition of gastric adenocarcinoma cell lines SNU-1 and N87

The inhibitor, STO-609 suppressed AMPK activity in the two gastric adenocarcinoma cell lines SNU-1 and N87, in a dose-dependent way (**Figure 1A**). STO-609 suppressed AMPK kinase activity in these two gastric adenocarcinoma cell lines as detected by abridged phosphorylation of AMPK at Thr172 (**Figure 1B**). STO-609 effectively inhibited proliferation of established gastric adenocarcinoma cell lines SNU-1 and N87 (**Figure 1C**). The effect of STO-609 on normal gastric epithelial cells, CCL-241 cells, was less significant and in keeping with a minimal toxic effect (**Figure 1D**). These findings show that STO-609 inhibits gastric adenocarcinoma cell proliferation independently of CaMKK-β/AMPK.

Short hairpin RNA (shRNA)-mediated knockdown of CaMKK-β and AMPK

Short hairpin RNA (shRNA), an artificial RNA molecule with a tight hairpin turn, was used to

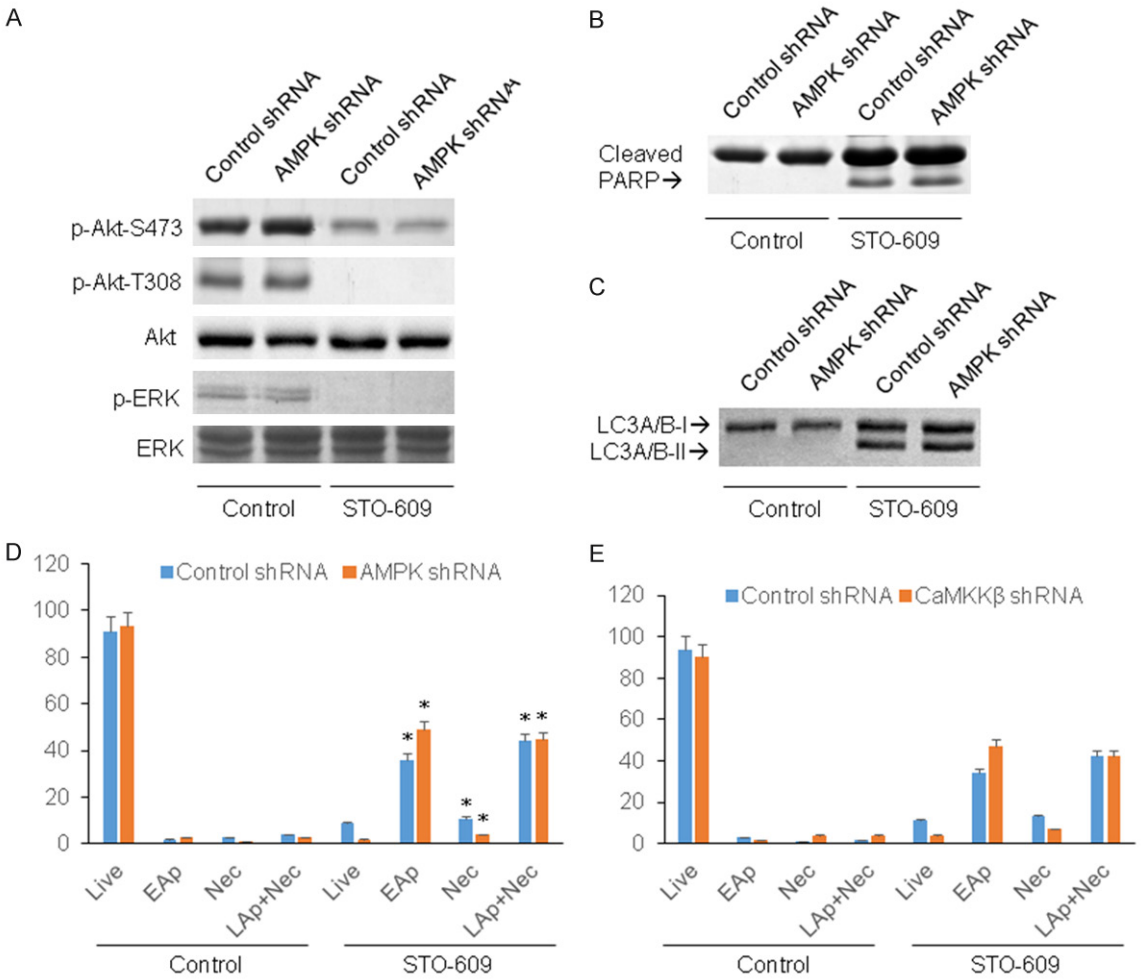

Figure 4. The anti-cancer effects of STO-609 are not AMPK-dependent. (A-C) Western blots demonstrating the effects of STO-609 on phosphorylation of Akt and ERK (A), apoptosis (B) and autophagy (C) in control or 'AMPK-silenced' gastric cancer cells. (D and E) apoptosis/necrosis examination of control or AMPK- and CaMKK-β-'silenced' gastric cancer cells incubated with 10 μM STO-609. DMSO, a vehicle of STO-609, was employed as a control. $*P \leq 0.001$. Results are representative of three individual experiments.

'silence' target gene expression. Using shRNA-mediated knockdown of CaMKK-β and AMPK, this method eliminated between 70% and 80% of the AMPK activity (**Figure 2A**).

To determine whether STO-609 required CaMKK-β or AMPK to inhibit cell proliferation, the gastric adenocarcinoma SNU-1 cells expressing control (nt), CaMKK-β and AMPK shRNA were treated with 10 μM STO-609. The results showed that STO-609 inhibited cancer cell proliferation regardless of CaMKK-β or AMPK (**Figure 2B**). The gastric adenocarcinoma cells that were CaMKK-β- or AMPK-'silenced' were more sensitive to the anti-proliferative actions of STO-609.

The Akt and ERK signaling pathways

As shown in **Figure 3A**, STO-609 inhibited Akt phosphorylation at serine 473 and threonine 308, the PI3K target sites. Similarly, STO-609 suppressed MEK activity towards ERK, as indicated by decreased ERK1/2 phosphorylation.

Gastric adenocarcinoma cell apoptosis

As shown in **Figure 3B**, STO-609-treated gastric adenocarcinoma cells exhibited caspase-dependent cleavage of Poly ADP ribose polymerase (PARP), indicating that STO-609 induced apoptosis. STO-609 increased autophagy as indicated by the augmented conversion

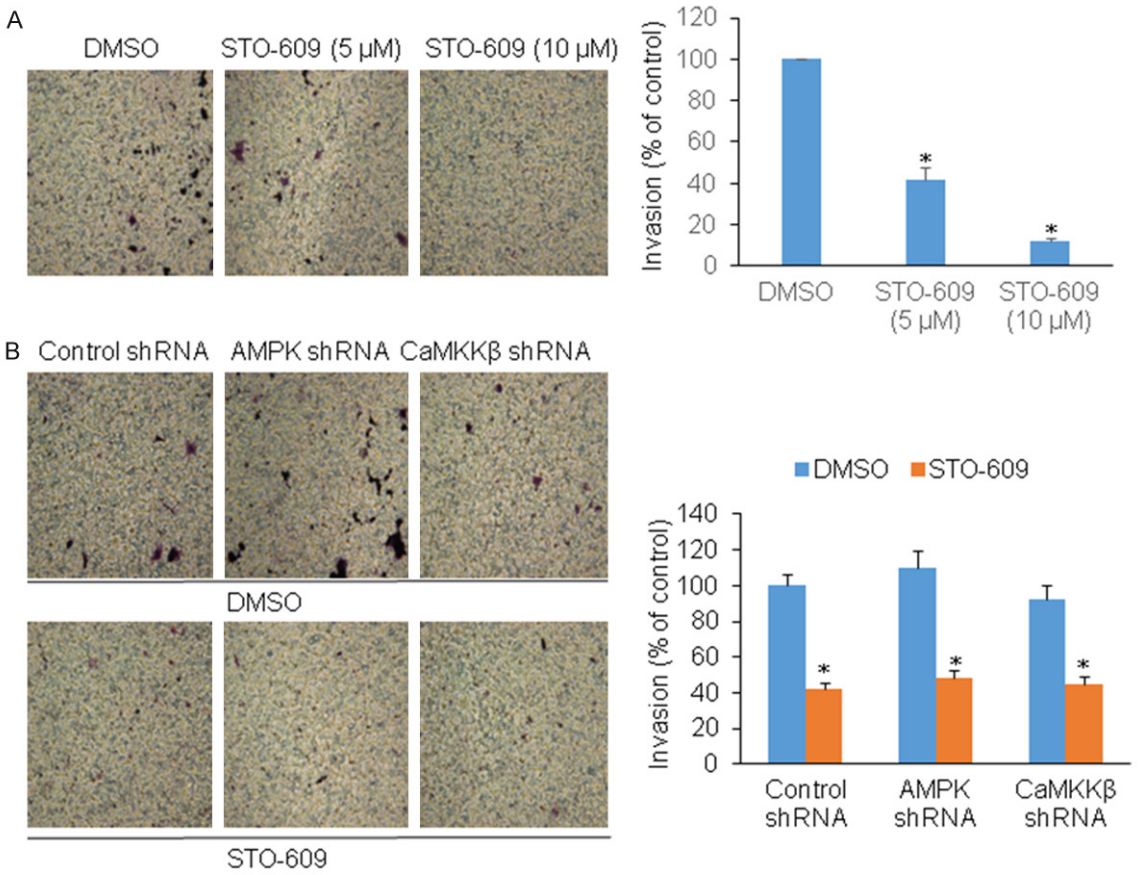

Figure 5. STO-609 inhibits gastric adenocarcinoma cell invasion, independent of CaMKKβ/AMPK. (A) Photomicrographs of gastric adenocarcinoma cells incubated with DMSO (vehicle) and STO-609 (5 and 10 μM), and (B) control, AMPK-knockdown, CaMKK-β-knockdown gastric adenocarcinoma cells treated with 10 μM STO-609 for 48 h. Cells were examined in an *in vitro* invasion assay. Each column indicates the mean (± SD) results. $P < 0.01$ vs control. The results are representative of three individual experiments.

of the microtubule-associated light chain 3 protein LC3A/B-I to LC3A/B-II (**Figure 3C**). A flow cytometry-based assay showed that STO-609 triggered cell death via both necrosis and apoptosis (**Figure 3D**).

The effects of STO-609 are AMPK-independent

Suppression of Akt phosphorylation by STO-609 both at Ser473 and Thr308 was comparable in gastric adenocarcinoma cells expressing control shRNA or AMPK shRNA (**Figure 4A**), indicating that the suppressive effects of STO-609 on PI3K activity were AMPK-independent. Similarly, STO-609 inhibited MEK activity towards ERK, as indicated by decreased ERK1/2 phosphorylation, in an AMPK-independent manner. STO-609 triggered autophagy, apoptosis, and necrosis in control- and AMPK- knockdown gastric adenocarcinoma cells (**Figure 4B-E**).

STO-609 inhibition of gastric adenocarcinoma cell invasion

STO-609 inhibited the invasive ability of gastric adenocarcinoma cells (**Figure 5A**) in a dose-dependent manner. To determine whether CaMKK-β and AMPK suppression was essential for this process, we employed CaMKK-β or AMPK knockdown gastric adenocarcinoma (SNU-1) cells. STO-609 inhibited the invasive ability of control, shRNA, AMPK and CaMKK-β knockdown SNU-1 cells (**Figure 5B**), suggesting that the suppressive effects of STO-609 on cell invasion is not AMPK-dependent.

Discussion

The role of physiologically activated calcium/calmodulin-dependent protein kinase kinase-beta (CaMKK-β) and AMP-activated protein kinase (AMPK) in malignancy is poorly under-

stood. The compound STO-609 inhibits CaMKK-β activity *in vitro*, making it a valuable pharmacological tool for assessing this kinase pathway [12].

This preliminary study has demonstrated a novel mechanism of STO-609-mediated gastric cancer cell death. We observed that STO-609 inhibited gastric cancer cell proliferation and invasion *in vitro* by inhibiting Akt/ERK signaling pathway activation independently of CaMKK-β or AMPK, with minimal effect on normal gastric epithelial cells. The results of this study indicate that STO-609, or similar compounds, may have a future role as an oncological therapeutic agent that inhibits cancer cells by multiple mechanisms, all of which are independent of CaMKK-β/AMPK.

In the present study, STO-609 was shown to have anti-proliferative effects in CaMKK-β- and AMPK-knockdown cells. Although STO-609 is an effective anti-proliferative agent *in vitro*, CaMKK-β and AMPK are not essential for its anti-proliferative actions. The findings of this study, therefore, also support a role for this pathway as a potential target in human cancer. Although this is a small *in vitro* study, these preliminary findings of the inhibitory actions of STO-609, on gastric adenocarcinoma cell apoptosis, necrosis, autophagy and cell invasion warrant further studies, particularly on the specific pathways for these effects.

Akt is an important factor in the control of proliferation and apoptosis via regulation of gene and protein expression and transcription [15-18]. Extracellular signal-regulated kinase (ERK) also plays an important role in cancer cell proliferation, survival and invasion [19-21]. Both activated Akt and ERK are involved in cell proliferation, as well as tumor angiogenesis [22, 23]. In the present study, we demonstrated that STO-609 inhibited the activation of both Akt and ERK independent of AMPK in gastric adenocarcinoma cells. It is possible that STO-609 may inhibit cancer cell proliferation and invasion by inhibiting the Akt/ERK signaling pathway; this pathway is not dependent on CaMKK-β and AMPK. Further studies on the role of this pathway in gastric adenocarcinoma and other malignancies are warranted.

In conclusion, this study has demonstrated that STO-609, the selective antagonist of CaMKK-β, is an effective cytotoxic agent that inhibits gastric adenocarcinoma cell proliferation *in vitro*. The inhibitory mechanisms include activation of the Akt/ERK signaling pathway and are independent of CaMKK-β/AMPK. The development of specific CaMKK-β/AMPK pathway pharmacological antagonists is awaited to enable future *in vivo* studies on the activated CaMKK-β/AMPK pathway in gastric adenocarcinoma.

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Disclosure of conflict of interest

None.

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