Original Research Article

Thieno [2, 3-d] pyrimidine inhibits gastric cancer cell proliferation via the down-regulation of bcl-2 and survivin expressions

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Abstract

Purpose: To investigate the effect of thieno [2, 3-d] pyrimidine on gastric cancer (GC) cell proliferation, and elucidate the mechanism of action involved.
Methods: Human GC cells (MKN1, MKN28 and SGC 7901) were cultured in RPMI-1640 medium supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin/ streptomycin solution at 37 °C for 24 h in a humidified atmosphere of 5 % $CO2$ and 95 % air. After attaining 60 - 70 % confluency, the cells were treated with serum-free medium and graded concentrations of thieno [2, 3-d] pyrimidine (0 – 12 µM) for 24 h. Normal cell culture without thieno [2, 3-d] pyrimidine served as control group. The cells were used in logarithmic growth phase. Cell viability and apoptosis were assessed using 3 (4,5 dimethyl thiazol 2 yl) 2,5 diphenyl 2H tetrazolium bromide (MTT), and flow cytometric assays, respectively. The levels of expression of ZNF139, B cell lymphoma 2 (bcl-2) and survivin in MKN1 cells and orthotopically transplanted mice were determined using Western blotting and real-time quantitative polymerase chain reaction (qRT-PCR).
Results: Treatment of MKN1, MKN28 and SGC 7901 cells with thieno [2, 3-d] pyrimidine for 72 h led to significant and dose-dependent reductions in their viabilities, as well as significant and dose-dependent increases in the number of apoptotic cells ($p < 0.05$). The results of qRT-PCR and Western blotting showed that ZNF139 mRNA and protein expressions in MKN1 cells were significantly down-regulated by thieno [2, 3-d] pyrimidine treatment ($p < 0.05$). Thieno [2, 3-d] pyrimidine treatment significantly and dose-dependently down-regulated the expressions of bcl 2 and survivin proteins in MKN1 cells and orthotopically transplanted mice ($p < 0.05$). It also significantly and dose-dependently inhibited the proliferation of GC cells in orthotopic mouse model of GC after 31 days of treatment ($p < 0.05$).
Conclusion: These results suggest that thieno [2, 3-d] pyrimidine suppresses the proliferation of GC cells via down-regulation of the expressions of ZNF139, bcl 2 and sur¬vivin. Thus, it has potentials for development for the management of gastric cancer.

Keywords: Thieno [2, 3-d] pyrimidine, Metastasis, Apoptosis, Cell proliferation, Expression

INTRODUCTION

Thienopyrimidines are bi-cyclic compounds with fused thiophene and pyrimidine rings [1]. These moieties act as scaffolds in the synthesis of pharmaceutically active compounds [2]. Thienopyrimidine-based derivatives exhibit varied pharmacological effects such as cGMP

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phosphodiesterase inhibitory [3], anti-inflammatory [4], antiviral [5], antimicrobial [6], and antitumor properties [7]. Gastric cancer (GC) is the fourth most common malignant tumor, and the second leading cause of cancer-related death worldwide [8]. The incidence of GC has increased on a global scale over the last few decades [8]. The incidence is highest in Southern America, Eastern Asia and Eastern Europe, and lowest in Northern America and Africa [9]. Chemotherapy and surgical resection are the usual strategies for treating GC [10,11]. However, GC is characterized by rapid metastasis and recurrence, thereby resulting in treatment failure, and poor prognosis [10]. Only 20 % of patients with GC survive beyond 5 years [11]. Hence, there is urgent need for new, non-toxic chemotherapeutic agents that can effectively inhibit GC cell proliferation and metastasis.

In mammalian cells, zinc finger proteins play key roles in the regulation of various cellular processes [12]. Some members of zinc finger protein family are involved in the development and metastasis of carcinomas [13]. The involvement of ZNF165 and ZNF217 in the pathogenesis of urinary bladder and colorectal and ovarian cancers have been reported [13]. The expression of ZNF139 has been shown to be upregulated in GC tissues [14].

The present study investigated the effect of thieno [2, 3-d] pyrimidine on GC cell *in vitro* and *in vivo*, and the mechanism involved.

EXPERIMENTAL

Materials

Human GC cell lines (MKN1, MKN28 and SGC-7901) were obtained from American Type Culture Collection (ATCC) (USA). Fetal bovine serum (FBS) and RPMI-1640 medium were purchased from Gibco (USA). Microplate reader and SYBR Green Supermix were products of Bio-Rad Laboratories, Inc. (USA). Annexin V/FITC kit and flow cytometer were obtained from BD Biosciences (USA). Trizol reagent and SuperScript Reverse transcription kits were products of Invitrogen, Thermo Fisher Scientific, Inc. (USA). Bicinchoninic acid (BCA) assay kit was purchased from Sangon Biotech Co., Ltd. (China). Polyvinylidene fluoride membranes were purchased from Roche Diagnostics (Switzerland). Anti-ZNF139, bcl-2, survivin and β-actin polyclonal antibodies were products of Abcam, (UK). Enhanced chemiluminescence system was obtained from GE Healthcare Life Sciences, (UK).

Cell culture

The MKN1, MKN28 and SGC-7901 cells were cultured in RPMI-1640 medium supplemented with 10 % FBS and 1 % penicillin/streptomycin solution at 37 °C for 24 h in a humidified atmosphere of 5 % CO_2 and 95 % air. After attaining 60 - 70 % confluency, the cells were treated with serum-free medium and graded concentrations of thieno [2, 3-d] pyrimidine (0 – 12 μM) for 24 h. Normal cell culture without thieno [2, 3-d] pyrimidine served as control group. Cells in logarithmic growth phase were selected and used in this study.

MTT assay

The viability of MKN1, MKN28 and SGC-7901 cells in the presence of thieno [2, 3-d] pyrimidine was assessed using MTT assay. The cells were seeded at a density of 1 x 10^6 cells/well in 96-well plates and cultured in Dulbecco's modified Eagle's medium (DMEM) for 24 h. Then, different concentrations of thieno [2, 3-d] pyrimidine (0 – 12 μM) were separately added to the cells and incubated for 72 h. At the end of the third day, 20 μL of MTT solution (5 mg/mL) was added to the wells, followed by incubation at 37 °C for 2 h.

The medium was finally replaced with 150 μL of 0.1 % dimethyl sulfoxide (DMSO) to completely dissolve the formazan crystals formed. The absorbance of the samples was read in a microplate reader at 485 nm. The assay was performed in triplicate. Cell viability was calculated as shown in Equation 1:

Cell viability (%) = Absorbance of the experimental group/Absorbance of the control group x 100 ……….(1)

Apoptosis assay

The GC cells were seeded at a density of 2.5 x 10^6 cells/well in 6-well plates and cultured for 24 h. Then, thieno [2, 3-d] pyrimidine (0 – 12 μM) was added to the medium and incubated for another 72 h, and thereafter washed with phosphate-buffered saline (PBS), and thoroughly mixed with 300 μL binding buffer. The cells were then stained with 5 μL each of Annexin V-fluorescein isothiocyanate and propidium iodide within 25 min at room temperature in the dark. Cell apoptosis was assessed using a flow cytometer fitted with argon laser operated at 485 nm.

Quantitative real time-polymerase chain reaction (qRT-PCR)

The MKN1 cells were treated with varied concentrations of thieno [2, 3-d] pyrimidine (0 – 12 µM) for 48 h. The levels of expression of Znf139, bcl-2 and survivin were measured using qRT-PCR. Total RNA was extracted from cells in each group with Trizol RNA extraction reagent, while cDNA synthesis kit was used to perform cDNA synthesis reaction according to the instructions of the manufacturer. Light Cycler 1536 RT-PCR detection system was used for the estimation of the mRNA expressions of these genes. Variation in the cDNA content was normalized using *β-a*ctin. The PCR reaction mixture (20 µl) consisted of 6.4 µl of dH_2O, 1.6 µl of gene-specific primer (10 µM), 2 µl of synthesized cDNA and 10 µl of SYBR Premix Ex Taq™ II. The Ct value of U6 was taken as the internal parameter, and $2^{-\Delta\Delta Ct}$ was used to calculate the relative expression levels of the proteins. The sequences of the primers used are shown in Table 1.

Table 1: Primer sequences used for qRT-PCR

Gene	Forward	Reverse
Znf139	CTT CCT GAG TTC TTG GTT TCG	CCT TTG ACC CAC TGG TTT ATG
Bcl-2	GGT GGG GTC ATG TGT GTGG	CGG TTC AGG TAC TCA GTC ATCC
survivin	GGA CCG CCT AAG AGG GCG TGC	AAT GTA GAG ATG CGG TGG TCC TT
*β-a*ctin	CAT GTA CGT TGC TAT CCA GGC	CTC CTT AAT GTC ACG CAC GAT

Western blotting

The MKN1 cells (5×10^8 cells/L) were incubated with thieno [2, 3-d] pyrimidine for 72 h. The cells were then washed twice with PBS and lysed with 250 µL of ice-cold radio-immunoprecipitation assay buffer (RIPA) containing protease and phosphatase inhibitors. The resultant lysate was centrifuged at 14,000 rpm for 20 min at 4 °C, and the protein concentration of the supernatant was determined using BCA method. A portion of total cell protein (10 µg) from each sample was separated on 10 % sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred to a fixed polyvinylidene fluoride membrane at 110 V and 90 °C for 120 min. Subsequently, non-fat milk powder (5 %) in Tris-buffered saline containing 0.2 % Tween-20 (TBS-T) was added with gentle shaking at 37 °C and incubated to block non-specific binding of the blot. Incubation of the blots was performed overnight at 4 °C with primary antibodies of rabbit polyclonal anti-ZNF139, survivin, bcl-2 and β-actin, each at a dilution of 1 to 800. Then, the membrane was washed thrice with PBS and further incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody for 1h at room temperature. The blot was developed using an X-ray film. Grayscale analysis of the bands was performed using Enhanced Chemiluminescence (ECL). The respective protein expression levels were normalized to that of β-actin which was used as a standard.

Establishment of mouse model of GC

Twenty adult male Balb/c mice aged 6 – 8 weeks, weighing 20 – 22 g (mean weight = 20 ± 2 g were obtained from Beijing HFK Bioscience Co., Ltd. The mice were housed in plastic cages under standard conditions and had free access to standard feed and water. Prior to commencement of study, the mice were acclimatized to the laboratory environment for 7 days. They were exposed to 12 h light/12 h dark cycle and maintained at a temperature of 25 °C, and 50 % humidity.

This study was approved by the Laboratory Animal Committee of China Medical University (no. STU/0017/134), and adhered strictly to the principles of Guide for the Care and Use of Laboratory Animals of the National Institute of Health [12].

Establishment of mice model of GC and grouping

The mice were anaesthetised using subcutaneous injection of pentobarbital sodium (6 %) and placed in a dorsal recumbent position. The fur around the surgical area was clipped and the area was swabbed with betadine. A 4 - 8 mm incision was made in the skin overlying the abdomen, and forceps were used to exteriorise the stomach. Then, 5×10^6 MKN1 cells in matrigel (50 µL) were injected into the serous side of the stomach, and the incision was closed using enterocoeliac sutures. The mice were then randomly assigned to four groups of 5 mice each: normal control group, negative control group, 5 mg/kg thieno [2, 3-d] pyrimidine group, and 10 mg/kg bwt thieno[2, 3-d] pyrimidine group. After tumor implantation in mice, the two treatment groups received 5 or 10 mg/kg thieno [2, 3-d] pyrimidine in DMSO, while mice in the normal and negative control groups received normal saline (5 mL/kg).

Statistical analysis

Data are expressed as mean ± SD, and statistical analysis was performed using SPSS (17.0). Groups were compared using Student *t*-test. Values of *p* < 0.05 were considered statistically significant.

RESULTS

Effect of thieno [2, 3-d] pyrimidine on the viability of GC cells

As shown in Figure 1, treatment of MKN1, MKN28 and SGC-7901 cells with thieno [2, 3-d] pyrimidine for 72 h led to significant and dose-dependent reductions in their viabilities (*p* < 0.05).
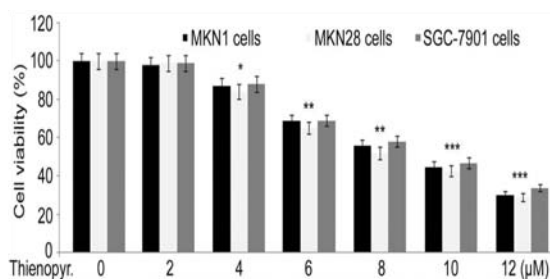

Figure 1: Effect of thieno [2, 3-d] pyrimidine on cell viability; *p* < 0.05, **p* < 0.01 and **p* < 0.001, when compared with control cells

Effect of thieno [2, 3-d] pyrimidine on apoptosis of GC cells

Treatment with thieno [2, 3-d] pyrimidine significantly and dose-dependently promoted apoptosis in GC cells (*p* < 0.05; Figure 2).
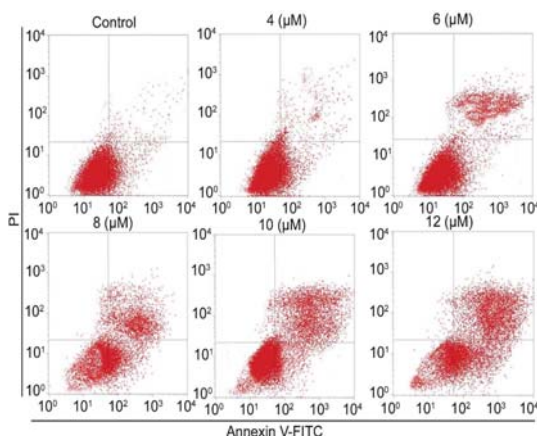

Figure 2: Effect of thieno [2, 3-d] pyrimidine on cell apoptosis

Effect of thieno [2, 3-d] pyrimidine on ZNF139 mRNA and protein expressions in MKN1 cells

The results of qRT-PCR and Western blotting showed that ZNF139 mRNA and protein expressions in MKN1 cells were significantly and dose-dependently down-regulated by thieno [2, 3-d] pyrimidine treatment. These results are shown in Figure 3.
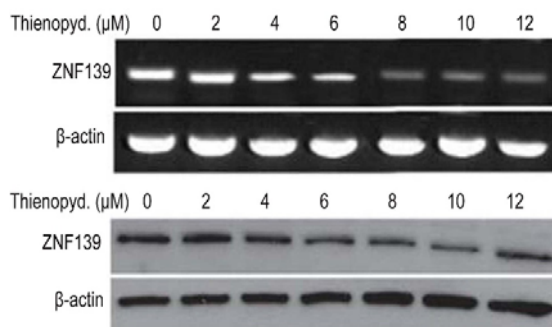

Figure 3: Effect of thieno [2, 3-d] pyrimidine on expressions of ZNF139 mRNA and protein in MKN1 cells. (**A**): Expression of ZNF139 mRNA as measured using qRT-PCR; and (**B**): ZNF139 protein expression as measured using Western blotting

Effect of thieno [2, 3-d] pyrimidine on the protein expressions of bcl-2 and survivin in MKN1 cells

Treatment of MKN1 cells with thieno [2, 3-d] pyrimidine significantly and dose-dependently down-regulated the expressions of bcl-2 and survivin proteins (*p* < 0.05; Figure 4).
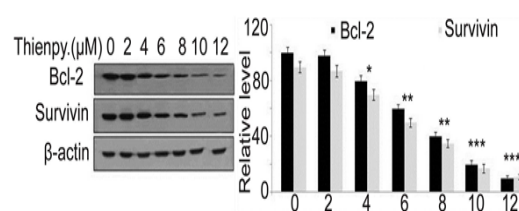
Figure 4: Levels of expression of bcl-2 and survivin in MKN1 cells after treatment with thieno [2, 3-d] pyrimidine. (**A**): Expressions of bcl-2 and survivin in MKN1 cells as measured using Western blotting; and (**B**): Expressions of bcl-2 and survivin in MKN1 cells as measured using qRT-PCR.

Effect of thieno [2, 3-d] pyrimidine on the proliferation of GC cells in mice

As shown in Table 2, treatment of the GC mice with thieno[2,3-d]pyrimidine caused a marked decrease in tumor growth after 31 days. However, the decrease in tumor growth was maximum in mice treated with thieno[2, 3-d] pyrimidine at the dose of 10 mg/kg than in mice

that received the drug at a dose of 5 mg/kg treatment group.

Effect of thieno [2, 3-d] pyrimidine on the expressions of bcl-2 and survivin proteins in orthotopically transplanted mice

Treatment of GC model mice with thieno [2, 3-d] pyrimidine significantly and dose-dependently down-regulated the expressions of bcl-2 and survivin proteins ($p < 0.05$; Figure 5).
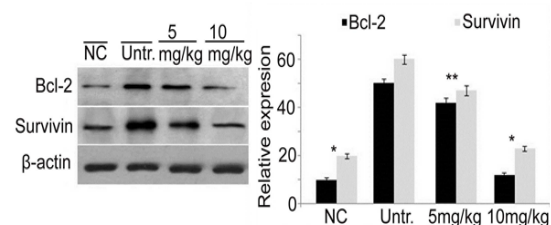

Figure 5: Effect of thieno [2, 3-d] pyrimidine on the expression levels of bcl-2 and survivin in GC model mice. **(A):** Expressions of bcl-2 and survivin in orthotopically transplanted mice as measured using Western blotting; and **(B):** Levels of expression of bcl-2 and survivin in orthotopically transplanted mice as measured using qRT-PCR

DISCUSSION

Gastric cancer (GC) is a leading cause of cancer death worldwide, and the advanced stage of the disease is correlated with the level of tumor invasion and metastasis [15]. The present study investigated the effect of thieno [2, 3-d] pyrimidine on the proliferation of GC cells, and the mechanism involved. Apoptosis is a highly regulated cellular process that plays an important role in getting rid of unwanted or aged cells. An imbalance in the regulation of apoptosis leads to tumor development [16,17]. Apoptosis is characterised by biochemical events which lead to changes in cells and cell death. These changes include blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation, and mRNA decay.

In this study, thieno [2, 3-d] pyrimidine treatment caused significant and dose-dependent reduction

in the viability of GC cells. Flow cytometric analysis revealed that the inhibitory effect of thieno [2, 3-d] pyrimidine on GC cell proliferation may be exerted via induction of apoptosis. An important member of zinc finger protein family involved in the regulation of transcription is ZNF139 [14].

The expression of ZNF139 has been reported to be markedly higher in cancer of the oesophagus and the gastroesophageal junction (GEJ) [18]. It is believed that ZNF139 promotes development of GC via enhancement of the expressions of ribonucleoproteins A2/B1 and fascin [19]. Studies have shown that the expression of ZNF139 is markedly higher in GC cells and contributes significantly to tumor progression [19]. In GC, ZNF139 promotes the expression of multidrug resistance protein-1 (MDR1), thereby promoting the resistance of the cells to chemotherapy [20]. In addition, ZNF139 has been shown to upregulate the expression of bcl-2, while suppressing bcl2-related X protein expression [20].

In this study, the exposure of MKN1 cells to thieno [2, 3-d] pyrimidine significantly and dose-dependently down-regulated mRNA and protein expressions of ZNF139. This suggests that thieno [2, 3-d] pyrimidine treatment may suppress GC cell proliferation via the down-regulation of protein expression of ZNF139. Upregulation of bcl-2 expression is associated with the anti-apoptotic potential of cancer cells, and involves regulation of mitochondrial redox potential [21]. The expression of survivin is associated with the suppression of caspase-3 activation, and therefore inhibition of cell apoptosis [22]. The results of this study showed that treatment of MKN1 cells with thieno [2, 3-d] pyrimidine significantly and dose-dependently down-regulated the expressions of bcl-2 and survivin proteins. These results suggest that thieno [2, 3-d] pyrimidine may inhibit the proliferation of GC cells, partly by inhibiting bcl-2 and survivin expressions. Treatment of GC model mouse with thieno [2, 3-d] pyrimidine also significantly and dose-dependently down-regulated the expressions of bcl-2 and survivin.

Table 2: Weight of tumor extracted from mice on day 31

Parameter	Group			
	Normal control	Negative control	5 mg/kg thieno [2, 3-d] pyrimidine	10 mg/kg thieno [2, 3-d] pyrimidine
Tumor weight mg)	0.0	1963.0	864.0	17.0

CONCLUSION

The results obtained in this study suggest that thieno [2, 3-d] pyrimidine suppresses the proliferation of GC cells via the down-regulation of the expressions of ZNF139, bcl-2 and survivin. Therefore, it can potentially be developed into a chemotherapeutic agent for gastric therapy.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Zhihui Lu, Li Chen, Fang Wu and Ni Guo performed the experimental work, carried out the literature survey and analysed the data. Fangyun Wan compiled the data. Xijuan Gu designed the study and wrote the manuscript. All the authors read the manuscript thoroughly and approved it for publication.

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