

Raltitrexed induces mitochondrial-mediated apoptosis in SGC7901 human gastric cancer cells

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Abstract. Raltitrexed is a specific inhibitor of thymidylate synthase (TS), which has been considered as a potential chemotherapeutic agent for the treatment of advanced gastric cancer. In the present study, the apoptosis mechanisms of raltitrexed in SGC7901 human gastric cancer cells were investigated. The cytotoxic activity of raltitrexed on SGC7901 cells was determined by cell counting kit-8 (CCK-8) assay. The CCK-8 assay indicated that raltitrexed inhibits SGC7901 cell growth in a dose- and time-dependent manner. The morphological changes were observed by fluorescent microscopy, and characteristic morphological changes, including nuclear shrinkage and apoptotic bodies, were observed following Hoechst 33258 staining. The effects on apoptosis, cell cycle, mitochondrial transmembrane potential and reactive oxygen species (ROS) were measured by flow cytometry. The analysis revealed that raltitrexed exerted a growth inhibitory effect by inducing time-dependent apoptosis and cell-cycle arrest at the G₀/G₁ phase. In addition, a compromised mitochondrial membrane potential and overproduction of ROS demonstrated the involvement of the mitochondrial signaling pathway. Raltitrexed-induced caspase-3-dependent apoptosis was identified using a caspase-3 activity assay and pretreatment with the caspase-3 inhibitor, Ac-DEVD-CHO (sequence, Ac-Asp-Glu-Val-Asp-CHO). The activity of caspase-3 was analyzed with a spectrometer. The protein expression levels of Bax, Bcl-2, cytochrome *c*, cleaved caspase-3 and TS were examined by western blot and the mRNA expression level of TS was detected by quantitative polymerase chain reaction. The analysis revealed that the protein levels of

Bax, cytochrome *c* and cleaved caspase-3 were significantly increased by raltitrexed, while Bcl-2 expression levels were reduced. Furthermore, raltitrexed increased the expression of the TS protein and mRNA in a time-dependent manner. These results indicate that raltitrexed induces the apoptosis of SGC7901 cells through the caspase-3-dependent mitochondrial signaling pathway and upregulates the expression of the TS protein and mRNA.

Introduction

Although the incidence of gastric cancer is decreasing in many regions, it remains the fourth most common type of malignant tumor, with the second highest mortality rate worldwide (1-3). In recent years, significant effort has been made to improve the diagnosis and clinical outcome for gastric cancer patients and the five-year survival rate for advanced gastric cancer in 2012 was ~60% (4). Previous studies have demonstrated that adjuvant chemotherapy results in a significant increase in five-year overall survival from 49.6 to 55.3% (5). However, the prognosis for advanced gastric cancer remains poor due to delayed diagnosis, drug resistance and the aggressive and heterogeneous nature of the disease. In addition, there are no universally accepted standard chemotherapy regimens (6,7). Therefore, it is important and necessary to develop novel efficacious therapies to treat advanced gastric cancer.

Raltitrexed is a specific inhibitor of thymidylate synthase (TS) that is chemically similar to quinazoline folate (8,9). *In vivo*, raltitrexed is metabolized into a series of polyglutamic acids. These metabolites are strong TS inhibitors that target DNA synthesis. Raltitrexed has been approved in Europe and China for the treatment of advanced colorectal cancer. A previous study demonstrated that the efficacy of raltitrexed alone, or in combination with oxaliplatin or irinotecan is comparable with that of fluorouracil (5-FU) or capecitabine, however, it is safer with regard to cardiac toxicity (10). Raltitrexed is also administered for the treatment of other solid tumors, including malignant pleural mesothelioma, pancreatic cancer, and head and neck neoplasms and has demonstrated good tolerance (7,11,12). Cardiac toxicity induced by 5-FU is a less common, although significant and potentially lethal side-effect. Unlike 5-FU, raltitrexed does

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not cause the accumulation of associated metabolites, which increase cardiotoxicity (13,14). Based on preliminary clinical observations, survival benefits were achieved in certain advanced gastric cancer patients receiving raltitrexed either alone or in combination with other therapeutic agents, such as paclitaxel and docetaxel.

Although TS is a major target of raltitrexed, the possible mechanisms of raltitrexed-induced apoptosis remain unknown in gastric cancer. The aims of the present study were to evaluate the effect of raltitrexed on SGC7901 human gastric cancer cells and to determine whether the potential mechanism of apoptosis is associated with caspase-3-dependent mitochondrial signaling pathway.

Materials and methods

Reagents. Raltitrexed was purchased from Jiangsu Zhengda Tianqing Pharmaceutical Co., Ltd. (Nanjing, China). Cell counting kit-8 (CCK-8), Annexin V-FITC Apoptosis Detection kit, propidium iodide (PI), Mitochondrial Membrane Potential assay kit, Reactive Oxygen Species (ROS) assay kit, Caspase-3 Activity assay kit, radio-immunoprecipitation assay (RIPA) lysis buffer, and ECL Plus were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Rabbit monoclonal anti-human antibodies against Bax, Bcl-2, cytochrome *c*, cleaved caspase-3, TS and β -actin were purchased from Cell Signaling Technology Inc., (Beverly, MA, USA). One-Step SYBR[®] PrimeScript[™] RT-PCR kit II was purchased from Takara Bio Inc., (Dalian, China).

Cell culture. The SGC7901 human gastric cancer cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI 1640 (Wisent Biotechnology, Nanjing, China) complete medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 mg/l streptomycin at 37°C in a humidified atmosphere of 5% CO₂ in air.

Cell proliferation assay. The effect of raltitrexed on cell proliferation was determined by CCK-8 assay. Briefly, cells at the logarithmic growth phase were seeded at a density of 4,000 cells/well into 96-well plates and incubated at 37°C for 24 h. Subsequently, cells were treated with different concentrations of raltitrexed (0.1, 0.5 and 2.5 μ g/ml) for 24, 48 and 72 h. Following raltitrexed treatment, 20 μ l CCK-8 reagent was added to each well and cells were incubated for a further 2 h. Absorbance values were measured at a wavelength of 450 nm in a Bio-Rad Model 680 microplate reader (Bio-Rad, Hercules, CA, USA).

Morphological observation of cells. SGC7901 cells were treated with or without 0.5 μ g/ml raltitrexed for 24, 48 and 72 h. Morphological observations were conducted with Hoechst 33258 (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) staining. Cells were fixed in 4% paraformaldehyde for 30 min. After washing twice with phosphate-buffered saline (PBS), nuclear staining was performed with Hoechst 33258 for 5 min at room temperature. The proportion of dead cells was visualized microscopically using an inverted fluorescence microscope (Olympus IX51, Olympus, Tokyo, Japan).

Flow cytometric analysis of apoptosis and the cell cycle. SGC7901 cells were treated with or without 0.5 μ g/ml raltitrexed for 24, 48 and 72 h. Following treatment, cells were centrifuged at 1,000 x g for 5 min in a 5415R Microcentrifuge (Eppendorf, Hamburg, Germany) and lightly resuspended in 195 μ l Annexin V-FITC Binding Buffer (from the apoptosis detection kit). Annexin V-FITC (5 μ l) and 10 μ l PI were applied for 30 min to stain the cells, which were maintained on ice. For cell cycle analysis, cells were harvested and fixed in 70% ice-cold ethanol at 4°C overnight. Following fixation, cells were centrifuged at 1,000 x g for 5 min, washed with cold PBS three times and stained with 50 μ g/ml PI and 100 μ g/ml RNase A at 37°C for 30 min in the dark. Finally, the apoptosis and cell cycle were immediately analyzed using flow cytometry (BD FACSCalibur[™]; BD Biosciences, San Jose, CA, USA).

Measurement of mitochondrial membrane potential. JC-1 dye (from the mitochondrial membrane potential assay kit) was used to assess the mitochondrial membrane potential by detecting fluorescence at a wavelength of 488 nm. Following treatment with or without 0.5 μ g/ml raltitrexed for 24 or 48 h, SGC7901 cells were collected by centrifugation at 600 x g for 5 min and resuspended in 1 ml PBS containing 5 μ g/ml JC-1 dye. Following incubation at 37°C for 20 min, cells were re-centrifuged at 600 x g for 5 min, then washed with PBS twice. The suspension was analyzed using flow cytometry.

Determination of ROS generation. The changes in intracellular ROS generation were determined by measuring the conversion of non-fluorescent 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; from the ROS assay kit) into fluorescent dichlorofluorescein (DCF). SGC7901 cells were treated with or without 0.5 μ g/ml raltitrexed for 24 or 48 h. Briefly, cells were cultured in 6-well plates containing raltitrexed and incubated with 10 μ M DCFH-DA at 37°C for 30 min. Rosup (1 μ l; from the ROS assay kit) was added and this served as the positive control group. The fluorescence intensity of DCF was analyzed at an excitation wavelength of 488 nm and an emission wavelength of 525 nm using flow cytometry.

Caspase-3 activity assay. The activity of caspase-3 was analyzed by determining the levels of p-nitroaniline (pNA) cleaved from the substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA). SGC7901 cells were treated with or without 0.5 μ g/ml raltitrexed for 24 or 48 h. The cell lysates were incubated with 2 mM Ac-DEVD-pNA at 37°C for 2 h according to the manufacturer's instructions. Following incubation, samples were analyzed using a U-2001 UV/Vis Spectrometer (Hitachi, Ltd., Tokyo, Japan) at a wavelength of 405 nm.

Western blot analysis. Raltitrexed was added at 0.5 μ g/ml into SGC7901 cells. After 24 and 48 h, cells were harvested and suspended on ice with RIPA lysis buffer containing 1 mM phenylmethanesulfonyl fluoride (Beyotime Institute of Biotechnology). Briefly, the gradient volume of standard protein was pipette into 96-well plates to prepare a standard curve. Then 200 μ l bicinchoninic acid (BCA) working reagent (Beyotime Institute of Biotechnology) was added to each

well. After incubation at 37°C for 30 min, absorbance values were determined at a wavelength of 562 nm in a microplate reader. The cell lysates were centrifuged at 12,000 x g for 15 min at 4°C and protein concentrations were measured using the BCA assay method. Proteins (50 µg) were separated on 10% SDS-PAGE by electrophoresis and transferred to polyvinylidene fluoride membranes (Merck Millipore, Darmstadt, Germany). After blocking with 5% non-fat milk in Tris-buffered saline with Tween-20 (TBST) solution for 1 h, the membranes were incubated with diluted primary antibodies (1:1,000) at 4°C overnight. The membranes were washed with TBST and incubated with the corresponding secondary antibodies. After washing with TBST three times, ECL Plus was used for detecting the target bands.

Quantitative polymerase chain reaction (qPCR) analysis. Following treatment with or without 0.5 µg/ml raltitrexed for 24 or 48 h, SGC-7901 cells were harvested and total RNA was extracted using TRIzol (Nanjing KeyGen Biotech Co., Ltd.). The fluorescent dye SYBR® Green I was applied for qPCR analysis using the ABI® 7300 Real-Time qPCR system (Applied Biosystems, Foster City, CA, USA). The primers used were as follows: Forward, 3'-GCAAAGAGTGATTGACACCATCAA-5'; and reverse, 3'-CAGAGGAAGATCTCTTGGATTCCAA-5' for TS and forward, 3'-CAGTCGGTTGGAGCGAGCAT-5'; and reverse, 3'-GGACTTCCTGTAAACAACGCATCT-5' for β-actin. Each qPCR reaction used 50 µl mixture in total, including 25 µl One-Step SYBR® RT-PCR buffer IV, 2 µl PrimeScript One-Step Enzyme mix II, 2 µl qPCR forward primer (0.4 µM), 2 µl reverse primer (0.4 µM), 4 µl total RNA and 14 µl RNase free dH₂O. Reverse transcription was performed at 42°C for 5 min, 95°C for 10 sec, followed by 40 cycles of qPCR amplification at 95°C for 5 sec and at 60°C for 31 sec. A melting curve analysis was conducted to detect the specificity of the qPCR products.

Statistical analysis. Data are expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed by one-way analysis of variance using the software SPSS v20.0 (IBM, Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference. The results are representative of three independent experiments, each in triplicate.

Results

Effect of raltitrexed on the inhibition of SGC7901 cells. The antiproliferative effect of raltitrexed on human gastric cancer SGC7901 cells was determined by a CCK-8 assay. The inhibitory rate of cells exposed to 0.1, 0.5 and 2.5 µg/ml raltitrexed for 24, 48 and 72 h was determined. The results indicate that raltitrexed decreased the cell viability in a dose- and time-dependent manner (Fig. 1). A concentration of 0.5 µg/ml was selected for the subsequent experiments.

Raltitrexed-induced morphological changes. Cell morphological changes were observed by Hoechst 33258 staining using fluorescence microscopy. SGC7901 cells treated with 0.5 µg/ml raltitrexed showed typical apoptotic

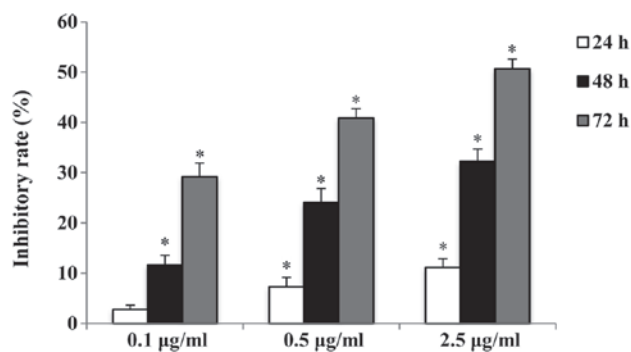


Figure 1. Effect of raltitrexed on inhibition rate in SGC7901 cells. Cell counting kit-8 assays revealed that raltitrexed inhibited the growth of SGC7901 cells in a dose- and time-dependent manner. Data are expressed as the mean ± standard error of the mean; *P<0.01 vs. the control.

morphology, including nuclear shrinkage, fragmentation, chromatin condensation and apoptotic bodies, particularly following 48 h of raltitrexed exposure (Fig. 2A).

Effect of raltitrexed on apoptosis. Flow cytometric analysis of Annexin V-FITC/PI staining showed that the early and late apoptosis rates of SGC7901 cells treated with raltitrexed were increased in a time-dependent manner compared with the rates observed in the control (Fig. 2B). The apoptosis rates of the 48- and 72-h treatment groups were increased to 28.52±1.82% and 47.27±1.61%, respectively (P<0.01). However, no significant apoptosis was observed after 24 h of raltitrexed exposure (P>0.05).

Effect of raltitrexed on the cell cycle. Cell cycle arrest is one of the predominant regulatory mechanisms of apoptosis. Cell cycle analysis was performed to examine the effects of raltitrexed by flow cytometry. The results show that raltitrexed significantly blocked the cell cycle at the G₀/G₁ phase in a time-dependent manner (Fig. 2C). Treatment with raltitrexed indicated an increase in the number of cells in the G₀/G₁ phase from 57.28±2.43% in the control to 65.34±1.86%, 74.24±2.83%, and 81.33±3.61% after 24, 48 and 72 h of exposure, respectively (P<0.01), with corresponding decreases in the S and G₂/M phases.

Effect of raltitrexed on mitochondrial membrane potential. Loss of mitochondrial membrane potential is important in apoptosis. The mitochondrial membrane potential was measured in the raltitrexed-treated groups using JC-1 staining and flow cytometric analysis. The green fluorescence of cells treated with raltitrexed was identified to be higher than that in the control, at 12.97±2.37% and 29.11±1.12% after 24 and 48 h of exposure, respectively (Fig. 3; P<0.01). Hence, raltitrexed treatment resulted in a decrease in the mitochondrial membrane potential.

Effect of raltitrexed on ROS generation. The level of intracellular ROS was determined by staining with DCFH-DA, and measuring fluorescence with a flow cytometer. It was demonstrated that raltitrexed increases the level of ROS from 0.15±1.38% (control) to 10.80±3.44% and 32.09±5.80% after 24 and 48 h of exposure, respectively (Fig. 4; P<0.01). The level

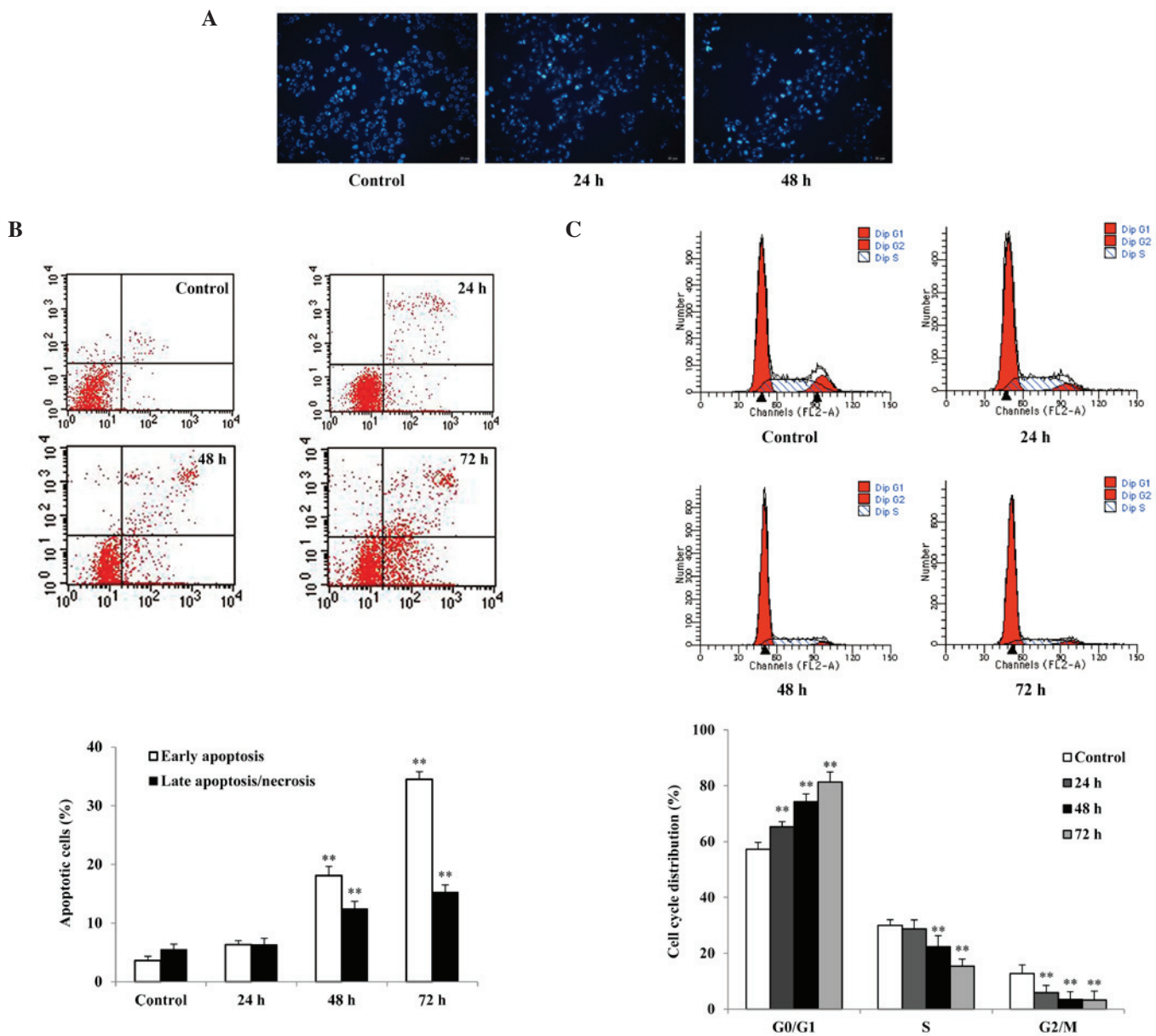


Figure 2. Effect of raltitrexed on apoptosis and cell cycle in SGC7901 cells. (A) Morphological observation of cells was performed using Hoechst 33258 staining. Magnification, x200. (B) Flow cytometric analysis of apoptosis was conducted with Annexin V-fluorescein isothiocyanate/potassium iodide (PI) staining. Dotted plots present Annexin V-FITC on the x axis, and PI on the y axis. (C) Flow cytometric analysis of cell cycle distribution was undertaken using PI staining. Data are expressed as the mean \pm standard error of the mean; * P <0.01 vs. the control.

of ROS in the positive control group (Rosup) was $93.08 \pm 4.06\%$. Therefore, the production of ROS was demonstrated to be associated with raltitrexed-induced apoptosis.

Caspase-3-dependent apoptosis induced by raltitrexed. To investigate whether raltitrexed-induced apoptosis is caspase-3-dependent, the activation of caspase-3 was measured with a Caspase-3 Activity assay kit. As shown in Fig. 5A, compared with that in the control, an increase of ~ 2.07 - 4.39 -fold was observed in the activity of caspase-3 after 24 and 48 h of exposure, respectively (P <0.01). In addition, the caspase-3 inhibitor, Ac-Asp-Glu-Val-Asp-CHO (Ac-DEVD-CHO) was used in the CCK-8 assay and it was revealed that $50 \mu\text{M}$ Ac-DEVD-CHO significantly reduces the inhibitory rate induced by $0.5 \mu\text{g/ml}$ raltitrexed (Fig. 5B; P <0.01).

Effect of raltitrexed on the apoptosis-associated protein expression. To investigate the mitochondria-dependent apoptosis induced by raltitrexed in SGC7901 cells, the protein expression levels of Bax, Bcl-2, cytochrome *c* and cleaved caspase-3 were measured using western blot analysis. As shown in Fig. 6, the expression levels of Bax, cytochrome *c* and cleaved caspase-3 significantly increased in a time-dependent manner (P <0.05, P <0.01), while the expression levels of Bcl-2 decreased in a time-dependent manner (P <0.05). These results indicate that raltitrexed induces apoptosis via activation of the mitochondria.

TS protein and mRNA expression levels. As TS is a major target of raltitrexed, the expression levels of TS protein and mRNA were determined by western blot analysis and qPCR, respectively. Compared with those in the control, the levels of TS protein and

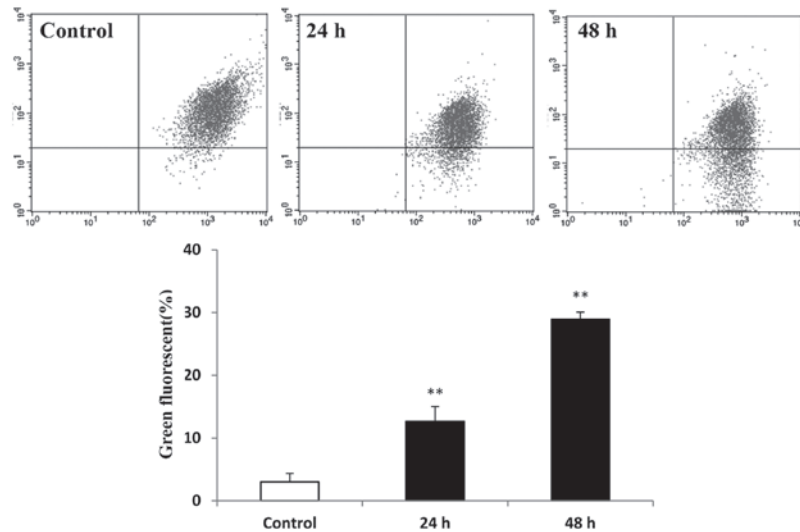


Figure 3. Effect of raltitrexed on mitochondrial membrane potential in SGC7901 cells. The mitochondrial membrane potential was examined by staining with JC-1. Dotted plot presents JC-1 monomers on the x axis and JC-1 aggregates on the y axis. Data are expressed as the mean \pm standard error of the mean; * P <0.01 vs. the control.

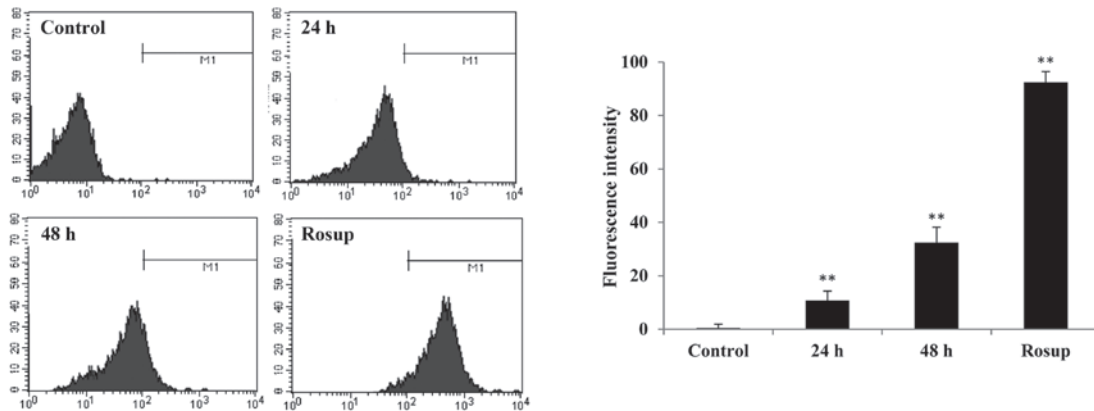


Figure 4. Effect of raltitrexed on reactive oxygen species (ROS) generation in SGC7901 cells. The level of ROS in cells was assayed by staining with dichloro-dihydro-fluorescein diacetate. In the graphs on the left, fluorescence intensity is presented on the x axis and cell number on the y axis. Data are expressed as the mean \pm standard error of the mean; * P <0.05, ** P <0.01 vs. the control.

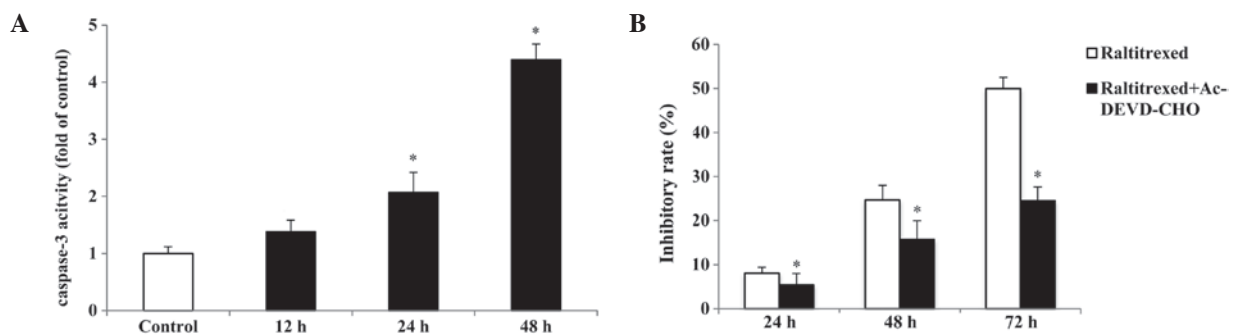


Figure 5. Effect of raltitrexed on caspase-3-dependent apoptosis in SGC7901 cells. (A) The activity of caspase-3 was measured by caspase-3 activity assay kit. (B) Inhibitory rate was analyzed by cell counting kit-8 assay. Data are expressed as the mean \pm standard error of the mean; * P <0.01 vs. the control.

mRNA expression significantly increased following treatment with raltitrexed for 48 h (Fig 7; P <0.05, P <0.01), however, there were no significant differences identified after a 24-h exposure (P >0.05). Melting curve analysis indicated that there was no non-specific amplification in the qPCR.

Discussion

Gastric cancer is one of the most common types of cancer worldwide and it is associated with numerous factors (15,16). In the early stages of gastric cancer, resection surgery offers

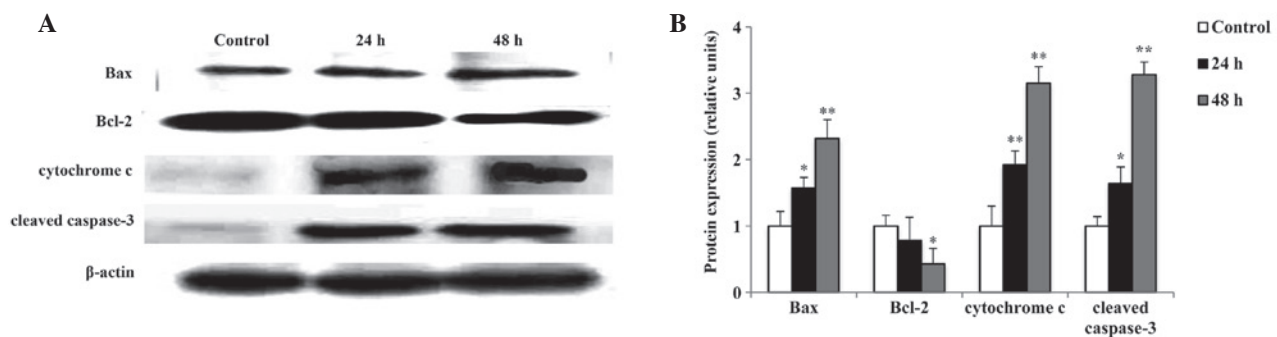


Figure 6. Effect of raltitrexed on the expression of the apoptosis regulators in SGC7901 cells. The levels of Bax, Bcl-2, cytochrome *c* and cleaved caspase-3 were measured by western blot analysis. Data are expressed as the mean \pm standard error of the mean; * $P < 0.05$, ** $P < 0.01$ vs. the control.

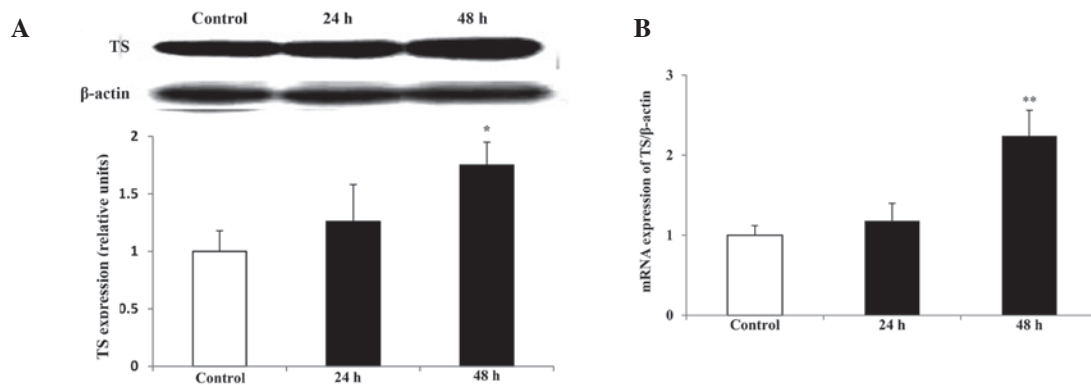


Figure 7. Effect of raltitrexed on the expression levels of TS protein and mRNA in SGC7901 cells. (A) The level of TS protein was determined by western blot analysis. (B) The level of TS mRNA was measured by quantitative polymerase chain reaction. Data are expressed as the mean \pm standard error of the mean; * $P < 0.05$, ** $P < 0.01$ vs. the control. TS, thymidylate synthase.

the possibility of a radical cure, however, certain patients are diagnosed in the middle or advanced stages when surgery loses its efficacy. For these patients, chemotherapy is the predominant treatment strategy. The chemotherapeutic agent, 5-FU is widely used for the treatment of gastric cancer due to its anti-tumor activity. However, drug resistance and side-effects have been encountered in numerous patients, therefore, it is necessary to establish alternatives with lower levels of toxicity. Raltitrexed is another chemotherapeutic agent and a folate antimetabolite, which specifically inhibits TS activity. It has been approved for patients with advanced colorectal cancer in Europe and China (10,17,18) and unlike 5-FU it rarely induces fluoropyrimidine-associated cardiotoxicity (19,20). For cancer patients with fluoropyrimidine-induced cardiotoxicity or a history of cardiac disease, raltitrexed may be a suitable alternative to 5-FU (18). In addition to patients with colorectal cancer, certain patients with advanced gastric cancer have benefited from treatment with raltitrexed and have demonstrated good tolerance to raltitrexed with low cardiac toxicity (21,22). However, the anticancer effect of raltitrexed for advanced gastric cancer remains unknown with regard to the pro-apoptotic mechanisms.

The present study demonstrated that raltitrexed increases the inhibitory rate of SGC7901 human gastric cancer cells in a dose- and time-dependent manner. Typical apoptotic morphological features were observed through staining with Hoechst 33258 and subsequent, flow cytometric analysis was consistent with these results, revealing that raltitrexed induced

time-dependent apoptosis and cell-cycle arrest at the G_0/G_1 phase. Apoptosis is a spontaneous process of programmed cell death, which is precisely regulated by organisms. Generally, there are three predominant signaling pathways in apoptosis, including the mitochondrion, the death receptor and the endoplasmic reticulum signaling pathways. Despite multiple cell types and apoptotic signaling pathways, the integration and amplification of apoptotic signaling usually occurs at the mitochondrial level (23). In the present study, the mitochondrial membrane potential and ROS generation were initially measured in SGC7901 cells treated with raltitrexed. The results indicated that raltitrexed significantly induced a mitochondrial membrane potential decrease and excessive generation of mitochondrial ROS in a time-dependent manner. Therefore, it may be inferred that raltitrexed-induced apoptosis is mediated by the mitochondrial signaling pathway in gastric cancer cells.

Mitochondria are involved in various cellular events of apoptosis, for example mitochondrial membrane potential decrease, ROS generation and the release of apoptosis-associated proteins (24-26). Loss of mitochondrial membrane potential is an early and specific event of the mitochondrial apoptosis signaling pathway; once it occurs, the apoptosis is irreversible. ROS that are produced in the mitochondria are generally considered to participate in mitochondria-mediated apoptosis either directly or indirectly, which results in the activation of caspase and DNA fragmentation (27). The *Bcl-2* gene family is critical in the signal transduction pathway of apoptosis (28,29).

A high *Bax/Bcl-2* ratio is usually recognized as a reliable basis for the induction of apoptosis. It affects mitochondria via the activation of a series of downstream genes. In the present study, the *Bax/Bcl-2* ratio was significantly increased in raltitrexed-treated SGC7901 cells compared with that in the controls. Under the stimulation of the above-mentioned factors, mitochondria release apoptosis-associated bioactive substances, such as cytochrome *c*. The release of mitochondrial cytochrome *c* is a primary step towards the intrinsic apoptosis signaling pathway (30,31). The binding of cytochrome *c* to apoptotic protease activating factor 1 results in the activation of pro-caspase-9 and active caspase-9 that initiates a caspase signaling cascade, which induces apoptosis (32). Activation of caspase-3 has been shown to be an indispensable aspect of the execution phase of apoptosis (33). The present study showed that treatment with raltitrexed induced a time-dependent increase in expression levels of cytochrome *c* and cleaved caspase-3. Subsequently, the caspase-3-dependent apoptosis was demonstrated by a caspase-3 activity assay and pretreatment with the caspase-3 inhibitor, Ac-DEVD-CHO. These results indicate the involvement of the caspase-3-dependent mitochondrial signaling pathway.

TS is the major rate-limiting enzyme for the *de novo* synthesis of thymine nucleotides. This process is necessary for DNA synthesis and repair. The present study determined that the expression level of TS protein gradually increases in line with the corresponding mRNA in a time-dependent manner. In a previous study of gastric cancer, the TS mRNA expression levels in the tumor and plasma were significantly lower in the raltitrexed-sensitive group than in the raltitrexed-resistant group (34). Therefore, reducing the expression of TS mRNA may be a possible method of increasing the sensitivity of cells to raltitrexed in a clinical setting (35,36).

In conclusion, the present study revealed that raltitrexed inhibited the growth of SGC7901 human gastric cancer cells and induced apoptosis via the caspase-3-dependent mitochondrial signaling pathway. The mechanisms included cell cycle arrest at the G₀/G₁ phase, compromised mitochondrial membrane potential, overproduction of ROS, upregulation of Bax, cytochrome *c* and cleaved caspase-3, and downregulation of *Bcl-2*. Furthermore, the expression levels of TS protein and mRNA were significantly increased by raltitrexed. However, the *in vitro* study is not sufficient to demonstrate that raltitrexed is a suitable treatment for gastric cancer. Further preclinical studies are to provide theoretical support for future clinical practice.

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