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• GASTRIC CANCER •

Effects of mifepristone on proliferation of human gastric adenocarcinoma cell line SGC-7901 *in vitro*

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Abstract

AIM: To explore the effects of mifepristone, a progesterone receptor (PR) antagonist, on the proliferation of human gastric adenocarcinoma cell line SGC-7 901 *in vitro* and the possible mechanisms involved.

METHODS: *In situ* hybridization was used to detect the expression of PR mRNA in SGC-7 901 cells. After treatment with various concentrations of mifepristone (2.5, 5, 10, 20 μmol/L) at various time intervals, the ultrastructural changes, cell proliferation, cell-cycle phase distribution, and the expression of caspase-3 and Bcl-X_L were analyzed using transmission electron microscopy (TEM), tetrazolium blue (MTT) assay, ^{3}H-TdR incorporation, flow cytometry, and reverse transcription-polymerase chain reaction (RT-PCR).

RESULTS: Mifepristone markedly induced apoptosis and inhibited cell proliferation of PR- positive SGC-7 901 cells revealed by TEM, MTT assay and ^{3}H-TdR incorporation, in a dose- and time-dependent manner. The inhibitory rate was increased from 8.98% to 51.29%. Flow cytometric analysis showed mifepristone dose-dependently decreased cells in S and G_2/M phases, increased cells in G_0/G_1 phase, reduced the proliferative index from 57.75% to 22.83%. In addition, mifepristone up-regulated the expression of caspase-3, and down- regulated the Bcl-X_L expression, dose-dependently.

CONCLUSION: Mifepristone effectively inhibited the proliferation of PR-positive human gastric adenocarcinoma cell line SGC-7 901 *in vitro* through multiple mechanisms, and may be a beneficial agent against human adenocarcinoma.

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INTRODUCTION

Gastric adenocarcinoma is the second most common cancer with the second highest mortality rate[1,2]. Presently, there is still no effective treatment means for patients with advanced gastric adenocarcinoma[3,4]. Chemotherapy or radiation therapy has generally shown some clinical response but little survival advantage and is not tolerated in many patients[5,6]. Therefore, there is a need to identify other therapeutic agents against the tumor.

Mifepristone is a progesterone receptor (PR) antagonist that has been widely used as the first- line drug for the termination of early pregnancy[7]. Interestingly, recent studies have proved that mifepristone could effectively inhibit the proliferation of PR-positive breast cancer[8-10], ovarian cancer[11,12], endometrial cancer[13], and prostate cancer[14] cells without serious side effects and drug resistance. However, the effects of mifepristone on gastric adenocarcinoma are still unknown. Therefore, the present study was undertaken to explore the effects of mifepristone on the proliferation of human gastric adenocarcinoma cell line SGC-7 901 *in vitro*. Results showed that mifepristone effectively inhibited the proliferation of cultured SGC-7 901 cells *in vitro* through multiple mechanisms.

MATERIALS AND METHODS

Cell culture and treatment

Human gastric adenocarcinoma cell line SGC-7 901, obtained from Wuhan University Type Culture Collection (Wuhan, China), was routinely maintained in phenol red-free RPMI1640 (Gibco BRL, Grand Island, NY) containing 100 mL/L fetal bovine serum (Hyclone, Logan, UT), 10^5 U/L penicillin and 100 mg/L streptomycin at 37 °C in a humidified atmosphere with 50 mL/L CO_2 in air. When cells were grown to approximately 50% confluence, medium was replaced with serum-free RPMI1640. After 24 h, fresh media containing 2.5, 5, 10, 20 μmol/L mifepristone (Sigma Chemical Co., St Louis, MO) were added, respectively. Control cells were treated with the same volumes of vehicle (ethanol). Unless otherwise indicated, the cells were harvested after 96 h of incubation.

In situ hybridization analysis of PR

The expression of PR mRNA in SGC-7 901 cells was detected by *in situ* hybridization (ISH) using an ISH detection kit for PR (Boster, Wuhan, China) according to the manufacturer's instructions. Unless otherwise stated, all steps were performed at room temperature. Briefly, after 24 h of culture on the RNAse-free slides, cells were washed 3 times with phosphate buffered saline (PBS, pH7.4), fixed with 40 g/L paraformaldehyde in PBS containing 0.1 g/L diethylpyrocarbonate (DEPC-water) for 30 min, washed 3 times with 0.01 mol/L PBS, and then incubated with 5 mL/L hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity. After being rinsed with 0.01 mol/L PBS, cells were digested with proteinase K (10 g/mL in 0.01 mol/L PBS) at 37 °C for 15 min. Further washes with 0.5 mol/L PBS were performed before pre-hybridization for 3 h at 37 °C in the pre-hybridization solutions in a humidified environment. Hybridization was then performed with digoxigenin-labeled cRNA antisense probe (5 g/mL) overnight at 37 °C in a moist chamber. Subsequently cells were washed for 10 min with 2 SSC (1 SSC = 150 mmol/L NaCl, 15 mmol/L sodium citrate, pH7.0), followed by 0.5 SSC for 15 min, and finally 0.2 SSC for

15 min. After treatment with blocking reagent for 30 min, cells were incubated with biotin-labeled mouse anti-digoxigenin antibody at 37 °C for 1 h, washed 4 times, for 5 min each time, with 0.5 mol/L PBS, and then treated with SABC solutions at 37°C for 20 min. Then cells were washed 3 times, for 5 min each time, with 0.5 mol/L PBS, incubated with biotin-labeled peroxidase (POD) at 37 °C for 20 min, washed three time with 0.5 mol/L PBS. Finally, cells were visualized with 3,3- diaminobezidine (DAB), counterstained with hematoxylin, dehydrated, cleared, mounted with neutralgum, and examined under an microscope. Brown-yellow deposits indicated the sites of hybridization. PR-positive breast cancer tissues were used as positive control, and probes were replaced by PBS as negative control.

Ultrastructural analysis

Harvested cells were washed 3 times with PBS, fixed for 2 h with 2.5 g/ L glutaraldehyde in PBS, and then post-fixed for 2 h at 4 °C with 1 g/L OsO_4 in PBS. Cells were dehydrated using gradually increasing concentrations of ethanol from 50% to 100%, and then embedded in Epon 812. The ultra-thin sections (60 nm) were stained with uranyl acetate and lead citrate prior to examination at 50 kV with a Hitachi 600 transmission electron microscrope (Hitachi Corp., Tokyo, Japan).

MTT assay

SGC-7 901 cells were seeded into 96-well plates at a density of 1×10^5/mL in RPMI1640. After 96 h of incubation with various concentrations of mifepristone, cell proliferation was measured by MTT (Sigma) reduction assay as described previously[15]. Absorbance at 570 nm (A_{570nm}) was assayed. The inhibitory rate (IR) of SGC-7 901 cells was calculated according to the equation as following: IR (%) = (A_{570nm} in control group-A_{570nm} in mifepristone-treated group)/A_{570nm} in control group×100%.

^{3}H-thymidine (^{3}H-TdR) incorporation

Cells were incubated at various time intervals without or with various concentrations of mifepristone, followed by treatment with 10 μCi ^{3}H-TdR (Amersham, Arlington Heights, IL) for an additional 6 h. Then, cells were washed twice with 100 mL/L trichloroacetic acid by centrifugation and resuspension, and were continuously incubated for 30 min at 60 °C with 0.5 mL of NaOH (0.3 mol/L). Finally, the cell lysates were collected, and the radioactivity was measured by a liquid scintillation counter (Beckman LS1 801, USA).

Cell cycle analysis by flow cytometry

The harvested cells were fixed with 700 mL/L ethanol at -20 °C for 30 min, and then stained with propidium iodide (Sigma) for 30 min in the dark. The stained cells were analyzed in a FACS Calibur flow cytometer (Becton Dickinson Labware, Lincoln Park, NJ) with excitation wavelength of 488 nm. The resulting histograms were analyzed by program MODFIT for cell distribution in cell cycle phase. Proliferative index (PI) was calculated according to the formula: PI (%) = ($S+G_2/M$)/ ($G_0/G_1+S+G_2/M$) ×100%.

Measurement of caspase-3 activity

Total proteins were extracted from harvested cells as described previously[16], and protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). An equal amount of cellular protein from extract of each group was added to a final volume of 100 μL of reaction mixture containing 0.2 mmol/L of a colorimetric caspase-3 substrate, acetyl-Asp-Glu-Val-Asp-ρ-nitroaniline (Ac-DEVD-ρNA; Calbiochem, San Diego, CA), followed by incubation at 30 °C for 10 min. Free ρ-nitroaniline (ρNA) released upon enzymatic cleavage was detected at 405 nm using a microplate reader (Bio-Rad). Caspase-3 activity correlated with the concentration of free ρNA generated in the reaction. Purified caspase-3 (Calbiochem) was used as positive control, whereas caspase-3 inhibitor I (Ac-DEVD- CHO, Calbiochem) was used as negative control.

RT-PCR analysis for Bcl-X_L

Total RNA was extracted from the cells using TRIzol reagent (Gibco BRL) according to the manufacturer's protocol. Two milligrams of total RNA were used for reverse transcription in a total volume of 20 μL with the SuperScript preamplification system (Promega, Madison, MI). Aliquots of 2 μL cDNA were subsequently amplified in a total volume of 50 μL using the GeneAmp PCR kit (Promega) following conditions recommended by the manufacturer. The sense and antisense primers for Bcl-X_L were 5'-AGGCAGGCGATGAGTTTGAAC-3' and 5'-GAACCACACC AGCCACA GTCA-3', respectively. The sense and antisense primers for -actin used as an internal control were 5'-ATCTGGCACC ACACCTTCTACAATGAGCT GCG-3' and 5'-CGTCA TACTCCTGCTTGCTGATCCACAT CTGC-3', respectively. The cycling conditions were 94 °C for 2 min, followed by 30 cycles of 92 °C for 30 s, at 62 °C for 30 s, and at 72 °C for 1 min and a final extension of 72 °C for 5 min. PCR products were separated on the 15 g/L agarose gel stained with ethidium bromide (EB) and viewed under ultraviolet light.

Statistical analysis

Data were expressed as mean±SD. Statistical analysis was performed using the Student's *t* test and the chi-square test. $P<0.05$ was considered statistically significant.

RESULTS

Expression of PR mRNA

In situ hybridization analysis showed that PR mRNA was highly expressed in the cultured SGC-7 901 cells, which was mainly localized in the cytoplasm of the cells (Figure 1).

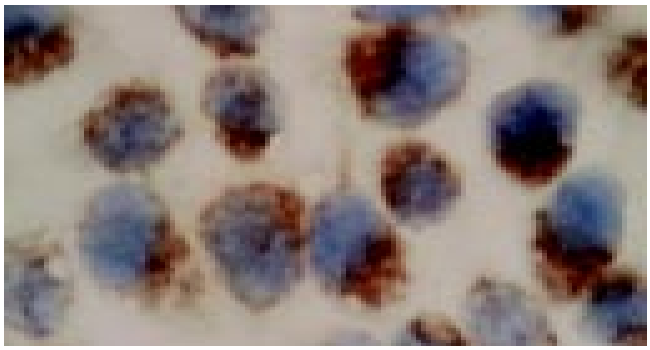

Figure 1 *In situ* hybridization (ISH) analysis of progesterone receptor (PR) expression in human gastric adenocarcinoma cell line SGC7 901 (ISH, ×1000).

Morphological changes

To assess the effect of mifepristone on the ultrastructural changes of SGC-7 901 cells, transmission electron microscopic analysis was performed. Results revealed that mifepristone dose-dependently induced apoptosis, which was especially remarkable at the 20 μmol/L concentration (Figure 2B). However, the irregular and enlarged nuclear, multiple nucleoli and increased nucleus-to-cytoplasm ratio were clearly seen in the cells of control group (Figure 2A).

MTT assay and ^{3}H-TdR incorporation

After 96 h of incubation with 2.5, 5, 10, 20 μmol/L mifepristone, MTT assay revealed A_{570nm} was markedly decreased in a dose-dependent manner, and the inhibitory rate (IR) of SGC-7 901

Table 1 Effects of mifepristone on cell proliferation, cell-cycle phase distribution and caspase-3 activity of SGC7 901 cells *in vitro* (mean±SD)

Concentration (μmol/L)	A_{570nm} (MTT)	Cell cycle phase distribution (%)			Caspase-3 activity (U)
		G_0/G_1	S	G_2/M	
0	1.125±0.048	42.25±4.20	35.68±3.98	22.07±3.01	1.28±0.28
2.5	1.024±0.030	49.47±5.68	30.82±4.36	19.71±2.41	2.79±0.36
5	0.896±0.035	52.23±6.22	28.68±3.64	19.01±1.36	5.04±0.29
10	0.678±0.026	65.80±5.63	25.93±3.01	8.27±1.10	9.46±0.20
20	0.548±0.031	77.16±8.25	15.54±2.54	7.29±0.82	15.23±0.41

cells by mifepristone was 8.98%, 20.36%, 39.73% and 51.29%, respectively (Table 1). Figure 3 shows that ^{3}H-TdR incorporation into DNA of SGC-7 901 cells was significantly decreased in a dose- and time-dependent manner.

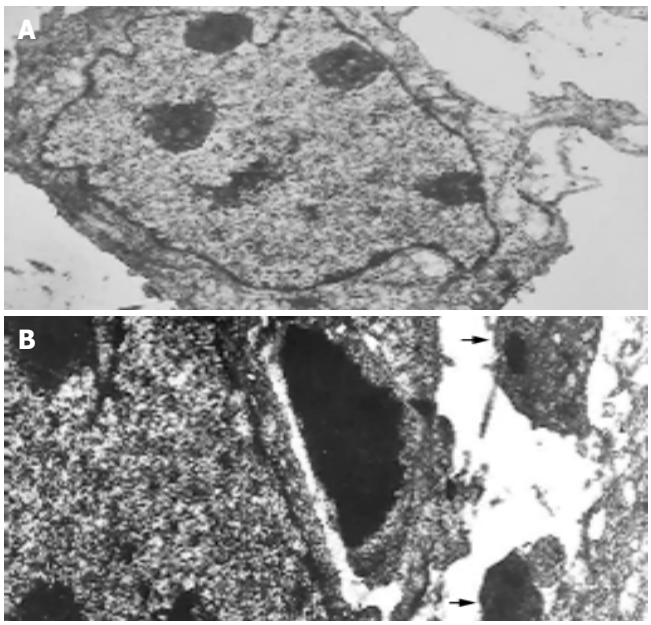

Figure 2 Transmission electron microscopic photographs of the SGC7901 cells cultured for 96 h in the absence(A) or the presence of 20 μmol/L mifepristone (B) *in vitro* (TEM, ×2000). Arrows indicate apoptotic bodies which were formed in the cells of mifepristone-treated group.

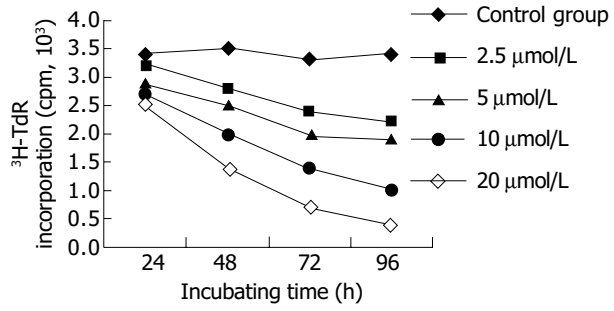

Figure 3 Effect of various concentrations of mifepristone on the ^{3}H-TdR incorporation of SGC7 901 cells at various time intervals *in vitro*.

Cell-cycle phase distribution

The effect of mifepristone on the cell-cycle phase distribution of SGC-7 901 cells was determined by flow cytometry. After treatment with mifepristone, there was a strong dose- dependent decrease in the percentage of S- and G_2/M-phase cells, and with a concomitant increase in the percentage of cells in the G_0/G_1 phases of the cell cycle (Table 1). Additionally, there is a significant decrease in the proliferative index (PI) of the mifepristone-treated cells (50.53%, 47.69%, 34.20% and 22.83%) as compared with control group (57.75%, P<0.01).

Expression of caspase-3 and Bcl-X_L

As shown in Table 1, mifepristone significantly up-regulated the activity of caspase-3 as compared with that in control group. Figure 4 shows the results of RT-PCR analysis for Bcl-x_L mRNA expression. Results indicated that mifepristone dose-dependently inhibited the expression of Bcl-X_L in the SGC-7 901 cells.

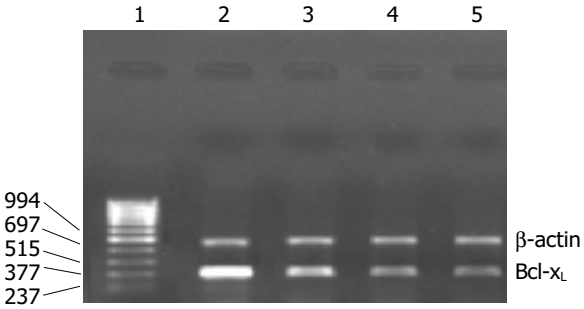

Figure 4 RT-PCR analysis of Bcl-X_L mRNA expression in the SGC7 901 cells cultured for 96 h in the absence or the presence of various concentrations of mifepristone *in vitro*. Lanes 1-5: Marker (bp), contro l, 5, 10, 20 μmol/L mifepristone, respectively.

DISCUSSION

Accumulating evidence demonstrates that PR level is closely associated with proliferation, invasion and metastasis of human gastric adenocarcinoma, as well as prognosis of patients[17-19]. Therefore, there has been increasing interest in the development of antiprogestins for tumor treatment. Mifepristone has been proved to be a potent and effective PR antagonist by competing with progesterone for PR binding, followed by binding to progesterone response element (PRE)[20]. In the present study, we proved that mifepristone effectively inhibited the proliferation of human gastric adenocarcinoma cell line SGC-7 901 *in vitro* through induction of apoptosis and arresting the cell cycle progession.

Previous studies[21] found that the determination of PR levels was primarily used as a marker of a tumor's responsiveness to mifepristone. To determine the expression of PR mRNA in the SGC-7 901 cells, *in situ* hybridization was performed. We found that PR mRNA was highly expressed in cultured SGC-7 901 cells. Meanwhile, the result is in agreement with the work of Cui *et al*.[22], who reported that the concentrations of PR protein in the cytoplasm and nuclei of cultured SGC-7901 cells were 20.3 fmoL/mg and 22.7 fmoL/mg, respectively, revealed by dextran-coated charcoal (DCC) assay. Thus, we speculate that the growth inhibitory effects of mifepristone in our study might be mediated, at least in part, by PR.

In our study, TEM, MTT assay and ^{3}H-TdR incorporation were used to evaluate the effect of mifepristone on the proliferation of SGC-7 901 cells *in vitro*. We found that mifepristone exerted significantly anti-proliferative effect on cultured SGC-7 901 cells *in vitro* in a dose- and time-dependent manner. The results are in agreement with those of previous studies on other tumor cell lines *in vitro*[9-11,23].

Although a number of studies have proved that mifepristone has the growth inhibitory effects on tumor cells, but the mechanisms remain unknown. These theoretically could be related with the changes of the dynamics of cell proliferation. The hypothesis was demonstrated by the work of Thomas *et al*.[24], who proved that mifepristone inhibited the proliferation of the MCF-7 human breast cancer cells by arresting them in the G_0/G_1 phase of the cell cycle. Recently, Peters *et al*.[25] reported that mifepristone up-regualted the expression of cell cycle protein $p21^{WAF/cip1}$ in medroxyprogesterone acetate-induced ductal mammary adenocarcinoma. Thus, we further explored the effects of mifepristone on the cell cycle of SGC-7 901 cell using flow cytometry. Results showed that mifepristone markedly increased the proportion of cells in G_0/G_1, and simultaneously decreased the percentage of cells in S- and G_2/M-phase. Taken together, it seems reasonable to conclude that the growth inhibitory effects of mifepristone on SGC-7901 cells is partially due to an accumulation of cells in G_0/G_1 phase.

To explore whether or not the apoptosis-related genes contributed to the inhibitory effect of mifepristone on the SGC-7 901 cells, we assessed the activity of caspase-3, an executioner of apoptosis[26,27], and mRNA level of Bcl-X_L, an anti-apoptotic gene. Our findings showed that mifepristone dose-dependently up-regulated caspase-3 activity and down-regulated Bcl-X_L mRNA expression. This result is supported by previous studies on prostate cells. El Etreby *et al*. reported that mifepristone significantly induced apoptosis in LNCaP prostate cancer cells in a time- and dose-dependent manner through down-regulation of Bcl-2 protein and induction of caspase-3 activity. Collectively, it is possible that the activation of caspase-3 and the degradation of Bcl-X_L are partially responsible for the antiproliferative effects of mifepristone on cultured SGC-7 901 cells.

In conclusion, the study demonstrated that mifepristone exerted marked antiproliferative effect on the PR-positive SGC-7 901 cells by inducing apoptosis, arresting cell cycle progression, up-regulating caspase-3 activity and down-regulating Bcl-X_L mRNA expression. These results indicate that mifepristone may be a useful agent against human gastric adenocarcinoma although further studies are clearly needed to prove the possibility.

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