Reversal of multidrug resistance in drug-resistant human gastric cancer cell line SGC7901/VCR by antiprogestin drug mifepristone

Da-Qiang Li, Zhi-Biao Wang, Jin Bai, Jie Zhao, Yuan Wang, Kai Hu, Yong-Hong Du

Abstract

AIM: To explore the reversal effect of mifepristone on multidrug resistance (MDR) in drug-resistant human gastric cancer cell line SGC7901/VCR and its mechanisms.

METHODS: Expression of multidrug resistance-associated protein (MRP) was detected using reverse transcription-polymerase chain reaction (RT-PCR). Flow cytometry was used to assay the expression of P-glycoprotein (P-gp), Bcl-2, Bax, and the mean fluorescent intensity of intracellular rhodamine 123 in the cells. Meanwhile, the protein levels of Bcl-2 and Bax were also detected by Western blotting analysis. The sensitivity of cells to the anticancer agent, vincristine (VCR), and the intracellular [3H]VCR accumulation were determined by tetrazolium blue (MTT) assay and a liquid scintillation counter, respectively.

RESULTS: Expression of MRP and P-gp in SGC7901/VCR cells was 6.04- and 8.37-fold higher as compared with its parental SGC7901 cells, respectively. After treatment with 1, 5, 10, and 20 μmol/L mifepristone, SGC7901/VCR cells showed a 1.34-, 2.29-, 3.11-, and 3.71-fold increase in the accumulation of intracellular VCR, a known substrate of MRP, and a 1.03-, 2.04-, 3.08-, and 3.68-fold increase in the retention of rhodamine 123, an indicator of P-gp function, respectively. MTT assay revealed that the resistance of SGC7901/VCR cells to VCR was 11.96-fold higher than that of its parental cells. The chemosensitivity of SGC7901/VCR cells to VCR was enhanced by 1.02-, 7.19-, 12.84-, and 21.17-fold after treatment with vincristine at above-mentioned dose. After 96 h of incubation with vincristine 10 μmol/L, a concentration close to plasma concentrations achievable in human, the expression of Bcl-2 protein was decreased to (9.21±0.65)% from (25.32±1.44)%, whereas the expression of Bax protein was increased to (19.69±1.13)% from (1.24±0.78)% (P<0.01). Additionally, the effects of mifepristone on the expression of Bcl-2 and Bax proteins in SGC7901/VCR cells were further demonstrated by Western blotting analysis.

CONCLUSION: Mifepristone has potent reversal effect on MDR in SGC7901/VCR via inhibiting the function of MRP and P-gp, modulating the expression of Bcl-2 and Bax proteins, and enhancing the sensitivity to anticancer agent VCR.

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MATERIALS AND METHODS

Cell culture and treatment

Human gastric cancer cell line SGC7901, and its drug-resistant counterpart SGC7901/VCR selected by stepwise exposure of parental SGC7901 cells to increasing concentrations of vincristine (VCR), were purchased from Wuhan University Type Culture Collection (Wuhan, China). Both cell lines were maintained in RPMI1640 medium (Gibco BRL, Grand Island, NY) supplemented with 100 mL/L heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 10 U/L penicillin and 100 mg/L streptomycin in a humidified incubator containing 50 mL/L CO2 at 37 °C. When cells were grown to approximately 50% confluence, the medium was then replaced with serum-free RPMI1640. After 24 h, fresh media containing 1.5, 10, and 20 μmol/L mifepristone (Sigma Chemical Co., St Louis, MO) was added, respectively. Control cells were treated with the same volume of vehicle (ethanol). Unless otherwise indicated, the cells were harvested after 96 h of incubation.

RT-PCR for MRP

Total RNA was extracted from the cultured cells using Trizol reagent (Gibco BRL) according to the manufacturer’s instructions. Two milligrams of total RNA was used for reverse transcription in a total volume of 20 μL with the SuperScript...
Detection of P-gp, Bcl-2, Bax by flow cytometry

The harvested cells were fixed with 40 g/L paraformaldehyde for 10 min, followed by treatment with 2 g/L Triton X-100 for 10 min. After incubation with normal rabbit serum for 10 min to block non-specific binding, the cells were incubated for 1 h at 4 °C with mouse anti-human monoclonal antibodies against P-gp, Bcl-2, Bax(Santa Cruz Biotechnology, Inc., USA) respectively, followed by treatment with FITC-conjugated goat anti-mouse IgG for 30 min at 4 °C. The percentage of positive cells were determined using the FACs Calibur flow cytometry (Becton & Dickinson) with an excitation wavelength of 488 nm.

Western blotting analysis of Bcl-2 and Bax

Western blotting analysis was made to detect Bcl-2 and Bax protein levels according to the published method with some modifications[17]. Briefly, proteins were extracted from the harvested cells using a lysis buffer containing 50 mmol/L HEPES, pH7.2, 100 mmol/L NaCl, 200 mL/L glycerol, 0.1 mmol/L EDTA, pH8.0, 2 g/L Triton X-100, 2 mmol/L phenylmethylsulfonyl fluoride (PMSF) and 1 mL/L dithiothreitol (DTT), and then quantitated using the Bio-Rad Detergent Compatible Protein Assay kit (Bio-Rad, Hercules, CA). Equal amounts of protein (10-20 µg) were resolved on a 100 g/L minigel by SDS-polyacrylamide gel electrophoresis. Proteins were transferred to a PVDF membrane(Millipore, Bedford, MA) using the Multiphor Novobilot electrophoresis transfer system, followed by immunoblotting using a monoclonal mouse anti-human antibody against Bcl-2 and Bax (Santa Cruz Biotechnology, Inc., USA), respectively. A horseradish peroxidase-conjugated secondary antibody (goat anti-mouse HRP, Amersham, Arlington Heights, IL) was used at a dilution of 1:3 000. The membranes were subsequently developed using Enhanced Chemiluminescence (ECL, Amersham) and exposed to film.

Intracellular [3H]VCR accumulation

Cells were incubated with 20 mmol/L [3H]VCR (specific activity 5.8 Ci/mmol, Amersham Pharmacia Biotech Co.) at 37 °C for 90 min in the absence or presence of various concentrations of mifepristone. Cells were then washed three times with ice-cold PBS and lyzed in distilled water by ultrasonication. Radioactivity of [3H]VCR in the cell extract was then determined with a liquid scintillation counter (Beckman LS1801, USA) and normalized to cellular protein content.

Rhodamine 123 retention assay

Retention of rhodamine 123(Sigma) was determined by flow cytometry as a functional index of P-gp activity. Cells (2x10^4) were treated with various concentrations of mifepristone for 24 h prior to the addition of 10 g/L rhodamine 123. After incubation at 37 °C for 1 h, cells were harvested and centrifuged at 300 g for 10 min. Cell pellets were resuspended with 500 µL of PBS and immediately used for flow cytometric analysis of rhodamine 123 retention.

Drug-sensitivity assay

The sensitivity of cells to VCR was determined using the MTT assay as described previously[13]. The drug concentration producing 50% inhibition of growth (IC_50) was determined graphically for VCR using the relative survival curves. The reversal effects of mifepristone were determined as the IC_50 value in the absence of mifepristone to that in the presence of mifepristone. Assays were performed in quadruplicate for at least three times.

Statistical analysis

Data were expressed as mean±SD. Statistical analysis of the data was performed using the Student’ s t test and the Chi-square test. P<0.05 was considered statistically significant.

RESULTS

Expression of MRP mRNA and P-gp protein

To examine the relationship between the levels of MRP and P-gp expression in SGC7901/VCR and SGC7901 cells and the changes in drug resistance, RT-PCR and flow cytometry were used to detect the expression of MRP mRNA and P-gp protein. As indicated in Figure.1, a 6.04-fold overexpression of MRP mRNA was found in SGC7901/VCR cells as compared with the parental line. The relative level of MRP mRNA expression in drug-resistant cells and drug-sensitive cells was 1.45±0.23 and 0.24±0.17, respectively. Similarly, the expression of P-gp was significantly increased in the SGC7901/VCR cells in comparison with the parental cells (57.64±8.56% vs 6.89±1.25%, 8.37-fold, P<0.005).

Intracellular [3H]VCR accumulation and rhodamine 123 retention

Intracellular accumulation of [3H]VCR, a known substrate of MRP, was measured in the presence or the absence of various concentrations of mifepristone in both cell lines. After treatment with 1, 5, 10, and 20 µmol/L mifepristone for 90 min, the
accumulation of intracellular VCR in SGC7901/VCR cells was enhanced by 1.34-, 2.29-, 3.11-, and 3.71-fold as compared with medium control, respectively (Table 1). It had been documented that the efflux of rhodamine 123 correlated with well P-gp expression. By this rational, we used rhodamine 123 to evaluate the function of P-gp. As shown in Table 1, after treatment with various concentrations of mifepristone for 24 h, the retention of rhodamine 123 in SGC7901/ VCR cells was increased by 1.03-, 2.04-, 3.08-, and 3.68-fold as compared with the medium control. In contrast, no significant increase in the intracellular rhodamine 123 retention and VCR accumulation was observed in mifepristone-treated SGC7901 cells.

**Expression of Bcl-2 and Bax**

Flow cytometric assay revealed that the expression of Bcl-2 protein was significantly increased, whereas the expression of Bax was decreased in SGC7901/VCR cells as compared with drug-sensitive SGC7901 cells (Table 2). Mifepristone when used at 10 µmol/L, a concentration close to plasma concentrations achievable in human, markedly up-regulated the expression of Bax and simultaneously down-regulated the expression of Bcl-2 in SGC7901/VCR cells (Table 3). Additionally, the results were further demonstrated by Western blotting analysis (Figure 2). Western blotting revealed that mifepristone dose-dependently modulated the expression of Bcl-2 and Bax proteins, which was especially remarkable at the 20 µmol/L concentration.

**Table 2** Flow cytometric analysis of Bcl-2 and Bax expression in human gastric cancer cell line SGC7901 and its drug-resistant counterpart SGC7901/VCR

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Bcl-2(%)</th>
<th>Bax(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGC7901</td>
<td>17.23±0.86</td>
<td>5.85±0.56</td>
</tr>
<tr>
<td>SGC7901/ VCR</td>
<td>25.32±1.44a</td>
<td>1.24±0.78a</td>
</tr>
</tbody>
</table>

aP <0.05 vs SGC7901 cell line.

**Table 3** Effect of mifepristone on Bcl-2 and Bax expression in SGC7901/VCR cells

<table>
<thead>
<tr>
<th>Mifepristone (µmol/ L)</th>
<th>Bcl-2(%)</th>
<th>Bax(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>25.32±1.44</td>
<td>1.24±0.78</td>
</tr>
<tr>
<td>10</td>
<td>9.21±0.65</td>
<td>19.69±1.13</td>
</tr>
</tbody>
</table>

P <0.05 vs control group.

**DISCUSSION**

Mifepristone is a potent antiprogestin agent that has been widely used as the first-line drug for the termination of early pregnancy[21,22]. Interestingly, recent studies[23-25] have proved that mifepristone exerts markedly anticancer effects and reversal effects on MDR in some cancer cells with no serious side-effects. Thus, there is an increasing interest in exploring the reversal effect of mifepristone on MDR in human gastric cancer cells. In the present study, we reported for the first time that mifepristone effectively reversed MDR in SGC7901/VCR via multiple mechanisms.

Previous studies[26-28] have proved that MDR in gastric cancer cells is closely related to overexpression of two ATP-dependent transporter proteins, P-gp encoded by MDR1 gene and MRP identified by Cole et al.[16] from adriamycin-selected MDR lung cancer cell line H69/ ADR. Both proteins belong to the ATP-binding cassette(ABC) protein superfamily, and efflux anticancer agents out of cells and therefore decrease their intracellular accumulation. Thus, we firstly determined the relationship between the levels of P-gp and MRP expression in SGC7901/VCR and its parental cells and the changes of drug resistance. Results showed that the expression of P-gp and MRP in SGC7901/VCR cells was 8.37- and 6.04-fold higher as compared with its parental counterparts. The data indicate that the overexpression P-gp and MRP confers, at least in part, the MDR phenotype of VCR-selected SGC7901/VCR cells.

To determine the effects of mifepristone on the function of P-gp and MRP, we further investigated the accumulation of intracellular VCR, a substrate of MRP, and the retention of rhodamine 123, an indicator of P-gp function, in both cell lines. Results revealed that mifepristone dose-dependently enhanced the intracellular VCR accumulation and rhodamine 123 retention in SGC7901/VCR cells. In contrast, mifepristone had no significant effects on the drug-sensitive SGC7901 cells. The results were further proved by the drug sensitivity assay. We found that, after incubation with 1, 5, 10, and 20 µmol/L mifepristone for 96 h, the sensitivity of SGC7901/VCR cells to VCR was enhanced by 1.02, 7.19, 12.84, and 21.17 times, whereas
no significant changes in the sensitivity to VCR were observed in mifepristone-treated SGC901 cells. The findings are in agreement with those of previous studies on other cancer cell lines in vitro[13,14]. Taken together, it seems reasonable to conclude that mifepristone can inhibit the function of P-gp and MRP and therefore enhance the sensitivity of cells to anticancer agent VCR.

Although overexpression of the P-gp and MRP plays an important role in the MDR of gastric cancer, this does not explain all of the MDR [29]. Recent studies have shown that overexpression of anti-apoptotic proteins, such as Bcl-2, Bcl-xL, and Mcl-1, induces cancer cells resistance to chemotherapeutic agents in cancer cells that act by apoptosis, whereas high levels of pro-apoptotic proteins, Bcl-xS and Bax, contribute to sensitize MCF-7 breast cancer cells to etoposide (VP16), taxol and epirubicin. These data were also proved by the work of Zhao et al. [19], who reported that the Bax gene-transfected SGC7901/VCR cells were more sensitive to adriamycin and VCR than mock vector transfected cells. In a word, Bcl-2/Bax pathway may be an alternative mechanism of drug resistance in gastric cancer. In this study, we proved that mifepristone when used at 10 μmol/L, a concentration close to plasma concentrations achievable in human, significantly up-regulated the expression of Bcl-2 and simultaneously down-regulated the expression of Bax in SGC7901/VCR cells. In addition, the modulating effects of mifepristone on the expression of Bcl-2 and Bax proteins in SGC7901/VCR cells were further demonstrated by Western blotting analysis. These results may partly explain the reversible effects of mifepristone on SGC7901/VCR.

In conclusion, mifepristone exerts potent reversal effect on MDR in SGC7901/VCR via inhibiting MRP- and P-gp-mediated drug transporter, modulating the expression of apoptosis-related genes Bcl-2 and Bax, and enhancing the sensitivity of cells to anticancer agents such as VCR. These results indicate that mifepristone may be a promising chemosensitizer likely allowing to reverse the MDR of human gastric cancer cells although further studies are clearly needed to prove the possibility.

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