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• GASTRIC CANCER •

# Effect of mitogen-activated protein kinase signal transduction pathway on multidrug resistance induced by vincristine in gastric cancer cell line MGC803

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## Abstract

**AIM:** To investigate the correlation between mitogen-activated protein kinase (MAPK) signal transduction pathway and multidrug resistance (MDR) in MGC803 cells.

**METHODS:** Western blot was used to analyze the expression of MDR associated gene in transient vincristine (VCR) induced MGC803 cells, which were treated with or without the specific inhibitor of MAPK, PD098059. Morphologic analysis of the cells treated by VCR with or without PD098059 was determined by Wright-Giemsa staining. The cell cycle analysis was performed by using flow cytometric assay and the drug sensitivity of MGC803 cells which were exposed to VCR with or without PD098059 was tested by using MTT assay.

**RESULTS:** Transient exposure to VCR induced P-gp but not MRP1 or GST-π expression in MGC803 cells and the expression of P-gp was inhibited by PD098059. Apoptotic bodies were found in the cells treated with VCR or VCR+PD098059. FCM results indicated that more MGC803 cells showed apoptotic phenotype when treated by VCR and PD098059 (rate: 31.23%) than treated by VCR only (rate: 18.42%) ($P<0.05$). The $IC_{50}$ (284±13.2 μg/L) of MGC803 cells pretreated with VCR was 2.24-fold as that of negative control group (127±17.6 μg/L) and 1.48-fold as that of the group treated with PD098059 (191±27.9 μg/L).

**CONCLUSION:** This study shows that the expression of P-gp can be induced by transient exposure to VCR and this induction can be prevented by PD098059, which can block the activity of MAPK. MAPK signal transduction pathway may play some roles in modulating MDR1 expression in gastric cancer.

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## INTRODUCTION

Multidrug resistance (MDR) is a major factor in the failure of many forms of chemotherapy[1-4]. Several different molecular mechanisms will switch on in MDR cells, the most investigated mechanisms with known clinical significance are: (1) activation of transmembrane proteins effluxing different chemical substance from the cells, including mainly P-glycoprotein (P-gp) encoded by MDR1 and multidrug resistance related protein (MRP); (2) activation of the enzymes of the glutathione detoxification system (especially GST-π); (3) alteration of the genes and proteins involved in the control of apoptosis (especially p53 and Bcl-2)[5-14]. MDR associated genes are expressed in a large proportion of human tumors, and its expression in several different forms of cancer was shown to be associated with a lack of response to combination chemotherapy. MDR1 expression is usually low or undetectable prior to treatment, but it is frequently increased during the progression of the disease and, most noticeably, after chemotherapy[15-20]. The increased expression of MDR1 mRNA can be found in some drug-sensitive cancer cells by transient exposure to different chemotherapeutic drugs[21-24].

The signal transduction pathway of the mitogen-activated protein kinase (MAPK) plays a critical role in cell proliferation, differentiation and apoptosis. The ERK1/2 (Ras/Raf-1/MEK1/2/ERK1/2) signal transduction pathway is a subfamily of MAPK. The expression of MDR and the activation of MAPK are increased in cancer cells after treatment with various therapeutic drugs. The selective inhibitor of MEK1/2, PD098058, has been shown to significantly reverse the drug resistance of drug resistant cell line L1210/VCR[25]. The mechanism is unclear. Whether MAPK plays a role in MDR, and whether the alteration of MEK can regulate the expression of MDR need to be elucidated.

Our study was to observe the expressions of associated genes of MDR of human gastric cancer cell line MGC803 by their transient exposure to vincristine (VCR) and the effect on MDR by the specific inhibitor of MEK1/2, PD098059.

## MATERIALS AND METHODS

### *Reagents*

Human gastric cancer cell line MGC803 was obtained from Tumor Research Institute (China Medical University, Shenyang). RPMI1640 medium was the product of Gibco (USA). Chemical drug vincristine was purchased from Hualian Co. (Shanghai, China). PD098059 was the product of Promega (USA). Rabbit anti-human P-gp, MRP1, GST-π polyclonal antibody were products of Oncogen (USA). Alkaline phosphatase- conjugated goat anti-rabbit IgG was purchased from Zhongshan Co. (Beijing).

### *Morphological analysis of cells*

After treated with VCR (20 μg/L) or VCR (20 μg/L)+ PD098059 (10 μmol/ L) for 24 h, 48 h, MGC803 cells were analyzed by wright-Giemsa staining, and the morphology of cells was examined under optic microscope.

### Cell cycle analysis

MGC803 cells ($1\times10^8$/L) were seeded into12-well plates and cultured in 1 mL RPIM medium. After cultured for 4 h, cells were treated with VCR (20 μg/L), PD098059 (10 μmol/L) or VCR (20 μg/L) + PD098059 (10 μmol/L) for 24 h, 48 h, 96 h. Cells were harvested and washed with ice-cold PBS twice, centrifuged (120×g, 5 min) and supplemented with ice-cold 70 mL/L ethanol overnight. Cells were treated with RNase (200 mg/ L) at 37 ℃ for 1 h after washed with ice-cold PBS twice, then centrifuged (120 *g*, 5 min), treated with PI (20 mg/L) for 30 min in dark room at 4 ℃. Cell cycle was analyzed by flow cytometer and CELLQuest software.

### MTT assay of drug sensitivity

Cells ($1\times10^8$/L) pretreated with VCR (20 μg/L) for 72 h were plated into 96-well plates and cultured in 100 μL RPMI medium. After cultured for 4 h, cells were divided into two groups: one group was treated with various concentrations of VCR (1 μg/L, 10 μg/L, 100 μg/L, 1 000 μg/L), the other group was treated with a fixed concentration of PD098059 (10 μmol/L) and various concentration of VCR (1 μg/L, 10 μg/L, 100 μg/L, 1 000 μg/L). The unpretreated MGC803 was treated with various concentration of VCR (1 μg/L, 10 μg/L, 100 μg/L, 1 000 μg/L) as negative control group. After treated for 72 h, 20 μL of 5 g/L MTT [3-(4,4- diemththiazol-2-yl)2,5-diphenylterazolium bromide] in PBS was added to each well, incubated for 4 h at 37 ℃ and the formed formazan crystals were dissolved in 100 μLof DMSO. The absorbance was recorded at 570 nm on a microplate reader (BIORAD). Drug sensitivity is expressed as $IC_{50}$ for cells, which the concentration of drugs that caused a 50% reduction in the at 570 nm relative to untreated cells (controls).

### Western blot analysis

MGC803 cells were harvested after treated with VCR (20 μg/L) or VCR (20 μg/L)+ PD098059 (10 μmol/L) for 24, 48, 72 h. A total of $2\times10^7$ cells were lysed in 200 μL RIPA buffer containing phenylnethy fluoride (PMSF, 100 mg/ L), Aprotinin (2 mg/ L), 50 mmol/ L Triscl pH 7.4, 150 mmol/ L NaCl, 1 g/L SDS, 10 g/LTriton-100, 1 mmol/ L EDTA pH 8.0. Protein samples were sonicated on ice, lysed for 40 min at 4 ℃, then centrifuged (15 400 *g*, 20 min) at 4 ℃. The supernatant was transferred to a new tip on ice and then the amount of the protein calculated. Protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDSPAGE), transferred to a PVDF membrane. The membrane was incubated in a blocking solution containing 50 g/L fat free milk powder for 1 h, then probed with rabbit anti-human P-gp polyclonal antibody overnight, and incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG for 2 h. The membrane was then stained by blue tetrazolium (NBT) and 5' -bromo-4-chloro-3 - indolyphosphate (BCIP) solution. The integrated density value (IDV) was analyzed by Fluorchem software.

### Statistical analysis

Data were analyzed by chi square test. $P<0.05$ was considered as significant.

## RESULTS

### Apoptosis of cells treated with VCR and PD098059

The apoptotic bodies were observed in the MGC803 cells after treated with VCR (20 μg/L) or VCR (20 μg/L)+ PD098059 (10 μmol/L) for 48 h (Figure 1A, B, C).

The apoptosis of MGC803 cells was detected by flow cytometric analysis. The rate of apoptotic cells treated with VCR for 72 h was 18.41%, and that treated with VCR and PD098059 for 72 h was 35.61%. There was a significant difference between them ($P<0.05$). The apoptotic rates of MGC803 cells untreated and treated with PD098059 only were 8.46% and 6.26%. There was no significant difference between them ($P>0.05$) (Figure 2).

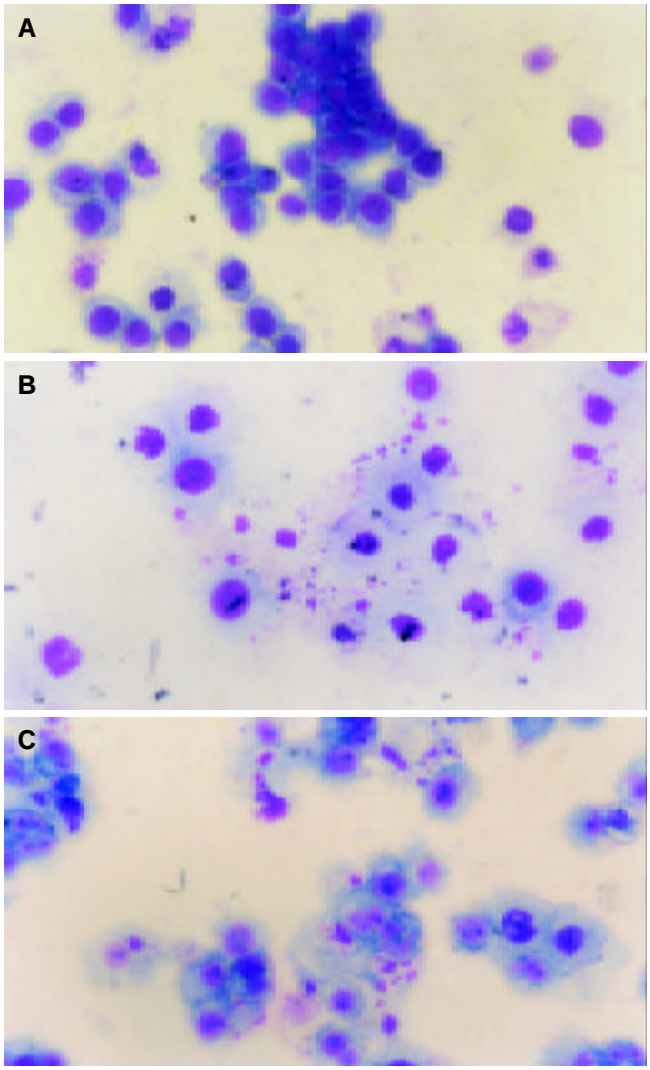


**Figure 1** A: Untreated MGC803 Cells( apoptotic bodies could not be found), B: MGC803 cells treated with VCR 48 h (apoptotic bodies could be found), C: MGC803 cells treated with VCR+PD098059 48 h (apoptotic bodies could be found).

### Drug sensitivity of cells treated with VCR and PD098059

The $IC_{50}$ of MGC803 pretreated with VCR for 72 h followed by treatment with various concentrations of VCR was 284±13.2 μg/L. It was 2.24-fold as that of negative control group (127±17.6 μg/ L), and 1.48-fold as those of cells treated with various concentrations of VCR (191±27.9 μg/L). And, the concentration of PD098059 was fixed. It showed that the drug-resistance of MGC803 pretreated with VCR was increased and PD098059 could reverse the drug resistance induced by VCR partially.

### Expression of MDR1, MRP1 and GST-*p*

Western blot was used to detect the expression of MDR associated genes. The expression of P-gp in MGC803 cells gradually increased after treated with VCR for 24-72 h (Table 1, Figure 3). But the expression of MRP1 and GST-π did not increase significantly (Table 1, Figure 3). The expression of P-gp was inhibited when MGC803 cells were treated with VCR and PD098059 for 24-72 h (Table 1, Figure 3).

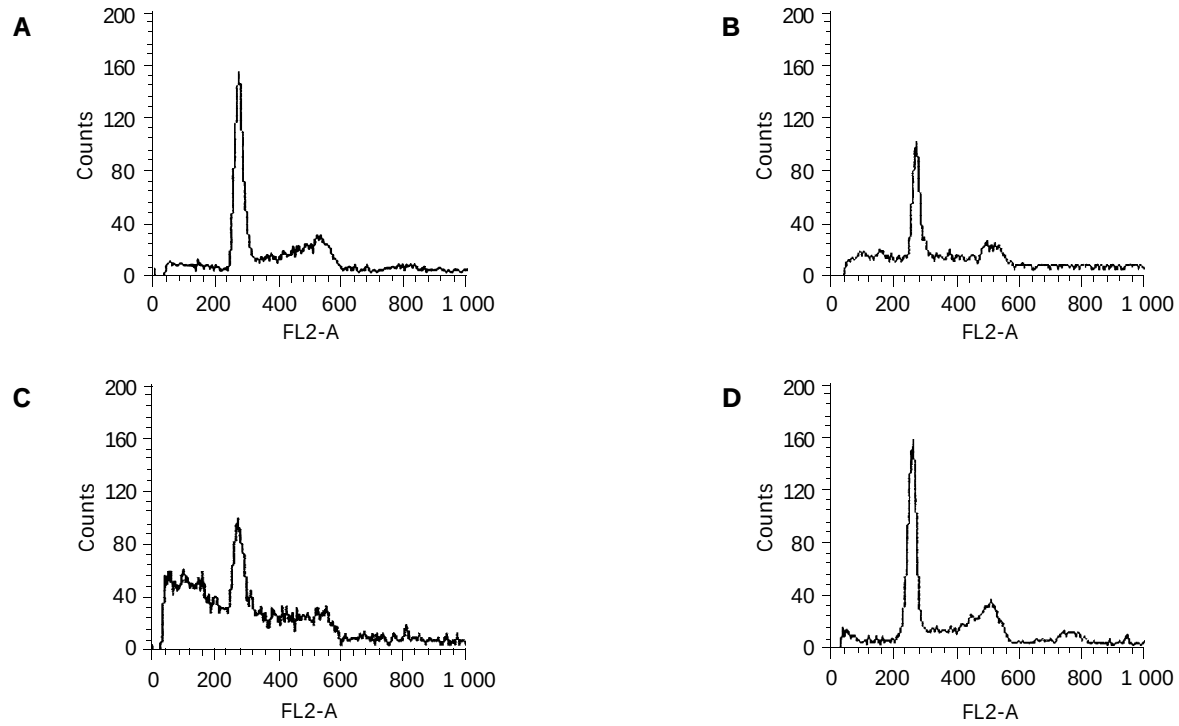


**Figure 2** Cell cycle analysis of MGC803. A: Untreated MGC803 cells at 72 h ($G_0G_1$: 48.20%; $G_2$M: 28.18%, S: 14.16%, apoptosis: 8.46%), B: MGC803 cells treated with VCR (20 ng / mL) after 72 h ($G_0G_1$: 37.62%; $G_2$M: 20.05%, S: 24.92%, apoptosis: 18.41%), C: MGC803 cells treated with VCR (20 ng / mL) +PD098059 (10 nmol / mL) after 72 h ($G_0G_1$: 32.53%; $G_2$M: 13.91%, S: 18.95%, apoptosis: 35.61%), D: MGC803 cells treated with PD098059 (10 nmol / mL) only after 72 h ($G_0G_1$: 49.27%; $G_2$M: 27.56%, S: 16.91%, apoptosis: 6.26%).

**Table 1** IDV of expression of MDR associated gene

| Group | VCR (MDR1) | VCR (MRP1) | VCR (GST-π) | VCR+PD098059 (MDR1) |
|---|---|---|---|---|
| Untreated MGC803 cells | 116±7.5 | 38±14.5 | 122±16 | 54±9.5 |
| Positive control | 192±8.6 | 64±13.2 | 144±21 | 105±9.5 |
| 24 h | 159±9.5 | 34±9.3 | 123±19.5 | 63±16.7 |
| 48 h | 172±7.6 | 30±11.5 | 110±15.6 | 64±21.1 |
| 72 h | 196±15.1 | 33±15.5 | 110±13.6 | 58±9.5 |


**Figure 3** The expression of associated gene of MDR in MGC803 cells treated with VCR or VCR+PD098059 24h-72 h. A: The expression of MDR1 of MGC803 cells treated with VCR increased. B,C: The expression of MRP1 and GST-π of MGC803 cells treated with VCR did not increase. D: The expression of MDR1 was inhibited when MGC803 cells treated with VCR and PD098059. E: The protein level of β-actin was detected to assess the loading amount in each well in SDS-PAGE. Lane 1, untreated cells; Lane 2, positive control cells; Lane 3, cells treated for 24 h; Lane 4, cells treated 48 h; Lane 5, cells treated for 72 h.

## DISCUSSION

Different tumors are different in sensitivity to chemotherapeutic drugs, and drug resistance can be induced by chemotherapy. The failure of cancer chemotherapy is mainly due to the overexpression of associated genes of MDR. Cytotoxic drug resistant cell lines have been induced after long-term exposure to gradient concentrations of cytotoxic drugs. Whether the expression of MDR could be induced by transient exposure to chemotherapeutic drugs has aroused. Chaudhary *et al* have found that the expressions of MDR1 mRNA in drug sensitive leukemia cell line K562 increased by short-term exposure to different chemotherapeutic drugs[21]. Schondorf *et al* also found that MDR1-mRNA was detectable in each cell line when short-term cultures of 6 established ovarian cancer cell lines were exposed to one of three anticancer drugs at concentrations equivalent to the clinically achievable plasma peak concentration. The method described here was easy to perform and could be of striking value in predicting the development of tumor chemoresistance[24]. Our results of western blot showed that the expression of P-gp in MGC803 cells increased significantly but the expressions of MRP1 and GST-π did not increase after induction by VCR for 72 h and the MTT assay showed that the drug resistance of MGC803 cells pretreated with VCR was 2.24-fold as that of untreated MGC803 cells. It suggested that the expression of MDR1 in MGC803 cells could be induced after transient exposure to VCR.

Mitogen-activated protein kinases (MAPKs), found in all eukaryotes, are common participants in signal transduction pathway from the membrane to the nucleus, and play an important role in cell proliferation, differentiation and apoptosis[26-28]. The mammalian MAPK family includes ERK1/2, JNK/SAPKs, ERK4 and *etc*. ERK1/2 is the most important subgroup among them. ERK1/2 signal transduction pathways contain at least three protein kinases. They are Raf-1, MAPK/ERK kinases1/2(MEK1/2) and ERK1/2. Raf-1 is activated by Ras, then phosphorylates two residues, either serine or threonine, to activate MEK1/2[29,30]. MEK1/2 activates ERK1/ERK2 by

phosphorylating a tyrosine and a threonine residues[31].

Signal transduction pathways play critical roles in pathogenesis and progress of tumor. The activation of MAPK and expression of MDR can be induced by anti-cancer drugs[32]. But the relation between them is not clear. Kisucka *et al* found PD098059 significantly reduced the survival of murine vincristine resistant L1210/VCR cells with a decrease of $LC_{50}$ to vincristine from 2.65 mmol/ L to 0.67 mmol/L. The result of the study demonstrated that the inhibitor of MEK1/2 signaling pathway was a reversal agent of VCR resistance in L1210/VCR cells, but the precise mechanism of PD098059 in modulation of MDR is not resolved yet, and the role of ERK-mediated phosphorylation cascade could be considered[25]. Ding *et al* found that PD098059 re-sensitised the Taxol resistant human ovarian cancer cell line A1847/TX at least 20-fold, but when MDR1 cDNA was stably expressed in the wild-type cell line to generate a highly Taxol-resistant sub-line, 1847/MDR5, MAPK kinases again became activated. This result demonstrated that the increased activity of the signaling pathway in the Taxol-resistant lines was directly attributable to MDR1 overexpression and was not due to the effects of Taxol itself, and that MAPK regulated the expression of MDR1[33]. It has been found that the expression of MDR1 can block apoptosis induced by the Fas ligand cascade. Expression of MDR1 resulted in a decrease in the rate of production of active caspase 3, a key effector caspase in the apoptotic cascade, upon Fas ligation[11]. The ERK1/2 pathway has also been shown to inhibit caspase-3 activation[34]. Another possibility is that MDR1 may have additional physiological functions, for instance, MDR1 induces novel $Na^+$ and $Cl^-$ dependent pathway for transmembrane $H^+$ efflux that results in intracellular alkalization[35]. Apoptosis induced by chemotherapeutic drugs is prevented by intracellular acidification and the induction of apoptotic events such as DNA laddering can be inhibited by increasing the intracellular pH in this manner. In a recent study, Wittstein *et al* demonstrated that inhibition of the ERK1/2 pathway using PD098059 resulted in re-alkalinisation of vascular endothelial cells in perfusion experiments[36]. Thus alterations in cellular pH may provide the stimulus for a variety of signaling pathways and responses mediated by both MDR and ERK1/2. The relation between MAPK and MDR1 is not clear yet. MAPK signal pathway may combine with other different mechanisms to modulate the expression of MDR1. Our study showed that PD098059 could reduce the drug resistance and enhance the killing action of VCR, and rates of apoptosis of MGC803 cells which were treated with VCR only increased from 18.41% to 35.61% when treated with PD098059 and VCR. It showed that PD098059 could reverse the drug resistance partially by reducing the $IC_{50}$ of MGC803 cells pretreated by VCR from (287±13.2) μg/L to (191±27.9) μg/L. At the same time, the expression of P-gp was inhibited by PD098059. It was suggested that when MGC803 cells were treated with VCR, the stimulation may be transducted by activation of MAPK signaling pathway to MDR1 gene and the expression of MDR1 increased. As a result, VCR was transported out of cells and multidrug resistance developed. The inhibitor of MEK1/2, PD098059 could reduce the expression of mdr1 and drug resistance of cells exposed to VCR by blocking the ERK1/2 signal transduction pathway. The precise mechanism between MDR1 and MAPK signal transduction pathway needs further study.

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