

## Antisense expression of PKC $\alpha$ improved sensitivity of SGC7901/VCR cells to doxorubicin

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### Abstract

**AIM:** To explore whether antisense blocking of protein kinase C alpha (PKC $\alpha$ ) would reverse multi-drug resistance (MDR) in the vincristine (VCR)-resistant human gastric cancer cell line SGC7901/VCR.

**METHODS:** SGC7901/VCR cells expressing antisense PKC $\alpha$ , SGC7901/VCR/aPKC, were established by transfection with a recombinant plasmid reversely inserted with PKC $\alpha$  cDNA. Empty vector (PCI-neo)-transfected cell clones, SGC7901/VCR/neo, served as the control. Western blot method was used to detect PKC $\alpha$  content in SGC7901, SGC7901/VCR, SGC7901/VCR/neo and SGC7901/VCR/aPKC cells, using PKC $\alpha$ -specific antibody. The sensitivity of SGC7901, SGC7901/VCR, SGC7901/VCR/neo and SGC7901/VCR/aPKC cells to doxorubicin (DOX) *in vitro* was determined by MTT assay. The uptake of DOX in these cells was detected with fluorescence spectrophotometer.

**RESULTS:** Western blot analysis showed that the PKC $\alpha$

protein level was about 8.7-fold higher in SGC7901/VCR cells than that in SGC7901 cells, whereas the protein expression of PKC $\alpha$  was reduced by 78% in SGC7901/VCR/aPKC cells when compared with the SGC7901/VCR cells. SGC7901/VCR/aPKC cells had a 4.2-fold increase in DOX cytotoxicity, accompanied by a 1.7-fold increase of DOX accumulation in comparison with SGC7901/VCR cells.

**CONCLUSION:** PKC $\alpha$  positively regulates MDR in SGC7901 cells, and inhibition of PKC $\alpha$  can partially attenuate MDR in human gastric cancer cells.

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**Key words:** Multi-drug resistance; Protein kinase C alpha; SGC7901; Gastric cancer

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### INTRODUCTION

Resistance of cancer cells to chemotherapeutic drugs is a major problem in clinical treatment of cancer. Multi-drug resistance (MDR) is the most important form of drug resistance characterized by decreased cellular sensitivity to a broad range of chemotherapeutic drugs, including anthracyclines, vinca alkaloids, epipodophyllotoxins, taxanes, antibiotics and some of the new topo- I inhibitors<sup>[1,2]</sup>. Classical MDR is mainly caused by overexpression of P-glycoprotein (Pgp), which is coded by the MDR1 gene and functions as an ATP-dependent drug-efflux membrane transporter that rapidly extrudes a variety of hydrophobic anticancer drugs from exerting cytotoxic effect<sup>[3,4]</sup>. Additionally, studies have shown that MDR is also accompanied by changes in the activity of protein kinase C (PKC).

Activation of PKC results in phosphorylation of Pgp and a decrease in drug accumulation, while inhibition of PKC can partially reverse the MDR phenotype<sup>[5,6]</sup>.

PKC comprises a family of at least 12 distinct serine/threonine kinase isoenzymes which are found in varying ratios in the cytosolic and membrane fractions of cells, depending on the type of tissue and its physiological state<sup>[6,7]</sup>. The family of PKC is composed of three subclasses: classical, novel and atypical PKC, each having different isoforms. The members of the classical PKCs ( $\alpha$ ,  $\beta$  I,  $\beta$  II and  $\gamma$ ) bind phorbol esters and are  $\text{Ca}^{2+}$  dependent. The novel PKCs ( $\delta$ ,  $\epsilon$  and  $\eta$ ) do not depend on  $\text{Ca}^{2+}$  but bind phorbol esters. The third subfamily includes the atypical PKCs ( $\tau$ ,  $\zeta$ ,  $\lambda$  and  $\mu$ ), which do not bind to either  $\text{Ca}^{2+}$  or phorbol ester. It is likely that each isoform has a specific role in a given cell<sup>[5,7]</sup>. PKC regulates numerous cell processes including proliferation, apoptosis and differentiation by phosphorylating proteins in response to transmembrane signals from hormones, growth factors, neuro-transmitters and pharmacological agents<sup>[5]</sup>. Recently accumulated evidence indicated that PKC, especially classical PKC, plays a significant role in the formation of cancer MDR<sup>[5,6]</sup>. The isoenzymes of PKC possess distinct differences in localization in different cells, and research on distinct function of isoforms in cancer MDR has important significance<sup>[5,6]</sup>.

Among those isoforms of PKC, PKC $\alpha$  is likely to play a decisive role in maintaining MDR phenotypes in some cancer cells, and may therefore represent potential novel targets for the treatment of cancers<sup>[6,8-12]</sup>. In an effort to see whether down-regulation of a single isoform of PKC could affect drug resistance, vincristine (VCR)-resistant human gastric carcinoma cell line SGC7901/VCR was transfected with an expression vector containing the cDNA for PKC $\alpha$  in the antisense orientation, and their expression of PKC $\alpha$ , doxorubicin (DOX) sensitivity and DOX accumulation were determined.

## MATERIALS AND METHODS

### Materials

MTT and DOX were purchased from Sigma, VCR from the Twelfth Shanghai Pharmaceutical Product Factory, Lipofectamine 2000 from Invitrogen, nitrocellulose membranes and 3', 3'-diaminobenzidine (DAB) from Sigma, G418 and rabbit polyclonal anti-PKC $\alpha$  from Gibco-BRL. Plasmid pSP64-PKC $\alpha$  was kindly provided by Dr. P.J. Parker (Imperial Cancer Foundation, England). The eukaryotic expression vector, plasmid PCI-neo, was kindly provided by Dr. Jun-Jie Xu (Institute of Microbiology and Epidemiology, Chinese Academy of Military Medical Sciences). The human gastric cancer cell line SGC7901, and its VCR-resistant counterpart SGC7901/VCR selected by stepwise exposure of parental SGC7901 cells to increasing concentrations of VCR, were purchased from Wuhan University Type Culture Collection (Wuhan, China).

### Cell culture

Both SGC7901 and SGC7901/VCR cells were grown in

RPMI-1640 medium supplemented with 10% fetal calf serum at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. The SGC7901/VCR cells were cultured in the presence of 0.8  $\mu\text{mol/L}$  VCR and grown in drug-free medium 2 wk before the experiments.

### Plasmid construction

The full-length cDNA encoding PKC $\alpha$  cDNA (2.3 kb) was recovered from Sal I sites in pSP64-PKC $\alpha$  plasmid and subcloned into Sal I sites of PCI-neo plasmid. The recombinant plasmid with the PKC $\alpha$  cDNA in the antisense orientation was confirmed by BamHI and/or Sal I restriction digestion, and designated PCI-neo-aPKC $\alpha$ .

### Cloning of cells transfected with antisense vector PCI-neo-aPKC $\alpha$

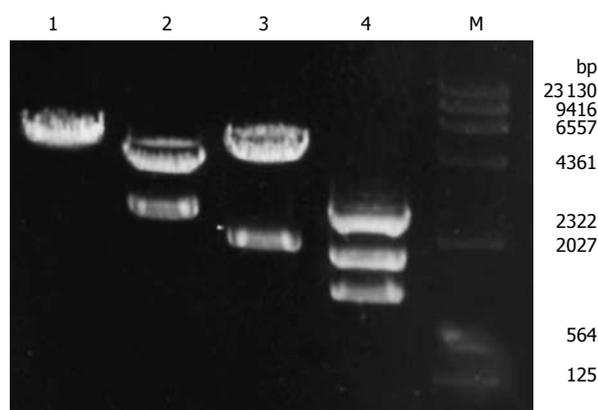
The process was performed as described by Wang *et al.*<sup>[13]</sup>. Briefly, SGC7901/VCR cells were seeded in six-well plates to 70%-80% confluence. Empty vector PCI-neo and antisense vector PCI-neo-aPKC $\alpha$  were transfected into SGC7901/VCR cells *via* Lipofectamine 2000. The two kinds of transfected cells were named SGC7901/VCR/neo and SGC7901/VCR/aPKC respectively. After two days of transfection, cells were selected by culture medium containing G418 (400 mg/L) for 2 wk. The single clone was picked out using limiting dilution method and was expanded and maintained in medium containing 400 mg/L G418 until 1 wk prior to experiments.

### Western blot analysis

The whole cell lysates were extracted with lysis buffer containing 1% Triton-100, 50 mmol/L NaCl, 50 mmol/L sodium fluoride, 20 mmol/L Tris (pH 7.4), 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L sodium vanadate, 0.2 mmol/L phenylmethylsulfonyl fluoride, and 0.5% NP40. An aliquot was taken for protein determination, and the remainder was mixed (1:1) with 2  $\times$  SDS sample buffer and boiled for 5 min. Samples were run on 10% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. After blocking, the membranes were incubated with rabbit polyclonal antibody against PKC $\alpha$  isoform at a dilution of 1:1000. Blots were washed three times with PBST to block nonspecific binding sites and incubated with secondary antiserum (goat anti-rabbit IgG conjugated to horseradish peroxidase) for 1 h at 37°C, then washed three times with 50 mmol/L Tris-HCl (pH 6.8). Color was developed with DAB as the substrate.

### Cytotoxicity assay

The cells in exponential growth were seeded in 96-well plates at a density of  $2 \times 10^4$  cells per well and 24 h later graded DOX (at the concentrations of 0, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, 10  $\mu\text{mol/L}$ ) were added, respectively. The total medium volume of each well was 200  $\mu\text{L}$ . Three days after drug addition, 10  $\mu\text{L}$  MTT (5.0 g/L in PBS) was added. After 4 h of incubation, supernatants were removed and replaced by 150  $\mu\text{L}$  DMSO. After formazan solubilization, the absorbance at 570 nm



**Figure 1** Restriction map of recombinant plasmid PCI-neo-aPKC $\alpha$ . Lane 1: PCI-neo digested with *Sal* I *Eco*R1; Lane 2: PCI-neo-aPKC $\alpha$  digested with *Bam*H1; Lane 3: PCI-neo-aPKC $\alpha$  digested with *Sa* I; Lane 4: PCI-neo-aPKC $\alpha$  digested with *Bam*H1 + *Sal* I; Lane M:  $\lambda$  DNA *Hind*III Marker.

was recorded using an automated microplate reader. IC<sub>50</sub> (concentration resulting in 50% inhibition of cell growth) values for DOX were calculated as 100% from plotted results using untreated cells.

### Intracellular DOX accumulation

The assay was performed as described by our previous report<sup>[2]</sup>. Briefly,  $1.0 \times 10^6$  cells were exposed to 4.0  $\mu$ mol/L DOX for 60 min. Following incubation, the cells were washed twice with ice-cold PBS, then suspended in 6.0 mL of 50% ethanol -0.3 mol/L hydrochloride, extracted for 2 h, centrifugated at  $100 \times g$  for 10 min, and the supernatant was collected. DOX-associated mean fluorescence intensity (MFI) was measured by fluorescence spectrophotometer at an excitation wavelength of 488 nm and an emission wavelength of 590 nm. The DOX standard solution ( $0.1$ - $1.0 \times 10^3$  nmol/L) was prepared, and a standard work curve with related quotient was constructed.

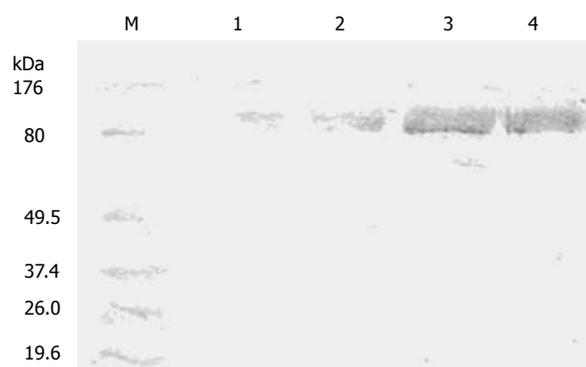
### Statistical analysis

Data were expressed as mean  $\pm$  SD. Student's *t* test was used to assess statistical significance of differences. *P* < 0.05 was considered statistically significant.

## RESULTS

### Establishment of SGC7901/VCR cells expressing antisense PKC $\alpha$

To construct the PKC $\alpha$  antisense expression plasmid, the full-length cDNA of PKC $\alpha$  (2.3 kb) recovered from *Sal* I sites in pSP64-PKC $\alpha$  plasmid was subcloned into *Sal* I sites of the eukaryotic expression vector PCI-neo (5.5 kb). A recombinant plasmid with PKC $\alpha$  cDNA in the antisense orientation was obtained and designated PCI-neo-aPKC $\alpha$ , which contains the neomycin resistance gene neo for drug selection. Two fragments were obtained by digesting PCI-neo-aPKC $\alpha$  with *Sal* I, one was PKC $\alpha$  cDNA fragment about 2.3 kb and the other was PCI-neo cDNA fragment about 5.5 kb (Figure 1). And the inserted fragment was verified by sequencing (data not shown).



**Figure 2** Western blot identification of PKC $\alpha$  protein in SGC7901, SGC7901/VCR, SGC7901/VCR/neo and SGC7901/VCR/aPKC cells. Lane M: Benchmark™ Marker; Lane 1: SGC7901 cells; Lane 2: SGC7901/VCR/aPKC cells; Lane 3: SGC7901/VCR/neo cells; Lane 4: SGC7901/VCR cells.

SGC7901/VCR cells were transfected with either PCI-neo or PCI-neo-aPKC $\alpha$ , respectively, and G418-resistant clones were isolated and amplified after G418 selection. Empty vector-transfected cell clones were named SGC7901/VCR/neo, which together with the parental cell line served as the controls for these experiments. Clones transfected with the PKC $\alpha$  antisense expression plasmid (PCI-neo-aPKC $\alpha$ ) were named SGC7901/VCR/aPKC.

Western blot analysis was performed to assess protein abundance of PKC $\alpha$  in SGC7901, SGC7901/VCR, SGC7901/VCR/aPKC and SGC7901/VCR/neo cells. As shown in Figure 2, the PKC $\alpha$  protein level was about 8.7-fold higher in SGC7901/VCR cells than that in SGC7901 cells. Transfection of SGC7901/VCR cells with the control vector did not alter PKC $\alpha$  expression, whereas the protein expression of PKC $\alpha$  was reduced by 78% in SGC7901/VCR/aPKC cells when compared with the SGC7901/VCR cells.

### Modulating effect of antisense expression of PkC $\alpha$ on MDR

Using the MTT assay, the *in vitro* cytotoxicity of DOX in SGC7901, SGC7901/VCR, SGC7901/VCR/neo and SGC7901/VCR/aPKC cells was examined. As shown in Table 1, SGC7901/VCR cells were 23.5 times more resistant to DOX in comparison with SGC7901 cells. The IC<sub>50</sub> in SGC7901/VCR/aPKC was 4.2-fold lower than that in SGC7901/VCR cells, indicating that antisense PkC $\alpha$  could partially reverse resistance to DOX in multi-drug resistant SGC7901 cells.

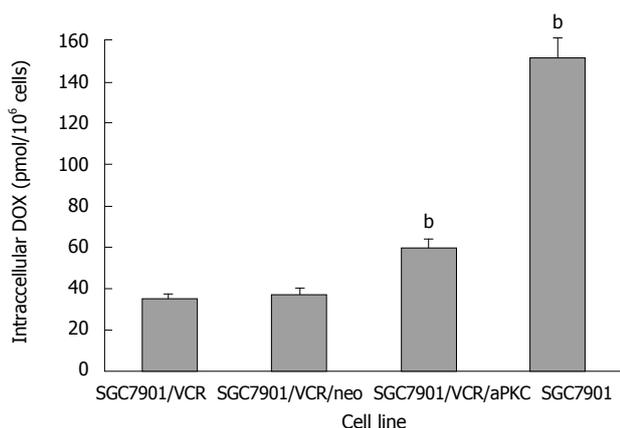
### Effect of antisense expression of protein kinase C $\alpha$ on intracellular accumulation of DOX in multi-drug resistant SGC7901 cells

DOX accumulation in SGC7901, SGC7901/VCR, SGC7901/VCR/neo cells and SGC7901/VCR/aPKC was measured by fluorescence spectrophotometry after incubation of cells with 4.0  $\mu$ mol/L DOX for 60 min. As shown in Figure 3, SGC7901/VCR cells accumulated 4.3-fold less DOX than SGC7901 cells, whereas SGC7901/VCR/aPKC cells showed 1.7-fold more drug retention than SGC7901/VCR cells.

**Table 1** Modulating effect of antisense expression of protein kinase C $\alpha$  on resistance to DOX in multi-drug resistant SGC7901 cells

Cell lines	IC <sub>50</sub> of DOX ( $\mu\text{mol/L}$ )	Resistance index (RI)
SGC7901	0.076 $\pm$ 0.0074	1.0
SGC7901/VCR	2.4 $\pm$ 0.14 <sup>b</sup>	31.6
SGC7901/VCR/neo	2.3 $\pm$ 0.19 <sup>b</sup>	30.3
SGC7901/VCR/aPKC	0.58 $\pm$ 0.076 <sup>b</sup>	7.6

<sup>b</sup> $P < 0.01$  vs IC<sub>50</sub> of SGC7901. The data are presented as mean  $\pm$  SD.



**Figure 3** Accumulation of intracellular DOX in SGC7901, SGC7901/VCR, SGC7901/VCR/neo and SGC7901/VCR/aPKC cells. Cells were incubated with 4.0  $\mu\text{mol/L}$  DOX for 60 min. Each point represents the mean  $\pm$  SD from three experiments. <sup>b</sup> $P < 0.01$  vs SGC7901/VCR cells.

## DISCUSSION

MDR to cancer treatment has been studied for more than 20 years. No useful method of reversing MDR suitable for clinical use has yet emerged from this large quantity of work. The reason could be complicated mechanisms involved. There are several ways for cancer cells to develop MDR<sup>[4]</sup>. The most investigated mechanisms with known clinical significance are: (1) activation of transmembrane proteins effluxing different chemical substances from the cells, in which Pgp is the best known efflux pump; (2) activation of the enzymes of the glutathione detoxification system; (3) alterations of the genes and the proteins involved in the control of apoptosis (especially p53 and Bcl-2)<sup>[10]</sup>. Recent studies have indicated a role for PKC in the regulation of the MDR phenotype. A number of studies have demonstrated that PKC activity was elevated in MDR-selected cell lines, and several PKC inhibitors are able to partially reverse MDR and inhibit Pgp phosphorylation<sup>[5,6]</sup>. Pgp is phosphorylated by PKC and that phosphorylation positively modulates its transport function<sup>[14]</sup>. The PKC family consists of at least 12 isoforms, and different isoforms of PKC possess distinct differences in expression and function in different MDR cells<sup>[12-15]</sup>. The elevated PKC activity level of the MDR cancer cells was frequently a result of increased PKC $\alpha$  expression in most cancers<sup>[8-12]</sup>, but in some cancers it was due to enhanced expression of other isoforms, such as PKC $\beta$  I, PKC $\gamma$  or PKC $\eta$ <sup>[15-18]</sup>.

SGC7901/VCR is an established VCR-resistant cell line selected by stepwise exposure of parental SGC7901 cells to increasing concentrations of VCR. Previous reports have proved that the SGC7901/VCR cells possess the characteristics of classical MDR with overexpression of Pgp, and the SGC7901/VCR cell line has been successfully used as an *in vitro* MDR reversal model by several study groups, including ours<sup>[19-23]</sup>. The recent report showed that the expression of PKC $\alpha$  was significantly higher in SGC7901/VCR cells than in SGC7901 cells, and there was no significant difference in the expression of PKC $\beta$  I, PKC $\beta$  II and PKC $\gamma$  between SGC7901/VCR and SGC7901 cells<sup>[10]</sup>.

In the present study, we observed that the expression of PKC $\alpha$  was significantly higher in SGC7901/VCR cells than in SGC7901 cells, which was consistent with the previous report by Han *et al*<sup>[10]</sup>. To investigate the possible effect of PKC $\alpha$  on MDR for chemotherapeutic drug in human gastric cancer cells, we established SGC7901/VCR cells stably expressing antisense PKC $\alpha$ . We demonstrated that down-regulation of the predominant PKC $\alpha$  isoform expressed in SGC7901/VCR cells by the expression of antisense PKC $\alpha$  led to greater DOX accumulation and partial reversal of resistance to DOX. These data support the thesis that MDR in this cell line is modulated, in part, by PKC $\alpha$ . These results suggest that a more effective means of reducing PKC $\alpha$  activity, possibly with RNA interference, might be a more efficient way of increasing drug sensitivity in this MDR cell line.

## COMMENTS

### Background

Protein kinase C (PKC) constitutes a family of closely related protein serine/threonine kinase which are sub-grouped into classical ( $\alpha$ ,  $\beta$  I,  $\beta$  II, and  $\gamma$ ), novel ( $\delta$ ,  $\epsilon$ ,  $\eta$ , and  $\theta$ ), and atypical ( $\iota$  and  $\zeta$ ) isoforms. The members of the PKC family are involved in the regulation of numerous cell processes including proliferation, apoptosis, and differentiation. It is likely that each isoform has a specific role in a given cell.

### Research frontiers

A recent study confirmed that PKC $\alpha$ , PKC $\beta$  I, PKC $\beta$  II and PKC $\gamma$  were expressed in both human gastric carcinoma cell line SGC7901 and vincristine (VCR)-resistant human gastric carcinoma cell line SGC7901/VCR, but the expression of PKC $\alpha$  was significantly higher in SGC7901/VCR cells than that in SGC7901 cells. There was no significant difference in the expression of PKC $\beta$  I, PKC $\beta$  II and PKC $\gamma$  between SGC7901/VCR cells and SGC7901 cells. In this report, the authors transfected SGC7901/VCR cells with an expression vector containing the cDNA for PKC $\alpha$  in the antisense orientation to see whether down-regulation of PKC $\alpha$  could affect drug resistance in SGC7901/VCR cells.

### Innovations and breakthroughs

In the present study the authors observed that down-regulation of the predominant PKC $\alpha$  isoform expressed in SGC7901/VCR cells by the expression of antisense PKC $\alpha$  led to greater doxorubicin accumulation and partial reversal of resistance to doxorubicin. These data support the thesis that MDR in this cell line is modulated in part by PKC $\alpha$ .

### Applications

This study provided a new target against MDR in human gastric carcinoma, suggesting that a more effective means of reducing PKC $\alpha$  activity, possibly with RNA interference, might be a more efficient way of increasing drug sensitivity in this MDR cell line.

### Peer review

The manuscript entitled "Antisense expression of PKC $\alpha$  improved sensitivity of SGC7901/VCR cells to doxorubicin" by Wu *et al* examines the effects of anti-

sense inhibition of PKC $\alpha$  expression on the MDR phenotype of gastric cancer cells. The authors used antisense technology to establish a derivative of the multi-drug/vincristine-resistant SGC7901-VCR cell line with reduced expression of PKC $\alpha$ . Using this isogenic pair of cell lines, the authors conclude that PKC $\alpha$  deficiency leads to increased intracellular accumulation of DOX and partially restores sensitivity to the drug, thus reversing the MDR phenotype.

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