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# Chinese Herbs of Shenghe Powder Reverse Multidrug Resistance of Gastric Carcinoma SGC-7901

Jianhua Wang, Yuesheng Xia, Huan Wang, and Zengxia Hou

The objective of this study was to investigate the reversal effect of Chinese herbs of Shenghe Powder on the multidrug resistance of the human SGC-7901 gastric carcinoma cell line and vincristine-resistant cell line (SGC-7901/vincristine) and the possible mechanism. SGC-7901 and SGC-7901/vincristine were cultured in liquid medium RPMI 1640, with the addition of vincristine to the vincristine-resistant line. The reversal effect of Shenghe Powder (using verapamil as control) on the multidrug resistance of SGC-7901/vincristine cells was observed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide method. The expression rate of P-glycoprotein (P-gp), lymphoma/leukemia-2 (Bcl-2), and apoptosis ratio of SGC-7901 and SGC-7901/vincristine with added Shenghe Powder, verapamil, or verapamil plus Shenghe Powder was observed by flow cytometry. Shenghe Powder and verapamil decreased the multidrug resistance of SGC-7901/vincristine. The effect of Shenghe Powder (10 mg/L) was significantly higher than verapamil ( $P < .05$ ). The intracellular concentration of vincristine was increased by Shenghe Powder and verapamil. The vincristine concentration of SGC-7901/vincristine treated with Shenghe Powder was significantly higher ( $P < .05$ ). Shenghe Powder reduced the expression level of P-gp and Bcl-2 in SGC-7901/vincristine and increased the apoptotic percentage of tumor cells; Shenghe Powder had the more significant effect on apoptosis ( $P < .05$ ). In conclusion, Shenghe Powder increases the intracellular concentration of vincristine, consistent with the down-regulation of the expression of P-gp and Bcl-2. The reversal effect of Shenghe Powder was stronger than that of verapamil.

**Keywords:** multidrug resistance; Bcl-2; P-glycoprotein; Chinese herbs

Multidrug resistance (MDR) makes the treatment of tumors more difficult. Because many factors contribute to the occurrence of MDR, it is difficult to control by blocking only a single factor. The combination of 2 or more kinds of reversal reagents at the same time may be more effective. It is difficult for

many drugs that can effectively inhibit MDR in vivo to obtain an effective concentration in target cells.<sup>1-3</sup> This research explored the MDR reversal effect of Shenghe Powder (SHP) and its mechanism, with the goal of creating a new MDR-reversing drug. In this study, we employed this agent to treat MDR of the human gastric carcinoma SGC-7901 cell line, observed its MDR reversal effect, compared it with a traditional reversal reagent, and attempted to demonstrate its mechanism.

## Materials and Methods

The experiment was carried out at the Second Military Medical University from March 2003 to January 2004.

### Materials

SGC-7901 was purchased from the Second Military Medical University. Vincristine (VCR; Shanghai Hualian Pharmaceutical Company, Shanghai, China), P-glycoprotein (P-gp)-PE fluorescent antibody and Bcl-2-FITC fluorescent antibody (Immunotech Biotech Company, Beijing, China), DMSO (Beijing Chemical Company, Beijing, China), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide (MTT) (Sigma, St Louis, Missouri) were purchased from the Fuzhou Maixin Bio Tech Company (Fuzhou, Fujian, China). Verapamil was purchased from Shandong Qilu Pharmaceutical Company (Shandong, China). SHP mainly includes *Panax ginseng* C.A. Meyer (Araliaceae), *Scrophularia ningpoensis* Hemsl. (Scrophulariaceae), *Atractylodes macrocephala* Koidz. (Asteraceae), *Coix*

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*lacryma-jobi* Linn. var. *ma-yuen* (Roman.) Stapf ex Hook.f. (Poaceae), *Hedyotis diffusa* Willd. (Rubiaceae), *Cistanche sp.*, *Bufo gargarizans* (Cantor, 1842) (Bufonidae), and *Glycyrrhiza uralensis* Fisch. ex D.C. (Fabaceae). All Chinese herbs were purchased from Shanxi Company of Chinese Herbal Medicines and identified by Professor Yuesheng Xia.

## Methods

### SHP Extraction Method

The dried whole herbs (1000 g) were degreased by heating under reflux with industrial ethanol in a bath and then extracted 3 times with 10 L boiling distilled water for 1 hour each time. The decoctions were then filtered through carbasus, mixed, and concentrated to 1000 mL. The concentrated extract was mixed with 95% ethanol to make the ethanol content up to 80%. After standing overnight ( $20 \pm 2$  h), the precipitate was filtered, washed, and vacuum-dried to give a brown powder. Before experimentation, it was dissolved in phosphate-buffered saline (PBS) and filtered with a 0.22- $\mu$ m membrane. The percolate was made up to concentrations of 2.5, 5, and 10 mg/L and stored at  $-20^{\circ}\text{C}$ .

### Cell Culture

SGC-7901 was cultured in liquid medium (RMPI 1640), which contains 100 g/L calf serum, 100 U/L penicillin, and streptomycin. The culture flask's environment was kept at  $37^{\circ}\text{C}$ , with saturated humidity and 5%  $\text{CO}_2$ . The VCR-resistant cell line (SGC-7901/VCR) was cultured in the same environment as above, but VCR was added to the RMPI 1640. The MTT assay was chosen to detect the reversal ratio of SHP. The SGC-7901/VCR cells had been cultured in VCR-free medium for 2 weeks before the experiment. Cells in the exponential proliferation period ( $1-5 \times 10^5/\text{mL}$ ) were placed in a 96-well plate. SHP, verapamil, and SHP plus verapamil were added to 3 test groups with concentrations ordinarily diluted by 50%. A blank group had no added cells. The reversal reagent of the control group was replaced by RMPI 1640. Every group included 3 parallel samples. The MTT solution was added 4 hours before detection. Then the absorbency (A490 value) was measured, and the growth rates of cells were computed. The concentration that kept the cells' growth rates higher than 95% was designated the nontoxic concentration. The concentration that kept the cells' growth rates between 85% and 95% was the low-toxicity concentration.

Calculation of the growth rate was as follows ("A" denotes absorbency):

$$\text{Growth rate} = A_{\text{sample}}/A_{\text{control}} \times 100\%.$$

### Measurement of the Multidrug Resistance Reversal Ratio

After adding the reversal, control, and blank reagents to samples, the cells'  $\text{IC}_{50}$  values for VCR were measured and computed. The  $\text{IC}_{50}$  value was determined using POMS software. The reversal ratio was calculated as follows:

$$\text{Reversal ratio} = [1 - (A_{\text{sample}}/A_{\text{control}})/A_{\text{control}}] \times 100\%.$$

### Intracellular Vincristine Concentration

The intracellular concentration of VCR was measured by fluorescent detection.

The reversal reagent and VCR at different concentrations were added to groups of cells in the exponential proliferation period ( $1-5 \times 10^5/\text{mL}$ ); the cells were then cultured 3 hours in a flask at  $37^{\circ}\text{C}$ , with saturated humidity and 5%  $\text{CO}_2$ . The cells were then washed with PBS 3 times and suspended in 500 g/L alcohol with 0.3 N HCl (at  $4^{\circ}\text{C}$ ) overnight. Next morning, the samples were centrifuged, and the fluorescent density of the supernatant was measured (Metertech  $\Sigma$ 960 microplate reader, Taiwan). The VCR concentration-fluorescent density line was drawn according to the calendar date. The intracellular concentration of VCR was then calculated.

### Expression Rate of P-gp and Bcl-2

The expression rates of P-gp and Bcl-2 protein were measured by flow cytometry (FCM) (FC500, Beckman-Coulter Company, Fullerton, California). Cells in the exponential proliferation period ( $1-5 \times 10^5/\text{mL}$ ) were divided into 3 groups, which each had 3 parallel samples. After VCR was added, the samples were cultured for 48 hours. For measurement of the P-gp expression rate, P-gp-PE fluorescent antibody was added to samples. After the samples were cultured in darkness for 30 minutes, they were mixed, centrifuged (800-1000 r/min), and washed in PBS twice. Then, the supernatant was discarded and the cells were suspended in PBS. The samples were then ready for detection. For measurement of Bcl-2 protein expression rate, after VCR was added, the samples were cultured 48 hours. After collecting the cells and washing them with PBS twice, the cells were fixed by 10 g/L polyformaldehyde for 15 minutes, then washed with PBS twice. Then, 0.1% Triton-X100 (membrane penetrating reagent) was added to the samples for 5 minutes. After the samples were washed with PBS once, the first antibody of Bcl-2 was added for 30 minutes. After the samples were washed with PBS once, the second antibody of Bcl-2 was added for 30 minutes. After the samples were washed with PBS 3 times, they were ready for detection.

**Table 1. Reversal Ratio of SHP, Measured by MTT Assay ( $\bar{x} \pm s$ )**

Drug, mg/L	IC <sub>50</sub>	Reversal Ratio
VCR (control) 10	58.2 ± 0.59**	
SHP 2.5	16.3 ± 0.94*	3.16
5.0	15.1 ± 1.01**	3.89
10	13.9 ± 0.28*	4.27
Verapamil 8.0	17.4 ± 0.85**	2.96
SHP + verapamil	14.0 ± 0.31*	4.29

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide; SHP = Shenghe Powder; VCR = vincristine.

\* $P < .05$  vs VCR group.

\*\* $P < .05$  vs SHP 10 mg/L.

### Measurement of Apoptosis Percentage by Flow Cytometry

Cells were centrifuged, washed twice with PBS, fixed by 95% ethanol, and kept at 4°C overnight. Then, after the cells were centrifuged and washed twice with PBS, 1 mL propidium iodide (PI) dye liquid was added for 30 minutes. The samples were then measured by FCM. Coulter Multi System II software was used to analyze and compute the data.

### Statistical Analysis

SPSS 12.0 was used to perform the statistical analysis. Data are expressed as mean ± SD.

## Results

### Detection of MDR Reversal

Shenghe Powder at 2.5, 5, and 10 mg/L; verapamil at 8 mg/L; and SHP 10 mg/L + verapamil 8 mg/L significantly inhibited MDR and increased the sensitivity of the SGC-7901/VCR cell line to VCR. The reversal ratios were 3.16, 3.89, 4.27, 2.96, and 4.29 (Table 1). The reversal ratios of SHP (5, 10 mg/L) and SHP 10 mg/L + verapamil 8 mg/L were significantly higher than verapamil alone ( $P < .05$ ). Shenghe Powder is thus able to reverse the MDR of the cells of SGC-7901/VCR in a dosage-dependent manner in vitro.

### Effects of Shenghe Powder on Intracellular Concentration of Vincristine

The intracellular VCR concentration and fluorescent density curve were calculated using SPSS software. The data are shown in Table 2. The intracellular concentration of VCR of the SGC-7901/VCR cell line is less than that of the SGC-7901 cells. Shenghe Powder (10 mg/L) and SHP (10 mg/L) + verapamil (8 mg/L) both enhanced the intracellular concentration of VCR to different extents, but all 3 intracellular concentrations of VCR mentioned were less than that of SGC-7901. The concentration difference

between the SGC-7901/VCR cells from the SHP group and SGC-7901 cells was the least (Table 2).

### Measurement of Apoptosis Percentage

With the addition of SHP (5, 10 mg/L), the apoptosis percentage of SGC-7901 cells was the highest ( $P < .01$ ), and that of SGC-7901/VCR cells was the least (Table 3). Shenghe Powder improves the apoptosis percentage of SGC-7901/VCR cells significantly, and the effect of SHP (10 mg/L) was the strongest.

### Expression Rates of P-gp and Bcl-2 Proteins

The P-gp and Bcl-2 expression rates in SGC-7901 were the lowest, and P-gp and Bcl-2 expression rates in SGC-7901/VCR were the highest. This indicates that these 2 indices are suitable for observing the effects of MDR reversal (Table 4). Shenghe Powder at concentrations of 2.5, 5, and 10 mg/L inhibits P-gp and Bcl-2 expression rates significantly, in a dose-dependent manner.

## Discussion

Shenghe Powder, a compound Chinese medicine thought to be effective on tumors, has long been used in our hospital for cancer treatment. It is only in recent years that its antitumor effect has been systematically evaluated. Many experimental results indicated that the direct aqueous extract of SHP had a marked anti-tumor effect.<sup>4,7</sup> Its complex components may offer multitargeted treatment: It shows Ca<sup>2+</sup> channel-blocking activity,<sup>5</sup> and the present study shows that its possible MDR reversal mechanism is to block the combination with P-gp and the export of chemotherapy drugs.<sup>4,5</sup>

Since the first report of MDR in 1970, there have been increasing numbers of studies concerning MDR in recent years.<sup>8</sup> These experiments are mainly concerned with exploring the mechanism and reversal of MDR. Multidrug resistance, a complex process, is the most important factor preventing tumor cells from being attacked by chemotherapy drugs. Research has shown that many pathways in the cell membrane, cytoplasm, and cell nucleus contribute to the emergence of MDR, including abnormal intracellular transferring proteins, energy conversion, detoxification systems, DNA synthesis, and other factors. Present research indicates that several main mechanisms of MDR are as follows<sup>9-11</sup>:

- Multidrug resistance induced by abnormal transferral proteins, such as high expression rates of P-gp coded by the *mdr1* gene, breast cancer drug-resistant protein (BCRP), and lung drug-resistant protein (LRP)
- Multidrug resistance induced by enzymes, including activity of glutathione S-transferase (GST), protein kinase C (PKC), and Topo II

**Table 2. Intracellular Drug Concentration ( $\bar{x} \pm s$ , mg/L)**

VCR	Intracellular Drug Concentration				
	SGC-7901	SGC-7901/VCR	SHP 10	Verapamil 8	SHP 10 + Verapamil 8
1	0.60 ± 0.10*	0.30 ± 0.13	0.46 ± 0.13	0.27 ± 0.14	0.46 ± 0.14
2	1.14 ± 0.16*	0.37 ± 0.25	1.11 ± 0.24*	0.73 ± 0.20	1.12 ± 0.25*
4	1.90 ± 0.38*	0.86 ± 0.11	1.56 ± 0.15*	1.19 ± 0.27	1.59 ± 0.20*
6	3.42 ± 0.20**	1.43 ± 0.31	3.28 ± 0.26*	2.29 ± 0.27*	3.31 ± 0.27*

SHP = Shenghe Powder; VCR = vincristine.

\**P* < .05.

\*\**P* < .01 vs SGC-7901/VCR.

**Table 3. Apoptosis Rates of Tumor Cells Measured by Flow Cytometry ( $\bar{x} \pm s$ )**

Drug, mg/L	Apoptosis, %
SGC-7901/VCR (control)	2.2 ± 0.3
SGC-7901 + VCR 2.0	9.4 ± 1.2**
SHP 2.5	4.7 ± 0.5****
5.0	7.8 ± 0.3****
10	8.9 ± 0.7****
Verapamil 8.0	3.4 ± 0.6*
SHP 10 + Verapamil 8.0	9.7 ± 1.3****

SHP = Shenghe Powder; VCR = vincristine.

\**P* < .05.

\*\**P* < .01 vs SGC-7901/VCR.

\*\*\*\**P* < .05 vs verapamil 8.0.

**Table 4. Expression Rate of P-gp, Bcl-2 ( $\bar{x} \pm s$ )**

Drug, mg/L	P-gp, %	Bcl-2, %
SGC-7901/VCR (control)	98.7 ± 2.5	25.3 ± 1.7****
SGC-7901 + VCR 2.0	10.4 ± 0.6**	17.8 ± 0.7****
SHP 2.5	78.6 ± 2.5*	80.3 ± 0.9*
5.0	45.9 ± 2.6**	43.1 ± 2.6****
10	27.1 ± 4.8**	28.2 ± 3.1****
Verapamil 8.0	76.3 ± 1.4****	89.7 ± 0.5
SHP 10 + verapamil 8	92.7 ± 2.5	27.6 ± 3.0****

SHP = Shenghe Powder; VCR = vincristine.

\**P* < .05.

\*\**P* < .01 vs SGC-7901/VCR.

\*\*\*\**P* < .05 vs verapamil 8.0.

- Multidrug resistance induced by apoptosis regulation, high expression of Bcl-2 gene and Bcl-2 protein, mutation, or loss of p53 gene and abnormalities of other apoptosis regulatory genes such as c-myc, ras, fas/APO-1, and bcl/abl
- Other causes of MDR include some cellular factors related to MDR such as tumor necrosis factor (TNF), interleukin-2 (IL-2) and insulin-like growth factor 1 (IGF-1), enhancement of DNA repair, and so on.

Finding effective MDR reversing drugs is an important step toward improving chemotherapy effectiveness. In the past 20 years, researchers have investigated many MDR reversal reagents. These reversal reagents mainly include the following: Ca<sup>2+</sup> channel-blocking reagents, immunologic regulators, steroids, megestrol, estrogen resistance, antimalarial and antiarrhythmia drugs, enzyme inhibitors, antibiotics, anthracyclines, Tween-80, and others.<sup>12-14</sup>

Current MDR reversal drugs cannot yet obtain complete inhibition. The reasons are mainly as follows: It is difficult to control the toxic effects at the same time as reversing MDR, it is difficult for a reversal reagent to penetrate target cells, and a single drug effective on only 1 factor cannot inhibit MDR resulting from multiple factors. For the reasons listed above, it is also important to get a high concentration

of reversal drugs in target cells, to reduce side effects, to combine 2 or more kinds of different reversal drugs, and to find new drugs.<sup>15,16</sup>

Research on MDR presently focuses on seeking chemosensitizers with more targets. Up-regulation of resistance genes or down-regulation of target genes may occur rapidly in human solid tumors, within days of the start of treatment, and similar changes are present in pre- and postchemotherapy biopsy material. The molecular processes used by each tumor appear to be linked to the drug used, but there is heterogeneity between individual tumors, even those with the same histological type, in the pattern and magnitude of response to the same drugs. Many MDR inhibitors have been identified, but none of them has been proven clinically useful without side effects.

Efforts continue to discover nontoxic MDR inhibitors. Traditional Chinese medicine, being a natural component, has shown high efficiency and low toxicity in reversing MDR.<sup>17</sup> Shenghe Powder is a combination of Chinese medicines that has been used to treat cancer for many years. Enhancing the reversal reagent's ability to penetrate and accumulate in target cells has also been of special concern in recent years.<sup>5</sup>

This research shows that the reversal effect of SHP is significantly stronger than that of verapamil. The

measurement of expression rates of P-gp and Bcl-2 shows that it can reduce the level of P-gp and Bcl-2 significantly. Its possible mechanisms include (1) stimulating apoptosis of tumor cells by inhibiting expression of Bcl-2, (2) blocking P-gp-stimulated transport of drugs out of the cell, and (3) achieving high concentration in target cells, thus effectively blocking the MDR of SGC-7901/ VCR cells.

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