

# The reversal effect of magnetic $\text{Fe}_3\text{O}_4$ nanoparticles loaded with cisplatin on SKOV3/DDP ovarian carcinoma cells

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**Abstract:** To explore whether the magnetic nanoparticles of  $\text{Fe}_3\text{O}_4$  (MNPs- $\text{Fe}_3\text{O}_4$ ) loaded with cisplatin can reverse the diaminedichloro platinum (DDP) resistance to multidrug resistance of ovarian carcinoma cells and to investigate its mechanisms. The SKOV3/DDP cells were divided into DDP treatment (DDP group), MNPs- $\text{Fe}_3\text{O}_4$  treatment (MNPs- $\text{Fe}_3\text{O}_4$  group), DDP + MNPs- $\text{Fe}_3\text{O}_4$  treatment (DDP + MNPs- $\text{Fe}_3\text{O}_4$  group), and control group. After incubation with those conjugates for 48 h, the cytotoxic effects were measured by MTT assay. Apoptosis and the intracellular DDP concentration were investigated by flow cytometry and inductively coupled plasma atomic emission spectroscopy, respectively. The expression of apoptosis associated gene Bcl-2 mRNA was detected by reverse transcription polymerase chain reaction and the expressions of MDR1, lung resistance-related protein (LRP), and P-glycoprotein (P-gp) genes were studied by Western blot. Our results indicated that the 50% inhibition concentration ( $\text{IC}_{50}$ ) of the MNPs- $\text{Fe}_3\text{O}_4$  loaded with DDP was  $17.4 \mu\text{mol/l}$ , while the  $\text{IC}_{50}$  was  $39.31 \mu\text{mol/l}$  in DDP groups ( $p < 0.05$ ); Apoptosis rates of SKOV3/DDP cells increased more than those of DDP groups. Accumulation of intracellular cisplatin in DDP + MNPs- $\text{Fe}_3\text{O}_4$  groups was higher than those in DDP groups ( $p < 0.05$ ). Moreover, the expression of Bcl-2 mRNA and the protein expressions of MDR1, LRP, and P-gp were decreased when compared with those of DDP groups, respectively. Our results suggest that MNPs- $\text{Fe}_3\text{O}_4$  can reverse the DDP resistance to the ovarian carcinoma cell. The effects may be associated with over-expression of MDR1, LRP, P-gp, and Bcl-2, which can increase the intracellular platinum accumulation and induce the cell apoptosis.

**Keywords:** magnetic nanoparticles of  $\text{Fe}_3\text{O}_4$ , multidrug resistance reversal, SKOV3/DDP, MDR1, LRP, P-gp, Bcl-2

## Introduction

Ovarian cancer is the leading cause of death from gynecologic malignancies and the fourth most common cause of death due to cancer among women. Treatment for ovarian cancer includes maximal cytoreductive surgery followed by combination chemotherapy. Unfortunately, the initial response rate is not durable; the majority will experience disease recurrence. The poor five-year survival rates seen in epithelial ovarian cancer are at least partly attributed to the development of platinum resistance.<sup>1</sup> A major clinical obstacle in cancer therapy is the development of resistance to a multitude of chemotherapeutic agents, a phenomenon called multidrug resistance (MDR). Cisplatin (*cis*-diaminedichloro platinum [CDDP]) is one of the most potent antitumor agents to display high efficiency in the treatment of ovarian and testicular cancer. CDDP exerts its cytotoxicity on ovarian cancer and induces apoptosis.<sup>2</sup> Resistance to CDDP remains a major obstacle for the

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successful treatment of cancer. The mechanisms of CDDP resistance include reduced drug accumulation by changing the profile of uptake/efflux, inactivation of CDDP by increased levels of the intracellular thiols such as glutathione, metallothionein, or other sulfur-containing molecules, increased repair of CDDP adducts, increased tolerance to CDDP adducts, and failure of apoptotic response.

Reduced intracellular CDDP accumulation in resistant cells may ascribe to an inhibition of CDDP uptake, an increase in drug efflux, or both. Multidrug transporter P-glycoprotein (P-gp), which is encoded by the MDR1 gene, is a major organic anion transporter in tissues responsible for the excretion of xenobiotics (both drugs and toxins) by the biliary tract and proximal tubule of the kidney.<sup>3</sup> P-gp functions as transmembrane drug efflux pumps, decreasing intracellular drug accumulation. Lung resistance-related protein (LRP) is another MDR-related protein.

Human ovarian adenocarcinoma cells (SKOV3), detected by high expression of 110-kDa LRP protein and over-expression of P-gp,<sup>4</sup> were incubated in a medium containing DDP to maintain the resistant characteristics. Clinical studies have demonstrated that the increased level of LRP/major vault protein (MVP) may be an important factor contributing to intrinsic CDDP resistance in SKOV3 cells.<sup>5</sup>

Apoptosis is a key determinant of chemosensitivity in ovarian cancer.<sup>6-9</sup> It has been widely accepted that apoptosis is an active gene-directed cellular suicide mechanism and many human genes contribute to the regulation. Among them, Bcl-2 families draw particular attention because it is one of the key factors of the common final pathway involved in the regulation of cell apoptosis. Recent studies showed that over-expression of Bcl-2 caused increased MDR in cancerous cells. In the SKOV3/DDP cell line, over-expression of Bcl-2 was demonstrated but not in SKOV3 cell line alone.<sup>10</sup>

In spite of the mechanism for MDR being unveiled incompletely, avoiding the appearance of drug resistance and modulation of the MDR has been a great challenge to cancer therapy in the laboratory and clinic. Much effort has been extended to efficient cancer therapies. Unfortunately, many approved treatments for ovarian cancer have accumulative toxicities that leave patients more susceptible to adverse events during subsequent lines of therapy. It is generally accepted that any treatment which could increase the effective concentration of an intracellular chemotherapeutic agent should be pursued.<sup>11</sup>

Recently, the application of drug-coated polymer nanospheres and nanoparticles to inhibit related MDR has drawn much attention. As a promising drug delivery system,

magnetic nanoparticles of  $\text{Fe}_3\text{O}_4$  (MNPs- $\text{Fe}_3\text{O}_4$ ) have been studied for some years in our group.<sup>11-12</sup> In our previous studies, we prepared MNPs- $\text{Fe}_3\text{O}_4$  loaded with Adriamycin (ADM) and/or tetrandrine (Tet) to reverse MDR of K562/A02 cells. The cytotoxicity test in vitro revealed that MNPs- $\text{Fe}_3\text{O}_4$  exhibited excellent biocompatibility.<sup>13-14</sup> In previous work by our group,<sup>12</sup> we found that MNPs- $\text{Fe}_3\text{O}_4$  loaded with ADM could enhance the effective accumulation of ADM in K562/A02 cells. It is demonstrated that ADM polymerized with MNPs- $\text{Fe}_3\text{O}_4$  have shown more chemosensitizing activities than those of ADM alone.

In this work, we present an evaluation of the potential of MNPs- $\text{Fe}_3\text{O}_4$  as a candidate agent for treatment of ovarian cancer and to investigate the role of MDR1, LRP, P-gp, and Bcl-2 in intracellular platinum accumulation and cell apoptosis.

## Materials and methods

### Cell line and cell culture

The MDR human ovarian cancer cell line, SKOV3/DDP, was obtained from the Chinese Academy of Medical Sciences and Peking Union Medical College. The SKOV3/DDP cell line was cultured in a flask in RPMI-1640 containing 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA), 2  $\mu\text{mol/l}$  l-glutamine, 100 U/ml penicillin, and 100  $\mu\text{g/ml}$  streptomycin (Gibco BRL, Grand Island, NY) at 37 °C, 5%  $\text{CO}_2$  with high humidity. For the maintenance of the MDR phenotype, 2  $\mu\text{g/ml}$  cisplatin (Sigma Aldrich, St. Louis, MO) was added to the medium, which was passaged every 2-3 days using 0.05% trypsin (Gibco BRL) and 0.01% EDTA (Sigma Aldrich).

### Preparation of drug-loaded nanoparticles

The synthesis of MNPs- $\text{Fe}_3\text{O}_4$  was prepared by electrochemical deposition under oxidizing conditions (EDOC) based on our previous studies.<sup>11-12</sup> Before application in the present experiment, the magnetic nanoparticles were well distributed in RPMI-1640 medium (Sigma Aldrich) with 10% heated inactivated FBS freshly added by using ultrasound treatment in order to obtain MNPs- $\text{Fe}_3\text{O}_4$  colloidal suspension. CDDP conjugated with MNPs- $\text{Fe}_3\text{O}_4$  (DDP + MNPs- $\text{Fe}_3\text{O}_4$ ) was prepared by mechanical absorption polymerization as previously reported.<sup>15</sup> The temperature effect was also investigated and a temperature of 37 °C or 4 °C was chosen for our polymerization process. Briefly, different concentrations (V/V) of 25  $\mu\text{g/ml}$  MNPs- $\text{Fe}_3\text{O}_4$  were respectively added under mechanical stirring to 200  $\mu\text{l}$  of an aqueous medium with 20  $\mu\text{mol/l}$  DDP that, in the final nanoparticle and cell suspension, was 50  $\mu\text{mol/l}$  (pH = 7.4). At different temperatures

(37 °C, 4 °C), the overall polymerization process lasted for 24 h. The RPMI-1640 medium was regarded as blank control and cells with no intervention were negative control.

## MTT assay

For cell growth and viability assays,  $5 \times 10^4$ /ml cells were plated into Six-well flat-bottomed plates (Costar, Charlotte, NC), respectively. Different concentrations of DDP, MNPs-Fe<sub>3</sub>O<sub>4</sub>, and MNPs-Fe<sub>3</sub>O<sub>4</sub> loaded with DDP or RPMI-1640 were added into these cells and cultured to measure their growth and viability. Based on these results, different concentrations of DDP and MNPs-Fe<sub>3</sub>O<sub>4</sub> were combined to find the best combination concentration which can best kill tumor cells. After incubation for 48 h, 20 µl MTT solution (5 mg/ml) was added into each well at 37 °C in the dark for at least 4 h. Formazan crystals were solubilized in 200 µl dimethyl sulfoxide (DMSO) in every well and the reduction of MTT was quantified by absorbance at 540 nm using a plate reader (Model 550; Bio-Rad, Tokyo, Japan). The inhibition ratio of cells was determined as follows  $(1 - A \text{ of test cells} / A \text{ of black control}) \times 100\%$ . Each assay was repeated at least three times.

## Annexin V–PI assays for apoptosis

As described before, cells were incubated and harvested. For Annexin V–propidium iodide (PI) assays, cells were stained and evaluated for apoptosis by flow cytometry according to the manufacturer's protocol. Briefly,  $1 \times 10^6$  cells were stained with 5 µl Annexin V–fluorescein isothiocyanate (FITC) and 10 µl PI (5 µg/ml) in  $1 \times$  binding buffer (1.0 mmol/L HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], pH = 7.4, 140 mmol/L NaOH, 2.5 mmol/L CaCl<sub>2</sub>) for 20 min at room temperature in the dark. The apoptotic cells were determined using flow cytometry (FACSCalibur™; Becton-Dickinson, Franklin Lakes, NJ).

## Measurement of intracellular DDP accumulation

Briefly, cells ( $5 \times 10^4$ /ml) were incubated with DDP, MNPs-Fe<sub>3</sub>O<sub>4</sub>, MNPs-Fe<sub>3</sub>O<sub>4</sub> loaded with DDP or RPMI-1640 medium for 48 h at 37 °C in humidified 5% CO<sub>2</sub> atmosphere. After incubation, an aliquot was taken, washed twice with 2 mol/l iced-cold isotonic buffer, the pellet was resuspended in 33% HNO<sub>3</sub>, and then the DDP content was determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES) in SPECTRO GENESIS SOP (SPECTRO Analytical Instruments, Marble Falls, TX) equipment.

## Reverse transcription polymerase chain reaction

As described before, cells seeded at a density of  $5 \times 10^4$  cells/well in 6-well plates were treated with one of the following treatments: (a) DDP + MNPs-Fe<sub>3</sub>O<sub>4</sub>, (b) DDP, (c) MNPs-Fe<sub>3</sub>O<sub>4</sub>, (d) RPMI-1640 for 48 h, and harvested. Cells were dissolved in TRIzol reagent (Gibco BRL). Total RNA was extracted according to the manufacturer's instructions. The RNA A<sub>260</sub>/A<sub>280</sub> ratios were between 1.6 and 1.8. The primers for human Bcl-2 (forward: 5'-GGGAGAACAGGGTAC GATAA-3'; reverse: 5'-CCACCGAACT CAAAGAAGG-3'), and β-actin (forward: 5'-TATGACTTAGTTGC GTTACACC-3'; reverse: 5'-CCTTCAC CGTTCAGTTT-3') were used. The amplified polymerase chain reaction (PCR) products were 452 bp and 155 bp, respectively. The copy number for each sample was calculated and all the data were normalized to β-actin. Briefly, cDNA was synthesized from 1 µg of total cellular RNA using TaKaRa RNA PCR kit (AMV) (Ver. 3.0; Dalian, China). The newly synthesized cDNA was amplified by PCR (TaKaRa). The PCR conditions were 95 °C for 3 min and 35 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min. Control amplifications were conducted either without reverse transcription (RT) or without RNA. Following PCR amplification, the reaction products were electrophoresed at 100 V on 1.5% agarose gels with 0.5 µg/ml ethidium bromide (Sigma Aldrich)<sup>16</sup> and PCR fragments were visualized by UV illumination (GDS7500 Gel; UVP Inc., Upland, CA, USA). Densitometric analysis was performed using the electrophoresis image analysis system Smart View 2000 software (Furi, Shanghai, China).

## Western blot

SKOV3/DDP cells were treated with DDP, MNPs-Fe<sub>3</sub>O<sub>4</sub>, DDP + MNPs-Fe<sub>3</sub>O<sub>4</sub>, and RPMI-1640 medium for 48 h, respectively. Whole cell extracts were harvested on ice, washed in phosphate-buffered saline (PBS), and lysed in 100 µl of lysis buffer (30 mM Tris, pH 7.5, 150 mM NaCl, 1 mM PMSF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1% Nonidet P-40, and 10% glycerol) for 30 min at 48 °C, then centrifuged at 14,000 r/min for 10 min. The supernatant was collected and the amount of protein was measured using Bio-Rad protein assay (Bio-Rad, Hercules, CA). Equal amount (25 µg) of protein from each sample was separated in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels using modified radio immunoprecipitation assay (RIPA) buffer and transferred the proteins to a nitrocellulose membrane (Bio-Rad). Western blotting was performed with a 1:500–1:1,000 dilutions of monoclonal antibodies against

either anti-human P-gp (NeoMarkers, Fremont, CA), MDR1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), LRP (Santa Cruz Biotechnology Inc.) or  $\beta$ -action anti-body (Santa Cruz Biotechnology Inc.) in 5% nonfat dry milk, followed by peroxidase-conjugated IgG-HRP (Santa Cruz Biotechnology Inc.) as a secondary antibody (peroxidase-conjugated swine anti-rabbit). The blots were developed by enhanced chemiluminescence (ECL system, Amersham, UK).

## Statistical analysis

All data were presented as means  $\pm$  standard deviation in triplicate. All analyses were performed with Statistical Package for Social Science (SPSS Release 11.5; SPSS Inc., IL, USA). Differences were evaluated using Student's *t*-test or paired *t*-test and considered statistically significant for values of  $p < 0.05$ .

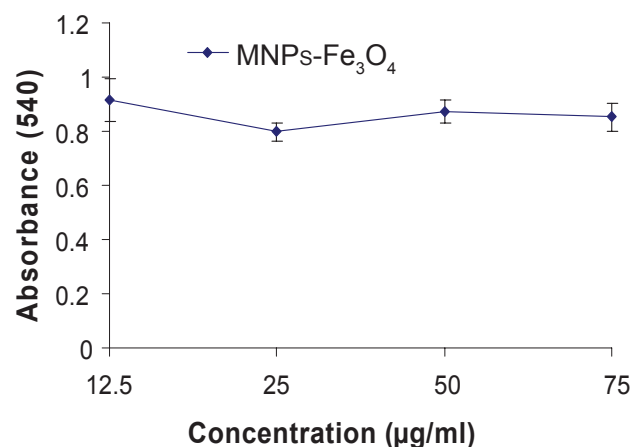
## Results

### Cell growth and inhibition

The MTT assay revealed that the growth and inhibition of DDP, MNPs-Fe<sub>3</sub>O<sub>4</sub>, or DDP + MNPs-Fe<sub>3</sub>O<sub>4</sub> to SKOV3/DDP cells (as shown in Figures 1, 2). It was indicated that MNPs-Fe<sub>3</sub>O<sub>4</sub> alone could hardly inhibit SKOV3/DDP cell proliferation when their doses were between 12.5  $\mu$ g to 75  $\mu$ g ( $p > 0.05$ ) (Figure 1). SKOV3/DDP cells were significantly resistant to DDP when the concentration of DDP was not less than 10  $\mu$ mol/l. The inhibition of SKOV3/DDP cells was in a dose-dependent manner and the 50% inhibition concentration (IC<sub>50</sub>) was 39.31  $\mu$ mol/l. Furthermore, we found the optimum concentration of MNPs-Fe<sub>3</sub>O<sub>4</sub> was 25  $\mu$ g/ml from a different concentration of MNPs-Fe<sub>3</sub>O<sub>4</sub> loaded with a different concentration of DDP. The inhibition of SKOV3/DDP cells in MNPs-Fe<sub>3</sub>O<sub>4</sub> loaded with DDP group was significantly higher than that in DDP group and the IC<sub>50</sub> of MNPs-Fe<sub>3</sub>O<sub>4</sub> loaded with DDP was 17.4  $\mu$ mol/l, which was 2.259-fold lower than that in the DDP groups ( $p < 0.05$ ), suggesting that the reverse rate was 2.259 (Figure 2).

### Annexin V-PI assays for apoptosis

Annexin V-PI double-staining assays demonstrated that MNPs-Fe<sub>3</sub>O<sub>4</sub> could hardly induce apoptosis of SKOV3/DDP cells compared to control group ( $p > 0.05$ ). Only (4.4%  $\pm$  0.36%) apoptosis of SKOV3/DDP cells were observed under MNPs-Fe<sub>3</sub>O<sub>4</sub>. When DDP was loaded with MNPs-Fe<sub>3</sub>O<sub>4</sub>, apoptosis rates of SKOV3/DDP cells in the DDP+MNPs-Fe<sub>3</sub>O<sub>4</sub> group was significantly higher than that in DDP group alone ( $p < 0.05$ ). The apoptosis rates were (42.07%  $\pm$  5.55%) and



**Figure 1** Growth inhibition rates of SKOV3/DDP cells incubating with different concentrations of MNPs-Fe<sub>3</sub>O<sub>4</sub> for 48 h by MTT assay.

**Note:**  $p > 0.05$ , when the concentration of MNPs-Fe<sub>3</sub>O<sub>4</sub> is less than 75  $\mu$ g (signal factor analysis of variance).

(26.93%  $\pm$  4.11%), respectively. There were all statistically significant when compared with (6.83%  $\pm$  0.64%) in the control group ( $p < 0.05$ ) (Figure 3).

### Intracellular DDP concentration

The intracellular DDP concentration in cells was explored by ICP-AES analysis when the SKOV3/DDP cells were incubated for 48 h. The intracellular DDP concentration of SKOV3/DDP cells in DDP with or without MNPs-Fe<sub>3</sub>O<sub>4</sub> groups was founded to increase by (0.057  $\pm$  0.003  $\mu$ mol/l) and (0.074  $\pm$  0.006  $\mu$ mol/l), respectively. There were statistically significant differences between them when compared ( $p < 0.05$ ) (Table 1).

### RT-PCR for Bcl-2 mRNA

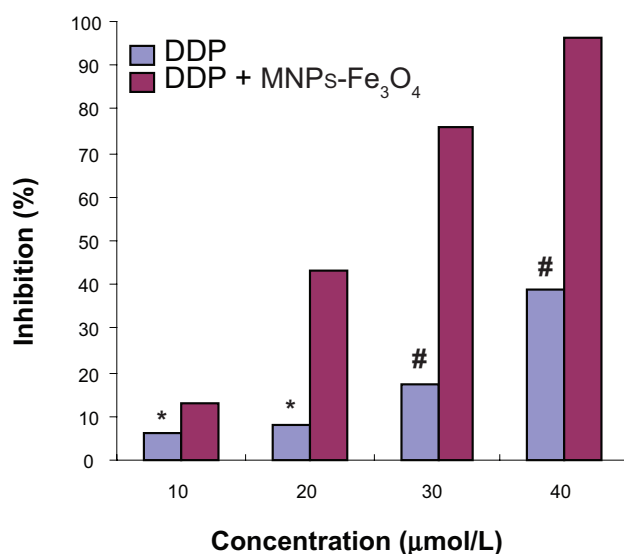
Based on computer-assisted image analysis, it appeared that single use of MNPs-Fe<sub>3</sub>O<sub>4</sub> had no obvious effect on Bcl-2 mRNA in SKOV3/DDP cells ( $p > 0.05$ ), but DDP + MNPs-Fe<sub>3</sub>O<sub>4</sub> reinforced downregulation of Bcl-2 mRNA content significantly ( $p < 0.05$ ), causing a 70-fold more decrease in Bcl-2 mRNA level and single use of DDP had less downregulation than the same concentration

**Table 1** Intracellular accumulation of DDP in SKOV3/DDP cells incubating with DDP or MNPs-Fe<sub>3</sub>O<sub>4</sub> for 48 h

Group	Intracellular DDP ( $\mu$ mol/l)
DDP	0.057 $\pm$ 0.003
DDP + MNPs-Fe <sub>3</sub> O <sub>4</sub>	0.074 $\pm$ 0.006*

**Note:** \* $p < 0.05$ , compared with DPP (signal factor analysis of variance).





**Figure 2** Growth inhibition rates of SKOV3/DDP cells incubating with different concentrations of DDP and DDP + MNPs-Fe<sub>3</sub>O<sub>4</sub> for 48 h by MTT assay, respectively.

**Notes:**  $p < 0.05$ , compared to DDP without MNPs-Fe<sub>3</sub>O<sub>4</sub> treated-SKOV3/DDP cells (signal factor analysis of variance);  $*p > 0.05$ , 10 μmol/l DDP compared to 20 μmol/l DDP (signal factor analysis of variance);  $*p < 0.05$ , when the concentration of DDP is not less than 20 μmol/l (signal factor analysis of variance).

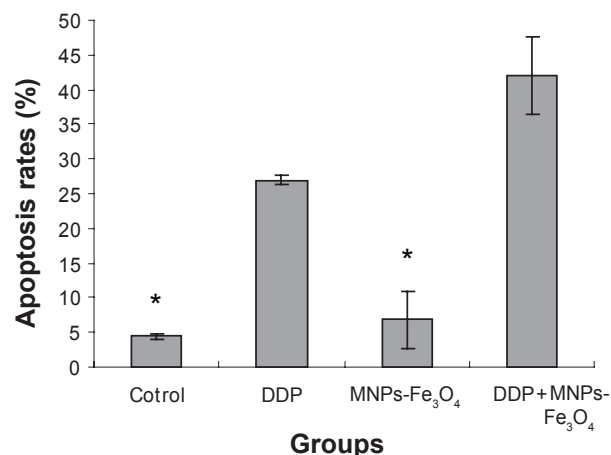
of DDP polymerized with MNPs-Fe<sub>3</sub>O<sub>4</sub> ( $p < 0.05$ ), suggesting that MNPs-Fe<sub>3</sub>O<sub>4</sub> can enhance the action of DDP in downregulation of Bcl-2 mRNA in SKOV3/DDP cells (Figure 4).

## Western blot

In order to examine the expression of the P-gp, LRP, and MDR1, we next performed Western blot analysis on whole cell protein extracts from cells treated for 48 h as described previously (Figure 5). Based on computer-assisted image analysis, it appeared that MNPs-Fe<sub>3</sub>O<sub>4</sub> themselves could not lower the protein of P-gp, LRP, and MDR1 in SKOV3/DDP cells ( $p > 0.05$ ), but they could downregulate the expression of P-gp, LRP, and MDR1 protein levels when loaded with DDP. We also found that the expressions of protein in DDP + MNPs-Fe<sub>3</sub>O<sub>4</sub> group were lower than those in DDP-alone groups ( $p < 0.05$ ), suggesting that MNPs-Fe<sub>3</sub>O<sub>4</sub> can enhance the accumulation of DDP in SKOV3/DDP cells.

## Discussion

It is well known that intrinsic and/or acquired chemoresistance is the major obstacle for successful treatment of patients with ovarian carcinoma.<sup>17</sup> A low five-year overall survival rate of only 53% for woman suffering from ovarian cancer is related to the development of resistance of tumor cells to



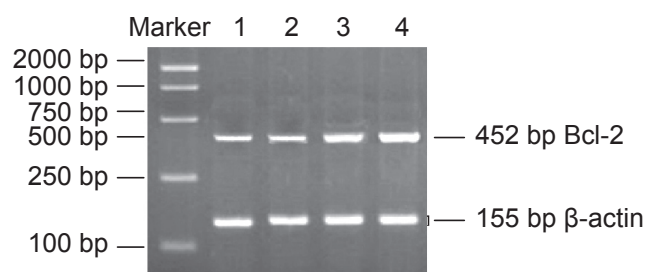
**Figure 3** Apoptosis of SKOV3/DDP cells incubating with 20 μmol/l DDP or DDP + 25 μg/ml MNPs-Fe<sub>3</sub>O<sub>4</sub> and Fe<sub>3</sub>O<sub>4</sub>-MNPs alone.

**Notes:**  $p < 0.05$ , compared to control with or without MNPs-Fe<sub>3</sub>O<sub>4</sub>-treated SKOV3/DDP cells (signal factor analysis of variance);  $*p > 0.05$ , MNPs-Fe<sub>3</sub>O<sub>4</sub> alone compared to control (signal factor analysis of variance).

standard chemotherapeutic agents.<sup>18–19</sup> Cisplatin was the first platinum-containing compound introduced into therapeutic trials for ovarian cancer. Resistance to cisplatin-based chemotherapy is a major cause of treatment failure in human ovarian cancer. Both intrinsic and acquired resistance to cisplatin occurs frequently.

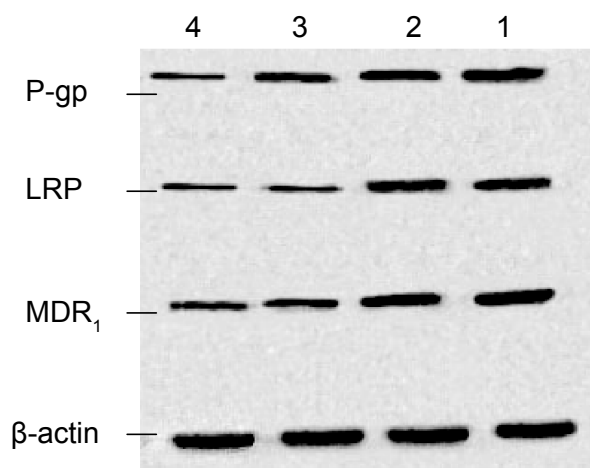
Many studies have shown that the development of drug resistance of cancer cells is related to inhibition of cell apoptosis. Moreover, the apoptotic rate increased with the concentration of cisplatin, suggesting that cisplatin induced apoptosis in a dose-dependent fashion. So, the first step to successful reversal drug resistance is to increase cisplatin concentrations in the ovarian cancer cell. Nanotechnology and nanoscience have developed rapidly during the last few decades. The most important clinical application of nanotechnology is probably in pharmaceutical development.<sup>20</sup> As demonstrated previously and as observed in this study, the intracellular cisplatin levels in DDP + MNPs-Fe<sub>3</sub>O<sub>4</sub> groups were found to be higher than those of DDP-alone groups, suggesting that MNPs-Fe<sub>3</sub>O<sub>4</sub> can increase cisplatin concentration in SKOV3/DDP cells and enhance the effective accumulation of anticancer agents in resistant cancer cells. We also found that the apoptosis rates of SKOV3/DDP cells were increased than those of DDP groups. All of these suggested that MNPs-Fe<sub>3</sub>O<sub>4</sub> could reverse the DDP resistance to the ovarian carcinoma cell (SKOV3/DDP), and the effects are ascribed to increase the intracellular DDP accumulation, and to induce apoptosis, which were demonstrated by flow cytometry.

As one of the most commonly used magnetic nanoparticles, MNPs-Fe<sub>3</sub>O<sub>4</sub> are obtained more easily than other MNPs



**Figure 4** The expression of Bcl-2 mRNA in SKOV3/DDP cells by different interferences for 48 h. Lane 4 was the negative control group; lane 3 was the MNPs-Fe<sub>3</sub>O<sub>4</sub> group; lane 2 was the DDP group; and lane 1 was the DDP + MNPs-Fe<sub>3</sub>O<sub>4</sub> group.

and they may aggregate in water or tissue fluid spontaneously with good biocompatibility and low toxicity,<sup>21–23</sup> which suggests MNPs-Fe<sub>3</sub>O<sub>4</sub> were safe for the use of drug carriers. Moreover, MNPs-Fe<sub>3</sub>O<sub>4</sub> are found readily to interact with proteins.<sup>24</sup> The mechanism of MNPs-Fe<sub>3</sub>O<sub>4</sub> in increasing the effective intracellular concentration of DDP is still unclear. Nanoparticles loaded with an anticancer drug could readily approach the cell membrane, leading to drug concentrations at the cell surface higher than those obtained with the same amount of drug diluted in a culture medium, leading in turn to higher intracellular drug concentration.<sup>25–26</sup> Not only have MNPs-Fe<sub>3</sub>O<sub>4</sub> the ability to block P-gp function, but they also have potency in aggregation and drug-capsulation.<sup>27–28</sup> The expression of P-gp and MDR-related proteins are associated with a poor prognosis in patients with ovarian cancer. P-gp and MDR protein (MRP) are known to be associated with MDR. P-gp, an integral membrane glycoprotein with a molecular mass of 170 kd, has been postulated to function as a pump to remove hydrophobic anticancer agents from drug-resistant cells. MRP, a 190-kd 1531-amino-acid membrane glycoprotein, is over-expressed in most non-P-gp-mediated multidrug resistant cell lines. MRP and P-gp are members of the adenosine triphosphate (ATP)-binding cassette super-family of membrane transporter proteins. It is generally accepted that increased levels of P-gp expression have been observed in some tumors at the time of relapse after initial chemotherapy. The 110-kd LRP frequently is over-expressed in multidrug resistant cells. For occurrence of MDR, a key role was P-gp, which effluxes chemotherapeutic agents out through an ATP-dependent transport leading to intracellular deficient drug concentrations.<sup>29</sup> Zhang and colleagues demonstrated that the SKOV3 cell line did not express P-gp, but over-expressed LRP/MRP. However, the SKOV3/DDP cell line has been proved to be resistant to



**Figure 5** The protein of P-gp, LRP, and MDR1 in SKOV3/DDP cells by different interferences for 48 h. Lane 1 was the negative control group; lane 2 was the MNPs-Fe<sub>3</sub>O<sub>4</sub> group; lane 3 was the DDP group; and lane 4 was the DDP+MNPs-Fe<sub>3</sub>O<sub>4</sub> group. Blots were stripped and re-probed with β-actin to indicate relative amounts of protein loaded.

CDDP and is characterized with high expression of P-gp, MRR1, and LRP.<sup>30,31</sup> It was also demonstrated that down-regulation of MDR1 gene expression or inhibiting the function of MDR1 has a reversal effect on SKOV3/DDP cells, which was resistant to DDP.<sup>32,31</sup> Our study demonstrated that MNPs-Fe<sub>3</sub>O<sub>4</sub> themselves could not lower the protein of P-gp, LRP, and MDR1 in SKOV3/DDP cells ( $p > 0.05$ ), but they could downregulate the expression of P-gp, LRP, and MDR1 protein levels when loaded with DDP. The expressions of proteins in the DDP + MNPs-Fe<sub>3</sub>O<sub>4</sub> group were also found to be lower than those in DDP-alone groups, suggesting that the mechanism of increasing the DDP concentration in SKOV3/DDP cell line may be associated with the downregulation of expression of MDR1 gene, MRP1, and LRP.

To further evaluate the role of Bcl-2 family proteins in reversal effect of MNPs-Fe<sub>3</sub>O<sub>4</sub> loaded with cisplatin on ovarian carcinoma cells, studies were performed using SKOV3 as model ovarian cancer cell lines. The Bcl-2 family proteins represent another class of key regulators of cell death and survival. Recent studies have shown that therapeutic agents may target proteins of the Bcl-2 family, affect mitochondrial outer membrane permeabilization, and initiate an apoptotic cell death.<sup>33</sup> The dormant period of cell increased and enhanced expression of Fas, Fas-L, and Bcl-2 protein may be the partial mechanisms of the cisplatin-resistance of SKOV3/DDP cells. Li and colleagues found that expression of Bcl-2 protein in SKOV3/DDP cells was higher than that in SKOV3 cells.<sup>16</sup> A more pronounced

difference of Bcl-2 expression and over-expressing of Bcl-2 were not insensitive to chemotherapy and may have been observed in SKOV3/DDP cells had the time been lengthened as was observed in other studies.<sup>16</sup> In contrast, in our study, there was only a slight effect on Bcl-2 mRNA in the enhanced group, but DDP + MNPs-Fe<sub>3</sub>O<sub>4</sub> reinforced downregulation of Bcl-2 mRNA content significantly ( $p < 0.05$ ), causing a 70-fold decrease in Bcl-2 mRNA level and single use of DDP had less downregulation than the same concentration of DDP polymerized with MNPs-Fe<sub>3</sub>O<sub>4</sub> ( $p < 0.05$ ), suggesting that MNPs-Fe<sub>3</sub>O<sub>4</sub> can enhance the action of DDP in downregulation of Bcl-2 mRNA in SKOV3/DDP cells.

## Conclusions

All these results suggest that MNPs-Fe<sub>3</sub>O<sub>4</sub> can reverse DDP resistance to the ovarian carcinoma cell, and the mechanism of chemoresistance may be associated with the downregulation of MDR1, P-gp, and LRP gene expression, which revealed a potential application for reversal of MDR in human ovarian cancer cells. The results presented here warrant further investigation in animal tumor models and eventually in cancer patients.

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