Silibinin Restores Paclitaxel Sensitivity to Paclitaxel-resistant Human Ovarian Carcinoma Cells

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Abstract. *Background: Drug resistance and tumor metastasis are the main causes of treatment failure and mortality in cancer patients. Silibinin, a naturally occurring flavanone, has been shown to be a potent sensitizer for apoptosis induced by a variety of anticancer drugs. In this study, whether silibinin could overcome chemoresistance and reduce the invasiveness of A2780/taxol cells was investigated. Materials and Methods: A2780 and A2780/taxol cells were treated with silibinin alone and in combination with paclitaxel. Cell viability was determined by MTT assay while apoptosis and cell cycle progression were assessed by flow cytometric analysis. Matrigel invasion assays assessed the invasive activity. Protein and mRNA levels influenced by the treatment were studied by Western blots and quantitative real-time PCR. Results: Silibinin enhanced the sensitivity of A2780/taxol cells to paclitaxel, increased paclitaxel-induced apoptosis and G_2/M arrest consistent with the down-regulation of survivin and P-glycoproteins. A2780/taxol cells demonstrated a two-fold increase in invasiveness ability compared to A2780 cells, whereas the invasive potential was reduced dramatically by silibinin. Conclusion: These results suggest silibinin in combination with paclitaxel may be a beneficial chemotherapeutic strategy, especially in patients with tumors refractory to paclitaxel alone.*

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Key Words: Silibinin, paclitaxel, drug resistance, invasion, P-glycoprotein, survivin.

Taxol (paclitaxel) is one of the most effective chemotherapeutic drugs to date, used clinically for the treatment of many solid tumors. However, its clinical efficacy has been hampered due to the development of drug resistance. A common mechanism of resistance to paclitaxel is the overexpression of plasma membrane drug efflux proteins called P-glycoproteins (P-gp) that can expel chemotherapeutic agents from the cancer cell, thus decreasing their effectiveness (1, 2). However, inhibition of these pumps does not always reverse established clinical resistance to paclitaxel (3); P-gp mediated drug efflux is unlikely to be the only cause of paclitaxel resistance.

Growing evidence has indicated that survivin expression plays an essential role in drug resistance and that genetic or pharmacological modulation of survivin expression affects drug effectiveness in apoptosis induction (4, 5). Survivin is a bifunctional protein that suppresses apoptosis and regulates cell division during mitosis *via* the α-helix coiled-coil domain binding to mitotic spindle microtubules (6). It is likely that this protein can specifically contribute to the response of cells to microtubule-interacting agents, *e.g.* paclitaxel. Forced expression of survivin counteracts the apoptosis induced by paclitaxel (7, 8). Approaches designed to inhibit survivin expression may lead to sensitization of human tumors to paclitaxel.

Drug resistance and tumor metastasis are the main causes of treatment failure and mortality in cancer patients. Drug resistance concomitant with invasion/metastasis has been found in patient samples (9, 10). That cells have these properties is due either to selection of more aggressive cells or to an increase in metastatic potential following chemotherapeutic insults (11). Thus, there is an urgent need for novel treatment strategies to overcome drug resistance and tumor metastasis.

Silibinin, a flavonoid antioxidant from milk thistle (*Silybum marianum* L.), has been used extensively for many years for the treatment of liver conditions (12). It recently

Table I. *Primers used for PCR*.

Gene	Sequences of primers		Size (bp)*	GeneBank No.
MDR-1	Antisense	TCTGGAGGAAGACATGACCAG GTA		
	Sense	GGCACCAAAATGAAACC TGAATGT	96	NM000927.3
MMP-2	Antisense	CAGCCTAGCCAGTCGGATTTGA		
	Sense	GCGGCGGTCACAGCTACTTCT	106	NM004530.2
MMP-9	Antisense	CATAGGTCACGTAGCCCACTTT		
	Sense	CACGACGTCTTCCAGTACCGAA	115	NM004994.2
Survivin	Antisense	CTTTCTCAACGACCACCG		
	Sense	GTAGGTGACGGGGTGAC	110	NM001168.2
β-Actin	Antisense	TTGCCGACAGGATGCAGAA	101	NM001101.2
	Sense	GCCGATCCACACGGAGTACT		

*Size of amplified fragment.

received attention due to its chemopreventive and anticancer activity (13, 14). *In vitro* and *in vivo* studies have shown that silibinin is a potent sensitizer for apoptosis induced by a range of anticancer drugs (15, 16). In this study, we evaluate the effect of silibinin in increasing sensitivity of ovarian cancer cells to paclitaxel and reducing their invasive ability, and define the molecular mechanisms involved.

Materials and Methods

Cell line and reagents. The human ovarian carcinoma line A2780 was purchased from the Chinese Center for Type Culture Collection (CCTCC, Wuhan, ROC). The ovarian carcinoma paclitaxel-resistant line A2780/taxol was kindly provided by Dr. Zhang Wei (Guangxi Institute for Cancer Research, Nanning, ROC). All cell culture products were from Gibco (Grand Island, NY, USA). Paclitaxel (Taxol) was supplied by Bristol-Myers Squibb (Princeton, NJ, USA). Silibinin from Sigma Chemical (St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO; Sigma Chemical). The amount of DMSO in the experiments never exceeded 0.1%, and accordingly, the same amount of DMSO was added to control cultures. 3,4,5-Dimethylthiazol-2,5 diphenyl tetrazolium bromide (MTT) was purchased from Sigma Chemical. Anti-survivin antibody was from Cell Signaling Technology (Beverly, MA, USA). Anti-P-gp, MMP-9, MMP-2 and anti-actin primary and peroxidase-conjugated secondary antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The enhanced chemiluminescence (ECL) detection system was from Amersham Life Science Incorporated (Arlington Heights, IL, USA).

Cytotoxicity assay using MTT. A2780 (paclitaxel-sensitive) and A2780/taxol (paclitaxel-resistant) cells grew in RPMI-1640 (supplemented with 10% fetal bovine serum, FBS) and were plated on 96-well plates at a density of $5x10^3$ cells per well. At 70% to 80% confluence, cells were treated with DMSO alone (0.1% v/v; control), different doses of silibinin alone (25-200 μM), paclitaxel alone (1-10000 nM), or silibinin and paclitaxel in combination. After 72 h of these treatments, the number of viable cells was determined using the MTT dye as described elsewhere (17). IC_{50} (concentration resulting in 50% inhibition of cell growth) values for drugs were calculated relative to results using untreated cells (plotted as 100%).

Flow cytometric apoptosis assay and cell cycle analysis. A2780/taxol cells at a density of $5x10^5$/ml in exponential growth were exposed to 100 nM paclitaxel alone, 100 μM silibinin alone or both in combination for 24 h. Then the cells were collected and fixed in 70% ethanol at –20˚C. Cells were re-suspended in phosphate-buffered saline (PBS) containing 0.25 mg/ml RNase A (Sigma Chemical) at 37˚C for 30 min then stained with 100 μg/ml of propidium iodide (Sigma Chemical) for 30 min in the dark. Samples were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). Data acquisition and analysis were controlled by Modifit software (Becton Dickinson, San Jose, CA, USA).

Cell invasion assays. Matrigel-coated filter inserts (8 μm pore size) that fit into 24-well invasion chambers were obtained from Becton-Dickinson (Franklin Lakes, NJ, USA). Fresh media with 10% FBS was added to the lower compartment of the invasion chamber. A2780/taxol cells were added to the upper compartment of the invasion chamber in the presence or absence of drugs. After 24 h incubation, the migratory cells were counted in five random fields per insert under a microscope at x20 magnification.

Quantitative RT-PCR analysis. Following treatment of A2780/taxol cells with 100 nM paclitaxel or /and 50 μM, 100 μM silibinin for 72 h, total RNA was extracted from cells by the Trizol reagent (Invitrogen, Carlsbad, CA, USA) based on the supplier's protocol. Reverse transcription was performed at 37˚C for 1 h, 95˚C for 5 min using the MLV Kit (Invitrogen). Quantitative RT-PCR (QRT-PCR) was performed using SYBR green with TaqMan assay (Applied Biosystems, Foster City, CA, USA) on a Light Cycler (Roche Diagnostics, Nutley, NJ, USA). Specific primers for survivin, MDR-1, MMP-2, MMP-9 and β-actin were generated by Primer 3.0 software (Applied Biosystems). Table I shows the sequences used. The PCR cycling conditions were as follows: The cycling conditions comprised a denaturation step for 10 min at 95˚C, followed by 40 cycles of denaturation (94˚C for 15 s), annealing (60˚C for 30 s) and extension (72˚C for 30 s). The data were analyzed with Light Cycle software 4.0 (Roche Diagnostics).

Protein extraction and Western blot analysis. A2780/taxol cells were exposed to 100 nM paclitaxel or /and 50 μM, 100 μM silibinin for 72 h. At the end of the treatment period, cells were

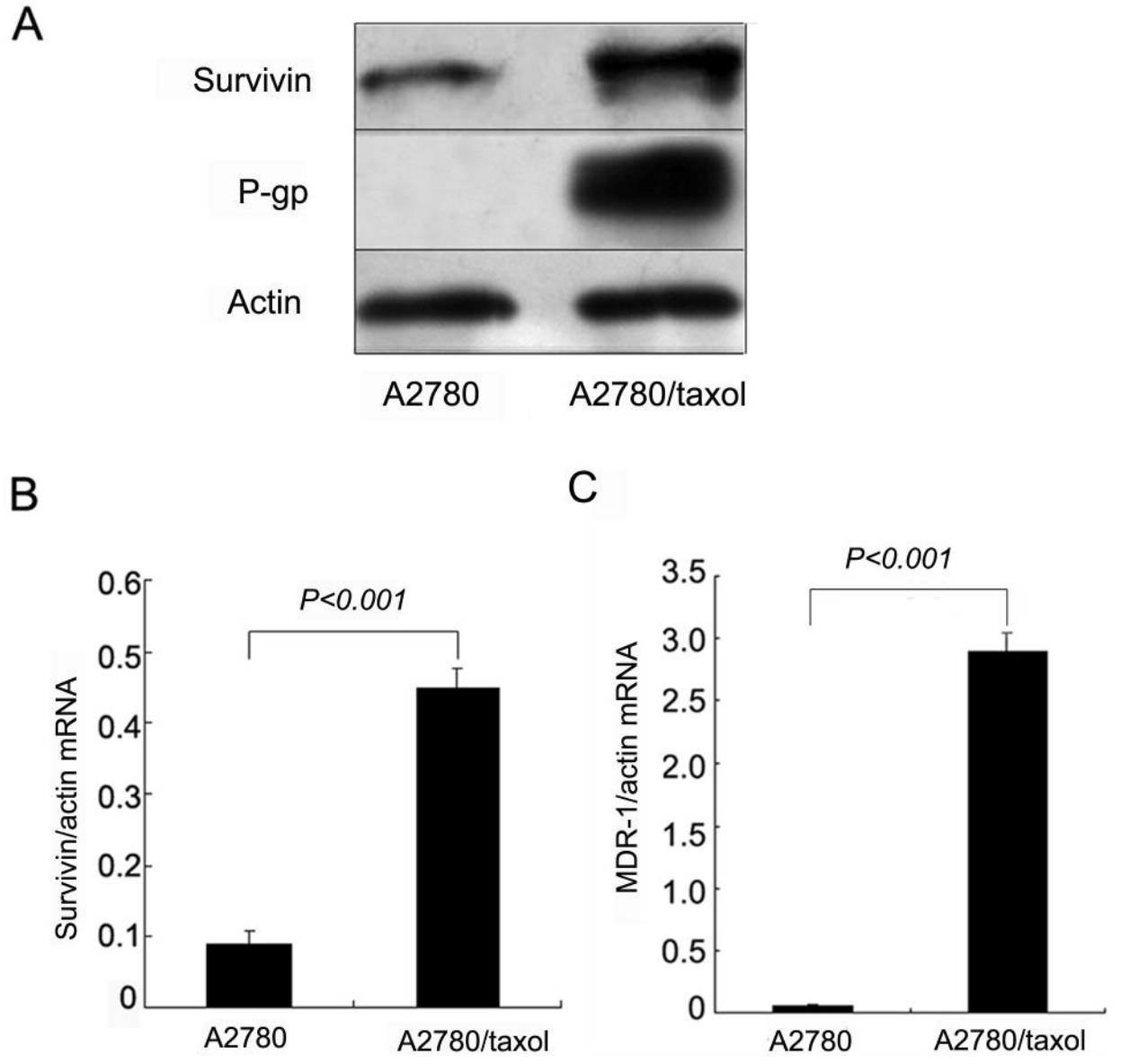

Figure 1. *Expression of survivin and MDR-1 in A2780 and A2789/taxol cells. A, Expression of survivin and P-glycoproteins (P-gp) protein was measured by Western blotting. B and C, Expression of survivin and MDR-1mRNA were measured by QRT-PCR. Values represented as bar graphs are mean of 3 experiments ±SD.*

harvested at 4˚C and lysed on ice in extraction buffer containing Tris-HCl (20 mM, pH 7.5), NaCl (150 mM), EDTA (1 mM), Triton X-100 (1%), sodium deoxycholate (0.5%) plus phenylmethyl sulfonylfluoride (PMSF, 1 mM), leupeptin (10 μg/ml) and aprotinin (30 μg/ml). Total protein (40 μg lysate per lane) was separated by 14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. Transferred membranes were blocked for 1 h in 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST). Membranes were then probed with specific primary antibodies followed by peroxidase-conjugated secondary antibody and visualized by the ECL detection system.

Statistical analysis. All statistical analysis was performed using the SPSS 11.5 software package for Windows (SPSS, Chicago, IL, USA). The results were expressed as means±SD. Student's two-tailed *t*-test was used to compare data between two groups. All experiments were performed at least three times. A p-value <0.05 was considered statistically significant.

Results

The expression of multidrug resistance-1 (MDR-1) and survivin in human ovarian carcinoma paclitaxel resistant cells (A2780/taxol). The expression of P-gp and survivin was

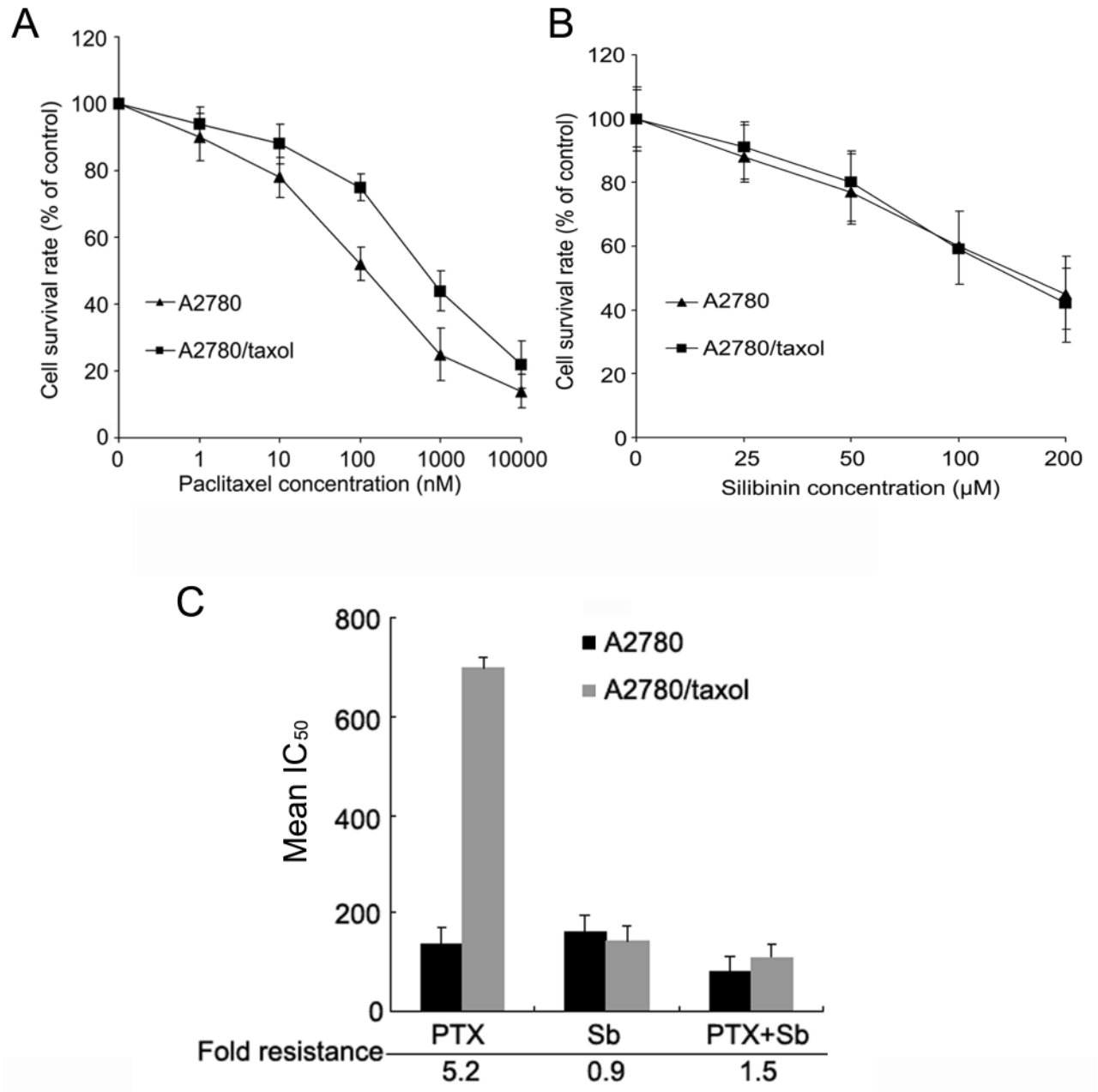

Figure 2. *Cytotoxicity of paclitaxel (PTX) and silibinin (Sb) in A2780 and A2789/taxol cells. A, Paclitaxel cytotoxicity in A2780 and A2780/taxol cells. B, Silibinin cytotoxicity in A2780 and A2780/taxol cells. C, Mean IC_{50} values for paclitaxel and silibinin in A2780 and A2780/taxol cells (nM for paclitaxel and μM for silibinin). The cell survival was determined by the MTT assay as described in the Materials and Methods. Values represented as bar graphs were means of 3 experiments ±SD.*

examined in A2780/taxol and A2780 cells by immunoblot analyses. As shown in Figure 1A, A2780/taxol cells expressed a higher level of survivin protein than did A2780 cells. P-gp was detected in A2780/taxol cells, but not in A2780 cells. We also found a significantly increasd expression of survivin (Figure 1B) and MDR-1(Figure 1C) mRNA in A2780/taxol cells by QRT-PCR ($p<0.001$).

Resistance of A2780/taxol cells to paclitaxel. Cells were treated with different concentrations of paclitaxel for 72 h and cell viability was measured by the MTT assay. As shown in Figure 2A and Figure 2C. A2780/taxol cells were resistant to paclitaxel-induced apoptosis in that the IC_{50} of paclitaxel was 5.2-fold higher in these cells compared to their parent cell line.

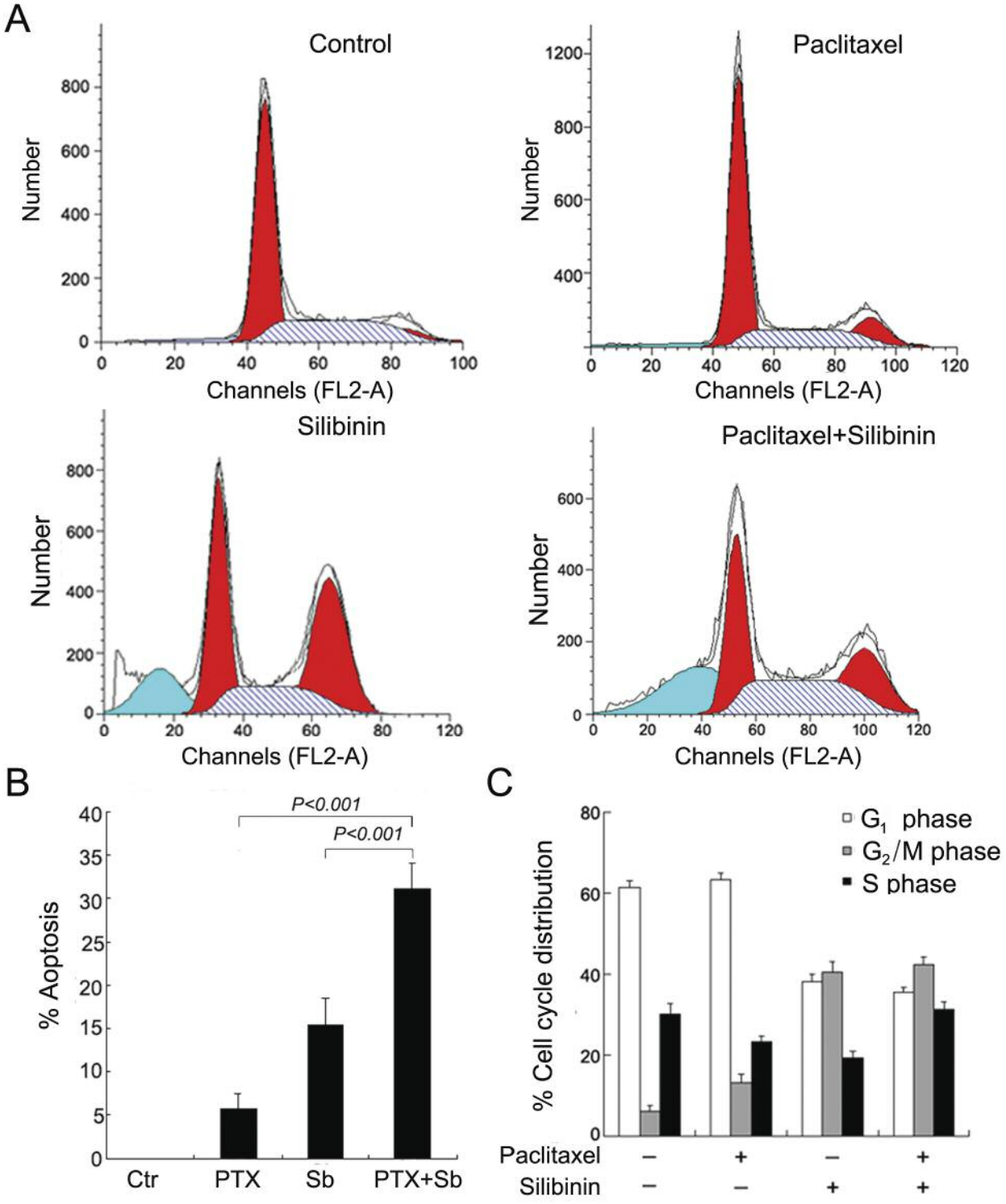

Figure 3. *Effects of silibinin on paclitaxel-induced apoptosis and cell cycle perturbations in A2780/taxol cells. A, Fluorescence-activated cell sorting analysis of A2780/taxol cells exposed to 100 nM paclitaxel and/or 100 μM/L silibinin for 24 h. B, Percentage of apoptotic cells in response to silibinin (100 μM), paclitaxel (100 nM), or their combination evaluated by FCM after 24 h exposure. C, Perturbations of A2780/taxol cells in each cell cycle phase, after exposure to 100 nM paclitaxel and/or 100 μM/L silibinin for 24 h. Data acquisition and analysis were controlled by Modifit software. Based on PI staining, cells in the sub-G_1 marker window were considered to be apoptotic. Values represented as bar graphs were means of 3 experiments ± SD.*

Table II. *Effect of different concentrations of silibinin on the sensitivity of A2780 and A2780/taxol cells to paclitaxel (Values represented Means±SD).*

Silibinin (μM)	Paclitaxel IC_{50} (nM)	
	A2780	A2780/taxol
0	138.15±18.12	699.06±23.58
25	126.23±16.45	541.68±30.65
50	118.61±21.98	387.42±19.36
100	93.19±17.53	206.89±25.71
200	78.18±15.13	117.33±21.82

IC_{50}: concentration resulting in 50% inhibition of cell growth.

Silibinin in paclitaxel sensitivity to paclitaxe-resistant ovarian carcinoma cells. To investigate whether silibinin was active against the resistant cells, the cytotoxicity of silibinin and combination of silibinin with paclitaxel in both A2780 and A2780/taxol cells was determined. The results showed that silibinin inhibited cell growth in a dose-dependent manner, accounting for up to 55% and 58% inhibition in A2780 and A2780/taxol cells respectively (Figure 2B); no significant difference in the mean IC_{50} values was obtained between the A2780 and A2780/taxol cells however (Figure 2C). Notably, the silibinin /paclitaxel combination resulted in a dramatic reversal of paclitaxel resistance in A2780/taxol cells. As shown in Table II, the IC_{50} for paclitaxel decreased with increasing concentrations of silibinin up to 200 μM. Silibinin reduced the IC_{50} of paclitaxel from 138.15±18.12 to 78.18±15.13 nM in A2780 cells, whereas it reduced the IC_{50} of paclitaxel from 699.06±23.58 to 117.33±21.82 nM in A2780/taxol cells. A2780/taxol cells exhibited a 5.2-fold resistance to paclitaxel, but were only 1.5-fold resistant to paclitaxel when the silibinin/paclitaxel combination was used (Figure 2C).

Effect of silibinin/paclitaxel combination on apoptosis and the cell cycle in A2780/taxol cells. To further confirm that the silibinin /paclitaxel combination resulted in reversal of paclitaxel resistance in A2780/taxol cells, the effects of this combination on apoptosis and mitotic arrest were assayed using flow cytometric (FCM) analysis. As shown in Figure 3, the exposure of A2780/taxol cells to 100 nM Paclitaxel alone for 48 h demonstrated their resistance to paclitaxel-induced apoptosis and G_2/M arrest. Treatment of cells with silibininn alone (100 μM) caused both G_1 and G_2/M arrests. However, the silibinin/paclitaxel combination significantly increased cell apoptosis more than either of the two drugs alone ($p<0.001$). The reduction of the proportion of cells in the G_1-phase and the accumulation of cells in the G_2/M-phase was also observed after the cells were exposed to 100 nM paclitaxel in the presence of 100 μM silibinin.

Effect of silibinin/paclitaxel combination on survivin and P-gp expression in A2780/taxol cells. To investigate the mechanism of silibinin sensitivity to paclitaxel resistant in A2780/taxol cells, the effects of different concentrations of silibinin on survivin and P-gp expression in A2780/taxol cells were evaluated. As shown in Figure 4, silibinin resulted in reduced survivin and P-gp expression in a dose-dependent manner in A2780/taxol cells.

Effect of silibinin on invasion ability. To determine whether silibinin can influence invasiveness of paclitaxel-resistant cells, A2780/taxol and A2780 cells were subject to Matrigel invasion assays. As can be seen in Figure 5A, A2780/taxol cells demonstrated a 2-fold greater in invasiveness as compared to the A2780 cells. In contrast, A2780/taxol cells treated with silibinin, alone or in combination with paclitaxel exhibited greater than 50% reduction in invasiveness as compared to untreated A2780/taxol cells.

Effect of silibinin on expression of MMP-9 and MMP-2 in A2780/taxol cells. MMPs play an important role in the invasion of cancer cells. In this study, the effects of silibinin on MMP-2 and MMP-9 expression in A2780/taxol cells were investigated. We found that silibinin significantly suppressed MMP-2 and MMP-9 expression (Figure 5B-D).

Discussion

In this study, we found that silibinin increased sensitivity of ovarian carcinoma cells to paclitaxel and overcame paclitaxel resistance by suppressing the expression of survivin and P-gp. Furthermore, drug-resistant cells demonstrated an increased invasiveness, which was reduced by treatment with silibinin consistent with the down- regulation of MMP-2 and MMP-9.

The emergence of chemoresistance is a major obstacle in treatments using paclitaxel. One strategy for reversing chemoresistance is the use of chemopreventive agents alongside standard chemotherapeutic protocols. Silibinin has been demonstrated to be an efficacious chemopreventive and chemotherapeutic agent (18, 19), without any toxic or adverse effects (20-22). Therefore, it should have potential clinical application in combination with chemotherapy. Our results demonstrate that silibinin resulted in a dramatic reversal of paclitaxel resistance and enhanced paclitaxel-induced apoptosis and G_2/M arrest in A2780/taxol cells.

To explore the mechanism of silibinin in overcoming paclitaxel resistance, we studied whether P-gp was involved in this effect. P-gp is an ATP-dependent drug pump and a common mechanism of resistance to paclitaxel (1). In our experiment, we found that P-gp was detected in A2780/taxol cells, but was absent from A2780 cells. Silibinin suppressed P-gp expression in A2780/taxol cells in a drug concentration -dependent manner, confirming the findings of other studies

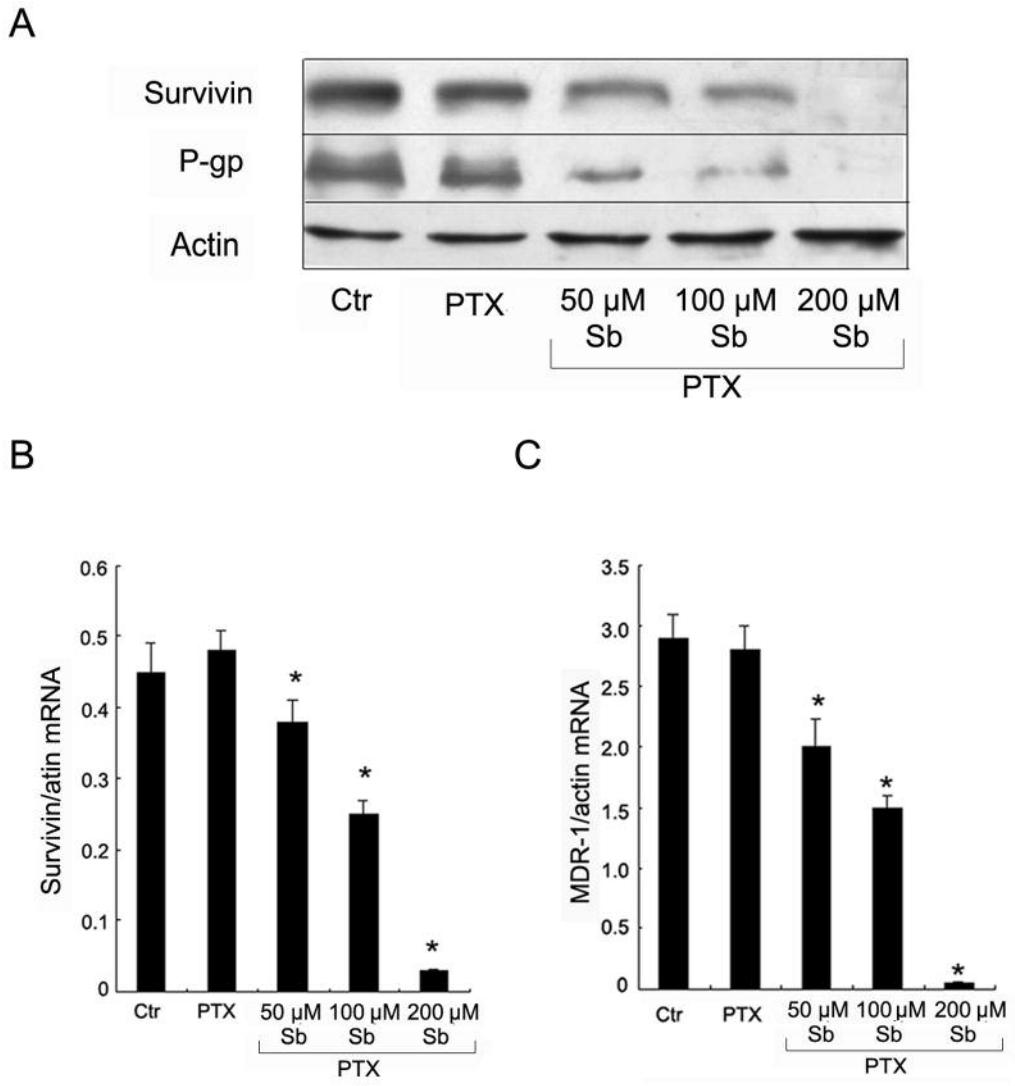

Figure 4. *Co-treatment with paclitaxel (PTX) and silibinin (Sb) caused greater attenuation of survivin and MDR-1 in A2780/taxol cells. Following treatment of A2780/taxol cells with 100 nM paclitaxel and/or 50 μM, 100 μM, and 200 μM silibinin for 48 h, A, Western blotting analyses of survivin and P-gp were performed on the cell lysates (the levels of β-actin served as the loading control); B and C, quantitative real-time PCR analyses of survivin and MDR-1 mRNA expression were carried out. Values represented as bar graphs were means of 3 experiments ± SD. * Statistically significant different from the control at p<0.001.*

that silibinin increases intracytoplasmic chemotherapeutic drug concentrations in P-gp-positive cells by inhibiting P-gp function (23, 24). Thereby, silibinin increased accumulation of chemotherapeutics in A2780/taxol cells (P-gp-positive cells) and raised the sensitivity of the cells to paclitaxel.

Survivin plays a great role in paclitaxel-induced microtubular stabilization and mitotic arrest (6, 7). Overexpression of survivin correlates with paclitaxel resistance (8). Although the molecular pathogenesis of survivin-mediated paclitaxel resistance is uncertain, proposed mechanisms have included: i) the suppression of caspase-mediated apoptosis, ii) an interaction with Smac/DIABLO, and iii) the stabilization of microtubules (25-27). In our experiment, we found that there was an overexpression of survivin in A2780/taxol cells in contrast to A2780 cells. However, silibinin reduced survivin expression and increased paclitaxel sensitivity. This suggests that silibinin enhances sensitivity to paclitaxel of ovarian carcinoma cells by down-regulating survivin expression.

In addition, tumor cells selected for drug resistance were more invasive than their parental cells (28). We observed drug resistant cells (A2780/taxol) to be more invasive than their parental cells; one might propose increased P-gp or survivin expression as part of the mechanism of increased aggressiveness. Silibinin reduced

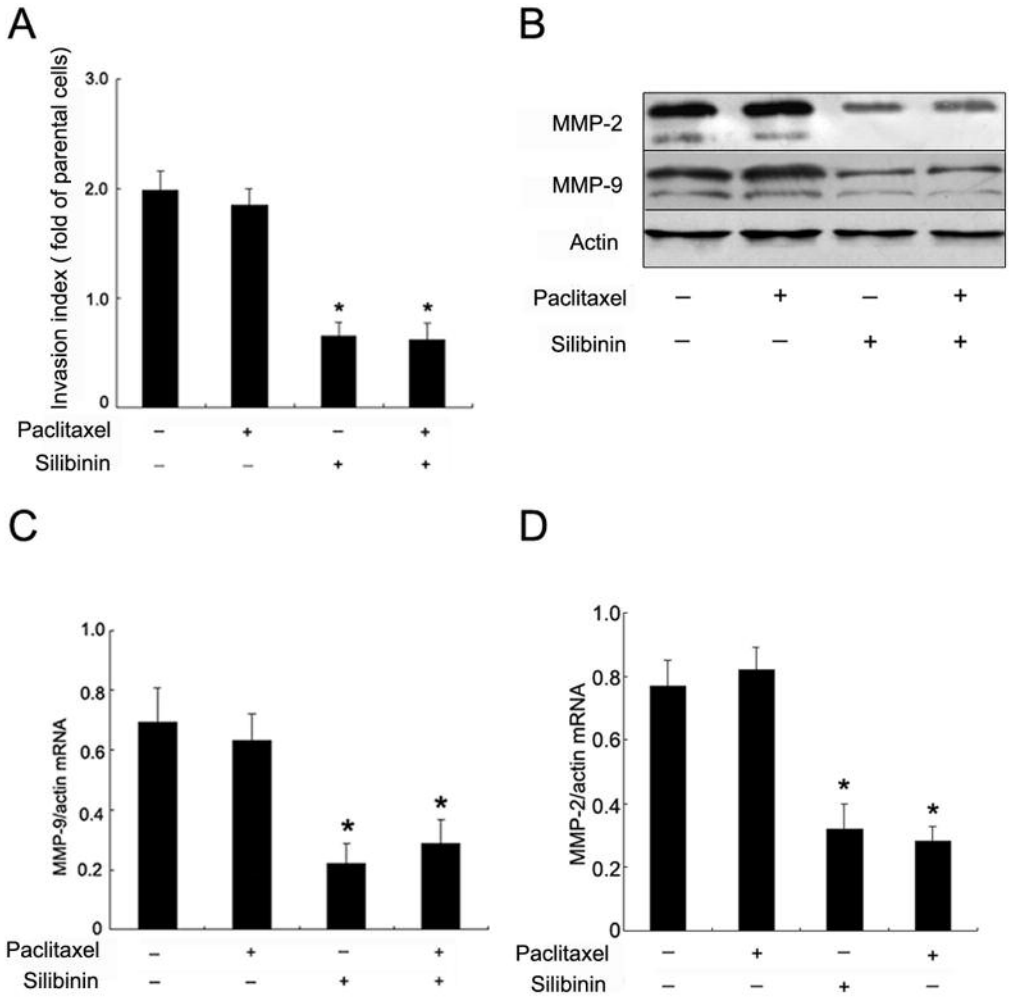

Figure 5. *Effects of silibinin on invasiveness and expression of MMP-2 and MMP-9 in A2780/taxol cells. A, Invasiveness of A2780/taxol cells compared to parental cells of A2780/taxol after different treatments: paclitaxel 100 nM alone, silibinin 100 μM alone or silibinin/paclitaxel in combination. Values represented as bar graphs were means of 3 experiments ±SD. B, Effects of silibinin on MMP-2 and MMP-9 protein expression as measured by Western blotting (the levels of β-actin served as the loading control). C and D, Effects of silibinin on MMP-2 and MMP-9 mRNA expression measured by QRT-PCR. Values represented as bar graphs were means of 3 experiments ±SD. * Statistically significant different from the control at p<0.001.*

invasiveness and the expression of two metalloproteinase proteins known to be associated with invasion. These results indicate that tumor cells exhibit drug resistance and potential invasive abilities in order to survive chemotherapy. Silibinin interferes with increased survival and tumor progression after drug exposure.

In conclusion, our study demonstrated that silibinin overcomed paclitaxel resistance, and reduced tumor invasiveness of paclitaxel-resistant cancer cells *in vitro*. If these effects are confirmed *in vivo*, silibinin in combination with paclitaxel may be a beneficial chemotherapeutic strategy, especially in patients with tumors refractory to paclitaxel alone.

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Received December 5, 2007
Revised January 29, 2008
Accepted February 11, 2008