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Sinomenine reverses multidrug resistance in bladder cancer cells *via* P-glycoprotein-dependent and independent manners

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P-Glycoprotein-mediated multidrug resistance is a frequent event during chemotherapy and a key obstacle for bladder cancer therapy. Search for strategies to reverse multidrug resistance is a promising approach to improve the management of bladder cancer. In the present study, we reported a novel P-glycoprotein-mediated multidrug resistant cell model 253J/DOX, which was generated from human bladder cancer 253J cell line. Furthermore, we found that the multidrug resistant phenotype of 253J/DOX cells could be overcome by sinomenine, an alkaloid derived from the stem of *Sinomenium acutum*. Mechanistically, the chemosensitive effect by sinomenine was mediated by down-regulating P-glycoprotein expression, as well as triggering apoptotic pathways. The chemosensitive effect of sinomenine may make it a prime candidate agent to target bladder cancer.

1. Introduction

Bladder cancer is a common malignant disease of the human genitourinary system. 70% of patient presents with superficial tumours associated high tendency to recur after surgical resection. Intravesical chemotherapy has been shown to significantly reduce the frequency of tumour recurrence. The other 30% present with muscle-invasive disease associated with a high risk of death from distant metastases. For these patients, systemic chemotherapy treatment remains an important therapeutic approach to improve quality of life and to improve survival (Kaufman et al. 2009). However, accumulated evidence indicates that drug-resistant cancer cells are selected and developed during chemotherapy. These drug-resistant cells are probably the main sources of recurrent tumors emerging after chemotherapy. Selection of cancer cells with one chemotherapeutic drug usually results in cross-resistance to other structurally and mechanistically unrelated drugs. This phenomenon is known as multidrug resistance (MDR). The acquisition of MDR could be mediated by different ways including increased drug efflux, reduced drug uptake, detoxifying systems and defective apoptotic pathways (Gottesman et al. 2002). P-Glycoprotein (P-gp), a ATP-dependent transporter encoded by multidrug resistance gene 1 (MDR1, also called ABCB1), plays a vital role in resistance to natural-product hydrophobic drugs such as anthracyclines, Vinca alkaloids and taxanes (Luqmani, 2005). P-gp-mediated drug efflux confers classical MDR by reduction of intracellular drug concentrations and predicts poor outcomes after intravesical or systemic chemotherapy in bladder cancer (Hasegawa et al. 1995; Hoffmann et al. 2010; Serretta et al. 2003). Thus, reduction of P-gp expression in bladder cancer cells might help to reverse chemo-resistance.

Sinomenium acutum is a Chinese herbal plant traditionally used for the treatment of various diseases for hundreds of years (Zhao et al. 2012). Sinomenine (7,8-didehydro-4-hydroxy-3,7-dimethoxy-17-methylmorphinane-6-one, SIN), an alkaloid with pharmacological effects of anti-inflammation, anti-angiogenesis, anti-arthritis and immunosuppression, is derived from the stem of *Sinomenium acutum* (Li et al. 2006). Li et al. (2006) firstly reported that SIN inhibited a list of genes involving inflammation, cell adhesion, proliferation, apoptosis and angiogenesis in human synovial sarcoma cells, suggesting a possible anti-neoplastic effect. In the past five years, a promising anti-cancer effect of SIN in human lung, gastric and hepatocellular cancers through inhibiting cell proliferation and inducing apoptosis was demonstrated (Jiang et al. 2010; Lu et al. 2013; Lv et al. 2011; Zhou et al. 2012). In the present study, we report a novel P-gp-mediated multidrug resistant cell model derived from human bladder cancer 253J cell line, and the reversal effect of SIN on MDR phenotype and the underlying mechanisms.

2. Investigations and results

2.1. *Multidrug resistant phenotype and P-gp expression of 253J/DOX*

To indentify the drug resistant characteristics of 253J/DOX cells, we compared the chemosensitivity of 253J/DOX and its parental 253J cells to different chemotherapeutic drugs. As shown in Table 1, 253J/DOX cells were significantly resistant to DOX, THP and VCR, but not resistant to DDP. This drug resistant phenotype is accorded with the ABC transporter P-gp mediated classical multidrug resistance, which mainly leads to resistance

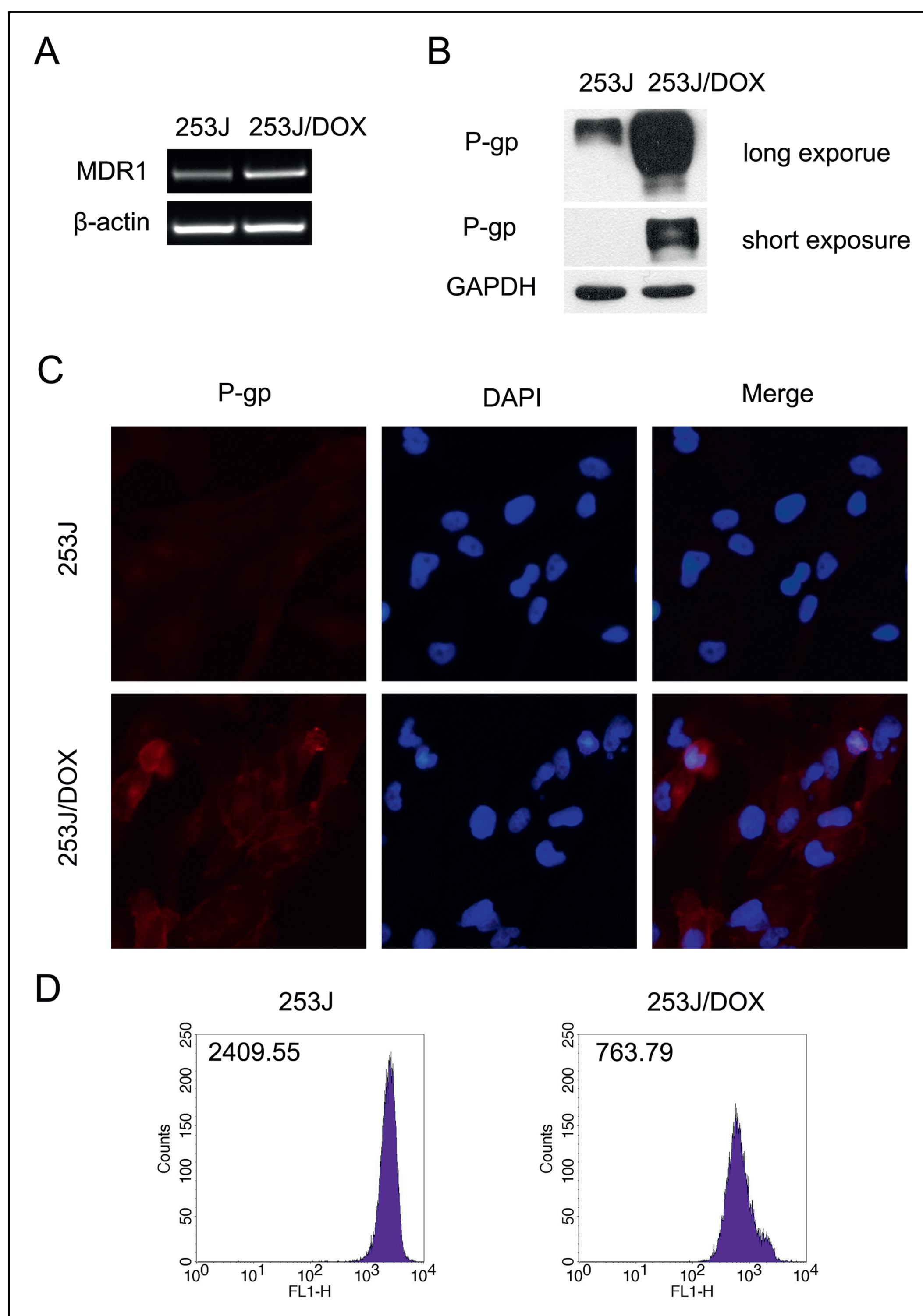

Fig. 1: P-gp expression in 253J and 253J/DOX cells. A. RT-PCR analysis of MDR1 mRNA expression in 253J and 253J/DOX cells. B. Western blot analysis of P-gp expression in 253J and 253J/DOX cells. C. Immunofluorescence staining analysis of P-gp expression in 253J and 253J/DOX cells. D. Comparison of P-gp-mediated efflux in 253J and 253J/DOX cells by Rhodamine 123 retention assay.

to anthracyclines, Vinca alkaloids and taxanes, but not to cisplatin. Next we compared the expression of MDR1 mRNA and P-gp protein in 253J/DOX and its parental 253J cells using RT-PCR and western blot. The results showed that both MDR1 mRNA and P-gp protein were up-regulated in 253J/DOX cells (Fig. 1A, B). This finding was also confirmed by immunofluo-

Table 1: IC50 (μg/ml) analyses of DOX, THP, VCR and DDP in 253J and 253J/DOX cells

	DOX	THP	VCR	DDP
253J	78.31 ± 5.121	0.03 ± 0.006	0.89 ± 0.009	3.70 ± 0.522
253J/DOX	15629.34 ± 832.870*	1.03 ± 0.071*	31.15 ± 2.125*	4.08 ± 0.235

* $P < 0.05$ vs 253J

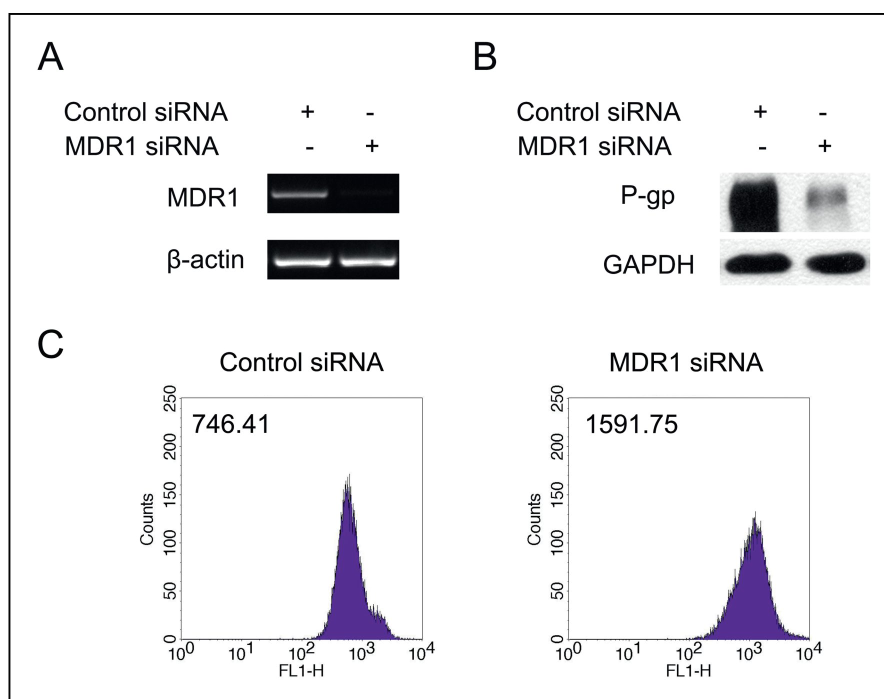

Fig. 2: Efficiency of MDR1-siRNA in 253J/DOX cells. 253J/DOX cells were transfected with control siRNA or MDR1 siRNA. After 48 h, cells were used for analysis. A. RT-PCR analysis of MDR1 mRNA expression in 253J/DOX cells transfected with control siRNA or MDR1 siRNA. B. Western blot analysis of P-gp expression in 253J/DOX cells transfected with control siRNA or MDR1 siRNA. C. Rhodamine 123 retention assay of P-gp-mediated efflux in 253J/DOX cells transfected with control siRNA or MDR1 siRNA.

rescence staining, which showed increased P-gp expression in 253J/DOX cells, which mainly located in cellular membrane (Fig 1C). In addition, we confirmed the ability of 253J/DOX cells to extrude Rhodamine 123 dye by analyzing mean fluorescence intensity as determined by flow cytometry. As seen in Fig. 1D, Rhodamine 123 mediated fluorescence intensity from 253J/DOX was significantly less than that from 253J cells (763.79 vs 2409.55), indicating increased P-gp-mediated efflux in 253J/DOX cells.

2.2. *Chemosensitization after knockdown of P-gp in 253J/DOX cells*

To further determine whether the multidrug resistant phenotype in 253J/DOX cells is mediated by P-gp, we employed RNAi strategy to knock down the expression of P-gp in 253J/DOX cells. As shown in Fig 2A and B, MDR1-siRNA significantly decreased the expression of MDR1 mRNA and P-gp protein in 253J/DOX 48 h after transfection. More importantly, treatment with MDR1-siRNA also inhibited Rhodamine 123 efflux (746.41 vs 1591.75, Fig. 2C) and significantly chemosensitized 253J/DOX cells to DOX, THP and VCR ($P < 0.05$, Table 2).

2.3. *Overcoming multidrug resistance of 253J/DOX cells by SIN*

In order to determine whether SIN exerts reversal effects on multidrug resistant phenotype, DOX (10 μg/ml), THP (0.1 μg/ml) were used alone or combined with SIN (100 or 200 μg/ml) to treat 253J/DOX cells. After 48 h of treatment, cell viability was detected by MTT assay and the inhibitory rate was evaluated. As shown in Fig. 3, SIN exerted a synergetic effect with conventional chemotherapeutic drugs and overcoming the multidrug resistant phenotype of 253J/DOX cells.

2.4. *Downregulation of P-gp expression by SIN*

To determine whether P-gp expression is involved in the reversal effect of SIN on multidrug resistant phenotype, P-gp expression in 253J/DOX cells treated with different concentrations of SIN was detected by western blot. We found that treatment with SIN down-regulated P-gp expression in a concentration-dependent manner (Fig. 4A, B). In addition, treatment with SIN consistently inhibited Rhodamine 123 efflux (656.48 *vs* 1312.62, Fig. 4C), indicating impaired P-gp-mediated efflux after SIN treatment.

2.5. *Induction of apoptosis by SIN*

In addition to inhibition of ABC transporters, induction of apoptosis is another strategy to reverse multidrug resistance. It is demonstrated that induction of apoptosis such as changing the balance of Bcl-2 family members, could also effectively chemosensitize cancer cells to anticancer drugs (Wu et al. 2011). To investigate whether SIN is able to overcome multidrug resistance through induction of apoptosis, we evaluated the effect of

Table 2: Effects of MDR1 siRNA on the IC50 (μg/ml) of DOX, THP and VCR in 253J/DOX cells

	DOX	THP	VCR
Control siRNA	13331.31 ± 97.209	0.98 ± 0.122	28.78 ± 3.046
MDR1 siRNA	4005.76 ± 182.870*	0.43 ± 0.095*	11.51 ± 2.482*

* $P < 0.05$ vs control siRNA

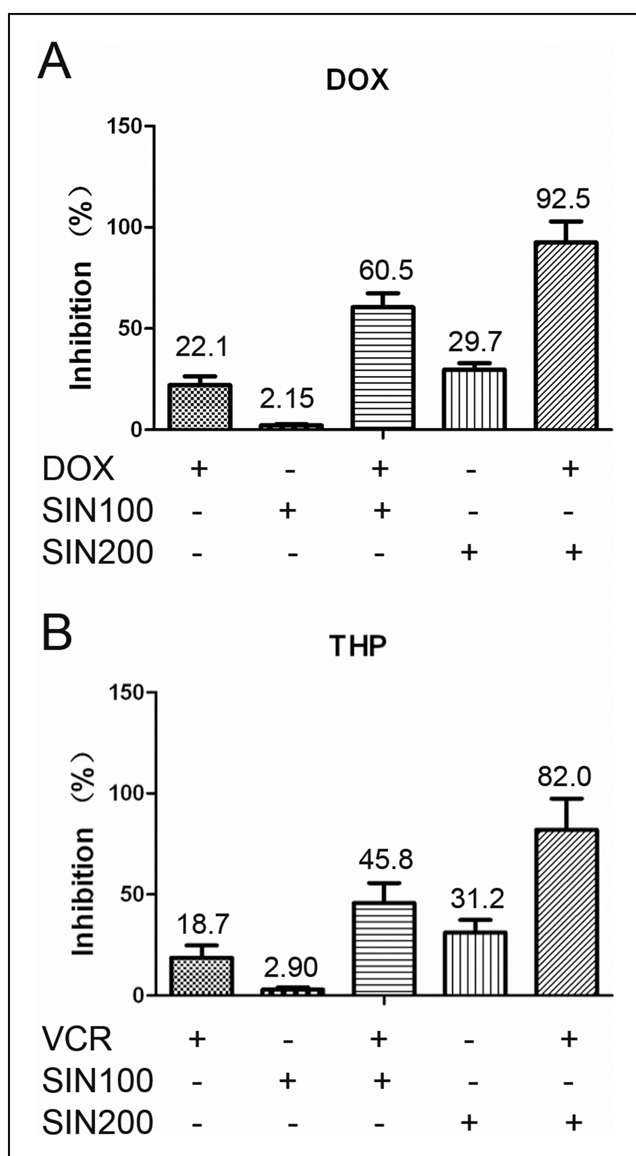

Fig. 3: Overcoming multidrug resistance of 253J/DOX cells by SIN. DOX (10 μg/ml), THP (0.1 μg/ml) were used alone or combined with SIN (100 or 200 μg/ml) to treat 253J/DOX cells. After 48 h of treatment, cell viability was detected by MTT assay and the inhibitory rate was evaluated.

SIN on apoptosis in 253J/DOX cells. MTT assay and annexin V/PI staining indicated that SIN inhibited cell viability and induced apoptosis respectively (Fig. 5A, B). This was consistent with the following western blot experiment, which showed an increased cleavage of PARP, a substrate of caspases (Fig. 5C). Finally, we found that treatment with SIN induced pro-apoptotic protein Bax expression in 253J/DOX cells. Thus, we conclude that SIN could induce apoptosis and reverse multidrug resistance in 253J/DOX cells besides decreasing P-gp expression and increasing drug efflux.

3. Discussion

It is well known that P-gp-mediated MDR is a frequent event and is one of the obstacles for cancer therapy. As a stress-inducible gene, the expression of MDR1 can be induced by several types of stimuli, including P-gp substrate drugs, non-P-gp substrate drugs and ultraviolet light radiation. Selection of cancer cells in culture by chemotherapeutic drugs such as doxorubicin, vincristine and cisplatin often induces P-gp over-expression and results in MDR (Balayssac et al. 2009; Hu et al. 2000; Li et al. 2009). In bladder cancer, the expression of MDR1 mRNA in recurrent and residual tumors after doxorubicin chemotherapy was higher than that in untreated primary tumors (Tada et al. 2002). The aberrant expression of P-gp in bladder cancer is correlated with drug resistance and predicts poor outcomes (Hoffmann et al. 2010; Rioja et al. 2011). In the present study, we exposed human bladder cancer 253J cells to DOX *in vitro* and obtained a novel multidrug resistant subline 253J/DOX, which was significantly resistant to P-gp substrate DOX, THP and VCR, but not to non-P-gp substrate DDP. Downregulation of increased P-gp expression in 253J/DOX cells by RNAi chemosensitized cells to drugs, suggesting that the multidrug resistant phenotype in 253J/DOX cells is mediated by P-gp.

SIN is an alkaloid with pharmacological effects of anti-inflammation, anti-angiogenesis, anti-arthritis and immunosuppression (Li et al. 2006). However, recent evidence indicated that it is a potential anti-neoplastic agent. It was demonstrated that SIN inhibited cell proliferation and induced apoptosis in human lung, gastric and hepatocellular cancers (Jiang et al. 2010; Lu et al. 2013; Lv et al. 2011; Zhou et al. 2012). The effect of SIN and other compounds extracted from *Sinomenium acutum* on chemosensitivity to paclitaxel was examined in human uterine sarcoma MES-SA/DR5cell line and human colorectal cancer HCT15 cell line (Min et al. 2006). The results showed that SIN did not change chemosensitivity of cancer cells to paclitaxel. However, in the present study, we observed a significant synergetic effect of SIN with conventional anti-cancer drugs and a reversal effect of SIN on MDR in bladder cancer 253J/DOX cells.

To further investigate the mechanisms of the reversal effect of SIN on MDR in 253J/DOX cells, we evaluated the role of SIN on P-gp expression and P-gp-mediated Rhodamine 123 efflux. Our findings revealed down-regulated P-gp expression in 253J/DOX cells and decreased Rhodamine 123 efflux after treatment with SIN. We concluded that down-regulation of P-gp expression might be at least in part responsible for reversal effect of SIN on MDR in 253J/DOX cells.

Recent evidence revealed that induction of apoptosis in MDR cells could also efficiently reverse MDR (Wei et al. 2011; Wu et al. 2011). Because SIN has been reported to induce apoptosis in several types of cancer cells, we hypothesized that SIN may reverse MDR *via* triggering apoptotic pathways. In the present study, we found that treatment with SIN induced apoptosis in 253J/DOX cells and induced expression of pro-apoptotic protein Bax. Thus, we conclude that SIN could reverse MDR in 253J/DOX cells not only by decreasing drug efflux through down-regulating P-gp expression but also by inducing apoptosis through up-regulating the expression of Bax protein.

In summary, we generated and identified a novel P-gp-mediated multidrug resistant cell model from human bladder cancer 253J cell line. The multidrug resistant phenotype can be overcome by SIN in both P-gp-dependent and independent manners. The chemosensitive effect and apoptotic effect of SIN may make it a prime candidate of agents to treat bladder cancer in future.

4. Experimental

4.1. Chemicals and reagents

Doxorubicin (DOX), pirarubicin (THP), vincristine (VCR), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and verapamil were purchased from Sigma-Aldrich Chemical Co. (St. Louis, Mo., USA). The culture medium Dulbecco's Modified Eagle Medium (DMEM) was obtained from GIBCO Co. (GrandIsland, N.Y., USA). Sinomenine hydrochloride (98% purity), kindly provided by the Hunan Zhengqing Pharmaceutical Group Co. Ltd. (Changsha, China), was directly dissolved in DMEM. Lipofectamine 2000 was purchased from *Invitrogen* Co. (Carlsbad, Calif., USA). Primary antibodies against P-gp and Bax were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, Calif., USA). Primary anti-

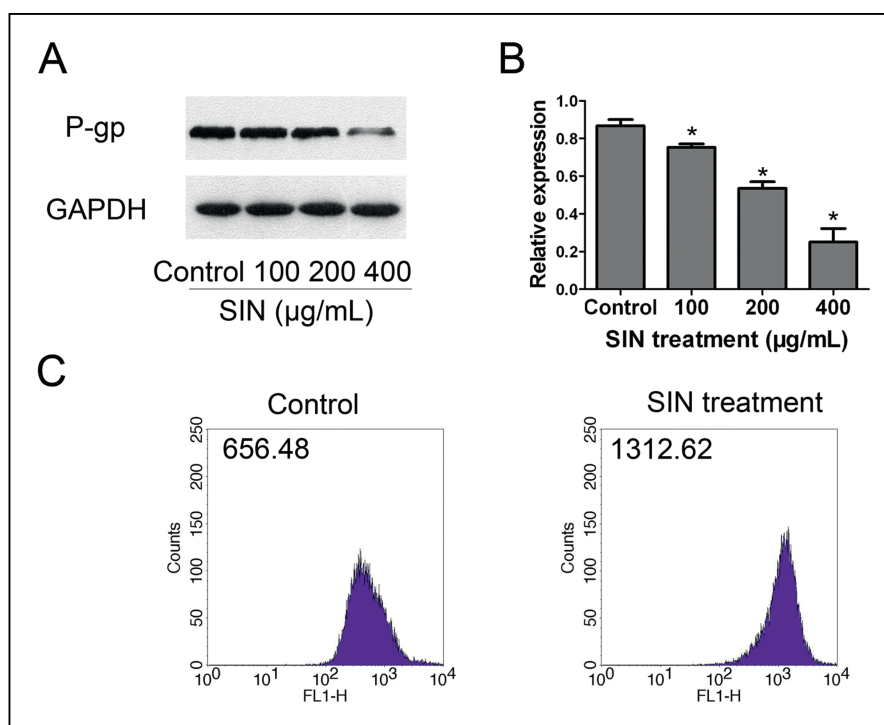

Fig. 4: Downregulation of P-gp expression by SIN. A, B. Western blot analysis of P-gp expression in 253J/DOX cells treated with various concentration of SIN for 48 h and GAPDH was detected as an internal control: representative western blot results (A), Quantitative analyses of expression relative to GAPDH (B). C. Rhodamine 123 retention assay of P-gp-mediated efflux in 253J/DOX cells treated with or without 200 μg/ml SIN.

body against PARP and Horseradish peroxidase and tetraethyl rhodamine isothiocyanate-conjugated secondary antibodies were purchased from Cell Signaling Technology Inc.(Beverly, Mass., USA).

4.2. Cell culture

Human bladder cancer 253J cell line was kindly provided by Dr. Leland W.K. Chung (Cedars-Sinai Medical Center, Los Angeles, Calif., USA). Cells were propagated in DMEM containing 10% FBS and maintained at 37 °C in a humidified incubator with 5% CO_2. A multidrug resistant subline of 253J was obtained by culturing the cells in gradually increasing doses of DOX. After 2 months, cells can grow in 0.05 μg/ml DOX. After a further 4 months, cells can grow in 0.1 μg/ml DOX. Then the drug resistant phenotype was stabilized by culturing the cells in medium containing 0.1 μg/ml DOX for more than 6 months. All cells used for experiments were grown in drug-free medium for at least 1 week.

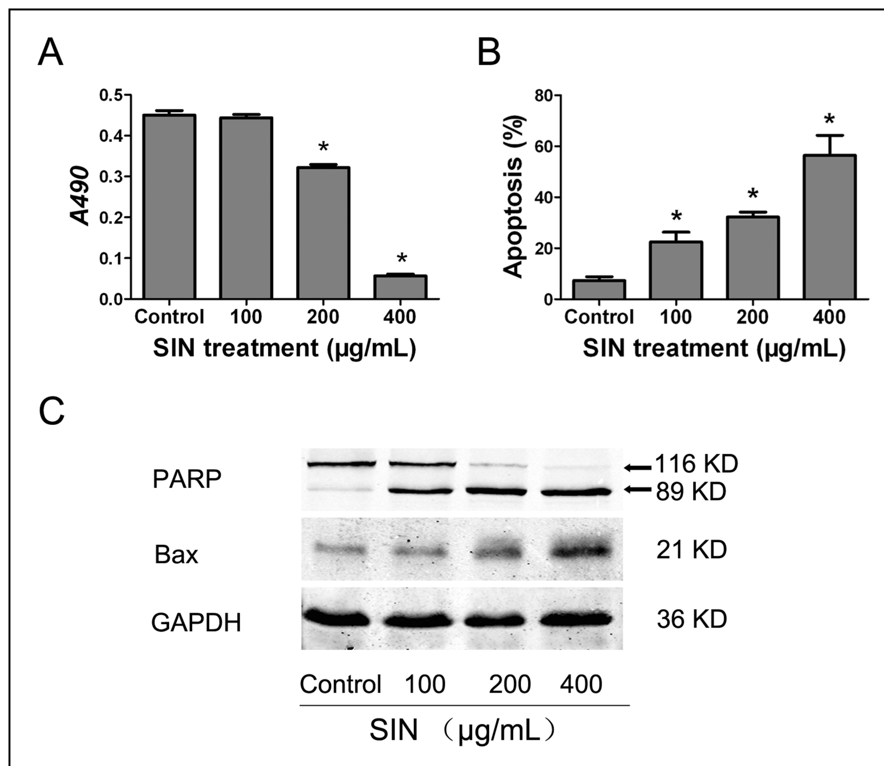

Fig. 5: Induction of apoptosis by SIN. A. MTT assay of cell viability of 253J/DOX cells treated with various concentration of SIN for 48 h. B. Flow cytometry analysis of apoptotic index of 253J/DOX cells treated with various concentration of SIN for 48 h. C. Western blot analysis of PARP cleavage and Bax expression in 253J/DOX cells treated with various concentration of SIN for 48 h.

4.3. MTT Assay

Cells were plated in 96-well culture plates in 0.2 ml of medium supplement containing 10% FBS. After culture for 24 h, the supernatant was replaced with fresh medium containing various concentrations of DOX (0, 1, 10, 50, 100 and 250 μ g/ml) or THP (0, 0.1, 1, 5, 10 and 20 μ g/ml) or VCR (0, 0.1, 0.5, 1, 5 and 10 μ g/ml) or DDP (0, 0.5, 1, 2, 4 and 8 μ g/ml) or SIN (0, 100, 200 and 400 μ g/ml). The cells were incubated for 48 h, followed by supplementation with 20 μl MTT (5 mg/ml) for each well and incubation for 4 h. Viable cell-mediated reaction products were recorded using a 96-well microplate reader (Bio-Rad, Hercules, Calif., USA) at a wavelength of 490 nm. The value of half-maximal inhibitory concentration (IC50) was calculated using the nonlinear regression of the dose-log response curves by SOFTmaxPro, and was used to evaluate chemosensitivity to anticancer drugs. To evaluate the chemosensitizing effect of SIN, cells were treated with single drug only or combination of conventional chemotherapeutic drug and SIN. Inhibitory rate of cell viability was calculated according to MTT assays.

4.4. Reverse-transcription polymerase chain reaction

The Reverse-Transcription Polymerase Chain Reaction (RT-PCR) was performed as we previously described(Chen et al., 2012). Primer sequences were listed as follows: MDR1: 5'-GTGTCCGTGGATCACAAGCC-3' and 5'-GCGAGCCTGGTAGTCAATGC-3'; β-actin: 5'-ATCATGTT TGAGACCTTCAACA-3' and 5'-CATCTCTTGCTCGAAGTCCA-3'.

4.5. Western blot

Total protein was extracted from cells with RIPA lysis buffer containing protease inhibitors. The protein concentration of lysates was analyzed by the Bradford method. 30 μg of protein was separated by electrophoresing on 12% SDS-polyacrylamide gel and blotted onto nitrocellulose filter membranes. The membranes were blocked with 5% nonfat-milk in Tris-buffered saline and Tween-20 for 1 h at room temperature. The blots were then incubated with primary antibodies overnight at 4 °C and horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Immunoreactive signals were detected using the ECL detection system followed by exposure to x-ray film. The relative intensity of each band was determined by using Quantity One software (Bio-Rad). Immunoblotting against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed as an internal control.

4.6. Immunofluorescence staining

Cells were plated on slides overnight and then fixed in 4% paraformaldehyde for 30 min at room temperature, washed with PBS and blocked for 1 h in 3% bovine serum albumin, followed by incubation with anti-P-gp antibody (1: 150) overnight at 4 °C. Then, the cells were washed and incubated with secondary antibody (anti-mouse-tetraethyl rhodamine isothiocyanate, 1: 150) in the dark at 37 °C for 30 min. The nuclear DNA was stained with 4',6-diamidino-2-phenylindole (DAPI). Finally, stained cells were visualized under an Olympus IX-50 fluorescence inverted microscope (Olympus, Tokyo, Japan).

4.7. Rhodamine-123 retention assay

Cells were digested with trypsin and washed once with PBS, followed by incubation with PBS containing 10 mM rhodamine-123 at 37 °C for 30 min. Then the supernatant containing rhodamine-123 was removed, and the cells were incubated in fresh PBS at 37 °C for another 30 min. The fluorescence of Rhodamine 123 was measured using flow cytometry at excitation and emission wave lengths of 507 and 529 nm, respectively.

4.8. siRNA and transfection

Human MDR1 small interference (siRNA) duplexes were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, Calif., USA). A scrambled siRNA oligonucleotide lacking known human coding cDNA was used as a negative control. Transfection of siRNAs using Lipofectamine 2000 was performed in accordance with the manufacturer's instructions. Cells were cultured for 48 h after transfection and then harvested for further experiments.

4.9. Flow cytometry for apoptotic index

Cells were exposed to various concentration of SIN (0, 100, 200, and 400 μg/ml) for 48 h. The cells were collected and subjected to annexinV and propidiumiodide (PI) staining using an Annexin V-FITC Apoptosis Detection Kit. Apoptotic index were then analyzed by flow cytometry.

4.10. Statistical analyses

Statistical analyses were carried out using SPSS 16.0 (SPSS lnc., Chicago, USA). Quantitative data were presented as mean ± SD. Student's t test was used when the difference between 2 groups was compared. Significance was assumed for P values < 0.05.

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LETTER TO THE EDITOR

Does resveratrol inhibit NF-κB activity?

Dear Editor,

With great interest I have read the paper by Ren and co-workers (Ren et al., 2013) entitled “Resveratrol inhibits NF-κB signaling through suppression of p65 and IκB kinase activities” which has recently been published in your journal. In essence, the authors of the study report a decrease of NF-κB transcriptional activity in the presence of resveratrol. The authors show that this is not associated with diminished DNA binding of the transcription factor and also not caused by altered protein amounts or impaired nuclear translocation of NF-κB. The conclusion that NF-κB activity is modulated by resveratrol is based on luciferase reporter assay results which have been generated using a plasmid encoding firefly luciferase under the control of a NF-κB-responsive promoter. It is well known that resveratrol is a potent inhibitor of the firefly luciferase enzyme (Bakhtiarova et al. 2006; Braeuning and Vetter, 2012). The authors of the latter publications have demonstrated that the IC50 of resveratrol for the inhibition of firefly luciferase is about 1-2 μM. In their study, Ren et al. used 10-40 μg/ml resveratrol, corresponding to molar concentrations of approximately 44–175 μM. Therefore, the observed decrease in NF-κB-dependent luciferase reporter activities is most likely caused by a direct inhibition of the firefly luciferase enzyme by resveratrol and not by diminished luciferase transcription due to altered transcriptional activity of NF-κB. Unfortunately, Ren et al. draw their conclusion solely on the luciferase assay and do not present additional lines of evidence to support their hypothesis, for example data on the mRNA expression of endogenous NF-κB target genes. In the present form, the study by Ren et al. is not suited to prove an influence of resveratrol on NF-κB transcriptional activity.

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