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Silibinin suppresses bladder cancer cell malignancy and chemoresistance in an NF-κB signal-dependent and signal-independent manner

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Abstract. Because bladder cancer (BCa) is the 9th most common malignant tumor and 13th leading cause of death due to cancer, therapeutic approaches have attracted a great deal of attention from both clinicians and BCa patients. Although the development of surgery and targeted drugs has brought new challenges for the traditional concept of BCa therapy, various types of chemotherapy remain the final treatment method for many BCa patients. However, chemoresistance inevitably appears, leading to the failure of chemotherapy. Silibinin, a polyphenolic flavonoid component isolated from the fruits or seeds of milk thistle, has been reported to play important roles in inhibiting tumor chemoresistance in breast cancer and head and neck squamous cell carcinomas. Our previous study indicated that silibinin inhibited BCa progression in some mechanisms but with no conclusion of chemoresistance inhibition. Therefore, in the present study, we dissected the role of silibinin in BCa progression and chemoresistance. Our results revealed that in BCa, chemodrug-induced chemoresistance was reversed in the presence of silibinin. Further mechanistic study indicated that silibinin suppressed chemoresistance and BCa malignancy in an NF-κB-dependent and -independent manner. In addition, all of the inhibitory effects were dose-dependent. Thus, our results provide a potential use for silibinin in BCa therapeutics.

Introduction

Worldwide, bladder cancer (BCa) is the 9th most common cancer and the 13th leading cause of death due to cancer (1), and it has attracted increasing attention from clinicians over the past several decades. The risk factors for BCa include but are not limited to genetic predisposition and acquired exposure, the mechanism of which is still unclear (2-5). Clinically, according to the pathological characteristics, BCa includes superficial and invasive types, which are treated using different therapeutic procedures. Briefly, the recommended therapeutic guideline for superficial BCa is transurethral resection of bladder tumor (TUR-bt), followed by intravesical chemo- or immunotherapeutics. Unfortunately, 70% of superficial BCa will inevitably progress to the invasive type followed by enhanced tumor malignancy. For invasive BCa, cystectomy plus adjuvant or neoadjuvant chemotherapy is accepted by clinicians and patients (6). Thus, chemotherapy is a vital and irreplaceable regimen for treating invasive BCa, regardless of whether initial invasive BCa or invasive BCa progressed from the superficial type. Many studies have indicated that cisplatin-based regimens play effective roles in BCa therapy (7,8). However, chemoresistance to cisplatin-containing regimens inevitably appears in the battle against BCa, the mechanism of which is still unknown.

Previous reports have identified the important roles of NF-κB signaling in the initiation and progression of BCa, e.g., promoting the process of epithelial to mesenchymal transition (EMT) (9), secreting MMP2/MMP9 and inhibiting apoptosis. In addition, the promoter of MDR1 (ABCB1), a multiple drug-resistant gene, contains an NF-κB binding site, indicating that MDR1 was monitored by this signaling pathway (10).

Silibinin, a polyphenolic flavonoid component isolated from the fruits or seeds of milk thistle (*Silybum marianum*), has been clinically used to treat various diseases, and it has been suggested that this reagent exhibited protective effects for patients with liver or heart disease. The inhibitory effects of silibinin against cancer were also indicated (11-17) in tumors such as breast cancer and head and neck squamous cell carcinomas. Our previous study indicated that silibinin inhibited BCa progression (18), leading us to hypothesize a link between silibinin and BCa chemoresistance.

In the present study, we hypothesized that silibinin may play inhibitory roles in the chemoresistance of BCa cells, possibly involving the NF-κB signaling pathway. Our results revealed that silibinin inhibited the progression and reversed the chemoresistance of BCa cells in an NF-κB-dependent and

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-independent manner, thus providing a potential therapeutic use for silibinin in patients with BCa.

Materials and methods

Cell culture. Human BCa cell lines T24 and J82 were obtained from the ATCC (American Type Culture Collection, Manassas, VA, USA) and cultured in DMEM supplemented by 10% FBS (Invitrogen, Carlsbad, CA, USA). Cells were cultured in incubators (Thermo Scientific, Germany) in an atmosphere with 5% CO_2 at 37˚C.

To obtain stable, cisplatin-resistant cell lines, we monitored the IC_{50} of cisplatin and obtained values of 58 μM for T24 and 49 μM for J82. Second, the cisplatin-resistant index (RI) was evaluated by MTT; the RIs of the cell lines were 21.35 and 28.75 for T24 and J82, respectively. The cultured parental T24 and J82 cells were supplemented with 20 μM cisplatin. The medium was refreshed every two days to remove the dead cells, and the cells were washed three times with sterile PBS (pH 7.2). This treatment was administered for more than three months to obtain stable cisplatin-resistant T24/J82 cells (tagged with $T24^R$/$J82^R$).

To inhibit NF-κB signaling, pyrrolidine dithiocarbamate (PDTC) (Sigma-Aldrich, USA) (19), an inhibitor of the NF-κB pathway, was used; the final concentration was 10 μM in the medium for the last 24 h before analysis.

Wound healing assay. Wound healing assays were carried out by scratching a 6-well dish with a 10-μl pipette tip when the dish was at 80% confluence (including parental cell and cisplatin-resistant cell lines). The width of the scratches was compared at 0, 6, 12 and 24 h after scratching.

Western blot analysis. Pretreated cells were harvested at 80% confluency and washed three times with cold PBS. Total cellular protein lysates were prepared with RIPA buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% NP40 and 0.5% sodium deoxycholate] containing proteinase inhibitors [1% inhibitor cocktail and 1 mM PMSF, both from Sigma, (St. Louis, MO, USA)]. Then, 30 μg of protein was separated on 10% SDS-PAGE gels and transferred to nitrocellulose membranes. The membranes were blocked at room temperature for 1 h with 5% skim milk in Tris-buffered saline (pH 7.6, TBS). Polyclonal primary antibodies were applied at different dilutions (Table I) in 5% skim milk in TBS at 4˚C overnight, followed by TBST (with Tween-20) washes. Membranes were incubated with fluorescent secondary antibodies (LI-COR, Rockford, IL, USA) coupled to the first antibody at room temperature in the dark for 1 h, then washed with TBST, dried with neutral absorbent paper and scanned with the Odyssey Detection system (LI-COR). MG-132 (Sigma-Aldrich) was used to inhibit the proteasome-dependent degradation when necessary (10 μM, 4 h before the protein harvest). GAPDH was used as a loading control (for total cell fraction).

Real-time PCR. Cellular total RNA was isolated using TRIzol reagent (Invitrogen) and quantified by absorbance at 260 nm. RNA (2 μg) was reverse transcribed using Revert Aid™ First Strand cDNA Synthesis kit (MBI Fermentas, St. Leon-Rot, Germany) strictly according to the manufacturer's protocol. For real-time PCR, we used the SYBR Premix Ex Taq™ II system (Takara Biotechnology Co., Ltd., Dalian, China) and the Bio-Rad CFX96TM Real-time system (Bio-Rad, CA, USA). Primers are listed in Table II. Briefly, 12.5 μl of SYBR Premix Ex Taq II, 1 μl of primer (F and R, respectively), 200 ng of cDNA and 9.5 μl of distilled and deionized water were mixed together, followed by two-stage, pre-denaturation at 95˚C, 30 sec, one repeat; and PCR reaction, at 95˚C, 5 sec followed by 60˚C, 30 sec, 30 repeats; and the third stage as dissociation, 95˚C, 15 sec followed by 60˚C, 30 sec, and another 95˚C, 15 sec. GAPDH was used as the loading control.

Table I. Information on the antibodies.

Gene ID	Antibody	Dilutions	Species	Supplied by
NM_004360.3	E-cadherin	1:600	*Homo*	Santa Cruz
NM_001792.3	N-cadherin	1:300	*Homo*	Santa Cruz
NM_003380.3	Vimentin	1:300	*Homo*	Santa Cruz
NM_004530.4	MMP2	1:400	*Homo*	Santa Cruz
NM_004994.2	MMP9	1:400	*Homo*	Santa Cruz
NM_002046.4	GAPDH	1:15,000	*Homo*	Abcam
NM_000927.4	ABCB1	1:400	*Homo*	Santa Cruz
	β-actin	1:300	*Homo*	Santa Cruz

Cell viability assay (MTT assay). Cell viability was assessed using a tetrazolium-based assay. Pretreated cells were incubated in the absence or presence of cisplatin/doxorubicin for the indicated times, and then washed once with PBS and incubated with 0.5 mg/ml of MTT at 37˚C for 1 h. The reagent was reduced by living cells to form an insoluble blue formazan product. After incubation, cells were lysed with DMSO. Colorimetric analysis using a 96-well microplate reader was performed at a wavelength of 490 nm. The experiments were performed in triplicate.

Cell migration/invasion assay. Migration/invasion ability was demonstrated by Boyden chamber assay. Chambers with 8-μm-diameter pores were obtained from Millipore (Millipore, Switzerland). For the migration assay, 0.2 ml of FBS-free DMEM medium suspension with 10,000 cells was added to the upper chamber in a 24-well plate, and 0.8 ml of FBS-free DMEM was added to the lower chamber. After 12 h of incubation, the chambers were washed with PBS (pH 7.4) three times to remove the cells in the upper chamber, fixed with 4% formalin for 15 min, and then stained with crystal violet (0.01% in ethanol) for 25 min followed by washing three times with PBS. The cells were counted using an inverted microscope, five images were randomly taken at 200x magnification, and the average number of cells was analyzed. For the invasion assay, the cell suspension (10,000 cells/well) in the upper chamber contained 0.2 ml mixture of FBS-free DMEM/Matrigel at an 8/1 ratio (Matrigel, Sigma, USA). Cells were incubated for 36 h, and the remainder of the protocol was conducted in a similar manner to the migration assay.

Table II. Primers for real-time PCR.

Gene ID	Gene	Primers
NM_002046.4	*GAPDH*	F: AAC AGC GAC ACC CAT CCT C R: CAT ACC AGG AAA TGA GCT TGA CAA
NM_004360.3	*E-cadherin*	F: TGC CCA GAA AAT GAA AAA GG R: GTG TAT GTG GCA ATG CGT TC
NM_001792.3	*N-cadherin*	F: ACA GTG GCC ACC TAC AAA GG R: CCG AGA TGG GGT TGA TAA TG
NM_003380.3	*Vimentin*	F: GAG AAC TTT GCC GTT GAA GC R: GCT TCC TGT AGG TGG CAA TC
NM_004530.4	*MMP2*	F: CTC ATC GCA GAT GCC TGG AA R: TTC AGG TAA TAG GCA CCC TTG AAG A
NM_004994.2	*MMP9*	F: TGA CAG CGA CAA GAA GTG R: CAG TGA AGC GGT ACA TAG G
NM_000927.4	*ABCB1*	F: GTC CCA GGA GCC CAT CCT R: CCC GGC TGT TGT CTC CAT A

Cell proliferative capacity assay. A 5-bromo-2-deoxyuridine (BrdU) incorporation assay was used to analyze tumor proliferative ability. Briefly, pretreated cells were plated on 8-well glass plates (Millipore) until 50-70% confluency. BrdU was added to the medium (3 μg/ml), followed by 4 h of incubation and rinsing 3X with PBS over 10 min to remove residual free BrdU. Cells were then fixed with 4% paraformaldehyde for 45 min, followed by rinsing 5X with PBS over 20 min. Then, 0.1% Triton X-100 was used to permeabilize the cell membrane for 15 min, and 2 N HCl was added for 25 min to separate DNA into single strands and thus allowing primary antibody access to the incorporated BrdU. Cells were then rinsed 3X with PBS over 10 min, and non-specific epitopes were blocked by 10% BSA for 20 min. Anti-BrdU antibody (1:200) in 10% BSA was added and incubated overnight at 4˚C. Cells were rinsed 5X with PBS, followed by incubation with TRITC-labeled secondary antibody for 1 h at room temperature, and finally rinsed 3X with PBS to remove the free antibody. The fluorescence intensity of TRITC was monitored with a SuperMicro Orifice Plate spectrophotometer (BioTek, USA) at 547 nm.

Immunofluorescence staining for nuclear translocation of NF-κB. After the designated treatment, the pretreated cells were washed three times with cold PBS (pH 7.4), followed by fixing with 4% paraformaldehyde for 15 min, permeabilization in 0.5% Triton X-100 for 10 min, and incubation in 1% BSA blocking solution for 1 h. Fixed cells were incubated overnight at 4˚C with rabbit anti-human-P65 in 1% BSA. Cells were washed and incubated with mouse anti-rabbit TRITC (Red) IgG antibody (Santa Cruz Biotechnology, USA) diluted 1:100 in blocking buffer for 1 h. Nuclei were stained with DAPI for 5 min. Cells were examined with a fluorescence microscope equipped with narrow bandpass excitation filters to individually select for red and blue fluorescence. Cells were observed through the Image-Pro Plus system™ mounted on a fluorescent microscope (Olympus, Japan). Each experiment was repeated three times.

Statistical analysis. ANOVA test was used to analyze the statistical discrepancy in >3 groups. Student's t-test was used to detect any statistically significant difference between 2 groups. P-values <0.05 were considered significant.

Results

Stable chemoresistant cell lines induced by cisplatin manifest enhanced migration/invasion and proliferation capacity. Chemoresistance is considered a vital obstacle in the battle against BCa and leads to the failure of BCa chemotherapy (20). Cisplatin, which is one of the major reagents in the chemotherapeutic regime for BCa, is recommended as first-line treatment in the clinic (21). Therefore, cisplatin resistance is ubiquitous in BCa patients, and dissecting the underlying mechanism potentially brings benefits to BCa patients. Cisplatin was used to treat BCa cell lines T24/J82 to obtain stable cisplatin-resistant cell lines (tagged by $T24^R$ and $J82^R$, respectively), as indicated in the Materials and methods. Compared with parental T24/J82, $T24^R/J82^R$ manifested cisplatin-resistance demonstrated by MTT assay (Fig. 1A). In addition, the *in vitro* analysis suggested that the wound-healing time was shorter for these cisplatin-resistant BCa cells (Fig. 1B), accompanied by enhanced proliferation (Fig. 1C) and migration /invasion (Fig. 1E) ability.

EMT markers are induced in $T24^R/J82^R$ cells, accompanied by elevated expression of ABCB1 (MDR1). The enhanced capacity of migration/invasion and proliferation of $T24^R/J82^R$ prompted us to monitor the expression of related genes, including EMT markers and matrix metalloproteinase (MMP). Both western blot analysis (Fig. 2A) and real-time PCR (Fig. 2B) indicated the elevated expression of

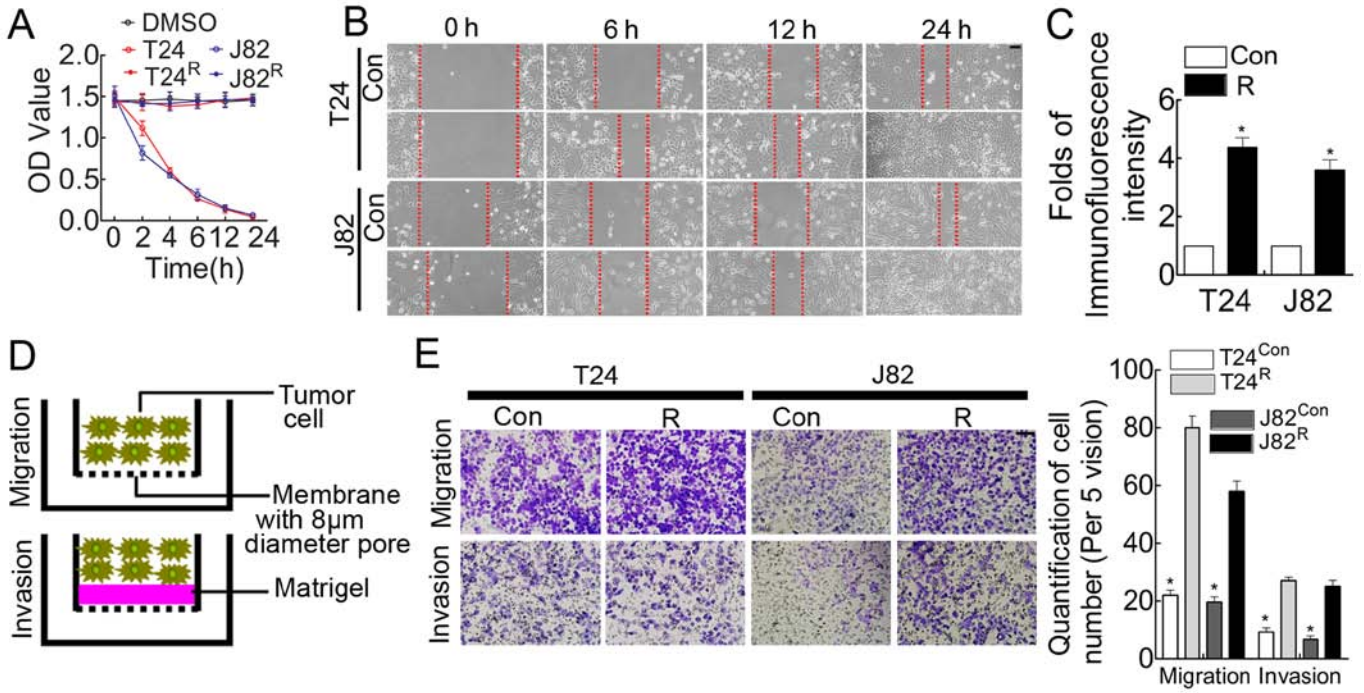

Figure 1. Cisplatin-resistant BCa cell lines T24/J82 manifest enhanced migration/invasion and proliferation capacities. (A) MTT assay showed that compared with parental T24/J82, cisplatin-resistant T24/J82 ($T24^R/J82^R$) manifested enhanced cisplatin-resistant capacity. (B) Wound healing assay indicated that $T24^R/J82^R$ exhibited shorter wound healing times comparing with parental T24/J82; bar, 100 μm. (C) Quantification of BrdU incorporation suggested that the proliferation capacity of $T24^R/J82^R$ was enhanced almost 4-fold relative to parental T24/J82; $^*P<0.05$. (D) Cartoon of Boyden chamber assay. (E) Boyden chamber assay suggested the increased migration/invasion ability of $T24^R/J82^R$ compared with T24/J82. Left, representative figures; bar, 100 μm. Right, quantification of the Boyden chamber assay; $^*P<0.05$.

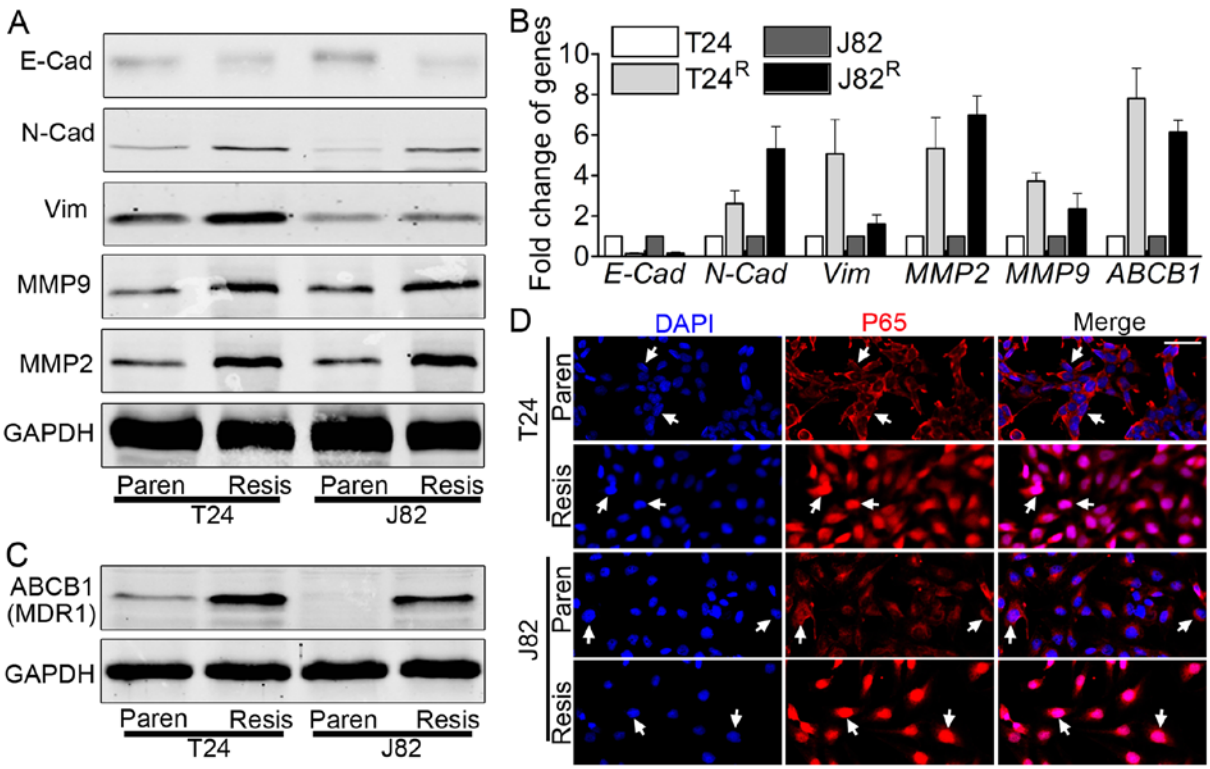

Figure 2. Epithelial to mesenchymal transition (EMT) is enhanced in $T24^R/J82^R$ cells, accompanied by increased expression of ABCB1 and NF-κB nuclear translocation. (A) Western blot analysis showed elevated expression of N-cadherin, vimentin, MMP2 and MMP9 but decreased expression of E-cadherin in $T24^R/J82^R$ vs T24/J82, indicating the process of EMT. (B) Real-time PCR results were consistent with western blot analysis results, including elevated expression of N-cadherin, vimentin, MMP2 and MMP9 but decreased E-cadherin. In addition, the expression of ABCB1, the multiple drug-resistant gene, was also elevated in $T24^R/J82^R$ vs T24/J82. (C) Western blot analysis suggested that the expression of the multiple drug-resistant gene, ABCB1, and also MDR1, was elevated as expected in $T24^R/J82^R$. (D) Immunofluorescence staining revealed the nuclear translocation of NF-κB (P65) in $T24^R/J82^R$, as the white arrow indicates; bar, 100 μm. Paren=Parental; Resis=Resistant.

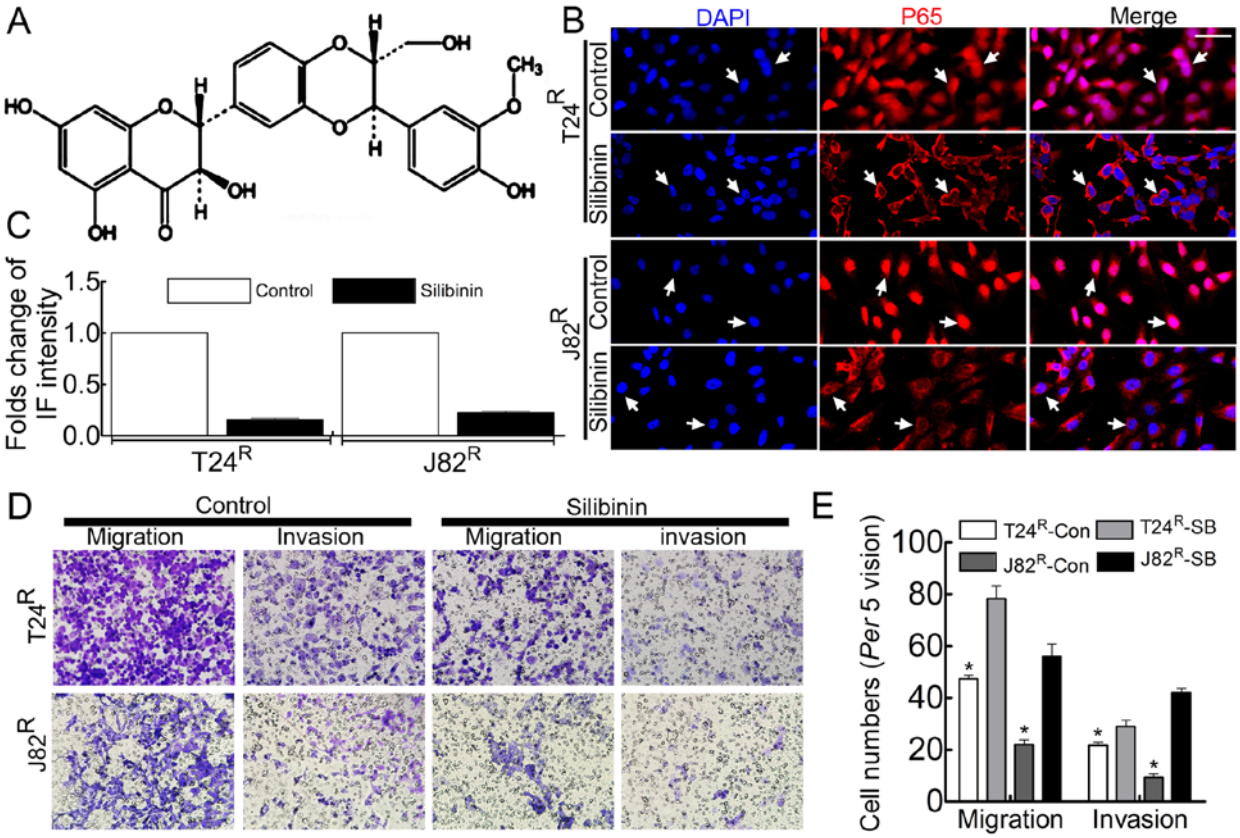

Figure 3. The malignant behavior of $T24^R/J82^R$ is inhibited by silibinin (SB). (A) The chemical structure of silibinin. (B) Immunofluorescence staining suggested that the nuclear translocation of NF-κB can be inhibited by silibinin, as indicated by white arrows; bar, 100 μm. (C) Quantification of BrdU incorporation revealed that the proliferation capacity of $T24^R/J82^R$ was attenuated in the presence of silibinin. (D) Representative images of the Boyden chamber assay suggested that compared with the control, silibinin significantly inhibited the malignant behavior of $T24^R/J82^R$; bar, 100 μm. (E) Quantification of the Boyden chamber assay, indicating the attenuated migration/invasion capacity of $T24^R/J82^R$ in the presence of silibinin; $^*P<0.05$.

EMT-related markers, e.g., N-cadherin, vimentin, MMP2 and MMP9, with decreased expression of E-cadherin. ABCB1, also called MDR1, plays a vital role in BCa chemoresistance (20). Therefore, the expression of ABCB1 was monitored in $T24^R/J82^R$ by western blot analysis and real-time PCR. ABCB1 was elevated in $T24^R/J82^R$ vs. T24/J82, as shown by blot analysis (Fig. 2C) and real-time PCR (Fig. 2B).

NF-κB signaling is overactivated in $T24^R/J82^R$. Emerging evidence noted the importance of NF-κB signaling in tumorigenesis and cancer metastasis (22,23), as well as chemoresistance (24), leading us to link the cisplatin-induced phenomenon to this signaling pathway. As indicated in Fig. 2D, immunofluorescence staining suggested that $T24^R/J82^R$ cells significantly manifested P65 nuclear translocation, suggesting the activation of this signaling pathway. In a parallel experiment, nuclear lysates from $T24^R/J82^R$ indicated the accumulation of P65 in nuclei, as demonstrated by blot analysis (data not shown). Thus, we concluded that NF-κB signaling was overactivated in $T24^R/J82^R$ cells vs. parental T24/J82 cells.

Migration/invasion ability and proliferation of $T24^R/J82^R$ are attenuated in the present of silibinin, accompanied by inhibition of NF-κB signaling in $T24^R/J82^R$ cells. Silibinin has been used clinically to treat various liver diseases and has been marketed as a dietary supplement (25). Previous studies had noted that silibinin inhibited tumor growth by suppressing MMPs (26,27), VEGF, HIF-1α (25) and the process of EMT (28,29). In the present study, silibinin was used to treat BCa cell $T24^R/J82^R$, followed by Boyden chamber assay, BrdU incorporation and immunofluorescence staining to observe the malignant behavior. As expected, the enhanced proliferation capacity of $T24^R/J82^R$ cells was attenuated in the presence of 100 μM silibinin (Fig. 3B), accompanied by decreased migration/invasion ability (Fig. 3C and E). In addition, the nuclear translocation of NF-κB was significantly inhibited in the presence of silibinin in $T24^R/J82^R$ cells (Fig. 3D).

Silibinin suppresses the migration/invasion and proliferation capacity of $T24^R/J82^R$ cells by inhibiting the expression of EMT-related markers and ABCB1 in a dose-dependent manner. Previous results suggested that silibinin could inhibit cisplatin-induced migration/invasion and proliferation. In addition, immunofluorescence staining indicated that silibinin could inhibit the activity of the NF-κB signaling pathway. According to the previous studies, this phenomenon was accompanied by an alteration of related genes and might be involved in a related signaling pathway. Therefore, we monitored the expression of EMT-related markers and ABCB1 in the presence of silibinin. As indicated in Fig. 4A and B, silibinin treatment led to significant inhibition of EMT, e.g., decreased expression of vimentin, N-cadherin, MMP2, and MMP9, with increased E-cadherin expression.

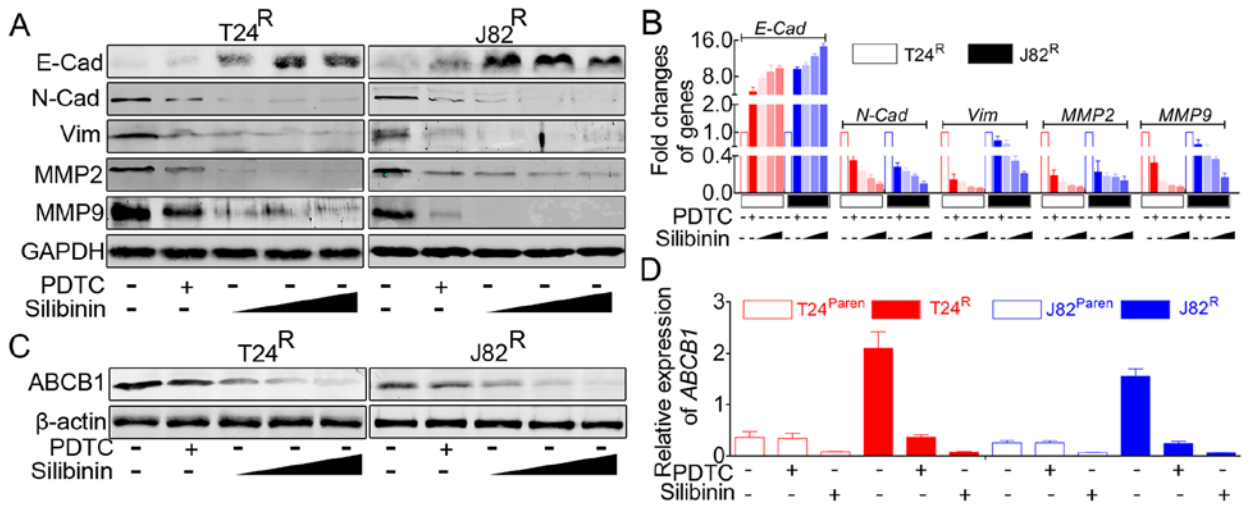

Figure 4. Silibinin inhibits T24^{R}/J82^{R} malignancy in an NF-κB-dependent and -independent manner, accompanied by attenuating the expression of ABCB1. (A) Western blot analysis suggested that PDTC, a classical inhibitor of NF-κB signaling, partially inhibited EMT, e.g., elevated expression of E-cadherin accompanied by decreased expression of N-cadherin, vimentin, MMP2 and MMP9; in addition, silibinin led to the reversal of EMT in a dose-dependent manner. (B) Real-time PCR indicated that the inhibition of NF-κB signaling by PDTC led to the reversal of EMT markers in T24^{R}/J82^{R}, and this EMT reversal can be enhanced by silibinin in a dose-dependent manner. (C) Western blot analysis showed that both PDTC and silibinin resulted in the decreased expression of ABCB1 but that silibinin possessed more powerful roles in a dose-dependent manner. (D) Real-time PCR revealed that PDTC could lead to the attenuation of ABCB1 in T24^{R}/J82^{R} but had no visible effects on parental T24/J82, whereas silibinin significantly inhibited the expression of ABCB1 in both cisplatin-resistant and parental T24/J82 cells.

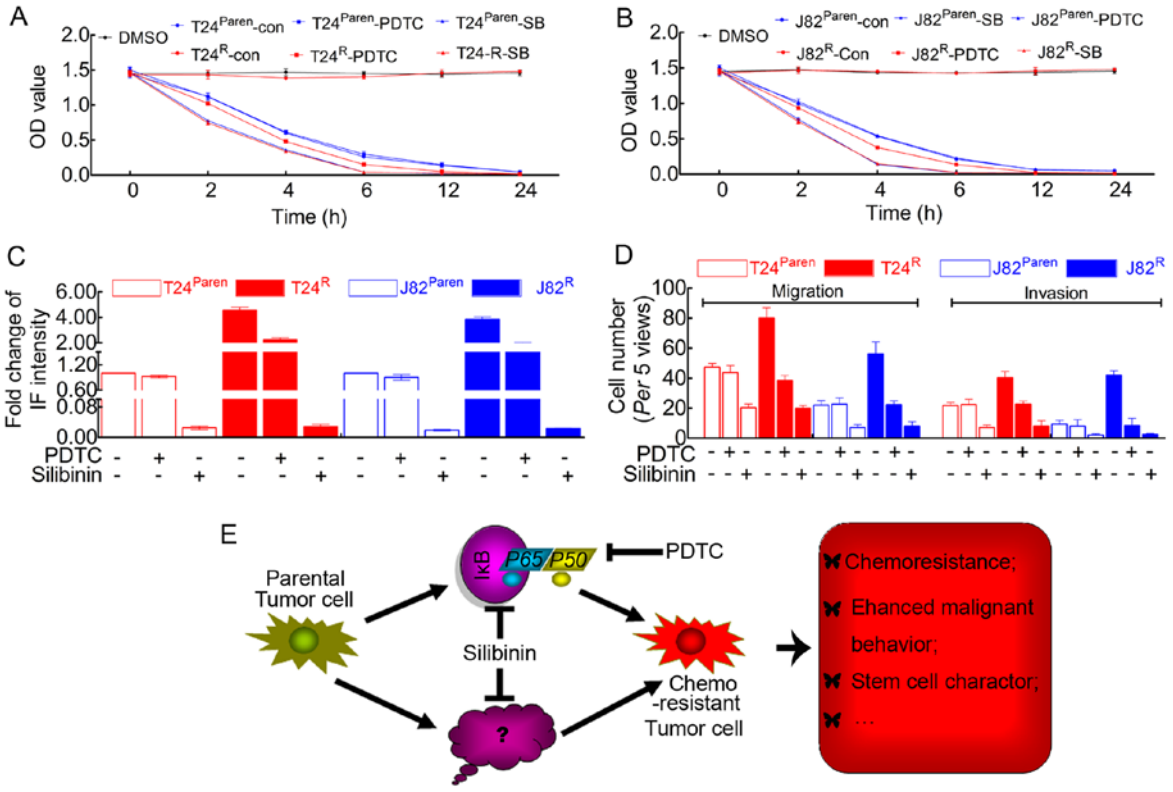

Figure 5. Silibinin exhibits more powerful roles in inhibiting cisplatin resistance, cell proliferation and migration/invasion. (A) MTT assay revealed that the cisplatin resistance of T24^{R} was destroyed in the presence of silibinin. Silibinin induced the enhanced cisplatin sensitivity of parental T24. In addition, PDTC attenuated the cisplatin resistance of T24^{R} but had no visible effect on parental T24. (B) MTT assay revealed that the cisplatin resistance of J82R was destroyed in the presence of silibinin. Silibinin induced the enhanced cisplatin sensitivity of parental J82. In addition, PDTC attenuated the cisplatin resistance of J82R but had no visible effect on parental J82. (C) Quantification of BrdU incorporation suggested that silibinin possessed more powerful effects on inhibiting BCa cell proliferation in both cisplatin-resistant cell lines and parental cell lines. However, PDTC seemed to have little effect on parental T24/J82, although the proliferative capacity of T24^{R}/J82^{R} was inhibited by this reagent. (D) Quantification of the Boyden chamber assay suggested that silibinin significantly attenuated the migration/invasion capacity of both parental and cisplatin-resistant T24/J82 cells. In addition, malignancy of T24^{R}/J82^{R} but not parental T24/J82 was inhibited by PDTC. (E) In summary: The prolonged time of cisplatin treatment inevitably resulted in drug resistance, the mechanism of which included but was not limited to the activation of NF-κB signaling, leading to the failure of chemotherapeutics. Like PDTC, silibinin was able to inhibit NF-κB signaling, which reversed the malignant behavior of drug-resistant cell lines. However, silibinin also attenuated chemoresistance and tumor cell malignancy in an NF-κB-independent manner, which is still unknown [tagged by ? in (E)].

In addition, silibinin suppressed the expression of ABCB1 in $T24^R/J82^R$ (Fig. 4C and D). As indicated in Fig. 4, the effects of silibinin on $T24^R/J82^R$ manifested in a dose-dependent manner, at doses from 100 μM, 200 μM to 400 μM, but revealed the most powerful role of PDTC.

Forced inhibition of NF-κB signaling in $T24^R/J82^R$ also leads to decreased expression of EMT-related markers and ABCB1. Previous reports indicated that forced inhibition of NF-κB signaling in BCa cells resulted in the reversal of EMT (30). Herein, in parallel, we used a specific inhibitor of NF-κB signal to repeat this process. In accordance with previous reports, our data indicated that PDTC effectively attenuated the activation of NF-κB signaling (data not shown), accompanied by decreased expression of EMT-related markers (Fig. 4A and B). In addition, as mentioned above, ABCB1 was one of the target genes of NF-κB signaling, and inhibiting this pathway led to the decreased expression of ABCB1 (Fig. 4C and D).

The suppressive roles of silibinin on $T24^R/J82^R$ cells presented NF-κB-dependent and -independent mechanisms. The previous results suggested that silibinin manifested more powerful inhibitory roles on $T24^R/J82^R$ cells than on parental T24/J82 cells. Moreover, as stated above, NF-κB signaling was inactivated in parental T24/J82 cells, suggesting that PDTC had no visible inhibitory roles on the malignancy and proliferative capacity of parental T24/J82 cells. This led us to ask whether the inhibitory roles of silibinin on $T24^R/J82^R$ involved NF-κB signaling or whether they were unrelated phenomena. Our results suggested that in $T24^R/J82^R$ cells, silibinin had a stronger inhibitory role than did PDTC in the tumor cell expression of EMT markers (Fig. 4A and B), ABCB1 (Fig. 4C), cisplatin sensitivity (Fig. 5A and B), proliferation capacity (Fig. 5C) and migration/invasion capacity (Fig. 5D), suggesting that the inhibitory roles of silibinin were partially independent of NF-κB signaling. Combined with the NF-κB signal inhibition induced by silibinin, we concluded that silibinin suppressed BCa cell line $T24^R/J82^R$ in an NF-κB-dependent and -independent manner.

Forced inhibition of NF-κB signaling has no visible inhibitory roles on parental T24/J82 cells. Our data indicated that inhibition of NF-κB signaling by PDTC in $T24^R/J82^R$ cells led to the attenuation of malignancy and reversal of EMT. In contrast, NF-κB signaling was suppressed in parental T24/J82 cells. In parallel, as indicated in Fig. 5C and D, PDTC had no visible effect on the migration/invasion and proliferation of parental T24/J82.

Discussion

Chemoresistance, especially acquired chemoresistance, is considered a vital obstacle in the battle against cancer and can lead to the failure of cancer therapy (20,31). Chemotherapy, in accordance with radiotherapy, is the final regimen for BCa patients. In addition, in both neoadjuvant chemotherapy and traditional chemotherapy for BCa, cisplatin is the one irreplaceable reagent. Thus, cisplatin resistance ubiquitously appears in BCa patients receiving chemotherapy, which is why it is important to investigate this mechanism further as done in recent decades. To dissect this mechanism, the present study was performed.

First, we used cisplatin treatment to obtain chemoresistant cell lines as described in the Materials and methods. Our results suggested that we efficiently obtained the stable cisplatin-resistant cell lines $T24^R/J82^R$ (Fig. 1A). In addition, in accordance with our hypothesis, these $T24^R/J82^R$ cells manifested enhanced wound healing (Fig. 1B), proliferation (Fig. 1C) and migration/invasion (Fig. 1E) capacity. NF-κB signaling was activated in these $T24^R/J82^R$ cells (Fig. 2B), accompanied by the process of EMT (Fig. 2A) and elevated expression of ABCB1 (Fig. 1C). Although our unpublished data partially revealed the mechanism responsible for cisplatin-induced chemoresistance, much work remains to be done.

Silibinin, a polyphenolic flavonoid component isolated from the fruits or seeds of milk thistle (*Silybum marianum*), has been clinically used to treat various liver diseases and has been marketed as a dietary supplement (32). Our previous results indicate that silibinin suppressed BCa by acting on tumor cell mitochondria (18) or another mechanism (33). Therefore, we hypothesized that silibinin might play important roles in the process of EMT, which has been previously reported in other tumors (28,29). Our results indicate that silibinin significantly suppresses the nuclear translocation of NF-κB, inhibiting NF-κB signaling (Fig. 3D). In accordance with our hypothesis, this inhibition is accompanied by an attenuated proliferation capacity (Fig. 3B) and migration/invasion capacity (Fig. 3C and E) and the reversal of EMT (Fig. 4A and B). The process by which silibinin inhibits the NF-κB signal is still unknown, but this result indicates that silibinin can potentially be used in BCa therapy.

As indicated by our data, silibinin inhibits the malignancy of BCa cells in a dose-dependent manner, e.g., the concentration of silibinin ranged from 100 to 400 μM, accompanied by decreased expression of EMT-related markers and ABCB1 (Fig. 4). Silibinin protects against heart disease in older patients and is used as a key ingredient in a Chinese herbal formula for managing age-related disease, which is also effective in a dose-dependent manner. Thus, silibinin is suitable for older BCa patients, especially those with heart disease.

Compared with PDTC, which is a specific inhibitor of NF-κB signaling, silibinin manifests more powerful inhibitory roles that affect BCa cells, especially parental T24/J82 cells (Figs. 4D and 5A-D). This indicates that the inhibitory roles of silibinin acting upon parental BCa cells might be independent of NF-κB signaling, with silibinin inhibiting the malignancy of BCa cells in a more ubiquitous manner. Taken together, these results lead us to conclude that silibinin suppresses BCa cell malignancy in an NF-κB-dependent and -independent manner.

In conclusion, as indicated in Fig. 5E, cisplatin treatment activates the NF-κB signaling pathway in an unknown manner, leading to the enhanced malignancy of BCa cells, which can be inhibited by PDTC. However, silibinin inhibited BCa progression not only by suppressing NF-κB signaling but also via other mechanisms, resulting in an enhanced therapeutic effect for BCa patients. Although the exact mechanisms are still unknown, silibinin exhibits numerous benefits and could be incorporated into various forms of therapy for BCa patients.

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