

# Vinblastine differs from Taxol as it inhibits the malignant phenotypes of NSCLC cells by increasing the phosphorylation of Op18/stathmin

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**Abstract.** Taxol (paclitaxel) and vinblastine (VBL) are both efficacious chemotherapeutic agents that target the microtubules of tumor cells, but each functions in a mutual antagonistic manner. Op18/stathmin is a small molecular phosphoprotein which promotes depolymerization of microtubules. Non-small cell lung cancer (NSCLC) NCI-H1299 cells were employed to compare the curative effects of VBL and Taxol and explore the correlation between drug sensitivity and Op18/stathmin signaling. The present study found that VBL obviously promoted cellular apoptosis and initiated activation of caspase 3 and 9, and inhibited cell proliferation and colony formation, as well as cell migration in the NCI-H1299 cells in contrast with Taxol. VBL did not affect the expression of Op18/stathmin, but increased its phosphorylation at all 4 serine sites. Conversely, Taxol mainly decreased the expression of Op18/stathmin and the phosphorylation at Ser25 and Ser63 sites. Silencing of Op18/stathmin by RNA interference (RNAi) led to a great reduction in the differences in the cell proliferation inhibition between VBL and Taxol. VBL treatment notably weakened the expression of PP2A, Bcl-2, NF- $\kappa$ B and interleukin-10 (IL-10) and autocrine IL-10 compared with Taxol; whereas PP2A was substantially increased following Taxol induction. High expression of Op18/stathmin was found to be negatively correlated with the sensitivity of Taxol in the NSCLC cells, but had a minor impact on VBL cytotoxicity.

These findings revealed that both VBL and Taxol induce cell apoptosis through Op18/stathmin, but the mechanisms are completely different. VBL is an attractive alternative to the treatment of Taxol-resistant tumors with high expression of Op18/stathmin.

## Introduction

Chemotherapeutic agents that target microtubules are mainly divided into two categories based on their functions. One agent, Taxol (paclitaxel), is a microtubule stabilizer which leads to obstacles in chromosome separation and the cell cycle process by promoting microtubule polymerization. Taxol is widely prescribed for the treatment of solid tumors including advanced ovarian and breast cancer, non-small cell lung cancer (NSCLC) and Kaposi's sarcoma (1,2). The other is vinblastine (VBL), a microtubule destabilizer vinca alkaloid, which causes the dysfunction of cell proliferation by predominantly binding to  $\alpha/\beta$  tubulin in order to disassemble microtubules. VBL is commonly applied to treat malignant lymphoma, osteosarcoma, breast, ovarian, gastric and lung cancer, Hodgkin's disease and choriocarcinoma (3,4). Thereby, both Taxol and VBL interact with tubulin while in opposite patterns.

Op18/stathmin is a small molecular biomarker and is highly expressed in many carcinomas. Op18/stathmin integrates and relays intracellular and extracellular stimuli to regulate the equilibrium of microtubule dynamics through phosphorylated inactivation and dephosphorylated activation at 4 serine sites including Ser16, Ser25, Ser38 and Ser63, which is vital to the maintenance of malignant phenotypes in tumors (5-7). Our previous study confirmed that high expression of Op18/stathmin was significantly correlated with Taxol resistance in 5 randomly selected human epithelial-derived carcinoma cell lines, including nasopharyngeal carcinoma CNE1, gastric MGC, breast cancer MCF-7, and hepatocellular carcinoma Hep3B-2 cells, as well as in NSCLC NCI-H1299 cells (8).

The present study aimed to compare the curative effects of VBL and Taxol in non-small cell lung cancer cells and to explore the correlation between drug sensitivity and Op18/stathmin signaling.

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## Materials and methods

**Cell culture.** NSCLC NCI-H1299 and A549 cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin/streptomycin and maintained at 37°C in 5% CO<sub>2</sub>. Both cell lines were presented as a gift from Dr Yongguang Tao from the Cancer Research Institute of Central South University. NCI-H1299 was confirmed to be a Taxol-resistant cell line with high expression of Op18/stathmin in our previous study (8).

**Antibodies and chemical reagents.** Primary antibodies included rabbit polyclonal anti-stathmin (Calbiochem, La Jolla, CA, USA), rabbit monoclonal anti-stathmin (phospho-Ser16, -Ser25, -Ser38 and -Ser63) (Abcam, Cambridge, MA, USA), mouse monoclonal anti-caspase 8, rabbit monoclonal anti-caspase 9, rabbit monoclonal anti-PP2A C subunit (Cell Signaling Technology, Danvers, MA, USA), rabbit anti-phosphoserine (Invitrogen, Carlsbad, CA, USA), part antibodies including rabbit polyclonal anti-Bcl-2, rabbit polyclonal anti-NF-κB, mouse monoclonal anti-β-actin, mouse monoclonal anti-caspase 3, mouse monoclonal anti-interleukin-10 (IL-10), which were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Secondary antibodies included rabbit anti-mouse HRP-conjugated IgG and goat anti-rabbit HRP-conjugated IgG (Santa Cruz Biotechnology, Inc.).

Taxol (sc-201439) and VBL (sc-491749) were also obtained from Santa Cruz Biotechnology, Inc., and were dissolved in dimethyl sulfoxide (DMSO) at appropriate concentrations, and were stored at -20°C.

**Assessments of cell apoptosis.** Upon reaching 80% confluency, the cells were treated with Taxol or VBL (100 nM) for 24 h, and the solvent DMSO was used as control. Then, the treated cells were harvested and stained with 500 μl specific binding buffer containing 5 μl Annexin V-FITC and 5 μl propidium iodide for 10 min at room temperature in the dark. Analysis of cell apoptosis was performed by flow cytometry at the The Second Xiangya Hospital Affiliated with Central South University.

**MTT assays.** Cells (5,000/well) in a logarithmic growth phase were planted into 96-well plates and treated with Taxol or VBL (100 nM). Next, 10 μl of 5 mg/ml 3-(4,5-dimethylthiazol-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added to each well at time points of 0, 24, 48 and 72 h, and the cells were subsequently incubated at 37°C for 4 h. After the culture supernatants were removed, 100 μl DMSO was added for 10 min, and the absorbance was measured at a 490-nm wavelength. Six parallel wells were performed.

Cellular proliferative viability was calculated using the following formulation: Relative proliferative percentages (%) = (OD<sub>treatment</sub>/OD<sub>control</sub>) × 100%. The percentage of the DMSO control was designed as 100% at all time points.

**Colony formation assays.** Cells (2,000/well) were cultured in media with 10 nM Taxol or VBL in 6-well plates for 1-2 weeks. The culture was suspended when obvious colonies were observed by the naked eye. Cells were fixed with

methanol, and then stained with 0.1% crystal violet for 30 min. Colonies with >50 cells/colony were counted using an inverted microscope. The experiment was carried out in triplicate independently. The relative colony formation efficiency (%) = (colony number/2,000) × 100%.

**Wound healing assays.** Cells were grown in 6-well plates to a monolayer. The cell monolayer was then wounded by creating a scratch using a 200-μl pipette tip. Old medium was replaced with fresh medium containing 100 nM Taxol or VBL. The status of wound healing was monitored at time points of 0, 12, 24 and 36 h, and images were captured for assaying the capability of cell migration in a 2-dimensional plane.

**Transwell assays.** Capability of cell migration in a 3-dimensional space was assessed by Transwell assays according to the protocol described in a previous study (8). Approximately 4 × 10<sup>4</sup> cells were seeded in 200 μl RPMI-1640 medium with 0.2% FBS in the upper chambers of a Transwell, while 800 μl media with 10% FBS was placed in the lower chambers. Twenty-four hours later, the media were removed and the cells were wiped off from the upper chambers using a cotton swab. The remaining migrated cells on the back of the bottom membrane in the upper chamber were fixed with 100% methanol and stained with 0.1% crystal violet. Finally, the Transwell plates were mounted and observed under an inverted microscope, and the migrated cells were counted and images were captured.

**Western blot analysis.** Protein extracts were collected according to our previous studies (9,10). Briefly, cells were lysed with lysis buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 2% SDS, 5 mM DTT, 10 mM PMSF), and then denatured in boiling water for sonication. Protein concentrations were determined using BCA protein assay reagent (Pierce, Rockford, IL, USA).

Protein extracts (50 μg) were separated by 10% SDS-PAGE, electrotransferred to nitrocellulose membrane, blocked with buffer containing 5% non-fat milk and incubated with primary antibodies overnight at 4°C and HRP-conjugated secondary antibodies for 1 h at room temperature and developed with an enhanced chemiluminescence detection kit (Thermo Fisher Scientific, Waltham, MA, USA).

**IP-western blot assays.** Cells were lysed in immunoprecipitation (IP) lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 10% NP-40, 1 mM EDTA, 10% glycerol, 10 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM DTT, 1 mM PMSF and protease inhibitor cocktail tablet), and then the supernatant was collected for detection of the total level of phospho-Op18/stathmin.

Magnetic immunobeads (cat#: 10002D; Thermo Fisher Scientific) were used to bind the anti-stathmin antibody and pull-down Op18/stathmin for western blot analysis as previously described (9).

**Autocrine IL-10 detection.** Equal number of cells were planted in 6-well plates and cultured. The old medium was replaced with fresh medium without phenol red indicator in the presence of Taxol or VBL (100 nM) when cells reached 80% confluency. Twenty-four hours later, the media were collected and centrifuged at 13,000 rpm for 30 min. Autocrine IL-10 in the

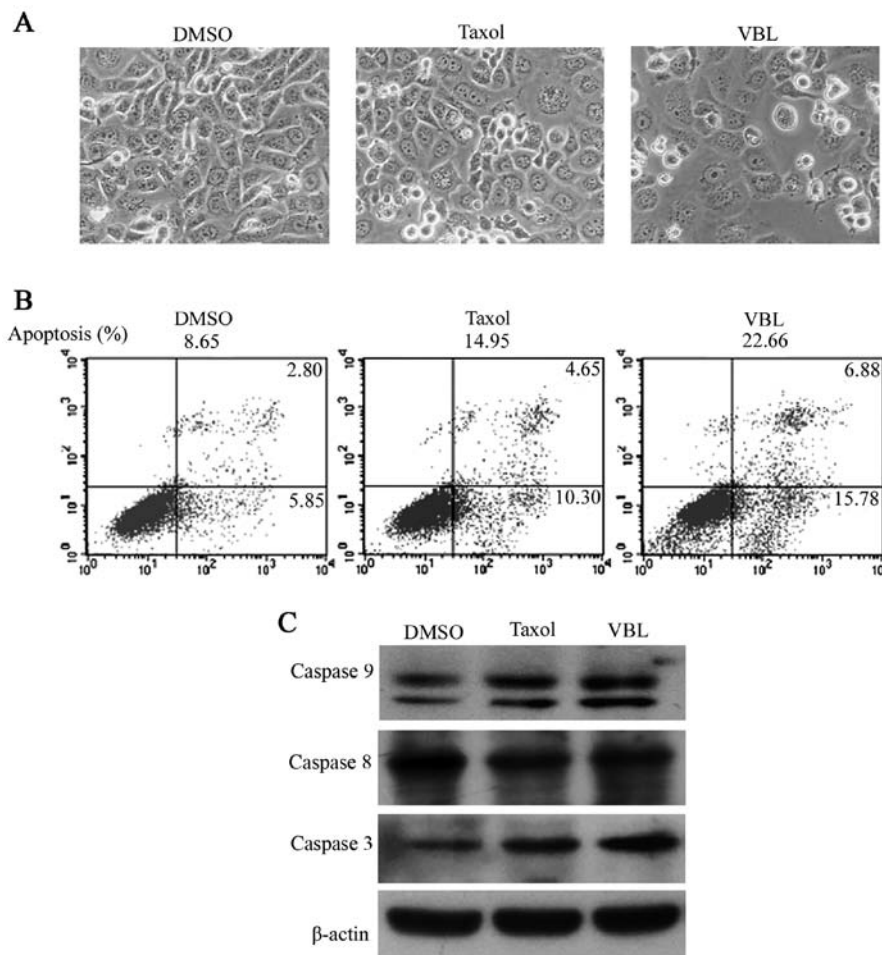


Figure 1. VBL induces a higher cell apoptotic ratio than Taxol. (A) Morphological images show the condition of cellular growth following treatment with DMSO, Taxol and VBL. (B) Analysis of cellular apoptosis by FCM. (C) Western blotting showing the expression of caspase 3, 8 and 9 in the three treatment groups. VBL, vinblastine; Taxol, paclitaxel.

separated supernatant was detected by ELISA using the human IL-10 ELISA kit (Cusabio, Wuhan, China). All experiments were carried out in triplicate.

**Construction of the RNA interference plasmid targeting *Op18/stathmin*.** Plasmids pGCsilencer-U6/Neo/GFP-RNAi (RNAi) with the RNAi gene targeting the coding region of *Op18/stathmin* at 5'-AGAGAACTGACCCACAAA-3' (GenBank no. 53305, sequence 374-393) and pGC-silencer-U6/Neo/GFP-non (Non) with an inserted non-coding sequence and pGC-silencer-U6/Neo/GFP (Blank) without any fragments inserted, were constructed at an early stage and were introduced into cells by Lipofectamine 2000 (Invitrogen) as described in our previous studies (8,10).

**Statistical analysis.** All statistical calculations were performed using the statistical software program SPSS 17.0. Differences with p-values <0.05 were considered statistically significant.

## Results

**NCI-H1299 cells are more sensitive to VBL than to Taxol.** Morphological images showed that cells grew to nearly complete confluency in the control DMSO, whereas cell

distribution became gradually dispersed with the appearance of a few translucent floating cells in the Taxol treatment cell group. A large number of suspended cells emerged and only a minority of cells adhered to the wall at a low density in the VBL treatment group (Fig. 1A).

Cell apoptotic ratios were 8.65, 14.95 and 22.66% following treatment with the solvent (DMSO), Taxol and VBL, respectively, in the NCI-H1299 cells as detected by FCM. The apoptotic ratio was significantly higher for VBL treatment compared with Taxol (Fig. 1B).

Western blot analysis showed that both Taxol and VBL increased the expression levels of caspase 3 and 9, but to a greater extent following VBL treatment, while no obvious changes in caspase 8 were observed in the three treatment groups (Fig. 1C).

**VBL significantly inhibits cell proliferation and colony formation compared with Taxol.** By contrast with DMSO, the relative cell proliferation percentages were 84.24, 55.11 and 32.00%, respectively, for VBL treatment at time points of 24, 48 and 72 h, while these percentages were 95.86, 91.47 and 86.46%, respectively, following Taxol treatment. The representative curve steeply declined with increasing exposure time to VBL, whereas, it declined slowly in the presence of Taxol (Fig. 2A).

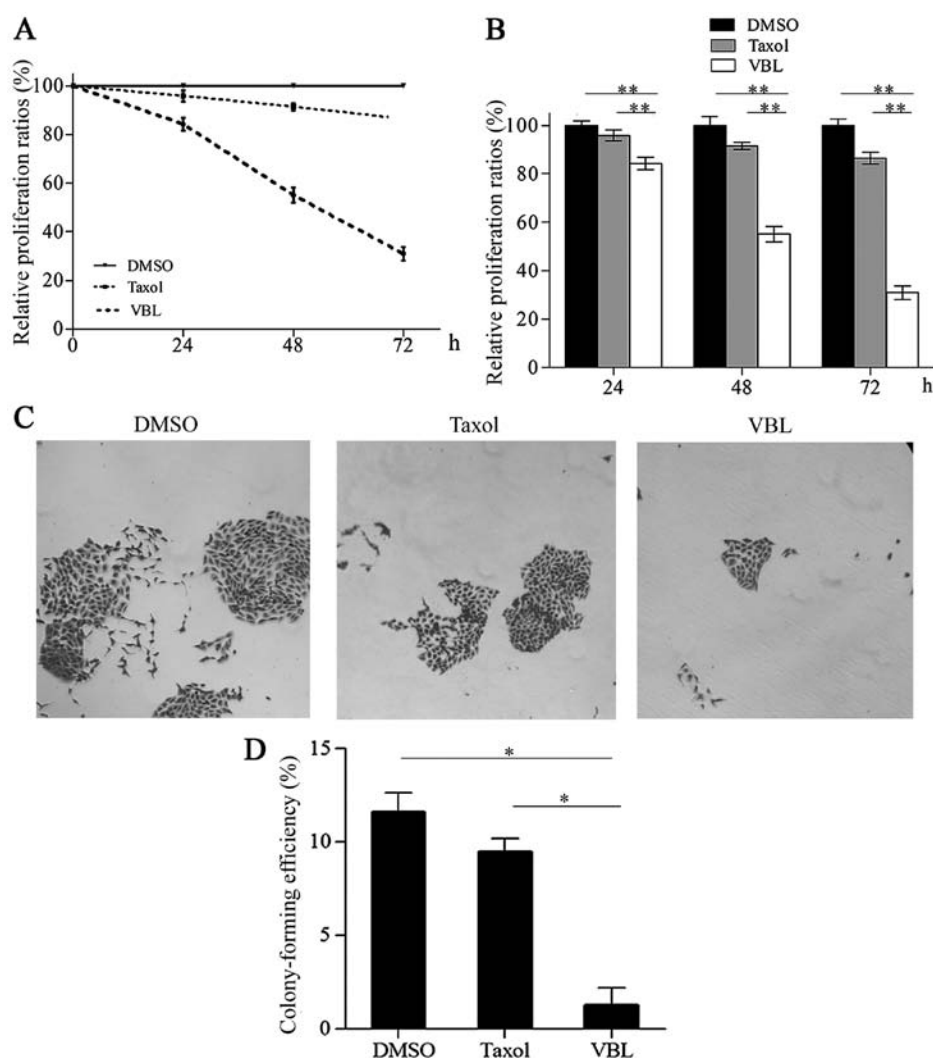


Figure 2. VBL significantly inhibits cell proliferation and colony formation compared with Taxol. (A) Curves represent relative proliferative trends of NCI-H1299 cells in terms of the different treatments. (B) Histograms illustrate statistical differences in cellular proliferation between the VBL treatment group and the other two treatment groups. (C) Images show the status of colony formation for the different treatment groups. (D) VBL significantly inhibited colony formation compared with Taxol; \* $p < 0.05$ , \*\* $p < 0.01$ . VBL, vinblastine; Taxol, paclitaxel.

Statistical differences existed in the proliferation percentages between VBL and that of the other two groups for Taxol and DMSO at 24, 48 and 72 h ( $p < 0.01$ ) (Fig. 2B).

Colony formation assays revealed that only a few small colonies appeared in the VBL treatment group, while some relative larger colonies emerged in the Taxol treatment group. There were a large number of large colonies which generally merged into an extensive one with unclear borders in the control DMSO group (Fig. 2C). The mean colony formation efficiency was 11.60, 9.47 and 1.07%, respectively, in the DMSO, Taxol and VBL treatment group. VBL significantly reduced the percentage of colony formation in comparison with the Taxol and DMSO treatment groups ( $p < 0.05$ ) (Fig. 2D).

*VBL notably suppresses cell migration in contrast with Taxol.* The scratch-wound trails obviously became narrow with time in the three groups, and were completely recovered at time point of 36 h in the DMSO treatment group, while the scratched areas nearly healed for Taxol induction. However, a large area of blank region still remained in the cells treated with VBL (Fig. 3A).

Transwell assay analysis revealed that the mean number of invading cells was 27, 16 and 8, respectively, in the DMSO, Taxol and VBL treatment group. The number of migrated cells was greatly decreased following treatment with VBL when compared to the Taxol treatment group, whereas Taxol also inhibited cell migration in contrast with DMSO (Fig. 3B). Differences in the number of migrating cells were significant between the VBL group and the other two treatment groups; VBL was the most notable in inhibiting cell migration of a 3-dimensional space ( $p < 0.01$ ) (Fig. 3C).

*VBL promotes the phosphorylation of Op18/stathmin, while Taxol decreases the expression and phosphorylation of Op18/stathmine.* Western blot analysis revealed that the expression of Op18/stathmin was decreased by Taxol, but was not obviously affected by VBL (Fig. 4A). IP-western blotting showed that Taxol decreased the phosphorylation of Op18/stathmin; however, VBL upregulated the total level of phosphorylated Op18/stathmin (Fig. 4B).

VBL universally increased the level of phosphorylated Op18/stathmin at all 4 serine sites including Ser63, Ser38,

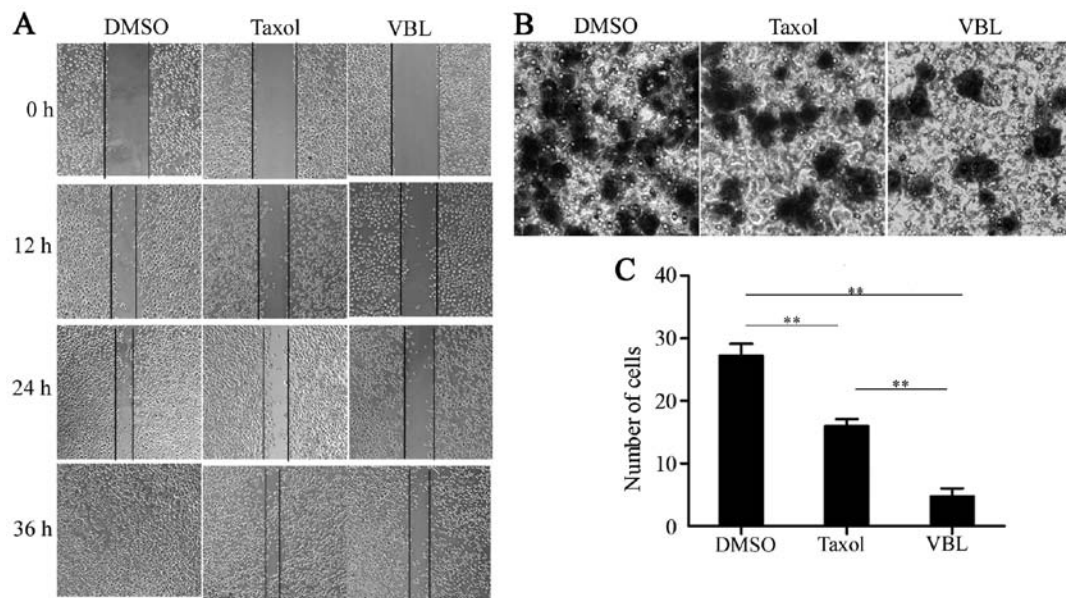


Figure 3. VBL notably inhibits cell migration in contrast with Taxol. (A) Wound healing assays demonstrated the status of cell migration in a 2-dimensional plane. (B) Transwell assay analysis shows the cell aggregation in 3 dimensional space. (C) Histograms show the statistical differences in migrated cells in the different treatment groups; \*\* $p < 0.01$ . VBL, vinblastine; Taxol, paclitaxel.

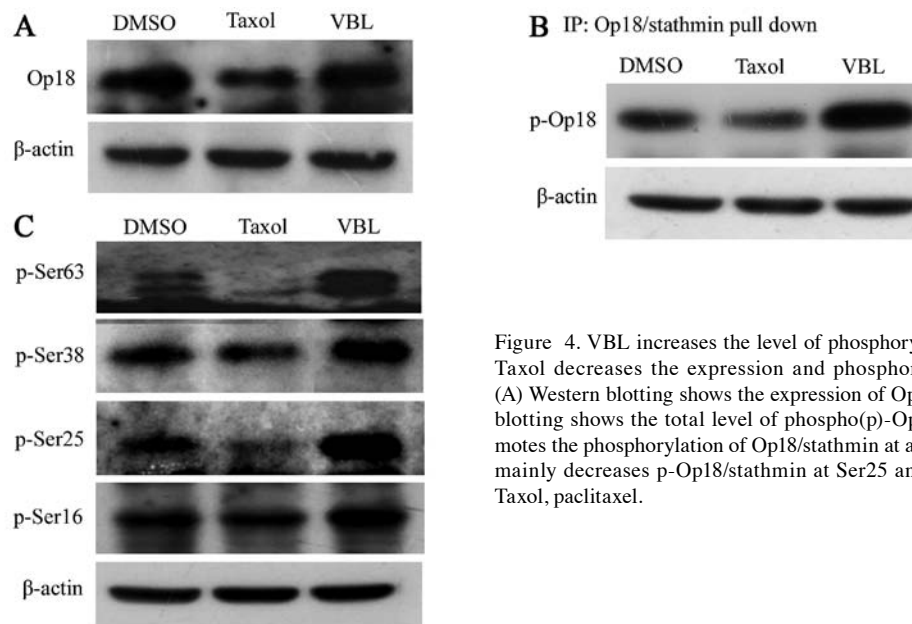


Figure 4. VBL increases the level of phosphorylated Op18/stathmin, while Taxol decreases the expression and phosphorylation of Op18/stathmin. (A) Western blotting shows the expression of Op18/stathmin. (B) IP-western blotting shows the total level of phospho(p)-Op18/stathmin. (C) VBL promotes the phosphorylation of Op18/stathmin at all 4 serine sites, while Taxol mainly decreases p-Op18/stathmin at Ser25 and Ser63. VBL, vinblastine; Taxol, paclitaxel.

Ser25 and Ser16, while Taxol mainly reduced phosphorylated Op18/stathmin at Ser25 and Ser63 sites, particularly, p-Ser63-Op18/stathmin was almost completely inhibited, yet Taxol did not influence the phosphorylation of Op18/stathmin at Ser16 and Ser38 (Fig. 4C).

*Op18/stathmin RNAi reduces the difference in cell proliferation inhibition between VBL and Taxol.* MTT analysis demonstrated that the proliferation ratios were 99.7, 96.79 and 94.48%, and 68.73, 54.38 and 50.88%, at time points 24, 48 and 72 h in the non-silenced and the RNAi groups respectively in comparison with the DMSO group. The representative curve of the RNAi group declined rapidly, the curve of the

non-silenced group nearly overlapped the one of the Blank. After co-treatment with Op18/stathmin RNAi and VBL or Taxol, the relative proliferative ratios were 60.95, 40.32 and 27.16%, and 58.69, 33.20 and 24.66%, respectively, at the three time points, while the corresponding curves were highly similar between VBL and Taxol, which declined steeply compared with the one for single RNAi (Fig. 5A). Histograms showed that the difference was not significant between the Blank and the non-silenced group, but was increased between the RNAi groups and the blank control ( $p < 0.01$ ). The combination of RNAi and VBL or Taxol significantly inhibited cell proliferation in contrast with RNAi alone treatment ( $p < 0.01$ ), whereas Op18/stathmin RNAi reduced the difference in cell

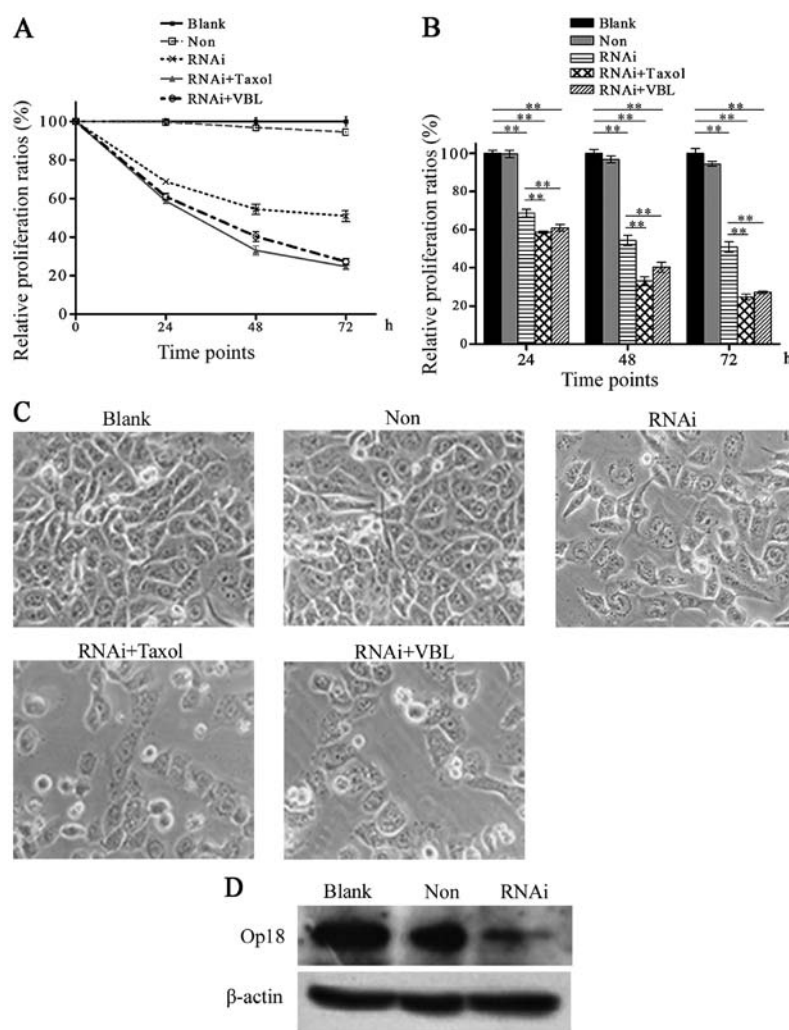


Figure 5. Op18/stathmin RNAi reduces the difference in cell proliferation inhibition between VBL and Taxol. (A) Curves represent the trends of cell proliferation for the different treatment groups. (B) Statistical differences are illustrated. (C) The conditions of cell growth are captured by images. (D) Op18/stathmin RNAi effectively inhibited the expression of Op18/stathmin; \* $p < 0.05$ , \*\* $p < 0.01$ . VBL, vinblastine; Taxol, paclitaxel.

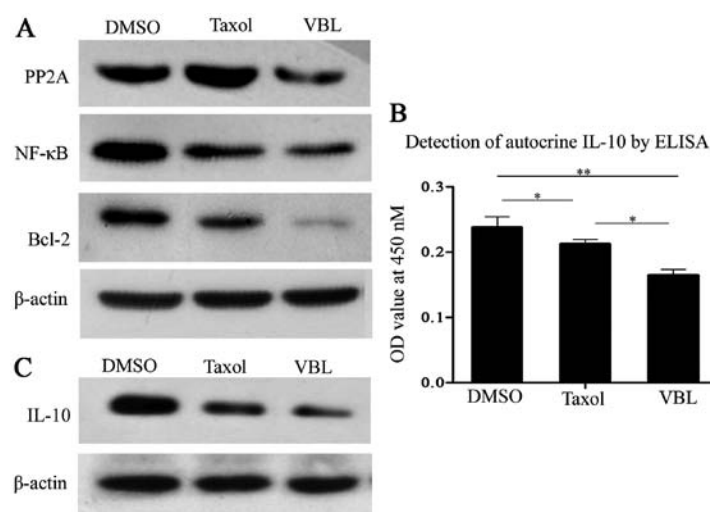


Figure 6. VBL decreases the expression of PP2A, Bcl-2, NF-κB and IL-10 and autocrine IL-10 in comparison with Taxol. (A) Western blotting shows the expression levels of PP2A, NF-κB and Bcl-2. (B) Detection of autocrine IL-10 from the culture supernatant by ELISA. (C) Western blot analysis shows the expression of IL-10 in the DMSO, Taxol and VBL treatment groups; \* $p < 0.05$ , \*\* $p < 0.01$ . VBL, vinblastine; Taxol, paclitaxel.

proliferation inhibition between VBL and Taxol at all time points (Fig. 5B).

Cell growth images show that cell proliferation was obviously inhibited by RNAi, while the inhibition was increased

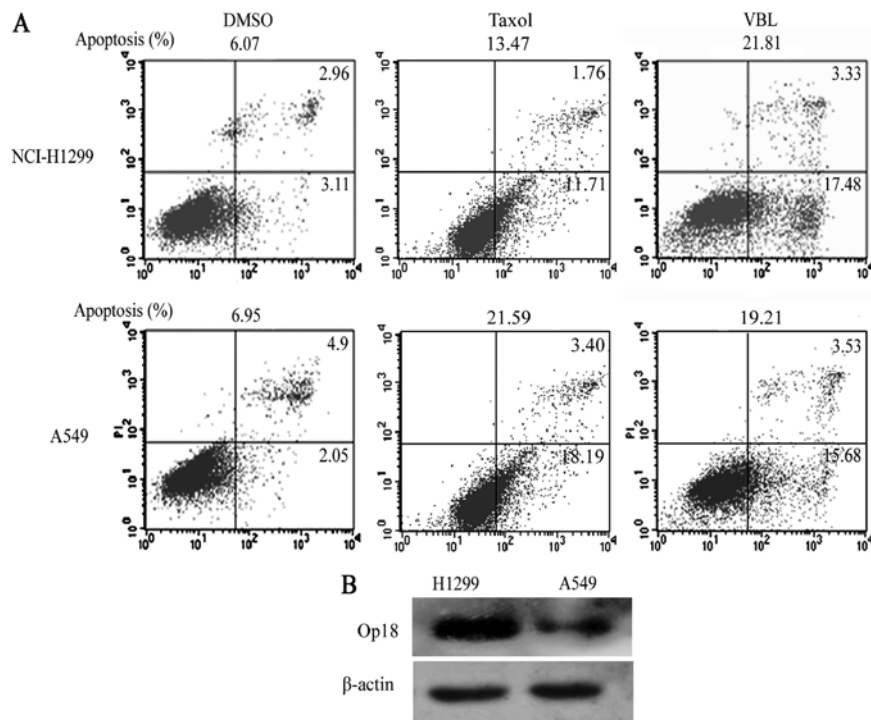


Figure 7. High expression of Op18/stathmin is negatively relevant to the sensitivity of Taxol in different NSCLC cells, but only exerted few impacts on VBL cytotoxicity. (A) FCM shows cellular apoptotic ratios in NCI-H1299 and A549 cells. (B) Western blotting shows the expression of Op18/stathmin. VBL, vinblastine; Taxol, paclitaxel.

following co-treatment of RNAi and VBL or Taxol. Cells became much sparser in the co-treated groups with RNAi than the RNAi alone treatment group (Fig. 5C).

Western blot analysis showed that Op18/stathmin RNAi effectively inhibited the expression of Op18/stathmin in the NCI-H1299 cells (Fig. 5D).

*VBL markedly weakens the expression levels of PP2A, Bcl-2, NF-κB and IL-10 and autocrine IL-10 in comparison with Taxol.* Western blot analysis showed that Taxol notably enhanced the expression of protein phosphatase 2A (PP2A), but PP2A was substantially decreased by VBL, while both Taxol and VBL treatments decreased the expression of nuclear factor-κB (NF-κB), B-cell lymphoma-2 (Bcl-2). However, the inhibitory effects were more notable for VBL induction than Taxol (Fig. 6A).

ELISA demonstrated that autocrine IL-10 from cell culture supernatants was decreased by Taxol and VBL, but VBL induced more obvious inhibitory effects when compared with Taxol. Histograms showed that the differences were significant between the VBL and the other two treatment groups (Fig. 6B).

Western blot analysis showed that both Taxol and VBL inhibited the expression of IL-10 in the NCI-H1299 cells, but VBL was more effective than Taxol (Fig. 6C).

*High expression of Op18/stathmin is negatively correlated with the sensitivity of Taxol in the different NSCLC cells, but only exerts a mild impact on VBL.* FACS showed that cellular apoptotic percentages were 6.07, 13.47 and 21.81%, respectively, in the NCI-H1299 cells in the DMSO, Taxol and VBL treatment groups, while they were 6.95, 21.59 and 19.21% respectively in A549 cells in the corresponding groups. NCI-H1299 cells

presented notable resistance to Taxol when compared with VBL, but the sensitivity of Taxol was similar to VBL in the A549 cells (Fig. 7A).

Western blot analysis demonstrated that NCI-H1299 cells highly expressed Op18/stathmin compared with the A549 cells (Fig. 7B).

## Discussion

Op18/stathmin is thought to be closely associated with cellular proliferation, differentiation and drug resistance of tumors (11-14). Our previous studies demonstrated that Op18/stathmin is regulated by multiple kinases including cyclin dependent 2 (CDC2) and extracellular signal regulated kinase (ERK), and that high expression of Op18/stathmin is positively correlated with the resistance of Taxol in different epithelial-derived carcinoma cells (8-10).

The present study demonstrated that NCI-H1299 cells were more sensitive to VBL than to Taxol. VBL obviously promoted cell apoptosis through initiating the activation of caspase 3 and 9, and inhibited cell proliferation, colony formation and cell migration in contrast with Taxol. VBL mainly decreased the activity of Op18/stathmin by triggering the phosphorylation of Op18/stathmin at all 4 serine sites. On the contrary, Taxol decreased the expression of Op18/stathmin and the phosphorylation at Ser25 and Ser63. In short, VBL, completely different from Taxol, mediates the Op18/stathmin signaling pathway by increasing the phosphorylation of Op18/stathmin. Other studies also confirmed that the phosphorylation of Op18/stathmin at 4 serine residues is involved in cell apoptosis induced by exogenous tumor necrosis factor (TNF) in mouse fibrosarcoma L929 cells, and wee-1 knockdown

promoted the sensitivity to VBL through augmenting phosphorylation of Op18/stathmin at Ser25 and Ser63 in human breast carcinoma BT20ST1 cells. Blocking ERK by PD98059 enhanced the sensitivity to Taxol through decreasing the expression of Op18/stathmin and the phosphorylation in NCI-H1299 cells (9,15,16).

Previous studies demonstrated that silencing of Op18/stathmin by RNAi promoted the sensitivity of breast cancer BT549 cells to Taxol and VBL as well as the sensitivity of nasopharyngeal carcinoma CNE1 cells to Taxol and high grade differentiated CNE1 xenografted tumors in nude mice (16,17). The present study found that Op18/stathmin RNAi reduced the differences in cellular proliferation inhibition between VBL and Taxol, which implies that Op18/stathmin signaling mediates the sensitivities of both VBL and Taxol.

PP2A is an important serine/threonine phosphatase acting to dephosphorylate Op18/stathmin (18). NF- $\kappa$ B is a transcription factor involved in cell survival and proliferation, which interacts with Op18/stathmin to promote tumor growth and predicts poor prognosis of pancreatic cancer (19-21). IL-10 is a pleiotropic cytokine which plays a vital role in tumor growth and drug resistance, of which high expression is associated with poor prognosis in tumor patients (22-24). Bcl-2 is a typical protein against cellular apoptosis induced by chemotherapeutics (25,26). The present study showed that the expression of PP2A was increased by Taxol, but decreased by VBL, which coincided with the status of phospho-Op18/stathmin for Taxol or VBL induction, while both Taxol and VBL inhibited the expression levels of NF- $\kappa$ B, Bcl-2 and IL-10 as well as autocrine IL-10 in NCI-H1299 cells, but the inhibitory effects were more obvious for VBL than for Taxol.

It has been reported that overexpression of Op18/stathmin markedly decreases microtubule binding with Taxol and increased binding with VBL, downregulated the sensitivity to Taxol, but to VBL to a lesser extent in human breast cancer cells (27,28). Others have found that high expression of Op18/stathmin increased the sensitivity to vindesine and vincristine (one of vinca alkaloids) in human lung carcinoma cells, while crystallographic analysis showed that Op18/stathmin increased VBL binding to tubulin. Conversely, VBL also increased Op18/stathmin binding to tubulin *in vitro* (29-31). The present study indicated that high expression of Op18/stathmin was negatively associated with the sensitivity of Taxol in different NSCLC cell lines, but exerted slight impact on VBL cytotoxicity, which is an attractive alternative for the treatment of Taxol-resistant tumors with highly expressed Op18/stathmin.

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