

Down-regulation of P-Glycoprotein Is Associated with Resistance to Cisplatin and VP-16 in Human Lung Cancer Cell Lines

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Abstract. Aim: To investigate whether down-regulation of P-glycoprotein (P-gp) is correlated to resistance to cisplatin and VP-16 in four histopathological subtype cell lines of lung cancer (SK-MES-1, SPCA-1, NCI-H-460 and NCI-H-446). Materials and Methods: After pretreatment with or without verapamil, the P-gp expression was examined by means of RT-PCR and immunofluorescence. Cell survival on treatment with cisplatin and VP-16 was determined by MTT assay. Results: The expression of P-gp was clearly inhibited by verapamil in all four cell lines. Following pretreatment with verapamil, NCI-H-446 was more sensitive to cisplatin, while SPCA-1, NCI-H-460 and NCI-H-446 were more sensitive to VP-16 compared to the control. Conclusion: Down-regulation of P-gp is associated with intrinsic resistance to cisplatin in the NCI-H-446 and to VP-16 in SPCA-1, NCI-H-460 and NCI-H-446 cell lines. These findings indicate that down-regulation of P-gp may be helpful for the reversion of drug resistance in some lung cancer cell line subtypes.

Lung cancer is one of most prevalent types of cancer and the leading cause of cancer mortality in the world (1). Chemotherapy treatment for lung cancer prolongs patient survival, but drug resistance, and especially intrinsic multidrug resistance, limit the chance of successful chemotherapy (2). Resistance to anticancer agents is frequently observed both in small cell lung cancer (SCLC) and in non-small cell lung cancer (NSCLC) (3).

Resistance to chemotherapy is mediated via a range of cellular alterations including reduced drug accumulation, changes in the level of protein targets, mutations that diminish the binding of the drug to the target, increased trapping of the drug in acidic vesicles, alterations in the metabolism of drugs, increased tolerance of cellular damage and diminished apoptotic signaling (4, 5). The reduction of drug accumulation occurs through a series of proteins, among which P-glycoprotein (P-gp) is the most important P-gp, encoded by the *MDR1* gene, is a 170 kDa phosphorylated glycoprotein and a member of the ATP-binding cassette (ABC) superfamily of membrane transporters (6). In tumor cells, the overexpression of P-gp is responsible for the efflux of various hydrophobic chemotherapeutic agents from the cells, which decreases their intracellular accumulation and leads to a low efficacy of chemotherapy and to multidrug resistance (7). P-gp is localized in the plasma membrane of resistant cancer cells, and can bind and transport a great variety of structurally and functionally unrelated antitumor drugs, such as vinblastine, vincristine, doxorubicin, daunorubicin, VP-16, teniposide, paclitaxel and many others in an ATP-dependent manner (8, 9). It has been suggested that P-gp is one of the important factors in the intrinsic chemoresistance of lung cancer cells (10).

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P-gp appears to be involved in drug resistance in human cancer; it is therefore an urgent matter to find mediators which can reverse the drug resistance mediated by P-gp (11). Verapamil, a phenylalkylamine L-type calcium channel antagonist, is a first-generation inhibitor of P-gp. It has been reported that verapamil can inhibit the expression of P-gp in several P-gp-positive cell lines, including human leukaemic, hamster lung cancer and human breast cancer cell lines (12-14). However, few studies relate to human lung cancer, and particularly to different histopathological subtypes. The aim of this study, therefore, was to investigate the expression of P-gp pretreated by verapamil and the effect of the down-regulation of P-gp in intrinsic drug resistance to cisplatin and VP-16 in different histopathological subtypes of lung cancer cell lines [squamous carcinoma, adenocarcinoma, large cell lung cancer (LCLC) and (SCLC)].

Materials and Methods

Cell culture and treatment. Histopathological subtypes of four human lung cancer cell lines, SK-MES-1, SPCA-1, NCI-H-460 and NCI-H-446 (squamous carcinoma, adenocarcinoma, LCLC and SCLC, respectively), were obtained from the American Type Culture Collection (Manassas, VA, USA). SK-MES-1 was maintained in EMEM (GIBICO, Carlsbad, CA, USA), and SPCA-1, NCI-H-460 and NCI-H-446 were cultured in RPMI-1640 medium (HyClone, Logan, UT, USA), supplemented with 10% foetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO₂. Before the experiments, the cells of each histopathological type were divided into an experimental group and a control group, depending on the addition of verapamil (10 µM; Sigma, St.Louis, MO, USA) or not for 24 h. All experiments were performed with exponentially growing cells and were repeated at least 3 times.

RNA isolation and RT-PCR. Total RNA extraction and RT-PCR were carried out as previously described (15). In brief, total cytoplasmic RNA was extracted from the cells of the two groups by using RNAiso plus (TaKaRa, Japan) according to the manufacturer's instructions. RT-PCR was performed with an RNA PCR Kit (AMV, Ver.3.0, TaKaRa, Japan). The housekeeping gene *GAPDH* was used as an internal control to confirm equal loading in each experiment and was amplified from the same cDNAs. The primers specific to *MDR1* and *GAPDH* are shown in Table I. Reverse transcription was performed at 50°C for 30 minutes, and terminated at 99°C for 5 minutes and 5°C for 5 minutes. The cDNA generated from this experiment was then subjected to PCR to amplify the P-gp transcript. The PCR cycle started with 1 cycle of 3 minutes at 94°C, 33 cycles of 45 seconds at 94°C, 45 seconds at 58°C, and 1 minute at 72 °C; this was followed by a final incubation at 72°C for 15 minutes. A total of 5 µl of PCR product was separated on a 2% agarose gel and stained with ethidium bromide for visualization. The relative abundance of each PCR product was determined by quantitative analysis of digital photographs of gels, using Labworks 4.6 software (UVP Products, Upland, CA, USA). The integral optical density (IOD) values of *MDR1* and *GAPDH* were measured. The ratio IOD_{*MDR1*}/IOD_{*GAPDH*} was used to represent the relative mRNA level of P-gp.

Table I. Sequence of primers and products for RT-PCR.

Gene	Sequence of primers	Products (bp)
<i>MDR1</i>	5' ATATCAGCAGCCCACATCAT 3' 5' GAAGCACTGGGATGTCCGGT 3'	154
<i>GAPDH</i>	5' GCCAAAAGGGTCATCATCTC 3' 5' GTAGAGGCAGGGATGATGTTC 3'	287

Table II. IC₅₀s of cisplatin and VP-16 in verapamil-pretreated and untreated lung cancer cell lines.

Cell line	IC ₅₀ of cisplatin (µM)		IC ₅₀ of VP-16 (µM)	
	Control	Verapamil	Control	Verapamil
SK-MES-1	8.28±0.61	5.36±1.41	14.76±1.47	13.08±1.58
SPCA-1	2.28±0.13	1.95±0.16	67.39±4.3	50.69±2.25*
NCI-H-460	4.82±0.95	3.58±1.01	62.37±2.88	45.79±4.47*
NCI-H-446	4.74±0.38	3.33±0.24*	56.35±3.15	43.61±1.64*

* Relative to the control group ($p < 0.05$, CI: 95%).

Immunofluorescence. The cells in the exponential phase were dispensed onto the coverglass in 6-well plates at a density of 1×10⁵ cells per well. The cells of the two groups were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min, and permeabilized in PBS containing 0.1% Triton X-100 and 5% bovine serum albumin (BSA) for 1 h before the detection of P-gp with immunofluorescence. The media, with anti-P-gp goat polyclonal antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) at a dilution of 1:400, were introduced into the culture chamber and the cells were incubated at 4°C overnight. After being washed with PBS three times, the cells were stained with anti-goat fluorescein isothiocyanate (FITC) green-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.) at a dilution of 1:200 at 37°C for 1 h. Cell images were subsequently captured with a fluorescence microscope and analyzed by using Northern exposure image analysis/archival software (Mississauga, Ontario, Canada).

Cell viability assay. Cell viability was measured with the MTT assay (16). Briefly, cells in the exponential phase were dispensed in 96-well plates overnight at a density of 1×10⁴ cells per well. The cells were incubated with cisplatin or VP-16 at different concentrations (cisplatin: 0, 2, 4, 6 and 8 µM; VP-16: 0, 20, 40, 60 and 80 µM) and after a 48-h incubation, 20 µl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) reagent was added into each well and cultured at 37°C in a 5% CO₂ incubator for 4 h. The medium was discarded, 100 µl of dimethyl sulfoxide (DMSO) were added into each well, and the mixture was incubated for 10 min. The optical density of each well was measured with a Multiskan Ascent (Thermo Fisher Scientific, MA, USA). For analysis, the sensitivity to cisplatin and VP-16, the cell viability and IC₅₀ were calculated via the following equations: cell viability=mean optical density of the anticancer drug-treated group/mean optical density of the control group×100%; IC₅₀=concentration of cisplatin/VP-16 at 50% cell viability.

Statistical analysis. All data are given as means \pm SE. Statistical comparisons were made by using the independent-samples *t*-test. All analyses were performed with SPSS 16.0 software. A *p* value of <0.05 (CI: 95%) was considered to indicate statistical significance.

Results

Down-regulation of P-gp by verapamil at the mRNA level in different lung cancer cell lines. To examine the effect of verapamil on MDR1 mRNA expression, RT-PCR analysis was carried out on the four cell lines pretreated with or without verapamil. As shown in Figure 1, verapamil pretreatment caused a decrease in P-gp at the mRNA level in all four cell lines. As compared with the control group, there was a significant decrease in the verapamil-pretreated group for all four cell lines, the result being ~0.6- to 8-fold lower than that for the control group (all *p* values<0.05). However, the levels of reduction observed for the cell lines differed. For example, *MDR1* for NCI-H-446 was ~8-fold lower than that for the control group (0.06 \pm 0.01 vs. 0.47 \pm 0.03), whereas the reduction was only 0.6-fold for the SK-MES-1 cell line (0.34 \pm 0.04 vs. 0.56 \pm 0.07).

Downregulation of P-gp by verapamil at the protein level in different lung cancer cell lines. To investigate whether the down-regulation of P-gp at the mRNA level was related to its protein expression for the cells pretreated with verapamil, immunofluorescence assays were carried out on the four cell lines that were additionally pretreated with or without verapamil. As shown in Figure 2, the immunofluorescence results demonstrated that P-gp was localized on the membranes of the cells. Similarly to the P-gp mRNA results, the fluorescence intensity of the pretreated cells was clearly weaker than that of the control group for all four cell lines. The normalized fluorescence intensities per cell in the experimental group were ~2- to 3-fold lower than that of the control group (17.26 \pm 1.93 vs. 36.34 \pm 1.32, 17.52 \pm 3.68 vs. 33.46 \pm 1.63, 18.49 \pm 1.01 vs. 47.89 \pm 4.13 and 16.44 \pm 1.01 vs. 47.87 \pm 5.15 for SK-MES-1, SPCA-1, NCI-H-460 and NCI-H-446 cell lines, respectively, all *p*-values <0.05, CI: 95%).

Effects of down-regulation of P-gp on the resistance to cisplatin and VP-16 in different lung cancer cells. To determine the effects of the down-regulation of P-gp on the resistance to cisplatin and VP-16 in the four cell lines, the cell viabilities were determined by MTT assay, and the IC₅₀s of cisplatin and VP-16 for the cell lines pretreated with or without verapamil were calculated. As shown in Table II and Figure 3, in comparison with the control group, verapamil pretreatment caused a significant decrease in the IC₅₀ of cisplatin only in the NCI-H-446 cell line (3.33 \pm 0.24 vs. 4.74 \pm 0.38, *p*<0.05, CI: 95%), and not in the other three cell lines. The IC₅₀ of VP-16 significantly decreased in the verapamil-pretreated cell lines of SPCA-1 (50.69 \pm 2.25 vs.

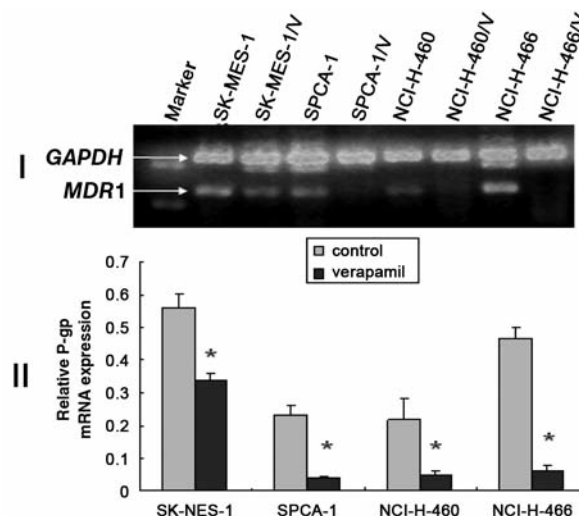


Figure 1. RT-PCR results for *MDR1* mRNA in four cell lines pretreated with or without verapamil, at 10 μ M for 24 h (experimental or control group) (SK-MES-1/V, SPCA-1/V, NCI-H-460/V and NCI-H-446/V, pretreated with verapamil). Bar graph showing the relative levels of P-gp mRNA evaluated by using the ratio IOD *MDR1*/IOD *GAPDH* for the four cell lines. **p*<0.05 relative to control group (CI: 95%).

67.39 \pm 4.3), NCI-H-460 (45.79 \pm 4.47 vs. 62.37 \pm 2.88) and NCI-H-446 (43.61 \pm 1.64 vs. 56.35 \pm 3.15), all *p*-values<0.05 (CI: 95%), but not for the SK-MES-1 cell line. This indicated that the down-regulation of P-gp is associated with the resistance to cisplatin in the NCI-H-446 cell line and to VP-16 in the SPCA-1, NCI-H-460 and NCI-H-446 cell lines.

Discussion

Drug resistance remains a major challenge for successful cancer chemotherapy (17). P-gp is one of the possible causes of drug resistance and failure of cancer chemotherapy (18). Methods to overcome the drug resistance mediated by P-gp are therefore desirable. This has led to the development of inhibitors during the past two decades (19). In the present study, the effects of verapamil on the expression of P-gp, the correlation between the down-regulation of P-gp and the resistance to cisplatin and VP-16 in lung cancer cell histopathological subtypes were investigated. The results demonstrated that verapamil inhibited the expression of P-gp significantly in all the four cell lines, at both mRNA and protein levels. The down-regulation of P-gp increased the chemosensitivity to cisplatin in the NCI-H-446 cell line, and to VP-16 in the SPCA-1, NCI-H-460 and NCI-H-446 cell lines. The findings indicated that verapamil is an effective inhibitor of P-gp, and the down-regulation of P-gp is associated with chemoresistance to cisplatin and VP-16 in lung cancer.

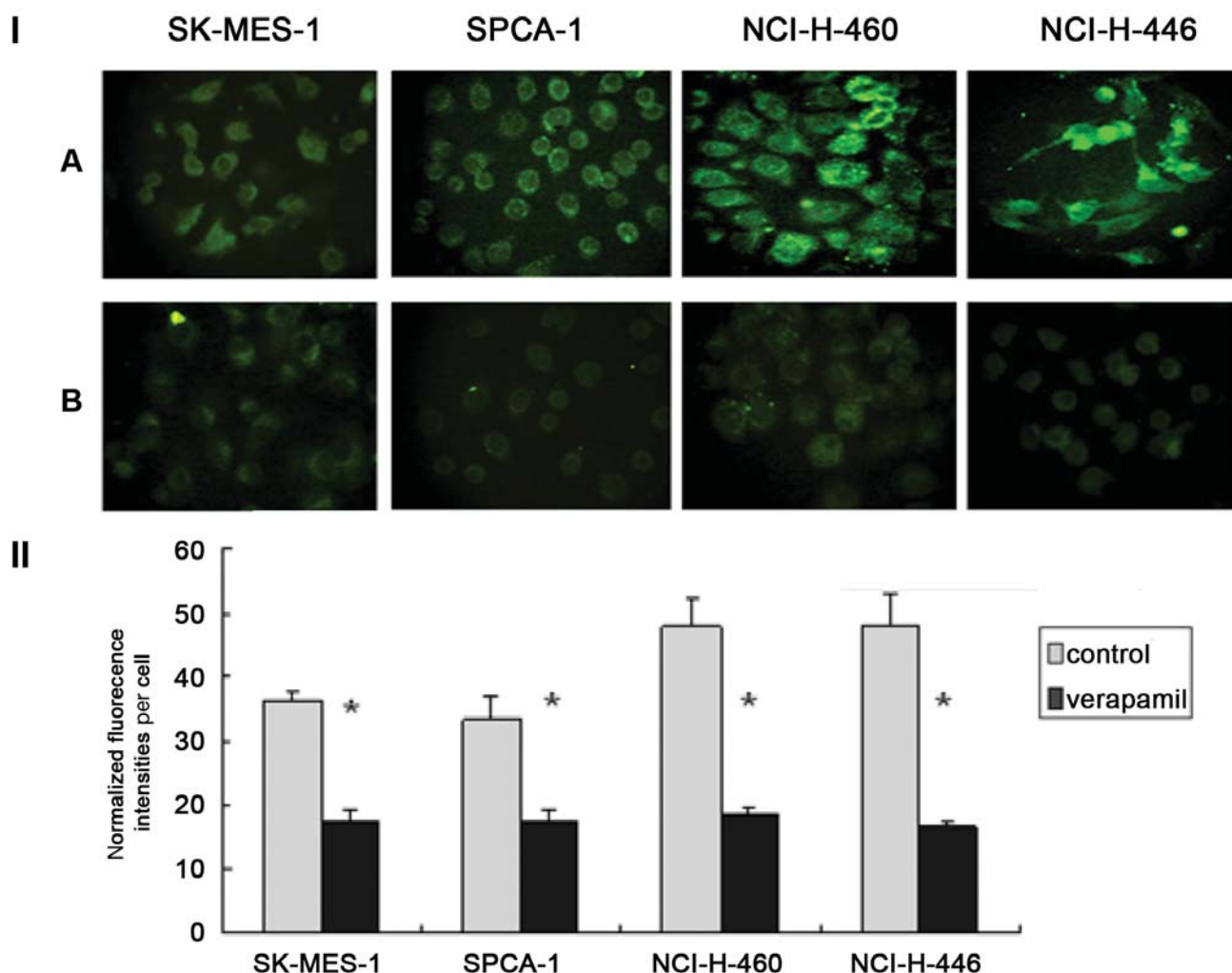


Figure 2. P-gp immunofluorescence results for the cell lines SK-MES-1, SPCA-1, NCI-H-460 and NCI-H-446 pretreated with or without verapamil. A, control group; B, pretreated with verapamil. The average expression of P-gp per cell reflected by normalized fluorescence intensities decreased on pretreatment with verapamil (II). The normalized fluorescence intensity per cell was determined as the number of cells in a region divided by the fluorescence intensity in that region. * $p < 0.05$ relative to the control group (CI: 95%).

Thus, the current study revealed that verapamil inhibits the expression of P-gp in human lung squamous carcinoma, adenocarcinoma, LCLC and SCLC cell lines (SK-MES-1, SPCA-1, NCI-H-460 and NCI-H-446), indicating that verapamil is potentially an appropriate inhibitor of P-gp in lung cancer. Many other reports have described that verapamil can inhibit the expression of P-gp. Sulova *et al.* (14) found that verapamil induced a depression of P-gp expression in the vincristine resistance cell line L1210/VCR. Takara *et al.* (12) reported that verapamil can decrease the expression of P-gp in the HeLa cell subline Hvr100-6, mainly *via* the down-regulation of P-gp mRNA. As concerns the mechanism, Hosey considered that verapamil can block L-type calcium channels and also alter the Na^+ - Ca^{2+} exchanger transport activity (20).

This may induce alterations in calcium homeostasis and affect the expression of P-gp (14, 20). However, the current study indicated that the degree of P-gp reduction differed for the four cell lines pretreated with verapamil, which suggested that selection of the inhibitor of P-gp should be based on the lung cancer cell line subtype.

The accumulated evidence indicates that P-gp is associated with chemotherapy resistance to many anticancer drugs, such as vincristine, doxorubicin, actinomycin D, mitomycin C, cisplatin and VP-16 (21-24). The current study revealed that the down-regulation of P-gp was able to increase the chemosensitivity to cisplatin in the SCLC cell line (NCI-H-446) and to VP-16 in the adenocarcinoma, LCLC and SCLC cell lines (SPCA-1, NCI-H-460 and NCI-

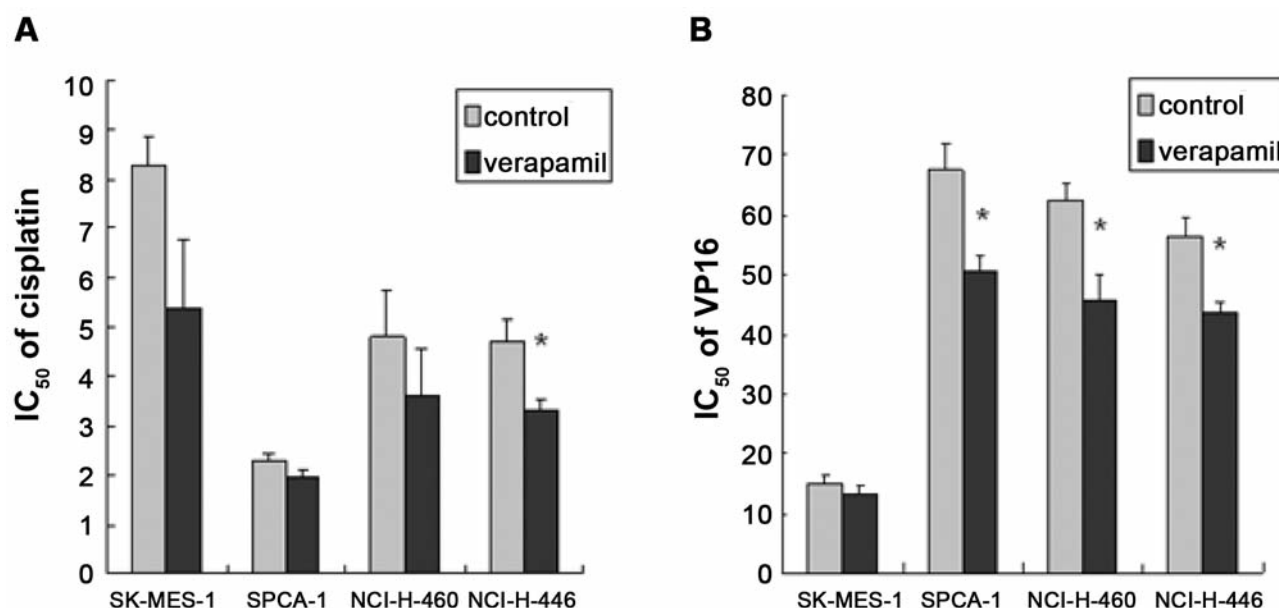


Figure 3. IC₅₀ values of cisplatin (A) and VP-16 (B) for four cell lines pretreated with or without verapamil (10 μ M) for 24 h as MTT viability assay. * $p < 0.05$ relative to control group.

H-446). Similarly to the current results, Triller *et al.* (22) observed that the expression of P-gp correlated with the resistance to cisplatin in SCLC cells. Murphy *et al.* also found that P-gp plays an important role in the resistance to VP-16 in SCLC and NSCLC cell lines (25). All these data demonstrate that P-gp-mediated resistance is important in lung cancer chemotherapy. The mechanisms of resistance to cisplatin or VP-16 mediated by P-gp are possibly similar: with the energy derived from ATP, P-gp can bind and transport the anticancer drug out of the cells, so as to decrease the intracellular concentration of the drug and lead to drug resistance (26). The different responses to the two drugs among the various subtypes of lung cancer may be related to the specific characteristics of each cell. Further investigations on the possible mechanism of cisplatin resistance in NSCLC may therefore be of benefit for the treatment of human lung cancer.

In conclusion, verapamil significantly inhibited the expression of P-gp in all four investigated cell lines. The down-regulation of P-gp is associated with resistance to cisplatin in the NCI-H-446 cell line and to VP-16 in the NCI-H-460, SPCA-1 and NCI-H-446 cell lines. These findings indicate that the down-regulation of P-gp may be helpful for the reversion of drug resistance in some lung cancer cell subtypes.

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