

## Co-treatment by docetaxel and vinblastine breaks down P-glycoprotein mediated chemo-resistance

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### ABSTRACT

**Objective(s):** Chemoresistance remains the main causes of treatment failure and mortality in cancer patients. There is an urgent need to investigate novel approaches to improve current therapeutic modalities and increase cancer patients' survival. Induction of drug efflux due to overexpression of P-glycoproteins is considered as an important leading cause of multidrug resistance. In this study, we investigated the role of combination treatments of docetaxel and vinblastine in overcoming P-glycoprotein mediated inhibition of apoptosis and induction of cell proliferation in human non-small cell lung carcinoma cells.

**Materials and Methods:** Cell proliferation and apoptosis were assessed using MTT assay and DAPI staining, respectively. P-glycoprotein expression was evaluated in gene and protein levels by Real-time RT-PCR and Western blot analysis, respectively.

**Results:** Combination treatment of the cells with docetaxel and vinblastine decreased the IC<sub>50</sub> values for docetaxel from (30±3.1) to (15±2.6) nM and for vinblastine from (30±5.9) to (5±5.6) nM ( $P\leq 0.05$ ). P-glycoprotein mRNA expression level showed a significant up-regulation in the cells incubated with each drug alone ( $P\leq 0.001$ ). Incubation of the cells with combined concentrations of both agents neutralized P-glycoprotein overexpression ( $P\leq 0.05$ ). Adding verapamil, a P-glycoprotein inhibitor caused a further increase in the percentage of apoptotic cells when the cells were treated with both agents.

**Conclusion:** Our results suggest that combination therapy along with P-glycoprotein inhibition can be considered as a novel approach to improve the efficacy of chemotherapeutics in cancer patients with high P-glycoprotein expression.

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### Introduction

Lung cancer is the most common cause of morbidity and mortality worldwide. This cancer was estimated to account for 27% of all cancer deaths between men and women in 2015 (1, 2). Among the variety of therapeutic approaches, chemotherapy is a fundamental treatment resulting in a median survival of approximately 10 months in non-small cell lung cancer (NSCLC) (3). Chemotherapeutic agents including docetaxel and vinblastine have been applied as first-line treatments against lung cancer in the past decade (4). Docetaxel is a microtubule inhibitor agent that is classified as the second generation of taxanes (5). Vinblastine is a member of vinca alkaloids, another group of microtubule inhibitor agents, which are widely applied in the treatment of NSCLC (6). Combination chemotherapy

is a recent approach in the treatment of cancer patients. In recent protocols, docetaxel and vinblastine were widely used in combination with cisplatin or carboplatin (4). Combined chemotherapy (7-9) has important theoretical advantages including affecting different phases of the cell cycle that results in increasing the number of cells exposed to cytotoxic effects, reducing the possibility of drug resistance, lowering the IC<sub>50</sub> dose of each agent, decreasing the side-effects and improving the quality of life in cancer patients (10, 11). Specific therapeutic approaches including chemotherapy with taxanes and vinca alkaloids are effective in the treatment of diverse cancers; however, chemoresistance is still an obstacle on the way of induction of apoptosis. Multidrug resistance (MDR) empowers cancer cells to exhibit resistance to different drugs concurrently (8, 9, 12-16) by

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different mechanisms including increased drug efflux, decreased drug uptake, drug metabolism, modifications of drug targets, increased DNA repair, and apoptosis defects (8, 9, 12, 15-22). A decrease in intracellular accumulation of the chemotherapeutic agents by drug efflux can be induced by the overexpression of ATP binding cassette (ABC) transporters, thereby conferring resistance to taxanes and vinca alkaloids. P-glycoprotein (P-gp), a member of ABC superfamily, plays a key role in the mechanisms of drug resistance. This 170-kDa protein is expressed by *mdr-1* gene. Additionally, high expression of P-gp in lung tumors has been demonstrated in different studies (9, 12, 15, 23, 24). Thus, to circumvent chemoresistance and increase the intracellular concentrations of chemotherapeutics, several ABC transporter inhibitors have been developed (12, 15, 25).

In this study, we investigated the role of combination treatment of H1299 lung cancer cells with docetaxel and vinblastine in alteration of the anti-proliferative and apoptotic effects of these agents. By inhibiting the activity of P-gp using a specific inhibitor, verapamil, we also studied the impact of P-gp expression on the effectiveness of chemotherapeutics; when we applied these agents alone or in combination. Our findings illustrated that co-treatment of the cells with both therapeutics enhances the efficacy of each agent in induction of cell death. We predict that identifying patients with high P-gp activity and then inhibiting its activity can provide an important adjuvant that increases the efficacy chemotherapeutic agents in cancer patients.

## Materials and Methods

### Materials

Docetaxel (Taxotere®-20 mg) was purchased from Sanofi-Aventis (Paris, France). Verapamil hydrochloride (VH) was obtained from Recordati (Milano, Italy). Vinblastine (VBL-10 mg) was purchased from Gedeon Richter Ltd. (Budapest, Hungary). RPMI-1640 Medium and penicillin-streptomycin were obtained from Sigma-Aldrich (St. Louis, MO, USA). FBS was gained from Invitrogen (Auckland, New Zealand). Primers were provided by MWG Biotech (Ebersberg, Germany). RNA isolation kit (RNX-Plus) was obtained from CinnaGen Co. (Tehran, Iran) and REVERTA-L RT reagents kit was purchased from Central Research Institute of Epidemiology of Russia (Moscow, Russia). Power SYBR® Green PCR Master Mix (5 ml) was obtained from Applied Biosystems (Warrington, UK). MTT and DAPI were obtained from Sigma-Aldrich (St. Louis, MO, USA). Anti-P-gp [JSB-1] and Anti-Beta Actin [mAbcam 8226] antibodies were purchased from Abcam (Cambridge, MA, USA). Anti-mouse IgG (H&L) HRP conjugated secondary antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). PVDF membrane was provided from Millipore

Corporation (Billerica, MA, USA). ECL kit was purchased from Amersham Biosciences (Freiburg, Germany). Prestained Protein Ladder was obtained from Fermentas (Hanover, MD, USA). X-ray films were purchased from Fuji Photo Film Company (Ltd., Tokyo, Japan).

### Cell culture

The H1299 human NSCLC cells (Pasteur Institute Cell Culture Collection Tehran, Iran) were grown in RPMI-1640 medium supplemented with 10% (v/v) FBS, 100 U/ml penicillin and 100 µg/ml streptomycin and incubated at 37 °C in 5% CO<sub>2</sub>.

### Single and combined treatment of H1299 cells

In single drug treatment, H1299 cells (5×10<sup>3</sup> cells/well) were seeded in 96-well plates and then were incubated with increasing serial concentrations of vinblastine and docetaxel alone for 24 and 48 hr to study the drug efficacy in induction of apoptosis. Consequently, the IC<sub>50</sub> values of docetaxel and vinblastine were determined for H1299 cells. In combination treatments, we applied variable concentrations of one agent along with IC<sub>50</sub> value of another agent to determine the effects of vinblastine/docetaxel combinations on cell death.

### Cell viability assay

To perform MTT assay, after incubation of the cells with desired concentrations of chemotherapeutics, the medium was replaced with (200 µl) of fresh media containing 50 µl of MTT solution (2 mg/ml in PBS), then the cells were incubated for an additional 4 hr at 37 °C. Then media/MTT mixture was removed and 200 µl of DMSO with 25 µl of Sorenson's glycine buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5) was added to each well. After 10 min of shaking in the plate thermo-shaker, the absorbance of each well was measured using a microplate reader (Biotek, ELx 800- USA) at 570 nm. MTT solution with DMSO (without the cells and medium) was applied as a blank control.

### Determination of IC<sub>50</sub> of vinblastine and docetaxel against H1299 cells

Plots of percent cytotoxicity index (%CI= [1-(OD<sub>treated</sub>/OD<sub>control</sub>)] × 100) versus drug concentrations were designed from each experiment. IC<sub>50</sub> of vinblastine and docetaxel was determined from each plot by calculating the slope and intercept.

### Quantification of apoptotic nuclei using DAPI staining

Cells (25×10<sup>4</sup> cells/well) were seeded in 6-well plates. After single and combination treatments of the cells with drugs for 48 hr, the cells were washed with PBS and then fixed in 4% paraformaldehyde for 20 min and permeabilized in 0.1% Triton X-100 for

15 min. Fixed cells were stained with DAPI (1:500 dilution; in PBS) for 15 min. Nuclear morphology of the cells was observed by fluorescence microscopy. Nuclei were considered to have the normal or apoptotic phenotypes. The condensed chromatin gathering at the periphery of the nuclear membrane, or a total fragmented morphology of nuclear bodies, is the main evidence for identifying apoptotic nuclei. Triplicate samples were prepared for each treatment, and at least  $3 \times 10^2$  cells were counted in random fields for each sample. Then the percentage of apoptotic nuclei was calculated (8, 9, 26, 27).

### RNA isolation and semi-quantitative RT-PCR analysis

Total RNA was extracted using the RNA isolation kit (RNX-Plus™ CinnaGen Co) 24 hr after incubation with different concentrations of each chemotherapeutic agent alone and in combination. Total extracted RNA was quantified by optical density measurement (A260/A280 ratio) with NanoDrop 1000 Spectrophotometer (Wilmington, DE, USA) and qualified by agarose gel electrophoresis. cDNA synthesis was performed using REVERTAA-L (RT reagents kit). Samples were then stored at  $-70^\circ\text{C}$ . The RT-PCR control was prepared without adding RNA. Real-time RT-PCR was performed in a total volume of (25  $\mu\text{l}$ ) using the iQ5 Optical System (Bio-Rad Laboratories, Inc., CA-USA). Each well contained (1  $\mu\text{l}$ ) of cDNA, (5.75  $\mu\text{M}$ ) of each primer and (12.5  $\mu\text{l}$ ) of 2X Power SYBR Green PCR Master Mix. The internal control was the constitutively expressed housekeeping human glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). Primers were designed by published Gene Bank sequences utilizing Beacon Designer™ 5.01 software. Primers for human *mdr-1* were as follows: F: 5'-ATGACCAGGTATGCCTATTATTAC-3' and R: 5'-CACATCAAACCAGCCTATCTC-3'. The primers for human *GAPDH* were as follows: F 5'-AAGCTCATTCTGCTATGACAACG-3' and R: 5'-TCTTCTCTTGCTCTTGCTGG-3'. Samples were assayed in triplicate on the 7500 Real Time PCR System (Applied Biosystems). All reactions were performed in triplicate in the presence of a negative control. Interpretation of the results was performed using the Pfaffle method and the CT values were normalized with respect to *GAPDH* expression.

### Western Blot analysis

The cells were treated with chemotherapeutic agents alone and in combination for 48 hr and were lysed with ice-cold RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5 % sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCL and pH 8) and protease inhibitor cocktail (Roche). The protein concentrations were measured at 280 nm using a NanoDrop Spectrophotometer (ND-1000 Wilmington, DE, USA).

Equal amounts of each protein sample lysate (50  $\mu\text{g}$  of protein) were subjected for electrophoresis on 8% SDS-polyacrylamide gel and subsequently transferred to PVDF membrane. Non-specific binding sites on the membrane were blocked with 5 % (w/v) non-fat dried milk in  $1 \times$  TBS-T buffer containing 50 mM Tris-base pH 7.6, 150 mM NaCl, 0.1 % (v/v) Tween 20 for 1 hr at room temperature. After incubating with anti-P-gp primary antibody (1:2000 dilution) and anti- $\beta$ -actin antibody (1:1000 dilution) as a loading control, the membrane was incubated with HRP-conjugated goat anti-mouse IgG secondary antibody (1:3000 dilution) for 1 hr at room temperature. The protein bands were visualized using ECL Plus detection system on X-ray films. The results of western blot were quantified using the band densitometry analysis with ImageJ software.  $\beta$ -actin (approximately 42 kDa) was applied as a loading control for our analysis. The intensity of each protein band was compared with that of  $\beta$ -actin and relative intensity ratios were calculated.

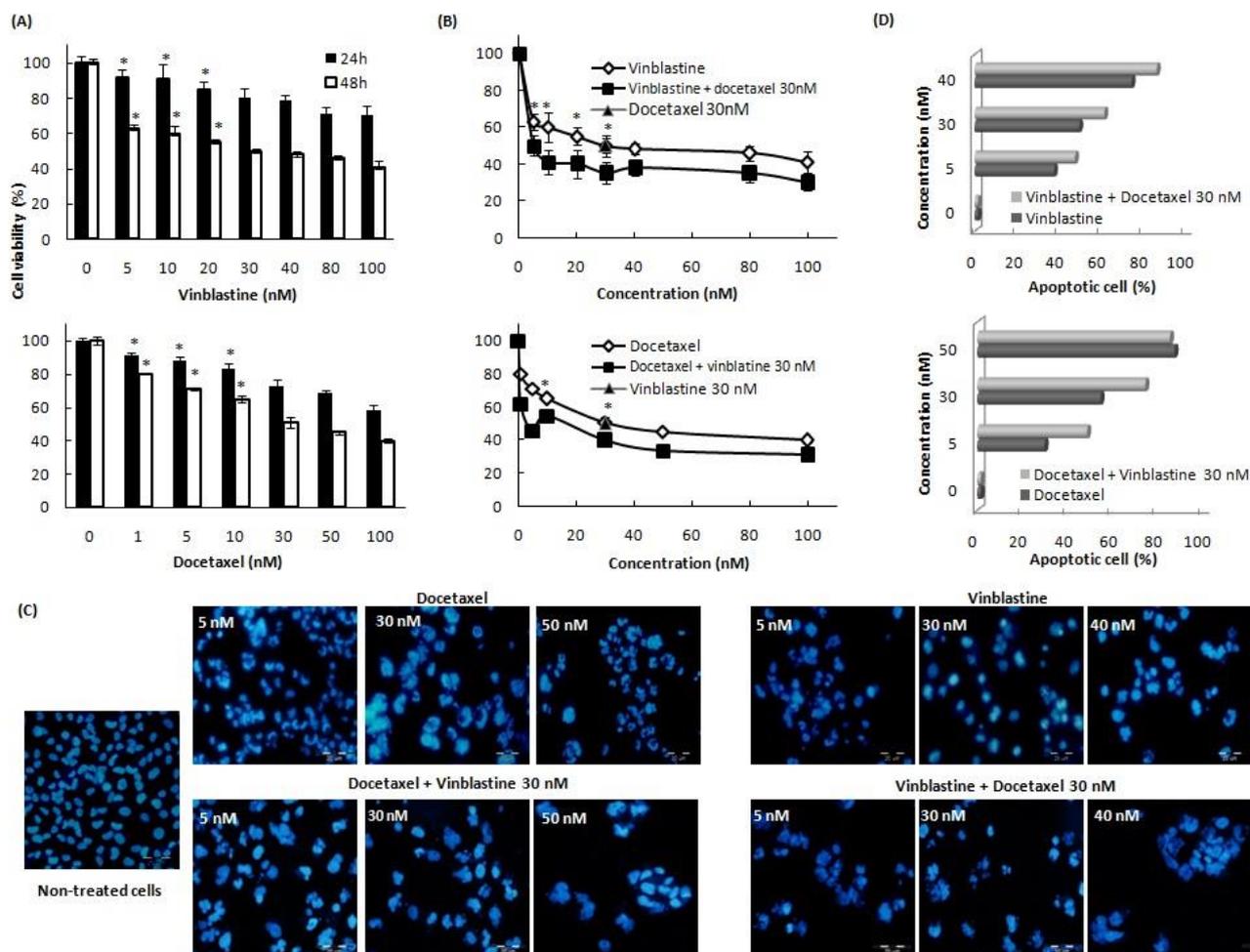
### Statistical analysis

Each set of experiments were conducted as three independent experiments. The results were reported as means  $\pm$  SD from three independent experiments. Statistical analysis was performed using SPSS software (IBM SPSS Statistics 19.0). Groups of treatment, at various doses or time points, were compared using one-way ANOVA. A value of  $P \leq 0.05$  was considered statistically significant.

## Results

### Cytotoxic effects of vinblastine and docetaxel on H1299 cells

The cytotoxicity of vinblastine and docetaxel was measured by both MTT assay and morphologic examination using DAPI staining. To determine the optimal concentration and  $\text{IC}_{50}$  value of each chemotherapeutic agent, H1299 cells were incubated with increasing concentrations of vinblastine and docetaxel for 24 and 48 hr. Treating the cells with 30 nM vinblastine decreased the viability of the cells to  $80 \pm 6\%$  after 24 hr and to  $50 \pm 1.5\%$  after 48 hrs ( $P \leq 0.05$ ) (Figure 1A). Docetaxel (30 nM) also decreased the cell viability to  $73 \pm 4\%$  after 24 hr and  $50 \pm 3\%$  after 48 hrs ( $P \leq 0.05$ ) (Figure 1A). Both chemotherapeutic agents showed significant anti-proliferative effects on the cells with the same value of  $\text{IC}_{50}$  (30 nM) in a dose- and time-dependent manner ( $P \leq 0.05$ ). Incubation of the cells for 48 hr with docetaxel (5-50 nM) caused 30-68% cell death while vinblastine (5-40 nM) caused 38-75% apoptotic cells when we stained the cells with DAPI (Figures 1C and D). These findings indicate that vinblastine induces a slightly higher apoptotic response, in H1299 cells, in comparison with docetaxel (Figures 1C and D).



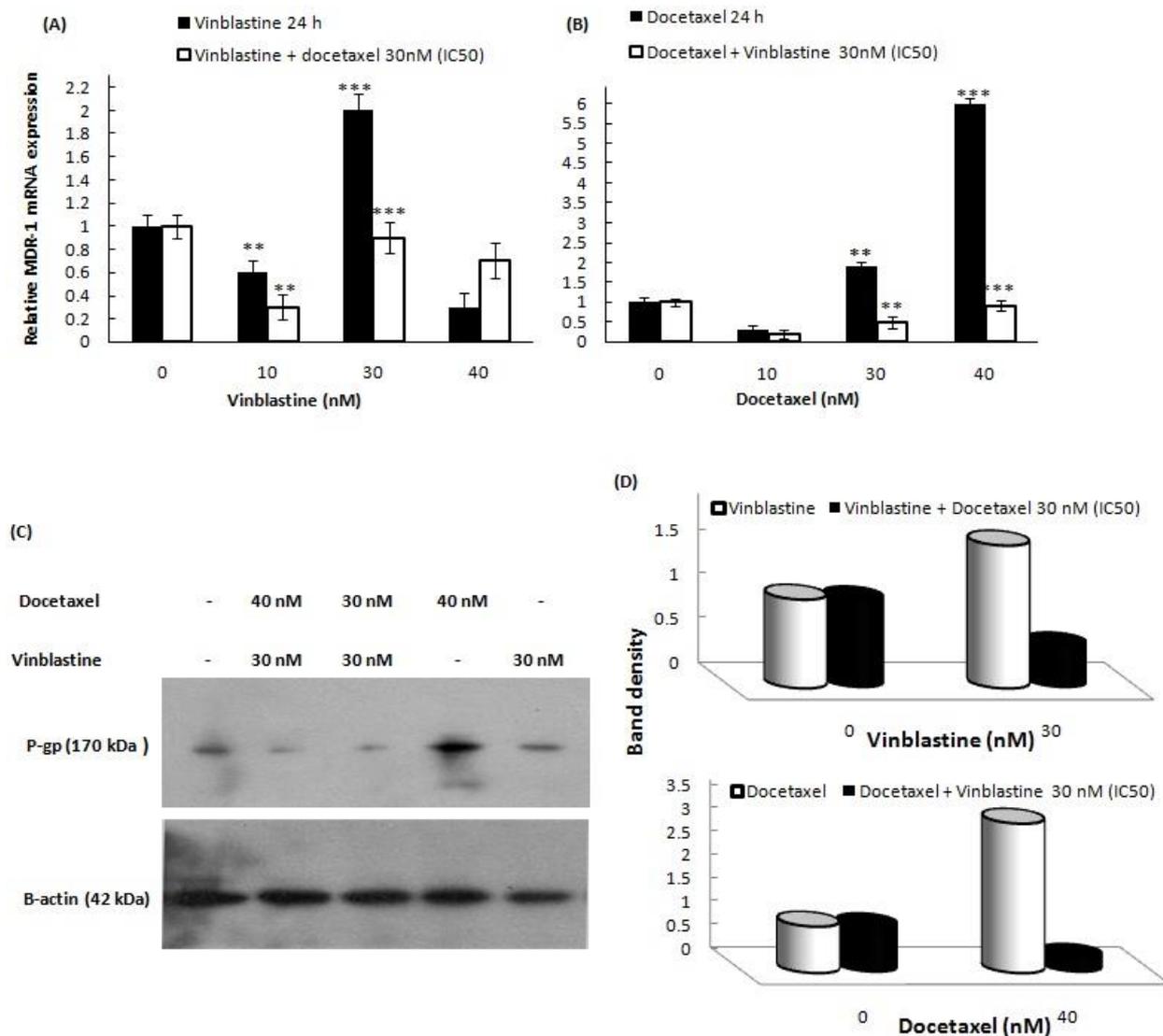
**Figure 1.** Effects of docetaxel and vinblastine alone and in combination on proliferation and apoptotic response in H1299 cells. Panel (A) shows cell viability (%) of the cells after 24 and 48 hrs incubation with vinblastine (0-100 nM) or docetaxel (0-100 nM). Panel (B) shows MTT results of incubation of the cells with combination of docetaxel and vinblastine. The cells were incubated with different concentrations of vinblastine (0-100 nM) and docetaxel (30nM) or different concentrations of docetaxel (0-100 nM) and vinblastine (30 nM) for 48 hrs. Results are expressed relative to the cell density of non-treated cells as positive control and cell viability (%) was measured using MTT assay. Each value indicates mean  $\pm$  SD for three independent experiments. Statistically significant differences are indicated by: \* ( $P \leq 0.05$ ). Panel (C) indicates morphology of the cells after single and combination therapy. The cells were incubated with different concentrations of docetaxel (0, 5, 30 and 50 nM), vinblastine (0, 5, 30 and 40 nM), and their combinations for 48 hrs, and then stained with DAPI. Pictures show the number of cells when treated with docetaxel/vinblastine in comparison with non-treated cells. D) Bar charts represent the number of apoptotic cells which shown as mean  $\pm$  SD calculated from counting a minimum of  $3 \times 10^2$  cells in random fields of triplicate wells from three independent experiments. Statistically significant differences are indicated by: ( $P \leq 0.05$ )

### Combination treatment reduces the $IC_{50}$ values of vinblastine and docetaxel

We also studied whether or not combination treatment enhances induction of apoptosis by docetaxel and vinblastine. A combination treatment of the cells with vinblastine (10 nM) and docetaxel at (30 nM) ( $IC_{50}$ ) for 48 hr, elevated the cytotoxicity of vinblastine from  $40 \pm 8\%$  to  $59 \pm 7\%$  ( $P \leq 0.05$ ) (Figure 1.B). Incubation of the cells with docetaxel (5 nM), along with  $IC_{50}$  value of vinblastine, showed that the cell viability decreased from  $71 \pm 0.7\%$  to  $46 \pm 3\%$  ( $P \leq 0.05$ ) (Figure 1.B). Combination treatment of the cells with both agents caused a marked decrease in

$IC_{50}$  values for docetaxel by two folds and for vinblastine by 6 folds ( $P \leq 0.05$ ).

Our results from DAPI staining showed that treatment of the cells with a combination of docetaxel (30 nM) and vinblastine (0-40 nM) caused a significant increase in the percentage of apoptotic cells when we applied different concentrations of vinblastine ( $P \leq 0.05$ ). However, the highest impact of combination treatment on induction of apoptosis was obtained when we applied (30 nM) of vinblastine along with (0-50 nM) of docetaxel ( $P \leq 0.05$ ) (Figures 1C and D).



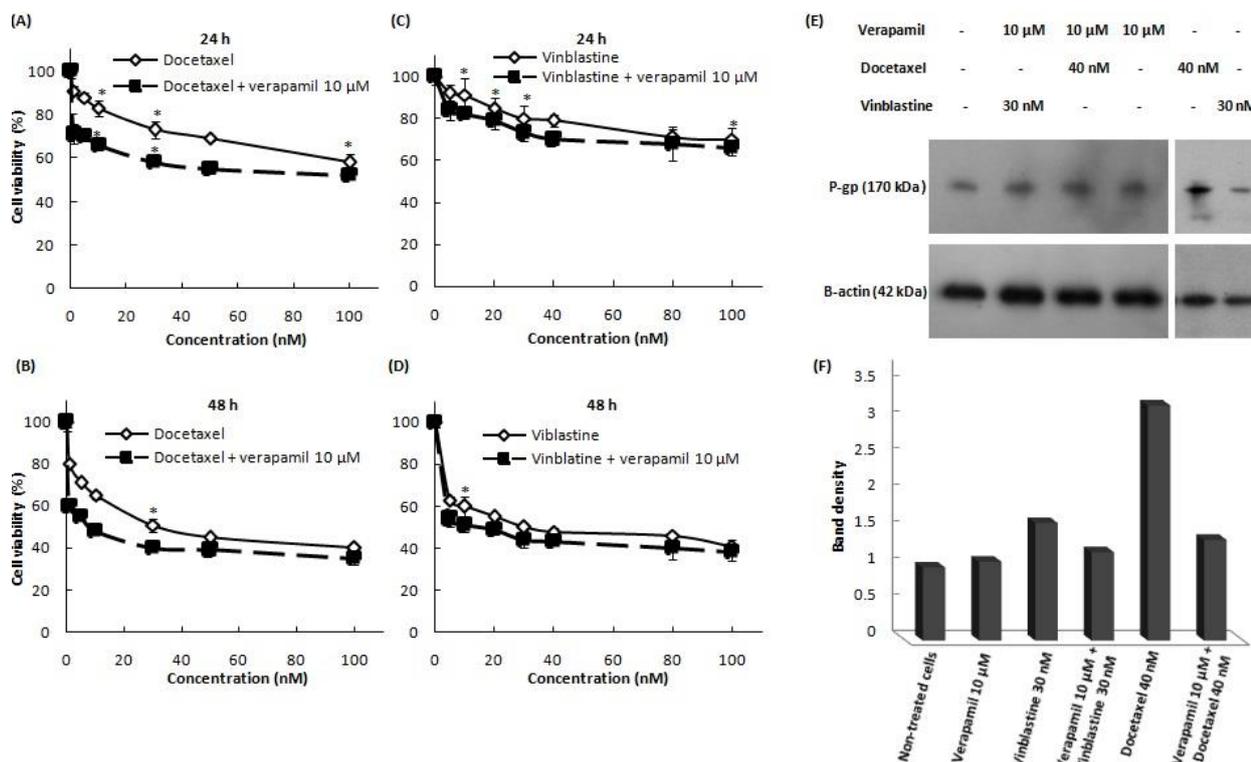
**Figure 2.** Quantification of P-glycoprotein gene/protein levels in H1299 cells incubated with docetaxel, vinblastine and their combinations. Panels (A and B) show the relative mRNA levels of *mdr-1* compared to *GAPDH* as an internal control. The cells were incubated with vinblastine or docetaxel (0, 10, 30 and 40 nM) alone and in combination for 24 hrs, respectively. The results are means ± SD from three independent experiments. Statistically significant differences are indicated by: \*\* ( $P \leq 0.01$ ); \*\*\* ( $P \leq 0.001$ ). Panel (C) shows the expression of P-gp in various cell lysates analyzed by Western blotting. The cells were treated with vinblastine (30 nM), docetaxel (40 nM) and their combinations for 48 hrs.  $\beta$ -actin protein was used a loading control. The results are representative of three independent experiments. Data were quantified by densitometric analysis using ImageJ software. Panel (D) indicates the densitometric ratio of P-gp/ $\beta$ -actin for each treatment. Statistically significant differences are indicated by: ( $P \leq 0.05$ )

**Co-treatment of the cells with both agents down-regulates P-gp gene and protein expression**

P-gp expression was evaluated at both mRNA and protein levels in H1299 cells when we applied docetaxel and vinblastine alone and in combination. Real-time RT-PCR analysis revealed that *mdr-1* expression level was increased up to 6 folds with docetaxel (40 nM) and up to 2 folds with vinblastine (30 nM) after 24 hr ( $P \leq 0.001$ ) (Figures 2A and B). Moreover, the enhancement of *mdr-1* gene expression upon treatment with docetaxel, but not vinblastine, was dose-dependent (Figures 2A and B). Combination treatment of the cells with docetaxel

and vinblastine decreased mRNA *mdr-1* expression level in a dose-dependent manner ( $P \leq 0.05$ ) (Figures 2A and B). Consequently, combination treatment of the cells with both agents caused a marked decrease in *mdr-1* gene expression compared to applying each agent alone ( $P \leq 0.05$ ) (Figures 2A and B).

Our results from Western blot analysis were consistent with Real-time RT-PCR results, which showed that H1299 cells expressed high levels of P-gp relative to non-treated cells when they were incubated with vinblastine (30 nM) or docetaxel (40 nM) for 48 hr ( $P \leq 0.05$ ) (Figures 2C and D). Incubation of the cells with combinations of



**Figure 3.** Combination effects of verapamil with chemotherapeutic agents on proliferation, apoptosis induction, and P-glycoprotein expression in H1299 cells. First, the cells were pre-treated with (10 μM) of verapamil and after 1 hr were challenged with vinblastine or docetaxel to measure cell survival and analyze whether drug resistance can be reversed. Verapamil pre-treated cells were incubated with docetaxel (0-100 nM) (A and B) or with vinblastine (0-100 nM) (C and D) for 24 and 48 hrs. The results are expressed as mean±SD values for three independent experiments. Statistically significant differences are indicated by: \* ( $P \leq 0.05$ ). E) Effects of verapamil on P-gp expression assessed by Western blot analysis in H1299 cells. The cells were harvested for protein isolation after treatment with docetaxel (40 nM), vinblastine (30 nM), combination of verapamil (10 μM) and docetaxel (40 nM) or vinblastine (30 nM) for 48 hrs. β-actin protein was used a loading control. Results shown are from three experiments performed with three different protein extractions, and were quantified by densitometric analysis using ImageJ software. F) Densitometric ratio of P-gp/β-actin for each treatment. Statistically significant differences are indicated by: ( $P \leq 0.05$ )

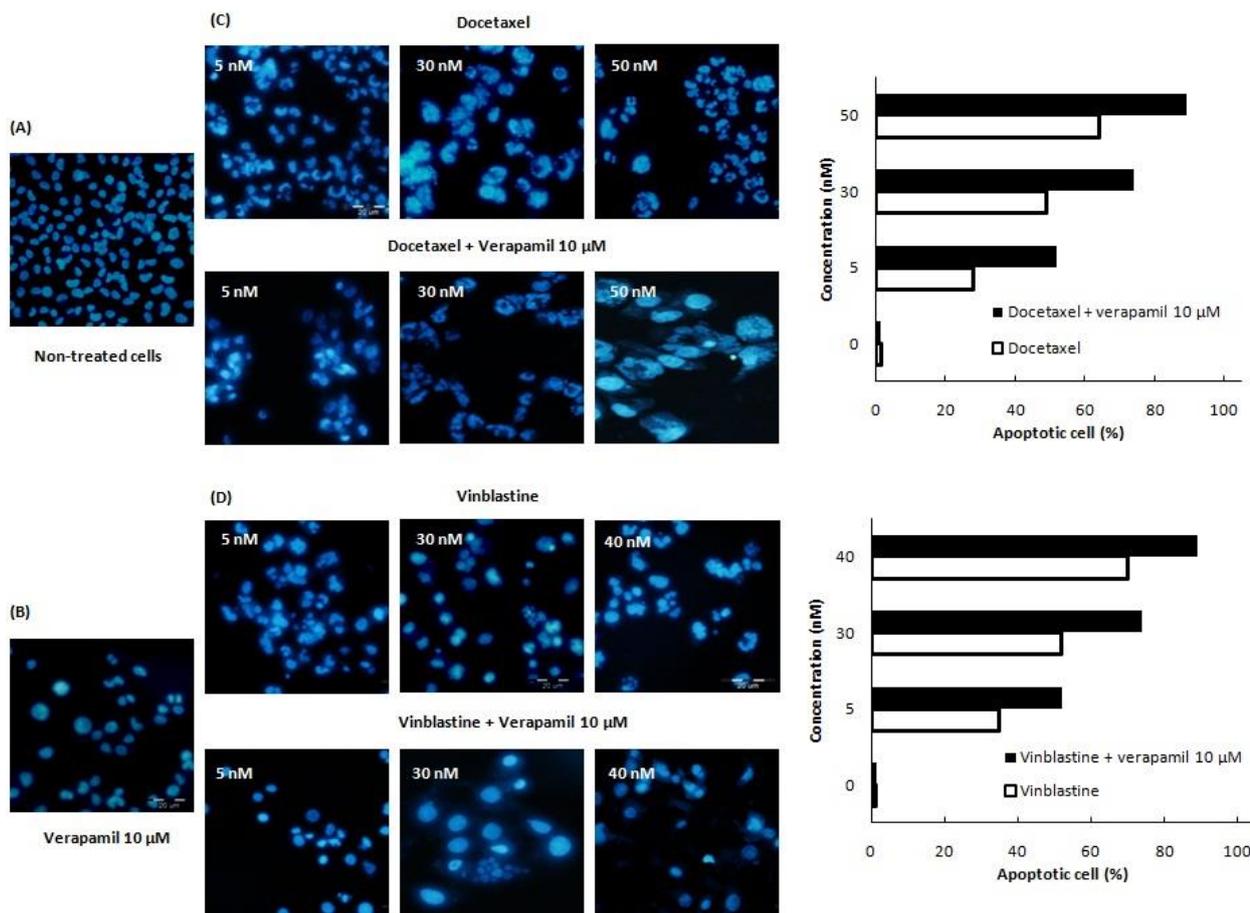
vinblastine and docetaxel for 48 hrs attenuated P-gp expression level significantly ( $P \leq 0.05$ ) (Figures 2C and D). These results suggest that co-treatment of the cells with both agents attenuated P-gp level compared to applying each agent alone (Figures 2C and D).

#### **Inhibition of P-gp activity enhances the efficacy of chemotherapeutic agents**

To evaluate the effect of verapamil, a P-gp inhibitor, on the effectiveness of chemotherapeutic agents, we first optimized verapamil concentration for inhibiting P-gp in our cancer cells. Our results indicated that verapamil, by itself, did not have significant anti-proliferative effect on the cells (The data is not shown). Considering previous studies, we chose (10 μM) of verapamil to perform subsequent experiments (28-31). To investigate whether chemo-sensitivity of H1299 cells can be restored by verapamil, we examined the combined effects of docetaxel/ verapamil and vinblastine/ verapamil on cellular proliferation and apoptotic response. Our results from MTT assay demonstrated that the

viability of the cells decreased from  $91 \pm 2\%$  to  $71 \pm 4.5\%$  and  $80 \pm 0.4\%$  to  $60 \pm 1.5\%$  when we applied (1 nM) of docetaxel along with (10 μM) of verapamil for 24 and 48 hr, respectively ( $P \leq 0.05$ ) (Figures 3A and B). The combination of vinblastine (10 nM) with verapamil (10 μM) for 24 and 48 hr reduced cell viability from  $90 \pm 8.2\%$  to  $82 \pm 2.3\%$  and  $60 \pm 4.6$  to  $51 \pm 3.3\%$ , respectively ( $P \leq 0.05$ ) (Figures 3C and D). Adding verapamil (10 μM) to combined concentrations of both agents caused a significant further decrease in cell viability (The data is not shown).

Although calcium channel blockers including verapamil inhibit P-gp function, it is not reported that they affect its gene expression (32). Therefore, we examined the effects of verapamil on P-gp by Western blot analysis. Similar to the other two chemotherapeutics, verapamil also increased the P-gp level when we applied each of them alone. Incubation of the cells with a combination of verapamil (10 μM) and docetaxel (40 nM) or vinblastine (30 nM) for 48 hrs markedly reduced P-gp expression ( $P \leq 0.05$ ) (Figures 3E and F). These results suggest that verapamil is capable of reversing



**Figure 4.** Morphology of H1299 cells after single and combination treatment to detect apoptotic nuclei. Bar charts represent the number of apoptotic cells which shown as mean  $\pm$  SD calculated from counting a minimum of 300 cells in random fields of triplicate wells from three independent experiments. Statistically significant differences are indicated by: ( $P \leq 0.05$ )

docetaxel and vinblastine-induced resistance in H1299 cells.

The results from DAPI staining also revealed that verapamil increased the percentage of apoptotic cells from  $65 \pm 2\%$  to  $89 \pm 1.5\%$  with docetaxel (50 nM). In addition, vinblastine (30 nM) induced  $52 \pm 4\%$  apoptotic cells, while in combination with verapamil (10  $\mu$ M) this percentage increased to  $74 \pm 3\%$  ( $P \leq 0.05$ ) (Figures 4A-D). Applying verapamil (10  $\mu$ M) to the combined treatment of the cells with vinblastine, and docetaxel significantly enhanced the percentage of apoptotic cells ( $P \leq 0.05$ ) (The data is not shown).

### Discussion

Chemotherapy is a common modality of cancer treatment. However, in many cases the efficiency of therapeutics in induction of cell death depends on different factors including drug action, drug dosage, genetic signature, and drug resistance (33).

Resistance to currently-available chemotherapeutic agents is mainly responsible for cancer recurrence, relapse and metastasis (8, 9, 12, 15, 16,

22, 34, 35). Increasing drug efflux by P-gp plays the central role in chemoresistance (9, 12, 15, 22, 36). Several studies showed poor prognosis and treatment outcomes related to enhanced expression of P-gp in lung cancer patients (23, 24, 37). Because of the crucial role of P-gp in the development of chemoresistance, many efforts have been made to inhibit P-gp function and improve the efficacy of existing chemotherapeutic agents (12, 38). The findings of this study also confirmed that combination therapy can be applied as a novel approach to overcome MDR (8, 9, 39).

Our data showed that both docetaxel and vinblastine induced cytotoxicity in cancer cells in a time and dose-dependent manner, which is consistent with previous studies (9, 29, 40).

Also, combinations of docetaxel and vinblastine reduced the  $IC_{50}$  values of each agent. Several studies demonstrated the efficacy of combined administration of taxanes and vinca alkaloids for the treatment of lung cancer patients (41-44). DAPI staining also revealed that combined treatment of H1299 cells with docetaxel and vinblastine induced

more apoptotic responses that was consistent with our MTT results.

Detection of *mdr-1* gene and protein expressions, after both single and combination treatments of the cells, demonstrated that docetaxel and vinblastine-induced cytotoxicity can be strongly antagonized by P-gp activity. In this study, we showed a significant increase in P-gp gene expression level when the cells were incubated with docetaxel or vinblastine alone ( $P \leq 0.05$ ). Western blot analysis also showed that vinblastine or docetaxel increased P-gp level ( $P \leq 0.05$ ). These results imply that both transcriptional and translational activation of *mdr-1*/P-gp expression caused MDR phenotype, which is induced by these chemotherapeutic agents. Several studies have revealed that *mdr-1* promoter responds directly to treatment with cytotoxic agents including docetaxel (45, 46). Also, P-gp function is altered by undergoing post-translational modifications. Our findings indicated that combinations of vinblastine and docetaxel decreased *mdr-1* gene, P-gp expression, and cell viability.

Adjuvant therapy using P-gp inhibitors can help to improve the chemosensitivity of tumor cell response by increasing the intracellular concentration of chemotherapeutic agents (12, 15, 25). There are many diverse modulators that can reverse MDR phenotype by potential mechanisms including affecting P-gp synthesis (12, 47), conformation (48), phosphorylation (49), as well as disruption of P-gp activity by direct blocking of drug binding site (50).

To investigate the role of P-gp in altering the efficacy of the agents, we applied verapamil, a calcium channel blocker that inhibits the activity of P-gp in drug efflux from the cells (51). In our study, verapamil enhanced the cytotoxic effects of docetaxel or vinblastine significantly, which is consistent with another study reporting that verapamil reverses drug resistance of A549/D16 and A549/V16 sublines to docetaxel or vincristine (29). Furthermore, Ross *et al* demonstrated that verapamil enhances daunorubicin cytotoxicity in all concentrations of acute myeloid leukemia cells. Also, verapamil caused greater than 20% augmentation of daunorubicin accumulation or retention (30). Our Western blot analysis revealed that verapamil alone increases P-gp expression. Indeed, it has been found that verapamil cannot increase *mdr-1* transcription, and enhancement in *mdr-1* expression is mediated post-transcriptionally in the nucleus (32).

Our findings are in agreement with several studies reporting that verapamil induces P-gp expression in different cell lines (32, 52), but a reversible decrease of P-gp expression in human

leukemic cell lines (53). This discrepancy can be explained by the type of applied cell lines with different mechanisms of action, treatment protocols, and experimental systems used.

Furthermore, when H1299 cells were exposed to verapamil prior to treatment with docetaxel or vinblastine, P-gp expression decreased considerably in comparison with vinblastine or docetaxel alone. Our results are in accordance with different studies reporting that verapamil induces enhancement of antitumor activity and cellular uptake of chemotherapeutics due to decreased *mdr-1*/P-gp expression (54-56). Inhibition of P-gp activity restored the sensitivity of H1299 cells to docetaxel and vinblastine. These findings imply that P-gp plays the main role in the sensitivity of H1299 cells to the microtubule inhibitor agents, docetaxel and vinblastine.

## Conclusion

Our results revealed that combined treatments of docetaxel and vinblastine enhances the efficacy of each agent in the induction of apoptosis in lung cancer cells. This work suggests that identifying cancer patients with high P-gp expression, and then inhibiting its activity, can improve the efficacy of treatment in patients with lung cancer.

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## Conflict of interest

The authors declare that they have no conflicts of interest.

## References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. *CA Cancer J Clin* 2015; 65:5-29.
2. Pallis AG. A review of treatment in non-small-cell lung cancer. *Eur Oncol Haematol* 2011; 7:27-31.
3. Pallis AG, Syrigos K, Kotsakis A, Karachaliou N, Polyzos A, Chandrinos V, *et al*. Second-line paclitaxel/carboplatin versus vinorelbine/carbo-platin

- in patients who have advanced non-small-cell lung cancer pretreated with non-platinum-based chemotherapy: a multicenter randomized phase II study. *Clin Lung Cancer* 2011; 12:100-105.
4. Molina JR, Adjei AA, Jett JR. Advances in chemotherapy of non-small cell lung cancer. *Chest* 2006; 130:1211-1219.
  5. Ringel I, Horwitz SB. Studies with RP 56976 (taxotere): a semisynthetic analogue of taxol. *J Natl Cancer Inst* 1991; 83:288-291.
  6. Duflos A, Kruczynski A, Barret JM. Novel aspects of natural and modified vinca alkaloids. *Curr Med Chem Anticancer Agents* 2002; 2:55-70.
  7. Miles D, von Minckwitz G, Seidman AD. Combination versus sequential single-agent therapy in metastatic breast cancer. *Oncologist* 2002; 7:13-19.
  8. Ghanbari P, Mohseni M, Tabasinezhad M, Yousefi B, Saei AA, Sharifi S, et al. Inhibition of survivin restores the sensitivity of breast cancer cells to docetaxel and vinblastine. *Appl Biochem Biotechnol* 2014; 174:667-681.
  9. Samadi N, Ghanbari P, Mohseni M, Tabasinezhad M, Sharifi S, Nazemieh H, et al. Combination therapy increases the efficacy of docetaxel, vinblastine and tamoxifen in cancer cells. *J Cancer Res Ther* 2014; 10:715.
  10. Chou TC. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacol Rev* 2006; 58:621-681.
  11. Aoudjit F, Vuori K. Integrin signaling in cancer cell survival and chemoresistance. *Chemother Res Pract* 2012; 2012.
  12. Nourbakhsh M, Jaafari MR, Lage H, Abnous K. Nanoliposomes-mediated MDR1 siRNA delivery reduces doxorubicin resistance in breast cancer cells and silences MDR1 expression in xenograft model of human breast cancer. *Iran J Basic Med Sci* 2015; 18:385.
  13. Stavrovskaya AA. Cellular mechanisms of multidrug resistance of tumor cells. *Biochemistry (Mosc)* 2000; 65:95-106.
  14. Fojo AT, Menefee M. Microtubule targeting agents: basic mechanisms of multidrug resistance (MDR). *Semin Oncol* 2005; 32:S3-8.
  15. Komeili-Movahhed T, Fouladdel S, Barzegar E, Atashpour S, Ghahremani MH, Ostad SN, et al. PI3K/Akt inhibition and down-regulation of BCRP re-sensitize MCF7 breast cancer cell line to mitoxantrone chemotherapy. *Iran J Basic Med Sci* 2015; 18:472.
  16. Sharifi S, Barar J, Hejazi MS, Samadi N. Roles of the bcl-2/bax ratio, caspase-8 and 9 in resistance of breast cancer cells to Paclitaxel. *Asian Pac J Cancer Prev* 2014; 15:8617-8622.
  17. Ambudkar SV, Dey S, Hrycyna CA, Ramachandra M, Pastan I, Gottesman MM. Biochemical, cellular, and pharmacological aspects of the multidrug transporter 1. *Annu Rev Pharmacol Toxicol* 1999; 39:361-398.
  18. Townsend DM, Tew KD. The role of glutathione-S-transferase in anti-cancer drug resistance. *Oncogene* 2003; 22:7369-7375.
  19. Eastman A, Schulte N. Enhanced DNA repair as a mechanism of resistance to cis-diamminedichloroplatinum (II). *Biochemistry* 1988; 27:4730-4734.
  20. Kavallaris M, Kuo DY, Burkhart CA, Regl DL, Norris MD, Haber M, et al. Taxol-resistant epithelial ovarian tumors are associated with altered expression of specific beta-tubulin isoforms. *J Clin Invest* 1997; 100:1282-1293.
  21. Sethi T, Rintoul RC, Moore SM, MacKinnon AC, Salter D, Choo C, et al. Extracellular matrix proteins protect small cell lung cancer cells against apoptosis: a mechanism for small cell lung cancer growth and drug resistance *in vivo*. *Nat Med* 1999; 5:662-668.
  22. Sabzichi M, Hamishehkar H, Ramezani F, Sharifi S, Tabasinezhad M, Pirouzpanah M, et al. Luteolin-loaded phytosomes sensitize human breast carcinoma MDA-MB 231 cells to doxorubicin by suppressing Nrf2 mediated signalling. *Asian Pac J Cancer Prev* 2013; 15:5311-5316.
  23. Abe Y, Ohnishi Y, Yoshimura M, Ota E, Ozeki Y, Oshika Y, et al. P-glycoprotein-mediated acquired multidrug resistance of human lung cancer cells *in vivo*. *Br J Cancer* 1996; 74:1929-1934.
  24. Volm M, Mattern J, Samsel B. Overexpression of P-glycoprotein and glutathione S-transferase-pi in resistant non-small cell lung carcinomas of smokers. *Br J Cancer* 1991; 64:700.
  25. Krishna R, Mayer LD. Multidrug resistance (MDR) in cancer: mechanisms, reversal using modulators of MDR and the role of MDR modulators in influencing the pharmacokinetics of anticancer drugs. *Eur J Pharm Sci* 2000; 11:265-283.
  26. Samadi N, Gaetano C, Goping I, Brindley D. Autotaxin protects MCF-7 breast cancer and MDA-MB-435 melanoma cells against Taxol-induced apoptosis. *Oncogene* 2008; 28:1028-1039.
  27. Samadi N, Bekele RT, Goping IS, Schang LM, Brindley DN. Lysophosphatidate induces chemoresistance by releasing breast cancer cells from taxol-induced mitotic arrest. *PLoS One* 2011; 6:e20608.
  28. Mallon R, Feldberg LR, Lucas J, Chaudhary I, Dehnhardt C, Santos ED, et al. Antitumor efficacy of PKI-587, a highly potent dual PI3K/mTOR kinase inhibitor. *Clin Cancer Res* 2011; 17:3193-203.
  29. Chiu L-Y, Ko J-L, Lee Y-J, Yang T-Y, Tee Y-T, Sheu G-T. L-type calcium channel blockers reverse docetaxel and vincristine-induced multidrug resistance independent of ABCB1 expression in human lung cancer cell lines. *Toxicol Lett* 2010; 192:408-418.
  30. Ross DD, Wooten P, Sridhara R, Ordonez J, Lee EJ, Schiffer C. Enhancement of daunorubicin accumulation, retention, and cytotoxicity by verapamil or cyclosporin A in blast cells from patients with previously untreated acute myeloid leukemia. *Blood* 1993; 82:1288-99.
  31. Ferrao PT, Frost MJ, Siah S-P, Ashman LK. Overexpression of P-glycoprotein in K562 cells does not confer resistance to the growth inhibitory effects of imatinib (STI571) *in vitro*. *Blood* 2003; 102:4499-4503.
  32. Herzog C, Tsokos M, Bates S, Fojo A. Increased mdr-1/P-glycoprotein expression after treatment of human colon carcinoma cells with P-glycoprotein antagonists. *J Biol Chem* 1993; 268:2946-2952.

33. McGrogan BT, Gilmartin B, Carney DN, McCann A. Taxanes, microtubules and chemoresistant breast cancer. *Biochim Biophys Acta* 2008; 1785:96-132.
34. Huanwen W, Zhiyong L, Xiaohua S, Xinyu R, Kai W, Tonghua L. Intrinsic chemoresistance to gemcitabine is associated with constitutive and laminin-induced phosphorylation of FAK in pancreatic cancer cell lines. *Mol Cancer* 2009; 8:21.
35. Samadi N, Bekele R, Capatos D, Venkatraman G, Sariahmetoglu M, Brindley DN. Regulation of lysophosphatidate signaling by autotaxin and lipid phosphate phosphatases with respect to tumor progression, angiogenesis, metastasis and chemoresistance. *Biochimie* 2011; 93:61-70.
36. Bell DR, Gerlach J, Kartner N, Buick R, Ling V. Detection of P-glycoprotein in ovarian cancer: a molecular marker associated with multidrug resistance. *J Clin Oncol* 1985; 3:311-315.
37. Yabuki N, Sakata K, Yamasaki T, Terashima H, Mio T, Miyazaki Y, et al. Gene amplification and expression in lung cancer cells with acquired paclitaxel resistance. *Cancer Genet Cytogenet* 2007; 173:1-9.
38. Patil YB, Swaminathan SK, Sadhukha T, Ma L, Panyam J. The use of nanoparticle-mediated targeted gene silencing and drug delivery to overcome tumor drug resistance. *Biomaterials* 2010; 31:358-365.
39. Tsakalozou E, Eckman AM, Bae Y. Combination effects of docetaxel and doxorubicin in hormone-refractory prostate cancer cells. *Biochem Res Int* 2012; 2012.
40. Chan DC, Earle KA, Zhao TL, Helfrich B, Zeng C, Baron A, et al. Exisulind in combination with docetaxel inhibits growth and metastasis of human lung cancer and prolongs survival in athymic nude rats with orthotopic lung tumors. *Clin Cancer Res* 2002; 8:904-912.
41. Nelli F, Naso G, De Pasquale Ceratti A, Saltarelli R, D'auria G, Lugini A, et al. Weekly vinorelbine and docetaxel as second-line chemotherapy for pretreated non-small cell lung cancer patients: a phase I-II trial. *J Chemother* 2004; 16:392-9.
42. Schmittel A, Siehl JM, Schulze M, Schulze K, Thiel E, Keilholz U. Phase II trial of carboplatin, paclitaxel plus vinorelbine in non-small cell lung cancer patients. *Anticancer Res* 2005; 25:1333-1336.
43. Dimitroulis J, Toubis M, Antoniou D, Marosis C, Armenaki U, Stathopoulos G, et al. Alternate paclitaxel-gemcitabine and paclitaxel-vinorelbine biweekly administration in non-small cell lung cancer patients: A phase II study. *Anticancer Res* 2006; 26:1397-1402.
44. Tester W, Ackler J, Tijani L, Leighton J. Phase I/II study of weekly docetaxel and vinblastine in the treatment of metastatic hormone-refractory prostate carcinoma. *Cancer J* 2006; 12:299-304.
45. Chin K-V, Chauhan SS, Pastan I, Gottesman MM. Regulation of mdr RNA levels in response to cytotoxic drugs in rodent cells. *Cell Growth Differ* 1990; 1:361-365.
46. Kohno K, Sato S-i, Takano H, Matsuo K-i, Kuwano M. The direct activation of human multidrug resistance gene (MDR1) by anticancer agents. *Biochem Biophys Res Commun* 1989; 165:1415-1421.
47. Juliano RL, Ling V. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim Biophys Acta* 1976; 455:152-162.
48. Beck WT. The cell biology of multiple drug resistance. *Biochem Pharmacol* 1987; 36:2879-2887.
49. Meyers M. Protein phosphorylation in multidrug resistant Chinese hamster cells. *Cancer Commun* 1989; 1:233.
50. Hofslie E, Nissen-Meyer J. Reversal of multidrug resistance by lipophilic drugs. *Cancer Res* 1990; 50:3997-4002.
51. Karwatsky J, Lincoln MC, Georges E. A mechanism for P-glycoprotein-mediated apoptosis as revealed by verapamil hypersensitivity. *Biochemistry* 2003; 42:12163-12173.
52. Anderle P, Niederer E, Rubas W, Hilgendorf C, Spahn-Langguth H, Wunderli-Allenspach H, et al. P-glycoprotein (P-gp) mediated efflux in Caco-2 cell monolayers: The influence of culturing conditions and drug exposure on P-gp expression levels. *J Pharm Sci* 1998; 87:757-762.
53. Muller C, Bailly JD, Goubin F, Laredo J, Jaffrézou JP, Bordier C, et al. Verapamil decreases P-glycoprotein expression in multidrug-resistant human leukemic cell lines. *Int J Cancer* 1994; 56:749-754.
54. Osman A-MM, Mohamad MA, Abdel-Wahab A-HA, Sayed-Ahmad MM. Modulation by verapamil of doxorubicin induced expression of multidrug resistance gene (mdr-1/P-glycoprotein) in murine tumour cells. *J Egypt Natl Cancer Inst* 2000; 12:221-227.
55. Al-Shabanah O, Osman A-M, Al-Harbi M, Al-Bekairi A, Al-Gharably N, Aziz S. Diltiazem potentiation of doxorubicin cytotoxicity and cellular uptake in Ehrlich ascites carcinoma cells. *Chemotherapy* 2009; 41:368-377.
56. Hori Y, Kawamoto K, Yamazaki N, Kumazawa H, Yamashita T, Kumazawa T. Establishment of a cisplatin-resistant KB cell line and its characterization. *Acta Otolaryngol Suppl* 1993; 113:142-148.