ANTICANCER RESEARCH *39*: 5461-5471 (2019)
doi:10.21873/anticanres.13739

Apatinib Reverses Paclitaxel-resistant Lung Cancer Cells (A549) Through Blocking the Function of ABCB1 Transporter

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Abstract. *Background/Aim: Multidrug resistance (MDR) is often associated with overexpression of P-glycoprotein (ABCB1) in cancer cells. Apatinib is a novel Vascular endothelial growth factor receptor-TKI (VEGFR-TKI) which inhibits the function of ABCB1 in certain cancers. This study aimed to investigate the effect of apatinib on the reversal of paclitaxel (PTX) resistance in A549 lung cancer cells (A549/PTX) and related mechanisms. Materials and Methods: A549/PTX cells were treated with apatinib alone, PTX alone, or PTX and apatinib. Cell viability was measured by the CCK8 assay. Apoptosis rate, cell-cycle arrest, Rhodamine efflux and reactive oxygen species (ROS) generation were determined by flow cytometry. The intracellular paclitaxel concentration was measured by ultra performance liquid chromatography (UPLC). Protein levels were analyzed by western blotting. Results: A549/PTX cells had significant resistance to PTX and higher expression of ABCB1 compared to A549 cells. Apatinib increased the cytotoxicity of PTX, enhanced PTX-induced apoptosis and cycle arrest, and triggered intracellular ROS generation in A549/PTX cells. In addition, apatinib treatment increased the concentration of intracellular PTX in A549/PTX cells. Apatinib-PTX combination inhibited AKT and ERK pathways. Conclusion: Apatinib reverses the drug resistance to PTX in A549 PTX-resistant cells through inhibiting the function of ABCB1 and resumes anti-cancer effects.*

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Key Words: Apatinib, ABCB1, NSCLC, drug resistance.

Lung cancer is one of the most common and deadly cancers in the world, with a 5-year survival rate of less than 15%. Among all lung cancer cases, non-small cell lung cancer (NSCLC) accounts for about 85%. Patients with advanced NSCLC survive only for 9-12 months (1-3). Chemotherapy is the primary treatment modality for late stage patients (4). However, many lung cancer patients do not respond to chemotherapy or become resistant to one or more therapeutic drugs. This leads to increase of drug dosage, which in turn increase cytotoxicity and has undesirable effects to normal cells/tissues (5).

Multidrug resistance (MDR), the ability of tumor cells to develop resistance to a spectrum of structurally dissimilar drugs, remains a major challenge for the treatment of NSCLC patients with chemotherapeutic compounds (6). Studies over the past few decades have shown that drug efflux, drug delivery protein mutations, and many other factors may lead to MDR in tumors, (7-9) among which overexpression of ATP-binding cassette (ABC) transport proteins is the most important one (10). Among the ABC transporters, ABCB1 is most widely studied. It is encoded by the *ABCB1* gene and is a member of the largest subfamily of transmembrane transporters (11, 12) ABCB1 is an ATP hydrolase that binds the drug in the presence of ATP and pumps it out of the cell, resulting in a decrease of intracellular drug levels (13). Studies have shown that overexpression of ABCB1 on the surface of tumor cells usually leads to MDR to various chemotherapeutic medications including taxanes, anthracyclines, vinca alkaloids and epipodophyllotoxin (14, 15). Therefore, ABCB1 has become an important target for developing resistance reversal agents (16) and many ABCB1 inhibitors have been developed and intensively studied since 1981 (17, 18). Although many have gone through clinical trials, there is still no mature clinical therapy using this strategy. New and more effective inhibitors are needed (19, 20).

Apatinib is a novel small molecule anti-angiogenic agent and a tyrosine kinase inhibitor (TKI) that selectively inhibits

vascular endothelial growth factor receptor-2 (VEGFR-2). It can block the signal transduction of vascular endothelial growth factor (VEGF) and its receptor, thereby strongly inhibiting angiogenesis in tumor cells and exerting anti-tumor effects (21). Clinical trials show that patients with advanced gastric cancer who failed standard chemotherapy obtained a desirable therapeutic effect and higher survival rate by apatinib treatment (22). In addition to acting on VEGF pathway, apatinib has been reported to reverse ABC transporter-mediated multidrug resistance. Apatinib could act on cells overexpressing ABCB1, and greatly improve the cytotoxicity of ABCB1 substrates (23).

Herein, we first showed the reversal effect of apatinib on PTX resistance in lung cancer cells, A549/PTX, the cell line derived from A549, the most commonly used lung cancer cell line in vitro. We successfully established A549 PTX resistant cell line A549/PTX. Subsequently we determined that expression of ABCB1 was higher in A549/PTX cells compared to their parental A549 cells. We further demonstrated that apatinib was able to reverse the drug-resistant phenotype of A549/PTX and sensitize them to treatment. Finally, we explored the reversal mechanisms and offered a perspective for its potential clinical usage in treating chemotherapy drug resistance patients with non-small cell lung cancers.

Materials and Methods

Materials. Apatinib, PTX and verapamil were purchased from American Selleck Co., Ltd. (Houston, TX, USA); RPMI-1640 medium and fetal bovine serum were purchased from BI Company (Israel); CCK8 reagent was purchased from Beijing Suobao Technology Co., Ltd. (China); cell cycle, apoptosis and reactive oxygen test kits were purchased from Jiangsu Kaiji Biotechnology Co., Ltd. (China); AKT, ERK1/2, ABCB1 and other antibodies were purchased from Cell Signaling Technology (CST, Danvers, MA).

Cell lines and cell culture. Human lung cancer A549 cells were purchased from the Cell Culture Bank of the Chinese Academy of Sciences' Type Culture Collection Committee (Shanghai).PTX-resistant A549/PTX cells were produced by the induction of a PTX concentration gradient. The A549 and the PTX-resistant cell line A549/PTX were cultured in 60 mm culture dishes containing RPMI-1640 with 10% fetal bovine serum at 37˚C in a humidified atmosphere of 5% CO_2.

Cell viability assays. A549 and A549/PTX cells were seeded into flat-bottomed 96-well plates at an initial density of 3500 cells per well before treatment. After 24 h, cells were exposed to varying concentrations of apatinib (0, 2, 4, and 8 μM), verapamil (8 μM), or their combination with varying concentrations of PTX (19683 nM to 3 nM, 3-fold diluted). After incubation at 37˚C for 72 h, 10 μl of the CCK-8 was added into each well for 4 h. Cell viability was monitored through measurement of the absorbance at 450 nm on an ELISA reader. Each set of experiments was repeated three times independently with six replicated wells. SPSS software was employed to calculate IC_{50} values for each group.

Table I. *Apatinib reduces paclitaxel IC_{50} values in A549/PTX cells. The data represent three different experiments and are shown as mean±SD (n=3).*

	A549/PTX	
Drug	IC_{50}±SD (μM)	Fold reversal
Paclitaxel	1.332±0.122	1
+ 2 μM Apatinib	0.370±0.036**	3.6
+ 4 μM Apatinib	0.119±0.023**	11.19
+ 8 μM Apatinib	0.009±0.004**	148
+ 8 μM Verapamil	0.016±0.003**	83.25

**: indicates $p<0.01$ compared with the Paclitaxel group.

Apoptosis analysis. A549/PTX cells were placed in 6-well plates (4.0×10^5 cells/well) and incubated for 24 h. Then, they were cultured with 0.5 μM PTX with or without 8 μM apatinib for 48 h. Cells were then collected and washed twice with cold PBS, suspended in 100 μl of binding buffer at a concentration of 1×10^6 cells/ml, and were incubated with fluorescein isothicocyannte (FITC)-conjugated annexin V reagent and propidium iodide (PI) in binding buffer for 20 min at room temperature as described by the manufacturer. Cells were then analyzed *via* flow cytometry (488 nm excitation and 600 nm emission filters) using a BD FACS Calibur flow cytometer (BD FACS Canto™, BD Biosciences). The apoptotic cells were identified by the localization of Annexin V and PI.

Cell cycle assay via propidium iodide staining assay. A549/PTX cells were first seeded in 6 cm dishes at 6×10^5 cells and incubated for 24 hours. Then A549/PTX cells were treated in four groups for 24 h (negative control group, 0.5 μM PTX group, 8 μM apatinib group, and 8 μM apatinib combined with 0.5 μM PTX group). Cells were harvested and washed with PBS. The cells were then collected by a centrifuge at 2000 rpm/min for 5 min, and fixed in 1 ml of 70% ethanol at 4˚C overnight. On the second day, ethanol was discarded and the cells were washed again two times with ice-cold PBS, and then 500 μl of pre-formulated PI/RNase A were added. They were stained at room temperature for 30-60 min in the dark. Cells were then harvested, washed, and suspended in PBS to a final concentration of 1×10^6/ml and analyzed by BD FACS Aria flow cytometry (488 nm excitation). Cell cycle distribution was measured by flow cytometry using the ability of intracellular DNA to bind to a fluorescent dye (propidium iodide PI).

Reactive oxygen species (ROS) measurement. A549/PTX cells were first seeded in 6-well plates (4.0×10^5 cells/well) and incubated for 24 h. Cells were treated for 24 h as described above, collected, and suspended in diluted DCFH-DA (1:1,000 with the serum free medium) to a cell concentration of approximately 1×10^6 per milliliter and incubated for 20 min in a 37˚C cell culture incubator. Cells were being mixed every 3-5 min to ensure that the probe fully reacted with the cells. The cells were then washed three times with serum-free medium to completely remove DCFH-DA that did not enter the cells. The intensity of intracellular fluorescence was measured by flow cytometry, and then the levels of ROS in cells among different groups were compared.

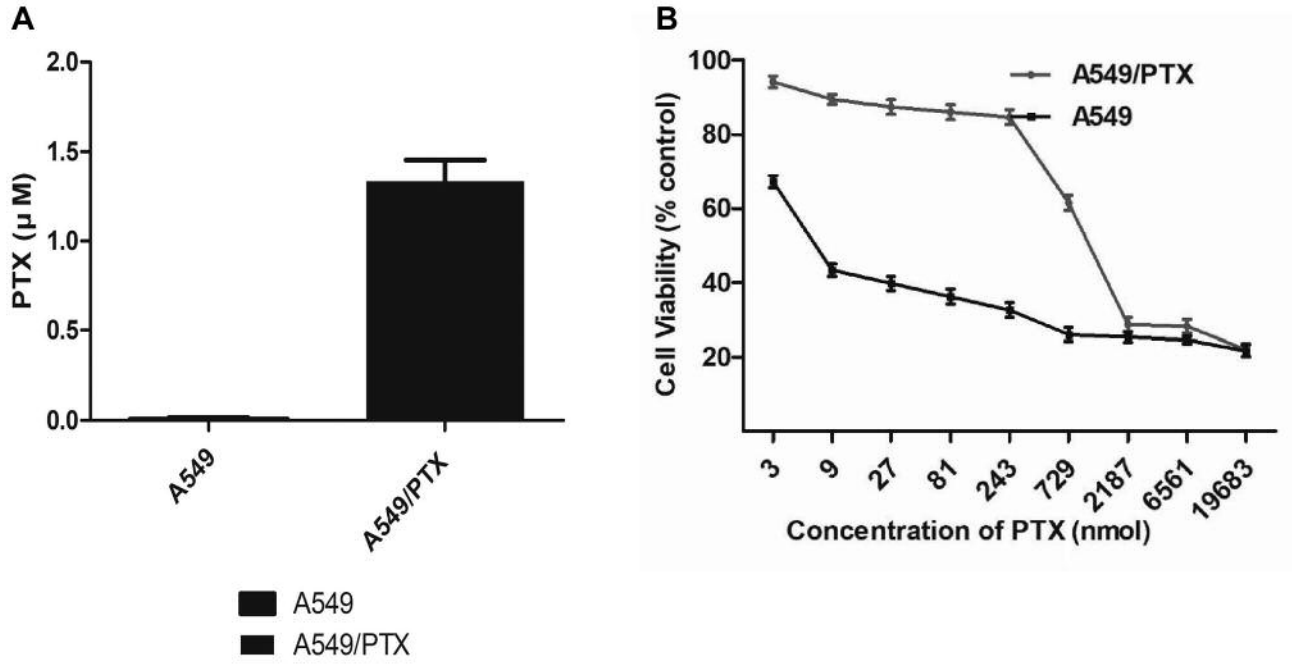

Figure 1. *PTX resistant A549/PTX cell line model. The cells were treated with various concentrations of PTX for 72 h. Cell growth was determined using the CCK8 assay (A) IC_{50} values of paclitaxel for A549 and A549 /PTX cells. (B) A549 and A549/PTX cell survival curves. The data are representative of three different experiments and are shown as mean±SD (n=3).*

Rhodamine accumulation assay. The function of P-gp was evaluated by examining the intracellular accumulation and efflux of Rh123 as previously described (24; 25). A549/PTX cells were treated with 2, 4 or 8 μM apatinib for 24 h. Then the culture medium was replaced with a new one supplemented with rhodamine at a final concentration of 1 μg/ml, and the cells were further incubated for 1 h. Subsequently, the culture medium was removed and cells were washed two times with PBS. Finally, the cells were suspended into 250 μl PBS, followed by FACS to examine green fluorescence intensity. A549/PTX cells were saturated with rhodamine for 1 h, and the fluorescence intensity of rhodamine in the cells was measured by flow cytometry to obtain the total cellular content of rhodamine. The intracellular rhodamine retention rate reflects the efflux function of the ABCB1 transporter.

PTX accumulation assay. The experiment was conducted in four groups: A549 group, A549/PTX group, A549/PTX 8μM apatinib group, and A549/PTX 8 μM verapamil group. First, the cells were cultured in 100 mm dishes. Following attachment of cells, apatinib or verapamil was added to the respective two groups of the cells. After 24 h, the medium was replaced with new one supplemented with 20 μM PTX and incubation continued for an additional 3 hours. In the end, the medium was removed and the cells were collected and washed three times with ice-cold PBS. Then, 3×10^6 cells of each group were resuspended in 1ml of PBS. Following sonication to break down the cells in each group, 7 ml of ethyl acetate was added to each tube, and the mixture was ultracentrifuged at a low temperature for 15 min. Aliquots of 6 ml of supernatants were dried with a nitrogen blow dryer. Then 120 μl of chromatographic grade methanol was added and the mixture was ultracentrifuged for another 15 min. An aliquot of 50 μl of the supernatant from each group was taken to the loading bottle, and the concentration of paclitaxel in each group was measured by UPLC.

Western blot analysis. Cells collected with trypsin/EDTA were washed twice with PBS and lysed on ice with RIPA buffer containing protease inhibitors. The cell lysate was centrifuged at 11000 rpm for 15 min at 4˚C and protein concentrations were measured by BCA. Then the supernatant was mixed with 6× loading buffer on a 5:1 ratio, and the protein was boiled in a water bath at 100˚C for 10 min. Equal amounts of protein of cell lysates were separated by SDS-PAGE and electrophoretically transferred onto nitrocellulose (NC) membranes. The NC membranes were then sealed in Tris-buffered saline (TBS) containing 5% non-fat milk for 2 h at room temperature, incubated with the diluted primary antibodies at 4˚C overnight, and then washed 3 times with TBST for 10 min each time. Then, NC membranes were incubated with goat anti-mouse or anti-rabbit HRP-conjugated secondary antibody at room temperature for 2 h, followed by three 10 min washes with TBST. The levels of protein expression were examined by chemiluminescence.

Statistical analyses. Data analysis was performed using the statistical software SPSS 19.0. For continuous variables the data were expressed as mean±standard deviation (Mean±SD). The Students *t*-test was used to compare mean difference between two groups. The significant difference was defined as $p<0.05$ (*$p<0.05$, **$p<0.01$).

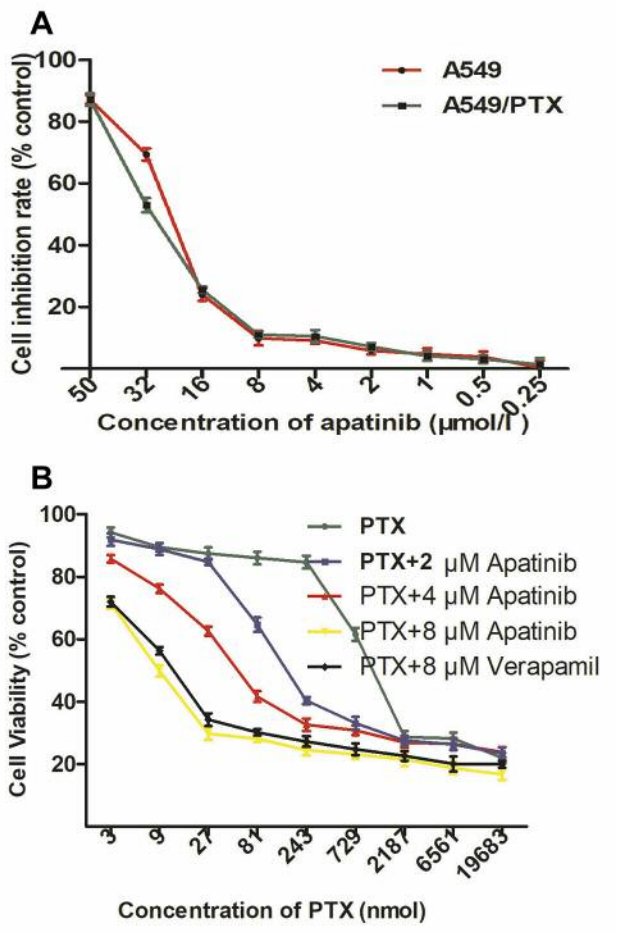

Figure 2. *Apatinib reverses drug resistance. Cells were treated with the indicated drugs for 72 h and subjected to the CCK8 assay. (A) Growth inhibition curve of apatinib on A549 and A549/PTX cells. (B) Apatinib increases the inhibitory effect of PTX on the proliferation of A549/PTX cells. The data are representative of three different experiments and are shown as mean±SD (n=3).*

Results

Establishment and confirmation of PTX-resistant A549/PTX cell line. By drug induction, we obtained the PTX-resistant cell line A549/PTX for non-small cell lung cancer. We determined the IC_{50} values of PTX in the A549/PTX and its parental cell line (A549). The mean IC_{50} value of PTX was 160-fold higher in A549/PTX cells than A549, suggesting that a highly resistant NSCLC PTX-resistant cell line A549/PTX was successfully established (Figure 1).

Apatinib reversed A549/PTX cells resistance. First, we determined the cytotoxicity of apatinib for A549 and A549/PTX cells by the CCK8 method. Apatinib had similar IC_{50} values for both A549 and A549/PTX cells. At 8 μM,

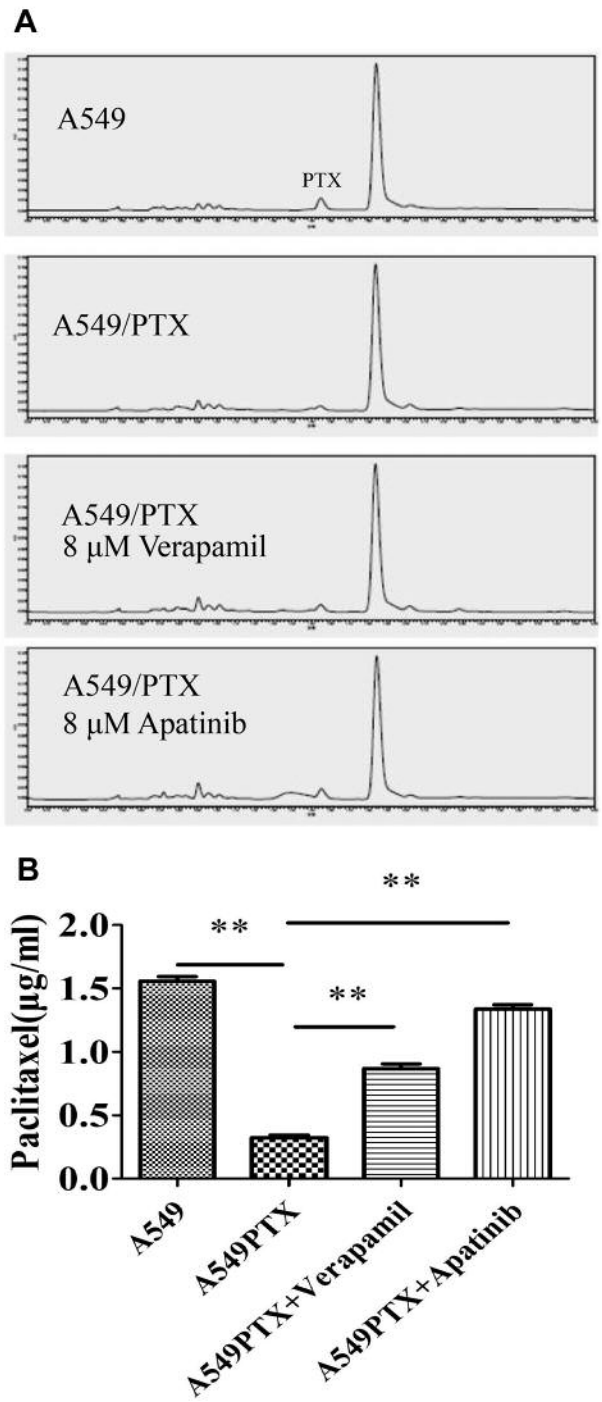

Figure 3. *Effect of apatinib on intracellular PTX accumulation. (A) UPLC tracer of PTX accumulation in cells. The accumulation of PTX in A549/PTX cells is remarkably lower than that in A549 cells. Apatinib greatly increased the accumulation of PTX in A549/PTX cells and it was more effective than verapamil at the same concentration. The peak marked as "PTX" (and the same positions for other groups) is the peak time point of PTX. Peak area represents the concentration of PTX. (B) The amount of PTX accumulation in each group of cells. The bars represent data from three different experiments and mean±SD (n=3). **p<0.01 compared with the other group.*

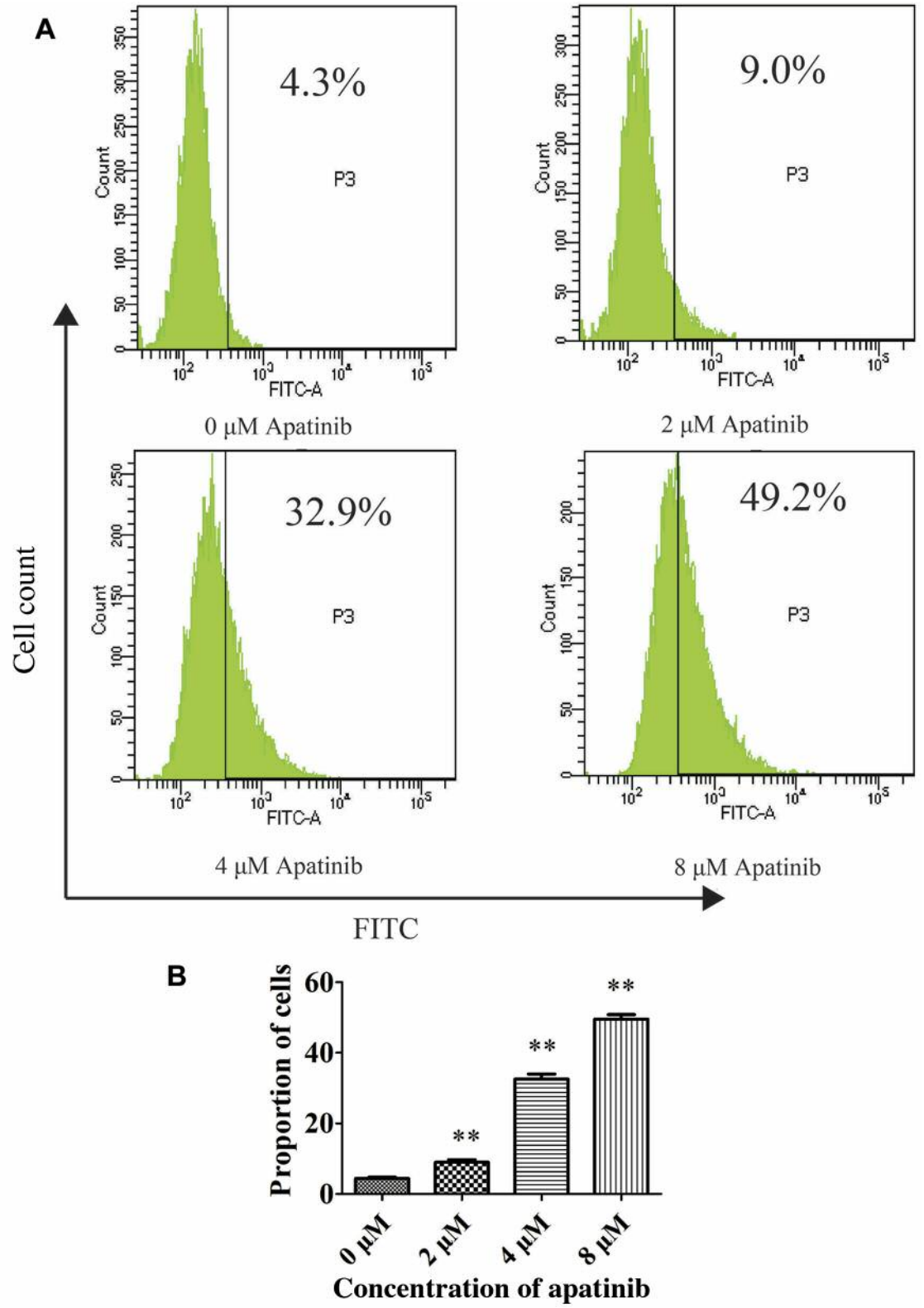

Figure 4. *Effect of apatinib on rhodamine retention in A549/PTX cells. (A) Apatinib enhances the retention of rhodamine in A549/PTX cells (B) Proportion of highly fluorescent cells (P3) in each group. The bars represent data from three different experiments and mean±SD (n=3). **p<0.01 compared with the control group.*

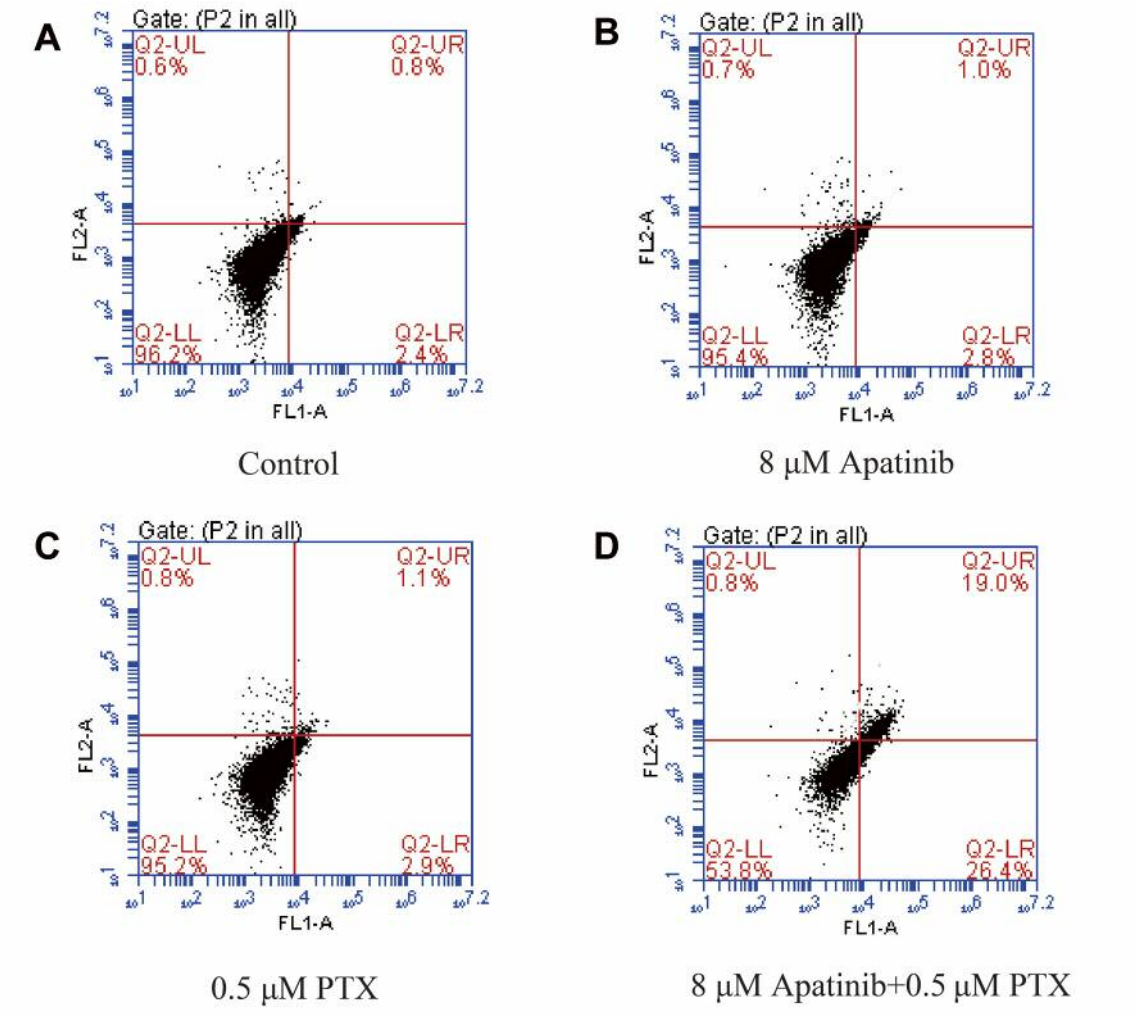

Figure 5. *Apatinib can potentiate PTX-induced apoptosis in A549/PTX cells. The cells were treated with apatinib (8 μM) and/or PTX (0.5 μM) for 48 h, stained with annexin V–FITC, and analyzed by flow cytometry.*

apatinib did not have significant cytotoxic effects on these cell lines and more than 90% of the cells survived. Based on this, apatinib was used at concentrations ranging from 2 to 8 μM. As shown in Figure 2, treatment with apatinib significantly reduced the IC_{50} value of PTX in A549/PTX cells in a dose-dependent manner. At 2 μM, 4 μM, and 8 μM, apatinib reduced the IC_{50} of PTX by 3.6-fold, 11.19-fold, and 148-fold, respectively. As a positive control, 8 μM verapamil reduced the IC_{50} of PTX by 83.25 times. These results showed that apatinib could reverse the resistance of A549/PTX cells to PTX and had a stronger reversal effect than verapamil (Table I).

Apatinib significantly increased the accumulation of PTX in A549/PTX cells. We used UPLC to measure the intracellular PTX content. Based on the peak area of the liquid chromatography, the PTX content was determined by a standard curve. As shown in Figure 3, the accumulation of PTX was significantly lower than that in A549 cells, suggesting that A549/PTX cells had increased ability of PTX efflux which may be an important cause of drug resistance in A549/PTX cells. However, in apatinib treated A549/PTX cells, the accumulation of PTX was significantly increased, at levels higher than those obtained following treatment with the known drug efflux pump inhibitor verapamil at the same concentration.

Apatinib enhanced the retention of rhodamine in A549/PTX cells. Rhodamine, a known specific ABCB1 substrate, is often used to detect the function of ABCB1 (24; 25). To explore the mechanism by which apatinib increases the concentration of PTX in drug-resistant cells, we used the rhodamine efflux

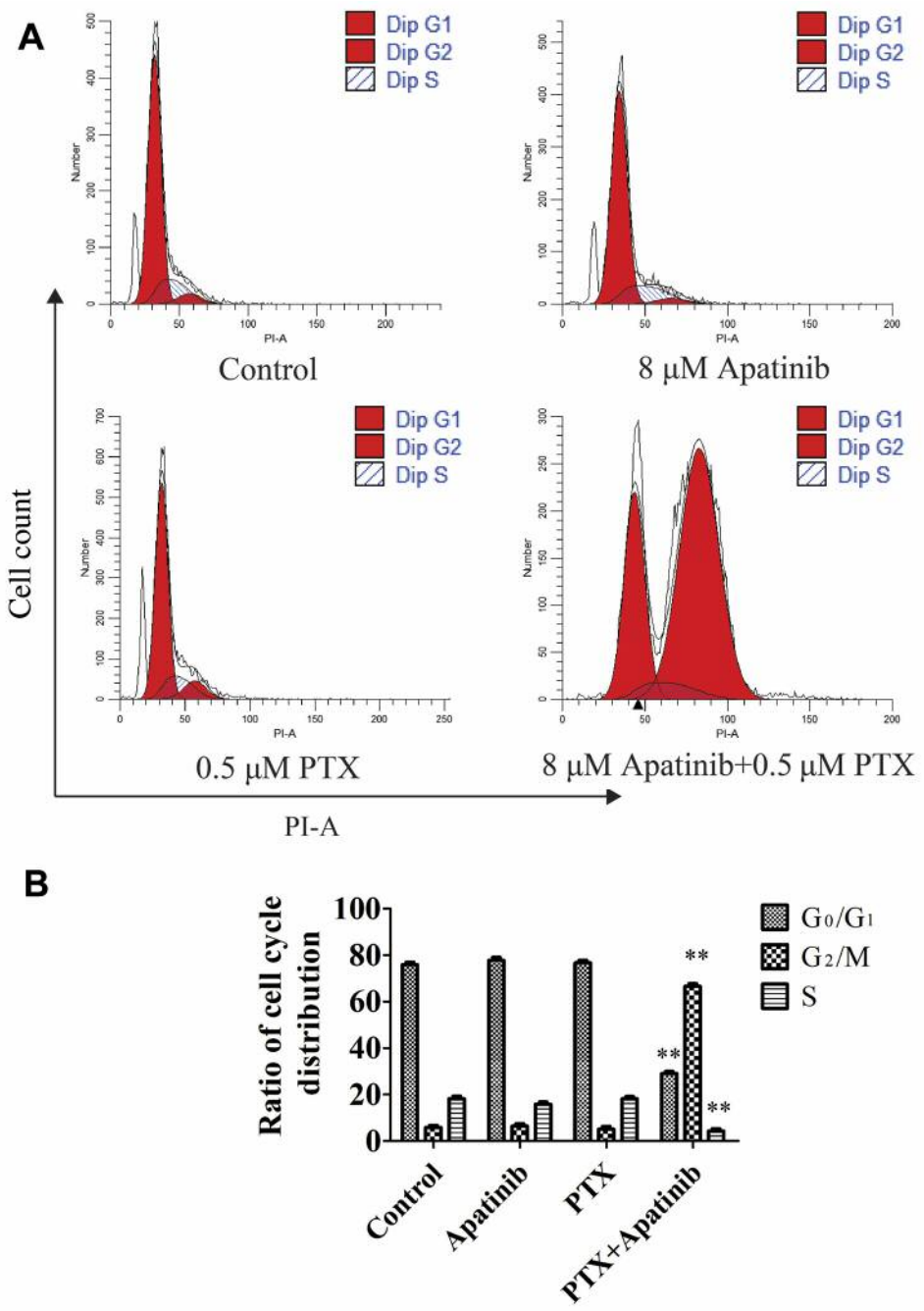

Figure 6. *Effect of apatinib and PTX on the cell-cycle of A549/PTX. (A) The cell cycle distribution profiles of the cells treated with apatinib (8 μM) and/or PTX (0.5 μM) were determined by flow cytometry. The first red peak in the cell-cycle diagram represents the G_1 phase, and the second red peak represents the G_2 phase. (B) Distribution of cells in each phase of the cell cycle in each group. The data represent experiments from three replicates shown as mean±SD (n=3). **p<0.01 compared with the control group.*

assay. As shown in Figure 4, the intracellular rhodamine retention rate increased significantly along with the increasing concentration of apatinib. This indicated that apatinib led to a decrease of ABCB1 activity in the cells, which cannot effectively discharge rhodamine out of cells.

Apatinib enhanced PTX-induced apoptosis, cell-cycle arrest and ROS generation in A549/PTX cells. We used flow cytometry to investigate whether apatinib increased PTX-induced apoptosis, cycle arrest, and intracellular ROS generation in A549/PTX cells. As shown in Figure 5, no

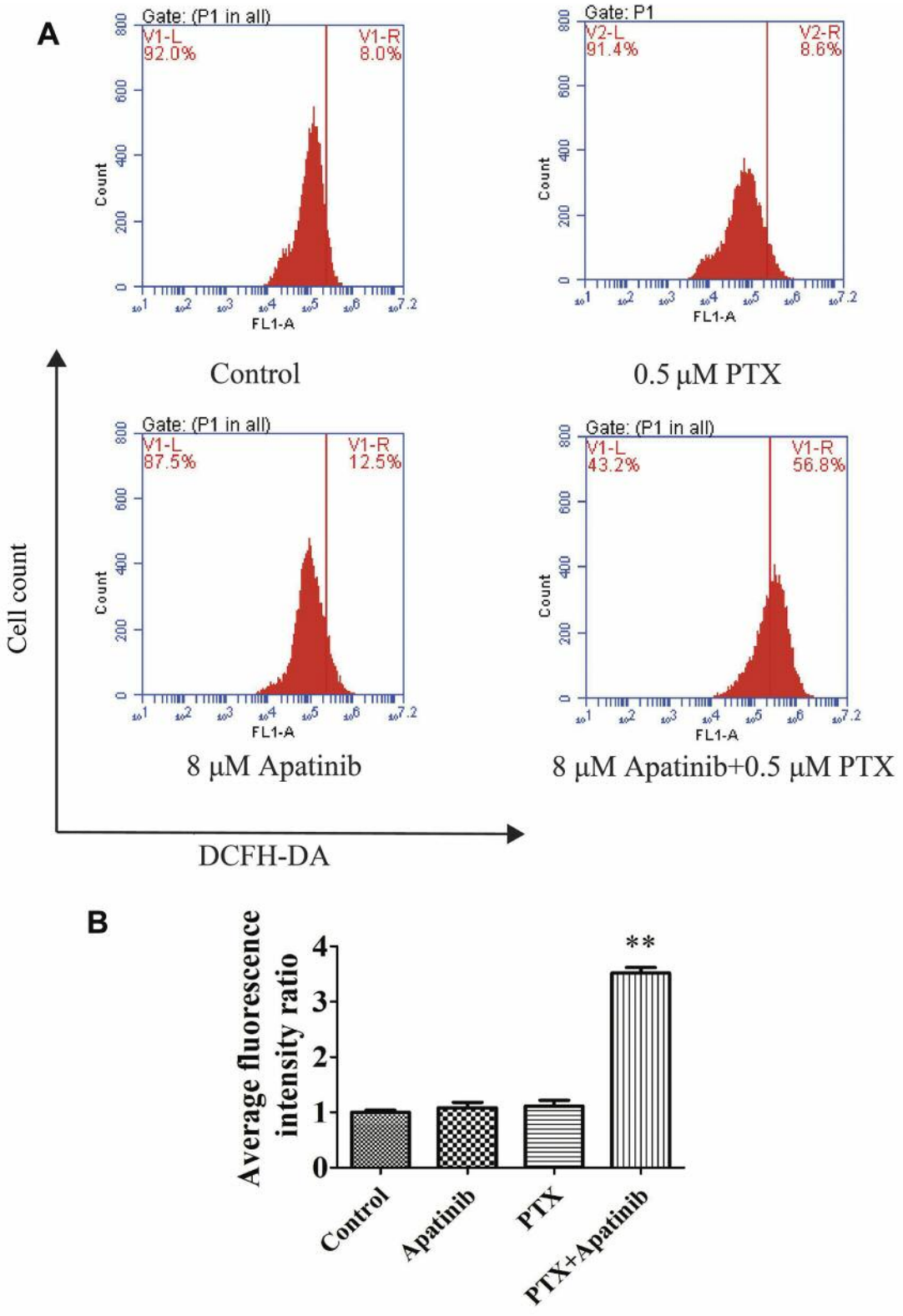

Figure 7. *Effect of apatinib and PTX on ROS level in A549/PTX cells. (A) Eight μM apatinib only or 0.5 μM PTX only had no effect on ROS level in A549/PTX cells. Apatinib can increase PTX-induced intracellular ROS levels. (B) Average fluorescence intensity of each group. The data are representative of three different experiments and are shown as mean±SD (n=3). **p<0.01 compared to the control group.*

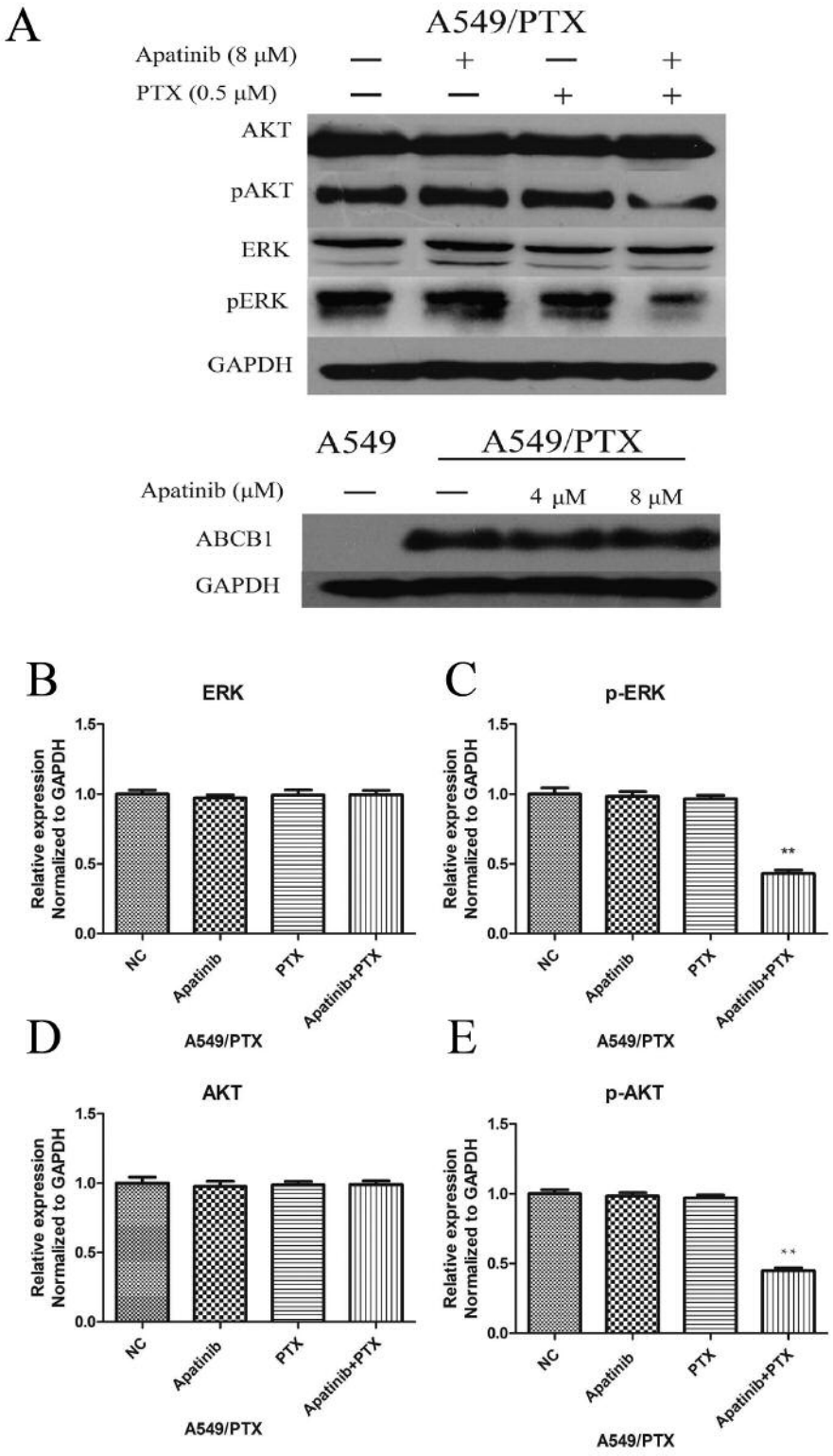

Figure 8. *Effects of apatinib on ABCB1 expression and the combination treatment of PTX and apatinib on AKT/ERK pathway. (A) ABCB1 is highly expressed in A549/PTX, but not in A549 cells. Apatinib did not reduce ABCB1 expression in A549/PTX. Combination treatment of PTX and apatinib reduced the expression levels of pERK and pAKT (C, E), but little effect was seen for the total ERK and AKT by western blot assay (B) (D). Eight μM apatinib only or 0.5 μM PTX only had no effect on the expression of total ERK, total pERK, AKT, and pAKT (B-E)(D)(E). The data represent three different experiments and are shown as mean±SD (n=3). **p<0.01 compared with the control group.*

obvious apoptosis was observed with 8 μM apatinib or 0.5 μM PTX alone. However, when the two were combined, there were significantly increased early apoptosis and late apoptosis. After incubation with 0.5 μM PTX alone, the proportion of cells in the G_1, G_2/M and S phases were 76.69%, 5.10%, and 18.21%, respectively, which were not significantly different from those in the negative control group. However, when combined with 8 μM apatinib, the proportions changed to 29.03%, 66.65%, and 4.32%, respectively. The increase in the proportion of cells in the G2/M phase was dramatic (Figure 6). Apatinib alone (8 μM) did not have significant effects on apoptosis and cell cycle distribution of A549/PTX cells. Additionally, the combined application also significantly increased ROS levels in the cells (Figure 7).

Apatinib-PTX combination inhibits AKT and ERK pathways. Treatment of A549/PTX cells with 0.5 μM PTX and 8 μM apatinib for 24 h, decreased significantly the expression of pAKT and pERK without having an effect on the levels of total AKT and ERK (Figure 8). This suggests that the combination of the drugs had an inhibitory effect on PI3K/AKT and MAP kinase/ERK pathways. However, the application of 0.5 μM apatinib or 0.5 μM PTX alone had no effect on the expression of total AKT, total ERK, pAKT, and pERK. Apatinib did not have an impact on ABCB1 expression in A549/PTX cells (Figure 8).

Discussion

Paclitaxel (PTX) is a widely used chemotherapeutic drug that has shown good results in the treatment of various malignancies including lung cancer. However, the acquired drug resistance often leads to failure of lung cancer treatment (5). Therefore, it is critical to find new drugs that can reverse drug resistance and improve the efficacy of clinical therapy. One of the mechanisms by which tumor cells develop resistance to chemotherapeutic drugs is through the overexpression of multidrug resistance proteins such as ABCB1 (13). They increase the efflux of the drugs, resulting in a decrease of effective intracellular drug concentration.

Apatinib is an anti-angiogenic small molecule, which selectively binds to VEGFR-2 and inhibits tumor angiogenesis and tumor growth (21). However, it has also been shown that it inhibits MDRs such as ABCB1. Mi *et al.* have found that MCF-7/Adr, KBv200 and S1-M1-80 resistant cells have high expression of ABCB1 and apatinib could sensitize the drug-resistant cells through inhibition of ABCB1 and subsequent increase in the intracellular drug concentration. Some *in vivo* studies have also shown that apatinib has a synergistic anti-tumor effect with doxorubicin, mitoxantrone and vincristine on the transplantation model of mouse drug-resistant cells (22; 23).

In the present study, we first showed the reversal effect of apatinib on PTX resistance lung cancer cells. We demonstrated that apatinib reversed drug resistance in A549/PTX cells in a dose-dependent manner. At the same concentration, the reversal effect of apatinib was stronger than that of the well-known ABCB1 inhibitor Verapamil. Using verapamil as a positive control, (26) we showed that the reversal effect was achieved by inhibiting the function of ABCB1 and that apatinib had a synergetic effect with PTX in promoting apoptosis, causing G_2/M phase arrest, and increasing intracellular ROS levels in A549/PTX cells. Consistent with our hypothesis and previous reports, ABCB1 was greatly increased in A549/PTX cells compared to A549 cells. Apatinib did not have an effect on ABCB1 expression; however, combined apatinib with PTX inhibited PI3K/AKT and MAP kinase/ERK pathways; one of the molecular mechanisms to inhibit cell proliferation (27; 28). However, further studies are needed to prove whether apatinib can successfully reverse drug resistance of lung cancer in animals and human subjects through clinical trials.

Conclusion

Apatinib can increase the concentration of PTX in A549/PTX cells, making resistant cells sensitive to PTX again. This effect, which takes place mostly through the inhibition of the transport function of ABCB1, provides a new possible approach to manage chemotherapy-resistant lung cancer.

Conflicts of Interest

The Authors declare no conflict of interests regarding this study.

Authors' Contributions

SYA conceived and designed the experiments. SYA performed the experiments, analyzed the data and created the figures. ZQC and SYA wrote the paper. SYA, CXS, XZW, WJ and OM discussed the data. ZXJ and ZFS supervised the whole experimental work and revised the manuscript. All Authors read and approved the manuscript.

Acknowledgements

The Authors are grateful to the National Natural Science Foundation of China for funding. This work was funded by the National Natural Science Foundation of China (Nos. 81472835 and 81670091).

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Received August 5, 2019
Revised August 20, 2019
Accepted August 21, 2019