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ORIGINAL ARTICLE

# In vitro sequence-dependent synergism between paclitaxel and gefitinib in human lung cancer cell lines

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**Abstract**
*Purpose* In clinical trials, the epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) administered concomitantly with first-line cytotoxicity chemotherapy failed to confer a survival benefit to patients with non-small-cell lung cancer (NSCLC). The aim of this study was to test whether paclitaxel followed by gefitinib is superior to other treatment schedules of NSCLC cell lines and to clarify the underlying mechanisms.
*Methods* Human lung cancer cell lines with wild-type and mutant-type EGFR genes were used as in vitro models to define the differential effects of various schedules of paclitaxel with gefitinib treatment on cell growth, signaling pathway, and cell cycle distribution.
*Results* Sequence-dependent antiproliferative effects differed between EGFR-TKI-resistant and EGFR-TKI-sensitive lung cancer cell lines. Exposure to paclitaxel resulted in an increased pEGFR level. This increase in phosphorylation was inhibited by subsequent exposure to gefitinib, whereas during the reverse sequence, the inhibition effect was reduced. After paclitaxel exposure, a higher level of pEGFR was observed in mitotic than in interphase cells. The sequence of paclitaxel followed by gefitinib resulted in greater anti-VEGF activity than did the reverse sequence. We confirmed that gefitinib arrested cells in G1, and paclitaxel arrested them in S phase. The sequence of paclitaxel followed by gefitinib arrested cells in G1, whereas the reverse sequence arrested cells in S and G2 phases.
*Conclusions* These findings suggest that the sequence of paclitaxel followed by gefitinib may be superior to other sequences in treating NSCLC cell lines. Our results also provide molecular evidence to support clinical treatment strategies for patients with lung cancer.

**Keywords** Lung cancer · Epidermal growth factor receptor tyrosine kinase

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## Introduction

Lung cancer is the leading cause of cancer deaths in developed countries, and non-small-cell lung cancer (NSCLC) accounts for 80–85% of lung cancer cases [1]. Platinum-based doublet chemotherapy is the mainstay of treatment for advanced NSCLC, with good performance status (PS) [2, 3]. Current data suggest that NSCLC chemotherapy has reached a therapeutic plateau and that new combinations of available cytotoxic agents are unlikely to confer clinically relevant survival improvement [4]. Reports using non-platinum-based doublets have yielded results similar to those of platinum-based doublets, with no differences in toxicity [5]. In the second-line setting, docetaxel and pemetrexed have shown equivalent benefits and are currently approved for second-line treatment of advanced NSCLC [6, 7]. However, the response rate is <10%, and median survival is only about 8 months with either of these agents. Better-tolerated treatment strategies for patients with advanced NSCLC are clearly needed. Gefitinib and erlotinib are orally active, reversible Her-1/EGFR tyrosine kinase inhibitors (EGFR-TKIs). In the phase III trial

INTEREST, gefitinib showed non-inferior survival to docetaxel in pretreated patients with advanced NSCLC [8]. A higher response rate to EGFR-TKIs has been correlated with activating mutations in the EGFR gene [9, 10]. These mutations lead to increased growth factor signaling and confer susceptibility to specific EGFR inhibitors. The IPASS trial indicated that gefitinib is superior to carboplatin–paclitaxel as an initial treatment for patients with advanced NSCLC harboring the EGFR mutation [11]. The EGFR mutation rate was higher in Asian than in Western patients [12].

Paclitaxel is a well-established anticancer agent with activity against a variety of solid tumors. It is a mitotic inhibitor that promotes the polymerization and stabilization of tubulin in microtubules [13].

A key issue in novel treatment modalities against NSCLC is the integration of EGFR-TKI with cytotoxic chemotherapy. Based on preclinical experiments, the combination of gefitinib with chemotherapy was expected to improve survival [14, 15]. However, the concomitant use of gefitinib with cytotoxic chemotherapy (cisplatin–gemcitabine in INTACT-1 and carboplatin–paclitaxel in INTACT-2) showed no survival improvement over chemotherapy alone [16, 17]. The failure to achieve the expected positive results may have been caused by either the lack of a predictive marker of response to EGFR-TKI in combination of chemotherapy or the sequence dependency of the antiproliferative effects of combined chemotherapy and gefitinib. Hence, we hypothesized that gefitinib may be best used in sequence with chemotherapy.

In the present study, we used human lung cancer cell lines with wild-type and mutant-type EGFR genes as in vitro models to define the differential effects of various schedules of cytotoxic drug and anti-EGFR-agent combinations on cell growth, signaling pathways, and cell cycle distribution. Specifically, we tested the EGFR inhibitor gefitinib and cytotoxic drug paclitaxel.

## Materials and methods

### Drugs

Pure gefitinib, kindly provided by AstraZeneca (London, UK), was dissolved in dimethyl sulfoxide (DMSO) at 20 mM as stocking solution. Paclitaxel was purchased from Sigma (St. Louis, MO, USA) and was dissolved in DMSO at 1 mM as stocking solution. The drugs were diluted with culture medium before use.

### Cell line

Human non-small cell lung carcinoma cells A549 were obtained from American Type Culture Collection (Manassas, VA) and maintained in our lab. Human lung adenocarcinoma cells H1975 (ATCC) and PC9 were kindly provided by Dr. Tony Mok, Chinese University of Hong Kong. A549, H1975, and PC9 were grown in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 UI/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere with 5% $CO_2$ and harvested with trypsin–EDTA when the cells were in exponential growth. A549 cells express wild-type EGFR, harbor the K-ras mutation and are resistant to EGFR-TKI. PC9 cells are derived from a lung adenocarcinoma harboring an EGFR exon 19-frame deletion and are highly sensitive to EGFR-TKI. H1975 cells carry the EGFR L858R-T790M mutation and are resistant to EGFR-TKI. A549, H1975, and PC9 cells are highly sensitive to paclitaxel.

### Evaluation of antiproliferative effects

Cell viability was determined via MTT assay, in accordance with the method of Mosmann and Carmichael [18, 19], and linearity between cell numbers and OD values was established. The antiproliferative activity of single-agent treatment was assessed in single monolayer culture condition, with cells grown in 96-well plates. The number of cells per well was A549, 2,000 cells; PC-9, 2,000 cells; and H1975, 3,500 cells. The IC50 value was the concentration resulting in 50% cell growth inhibition by a 72-h exposure to drug compared with untreated control cells. To evaluate the antiproliferative effects of the combined treatment, cells were treated with three different sequences: I, pretreated with paclitaxel 24 h, aspirated and washed once with PBS, followed by gefitinib for 48 h; II, pretreated with gefitinib 48 h, aspirated and washed once, followed by paclitaxel for 24 h; III, treated concomitantly with paclitaxel and gefitinib for 48 h and incubated in drug-free medium for 24 h. The different drug doses were combined using constant ratios of the IC50 values calculated from the previous cytotoxicity tests. Thus, we used 0.125, 0.25, 0.5, 1, 2, and 4 times the IC50 dose in paclitaxel and gefitinib combination doses to calculate the CI value. The concentration ranges of gefitinib used in PC9 are physiological achievable. The lowest concentration of gefitinib used in A549 and H1975 are proximate to the peak plasma drug concentrations in patients [20]. The results of sequential treatment with paclitaxel and gefitinib were analyzed according to the method of Chou and Talaly [21]. The resulting Combination Index (CI) was a quantitative measure of the degree of interaction between different drugs, with $CI > 1.1$, $CI = 0.9–1.1$, and $CI < 0.9$ indicating antagonistic, additive, and synergistic effects, respectively. The CI value was calculated using CompuSyn software (ComboSyn, Inc., Paramus, NJ, USA).

### Western blot analysis

Cells were seeded at $1 \times 10^5$ per plate on 10-cm plates and left to settle overnight. Cells were treated by adding fresh medium containing drugs for the desired times. Media were removed from each of the wells and centrifuged at 1,000*g* for 5 min to pellet any cells that had become detached from the plate surface. Cells that remained attached to the plate were washed twice with ice-cold PBS and then scraped into Ripa buffer. Lysates were sonicated and centrifuged. The protein concentration of each lysate was determined using the bicinchoninic acid assay. Lysates (30 μg protein/well) were resolved on 3–8% acrylamide Tris–acetate gels, and the proteins were transferred to PVDF membranes, blocked for 1 h at room temperature in 5% w/v non-fat milk diluted in TBST and then incubated with appropriate primary antibodies under the conditions recommended by the manufacturers. Primary antibodies: pY1068 EGFR, ps473AKT, AKT, ERK1/2, pERK1/2, PTEN, and $\beta$-actin were purchased from Cell Signaling Technology, EGFR antibody from Santa Cruz. The blots were then washed with TBS-T for 30 min and incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. Antibody binding was detected using an enhanced chemiluminescence system.

For quantification of protein levels, films were scanned and analyzed with labwork software. The relative protein levels were counted using a comparison to untreated control.

### Flow cytometric analysis of cell cycle distribution

Cells were seeded into six-well plates at a density of $1 \times 10^5$ per well. After 24 h, the wells were treated with paclitaxel and gefitinib, alone or sequentially, at IC50 levels. At the end of the experiments, adherent cells were trypsinized, counted, washed, and resuspended, along with the corresponding floating cells. The cells were then pelleted and fixed by dropwise addition of 70% ice-cold ethanol, stored in PBS, and then resuspended in propidium iodide solution (180 μg/ml) and analyzed using flow cytometry.

### Measurement of VEGF culture medium

Vascular endothelial growth factor (VEGF) culture medium was measured using ELISA (Quantikine®, R&D Systems, Minneapolis, MN, USA). H1975 cells were treated with paclitaxel and gefitinib (IC50 dose) using different sequences. After 24 h serum starvation, IC50 doses of paclitaxel and gefitinib were added to the cells in the same sequence. After 72 h, aliquots of supernatant were removed from the dishes, and ELISA was performed on the VEGF medium.

**Table 1** IC50 values for each drug were calculated by performing dose response experiments with gefitinib and paclitaxel

| IC50 | A549 | H1975 | PC-9 |
|---|---|---|---|
| Paclitaxel | 2.71 ± 0.071 nM | 1.68 ± 0.109 nM | 2.52 ± 0.99 nM |
| Gefitinib | 9.47 ± 0.09 μM | 7.88 ± 0.94 μM | 17 ± 0.07 nM |

Each test were repeated in triplicate

### Statistics

Results are presented as mean ± SE of at least three experiments. Student's *t* test was used to assess the statistical significance of differences, with a significance level of $P \leq 0.05$ used in this study.

## Results

### Sequence-dependent antiproliferative effects differed between EGFR-TKI-resistant and EGFR-TKI-sensitive lung cancer cell lines

In our evaluation of the antiproliferative effects of paclitaxel and gefitinib treatment on A549, H1975, and PC-9 cells, we observed dose–response growth inhibition effects. Table 1 summarizes the IC50 of these two drugs. We evaluated the growth inhibition effect on PC-9, A549, and H1975 of three different sequences of combined paclitaxel and gefitinib treatments. As shown in Fig. 1, in all of the cell lines, the antiproliferative effects of the paclitaxel–gefitinib sequence was more potent than either the gefitinib–paclitaxel sequence ($P < 0.05$) or concomitant administration of the two drugs ($P < 0.05$ in median and low dose combination, $P > 0.05$ in high dose combination). In EGFR-TKI-resistant A549 and H1975 cell lines, a clear synergistic growth inhibitory effect at every drug concentration was found only with the sequence of paclitaxel followed by gefitinib (CI < 0.9). In contrast, the sequence of gefitinib followed by paclitaxel resulted in an antagonistic interaction (CI > 1.1). Simultaneous administration of the two drugs resulted in additive and antagonistic effects (Fig. 2a, b). In PC-9 cells, which are highly sensitive to TKI, paclitaxel–gefitinib sequence resulted in synergistic effects. Concomitant administration of the drugs resulted in synergistic and additive effects. The gefitinib–paclitaxel sequence resulted in additive and antagonistic effects as the drug concentration was increased (Fig. 2c). Each experiment was repeated in triplicate.

### Paclitaxel-activated EGFR and the downstream signaling pathway, which were inhibited by subsequent gefitinib

To gain insight into the mechanisms involved in regulating the interaction between paclitaxel and gefitinib, we studied

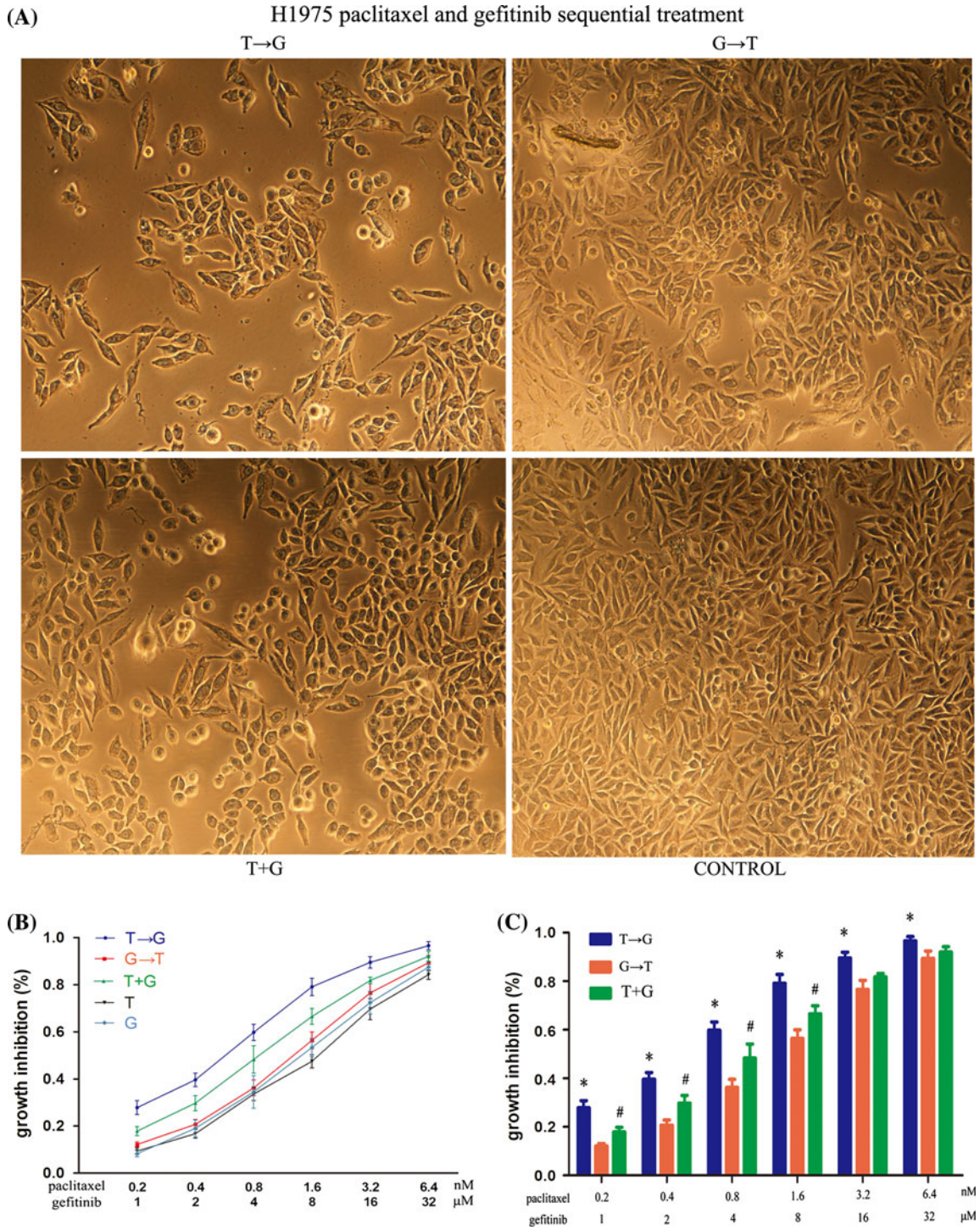


**Fig. 1** The antiproliferative effects between paclitaxel and gefitinib was sequence dependent. The antiproliferative effects of the paclitaxel–gefitinib sequence was more potent than either the gefitinib–paclitaxel sequence or concomitant administration of the two drugs. **a** H1975 cells were exposed to IC50 dose of paclitaxel and gefitinib with different sequences. **b** H1975 cells were exposed to sequential paclitaxel and gefitinib using constant ratios of the IC50 dose. T → G, paclitaxel followed by gefitinib. G → T, gefitinib followed by paclitaxel. T + G, paclitaxel and gefitinib concomitant. *T*, paclitaxel single. *G*, gefitinib single. **c** $^{*}P < 0.05$, T → G versus G → T. $^{\#}P < 0.05$, T → G versus T + G

the effect of treatment on the singling activation of EGFR, AKT, and ERK. Cells were exposed to threefold IC50-value doses of paclitaxel. To evaluate the effect of paclitaxel and gefitinib sequential treatment on the cell-signaling pathway, cells were exposed to the IC50 dose of paclitaxel and gefitinib. As shown in Fig. 3, exposure to

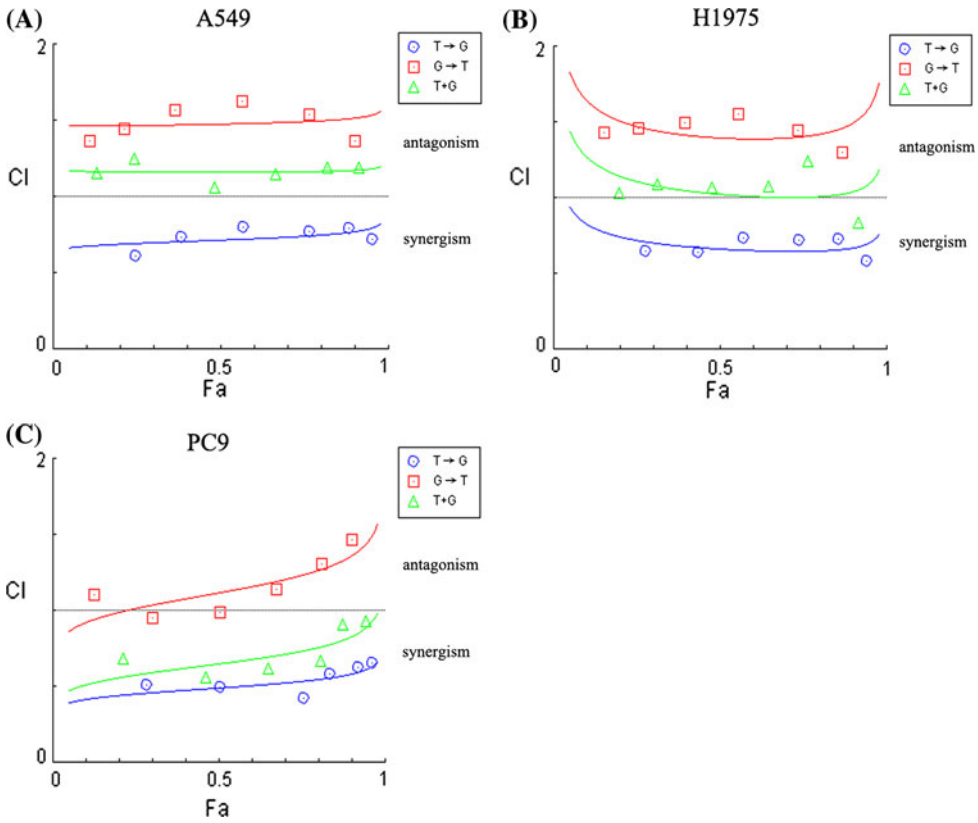


**Fig. 2** Sequence-dependent cytotoxic synergism of paclitaxel and gefitinib in lung cancer cell lines. **a** A549. **b** H1975. **c** PC-9. Points, mean values of three different experiments. *Open circle* T → G, paclitaxel followed by gefitinib. *Open square* G → T, gefitinib followed by paclitaxel. *Triangle* T + G, paclitaxel and gefitinib concomitant. The sequence T → G produced the most potent cytotoxic growth inhibition. Only the T → G sequence resulted in synergism at all drug concentrations

paclitaxel induced EGFR phosphorylation. In A549, we observed an increased pEGFR level 24 h after paclitaxel treatment and lasting to 72 h, with pAKT and pErk1/2 phosphorylation levels also increased (Fig. 3a). Paclitaxel-treated H1975 cells showed an initial increase in the pEGFR level lasting 24 h before decreasing to the basal level at 48 h, with the pAKT level also increasing over 48 h (Fig. 3b). Paclitaxel-treated PC-9 cells also showed an initial increase in the pEGFR level lasting 24 h before decreasing below the basal level (Fig. 3c).

We selected H1975 for sequential analysis, as these cells showed a median level of EGFR activation. As shown in Fig. 4, we found that gefitinib significantly inhibited EGFR phosphorylation ($P < 0.01$). When H1975 cells were exposed to paclitaxel alone, pEGFR and pAkt levels significantly increased after paclitaxel exposure for 24 h ($P = 0.01$). The increase in pEGFR was blocked by adding gefitinib (Fig. 4a, lane 3). In the reverse sequence, after 48 h, gefitinib had inhibited the pEGFR level to 0.3 compared to the untreated control level of pEGFR (Fig. 4a, lane 6), and subsequent paclitaxel exposure for 24 h enhanced the EGFR phosphorylation level to 0.8. The pAKT level also increased to 1.4 compared to the untreated control level of pAKT. The paclitaxel–gefitinib sequence produced significantly greater pEGFR inhibition than did the opposite sequence ($P = 0.04$) (Fig. 4b). These findings suggest that EGFR and its downstream pathway activation may represent a survival response of tumor cells following paclitaxel treatment and that gefitinib subsequently inhibits phosphorylation and may block the initial survival response and promote apoptosis. In contrast, the reverse sequence of gefitinib treatment followed by paclitaxel interferes with the inhibition of pEGFR by gefitinib.

## Paclitaxel activated the EGFR signaling pathway in mitotic cells

Because other studies have shown that mitosis is crucial to paclitaxel-mediated cytotoxicity [22], we hypothesized that the EGFR signaling pathway might have a role in the cell cycle and tested the effects of EGFR signaling activation by paclitaxel on mitotic cells. The mitotic cells were obtained by mechanical shake-off as described by Tobey [23]. As shown in Fig. 5a, 50% of the A549 cells obtained by mechanical shake-off after 8-nM paclitaxel treatment were tetraploid, 30% were aneuploid, and 18% were diploid. Western blot analysis demonstrated that pEGFR was activated in unsynchronized cells, and we observed a greater activation of pEGFR in mitotic cells following paclitaxel treatment (Fig. 5b, c). These results indicate that mitotic cells resulting from paclitaxel treatment may be more sensitive to subsequent EGFR-TKI due to the higher level of pEGFR activation.

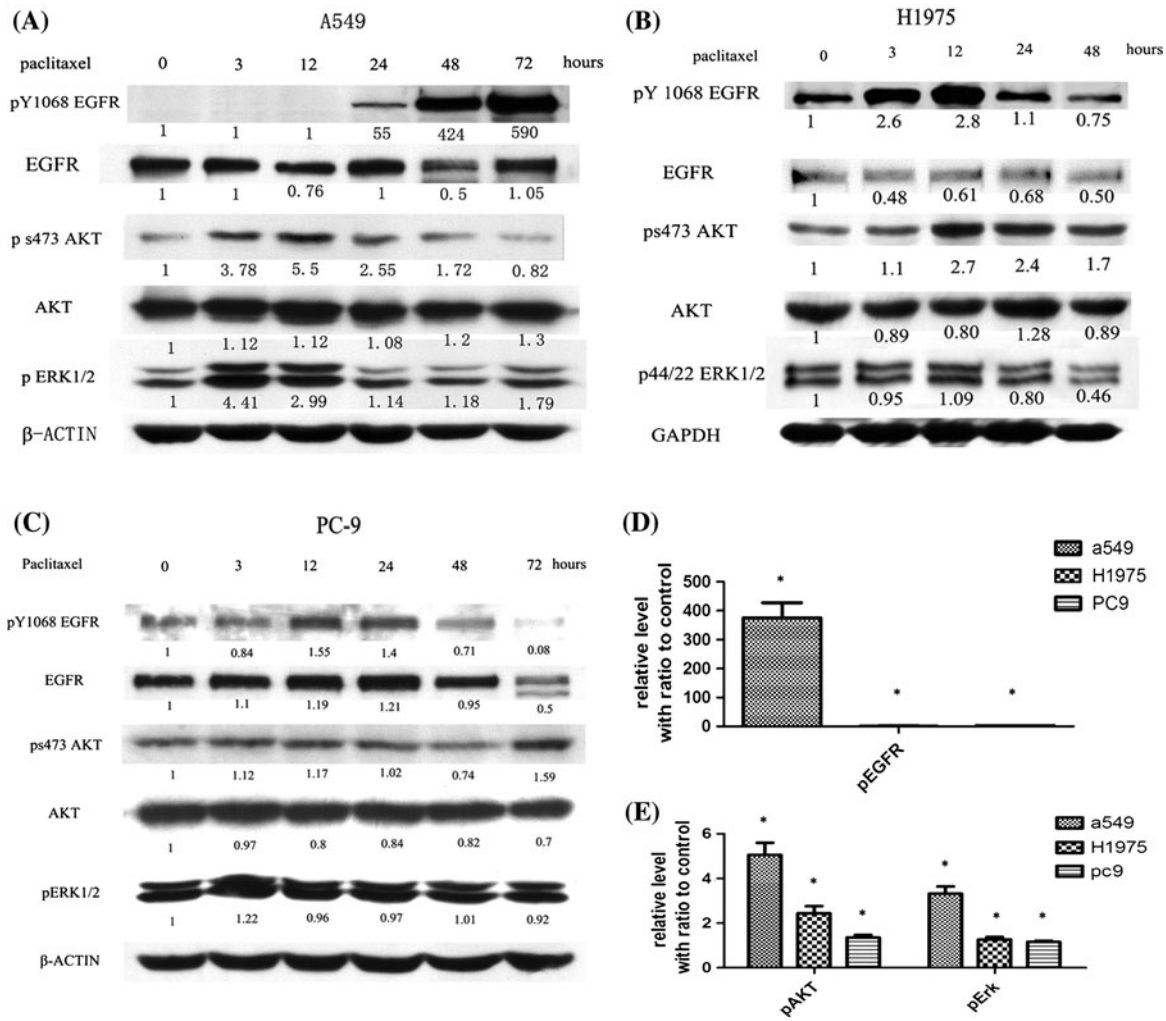


**Fig. 3** Time course of the activation of EGFR and downstream signaling pathway after paclitaxel exposure. The dose of paclitaxel was threefold IC50. **a** A549, **b** H1975, **c** PC-9. Cells were treated for the indicated time. The *numbers* below represent relative expression level compared with the untreated control (**d**, **e**) *Asterisk* represents significantly different from controls ($P < 0.05$)

### The paclitaxel–gefitinib sequence resulted in greater anti-VEGF activity

VEGF is correlated with tumor vasculature, and we evaluated the effect of sequential treatment on VEGF. Figure 5d shows that gefitinib alone lowered the VEGF level beyond that of the control ($P < 0.001$). Paclitaxel alone had no effect on VEGF. The combination of paclitaxel and gefitinib showed anti-VEGF activity, and the paclitaxel followed by gefitinib sequence resulted in significantly greater anti-VEGF activity than did the opposite sequence ($P = 0.02$).

### Cell cycle modulation by paclitaxel and gefitinib

DNA flow cytometry studies were performed to evaluate the effect of single and sequential paclitaxel and gefitinib treatments on cell cycle distribution and determine whether their cell cycle modulation activity might provide clues to optimizing the drug sequence. As shown in Table 2, gefitinib exposure caused cells to accumulate in the G1 phase, but after subsequent treatment with paclitaxel, the cells were blocked in the S phase. In contrast, paclitaxel exposure blocked cells in the S phase, whereas subsequent exposure to gefitinib arrested them in the G1 phase. The sequential and concurrent administration of paclitaxel and gefitinib resulted in cell cycle distributions indicative of overlapping effects from both agents.

## Discussion

We investigated the optimal schedule of combined treatment with paclitaxel and gefitinib in three human lung cancer cell lines and found that the potentiation of antiproliferative activity of paclitaxel and gefitinib in combination was

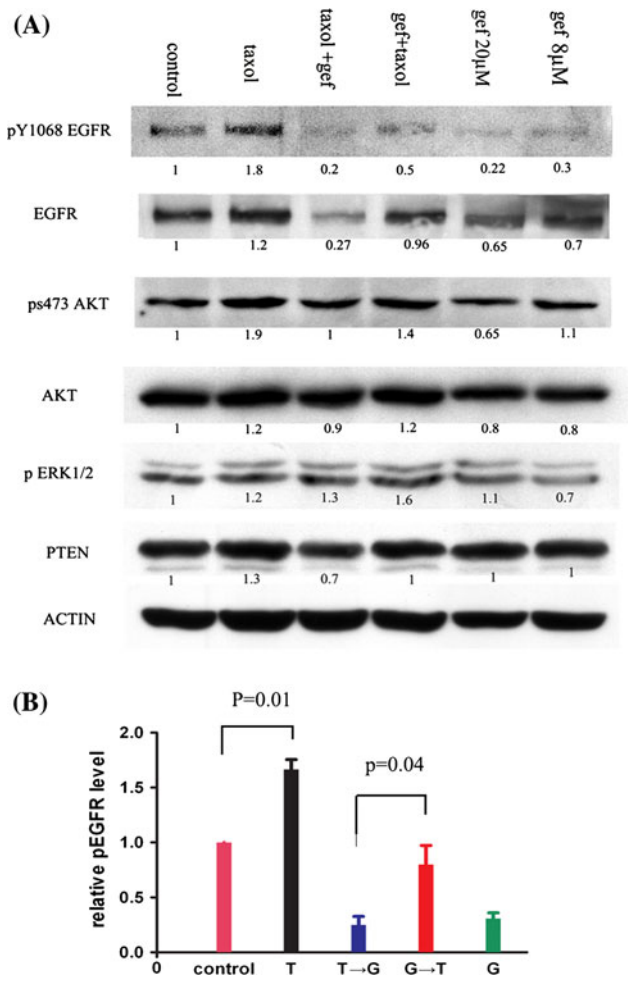


**Fig. 4** H1975 cells were treated with paclitaxel and gefitinib alone or sequentially. **a** *Lane 1* untreated control; *lane 2* paclitaxel only for 24 h; *lane 3* paclitaxel 24 h followed by gefitinib 48 h; *lane 4* gefitinib 48 h followed by paclitaxel 24 h; *lane 5* gefitinib 20 μM for 24 h; *lane 6* gefitinib 8 μM 24 h. The *numbers* below represent relative expression level compared with untreated control. **b** Relative pEGFR level

sequence dependent. We demonstrated that the sequence of paclitaxel followed by gefitinib resulted in a synergistic effect in the three cell lines. Exposure to gefitinib first protected cells from the subsequent paclitaxel treatment. The sequence-dependent antiproliferative effects appear to result from effects on cell cycle progression, growth factor signaling modulation, and anti-VEGF activity.

Paclitaxel binds to $\beta$-tubulin and stabilizes cellular microtubules, stimulates microtubule polymerization. The cell fate of antimitotic drug–induced mitotic arrest display profound variation at different cell line and different drug concentration. The cancer cells either die in mitosis or exit mitosis by slippage into a tetraploid G1 state. From which, they either die, arrest in G1, or initiate a new round of mitosis [24–26]. Two competing networks were proposed to dictate the cell fate, one involving cell death pathway activation, the other protecting cyclin B1 from degradation [25, 27]. In other words, slippage and apoptosis can be viewed as two competing pathways. Slippage is thought to occur by gradual proteolysis of cyclin B1 [28]. Paclitaxel only cause low levels of cell killing in mitotic phase. After withdrawing paclitaxel, blocked mitotic cells slip into interphase. Most cell death occurred after mitosis exit [22, 29]. Slippage is promoted by paclitaxel through the induction of mitotic checkpoint override [30]. Premature exit from mitotic arrest due to a weakened ablated spindle assembly checkpoint (SAC) is known to decrease sensitivity to spindle-perturbing drugs [31].

The effect of EGFR-TKI was not just cytostatic, it also induced apoptotic death in cells with EGFR-activating mutation [32, 33]. In higher doses, The EGFR-TKI increased G1 arrest and apoptosis in cells with wild-type EGFR [34, 35]. EGFR-TKI have shown modest effect in unselected patients, but produced dramatic effect in patients with EGFR-activating mutation. Mitosis is crucial to paclitaxel-mediated cytotoxicity. Exposure to gefitinib first induces G1 arrest and protects cells from subsequent paclitaxel-mediated cytotoxicity.

Chemotherapy-induced activation of cell survival pathways has been increasingly observed with cytotoxicity drugs [22], and targeting these survival signals will be important in the design of innovative combination therapies. Paclitaxel has been shown to activate signaling transduction cascades involved in apoptosis, such as JNK, Raf-1, and Bcl-2 family members [36, 37]. In contrast, paclitaxel also activates cell survival pathways, such as the MEK-ERK pathway and PI3K-AKT cascade, which lead to cell proliferation [38, 39].

We found that treatment schedule has an important effect on cell growth factor signaling. Paclitaxel induced an increase in the EGFR phosphorylation level. The exact mechanisms underlying the increased EGFR phosphorylation level remain to be determined. In H1975, exposure to paclitaxel first increased the $p^{Y1068}$EGFR and the downstream signaling levels. Subsequent exposure to gefitinib blocked these increase and was associated with a decrease in $p^{Y1068}$EGFR. In contrast, exposure to gefitinib first decreased $p^{Y1068}$EGFR, and subsequent treatment with paclitaxel led to a 2.5-fold increase in $p^{Y1068}$EGFR, 40% increase in $p^{S473}$AKT, and 23% increase in pErk1/2, compared to the reverse sequence. In mitotic cells, the pEGFR level increased over that of unsynchronized cells after paclitaxel treatment, which also rendered them more sensitive to subsequent gefitinib treatment. Thus, the sequence of paclitaxel followed by gefitinib seemed to be superior to the reverse schedule, based on factors related to both cell kinetics and growth signaling.

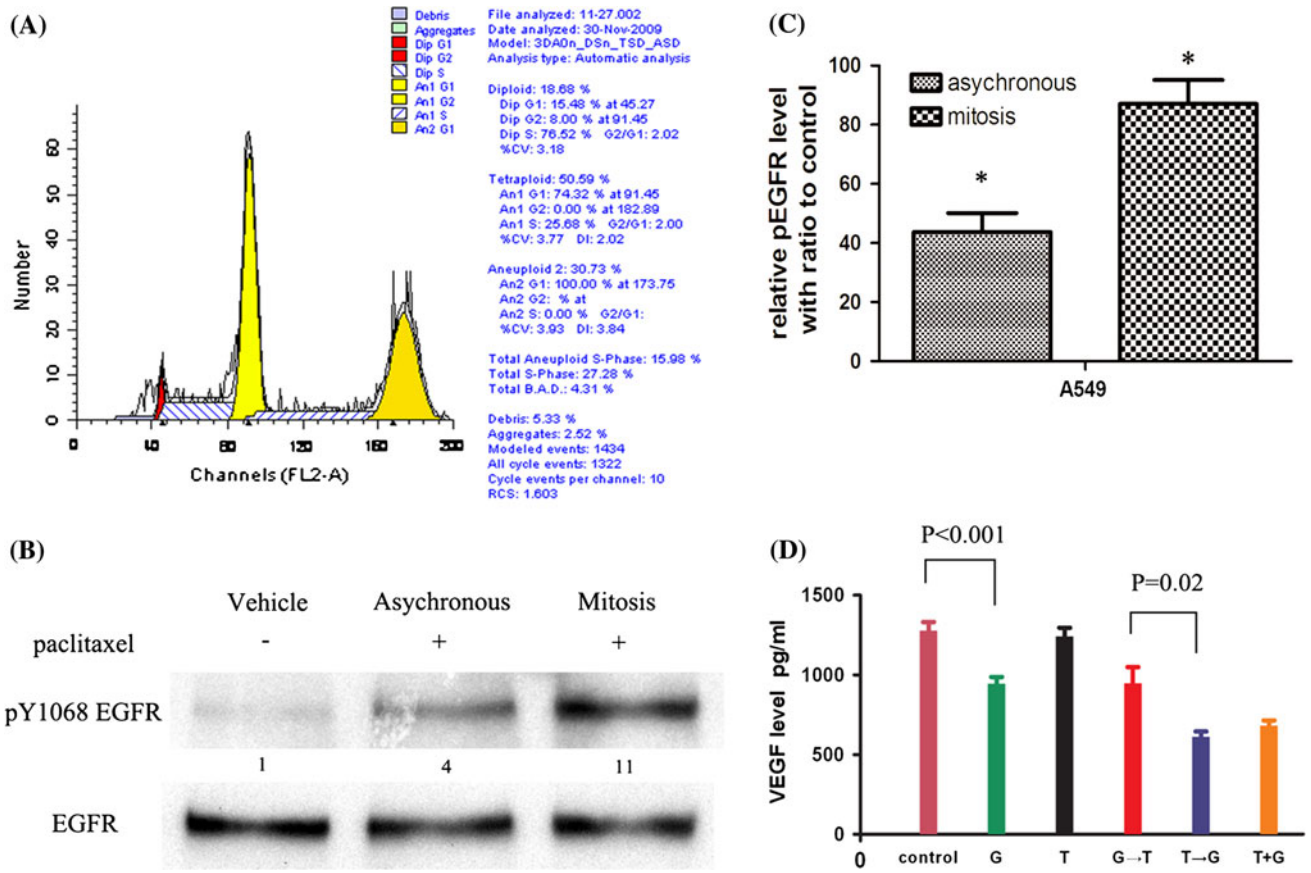


**Fig. 5** **a** Cell cycle distribution of mitotic A549 cells obtained by mechanical shake-off was analyzed using flow cytometry. Half of the cells were tetraploid. **b** Western blot analysis demonstrated higher activation level of pEGFR in mitotic than in asynchronous cells after paclitaxel treatment. **c** *Asterisk* represents significantly different from controls ($P < 0.05$). **d** Sequence-dependent anti-VEGF activity. H1975 cells were treated with sequential paclitaxel and gefitinib. ELISA values were normalized to the number of cells present. The level of VEGF secreted into medium decreased with gefitinib treatment alone ($P < 0.001$). The schedules were paclitaxel followed gefitinib ($P < 0.001$), gefitinib followed by paclitaxel ($P = 0.05$), paclitaxel and gefitinib concomitant ($P < 0.001$) compared with the control. Paclitaxel alone had no effect on VEGF. The VEGF level after treatment with paclitaxel followed by gefitinib was lower than that for gefitinib followed by paclitaxel ($P = 0.02$, Student's $t$ test)

As the EGFR-TKIs gefitinib and erlotinib are active in patients with NSCLC harboring EGFR mutations [11, 40], combining them in treatment with chemotherapy was seen as promising. However, in the INTACT-1, INTACT-2, TALENT, and TRIBUTE clinical trials, the addition of gefitinib or erlotinib to first-line chemotherapy failed to improve survival [16, 17, 41, 42]. Gandara et al. [43, 44] have proposed two hypotheses that seem most likely to explain these negative results. First, patients were not selected on the basis of a predictive response marker, although several studies have reported that mutation activation in the EGFR tyrosine kinase domain was associated with a dramatic response to gefitinib and erlotinib. Second, the potentiation of antagonistic interaction between concurrent EGFR-TKI and chemotherapy may have played a role, as in vitro and in vivo studies of concurrent administration have suggested antagonism. Davies et al. [45] have proposed the pharmacodynamic separation model, EGFR-TKIs primarily cause cell cycle arrest and accumulation of cells in G1 and thus, when administered concurrently with chemotherapy, can interfere with cell cycle-specific cytotoxicity. Like taxoxifen, which induce G1 arrest, produced a negative interaction when combined concurrently with chemotherapy. Gumerlock et al. [46] have compared sequential administration of EGFR-TKI and chemotherapy with concurrent treatment in A549 and Calu cells. The G1 arrest resulting from pretreatment with EGFR-TKIs blocks subsequent chemotherapy effects, and continuous concurrent administration of the combination is less effective than intermittent or sequential pulse delivery. Because schedule effects often play a role in the outcome of treatment, the design of combination protocols requires careful consideration. Several studies have shown that different sequences of a cytotoxic drug with a targeted drug

**Table 2** Cell cycle distribution after drug exposure

| Cells and treatment | G1 phase | S phase | G2/M phase |
|---|---|---|---|
| *H1975* | | | |
| Vehicle control | 56 | 25.8 | 17.7 |
| Gefitinib | 89 | 3 | 6.4 |
| Paclitaxel | 54 | 35 | 10.4 |
| Paclitaxel followed by gefitinib | 80 | 16.2 | 3.7 |
| Gefitinib followed by paclitaxel | 51.1 | 26.2 | 22.7 |
| Paclitaxel and gefitinib concurrent | 53.4 | 31.8 | 14.7 |
| *A549* | | | |
| Vehicle control | 69.5 | 24 | 5.9 |
| Gefitinib | 78.7 | 16 | 5.0 |
| Paclitaxel | 54.3 | 44.5 | 1.5 |
| Paclitaxel followed by gefitinib | 69.2 | 24 | 6.8 |
| Gefitinib followed by paclitaxel | 64.6 | 25.6 | 9.9 |
| Paclitaxel and gefitinib concurrent | 61.3 | 30.3 | 8.4 |
| *PC-9* | | | |
| Vehicle control | 61.6 | 24.1 | 14.3 |
| Gefitinib | 85.3 | 8.5 | 6.2 |
| Paclitaxel | 71 | 26.5 | 2.0 |
| Paclitaxel followed by gefitinib | 91 | 6.2 | 2.4 |
| Gefitinib followed by paclitaxel | 39 | 28 | 32 |
| Paclitaxel and gefitinib concurrent | 90 | 7.7 | 1.54 |

cause different antiproliferative effects [47–49]. In Fast-Act, a randomized phase II trial of sequential erlotinib and chemotherapy as first-line treatment of patients with advanced NSCLC, gemcitabine plus cisplatin or carboplatin (GC) were sequentially followed by erlotinib (GC-E) versus GC followed by a placebo (GC-P). The preliminary results showed that GC-E significantly improved progression-free survival (PFS), compared with GC-P [50]. These studies suggest that the best schedule of EGFR-TKI and a cytotoxic drug to treat NSCLC may be sequential: a cytotoxic drug followed by EGFR-TKI.

In conclusion, this study demonstrated that the most advantageous schedule of paclitaxel and gefitinib to treat NSCLC in vitro is the sequence of paclitaxel followed by gefitinib. We also characterized the molecular mechanisms involved in the synergistic effect between paclitaxel and gefitinib against NSCLC cells. These results have implications for the development of combination regimens in clinical trials.

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**Conflict of interest statement** Yi-long Wu has received speaking honoraria from AstraZeneca, Eli Lilly, F. Hoffmann-La Roche and Pfizer. No other potential conflict of interest relevant to this article was reported.

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