J Lung Cancer 2009;8(2):92-98

Combining Erlotinib with Cytotoxic Chemotherapy May Overcome Resistance Caused by T790M Mutation of EGFR Gene in Non-Small Cell Lung Carcinoma

Purpose: T790M is a mechanism underlying acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs). We hypothesized that a synergistic combination of cytotoxic drugs and EGFR-TKIs may overcome resistance. **Materials and Methods:** The antiproliferative effects and cell cycle distributions following treatments with Erlotinib (E) and cytotoxic drugs (C) were studied using a lung cancer cell line (NCI-H1975) harboring two mutations (L858R and T790M) in the EGFR gene. The cell viability assay and cell cycle analysis were conducted via an MTT assay and flow cytometry. The results of the treatments in different sequences were assessed using the combination index. **Results:** Antagonisms were noted when erlotinib was administered before cytotoxic drugs (EC sequence), whereas synergisms were observed when pre-treatment with cytotoxic drugs was administered before erlotinib (CE sequence). Treatment in the EC sequence arrested the cells in G0/G1 phase and reduced the apoptotic fraction. However, treatment in the CE sequence arrested the cells in the G2/M and S phase and a trend toward higher fractions of apoptotic cell death was observed. **Conclusion:** Our studies demonstrated a schedule-dependent effect of cytotoxic drugs and erlotinib in an NSCLC cell line with the T790M mutation. Sequential treatment may overcome EGFR-TKI resistance. **(J Lung Cancer 2008;8(2):92–98)**

Key Words: Erlotinib, Drug therapy, Non-small-cell lung carcinoma, T790M

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Received: July 27, 2009
Revised: August 31, 2009
Accepted: September 3, 2009

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This study was supported by the Chonnam National University Grant 2005-0699.

INTRODUCTION

Since the year 2000, lung cancer has been the leading cause of cancer death in South Korea, as in many other parts of the world. Many cytotoxic agents have been developed for lung cancer, but the platinum doublets with third-generation chemotherapeutic agents have achieved plateaus in efficacy. In the Eastern corporative oncology group's 1594 trial, in which chemotherapy regimens for advanced non-small cell lung cancer (NSCLC) were compared, no difference was detected in efficacy and survival between the four arms using different platinum doublet regimens (1).

Recently, epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) were developed and employed for the treatment of refractory NSCLC. EGFR gene mutations (2) and amplifications (3) are known to be independent predictors of response to EGFR-TKIs.

The combination of EGFR-TKIs and cytotoxic drugs may prove to be a useful strategy against lung cancers with wild-type

EGFR. However, clinical trials (4-7) with combinations of cytotoxic drugs and EGFR-TIKs failed to show improved survival rates as compared to platinum-based doublets. An antagonism between EGFR-TKIs and cytotoxic chemotherapy drugs was proposed as a possible explanation for the negative results.

Paclitaxel and docetaxel share a common mechanism of action via the stabilization of the mitotic spindle. Thus, cancer cells treated with taxanes evidenced accumulation in the G2 and M phases of the cell cycle (8). Gemcitabine inhibits ribonucleotide reductase and DNA polymerases, thereby interfering with DNA synthesis (9).

Treatment with EGFR-TKIs in cell lines expressing wild-type EGFR typically results in a G1 cell cycle arrest, whereas pronounced apoptosis is characteristic of cell lines with EGFR mutations (10). Several previous studies have demonstrated that these cell cycle effects result in a negative interaction between concurrently administered chemotherapy and EGFR-TKIs in human lung cancer cell lines with mutant K-ras (11) and in a human esophageal squamous cancer cell line (12).

Despite the dramatic responses to EGFR-TKI, the majority of responders ultimately relapse. A second mutation, T790M, is one mechanism of acquired resistance to EGFR-TKIs (13). In order to overcome the acquired resistance, second generation EGFR inhibitors have been developed and are being studied. We hypothesized that a synergistic combination of cytotoxic drugs and EGFR-TKI may overcome resistance in cell lines harboring T790M.

MATERIALS AND METHODS

1) Reagents

Docetaxel, paclitaxel, and gemcitabine were provided by Sanofi Aventis Korea, BMS Pharmaceuticals Korea, and Lilly Korea. Erlotinib was provided by Hoffmann-La Roche.

2) Cell lines

The human lung cancer cell line NCI-H1975 derived from pulmonary adenocarcinoma was acquired from the American Type Culture Collection (Rockville, MD, USA). This cell line harbors two mutations (L858R and T790M) in the EGFR gene (13). We selected this cell line to observe the combination effect of EGFR-TKI and cytotoxic drugs with an EGFR-TKI resistant cell line, as the T790M mutation of the EGFR gene is known to result in gefitinib resistance. Cells were maintained in RPMI 1640 (Gibco BRL, Grand Island, NY, USA), supplemented with 10% heat-inactivated fetal bovine serum, 20 mmol/L HEPES (pH 7.4), penicillin (100 IU/mL), streptomycin (100 μg/mL), and 4 mmol/L glutamine (Invitrogen Corp., Carlsbad, CA, USA) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

3) Evaluation of antiproliferative effects

The antiproliferative activity of single agent treatment was evaluated under monolayer culture conditions by plating NCI-H1975 lung cancer cells in 48 multi-well cluster dishes (Becton Dickinson, Franklin Lakes, NJ, USA). After 24 hours, the cells were treated with different concentrations of erlotinib, docetaxel, paclitaxel, and gemcitabine each day for 3 days. For combination treatments, the first reagent was administered for 32 hours, followed by the second reagent for 40 hours.

We utilized a tetrazolium dye (3,(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; MTT Sigma Aldrich Company, St. Louis, MO, USA) assay to evaluate the cytotoxicity of a variety of drug concentrations. In brief, cancer cells were seeded into cells ($3\sim5\times10^3$ per well) on 48-well plates at appropriate densities. After successful seeding, the cells were exposed to a variety of treatments, as detailed above, for 72 hours. After 72 hours of incubation at 37°C, 20 μL of MTT solution (5 mg/mL in PBS) was added to each well; the plates were then incubated for an additional 4 hours at 37°C. After centrifuging the plates at 200×*g* for 5 minutes, the medium was aspirated from each well and 200 μL of dimethyl sulfoxide (DMSO) was added to each well in order to dissolve the formazan. Optical density was measured at 560 nm using a Delta Soft ELISA analysis program interfaced with a Bio-Tek Microplate Reader (EL-340; Bio- Metallics, Princeton, NJ, USA). Each experiment was conducted in three replicate wells for each drug concentration, and was independently conducted three or four times. The 50% inhibitory concentration (IC_{50}) value was defined as the concentration needed for a 50% reduction in the absorbance, as calculated on the basis of the dose-effect curves.

The results of the sequential treatment with cytotoxic drugs and erlotinib were analyzed according to the method of Chou and Talalay (14), using the Calcusyn software program (Biosoft, Cambridge, UK). The resultant Combination Index (CI) is a

quantitative measure of the degree of interaction between different drugs. When CI is equal to 1, it denotes additivity; when the CI is greater than 1, antagonism; CI values between 1 and 0.7 indicate slight synergism; CI values of 0.7 to 0.3, synergism; CI values less than 0.3, strong synergism.

4) Flow cytometric analysis of cell cycle distribution and cell death

In order to evaluate the effects on cell cycle and the induction of apoptosis, NCI-H1975 cells were plated in 100 mm tissue culture dishes (Becton Dickinson, Franklin Lakes, NJ, USA) and treated with the indicated concentrations of either erlotinib, docetaxel, paclitaxel, gemcitabine alone, or a

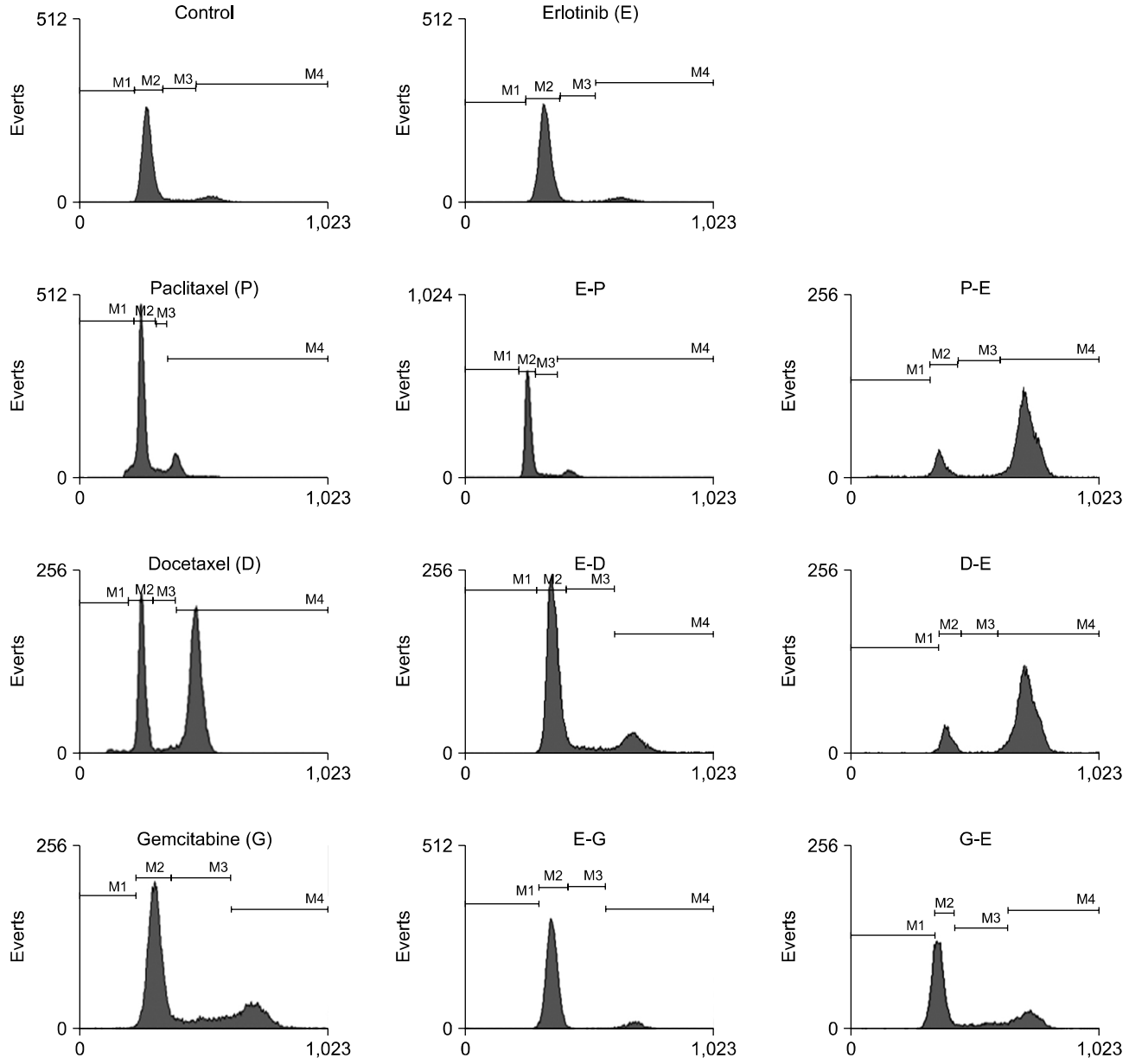

Fig. 1. DNA histograms and percentage in cell cycles after treatment with individual drugs. Pretreatment with erlotinib (E) abrogated the effects of cytotoxic drugs. Treatment with cytotoxic drugs (paclitaxel, P; docetaxel, D; or gemcitabine, G) followed by erlotinib (E) resulted in enhanced accumulation of G2/M or S fractions.

sequence of a first drug for 32 hours followed by the second reagent for 40 hours. After an additional 24 hours, both adherent and detached cells were harvested.

After harvesting, the cells were trypsinized, rinsed in ice-cold phosphate-buffered saline (PBS, pH 7.4) and fixed with 70% cold ethanol at 4°C overnight. Prior to the flow cytometric assays, the cells were washed twice with ice-cold PBS and the fixed cells were resuspended in PBS-containing 0.1 mg/mL RNAase at room temperature for 30 minutes prior to the addition of propidium iodide (Sigma-Aldrich, St. Louis, MO, USA) to a final concentration of 50 μg/mL. Cell-cycle analysis was conducted using an Epics Profile II flowcytometer (Beckman Coulter, Fullerton, CA, USA) with MULTICYCLE software (Phoenix Flow Systems, San Diego, CA, USA). At

Table 1. Cell Cycle Distribution of NCI-H1975 Cells after Treatment with Cytotoxic Drugs and Erlotinib

%	Sub-G1	G0/G1	S	G2/M
Control	0.4±0.5	78.0±3.4	5.5±0.9	16.3±2.9
Erlotinib	0.4±0.3	84.8±4.1*	2.3±1.0*	12.4±3.4
Paclitaxel	5.0±0.2‡	66.3±2.5†	8.0±1.4*	21.0±0.6*
Docetaxel	2.1±0.1‡	32.3±2.9‡	3.5±0.1†	62.3±2.8‡
Gemcitabine	2.1±1.4	56.6±4.1‡	18.1±8.3	23.4±5.6*

Data represent mean±standard deviation. Proportions after treatments with each reagent were compared with those of control. *$p<0.05$, †$p<0.01$, ‡$p<0.001$.

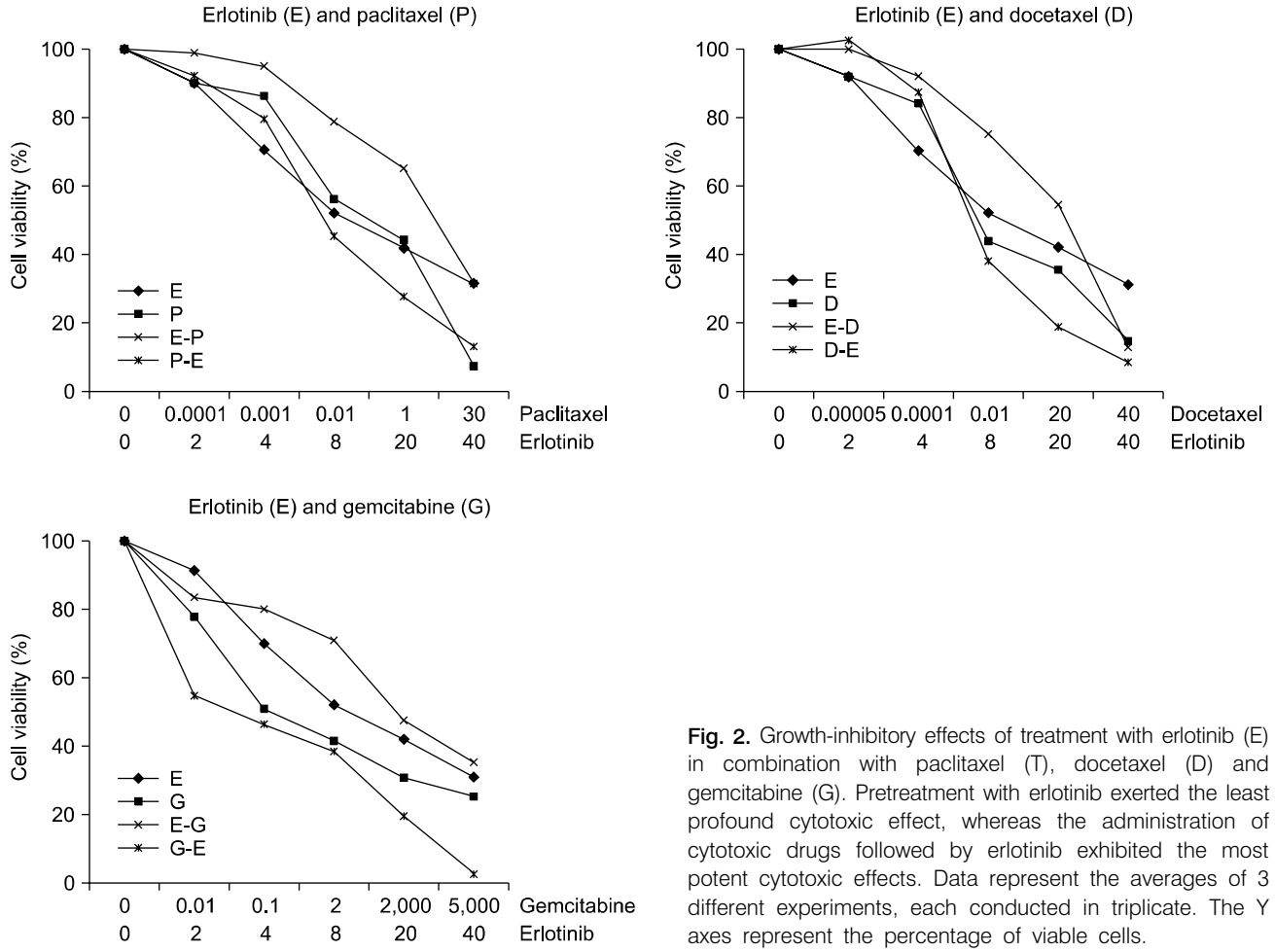

Fig. 2. Growth-inhibitory effects of treatment with erlotinib (E) in combination with paclitaxel (T), docetaxel (D) and gemcitabine (G). Pretreatment with erlotinib exerted the least profound cytotoxic effect, whereas the administration of cytotoxic drugs followed by erlotinib exhibited the most potent cytotoxic effects. Data represent the averages of 3 different experiments, each conducted in triplicate. The Y axes represent the percentage of viable cells.

least 20,000 events were analyzed, and the percentages of cells in each cell cycle were calculated. Aggregates of cell debris at the origin of the histogram were excluded from the sub-G1 cells (15).

RESULTS

1) Antiproliferative and cell cycle effects of individual drugs

The growth inhibitory effects of erlotinib and cytotoxic drugs on lung cancer cells were assessed via an MTT assay. A dose-dependent growth inhibitory effect was noted. The 50% inhibitory concentration (IC_{50}) of all drugs was tested with the NCI H-1975 cancer cell line. The IC_{50} values of erlotinib were 9.7±0.19 μM. The IC_{50} values of paclitaxel, docetaxel, and gemcitabine were 0.35±0.06 μM, 0.25±0.07 μM, 0.18±0.04 μM.

Flowcytometric analysis after treatment with erlotinib and cytotoxic drugs evidenced different patterns (Fig. 1). In this resistant cell line harboring the T790M mutation, erlotinib treatment resulted in no detectable sub-G1 accumulation, an indicator of apoptosis. However, treatment with docetaxel and paclitaxel resulted in significant increases in the S and G2/M fractions. Gemcitabine resulted in a significant accumulation of sub-G1 fractions (Table 1).

2) Antiproliferative effects according to combination sequences

Various combinations of cytotoxic drugs and erlotinib evidenced different antiproliferative effects on the NCI-H1975 cell line. The growth inhibitory effects of combination treatments are shown in Fig. 2. Pretreatment with erlotinib evidenced the least profound cytotoxic effect, whereas treatment with cytotoxic drugs followed by erlotinib evidenced the most profound cytotoxic effects.

As is shown in Table 2, antagonisms (CI > 1) were noted when erlotinib was administered before cytotoxic drugs (EC sequence), whereas synergisms (CI < 1) were noted when cytotoxic drugs were pre-treated before EGFR-TKIs (CE sequence).

3) Flowcytometric cell cycle analysis according to combination sequences

Flow cytometric analyses of cell cycle distributions revealed that treatment in the EC sequence arrested the cells in the G0/G1 phase and reduced the apoptotic fraction. However, treatments in the CE sequence arrested the cells in the G2/M phase and a trend toward higher fractions of apoptotic cell death were noted (Table 3).

Despite an apparent lack of cytotoxicity from single-agent erlotinib, combined cytotoxic drug treatments in CE sequences resulted in additive sub-G1 accumulation as compared to what

Table 2. Effects on NCI-H1975 Cell Growth with Combination Treatment of Erlotinib and Cytotoxic Drugs

CI at IC_{50}	Erlotinib followed by cytotoxic drugs	Cytotoxic drugs followed by Erlotinib
Paclitaxel	1.690±0.133	0.707±0.002
Docetaxel	1.340±0.007	0.952±0.281
Gemcitabine	1.535±0.122	0.334±0.111

Data represent mean±standard deviation. CI values were calculated according to the Chou and Talalay method using Calcusyn software. CI: combination index, IC_{50}: inhibitory concentration.

Table 3. Cell Cycle Distribution of NCI-H1975 Cells after Treatment with Cytotoxic Drugs and Erlotinib

%	Sub-G1	G0/G1	S	G2/M
E→T	0.5±0.1	75.1±0.8	10.1±0.9	15.0±0.3
T→E	6.7±5.7	15.8±3.9†	6.2±3.9	71.7±13.6*
E→D	0.4±0.2	71.8±2.0	8.7±2.1	19.3±2.0
D→E	3.3±3.4	10.5±2.8‡	3.8±3.3*	82.5±3.8‡
E→G	0.6±0.7	83.8±4.6	3.8±2.6	12.1±2.0
G→E	7.5±5.6	41.6±13.3‡	18.2±3.9‡	33.5±4.3‡

E→T denotes pretreatment with Erlotinib followed by Paclitaxel, while T→E denotes Paclitaxel treatment followed by Erlotinib. Comparisons of values were made between pairs of treatment sequences; E followed by cytotoxic drugs vs. cytotoxic drugs followed by E. E: erlotinib, T: paclitaxel, D: docetaxel, G: gemcitabine. *$p<0.05$, $^{\dagger}p<0.01$, $^{\ddagger}p<0.001$.

was observed in the EC sequences.

DISCUSSION AND CONCLUSION

The schedule-dependent cytotoxic effects of cytotoxic drugs and EGFR-TKIs have been addressed in previous studies. However, these studies have used esophageal squamous carcinoma cell lines (12) or a lung cancer cell line with the K-ras mutation (11). Li et al. (16) observed antagonism of erlotinib pre-exposure before pemetrexed. Solit et al. (17) also reported schedule-dependent efficacy in an NSCLC xenograft model with paclitaxel and gefitinib.

In the current study, using a EGFR-TKI-resistant lung cancer cell line with the T790M mutation, we also demonstrated a negative interaction of erlotinib on the effect of cytotoxic drugs. However, we could observe significant enhancement of the antitumor effect when we administered cytotoxic drugs followed by erlotinib. Erlotinib, while minimally active as a single agent in this resistant cell line, nevertheless potentiated the activity of docetaxel, paclitaxel, and gemcitabine in the schedule of cytotoxic drugs followed by erlotinib.

When erlotinib was administered before cytotoxic drugs, cell accumulation in G1 was observed, potentially limiting cytotoxic drug-induced damage during the S-through M-phases of the cell cycle. By way of contrast, when cytotoxic drugs were administered first, M or S-phase accumulation was observed. The addition of erlotinib to cells that have a continued arrest period might increase the incidence of entry into apoptotic pathways. However, as we examined this interaction with a single cell line only, further validation will be needed with other cell lines.

Upon these theoretical bases, subsequent clinical studies combining cytotoxic drugs and EGFR-TKIs utilize an intermittent dosing schedule (18). Pharmatokinetic separation was noted when docetaxel was administered after erlotinib and its metabolites were cleared (19). In a recently published randomized phase 2 trial, Riely et al. (20) reported higher efficacy of pharmacodynamic separation using erlotinib and carboplatin/paclitaxel in patients with NSCLC.

In conclusion, our study demonstrated the schedule-dependent effect of cytotoxic drugs and erlotinib in the NSCLC cell line with T790M mutation, which is a second EGFR mutation detected in progressed NSCLC following treatment with EGFR-TKIs. The sequential treatment of cytotoxic chemotherapy followed by EGFR-TKI may overcome EGFR-TKI resistance.

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