Schedule-dependent antitumor activity of the combination with erlotinib and docetaxel in human non-small cell lung cancer cells with EGFR mutation, KRAS mutation or both wild-type EGFR and KRAS

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Abstract. Erlotinib is used as a standard treatment for recurrent advanced non-small cell lung cancer (NSCLC). Epidermal growth factor receptor (EGFR) mutations in NSCLC have been shown to be a predictive factor of erlotinib, although the relationship between K-ras oncogene (KRAS) mutations and erlotinib resistance is controversial. Recently, *in vitro* sequence-dependent interactions of erlotinib and docetaxel have been studied on as a novel therapeutic approach against NSCLC. The purpose of the present study was to determine the optimum novel regimen of erlotinib and docetaxel against NSCLC cells which have EGFR mutation (HCC827 cells), KRAS mutation (A549 cells) or both wild-type (NCI-H292 cells). First, we analyzed the effects of *in vitro* combination for cell proliferation-inhibition using a combination index. In all cell lines, docetaxel followed by erlotinib treatment showed nearly additive effects. On the other hand, erlotinib followed by docetaxel treatment showed remarkable antagonistic interactions. Second, we examined the effect of combinations on the *in vitro* apoptosis induction. Erlotinib followed by docetaxel treatment reduced apoptosis induction compared with docetaxel alone; in contrast, docetaxel followed by erlotinib treatment had no inhibitory effects on docetaxel-induced apoptosis in any of the cell lines. Finally, an *in vivo* tumor growth inhibition test was performed using xenograft models. Docetaxel followed by erlotinib administration resulted in significant tumor growth inhibition compared with erlotinib or docetaxel monotherapy in all models. In conclusion, we demonstrated that docetaxel followed by erlotinib therapy was a potentially optimum regimen against NSCLC regardless of the mutation status of EGFR and KRAS.

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Introduction

Non-small cell lung cancer (NSCLC) is the most common type of lung cancer, accounting for ~80% of cases. Despite recent improvements in diagnosis and first-line treatment, prognosis remains very poor, with an overall 5-year survival probability of around only 15%. A substantial fraction of patients who received first-line treatment do progress and should be offered second-line treatment. Erlotinib, docetaxel and pemetrexed monotherapies are widely used second-line treatments, however the response rates were limited to only 8.9, 8.8 and 9.1%, respectively (1,2).

Docetaxel is an antineoplastic agent belonging to the taxoid family. Docetaxel promotes the assembly of microtubules from tubulin dimers and stabilizes microtubules by preventing depolymerization. This stability causes cell cycle arrest at the G2/M phase and induces apoptosis in tumor cells (3).

Erlotinib is an oral, small-molecule tyrosine kinase inhibitor (TKI) that reversibly binds to the intracellular tyrosine kinase domain of epidermal growth factor receptor (EGFR). This blocks autophosphorylation of EGFR with subsequent inhibition of the downstream signaling pathways to promote signaling pathways. Erlotinib causes cell growth arrest in the G1 phase and induces apoptosis in tumor cells (4).

Remarkable response rates of EGFR TKI are associated with the occurrence of EGFR-activating mutations (5). The frequency of EGFR mutations is 10-30% in NSCLC (6,7). Besides EGFR mutation, the mutations of KRAS which constitutively activate RAF/MAPK signaling are detected in up to 21% of Caucasian NSCLC patients (6). EGFR mutations and KRAS mutations are generally mutually exclusive (8). The recent TRUST open-access erlotinib study in Europe showed that the KRAS mutation was associated with decreased survival, though without statistical significance (9). On the other hand, the magnitude of benefit with erlotinib was similar in both *KRAS*-mutant and *KRAS* wild-type patients in

the SATURN study of maintenance erlotinib following first-line chemotherapy. Thus, a conclusion on the association between KRAS mutations and erlotinib resistance has not been reached even now.

Sequential treatment of EGFR TKI and paclitaxel or docetaxel has been reported as a promising therapeutic approach against NSCLC, which indicates that pharmaco-dynamic separation is important for effective interactions of the two drugs (10,11). However, there is no evidence on the relativity between sequence-dependent interactions of these two drugs and EGFR or KRAS mutation status in NSCLC.

Therefore, we tried to investigate the optimum regimen of erlotinib in sequential combination with docetaxel against NSCLC cell lines which have EGFR mutation, KRAS mutation or both wild-type.

Materials and methods

Chemicals. Erlotinib was provided by F. Hoffman-La Roche Ltd. (Basel, Switzerland). Erlotinib was dissolved in DMSO for the *in vitro* assay and in 6% Captisol® (Cydex Inc., Lenexa, KS, USA) solution for the *in vivo* experiment. Captisol was dissolved in distillated water. Docetaxel (Sigma, Tokyo, Japan) was dissolved in DMSO for the *in vitro* assay and in 50% Tween-80/cremophol solution for the *in vivo* study.

Cell lines. Human NSCLC cell lines, HCC827, NCI-H292 and A549, were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in an ATCC-recommended medium at 37˚C in 5% CO_2. The HCC827 and NCI-H292 cells were maintained in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS (Japan Bioserum, Hiroshima, Japan). The A549 cells were maintained in DMEM/F12 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS.

In vitro cell proliferation-inhibition assays of monotherapy. The anti-proliferative activity of single drugs, erlotinib and docetaxel, was evaluated using the tetrazolium dye [3-(4,5-Dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide, MTT; Dojindo, Tokyo, Japan] assay. After cells were treated with each drug for 4 days in 96-well clear plates, 10 μl of MTT solution was added to each well and incubated for 2-5 h at 37˚C. The optical density of each well was measured at 450 and 600 nm with a Benchmark plus microplate reader (Bio-Rad, Hercules, CA, USA). Each experiment was performed in triplicate for each drug concentration and was independently performed 2 or 3 times. The cytotoxicity of the drug was expressed as the percentage of cell viability which was calculated as: [(mean absorbance of drug-treated wells - mean absorbance of cell-free wells)/(mean absorbance of vehicle wells - mean absorbance of cell-free wells)] x100.

In vitro cell proliferation-inhibition assays of sequential combinations. To evaluate the schedule-dependent interactions of erlotinib and docetaxel, experiments were performed in two alternative schedules. In one schedule, the cells were treated with docetaxel followed by a washout and then additionally treated with erlotinib. The medium was washed out and the culture was continued in a drug-free medium. The duration of treatment for docetaxel, erlotinib and drug-free medium was 24, 48 and 48 h for HCC827 cells; 24, 72 and 24 h for NCI-H292 cells and 6, 72 and 24 h for A549 cells, respectively. The alternative schedule for the reverse sequence of erlotinib followed by docetaxel was performed. At the end of the treatment, the proliferation of cells was evaluated by MTT assay. The experiments were performed in triplicate and independently repeated 2 or 3 times. As previously described (12), the effects of sequential combination treatment were analyzed according to the Chou and Talalay method by using XLfit software (ID Business Solutions Ltd.) and evaluated using a combination index (CI) interpreted as: <0.7, synergistic; 0.7-1.3, additive; >1.3, antagonistic (13-15).

In vitro cell cycle analysis of sequential combinations. For the evaluation of schedule-dependent cell cycle distributions, two alternative schedules were used. The cells were treated with docetaxel for 1 day followed by a washout and additionally treated with erlotinib for 2 days. The alternative schedule for the reverse sequence of erlotinib followed by docetaxel was performed. At the end of the treatment, the cells were harvested and the DNA content was examined by propidium iodide staining using the Cycletest Plus DNA reagent kit (BD Biosciences, San Jose, CA) following the manufacturer's recommendations. Flow cytometric analysis was performed by FACScan (BD Biosciences) and the percentage of cells in the sub-G1 region of the cell cycle, an indicator of the proportion of apoptotic cells, was estimated from the DNA content histogram using CellQuest software (BD Biosciences).

Human NSCLC xenograft models of sequential combinations. Male 5-week-old BALB/c-nu/nu (CAnN.Cg-Foxn1<nu>/CrlCrlj nu/nu) mice were purchased from Charles River Japan, Inc. (Yokohama, Japan). All animals were housed in a pathogen-free environment under controlled conditions (temperature: 20-26˚C, humidity: 40-70%, light-dark cycle: 12-12 h). Chlorinated water and irradiated food were provided *ad libitum*. The animals were allowed to acclimatize and recover from shipping-related stress for one week prior to the study. The health of the mice was monitored daily.

A cell suspension of NSCLC cells (10^6 viable cells/mouse) was subcutaneously inoculated into the right flank of each mouse. When the tumor grew to 200-300 mm^3, the mice were randomly divided into four groups of four (HCC827 and NCI-292 models) or five (A549 model) mice, and administered either intravenous docetaxel (50 mg/kg, HCC827 model; 10 mg/kg, NCI-H292 and A549 models) on day 1 or oral erlotinib (2 mg/kg/day, HCC827 model; 75 mg/kg, NCI-H292 and A549 models) on days 2-10. In the sequential combination therapy, docetaxel and erlotinib were administered at the same dose and schedule as each drug alone. The schedule of drug treatment was the same as that of the clinical setting (11). Saline or captisol (6%) was administered in the same schedule as the control. Tumor diameter was measured twice a week using calipers and tumor volume was calculated as: $ab^2/2$ mm^3, where a is the length and b is the width of the tumor. Day 1 denotes the first day of the treatment and the drug effects were estimated on day 11.

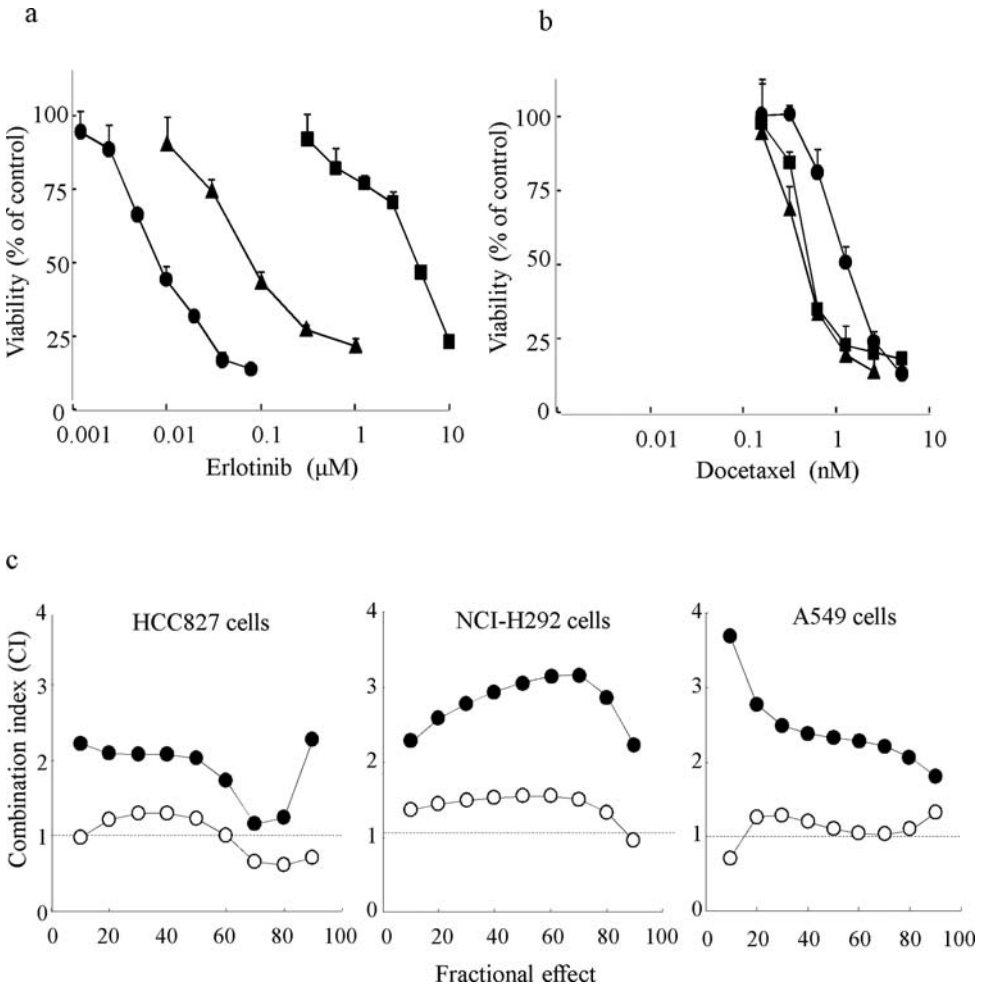

Figure 1. Effects of erlotinib and docetaxel on proliferation of NSCLC cells *in vitro*. The cells were treated with the indicated concentrations of erlotinib (a) or docetaxel (b) alone for 4 days (HCC827 cells, circle; NCI-H292 cells, triangle; and A549 cells, square), and the proliferation of cells were determined by MTT assay. Each point represents mean ± SD of triplicates. (c) As described in Materials and methods, the cells were treated either alone or in sequential combination with erlotinib and docetaxel at a fixed molar ratio, erlotinib: docetaxel = 16:1 (HCC827 cells), 500:1 (NCI-H292 cells) and 1000:1 (A549 cells), and the proliferation of cells were determined by MTT assay. Docetaxel followed by erlotinib treatment; open circle, erlotinib followed by docetaxel treatment; closed circle. The combination index (CI) for each fraction-affected value was calculated using the Chou-Talalay method.

The protocol was reviewed by the Institutional Animal Care and Use Committee of Chugai Pharmaceutical Co., Ltd. Animal experiments were performed in accordance with the 'Guidelines for the Accommodation and Care of Laboratory Animals' of Chugai Pharmaceutical Co., Ltd.

Statistical analysis. The Wilcoxon test was used to detect the statistical differences in tumor volume. $P<0.05$ were considered to be significant. Statistical analyses were performed using the SAS preclinical package (version 8.2; SAS Institute Inc., Cary, NC, USA).

Results

In vitro schedule-dependent anti-proliferative activity of erlotinib and docetaxel in NSCLC cells. We examined the *in vitro* cell growth inhibition of erlotinib or docetaxel alone on NSCLC cell lines using an MTT assay. For this purpose, three cell lines were tested, namely, HCC827 cells (EGFR mutation Del746-750), NCI-H292 cells (EGFR wild-type/KRAS wild-type), and A549 cells (KRAS mutation G12S). The IC_{50} value for erlotinib was 0.008 μM for HCC827 cells, 0.08 μM for NCI-H292 cells and 4.8 μM for A549 cells (Fig. 1a). The IC_{50} value for docetaxel was 1.3 nM for HCC827 cells, 0.4 nM for NCI-H292 cells and 0.5 nM for A549 cells (Fig. 1b). There was marked difference in erlotinib sensitivity, on the other hand, little difference was observed in docetaxel sensitivity between the three cell lines. To evaluate the schedule-dependent interactions of erlotinib and docetaxel, a combination index (CI) was determined using the Chou-Talalay method. The CI value for docetaxel followed by erlotinib treatment ranged from 0.7 to 1.3 on every fraction, suggesting that the combination effects of docetaxel followed by erlotinib treatment were additive in the three cell lines (Fig. 1c). In contrast, the reverse sequence of erlotinib followed by docetaxel treatment resulted in antagonistic effects in the three cell lines (CI>1.3) (Fig. 1c). Therefore, the sequential effect of docetaxel followed by erlotinib treatment was considered additive and the reverse sequential effect of erlotinib followed by docetaxel treatment was considered antagonistic in NSCLC cells regardless of the mutation status of EGFR and KRAS.

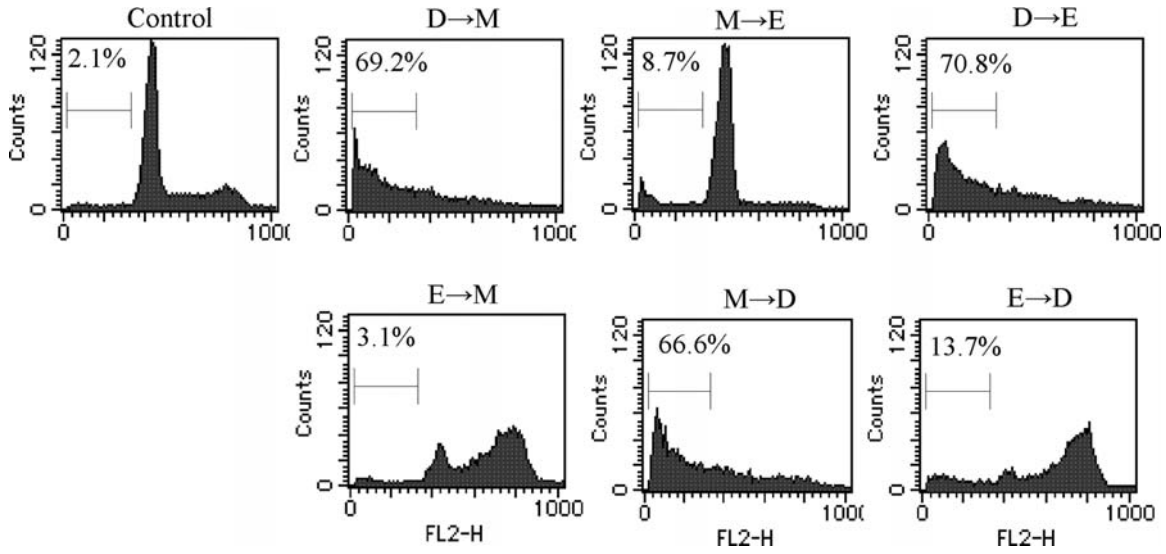

Figure 2. Effects of apoptosis induction in sequential combination with erlotinib and docetaxel in HCC827 cells. The cells were treated with 10 nM docetaxel, 0.1 μM erlotinib or drug-free medium as specified, and the cell cycle distributions were determined by flow cytometric analysis. DNA histograms and apoptotic cells (% of sub-G1 content) from a representative run are shown. Arrows indicate sequential administration of agents. Upper panels: docetaxel followed by erlotinib treatment and each drug control. Lower panels: erlotinib followed by docetaxel treatment and each drug control. D, docetaxel; E, erlotinib; and M, drug-free medium.

Table I. Apoptosis induction in sequential combination with erlotinib and docetaxel in NCI-H292 and A549 cells.[a]

	Control	D→M	M→E	D→E	E→M	M→D	E→D
NCI-H292 cells	2.5%	43.6%	2.0%	60.5%	4.5%	35.2%	3.3%
A549 cells	1.1%	30.0%	1.7%	30.2%	1.2%	57.5%	19.6%

[a]The cells were treated with 10 nM docetaxel, erlotinib (1 μM, NCI-H292 cells and 10 μM, A549 cells) or drug-free medium as specified, and the cell cycle distributions were determined by flow cytometric analysis. Apoptotic cells (% of sub-G1 content) from a representative run are shown. D, docetaxel; E, erlotinib; and M, drug-free medium.

In vitro schedule-dependent apoptosis induction of erlotinib and docetaxel in NSCLC cells. Sub-G1 phase accumulation of cell cycle distributions, an indicator of apoptosis, was determined by flow cytometric analysis. Representative series of histograms in HCC827 cells are shown in Fig. 2. When cells were treated with erlotinib alone, the cell accumulation in G1 phase which indicates G1-arrest was observed in all of the three cell lines (data not shown). When cells were treated with docetaxel alone, sub-G1 content was markedly increased in the three cell lines (Fig. 2 and Table I; M→D and D→M). In sequential combinations, almost the same level of sub-G1 content was observed in docetaxel followed by erlotinib treatment and docetaxel single treatment in HCC827 cells and A549 cells (Fig. 2 and Table I; D→E), suggesting that docetaxel followed by erlotinib combination induced an additive effect on apoptosis induction. Especially in NCI-H292 cells, subG1 content of the sequential treatment was more than that of docetaxel alone, suggesting that the docetaxel followed by erlotinib combination induced a supra-additive response. In contrast, the reverse sequence, erlotinib followed by docetaxel treatment, resulted in markedly decreased sub-G1 content compared with docetaxel alone (Fig. 2 and Table I; E→D), suggesting that the erlotinib to docetaxel combination was antagonistic in the three cell lines. Thus, the schedule-dependent interactions for apoptosis induction in the combination of erlotinib with docetaxel were consistent with that of anti-proliferative activity in the three cell lines.

Antitumor effects of sequential treatment of docetaxel followed by erlotinib in NSCLC xenograft models. To evaluate the effect of the sequential treatment of docetaxel followed by erlotinib on *in vivo* tumor growth, we conducted xenograft model experiments using HCC827, NCI-H292 and A549 cells. Tumor growth was significantly inhibited by erlotinib and docetaxel monotherapy in the three cell lines ($P<0.05$, Fig. 3). On the other hand, the sequential combination treatment with docetaxel followed by erlotinib showed a significantly stronger tumor growth inhibition than either drug monotherapy in the three cell lines ($P<0.05$, Fig. 3). The body weight loss in each group on day 11 was <10% compared to the body weight at the start of administration (Table II). These results show that erlotinib enhanced the antitumor activity of docetaxel without affecting normal tissue with severe toxicity in NSCLC xenograft models, regardless of EGFR or KRAS mutation status.

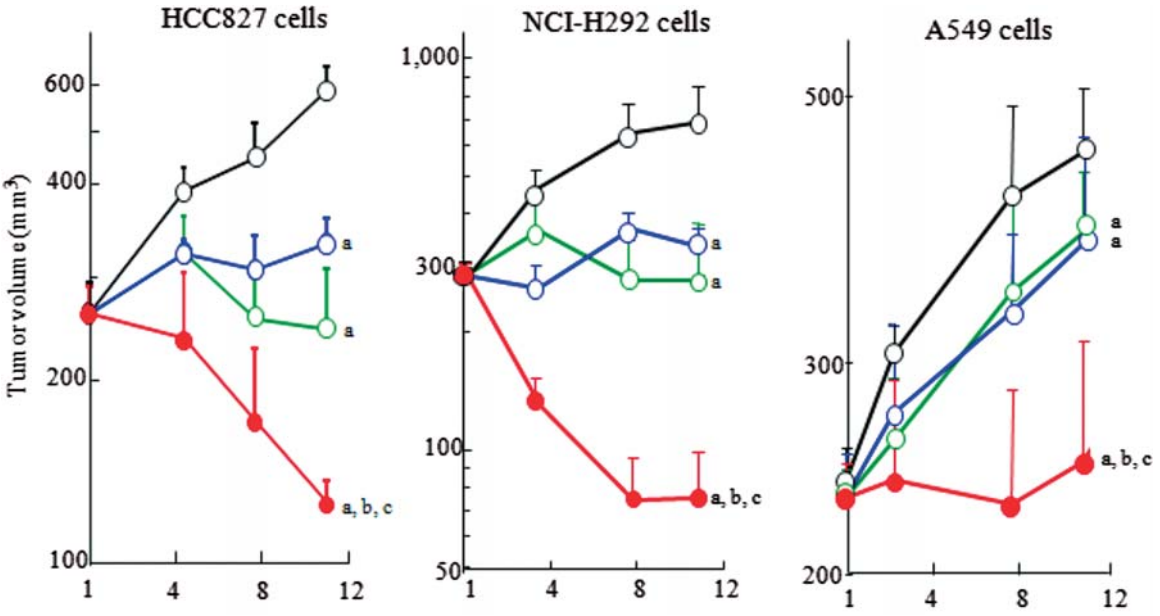

Figure 3. Antitumor activity of erlotinib in sequential combination with docetaxel in NSCLC tumor xenograft models. Nude mice bearing (s.c.) NSCLC xenografts were treated with erlotinib daily from day 2-10 (brown lines), docetaxel on day 1 (green lines) and a sequential combination of both drugs (red lines) and vehicle (black lines). Each point represents the mean ± SD of tumor volume per group. Statistical differences ($P<0.05$) on day 11 are shown vs. the control (a), erlotinib (b), and docetaxel (c) (Wilcoxon test).

Table II. Body weight of mice treated with erlotinib in sequential combination with docetaxel in NSCLC tumor xenograft models.[a]

	HCC827 cells		NCI-H292 cells		A549 cells	
	Day 1	Day 11	Day 1	Day 11	Day 1	Day 11
Control	27.9±1.8	26.2±2.6	24.2±1.7	24.9±2.0	25.8±1.6	25.9±1.6
Erlotinib alone	25.9±1.8	23.6±1.4	25.8±1.1	24.7±2.1	25.8±0.7	26.1±0.8
Docetaxel alone	26.5±1.0	25.7±1.3	23.9±0.9	24.1±1.9	25.6±1.2	23.7±1.3
Docetaxel to erlotinib	28.0±1.1	26.1±1.3	24.8±0.8	22.0±1.5	26.5±1.7	23.4±2.1

[a]Values represent the mean ± SD of body weight per group.

Discussion

No second-line combination chemotherapy has been shown to provide a survival benefit, due to excessive toxicities and the rapidly declining clinical condition of these patients. Randomized clinical phase III trials with continuous daily administration of EGFR TKI in combination with conventional chemotherapy, such as carboplatin + paclitaxel and cisplatin + gemcitabine, have failed to improve survival in patients with advanced NSCLC (16,17). In these studies, the mechanism of antagonistic interactions of EGFR TKI and chemotherapy was considered to be that EGFR TKI-induced G1 arrest of tumor cells might obviate the effects of concurrently administered cytotoxic agents. Recently, to conquer the antagonistic effects in concurrent combination therapy of EGFR TKI and chemotherapeutic agents, a novel approach, sequential combination of EGFR TKI and chemotherapy, is being investigated (10,11,18). Mahaffey *et al* reported *in vitro* sequence-dependent efficacy of erlotinib and docetaxel in KRAS-mutated NSCLC cell lines (11).

In the present study, we used three NSCLC cell lines: HCC827 cells (Del746-750 EGFR/wild-type KRAS), NCI-H292 cells (wild-type EGFR/wild-type KRAS) and A549 cells (wild-type EGFR/G12S KRAS). The HCC827 cells were the most sensitive (IC_{50}: 0.008 μM), NCI-H292 cells were moderately sensitive (IC_{50}: 0.08 μM) and A549 cells were the least sensitive (IC_{50}: 4.8 M) to erlotinib in an *in vitro* cell proliferation assay. The marked difference in the erlotinib sensitivity between the three cell lines is consistent with several studies in which EGFR TKI monotherapy has been shown to be favorable to the presence of activating EGFR mutations and absence of KRAS mutations in NSCLC (5,6,9,19). Cell proliferation assays and cell cycle assays were performed to examine the schedule-dependent interactions of erlotinib and docetaxel. Erlotinib potentiated the effect of docetaxel when cells were treated with docetaxel followed by erlotinib. In contrast, when cells were treated with erlotinib followed by docetaxel, antagonistic interactions were observed. Our results of sequence-dependent apoptosis induction in KRAS-mutated A549 cells were consistent with the results of

Mahaffey *et al* (11). In mouse xenograft models using the 3 human NSCLC cell lines, erlotinib administration after docetaxel significantly enhanced the antitumor activity of docetaxel without severe body weight loss. These *in vitro* and *in vivo* results indicate that sequential treatment of docetaxel followed by erlotinib could provide a potential therapeutic benefit in NSCLC regardless of the mutation status of EGFR or KRAS or cell intrinsic sensitivity to erlotinib.

Schedule-dependent anti-proliferation and apoptosis induction of these two drugs have been reported in not only NSCLC but also head and neck carcinoma and esophageal squamous epithelial carcinoma (19). Although the mechanisms of the combination effects remain unclear, changes in the cell cycle distributions may explain the mechanism of sequential interactions. In docetaxel followed by erlotinib treatment, docetaxel induces the accumulation of M phase population, which is considered sensitive to erlotinib; hence, this sequential combination treatment enhances antitumor activity. Conversely, in the reverse sequence, erlotinib caused G1 arrest in tumor cells leading to a reduction in M phase entry, which is crucial for docetaxel-mediated cytotoxicity.

Almost 70% of NSCLC patients have locally advanced or metastatic disease at the time of diagnosis. Frequent genetic heterogeneities were found in primary tumors and corresponding metastatic sites in advanced NSCLC (20). Especially, a discordance of genetic alteration of EGFR and KRAS between primary tumors and corresponding metastatic lesions was observed in several studies, which showed 27% and 64% inconsistency in EGFR and KRAS mutational status, respectively (21,22). Thus, the sequential-therapy investigated in the present study, being unaffected by differences in the mutational status of EGFR and KRAS from primary to metastatic tumors, could be a useful regimen.

A phase II study with a combination of docetaxel and intermittent dosing of erlotinib has been conducted in the US (11,23). In this 3-week cycle of trial, docetaxel (70-75 mg/m^2) was intravenously administered on day 1 and erlotinib (150-200 mg) was orally administered on days 2-16 in patients with advanced NSCLC. The sequential combination showed a favorable response rate and time to progression compared to prior trials of the single agents. The results of our study were consistent with the results of this phase II clinical trial.

In conclusion, we demonstrated schedule-dependent cell growth inhibition and apoptosis induction of erlotinib plus docetaxel in both *in vitro* and *in vivo* NSCLC models. We found that the docetaxel followed by erlotinib treatment was an optimum regimen against NSCLC regardless of the mutation status of EGFR and KRAS.

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