Dose-dependent and sequence-dependent cytotoxicity of erlotinib and docetaxel in head and neck squamous cell carcinoma

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The purpose of this study was to determine whether the efficacy of taxoid treatment combined with epidermal growth factor receptor (EGFR) inhibition is dose and sequence dependent in head and neck squamous cell carcinoma. Three head and neck squamous cell carcinoma cell lines, chosen on the basis of their diverse EGFR expression levels, were treated with docetaxel, erlotinib, or both. The combination index was calculated using the Chou–Talalay equation. Propidium iodide staining with fluorescence-activated cell sorting analysis was used to evaluate the effects of drugs on cell cycle changes. Western blot analysis was used to determine the effects of agents on cell signaling pathways. Administration of low-dose docetaxel (0.1–3 nmol/l) concurrently or before erlotinib had additive cytotoxic effects in two cell lines but was antagonistic in one line, whereas low-dose docetaxel after erlotinib was synergistic in all cell lines. In contrast, high-dose docetaxel (40 nmol/l) resulted in more apoptosis when given before, rather than after or concurrently with, erlotinib. Low-dose docetaxel induced an accumulation of cells in the sub-G0 phase of the cell cycle with no mitotic arrest or apoptosis, whereas high-dose docetaxel induced mitotic arrest and apoptosis. The low and high doses of docetaxel had opposite effects on EGFR expression: a decrease and an increase, respectively. The dose of docetaxel affects sequence-dependent cytotoxicity when docetaxel is combined with an EGFR inhibitor. The mechanism for this difference is a combination of the dose-dependent effects of docetaxel on the mode of cell death and on EGFR expression.

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Introduction

All epithelial tumors pose formidable challenges in clinical practice, but the anatomy of the head and neck region makes tumors located there particularly difficult to treat. Approximately 45 000 new cases of head and neck cancer are diagnosed in the United States each year, with an estimated worldwide incidence of 500 000 [1]; the majority of these are of squamous histology. Although the survival rate after treatment for advanced head and neck squamous cell carcinoma (HNSCC) has improved in certain subsets of patients, the overall 5-year survival rate has been relatively static for the past three decades at 50–60% [1]. The incidence and survival statistics for head and neck cancer are only half of the story, however. Cancer and its treatment can distort the anatomy and physiology of the head and neck region and have a profound impact on important functions such as chewing, swallowing, speaking, sight, smell, and hearing. These functional losses are often accompanied by distortions in facial appearance that contribute to social isolation and poor self-image in the patients who survive. Although novel approaches with concurrent chemoradiation therapy have improved locoregional control in advanced HNSCC, locoregional and distant recurrence remain common in patients with advanced disease at presentation, and the vast majority of these cannot be cured. Thus, there is a great need to improve systemic therapy in patients with these tumors to both improve cure rates and reduce morbidity.

Epidermal growth factor receptor (EGFR) is a potential therapeutic target in HNSCC. Both EGFR and its ligand, transforming growth factor α, are expressed commonly in HNSCC tumors [2]. Mechanisms of increased EGFR expression include increased gene copy number [3] and decreased downregulation. Activation of EGFR can occur by autocrine or paracrine ligand stimulation and by transactivation by other ErbB family members, G-protein-coupled receptors, or other receptor and nonreceptor tyrosine kinases (reviewed in Ref. [4]); activating EGFR mutations in HNSCC is very rare [3,5,6]. Inhibition of EGFR is a novel treatment strategy for HNSCC that has been shown in preclinical and early clinical studies to be efficacious to various extents

[4,7–9]. In heavily pretreated patients with recurrent or metastatic HNSCC, single-agent therapy with the EGFR inhibitor erlotinib resulted in a response rate of only 4.3% [10]. EGFR inhibition with cetuximab, however, enhanced the locoregional control of HNSCC treated with radiotherapy and increased patient survival [11].

EGFR inhibition may also improve the efficacy of chemotherapy. Docetaxel is a member of the taxoid family that inhibits microtubule depolymerization and blocks cells in the M phase of the cell cycle. Docetaxel has antitumor activity against a wide range of tumor types in patients. The taxoids, which also include paclitaxel, are the most efficacious single agents identified thus far for HNSCC treatment [12–14]. The combination of a taxoid with EGFR inhibition has potent additive antitumor effects in cancer cells in culture [15,16] and in tumor xenografts in mice [17,18]. Cetuximab has been added to the taxoid paclitaxel in patients with metastatic HNSCC [19] and to induction chemotherapy with paclitaxel and carboplatin in patients with locally advanced HNSCC [20]. Erlotinib has been combined with docetaxel and cisplatin [21] and with cisplatin alone [22] in patients with recurrent or metastatic HNSCC. All four of these trials were single-arm, phase II studies, and all demonstrated that the regimens were tolerable and had promising clinical activity. In addition, the EXTREME trial recently demonstrated that adding cetuximab to first-line cisplatin chemotherapy in patients with metastatic HNSCC resulted in increased survival [23].

Despite similar preclinical data for non-small cell lung cancer (NSCLC) [24], three large clinical trials have shown that the addition of EGFR inhibitors to chemotherapy does not improve patient survival [25–27]. On the basis of the results from these clinical trials as well as an in-vitro study that showed antagonism [28], there is concern that EGFR inhibitors could be antagonistic to cytotoxic chemotherapy when given concurrently [29,30]. Data from in-vitro and animal studies have suggested that the order in which these agents are given may affect their efficacy [28,31]. Some of the studies that have examined the combination of taxoids with EGFR inhibitors in HNSCC cancer cells in culture have used sequential treatment (paclitaxel or docetaxel on day 1 followed by EGFR inhibitor on days 2–6) [32,33]; others have used concurrent treatment *in vitro* [15,34]. Most in-vivo studies have used concurrent therapy [24,34]. These schedules have resulted in enhanced antitumor effects. No published study has, however, examined the effects of treatment sequence on the efficacy of the combination of a taxoid with EGFR inhibition in HNSCC. To address this gap in knowledge, we compared several treatment sequences of erlotinib and docetaxel in HNSCC *in vitro* using the Chou–Talalay equation to determine each sequence's effect on cytotoxicity. We also varied the dose of docetaxel because the dose can influence gene expression and the mechanism of cancer cell death [35,36].

Methods

Materials

Erlotinib was provided by OSI Pharmaceuticals and was prepared as a 10 mmol/l stock solution in dimethyl sulfoxide. Docetaxel was provided by Aventis and prepared as a 10 mmol/l stock solution in 96% ethanol.

Cell cultures

The eight human HNSCC cell lines used in this study [37] were obtained from Dr J. Myers and Dr G. Clayman of The University of Texas MD Anderson Cancer Center (Houston, Texas, USA). Cells were grown in monolayer cultures in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 2 mmol/l glutamine at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Transformed human keratinocytes (HOK16b) were obtained from American Type Culture Collection and maintained in keratinocyte medium.

Western blot analysis

Detached cells from each cell culture plate were collected by centrifugation, washed in phosphate-buffered saline (PBS), and added to the cell lysate from their corresponding plates. Adherent cells were rinsed with ice-cold PBS and lysed in the cell culture plate for 20 min on ice in a lysis buffer consisting of 50 mmol/l Trizma base (pH 8; Sigma Chemical, St Louis, Missouri, USA), 1% Triton X-100, 150 mmol/l NaCl, 20 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mmol/l phenylmethanesulfonyl fluoride, and 1 mmol/l sodium vanadate. Lysates were spun in a centrifuge at 14 000 rpm for 5 min and the supernatant was collected. Equal protein aliquots were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, immunoblotted with primary antibody, and detected with horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories, Hercules, California, USA) and ECL reagent (Amersham Biosciences, Piscataway, New Jersey, USA). The antibodies used were those against total EGFR, phosphorylated EGFR (Y1068 and Y845) (all from Cell Signaling Technology, Beverly, Massachusetts, USA). Beta-actin was used as a loading control, and the anti-β-actin antibody was obtained from Sigma Chemical.

MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay was used to assess the cytotoxicity of the drugs and drug combinations. Cells were plated into 96-well plates and incubated for 24 h using the conditions described above for standard cell culture maintenance. The cells were then exposed to erlotinib, docetaxel, or both at various concentrations and times, as indicated in the figures. In experiments where the cell medium was

changed to remove or add drugs, it was changed in all treatment groups to eliminate the effects of medium change on cell proliferation. Eight wells were treated at each concentration and the results averaged. After treatment, 25 µl of MTT was added to each well, the wells were incubated for 3 h, and 100 µl of Me_2SO was added. The absorbance of individual wells was read at a wavelength of 570 nm.

Determination of synergism and antagonism

The combination index (CI) was calculated using the Chou–Talalay equation, which takes into account both the potency [*Dm* or inhibitory concentration 50% (IC_{50})] and shape of the dose–effect curve [38–40]. The general equation for the classic isobologram (CI = 1) is given by $CI = (D)_1/(Dx)_1 + (D)_2/(Dx)_2$, where $(Dx)_1$ and $(Dx)_2$ are the doses (or concentrations) for D_1 (erlotinib) and D_2 (docetaxel) alone that result in *x%* inhibition, and $(D)_1$ and $(D)_2$ are the doses of erlotinib and docetaxel in combination that result in *x%* inhibition (i.e. that are isoeffective). CI values of less than 1, 1, and greater than 1 indicate synergism, additive effect, and antagonism, respectively. The inputs were the concentrations of single inhibitors, the combination doses at fixed ratios, and the fractional inhibition, that is, the fraction affected (*Fa*) of single drugs and combinations [*Fa* = (drug A control – drug A treated)/drug A control]. The fraction of unaffected cells (*Fu*) was calculated as *Fu* = 1 – *Fa*. The values of $(Dx)_1$ or $(Dx)_2$ can be readily calculated from the median-effect equation of Chou *et al.* [38,39]: $Dx = Dm[Fa/(1-Fa)]^{1m}$, where *Dm* is the median-effect dose that is obtained from the antilog of the X-intercept of the median-effect plot, $X = \log(D)$ versus $Y = \log[fa/(1-fa)]$ or $Dm = 10 - (Y\text{-intercept})/m$, and *m* is the slope of the median-effect plot. Calcusyn software (Biosoft, Ferguson, Missouri, USA) was used to calculate *m*, *Dm*, *Dx*, and CI values. From $(Dm)_1$, $(Dx)_2$, and $(D)_1 + (D)_2$, isobolograms can be constructed on the basis of the first equation.

Cell cycle and apoptosis analysis

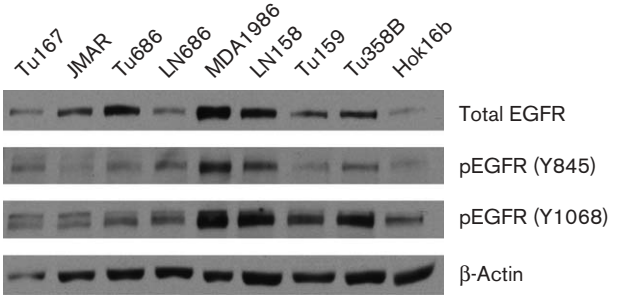
Subconfluent cells were treated with erlotinib, docetaxel, or both at the concentrations and times indicated in the figures. For cell cycle analysis, the cells were harvested, washed in PBS, fixed in 1% paraformaldehyde, rewashed in PBS, resuspended in 70% ethanol at –20°C overnight, washed twice more with PBS, and stained with 20 µg/ml propidium iodide. DNA content was analyzed on a cytofluorimeter by fluorescence-activated cell sorting analysis (Becton Dickinson and Company, San Jose, California, USA) using ModFit software (Verity Software House, Turramurra, New South Wales, Australia).

Results

Selection of cell lines

To assess whether the order in which cancer cells are exposed to EGFR inhibitors and taxoids affects cytotoxicity, we treated cells with erlotinib and docetaxel using a variety of schedules. We chose three human HNSCC cell lines from a panel of eight based on their distinct basal EGFR expression and activation levels: Tu167, LN158, and MDA1986 (Fig. 1).

Fig. 1



Baseline expression of epidermal growth factor receptor (EGFR) and activated EGFR in head and neck squamous cell carcinoma (HNSCC) cell lines and keratinocytes. HNSCC cells and HOK16b keratinocytes were grown under standard culture conditions, lysed, and analyzed by western blotting for expression of EGFR, phosphorylated EGFR (pEGFR; Y1068 and Y845), and β-actin.

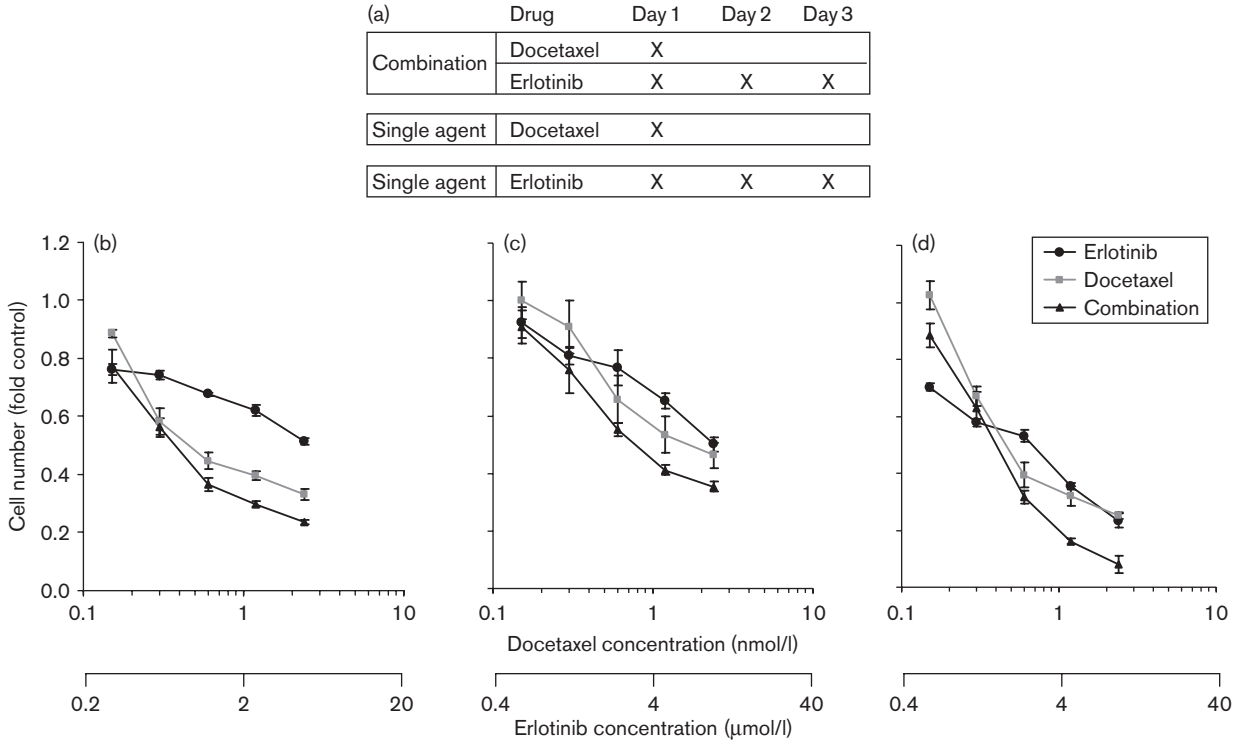
Administration of docetaxel concurrently with or before erlotinib has additive cytotoxic effects

To evaluate the cytotoxic effects of docetaxel and erlotinib administered concurrently, cells were incubated with both drugs for 24 h; the docetaxel was then removed, and erlotinib was continued for an additional 48 h. Doses were chosen based on the IC_{50} values of the drugs given as single agents. For single-agent controls, cells were treated with docetaxel alone for 24 h (then no treatment for the remaining 48 h) or erlotinib alone for 72 h (Fig. 2a); in all cases the cytotoxicity was measured after 72 h. This combination was additive in LN158 and Tu167 cells but antagonistic in MDA1986 cells; the CI values (*Fa* 0.5, ± SD) were 1.0 ± 0.21, 1.0 ± 0.30, and 1.7 ± 0.20, respectively. Cytotoxicity curves are shown in Fig. 2b–d. Additional experiments in which both agents were given concurrently for 72 h also demonstrated additive effects (data not shown). On the basis of data from NSCLC studies, which suggested that docetaxel given before erlotinib enhances apoptosis [28], we also treated cells with docetaxel alone for 24 h and then with erlotinib alone for 48 h (Fig. 3a). The results were similar to those for docetaxel and erlotinib administered concurrently in that this combination was additive in LN158 and Tu167 cells and antagonistic in MDA1986 cells (Fig. 3b–d). The CI values (*Fa* 0.5, ± SD) were 0.78 ± 0.14, 1.2 ± 0.40, and 1.6 ± 0.45, respectively.

Administration of erlotinib before docetaxel is synergistic

To evaluate the cytotoxic effects of erlotinib administered before docetaxel, we used two different schedules,

Fig. 2



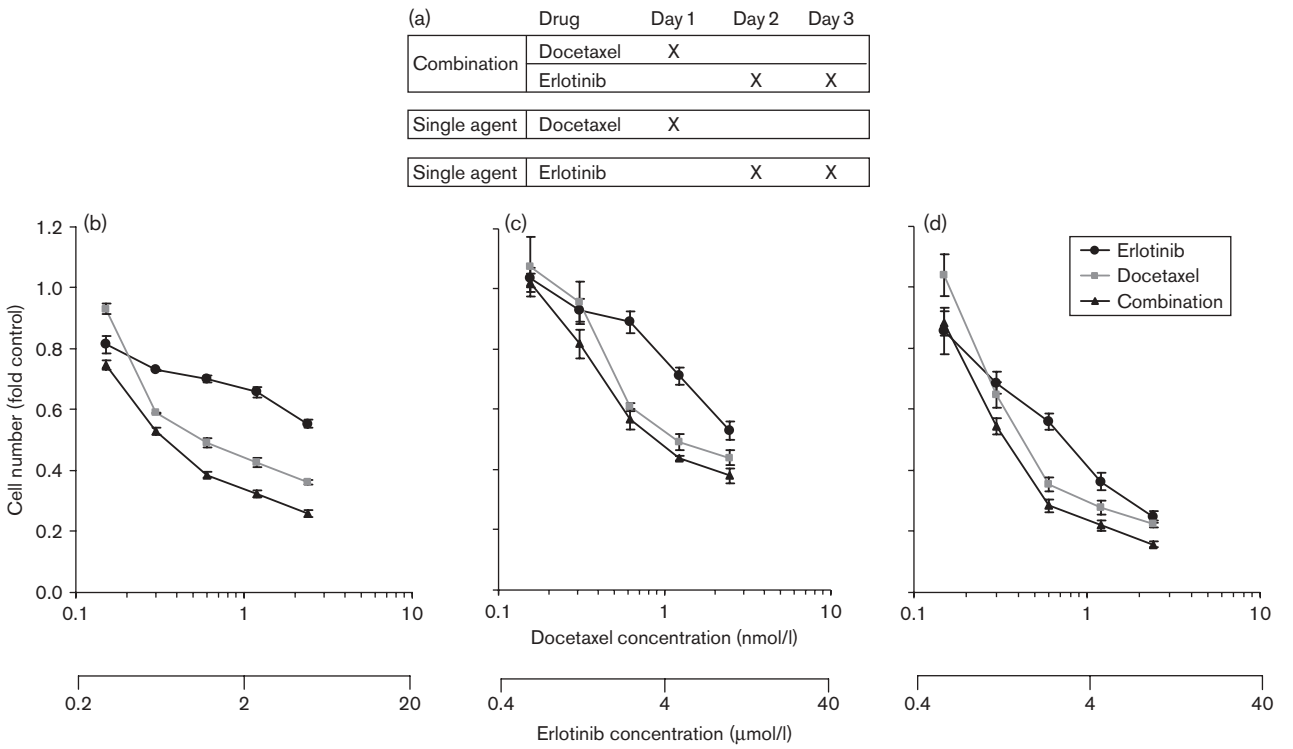
Administration of docetaxel and erlotinib concurrently has additive cytotoxic effects. (a) Head and neck squamous cell carcinoma cells were incubated with both drugs for 24 h; the docetaxel was then removed, and erlotinib was continued for an additional 48 h. For single-agent controls, cells were treated with docetaxel alone for 24 h or erlotinib alone for 72 h. (b–d) The number of remaining viable cells was estimated by MTT assay. The combination was additive in Tu167 (b) and LN158 (c) and antagonistic in MDA1986 cells (d).

one in which erlotinib was administered before concurrent treatment and one in which erlotinib was administered and then removed before treatment with docetaxel alone. In the first schedule, cells were incubated with erlotinib alone for 24 h and then with both agents for an additional 48 h. For single-agent controls, cells were incubated without drugs for 24 h then with docetaxel alone for 48 h or erlotinib alone for 72 h. In all cases, the cytotoxicity was measured after 72 h (Fig. 4a). In all three cell lines, there was clear synergy (Fig. 4b–d). The CI values (*Fa* 0.5, ± SD) were 0.19 ± 0.01, 0.43 ± 0.13, and 0.33 ± 0.05 in Tu167, LN158, and MDA1986 cell lines, respectively. In the second schedule, cells were incubated with erlotinib alone for 24 h and then with docetaxel alone for 48 h. For single-agent controls, cells were incubated without drug for 24 h then with docetaxel alone for 48 h or erlotinib alone for 24 h (then no treatment for the remaining 48 h). In all cases the cytotoxicity was measured after 72 h (Fig. 5a). Again, there was synergy in all cell lines (Fig. 5b–d); the CI values (*Fa* 0.5, ± SD) were 0.16 ± 0.06, 0.66 ± 0.28, and 0.41 ± 0.15 in Tu167, LN158, and MDA1986 cell lines, respectively. These data demonstrate that the administration of erlotinib before docetaxel in HNSCC cells *in vitro* enhances cytotoxicity. We were, however, concerned that in clinical practice, pretreatment with erlotinib would often last longer than 24 h, which could lead to a greater degree of cell cycle arrest and affect the subsequent activity of docetaxel. To assess this possibility, we incubated HNSCC cells with erlotinib for 72 h and then added docetaxel for an additional 24 h. The results were similar to those in which erlotinib was given for 1 day before docetaxel was added: CI values were 0.53 ± 0.13, 0.61 ± 0.15, and 0.85 ± 0.12 in the Tu167, LN158, and MDA1986 cell lines, respectively. This again demonstrates that erlotinib given first results in synergy.

Effects of docetaxel on cell cycle distribution and apoptosis are dose dependent

To investigate the mechanism for the enhanced effects of concurrent docetaxel and erlotinib, we incubated cells with vehicle control, erlotinib, docetaxel, or both agents concurrently, and the cell cycle distribution was analyzed by fluorescence-activated cell sorting. Docetaxel concentrations were chosen based on the IC_{50} for the combination when the drugs are given concurrently for 72 h (data not shown). Erlotinib concentrations were chosen based on concentrations that are clinically achievable and lead to inhibition of EGFR *in vitro*. In LN158 cells, erlotinib had no noteworthy effect on the cell cycle at 24 and 48 h.

Fig. 3



Administration of docetaxel before erlotinib alone has additive cytotoxic effects. (a) Head and neck squamous cell carcinoma cells were incubated with docetaxel alone for 24 h; the docetaxel was then removed, and erlotinib was added for 48 h. For single agent controls, cells were treated with docetaxel alone for 24 h or erlotinib alone for 48 h. (b–d) The number of remaining viable cells was estimated by MTT assay. The combination was additive in Tu167 (b) and LN158 (c), and antagonistic in MDA1986 cells (d).

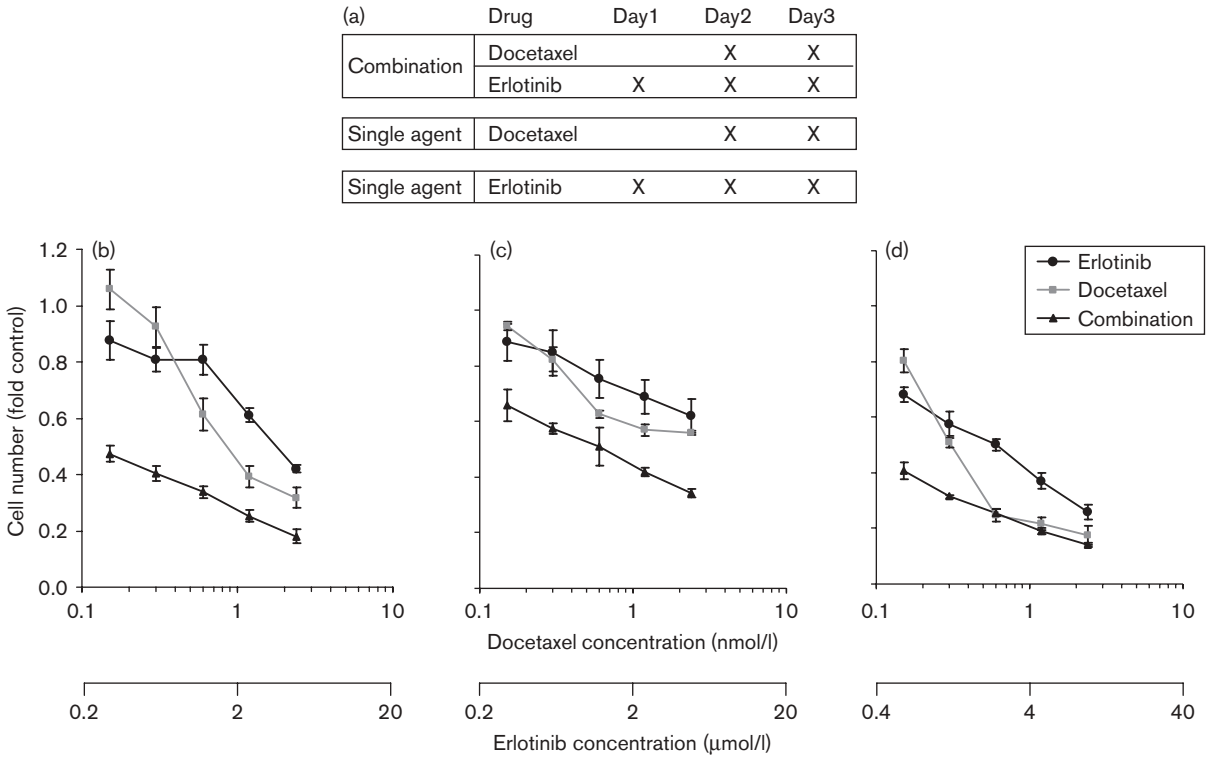
At 72 h, there was a slight increase in the proportion of cells in the sub-G0 fraction (Fig. 6a). The sub-G0 fraction represents apoptotic and necrotic cells as well as cellular debris resulting from cell death. MDA1986 cells, which are more sensitive to erlotinib than are LN158 cells, underwent G1 arrest, and the proportion of cells in the G0/G1 phase increased from 49 to 75% after incubation with erlotinib for 24 h (Fig. 6b). Docetaxel caused a substantial increase in the proportion of cells in the sub-G0 fraction. We were, however, unable to demonstrate G2/M arrest, changes in β-tubulin expression, PARP cleavage (Fig. 6c), or morphologic changes consistent with apoptosis (data not shown) with this dose of docetaxel. These data, in combination with the MTT data presented in Figs 2–5, demonstrate that this low dose of docetaxel leads to cytotoxicity via necrosis and not cell cycle arrest and apoptosis.

As we were unable to demonstrate G2/M arrest or apoptosis with these low doses of docetaxel (0.5–1 nmol/l), we increased the drug concentrations but kept both well within the range of known plasma values in humans. Standard erlotinib doses of 150 mg/day result in a maximum plasma concentration of 2.1 μg/ml (4.9 μmol/l) at steady state, a steady state serum concentration of about 2 μmol/l, and a half-life of about 18 h [41]. The peak plasma concentration for docetaxel is 3.67 μg/ml (4.2 μmol/l) after a standard infusion of 100 mg/m^2 [42]. When we treated cells with 40 nmol/l docetaxel for 24 h, we observed the expected mitotic arrest, an increase in the proportion of cells in the sub-G0 population (Fig. 6d), morphologic changes consistent with apoptosis (data not shown), a decrease in β-tubulin and an increase in cleaved PARP expression (Fig. 6c). LN158 cells, which are resistant to erlotinib, were unaffected by 2 μmol/l erlotinib (Fig. 6d), whereas MDA1986 cells, which are sensitive to erlotinib, underwent G0/G1 arrest (Fig. 6b). Our results are consistent with the distinct effects of low-dose and high-dose docetaxel in breast cancer cells described by Hernandez-Vargas *et al.* [35]: 2–4 nmol/l docetaxel led to accumulation of cells in the sub-G0 fraction and subsequent necrosis, whereas 100 nmol/l docetaxel resulted in G2/M arrest and apoptosis.

Sequence-dependent effects of erlotinib and docetaxel are dose dependent

We hypothesized that because low and high doses of docetaxel have distinct biological effects, the docetaxel dose would affect the activity of the sequence-dependent combination with erlotinib. Chou–Talalay analysis is not

Fig. 4



Administration of erlotinib before docetaxel addition has synergistic cytotoxic effects. (a) Head and neck squamous cell carcinoma cells were incubated with erlotinib alone for 24 h and then with both agents for an additional 48 h. For single agent controls, cells were treated with docetaxel alone for 48 h or erlotinib alone for 72 h. (b–d) The number of remaining viable cells was estimated by MTT assay. The combination was synergistic in all three cell lines: Tu167 (b), LN158 (c), and MDA1986 (d).

possible at doses that far exceed the IC_{50}, so we examined the effects of the drug sequences with high doses of docetaxel on the cell cycle instead. The cell cycle profiles from the cells treated with the two agents concurrently or with erlotinib followed by docetaxel were similar to that of docetaxel alone (Fig. 6d). When docetaxel was administered before erlotinib, however, there was a substantial increase in the proportion of cells in the sub-G0 phase (75 vs. 30% for cells treated with both the drugs concurrently) and morphologic changes consistent with massive apoptosis (data not shown). Similar sequence-dependent effects were observed in MDA1986 cells (data not shown).

Docetaxel has distinct effects on epidermal growth factor receptor expression

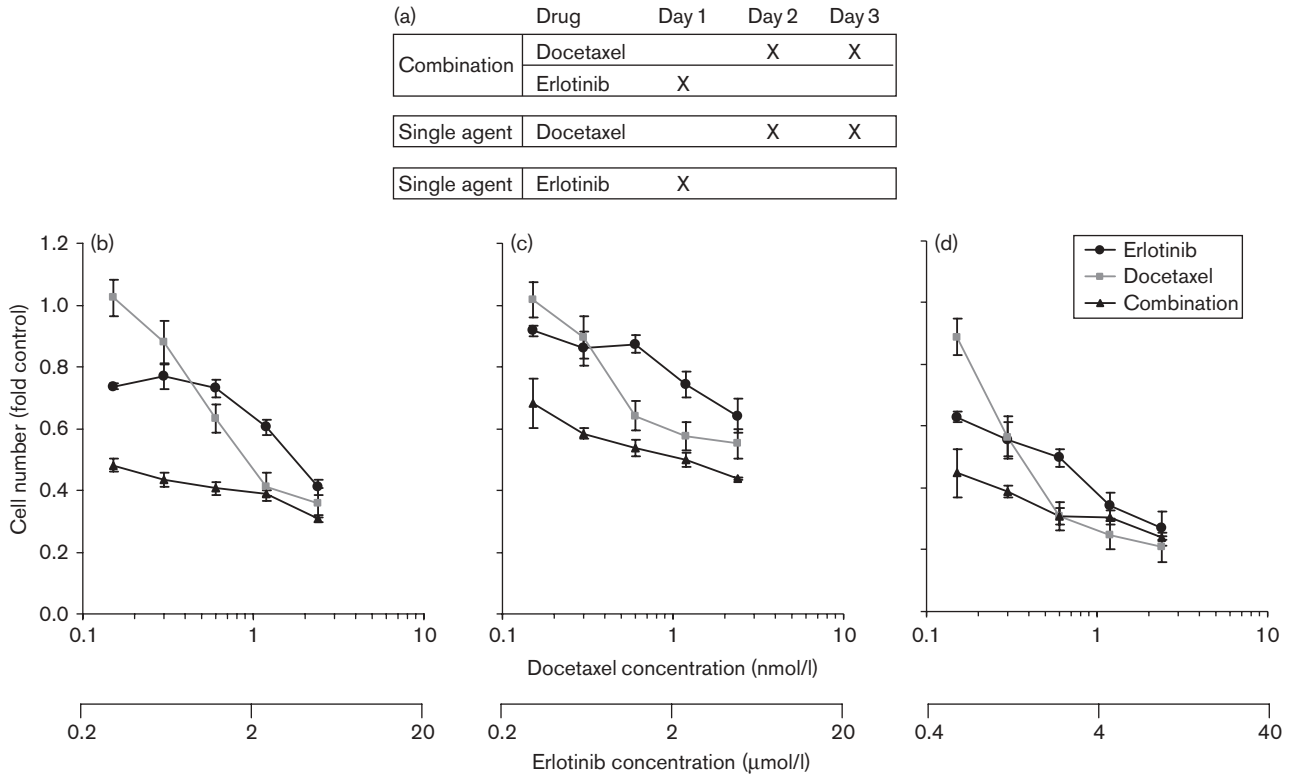
We examined the effect of docetaxel on EGFR expression, activation, and signaling because it has been shown to decrease EGFR expression [43] and enhance EGF binding [44] in cancer cells. Treatment of LN158 cells with 0.5 nmol/l docetaxel for 24 h led to a 1.4-fold increase in total EGFR expression versus control (Fig. 6e), whereas 40 nmol/l docetaxel for 24 h resulted in a 0.5-fold decrease in total EGFR expression (Fig. 6f). In MDA1986 cells, 0.5 nmol/l docetaxel for 24 h led to a 1.6-fold increase in total EGFR expression versus control and 40 nmol/l docetaxel for 24 h resulted in no significant change in total EGFR expression (Fig. 6c). This demonstrates that HNSCC cells respond to low-dose and high-dose docetaxel with distinct signaling effects and suggests that the modulation of EGFR expression affects subsequent sensitivity to EGFR inhibition.

Discussion

The combination of taxoids with EGFR inhibition has potent additive antitumor effects in HNSCC cells *in vitro* and in mice [16,34] and promising clinical activity in early studies in HNSCC patients [19–22]. Several studies have, however, demonstrated that the order in which cytotoxic chemotherapy and targeted agents are administered can significantly alter cytotoxicity. In this study, we sought to determine the optimal timing of docetaxel and erlotinib administration in HNSCC by examining different schedules and investigating the underlying mechanisms for the observed biological effects.

Initially, we started with doses of erlotinib and docetaxel that encompassed the IC_{50} values for the single agents. We were surprised to discover that the addition of

Fig. 5

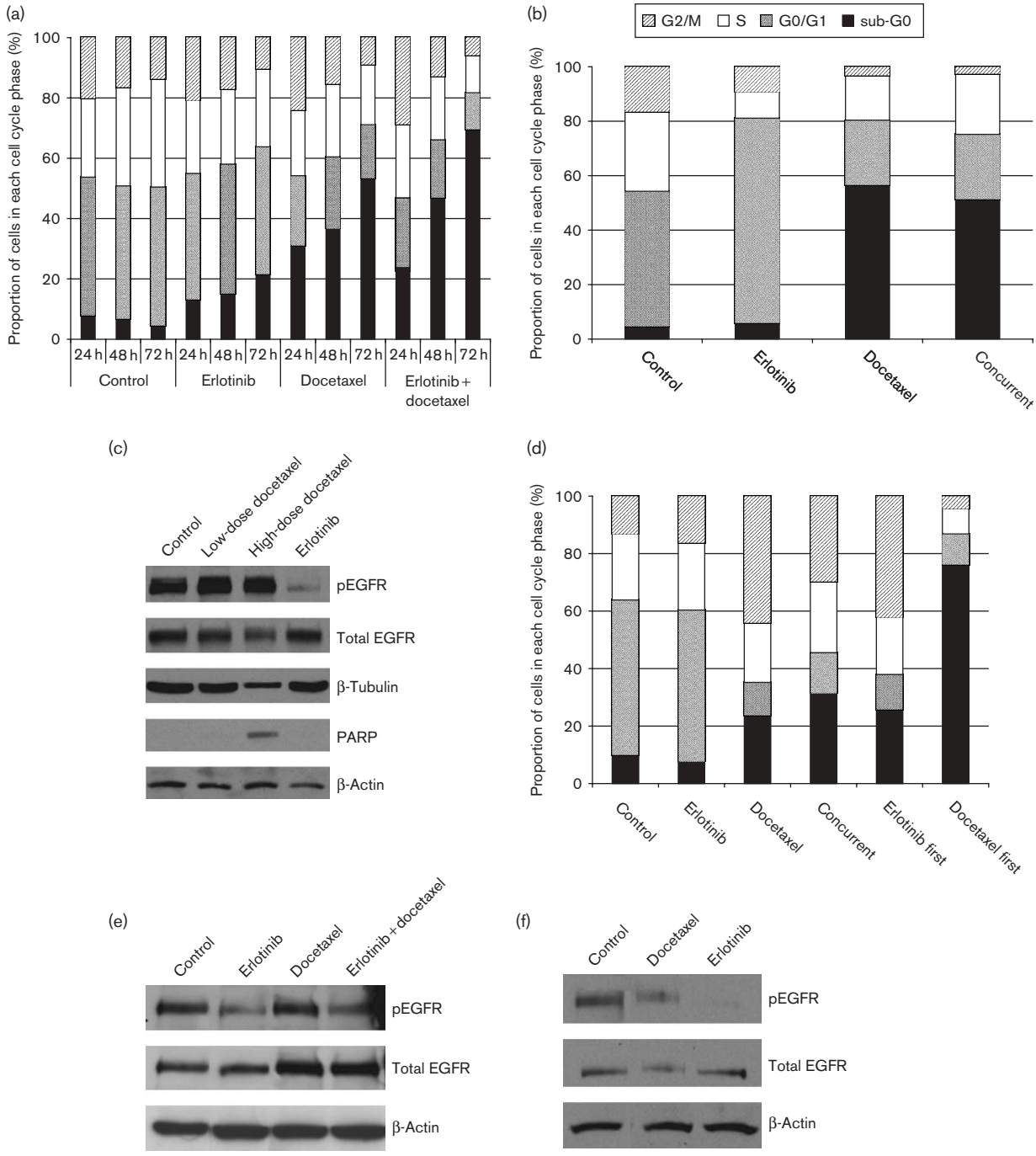


Administration of erlotinib before docetaxel alone has synergistic cytotoxic effects. (a) Head and neck squamous cell carcinoma cells were incubated with erlotinib alone for 24 h and then docetaxel alone for 48 h. For single agent controls, cells were treated with docetaxel alone for 48 h or erlotinib alone for 24 h. (b–d) The number of remaining viable cells was estimated by MTT assay. The combination was synergistic in all three cell lines: Tu167 (b), LN158 (c), and MDA1986 (d).

erlotinib concurrently or after docetaxel generally had an additive cytotoxic effect and that administering docetaxel after erlotinib was synergistic. These results are the opposite of those found by several other investigators using a variety of tumor cell types, chemotherapeutic agents, and targeted agents. We hypothesized that the discrepancy could be accounted for by the distinct biological effects that docetaxel can have at low versus high doses. In keeping with recently published results from studies using breast cancer cells [35], we found that low-dose docetaxel led to an accumulation of cells in the sub-G0 fraction with no evidence of G2–M arrest or early apoptosis, whereas high-dose docetaxel led to G2–M arrest and apoptosis in addition to an accumulation of cells in the sub-G0 fraction. Adding erlotinib concurrently or before high-dose docetaxel only minimally altered the cell cycle distribution compared with docetaxel alone, whereas erlotinib added after high-dose docetaxel led to a profound increase in the number of cells in the sub-G0 fraction. The dose of docetaxel also had distinct molecular effects in that EGFR expression was increased in HNSCC cells treated with low-dose docetaxel but decreased in those treated with high-dose docetaxel.

Several studies that have examined the sequence dependency of chemotherapeutic and targeted agents suggest that the agents' effects on the cell cycle may be the underlying mechanism for the observed differences. In NSCLC cells in culture, the combination of docetaxel (50 nmol/l, 18 h) and erlotinib (1 μmol/l, 24 h) enhanced apoptosis compared with the single agents. Sequential treatment was more efficacious than concurrent treatment. The percentage of cells undergoing apoptosis was 15–24% when erlotinib was followed by docetaxel and 21–42% when docetaxel was followed by erlotinib [45]. The results were similar when NSCLC cells were exposed to EGFR inhibition before treatment with the cell cycle-specific agents paclitaxel, vinblastine, bortezomib, and pemetrexed [31,46]. Likewise, treatment of breast cancer cells with 17-AAG before paclitaxel caused a G1 phase arrest and antagonism, whereas the reverse sequence led to enhanced apoptosis [47]. In bladder cancer cell lines that are sensitive to the EGFR inhibitor gefitinib, its administration after docetaxel enhanced apoptosis, whereas treatment with gefitinib followed by docetaxel did not [48]. These data are consistent with our finding in HNSCC cells that EGFR inhibition after high-dose docetaxel treatment enhanced the biological

Fig. 6



Effects of docetaxel on cell cycle distribution, apoptosis, and gene expression are dose dependent. (a) LN158 cells were treated with vehicle control, 1 μmol/l erlotinib, 0.5 nmol/l docetaxel, or both agents concurrently for 24, 48, or 72 h. DNA content was analyzed on a cytofluorimeter by fluorescence-activated cell sorting analysis (FACS) using ModFit software. (b) MDA1986 cells were treated with vehicle control, 2 μmol/l erlotinib, 1 nmol/l docetaxel, or both agents concurrently. DNA content was analyzed by FACS. (c) MDA1986 cells were treated for 24 h with vehicle control, 1 μmol/l erlotinib, 0.5 nmol/l docetaxel, or 40 nmol/l docetaxel; lysed; and analyzed by western blotting. (d) LN158 cells were treated with vehicle control, 2 μmol/l erlotinib, 40 nmol/l docetaxel, both agents concurrently, erlotinib followed by docetaxel, or docetaxel followed by erlotinib for 24 h. DNA content was analyzed by FACS. (e) LN158 cells were treated for 24 h with vehicle control, 1 μmol/l erlotinib, 0.5 nmol/l docetaxel, or both agents concurrently; lysed; and analyzed by western blotting. Quantification of these blots revealed that erlotinib, docetaxel, and both agents concurrently resulted in total EGFR expression levels (normalized for β-actin expression) that were 0.97-fold, 1.43-fold, and 1.43-fold that of control, respectively. (f) LN158 cells were treated for 24 h with vehicle control, or 40 nmol/l docetaxel, 2 μmol/l erlotinib; lysed; and analyzed by western blotting. Docetaxel and erlotinib resulted in total EGFR expression levels (normalized for β-actin expression) that were 0.50-fold and 0.76-fold that of control, respectively. pEGFR, phosphorylated epidermal growth factor receptor.

effects, but EGFR inhibition prior to docetaxel did not. The explanation proposed for this sequence dependence in NSCLC cells is that the inhibition of EGFR could cause cell cycle arrest that would make the cells less sensitive to the cell cycle-specific effects of docetaxel [31,45]. In both our HNSCC cells and in NSCLC cells [31], however, this sequence dependence with high-dose taxanes was also observed in cell lines that are resistant to EGFR inhibition (i.e. A549, LN158), suggesting that another mechanism contributes to the sequence-dependent effects.

When HNSCC cells are exposed to low concentrations of docetaxel, its effects are not cell cycle specific owing to a distinct mechanism of cell death. Most studies of taxanes have identified the mechanism of cell death as mitotic slippage. The taxane binds to the β-subunit of tubulin, leading to microtubule polymerization, stabilization, and cell cycle arrest in mitosis. The cells then enter a tetraploid phase and undergo apoptosis. This process is clearly cell cycle dependent. Cells treated with docetaxel, however, can also undergo nonapoptotic cell death, the most prominent type being mitotic catastrophe followed by necrosis. The degree to which cells undergo apoptosis versus mitotic death (and other nonapoptotic cell death mechanisms) varies by dose and cell type [36]. Our results are consistent with these observations in that the lower doses of docetaxel led to the accumulation of cells in the sub-G0 fraction with no evidence of apoptosis, which indicates aberrant mitosis and aneuploidy, with subsequent necrosis. Additionally, we did not observe any effect of low-dose docetaxel on β-tubulin levels. In contrast, the higher doses of docetaxel led to decreased β-tubulin levels, G2–M arrest, apoptosis, and an accumulation of cells in the sub-G0 fraction. These distinct dose-dependent biological responses to docetaxel are mirrored in the distinct and opposite effects of treatment sequence with erlotinib on cytotoxicity; synergy was observed when erlotinib was given before low-dose docetaxel, but the rate of apoptosis was higher when erlotinib was given after, rather than before, high-dose docetaxel.

Altered expression of EGFR and other genes also may contribute to the sequence-dependent and dose-dependent cellular effects of erlotinib and docetaxel in HNSCC cells. Molecular profiling of breast cancer cells showed differences in gene expression according to the dose of docetaxel [35]. Likewise, we observed a dose-dependent effect of docetaxel on EGFR expression. Others have also observed that high-dose docetaxel led to a decrease in EGFR expression in HNSCC cells in culture [43]. Interactions between taxanes and the EGFR pathway have been observed in other epithelial cancers. For example, breast cancer cells bind more EGF after exposure to docetaxel [44]. Paclitaxel led to increased activation of both EGFR and AKT, as well as enhanced cytotoxicity when given concurrently with gefitinib in NSCLC [49]. The distinct effects of docetaxel on EGFR expression in HNSCC cells may also explain the antagonistic effect observed in MDA1986 cells treated with low-dose docetaxel followed by erlotinb (vs. additive effects in LN158 and Tu167 cells). In NSCLC cell lines, synergy between gefitinib and cisplatin was observed only in those cell lines in which there was an increase in activated EGFR and AKT expression after cisplatin exposure. Interestingly, low-dose docetaxel did lead to a significant increase in EGFR in LN158 cells but not in MDA1986 cells.

As we and others have observed differences in biological effects at different docetaxel doses, it is important to know the concentration of docetaxel that tumors are exposed to in patients. Although this value cannot be determined directly, some preclinical and clinical data do address this in an indirect way. After a 1–2-h infusion of docetaxel (100 mg/m^2), the peak plasma concentration in humans was found to be 3.67 μg/ml (4.2 μmol/l) [42], which is similar to the maximum plasma concentration found after a weekly dose (35 mg/m^2) of 4.9 μg/ml (5.6 μmol/l) infused over 30 min [50]. In patients treated with either the standard dose (100 mg/m^2 every 3 weeks) or the lower weekly dose (35 mg/m^2), the plasma level is higher than 10 nmol/l for at least 24 h [42]. The level of free drug in plasma is, however, approximately one order of magnitude lower than the total drug level [50]. Animal studies have demonstrated that the peak level of docetaxel in tumor tissue is less than 10% that in plasma, although the area under the curve is higher [51]. This observation suggests that human tumor tissue may have prolonged exposure to low doses of docetaxel after a single infusion. Thus, although administering erlotinib and docetaxel at different times is clinically feasible [28], the prolonged exposure of tissue to docetaxel may make it difficult to separate the exposure of tumors to docetaxel from their exposure to erlotinib.

The majority of studies examining the effects of drug sequence have been performed *in vitro*. In-vivo studies are needed because there are biological effects, such as those on angiogenesis and stromal tissue, which cannot be addressed *in vitro*. In-vivo studies incorporate a more complex pharmacokinetic profile that more closely mirrors that seen in patient tumors. Owing to the 'oncogenic shock' that follows even transient inhibition of important oncogenes, the effects of pulsatile treatment should also be examined [52]. In mice bearing breast cancer xenografts, pulsatile, high-dose administration of gefitinib before paclitaxel administration had a superior antitumor effect compared with concurrent, continuous, or high-dose gefitinib after paclitaxel [53].

In conclusion, ours is the first report to demonstrate that the dose of docetaxel can affect the sequence-dependent cytotoxicity of combined docetaxel and EGFR inhibition

therapy. The cause of the dissimilar effects of the different sequences we observed is likely a combination of the dose-dependent effects of docetaxel on the mode of cell death (mitotic arrest with apoptosis vs. mitotic catastrophe with necrosis) and on EGFR expression. The profound synergy observed in some of the sequence combinations is promising for clinical development. Further study of these processes in animals is warranted to rationally design clinical trials using erlotinib and docetaxel combinations in HNSCC patients.

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