

## Enhancement of Docetaxel-Induced Cytotoxicity by Blocking Epidermal Growth Factor Receptor and Cyclooxygenase-2 Pathways in Squamous Cell Carcinoma of the Head and Neck

Mi Sun Choe, Zhuo Chen, Carmen M. Klass, Xin Zhang, and Dong M. Shin

**Abstract** **Purpose:** The addition of molecular targeted agents to enhance the cytotoxicity of chemotherapeutic agents is a promising strategy in cancer treatment. The combination of cyclooxygenase-2 inhibitors and epidermal growth factor receptor tyrosine kinase inhibitors, such as celecoxib and ZD1839 (gefitinib), was reported to achieve synergistic cell growth inhibition in squamous cell carcinoma of the head and neck. Therefore, we postulated that the addition of celecoxib and ZD1839 to docetaxel, a cytotoxic agent, might further increase antitumor activity. **Experimental Design:** The combination of celecoxib, ZD1839, and docetaxel was studied for its effect on cell growth and apoptosis by cell growth inhibition and Annexin V assays. The relevant molecular targets of these agents and apoptotic markers were examined by immunoblotting analyses in the presence or absence of these three drugs. Morphologic changes of the microtubule cytoskeleton, a known target of docetaxel, were also evaluated by staining for  $\alpha$ -tubulin after the combination treatment. **Results:** We showed that this triple combination significantly enhanced cell growth inhibition and docetaxel-induced apoptosis. Docetaxel mainly induced caspase-8 activation, whereas the addition of celecoxib and ZD1839 augmented the caspase-8 activation and enhanced caspase-9 activation. One of the underlying mechanisms for augmentation of docetaxel-induced apoptosis by celecoxib and ZD1839 is to further inhibit the activation of prosurvival pathway molecules, such as extracellular signal-regulated kinase and AKT, and the promotion of aberrant apoptosis. **Conclusions:** Our studies suggest that the combination of docetaxel with a cyclooxygenase-2 inhibitor and an epidermal growth factor receptor tyrosine kinase inhibitor may further improve efficacy of docetaxel and other taxane-based therapies in squamous cell carcinoma of the head and neck.

The recent development of targeted agents for cancer therapy has changed the practice of hematology and oncology. Although targeted agents have frequently only modest anti-tumor activity as single agents, the combination with conventional cytotoxic drugs is an attractive new treatment strategy. Preclinical and clinical studies suggest synergy of targeted agents with conventional cytotoxic drugs, which could lead to not only less toxic but also more effective cancer treatment options (1).

Docetaxel, a widely used chemotherapy drug, is derived from the European yew tree and belongs to the family of taxanes (2). It is widely used in the treatment of various solid tumors (e.g.,

advanced breast cancer, non-small cell lung cancer, ovarian cancer, and head and neck cancer; refs. 2, 3). Taxanes bind to  $\beta$ -tubulin and stabilize microtubules. Treatment with taxanes results in the arrest of the mitotic spindle checkpoint and apoptosis (4–6). At high concentrations, they are known to promote microtubule polymerization, whereas at lower concentrations, taxanes suppress microtubule dynamics, resulting in mitotic arrest and aberrant mitosis (6). Although Bcl-2 phosphorylation induced by the activated c-Jun NH<sub>2</sub>-terminal kinase/stress-activated protein kinase pathway and deregulation of certain signaling pathways seems to attribute to the taxane-induced cell death (7, 8), the precise mechanisms of microtubule stabilization and consequent cell death has not been clearly determined.

Up-regulation of activated AKT has been shown to correlate with worse prognosis in squamous cell carcinoma of the head and neck (SCCHN; refs. 9, 10). Recent studies have shown that the apoptosis induced by taxanes is enhanced by cotreatment with extracellular signal-regulated kinase (ERK) 1/2 or AKT inhibitors (11–14). ERK1/2a is a serine-threonine protein kinase and one of the mitogen-activated protein kinases (MAPK). In general, ERK is involved in cell cycle progression, survival, differentiation, and proliferation, whereas other MAPKs, c-Jun NH<sub>2</sub>-terminal kinase/stress-activated protein kinases, and p38 MAPK are activated by various

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noxious stimuli and have been implicated mainly in cell death (15). Moreover, AKT is a downstream target of phosphatidylinositol 3-kinase. It is also a serine-threonine kinase and known to inhibit apoptosis and to promote cell survival and growth (15).

Among those molecular targeted agents, cyclooxygenase-2 inhibitors (COX-2I) and epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKI) are promising in treatment of head and neck cancer (16, 17). COX-2 and EGFR have been strongly implicated in head and neck carcinogenesis (18). The inhibition of these pathways using specific inhibitors has shown promising results both *in vitro* and *in vivo*. Celecoxib is one of the selective COX-2Is and displays an antineoplastic effect by inducing cell cycle inhibition, apoptosis, antiangiogenesis, and decreased metastatic potential (19). COX-2Is are shown to augment taxane-mediated inhibition of tumor growth in an experimental model of lung cancer (20).

ZD1839 (Iressa or gefitinib) is an EGFR-TKI competing with ATP for the binding with EGFR and thus blocking EGFR-induced signaling pathway (21). It inhibits activation of ERK and AKT and induces growth arrest of tumor cells. EGFR-TKIs have shown promising synergistic effects in combination with various cytotoxic drugs, including taxanes, against various human tumors *in vitro* and *in vivo* (22–24).

Recently, COX-2 and EGFR pathways were reported to be tightly connected, and the combinations of COX-2I and EGFR inhibitor were shown to have a synergistic effect in treatments of cancer (18, 25, 26). Combined treatment with a COX-2I and an EGFR-TKI has shown synergistic growth inhibition *in vivo* and *in vitro*, due to further inhibition of EGFR-mediated pathways, including ERK and AKT (17, 27, 28). This combination also effectively inhibits the angiogenesis. We have also shown that the combination of celecoxib and ZD1839 can induce synergistic or growth inhibition of SCCHN cell lines (17, 27). A recently reported phase I trial investigated the safety of the combination of celecoxib and gefitinib in 19 patients with recurrent or metastatic SCCHN. No dose-limiting toxicities were encountered, and a partial response rate of 22% is encouraging in this chemotherapy-resistant population (29). Therefore, we hypothesize that the addition of celecoxib and ZD1839 to docetaxel may show more augmentation of cytotoxic effect of this drug, which has been shown by the current study. In addition, we elucidated the signal transduction pathways that led to apoptosis induced by the three-drug combination.

## Materials and Methods

**Cell lines.** Six SCCHN cell lines (Tu177, Tu212, Tu686, 686LN, 886LN, and SqCC/Y1) were used for this study. The Tu177 cell line was established from a patient with laryngeal carcinoma. Tu212 was established from a primary hypopharyngeal tumor. 686LN and 886LN were established from the lymph node metastases of a squamous cell carcinoma of the tongue and larynx, respectively. SqCC/Y1 was established from a squamous cell carcinoma of the oral cavity. These cell lines were obtained from Dr. Gary L. Clayman (University of Texas M. D. Anderson Cancer Center, Houston, TX), Dr. Peter G. Sacks (College of Dentistry, New York University, New York, NY), and Dr. Shi-Yong Sun (Emory University Winship Cancer Institute, Atlanta, GA). They were cultured in DMEM/Ham's F-12 (1:1), supplemented with 10% heat-inactivated fetal bovine serum and

antibiotics (streptomycin, penicillin G, and amphotericin B) and maintained in a 37°C, 5% CO<sub>2</sub>, humidified incubator.

**Reagents.** Docetaxel and celecoxib were obtained from Aventis Pharmaceuticals, Inc. and Pfizer Pharmaceuticals, Inc., respectively. ZD1839 was provided by AstraZeneca Pharmaceuticals. All three drugs were dissolved in sterile DMSO (Sigma-Aldrich) in appropriate concentrations and stored at –20°C until use. The stock solutions were then diluted in culture medium to the desired final concentration. In all cases, final concentrations of DMSO were <0.1% in the cell culture medium. The ERK inhibitor U0126 was purchased from Promega.

**Cell growth assay.** SCCHN cells were seeded at a density of  $3 \times 10^3$  cells per well into 96-well plates in triplicate. Twenty-four hours later, drugs were added in various range of concentrations as single agents (0.1–100 nmol/L for docetaxel, 5–100  $\mu$ mol/L for celecoxib, and 0.01–50  $\mu$ mol/L for ZD1839) and incubated for 72 h. In subsequent experiments, the single concentrations for each drug were carefully chosen to achieve 10% to 40% of inhibition in each cell line, Tu177, Tu212, Tu686, 686LN, 886LN, and SqCC/Y1, which resulted in a range from 0.5 to 1 nmol/L docetaxel, 20 to 25  $\mu$ mol/L celecoxib, and 0.5 to 1  $\mu$ mol/L ZD1839. Afterwards, each of the cell lines was treated with single, double, or triple drug combinations using the chosen fixed concentrations for 72 h. Cell growth inhibition was measured by determining cell density with the sulforhodamine B assay (30). The percentage of inhibition was determined by comparison of cell density in the drug-treated cells with that of the untreated control cells. All experiments were repeated at least thrice. For the combination treatment of docetaxel and U0126 in Tu212 cell lines, 10  $\mu$ mol/L U0126 was used and the experiment was done in the same way as other growth inhibition assays.

**Annexin V assay for assessment of apoptosis.** The effects of each single and combination drugs on apoptosis were analyzed in three cell lines, Tu177, Tu212, and SqCC/Y1, using flow cytometry. A total of  $2.5 \times 10^5$  cells in exponentially growing phase were seeded in 60-mm<sup>2</sup> dishes. After 24 h, they were treated with single, double, or triple drugs using the same concentrations as for the growth inhibition assay. After 72 h of treatment, both adherent and floating cells were collected and analyzed with Annexin V assay according to the manufacturer's instruction (BD PharMingen). Pelleted cells were briefly washed with PBS and resuspended in an Annexin binding buffer (BD PharMingen). Cells were then incubated with Annexin V-phycoerythrin (BD PharMingen) and 7-amino-actinomycin (BD PharMingen) for 15 min at room temperature. After incubation, the stained cells were analyzed using a FACSCalibur benchtop flow cytometer (Becton Dickinson). Cells with no drug treatment were used as a control. The experiments were repeated at least thrice independently, and average apoptosis rate was determined and graphed using FlowJo software (TreeStar, Inc.).

**Immunoblotting analysis.** To elucidate the underlying mechanism of enhanced growth inhibition and apoptosis, we tested the Tu212 cell line using immunoblotting. Tu212 cells were seeded at a density of  $6 \times 10^5$  cells in 100-mm<sup>2</sup> dishes for 24 h before the treatment. After 72 h of incubation with single, double, or triple drug combinations, cells were lysed and 75  $\mu$ g proteins from each sample were separated on 8% to 15% SDS-polyacrylamide gels and then transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween 80 (TTBS buffer) and incubated with relevant primary antibodies, including poly(ADP-ribose) polymerase (PARP), caspase-9, AKT, phosphorylated AKT, ERK, and phosphorylated ERK (antirabbit; Cell Signaling); caspase-8 (antimouse; Cell Signaling); EGFR and phosphorylated EGFR (antirabbit; Santa Cruz Biotechnology); p27 (antimouse; Santa Cruz Biotechnology); caspase-3 (antimouse; Imgenex); and horseradish peroxidase-conjugated secondary antibodies (Promega). The binding signal was visualized using an enhanced chemiluminescence kit (Amersham).

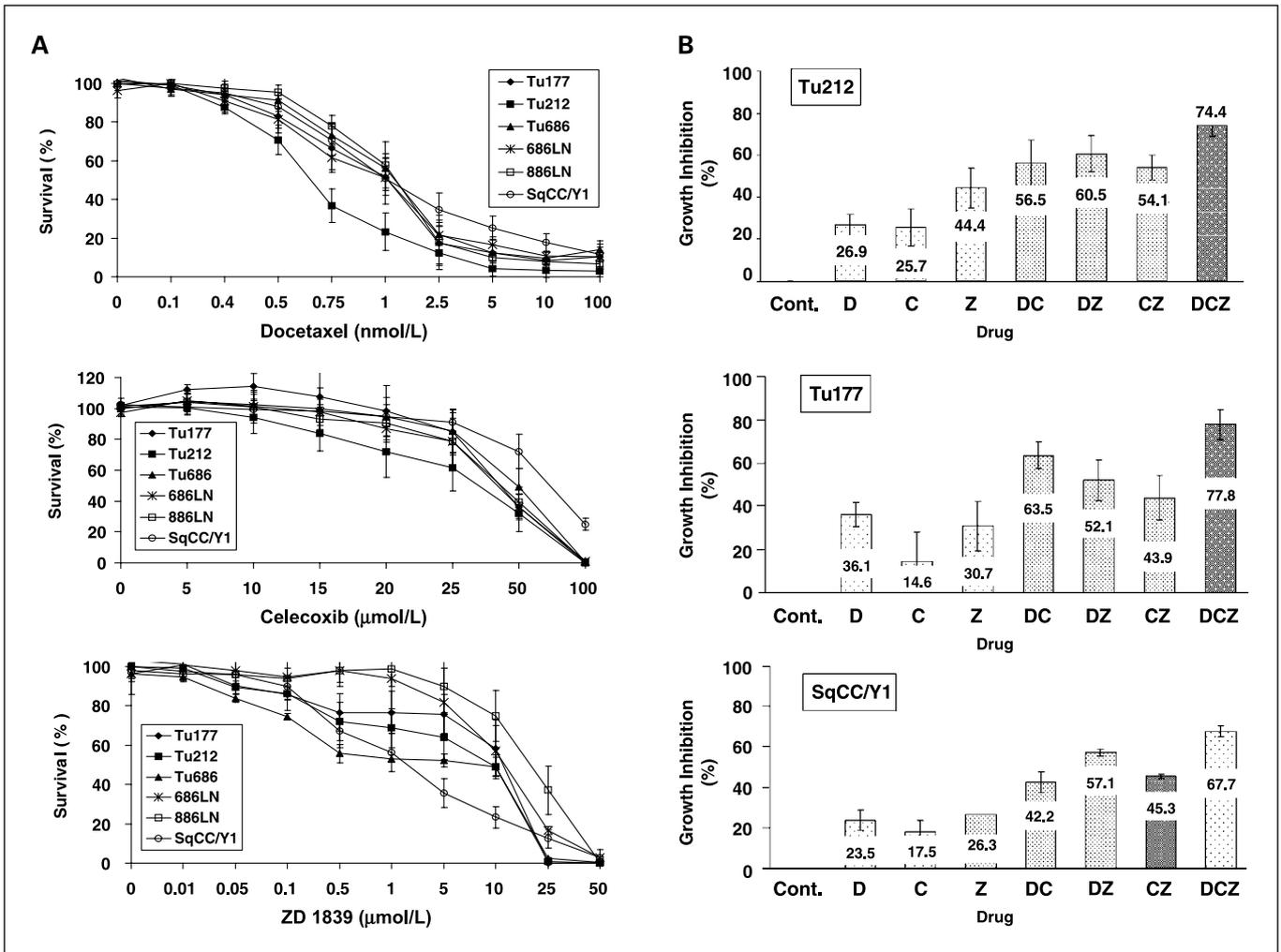
**Immunofluorescence and microscopy.** The morphologic change of microtubules caused by drug treatment was evaluated by immunofluorescence. Exponentially growing cells were plated on 12-mm glass

coverslips at a density of  $15 \times 10^3$ . Twenty-four hours after seeding, drugs were added at the same concentration as used in the growth inhibition assays and incubated for an additional 24 or 72 h. After drug treatment, cells were rinsed in PBS and fixed with 37°C PHEMO buffer [3.7% (w/v) formaldehyde, 0.05% (w/v) glutaraldehyde, 0.5% Triton X-100, 25 mmol/L HEPES, 68 mmol/L PIPES, 15 mmol/L EGTA, 3 mmol/L MgCl<sub>2</sub>, 10% DMSO] for 10 min at room temperature. The coverslips were rinsed thrice with PBS and blocked with 10% goat serum/PBS for 10 min at room temperature. The coverslips were incubated with rat monoclonal anti- $\alpha$ -tubulin antibody (Chemicon) as the primary antibody. Alexa Fluor-conjugated antirat antibody (Molecular Probes, Inc.) was used as a secondary antibody. DNA was counterstained with 5 nmol/L SYTOX Green (Invitrogen Corp.) in PBS. Coverslips were mounted onto glass slides using mounting medium (Biomedica Corp.). Coverslips were examined with a Zeiss axioplasm laser scanning confocal microscope. Mitotic index, mitotic spindle organization, and microtubule interphase morphology were determined on cells at 24 or 72 h of drug treatment. Experiments were repeated twice.

**Statistical analysis.** Effects of the combined treatments on growth inhibition and apoptosis were analyzed statistically using *t* test. *P* value <0.05 was considered as statistically significant.

**Results**

*The addition of celecoxib and ZD1839 combination significantly enhances the growth-inhibitory effect of docetaxel.* To study the sensitivity of SCCHN cells to each single drug, we initially tested six SCCHN cell lines, Tu177, Tu212, Tu686, 686LN, 886LN, and SqCC/Y1, using a range of concentrations of docetaxel (0.1-100 nmol/L), celecoxib (5-100  $\mu$ mol/L), and ZD1839 (0.01-50  $\mu$ mol/L). All three drugs inhibited SCCHN cell growth in a dose-dependent manner (Fig. 1A). In subsequent combination experiments, the chosen concentrations of docetaxel, celecoxib, and ZD1839 as single agents induced growth inhibition rates of 23% to 36%, 11% to 26%, and 21% to 44%, respectively (Fig. 1B). Results in all tested six cell lines were similar; the three cell lines shown are representative. The combination of celecoxib with ZD1839 (CZ) induced 34% to 54% of growth inhibition in each cell line compared with each single drug in all tested cell lines (*P* < 0.02). The double combination of docetaxel with celecoxib (DC) and docetaxel with ZD1839 (DZ) showed growth



**Fig. 1.** Inhibition effect on SCCHN cell growth by single and combination drug treatment. *A*, six SCCHN cell lines, Tu177, Tu212, Tu686, 686LN, 886LN, and SqCC/Y1, were treated with docetaxel (0.1-100 nmol/L), celecoxib (5-100  $\mu$ mol/L), and ZD1839 (0.01-50  $\mu$ mol/L) as single drugs. *B*, three SCCHN cell lines, Tu212, Tu177, and SqCC/Y1, were treated with each single (D, C, and Z), double (DC, DZ, and CZ), and triple (DCZ) drug combination for 72 h. Each experiment was repeated thrice. Points and columns, mean; bars, SD.

inhibition rates of 42% to 64% and 41% to 61%, respectively, with a significant *P* value ( $P < 0.005$ ) compared with single treatment of docetaxel. The triple combination of docetaxel, celecoxib, and ZD1839 (DCZ) induced 64% to 78% of growth inhibition in each cell line with a significant *P* value ( $P < 0.05$ ) compared with the double drug combinations DC and DZ or the single drug docetaxel.

Augmented apoptosis contributes to the enhanced growth inhibition in SCCHN cells treated with the combination of docetaxel, celecoxib, and ZD1839. To examine whether observed growth inhibition is due to enhanced apoptosis, we evaluated the apoptosis rates of each single, double, and triple drug treatments in three cell lines, Tu177, Tu212, and SqCC/Y1, using an Annexin V assay using the same concentrations as the growth inhibition assays. Treatment of cells with the triple drug combination (DCZ) induced a significantly higher rate of apoptosis compared with single drugs (Fig. 2). Single treatment of docetaxel showed a 7% to 16% apoptosis rate, which were 1.3-, 1.7-, and 2.0-fold higher than the control cells in Tu177, Tu212, and SqCC/Y1 cell lines, respectively. Single celecoxib or ZD1839 showed similar apoptotic rates as the control cells in these cell lines. However, the combination of celecoxib and ZD1839 showed a slight increase in apoptosis when compared with each single agent. Although the addition of either celecoxib or ZD1839 to docetaxel did not result in significantly enhanced

apoptosis, the addition of celecoxib and ZD1839 (CZ) to docetaxel significantly enhanced the rate of apoptosis in all three cell lines ( $P < 0.04$ ). The triple combination (DCZ) showed 3.6-, 3.1-, and 5.1-fold higher apoptosis rates than the controls in Tu177, Tu212, and SqCC/Y1 cell lines, respectively (Fig. 2A).

Apoptosis induced by low-dose docetaxel treatment is mainly through activation of caspase-8, but the addition of celecoxib and ZD1839 results in further enhanced caspase-9 activation. To further elucidate mechanisms of cell death induced by treatment with docetaxel, celecoxib and gefitinib cell lysates were evaluated by immunoblotting (Fig. 2B). Cleaved PARP, caspase-8, and caspase-3 levels peaked at 48 h of docetaxel treatment (12 h; data not shown). Docetaxel did not add to the activation of caspase-9. These results suggest that the death receptor pathway plays a main role in docetaxel-induced apoptosis in the Tu212 cell line. Combination of docetaxel and celecoxib (DC) showed neither further activation of caspase-8 or caspase-3 nor an increase in PARP cleavage, which corresponds to our findings in the Annexin V apoptosis assay. The addition of ZD1839 to docetaxel alone or docetaxel and celecoxib resulted in activation of caspase-3, caspase-8, and PARP as early as after 24 h. Only the triple combination showed enhanced caspase-9 cleavage, suggesting that the triple combination induced apoptosis through the mitochondrial pathway as well as the death receptor pathway.

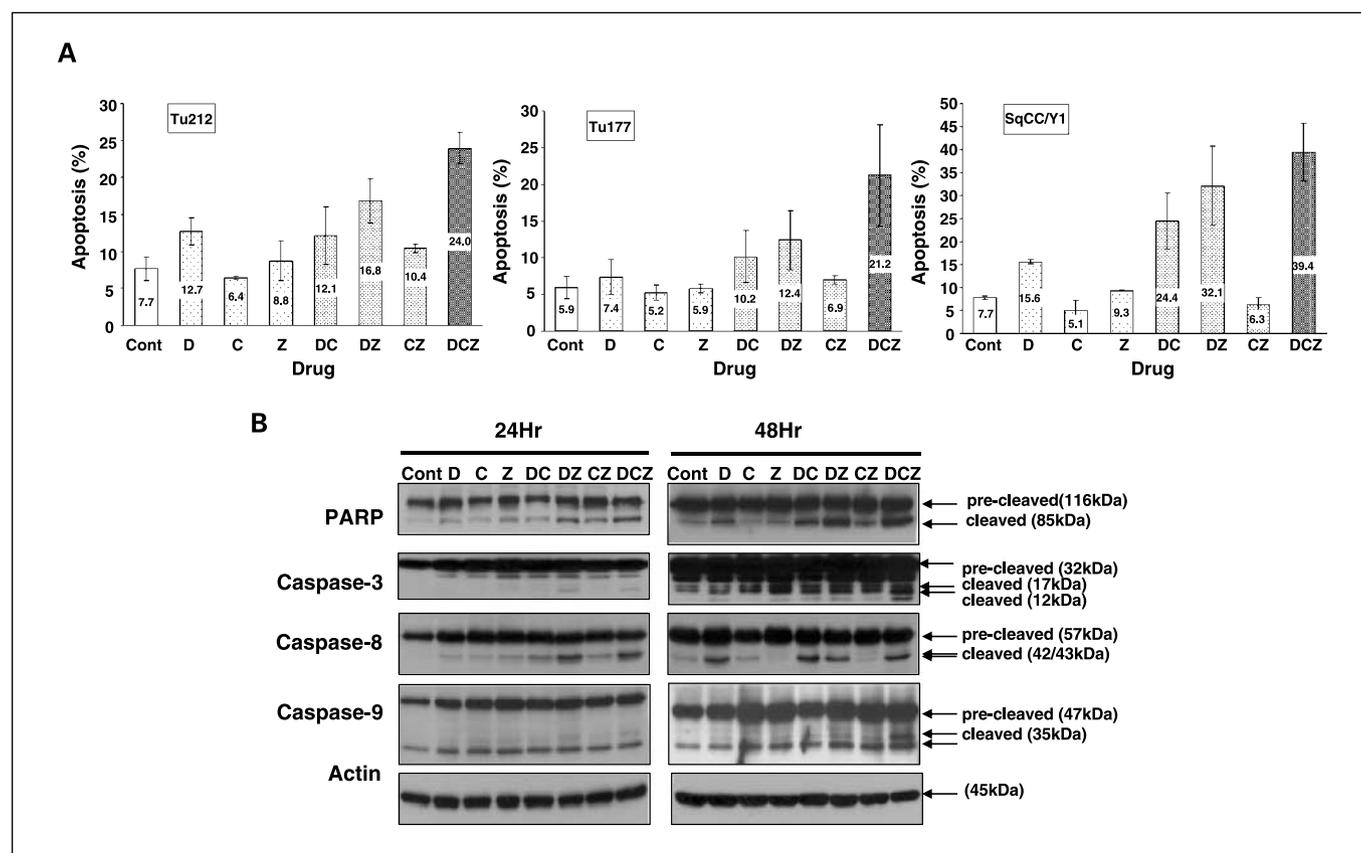
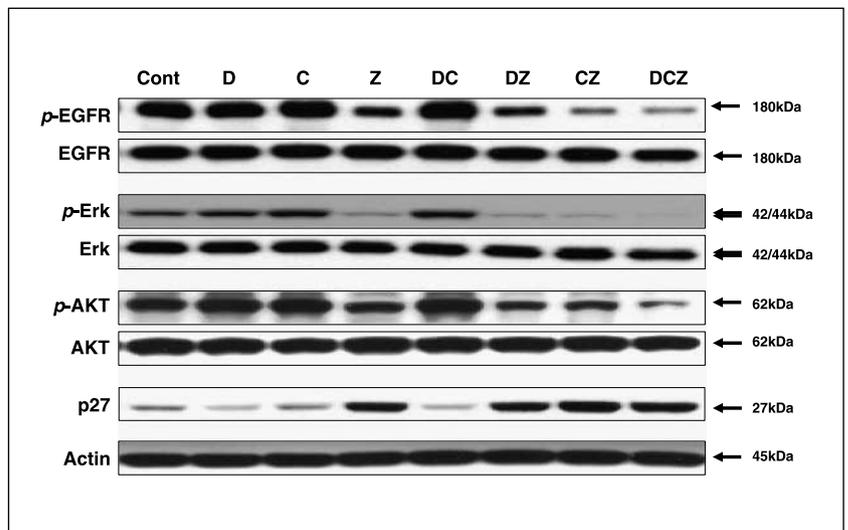


Fig. 2. Effect of the combined drugs on apoptosis. The effects of each single drug and the combinations on apoptosis were analyzed in three SCCHN cell lines (Tu212, Tu177, and SqCC/Y1) using flow cytometry. After 72 h of treatment, adherent and floating cells were collected and incubated with Annexin V-phycoerythrin and 7-amino-actinomycin. Experiments were repeated thrice. Average apoptotic rate was determined and graphed using the computer software FlowJo. A, average apoptotic rate in each of the cell lines (Tu212, Tu177, and SqCC/Y1) after treatment with single (D, C, and Z), double (DC, DZ, and CZ), and triple (DCZ) drug combinations. B, Tu212 cells were used to analyze the effects of the single or the combinations on apoptosis-related proteins using immunoblotting. We assayed PARP cleavage and activation of caspase-3, caspase-8, and caspase-9 for 24 and 48 h after each of the drug treatments to observe a time course of protein activation.

**Fig. 3.** Immunoblotting of proteins relevant to EGFR signaling pathways on Tu212 cell line. Tu212 cell line was used to analyze the effects of the single or combinational drugs on EGFR signaling pathway markers [phosphorylated EGFR (*p-EGFR*)/EGFR, phosphorylated ERK (*p-ERK*)/ERK, phosphorylated AKT (*p-AKT*)/AKT, p27, and actin for control] using immunoblotting.

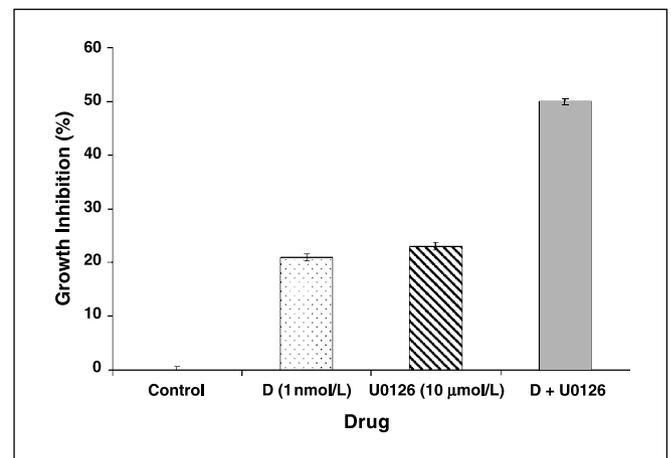


**Combined docetaxel with celecoxib and ZD1839 further blocks EGFR-mediated signaling pathways.** ERK and AKT are an important downstream target of the EGFR pathway. The serine-threonine kinases ERK and AKT are known to inhibit apoptosis in several ways (e.g., by inhibition of caspase activation; ref. 15). To elucidate the effects of single or combined drugs on the EGFR signaling pathway, the levels of phosphorylated EGFR, phosphorylated ERK1/2, and phosphorylated AKT in Tu212 cell line were studied by immunoblotting. As shown in Fig. 3, ZD1839 inhibited the activation of EGFR and its downstream signaling mediators ERK and AKT effectively, whereas the inhibitory effects on these signaling pathways by docetaxel and celecoxib were negligible. The combination of celecoxib and ZD1839 showed enhanced inhibition of EGFR phosphorylation and to a lesser degree of ERK and AKT activation. The triple combination showed the strongest inhibitory effect on EGFR-mediated pathways compared with either single or double drug treatment. We therefore speculated that the augmented apoptosis by the triple drug combination is through inhibition of ERK and AKT pathways by EGFR inactivation.

**Inhibition of ERK pathway augments docetaxel-induced cell growth inhibition.** To test whether the enhanced apoptosis by celecoxib and ZD1839 combination was attributed to its inhibitory effect on ERK pathway, we used the MAPK/ERK kinase inhibitor, U0126. It effectively blocked ERK activation at a concentration of 10  $\mu\text{mol/L}$  in Tu212 cells (data not shown). Addition of U0126 to docetaxel resulted in enhanced growth inhibition (Fig. 4), suggesting that activation of the ERK pathway seems to play an important role in docetaxel-mediated cytotoxicity.

**Addition of celecoxib and ZD1839 increases aberrant mitosis by docetaxel.** Microtubule organization was assessed by confocal microscopy. In untreated Tu212 cells, a well-organized microtubule cytoskeleton was observed (Fig. 5). Treatment with high-dose docetaxel (20 nmol/L) for 24 h resulted in prominent microtubule bundles and aberrant mitotic cells (multinucleated; Fig. 5). However, the low concentration of docetaxel (0.5 nmol/L) used in this experiment did not show significant changes in microtubule distribution and morphology, although aberrant mitotic figures were frequently noted (1.2% in

docetaxel treated cells versus 0.3% in control cells). Cells in M phase were also increased compared with the control (4.2% in docetaxel treated versus 2.6% in control), reflecting M phase arrest. The treatment of ZD1839 induced bundled appearing microtubules at 72 h of treatment (data not shown). Celecoxib also showed some bundled microtubules (Fig. 6). The combination of celecoxib and ZD1839 further impaired normal microtubular bundling at 72 h and cells in M phase were reduced to 1.6% at 24 h, supporting the cytostatic function of these two drugs (control, 2.6%; celecoxib, 2.6%; and ZD1839, 2.2%; Fig. 6). Correlating with these findings, immunoblotting showed increased expression of p27, which leads to G<sub>1</sub> cell cycle arrest, by ZD1839 treatment, either alone or combined with other drugs (Fig. 3). Interestingly, addition of celecoxib and ZD1839 to docetaxel increased the number of aberrant mitoses (8.0% in DCZ versus 1.2% in docetaxel) at 24 h of treatment, although single agents, celecoxib or ZD1839, or the combination of celecoxib and ZD1839 did not show any change in the number of aberrant mitoses compared with control cells (Fig. 6). These findings suggest that the profound



**Fig. 4.** Effect of ERK inhibition on docetaxel-induced growth inhibition. To test the effect of ERK pathway in docetaxel (D) treatment on Tu212 cell line, MAPK/ERK kinase inhibitor, U0126 (10  $\mu\text{mol/L}$ ), in combination with docetaxel (1 nmol/L) was used to evaluate growth inhibition of Tu212 cells.

inhibitory effect of the triple drug combination on cell growth is in part due to further deterioration of microtubule functionality and augmented aberrant mitosis. After 72 h of treatment, the viable cells treated with celecoxib and ZD1839 or the triple combination showed similar features with coarse microtubular bundles and rounded cell nests (Fig. 6).

## Discussion

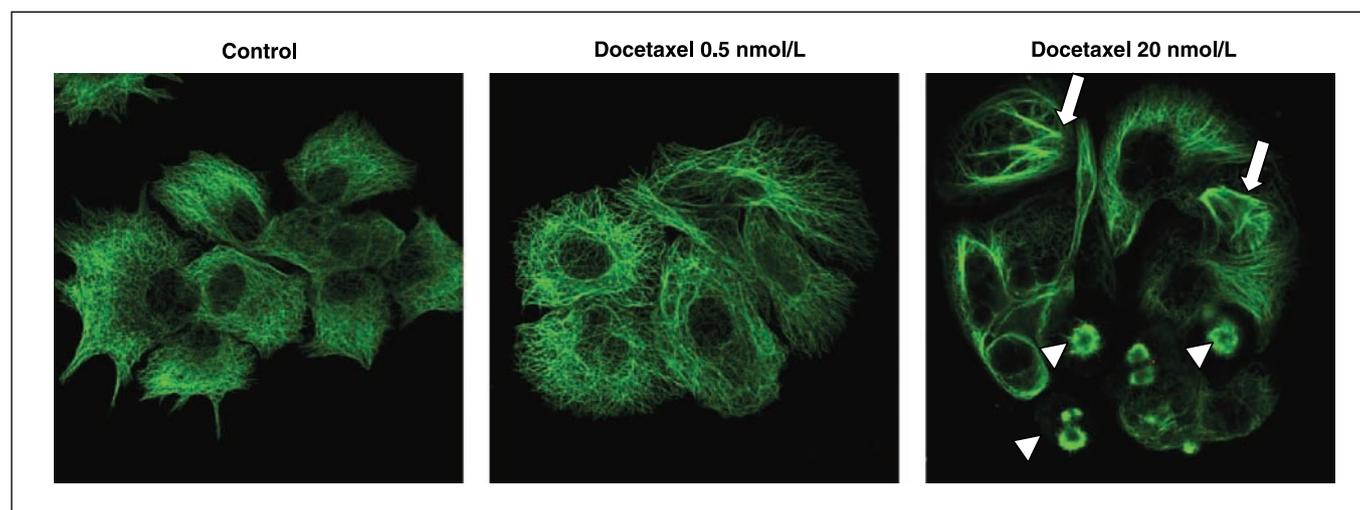
This is the first report of preclinical efficacy of the triple drug combination docetaxel, celecoxib, and ZD1839. We were able to show that this effect is mediated via the EGFR pathway and that inhibition of ERK increases growth inhibition mediated by impaired microtubular function leading to aberrant mitosis.

Six different SCCHN cell lines when treated with either docetaxel, celecoxib, or ZD1839 alone or in combination showed significant cell growth inhibition. Both celecoxib and ZD1839 have shown to enhance the effect of cytotoxic drugs or radiation. Altorki et al. (31) showed that the addition of celecoxib to paclitaxel and carboplatin in non-small cell lung cancer patients enhanced the response rate. Another selective COX-2I, JTE-522, also enhanced the effect of docetaxel in non-small cell lung cancer cell line *in vivo* and *in vitro* (32). In our experiment, the combination of docetaxel and celecoxib showed significantly higher growth inhibition in all cell lines tested compared with each single drug. However, the addition of celecoxib to docetaxel did not significantly increase the apoptotic rate nor activate caspases.

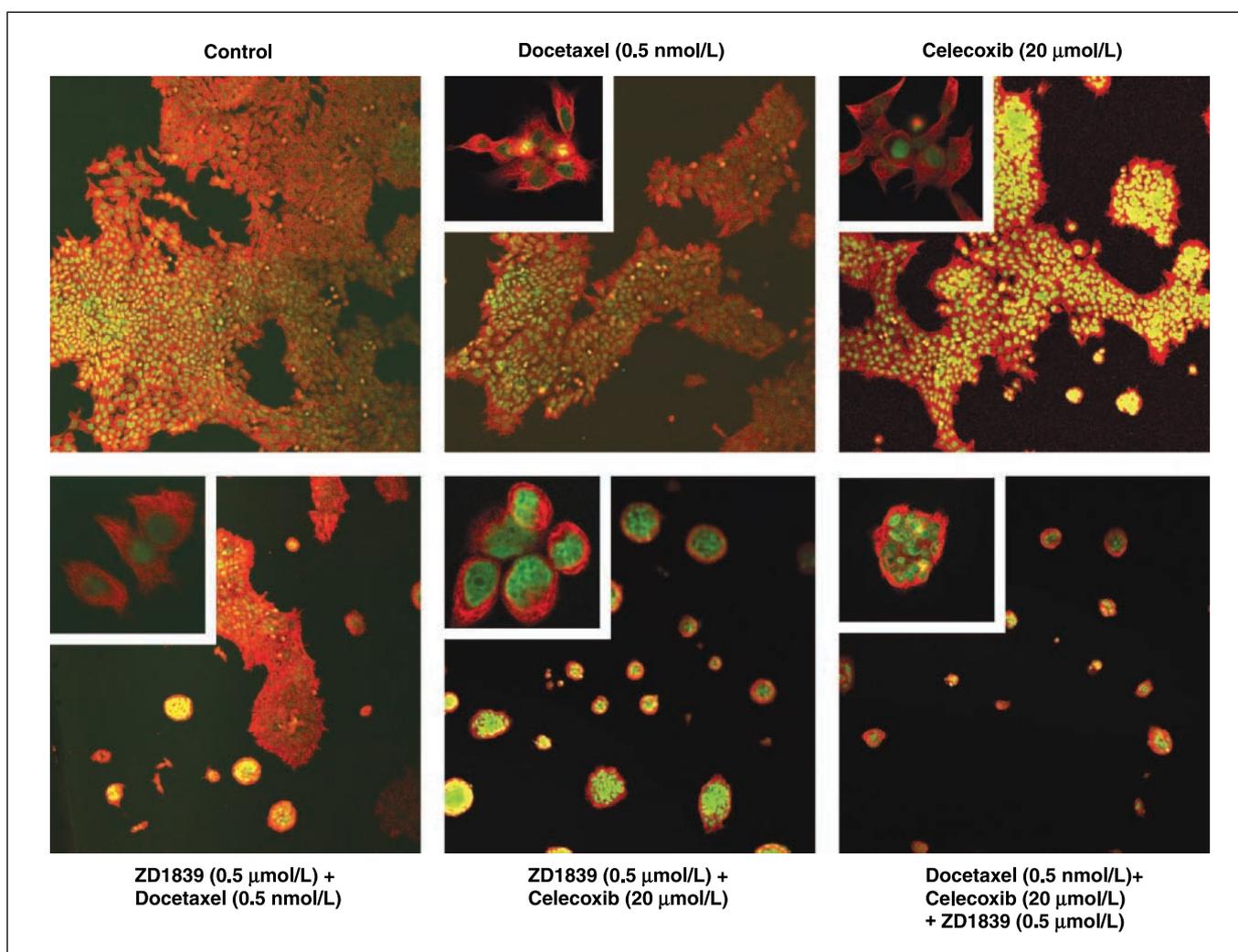
ZD1839 has been well shown to enhance the effect of taxanes (22, 24). Consistent with these findings, we also observed a significant enhancement of growth inhibition compared with each single drug. Combination of ZD1839 with docetaxel resulted in a higher rate of apoptosis than the combination of docetaxel and celecoxib and led to earlier caspase activation. The triple drug combination showed the highest rate of growth inhibition when compared with drug doublets (64-78% with drug triplets versus 34-64% with drug doublets; Fig. 1). Furthermore, only the triple combination showed activation of caspase-8 as well as activation of caspase-9. Therefore, the

addition of celecoxib and ZD1839 combination to docetaxel enhances docetaxel-induced cytotoxicity (Fig. 1).

Part of this growth inhibition can be explained with apoptosis. The proteolytic cleavage of PARP is an early biochemical event during apoptosis and a hallmark of caspase activation. Apoptotic cell death is categorized in two main phases: induction and execution (33). The induction phase is initiated by different mechanisms that either involve cell surface (extrinsic) signaling and subsequent signal transduction mechanisms or activate intracellular (intrinsic) pathways, which result in the release of mitochondrial components. Caspase-8 and caspase-9 are generally known to be initiator caspases (34). Once activated by cleavage, these caspases can activate downstream caspases, such as caspase-3. Treatment with DZ and DC results in high levels of caspase-3 and caspase-8 activation as early as 24 h. We were able to show that low-dose docetaxel in combination with ZD1839 and celecoxib induces the extrinsic apoptotic pathway most potently. This occurs as early as 24 h after addition of drugs (Fig. 2). This is supported by the downstream effector caspase-3 cleavage pattern, which shows the same pattern of up-regulation. However, triple combination drug treatment with DCZ does not only activate the extrinsic pathway but also the intrinsic apoptotic pathway to a higher degree than the other drug combinations tested, resulting in up-regulation of caspase-9. The caspases involved in taxane-induced apoptosis have been controversial and seem to be cell line specific (35, 36). Perkins et al. (36) reported that the mitochondrial pathway operated mainly by caspase-9 is the main cause of apoptosis in acute myelogenous leukemia cells treated with paclitaxel. However, Goncalves et al. (37) reported that caspase-8 played a main role in paclitaxel-induced apoptosis in a colon cancer cell line, which is consistent with the findings in our SCCHN cell lines. In the human oral squamous cell carcinoma cell line, HSC-3 activation of caspase-3, caspase-8, and caspase-9 has been found secondary to treatment with docetaxel (38). Single-agent treatment with docetaxel did result in activation of caspase-3 and caspase-8 only but not caspase-9. Celecoxib induces apoptosis in human lymphoma cell lines via caspase-9 through a mitochondrial



**Fig. 5.** Effect of docetaxel on the organization of the microtubule cytoskeleton in Tu212 cell line. Cells were incubated with no drug (*Control*), 0.5 nmol/L docetaxel, or 20 nmol/L docetaxel for 24 h. The cellular microtubule network ( $\alpha$ -tubulin; *green*) was imaged using a Zeiss LSM510 laser scanning confocal microscope system. Arrows, microtubule bundle formation; arrowheads, aberrant mitotic cells.



**Fig. 6.** Effect of the drug combination on microtubule organization in Tu212 cells. Cells were incubated with no drug (*Control*); 0.5 nmol/L docetaxel; 20  $\mu$ mol/L celecoxib; 0.5  $\mu$ mol/L ZD1839 + 0.5 nmol/L docetaxel; 0.5  $\mu$ mol/L ZD1839 + 20  $\mu$ mol/L celecoxib; and 0.5 nmol/L docetaxel + 20  $\mu$ mol/L celecoxib + 0.5  $\mu$ mol/L ZD1839 for 24 h (*inset*) or 72 h. Red, microtubules; green, chromatin.

signaling pathway (39). In our studies, however, we could not detect further up-regulation of cleaved caspase-9 by treatment with celecoxib (39). This might be secondary to lower celecoxib doses of 20  $\mu$ mol/L that were used in our experiment when compared with the 75 to 100  $\mu$ mol/L used in studies of Jendrossek et al. (39). The low doses at which the single agents were used in our experiment do not show large effects on caspase activation or on apoptosis in general but do seem to have synergistic effects when combined. It seems that in Tu212 cells, apoptosis is primarily mediated by the extrinsic pathway, as evidenced by an increase in caspase-8 activation. The substantial increase in the rate of apoptosis in HNSCC cells treated with the drug triplet DCZ seems to correlate with enhanced caspase-9, caspase-8, and caspase-3 activation, suggesting a role for extrinsic and possibly intrinsic apoptotic pathways.

Similar to previous findings, the combination of EGFR-TKI and COX-2I inhibited EGFR activation more potently than treatment with the EGFR inhibitor alone. EGFR-TKIs have been shown to up-regulate p21 and p27 and were reported as critical events (40). Consistent with this report, we also observed

up-regulation of p27 by ZD1839 treatment and further up-regulation by the combination of celecoxib and ZD1839. Up-regulation of activated AKT has been shown to correlate with worse prognoses in SCCHN (9, 10). We were able to show the most potent down-regulation of AKT activation by the triple drug combination. Treatment with DCZ led to an almost complete suppression of phosphorylated ERK expression. This suggests that cell survival in Tu212 cells is dependent on these two potent survival pathways, which are fatally disrupted by treatment with DCZ.

We also investigated whether other nonapoptotic mechanisms were responsible for the observed growth inhibition. We analyzed cell cycle effects of docetaxel and ZD1839 (data not shown). As reported by other groups as well, single-agent treatment with docetaxel results in  $G_2$ -M arrest (41).  $G_0$ - $G_1$  arrest was observed in cells treated with ZD1839 (40). Combination treatment of the two drugs resulted in down-regulation of  $G_0$ - $G_1$  arrest and the  $G_2$ -M arrest, suggesting that the effect of these two drugs on the cell cycle neutralize each other (data not shown). We have shown previously that celecoxib also causes accumulation of cells in  $G_0$ - $G_1$  and the combination of celecoxib and

ZD1839 augments this effect (17). This suggests an alternative mode for cell death in cells treated with docetaxel, celecoxib, and ZD1839. Cell cycle progression can be blocked by taxanes through centrosomal impairment, induction of abnormal spindles, and suppression of spindle microtubule dynamics (42). The occurrence of aberrant mitosis or mitotic slippage has been described as an alternative nonapoptotic cause of cell death in taxane-treated cells. Mitotic slippage described a state in which the cell cycle-arrested cell will undergo aberrant mitosis, which results in a polyploidy G<sub>1</sub>-like state, resulting in cell death. The induction of mitotic arrest through the microtubule stabilization is well established to be a main cytotoxic event caused by taxanes (7, 43, 44). In addition, the ERK pathway is known to play an important role in mitotic progression. However, at the low dose of 1 nmol/L docetaxel, we were not able to detect any effects on microtubule formation or stability, whereas these effects were present at a higher dose of 20 nmol/L. However, even at the very low dose of docetaxel, an increase in the rate of aberrant mitosis was detected, which suggests a pivotal role for this mechanism of cell death in low-dose docetaxel-treated cells. Shapiro et al. (45) reported the localization of active ERK on spindle microtubules, spindle poles, and kinetochores and suggested a role of ERK in regulating spindle assembly, possibly through regulation of motor activity or microtubule polymerization. We also showed that the combination of celecoxib and ZD1839 induced impaired assembly of microtubules, and addition of docetaxel further enhanced the number of aberrant mitoses (Fig. 6). This supports a role of ERK in microtubule homeostasis and the rationale of using ERK pathway inhibitors in combination with docetaxel. Mitotic catastrophe as the main cause of cell death in cells treated with low doses of docetaxel that were not able to induce a high rate of apoptosis has been reported in a breast cancer cell line (46). Although for a long time it was thought that apoptosis was the main cause of taxane-related cytotoxicity (47), recent evidence indicates that dose- and cell line-specific mixtures of apoptotic and mitotic cell death are observed in taxane-treated cells (46, 48, 49).

The precise mechanism by which docetaxel induced mitotic arrest is linked to cell death has not been determined, although

activation of the c-Jun NH<sub>2</sub>-terminal kinase pathway and phosphorylation of Bcl-2 have been suggested to be pivotal events (50). On the other hand, inhibition of the ERK or AKT pathway has shown significant augmentation of taxane-induced apoptosis in various cell lines (11, 13). This reflects that these two pathways may play a role in rescuing the cancer cells when exposed to cytotoxic agents, thus compromising the effect of taxanes. Because both ERK and AKT have been well documented to inhibit apoptosis in several aspects (15), we specifically inhibited the ERK pathway in docetaxel treatment using MAPK/ERK kinase inhibitor U0126 to test whether the mechanism of the enhanced cytotoxicity by celecoxib and ZD1839 can be attributed to inhibition of ERK pathway in HNSCC cell lines. The cotreatment of docetaxel and U0126 enhanced growth inhibition and confirmed that the ERK pathway plays a prosurvival role in Tu212 cells. There are two possible explanations for this additive inhibitory effect. First, ERK- and docetaxel-mediated growth inhibition may be two paralleled events, which independently result in cell growth inhibition, and second, blocking ERK pathway and addition of docetaxel may lead to the same effect on the same target. The fact of enhancement of the number of aberrant mitoses by both EGFR-TKI/COX-2I and docetaxel supports the second explanation. However, we cannot rule out the first possibility. This is supported by our observation that addition of celecoxib to docetaxel did not enhance docetaxel-induced apoptosis (Fig. 2) and did not inhibit the ERK activation any further than single agent docetaxel alone (Fig. 3).

Our data suggest that inhibition of the ERK or AKT pathway may generally block cell survival pathways likely by facilitation of aberrant mitosis. Our study indicates that the combination of docetaxel, celecoxib, and ZD1839 inhibits head and neck cancer growth effectively through augmentation of docetaxel-induced cytotoxicity by promoting aberrant mitosis.

In conclusion, the addition of celecoxib and ZD1839 to docetaxel enhanced the cytotoxicity of docetaxel in head and neck cancer cell lines, suggesting promising clinical implication, and deserved further *in vivo* study.

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## Enhancement of Docetaxel-Induced Cytotoxicity by Blocking Epidermal Growth Factor Receptor and Cyclooxygenase-2 Pathways in Squamous Cell Carcinoma of the Head and Neck

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