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Combined Inhibition of Epidermal Growth Factor Receptor and Cyclooxygenase-2 as a Novel Approach to Enhance Radiotherapy

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Abstract

Targeting epidermal growth factor receptor (EGFR) is a promising approach to increasing radiosensitivity of head and neck cancers but treatment resistance remains an important clinical problem. We hypothesize that combined EGFR and cyclooxygenase-2 (COX-2) inhibition, using small molecule inhibitors erlotinib and celecoxib, respectively would further increase the antitumor activity of radiotherapy. The effects of combinations of celecoxib, erlotinib and ionizing radiation (IR) on cell growth, cell cycle progression and apoptosis of head and neck cancer cell lines were assessed *in vitro* by cell viability, clonogenic survival, flow cytometry and Annexin V assays and *in vivo*. The effects of celecoxib, erlotinib and IR on primary and downstream molecular targets were analyzed by immunoblotting & ELISA assays. Compared to single or double agent approaches, concurrent celecoxib, erlotinib and IR was the most effective regimen at reducing clonogenic survival, increasing apoptosis and inhibiting tumor growth *in vivo*. Concurrent treatment with celecoxib and erlotinib ± IR inhibited multiple prosurvival proteins including p-ERK1/2, p-EGFR, p-AKT, p-STAT3, COX-2 and PGE-2. The combination of celecoxib, erlotinib and IR is a promising strategy to overcoming resistance to combined EGFR inhibition and IR alone.

Keywords

Cyclooxygenase-2; Epidermal growth factor receptor; Ionizing radiation; Celecoxib; Erlotinib; Head and neck cancer

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Introduction

The epidermal growth factor receptor (EGFR) is a 170-kDa transmembrane protein with intrinsic tyrosine kinase activity [1]. Following ligand binding and dimerization, EGFR activation leads to phosphorylation and activation of downstream signaling cascades, including Ras/Raf/Erk/MAPK and PI3K/Akt that regulate cell proliferation, angiogenesis, invasion and metastasis [2]. These pathways are also implicated in radiation resistance [3]. Overexpression of EGFR has been documented in 80 to 90% of patients with head and neck squamous cell carcinoma (HNSCC), correlating with an increased risk of locoregional recurrence, distant metastasis and mortality [3,4]. Two major strategies to inhibit EGFR have undergone intensive preclinical and clinical evaluation [2]. The monoclonal antibodies cetuximab and panitumumab bind the extracellular domain of EGFR to prevent ligand binding, dimerization and receptor activation [5]. Small-molecule receptor tyrosine kinase inhibitors erlotinib, gefitinib and lapatinib reversibly bind to the intracellular ATP-binding site in the tyrosine kinase domain of EGFR, inhibiting receptor autophosphorylation and downstream signaling [6].

Combining epidermal growth factor receptor inhibitors with radiation has been shown to be more effective than either treatment alone for head and neck squamous cell carcinoma in multiple preclinical studies [7,8]. However, the majority of patients treated with concurrent cetuximab and radiation in a phase III trial recurred in the irradiated neck, suggesting that treatment resistance remains a major concern [9,10].

In addition to the EGFR pathway, cyclooxygenase-2 (COX-2) is another promising target in HNSCC [11]. COX-2 is overexpressed in head and neck cancers and is implicated in carcinogenesis [12,13]. Celecoxib, a selective COX-2 inhibitor has been shown to induce cell cycle inhibition, apoptosis, suppression of angiogenesis and decreased metastatic potential [14-16]. There is significant crosstalk between COX-2 and EGFR signaling pathways and preclinical studies suggest that combined inhibition of both pathways results in synergistic growth inhibition, decreased angiogenesis and reduced metastatic potential [17-24]. The combination of celecoxib and gefitinib was recently tested in a phase I trial of 19 patients and shown to be an active and well-tolerated regimen in recurrent and metastatic HNSCC [25]. Based on these compelling preclinical and clinical data, we hypothesized that adding celecoxib would further enhance the therapeutic ratio of concurrent EGFR-inhibition and radiation for HNSCC.

Materials and Methods

Cell culture and reagents

Three HNSCC cell lines, SQ20B, SCC61 and SCC25 were used in this study. Previous studies demonstrated that SQ20B ($D_0=2.5$) is relatively radioresistant, SCC25 ($D_0=1.4$) has intermediate radiosensitivity and SCC61 ($D_0=1.0$) is considered relatively radiosensitive [26]. Cells were cultured in DMEM/ F12 media supplemented with 20% fetal bovine serum, 0.4 $\mu\text{g/mL}$ hydrocortisone, 4 mmol/L L-glutamine, 100 units/ mL penicillin and 100 mg/mL streptomycin and incubated in a 37°C, 5 % CO₂, humidified incubator. Human primary keratinocytes were a gift from Dr. Rong Du (Columbia University, New York, NY)

Experimental treatment

Erlotinib was obtained from OSI Pharmaceuticals while celecoxib was obtained from Pfizer, Inc. Both agents were dissolved in DMSO at concentrations of 50mM and 100 mM and stored at -20°C until use. Prior to experimental treatment, stock solutions were diluted in culture medium to achieve the desired concentration with a final DMSO concentration of <0.1%. Treatment was given for 24 hours prior to ionizing radiotherapy or sham

radiotherapy. Experimental radiation was performed on a 600C 6MV linear accelerator (Varian) at a dose rate of 3.55 Gy/minute.

Immunoblotting analysis

Effects of erlotinib, celecoxib and radiation on cell signaling were assessed by immunoblotting. Cells were washed with cold PBS and lysed in lysis buffer (Cell Signaling Technology) using a standard protocol. The protein concentration was analyzed by a protein assay kit with bovine serum albumin standards according to the manufacturer's instructions (Bio-Rad Laboratories). Cell lysates were separated by SDS-PAGE and transferred onto a nitrocellulose membrane (Hybond-C, Amersham Pharmacia Biotech, Inc.). Following blocking with PBS-Tween containing 5 % nonfat dry milk for 1 h, membranes were incubated overnight at 4°C with anti-phospho-Tyr 1068-EGFR antibody, anti-EGFR antibody, anti-COX-2 antibody, anti-phospho-ERK1/2 antibody, anti-ERK1/2 antibody, anti-phospho-AKT antibody, anti-AKT antibody, anti-phospho-STAT3 antibody, anti-STAT3 antibody followed by incubation with horseradish peroxidase conjugated secondary antibody. All antibodies for *in vitro* experiments were purchased from Cell Signaling Technology. Immunoreactive bands were detected by an enhanced chemiluminescence kit. The membrane was stripped with stripping buffer (Thermo Scientific) at room temperature for 15 min and re probed with anti-actin antibody as a loading control.

Cell growth assay

Inhibition of cell proliferation was evaluated by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma). Briefly, cells were seeded in 96-well tissue culture plates at a density of 3×10^3 cells per 100 μ L/well. Four wells were assigned to each experimental treatment. After 24 hours for attachment, cells were treated with erlotinib (0-6 μ mol/L) and celecoxib (0-100 μ mol/L). After 72 hours of treatment, 10 μ L of MTT solution were added to each well for two hours incubation. Absorbance at 490 nm was recorded using microplate reader.

Clonogenic assay

The effects of celecoxib, erlotinib and radiation on clonogenic survival were analyzed. Cells were seeded into 6-well plates at concentration of 1000/well in triplicate. After 24 hours, erlotinib and celecoxib were added as either single or double agents with or without ionizing. Cells were incubated for 10-14 days to form visible colonies. The colonies were fixed in 70% ethanol and stained in Giemsa solution. The colonies containing above 50 cells were counted.

Annexin V assay for apoptosis

The effects of drugs and radiation on apoptosis were analyzed. A total of 1×10^5 cells were plated in 25cm² cell culture flasks. Celecoxib, erlotinib and radiation was administered 24 hr later. At 72 h after radiation, cells were trypsinized and washed with PBS. Pelleted cells were stained with both Annexin V-FITC and propidium iodide (PI) for 15 minutes at room temperature. After incubation, stained cells were analyzed using a FACScan flow cytometer with CellQuest software.

Cell cycle analysis

Cell cycle distribution was analyzed by flow cytometric analysis. In brief, cells grown in 25cm² cell culture flasks were harvested by trypsinization and fixed with 70% ethanol. Cells were stained for total DNA content with a solution containing 50 μ g/mL propidium iodide and 100 μ g/mL RNase I in PBS for 30 min at 37°C. Cell cycle distribution was then analyzed with a FACScan flow cytometer.

Enzyme-linked Immunoassay

We measured prostaglandin E2 (PGE2) concentration in cell culture media, the media were collected at 72 hours measured by PGE2 ELISA kit following the manufacturer's protocol (Thermo Scientific). The PGE2 concentrations were calculated using a standard curve that was generated from PGE2 standards provided by the manufacturer.

Statistical analysis

Results are presented as mean \pm standard deviation of at least three experiments. Effects of the various treatments (radiation, erlotinib and/or celecoxib) on PGE-2 levels, apoptosis, cell cycle and clonogenic survival were analyzed statistically using a paired 2-sided test compared to controls. In order to determine if treatment effects on clonogenic survival are additive or synergistic, three-way analysis of variance (ANOVA) with interactions was performed. Tests for the effect of radiation (0, 2, 4 or 6 Gy), erlotinib (present or absent), celecoxib (present or absent), and their interactions were carried out. Data, counts of tumor colonies, were transformed on a \log_{10} scale before proceeding with ANOVA. A p value of <0.05 was considered statistically significant.

Human phase I study

Since both erlotinib and celecoxib are widely used in clinical practice, we designed a phase I trial (MSSM GCO 06-0509; NCT00970502) to test the hypothesis that celecoxib and erlotinib enhance radiation response in patients with radioresistant head and neck cancers. Patients with poor prognosis recurrent head and neck cancer who failed previous radiation but had no distant metastases were eligible. Following IRB approval, between March 2007 and December 2009, 14 patients were treated with celecoxib (200 mg BID to 600 mg BID), erlotinib (150 mg QD) starting 14 days prior to and concurrent with reirradiation (59.4 Gy for high risk microscopic disease and 70.4 Gy for gross disease in 2.2 Gy per fraction). All enrolled patients provided written informed consent.

The starting dose was celecoxib 200 mg BID, escalated to 400 mg BID and 600 mg BID using a 3+3 design with an expanded cohort at the maximally tolerated dose. Response was assessed by CT and PET scans performed every 3 to 4 months. Post-treatment pathology was not routinely obtained.

Immunohistochemistry

Pretreatment tumor samples were available in 11 patients, embedded into paraffin, and stained with mouse monoclonal human anti-Cox2 and anti-EGFR antibodies (Dako, Belgium). Briefly, tissues were fixed to glass slides with 10% formalin, deparaffinized in a xylene bath, and dehydrated with ethyl alcohol. Prior to immune staining, slides were bathed in 95°C water bath and allowed to cool in buffer for 20 minutes. Slides were blocked in swine serum and primary antibody was applied at a 1:100 dilution for 1 hour at room temperature. Samples were then stained with secondary, peroxidase-labeled horse anti-mouse antibody at a 1:100 dilution for 1 hour at room temperature followed by application of DAB chromogen and counterstaining with hematoxylin. Slides were scored for COX-2 and EGFR staining by a pathologist (M.R.) with subspecialty training in head and neck cancer pathology who was blinded to outcome.

Results

Association between baseline EGFR and COX-2 expression and sensitivity to radiation, erlotinib and celecoxib in HNSCC cell lines

We first tested the baseline levels of phosphorylated EGFR (p-EGFR)/EGFR, COX-2, phosphorylated AKT (p-AKT)/AKT, phosphorylated ERK (p-ERK)/ERK, phosphorylated STAT3 (p-STAT3)/STAT3 in SQ20B, SCC61, SCC25 HNSCC cells. Compared to human primary keratinocytes, increased EGFR expression is observed in all three cancer cell lines. EGFR levels, activated p-EGFR, p-AKT, p-ERK and p-STAT-3 levels were highest in SQ20B cells. In contrast, COX-2 was expressed by all three HNSCC cell lines but was markedly decreased in SQ20B compared against human primary keratinocytes (Figure 1a). Compared to normal keratinocytes, there was increased expression of EGFR and decreased expression of COX-2 in SQ20B cells. Among this panel of HNSCC, elevated EGFR correlated with radioresistance and was inversely correlated with baseline COX-2 levels.

We then tested sensitivity of HNSCC cells to erlotinib (0 to 6 $\mu\text{mol/L}$) and celecoxib (0 to 100 $\mu\text{mol/L}$). The results showed that both drugs reduced cell viability in all three cancer cell lines in a dose-dependent manner (Figure 1b-c). Neither baseline EGFR nor COX-2 expression strongly predicted sensitivity to erlotinib or celecoxib.

Effect of radiation on EGFR and COX-2 signaling pathways

To elucidate the molecular mechanisms of the inhibitory effects of erlotinib and celecoxib on irradiated HNSCC cells, we examined changes in protein expression levels of p-EGFR/EGFR, p-ERK/ERK, p-AKT/AKT and p-Stat3/Stat3 by immunoblotting. Ionizing radiation resulted in a significant increase in COX-2 in SQ20B cells while p-EGFR was increased in SCC25 cells 72 hours after treatment (Figure 2). Thus following radiation, both cell lines demonstrate significantly increased activated EGFR and COX-2.

In SQ20B cells, 3 $\mu\text{mol/L}$ erlotinib with or without 50 $\mu\text{mol/L}$ celecoxib resulted in significant decreases in p-EGFR, p-Akt, p-ERK and p-Stat3 within 20 minutes of treatment (Figure 2a, left panel). At 72 hours after treatment, combined treatment with erlotinib and celecoxib was significantly more effective at reducing expression of p-EGFR, COX-2 and possibly p-Stat3 than either treatment alone, particularly in irradiated SQ20B cells (Figure 2a, right panel and Figure 3a). In the SCC25 cells, 3 $\mu\text{mol/L}$ erlotinib with or without 50 $\mu\text{mol/L}$ celecoxib also decreased p-EGFR, p-Akt, p-ERK but had no effect on p-Stat3 (Figure 2b, left panel). At 72 hours after treatment, combined treatment with erlotinib and celecoxib resulted in reduced expression of p-EGFR, COX-2 and p-Akt in SCC25 cells (Figure 2b, right panel and Figure 3a). Celecoxib alone increased p-EGFR and p-ERK at 72 hours after treatment in the SCC25 cell line, which was effectively reversed by erlotinib (Figure 2b, right panel). In the SCC61 cell line, the effects of erlotinib \pm celecoxib were limited to reduced COX-2 and p-ERK (Figure 2c and Figure 3a). Taken together, these results suggest that combined erlotinib and celecoxib effectively inhibit multiple pro-survival proteins downstream of EGFR following ionizing radiation.

Combination of erlotinib and celecoxib inhibits radiation-induced PGE-2 production

Prostaglandin-2 (PGE₂) is a major product of COX-2. To examine the effect of celecoxib on downstream targets of COX-2, we measured PGE₂ levels by enzyme immunoassay in cultured media collected from treated cells. Following 2 Gy of ionizing radiation, PGE-2 production increased, particularly in SCC25 cells ($p=0.04$) with higher baseline COX-2 levels. Both 3 $\mu\text{mol/L}$ erlotinib and especially 50 $\mu\text{mol/L}$ celecoxib significantly decreased PGE-2 production in all three cell lines compared to controls (Figure 3b, $p<0.05$). Celecoxib

was highly effective at inhibiting PGE-2 at from 2.5 to 50 $\mu\text{mol/L}$ in all three cancer cell lines (supplemental Figure 1).

Erlotinib induces G1 arrest with or without celecoxib or IR

To determine whether growth inhibition of HNSCC by erlotinib and celecoxib is due to cell cycle arrest, we performed flow cytometry for cell cycle. These experiments demonstrated that 3 $\mu\text{mol/L}$ erlotinib both alone or in combination with 50 $\mu\text{mol/L}$ celecoxib or 2 Gy radiation, was effective at inducing G1 arrest compared to controls (Figure 4, $p < 0.05$). In contrast, neither celecoxib nor radiation had significant effects on cell cycle arrest.

Combined erlotinib and celecoxib induces apoptosis in combination with IR

To determine the effect of erlotinib and celecoxib on apoptosis, we performed the Annexin V assay on cells treated with 50 $\mu\text{mol/L}$ celecoxib, 3 $\mu\text{mol/L}$ erlotinib and/or 2 Gy radiation. There was significantly increased apoptosis following combined erlotinib, celecoxib and IR compared to IR \pm single agents (Figure 4, $p < 0.05$).

Combined erlotinib and celecoxib inhibits clonogenic survival in combination with radiation

Based on cell viability data, we performed confirmatory clonogenic assays using moderately effective single agent doses of celecoxib and erlotinib on SQ20B, SCC61 and SCC25. Clonogenic analysis demonstrated that the combination of 25 μM celecoxib and 0.5 μM erlotinib markedly decreased clonogenic survival compared to either drug alone in all three cell lines, particularly in the SQ20B and SCC25 cell lines ($p < 0.05$). Further, combined celecoxib and erlotinib significantly decreased clonogenic survival in combination with IR (0 to 6 Gy), particularly in the less radiosensitive SQ20B and SCC25 cell lines compared to erlotinib and IR alone (Figure 5, $p < 0.05$).

Particularly in the SQ20B cell line, combined erlotinib and celecoxib had a strongly synergistic effect ($p < 0.001$) in combination with radiation. In the SCC25 and SCC61 cell lines, the effects of combined therapy were additive rather than supraadditive. Similar trends were observed with higher doses of celecoxib (50 μM), erlotinib (3 μM) and radiation (0 to 6 Gy).

Preliminary clinical responses following erlotinib, celecoxib and reirradiation from a human phase I trial

We have completed patient accrual with 14 patients treated with combined erlotinib, celecoxib and reirradiation. At a median follow-up of 15 months in surviving patients, 4 patients remain alive and free of recurrence, 4 patients had a local failure, 2 patients had a distant failure, 1 patient had a local and distant failure and 3 patients were free of cancer but succumbed to non-cancer related deaths. A durable complete response of one patient with unresectable T4bN1 hypopharynx cancer who failed prior chemoradiation and surgery is shown (Figure 6a-b). This patient had regions that were strongly positive for EGFR and was also focally positive for COX-2 (Figure 6c). Although there was no specific correlation between baseline EGFR levels and response, all 3 patients with squamous cell carcinoma that remain alive and free of cancer had evidence of positive COX-2 immunostaining.

Discussion

EGFR expression is detectable in 80%-90% of HNSCC and is further upregulated after exposure to radiation, which contributes to radioresistance [1,27]. Recent clinical studies have validated extensive preclinical research suggesting that inhibiting EGFR will result in clinically significant radiosensitization without significantly increasing radiation-induced

toxicity [7,9]. In the recently reported randomized trial comparing radiotherapy ± cetuximab for stage III-IVB HNSCC, the 3-year locoregional control and overall survival with concurrent cetuximab and radiotherapy was 47% and 45% respectively [9]. However, cetuximab failed to reduce the risk of distant metastasis [9]. Along with emerging preclinical data, these results suggest that resistance to EGFR inhibition remains a major impediment to cure for many patients with locally advanced HNSCC [10].

This is the first report demonstrating that combined treatment with EGFR and COX-2 inhibitors significantly increase radiosensitivity. Targeting both EGFR and COX-2 is a conceptually attractive and clinically feasible therapeutic approach for HNSCC [25]. Within 20 minutes of erlotinib administration, reduction in p-EGFR, p-Akt, p-ERK was observed. These changes in protein levels were maintained at 72 hours. Combined erlotinib and celecoxib resulted in greater inhibition of p-EGFR and p-AKT at 72 hours, particularly in the SCC25 cell line with high baseline COX-2 expression. In particular, activation of AKT has been strongly associated with resistance to therapy [2]. Of particular interest, exposure to 2 Gy of ionizing radiation was shown to induce multiple prosurvival pathways at 72 hours after treatment including p-EGFR, p-ERK, COX-2, PGE-2. Importantly, these were all effectively inhibited by erlotinib and celecoxib. Both celecoxib and erlotinib are approved small molecule inhibitors that are in routine clinical use. Prior studies demonstrated supraadditive growth inhibition following treatment with EGFR and COX-2 inhibitors in HNSCC models [17,24]. Multiple mechanisms appear to be involved at the cellular and whole organism levels [18,21-23]. PGE-2 produced by COX-2 has been shown to transactivate EGFR, MAPK and Akt by activating the cyclic AMP/protein kinase A pathway and promoting the expression of the EGFR ligand amphiregulin [21-23]. Further PGE-2 may promote tumor progression by inducing myeloid derived suppressor cells [28].

The marked increase in PGE-2 secretion following radiation is a pro-survival response resulting in transactivation of EGFR, upregulation of AKT and promoting resistance to apoptosis [22,29,30]. Our study confirms the effect of celecoxib in reducing PGE-2, particularly in cells treated receiving concurrent radiation, which translates into more effective inhibition of EGFR-resistance pathways including ERK, Akt and Stat3. As a result of these significant alterations in cell signaling, erlotinib and celecoxib treated cells exhibited decreased proliferation, G1 arrest and increased apoptosis. The combination of celecoxib, erlotinib and radiation resulted in at least additive inhibition of HNSCC cell growth. Moreover, these *in vitro* observations were confirmed in animal models. Further, it should be noted that doses of celecoxib tested *in vitro* are much higher than doses given to patients. As a result, it is probable that some of the growth inhibitory effects observed at these doses can be attributed to off target effects. However, celecoxib is highly effective at inhibiting PGE-2 even at low μ M doses readily achievable in patients.

Other potential biological effects of combined inhibition of EGFR and COX-2 have been well described *in vivo* by other investigators [18,24]. Celecoxib mediated reduction in VEGF expression and angiogenesis inhibition is another potential mechanism of overcoming resistance to anti-EGFR monotherapy [29,31]. Further, there is robust data that COX-2 inhibitors inhibit myeloid derived suppressor cells implicated in tumor-mediated immune suppression and tumor vasculogenesis [32]. Recent preclinical data strongly supports a role for EGFR and COX-2 in preventing metastasis. An 18-gene signature for a mouse model of breast cancer with predilection for lung metastasis included the EGFR-receptor ligand ephregulin, COX-2 and matrix metalloproteinases 1 and 2 [33]. Provocative preclinical data demonstrates that combined celecoxib and cetuximab was effective at preventing lung colonization by highly virulent breast cancer cells while monotherapy was less effective [19]. Recent data from our laboratory suggests an important role for both

increased COX-2 gene expression and EGFR activation via elevated urokinase plasminogen activator receptor in the T-HEp3 head and neck cancer model [34].

Based on these promising *in vitro* and *in vivo* data suggesting potential benefits of a combined EGFR and COX-2 inhibition strategy, our group designed and recently completed accrual of a phase I trial of concurrent erlotinib, celecoxib and reirradiation in recurrent head and neck cancer (NCT00970502). A prior phase I trial investigating concurrent gefitinib and celecoxib in HNSCC demonstrated an extremely promising 22% response rate, suggesting a supraadditive effect [25]. This study confirmed that concurrent COX-2 and EGFR inhibition is feasible and well tolerated and is a highly attractive strategy to achieve radiosensitization in patients with HNSCC. Importantly, significant decreases in urinary PGE-2 have been detected at the FDA approved celecoxib dose of 400 mg twice a day [35]. Importantly, elevated urinary prostaglandin has recently been associated with poor prognosis in smoking-associated HNSCC [36]. Preliminary results confirm evidence of significant clinical activity in a subset of patients with poor prognosis patients with recurrent head and neck cancer that failed prior radiation. The demonstration of complete clinical responses in 3 patients with documented COX-2 expression certainly supports the notion that celecoxib may enhance sensitivity to combined EGFR inhibition and radiation in a subset of treatment refractory patients. Consistent with the recent analysis of the Bonner trial, we paradoxically observed greater benefit for combined EGFR inhibition and radiotherapy in patients with weaker EGFR staining [37]. Further exploration of biomarkers that can prospectively predict which patients would benefit from novel combinations of radiation, EGFR inhibitors and other biological targeted agents is clearly warranted

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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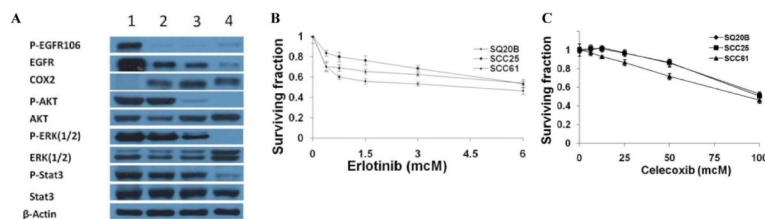


Figure 1.

Baseline protein levels and sensitivity to erlotinib and celecoxib.

- Immunoblotting of proteins relevant to EGFR and COX-2 signaling pathways on untreated SQ20B, SCC61 and SCC25 head and neck squamous cell lines. These levels are compared to WI-38 fibroblasts. Proteins analyzed by immunoblotting are phosphorylated EGFR (p-EGFR)/EGFR, COX-2, phosphorylated AKT (p-AKT)/ AKT, phosphorylated ERK (p-ERK)/ERK, phosphorylated STAT3 (p-STAT3)/STAT3 and actin for control.
- Cell viability assay assessed by the MTT assay for three HNSCC cell lines, SQ20B, SCC61 and SCC25, treated with erlotinib (0-6 μM) for 24 hours. Each experiment was performed in triplicate. Points represent the mean and error bars represent the standard error.
- Results for the MTT cell viability assay for SQ20B, SCC61 and SCC25 cells treated with celecoxib (0-100 μM) for 24 hours. Each experiment was performed in triplicate. Points represent the mean and error bars represent the standard error.

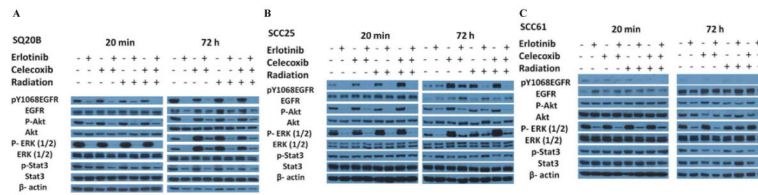


Figure 2.

Effect of single and combination treatment on EGFR-related protein levels in SQ20B, SCC25 and SCC61 cells. The effects of each single treatment (erlotinib, celecoxib and ionizing radiation) and the combinations on p-EGFR/EGFR, p-AKT/AKT, p-ERK/ERK, p-STAT3/STAT3 and actin were assessed by immunoblotting. We assessed protein levels at 20 minutes and 72 hours after treatment to observe a time course of protein activation. Differences in protein levels were assessed by densitometry and are shown in Supplemental figures.

- a. SQ20B
- b. SCC25
- c. SCC61

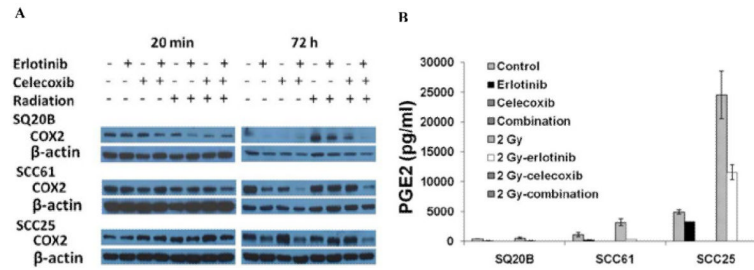


Figure 3.

Effect of combination treatment on COX-2 protein levels and PGE-2 levels.

- The effects of each single treatment (erlotinib, celecoxib and ionizing radiation) and the combinations on COX-2 and actin were assessed by immunoblotting at 20 minutes and 72 hours after treatment in SQ20B, SCC61 and SCC25. Differences in protein levels were assessed by densitometry and are shown in Supplemental figures.
- The effects of each single treatment (erlotinib, celecoxib and ionizing radiation) and the combinations on PGE-2 concentration assessed by enzyme-linked immunosorbent assay in SQ20B, SCC61 and SCC25.

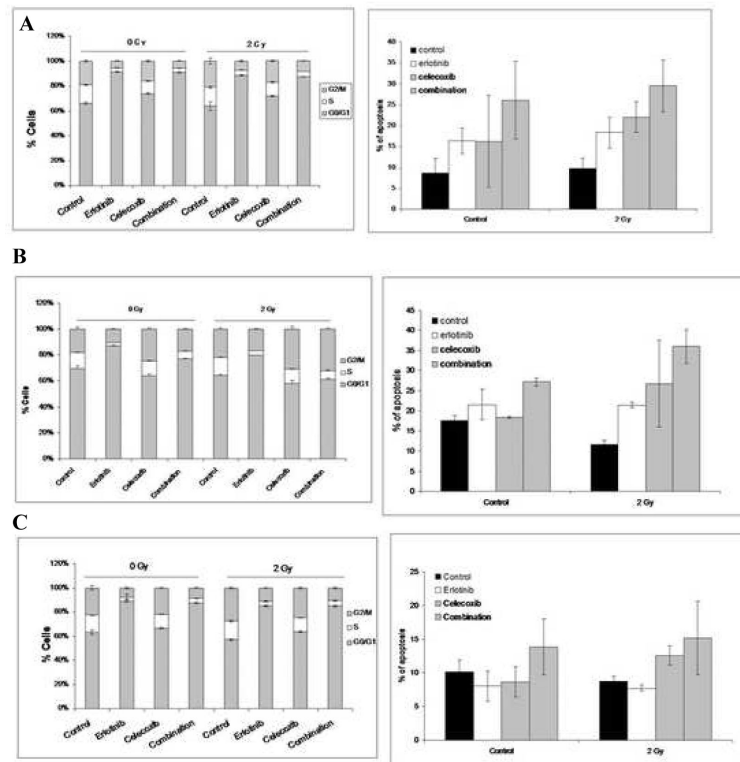


Figure 4.

Effects of combination treatment on cell cycle arrest and apoptosis (*Left panels*). Effects of single treatment (erlotinib, celecoxib and ionizing radiation) and combinations therapy on cell cycle head and neck cancer cells assessed by flow cytometry using propidium iodide staining. (*Right panels*) Effects of single treatment (erlotinib, celecoxib and ionizing radiation) and the combinations on apoptosis in head and neck cancer cells assessed by flow cytometry. After 72 hours of treatment, adherent and floating cells were collected and incubated with Annexin V-phycoerythrin. Each experiment was performed in triplicate. Each experiment was performed in triplicate. Points represent the mean and error bars represent the standard deviation.

- a. SQ-20B
- b. SCC-25
- c. SCC-61

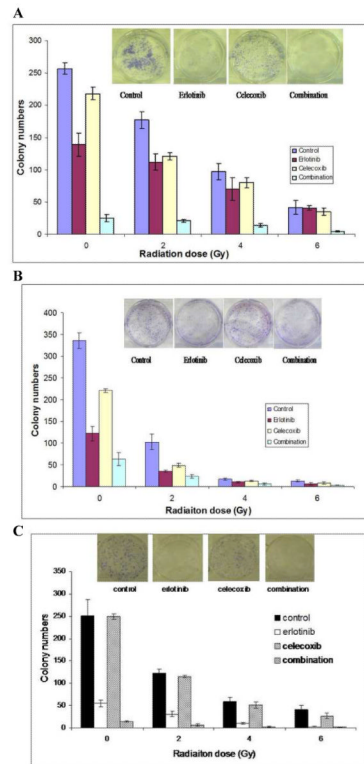


Figure 5.
Effects of erlotinib, celecoxib and ionizing radiation on clonogenic survival in head and neck cancer cells
a. SQ-20B
b. SCC-61
c. SCC-25

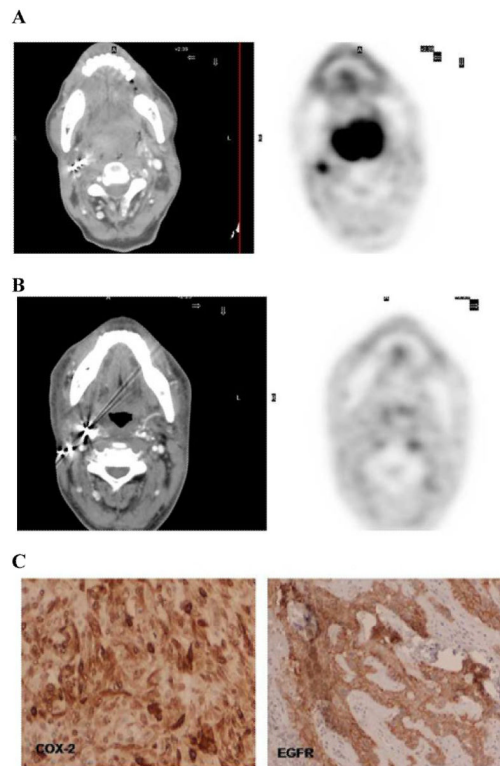


Figure 6.

Complete clinical and radiographic response following concurrent erlotinib, celecoxib and reirradiation in a patient with recurrent head and neck cancer who failed prior chemoradiation, total laryngectomy and neck dissection.

a. Pretreatment CT and PET demonstrated a massive tumor in the oropharyngeal wall, reconstructed pharynx and right cervical lymph node.

b. Following combined treatment, the patient had a complete response on CT and PET. The patient remains alive and free of recurrent disease 10 months after treatment.

c. Immunohistochemical staining of pretreatment biopsy samples demonstrates focal strong COX-2 positivity and strong EGFR positivity.