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# 폐암세포에서 표피성장인자 수용체-티로신 키나아제 억제제와 선택적인 사이클로옥시게나제-2 억제제의 병합치료 효과

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서울대학교 대학원

의학과 내과학 전공

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# The effects of combined treatment with epidermal growth factor receptor-tyrosine kinase inhibitor and selective cyclooxygenase-2 inhibitor on lung cancer cells

by

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# Abstract

## The effects of combined treatment with epidermal growth factor receptor-tyrosine kinase inhibitor and selective cyclooxygenase-2 inhibitor on lung cancer cells

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**Introduction:** To overcome the acquired resistance to epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs), various strategies have been explored in preclinical and clinical setting. Cyclooxygenase (COX)-2 inhibitors have been reported to suppress cell growth and to lead to apoptosis of various cancer cells by EGFR down-regulation. In the present study, we assessed whether the combination of celecoxib, a COX-2 inhibitor, and EGFR-TKIs could overcome the acquired resistance in lung cancer cells.

**Materials and Methods:** The *EGFR*-mutated lung cancer cell lines (HCC827 and PC-9) and drug-resistant cell lines (HCC827/GR, HCC827/ER, PC-9/GR and PC-9/ER) were used. Celecoxib and COX-2 siRNA were used as COX-2 inhibitor. Reversible EGFR-TKIs, gefitinib

and erlotinib, and EGFR siRNA were used as EGFR inhibitor. Western blotting was employed to investigate the expression of proteins involved with EGFR signaling.

**Results:** Addition of celecoxib treatment enhances sensitivity to EGFR-TKIs in parental HCC827 and PC-9 cells harboring with *EGFR* activating mutation. Combined celecoxib and gefitinib treatment overcame gefitinib resistance via the inhibition of the phosphorylation of MET, EGFR and Akt in HCC827/GR cells. In HCC827/ER cells, combination treatment with erlotinib and celecoxib inhibited the expression of AXL, p-Akt and Erk. We evaluated the ability of combination treatment with gefitinib or erlotinib, and celecoxib to inhibit the proliferation of PC-9 cells with an *EGFR* T790M mutation. These combinations showed an additive growth inhibition in PC-9/GR cells and a synergistic growth inhibition in PC-9/ER cells through the suppression of EGFR and Akt activities.

**Conclusions:** The combination of EGFR-TKIs and celecoxib may be a new strategy to overcome the acquired resistance to EGFR-TKIs in lung cancer.

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**Keywords:** lung cancer, epidermal growth factor receptor mutation, epidermal growth factor receptor-tyrosine kinase inhibitor, selective cyclooxygenase-2 inhibitor

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# List of abbreviations and symbols

Overall survival, OS; Non-small cell lung cancer, NSCLC; Epidermal growth factor receptor, EGFR; Tyrosine kinase inhibitors, TKIs; Progression-free survival, PFS; Fibroblast growth factor receptor, FGFR; Cyclooxygenase, COX; Prostaglandin, PG; Prostaglandin E2, PGE2; Fetal bovine serum, FBS; Gefitinib-resistant HCC827, HCC827/GR; Erlotinib-resistant HCC827, HCC827/ER; Gefitinib-resistant PC-9, PC-9/GR; Erlotinib-resistant PC-9, PC-9/ER; Combination index, CI; Small interfering RNAs, siRNAs; Median inhibitory concentration, IC50; Phosphoinositide 3-kinase, PI3K; Tyro-Axl-Mer, TAM; Receptor tyrosine kinase, RTK

# Introduction

Lung cancer is the leading cause of cancer-related mortality, with an estimated 1.4 million deaths in 2008 globally [1]. Traditional chemotherapy provides the limited gains as it prolongs overall survival (OS) by only a few months in advanced non-small cell lung cancer (NSCLC) patients compared with supportive care [2]. In order to overcome this problem, recent research efforts have focused on the development of targeted agents for the treatment of advanced NSCLC. Given the importance of epidermal growth factor receptor (EGFR) in the development and progression of NSCLC [3, 4], EGFR-targeted agents have been developed, including the small molecules, selective and reversible EGFR-tyrosine kinase inhibitors (TKIs), gefitinib (IRESSA, AstraZeneca) and erlotinib (TARCEVA, Roche). EGFR-TKIs have been widely used for patients with NSCLC, especially whose with *EGFR*-mutant NSCLC. Activating *EGFR* mutations, such as deletions in exon 19 and point mutations in exon 21, are considered the most reliable predictive factors of outcome after the treatment of NSCLC with EGFR-TKIs [5, 6].

However, despite the treatment of EGFR-TKI leads to a significant clinical benefit in patients with *EGFR*-mutant NSCLC, most of them will inevitably develop the acquired resistance after a progression-free survival (PFS) of about 10 months [7, 8]. Several mechanisms of the acquired resistance to EGFR-TKIs have been identified as follows: (i) the secondary mutation in the EGFR kinase domain (T790M, 50-60%);

(ii) activation of alternative pathways such as MET, HER2, fibroblast growth factor receptor (FGFR), and AXL (1-25%); and (iii) histologic transformation to mesenchymal cell or small cell features (5-10%) [9, 10]. Various strategies to overcome the acquired resistance to EGFR-TKIs have been explored in preclinical and clinical settings, including "next generation" EGFR-TKIs and rational combinations of targeted agents [9]. Among them, the third-generation EGFR-TKIs have now been developed and have yielded promising results for patients harboring T790M [9, 10]. However, to date, there are no effective therapies for patients with acquired resistance that does not involve *EGFR* T790M. One reasonable strategy for overcoming the acquired resistance in patients without T790M is an EGFR-TKIs based combination therapy.

Cyclooxygenase (COX) is a rate-limiting enzyme that converts arachidonic acid to prostaglandin (PG) [11]. There are two isoforms of COX: COX-1, a constitutive enzyme expressed in many normal tissue types, and COX-2, an inducible enzyme that is overexpressed in inflammatory and many neoplastic tissues [12-14]. COX-2 is involved in various aspects of cancer formation and progression, primarily through PG synthesis [15-18]. COX-2 is frequently expressed in tissue samples from NSCLC, and some *in vitro* studies have suggested that selective COX-2 inhibitors inhibit cancer cell growth and induce apoptosis in NSCLC [19-21].

Preclinical evidence suggests that there is a direct interaction between

EGFR signaling and COX-2 activity. In colon cancer models, activation of EGFR signaling leads to increased transcription of COX-2 and enhanced synthesis of PG, whereas prostaglandin E2 (PGE2), one of the major products of COX-2, can activate EGFR signaling [22-24]. In vitro studies have shown that simultaneously targeting both EGFR and COX-2 produces a synergistic effect in colon, head and neck, and breast cancer [25-28]. In addition, recent studies have found that the efficacy of combined COX-2 inhibitor and EGFR-TKIs is significantly greater in NSCLC cells with *EGFR* mutations [29, 30]. However, there are insufficient data on whether the acquired resistance to EGFR-TKI in NSCLC could be overcome by dual blockade of COX-2 and EGFR.

This study aimed to assess the combined effect of COX-2 inhibitor and EGFR-TKIs in NSCLC cells with acquired resistance to EGFR-TKIs.

# Materials and Methods

## Cell lines and reagents

The *EGFR*-mutated NSCLC cell lines HCC827 and PC-9 were used. Both HCC827 cells and PC-9 cells are known to contain a deletion in exon 19 (delE746-A750) of *EGFR* and be highly sensitive to gefitinib and erlotinib. HCC827 cells were purchased from the American Type Culture Collection (Rockville, MD), and PC-9 cells were a gift from F. Koizumi and K. Nishio (National Cancer Center Hospital, Tokyo, Japan). Cells were cultured in RPMI 1640 containing 10% fetal bovine serum (FBS) and 1% penicillin- streptomycin at 37°C in an atmosphere of 5% $CO_2$. All the cell culture materials were obtained from WelGENE (Daegu, Korea). Gefitinib, erlotinib, and selective COX-2 inhibitor, celecoxib were purchased from Selleck Chemicals (California, USA). Drugs were dissolved in DMSO at 50 mM and stored at −20°C. The final DMSO concentration in all experiments was <0.1% in medium.

## Establishment of gefitinib- and erlotinib-resistant cell lines

Gefitinib- and erlotinib-resistant cells were developed by chronic, repeated drug exposure. Briefly, both HCC827 cells and PC-9 cells were exposed to 10 nmol/L of gefitinib or erlotinib for 48 hours in medium containing 10% FBS. They were then washed and cultured in drug-free medium until surviving cells were 80% confluent. These cells were then re-exposed to increasing concentrations of gefitinib or erlotinib. Cells that were able to grow in 1 μmol/L gefitinib or erlotinib were obtained over an 8 month period after initial exposure. The established resistant cell lines were maintained in medium containing 1 μmol/L of gefitinib or erlotinib. Gefitinib- and erlotinib-resistant HCC827 cells are referred to as HCC827/GR and HCC827/ER cells, respectively. Also, gefitinib- and erlotinib-resistant PC-9 cells are referred to as PC-9/GR and PC-9/ER cells, respectively. For all *in vitro* studies, resistant cells were maintained in drug-free medium for at least 1 week before experiments, to eliminate the effects of the drugs.

## Cell viability assay

To perform the MTT assay, cells ($0.5 \times 10^4$/well) were plated in 96-well sterile plastic plates and allowed to attach overnight. Cells were exposed to varying doses of gefitinib, erlotinib, and celecoxib in medium containing 1% FBS. After 72 hours, 15 μL of MTT solution (0.5 mg/mL) was added to each well and plates were incubated for 4

hours. After the culture medium was aspirated off and 200 μL DMSO was added to each well. Absorbance at 595nm was read spectrophotometrically using a microplate reader. The combination effect was evaluated by MTT assay. Combination index (CI) values were processed using the CalcuSyn software version 2.1 (Biosoft, Cambridge, UK). CI values <1, =1, and >1 indicated synergism, additive effect, and antagonism, respectively.

## Western blot

Cell lysates were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% skim milk–PBS–0.1% Tween 20 for 1 hour at room temperature before being incubated overnight with primary antibodies diluted 1:1,000 in 5% skim milk–PBS–0.1% Tween 20. The membranes were then washed three times in PBS–0.1% Tween 20 and incubated with horseradish peroxidase-conjugated secondary antibodies diluted 1:1,000 in 5% skim milk for 1 hour. After successive washes, the membranes were developed using an ECL kit and analyzed by the ImageQuant™ LAS 4000 image analysis system (GE Healthcare, Uppsala, Sweden). Antibodies specific for p-EGFR (Tyr1173), MET and AXL were obtained from Santa Cruz Biotechnology; those for EGFR, COX-2, p-MET (Tyr1234/1235), Akt, p-Akt (Ser473), Erk, p-Erk (Thr202/Tyr204), and GAPDH were purchased from Cell Signaling

Technology; and those for p-AXL were purchased from R&D system.

## RNA interference

Silencer-validated small interfering RNAs (siRNAs) for EGFR (sc-29301), COX-2 (sc-29279), and control (sc-37007) were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). HCC827 cells and PC-9 cells were seeded into 60 mm dishes and allowed to attach overnight. Transfection of siRNAs was carried out using the Oligofectamine™ reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's specifications. After transfection, the suppression of targeted proteins was determined by Western blotting. The viability of cells was determined by MTT assay.

HCC827 cells and PC-9 cells were transfected with siRNA specific for EGFR and COX-2 following manufacturer’s instructions. One day prior to transfection, HCC827 cells and PC-9 cells were cultivated in RPMI 1640 with 10 % FBS. Cells were then incubated with a complex formed by siRNA (10 nM), transfection reagent (Lipofectamine 2000, Gibco) and transfection medium (Opti-MEM 1, Gibco) for 72 hours at 37 °C. Scrambled siRNA sequence was used as a control. Transfection efficiency was confirmed by western blot.

## Statistical analysis

All cell viability experiments were repeated at least three times. Cell viability data are presented as mean ± standard deviation as a percentage of control. The Mann–Whitney U-test was used for comparisons, and a p value of $<0.05$ was considered statistically significant.

# Results

## Addition of celecoxib treatment enhances sensitivity to EGFR-TKIs in cells with *EGFR* activating mutations

The proliferation of NSCLC cell lines HCC827 and PC-9 was inhibited by gefitinib, erlotinib and celecoxib, and the effect was dose-dependent (Figure 1 and 2). The median inhibitory concentration (IC50) of gefitinib, erlotinib and celecoxib in the HCC827 and PC-9 cells were 13.4 nmol/L, 36.8 nmol/L and 38.3 μmol/L, respectively, and 3.7 nmol/L, 26.0 nmol/L and 15.1 μmol/L, respectively. The combined effect was evaluated on the basis of the CI. The combination of celecoxib and gefitinib or erlotinib manifested a synergistic effect on the viability of HCC827 and PC-9 cells in most combination concentrations (Figure 3 and 4).

We performed immunoblotting to determine changes in EGFR down-signaling protein expression in HCC827 and PC-9 cells after treatment with gefitinib, erlotinib, celecoxib, or an each combination (Figure 5). In HCC827 cells, a significant decrease in the expression levels of COX-2, p-EGFR, and p-Akt proteins was observed after treatment with any of the single EGFR-TKI agents (10 nmol/L) or combinations with celecoxib (30 μmol/L). Single drug treatment with celecoxib did not affect the expression levels of COX-2 and p-EGFR.

No significant changes in the expression levels of COX-2, p-EGFR, and p-Akt proteins were observed after treatment with any of the single-agent concentrations (EGFR-TKI 1 nmol/L and celecoxib 20 μ mol/L) tested in PC-9 cell line. However, combined gefitinib or erlotinib with celecoxib treatment significantly inhibited the phosphorylation of EGFR and Akt, as well as the expression of COX-2.

Interestingly, there was significant down-regulation of EGFR and COX-2 in both cell lines following combined treatment with EGFR-TKI and celecoxib compared to that following treatment with either drug alone. To identify the cross-activity of EGFR-TKI and celecoxib, we transfected both HCC827 and PC-9 cells with EGFR or COX-2 siRNAs (Figure 6). COX-2 expression was markedly suppressed when both HCC827 and PC-9 cells were transfected with EGFR siRNA. In addition, immunoblotting revealed that COX-2 siRNA effectively suppressed the *EGFR* gene.

These results indicate that the combination of EGFR-TKI and celecoxib has a synergistic effect on the viability of *EGFR*-mutated NSCLC cells, and that this effect is mediated through the inhibition of the EGFR downstream pathway.

## Combined treatment with celecoxib and gefitinib overcomes the drug resistance caused by *MET* gene

## amplification

A gefitinib-resistant subline that was derived from the parental drug-sensitive HCC827 cell line was established by continuous exposure of cells to gefitinib more than a period of 8 months. The resistant subline was designated HCC827/GR and exhibited a 1,000-fold higher resistance to gefitinib than the parental cells (gefitinib IC50, <0.01 μ mol/L in HCC827 cells and >10 μmol/L in HCC827/GR cells; Figure 7). Also, HCC827/GR cells showed a 2-fold higher resistance to celecoxib than the parental cells (celecoxib IC50, <40 μmol/L in HCC827 cells and >80 μmol/L in HCC827/GR cells; Figure 7). Consistent with a prior study, we observed increased the phosphorylation of MET and gene amplification in HCC827/GR cells (data not shown).

When HCC827/GR cells were treated with gefitinib and celecoxib, celecoxib restored sensitivity to gefitinib (Figure 8). A similar synergistic effect was observed across the full range of doses tested.

In immunoblotting, high dose gefitinib (1 μmol/L) inhibited the phosphorylation of MET, EGFR and Akt in HCC827/GR cells, but no significant change of protein expression was observed when low-dose gefitinib (0.01 μmol/L) was used. Combined celecoxib (30 μmol/L) and low dose gefitinib (0.01 μmol/L) treatment overcame gefitinib resistance via the inhibition of the phosphorylation of MET, EGFR and Akt, which was maintained in the presence of gefitinib alone (Figure 9).

## Combined treatment with celecoxib and erlotinib overcomes the AXL-mediated drug resistance

A previous study reported that increased activation of AXL led to EGFR-TKI resistance in HCC827/ER cells (31). Erlotinib resistant subline exhibited IC50 values for erlotinib that were more than 1,000-fold larger than those of the parental HCC827 cells (erlotinib IC50, <0.01 μmol/L in HCC827 cells and >10 μmol/L in HCC827/ER cells; Figure 10). Also, HCC827/ER cells showed markedly higher resistance to celecoxib than the parental cells (celecoxib IC50, <20 μmol/L in HCC827 cells and >100 μmol/L in HCC827/ER cells; Figure 10). The combination of erlotinib and celecoxib effectively inhibited the growth of HCC827/ER cells, where as neither of the agents alone led to growth inhibition (Figure 11). These combinations showed a synergistic growth inhibition.

In HCC827/ER cells, high dose erlotinib (1 μmol/L) inhibited the phosphorylation of Akt and Erk in HCC827/ER cells and the protein expression of AXL, but no significant change of protein expression was observed when low-dose erlotinib (0.01 μmol/L) was used. Combination treatment with low dose erlotinib (0.01 μmol/L) and celecoxib (30 μmol/L) inhibited the expression of AXL, p-Akt and Erk (Figure 12).

## Celecoxib treatment enhances sensitivity to EGFR-TKIs in T790M-mediated resistant cells

As previously reported, PC-9/GR and PC-9/ER cells were generated by stepwise selection using increasing doses of gefitinib or erlotinib over a period of 8 months. These resistant cells acquired the T790M mutation (32). These resistant sublines presented IC50 values for EGFR-TKIs that were more than 1,000-fold larger than those of the parental PC-9 cells (Figure 13 and 14). We evaluated the ability of combination treatment with gefitinib or erlotinib, and celecoxib to inhibit the proliferation of NSCLC cells with an *EGFR* T790M mutation. The addition of celecoxib enhanced the ability of EGFR-TKIs to induce growth inhibition (Figure 15 and 16). These combinations showed an additive growth inhibition in PC-9/GR cells and a synergistic growth inhibition in PC-9/ER cells.

To investigate the mechanism by which celecoxib enhanced the antitumor activities of the EGFR-TKIs, the activities of EGFR and its downstream molecules were examined. The inhibitory effect of single treatment with celecoxib (20 μmol/L), gefitinib (0.001 μmol/L), or erlotinib (0.001 μmol/L) on EGFR and Akt activities was modest, whereas the combination of celecoxib and gefitinib or erlotinib substantially suppressed EGFR and Akt activities (Figure 17 and 18).

# Discussion

In present study, we showed that the addition of celecoxib could overcome the acquired resistance to EGFR-TKIs. Several resistance mechanisms to EGFR-TKIs were observed in gefitinib- or erlotinib-resistant cell lines, such as secondary mutation (T790M) and bypass signaling (MET or AXL). Our results demonstrated that combination treatment with celecoxib and EGFR-TKI has a synergistic effect in NSCLC cells with resistance to EGFR-TKIs via the bypass signals of MET or AXL, as well as secondary T790M mutation.

Previous studies reported that celecoxib could inhibit EGFR activity in lung cancer cells [29, 30, 33]. Chen et al. indicated that celecoxib combined with gefitinib led to stronger inhibition of EGFR signal transduction in NSCLC [33]. Recent two studies showed that the efficacy of the addition of celecoxib to EGFR-TKI is significantly greater in NSCLC cells with *EGFR* mutations than in NSCLC cells with wild-type *EGFR* [29, 30]. Consistent with previous studies, combined treatment of celecoxib with EGFR-TKI showed additive or synergistic growth inhibitions in NSCLC cells harboring *EGFR* mutations, but not in A549 cells with wild-type *EGFR* in our study (data not shown). Different combined effect among lung cancer cells with various *EGFR* mutational status might be related to the basal status of EGFR activity and cellular dependency to EGFR signaling. Mutations in the *EGFR* gene activate the EGFR signaling pathway and promote the cellular dependency on EGFR-derived proliferation and

survival. Therefore, the addition of celecoxib to EGFR-TKIs leading to more complete inhibition of EGFR signals could cause significant anti-proliferative and pro-apoptotic effects to cells with mutant *EGFR*.

MET is a transmembrane tyrosine kinase receptor which are involved in the regulation of cell proliferation, migration, invasion, and angiogenesis [34]. Amplification of *MET* has been reported in approximately 5%–22% of NSCLC with acquired resistance to EGFR-TKIs. MET mediates the resistance to EGFR inhibitors through downstream activation of the phosphoinositide 3-kinase (PI3K)/AKT pathway by heterodimerization with ERBB3. Engelman et al. described that gefitinib-resistant HCC827 cells maintain the phosphorylation of ERBB3 and AKT in the presence of gefitinib [35]. However, gefitinib plus PHA665752, a selective small molecule inhibitor of c-Met, or MET-specific short hairpin RNA suppressed the phosphorylation of ERBB3 and AKT, and restored sensitivity to gefitinib. Also, another study using animal models of EGFR TKI-resistant NSCLC showed that dual inhibition of MET and EGFR significantly attenuates Akt phosphorylation and results in tumor regression, whereas treatment of either agent alone did not [36].

We produced a gefitinib-resistant HCC827 cell line (HCC827/GR), which is similar to those established by Engelman and colleagues [35]. In our study, HCC827/GR cell line expressed a high level of MET activity through the amplification of *MET*, and its growth was significantly inhibited by the combined treatment with gefitinib and

celecoxib. These findings suggest that celecoxib has potential effectiveness to overcome the resistance mediated by the MET bypass signal. Several earlier studies also suggested that selective COX-2 inhibitor inhibits c-MET induction in benign or tumorous conditions [37-40]. Tuynman et al. reported that selective COX-2 inhibition significantly decreases both COX-2 and MET expression levels and results in anti-proliferative and pro-apoptotic effects *in vitro* and in patients with esophageal adenocarcinoma [38]. Celecoxib also effectively suppressed the *in vivo* tumor growth in an orthotopic liver cancer model through the inhibition of c-MET expression [40].

AXL belongs to the TAM (Tyro-Axl-Mer) family of receptor tyrosine kinase (RTK) [41]. AXL receptor homodimerization or heterodimerization with other RTKs, such as EGFR, leads to increased cell motility and survival via the activation of downstream effectors. Recently, several study groups reported that activation of AXL could mediate drug resistance to EGFR-targeted therapy in lung cancer [31, 42-43]. In these studies, combined inhibition of AXL and EGFR restored therapeutic efficacy. Previously, we established an erlotinib-resistant HCC827 cell line (HCC827/ER), which expressed a high level of AXL activity [31, 44]. Our current results presented that celecoxib treatment inhibits both AXL and EGFR in HCC827/ER cells. Although AXL-mediated signaling is correlated with acquired resistance to erlotinib, both EGFR- and AXL-signals may involve in cell survival and proliferation. Therefore, the addition of celecoxib to erlotinib

synergistically suppressed cell proliferation in HCC827/ER. To our knowledge, our study is the first to demonstrate that celecoxib can reduce the activity of AXL in AXL-mediated resistant lung cancer cell line.

Threonine 790 is a gatekeeper for the ATP-binding pocket in EGFR. The T790M mutation results in drug resistance by reduced affinity for EGFR-TKIs or enhanced ATP binding affinity. In clinical setting, the *EGFR* T790M mutation was present in approximately 50 to 60 % of resistant cases [47]. In previous study, we found that the acquisition of the *EGFR* T790M mutation leads to resistance to both gefitinib and erlotinib in PC-9 cells [45]. Regales et al. suggested that dual targeting of EGFR by the combination of cetuximab and BIBW-2992 could overcome T790M-mediated drug resistance through efficient depletion of both phosphorylated and total EGFR [46]. Our study also evaluated the efficacy of dual targeting by the combination of celecoxib and gefitinib or erlotinib in PC-9 cells harboring T790M mutation. Interestingly, this combination strategy showed a synergistic or an additive efficacy in drug-resistant PC-9 cell lines. This phenomenon may be explained from preclinical data that tyrosine kinase activity is maintained although the EGFR T790M can substantially suppress the efficacy of ATP-competitive reversible inhibitors, gefitinib and erlotinib [48]. Our results suggest that dual EGFR targeting may be resonable.

In summary, celecoxib enhances the efficacy of EGFR-TKIs to overcome drug resistance by suppression of bypass signals as well as

EGFR signals. This combination leads to more complete inhibition of EGFR and Akt signals that may not be achievable by treatment of EGFR-TKI alone in cancer cells with drug resistance. Present study supports that the combination treatment with celecoxib and EGFR-TKI may be one of the strategies for treating patients with acquired resistance to EGFR-TKIs via various mechanisms.

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overcome a major drug resistance mutation in mouse models of EGFR mutant lung cancer. J Clin Invest 2009;119:3000-10.

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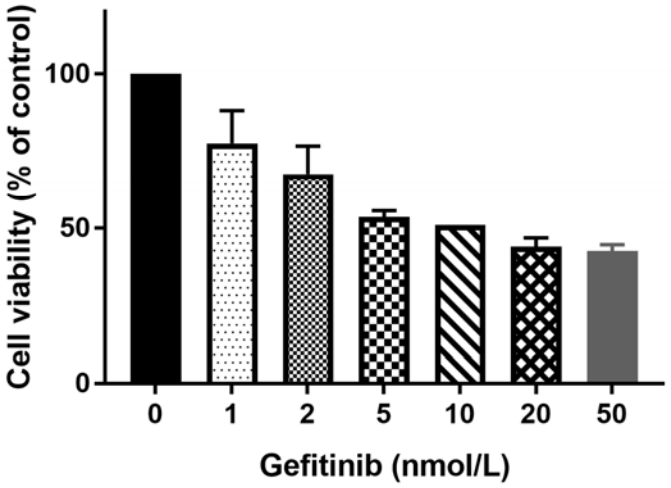
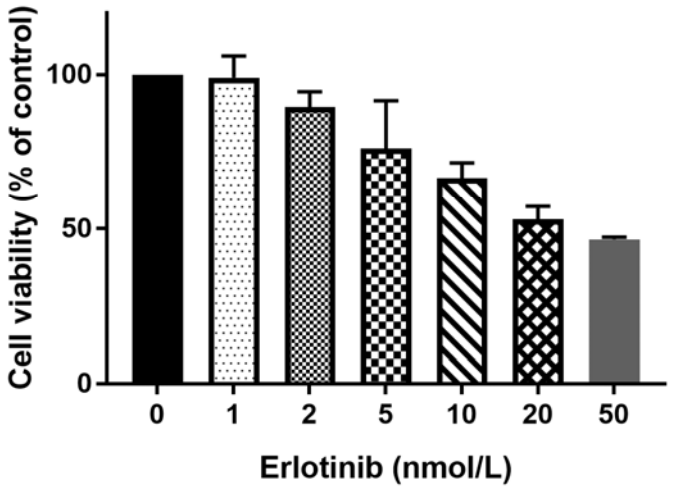
A


Cell viability (% of control)
100
50
0
0
1
2
5
10
20
50
Gefitinib (nmol/L)

B


Cell viability (% of control)
100
50
0
0
1
2
5
10
20
50
Erlotinib (nmol/L)

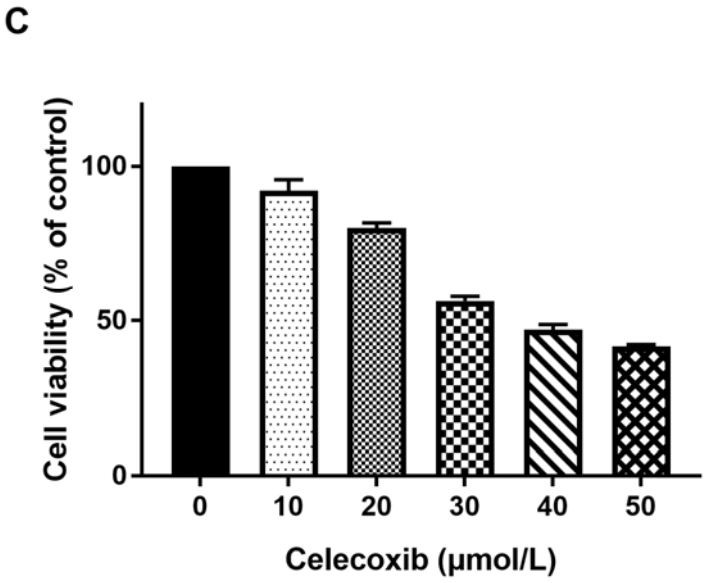
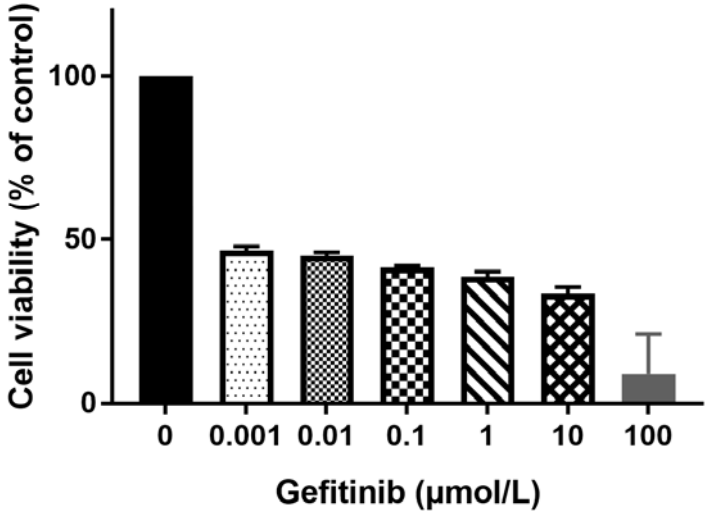
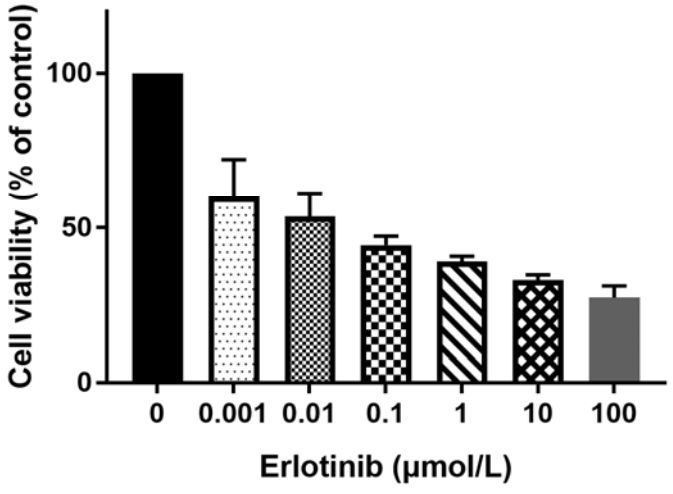



Figure 1. Effects of EGFR-TKI and celecoxib in parental HCC827 cells. Cells were treated with the indicated doses of gefitinib (A), erlotinib (B), or celecoxib (C) for 72 hours in medium containing 1% FBS. Cell viability was determined using the MTT assay.

A


Cell viability (% of control)
100
50
0
0
0.001
0.01
0.1
1
10
100
Gefitinib (μmol/L)

B


Cell viability (% of control)
100
50
0
0
0.001
0.01
0.1
1
10
100
Erlotinib (μmol/L)

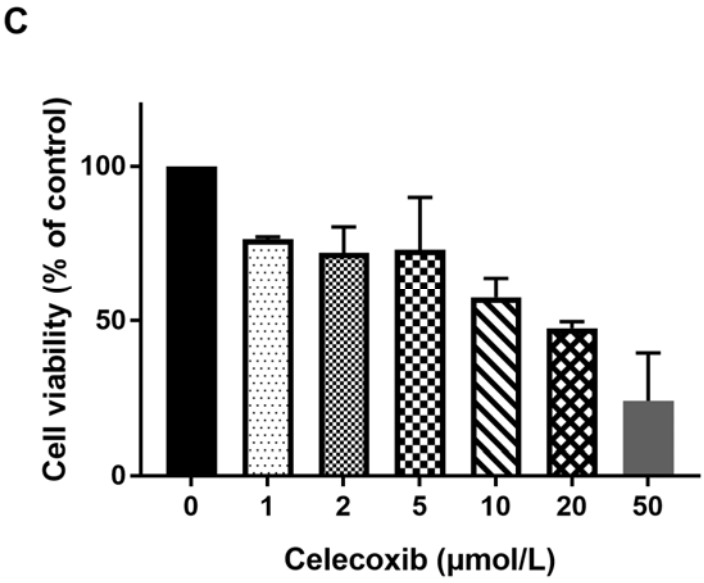



Figure 2. Effects of EGFR-TKI and celecoxib in parental PC-9 cells. Cells were treated with the indicated doses of gefitinib (A), erlotinib (B), or celecoxib (C) for 72 hours in medium containing 1% FBS. Cell viability was determined using the MTT assay.

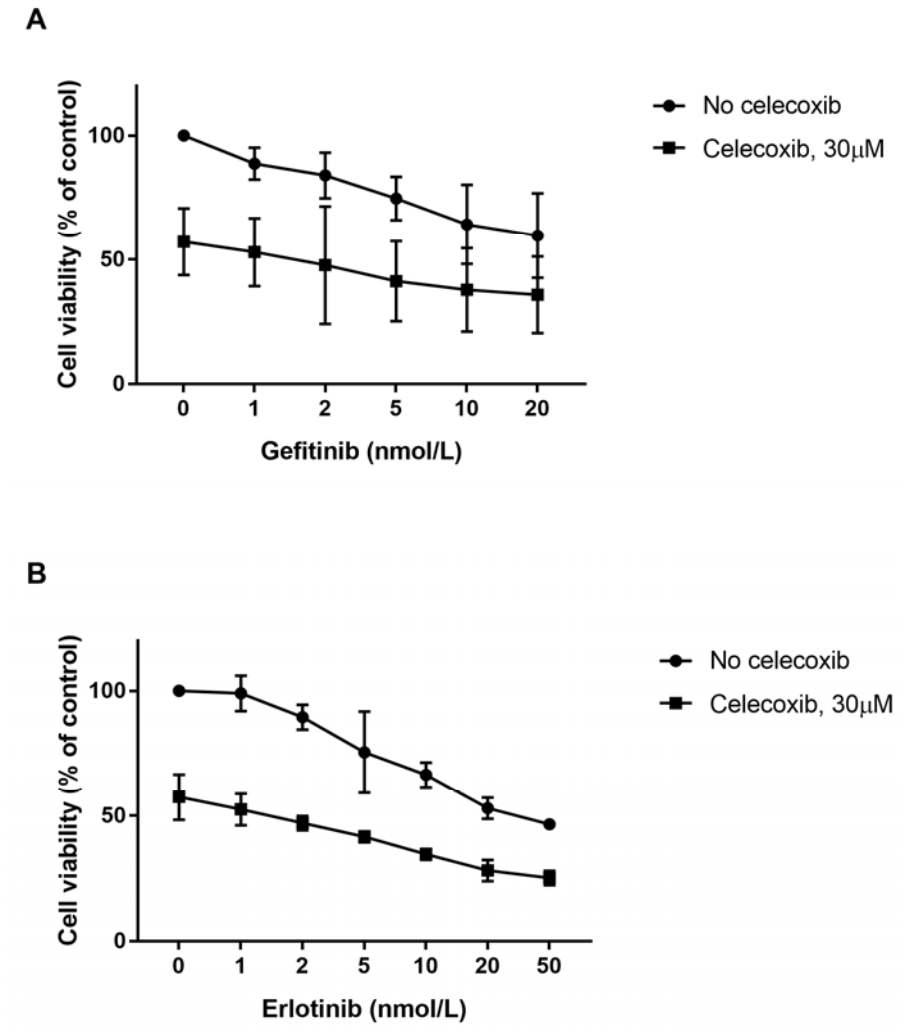



Figure 3. Combination effects of EGFR-TKI and celecoxib in parental HCC827 cells. Cells were treated with the indicated doses of gefitinib (A) or erlotinib (B), and the fixed dose of celecoxib (30 μmol/L) for 72 hours in medium containing 1% FBS. Cell viability was determined using the MTT assay.

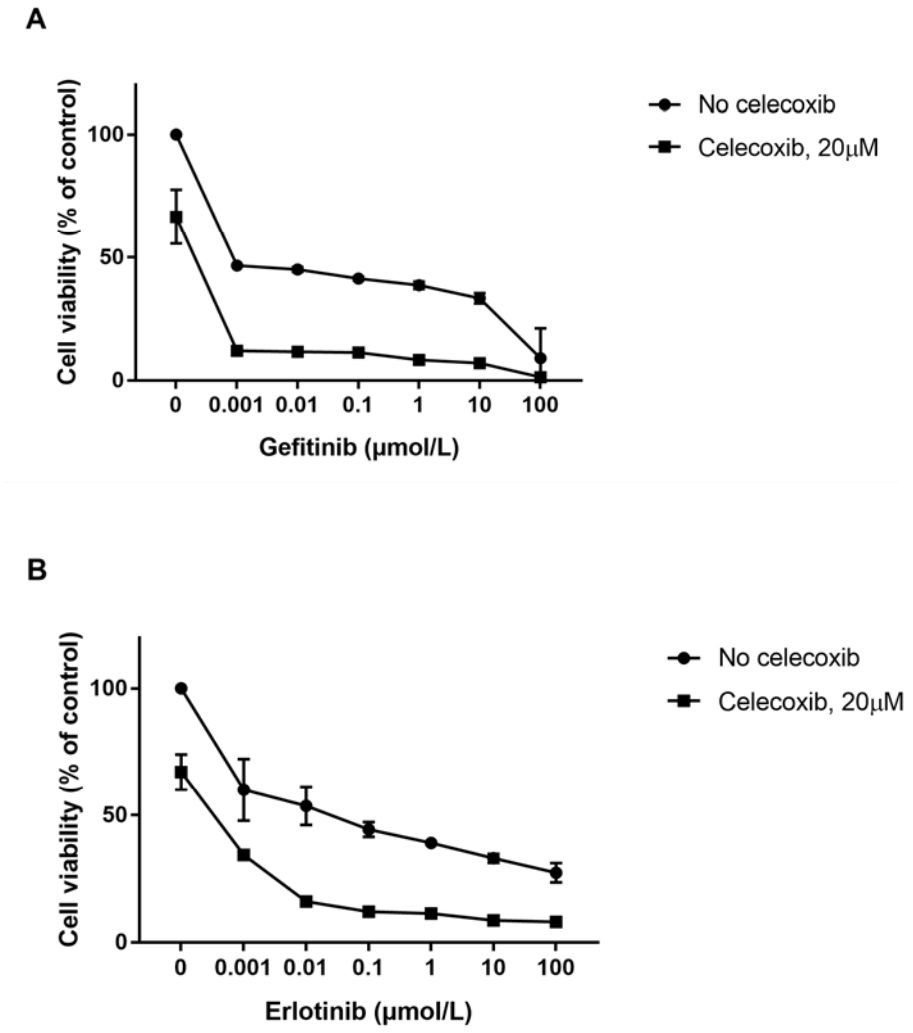



Figure 4. Combination effects of EGFR-TKI and celecoxib in parental PC-9 cells. Cells were treated with the indicated doses of gefitinib (A) or erlotinib (B), and the fixed dose of celecoxib (20 μmol/L) for 72 hours in medium containing 1% FBS. Cell viability was determined using the MTT assay.

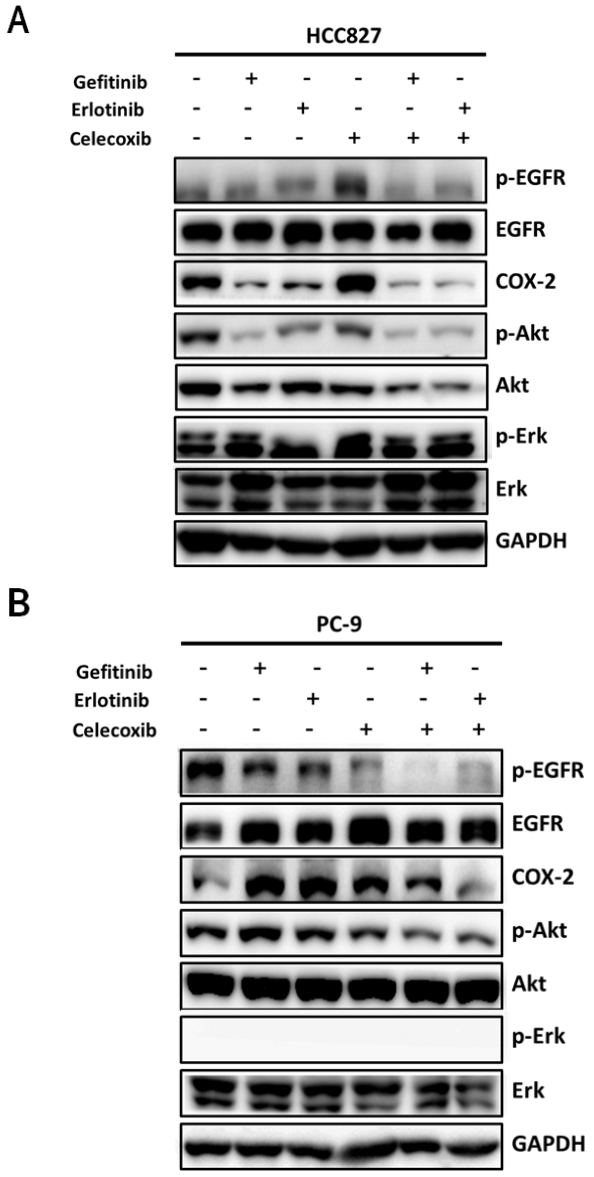



Figure 5. Protein expression of EGFR and downstream molecules in parental HCC827 (A) and PC-9 (B) cells.

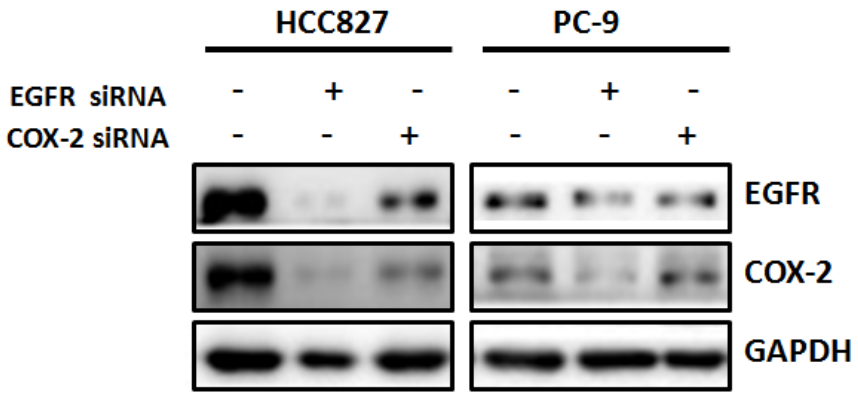
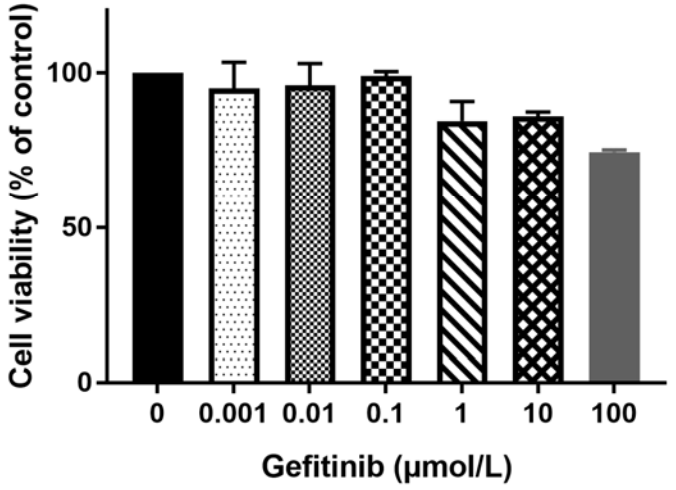



Figure 6. Transfection of EGFR and COX-2 specific-siRNAs in parental HCC827 and PC9 cells. Cells were transfected with the indicated siRNA and gene knockdown was confirmed by Western blotting.

A


Cell viability (% of control)
100
50
0
0
0.001
0.01
0.1
1
10
100
Gefitinib (μmol/L)

B

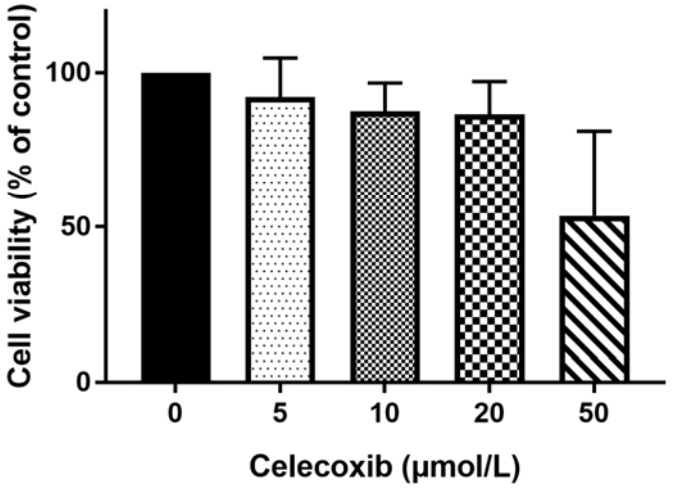

Cell viability (% of control)
100
50
0
0
5
10
20
50
Celecoxib (μmol/L)

Figure 7. Effects of gefitinib and celecoxib in HCC827/GR cells. Cells were treated with the indicated doses of gefitinib (A), or celecoxib (B) for 72 hours in medium containing 1% FBS. Cell viability was determined using the MTT assay.

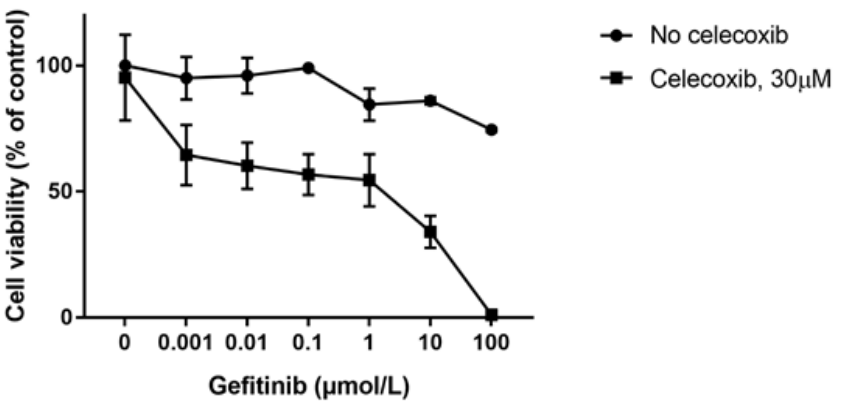



Figure 8. Combination effects of gefitinib and celecoxib in HCC827/GR cells. Cells were treated with the indicated doses of gefitinib, and the fixed dose of celecoxib (30 μmol/L) for 72 hours in medium containing 1% FBS. Cell viability was determined using the MTT assay.

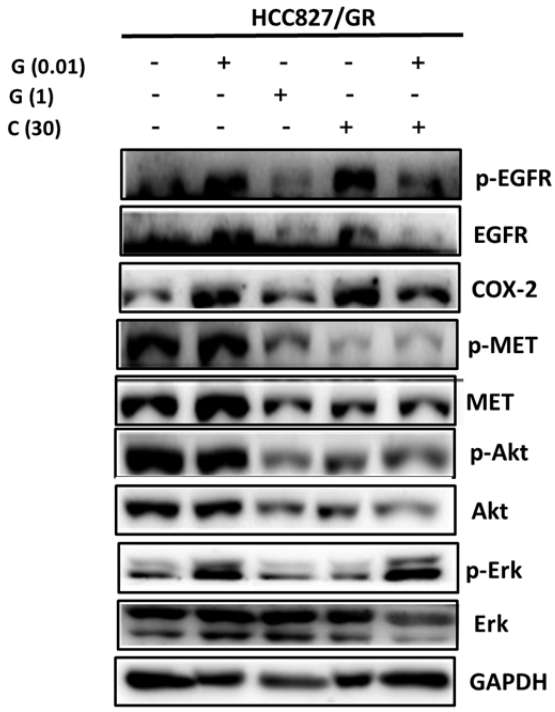
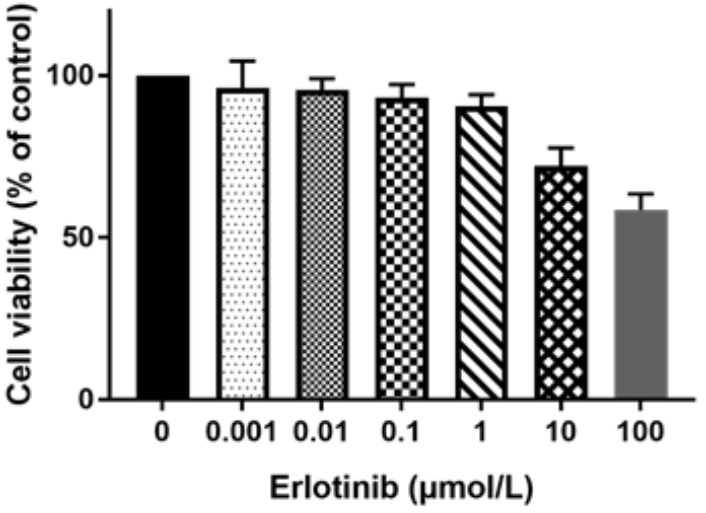



Figure 9. Protein expression of EGFR and downstream molecules in HCC827/GR cells.

A


Cell viability (% of control)
100
50
0
0
0.001
0.01
0.1
1
10
100
Erlotinib (μmol/L)

B

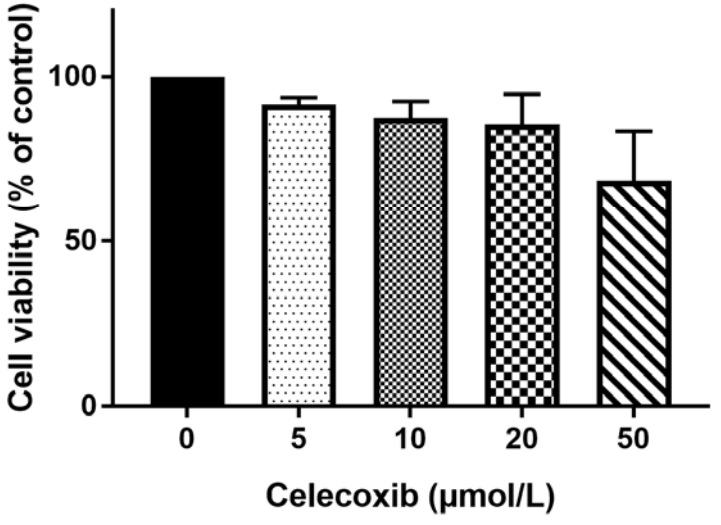

Cell viability (% of control)
100
50
0
0
5
10
20
50
Celecoxib (μmol/L)

Figure 10. Effects of erlotinib and celecoxib in HCC827/ER cells. Cells were treated with the indicated doses of erlotinib (A), or celecoxib (B) for 72 hours in medium containing 1% FBS. Cell viability was determined using the MTT assay.

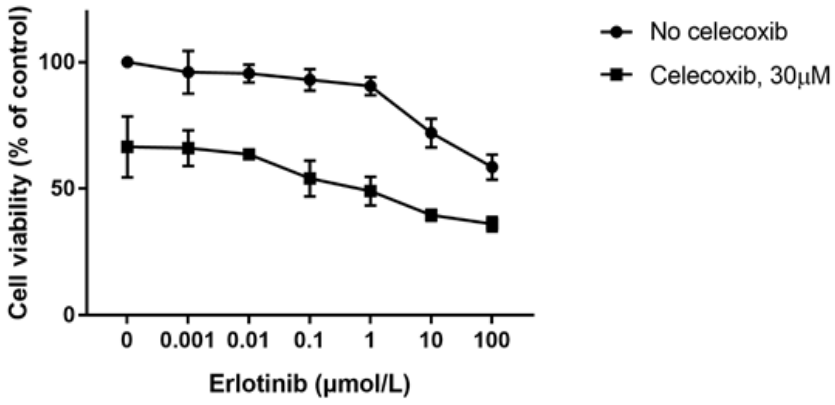



Figure 11. Combination effects of erlotinib and celecoxib in HCC827/ER cells. Cells were treated with the indicated doses of erlotinib, and the fixed dose of celecoxib (30 μmol/L) for 72 hours in medium containing 1% FBS. Cell viability was determined using the MTT assay.

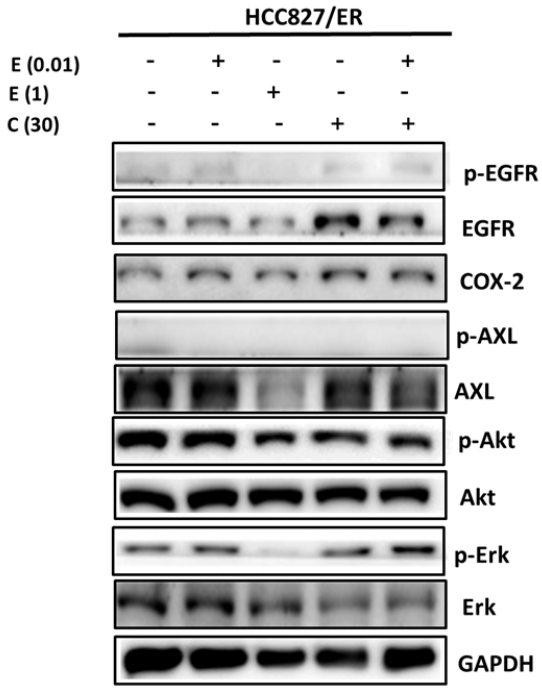
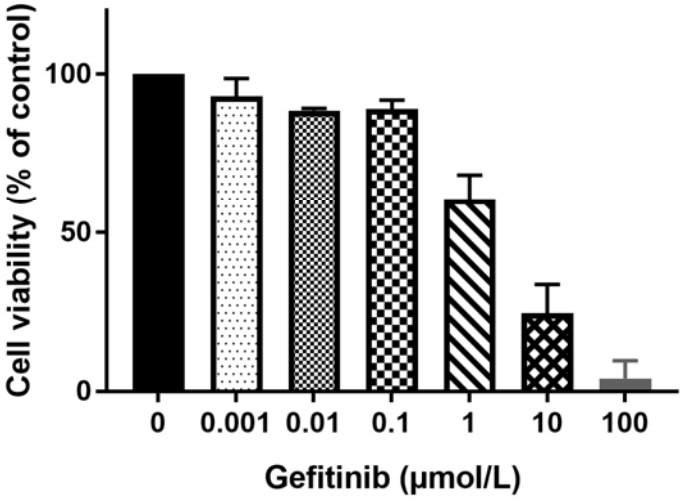



Figure 12. Protein expression of EGFR and downstream molecules in HCC827/ER cells.

A


Cell viability (% of control)
100
50
0
0
0.001
0.01
0.1
1
10
100
Gefitinib (μmol/L)

B

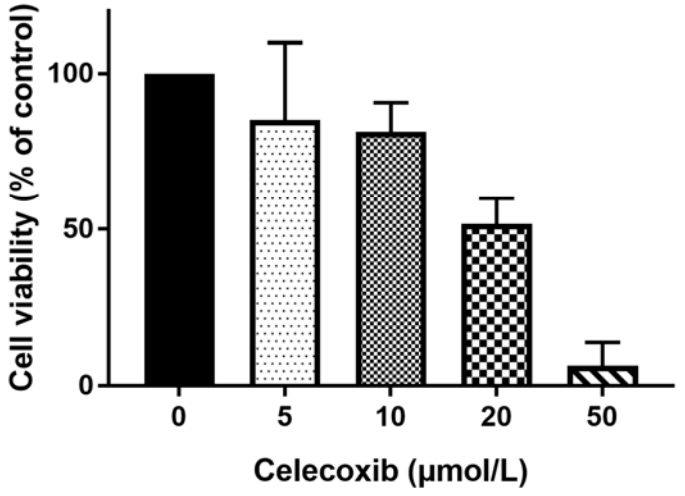
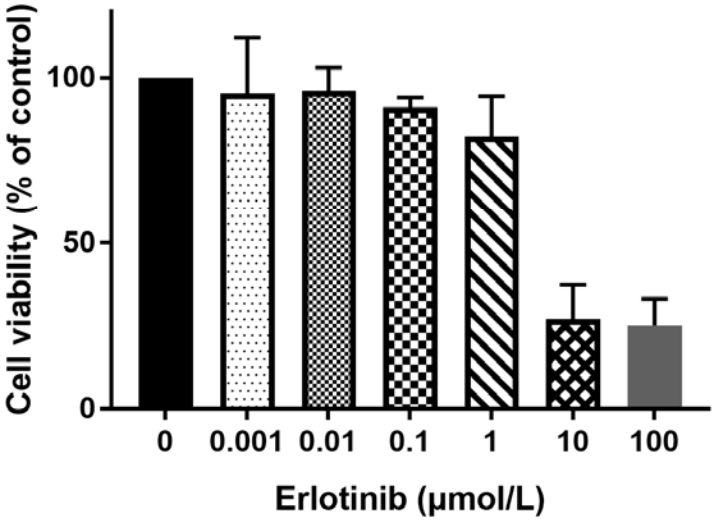

Cell viability (% of control)
100
50
0
0
5
10
20
50
Celecoxib (μmol/L)

Figure 13. Effects of gefitinib and celecoxib in PC-9/GR cells. Cells were treated with the indicated doses of gefitinib (A), or celecoxib (B) for 72 hours in medium containing 1% FBS. Cell viability was determined using the MTT assay.

A


Cell viability (% of control)
100
50
0
0
0.001
0.01
0.1
1
10
100
Erlotinib (μmol/L)

B

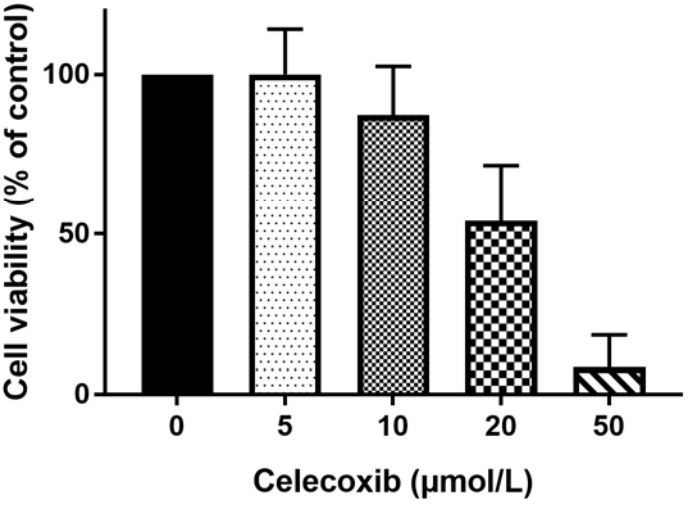

Cell viability (% of control)
100
50
0
0
5
10
20
50
Celecoxib (μmol/L)

Figure 14. Effects of erlotinib and celecoxib in PC-9/ER cells. Cells were treated with the indicated doses of erlotinib (A), or celecoxib (B) for 72 hours in medium containing 1% FBS. Cell viability was determined using the MTT assay.

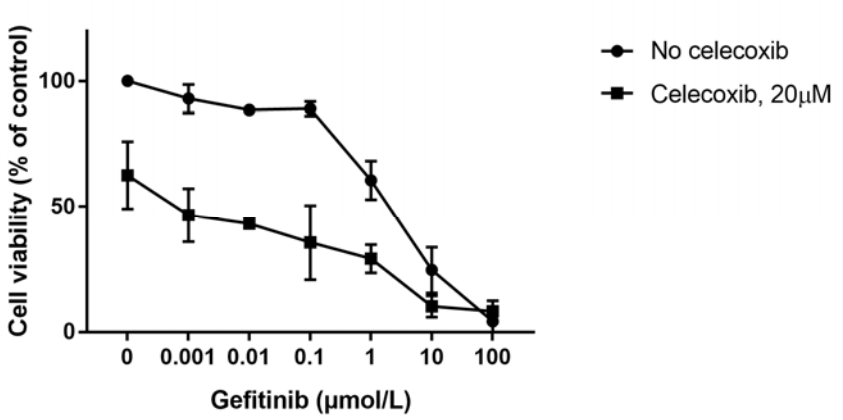



Figure 15. Combination effects of gefitinib and celecoxib in PC-9/GR cells. Cells were treated with the indicated doses of gefitinib, and the fixed dose of celecoxib (20 μmol/L) for 72 hours in medium containing 1% FBS. Cell viability was determined using the MTT assay.

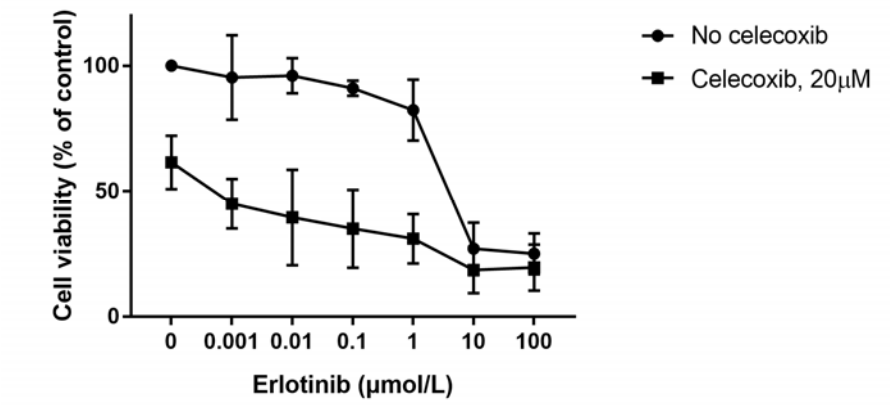



Figure 16. Combination effects of erlotinib and celecoxib in PC-9/ER cells. Cells were treated with the indicated doses of erlotinib, and the fixed dose of celecoxib (20 μmol/L) for 72 hours in medium containing 1% FBS. Cell viability was determined using the MTT assay.

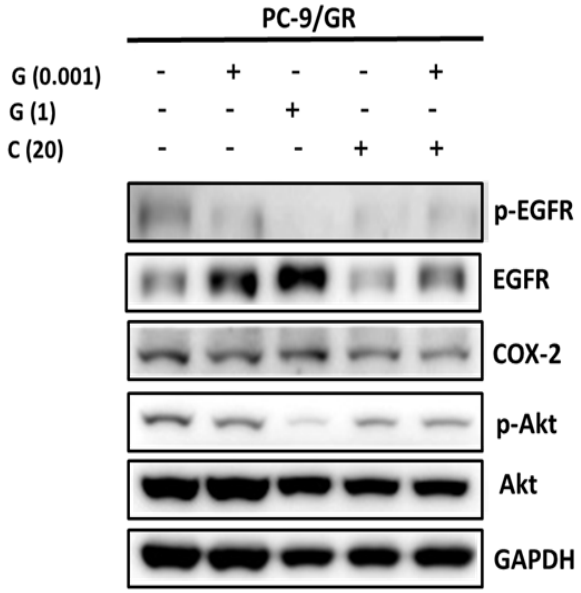



Figure 17. Protein expression of EGFR and downstream molecules in PC-9/GR cells.

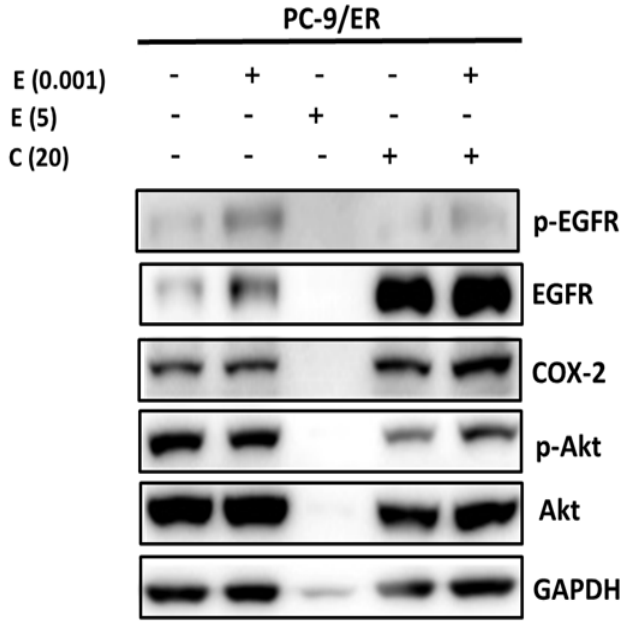



Figure 18. Protein expression of EGFR and downstream molecules in PC-9/ER cells.

# 국문초록

**목적**: 폐암에서 표피성장인자 수용체-티로신 키나아제 억제제에 대한 획득내성을 극복하기 위한 다양한 치료전략들이 전임상과 임상단계에서 연구되고 있다. 선택적인 사이클로옥시게나제-2 억제제는 표피성장인자 수용체를 억제함으로써 다양한 암세포들의 성장을 억제하고 세포사멸을 유도하는 것으로 보고되고 있다. 본 연구에서는 폐암세포를 이용하여 표피성장인자 수용체-티로신 키나아제 억제제와 선택적인 사이클로옥시게나제-2 억제제의 병합치료가 표피성장인자 수용체-티로신 키나아제 억제제에 대한 획득내성을 극복할 수 있는지 알아보고자 하였다.

**실험 방법**: 표피성장인자 수용체-티로신 키나아제 억제제에 감수성이 있다고 알려진 표피성장인자 수용체 엑손 19 결손 돌연변이를 가지고 있는 폐암 세포주인 HCC827 세포와 PC-9 세포, 그리고 표피성장인자 수용체-티로신 키나아제 억제제인 게피티니브와 얼로티니브에 노출시켜 각각의 약제에 내성을 갖게 된 HCC827/GR, HCC827/ER, PC-9/GR, PC-9/ER 세포를 배양 후 표피성장인자 수용체-티로신 키나아제 억제제인 게피티니브 또는 얼로티니브, 그리고 선택적인 사이클로옥시게나제-2 억제제인 세레콕시브를 단독 혹은 병합 처리하고 72시간 뒤 세포생존율을 구하고 병합치료의 효과를 평가하였다. 그리고 표피성장인자 수용체의 신호전달경로에 관여하는 단백질 발현의 변화를 관찰하였다.

**결과**: 표피성장인자 수용체-티로신 키나아제 억제제에 감수성이 있는 HCC827 세포와 PC-9 세포의 경우 표피성장인자 수용체-티로신

키나아제 억제제와 사이클로옥시게나제-2를 병합했을 때 상승효과가 있음을 확인하였다. 표피성장인자 수용체-티로신 키나아제 억제제의 일종인 게피티니브에 획득내성을 가지는 HCC827/GR 세포에서 게피티니브와 세레콕시브의 병합치료는 p-MET, p-EGFR 그리고 p-Akt의 발현을 감소시킴으로써 게피티니브에 대한 내성을 극복할 수 있었다. 얼로티니브에 획득내성을 가지는 HCC827/ER 세포의 경우 얼로티니브와 세레콕시브의 병합치료는 AXL, p-Akt 그리고 Erk의 발현을 감소시킴으로써 상승효과를 나타내었다. 게피티니브와 얼로티니브에 노출시켜 각각의 약제에 내성을 갖게 된 PC-9/GR, PC-9/ER 세포의 경우 획득내성 기전으로 이차 돌연변이 T790M을 갖고 있음을 확인하였다. 게피티니브 또는 얼로티니브, 그리고 세레콕시브의 병합치료는 EGFR과 Akt의 활성을 억제함으로써 PC-9/GR 세포의 경우 부가효과를 나타내었고, PC-9/ER 세포의 경우 상승효과를 나타내었다.

**결론**: 폐암에서 표피성장인자 수용체-티로신 키나아제 억제제와 선택적인 사이클로옥시게나제-2 억제제의 병합치료는 표피성장인자 수용체-티로신 키나아제 억제제에 대한 획득내성을 극복할 수 있는 새로운 치료전략으로서 가능성이 있다.

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**주요어**: 폐암, 표피성장인자 수용체 돌연변이, 표피성장인자 수용체-티로신 키나아제 억제제, 사이클로옥시게나제-2 억제제

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