Combined lapatinib and cetuximab enhance cytotoxicity against gefitinib-resistant lung cancer cells

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Abstract

Although non-small cell lung cancer (NSCLC) cells with somatic mutations in their epidermal growth factor receptors (EGFR) initially show a dramatic response to tyrosine kinase inhibitor (TKI), these cells eventually develop resistance to TKI. This resistance may be caused by a secondary T790M mutation in the EGFR tyrosine kinase, which leads to the substitution of methionine for threonine in 790. In this study, we show that a combination of lapatinib and cetuximab overcomes gefitinib resistance in NSCLC with the T790M mutation. We observed that T790M lung cancer cells were resistant to gefitinib, and Stat3 was persistently activated in the resistant cells. A reversible EGFR and HER2 TKI, lapatinib, decreased Stat3 activation by blocking heterodimerization of EGFR and HER2, which led to a modest increase in the inhibitory effect on gefitinib-resistant T790M cells. In addition to lapatinib, the anti-EGFR antibody, cetuximab, induced down-regulation of EGFR and apoptotic cell death in T790M cells. Finally, combined lapatinib and cetuximab treatment resulted in significantly enhanced cytotoxicity against gefitinib-resistant T790M cells *in vitro* and *in vivo*. Taken together, these data suggest that treatment with a combination of lapatinib and cetuximab, which induces dimeric dissociation and EGFR down-regulation, appears to be an effective strategy for treatment of patients with EGFR TKI-resistant NSCLC. [Mol Cancer Ther 2008;7(3):607 – 15]

Introduction

Epidermal growth factor (EGF) receptor (EGFR) is a major target of molecular anticancer therapy. Two treatment approaches involving the use of monoclonal antibodies and receptor tyrosine kinase inhibitors (TKI) have been developed, and both of these approaches have shown benefits in clinical trials (1). Although anti-EGFR therapies are active in some patients, in nearly all patients, the disease eventually becomes resistant to therapy. Therefore, a better understanding of the mechanisms underlying the resistance to anti-EGFR therapies is critical to improve the efficacy of these treatments. The mechanisms that mediate resistance to anti-EGFR therapies include autocrine and paracrine production of ligands, secondary mutations, the constitutive activation of downstream pathways, and the activation of alternative pathways such as angiogenesis (2–4). Recent studies have shown that a single secondary mutation that results in the substitution of methionine for threonine at position 790 in exon 20 (T790M) confers resistance to gefitinib in lung cancer cells (4, 5). The T790M mutation is observed in ∼50% of patients whose disease progresses after an initial response to gefitinib or erlotinib. It is suggested that the T790M mutation confers resistance by inducing steric hindrance of erlotinib binding as a result of the presence of a bulkier methionine side chain in the ATP kinase binding pocket (4). This T790M mutation is structurally analogous to other TKI-resistant mutations, such as T351I in Bcr-Abl and T670I in c-Kit, which also cause steric hindrance resulting in the inhibition of TKI binding (6–8). Besides T790M, recent studies have shown that amplification of c-MET also confers the resistance to EGFR TKI (9).

Elucidation of the molecular mechanisms leading gefitinib or erlotinib resistance has led to the development of alternative strategies that can circumvent resistance to EGFR TKIs. These strategies have included development of a second generation of EGFR TKIs that include EKB-569 and CL-387,785 (which inhibit EGFR), HKI-272, and BIBW-2992 (which inhibits HER2 as well as EGFR; refs. 4, 10–12). Unlike first-generation EGFR TKIs, most second-generation TKIs are irreversible inhibitors that form a stable covalent bond with cysteine residues present in EGFR or HER2. Based on their irreversible and specific binding activities, previous studies have suggested that irreversible TKIs may be able to overcome the gefitinib or erlotinib resistance caused by T790M (4, 13, 14). Although it is unclear if the inhibition of HER2 in addition to that of EGFR plays a role in overcoming the resistance, inhibitors with dual activity may have therapeutic advantages over compounds that only inhibit EGFR because heterodimerization of EGFR and HER2 has a greater effect on EGF signal activation than EGFR homodimerization.

Lapatinib (GW572016, Tykerb) is a small molecule that is administered orally and functions as a dual reversible inhibitor of EGFR and HER2 tyrosine kinases (15). Lapatinib has been shown to significantly inhibit the proliferation of cancer cells showing EGFR and/or HER2

overexpression both *in vitro* and *in vivo* (16–18). However, the effects of lapatinib on lung cancer are largely unknown. Cetuximab (Erbitux) is a chimeric monoclonal antibody that competes with receptor ligands for binding to EGFR (19, 20). Cetuximab undergoes internalization and, in some cells, induced receptor degradation and down-regulation (21–23). Recent studies have shown that, when combined with gefitinib or erlotinib treatment, cetuximab is also effective in cancer cells expressing mutant EGFR (24). Moreover, treatment with a combination of gefitinib and cetuximab has shown a synergistic effect against EGFR-dependent tumor cells both *in vitro* and *in vivo*, suggesting that combined treatment with these drugs causes a complete blockade of EGF signaling (25, 26).

In this study, we show that active heterodimerization of EGFR and HER2 is blocked by lapatinib and that EGFR is down-regulated by cetuximab in gefitinib-resistant T790M lung cancer cells. These findings indicate that combined treatment with lapatinib and cetuximab results in enhanced cytotoxicity against T790M cells *in vitro* and *in vivo*. Given that receptor dimerization and EGFR expression are the main signals on EGF activation, treatment with a combination of lapatinib and cetuximab may be an effective strategy for EGFR targeted therapy.

Materials and Methods

Tissue Procurement and Mutational Analyses of the EGFR Gene

Tumor specimens obtained before gefitinib treatment and at the time that progression of the disease occurred despite gefitinib treatment were obtained by fine-needle biopsies. The protocols used in this study were approved by the Institutional Review Board of Seoul National University Hospital and informed consent was received from all patients included in this study. Genomic DNA was extracted from the tumor specimens, and EGFR (exons 18-24) was then sequenced using primers and methods as described previously (27). All sequencing reactions were done in both forward and reverse directions, and all mutations were confirmed at least twice from independent PCR isolates as described previously (27).

Cell Culture and Reagents

H1975 human cancer cells and COS7 monkey kidney cells were grown at 37°C under 5% CO_2 in either RPMI 1640 or DMEM containing 10% fetal bovine serum (WELGENE, Inc.). H1975 and COS7 cells were purchased from American Type Culture Collection. Gefitinib was a gift from AstraZeneca, and lapatinib was a gift from GlaxoSmithKline. Cetuximab and trastuzumab were kindly provided by Merck and Roche, respectively. Stock solutions were prepared in DMSO, and then stored at -20°C. Gefitinib and lapatinib were diluted in fresh medium before each experiment, and the final concentration of DMSO was <0.1%. EGF was purchased from Sigma-Aldrich.

EGFR Mutant Constructs and Transfection

The cDNA for human EGFR was kindly provided by Dr. Y. Yarden (Weizmann, Institute of Science). Point mutations of E709K, G719A, T790M, L858R, A859T, or T790M/L858R were induced in the cDNA using a QuickChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's protocol. Each mutant construct was confirmed by sequencing and then transfected into the COS7 cell line using LipofectAMINE 2000 to generate stable cell lines after selection with G418 following the method described by the manufacturer (Invitrogen).

Western Blotting and Immunoprecipitation

Antibodies against phosphorylated EGFR (pY858 and pY1068), phosphorylated signal transduction and activation of transcription 3 (Stat3; pY705), phosphorylated Akt (pS473), phosphorylated mitogen-activated protein kinase (MAPK; Thr^{202}/Tyr^{204}), HER2, Stat3, Akt, and MAPK were purchased from Cell Signaling Technology. Anti-EGFR, poly(ADP-ribose) polymerase, caspase-3, cyclin D, cyclin E, cyclin A, cyclin B, p27, and p21 antibody were obtained from Santa Cruz Biotechnology. Cultured cells that had reached ∼70% to 80% confluence were used for protein analyses. To analyze the EGFR signaling in various mutants, cells were starved for 24 h, and then treatment drugs were added for 3 h followed by stimulation with EGF (100 ng/mL) for 30 min before cell harvest. For the analysis of apoptosis signaling as a result of drug treatments, cells were treated with different concentrations of lapatinib or cetuximab for 24, 48, or 72 h. Cells were then lysed in RIPA or NP40 to induce immunoprecipitation following a previously described method (28) and then analyzed by Western blotting using an enhanced chemiluminescence system.

Growth Inhibition Assay

The viability of cells was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich) assays. Briefly, cells were seeded in complete growth medium in 96-well plates at a density of 3,000 per well (COS7-L858R/T790M and H1975). Following overnight incubation, cells were grown for 24 h in medium supplemented with or without 0.1%, 1%, and 10% serum and/or EGF and then incubated for an additional 72 h in the presence of the treatment drugs.

Annexin V Staining

H1975 cells were treated with 0.5 and 5 μmol/L lapatinib and 1 and 10 μg/mL cetuximab for 72 h, at which point cells were collected and stained with Annexin V-phycoerythrin and 7-aminoactinomycin D (Becton Dickinson). Apoptotic cell death was measured by counting the number of cells that stained positive for Annexin V-phycoerythrin as assessed by fluorescence-activated cell sorting analysis.

H1975 Xenografts

Animal experiments were carried out in the animal facility of the Seoul National University in accordance with institutional guidelines. Six- to 8-week-old female BALB/c athymic (*nu+/nu+*) mice were purchased from Central Lab Animal, Inc. (Seoul, Korea). The initial body weight of the animals at the time of arrival was between 18 and 20 g. Mice were allowed to acclimatize to local conditions for 1 week before being injected with cancer cells. Tumors

were induced by injecting H1975 cells (5×10^6) subcutaneously into the right flank of mice. The tumors were then measured twice a week using calipers, and the tumor volume (mm^3) was calculated according to following formula: [(width^2 × height) / 2]. When tumors had reached a volume of 50 to 100 mm^3, treatment with either gefitinib, lapatinib, cetuximab, a combination of lapatinib and cetuximab, or a vehicle control was initiated. Gefitinib and lapatinib were administered via oral gavage at a concentration of 50 to 150 mg/kg in 0.5% Tween80 (Sigma) in sterile milli-Q water Monday to Friday for 3 weeks. A dose of 10 mg/kg of cetuximab was given i.p. twice a week. Xenograft tumors were excised from euthanized mice and snap frozen in liquid nitrogen, and the lysates were then prepared in RIPA using a Dounce homogenizer. Total protein levels were determined, and detection of the EGFR levels was assessed by Western blot as described previously (29).

Statistical Analysis

An unpaired two-tailed *t* test was used to determine the significance of change in levels of cell viability and apoptosis between different treatment groups. Statistical analysis to compare tumor sizes in xenograft-bearing mice was done with ANOVA. Differences between groups were considered statistically significant if $P < 0.05$.

Results

Secondary T790M Mutation Confers Resistance to Gefitinib-Sensitive L858R Mutant Cells

We identified a patient with a T790M secondary mutation in the EGFR tyrosine kinase domain. This patient with a L858R mutation initially responded to gefitinib; however, after 8 months of treatment, her symptoms worsened and a computed tomographic scan showed that the disease had progressed. Tumor tissues obtained at the time of progression confirmed the presence of adenocarcinoma, and EGFR sequencing revealed an additional C-to-T mutation at nucleotide 2,369 of exon 20, indicating a T790M mutation. To test and compare the sensitivity of T790M cells to gefitinib, various EGFR mutants, including T790M mutants, were derived from non-small cell lung cancer (NSCLC) patients. L858R mutants were included as a gefitinib-sensitive mutation to provide a reference, whereas E709K, G719A, and A859T mutants were derived from patients that did not respond to gefitinib (27). Next, each of these mutants was transfected into COS7 cells, which have low levels of endogenous EGFR (Fig. 1*A*). Because these EGFR tyrosine kinase mutations reside near the ATP cleft, where gefitinib binds, it was expected that these mutant cells would show altered sensitivity to EGFR inhibitors. EGF-induced autophosphorylations of EGFR were measured in cells pretreated with various concentrations of gefitinib. When the sensitive L858R cells were compared with cells containing the resistant E709K, G719A, and A859T mutations, a descending level of sensitivity to gefitinib was observed, with the least amount of sensitivity being observed in cells containing the T790M mutation.

All mutants, with the exception of the T790M mutant, were found to be completely inhibited by gefitinib at a concentration of 100 nmol/L.

To confirm the functional effect of the EGFR T790M mutation, COS7 cells were stably transfected with wild-type, L858R, T790M, or a combination of L858R and T790M DNA. In the absence of gefitinib, cells with either mutant expressed similar levels of total EGFR and phosphorylated EGFR, which suggests that the presence of the T790M mutation does not substantially alter the production, degradation, or activation of the scaffold EGFR molecule. Moreover, following treatment with gefitinib, cells with either the T790M mutation alone or both L858R and T790M mutations showed persistent EGFR activation (Fig. 1*B*). Similarly, T790M-transfected PC9 cells with an in-frame deletion of exon 19 (delE746-A750) showed persistent EGFR activation after gefitinib treatment (data not shown). Consistent with our observations, the T790M mutation has been reported in patients resistant to gefitinib and erlotinib therapy (4, 5). To better understand how T790M induces gefitinib resistance at a molecular level, we examined the effects of gefitinib on Stat3, Akt, and MAPK, the main downstream signaling effectors of EGFR. Following gefitinib treatment, phosphorylated Akt and MAPK levels were not significantly different between gefitinib-sensitive cells containing the L858R mutation and resistant cells containing the T790M mutation or both L858R and T790M mutations. Conversely, phosphorylation of Stat3 was not inhibited by gefitinib in cells containing the T790M mutation or cells containing both L858R and T790M mutations, whereas it was blocked in cells transfected with only the gefitinib-sensitive L858R mutation (Fig. 1*C*). Additionally, transfected PC9 cells containing the delE746-A750 mutation showed persistent Stat3 phosphorylation after gefitinib treatment, similar to what was observed in the T790M-transfected cells (data not shown). Together, these data suggest that the secondary T790M mutation confers resistance to gefitinib-sensitive L858R mutant lung cancer cells via persistent Stat3 activation.

Lapatinib Inhibits the Heterodimerization of EGFR and HER2, Which Leads to a Growth Inhibitory Effect on Gefitinib-Resistant H1975 Cells

Next, because Stat3 is a major downstream signal of EGFR activation, we speculated that the constitutively activated ligand-receptor interaction might contribute to gefitinib resistance. Based on this speculation, we screened several EGFR targeting agents that can affect ligand-receptor interactions. We found that, compared with gefitinib or trastuzumab, lapatinib significantly inhibited the phosphorylation of Stat3 in H1975cells that harbored both L858R and T790M mutations of the EGFR (Fig. 2*A*). We also noticed that cetuximab modestly decreased Stat3 phosphorylation. It was reported previously that Stat3 is a critical mediator of the oncogenic effects of EGFR mutation in NSCLC among the various downstream signals following EGFR activation (30). Our data also support that Stat3 is one of key signals in gefitinib-resistant T790M cells.

Next, we used a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay to determine if lapatinib was able to inhibit the growth of stably transfected COS7-L858R/T790M cells and H1975 cells (Fig. 2B). The IC$_{50}$ of lapatinib was between 1 and 2 µmol/L, and cells treated with lapatinib showed enhanced cytotoxicity compared with those treated with gefitinib. Consistent with the results of the *in vitro* experiment, lapatinib treatment delayed tumor growth in H1975 tumor-bearing mice (Fig. 2C).

We then attempted to determine the mechanism by which lapatinib inhibits the phosphorylation of Stat3 in gefitinib-resistant cells. Gefitinib is known to induce the dimeric dissociation or inactive dimerization of receptors by inhibiting EGFR tyrosine kinase activity (31). In gefitinib-resistant T790M cells, we found that the hetero-dimerization of EGFR and HER2 was maintained in the presence of gefitinib, which resulted in the persistent activation of Stat3 (Fig. 2D). Conversely, lapatinib treatment significantly reduced the heterodimerization of EGFR and HER2, which subsequently diminished the phosphorylation of tyrosine residues. Therefore, it is likely that the activation of Stat3 in gefitinib-resistant T790M cells is mediated by active heterodimerization of EGFR and HER2, which is effectively blocked by lapatinib. Phosphorylation of Akt and MAPK were also significantly inhibited by treatment with 1 µmol/L lapatinib.

Cetuximab in Combination with Lapatinib Enhances Cytotoxicity against T790M NSCLC

We next did cell growth inhibition with gefitinib or lapatinib by culturing cells with or without serum and/or EGF to examine whether inhibition of ligand binding affects cell growth in H1975 cells. The maximum growth inhibition was observed when cells were grown with lapatinib in the absence of serum and EGF, which indicates that T790M H1975 cells may partially retain ligand-dependent growth (Fig. 3A). These data prompted us to test the combination effect of treatment with anti-EGFR antibody to cetuximab because cetuximab competes for binding with the ligand to EGFR. In experiments comparing the cell growth inhibition of lapatinib-treated cells and cells treated with a combination of lapatinib and cetuximab, we observed that the growth inhibition effects on H1975 cells was further inhibited in a dose-dependent
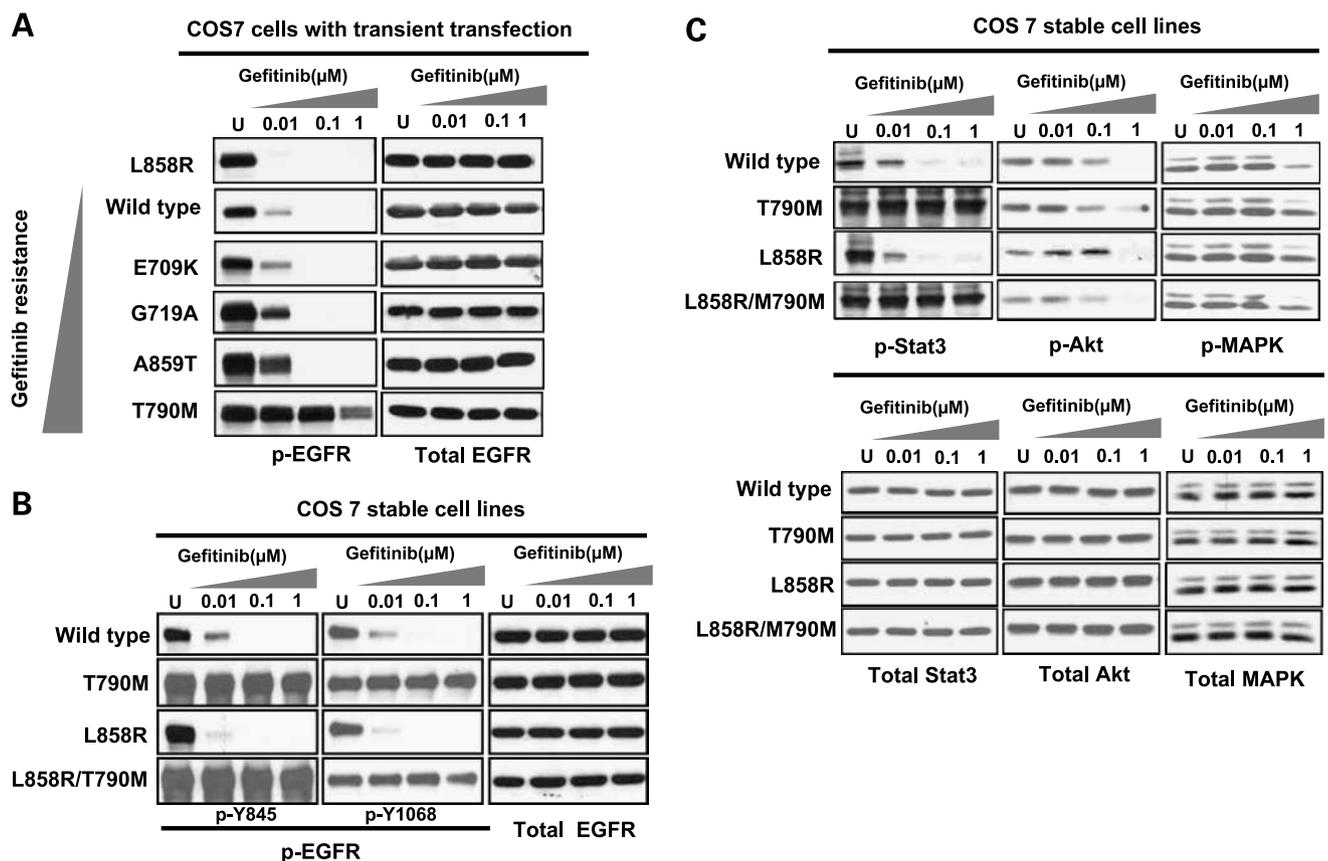

Figure 1. EGFR T790M mutants are resistant to gefitinib. **A,** COS7 cells were transiently transfected with plasmids encoding for wild-type EGFR or for various EGFR mutants. Before harvesting, the cells were serum starved for 24 h and then grown in the presence of varying concentrations of gefitinib for 3 h followed by 30 min of EGF (100 ng/mL) stimulation. Western blots are shown for phosphorylated EGFR (pY1068) and total EGFR. **B,** Western blots for phosphorylated EGFR (pY858 and pY1068) and total EGFR are shown for the COS7 cell lines expressing wild-type, T790M, L858R, or L858R/T790M. **C,** whole-cell extracts were Western blotted with phosphorylated and total Stat3, Akt, and MAPK.
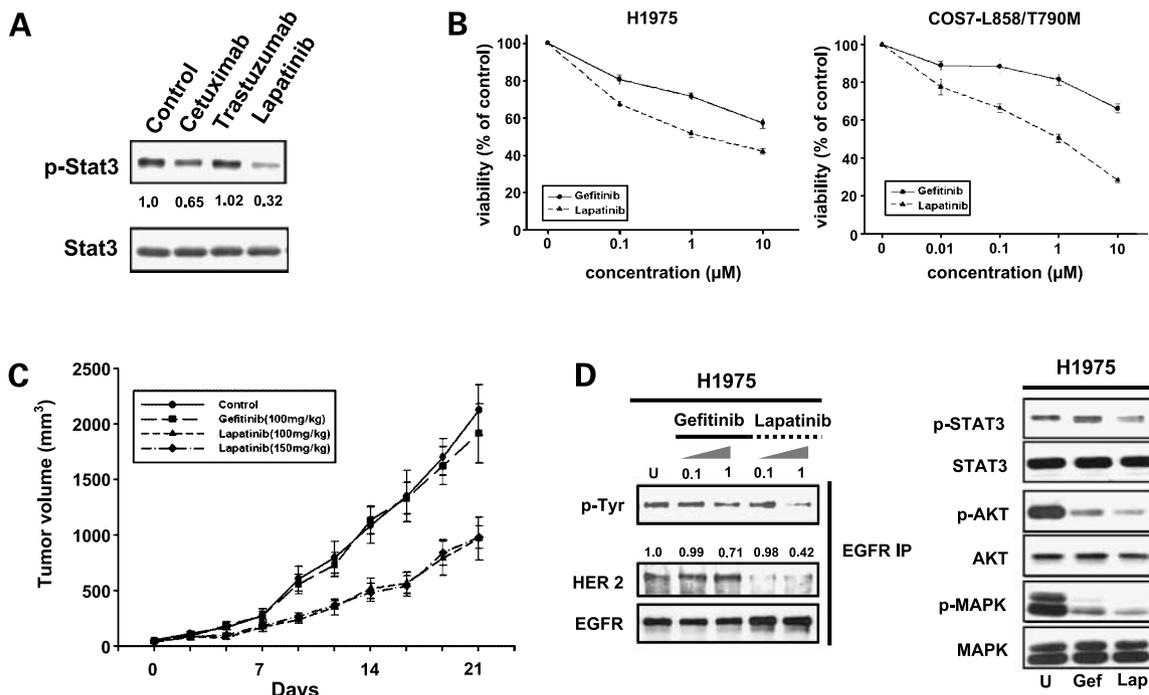
Figure 2. Lapatinib inhibits Stat3 and the heterodimerization of EGFR and HER2 in gefitinib-resistant T790M cells. **A,** COS7-L858R/T790M cells were grown for 24 h in the presence of cetuximab (10 ng/mL), trastuzumab (10 ng/mL), or lapatinib (1 μmol/L). Western blots are shown for both phosphorylated and total Stat3. Values indicate the density of phosphorylated Stat3. **B,** the two EGFR L858R/T790M expressing the indicated cell lines, H1975 and COS7-L858-L858R/T790M, were grown under the indicated conditions with gefitinib or lapatinib. After 72 h of treatment, the cell viabilities were measured via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. The percentage of viable cells is shown relative to that of the untreated controls. Representative of three independent experiments. **C,** H1975 cells were injected s.c. into nude mice with randomization ($n = 8$). Once the tumors reached >50 mm^3, gefitinib (100 mg/kg p.o.) and lapatinib (100 and 150 mg/kg p.o.) were administered daily for 3 wks. Bars, SE. Repeated measures of ANOVA indicated statistically significant effects ($P < 0.005$) in all groups. **D,** H1975 cells were exposed to the indicated concentrations of gefitinib and lapatinib for 24 h. Equal amounts of total protein were then immunoprecipitated with antibodies against phosphorylated Tyr, total HER2, and EGFR. Representative of three independent experiments. Values indicate the density of phosphorylated Tyr (*left*). Whole-cell extracts were Western blotted with phosphorylated and total Stat3, Akt, and MAPK.

manner (Fig. 3*B, left*). In contrast, a combination of gefitinib and cetuximab did not show synergistic effects, suggesting that inhibition of both EGFR and HER2 tyrosine kinase by lapatinib may contribute to a synergism with cetuximab (Supplementary Fig. S1).[3] Annexin V staining showed that lapatinib alone induced apoptotic cell death in a dose-dependent manner, but cetuximab did not. However, treatment with a combination of lapatinib and cetuximab resulted in a greater induction of apoptosis. For example, treatment with 0.5 μmol/L lapatinib and 5 μg/mL cetuximab induced apoptosis in 18% of cells, whereas treatment with lapatinib or cetuximab alone resulted in apoptosis in 6.5% and 4% of cells, respectively (Fig. 3*B, right*).

To further confirm the previous data, we did Western blotting to determine the level of phosphorylation and total EGFR, Stat3, Akt, and MAPK (Fig. 3*C*). In terms of EGFR inhibition, lapatinib tended to inhibit phosphorylated

EGFR, whereas cetuximab significantly decreased the level of EGFR. Treatment with a combination with lapatinib and cetuximab significantly decreased both phosphorylated EGFR and total EGFR. Activated downstream signals, including Stat3, were also significantly inhibited by treatment with a combination of lapatinib and cetuximab. In addition, poly(ADP-ribose) polymerase cleavage and activated caspase-3 were increased as a result of treatment with a combination of lapatinib and cetuximab, which is consistent with the results of the apoptotic assay. The cell proliferation-related cyclins D, E, A, and B were all decreased after treatment with the combination therapy, whereas cell cycle inhibitors p21 and p27 were increased after treatment with a combination of lapatinib and cetuximab. Finally, we tested the effects of treatment with a combination of lapatinib and cetuximab on gefitinib-resistant H1975 xenografts that expressed the L858R/T790M mutant EGFR (Fig. 3*D, left*) and found that treatment with a combination of lapatinib and cetuximab completely inhibited tumor growth in the H1975-bearing xenograft. The growth inhibition effect of cetuximab alone was also notable and appeared to be greater than that of

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[3] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
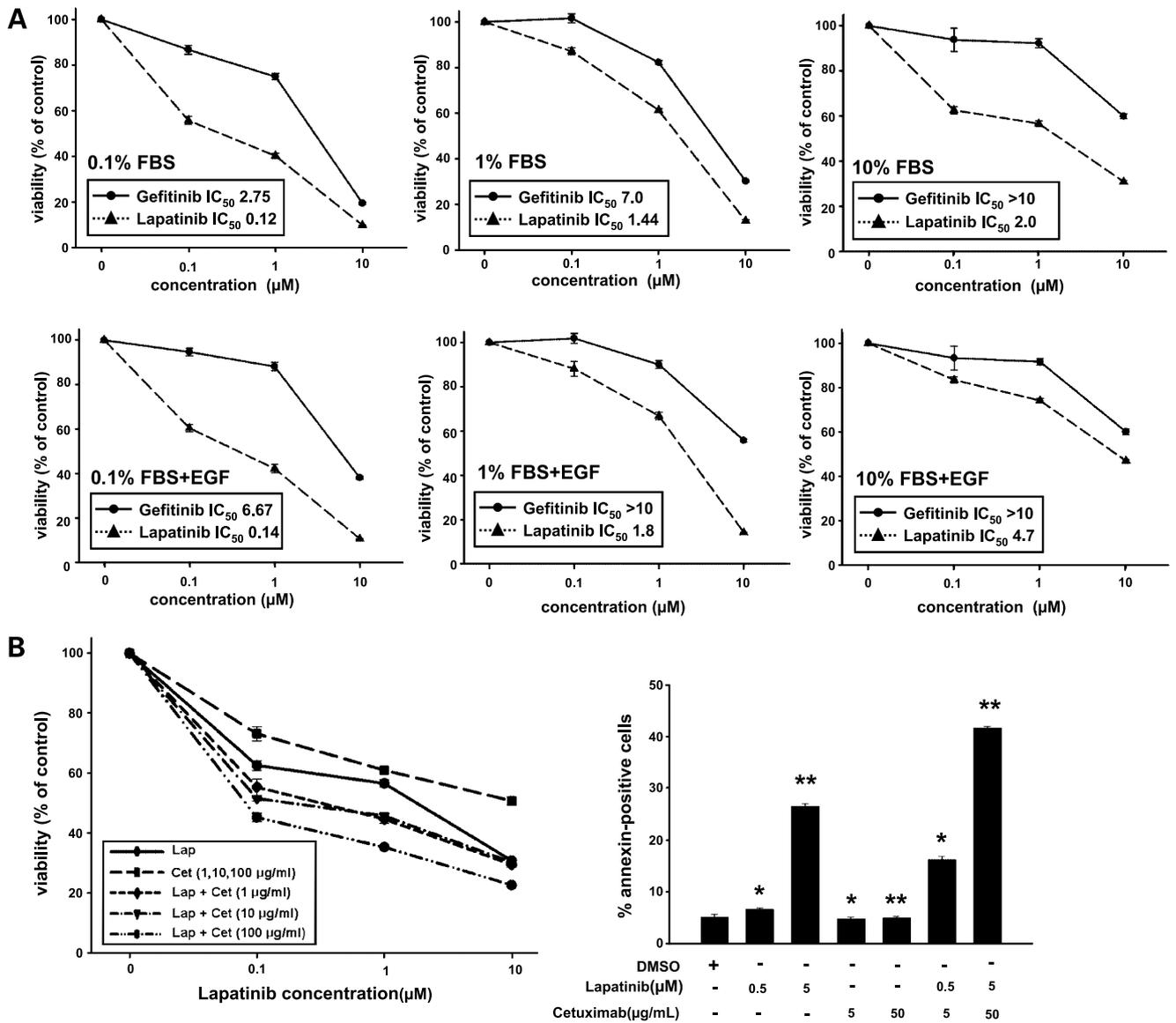
Figure 3. Cetuximab in combination with lapatinib enhances cytotoxicity against T790M NSCLC. **A,** H1975 cells were treated with gefitinib or lapatinib with or without 0.1%, 1%, and 10% serum and/or EGF (30 ng/mL). After 72 h of treatment, cell viabilities were assessed via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. **B,** cells were treated with a combination of lapatinib and cetuximab (1, 10, and 100 ng/mL) for 72 h, after which the cell viabilities were determined via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays (*left*). The cells were treated with lapatinib, cetuximab, or a combination of both at the indicated concentrations. The cells were collected, stained with Annexin V-phycoerythrin (*AnnV PE*), and analyzed via flow cytometry (*right*). Columns, mean percentage of cells staining positive for Annexin V-phycoerythrin; bars, SD. * and **, $P < 0.05$, combination versus singles.

lapatinib alone. Because the cetuximab-induced antitumor effect may involve an immune mechanism unrelated to the inhibition on EGFR, we tested the effect of drug treatment on EGFR and downstream molecules. Treatment with cetuximab alone reduced phosphorylated and total EGFR; however, phosphorylated and total EGFR were more reduced as a result of combination treatment in H1975-bearing xenografts (Fig. 3*D, right*), suggesting that the antitumor activity of the combination approach is mediated by inhibition of EGFR signaling. Taken together, these data show that treatment with a combination of lapatinib and cetuximab significantly inhibited the growth of T790M H1975 cells.

Discussion

Understanding genetic alterations of tumors is essential for the successful development of novel strategies to circumvent resistance to TKIs. Currently, it is known that irreversible EGFR TKIs, such as HKI-272 and HKI-357,
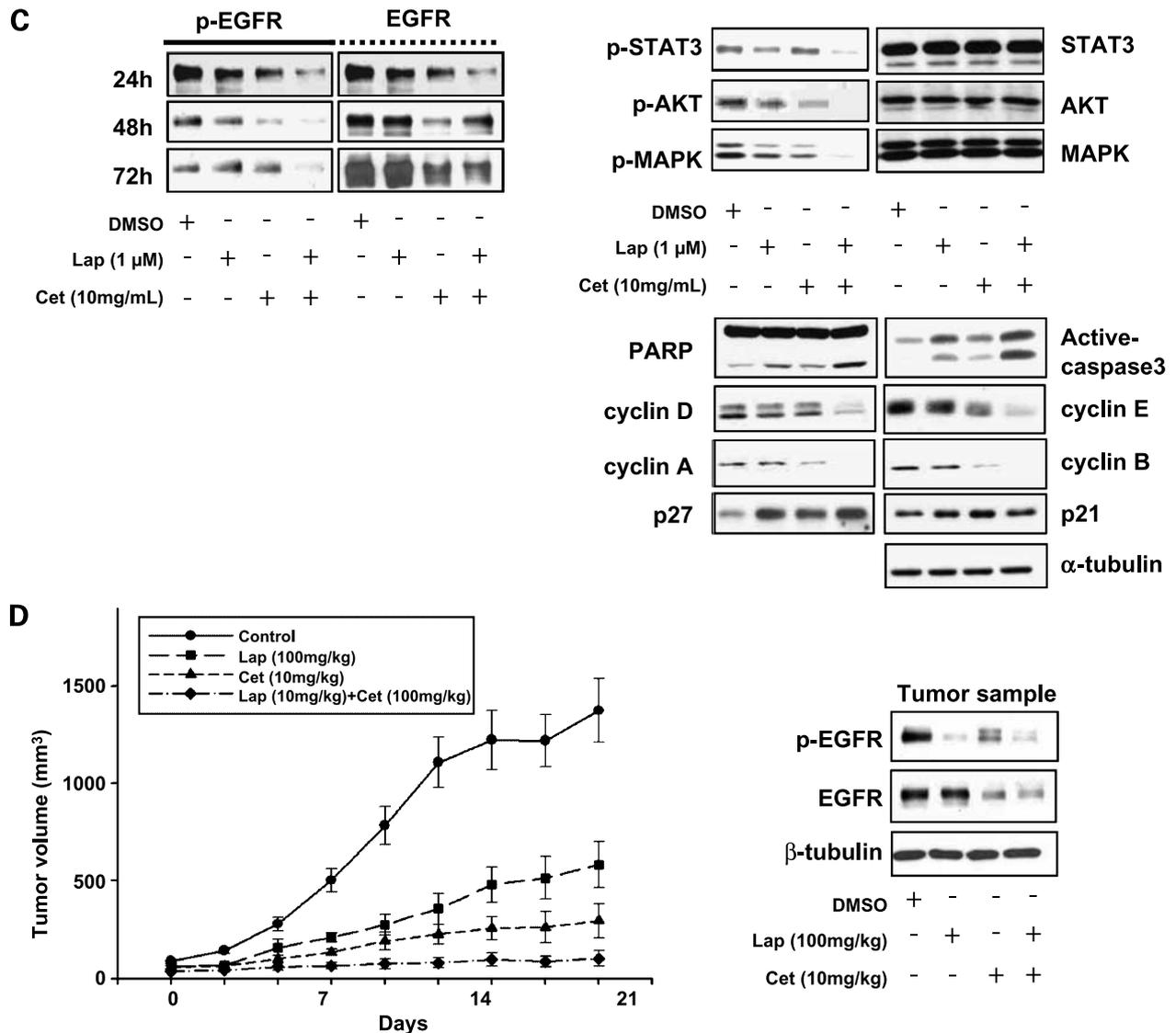
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	p-EGFR				EGFR			
24h								
48h								
72h								

DMSO	+	-	-	-	+	-	-	-
Lap (1 μM)	-	+	-	+	-	+	-	+
Cet (10mg/mL)	-	-	+	+	-	-	+	+

p-STAT3 — STAT3
p-AKT — AKT
p-MAPK — MAPK

DMSO	+	-	-	-	+	-	-	-
Lap (1 μM)	-	+	-	+	-	+	-	+
Cet (10mg/mL)	-	-	+	+	-	-	+	+

PARP — Active-caspase3
cyclin D — cyclin E
cyclin A — cyclin B
p27 — p21
— α-tubulin

D

Control
Lap (100mg/kg)
Cet (10mg/kg)
Lap (10mg/kg)+Cet (100mg/kg)

Tumor sample

p-EGFR
EGFR
β-tubulin

DMSO	+	-	-	-
Lap (100mg/kg)	-	+	-	+
Cet (10mg/kg)	-	-	+	+

Figure 3 *Continued.* **C,** cells were treated with lapatinib (1 μmol/L), cetuximab (10 ng/ml), or a combination of both for 72 h. Western blots are shown for the indicated proteins. **D,** H1975 cells were injected s.c. into nude mice with randomization ($n = 8$). Treatment with lapatinib (100 mg/kg p.o. daily for 3 wks) and cetuximab (10 mg/kg i.p. twice weekly for 3 wks) was initiated once the tumors achieved a volume of 50 mm³. Bar, SE. Repeated measures of ANOVA indicated statistically significant effects ($P < 0.05$) in all groups. Tumor samples treated with lapatinib, cetuximab, or a combination thereof were collected at the end point and homogenized. Western blots are shown for phosphorylated and total EGFR. α-Tubulin was employed as a loading control.

may overcome gefitinib resistance in T790M cells by irreversibly binding to the ATP-binding cleft, even in the presence of the T790M mutation. Although irreversible binding capacity of those TKIs appears to be the main reason for overcoming gefitinib resistance, it should also be noted that these TKIs target not only EGFR but also HER2. Down-regulation of HER2 causes a loss of viability in T790M cells, suggesting the importance of HER2 inhibition in overcoming resistance to EGFR TKI (14). Similar to these results, our study showed that lapatinib dissociated hetero-dimerization of EGFR and HER2 in T790M cells, whereas gefitinib-resistant T790M cells maintained EGFR/HER2

heterodimerization in spite of gefitinib treatment. Lapatinib is a reversible dual TKI for both EGFR and HER2 that binds to an inactive conformation of EGFR that is quite different from the active structure bound by the selective EGFR inhibitor, erlotinib, which suggests that this TKI has a different mechanism of actions (32). Therefore, besides irreversibility of TKIs, our data also suggest that inhibition of HER2 may play a role in reversing the resistance in terms of receptor dedimerization. Moreover, lapatinib has a very slow off-rate from purified intracellular domains of EGFR and HER2 compared with erlotinib or gefitinib. Lapatinib is currently under active clinical investigation and has shown

promising activity against HER2-overexpressing breast cancer cells. However, the efficacy of lapatinib has not been investigated in NSCLC, and its role in EGFR TKI-resistant NSCLC is unknown. In this study, we showed that lapatinib revealed moderately enhanced cytotoxicity against gefitinib-resistant T790M cells *in vitro* and *in vivo*. Based on its dedimerization effect on EGFR and HER2, lapatinib may partially attenuate the resistance to EGFR TKI in T790M lung cancer cells.

EGFR mutant cells are constitutively activated by autophosphorylation of mutant tyrosine kinase, thereby transducing proliferation signals to cancer cells (33, 34). These signals are the therapeutic basis of the use of TKI for EGFR mutant cells, because the growth of EGFR mutant cells is primarily dependent on tyrosine kinase activity regardless of ligands binding. In particular, it is reported previously that T790M mutant cells are totally independent of ligand binding, which means that the growth of T790M cells are primarily mediated by autophosphorylated tyrosine kinase (13). However, in this study, we observed that the presence of serum or EGF reduced the antiproliferative effect of lapatinib in T790M cells, which suggests that these cells may retain ligand-dependent growth in part; thus, the competition of cetuximab for the binding of ligands may be responsible for its effectiveness in combination therapy. The antitumor mechanisms of cetuximab include inhibition of ligands binding, receptor internalization, and degradation or down-regulation of the receptor. However, there is a controversy about the efficacy of cetuximab in EGFR mutant cells. Initial experiments have shown that cetuximab is less effective than gefitinib in the treatment of EGFR mutants (35, 36). Conversely, recent studies have shown that cetuximab inhibited the tumor growth of L858R/ T790M H1975 cells by down-regulating EGFR (24, 29). In this study, we also found that cetuximab down-regulated mutant EGFR in T790M cells; furthermore, treatment with a combination of cetuximab and lapatinib significantly inhibited tumor growth both *in vitro* and *in vivo* (Fig. 3*B-D*). Various biochemical studies and apoptotic assay have shown the synergistic activity of combined lapatinib and cetuximab treatment in gefitinib-resistant T790M cells. Taken together, we propose that combination of lapatinib and cetuximab is mechanistically cooperative because lapatinib affects receptor heterodimerization and cetuximab primarily reduces the level of EGFR proteins (Fig. 4). In gefitinib-resistant T790M lung cancer cells, lapatinib reduced Stat3 activation by blocking the heterodimerization of EGFR and HER2, and cetuximab caused a complete down-regulation of EGFR. The combination of lapatinib and cetuximab completely blocked the activation of downstream signals including Stat3, resulting in enhanced cytotoxicity against in T790M cells. Therefore, this combination provides a therapeutic concept of a complete blockade of EGFR signaling for cancer therapy. Because both drugs are currently being used in clinics, these findings can be directly tested in future clinical investigations. Our current observations also raise important clinical issues. First, the combination of lapatinib and
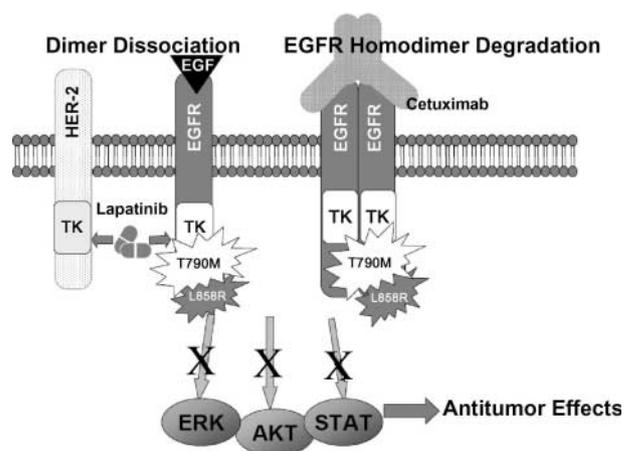

Figure 4. Proposed model showing the effects of lapatinib and cetuximab combination treatment in gefitinib-resistant T790M cells. T790M lung cancer cells were resistant to gefitinib, and Stat3 was persistently activated in these cells. Lapatinib reduced Stat3 activation by blocking the heterodimerization of EGFR and HER2, and cetuximab caused a complete down-regulation of EGFR. Therefore, the combination of lapatinib and cetuximab, which induces dimeric dissociation and EGFR down-regulation, results in antitumor effects on T790M cells.

cetuximab needs to be investigated in NSCLC patients whose disease has progressed after treatment with gefitinib. Second, this combination should also be tested to determine whether lapatinib is superior to other EGFR TKIs when combined with cetuximab.

In conclusion, these data suggest that treatment with a combination of lapatinib and cetuximab is promising for the management of NSCLC patients that exhibit resistance to EGFR TKI.

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