An Alternative Inhibitor Overcomes Resistance Caused by a Mutation of the Epidermal Growth Factor Receptor

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Abstract

Mutations of the epidermal growth factor receptor (*EGFR*) gene have been identified in non–small cell lung cancer specimens from patients responding to anilinoquinazoline EGFR inhibitors. However, clinical resistance to EGFR inhibitor therapy is commonly observed. Previously, we showed that such resistance can be caused by a second mutation of the *EGFR* gene, leading to a T790M amino acid change in the EGFR tyrosine kinase domain and also found that CL-387,785, a specific and irreversible anilinoquinazoline EGFR inhibitor, was able to overcome this resistance on the biochemical level. Here, we present the successful establishment of a stable Ba/F3 cell line model system for the study of oncogenic EGFR signaling and the functional consequences of the EGFR T790M resistance mutation. We show the ability of gefitinib to induce growth arrest and apoptosis in cells transfected with wild-type or L858R EGFR, whereas the T790M mutation leads to high-level functional resistance against gefitinib and erlotinib. In addition, CL-387,785 is able to overcome resistance caused by the T790M mutation on a functional level, correlating with effective inhibition of downstream signaling pathways. Similar data was also obtained with the use of the gefitinib-resistant H1975 lung cancer cell line. The systems established by us should prove useful for the large-scale screening of alternative EGFR inhibitor compounds against the T790M or other EGFR mutations. These data also support the notion that clinical investigations of compounds similar to CL-387,785 may be useful as a treatment strategy for patients with resistance to EGFR inhibitor therapy caused by the T790M mutation. (Cancer Res 2005; 65(16): 7096-101)

Introduction

Recurrent, oncogenic mutations of the epidermal growth factor receptor (*EGFR*) gene were recently identified in patients responding to the anilinoquinazoline EGFR inhibitors, gefitinib and erlotinib (1, 2). These mutations are small deletions that affect amino acids 747 to 750, or point mutations, such as the most common L858R mutation. Despite the initial success of these compounds in patients with oncogenic EGFR mutations (3, 4), resistance seems to emerge over time in the majority of patients. We and others identified a single bp change leading to a threonine to methionine (T790M) amino acid alteration in the ATP-binding pocket of the EGFR as a likely common mechanism of acquired resistance to both gefitinib and erlotinib in patients with secondary resistance to EGFR inhibitor therapy (5, 6). Based on structural modeling studies, we also identified the likely mechanism of resistance as steric hindrance caused by the introduction of the bulkier methionine residue interfering with drug binding and showed high-level resistance against gefitinib as well as a number of other EGFR inhibitors in biochemical studies utilizing transient transfection studies with different EGFR constructs. In preliminary studies, we also found that an alternative, irreversible anilinoquinazoline EGFR inhibitor, CL-387,785 (7), overcame resistance at the biochemical level, suggesting opportunities for the development of effective, second-generation EGFR inhibitors. The transient systems used in these studies had limited utility for the better understanding of the functional consequences of the emergence of the T790M mutation as well as the further study of alternative EGFR inhibitors. In the current study, we developed Ba/F3 stable cell lines and utilized an EGFR mutant lung cancer cell line with a *de novo* T790M resistance mutation (H1975) to further study the functional correlates and signaling consequences of the EGFR T790M mutation as well as better delineation of the efficacy of CL-387,785 to overcome resistance rendered by the presence of this mutation.

Materials and Methods

Reagents. Gefitinib, erlotinib, and cetuximab were purchased from a commercial supplier. CL-387,785 was purchased from Calbiochem (Darmstadt, Germany). Stock solutions for gefitinib, erlotinib, and CL-387,785 were prepared in DMSO and stored at −20°C. Cetuximab was stored at 4°C. The drugs were diluted in fresh media before each experiment, and the final DMSO concentration was <0.5%.

Cell culture. Ba/F3 cell lines were maintained in RPMI supplemented with 10% fetal bovine serum (FBS) and 5% WEHI-conditioned medium as the source of interleukin-3 (IL-3). NIH-H1975 cell lines were maintained in RPMI 1640 supplemented with 10% FBS. Both lines were grown at 37°C in a humidified atmosphere with 5% CO_2.

Epidermal growth factor receptor mutant constructs and transfections. EGFR mutant constructs were generated as previously described (5) and utilized to generate stable Ba/F3 cell lines using electroporation (Amaxa, Cologne, Germany) followed by selection in 1 mg/mL G418.

Cell proliferation and growth inhibition assay. Cell counts were done at daily intervals using trypan blue dye exclusion. Growth inhibition was assessed by MTS assay using CellTiter 96 AQueous One solution proliferation kit (Promega, Madison, WI). For Ba/F3 stable lines, cells were washed thrice with RPMI 1640 only and resuspended in RPMI 1640 supplemented with 10% FBS and 20 ng/mL EGF (Sigma, St. Louis, MO). Then, cells were transferred to triplicate wells at 10,000 cells/well in 96-well flat-bottomed plates with various concentrations of

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inhibitors and the cells were incubated for 48 hours. H1975 cells were plated at 6,000 cells/well; 24 hours after plating, cell culture media was replaced with RPMI 1640 supplemented with 10% FBS with specified concentrations of inhibitors and then incubated for an additional 48 hours.

Antibodies and Western blotting. To determine the phosphorylation level of EGFR, Ba/F3 stable cells were treated with serum starvation for 6 hours and then were stimulated with 100 ng/mL EGF for the indicated periods. To assess the phosphorylation level of other proteins, Ba/F3 stable lines were washed thrice with RPMI 1640 only and resuspended in RPMI 1640 supplemented with 10% FBS, 20 ng/mL EGF, and gefitinib or CL-387,785 at increasing concentrations for 6 hours. Whole cell extracts were separated on 8% SDS-polyacrylamide gels, transferred to nitrocellulose, and analyzed with the use of Western Lightning Chemiluminescence Reagent (Perkin-Elmer Life Science, Wellesley, MA). Total EGFR and total STAT5 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Total extracellular signal-regulated kinase 1/2 (ERK 1/2) antibody was purchased from BD Transduction Laboratories (Lexington, KY). Phospho-EGFR (pTyr1068), phospho-STAT5 (pTyr694), phospho-Akt (pS473), phospho-ERK 1/2 (pT202/pY204), and total Akt antibodies were purchased from Cell Signaling Technology (Beverly, MA). Antibodies were used according to the conditions recommended by the manufacturer.

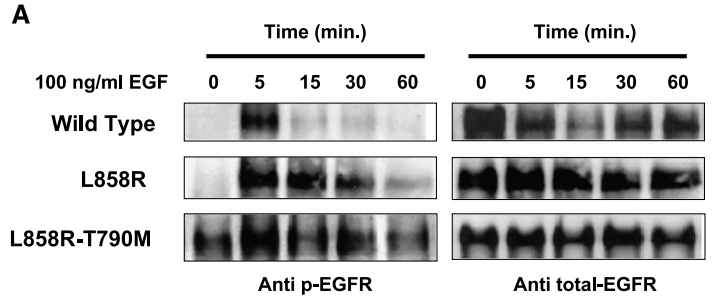
Apoptosis analysis. Ba/F3 stable cells were washed thrice with RPMI 1640 only and resuspended in RPMI 1640 supplemented with 10% FBS, 20 ng/mL EGF, and 1 mg/mL G418, plated in triplicate at a density of 2×10^5 cells/mL in six-well plates with or without inhibitors, and then incubated for 24 hours. H1975 cells were plated in triplicate at a density of 1×10^5 cells/mL in six-well plates in RPMI 1640 supplemented with 10% FBS. The next day, gefitinib or CL-387,785 was added to the medium and cells were incubated for another 48 hours. Apoptosis was assessed using an Annexin V-FLUOS staining kit (Roche, Basel, Switzerland) according to the instructions of the manufacturer.

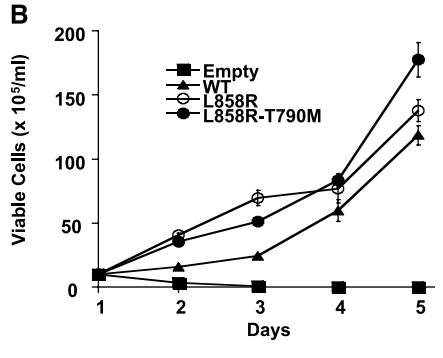
Statistical analysis. The Welch t test was used to determine statistical significance.

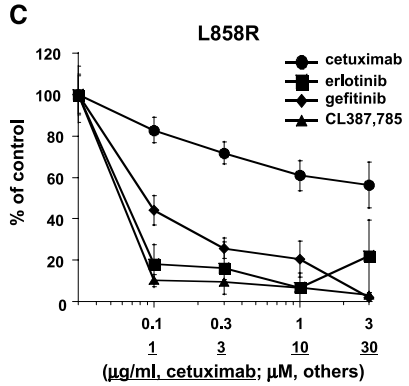
Results

Ba/F3 cells stably expressing epidermal growth factor receptor mutants grow in an interleukin-3–independent fashion. To determine the functional significance of the EGFR T790M mutation, expression constructs containing wild-type EGFR, EGFR-L858R (Ba/F3-L858R), or EGFR-L858R-T790M (Ba/F3-L858R-T790M) were introduced into Ba/F3 cells. Ba/F3 is a murine proB cell line that requires IL-3 for growth and can be rendered growth factor independent by the introduction of a variety of activated kinases, including BCR-ABL and FLT-3 mutants (8, 9). Immunoblot analysis of stably transfected cells showed that EGFR became phosphorylated 5 minutes after EGF stimulation in all transfected cell lines (Fig. 1*A*). Ba/F3-L858R and Ba/F3-L858R-T790M cells showed prolonged ligand-dependent activation upon EGF stimulation compared with the wild-type receptor consistent with prior reports (10). Ba/F3 cells transfected with L858R-T790M showed ligand-independent activation even in the absence of EGF stimulation, although EGF stimulation led to increased levels of phosphorylation compared with the levels observed in the absence of EGF. Furthermore, Ba/F3 lines stably expressing EGFR constructs proliferated in the absence of IL-3, whereas Ba/F3 empty control cells failed to proliferate (Fig. 1*B*). These data indicate that the activation of EGFR leads to IL-3–independent growth consistent with the role of activated and mutant EGFRs as potent oncogenes and suggests that this system can be a useful tool for drug screening and downstream signaling analysis.

CL-387,785 inhibits the growth of, and induces apoptosis in, gefitinib-resistant L858R-T790M mutant Ba/F3 cells. We did MTS assays to investigate whether CL-387,785 was able to inhibit the growth of stably transfected Ba/F3 cells. We observed that both gefitinib and erlotinib inhibited the growth of Ba/F3-L858R but not







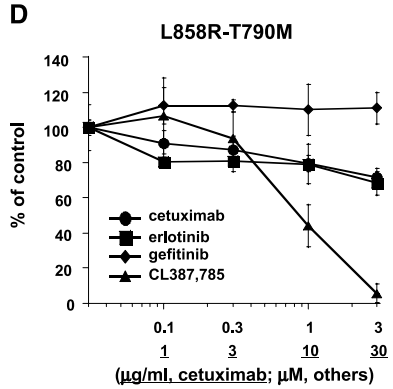


Figure 1. EGFR phosphorylation, IL-3–independent growth, and response to EGFR inhibitors of Ba/F3 cells expressing wild-type EGFR, L858R, or L858R-T790M. *A,* time course of expression and phosphorylation of EGFR (*left*). Total EGFR expression is shown on the right (*control*). *B,* growth of Ba/F3 cells expressing wild-type EGFR, L858R or L858R-T790M, or Ba/F3 cells transduced with vector only (*Empty*). Cells were seeded at a density of 1×10^5/mL and counted daily. *C* and *D,* dose-dependent growth inhibition of Ba/F3 cells expressing L858R (*C*) and L858R-T790M (*D*) detected by the MTS assay. *Bars,* SD.

Ba/F3-L858R-T790M cells (Fig. 1*C* and *D*), whereas cetuximab was only modestly effective in both cell lines, consistent with a previous report (11). The partial inhibition by cetuximab was not significantly affected by the presence of or absence of the T790M mutation. However, CL-387,785 inhibited the Ba/F3-L858R-T790M with a cellular IC_{50} of ~1 μmol/L (Fig. 1*D*). The addition of IL-3 to the growth media restored cell growth in the presence of CL-387,785, suggesting that this inhibition was not due to nonspecific toxicity (data not shown). To examine whether the mechanism of this growth inhibition of CL-387,785 was caused by apoptosis, we did Annexin V apoptosis assays. As shown in Fig. 2, whereas both gefitinib and Cl-387,785 led to massive apoptosis of Ba/F3-L858R cells, only CL-387,785 induced significant apoptosis in Ba/F3-L858R-T790M cells, whereas gefitinib was completely ineffective.

Phosphorylation of epidermal growth factor receptor and its downstream targets, STAT5, AKT, and ERK, were inhibited by CL-387,785. To clarify the molecular mechanisms underlying the differential cellular sensitivity to treatment with gefitinib and CL-387,785 in Ba/F3 cells stably expressing L858R and L858R-T790M, we examined the effects of gefitinib or CL-387,785 on the phosphorylation of EGFR and its main downstream signaling effectors, STAT5, AKT, and ERK 1/2. In Ba/F3-L858R cells, both gefitinib and CL-387,785 inhibited phosphorylation of EGFR and all of its examined downstream signaling effectors (Fig. 3*A* and *C*). In contrast, although gefitinib failed to inhibit EGFR, AKT, and ERK 1/2 phosphorylation even at concentrations as high as 10 μmol/L, CL-387,785 potently inhibited EGFR, STAT5, AKT, and ERK 1/2 phosphorylation in Ba/F3-L858R-T790M cells. The IC_{50} of CL-387,785 for phosphorylation of EGFR and its downstream targets was ~1 μmol/L, which correlated strongly with the results of the growth inhibition assays done (Fig. 1*D*). These data indicate that CL-387,785 effectively inhibits phosphorylation of EGFR and its downstream targets, resulting in the induction of apoptosis of Ba/F3-L858R-T790M cells. Interestingly, STAT5 phosphorylation was also partially inhibited by gefitinib in these gefitinib-resistant cells (Fig. 3*A*, *right*), suggesting that AKT and/or ERK might possibly play a more important role in cellular resistance to gefitinib than alterations in STAT5 signaling.

CL-387,785 inhibits growth and induces apoptosis in the gefitinib-resistant cell line H1975. Recently, it was reported that one adenocarcinoma lung cancer cell line, H1975, carries a *de novo* L858R-T790M EGFR mutation and is resistant to gefitinib and erlotinib (6). We next explored the differential effect of gefitinib versus CL-387,785 in this line. In MTS assays, we showed that H1975 cells were resistant to gefitinib, with an apparent IC_{50} of 4 to 5 μmol/L. In contrast, these cells were at least 10-fold more sensitive to treatment with CL-387,785 with an IC_{50} of ~0.3 μmol/L (Fig. 4*A*). Furthermore, apoptosis assays revealed that H1975 cells were completely resistant to treatment with gefitinib at a concentration of 1 μmol/L but remained sensitive to 1 μmol/L CL-387,785 (Fig. 4*B*). The mean percentage of apoptotic cells was 8.84 ± 2.88% (control), 8.73 ± 2.30% (gefitinib), and 42.1 ± 9.47% (CL-387,785; n = 7). The differences between gefitinib and CL-387,785 treatment with regards to the induction of apoptosis were statistically significant (gefitinib versus CL-387,785, P = 0.0001; Welch t test). To confirm the signaling mechanisms obtained by Ba/F3 cell line model system, we examined the effects of CL-387,785 on the phosphorylation of EGFR and its main downstream signaling effectors in H1975 cells. Whereas gefitinib failed to inhibit EGFR, AKT, and ERK 1/2 phosphorylation

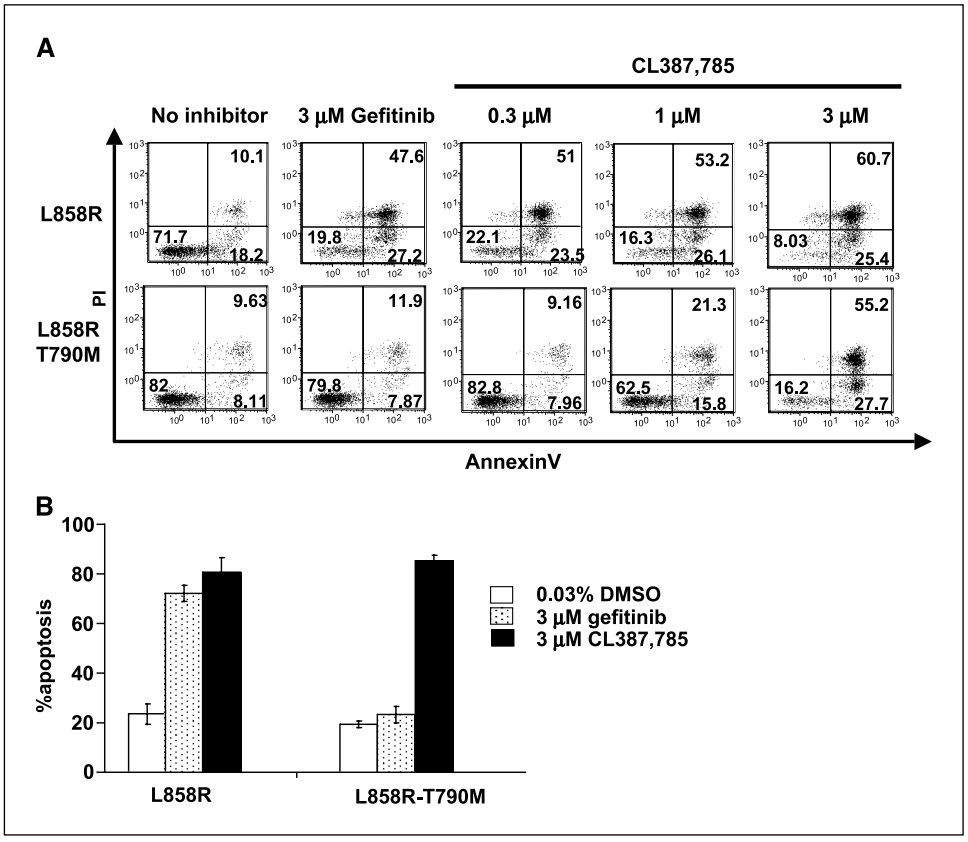


Figure 2. CL-387,785 induces apoptosis in gefitinib-resistant Ba/F3-L858R-T790M cells. *A,* Annexin V apoptosis assay. Representative flow cytometry histograms are shown—the numbers represent percent cells in the appropriate quadrant. *Left bottom quadrant,* viable cells; *right bottom quadrant,* early apoptotic cells; *right top quadrant,* late apoptotic cells. *B,* quantification of apoptosis. Ba/F3-L858R or Ba/F3-L858R-T790M cells were grown in the absence or presence of gefitinib or CL-387,785 for 24 hours. *Bars,* SD (n = 3).

at concentrations as high as 3 μmol/L, CL-387,785 completely inhibited EGFR, STAT5, AKT, and ERK 1/2 phosphorylation at 1 μmol/L, consistent with the results of the growth inhibition assays and Annexin V assays (Fig. 4*A* and *B*). Again, STAT5 phosphorylation was also partially inhibited by gefitinib (Fig. 4*C*, *right*). These data indicate that CL-387,785 is capable of overcoming resistance caused by the T790M mutation in stable expression model systems and native lung cancer cells harboring the T790M gefitinib-resistant mutation in an identical fashion.

Discussion

We have successfully developed cell model systems for the study of oncogenic EGFR signaling, mechanisms of resistance to EGFR inhibitors as well as the testing of compounds potentially capable of overcoming such resistance. The Ba/F3 cell line system has previously been shown to be extremely useful for the study of other oncogenic tyrosine kinases (8, 9), and our ability to generate IL-3–independent growth in cells transfected with different EGFR constructs suggests that it should prove similarly useful for the study of oncogenic signaling of HER family members. Our observations of prolonged EGFR activation in cells carrying L858R-EGFR confirms prior findings (10). The mechanism of this prolonged activation remains unclear, but we believe that model systems such as the one developed by us will prove ideal for the further study of putative mechanisms, such as altered interaction with the cellular degradation, internalization, or dephosphorylation machinery.

As shown in Fig. 1, it seems that Ba/F3 cells transfected with the EGFR-T790M constructs show a baseline level of ligand-independent activation even in the absence of EGF stimulation as opposed to wild-type or L858R-transfected cells. Whereas this observation will require further confirmation in other cell model systems, it seems intriguing in the light of the recent observations that the T790M mutation can be infrequently detected in untreated tumor specimens (12) as well as in the H1975 non–small cell lung cancer cell line (6), possibly suggesting that this mutation might not only confer resistance to anilinoquinazoline EGFR inhibitors but might also have some oncogenic potential per se (13). Ligand-independent activation of the T790M mutant receptor could certainly contribute to increased signaling leading to uncontrolled proliferation and enhanced oncogenic potential.

Recently, we and others have described a point mutation, T790M, in the EGFR ATP-binding pocket as a mechanism of secondary resistance to anilinoquinazoline EGFR inhibitors (5, 6). This mutation is analogous to resistance mutations affecting the tyrosine kinase binding domain of imatinib-resistant bcr-abl or c-kit mutations in chronic myeloid leukemia and gastrointestinal stromal cell tumors (14, 15). Our results with the Ba/F3 cells further confirm our initial observations that the presence of the T790M mutation confers high-level biochemical and functional resistance to both gefitinib and erlotinib. Whereas the analogous abl kinase T315I mutation seems highly resistant to all other alternative bcr-abl inhibitors tested thus far (16), we found that an irreversible anilinoquinazoline inhibitor, CL-387,785, maintained activity against the T790M mutant receptor. These functional changes were accompanied by predictable downstream signaling correlates.

The H1975 non–small cell lung cancer cell line was recently described to carry a *de novo* T790M mutation in addition to a L858R mutation (6). We tested this cell line for its sensitivity to gefitinib and CL 387,785 and consistent with our prior results found that whereas

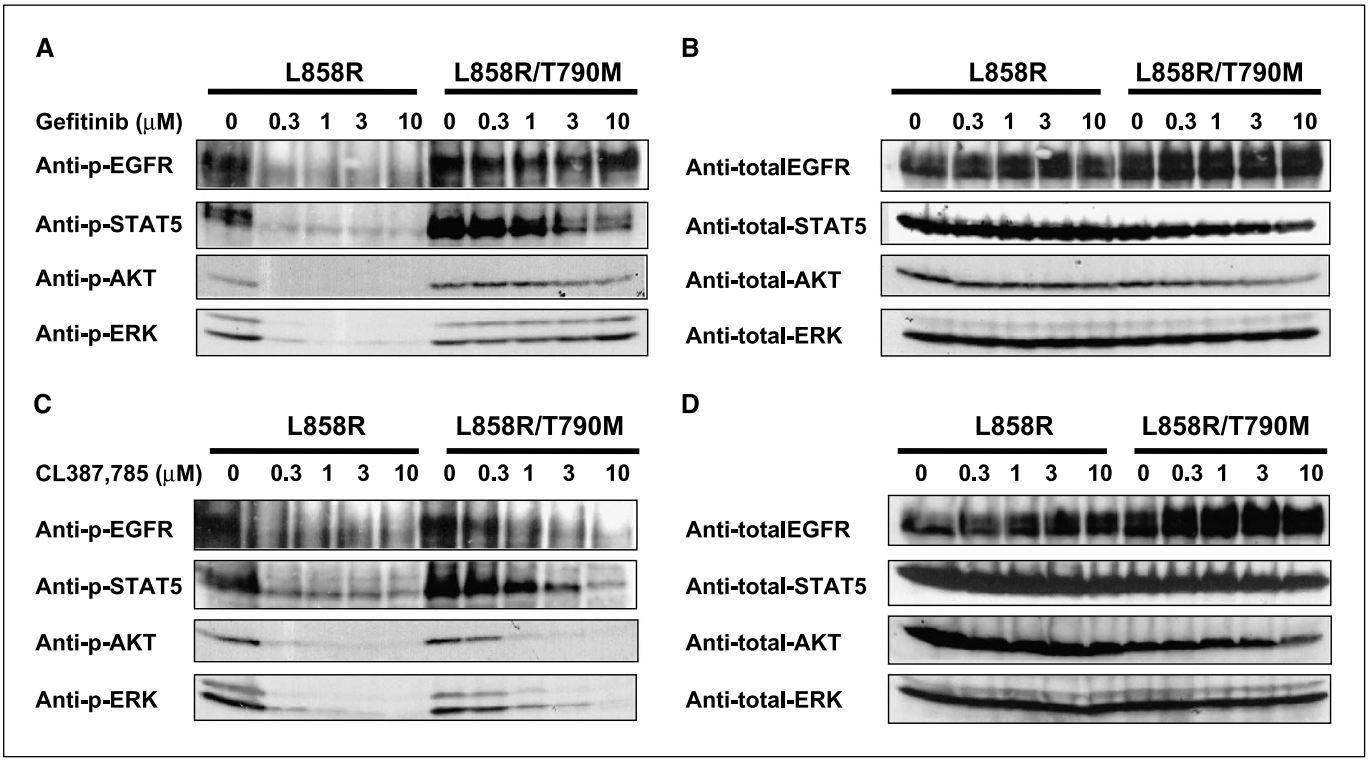


Figure 3. Dose response of gefitinib and CL-387,785 on phosphorylation of EGFR, STAT5, AKT, and ERK 1/2 in Ba/F3-L858R or Ba/F3-L858R-T790M cells. Cells were treated with gefitinib or CL-387,785 at indicated concentrations for 6 hours in the absence of IL-3 and in the presence of 20 ng/mL EGF. Western blots are shown for phospho- (*A* and *C*) and total (*B* and *D*) EGFR, STAT5, ERK 1/2, and AKT.

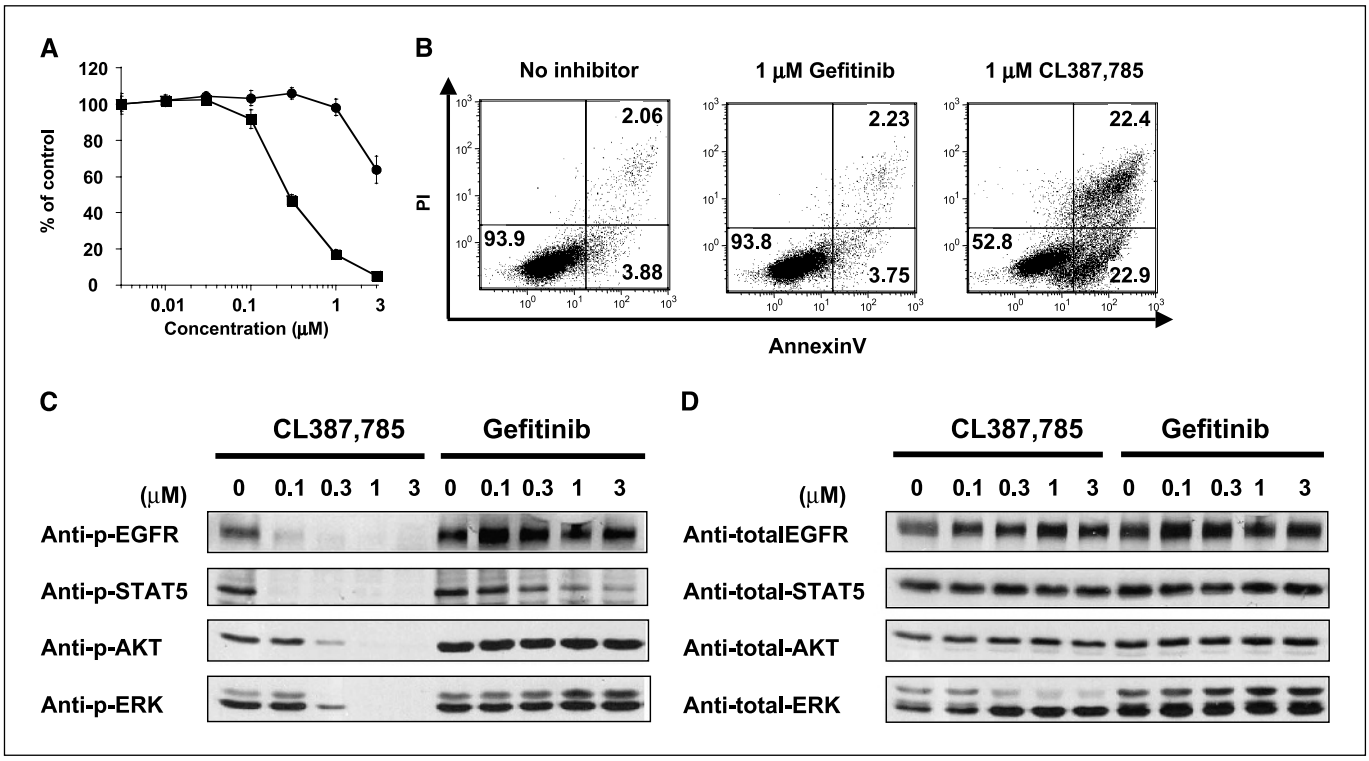


Figure 4. CL-387,785 induces apoptosis in gefitinib-resistant H1975 cells, accompanied by the inhibition of EGFR, STAT5, AKT, and ERK 1/2 phosphorylation. *A,* dose-dependent growth inhibition of H1975 cells treated with gefitinib (—●—) or CL-387,785 (—■—) detected by MTS assay. *B,* representative flow histogram of Annexin V apoptosis assay. H1975 cells were grown in the absence or presence of gefitinib or CL-387,785 for 48 hours. *C* and *D,* dose response of CL-387,785 and gefitinib on phosphorylation of EGFR and its downstream signaling. Cells were treated with CL-387,785 and gefitinib in RPMI 1640 supplemented with 10% FBS at indicated concentrations for 6 hours. Whole cell extracts were immunoblotted with phospho- (*C*) and total (*D*) EGFR, STAT5, ERK 1/2, and AKT.

these cells are highly resistant to gefitinib therapy, they retain sensitivity to treatment with CL-387,785 and this sensitivity is reflected by inhibition of downstream signaling events. Identifying this sensitivity in non–small cell lung cancer cells corroborates our prior findings obtained in other cellular systems (5), and further shows the potential clinical application of similar compounds.

Our results utilizing stable cell line model systems and functional assays further confirm the high-level resistance conferred by the T790M mutation to gefitinib and erlotinib and show retained sensitivity against CL-387,785, an irreversible anilinoquinazoline inhibitor compound. The functional assays established in these studies should prove useful for the screening of other alternative EGFR inhibitors and inhibitors of downstream pathways, such as inhibitors of STAT, PI3kinase, and mitogen-activated protein kinase signaling as well as combinations thereof. The Ba/F3 model system could also be utilized for the functional evaluation of other EGFR resistance mutations to be discovered. The identification of mechanisms of resistance, as well as the identification of alternative inhibitors capable of overcoming resistance, should have immediate clinical implications. The critical need for EGFR mutant non–small cell lung cancers to reactivate the oncogenic EGFR pathway suggests critical dependence on the activity of this pathway, as supported by the concept of "oncogene addiction" (17, 18). These findings suggest that if active inhibitors could be defined, such as CL-387,785, these should retain their clinical utility similar to the recent success of second-generation bcr-abl and c-kit inhibitors in the treatment of chronic myeloid leukemia and GIST (16, 19, 20). Such information might also lead to changes in upfront therapy aimed at preventing the emergence of resistance.

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