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Synergistic cytotoxicity of afatinib and cetuximab against EGFR T790M involves Rab11-dependent EGFR recycling

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ABSTRACT

EGFR is an important therapeutic target for non-small cell lung cancers (NSCLCs). Tyrosine kinase inhibitors (TKIs), such as gefitinib and erlotinib, are effective in cases with EGFR-activating mutations. However, most such cases become resistant through a secondary EGFR mutation, T790M. While the second-generation TKI afatinib has a higher affinity for double-mutant EGFRs, better efficacy is needed. Combining afatinib with the anti-EGFR monoclonal antibody cetuximab improves clinical outcomes, but the mechanism is unclear. Here we examined this effect using erythroleukemic K562 cells. The activating EGFR mutation L858R is sensitive to first-generation TKIs, and adding T790M confers resistance to these drugs. This double-mutant EGFR was moderately sensitive to afatinib, but responded weakly to cetuximab. Combined afatinib and cetuximab synergistically increased their cytotoxicity for K562 cells expressing the double-mutant EGFR. Apoptosis in these cells followed induction of the pro-apoptotic protein BIM. Unexpectedly, afatinib caused redistribution of EGFR to the cell surface through Rab11a-dependent recycling. Cetuximab reduced cell-surface EGFR, and total EGFR decreased synergistically when cetuximab was combined with afatinib. Our results suggest that the synergistic effect exerted by afatinib and cetuximab on NSCLCs is associated with BIM induction and alterations in EGFR status.

1. Introduction

NSCLCs account for more than 80% of all lung cancer cases and are the leading cause of cancer-related deaths worldwide. Aberrant EGFR signaling, caused by activating somatic *EGFR* mutations, is implicated in the oncogenesis of a subset of NSCLCs [1,2]. L858R and exon 19 deletion (Ex19del) represent 90% of these mutations. These NSCLCs are responsive to two EGFR-TKIs, gefitinib and erlotinib, which have higher affinity for the mutants' ATP-binding pocket than for wild-type EGFR's [3–6]. The EGFR-TKIs inhibit EGFR's kinase activity, autophosphorylation, and downstream signaling, including the PI3K, Akt, and MAPK pathways; apoptosis via the induction of pro-apoptotic Bcl-2 family protein BIM follows [7]. BIM is implicated in the activity of various anti-cancer drugs, and its induction is tightly linked to TKI-sensitivity [8].

Acquisition of resistance is a serious setback for NSCLC patients receiving EGFR-TKIs [9]. Although 60–70% of patients initially respond to first-generation TKIs, many eventually acquire resistance, conferred by a new activating mutation. This EGFR gatekeeper mutation, T790M, occurs in approximately 60% of resistant cases [10]. To overcome this clinical resistance, second-generation TKIs with a higher affinity for EGFRs harboring T790M have been developed. Afatinib (BIBW2992) is a pan-HER inhibitor that blocks not only EGFR, but also HER2 and HER4 [11]. It binds covalently to EGFR's activation site, inhibiting ATP binding to EGFRs bearing a single Ex19del or L858R mutation or to double mutants carrying T790M. Afatinib is a promising treatment for

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patients who become resistant to erlotinib or gefitinib [12,13]; however, better efficacy is needed to overcome TKI resistance.

Monoclonal antibodies such as cetuximab (Erbitux) are also used to target EGFR [14]. Clinically, cetuximab is an effective treatment for colorectal or head and neck cancers. Cetuximab binds EGFR's extracellular domain III, preventing its ligand binding, conformational activation, and receptor dimerization, which leads to EGFR's endocytosis and downregulation. Under physiological conditions, internalized EGFR is translocated to early endosomes, then either returned to the surface by recycling endosomes, assisted by Rab-family proteins, or ubiquitinated and captured by the endosomal sorting complex required for transport (ESCRT) [15,16]. Antibody-mediated EGFR downregulation uses the latter pathway, and lysosome-dependent EGFR degradation supports cetuximab's therapeutic effect. However, cetuximab alone is not effective for most NSCLC cases, indicating that other EGFR-targeting drugs are needed.

Combining EGFR-TKIs with other agents could potentially overcome the drug resistance, and afatinib–cetuximab combination treatment is a promising new strategy for TKI-resistant tumors [17]. The combination induces tumor shrinkage in a transgenic mouse model of EGFR T790M-positive lung cancer [18], but it is unclear how these drugs induce apoptosis. Here, we investigated the physiological basis of this combination therapy using human K562 cells expressing mutant EGFRs. Our results reveal a cell-based rationale for this therapy and shed light on the drugs' synergistic effect.

2. Materials and methods

2.1. Cells

K562, HeLa, COS-7 (RIKEN BioResource), NCI-H1975 ($EGFR^{L858R+T790M}$, American-Type Culture Collection) and PLAT-A were cultured in RPMI1640 or DMEM containing 10% fetal calf serum (FCS) and antibiotics at 37 °C in 5% CO_2. Plasmids were transferred into cells using FuGENE HD (Roche Diagnostics). Retroviruses were transferred into K562 cells by spinfection.

2.2. Plasmids

Human EGFR cDNA was subcloned into a retrovirus vector, pMXs-Puro-3xHA-$hEGFR^{WT}$. Mutated EGFR-expression vectors for L858R (pMXs-Puro-3xHA-$hEGFR^{L858R}$), T790M (pMXs-Puro-3xHA-$hEGFR^{T790M}$), and L858R+T790M (pMXs-Puro-3xHA-$hEGFR^{L858R+T790M}$) were generated by a PCR-based method. Mutant Rab11a ($Rab11a^{S25N}$: pBabe-GFP-Rab11a-S25N) and VPS4A ($VPS4A^{E228Q}$: pBabe-Puro-hVPS4E228Q) expression plasmids were newly prepared and verified by DNA sequencing.

2.3. Reagents and antibodies

Imatinib mesylate (Enzo Life Sciences), gefitinib, erlotinib hydrochloride salts (Toronto Research Chemicals), afatinib (Boehringer-Ingelheim), cycloheximide (Wako), cetuximab (Bristol-Myers Squibb), and rituximab (anti-human CD20, Chugai Pharmaceutical) were used. Anti-HA (Roche Diagnostics), anti-phospho-EGFR (Tyr1068), anti-phospho-Akt (Ser473), anti-Akt, anti-phospho-ERK (p-ERK), and anti-ERK (Cell Signaling Technology) were used for western blots. For flow cytometry, PE-conjugated anti-EGFR (Abcam) and anti-mouse IgG1, κ-isotype control antibodies (BD Biosciences) were used. For confocal microscopy, anti-EGFR, anti-EEA1 (Santa Cruz Biotechnology), anti-mouse IgG-conjugated AlexaFluor488, anti-goat IgG-conjugated AlexaFluor594 (Life Technologies) were used.

2.4. Flow cytometry and sorting

Cell suspensions were incubated with an anti-EGFR antibody. Dead cells were excluded with 1 μg/ml 7-AAD (Sigma–Aldrich). Surface staining was analyzed using a FACS Canto II Flow Cytometer with FACS Diva software, and cell sorting was performed with a FACS AriaII (BD Biosciences).

2.5. Cell viability and apoptosis assay

Cells were seeded at 5×10^3 cells/0.2 mL in 96-well plates, with 3 μM imatinib and the indicated doses of gefitinib, erlotinib, afatinib, or cetuximab. Cells were incubated for 72 h, then counted with a Cell Counting Kit-8 (Dojindo). For apoptosis assays, 2×10^5 cells were cultured in 6-well plates for 48 h with 3 μM imatinib, with or without TKIs or cetuximab. Cells were stained with 7-AAD and Annexin V (BioLegend), analyzed by FACS, and the percentage of Annexin $V^+/7\text{-}AAD^-$ cells was determined.

2.6. Western blotting

Western blotting was performed using standard methods, and blots were probed with HRP-conjugated secondary antibodies.

2.7. Immunofluorescence and confocal microscopy

Cells grown on 4-chambered slides (Thermo Scientific) were deprived of FCS for 16 h. The medium was then replaced with fresh medium containing 3 μM imatinib and 50 μg/ml cycloheximide, with or without 0.1 μM afatinib. After 3 h, cells were washed and fixed with 4% paraformaldehyde (Wako Pure Chemicals) in PBS. Samples were stained with anti-EGFR and anti-EEA1, then with secondary antibodies and DAPI (Dojindo), and finally analyzed by confocal microscopy (Carl Zeiss LSM510).

2.8. Statistical analysis

Statistical differences were analyzed by paired Student *t*-tests (MS Excel). A *P*-value <0.05 was regarded as statistically significant.

3. Results

3.1. K562 cells expressing exogenous EGFRs acquire EGFR-dependence in the presence of imatinib

To study EGFR-TKI's effects, we needed an EGFR-negative cancer cell line to use as an evaluation system. Our screen for EGFR-negative human cell lines showed that most, including HeLa and COS-7, expressed considerable EGFR, but the erythroleukemic line K562 lacked endogenous EGFR (Fig. 1A), HER2, or HER3, expression (data not shown). We then introduced wild-type EGFR ($EGFR^{WT}$), a point-mutated L858R EGFR ($EGFR^{L858R}$), or T790M-mutated EGFRs ($EGFR^{T790M}$ and $EGFR^{L858R+T790M}$) (Fig. 1B) into K562 cells. Sorted K562 cells with comparable levels (∼within 3-fold) of cell-surface EGFR were used for the analyses (Fig. 1C).

K562 cells carry the BCR-ABL gene fusion, which drives strong proliferation that can be efficiently blocked by the small-molecule inhibitor imatinib. Accordingly, a 72-h incubation with imatinib killed almost all parental K562 cells (Fig. 1D). Adding EGFR to K562 cells expressing $EGFR^{WT}$ significantly improved the survival rate, to 49%, compared to 4% in control, vehicle-treated cultures. Cells with the oncogenic mutation $EGFR^{L858R}$ showed slightly higher survival rates in response to EGFR, and even higher rates were obtained from cells expressing the double-mutant $EGFR^{L858R+T790M}$.

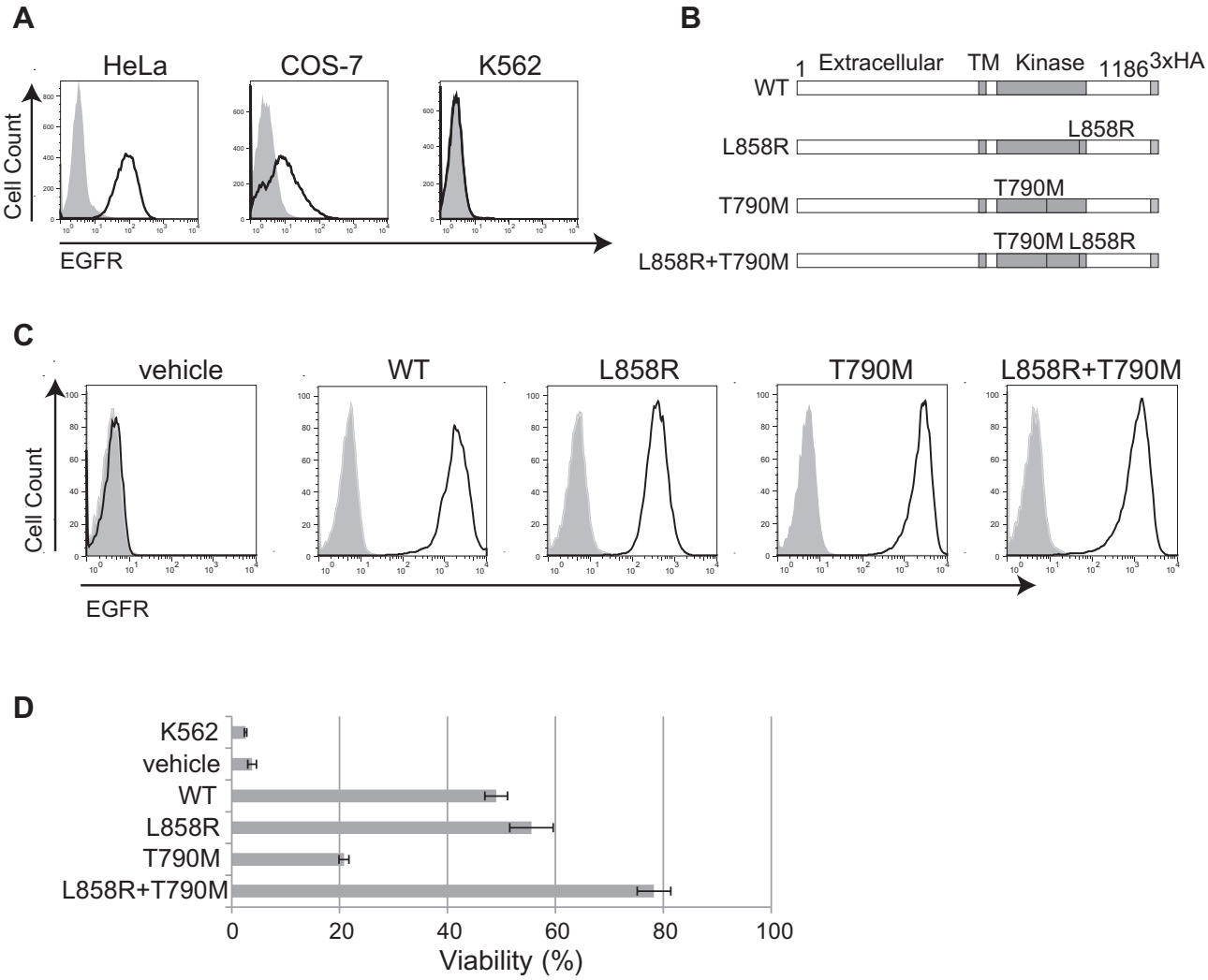

Fig. 1. EGFR-expressing K562 cells acquire EGFR-dependence in the presence of imatinib. (A) Flow cytometry analysis of EGFR in HeLa, COS-7, and K562 cells. Gray: class-matched IgG antibody control staining. (B) EGFR structures. The extracellular domain, transmembrane region (TM), and tyrosine kinase domain are indicated. The carboxy-terminus of each EGFR was tagged with three tandem HAs. L858R: leucine-to-arginine substitution of amino acid 858. T790M: threonine-to-methionine mutation of amino acid 790. L858R+T790M is the double mutant. (C) Flow cytometric analysis of EGFR expression in K562 cells: Cells were retrovirally transduced with WT and mutant EGFRs. Gray: class-matched control antibody. (D) Viability of K562 cells in the presence of 3 μM imatinib for 72 h.

Thus, the exogenous EGFRs expressed by the K562 cells delivered 'driver-like' signals that at least partially substituted for the imatinib-induced loss of BCR-ABL's driver function.

3.2. Gefitinib-resistant EGFR mutants are partially sensitive to afatinib

To examine the effectiveness of first-generation TKIs against K562 cells expressing mutant EGFRs, we incubated transfected cells with gefitinib and imatinib for 72 h. The viability of K562 cells expressing $EGFR^{WT}$ decreased dose-dependently with gefitinib (IC_{50} approximately 1 μM). As expected, K562 cells expressing $EGFR^{L858R}$ were much more sensitive to gefitinib, with almost no viable cells at 0.1 μM. In contrast, gefitinib had little effect on cells expressing $EGFR^{T790M}$ or $EGFR^{L858R+T790M}$ at concentrations up to 1 μM (Fig. 2A). Erlotinib was more potent: $EGFR^{WT}$- and $EGFR^{L858R}$-expressing cultures became non-viable at 0.1 μM, but as with gefitinib, 1 μM erlotinib had little effect on $EGFR^{T790M}$- or $EGFR^{L858R+T790M}$-expressing cells (Fig. 2B). Afatinib was cytotoxic for $EGFR^{WT}$- and $EGFR^{L858R}$-expressing cells at 0.01 μM, and it dose-dependently decreased the viability of $EGFR^{L858R+T790M}$-expressing cells (IC_{50} < 1 μM) (Fig. 2C). $EGFR^{T790M}$-expressing cells were more sensitive to afatinib than $EGFR^{L858R+T790M}$-expressing cells (IC_{50} < 0.1 μM). Thus, afatinib was cytotoxic to cells with the T790M mutation. Under the same culture conditions, cetuximab reduced the viability of K562 cells at 0.1 μM, but higher doses did not increase the effect (Fig. 2D). The cetuximab-induced loss of viability was most profound in cells expressing $EGFR^{T790M}$.

To examine the effect of combining afatinib and cetuximab, we used the K562 $EGFR^{L858R+T790M}$ cells. In the presence of low-dose cetuximab (0.1 μg/mL), afatinib (0.1 μM) decreased cell viability from 85% to 31% (Fig. 2E). Increasing the cetuximab did not change afatinib's dose–response curve. Next, we investigated the apoptosis of these cells. $EGFR^{L858R+T790M}$-expressing K562 cells were incubated under various conditions for 48 h, and then stained with AnnexinV/7AAD. Basal apoptosis, from the imatinib, was 10%. Gefitinib (0.1 μM) alone did not detectably increase apoptosis (Fig. 2F), while afatinib alone (0.1 μM) increased it to 19%. While cetuximab (0.1 μg/mL) had no noticeable effect on apoptosis after 48 h of incubation, cetuximab and afatinib together significant increased it, to 42%. Rituximab (control) had no effect on apoptosis. Thus, afatinib and cetuximab synergistically increased apoptosis in $EGFR^{L858R+T790M}$ K562 cells.

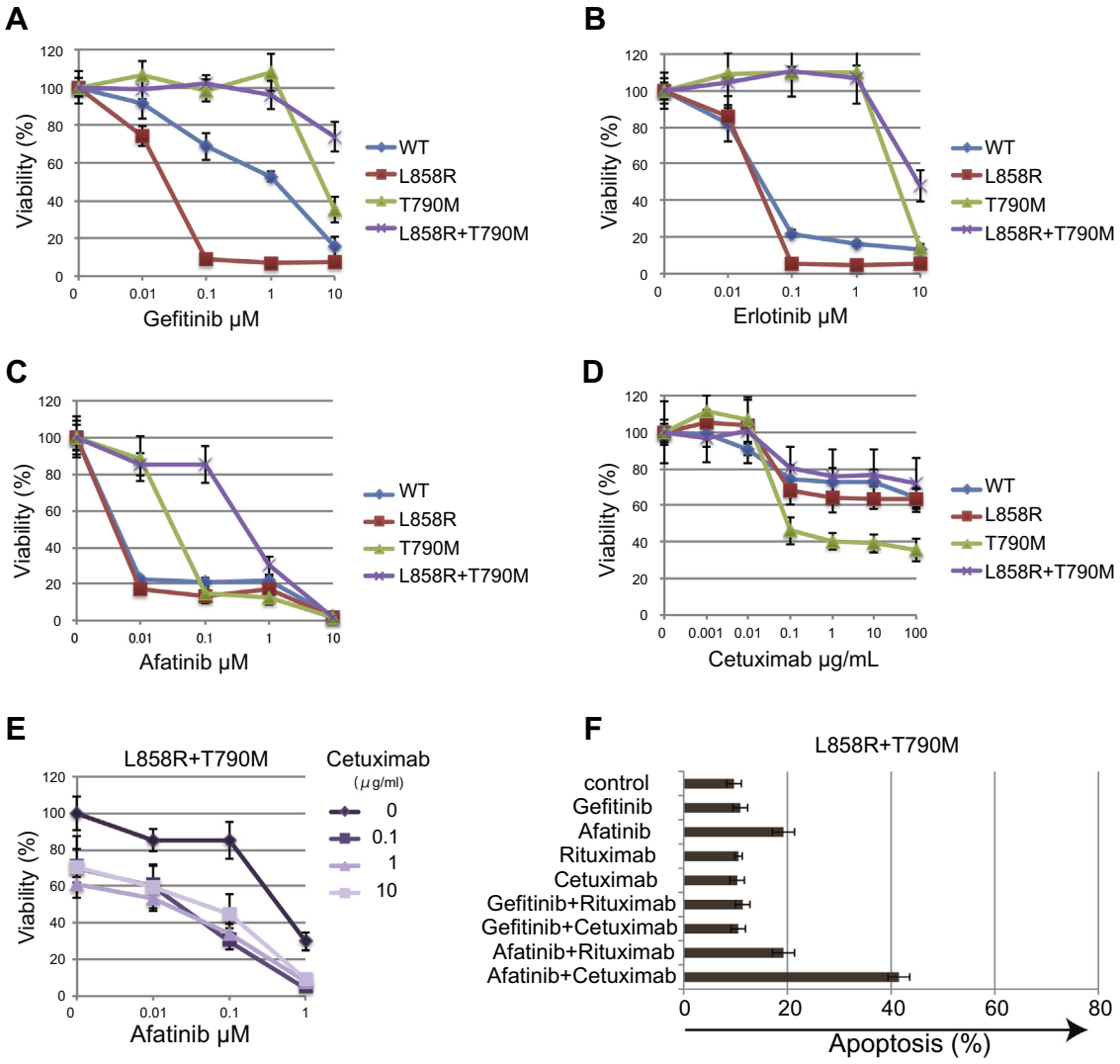

Fig. 2. EGFR-TKIs and cetuximab affect the viability of K562 cells. (A, B, C, and D) EGFR-expressing K562 cells were cultured with 3 μM imatinib and the indicated concentrations of gefitinib, erlotinib, afatinib, or cetuximab for 72 h and analyzed for viability. Viability (%) was expressed relative to that with imatinib alone. (E) Cetuximab augments afatinib's cytotoxicity. Viability of K562/EGFR$^{L858R+T790M}$ cells cultured with afatinib and cetuximab with imatinib. (F) Apoptosis induction of EGFR$^{L858R+T790M}$-expressing K562 cells. Cells incubated for 48 h with 3 μM imatinib and the indicated inhibitors were stained with 7-AAD and AnnexinV. The percentage of AnnexinV^{+}/7AAD^{-} cells was determined.

3.3. Afatinib and cetuximab treatment leads to BIM activation in EGFR $^{L858R+T790M}$ cells

To learn if the cytotoxic effect of afatinib and cetuximab on EGFR$^{L858R+T790M}$ cells was related to EGFR-mediated signaling, we examined EGFR and its downstream signaling pathways. First, we analyzed the effects of a short-term, 3-h incubation with cetuximab and afatinib, singly or combined, by western blotting. Afatinib almost completely inhibited EGFR phosphorylation without affecting total EGFR levels, whereas cetuximab did not affect either one (Fig. 3A). Afatinib also reduced Akt activation (p-Akt) by approximately 45%, whether or not cetuximab was present. Alone, cetuximab had little effect on p-Akt. ERK activation, was mildly inhibited by afatinib, and the combined treatment slightly enhanced this inhibition, but EGFR levels did not differ significantly between samples after 3 h of incubation. In contrast, after a 48-h incubation, EGFR levels decreased in the presence of afatinib or cetuximab added singly, and combination treatment reduced the EGFR to 48% of its original level (Fig. 3B).

Next we examined the drugs' effects on pro-apoptotic proteins. We found that BIM$_{EL}$, one of three BIM isoforms, was enhanced by afatinib, and adding cetuximab increased BIM$_{EL}$ even further (Fig. 3C). Notably, a faster-migrating band representing dephosphorylated BIM$_{EL}$ was induced 3.2-fold by the combined treatment. Likewise, the apoptosis-related proteins Caspase-3 and PARP were cleaved in the presence of afatinib, and the amounts of cleaved products increased significantly with afatinib–cetuximab treatment. Collectively, these results suggest that combined treatment with afatinib and cetuximab reduced Akt and ERK activation in the short term, and over a longer period, induced BIM and activated Caspase-3 and PARP.

3.4. Afatinib increases cell-surface T790M-mutant EGFRs through Rab11-dependent recycling

To learn more about the synergistic mechanism of afatinib and cetuximab, we examined whether EGFR-TKIs affected the level of cell-surface EGFR. Gefitinib induced little if any change in cell-surface EGFR^{WT}, EGFR^{L858R}, or EGFR$^{L848R+T790M}$ after 3 h of incubation (Fig. 4A). Similarly, afatinib did not affect EGFR^{WT} or EGFR^{L858R}; however, it significantly increased cell-surface EGFR$^{L858R+T790M}$ (Fig. 4B). Afatinib also increased cell-surface EGFR in NCI-H1975

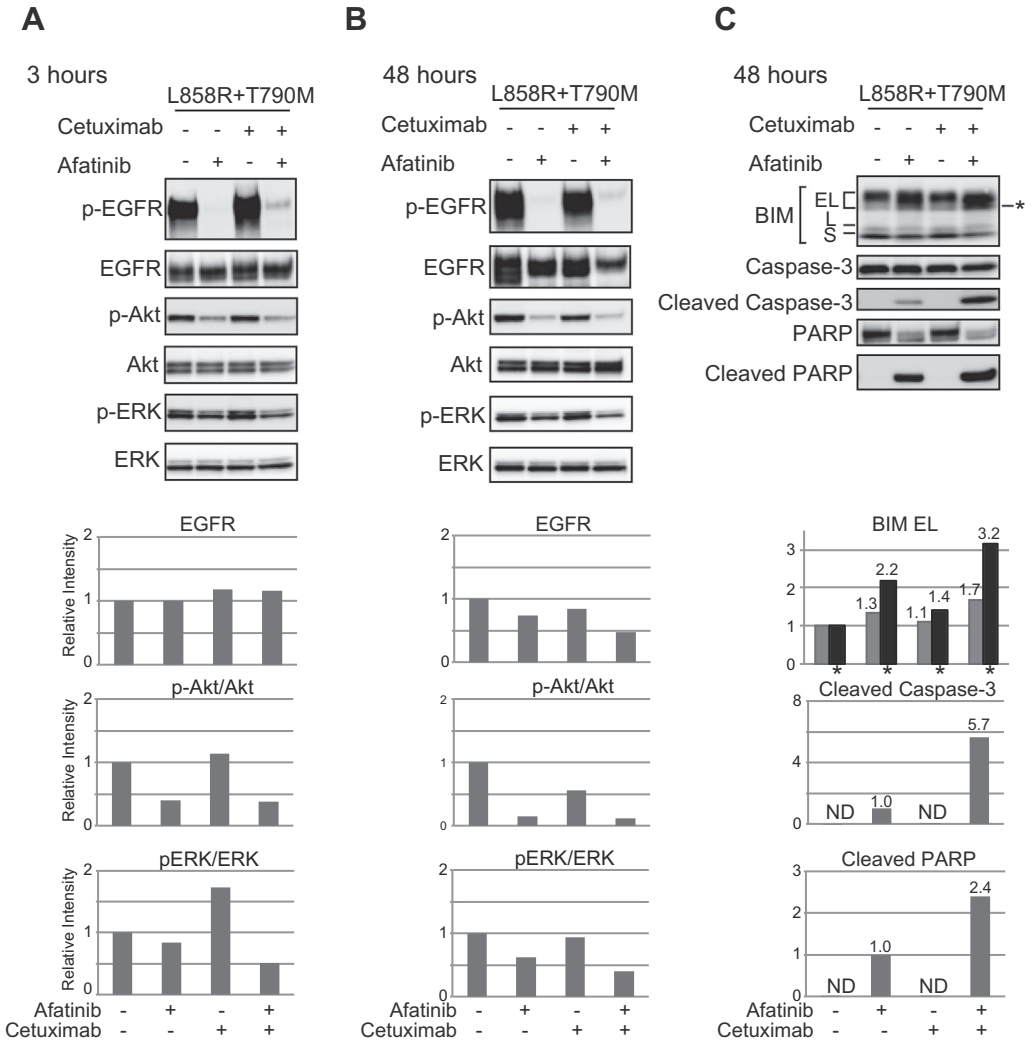

Fig. 3. Afatinib and cetuximab inhibit EGFR's downstream signaling and synergistically induce proapoptotic proteins. (A) and (B) $EGFR^{L858R+T790M}$-expressing K562 cells were incubated with afatinib (0.1 μM), cetuximab (0.1 μg/mL), or both for 3 h (A) or 48 h (B). Imatinib (3 μM) and cycloheximide (50 μg/mL) were added to each culture. Cell lysates were analyzed by western blots. The relative intensities of the western blot signals are shown. (C) $EGFR^{L858R+T790M}$-expressing K562 cells were treated with afatinib (0.1 μM), cetuximab (0.1 μg/mL), or both for 48 h. Three forms of BIM (EL, L, and S) are indicted. Asterisks and black columns indicate the faster-migrating dephosphorylated BIM_{EL}. Graphs show the relative intensities of western blot signals.

lung-cancer cells expressing $EGFR^{L858R+T790M}$ (Fig. 4C and D). Confocal microscopy showed that, in the absence of afatinib, $EGFR^{L858R+T790M}$ was partially located on the EEA1-positive endosomes; thus, $EGFR^{L858R+T790M}$ relocalized to the cell surface when afatinib was added.

Because these experiments were performed in the presence of cycloheximide, the afatinib-induced increase in cell-surface $EGFR^{L858R+T790M}$ did not require *de novo* synthesis. As expected, cetuximab reduced the cell-surface $EGFR^{L858R+T790M}$ after a longer incubation. In combination, the afatinib-dependent EGFR increase was diminished by cetuximab (Fig. 4E).

Next, to examine whether afatinib modified $EGFR^{L858R+T790M}$ recycling, we introduced $Rab11a^{S25N}$, a dominant-negative Rab11a mutant that potently inhibits recycling endosomes [19]. $Rab11a^{S25N}$ almost completely abolished the afatinib-induced increase in cell-surface $EGFR^{L858R+T790M}$ (Fig. 4F). To examine the role of the endosomal sorting system, we introduced a dominant-negative VPS4A mutant, $VPS4A^{E228Q}$, but it did not alter the afatinib-induced increase in cell-surface $EGFR^{L858R+T790M}$ (Fig. 4G), indicating that afatinib does not modify ESCRT-related membrane sorting. Thus, afatinib activates the recycling of $EGFR^{L858R+T790M}$ to increase its cell-surface expression, but not by affecting sorting.

4. Discussion

Here we demonstrated that cetuximab complements afatinib therapy by enhancing afatinib's ability to induce apoptosis. We found that afatinib and cetuximab together were strongly cytotoxic for gefitinib-resistant $EGFR^{L858R+T790M}$-expressing K562 cells, compared with either drug alone. This combination also induced BIM_{EL} in these cells. A likely mechanism underlying this synergistic effect is that the afatinib-induced redistribution of EGFR to the cell surface, through Rab11a-dependent recycling, increased cetuximab's binding to the receptor, thereby augmenting cetuximab's effect.

Afatinib's high affinity for EGFR was demonstrated by its almost complete cytotoxicity for both the $EGFR^{WT}$- and $EGFR^{L858R}$-expressing cells at a very low dose (0.01 μM). Afatinib's partial but significant cytotoxicity for $EGFR^{L858R+T790M}$-expressing cells

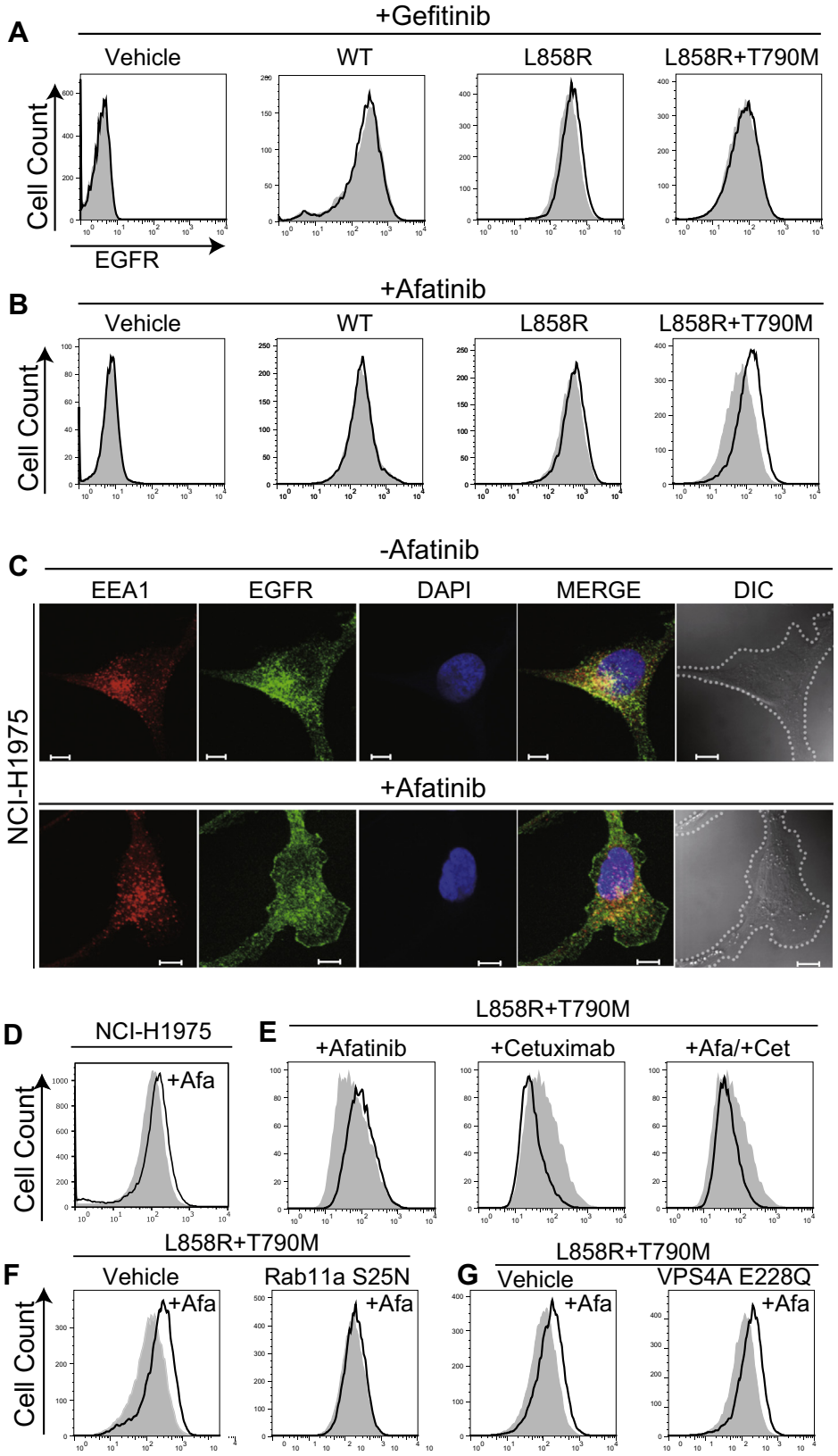

Fig. 4. Afatinib enhances cell-surface EGFR$^{L858R+T790M}$ through Rab11-dependent recycling. Flow cytometric analyses of cells cultured for 3 h at 37 °C with 0.1 μM gefitinib (A) or afatinib (B). (C) Confocal analysis of NCI-H1975 cells cultured for 3 h with or without 0.1 μM afatinib. Cells were fixed, loaded with anti-EGFR and anti-EEA1, and stained with anti-mouse IgG-conjugated Alexa Fluor 488 or anti-goat IgG-conjugated Alexa Fluor 594 antibodies and DAPI. DIC (Differential interference contrast) images of cells outlined with white dots are also shown. Scale bars, 10 μM. (D) Afatinib enhances NCI-H1975 cells' surface EGFR$^{L858R+T790M}$. (E) K562$^{L858R+T790M}$ cells were cultured for 48 h with 0.1 μM afatinib, 0.1 μg/mL cetuximab, or both. Cycloheximide was added. Gray: class-matched IgG control. EGFR$^{L858R+T790M}$-expressing K562 cells transduced with Rab11a-S25N (F) or VPS4A^{E228Q} (G) were cultured for 3 h with 0.1 μM afatinib.

provides the rationale for its use in combination therapy. In the short term, afatinib's cytotoxicity was associated with a significant inhibition of phosphorylated $EGFR^{L858R+T790M}$ and the suppression of downstream signaling, including Akt and ERK activation. In contrast, cetuximab alone only modestly inhibited the viability of cells expressing wild-type or mutant EGFR, and it minimally affected Akt and ERK activation, consistent with this drug's inability to kill NSCLCs efficiently as a single agent [20].

Afatinib and cetuximab inhibited the proliferation of K562 cells and induced their apoptosis, in agreement with a recent report showing that this combination shrinks $EGFR^{L858R+T790M}$-positive tumors *in vivo*. Furthermore, combined afatinib–cetuximab shows robust clinical activity in EGFR-mutant lung cancers with acquired gefitinib or erlotinib resistance, with or without T790M [17]. Short-term EGFR inhibition by afatinib probably inhibits intracellular signaling, but prolonged drug exposure appears to influence EGFR status. Here, the amount of total EGFR in K562 cells was severely reduced by the combination treatment. After a 48-h incubation with afatinib, the amount of EGFR declined, suggesting that EGFR's internalization, vesicular trafficking, and stability are influenced by its phosphorylation. Likewise, long-term cetuximab exposure decreased the amount of total and phosphorylated EGFR. The afatinib and cetuximab combination synergistically reduced the amount of EGFR, in agreement with another study showing that the afatinib–cetuximab combination almost depleted the EGFR and phospho-EGFR in transgenic mouse tumors and NCI-H1975 cells [18].

Based on our present findings, we speculate that altered intracellular EGFR trafficking by prolonged afatinib exposure increases its accessibility to cetuximab, which in turn decreases the EGFR amount and induces cytotoxicity. The incomplete depletion of $EGFR^{L858R+T790M}$ by the combined treatment may reflect the afatinib dose used in this study (1 μg/mL), which was much lower than in a previous *in vitro* study (20 μg/mL) [18]. Similarly, the reduction in NSCLC tumor size by cetuximab used alone in mice, reported in the same study, required the relatively high dose of 1 mg/mouse every three days. In our *in vitro* experiment, cetuximab almost reached its maximum effect at the low dose of 0.1 μg/mL when used simultaneously with 0.1 μM afatinib. Although the *in vivo* pharmacokinetics may differ from those observed *in vitro*, these results suggest that lower cetuximab dosages may be effective and worth trying in clinical trials of combination therapy, which should alleviate the severe skin rash and other adverse effects of this drug.

The cytotoxicity exerted by afatinib and cetuximab was associated with a BH3-only pro-apoptotic protein, BIM. In this study, afatinib but not gefitinib induced BIM in $EGFR^{L858R+T790M}$ cells. BIM induction by gefitinib is a critical step in the apoptosis of gefitinib-sensitive NSCLCs [7,21], and it appears to be a common requirement for efficient TKI responses for several oncogene-addicted cancers [8]. BCR-ABL-addicted cells are one such target, and imatinib-sensitive cell death is associated with BIM. The basal BIM observed here in the presence of imatinib may suggest that EGFR cannot fully compensate for the inactivation of BCR-ABL.

BIM induces apoptosis by activating Caspase-3 and PARP cleavage, and thus is a good 'TKI-response marker.' Furthermore, the knock-down of BIM expression attenuates or prevents apoptosis [21]. Our findings suggest that cetuximab enhances afatinib-dependent BIM induction. In support of this interpretation, the transcriptional up-regulation of BIM is controlled by the transcription factor FOXO3A, and is related to shutdown of the PI3K-Akt pathway [8], which agrees well with our finding that combined afatinib and cetuximab severely inhibited Akt's activation. Notably, a band representing dephosphorylated BIM_{EL} appeared in response to afatinib treatment. TKIs block the post-translational phosphorylation of BIM_{EL} devoid of serine-phosphorylation, which is attributed to ERK inhibition [21]. The increase in dephosphorylated BIM_{EL} is consistent with the afatinib–cetuximab-mediated reduction in ERK activation. However, cetuximab alone only caused a marginal increase in BIM. Full BIM induction requires the simultaneous inhibition of both the Akt and ERK pathways [22].

Afatinib increased the cell-surface $EGFR^{L858R+T790M}$ without *de novo* protein synthesis. This increase was sensitive to the disruption of Rab11-dependent recycling, but not to the loss of ESCRT protein VPS4A function. Rab11a regulates a slow recycling process, and therefore afatinib's ability to increase surface EGFR requires intact slow recycling [23]. $EGFR^{L858R+T790M}$ tends to reside within the cytoplasm rather than on the surface (Fig. 4C and [24]). When afatinib redistributes the receptor to the surface, cetuximab binding increases, leading to the efficient degradation of EGFR. Here, the decrease in cell-surface $EGFR^{L858R+T790M}$ by the combination treatment was less than that caused by cetuximab alone. The discrepancy between surface and total EGFR amounts after afatinib–cetuximab treatment can be attributed to the complicated regulation by the TKI. Our next goal is to elucidate the intracellular trafficking machinery regulated by $EGFR^{L858R+T790M}$. Interestingly, however, combined cetuximab–afatinib therapy was effective for T790M-negative NSCLCs in a clinical study [17], which cannot be explained by afatinib-dependent cell-surface EGFR upregulation. It is possible that these two drugs act on spatially different pools of EGFR *in vivo*.

The K562 cell system we report here provides several advantages for examining EGFR's addiction and drug responses. First, these human cells are more likely to have clinically relevant responses than the previously used BAF-B03 mouse cells [7]. Second, the lack of EGFR expression in K562 cells enables detailed analyses of EGFR-mediated signaling, cell survival, and death. Cells with a single T790M EGFR mutation, rare among cancers, can be easily established using K562 cells, to provide otherwise unavailable information. Other combination treatments, such as TKIs as along with mTOR blockers and histone deacetylase inhibitors, can also be investigated with our system. Third, K562's addiction to its driver mutation means that, in the presence of imatinib, it serves as a model system for testing small chemical inhibitors and antibodies. One limit of the system is suggested by our finding that EGFRs could not completely rescue the K562 cells, which indicates that BCR-ABL is a more potent oncogenic driver than the EGFRs. Collectively, our K562-based TKI evaluation system and our current findings on the combined treatment may clarify the mechanisms of tumor-cell resistance, and lead to new-generation therapies to overcome NSCLC's TKI resistance.

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