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Priority Report

Cancer Research

Chemogenomic Profiling Provides Insights into the Limited Activity of Irreversible EGFR Inhibitors in Tumor Cells Expressing the T790M EGFR Resistance Mutation

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Abstract

Reversible epidermal growth factor receptor (EGFR) inhibitors are the first class of small molecules to improve progression-free survival of patients with *EGFR*-mutated lung cancers. Second-generation EGFR inhibitors introduced to overcome acquired resistance by the T790M resistance mutation of EGFR have thus far shown limited clinical activity in patients with T790M-mutant tumors. In this study, we systematically analyzed the determinants of the activity and selectivity of the second-generation EGFR inhibitors. A focused library of irreversible as well as structurally corresponding reversible EGFR-inhibitors was synthesized for chemogenomic profiling involving over 79 genetically defined NSCLC and 19 EGFR-dependent cell lines. Overall, our results show that the growth-inhibitory potency of all irreversible inhibitors against the $EGFR^{T790M}$ resistance mutation was limited by reduced target inhibition, linked to decreased binding velocity to the mutant kinase. Combined treatment of T790M-mutant tumor cells with BIBW-2992 and the phosphoinositide-3-kinase/mammalian target of rapamycin inhibitor PI-103 led to synergistic induction of apoptosis. Our findings offer a mechanistic explanation for the limited efficacy of irreversible EGFR inhibitors in $EGFR^{T790M}$ gatekeeper-mutant tumors, and they prompt combination treatment strategies involving inhibitors that target signaling downstream of the EGFR. *Cancer Res; 70(3): 868–74. ©2010 AACR.*

Introduction

Reversible epidermal growth factor receptor (EGFR) inhibitors were the first targeted therapeutics approved for the treatment of non–small cell lung cancer (NSCLC; refs. 1, 2). The 4-amino-quinazoline scaffold allows specific binding in the ATP-binding cleft of mutationally activated ERBB kinases (3). Based on this scaffold, inhibitors such as gefitinib abrogate the oncogenic signaling of EGFR (4, 5), promote tumor shrinkage in *EGFR*-mutated patients, and thus, significantly extend progression-free survival of patients with late stage lung cancer (2, 6).

One of the greatest challenges for drug development is the emergence of *in cis* resistance mutations to tyrosine kinase inhibitors. For patients with *EGFR* mutant lung cancer with acquired drug resistance mutation of the gatekeeper (T790M; ref. 7), irreversible second-generation EGFR inhibitors (8) have been introduced but have thus far shown limited clinical efficacy (9, 10). We synthesized and studied the activity of a library of 19 structurally related ERBB inhibitors in genetically validated cellular models (11, 12).

Materials and Methods

A detailed description of the synthesis of all inhibitors and applied methods is given in the Supplementary Methods. All other compounds were purchased from commercial suppliers, dissolved in DMSO, and stored at −80°C. The collection of 84 NSCLC cells was established previously (11). Single nucleotide polymorphism arrays and mutational analyses were used for routine authentication of the cell lines (11). Ba/F3 cell lines were established and described previously (12) and tested for the expression of the mutant ERBB construct, using immunoblotting and Sanger sequencing. Viability

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R.K. Thomas and D. Rauh co-directed this project.

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assays were performed measuring cellular ATP content (CellTiter-Glo; Promega). Immunoblotting was performed using standard procedures (13). For apoptosis assays, cells were treated with the respective compound for 24 to 96 h, stained with Annexin V-FITC/PI and analyzed by flow cytometry on a Canto instrument (BD Biosciences). The modeling of RL58 into EGFR-T790M was performed using PyMol (DeLano Scientific LLC). The structure was modeled by mapping the coordinates onto the erlotinib structure bound to EGFR (PDB code: 1m17). For covalent bond formation, velocity was measured determining fluorescence in black 384-well plates with a TECAN Safire^2 plate reader over time (excitation, 368 nm; emission, 420 nm).

Results

To identify the critical moieties of ERBB inhibitors determining potency and specificity, we synthesized (Supplementary Fig. S1) a library of irreversible 4-amino-quinazolines and quinolines together with their reversible counterparts and characterized their activity in biochemical assays (Fig. 1A). To obtain compounds with high structural similarity and to facilitate the interpretation of structure-activity relations of each modification, we varied the inhibitor structures (Fig. 1A).

To determine the activity of these compounds, we used a NSCLC cell line panel that consists of 18% EGFR- and ERBB2-mutated cells (Fig. 1B; ref. 11). We first genetically validated the dependency on EGFR signaling in *EGFR*-mutated cells using lentiviral-mediated gene silencing of EGFR (Supplementary Fig. S2A). We next confirmed the activity of our synthesized compounds and observed different levels of dephosphorylation of EGFR in the EGFR-dependent PC9 cells after 8 hours of treatment (Supplementary Fig. S2B).

We next characterized cellular compound activity (Supplementary Table S1) and performed hierarchical clustering for all screened cell lines (n = 79/84; Fig. 1C). This analysis revealed clustering of three subgroups of compounds as defined by their potency and specificity to target EGFR and ERBB2 (Fig. 1C). The first group exhibited low activity in ERBB-dependent cells and extended activity in non–ERBB-dependent cells (RL45, RL13, RL14, and RL7; Fig. 1C). A second group of inhibitors, showed high activity in *EGFR*-mutated cell lines and limited activity in *ERBB2*-mutated and ERBB-independent cells (RL20, RL2, RL11, RL6, erlotinib, RL10, RL23, RL58, RL50, gefitinib, PD168393, and RL3; Fig. 1C). In a third group, high activity in *EGFR*- and *ERBB2*-mutated cell lines was coupled to limited activity in ERBB-independent cells (lapatinib, BIBW-2992). Of note, none of the analyzed inhibitors showed high activity in T790M-EGFR gatekeeper mutant cell lines (H1975, H820). Thus, our analysis revealed the genotype-dependent activity of our ERBB inhibitor library and shows the limited effect of different electrophilic side chain substitutions on the potency of ERBB inhibitors against tumors expressing the T790M mutation.

We next screened a collection of Ba/F3 cells (12) expressing known EGFR and ERBB2 mutations with or without concomitant expression of the T790M mutation. We measured cellular viability (Supplementary Table S2) and performed hierarchical clustering of the GI_{50} values. We observed high activity for all inhibitors in cells without a gatekeeper mutation with superior activity of irreversible inhibitors in the presence of insertion mutations and extracellular domain mutations of EGFR (Fig. 2A; Supplementary Fig. S3). Mirroring our results obtained in the panel of NSCLC cell lines, we observed a reduction of the activity of all inhibitors in the background of T790M mutations (Fig. 2A and B). Of note, the reduction of activity of irreversible inhibitors varied between the different concomitant mutations expressed with the EGFR gatekeeper T790M mutation in the Ba/F3 cell lines (Fig. 2A and B; Supplementary Table S2).

Dephosphorylation of the target tyrosine kinase was achieved at the nanomolar range in cells with single-activating EGFR mutations but not in cells coexpressing the T790M gatekeeper mutation (Fig. 2C). Thus, our results imply that although irreversible binding of EGFR inhibitors in gatekeeper-mutated EGFR is more potent than reversible inhibition, the potency of the inhibitors is dramatically reduced when compared with single activating EGFR mutations.

We next sought to determine the effect of the T790M mutation in EGFR on the velocity of covalent bond formation of an irreversible inhibitor in the ATP-binding pocket. For this purpose, we developed a fluorescent-based assay (Supplementary methods), which exploits the fact that covalent bond formation of the inhibitor with EGFR-Cys797 leads to a shift in fluorescence emission of the inhibitor (Fig. 3A). For PD168393 in $EGFR^{L858R + T790M}$, we observed a doubling of covalent bond-formation time (Fig. 3B), thus providing a mechanistic explanation for the limited ability of the irreversible inhibitors to inhibit the target.

We next validated the dependency on EGFR signaling in the $EGFR^{L858R + T790M}$–mutated H1975 cells through lentiviral-mediated gene silencing of EGFR (Supplementary Fig. S4). Based on our biochemical and cellular analyses, we speculated that the activity of irreversible inhibitors is primarily dictated by the initial reversible binding of the scaffold to the hinge region—a short flexible sequence of amino acids that connects the N-lobe and C-lobe of the kinase and forms key hydrogen bonding interactions with ATP-competitive inhibitors (Fig. 3A). These initial interactions may be critical for promoting the subsequent reaction of the electrophiles of such inhibitors with Cys797 of EGFR. Thus, we tested BIBW-2992, its reversible counterpart RL58, and erlotinib for their potency to dephosphorylate EGFR in H1975 cells (Fig. 3C). Confirming our hypothesis, RL58 dephosphorylated EGFR at concentrations 5-fold lower than erlotinib (Fig. 3C). Erlotinib and RL58 are similar in that they both contain a water-solubilizing moiety, although different, at the 7-position of a quinazoline core. Thus, the superior potency of RL58 might be explained by the 4-(dimethylamino)butanamide moiety found in the 6- position. The protonated tertiary amine of this moiety may form a charged interaction with the side chain of Asp800 located in a helix at the front lip of the ATP-binding pocket of EGFR (Fig. 3D), an additional interaction not observed for erlotinib.

Overall, our data derived from the covalent bond formation assays suggest that beyond the effect on the affinity

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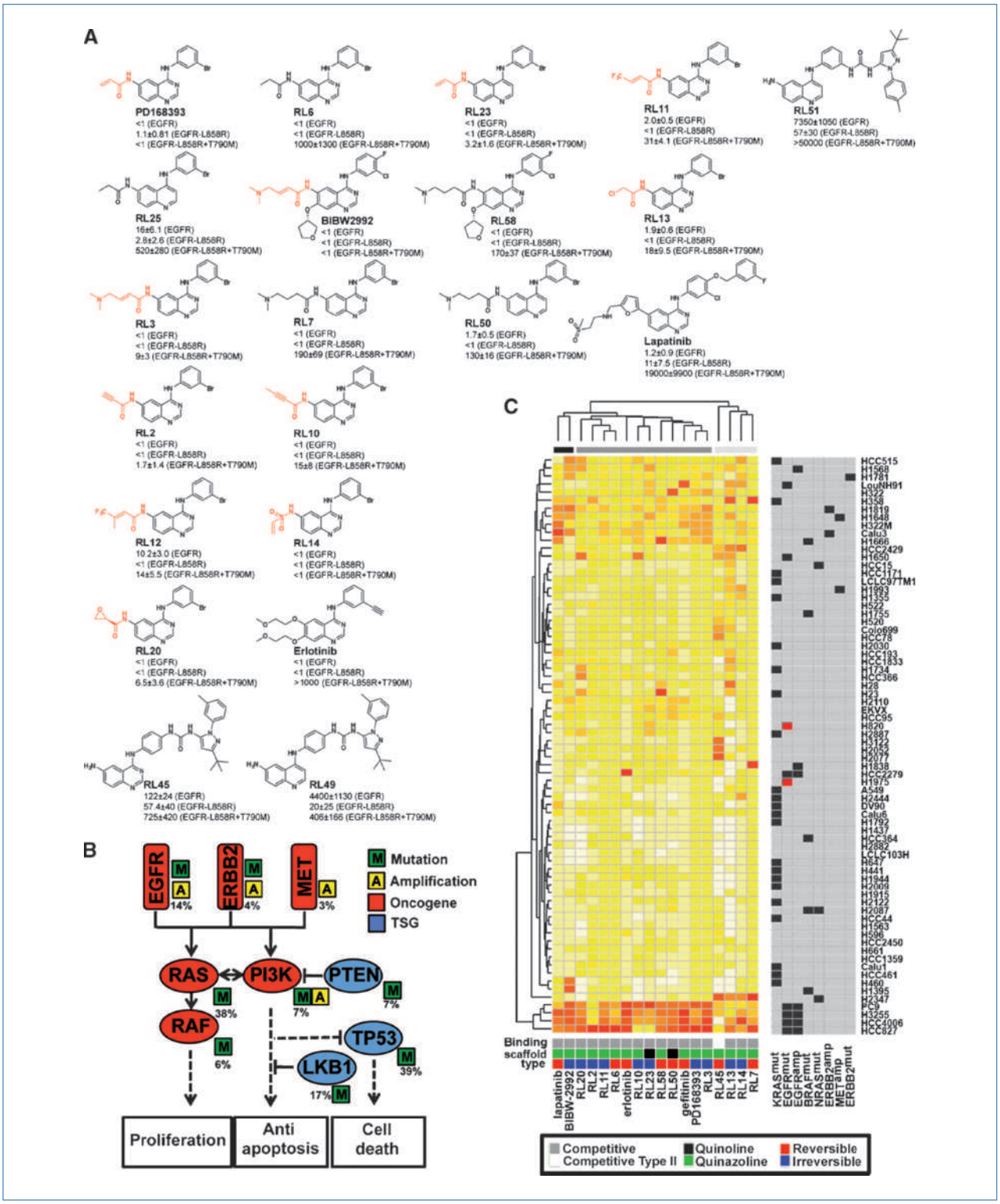

Figure 1. Profiling in NSCLC cell lines. A, the structures of ERBB inhibitors (*red,* reactive groups) and biochemical IC_{50}s (nmol/L). B, the prevalence of lesions involved in oncogenic signaling in lung cancer present in our cell line panel (20). C, a hierarchical cluster of cell lines and compounds, clustered according to GI_{50} values. Three groups defined by their selectivity are marked in the upper part of the cluster (group 1, light gray squares; group 2, gray squares; group 3, black squares). The presence (black squares, mutation; red squares, T790M mutation) or absence (gray squares) of selected lesions is also depicted (right). Binding mode (gray, competitive; white, competitive type II), scaffold type (green, quinazoline; black, quinoline), and type of inhibitor (red, reversible; blue, irreversible) are displayed.

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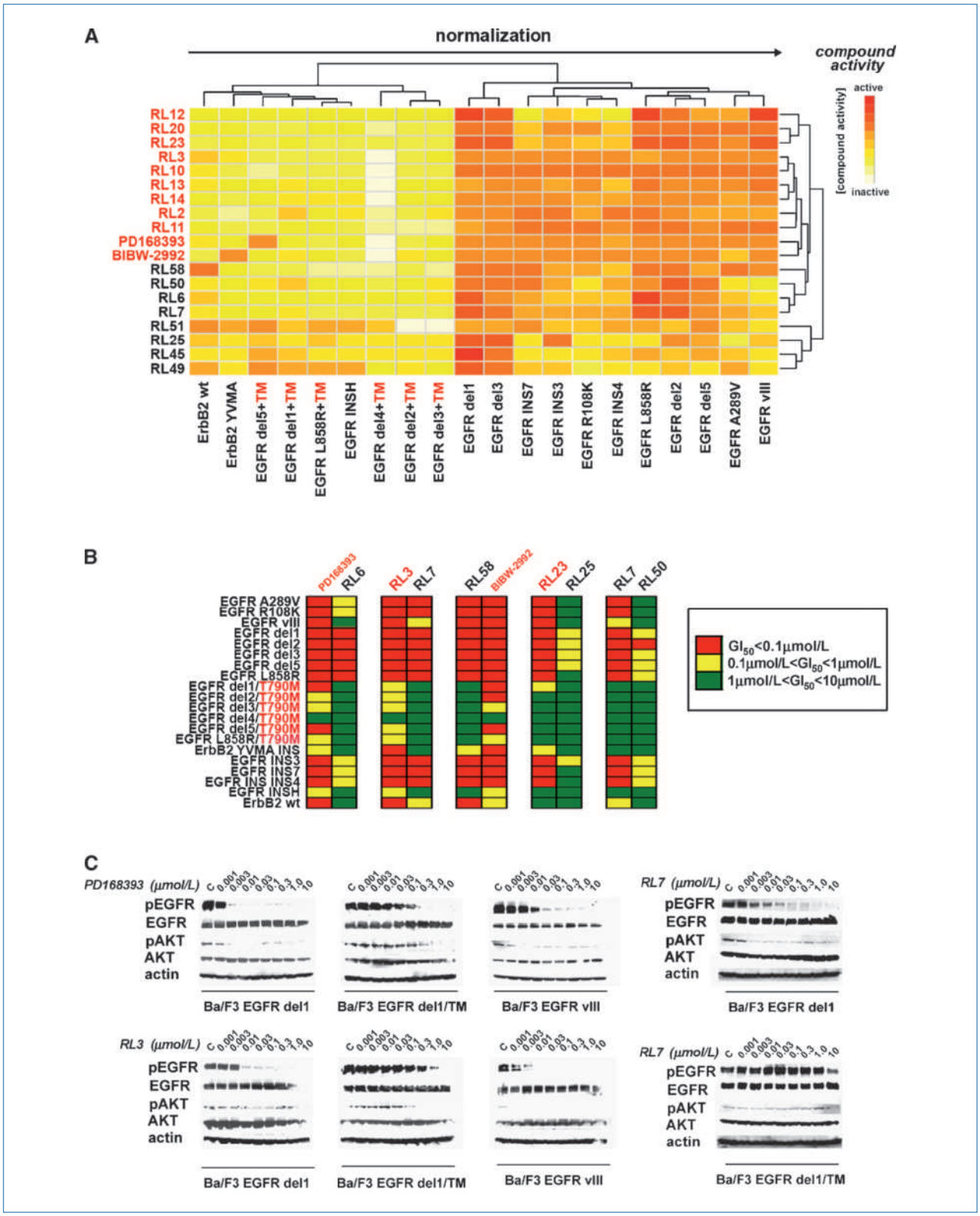

Figure 2. Profiling in ERBB-dependent Ba/F3 cell lines. A, a hierarchical cluster of Ba/F3 cell lines and screened compounds, clustered according to GI_{50} values. B, GI_{50} values across the screened Ba/F3 cell lines, for the ERBB-inhibitors. The respective range of compound activity is color-coded. C, activation status of EGFR and its signal transducer (AKT) were determined by immunoblotting after treatment of Ba/F3 cell lines (EGFR del1, EGFR del1/TM, EGFR vIII) with PD168393, RL3, or RL7.

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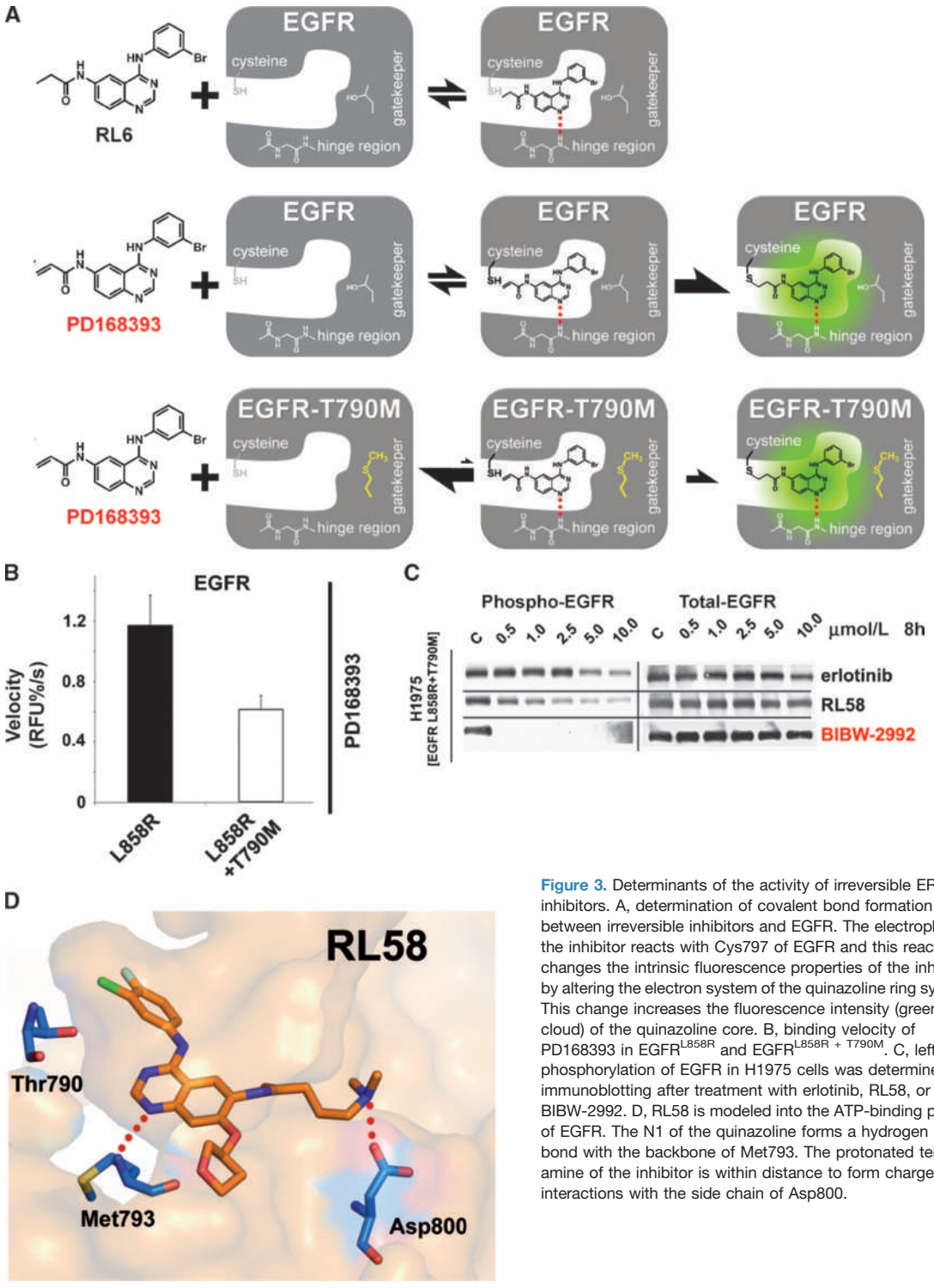

Figure 3. Determinants of the activity of irreversible ERBB inhibitors. A, determination of covalent bond formation between irreversible inhibitors and EGFR. The electrophile of the inhibitor reacts with Cys797 of EGFR and this reaction changes the intrinsic fluorescence properties of the inhibitor by altering the electron system of the quinazoline ring system. This change increases the fluorescence intensity (green cloud) of the quinazoline core. B, binding velocity of PD168393 in $EGFR^{L858R}$ and $EGFR^{L858R + T790M}$. C, left, phosphorylation of EGFR in H1975 cells was determined by immunoblotting after treatment with erlotinib, RL58, or BIBW-2992. D, RL58 is modeled into the ATP-binding pocket of EGFR. The N1 of the quinazoline forms a hydrogen bond with the backbone of Met793. The protonated tertiary amine of the inhibitor is within distance to form charged interactions with the side chain of Asp800.

for ATP (14), the loss of activity of irreversible ERBB inhibitors within the context of the T790M mutation (8), may also be due to steric hindrance. This hindrance disrupts the initial reversible binding of these inhibitors in the ATP binding site and delays covalent bond formation (Fig. 3B). However, this effect may be partially overcome by the introduction of additional protein-inhibitor interactions with amino acid side chains outside of the hinge region. This is highlighted

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by the ability of RL58 to retain high potency against $EGFR^{T790M}$ in both biochemical (Fig. 1A) and cellular (Fig. 3C) assays when compared with its irreversible counterpart BIBW-2992.

We speculated that inhibiting signaling downstream of EGFR might compensate for the limited activity of irreversible ERBB inhibitors (Fig. 4A) in T790M-mutated cells at clinically relevant concentrations. At clinically achievable doses (0.3 μmol/L), BIBW-2992 does not fully dephosphorylate either EGFR or the downstream signaling (Fig. 4B). Of note, inhibition of MEK (PD0325901) also leads to the dephosphorylation of EGFR, and dasatinib treatment does not lead to dephosphorylation of its primary target SRC in this setting (Fig. 4B). Induction of apoptosis achieved by BIBW-2992 alone (Fig. 4C) can be mimicked by dual inhibition with BIBW-2992 and the phosphoinositide-3-kinase (PI3K)/mammalian target of rapamycin inhibitor PI-103 (Fig. 4D), although PI-103 alone could induce apoptosis in these cells (ref. 15; Fig. 4D). Although statistically not significant, this combination is superior to BIBW-2992 single treatment as well as dual EGFR/MEK inhibition or dual EGFR/SRC inhibition at clinically relevant concentrations (Fig. 4B and D). Thus, our data suggests that erlotinib-resistant lung cancer might be overcome by combined treatment with irreversible EGFR inhibitors and PI3K inhibitors.

Discussion

Here, we show that the gatekeeper mutation T790M in EGFR slows down the covalent bond formation of irreversible inhibitors, reduces target inhibition, and limits the cytotoxic activity of such inhibitors in cells expressing the resistance mutation. Furthermore, our structural analysis of the reversible counterpart of BIBW-2992 provides evidence that the first step of the non–covalent binding of irreversible inhibitors in the binding pocket might be responsible for the potency of such inhibitors. Initial preclinical studies had provided a rationale for irreversible EGFR inhibitors in the setting of T790M-related acquired erlotinib resistance (16, 17). However, in virtually all of these cases, cytotoxicity could only be achieved at clinically unachievable concentrations or combination therapy was required to augment this activity (18). Our systematic

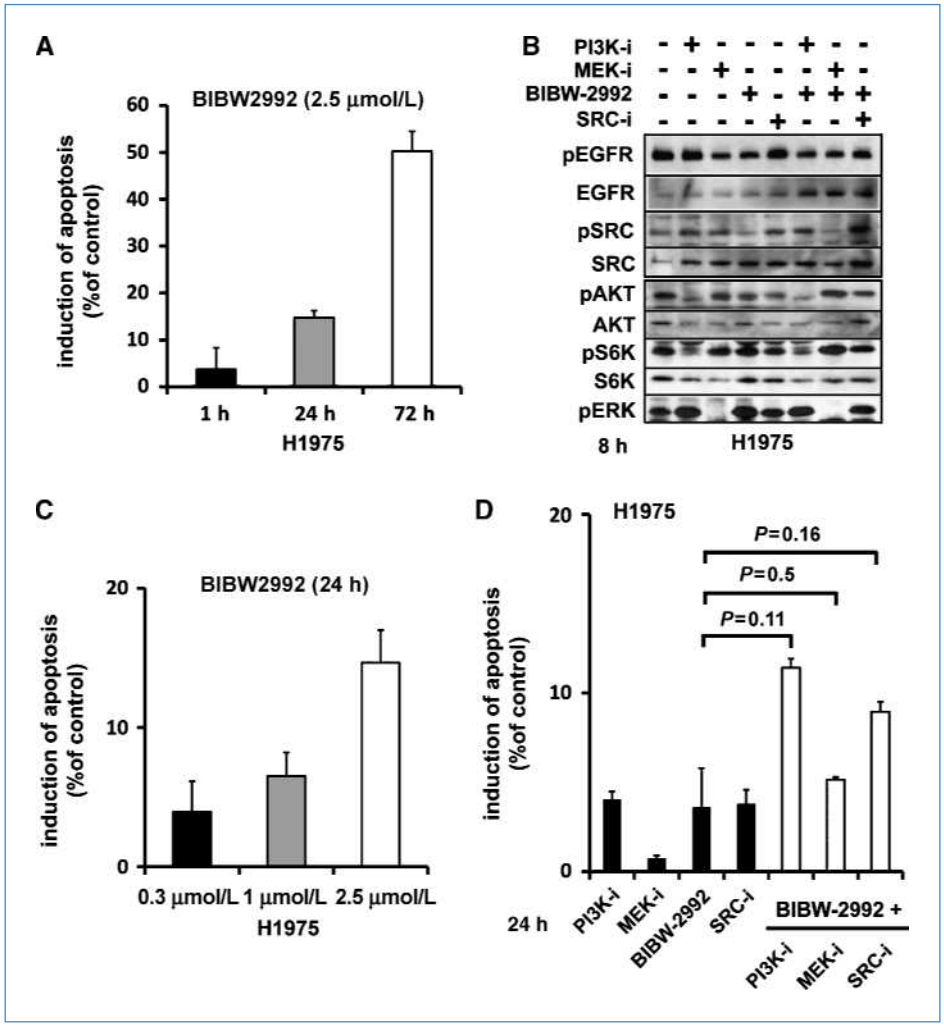

Figure 4. Combined treatment of EGFR and PI3K signaling. A, induction of apoptosis after treatment with increasing concentrations of BIBW-2992 (H1975). B, phosphorylation of EGFR and its signal transducers were determined by immunoblotting after treatment with BIBW-2992 0.3 μmol/L, PI-103 0.5 μmol/L (PI3K-i), PD0325901 0.25 μmol/L (MEK-i) or dasatinib 0.25 μmol/L (SRC-i) in H1975 cells. C, the time-dependent induction of apoptosis after treatment with BIBW-2992 in H1975 cells. D, the induction of apoptosis after treatment with BIBW-2992 (0.3 μmol/L), PI-103 (0.5 μmol/L, PD0325901 at (0.25 μmol/L) dasatinib (0.25 μmol/L), and combinations (H1975). *P*, the comparison of the respective combination to single-treatment BIBW-2992.

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approach is directed towards the T790M mutation in EGFR as a limiting factor in the efficacy of irreversible inhibitors being in line with previous reports (19).

In summary, we provide insights into the limited efficacy of second-generation ERBB inhibitors in erlotinib-resistant T790M gatekeeper-mutated cells, which might be overcome through combinatorial inhibition of EGFR and downstream PI3K signaling with a direct effect on the clinical evaluation of these drugs.

Disclosure of Potential Conflicts of Interest

R.K. Thomas: commercial research grant, AstraZeneca; commercial research support, Novartis; honoraria from speakers bureau, Roche and Infinity; consultant/advisory board, Sequenom. The other authors disclosed no potential conflicts of interest.

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