Priority Report

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


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<sup>T790M</sup> 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<sup>T770M</sup> 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).

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

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

M.L. Sos, H.B. Rode, and S. Heynck contributed equally to this work.
R.K. Thomas and D. Rauh co-directed this project.

Corresponding Authors: Daniel Rauh, Chemical Genomics Centre, Otto-Hahn-Str. 15, D-44227 Dortmund, Germany. Phone: 49-231-8742-6448; Fax: 49-231-8742-6478; E-mail: daniel.rauh@ccg.mpg.de and Roman Thomas, Max Planck Institute for Neurological Research, Gießeler Str. 50, D-50931 Cologne, Germany. E-mail: nini@nf.mpg.de.
doi: 10.1158/0008-5472.CAN-09-3106

©2010 American Association for Cancer Research.
assays were performed measuring cellular ATP content (Cell-Titer-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 Safire² 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 NSCLC cell line panel that consists of 18% EGFR- and ERBB2-mutation, we varied the inhibitor structures (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).

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 through lentiviral-mediated gene silencing of EGFR (Supplementary Fig. S4).

Overall, our data derived from the covalent bond formation assays suggest that beyond the effect on the affinity
Figure 1. Profiling in NSCLC cell lines. A, the structures of ERBB inhibitors (red, reactive groups) and biochemical IC_{50} values (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.
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 GI50 values. B, GI50 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.
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...
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.
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.

References


Acknowledgments

We thank Willem van Otterlo for helpful discussions and André Richters, Christian Grütter, and Armin Robahi for expert assistance during kinetic studies.

Grant Support

Deutsche Krebshilfe grant 107964, the Fritz-Thyssen-Stiftung grant 10.08.2.175, and the NFGNplus program of the German Ministry of Education and Research (BMBF; grant 03G09100 [R.K. Thomas]; the Alexander von Humboldt Foundation [J.R. Simard]; the German Ministry of Education and Research/BMBF, grant 01GS08102 [D. Rauch]. Schering Plough, Bayer-Schering Pharma, Merck-Serono, and Bayer CropScience.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 8/20/09; revised 11/7/09; accepted 12/3/09; published OnlineFirst 1/26/10.
Chemogenomic Profiling Provides Insights into the Limited Activity of Irreversible EGFR Inhibitors in Tumor Cells Expressing the T790M EGFR Resistance Mutation

Martin L. Sos, Haridas B. Rode, Stefanie Heynck, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-09-3106

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2010/01/26/0008-5472.CAN-09-3106.DC1

Cited articles
This article cites 20 articles, 8 of which you can access for free at:
http://cancerres.aacrjournals.org/content/70/3/868.full.html#ref-list-1

Citing articles
This article has been cited by 14 HighWire-hosted articles. Access the articles at:
/content/70/3/868.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.