**In Vitro and In Vivo Characterization of Irreversible Mutant-Selective EGFR Inhibitors That Are Wild-Type Sparing**

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**Abstract**

Patients with non–small cell lung carcinoma (NSCLC) with activating mutations in epidermal growth factor receptor (EGFR) initially respond well to the EGFR inhibitors erlotinib and gefitinib. However, all patients relapse because of the emergence of drug-resistant mutations, with T790M mutations accounting for approximately 60% of all resistance. Second-generation irreversible EGFR inhibitors are effective against T790M mutations *in vitro*, but retain affinity for wild-type EGFR (EGFR\(^{WT}\)). These inhibitors have not provided compelling clinical benefit in T790M-positive patients, apparently because of dose-limiting toxicities associated with inhibition of EGFR\(^{WT}\). Thus, there is an urgent clinical need for therapeutics that overcome T790M drug resistance while sparing EGFR\(^{WT}\). Here, we describe a lead optimization program that led to the discovery of four potent irreversible 2,4-diaminopyrimidine compounds that are EGFR mutant (EGFR\(^{mut}\)) selective and have been designed to have low affinity for EGFR\(^{WT}\). Pharmacokinetic and pharmacodynamic studies in H1975 tumor–bearing mice showed that exposure was dose proportional resulting in dose-dependent EGFR modulation. Importantly, evaluation of normal lung tissue from the same animals showed no inhibition of EGFR\(^{WT}\). Of all the compounds tested, compound 3 displayed the best efficacy in EGFR L858R/T790M-driven tumors. Compound 3, now renamed CO-1686, is currently in a phase I/II clinical trial in patients with EGFR\(^{mut}\)-advanced NSCLC that have received prior EGFR-directed therapy.

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**Introduction**

Despite clinical advancements in early detection and treatment, lung cancer remains by far the leading cause of cancer deaths among both men and women globally (1). In 2013, it is estimated that approximately 230,000 new cases of lung cancer were diagnosed in the United States alone with close to 160,000 deaths (2–4). The most common form of lung cancer is non–small cell lung cancer (NSCLC), and, in a subset of these patients, tumor growth is driven by activating mutations in the epidermal growth factor receptor (EGFR). The most common activating mutations, accounting for 85% to 90% of all EGFR mutations, are the in-frame deletion in exon 19 (DelE746-A750) and the L858R point mutation in exon 21 (5, 6). EGFR mutations occur in 10% to 15% of patients with NSCLC of Caucasian descent and 30% to 35% of patients with NSCLC of East Asian descent (5). Clinical features likely to be associated with EGFR mutations are nonsmoker and of East Asian ethnicity (7).

First-generation EGFR tyrosine kinase inhibitors (TKI) erlotinib and gefitinib have achieved marked responses as first-line therapies in patients with NSCLC that harbor activating EGFR mutations, with median prolonged progression-free survival (PFS) of approximately 1 year (8–10). However, all patients will eventually relapse because of the development of drug resistance to these reversible small molecule inhibitors. The most common drug-resistant mutation is the acquisition of a secondary T790M gatekeeper mutation in the catalytic domain of EGFR kinase, and it is seen in approximately 60% of resistant tumors (11). The T790M mutation increases the affinity of mutant EGFR for ATP, and as a result, erlotinib and gefitinib can no longer effectively compete with ATP, requiring higher concentrations of inhibitor to suppress EGFR signaling (8, 12). However, erlotinib and gefitinib are associated with dose-limiting toxicities such as diarrhea and rash/ acne in response to inhibition of wild-type EGFR (EGFR\(^{WT}\)) in intestine and skin, respectively (13).

To overcome the T790M drug-resistance mutation, several new EGFR TKI selectivity profiles have recently emerged. Second-generation EGFR TKIs afatinib, neratinib,
and dacomitinib have been developed to inhibit the T790M mutation and are in late-stage clinical development (2, 8, 14–16). Unlike the first-generation EGFR TKIs, these irreversible inhibitors target a unique cysteine at residue 797 in the EGFR catalytic domain. The U.S. Food and Drug Administration (FDA) has recently approved afatinib for the first-line treatment of patients with metastatic NSCLC with activating EGFR mutations. Preclinical studies have shown that afatinib potently inhibits T790M in xenograft tumor models. However, in a clinical trial afatinib failed to meet the primary end-point of overall survival in patients with NSCLC that had previously failed erlotinib and gefitinib (17). Both neratinib and dacomitinib also failed to offer overall survival benefits to EGFR mutant patients that have failed first-generation TKIs (18–20). Although these TKIs inhibit T790M, they are not mutant selective and seem to be more effective than erlotinib and gefitinib in inhibiting EGFRWT, and therefore, are hindered by dose-limiting toxicities (8, 17, 19, 20).

To avoid dose-limiting toxicities and overcome the T790M drug-resistant mutation, we developed third-generation EGFR TKIs that are selective for both activating and drug-resistant EGFR mutations, but importantly, are EGFRWT sparing (21). Recently, an irreversible inhibitor, WZ4002, was described that showed mutant-selective activity in preclinical models but did not progress to human clinical trials (22). Other irreversible and reversible EGFR mutant-selective inhibitors have recently been described (23–25). Through structure-based design we identified 4 irreversible inhibitors that are EGFR mutant-selective. Here we describe the in vitro and in vivo characterization of these inhibitors and show that they potently inhibit both the activating and T790M drug-resistant EGFR mutations, but have little affinity for EGFRWT, thus avoiding dose-limiting on-target toxicities associated with inhibition of EGFRWT. Our results showed that among all compounds evaluated, compound 3 had the overall desired EGFR mutant selectivity profile and was selected as a development candidate. More recently, in a companion manuscript, Walter and colleagues further evaluated compound 3 in additional EGFR wild type and drug-resistant EGFR mutations, but importantly, are EGFR mutant-selective. Here we describe the in vitro and in vivo characterization of these inhibitors and show that they potently inhibit both the activating and T790M drug-resistant EGFR mutations, but have little affinity for EGFRWT, thus avoiding dose-limiting on-target toxicities associated with inhibition of EGFRWT. Our results showed that among all compounds evaluated, compound 3 had the overall desired EGFR mutant selectivity profile and was selected as a development candidate. More recently, in a companion manuscript, Walter and colleagues further evaluated compound 3 in additional EGFR wild type and EGFR mutant cell lines, several xenograft tumor models, and induction of acquired resistance after prolonged exposure to compound 3 (26). The results are consistent and complementary to the data presented here supporting compound 3 as an EGFR mutant–selective drug.

Materials and Methods

Cell culture and antibodies

A431 human epidermoid carcinoma, H1975 human NSCLC, and HCC827 human NSCLC adenocarcinoma cells were obtained from the American Type Culture Center. A431 cells were grown in DMEM (Invitrogen) supplemented with 10% FBS (HyClone) and 1% penicillin–streptomycin (P/S; Lonza). H1975 and HCC827 cells were grown in complete RPMI 1640 (Invitrogen) supplemented with 10% FBS and 1% P/S. All cells were maintained and propagated as monolayer cultures at 37°C in a humidified 5% CO2 incubator. All cells were passaged for less than 6 months in our laboratory after receipt or resuscitation. These cells were authenticated on the basis of EGFR expression and inhibition of pEGFR by reference compounds such as afatinib, erlotinib, and WZ4002.

All primary antibodies (total EGFR: #2239S; phospho-EGFR: #3777S; EGFRL858R: #3197S) were obtained from Cell Signaling and used at 1:1,000. Secondary antibodies were used at 1:10,000. Goat anti-mouse IgG IRDye 800CW antibody (#926-32211) was obtained from LiCor Biosciences and goat anti-rabbit IgG Alexa Fluor 680 (#A21057) was obtained from Invitrogen.

Cell proliferation assays

Cells were plated in Growth Media supplemented with 5% FBS and 1% P/S at a density of 3,000 cells per well in 96-well tissue culture plates (Corning). Cells were treated with a dilution series of test compound for 72 hours. Cell viability was determined by CellTiter Glo (Promega) and results were converted to cell numbers using a standard curve. Growth inhibition (GI50) values were determined by Graph Pad Prism version 5.

Immunoblotting

Cells were grown in 12-well plates (Corning) to 90% confluence and then incubated in low-serum (0.1% FBS) media for 16 to 18 hours. Cells were then treated with a dilution series of test compound in low-serum (0.1% FBS) media for 1 hour. A431 cells were then stimulated with 50 ng/mL EGF (Peprotech) for 15 minutes. After treatment, cell monolayers were washed with cold PBS (Invitrogen) and immediately lysed by scraping into 60 μL cold Cell Extraction Buffer (Invitrogen) supplemented with complete protease inhibitors (Roche) and PhosphoSTOP (Roche) phosphatase inhibitors. Lysate protein concentrations were determined by BCA Assay (Pierce) and 50 to 60 μg of each lysate were separated by 4% to 12% gradient SDS-PAGE (Invitrogen), transferred to nitrocellulose membrane (Bio-Rad) and probed with specific antibodies. Protein signals were quantitated using Odyssey Infrared Imaging (Li-Cor Biosciences). Results were normalized to %DMSO control. Normalized data were fitted using a sigmoidal curve analysis program (Graph Pad Prism version 5) with variable Hill slope to determine the EC50 values.

Prolonged duration of action

To assess prolonged duration of action on EGFRL858R/T790M and EGFRDelE746-A750 proteins by compound 3, H1975 and HCC827 cells were treated with 500 or 1,000 nmol/L, respectively (~10-fold of EC50) of compound for 1 hour. Cells were then extensively rinsed to remove compound. Cell lysate was collected for the 0 hour time point. The remaining cells were incubated with complete medium (RPMI + 10% FBS) for the indicated times. Lysates were assessed for EGFR modulation and downstream signaling. HCC827 cells were also treated with erlotinib (100 nmol/L; ~10-fold of EC50) and assessed for pEGFR signaling.
Cellular time-dependent inhibition

H1975 and HCC827 cells were treated with approximately 10-fold EC_{50} of compound 3 for 0, 5, 15, 30, and 60 minutes. Lysates were assessed for EGFR modulation and downstream signaling by immunoblotting.

Target occupancy

To assess EGFR^{WT} occupancy, lysates from A431 cells treated with compound were incubated with 1 μmol/L of the biotinylated covalent probe compound for 1 hour at room temperature. The lysates were separated as described above, and the membranes were incubated with streptavidin-680 to detect binding of the biotinylated covalent probe to EGFR. For occupancy on drug-resistant EGFR^{L858R/T790M} protein, lysates from H1975 cells treated with compound were immunoprecipitated with an anti-total EGFR^{L858R} antibody that only recognizes EGFR {L858R} but not EGFR^{WT} protein (27). This antibody does not bind to EGFR^{L858R} when compound is bound to this protein (data not shown). Lysates were then eluted and were separated as described above, and the membranes were immunoblotted with anti-total EGFR^{L858R} to detect free EGFR.

Kinase selectivity panel

All compounds were tested against a 62 kinase panel at Reaction Biology Corporation (RBC), which includes representative kinases from each branch of the kinome tree. Compounds were tested in duplicate at a concentration of 1 μmol/L. Control compound was tested in a 10 dose IC_{50} curve with 1/3 serial dilution starting at a concentration of 20 μmol/L. All reactions were carried out at 100 μmol/L ATP. Kinases inhibited greater than 50% are indicated. ERBB2/HER2 and BLK were not included in the original 62 kinase panel but were subsequently screened (IC_{50-apparent}) at RBC.

In vivo studies

All the procedures related to animal handling, care, and the treatments in this manuscript were performed according to the guidelines approved by Institutional Animal Care and Use Committees (IACUC) following the guidance of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). H1975 xenograft studies were performed by Charles River Laboratories. Briefly, female nu/nu mice were implanted subcutaneously with 1 × 10^7 H1975 tumor cells in 50% Matrigel (0.2 mL injection volume) in the flank. Tumor measurements were recorded 3 times per week. Tumors were pair matched when they reached an average size of 100 to 150 mm^3. Group size was 8 mice. Test articles were administered intraperitoneally at 25 mg/kg daily for 15 days and control compounds were administered orally at indicated doses. Tumor volume was followed until tumors reached 1,500 mm^3 or until day 15.

For pharmacokinetic/pharmacodynamic studies, H1975 tumors in mice were allowed to reach ~500 mm^3 before being treated with test articles for 5 consecutive days. Tumors, plasma, and lung tissues were collected for pharmacokinetic and EGFR modulation analyses. Tumors and lungs were homogenized using 2.0 mL Precellys tubes for 30 seconds in a Precellys 24 Homogenizer (Precellys). Lysates were immunoblotted as described above.

Molecular modeling

The x-ray structures of EGFR^{WT} (pdb code: 1M17) and EGFR^{L858R/T790M} (pdb code: 2JIU) were initially used in docking studies. All EGFR x-ray structures were retrieved from the Protein Data Bank and the molecular modeling was conducted using Discovery Studio (Accelrys Inc.). The designed compounds were first docked into the EGFR protein as a reversible inhibitor, and then one of the best poses was chosen based on the distance and orientation of the electrophilic functional group. Consequently, the covalent bond between thiol (Cys 797) and the β carbon of the acrylamide was formed, and the bond geometry was optimized through the minimization using CHARMM in Discovery Studio.

Results

Identification of 2,4-diaminopyrimidine inhibitors that are EGFR mutant selective

Previously, we discovered a 4,6-diaminopyrimidine-based irreversible inhibitor targeting the unique cysteine (Cys 797) in the ATP-binding domain of EGFR that potently inhibited EGFR^{WT}, the drug-resistant EGFR^{L858R/T790M} mutation, and the activating EGFR^{DelE746-A750} mutation (28). We initiated a lead optimization program based on biochemical criteria, signaling, and cell proliferation to develop a potent EGFR mutant–selective irreversible inhibitor based on the previously discovered diaminopyrimidine scaffold (21). Structure-based design led to the discovery of 4 lead compounds that potently inhibited the drug-resistant EGFR mutation with minimal activity on EGFR^{WT} (Table 1). These compounds are based on a 2,4-diaminopyrimidine core structure and are structurally different from the quinazoline-based inhibitors, such as afatinib and dacomitinib, even though they target the same Cys 797 in the ATP-binding pocket. Mass spectrometry confirmed that these compounds covalently modified Cys 797 (Supplementary Table S1).

All 4 compounds have a meta acrylamide derived from the 4-position of the core pyrimidine, which targets the Cys 797 in the EGFR catalytic domain (Fig. 1). The linker at 4-position can be either an –O- or –NH-. Both lead to a conformation in which the electrophile reaches the targeted Cys as evidenced by the MALDI-TOF MS study (Supplementary Materials and Methods and Supplementary Table S1). The substitution at the 2-position was a para-substituted ortho-methoxyaniline. The methoxy group plays a role in the selectivity against other kinases with a larger residue in this position (i.e., tyrosine or phenyalanine). The para substitution on this aniline provided increased solubility and improved in vivo properties. The morpholine group in compounds 1 and 2, as well as the acyl/hydroxylacyl group on the piperazine in...
Compounds 3 and 4 were used to reduce the basicity of the para substitution groups in order to mitigate the potential hERG liability often observed from a basic amine at this position.

Selectivity for the gatekeeper EGFR T790M mutant was obtained through the 5-position substitution in the dia-minopyrimidine ring that points directly toward the methionine residue at position 790 (Fig. 1). Presumably, this resulted in a strong hydrophobic interaction between the 5-substituent and sulfur atom of methionine and afforded higher affinity for mutated EGFRT790M. In the EGFRWT kinase, the bulkier methionine residue is replaced by the smaller threonine residue at position 790, and therefore, it reduces the hydrophobic interaction.

### Table 1. Structures, signaling, and cell proliferation potencies

<table>
<thead>
<tr>
<th>Compound ID</th>
<th>Structure</th>
<th>pEGFR-EC50 (nmol/L)</th>
<th>Cell proliferation GI50 (nmol/L)</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td>L858R/T790M</td>
<td>Ratio WT/Mut</td>
</tr>
<tr>
<td>1</td>
<td>![Structure 1]</td>
<td>3.304 ± 104</td>
<td>48 ± 25</td>
</tr>
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<td>2</td>
<td>![Structure 2]</td>
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<td>35 ± 13</td>
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<td>3</td>
<td>![Structure 3]</td>
<td>&gt;4.331</td>
<td>58 ± 34</td>
</tr>
<tr>
<td>4</td>
<td>![Structure 4]</td>
<td>1.669 ± 370</td>
<td>75 ± 23</td>
</tr>
<tr>
<td>WZ4002</td>
<td>![Structure WZ4002]</td>
<td>807 ± 273</td>
<td>159 ± 100</td>
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<tr>
<td>Afatinib</td>
<td>![Structure Afatinib]</td>
<td>&lt;5</td>
<td>42 ± 29</td>
</tr>
<tr>
<td>Erlotinib</td>
<td>![Structure Erlotinib]</td>
<td>&lt;7</td>
<td>&gt;5000</td>
</tr>
</tbody>
</table>

**NOTE:** WT = A431 cells; Mut = H1975, L858R/T790M; ratio = WT over L858R/T790M.
compounds 3 and 4. In signaling assays, compounds 1 and erlotinib only inhibited EGFR WT but not the EGFR L858R/T790M proliferation inhibition (GI 50Minimal EGFR WT activity (EC 50Expected, afatinib potently inhibited EGFR WT and mutant mutant-selective research tool compound (22). As type over mutant EGFR. Potency and selectivity was determined by assessing the ratio of inhibition of wild was occupied by the smaller threonine residue, which reduces hydrophobic interactions, resulting in lower affinity; L, leucine; P, proline; M, methionine; C, cysteine. Position of residues are indicated.

between the gatekeeper residue and the 5-substituent, leading to lower affinity. Compound 4 was used to model binding to EGFR790M mutant (Fig. 1) and was also representative of the modeling of compound 3 binding and bonding to mutant EGFR (26).

The cellular models used to determine signaling and cell proliferation activity were A431 cells that overexpress and are dependent on wild-type EGFR for growth, and H1975 cells that harbor the activating L858R and drug-resistant T790M mutation (EGFRL858R/T790M). Selectivity was determined by assessing the ratio of inhibition of wild type over mutant EGFR. Potency and selectivity were compared with afatinib, erlotinib, and WZ4002, an EGFR mutant-selective research tool compound (22). As expected, afatinib potently inhibited EGFRWT and mutant EGFRL858R/T790M without any selectivity; whereas, erlotinib only inhibited EGFRWT but not the EGFRL858R/T790M mutant (Table 1). WZ4002 showed 5-fold selectivity over EGFRWT in signaling assays. All 4 lead compounds potently inhibited the drug-resistant EGFRL858R/T790M mutant in signaling (EC50 = 35–75 nmol/L) and cell proliferation inhibition (GI50 = 48–132 nmol/L), with minimal EGFRWT activity (EC50 > 1,600 nmol/L and GI50 > 600 nmol/L). In cell proliferation assays, all 4 compounds displayed similar selectivity over EGFRWT (10–15 fold); however, compounds 1 and 2 had weaker activity against both EGFRWT and EGFRL858R/T790M than compounds 3 and 4. In signaling assays, compounds 1 and 2 had similar potencies and selectivity over EGFRWT (69- to 72-fold). Although structurally very similar, compounds 3 and 4 showed different potencies against EGFRWT, with compound 3 approximately 3-fold less active against EGFRWT than compound 4 in signaling. Both compounds had similar potencies against EGFRL858R/T790M. As a result, selectivity over EGFRWT for compound 3 was >75-fold, whereas for compound 4 selectivity was only 22-fold. All 4 compounds were less active against EGFRWT than WZ4002, resulting in increased selectivity over EGFRWT in signaling. Of these 4 lead compounds, compound 3 had the overall desired selectivity profile. Compound 3 activity and mutant selectivity were further confirmed in 2 additional EGFR wild-type (H1299 and H358) and mutant-expressing (HCC827 and PC-9) NSCLC cells in vitro (26). Moreover, compound 3 showed potent activity in erlotinib-resistant cell lines such as HCC827-EPR, PC9-ER, and H3255/XLR cells, and against minor EGFR mutants (G719S and L861Q; ref. 26). Treatment with compound 3 induced apoptosis and was associated with increased PARP and BimEL protein expression (26).

**In vitro EGFR modulation**

To determine EGFR modulation against wild-type and EGFR mutations, we used the previously mentioned A431 and H1975 cells for EGFRWT and EGFRL858R/T790M activity, respectively, as well as HCC827 cells that harbor the activating EGFRD746-A750 mutation. Because compound 3 displayed the desired selectivity for mutant versus wild type, we used this inhibitor for further in vitro characterization and mechanism of action studies. Compound 3 potently inhibited proliferation in the mutant EGFR NSCLC cell lines HCC827 (EGFRD746-A750) and H1975 (EGFRL858R/T790M) with GI50 values of 7 ± 2 nmol/L (26) and 48 ± 17 nmol/L (Table 1), respectively. In comparison, the GI50 value for EGFRWT A431 cells was 602 ± 50 nmol/L (Table 1). In agreement with the inhibition of cell proliferation data, compound 3 potently and significantly inhibited the drug-resistant pEGERWT, pERK, and pS6RP confirmed that compound 3 selectively inhibited only the EGFR mutations while sparing wild-type EGFR (Fig. 2A and Supplementary Fig. S1 and Table S2). Similarly, EGFR downstream biomarkers pAKT, pERK, and pS6R confirmed that compound 3 selectively inhibited only the EGFR mutations while sparing wild-type EGFR (Fig. 2A and Supplementary Fig. S1 and Supplementary Table S2).

The covalent mechanism of action of irreversible inhibitors has enabled design of a companion pharmacodynamic assay that directly quantifies covalent bonding to EGFR protein after drug exposure. Therefore, a covalent biotinylated probe was developed to assess target occupancy by measuring unoccupied EGFRWT protein after compound exposure. Such an approach has been successfully used in our BTK clinical development program as a translational tool using a specific
BTK covalent biotinylated probe (29). A431 cells were treated with compound 3 in a dose-dependent manner and lysates were then assessed for EGFRWT occupancy (Fig. 2B, left and Supplementary Table S2). Compound 3 weakly occupied wild-type protein (EC50 > 4,260 nmol/L), which correlated with the lack of pEGFRWT inhibition in A431 cells (EC50 > 4,331 nmol/L). Because H1975 cells are heterogeneous for EGFR WT and EGFRL858R/T790M, we could not use the covalent biotinylated probe approach because this probe did not distinguish between wild-type and mutant EGFR proteins. Therefore, to assess target occupancy on mutant EGFRL858R/T790M protein in H1975 cell lysates, we used an L858R-specific antibody that only binds to unoccupied EGFR L858R/T790M protein (data not shown). In contrast to wild-type target occupancy, compound 3 potently occupied mutant EGFR L858R/T790M protein (EC50 = 34 ± 9 nmol/L; P = 0.0047; Fig. 2B, right and Supplementary Table S2), which correlated to inhibition of EGFRL858R/T790M kinase activity (EC50 = 58 ± 34 nmol/L). The data indicated that there is a direct one-to-one stoichiometric correlation between target occupancy and inhibition of EGFRL858R/T790M kinase activity. These results showed that compound 3 potently inhibited the drug-resistant and activating EGFR mutant proteins with limited EGFRWT activity.

**Prolonged and time-dependent inhibition**

One of the advantages of irreversible inhibitors is sustained inhibition of the targeted protein; recovery of activity can only occur after resynthesis of the targeted protein. To confirm the covalent mechanism of action in cells, H1975 cells were treated with 500 nmol/L (~10-fold EC50) of compound 3 for 1 hour. Excess compound was washed off and cells were incubated with complete compound-free medium. Recovery of EGFR modulation was evaluated over time (Fig. 3A, left and Supplementary Fig. S2A). Inhibition of pEGFR and molecular target occupancy by compound 3 were consistent with mutant protein degradation (t1/2 ~ 16 hours; Supplementary Fig. S3A) with approximately 50% of activity recovered at 16 hours. Furthermore, sustained inhibition of pERK was also consistent with inhibition of EGFR. Partial activity of pAKT returned after 2 to 8 hours with approximately 50% recovery at 16 hours and full recovery after 24 hours.

Prolonged duration of action on EGFR modulation by compound 3 was also evaluated in HCC827 cells at approximately 10-fold EC50 (1,000 nmol/L). The degradation rate (t1/2) of mutant EGFR protein in HCC827 cells was determined to be between 8 and 16 hours (Supplementary Fig. S3B). Inhibition of pEGFR and pAKT by compound 3 in this cell line is consistent with the protein degradation rate, with approximately 50% signal recovery at 8 to 16 hours (Fig. 3A, right and Supplementary Fig. S2B). However, pERK recovery was almost complete at 4 hours. These data confirmed that compound 3 provided prolonged inhibition of kinase activity hours after the drug was removed from cells, which is consistent with an irreversible compound. In contrast, the reversible inhibitor erlotinib showed a reversible response with complete pEGFR signal recovery 2 hours after compound was removed in HCC827 cells (Supplementary Fig. S4).

To determine time-dependent inhibition of EGFRL858R/T790M kinase, H1975 cells were treated with 500 nmol/L (~10-fold EC50) of compound 3 for 5 to 60 minutes and evaluated for EGFR modulation and downstream signaling (Fig. 3B, left and Supplementary Fig. S5A). Inhibition occurred rapidly with more than 50%
EGFR signaling modulation achieved within 15 minutes of compound exposure and complete inhibition at 30 minutes. Time-dependent inhibition was confirmed by pAKT and pERK inhibition in H1975 cells.

Time-dependent inhibition by compound 3 (1,000 nmol/L; 10-fold EC50) was also observed in HCC827 cells harboring the EGFR DelE746-A750 kinase with more than 50% inhibition by 5 minutes and complete inhibition at 30 to 60 minutes (Fig. 3B, right and Supplementary Fig. S5B). Similar time-dependent inhibition was observed for pAKT and pEGFR in HCC827 cells, whereas slower kinetic activity was observed for pERK; at 5 minutes only 20% of ERK was inhibited. ERK inhibition did not reach completion in HCC827 cells at 60 minutes, with approximately 20% of ERK activity remaining.

Selectivity profile of compound 3

To determine the selectivity of compound 3, activity against 62 protein kinases was first evaluated at 1 μmol/L. At this concentration, compound 3 inhibited 13 kinases greater than 50% at 100 μmol/L ATP (Table 2). FLT3 and the drug-resistant EGFR<sup>L858R/T790M</sup> mutant were the most strongly inhibited (>90%). Besides EGFR, 7 of 10 kinases with a cysteine in a homologous position as Cys 797 in EGFR were also inhibited >50% (Table 2 and Supplementary Table S3). Of the 2 remaining kinases that share Cys 797, BLK kinase was only inhibited 30% at 1 μmol/L and no data were available for ERBB2/HER2 as it was not part of the 62 kinase panel. To more accurately assess the biochemical activity of all 13 kinases inhibited >50% by compound 3, the IC<sub>50-apparent</sub> (nmol/L) values were determined at Km ATP concentration for each kinase (Table 2).

As expected, compound 3 inhibited EGFR<sup>L858R/T790M</sup> kinase most potently with an IC<sub>50-apparent</sub> < 0.5 nmol/L and >12-fold selectivity over EGFR<sup>WT</sup> (IC<sub>50-apparent</sub> = 6 nmol/L), consistent with the cellular selectivity seen with compound 3. Selectivity against the other kinases was at least 20- to 2000-fold. The IC<sub>50-apparent</sub> for FLT3 (223 nmol/L) indicated that compound 3 only weakly inhibited this kinase. The IC<sub>50-apparent</sub> was also determined for BLK and ERBB2/HER2 and both displayed weak inhibition with IC<sub>50-apparent</sub> of 623 and 72 nmol/L, respectively.
In vivo antitumor activity

To determine the in vivo potencies of these inhibitors, we assessed tumor growth inhibition in H1975 tumor-bearing mice (Fig. 4A). To maximize compound exposure, mice were treated with 25 mg/kg compound, delivered i. p., daily, for 15 consecutive days. Tumor-bearing mice were also treated with erlotinib (100 mg/kg, p.o.), WZ4002 (25 mg/kg, p.o.), and afatinib (20 mg/kg, i.p.) as controls. Compounds 1 and 2, with the morpholine substituent, displayed similar tumor growth inhibition (75% and 74%, respectively). Compound 3 displayed improved tumor growth inhibition (95%, $P = 2.2 \times 10^{-5}$), which was comparable to inhibition observed with afatinib (91%, $P = 5.3 \times 10^{-6}$) and WZ4002 (100%, $P = 2.0 \times 10^{-5}$). As expected, erlotinib had little activity (25% inhibition, $P = 0.08$) in the H1975 tumor model. Because compound 4 had a less than desired kinase selectivity profile (data not shown), it was not further evaluated for tumor growth inhibition. All tested compounds were well tolerated with no body weight loss in contrast to afatinib and erlotinib (Fig. 4B).

Selective in vivo EGFRmut modulation in H1975 tumor-bearing mice

To understand the pharmacokinetic and pharmacodynamic relationship in vivo, a multidose-level pharmacokinetic/pharmacodynamic study was conducted in H1975 tumor-bearing mice. H1975 tumors were grown to ~500 mm$^3$ in mice and treated daily with compound 3 at 3, 10, 30, and 100 mg/kg, p.o., for 5 consecutive days. For comparison, mice were also treated with erlotinib (100 mg/kg, p.o.) or afatinib (20 mg/kg, i.p.). Tumors were evaluated for pharmacokinetic at 1 and 6 hours post final dose (Fig. 5A and Supplementary Table S4). Dose-dependent exposure was observed with exposures ranging from a maximum of 4,065 ± 813 ng/g at 100 mg/kg to a minimum of 150 ± 14 ng/g at 3 mg/kg at 1 hour post final dose. At 6 hr post final dose, exposures decreased 5- to 7-fold. No compound was detected at 6 hours for the 3 mg/kg dose because of technical issues.

Tumors were evaluated for pEGFR inhibition and target occupancy at 6 hours post final dose (Fig. 5A and Supplementary Table S4) because pilot studies indicated that maximum pharmacodynamic modulation was achieved at 6 hours (data not shown). Dose-dependent EGFR modulation was observed with maximum pEGFR mutant inhibition (96% ± 1%) and EGFR mutant target occupancy (94% ± 1%) at 100 mg/kg (Fig. 5A and Supplementary Table S4). Despite a decrease in exposure 6 hours post final dose, maximum pharmacodynamic modulation was achieved at this time point, consistent with an irreversible mode of action for compound 3. As expected in the H1975 xenograft model, erlotinib had no effect on the tumor xenograft model, erlotinib had no effect on the pEGFR at the high dose of 100 mg/kg, whereas afatinib achieved inhibition of pEGFR (73% ± 1%) at 6 hours post final dose (Fig. 5B). The results indicated that the efficacious dose for compound 3 was 100 mg/kg.

To confirm selectivity against EGFRWT, lung tissues from drug-treated H1975 tumor-bearing mice were assessed for compound exposure and pEGFR inhibition (Fig. 5C and Supplementary Table S4). Lung and tumor tissues had similar exposure across all doses. To assess inhibition of EGFRWT, lung tissue from mice treated with the efficacious dose of 100 mg/kg were analyzed 6 hours post final dose. No inhibition of EGFRWT (0% ± 12%) was observed, whereas at a similar dose significant inhibition of EGFRWT/L858R/T790M mutant (96% ± 1%) was observed (see Fig. 5A). These results confirmed that compound 3 potently and selectively modulated the drug-resistant EGFRWT/L858R/T790M mutant but not EGFRWT in vivo.

Table 2. Kinase selectivity profile of compound 3 at 1 μmol/L

<table>
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<tr>
<th>Kinase</th>
<th>% Inhibition at 1 μmol/L</th>
<th>IC50-apparent (nmol/L)</th>
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<tr>
<td>FLT3</td>
<td>94%</td>
<td>223</td>
</tr>
<tr>
<td>EGFR (L858R/T790M)$^a$</td>
<td>91%</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>JAK3$^a$</td>
<td>87%</td>
<td>39</td>
</tr>
<tr>
<td>TXK$^a$</td>
<td>84%</td>
<td>486</td>
</tr>
<tr>
<td>Aurora A</td>
<td>82%</td>
<td>1,142</td>
</tr>
<tr>
<td>EGFR (WT)$^b$</td>
<td>81%</td>
<td>6</td>
</tr>
<tr>
<td>FAK/PTK2</td>
<td>77%</td>
<td>11</td>
</tr>
<tr>
<td>BMX/ETK$^a$</td>
<td>74%</td>
<td>28</td>
</tr>
<tr>
<td>CHK2</td>
<td>74%</td>
<td>36</td>
</tr>
<tr>
<td>ERBB4/HER4$^a$</td>
<td>66%</td>
<td>32</td>
</tr>
<tr>
<td>BTK$^a$</td>
<td>64%</td>
<td>10</td>
</tr>
<tr>
<td>TEC$^a$</td>
<td>62%</td>
<td>1,140</td>
</tr>
<tr>
<td>ITK$^a$</td>
<td>58%</td>
<td>87</td>
</tr>
<tr>
<td>BLK$^a$</td>
<td>30%</td>
<td>623</td>
</tr>
<tr>
<td>ERBB2/HER2$^a$</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

NOTE: Kinases inhibited >50% are indicated, except for BLK and ERBB2/HER2.
Abbreviation: nd, not determined.
$^a$Indicated kinases that share Cys 797 with EGFR kinase.

(Table 2). To confirm the biochemical potency, select kinases were also evaluated for inhibition of signaling in cells. Inhibition of pBTK, pJAK3, and pAUR-A was evaluated in Ramos, CTLL-2, and HeLa cells, respectively, with EC50 > 200 nmol/L, >1,000 nmol/L, and >1,000 nmol/L, respectively (data not shown). These data demonstrate that compound 3 is very selective, especially against kinases with a cysteine in a homologous position as Cys 797. The selectivity profile of compound 3 is similar to other irreversible EGFR inhibitors with afatinib and WZ4002 inhibiting 11 and 14 kinases, respectively, greater than 50% at 1 μmol/L (Supplementary Table S3). Excluding EGFRWT and EGFRL858R/T790M, afatinib showed greater than 50% inhibition in 6 of 10 kinases that share Cys 797 with EGFR, whereas WZ4002 and compound 3 inhibited 7 of 10 kinases.

In vivo antitumor activity

To determine the in vivo potencies of these inhibitors, we assessed tumor growth inhibition in H1975 tumor-bearing mice (Fig. 4A). To maximize compound exposure, mice were treated with 25 mg/kg compound, delivered i. p., daily, for 15 consecutive days. Tumor-bearing mice were also treated with erlotinib (100 mg/kg, p.o.), WZ4002 (25 mg/kg, p.o.), and afatinib (20 mg/kg, i.p.) as controls. Compounds 1 and 2, with the morpholine substituent, displayed similar tumor growth inhibition (75% and 74%, respectively). Compound 3 displayed improved tumor growth inhibition (95%, $P = 2.2 \times 10^{-5}$), which was comparable to inhibition observed with afatinib (91%, $P = 5.3 \times 10^{-6}$) and WZ4002 (100%, $P = 2.0 \times 10^{-5}$). As expected, erlotinib had little activity (25% inhibition, $P = 0.08$) in the H1975 tumor model. Because compound 4 had a less than desired kinase selectivity profile (data not shown), it was not further evaluated for tumor growth inhibition. All tested compounds were well tolerated with no body weight loss in contrast to afatinib and erlotinib (Fig. 4B).
contrast, afatinib and erlotinib inhibited EGFR<sup>WT</sup> by 68% and 48%, respectively, 6 hours post final dose (Fig. 5C).

**Discussion**

To date, patients with NSCLC who have failed treatment with TKIs and whose tumors have an EGFR T790M mutation have limited treatment options and represent a high-risk group with fatal disease and unmet need. Although chemotherapies, including cisplatin, carboplatin, gemcitabine, paclitaxel, docetaxel, and pemetrexed have shown activity in patients with EGFR mutations following failure of first-generation EGFR TKIs, these therapies have significant toxicity and patients invariably progress (30–32). So far, the second-generation inhibitors, including afatinib, neratinib, and dacomitinib, have provided limited therapeutic benefit in patients with drug-resistant NSCLC (17, 19, 20). These compounds are hindered by dose-limiting toxicities associated with EGFR<sup>WT</sup> inhibition, and therefore, cannot be adequately dosed to inhibit the T790M drug-resistant mutation. Moreover, these inhibitors do not prevent the emergence of the T790M drug-resistant mutation (8, 33). Therefore, there is a clinical need for next generation inhibitors that can overcome the drug-resistant EGFR mutation while sparing wild-type EGFR, and thus, avoid or reduce the dose-limiting toxicities that have restricted the therapeutic benefits of first- and second-generation EGFR inhibitors.

We initiated a lead optimization program to address this issue, and through structure-based design identified 4 lead 2,4-diaminopyrimidine irreversible inhibitors that potently inhibited the key T790M drug-resistant EGFR mutation, but were wild-type sparing. Moreover, these compounds also inhibited the most common activating EGFR mutations, and as such, can be used in both first- and later-line settings. Recently, another EGFR mutant-selective inhibitor, AZD9291, was described and is showing promising clinical activity (25).

Given the long protein half-life, strong clinical validation, clearly defined need for a mutant selectivity profile, and the presence of a poorly conserved cysteine in the ATP-binding pocket, EGFR represented an excellent target for selective covalent inhibition. Sequence analysis showed only 10 of approximately 500 human kinases shared the targeted cysteine 797 in the ATP binding site of the catalytic domain of EGFR. Three of 10 kinases, including EGFR, have the smaller leucine residue at position 792. However, 7 of 10 kinases possess larger side chain residues, such as tyrosine or phenylalanine, in this position, which provided an opportunity for selective inhibition of EGFR against kinases that share Cys 797. For example, modeling suggested that the o-methoxy substituent clashes with the tyrosine residue at position 476 in BTK. This selectivity effect of an o-methoxy group has been previously observed (22, 34). Selectivity against EGFR<sup>WT</sup> was conferred most likely by reducing hydrophobic interactions at the gatekeeper position between the...
5-substituent of the diaminopyrimidine ring with the smaller threonine residue of EGFR WT in the kinase domain. Compound 3 was highly selective with only 13 kinases, including EGFRWT and EGFRL858R/T790M, of a 62 kinase panel inhibited greater than 50% at a high concentration of 1 μmol/L. Determination of IC50app equal to or less than 50% as a measure of potency indicated that compound 3 only potently inhibited EGFR L858R/T790M kinase and that the other kinases were relatively weakly or not inhibited at relevant doses. Selectivity over EGFRWT was >12-fold and selectivity for the other kinase at least 20- to 2,000-fold. Cellular assessment of inhibition of signaling for select kinases (BTK, JAK3, and AUR-A) confirmed that compound 3 was highly selective for EGFR L858R/T790M kinase (data not shown). Kinase selectivity against a wider panel of 434 kinases confirmed the selectivity of compound 3, especially against other kinases that share Cys 797 with EGFR (26).

The long protein half-life of EGFR provides for prolonged duration of drug action that extends well beyond the time frame of systemic covalent drug exposure. Pharmacokinetic and pharmacodynamic correlation have shown that although compound exposure decreased approximately 5-fold at 100 mg/kg from 1 to 6 hours, maximum pharmacodynamic modulation (>90%) was observed at 6 hours post final dose in the H1975 tumor xenograft model. A multidose-level pharmacokinetic/
pharmacodynamic study showed that dosing compound 3 at 100 mg/kg once daily potently inhibited EGFR pathway signaling. Evaluation of normal lung tissues showed no inhibition of EGFRWT at the efficacious dose of 100 mg/kg once daily even though similar compound exposure was achieved in lungs as in the H1975 tumors. Immunohistochemical analysis of normal mouse skin from compound 3–treated animals also showed no inhibition of EGFRWT, whereas erlotinib–treated animals showed complete inhibition of EGFRWT (26, 35). These results confirmed that compound 3 selectively inhibits mutant EGFR and is wild-type sparing. Further studies have shown that pharmacodynamic inhibition persisted up to 24 hours post final dose in H1975 tumors, and as expected does not show inhibition in normal lung tissues (26, 35). These data underscore the benefits of covalent inhibitors with prolonged duration of activity, long after compound has been cleared. In H1975 tumors, the pharmacokinetic/pharmacodynamic relationship indicated that ≥80% inhibition of pEGFR and target occupancy were achieved at a total tumor concentration of >200 ng/mL.

Compound 3 showed increased tumor growth inhibition over the other compounds and was as efficacious as afatinib or WZ4002. This compound was further profiled for drug-like properties and displayed good kinase selectivity and safety profile with no major concerns for CYP inhibition and induction, hERG and off-target drug safety panel screening (CEREP; data not shown). Therefore, this compound was selected as a clinical candidate, designated CO-1686, and is currently being developed by Clovis Oncology. Recently, Walter and colleagues described tumor regression by CO-1686 as a single agent in mouse models of L858R/T790M and DelE746-A750 human–driver tumors at 100 mg/kg/day when administrated orally, either on a once or twice daily schedule (26).

CO-1686 is currently being evaluated in a phase I/II clinical trial in patients with EGFR-mutant advanced NSCLC who have received prior EGFR-directed therapy (NCT01526928). Recently, CO-1686 demonstrated a 67% RECIST partial response rate in evaluable T790M-positive patients (36). Metastasis shrinkage was observed at multiple organ sites, including brain and liver (37). CO-1686 was well tolerated at all doses with no dose-related signs of diarrhea or rash, consistent with no inhibition of EGFRWT (36, 37), and suggests that CO-1686 is significantly differentiated from other clinical EGFR inhibitors.

Disclosure of Potential Conflicts of Interest

T.C. Harding has ownership interest (including patents) in Clovis Oncology, Inc. M. Nacht has ownership interest in Celgene stock options. No potential conflicts of interest were disclosed by the other authors.

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Study supervision: R. Tjin Tham Sjin, R. Karp, M. Raponi, T.C. Harding, M. Nacht, W.F. Westlin

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Molecular Cancer Therapeutics

**In Vitro and In Vivo Characterization of Irreversible Mutant-Selective EGFR Inhibitors That Are Wild-Type Sparing**

Robert Tjin Tham Sjin, Kwangho Lee, Annette O. Walter, et al.


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