Discovery of a Mutant-Selective Covalent Inhibitor of EGFR that Overcomes T790M-Mediated Resistance in NSCLC

Annette O. Walter, Robert Tjin Tham Sjin, Henry J. Haringsma, Kadoaki Ohashi, Jing Sun, Kwangho Lee, Aleksandr Dubrovskiy, Matthew Labenski, Zhendong Zhu, Zhigang Wang, Michael Sheets, Thia St Martin, Russell Karp, Dan van Kalken, Prasoon Chaturvedi, Deqiang Niu, Mariana Nacht, Russell C. Petter, William Westlin, Kevin Lin, Sarah Jaw-Tsai, Mitch Raponi, Terry Van Dyke, Jeff Etter, Zoe Weaver, William Pao, Juswinder Singh, Andrew D. Simmons, Thomas C. Harding, and Andrew Allen

ABSTRACT

Patients with non-small cell lung cancer (NSCLC) with activating EGF receptor (EGFR) mutations initially respond to first-generation reversible EGFR tyrosine kinase inhibitors. However, clinical efficacy is limited by acquired resistance, frequently driven by the EGFR T790M mutation. CO-1686 is a novel, irreversible, and orally delivered kinase inhibitor that specifically targets the mutant forms of EGFR, including T790M, while exhibiting minimal activity toward the wild-type (WT) receptor. Oral administration of CO-1686 as single agent induces tumor regression in EGFR-mutated NSCLC tumor xenograft and transgenic models. Minimal activity of CO-1686 against the WT EGFR receptor was observed. In NSCLC cells with acquired resistance to CO-1686 in vitro, there was no evidence of additional mutations or amplification of the EGFR gene, but resistant cells exhibited signs of epithelial–mesenchymal transition and demonstrated increased sensitivity to AKT inhibitors. These results suggest that CO-1686 may offer a novel therapeutic option for patients with mutant EGFR NSCLC.

SIGNIFICANCE: We report the preclinical development of a novel covalent inhibitor, CO-1686, that irreversibly and selectively inhibits mutant EGFR, in particular the T790M drug-resistance mutation, in NSCLC models. CO-1686 is the first drug of its class in clinical development for the treatment of T790M-positive NSCLC, potentially offering potent inhibition of mutant EGFR while avoiding the on-target toxicity observed with inhibition of the WT EGFR. Cancer Discov; 3(12); 1404–15. ©2013 AACR.

Authors’ Affiliations: 1Clovis Oncology Inc., San Francisco, California; 2Celgene Avilomics Research, Bedford, Massachusetts; 3Division of Hematology-Oncology, Department of Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee; 4Mouse Cancer Genetics Program; and 5Center for Advanced Preclinical Research, Science Applications International Corporation-Frederick, Inc., National Cancer Institute, Frederick, Maryland
INTRODUCTION

Despite years of research and prevention strategies, lung cancer remains the most common cancer worldwide, with approximately 1.35 million new cases annually. Non-small cell lung cancer (NSCLC) accounts for almost 85% of all lung cancers (1). In addition, lung cancer continues to be the most common cause of cancer-related deaths worldwide, with a 5-year survival rate of less than 20% in patients in the United States (http://seer.cancer.gov).

Activating mutations in the EGF receptor (EGFR) are key drivers of NSCLC malignancy in 10% to 15% of patients of European descent and approximately 30% of patients of East Asian descent (2). Patients with the most common EGFR mutations (L858R mutation in exon 21 and delE746-A750 deletions in exon 19) typically have good responses to therapy with first-generation reversible EGFR tyrosine kinase inhibitors (TKI), such as erlotinib or gefitinib (3–5). Toxicity associated with both erlotinib and gefitinib includes skin rash and diarrhea related to inhibition of wild-type EGFR (WT EGFR) in skin and intestine, respectively (6).

Despite the impressive initial response to treatment, disease progression generally occurs after 9 to 14 months of erlotinib or gefitinib therapy, driven in approximately 60% of cases by a second-site EGFR point mutation that results in the substitution of threonine with methionine at amino acid position 790 [T790M (7–10)]. Research suggests that T790M mediates resistance to first-generation EGFR inhibitors by acting as a “gatekeeper” mutation, inducing steric hindrance in the ATP-binding pocket and preventing inhibitor binding (8, 11, 12). Additional work has indicated that T790M increases the affinity of EGFR for ATP, therefore out-competing ATP-competitive TKIs and restoring enzymatic activity in their presence (13).

Patients with mutant EGFR NSCLC who have failed treatment with first-generation EGFR TKIs and have acquired resistance through the T790M mutation have few treatment options. There are no targeted therapies for these patients, who are currently treated with cytotoxic chemotherapy that has limited efficacy, but significant toxicity, in the second- or third-line setting. Although second-generation irreversible HER-family TKIs, including dacomitinib (PF299804) and afatinib (BIBW2992), are able to inhibit T790M-mutant EGFR in vitro, in clinical trials these agents have not been shown to induce compelling responses in patients who have failed first-generation TKIs (14). Likely due to the potent inhibition of WT EGFR and its consequent toxicities, these agents cannot reach exposures in the clinic required to inhibit T790M in tumor tissue. To circumvent this problem, a covalent inhibitor that inhibits mutant EGFR, including T790M, more potently than the WT receptor, termed WZ4002, was described previously (15), but did not progress into human clinical trials. Recently, PKC412 (midostaurin), an indolocarbazole compound currently in development as a FLT-3 inhibitor in acute myeloid leukemia, was described to selectively inhibit T790M over WT EGFR in a noncovalent fashion (16). As a staurosporine-derived compound, PKC412 has a broad kinase inhibition profile (17), and its clinical application in T790M-positive NSCLC is therefore questionable, particularly as it does not potently inhibit activating EGFR mutations. Hence, there is currently a need for an EGFR TKI that is able to effectively treat T790M-positive lung tumors.
In the current study, we report the preclinical validation of a 2,4-disubstituted pyrimidine compound, CO-1686, that irreversibly and selectively inhibits mutant EGFR, in particular the T790M drug-resistance mutation, in NSCLC models. Oral administration of CO-1686 leads to tumor regressions in cell-based and patient-derived xenograft models as well as in a transgenic mouse model expressing mutant forms of human EGFR. In these models, acquired resistance to this third-generation EGFR TKI is not mediated by further second-site mutations or amplification of the EGFR gene, and resistant cells seem to have a reduced dependence on EGFR signaling compared with parental cells. CO-1686 is currently being evaluated in phase I/II clinical trials in EGFR-mutant NSCLC.

RESULTS

CO-1686 Is a Potent and Irreversible Inhibitor of EGFR In Vitro

Using a structure-based approach, we designed and developed a potent 2,4-disubstituted pyrimidine molecule, CO-1686, that covalently modified the conserved Cys797 in the ATP-binding pocket of the EGFR kinase domain (Fig. 1A). As shown in the structural model of CO-1686 in complex with EGFR<sup>T790M</sup>, the meta-acrylamide points to Cys797 and forms the covalent bond (Fig. 1B). To confirm that CO-1686 covalently modified the EGFR<sup>L858R/T790M</sup> kinase, we performed mass spectrometry. Incubation of CO-1686 with recombinant EGFR<sup>L858R/T790M</sup> protein resulted in a mass shift of EGFR<sup>L858R/T790M</sup> consistent with the formation of a covalent complex between CO-1686 and the EGFR<sup>L858R/T790M</sup> protein (Supplementary Fig. S1A). Pepsin digest analyses confirmed that CO-1686 modified the conserved Cys797 residue in the EGFR<sup>L858R/T790M</sup> kinase domain (Supplementary Fig. S1B and S1C).

To determine the selectivity and potency of CO-1686 in vitro, we performed kinetic studies using recombinant WT EGFR and mutant EGFR<sup>L858R/T790M</sup> kinases. When assessing a covalent inhibitor like CO-1686, potency is expressed by using the ratio ($k_{\text{inact}}/K_i$) of the inactivation rate constant ($k_{\text{inact}}$) with respect to the binding constant ($K_i$) as the amount of active EGFR enzyme changes over time. CO-1686 is a potent inhibitor of EGFR<sup>L858R/T790M</sup> kinase ($k_{\text{inact}}/K_i = (2.41 ± 0.30) \times 10^4$ (mol/L)<sup>-1</sup>s<sup>-1</sup>) and is approximately 22-fold more selective than WT EGFR ($k_{\text{inact}}/K_i = (1.12 ± 0.14) \times 10^3$ (mol/L)<sup>-1</sup>s<sup>-1</sup>; Table 1). As expected for a first-generation TKI, erlotinib potently inhibited WT EGFR ($K_i = 0.40 ± 0.03$ nmol/L), whereas it had little activity against EGFR<sup>L858R/T790M</sup> kinase ($K_i = 98.0 ± 8.1$ nmol/L). CO-1686 also demonstrated a favorable selectivity profile when profiled against 434 kinases (Supplementary Table S1A). Twenty-three targets (representing 14 different kinases) were identified to be inhibited more than 50% at 0.1 μmol/L CO-1686. EGFR del19+, T790M-, L858R/T790M-, and L858R-mutant kinases demonstrated the highest degree of inhibition, indicating the specificity of CO-1686; however, other kinase targets were observed to be inhibited at lower potency, including focal adhesion kinase (FAK), CHK2, ERBB4, and Janus-activated kinase 3 (JAK3). Specificity was also examined by apparent IC<sub>50</sub> determination in kinases known to possess a cysteine equivalent to C797 in EGFR (Supplementary Table S1B). In comparison with EGFR<sup>T790M</sup>, other C79-related kinases are observed to be...
Development of Covalent EGFR\textsuperscript{770M} Inhibitor in NSCLC

Inhibited by CO-1686 with an IC\textsubscript{50} greater than 6-fold, including members of the Tec-family and JAK3. In summary, CO-1686 is the first EGFR inhibitor in clinical development that is mutant-selective and inhibits T790M more potently than WT EGFR.

CO-1686 Potently and Selectively Inhibits Growth of NSCLC Cells Expressing Mutant EGFR and Induces Apoptosis

Selectivity and activity of CO-1686 against cells expressing EGFR mutations was demonstrated in a panel of cell lines (Fig. 2A and Supplementary Table S2). The effect of CO-1686 treatment on cell growth was determined in four NSCLC cell lines expressing mutant EGFR (HCC827, PC9, HCC827-EPR, and NCI-H1975) and in three cell lines expressing WT EGFR (A431, NCI-H1299, and NCI-H358). HCC827 and PC9 cell lines both harbor the EGFR\textsuperscript{L858R/T790M}–activating mutation in exon 19. HCC827-EPR is a resistant clone of HCC827 that acquired T790M in response to continuous exposure to erlotinib and the MET inhibitor PHA-665,752 (18). NCI-H1975 is another T790M-positive cell line that harbors the EGFR\textsuperscript{L858R/T790M} double mutation. CO-1686 potently inhibited proliferation in the mutant EGFR NSCLC cells with GI\textsubscript{50} values ranging from 7 to 32 nmol/L. In comparison, the GI\textsubscript{50} value for A431 cells, an epidermoid cell line that is driven by amplified WT EGFR (19), was 547 nmol/L. Two cell lines expressing WT EGFR in the presence of an NRAS or Kras mutation (NCI-H1299 and NCI-H358, respectively) were inhibited by CO-1686 at a concentration of 4275 and 1806 nmol/L, respectively. Similar results were obtained when determining effects of CO-1686 on EGFR signaling by immunoblot analysis in the WT-driven A431 cells compared with the EGFR-mutant cells. IC\textsubscript{50} values for inhibition of EGFR phosphorylation were above 2,000 nmol/L in the three WT EGFR-expressing cells, whereas CO-1686 inhibited p-EGFR with IC\textsubscript{50} values ranging from 62 to 187 nmol/L in the mutant EGFR-expressing cells (Supplementary Table S2) confirming the mutant-selective properties of CO-1686. CO-1686 inhibits cell proliferation and EGFR phosphorylation equally in the parental HCC827 (EGFR\textsuperscript{L858R}) as well as the erlotinib-resistant HCC827-EPR (del19/T790M) clone. Treatment with CO-1686 induces apoptosis in both cell lines as demonstrated by an increase in cleaved PARP and Bim-EL protein (Fig. 2B), irrespective of the T790M status. Erlotinib, on the other hand, has no effect in the T790M-positive HCC827-EPR cells (Fig. 2B). In addition, we treated PC-9/ER (del19/T790M) and H3255/XLR cells (L858R/T790M) with erlotinib and CO-1686 in standard growth inhibition assays. Both are polyclonal populations of cells that acquired T790M in response to continuous exposure to EGFR TKIs (20). Again, CO-1686 was superior to erlotinib in inhibiting the growth of these cells (Supplementary Fig. S2A and S2B). Collectively, the EGFR signaling and cell growth/apoptosis data indicate that CO-1686 selectively and potently affects cells harboring activating EGFR mutations as well as the T790M resistance mutation and has minimal activity in cells expressing WT EGFR.

CO-1686 Has Activity Against Minor EGFR Mutants In Vitro

We examined the effect of CO-1686 against other EGFR mutations occasionally found in lung cancer, such as G719S, an exon 19 insertion mutation (ex19ins: I744-K747insKIPVAI), and L861Q, in surrogate kinase assays. All of these except the exon 20 insertion have been associated with sensitivity to first-generation EGFR TKIs (21). Similar to erlotinib, CO-1686 was active against G719S, the exon 19 insertion, and L861Q, but not against the exon 20 insertion (Supplementary Fig. S3).

CO-1686 Demonstrates Antitumor Activity in NSCLC EGFR-Mutant Xenograft Models

Initial CO-1686 pharmacokinetics were evaluated in female NCRnu.nu mice (n = 3/group) following intravenous and oral
**CO-1686 Is WT EGFR Sparing In Vivo**

To demonstrate the mutant selectivity and WT-sparing properties of CO-1686 in vivo, CO-1686 potency was evaluated in the A431 xenograft model that has been previously shown to be dependent on WT EGFR for proliferation (19, 22). In this model, CO-1686 dosed at 50 mg/kg twice daily was compared with erlotinib and afatinib at their respective highest tolerated dose in mice [75 mg/kg once daily per os and 20 mg/kg once daily intraperitoneally (i.p.), respectively]. CO-1686 exhibited a minimal [36% tumor growth inhibition (TGI)] although significant ($P < 0.01$) reduction in tumor growth, whereas both erlotinib and afatinib administration resulted in tumor regression (Fig. 3D). A431 tumor lysates from animals in each treatment group were analyzed for phosphorylated WT EGFR at tyrosine 1068 (a known marker for EGFR activation; Fig. 4A and B). Compared with the vehicle control group, A431 tumors harvested from animals treated with 50 mg/kg twice-daily CO-1686 had no detectable reductions in EGFR phosphorylation (84% phosphorylated EGFR relative to vehicle; $P = 0.58$; Fig. 4B). In contrast, A431 tumors harvested from animals treated with 75 mg/kg once-daily erlotinib and 20 mg/kg once-daily afatinib had statistically significant reductions in EGFR phosphorylation (12% and 2% phosphorylated EGFR relative to vehicle, respectively; $P < 0.05$ in both cases; Fig. 4A and B). In addition, consistent with a lack of WT EGFR inhibition, CO-1686 administration did not cause significant mouse body weight alterations in body weight with either dosing schedule (Supplementary Fig. S5A and S5B). However, the twice daily CO-1686 administration schedule was statistically superior to once-daily day 15 postdosing ($P < 0.01$) and was therefore chosen as the optimal dosing regimen (Supplementary Fig. SSA).

**Figure 3.** In vivo antitumor efficacy of CO-1686 in lung (A) NCI-H1975, (B) LUM1868, (C) HCC827, and (D) squamous epidermoid A431 xenograft models. CO-1686 was administered orally, daily, or twice daily at concentrations ranging from 3, 10, 30, and 100 mg/kg/d. N = 10 animals/group. Data plotted as mean ± SEM.

Walter et al.
changes in the A431 xenograft experiment (Supplementary Fig. S6). Body-weight loss was observed in both erlotinib- and afatinib-treated groups (P < 0.01).

Sparing of WT EGFR downstream signaling by CO-1686 was also examined in mouse skin (Fig. 4C). Administration of CO-1686 at the efficacious dose of 100 mg/kg once daily for 5 days had no effect on phosphorylated mitogen-activated protein kinase (pMAPK) levels in normal mouse skin, whereas erlotinib and afatinib both inhibited MAPK phosphorylation (Fig. 4C).

These results demonstrate that CO-1686, contrary to first- and second-generation TKIs, spares WT EGFR in vivo at efficacious exposures that cause significant growth inhibition of tumors harboring mutant EGFR, including T790M.

**CO-1686 Demonstrates Antitumor Activity in Human EGFR<sup>L858R</sup>- and EGFR<sup>L858R/T790M</sup>-Expressing Transgenic Mice**

The efficacy of CO-1686 was examined in the bitransgenic EGFR<sup>L858R</sup>-CCSP<sup>rtTA</sup> genetically engineered mouse (GEM) model that develops lung adenocarcinoma upon human mutant EGFR transgene induction (23). Expression of mutant human EGFR<sup>L858R</sup> was induced in GEM mice by doxycyline feeding, and tumor development was monitored in animals by MRI of the lungs. In this model, diffuse adenocarcinoma dependent on human EGFR<sup>L858R</sup> expression develops over the course of several weeks of induction. Four weeks after induction, baseline MRI showed that neoplasms had developed in the lungs and animals...
were randomized to vehicle, CO-1686, or erlotinib treatment. Twenty-one days posttreatment initiation, animals underwent MRI to observe the impact of drug administration on tumor volume. Representative examples of posttreatment MRI scans are shown in Fig. 4E. The mean tumor volumes (MTV) of mice in the vehicle, afatinib, and CO-1686 groups were 780, 486, and 7 mm³, respectively. Complete or near-complete responses were thus observed in all mice treated with CO-1686. Immunohistochemical analysis performed on lung tumor sections from vehicle- and CO-1686–treated animals confirmed that treatment with CO-1686 greatly reduced both tumor burden and cell proliferation [hematoxylin and eosin (H&E) and Ki67 staining; Supplementary Fig. S7] in the transgenic models. Taken together, CO-1686 exhibits potent antitumor activity as a single agent in transgenic models with a mesenchymal cell signature in the COR clones, vimentin (VIM) expression was upregulated and E-cadherin (CDH1) expression was downregulated in the CO-1686–resistant clones at the protein level by EGFR immunoblot analysis (Fig. S8B) as well as at the protein level by EGFR immunohostochemistry analysis performed on lung tumor sections from vehicle- and CO-1686–treated animals confirmed that treatment with CO-1686 greatly reduced both tumor burden and cell proliferation [hematoxylin and eosin (H&E) and Ki67 staining; Supplementary Fig. S7] in the transgenic models. Taken together, CO-1686 exhibits potent antitumor activity as a single agent in transgenic models with one of the most common primary human EGFR mutations (L858R), as well as the most common EGFR mutation associated with acquired resistance (L858R/T790M). These data suggest that CO-1686 may have utility in both first-line and previously treated patients with EGFR-mutant NSCLC.

Acquired Resistance to CO-1686 Is Associated with EMT in T790M-Positive NCI-H1975 Cells

To address acquired resistance to this novel third-generation mutant-selective EGFR TKI, we continuously exposed NCI-H1975 cells harboring the L858R/T790M mutation for several months to increasing doses of CO-1686 until resistance developed. We isolated five independent CO-1686–resistant (COR) clones, designated as COR 1-1, COR 1-2, COR 10-1, COR 10-2, and COR 10-3. Cell viability was determined for all clones after treatment with a first-, second-, and third-generation EGFR TKI (erlotinib, afatinib, and CO-1686, respectively), and all CORs exhibited resistance to CO-1686, erlotinib, afatinib, and third-generation EGFR TKI (erlotinib, afatinib, and CO-1686, respectively), and all CORs exhibited resistance to all three types of inhibitors, whereas the parental cell line was resistant only to erlotinib (Table 2). The resistance phenotype was maintained for at least 3 months in the absence of CO-1686 treatment as determined by cell viability assays (data not shown). Comparing the cell morphology of COR cell clones with the parental NCI-H1975 cells, the resistant cells seemed to acquire a spindle-like morphology (Supplementary Fig. S8A). The mutational status of 19 common EGFR-siRNA knockdown of EGFR expression for viability (Fig. 5B). Averaged across the two EMT gene signature (Supplementary Fig. S9). To confirm the differential expression of EMT markers identified by RNA-Seq, we analyzed COR-1 and COR10-1 CO-1686–resistant cells using RNA-Seq. Analysis of genes differentially expressed in the CO-1686–resistant cell clones compared with the parental cell line using gene set enrichment analysis (MSigDB, Broad Institute, Cambridge, MA) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (Kanehisa Laboratories, Kyoto University, Kyoto, Japan) demonstrated a significant enrichment of genes involved in epithelial–mesenchymal transition (EMT) in COR-1 and COR10-1 (data not shown). EMT has previously been associated with EGFR TKI resistance in NSCLC (7, 25). In support of the results of the unsupervised analysis, comparison of differentially expressed genes in the COR cell lines with the 76-gene EMT signature developed by Byers and colleagues (26) indicated increases in the presence of a significant (P < 0.0001) overlap comparing the gene sets (Supplementary Table S3A and S3B). In addition, parental and resistant clones are clustered into distinct groups using hierarchical clustering with the Byers’ EMT gene signature (Supplementary Fig. S9).

To confirm the differential expression of EMT markers identified by RNA-Seq, we analyzed COR-1 and COR10-1 CO-1686–resistant clones by quantitative RT-PCR (qRT-PCR, Fig. 5C) and Western blot analysis (Supplementary Fig. S10). Consistent with a mesenchymal cell signature in the COR clones, vimentin (VIM) expression was upregulated and E-cadherin (CDH1) downregulated in the CO-1686–resistant clones at the protein and RNA level. qRT-PCR analysis of additional markers further supported EMT, including the upregulation of AXL, ZEB1, and MET.
Development of Covalent EGFR<sup>T790M</sup> Inhibitor in NSCLC

CDH5, and FN1 and the downregulation of the epithelial markers MIR200B (27), CLDN4, EPCAM, and CLDN7. EGFR expression was moderately reduced in the COR cell clones compared with the parental NCI-H1975 cell line; however, EGF ligand expression was induced as demonstrated by qRT-PCR (Fig. 5C).

Amplification of the MET gene is observed in approximately 5% to 10% of patients with NSCLC failing first-generation EGFR TKIs (10, 28). An additional subpopulation of EGFR TKI-resistant patients also upregulate the MET ligand, hepatocyte growth factor (HGF; ref. 29). Analysis of phospho-MET in the COR clones indicated that the HGF pathway was not activated following CO-1686 resistance in the NCI-H1975 cell line (Supplementary Fig. S10), and expression of the MET receptor was significantly reduced in the COR cell clones at the RNA level (Fig. 5C). ERBB2 amplification may be associated with acquired resistance, especially in patients without detected T790M mutations (10, 30). Activation of additional EGF receptor members ERBB2 and ERBB3 by phosphorylation was also not observed and actually seemed to be decreased in the COR clones compared with the parental NCI-H1975 cell line (Fig. 5C; Supplementary Fig. S10).

CO-1686–Resistant NSCLC Cell Lines Are Sensitive to AKT Inhibition

We next examined potential druggable targets overexpressed in CO-1686–resistant cell clones that when inhibited could potentially restore CO-1686 sensitivity. Higher basal levels of phosphorylated AKT (pAKT) were observed in the COR cell clones compared with the parental NCI-H1975 cell line (Fig. 5C).

**Figure 5.** A and B, CO-1686–resistant NCI-H1975 cell clones, COR1-1 and COR10-1 display a reduced dependence on EGFR signaling for survival. A, effect of EGFR siRNA transfection on EGFR protein expression in CO-1686-resistant clones (COR 1-1 and COR 10-1) and NCI-H1975 parental as determined by Western blotting. B, impact of EGFR siRNA knockdown on cell viability in NCI-H1975, COR1-1, and COR10-1 clones. Cell viability was determined 72 hours post-siRNA transfection and plotted relative to the siRNA control treated group (representing 100%). Data plotted as mean ± SEM. *, P < 0.05 and **, P < 0.005 comparing siCTRL and siEGFR groups. C, qRT-PCR analysis of EMT- and EGFR-related genes in COR1-1 and COR10-1 cell clones. Data plotted as fold change relative to parental NCI-H1975 cells ± SEM. All data shown were P < 0.05 comparing COR clones to parental. D, inhibition of AKT restores partial sensitivity of COR cell clones to CO-1686. COR10-1 was exposed to CO-1686, the AKT inhibitors MK-2206 (left) or GDC-0068 (right), or an equimolar combination of AKT inhibitor + CO-1686. 72 hours postdrug addition cell viability was determined by CellTiter-Glo. Data plotted as percentage viability relative to dimethyl sulfoxide (DMSO) (no drug) control.

ERBB2 amplification may be associated with acquired resistance, especially in patients without detected T790M mutations (10, 30). Activation of additional EGF receptor members ERBB2 and ERBB3 by phosphorylation was also not observed and actually seemed to be decreased in the COR clones compared with the parental NCI-H1975 cell line (Fig. 5C; Supplementary Fig. S10).
complete reduction of pAKT to negligible levels in the NCI-H1975 parental cell line (Supplementary Fig. S10). In comparison, the CO-1686 addition to COR cell clones, while reducing pAKT, did not completely inhibit pAKT levels (Supplementary Fig. S10). Examining AKT isoform RNA expression in the COR cell clones by RNA-seq and qRT-PCR indicated that AKT3 expression was upregulated 12- and 121-fold, respectively (P < 0.001; Fig. 5C). Consistent with the RNA data, AKT3 was also upregulated in the COR clones compared with the parental cell line at the protein level (Supplementary Fig. S10). AKT2 RNA was increased approximately 2-fold (P < 0.0001) by RNA-Seq and qRT-PCR analysis (Fig. 5C). No change was observed for AKT1 RNA expression (data not shown). Given the upregulation of AKT isoforms and continued AKT signaling in the presence of CO-1686 in the COR cell clones, we examined the impact of AKT inhibition on CO-1686 resistance using the AKT inhibitors MK-2206 (31) and GDC-0068 (32) that are currently in clinical development for multiple oncology indications. Although not effective when used as single agents (Fig. 5D), both MK-2206 and GDC-0068 restored partial drug sensitivity to the COR10-1 clone when used together in an equimolar fashion with CO-1686, with a combination index (CI) of 0.1, indicative of strong synergism. Synergy between AKT inhibition and CO-1686 was also observed in additional COR cell clones (data not shown).

The upregulation of AXL receptor tyrosine kinase has previously been reported in EGFR-resistant NSCLC (25, 26). Consistent with these reports, AXL was observed to be upregulated at the RNA (Supplementary Table S2; Fig. 5C) and protein levels (Supplementary Fig. S10) in the CO-1686-resistant COR cell clones compared with the parental cell line. In addition, growth arrest–specific 6 (GAS6), a ligand of AXL implicated in EGFR TKI resistance (25, 33), was significantly upregulated in COR cell clones as determined by RNA-Seq and qRT-PCR analysis (Fig. 5C). AXL kinase function was inhibited using XL-880, a VEGFR/MET/AXL inhibitor [foretinib; Exelixis (34)]. Pharmacologic inhibition of AXL kinase activity restored partial sensitivity of EGFR TKI–resistant COR cell clones to CO-1686 (Supplementary Fig. S11A); however, this effect was modest in comparison with previous reports (25), with a CI of 0.53 and 0.44 for COR1-1 and COR10-1, respectively, indicative of synergy. The combination of CO-1686 with R428, an AXL-selective small-molecule inhibitor (35), produced comparable results to XL-880 (data not shown). Given the multiple targets inhibited by small-molecule kinase inhibitors, the role of AXL inhibition in sensitizing COR cell clones to CO-1686 was specifically examined using siRNA to knockdown AXL expression (Supplementary Fig. S11B and S11C). Despite effective AXL knockdown at the mRNA and protein level (Supplementary Fig. S11B), only a modest reduction in CO-1686 GI50 in the COR1-1 and COR10-1 clones of 2.2- and 1.6-fold, respectively (Supplementary Fig. S11C), was observed. In addition, we generated stable pools of parental NCI-H1975 cells that overexpressed AXL at the mRNA and protein level (Supplementary Fig. S11D) and determined the GI50 for CO-1686. Compared with the parental cell line, NCI-H1975 cell populations overexpressing AXL had a slight increase in CO-1686 GI50 of approximately 2-fold (Supplementary Fig. S11E). To place this result into perspective, the CO-1686 GI50 of the COR cell clones 1-1 and 10-1 that overexpress AXL ~100-fold over the parental NCI-H1975 cell line (Fig. 5C) are more than 1,000 nmol/L, and the NCI-H1975 cell line engineered to overexpress AXL at 195-fold above the parental cell line is 52 nmol/L. Therefore, in our studies, although small-molecule inhibitors of AXL kinase can partially restore the sensitivity of COR cell lines to CO-1686, the specific role of AXL inhibition in this effect seems minimal and perhaps related to the broader spectrum of kinase inhibited by these agents.

DISCUSSION

T790M-driven lung cancer is a growing clinical problem given widespread screening of patients with lung adenocarcinoma for activating EGFR mutations and consequent recommended use of erlotinib or gefitinib as first-line therapy in mutant EGFR patients (National Comprehensive Cancer Network guidelines version 2.2013 NSCLC). Significantly, early clinical evidence suggests that a secondary T790M mutation can drive resistance to second-generation TKIs such as afatinib as well (36). CO-1686 has attractive relevant properties as a potential therapeutic for T790M-positive mutant EGFR NSCLC. It potently inhibits the kinase activity of EGFR carrying the T790M mutation, both in vitro and after oral administration in vivo (cell line xenograft and patient-derived xenograft). This inhibitory activity against a pathogenic mutant form of EGFR is not accompanied by meaningful WT EGFR inhibition, suggesting that drug tolerability may be superior to first- and second-generation TKIs, for which key toxicities of skin rash, diarrhea, and interstitial lung disease are attributed to WT EGFR inhibition (6, 37). CO-1686 is currently in a human dose-escalation study to evaluate safety, pharmacokinetics, and preliminary efficacy in previously treated patients with mutant EGFR NSCLC (ClinicalTrials.gov identifier: NCT01526928). Although still in dose-escalation phase with the maximal-tolerated dose not reached, objective RECIST (Response Evaluation Criteria in Solid Tumors) responses have been observed in patients with heavily pretreated T790M-positive NSCLC administered CO-1686 following the development of resistance to erlotinib (38). In addition, metastasis shrinkage has been observed at multiple organ sites, including both brain and liver metastases. Consistent with the preclinical data presented in this article, CO-1686 seems to be well-tolerated, with no evidence of dose-related diarrhea or rash.

CO-1686 potently inhibits single activating mutant forms of EGFR, both in vitro and in vivo (cell line xenograft and human EGFR transgenic mouse models). Assuming that this nonclinical activity translates into clinical efficacy in treatment-naïve mutant EGFR patients, an interesting question is whether the development of acquired resistance to CO-1686 will be slower than acquisition of resistance to first-generation TKIs, such that progression-free survival with CO-1686 would be superior to erlotinib. Initial studies of in vitro resistance to CO-1686 demonstrate enrichment of an RNA-based signature associated with EMT. A similar signature has been observed in cell lines resistant to EGFR and PI3K/AKT inhibitors (26). In support of the CO-1686 resistance data performed in the NCI-H1975 cell background, we have also generated CO-1686-resistant clones of the HCC827 cell line (EGFRΔL858R). EMT is also associated with resistance in these cell clones, with the downregulation of E-cadherin and upregulation of vimentin at the RNA and protein level (data not shown). In addition, recent work from two independent investigators using a tool-compound structurally related to CO-1686, termed CNX-2006 (Cельге), also
Development of Covalent EGFR<sup>790M</sup> Inhibitor in NSCLC

**Research Article**

indicated that EMT is associated with resistance in multiple cell backgrounds (39, 40). Recent articles indicate that resistance to WZ4002, a covalent EGFR inhibitor, is mediated by insulin-like growth factor-I receptor (IGF-IR) pathway activation due to the repression of IGFBP3 expression via promoter methylation (41) or genomic amplification of the MAPK1 gene (42). These specific mechanisms were not observed in our studies (data not shown). Two key next steps with CO-1686 are (i) to assess the kinetics of acquired EMT and compare with acquired T790M using in vitro and in vivo systems, and (ii) to examine the value of combinatorial therapy targeting the specific resistance pathways defined above. It is encouraging that resistance to CO-1686 has not yet been observed to be driven by direct EGFR pathway modification. This suggests that CO-1686 may be sufficient to functionally silence mutant EGFR signaling at clinically achievable concentrations, a key therapeutic goal in mutant EGFR NSCLC that has not been achieved with the currently available clinical TKIs due to acquired resistance within the pathway that leads to patient relapse. Moreover, the sparing of WT EGFR by CO-1686 may allow for evaluation of combination therapies with less potential for overlapping toxicities in patients.

**Methods**

Note that additional materials and methods are included in Supplementary Materials and Methods.

**Cell Culture**

NCI-H1975, HCC827, HCC-H1299, and NCI-H358 human NSCLC adenocarcinoma cells and A431 human epidermoid carcinoma cells were obtained from the American Type Culture Collection. PC-9 cells were a kind gift from Dr. F. Kozumi (National Cancer Center Research Institute and Shien-Lab, Tokyo, Japan). HCC827-EPR cells (18) were a kind gift from Dr. S. Kenichi (Aichi Cancer Center Hospital, Nagoya-shi, Japan). 293H cells were purchased from Invitrogen. PC-9/Ee and H3255/QLR cells were derived in the Pao Laboratory (20). Cell line identity was confirmed by short-tandem repeat analysis (Genetica) and cells were used for no longer than 6 months before being replaced. NCI-H1975, HCC827, HCC-H1299, NCI-H358, and PC-9 cells were grown in RPMI-1640 (Life Technologies) supplemented with 10% FBS (HyClone), 2 mmol/L L-glutamine, and 1% penicillin-streptomycin (Mediatech). A431 cells were grown in Dulbecco’s Modified Eagle Medium (DMEM; Life Technologies) supplemented with 10% FBS (HyClone), 2 mmol/L L-glutamine, and 1% penicillin-streptomycin (Mediatech). HCC827-EPR cells were grown in RPMI-1640 supplemented with 10% FBS, 2 mmol/L L-glutamine, 1% penicillin-streptomycin, 1 μmol/L erlotinib, and 1 μmol/L PHA-66752 (Selleck Chemical). PC-9/Ee and H3255/QLR cells were grown in the same media as above supplemented with 1 μmol/L erlotinib (Selleck). All cells were maintained and propagated as monolayer cultures at 37°C in a humidified 5% CO₂ incubator.

**Cell Proliferation Assays**

Cells were seeded at 3,000 cells per well in growth media supplemented with 5% FBS, 2 mmol/L L-glutamine, and 1% penicillin-streptomycin, allowed to adhere overnight, and treated with a dilution series of test compounds for 72 hours. Cell viability was determined by CellTiter-Glo (Promega), and results were represented as background-subtracted relative light units normalized to a dimethyl sulfoxide (DMSO)-treated control. Growth inhibition (GI₅₀) values were determined by GraphPad Prism 5.04 (GraphPad Software), MK-2206 and XL-880 compounds were obtained from Selleck Chemical. C.I. data were generated using CalcuSyn (Biosoft).

**Cell Signaling Analysis of HC827 and HCC827-EPR**

HCC827 and HCC827-EPR cells were seeded at 1.5 × 10⁴ cells per 10 cm² dish in RPMI-1640, 10% FBS, 2 mmol/L L-glutamine, and 1% penicillin-streptomycin and allowed to adhere overnight. Cells were treated with DMSO, 2 μmol/L CO-1686, or 2 μmol/L erlotinib for 72 hours and lysed. Lysis buffer contained 1× phenylmethylsulfonyl fluoride (Sigma), 1× cell extraction buffer (Life Technologies), 1× protease inhibitor cocktail (Enzo Life Sciences), 1× phosphatase inhibitor cocktails I and II (EMD Chemicals). Total protein concentration was determined using a standard Bradford assay and measured on a NanoDrop 2000 spectrophotometer (Thermo Scientific). Western blotting was performed on cell lysates normalized to 25 μg total protein in loading buffer (LI-COR). Normalized lysates were run on SDS-PAGE and transferred to a nitrocellulose membrane (Life Technologies). The membrane was incubated in Qantik signal enhancement solution (Thermo Scientific), blocked, and incubated overnight at 4°C with primary antibodies (1:1,000) from Cell Signaling Technology. Membranes were washed, incubated with IRDye secondary antibodies (LI-COR), washed again, and imaged on an Odyssey Fc (LI-COR).

**Analysis of Minor EGFR Mutations**

293H cells were transiently transfected with various pcDNA3.1(--) vectors using Lipofectamine 2000 (Invitrogen) and 2 μg DNA per sample as previously described (43). The EGFR G719S, ex19ins, ex20ins, T790M, and L858R + T790M mutations were introduced via site-directed mutagenesis as described previously (43). Following 6-hour treatment with DMSO, afatinib, or increasing doses of erlotinib or CO-1686 at various concentrations, cells were lysed. Immunoblotting was performed using corresponding lysates with antibodies against phospho-EGFR (Santa Cruz Biotechnology) or total EGFR (BD Biosciences). Secondary anti-goat antibody was obtained from Santa Cruz Biotechnology, and secondary anti-mouse antibody was obtained from Cell Signaling Technology.

**Xenograft Studies**

All the procedures related to animal handling, care, and treatment in this article 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). Cell line xenograft studies (NCI-H1975, HCC827, and A431) were performed by Charles River Laboratories. Briefly, NCRnu.nu mice were subcutaneously implanted with 1 × 10⁶ tumor cells in 50% Matrigel (injection volume of 0.2 mL/mouse). Once tumors reached 100 to 200 mm³, animals were dosed with compounds as outlined (N = 10 animals/group). The LUM1686 PDX xenograft study was performed by CrownBio. Briefly, LUM1686 PDX tumor fragments, harvested from donor mice, were inoculated into BALB/c nude mice. Administration of test compounds was initiated at a mean tumor size of approximately 160 mm³. Tumor growth was monitored over time to determine tumor growth inhibition of the experimental agent versus vehicle. The endpoint of the experiment was an MTV in the control group of 2,000 mm³. Percentage TGI was defined as the difference between the MTV of the designated control group and the MTV of the drug-treated group, expressed as a percentage of the MTV of the designated control group. The MTV in the treatment group(s) versus control group was compared using either Student t test for studies with only two groups or ANOVA followed by Tukey test for studies with more than two groups. Data are presented as mean ± SEM.

**Immunohistochemistry of Skin Tissues**

Phospho-p44/42 MAPK (Erk1/2, Thr202/Tyr204, Rabbit monoclonal antibody) was purchased from Cell Signaling Technology. Peroxidase-based immunohistochemistry detection system [EnVision+ System-HRP,
Rabbit (DAB+), antibody diluent, and serum-free protein block were purchased from Dako. Formalin-fixed paraffin-embedded tissue sections underwent antigen retrieval and endogenous peroxidase block, and were incubated with primary antibody overnight at 4°C. Following overnight incubation, slides were rinsed and incubated with a peroxidase-labeled polymer. The tissue sections were then rinsed and stained with 3,3’-diaminobenzidine (DAB) substrate-chromogen and counterstained with Hematoxylin Gill I (EMD Millipore) and bluing reagent (EMD Millipore). Electronic images were captured using a Leica DM1000 LED microscope (>40 objective) with a Leica DFC295 camera and Leica Application Suite (Leica Microsystems).

Disclosures

This project was funded in part with federal funds from the National Cancer Institute, NIH, under Contract No. HHSN26120080001E. W. Pao received additional funding from NIH NCI grants R01CA121210, P01CA129243, U54CA143798, and P30CA68485.

Received June 23, 2013; revised September 16, 2013; accepted September 19, 2013; published OnlineFirst September 24, 2013.

REFERENCES


Grant Support

No potential conflicts of interest were disclosed by the other authors.

Disclosure of Potential Conflicts of Interest

The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the United States Government.

Authors’ Contributions


Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H.J. Haringsma, S. Jaw-Tsa, W. Pao, A.D. Simmons


Direction of preparation of materials on which the study was conducted: J. Eter

Compound design: Z. Zhu

Development of methodology:

H.J. Haringsma is employed as Senior Executive Associate at Clovis Oncology, Inc. W. Pao has received commercial research support from Clovis Oncology, Inc. and is a consultant/advisory board member of the same. M. Nacht, W. Westlin, M. Raponi, Z. Weaver, W. Pao, J. Singh, A. Allen, are authors on other papers authored by other authors.

Disclaimer

No potential conflicts of interest were disclosed by the other authors.
Development of Covalent EGFR T790M Inhibitor in NSCLC

RESERACH ARTICLE


Downloaded from cancerdiscovery.aacrjournals.org on June 16, 2017. © 2013 American Association for Cancer Research.
Discovery of a Mutant-Selective Covalent Inhibitor of EGFR that Overcomes T790M-Mediated Resistance in NSCLC

Annette O. Walter, Robert Tjin Tham Sjin, Henry J. Haringsma, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/2159-8290.CD-13-0314

Supplementary Material
Access the most recent supplemental material at:
http://cancerdiscovery.aacrjournals.org/content/suppl/2013/09/24/2159-8290.CD-13-0314.DC1

Cited articles
This article cites 40 articles, 24 of which you can access for free at:
http://cancerdiscovery.aacrjournals.org/content/3/12/1404.full#ref-list-1

Citing articles
This article has been cited by 35 HighWire-hosted articles. Access the articles at:
http://cancerdiscovery.aacrjournals.org/content/3/12/1404.full#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.