A Constitutive EGFR Kinase Dimer to Study Inhibitor Pharmacology

Justin J. Kim, Ilse K. Schaeffner, David E. Heppner, Ciric To, Pasi A. Jänne, Tyler S. Beyett*, and Michael J. Eck*

Affiliations:
Department of Cancer Biology, Dana-Farber Cancer Institute, Boston, MA 02215, USA (JJK, IKS, TSB, MJE), Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115, USA (JJK, IKS, TSB, MJE), Department of Chemistry, University at Buffalo, Buffalo, NY 14260, USA (DEH), Department of Pharmacology and Therapeutics, Roswell Park Comprehensive Cancer Center, Buffalo, NY 14203 (DEH), Lowe Center for Thoracic Oncology, Dana-Farber Cancer Institute, Boston, MA 02215, USA (CT, PAJ), Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA 02215, USA (CT, PAJ), Department of Medicine, Harvard Medical School, Boston, MA 02115, USA (CT, PAJ).

TSB: Present address, Department of Pharmacology and Chemical Biology, Emory University, Atlanta, GA 30322.

*Corresponding Authors
Abstract

Lung cancer is commonly caused by activating mutations in the epidermal growth factor receptor (EGFR). Allosteric kinase inhibitors are unaffected by common ATP-site resistance mutations and represent a promising therapeutic strategy for targeting drug-resistant EGFR variants. However, allosteric inhibitors are antagonized by kinase dimerization, and understanding this phenomenon has been limited to cellular experiments. To facilitate the study of allosteric inhibitor pharmacology, we designed and purified a constitutive EGFR kinase dimer harboring the clinically relevant L858R/T790M mutations. Kinetic characterization revealed that the EGFR kinase dimer is more active than monomeric EGFR(L858R/T790M) kinase and has the same $K_{m,\text{ATP}}$. Biochemical profiling of a large panel of ATP-competitive and allosteric EGFR inhibitors showed that allosteric inhibitor potency decreased by $>500$-fold in the kinase dimer compared to monomer, yielding $IC_{50}$ values that correlate well with Ba/F3 cellular potencies. Thus, this readily purifiable constitutive asymmetric EGFR kinase dimer represents an attractive tool for biochemical evaluation of EGFR inhibitor pharmacology, in particular for allosteric inhibitors.
Significance Statement

Drugs targeting EGFR kinase are commonly used to treat lung cancers but are affected by receptor dimerization. Here, we describe a locked kinase dimer that can be used to study EGFR inhibitor pharmacology.
Introduction

Lung cancer is the second most diagnosed cancer and is the leading cause of cancer-related deaths in the United States. (Siegel et al., 2022) Major drivers of lung cancer include activating mutations in the epidermal growth factor receptor (EGFR). (Gridelli et al., 2015) EGFR is a receptor tyrosine kinase that primarily signals through the MEK/ERK and PI3K pathways to promote cell proliferation, differentiation, and survival. EGFR activation is driven by growth factor binding to the extracellular domains, which causes dimerization of intracellular kinase domains, autophosphorylation, and downstream signaling. (Uribe et al., 2021) The most common oncogenic activating point mutation is L858R, which is effectively treated by ATP-competitive tyrosine kinase inhibitors (TKIs) due to its decreased affinity for ATP, (Carey et al., 2006; Yun et al., 2007) however, the acquired resistance mutation T790M increases ATP binding affinity and renders first-generation reversible inhibitors ineffective. (Yun et al., 2008) Second- and third-generation irreversible inhibitors bind covalently to C797 and overcome T790M-dependent resistance but are susceptible to a tertiary resistance mutation, C797S, that prevents covalent adduct formation. (Zhou et al., 2009; Thress et al., 2015; Ramalingam et al., 2018) Patients who acquire both T790M and C797S become resistant to all currently approved therapies. (Tumbrink et al., 2021)

To overcome these acquired resistance mutations, mutant-selective allosteric inhibitors were developed. (Jia et al., 2016; de Clercq et al., 2019; To et al., 2019, 2022; Gero et al., 2022; Obst-Sander et al., 2022) Allosteric inhibitors stabilize the inactive (αC-helix out) conformation of EGFR by binding a different pocket than ATP and are therefore not affected by the T790M/C797S resistance mutations. However, EGFR dimerization induces the active (αC-helix in) conformation of the kinase domains, which closes the allosteric pocket and therefore antagonizes allosteric inhibitor binding. The early allosteric inhibitor EAI045 relied on co-administration of the dimerization-disrupting antibody cetuximab to sensitize cells to its inhibitory activity. (Jia et al., 2016) Subsequently developed allosteric inhibitors such as JBJ-09-063 are
sufficiently potent that they are active as single agents, but their potency is nevertheless affected by receptor dimerization owing to their allosteric mechanism of action. (To et al., 2019, 2022) Expression levels of EGFR and other ErbB-family members with which it can heterodimerize alter dimerization, as do levels of ligand, which leads to variability in cellular studies and also marked differences in potency in cellular assays as compared with biochemical assays with the monomeric EGFR kinase domain. (Jia et al., 2016; To et al., 2019) To better understand the effects of kinase dimerization on allosteric inhibition, we designed, purified, and characterized a constitutive EGFR(L858R/T790M) kinase dimer for biochemical studies of allosteric EGFR inhibitor pharmacology. This construct fuses two EGFR kinases in tandem in a single polypeptide chain. Interestingly, a similar tandem duplication of the EGFR kinase domain has also been found in lung cancer patients. (Gallant et al., 2015; Du et al., 2021) We find that this tandem kinase construct recapitulates the activating asymmetric dimer interaction in biochemical assays. Profiling of diverse allosteric inhibitors against this construct yields inhibitor potencies that closely track those measured in Ba/F3 cellular assays, demonstrating that it is a useful tool for inhibitor evaluation, in particular for the novel class of allosteric EGFR TKIs.
Materials and Methods

Compounds

All inhibitors were purchased from MedChemExpress or Selleck Chem and are ≥95% pure per vendors’ analyses except for EAI002, EAI045, JBJ-04-125-02, and JBJ-09-063 which were previously synthesized in-house for other studies. (Jia et al., 2016; de Clercq et al., 2019; To et al., 2019, 2022) BAY-33 was synthesized by PepTech Co. (>95% purity per the vendor’s analysis) utilizing the methods described for compound #33 in patent WO2019081486. Compound dilutions were prepared in DMSO and stored at -20 °C when not in use.

EGFR protein expression and purification

The EGFR dimer construct was purchased as a synthetic gBlock and began with a 6xHis tag followed by a tobacco etch virus (TEV)-protease cleavage site, an EGFR kinase domain (residues 696-1022) containing L858R and T790M mutations, a 6x(Ser-Gly) linker, and an EGFR kinase domain (residues 687-1022) containing L858R, T790M, and V948R mutations (Fig. 1A). The gene encoding the kinase dimer was cloned into pAC8 and recombinant baculovirus was generated and used to infect SF9 (RRID:CVCL_0549) insect cells infected. Cells were pelleted and resuspended in buffer composed of 50 mM Tris pH 8.0, 500 mM NaCl, 5% glycerol, and 1 mM tris(2-carboxyethyl) phosphine (TCEP), and protease inhibitor cocktail (Roche). Cells were lysed via sonication, clarified via ultracentrifugation at >200,000 g for 1 hr, and purified using Ni agarose beads. The resin was washed with lysis buffer supplemented with 40 mM imidazole and the protein eluted with lysis buffer supplemented with 200 mM imidazole. The eluted protein was further purified by size exclusion chromatography on a Superdex S200 column (Cytiva), concentrated, and flash frozen in liquid nitrogen.

Enzyme kinetics

Kinetic assays were performed using the HTRF KinEASE tyrosine kinase assay kit
(Cisbio, CAT 62TK0PEC) according to the manufacturer’s protocol. ATP at differing concentrations of up to 1 mM were dispensed into 384-well plates. Reactions were initiated with assay buffer containing purified EGFR at a final concentration of 100 nM for WT EGFR, 1 nM for EGFR(L858R) and EGFR(L858R/T790M) monomer, and 0.01 nM for EGFR(L858R/T790M) dimer dispensed using a Multidrop Combi dispenser into 384-well plates. Reactions were incubated at room temperature at varying time points between 1 minute and 20 minutes before being quenched using the detection reagent from the KinEASE assay kit. The FRET signal ratio was measured at 665 and 620 nm using a PHERAstar microplate reader and plotted as 665/620 nm *10^4. Initial rates of enzyme activity were obtained from the linear region of the time course plot and were plotted as varying ATP concentrations. The data were processed using GraphPad Prism and a Michaelis-Menten curve was fitted to the data to determine kinetic parameters. The assay was performed three independent times in triplicate. To determine the concentration of phosphorylated peptide from the FRET signal ratio, we generated a standard curve. We aliquoted 1 mM ATP into two tubes and mixed HTRF KinEASE tyrosine kinase assay buffer with 1 nM EGFR(L858R/T790M) monomer into one tube and assay buffer without enzyme into the other tube. Reactions were incubated for 30 minutes before quenching using the detection reagent from the KinEASE assay kit. Phosphorylated and unphosphorylated substrate were subsequently mixed in various ratios. The FRET signal ratio was measured at 665 and 620 nm using a PHERAstar microplate reader and plotted as 665/620 nm *10^4. Data were processed using GraphPad Prism and a simple linear regression fit to the data was used to determine the slope. The assay was performed three independent times in triplicate. Each biological replicate was calculated from the mean of the corresponding technical replicates and the data plotted is the average of the biological replicates ± standard deviation.

Kinase inhibition assays
Inhibition assays were performed using the HTRF KinEASE tyrosine kinase assay kit (Cisbio) according to the manufacturer’s protocol. Inhibitors (10 mM DMSO stocks) were dispensed into black 384-well plates using an HP D300e dispenser and normalized to a 1% final DMSO concentration. Assay buffer containing purified EGFR at a final concentration of 0.01 nM for both EGFR(L858R/T790M) monomer and dimer were dispensed using a Multidrop Combi dispenser into 384-well plates and incubated with the inhibitors at room temperature for 30 min. Reactions were initiated with 100 µM ATP using a Multidrop Combi dispenser and allowed to proceed for 30 min at room temperature before being quenched using the detection reagent from the KinEASE assay kit. The FRET signal ratio was measured at 665 and 620 nm using a PHERAstar microplate reader and plotted as 665/620 nm *10^4. Data were processed using GraphPad Prism a three-parameter dose-response model with a Hill slope constrained to 1 was fit to the data to determine potencies. The assay was performed three independent times in triplicate. Each biological replicate was calculated from the mean of the corresponding technical replicates and the data plotted is the average of the biological replicates ± standard deviation.

Cellular assays
Parental Ba/F3 cells were a generous gift from David Weinstock. Ba/F3 (RRID:CVCL_0161) cells harboring EGFR(L858R/T790M) were previously generated and characterized.(Zhou et al., 2009; Jia et al., 2016; To et al., 2019, 2022; Beyett et al., 2022) Ba/F3 cells were grown in RPMI 1640 media with 10% FBS and 1% penicillin and streptomycin. Cells were tested negative for Mycoplasma using the Mycoplasma Plus PCR Primer Set (Agilent, 302008) and were passaged for no longer than 4 weeks. Ba/F3 cells were plated, treated with increasing concentrations of inhibitors for 72 h, and growth inhibition assessed by Cell Titer Glo (Promega, G1111) per the manufacturer’s protocol. Cetuximab (Lilly; NDC 66733-948-23) treatment at 10 µg/mL was concurrent with inhibitor treatment.
Statistics

For all data, each biological replicate was calculated from the mean of the corresponding technical replicates. The data points in tables and graphs are the average of the biological replicates ± standard deviation. Correlation between enzyme and cellular potencies were determined in GraphPad Prism using a two-tailed Pearson correlation test for each X vs. every set of Y. Statistical significance for inhibitor potency change between monomeric and dimeric enzyme were determined using a one-sample T-test to compare the IC$_{50}$ to a hypothetical value of 1 (no change) to determine if a significant change occurred upon kinase dimerization. For all analyses, *p<0.05, **p<0.01, and ***p<0.001.

Results

To create the constitutively active dimer, we used a flexible polypeptide linker composed of repeating glycine and serine units to link two EGFR kinase domains guided by a crystal structure of an active, asymmetric EGFR kinase dimer. (Zhang et al., 2006) The N-terminal “activator” kinase domain contained the clinically relevant L858R/T790M mutations, and the C-terminal “receiver” kinase domain contained L858R/T790M/V948R. The V948R mutation prevents the C-terminal receiver kinase domain from flipping and functioning as an activator. V948R also stops the formation of dimers-of-dimers or higher order oligomers in which the receiver kinase could simultaneously function as an activator (Fig. 1A). Importantly, the construct was designed so that the kinase domain in the acceptor position included N-terminal sequences corresponding to the juxtamembrane latch that contributes to the stability of the asymmetric dimer. (Jura et al., 2009) The EGFR(L858R/T790M) dimer was expressed in SF9 insect cells and purified via immobilized metal affinity and size-exclusion chromatography. Since this dimer can be used to study ATP-competitive and allosteric inhibitors, we sought to understand its ATP affinity compared to other monomeric EGFR kinase variants. The kinetic
characterization revealed that the EGFR(L858R/T790M) dimer is 6.1 times more active than the EGFR(L858R/T790M) monomer (Fig. 1B, 1C, Table 1, Supp. Fig. 1, and Supp. Fig. 2).

Dimerization did not affect ATP affinity as indicated by a $K_{m,ATP}$ for the EGFR(L858R/T790M) monomer and dimer of 41.2±3.0 and 45.0±1.8 µM, respectively. The catalytic efficiency of the EGFR(L858R/T790M) dimer was greater than two-fold compared to the monomer, suggesting the increased activity is due to activation as a result of dimerization rather than simply two kinase active sites. Together, these data support our hypothesis that our dimeric EGFR(L858R/T790M) construct is in the active asymmetric dimer conformation.

As expected, allosteric EGFR inhibitors were approximately 100- to 1000-fold less potent in against the dimer compared to monomer (Fig. 2A). Since there is typically a disconnect between cellular and monomeric enzyme inhibition assay potencies, we compared the former to dimeric enzyme assay results. Cellular potencies for a panel of allosteric inhibitor derivatives of JBJ-09-063 correlated with the potencies for the EGFR(L858R/T790M) dimer but not the monomer, which are approximately 1000-fold greater (Fig. 2B, Supp. Table 1). This correlation is likely due to basal EGFR dimerization in Ba/F3 cells due to growth factors in the culture media, which promote the active conformation of the kinase and obstructs the allosteric site in the receiver kinase that is responsible for downstream signaling (Fig. 2C). Differential sensitivity to allosteric inhibitors has been observed for other cell lines and medias, likely due to differing levels of receptor dimerization.(To et al., 2022) Addition of the dimerization-disrupting antibody cetuximab to the culture media resulted in cellular potencies correlating with the EGFR(L858R/T790M) monomer instead of the dimer. We performed a Pearson correlation test to determine if these correlations were significant and found that potencies against cetuximab-treated cells significantly correlates with the monomeric enzyme and potencies of allosteric inhibitors against cells without cetuximab significantly correlates with the dimeric enzyme. Thus, our constitutive kinase dimer recapitulates the potencies observed in common Ba/F3 cellular assays and enables an easier prediction of cellular potency from a biochemical assay.
To broadly understand the effect of dimerization on kinase inhibitor potency, we profiled a panel of ATP-competitive and allosteric inhibitors in enzymatic inhibition assays using monomeric and dimeric EGFR(L858R/T790M) kinases (Fig. 3A, Supp. Table 2, Supp. Fig. 3). We hypothesized that allosteric and inactive conformation binding ATP-competitive inhibitors would display decreased potency against the dimeric enzyme construct. We also hypothesized that the potency of ATP-competitive inhibitors that structurally do not clash with the active conformation will not be affected by kinase dimerization. Like allosteric inhibitors, ATP-competitive inhibitors neratinib, lapatinib, JBJ-08-178-01, and BDTX-189 that bind the inactive conformation of the kinase also displayed a >100-fold decrease in potency against the dimeric kinase (Fig. 3B).(Son et al., 2022) ATP-competitive inhibitors, such as the osimertinib metabolite AZ5104, whose binding does not clash with the active, C-helix-in conformation were generally more potent inhibitors of the dimeric kinase (Fig. 3C).(Beyett et al., 2022) However, afatinib, poziotinib, and gefitinib displayed an approximately 10-fold decrease in potency against the dimer despite the fact that crystal structures show that these inhibitors bind the active conformation of the kinase. Nearly all potency fold changes with the EGFR dimer were significant compared with that of the monomeric kinase enzyme, with the three nonsignificant inhibitors exhibiting greater deviation (we note that for BDTX-189 p=0.052). Despite their statistical significance, many potency changes were only a few fold in either direction and are not expected to be biologically significant. These results demonstrate the utility of such a constitutive EGFR kinase dimer protein in the study of EGFR inhibitor pharmacology and provides a useful tool for the discovery and characterization of novel inhibitors of the dimeric form of the kinase.
Discussion

Allosteric inhibitors, a promising therapy for treating drug-resistant EGFR variants, are antagonized by dimerization. Initial studies of allosteric EGFR inhibitors found co-administration of the dimerization-disrupting antibody cetuximab to be required for in vivo efficacy. (Jia et al., 2016) Highly-potent allosteric inhibitors such as JBJ-04-125-02 and JBJ-09-063 disrupt EGFR dimers in cells and thus display single-agent efficacy but are still antagonized by dimerization. (To et al., 2019, 2022) Here, we describe a purifiable kinase domain dimer tool suitable for biochemical analyses of EGFR inhibitor pharmacology, especially allosteric inhibitors. Although we have not obtained a crystal structure, our kinetic and pharmacological results indicate formation of a constitutive asymmetric dimer interaction. Additionally, structure function studies of a similar duplication by Du et al. revealed that its activity was diminished by classic mutations known to disrupt the asymmetric dimer interaction. (Du et al., 2021) While we chose the L858R/T790M mutations due to their relevance to allosteric inhibitor development, we expect alternative mutations or different combinations of mutations in the activator and receiver kinases to be compatible with our designed construct. Since EGFR is known to heterodimerize with other members of the ErbB family such as HER2 and HER3, heterodimer constructs could also be made to study heterodimer pharmacology. (Olayioye et al., 2000)

While recently reported allosteric inhibitors do not require co-administration of cetuximab for efficacy, they are still antagonized by dimerization. Evaluation via cellular assays with and without cetuximab is preferred over biochemical assays with kinase domain monomers during development to understand the effects of dimerization on new allosteric inhibitors. This complicates further allosteric inhibitor discovery and development due to the lower throughput and increased cost associated with cellular assays compared to biochemical assays. We found that our dimeric kinase construct is inhibited similarly to Ba/F3 cells without cetuximab treatment making it easier to predict how biochemical potency will translate to cellular potency.
The high activity of our EGFR(L858R/T790M) kinase dimer allows for the use of sub-nanomolar or, in some assay formats, sub-picomolar enzyme concentrations, which is advantageous for large high-throughput screens. We also found that the ATP-affinity of the EGFR(L858R/T790M) dimer was the same as monomeric kinase. This observation suggests our dimeric construct will be of value to the study of ATP-competitive inhibitors, as differences in potency will be a result of protein conformation rather than different competitive effects with ATP. Conducting high-throughput screens with monomeric and dimeric EGFR(L858R/T790M) kinase domains may identify novel molecules that preferentially inhibit the dimeric form of EGFR kinase.

EGFR kinase domain duplication (EGFR-KDD) is a rare oncogenic variant found in gliomas and lung cancers that contains a duplication of the entire wild-type kinase domain with a portion of the juxtamembrane segment linking the two kinase domains. (Gallant et al., 2015; Du et al., 2021; Lee et al., 2022) It is likely that this produces a receptor with an intramolecular constitutive kinase dimer, similar to our synthetic dimer construct, that does not rely on ligand-induced dimerization for oncogenic signaling. Prior work on EGFR-KDD showed it was resistant to allosteric inhibition similarly to our synthetic kinase domain dimer. (To et al., 2022) EGFR-KDD is also reported to be less sensitive to afatinib treatment, and afatinib surprisingly displayed reduced potency against our kinase dimer compared to the monomer. This suggests that our constitutive kinase dimer may also represent a useful tool for the study of EGFR-KDD pharmacology.

Although the EGFR(L858R/T790M) dimer functionally and pharmacologically behaves like an asymmetric kinase dimer, a limitation is that it is a “locked” dimer with kinase domains tethered by a covalent linker. In cells, allosteric inhibitors disrupt intermolecular dimers, which is more difficult to achieve using our intramolecular dimer construct and may exaggerate the pharmacological effect of dimerization. However, our data show matching potencies between cellular and biochemical assays, suggesting that disruption our dimeric construct may be similar
to breaking a receptor dimer on a cell surface. Moreover, the covalent linker may play a similar role to that of the dimerization arm at the extracellular domain level of stabilizing the intermolecular dimer. Unlike EGFR-KDD that has a portion of the endogenous juxtamembrane segment connecting the kinase domains, our construct is linked by a flexible glycine and serine repeat segment. This has the potential to introduce artifacts in assay results, but we suspect is a minor concern given the high degree of similarity with prior work on allosteric inhibitors and EGFR-KDD pharmacology. In conclusion, we have described the creation and characterization of an EGFR(L858R/T790M) kinase dimer that represents a useful tool for the study of EGFR inhibitor pharmacology and future drug discovery and development.

**Data Availability**

This article contains no datasets generated or analyzed during the current study.

**Conflicts of Interest**

The Eck lab receives (or has received within the past two years) sponsored research support from Novartis, Takeda, and Springworks Therapeutics. P.A.J. has received consulting fees from AstraZeneca, Boehringer Ingelheim, Pfizer, Roche/Genentech, Takeda Oncology, ACEA Biosciences, Eli Lilly and Company, Araxes Pharma, Ignyta, Mirati Therapeutics, Novartis, LOXO Oncology, Daiichi Sankyo, Sanofi Oncology, Voronoi, SFJ Pharmaceuticals, Biocartis, Novartis Oncology, Nuvalent, Esai, Bayer, Transcenta, Silicon Therapeutics, Allorion Therapeutics, Accutar Biotech and AbbVie; receives post-marketing royalties from DFCI-owned intellectual property on EGFR mutations licensed to Lab Corp; receives or has received sponsored research funding from AstraZeneca, Astellas, Daichi-Sankyo, PUMA, Boehringer Ingelheim, Eli Lilly and Company, Revolution Medicines and Takeda and has stock ownership in Gatekeeper Pharmaceuticals. The remaining authors declare no conflicts of interest.
Author Contributions

Participated in research design: Kim, Schaeffner, Jänne, Beyett, Eck

Conducted experiments: Kim, Schaeffner, Heppner, To

Contributed new reagents or analytic tools: Heppner

Performed data analysis: Kim, Schaeffner, Beyett

Wrote or contributed to the writing of the manuscript: Kim, Beyett, Eck
References


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Figure Captions

**Fig. 1:** Design and characterization of the constitutive EGFR dimer. **A)** Design of the EGFR(L858R/T790M) dimer. **B)** Initial rates from ATP titration used to produce Michaelis-Menten kinetic plots (representative experiment shown from n=3). **C)** Michaelis-Menten kinetic plot for the EGFR(L858RR/T790M) dimer (n=3 in triplicate, plotted as biological replicate mean ± SD).

**Fig. 2:** Pharmacological characterization of inhibitors against EGFR. **A)** Potencies of allosteric inhibitors against the EGFR(L858R/T790M) dimer (n=3, mean ± SD). **B)** Correlation between monomeric (blue) or dimeric (red) enzyme and cellular potencies. Each point represents an allosteric inhibitor tested in enzymatic and cellular EGFR(L858R/T790M) assays in the presence or absence of the dimerization-disrupting antibody cetuximab. Two-tailed Pearson correlation analysis showed significant correlation between cellular potency without cetuximab and dimeric kinase (*p<0.05) and between cellular potency with cetuximab and monomeric kinase (**p<0.001). **C)** Allosteric inhibitor JBJ-09-063 bound to EGFR in the inactive conformation (PDB 7JXQ) superimposed with EGFR kinase in the active conformation induced by asymmetric kinase dimerization (PDB 2GS2). The inward position of the C-helix in the active conformation clashes with the inhibitor and explains why allosteric inhibitor potency is antagonized by kinase dimerization.

**Fig. 3:** Inhibitor profiling with monomeric and dimeric EGFR(L858R/T790M) kinase. **A)** IC_{50} fold changes between dimer and monomer enzymes (n=3 in triplicate, plotted as biological replicate mean ± SD). A one-sample T-test comparing the fold potency change to a hypothetical value of 1 (no change) was used to determine if a significant change occurred upon kinase dimerization (*p<0.05, **p<0.01, and ***p<0.001). **B)** Neratinib (PDB 2JIV) superimposed with active
conformation EGFR kinase (PDB 2GS2). Neratinib cannot bind to the active conformation due to steric clashes with the inward C-helix position highlighted by E762 and M766 side chains. This explains the reduced potency of neratinib against the active conformation dimer construct.

C) The osimertinib metabolite AZ5104 (PDB 7JXL) superimposed with active conformation EGFR kinase (PDB 2GS2). The C-helix in position of the active conformation does not sterically clash with AZ5104 and explains why its potency does not dramatically change when the kinase is in the dimeric form.

Table 1: Kinetic characterization of EGFR WT, L858R, L858R/T790M monomer, and L858R/T790M dimer variants. Data are shown as mean ± SD (n=3 in triplicate, plotted as biological replicate mean ± SD).

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<th>EGFR WT</th>
<th>L858R</th>
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<th>L858R/T790M dimer</th>
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Fig. 2

A

![Graph showing normalized activity (% vs. inhibitor (µM)).](image)

- EA045 monomer
- EA045 dimer
- JBJ-04-125-02 monomer
- JBJ-04-125-02 dimer
- JBJ-09-063 monomer
- JBJ-09-063 dimer

B

![Graph showing enzymatic potency (µM) vs. cellular potency (µM) without and with cetuximab for monomer and dimer enzyme.](image)

C

![Diagram showing AMP-PNP and C-helix inactive vs. active interactions with JBJ-09-063.](image)
Fig. 3

A

![Graph showing potency fold change for various drugs](image)

B

![Molecular structure of Neratinib and C-helix](image)

C

![Molecular structure of AZ5104 and C-helix](image)
Supplemental Information

A Constitutive EGFR Kinase Dimer to Study Inhibitor Pharmacology

Justin J. Kim\textsuperscript{1,2}, Ilse K. Schaeffner\textsuperscript{1,2}, David E. Heppner\textsuperscript{3,4}, Ciric To\textsuperscript{4,5,6}, Pasi A. Jänne\textsuperscript{4,5,6}, Tyler S. Beyett\textsuperscript{1,2,†}, and Michael J. Eck\textsuperscript{1,2,*}

Affiliations:
\textsuperscript{1}Department of Cancer Biology, Dana-Farber Cancer Institute, Boston, MA 02215, USA.
\textsuperscript{2}Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115, USA.
\textsuperscript{3}Department of Chemistry, University at Buffalo, Buffalo, NY 14260, USA.
\textsuperscript{4}Department of Pharmacology and Therapeutics, Roswell Park Comprehensive Cancer Center, Buffalo, NY 14203
\textsuperscript{5}Lowe Center for Thoracic Oncology, Dana-Farber Cancer Institute, Boston, MA 02215, USA.
\textsuperscript{6}Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA 02215, USA.
\textsuperscript{7}Department of Medicine, Harvard Medical School, Boston, MA 02115, USA.

\textsuperscript{†}Present address: Department of Pharmacology and Chemical Biology, Emory University, Atlanta, GA 30322.

*Corresponding Authors:

Michael J. Eck, MD, PhD
450 Brookline Avenue, LC4313
Boston, MA, 02215
Phone: (617) 632-5860
Email: michael_eck@dfci.harvard.edu

Tyler S. Beyett, PhD
1510 Clifton Rd NE
Rollins Research Center 5119
Atlanta, GA 30322
Email: tbeyett@emory.edu
Supplemental Figure 1. Time course plots of EGFR variants with varying ATP concentrations. 
A) Time course plot for wild-type EGFR (representative experiment shown from n=3, mean ± SD). B) Representative time course plot for EGFR(L858R) (representative experiment shown from n=3, mean ± SD). C) Representative time course plot for EGFR(L858R/T790M) monomer
Supplemental Figure 2. Enzyme kinetics of EGFR variants. A) Michaelis–Menten curve of the kinetics for wild-type EGFR (n=3, mean ± SD). B) Michaelis–Menten curve of the kinetics for EGFR(L858R) (n=3, mean ± SD). C) Michaelis–Menten curve of the kinetics for EGFR(L858R/T790M) monomer (n=3, mean ± SD). D) Michaelis–Menten curve of the kinetics for the EGFR(L858R/T790M) dimer (n=3, mean ± SD). E) Linear best-fit line standard curve used for determining enzyme kinetics of the EGFR variants (y = 5066x+600, n=3, mean ± SD).
Supplemental Figure 3. Dose-response curves used to determine the potencies in SI Table 1. 
A) Inhibition curves of EGFR inhibitors against the EGFR(L858R/T790M) monomer (representative experiment shown from n=3, mean ± SD) B) Inhibition curves of EGFR inhibitors against the EGFR(L858R/T790M) dimer (representative experiment shown from n=3, mean ± SD).
**Supplemental Table 1.** Potencies of various EGFR allosteric inhibitors against EGFR(L858R/T790M) Ba/F3 cells ± cetuximab, monomeric EGFR(L858R/T790M) kinase domain, and dimeric EGFR(L858R/T790M) kinase domain (n=1 in triplicate). Inhibitors with potencies >100 µM were not plotted.

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<th>Cellular IC$_{50}$ (µM) with cetuximab</th>
<th>Enzyme IC$_{50}$ (µM) monomer</th>
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Supplemental Table 2. Biochemical potencies (IC$_{50}$) of various EGFR inhibitors against EGFR(L858R/T790M) monomer and EGFR(L858R/T790M) dimer (n=3 in triplicate, mean ± SD).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>EGFR(L858R/T790M) Monomer IC$_{50}$ (nM)</th>
<th>EGFR(L858R/T790M) Dimer IC$_{50}$ (nM)</th>
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<tbody>
<tr>
<td>BI-4020</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
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<tr>
<td>EAI002</td>
<td>98 ± 11</td>
<td>&gt;10000</td>
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<tr>
<td>BDTX-189</td>
<td>0.3 ± 0.1</td>
<td>293 ± 69</td>
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<tr>
<td>JBJ-04-125-02</td>
<td>0.6 ± 0.1</td>
<td>420 ± 36</td>
</tr>
<tr>
<td>EAI045</td>
<td>1.7 ± 0.2</td>
<td>827 ± 102</td>
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<tr>
<td>JBJ-09-063</td>
<td>0.17 ± 0.03</td>
<td>66 ± 16</td>
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<tr>
<td>Neratinib</td>
<td>0.6 ± 0.1</td>
<td>40 ± 10</td>
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<tr>
<td>JBJ-08-178-01</td>
<td>2.3 ± 0.2</td>
<td>115 ± 17</td>
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<tr>
<td>Lapatinib</td>
<td>187 ± 24</td>
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<td>Afatinib</td>
<td>0.3 ± 0.1</td>
<td>2.6 ± 0.4</td>
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<td>Gefitinib</td>
<td>119 ± 12</td>
<td>760 ± 154</td>
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<tr>
<td>BLU-945</td>
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<td>1.1 ± 0.2</td>
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<td>Mobocertinib</td>
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<tr>
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<td>0.3 ± 0.1</td>
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<td>0.05 ± 0.01</td>
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<td>Osimertinib</td>
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<td>WZ4002</td>
<td>8.9 ± 3.5</td>
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<td>Almonertinib</td>
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<tr>
<td>Inhibitor</td>
<td>EGFR(L858R/T790M) Monomer IC\textsubscript{50} (nM)</td>
<td>EGFR(L858R/T790M) Dimer IC\textsubscript{50} (nM)</td>
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<td>BAY-33</td>
<td>6.0 ± 1.0</td>
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<td>Olmutinib</td>
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<td>Avitinib</td>
<td>0.4 ± 0.2</td>
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