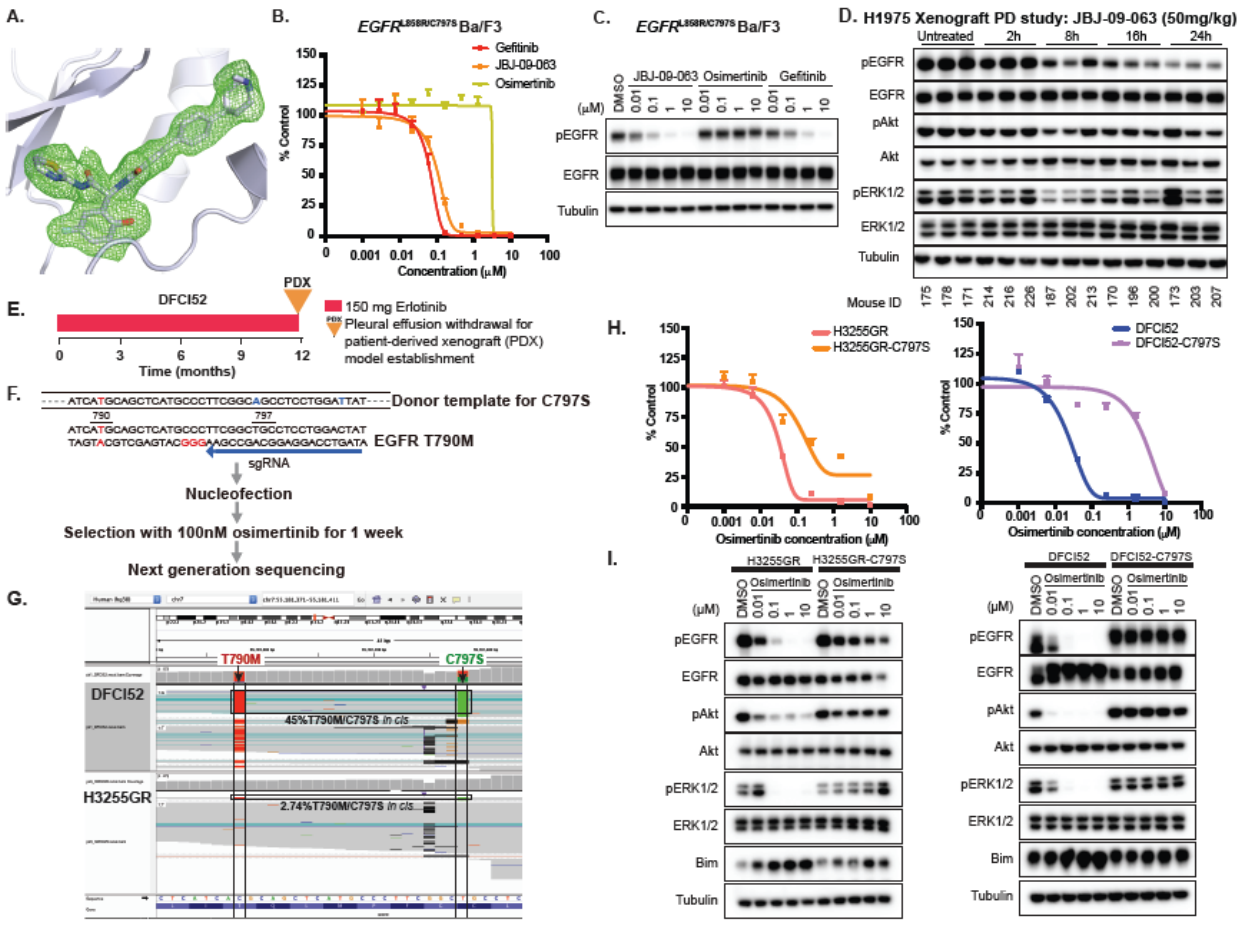


**Figure S1. Structure, potency, pharmacodynamic studies of JBJ-09-063 and the development and validation of H3255GR-C797S and DFIC52-C797S models *in vitro*.**

**(A)** Positive $F_o$-$F_c$ electron density at 3 sigma for JBJ-09-063. **(B)** Cell viability assay and **(C)** Western Blot analyses of *EGFR*$^{L858R/C797S}$ Ba/F3 cells treated with increasing concentrations of JBJ-09-063, osimertinib and gefitinib. **(D)** Pharmacodynamic study of JBJ-09-063 in lung tumor tissues of H1975 xenograft mice dosed with 50 mg/kg of the compound. Samples were harvested at 0 or 2, 8, 16 and 24h after dosing and samples were processed for Western Blotting analyses. **(E)** Treatment history of patient who was given the first-generation tyrosine kinase inhibitor, erlotinib (pink bar). Pleural effusion was subsequently drawn (orange arrow with PDX) to establish patient-derived xenograft (PDX) model. DFCI52 cell line was derived from

PDX tumors (mouse #135 derived from the effusion). **(F)** Workflow of *EGFR*$^{L858R/T790M/C797S}$ cell line generation: H3255GR and DFCI52 cells, which already harbor the *EGFR*$^{L858R/T790M}$ mutation, were transfected by nucleofection with a sgRNA designed to incorporate the C797S mutation *in cis* with EGFR$^{T790M}$. 100 nM osimertinib was used for selection for 1 week and samples were sent for next generation sequencing to determine the presence and frequency of C797S mutation. **(G)** Visualization of the presence and frequency of T790M and C797S allele variants *in cis* in H3255GR-C797S and DFCI52-C797S cells using Integrated Genome Viewer (IGV). **(H)** Cell viability assay and **(I)** Western Blot analyses of H3255GR vs. H3255GR-C797S and DFCI52 vs. DFCI52-C797S cells treated with increasing concentrations of osimertinib. Data shown is a representative experiment that was repeated at least three times. Cell viability was graphed as a percentage relative to DMSO control.

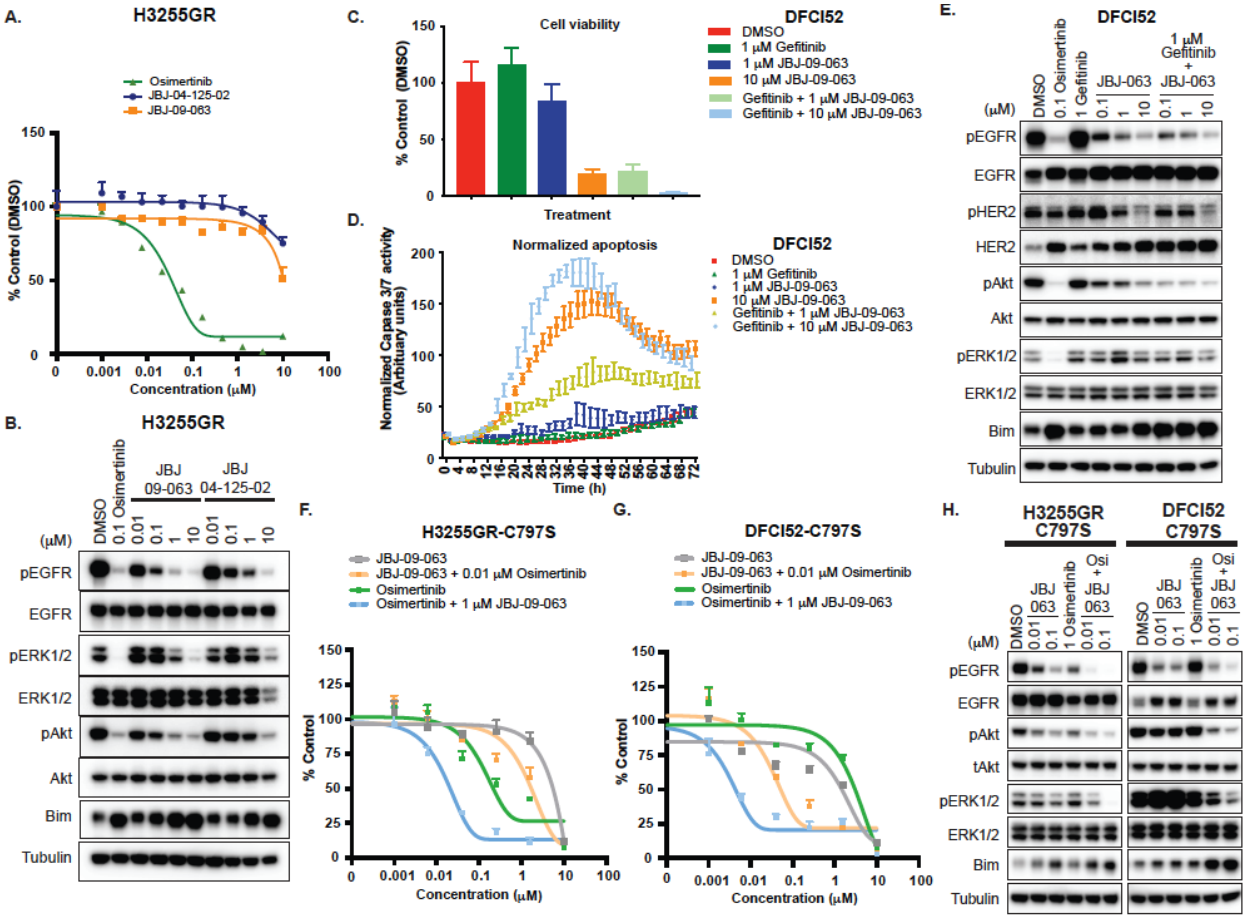


**Figure S2. JBJ-09-063 efficacy is reduced *in vitro* unless combined with gefitinib in H3255GR and DFCI52 cells or with osimertinib in H3255GR-C797S and DFCI52-C797S cells.**

**(A)** Cell viability and **(B)** Western **b**lot analyses of H3255GR cells treated with indicated concentrations of osimertinib, JBJ-09-063 and JBJ-04-125-02. **(C)** Cell viability, **(D)** apoptosis and **(E)** Western Blot analyses of DFCI52 cells treated with indicated concentrations of gefitinib, JBJ-09-063 or the combination of both agents. Cell viability of H3255GR-C797S **(F)** and DFIC52-C797S **(G)** cells treated with indicated concentrations of JBJ-09-063 and osimertinib as a single agent or in combination of both compounds. (**H)** Western Blot analyses of H3255GR-C797S and DFIC52-C797S cells treated with indicated concentrations of JBJ-09-063 and osimertinib as a single agent or in combination of both compounds. All cell viability assays were

graphed as a percentage of activity relative to DMSO control over indicated concentrations and all apoptosis experiments were graphed as normalized caspase 3/7 activity (in arbitrary units) over time. All studies shown here are representative experiments that were repeated at least three times.

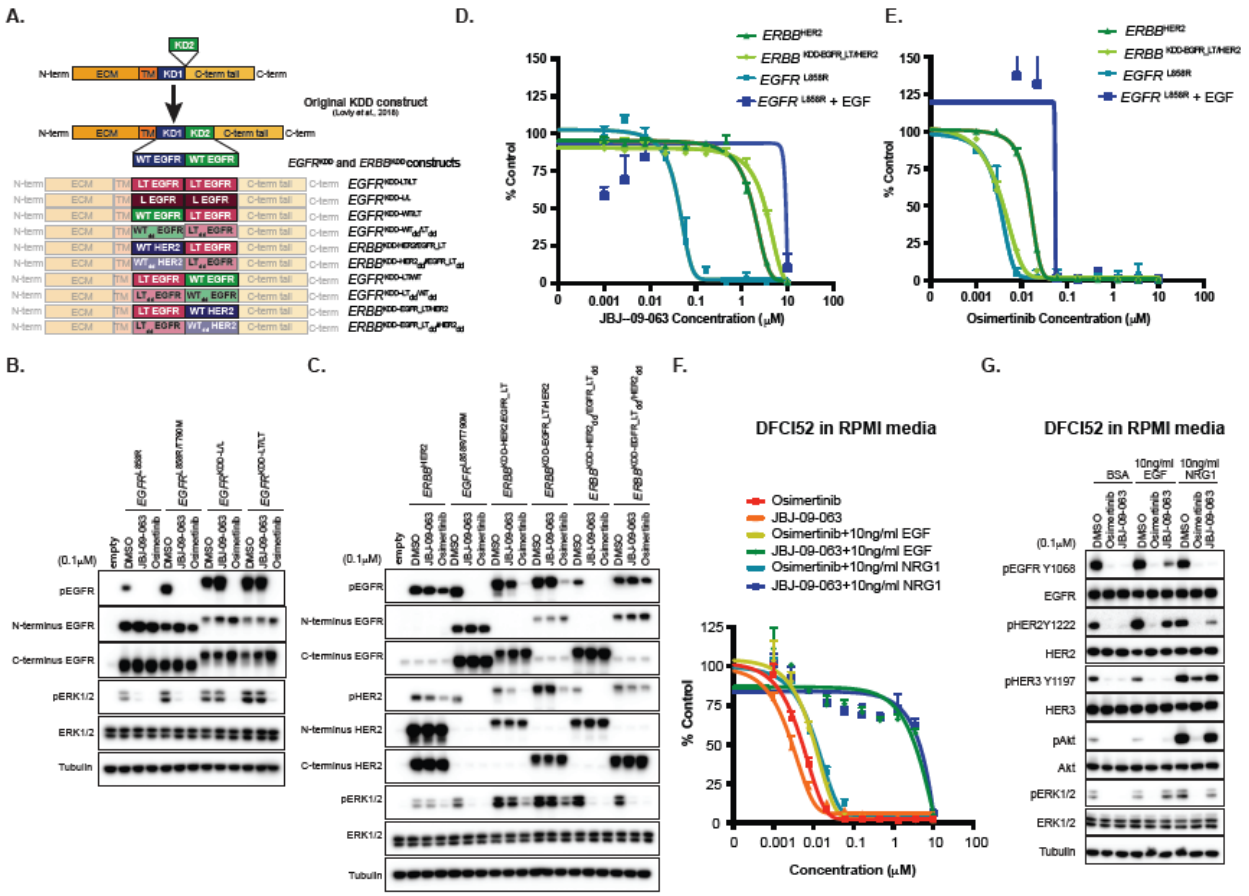


**Figure S3. Forced or ligand induced dimerization can impart resistance to JBJ-09-063.**

**(A)** Schematic representation of the original KDD construct which involves the insertion of a second kinase domain (KD2) after the first kinase domain (KD1) to generate a kinase domain duplication (KDD) mutation that induces intramolecular dimerization. Different combination of *EGFR*$^{WT}$, *EGFR*$^{L858R}$, *EGFR*$^{L858R/T790M}$, *HER2*$^{WT}$ kinase domains were assembled as KD1 and KD2 to make the EGFR$^{KDD}$ and ERBB$^{KDD}$ constructs. EGFR and ERBB dimerization-deficient mutants were generated as controls. N-term = amino-terminus, ECM = extracellular membrane domain, TM = transmembrane domain. C-term tail = carboxyl-terminus tail. Western Blot analyses of HEK293T/Cl.17 cells transiently transfected with **(B)** *EGFR*$^{L858R}$, *EGFR*$^{L858R/T790M}$, *EGFR*$^{KDD}$ constructs or **(C)** *ERBB*$^{HER2}$, *EGFR*$^{L858R/T790M}$ or *ERBB*$^{KDD}$ constructs and treated with

DMSO, osimertinib or JBJ-09-063. Cell viability of Ba/F3 cells stably infected with either *ERBB*$^{HER2}$, *ERBB*$^{\text{KDD-EGFR\_LT/HER2}}$ or *EGFR*$^{\text{L858R}}$ in the presence or absence of EGF treated with increasing concentrations of **(D)** JBJ-09-063 or **(E)** osimertinib and analyzed using Cell Titer Glo reagents. **(F)** Cell viability and **(G)** Western Blot analyses of DFCI52 cells cultured in RPMI media and treated with indicated concentrations of compounds in the presence or absence of EGF or NRG1. All data shown is a representative experiment that was repeated at least three times. Cell proliferation was graphed as a percentage relative to DMSO control.

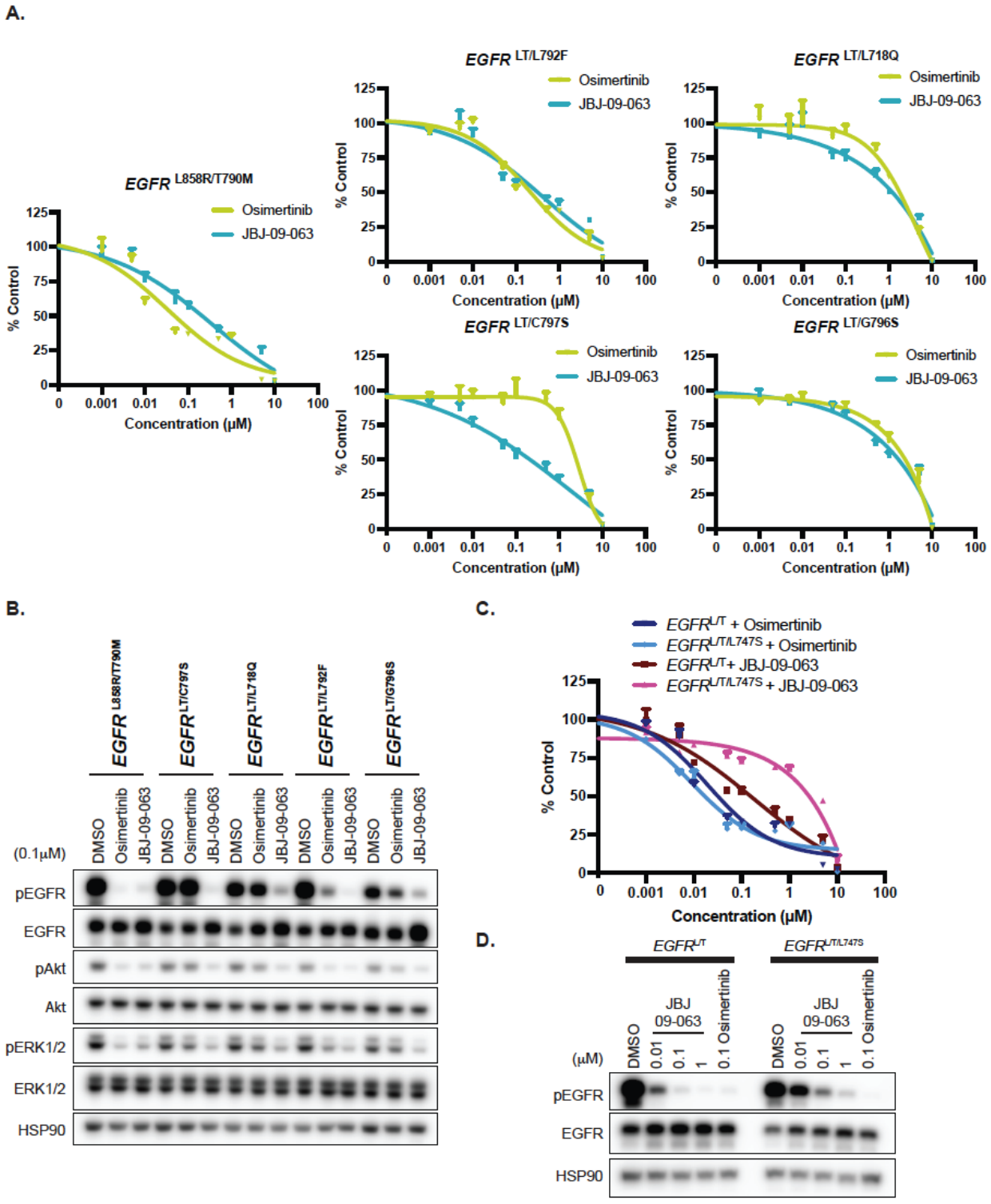


**Figure S4. JBJ-09-063 is effective in H1975 cells exogenously expressing the osimertinib resistant mutations while osimertinib is effective in H1975 cells exogenously expressing the JBJ-09-063 mutation, L747S.**

**(A)** Cell proliferation and **(B)** Western Blot analyses of *EGFR*$^{L858R/T790M,}$ *EGFR*$^{LT/C797S,}$ *EGFR*$^{LT/L718Q,}$ *EGFR*$^{LT/L792F,}$ and *EGFR*$^{LT/G796S}$ H1975 cells treated with DMSO, osimertinib or JBJ-09-063. (**C)** Cell growth inhibition and (**D)** EGFR phosphorylation activity of *EGFR*$^{L858R/T790M}$ or *EGFR*$^{LT/L747S}$ H1975 cells treated with JBJ-09-063 or osimertinib was measured by Cell Titer Glo assay and analyzed by Western Blot. Data shown is a representative experiment that was repeated at least three times. Cell viability was graphed as a percentage relative to DMSO control. LT=L858R/T790M.

**Table S1. Crystallographic data collection and refinement statistics**

| $EGFR^{T790M/V948R}$ AMP-PNP and JBJ-09-063 | |
|---|---|
| PDB Accession | 7JXQ |
| Resolution range | 56.66 - 1.83 (1.90 - 1.83) |
| Space group | P 1 $2_1$ 1 |
| Unit cell | 57.3 74.6 150.6 90 98.7 90 |
| Total reflections | 771509 (78463) |
| Unique reflections | 110113 (10972) |
| Multiplicity | 7.0 (7.2) |
| Completeness (%) | 99.31 (99.38) |
| Mean I/sigma(I) | 9.26 (1.01) |
| Wilson B-factor | 33.14 |
| R-merge | 0.095 (1.647) |
| R-meas | 0.102 (1.775) |
| R-pim | 0.038 (0.658) |
| CC1/2 | 0.999 (0.554) |
| Reflections used in refinement | 109988 (10943) |
| Reflections used for R-free | 2005 (198) |
| R-work | 0.190 (0.383) |
| R-free | 0.223 (0.383) |
| Number of non-hydrogen atoms | 10431 |
| macromolecules | 9602 |
| ligands | 288 |
| solvent | 541 |
| Protein residues | 1189 |
| RMS(bonds) | 0.008 |
| RMS(angles) | 1.01 |
| Ramachandran favored (%) | 98.12 |
| Ramachandran allowed (%) | 1.88 |
| Ramachandran outliers (%) | 0.00 |
| Rotamer outliers (%) | 2.16 |
| Clashscore | 3.44 |
| Average B-factor | 45.23 |
| macromolecules | 45.50 |
| ligands | 35.90 |
| solvent | 45.39 |

**Table S2. $IC_{50}$ of JBJ-04-125-02 and JBJ-09-063 in enzymatic assays, Ba/F3 cells, H1975 cells, and human cancer cells**

**Enzymes**

| Variant | JBJ-04-125-02 (nM) | JBJ-09-063 (nM) | Osimertinib (nM) |
|---|---|---|---|
| EGFR L858R | 0.767 | 0.147 | 1.05 |
| EGFR L858R/T790M | 0.317 | 0.063 | 0.440 |
| EGFR L858R/T790M/C797S | 0.32 | 0.083 | >1000 |
| EGFRLT/L747S | 1.188 | 0.396 | 0.683 |

**Ba/F3 cells overexpressing EGFR mutants**

| Cell line | JBJ-04-125-02 (μM) | JBJ-09-063 (μM) | Osimertinib (μM) | Gefitinib (μM) | JBJ-0-063 + EGF (μM) | Osimertinib + EGF (μM) |
|---|---|---|---|---|---|---|
| $EGFR^{WT}$ | - | 3.541 | 0.101 | - | - | - |
| $EGFR^{L858R}$ | 1.040 | 0.051 | 0.003 | 0.014 | 6.367 | 0.039 |
| $EGFR^{L858R/T790M}$ | 0.610 | 0.050 | 0.010 | >9.3 | 2.649 | 0.022 |
| $EGFR^{L858R/T790M/C797S}$ | 0.050 | 0.010 | 1.540 | >6.9 | - | - |
| $EGFR^{KDD-LT/WT}$ | - | 1.972 | 0.005 | - | - | - |
| $EGFR^{LT/L747S}$ | - | 0.126 | 0.003 | - | - | - |
| $ERBB^{KDD-EGFR\_LT/HER2}$ | - | 3.376 | 0.004 | - | - | - |
| $ERBB^{HER2}$ | - | 2.172 | 0.015 | - | - | - |
| $EGFR^{LT/L718Q}$ | - | 0.032 | 0.403 | - | - | - |
| $EGFR^{LT/L792F}$ | - | 0.005 | 0.014 | - | - | - |
| $EGFR^{LT/G796S}$ | - | 0.090 | 0.392 | - | - | - |

**H1975 cells overexpressing EGFR mutants**

| Cell line | JBJ-09-063 (μM) | Osimertinib (μM) |
|---|---|---|
| $EGFR^{L858R/T790M}$ | 0.210 | 0.048 |
| $EGFR^{L858R/T790M/C797S}$ | 0.212 | 2.483 |
| $EGFR^{LT/L747S}$ | 2.563 | 0.017 |
| $EGFR^{LT/L718Q}$ | 1.208 | 1.781 |
| $EGFR^{LT/L792F}$ | 0.318 | 0.217 |
| $EGFR^{LT/G796S}$ | 1.687 | 2.399 |

**Human cancer cells**

| Cell line | JBJ-04-125-02 (μM) | JBJ-09-063 (μM) | Osimertinib (μM) | Gefitinib (μM) | JBJ-09-063 + 10ng/ml EGF (μM) | Osimertinib + 10ng/ml EGF (μM) | JBJ-09-063 + 10ng/ml NRG1 (μM) | Osimertinib + 10ng/ml NRG1 (μM) | Afatinib (μM) | Osimertinib + 1μM JBJ-09-063 (μM) | JBJ-09-063 + 0.01μM Osimertinib (μM) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| A431 | - | 3.645 | - | 0.209 | - | - | - | - | 0.069 | - | - |
| H3255 (R10) | - | 0.009 | 0.003 | 0.001 | - | - | - | - | - | - | - |
| H3255GR | | 5.995 | 0.026 | | - | - | - | - | - | 0.123 | 6.691 |
| H3255GR (R10) | - | 0.005 | 0.003 | - | 4.486 | 0.047 | 5.514 | 0.01 | - | - | - |
| H3255GR-C797S | - | 5.1 | 0.213 | - | - | - | - | - | - | 0.025 | 2.023 |
| DFCI52 | 7.981 | 2.295 | 0.024 | >9.6 | - | - | - | - | - | 0.019 | 1.29 |
| DFCI52 (R10) | - | 0.003 | 0.005 | - | 2.988 | 0.009 | 3.525 | 0.009 | - | - | - |

**Table S3. Pharmacokinetic profile of JBJ-09-063**

| Drug | Route | Dose (mg/kg) | $T_{1/2}$ (hr) | $T_{max}$ (hr) | $C_{max}$ (ng/ml) | $C_{max}$ ($\mu$M) | $AUC_{last}$ (min*ng/mL) | $AUC_{last}$ ($\mu$M.hr) | AUCINF_obs (min*ng/mL) | AUC (%Extrap) | Cl_obs (mL/min/kg) | $MRT_{INF\_obs}$ (hr) | Vss obs (L/kg) | F (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| JBJ-09-063 | IV | 3 | 2.28 | 0.08 | 3397 | 6.11 | 147478 | 4.42 | 191945 | 23.03 | 15.74 | 2.52 | 2.37 | |
| | PO | 20 | | 6.67 | 647 | 1.16 | 143880 | 4.31 | | | | | | 14.63 |

## Table S4. Reagents and Antibodies

| Reagents | Source/Company | Catalog Number |
|---|---|---|
| **Antibodies** | | |
| Phospho-EGF Receptor (Tyr1068) (D7A5) XP rabbit antibody | Cell Signaling Technology | Cat#3777 |
| EGF Receptor (D38B1) XP rabbit antibody | Cell Signaling Technology | Cat#4267S |
| Phospho-AKT (SER473) (D9E) XP rabbit antibody | Cell Signaling Technology | Cat#4060L |
| AKT antibody | Cell Signaling Technology | Cat#9272L |
| Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP rabbit antibody | Cell Signaling Technology | Cat#4370L |
| p44/42 MAPK (Erk1/2) (137F5) rabbit antibody | Cell Signaling Technology | Cat#4695S |
| Monoclonal Anti-α-Tubulin antibody | Sigma Aldrich | Cat#T5168-.5ML |
| Phospho-EGFR (Tyr998) (C24A5) rabbit antibody | Cell Signaling Technology | Cat#2641S |
| EGF Receptor (N-terminal) rabbit antibody | Abgent | AP19833a |
| Phospho-HER2 (Tyr877) rabbit antibody | Cell Signaling Technology | Cat#2241S |
| c-*erb*B-2/HER-2/*neu* (Phospho-specific) Ab-18 (Clone PN2A) mouse monoclonal antibody | Thermo Scientific | Cat#MS-1072-P0 |
| HER2 (29D8) rabbit antibody | Cell Signaling Technology | Cat#2165S |
| HER2 (D8F12) XP rabbit antibody | Cell Signaling Technology | Cat#4290S |
| Bim (C34C5) rabbit antibody | Cell Signaling Technology | Cat#2933 |
| Phospho-HER3 (Tyr1197) (C56E4) rabbit antibody | Cell Signaling Technology | Cat#4561S |
| HER3 (D22C5) XP rabbit antibody | Cell Signaling Technology | Cat#12708S |
| HSP90 α/β rabbit antibody | Santa Cruz Biotechnology | Cat#S7947 |
| | | |
| **Chemicals, Peptides, and Recombinant Proteins** | | |
| EGF Recombinant Human Protein Solution | Thermo Fisher Scientific | PHG0311L |
| Mouse Recombinant Interleukin-3 | Prospec | CYT-371 |
| JBJ-04-125-02 | To and Jang *et al*., 2019 | |
| Osimertinib | Medchem Express | HY-15772 |
| Afatinib (BIBW2992) | Selleck Chemicals | S1011 |
| N-ethyl-N-nitrosourea (ENU) | Sigma Aldrich | N3385-1G |
| NMP | Sigma Aldrich | 494496 |
| PEG-300 | Sigma Aldrich | 90878 |
| HPMC | Sigma Aldrich | 09963 |
| Fugene® HD Transfection Reagent | Promega | E2311 |
| Gefitinib | Selleck Chemicals | S1025 |
| Nrg1 human recombinant protein | Abcam | ab50227 |
| | | |
| **Commercial Assays** | | |
| Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay | Promega | G1111 |
| CellEvent™ Caspase-3/7 Green ReadyProbes® Reagents | Thermo Fisher Scientific | R37111 |
| Duolink® Proximation Ligation Assay | Sigma Aldrich | DUO92101 |
| Duolink® in situ PLA probe Anti-Rabbit Minus | Sigma Aldrich | DUO92005 |

**Table S5. Sequences and primers for plasmids and cell lines generation**

**A.** EGFR mutant plasmids

| Method | Cloning | | Primer Sequences |
|---|---|---|---|
| | Construct | Mutation/Infusion fragment | |
| Mutagenesis | | EGFR L858R/T790M | 5'-ATCATGCAGCTCATGCCC-3'<br>5'-GAGTTGCACGGTGGAGGTG-3' |
| Mutagenesis | | EGFR L747S | 5'-GAGATGTTGCTTCTCTTGATTCCTTGATAGCGACGG-3'<br>5'-CCGTCGCTATCAAGGAATCAAGAGAAGCAACATCTC-3' |
| Mutagenesis | | EGFR G796S | 5'-GCTCATGCCCTTCAGCTGCCTCCTGGA-3'<br>5'-TCCAGGAGGCAGCTGAAGGGCATGAGC-3' |
| Mutagenesis | | EGFR L792F | 5'-AGCCGAAGGGCATGAACTGCGTGATGAGTTG-3'<br>5'-CAACTCATCACGCAGTTCATGCCCTTCGGCT-3' |
| PCR amplification | | EGFR TKD (partial) | 5'-GGTGACTCCTTCACACATACTC-3'<br>3'-GCTTTGCAGCCCATTTCTATC-3' |
| PCR amplification | | JP destination vector | 5'-GGAAGCTTTCTAGACCATTCG-3'<br>5'-GGTGTCGACTGATAACTTCG-3' |
| PCR amplification | | pDNR mutant insert | 5'-TTATCAGTCGACACCATGCGACCCTCCGGGACG-3'<br>5'-GTCTAGAAAGCTTCCCAATGCTCCAATAAATTCACTGC-3' |
| Mutagenesis | | EGFR L858R | 5'-GATTTTGGGCGGGCCAAACTG-3'<br>5'-TGTGATCTTGACATGCTGC-3' |
| Mutagenesis | | EGFR T790M | 5'-GCTCATCATGCAGCTCATGCC-3'<br>5'-TGCACGGTGGAGGTGAGGC-3' |
| Mutagenesis | | EGFR I941R | 5'-CATATGTACCCGCGATGTCTACATGATCATGGTCAAGTGC-3'<br>5'-GGTGGCTGAGGGAGGCGT-3' |
| Mutagenesis | | HER2 V956R | 5'-CATGATCATGCGCAAATGTTGGATG-3'<br>5'-TAGACATCAATGGTGCAG-3' |
| PCR amplification | $EGFR^X$:$EGFR^X$ | N-EGFR inf.fr. | 5'-CGGCCAATCTAGATGCGGCCGCATGCGACCCTCCGGGACG-3'<br>5'-GCTCCACAAGCAGAGAGCTCAGGAGGGGAGTCC-3' |
| PCR amplification | | C-EGFR inf.fr. | 5'-GAGCTCTCTGCTTGTGGAGCCTCTTACACCC-3'<br>5'-CTACCCGCTTCCATTGCTCAGCGGTGCTGTCCATCTGCACGA-3' |
| PCR amplification | $EGFR^X$:$HER2^X$ | N-EGFR inf.fr. | 5'-CGGCCAATCTAGATGCGGCCGCATGCGACCCTCCGGGACG-3'<br>5'-GCTCCACCAGCAGAGAGCTCAGGAGGGGAGTCC-3' |
| PCR amplification | | C-HER2 inf.fr. | 5'-GAGCTCTCTGCTGGTGGAGCCGCTGACAC-3'<br>5'-AAGCTGGGTCTCACACTGGCACGTCCAGAC-3' |
| PCR amplification | | Inf.fr. X | 5'-GCCAGTGTGAGACCCAGCTTTCTTGTACAAGTAC-3'<br>5'-CTACCCGCTTCCATTGCTCAGCGGTGCTGTCCATCTGCACG-3' |
| PCR amplification | $HER2^X$:$EGFR^X$ | N-HER2 inf.fr. | 5'-CGGCCAATCTAGATGCGGCCGCATGGAGCTGGCGGCCTTGTGC-3'<br>5'-GCTCCACAAGCCTGTGGTGGACCATGCCC-3' |
| PCR amplification | | C-EGFR inf.fr. | 5'-CCACCACAGGCTTGTGGAGCCTCTTACACCC-3'<br>5'-CTACCCGCTTCCATTGCTCAGCGGTGCTGTCCATCTGCACGA-3' |

*X stands for WT or any introduced mutation(s)

**B.** C797S CRISPR cell lines

| Oligo | Primer sequences |
|---|---|
| sgRNA<br>EGFR exon 20 | TAGTCCAGGAGGCAGCCGAA |
| Donor template<br>T790M-C797S | TCTGCCTCACCTCCACCGTGCAGCTCATCACGCAGCTCATGCCCTTCGGCAGCC<br>TCCTGGATTATGTCCGGGAACACAAAGACAATATTGGCTCCCAGTA |
| CRISPR sequencing primers | 5'-GGGCATCTGCCTCACCTC<br>3'- GCAGACCGCATGTGAGGAT |