Structural Basis for Inhibition of Mutant EGFR with Lazertinib (YH25448)

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Abstract. Lazertinib (YH25448) is a novel third-generation tyrosine kinase inhibitor (TKI) developed as a treatment for EGFR mutant non-small cell lung cancer. To better understand lazertinib inhibition at the molecular level, we determined crystal structures of lazertinib in complex with both WT and mutant EGFR and compared its binding mode to that of structurally-related EGFR TKIs. We observe that lazertinib binds with the novel pyrazole moiety involved in hydrogen bonds and van der Waals interactions consistent with drug potency and T790M mutant selectivity. Biochemical assays and cell studies confirm that lazertinib effectively targets EGFR(L858R/T790M) and to a lesser extent against HER2 as consistent with an improved toxicity profile. The molecular basis for lazertinib inhibition of EGFR reported here highlights new strategies for structure-guided design of tyrosine kinase inhibitors.

Keywords. Lung cancer, targeted therapy, kinase inhibitor, mutant, epidermal growth factor receptor, structural biology.

Introduction. Activating mutations within the epidermal growth factor receptor (EGFR) kinase domain, most prevalently L858R and exon19del, are common causes of non-small cell lung cancer (NSCLC) and often serve as predictive markers for the selection of EGFR tyrosine kinase inhibitors (TKIs) as effective targeted therapies.[1-2] Prolonged efficacy of first-generation TKIs (gefitinib and erlotinib) is eventually made limited due to drug resistance as a result of patients acquiring a second T790M “gatekeeper” mutation.[3] To produce a viable treatment option for T790M-positive NSCLC

tumors, drug development efforts have yielded the clinically-approved drug AZD9291 (osimertinib), which is selective for T790M-containing EGFR and made potent by forming an irreversible covalent bond to C797.[4-5] Despite promising indications, drug resistance to osimertinib is inevitable and caused in part by the acquisition of a third kinase domain mutation C797S that prevents formation of the potency-enabling covalent bond. More recently, osimertinib has been shown effective, and clinically-approved, as a front-line therapy in untreated patients harboring EGFR L858R and exon19del activating mutations.[6]

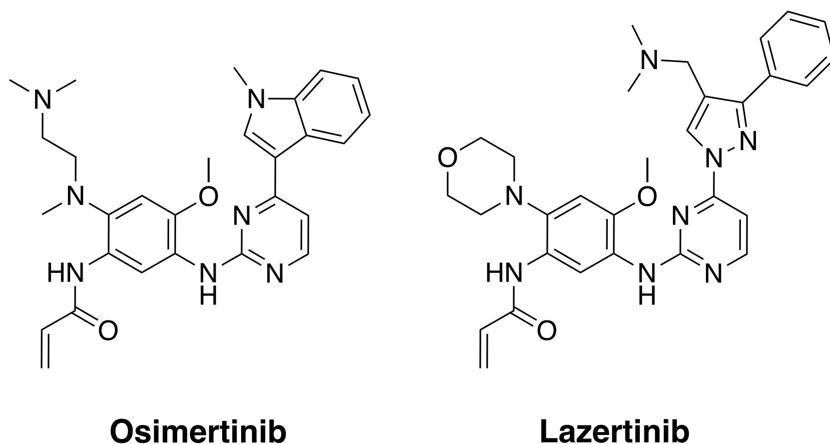


Figure 1. Chemical Structures of third-generation EGFR inhibitors osimertinib and lazertinib.

As osimertinib is the only approved third-generation EGFR TKI for L858R and exon19del EGFR mutant tumors, development efforts from Yuhan and Janssen biotech sought to produce a drug with improved medicinal chemistry properties. These efforts resulted in YH25448 (lazertinib), which is structurally related to osimertinib comprising an aminopyrimidine core and acrylamide warhead but is distinct with respect to the substituted pyrazole as well as morpholine groups (Figure 1).[7] Preclinical head-to-head

experiments confirm that lazertinib is superior to osimertinib in several key respects including *in vivo* efficacy against H1975 (L858R/T790M) xenograft mouse models, brain penetrance, target specificity, and dose-limiting toxicity.[8] These improvements have motivated clinical evaluation of lazertinib in a variety of trials as front-line (NCT04248829) or in combination with the antibody Amivantamab (NCT04077463), and is currently approved to treat T790M-containing NSCLC in the Republic of Korea.[7] Regardless of these improvements, drug resistance to lazertinib has been shown to be due to the acquisition of C797S mutation.[9-10] Despite the improved properties and positive clinical outlook, no crystal structures have been reported detailing the molecular basis for lazertinib inhibition of mutant EGFR.

Results and Discussion. To characterize the binding mode of lazertinib in complex with the EGFR kinase domain, we determined a 2.4 Å resolution X-ray co-crystal structure of lazertinib soaked into WT EGFR crystals (Figure 2 & S1, PDB ID 7UKV). WT kinase domains crystallize in the active "αC-helix in" conformation due to crystal packing of the kinase domains as asymmetric dimers.[11] As expected, lazertinib binds with the aminopyrimidine anchored to the hinge region by H-bonds to M793 and covalent bond formed at C797 as generally observed for third-generation TKIs.[12] Importantly, the unique pyrazole group extends away from the hinge in a conformation that positions the phenyl ring toward the K745-E762 salt bridge and the *N*,*N*-dimethylmethyleneamine (methyleneamine) in a H-bond with the DFG-motif D855 carboxylate.

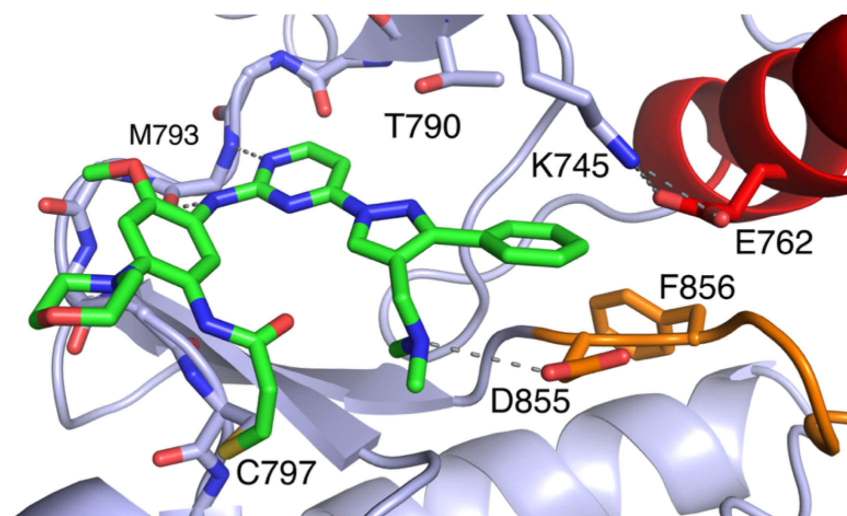


Figure 2. Lazertinib bound to WT EGFR. Binding mode of lazertinib in complex with WT EGFR with the kinase domain in the active αC-helix in conformation. P-loop cartoon removed for clarity. (PDB ID 7UKV).

Additionally, we determined a 2.6 Å resolution co-crystal structure of lazertinib in complex with EGFR containing the T790M mutation. This was accomplished by soaking crystals of EGFR(T790M/V948R), where the V948R variant prevents asymmetric dimer packing and stabilization of the inactive "αC-helix out" conformation. We succeeded in modeling lazertinib in two of the four copies of the asymmetric unit. Despite extensive efforts, we were unable to model the electron density observed in two kinase domain chains (A and D), which we have elected to leave without bound ligands. In one protein chain, we observe lazertinib binding in an identical conformation to the WT EGFR structure where the phenyl ring of the pyrazole binds near T790M exhibiting close contacts to the carbon of the T790M methionine (Figure 3A & S2, PDB ID 7UKW). Distinctly, the methyleneamine is observed in an intramolecular H-bond suggesting a role for this group in stabilizing the drug conformation. In the other chain, lazertinib is observed bound in an alternative "flipped" conformation, marked by a 180° rotation of the pyrazole

moiety (Figure 3B & S2). This conformation shows the methyleneamine simultaneously H-bonding with D855 and contacting the methionine thioether. Additionally, the pyrazole nitrogen is positioned toward the acrylamide carbonyl potentially indicating an intramolecular dipole-dipole interaction. The overall picture revealed by these co-crystal structures defines the molecular basis for lazertinib binding to EGFR where the pyrazole substitutions can produce diverse intra- and intermolecular interactions.

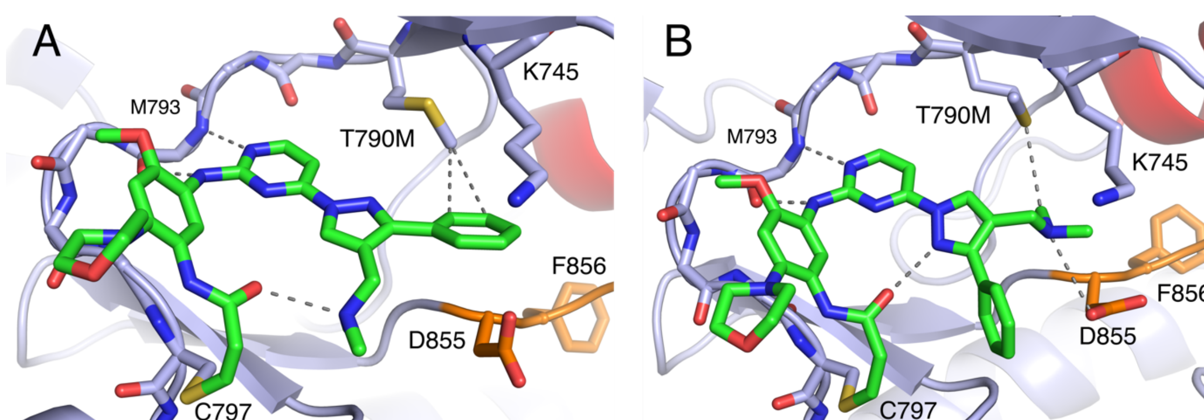
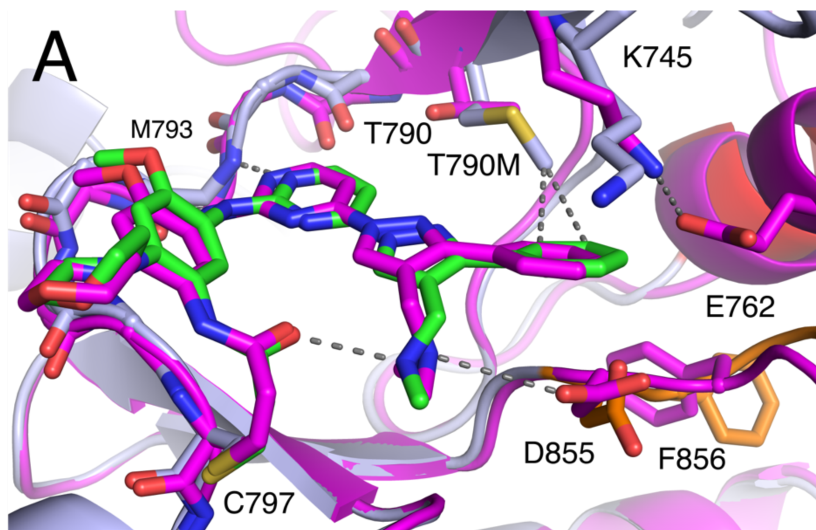


Figure 3. Lazertinib bound to inactive EGFR(T790M/V948R) with distinct conformations. A) Lazertinib bound with phenyl ring anchored within van der Waals distance to the T790M methionine (~3.8 Å) and methane amine involved in intramolecular H-bonding. B) A distinct conformation of lazertinib consisting of a "flipped" conformation with outward phenyl and methyleneamine donating a H-bond to D855. Other interactions seen in this structure include an intramolecular dipole-dipole pyrazole to carbonyl (3.2 Å) as well as van der Waals methyleneamine methyl to thioether (3.8 Å). P-loop cartoon removed for clarity. (PDB ID 7UKW)

The substituted pyrazole of lazertinib is unique among third-generation EGFR TKIs and most likely the basis for mutant-selectivity and improved medicinal chemistry properties. A comparison of our structures from the "αC-helix in" active WT (Figure 2) and "αC-helix out" inactive T790M (Figure 3) show very similar binding positions indicating that lazertinib is anchored to the EGFR kinase domain identically in both active and inactive states and independent of the Thr versus Met 790 gatekeeper residue (Figure

4A). We expect that this conformation of lazertinib is preferred as compared to the "flipped" conformation (Figure 3B) in the "αC-helix in" active state due to the requirement to anchor the positive methyleneamine near the K745-E762 salt bridge (Figure S3). Recent structural studies of EGFR(T790M) co-crystal structures with bound osimertinib revelated a novel binding mode where the *N*-methylindole directly interacts with T790M through van der Waals interactions responsible for osimertinib mutant selectivity.[13] We observe analogous intermolecular interactions in the case of the bound lazertinib phenyl showcasing that both inhibitors are made selective for T790M-containing EGFR through van der Waals interactions (Figure 4B). Additionally, versatile H-bonding methyleneamine moiety of lazertinib is distinctive potentially enabling the modest improvement in binding compared to osimertinib. Another informative comparison is the structurally-related imidazole-based covalent inhibitor LN2057 (Figure S4) that forms an H-bond with K745 enabling C797S mutant inhibition at the expense of a loss of selectivity, i.e., enhanced binding to WT EGFR.[14-15] By comparison, the lazertinib pyrazole substituents sterically block K745 potentially diminishing binding to WT EGFR (Figure 4C). The correlation of these differences in binding mode demonstrates how the pyrazole moiety of lazertinib affords distinct interactions with EGFR to enable T790M mutant selectivity.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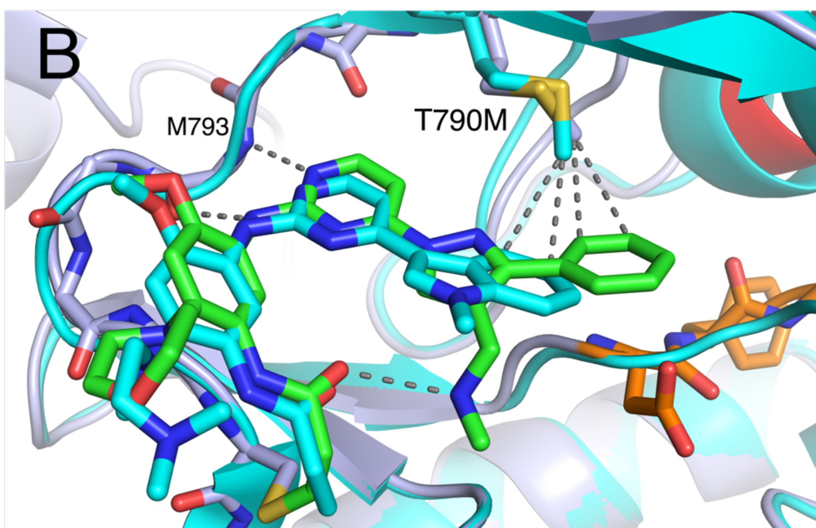
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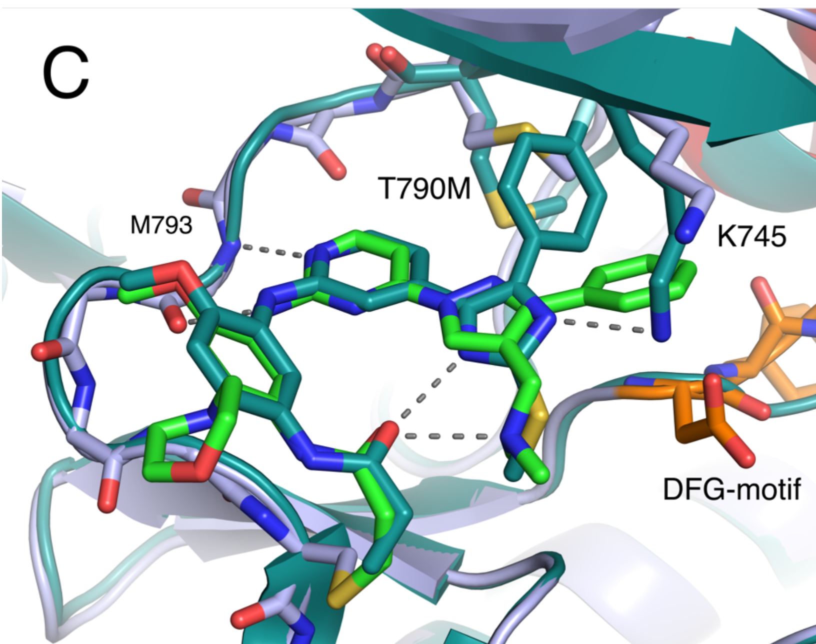
C
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K745
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Figure 4. Distinct binding mode features of third-generation EGFR TKIs A) Superposition of lazertinib in complex with active WT (magenta, PDB ID 7UKV) and inactive T790M/V948R (green, PDB ID 7UKW). B) Overlay of lazertinib in complex with T790M/V948R (green, PDB ID 7UKW) and osimertinib in T790M (cyan, PDB ID 6JX0) showing T790M van der Waals intermolecular interactions. C) Overlay of lazertinib (green PDB ID 7UKW) compared with LN2057 (forest green, PDB ID 6V6K) in complex with T790M/V948R highlighting the contrasting impact on steric repulsion versus H-bonding with K745, respectively.

To further understand the functional significance of the lazertinib binding mode, we conducted biochemical assays with purified kinase domains. The three inhibitors potently inhibit EGFR mutants L858R and L858R/T790M as consistent with previous studies.[8, 14] Their strong potency for the T790M-containing variant is most likely due to productive binding to the T790M through intermolecular van der Waals interactions (lazertinib and osimertinib, Figure 4B) or methionine pi-stacking (LN2057, Figure 4C). LN2057 is found more potent against WT EGFR compared to osimertinib and lazertinib, most likely due to added binding affinity afforded by the imidazole-K745 H-bond (Figure 4B).[15] It is also likely that enhanced binding from this H-bond direct for the observed higher potency against L858R. To compliment earlier studies, we assessed biochemical potencies for these three inhibitors against HER2. We observe that lazertinib exhibits significantly lower potency against HER2, which is proposed to limit adverse events and improve drug tolerability.[8] For completeness, we confirmed lazertinib forms a covalent bond at Cys-805 within the HER2 purified kinase domain with LC-MS/MS (Figure S5). These trends in biochemical assays showcase how structural differences between these inhibitors elicit differential effects on inhibitor potency and selectivity.

Table 1. Biochemical activity assays (Homogenous time-resolved fluorescence HTRF) of recombinant EGFR and HER2 kinase domains. IC_{50} values were measured from a single experiment in triplicate. The ATP concentration was 100 μM. Errors are reported as ± the standard error.

	Biochemical activity IC_{50} (nM)			
Compound	**WT EGFR**	**WT HER2**	**L858R EGFR**	**L858R/T790M EGFR**
Lazertinib	15±3	133±10	4.0±0.6	0.15±0.02
Osimertinib	29±7	44±9	1.0±0.1	0.58±0.08
LN2057	6.5±1[a]	12±2	0.26±0.03[a]	0.27±0.05[a]

[a]Data from ref. Wittlinger et al.,[16]

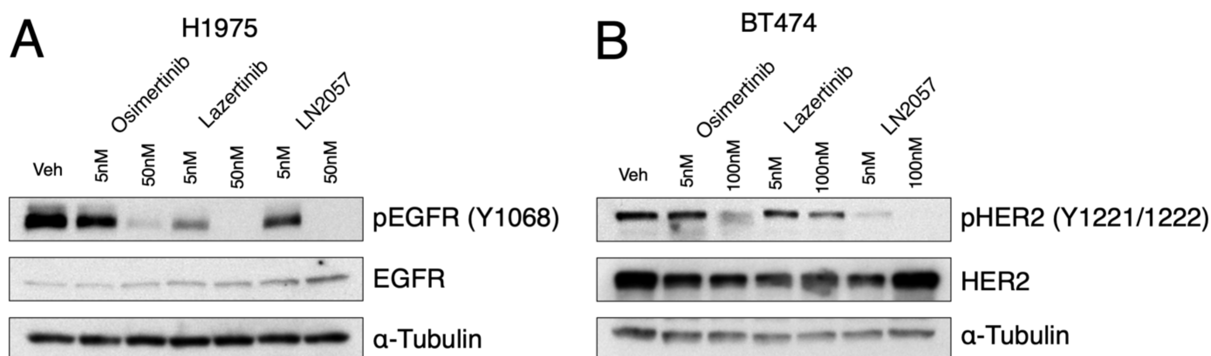

Figure 5. Lazertinib effectively ablates EGFR(L858R/T790M) phosphorylation in NSCLC H1975 cells and marginal inhibition of HER2 in BT474 breast cancer cells. A) Western blot analysis of phosphorylated EGFR (pY1068) after dose-dependent treatment of H1975 (L858R/T790M) lung adenocarcinoma cell lines with osimertinib, lazertinib, and LN2057 for 2 hours. B) Western blot analysis of phosphorylated HER2 (pY1221/1222) after dose-dependent treatment of BT474 breast cancer cells with osimertinib, lazertinib, and LN2057 for 6 hours. Western blots representative examples of three independent experiments.

Since enhanced efficacy against EGFR and diminished HER2 targeting is a proposed advantage for treatment of mutant EGFR NSCLC with lazertinib,[8] we were motivated to assess lazertinib inhibition in cellular contexts compared to osimertinib and LN2057. We first assessed dose-dependence of inhibition of EGFR(L858R/T790M) in H1975 NSCLC cells by osimertinib, lazertinib, and LN2057 by blotting for active EGFR (pY1068). After dosing 5 or 50 nM of these drugs for 2 hours, we observed that lazertinib

suppressed pY1068 to a greater extent compared to equivalent dosing of osimertinib and LN2057, as consistent with previous studies (Figure 5A).[8] Uniquely, lazertinib is observed to be notably less effective at inhibiting active HER2 (pY1221/1222) in BT474 HER2 overexpressing breast cancer cells, confirming the selective targeting of EGFR by lazertinib compared to osimertinib and LN2057 (Figure 5B). These findings are consistent with our biochemical activity assays (Table 1) indicating that lazertinib potently and selectively inhibits mutant EGFR(L858R/T790M) and WT EGFR while simultaneously affording limited activity against HER2.

In conclusion, we have determined the molecular basis of the novel EGFR TKI lazertinib bound to EGFR in X-ray co-crystal structures showcasing that the lazertinib pyrazole ring binds facilitates H-bonds and van der Waals interactions consistent with drug efficacy and T790M selectivity. Structural and functional correlation to osimertinib and LN2057 demonstrate the importance of productive intermolecular interactions with T790M. Additionally, we find that lazertinib does not H-bond with K745, which likely contributes to lower potency for WT EGFR. Another important feature to lazertinib is the lack of potency on HER2 that is often associated with dose-limiting adverse events,[17] as confirmed here in biochemical and cell-based studies. Our present structural analysis, however, does not reveal a discrete molecular origin for the preference of lazertinib for EGFR when compared to HER2. We speculate that differences in the sequence and dynamics of the HER2 kinase domain, as evident from reported crystal structures,[18] negatively impacts lazertinib reversible binding to HER2 and not osimertinib and LN2057 and motivates future structural studies to understand the structural basis for EGFR kinase

specificity. Results from these studies define the binding mode of a novel third-generation mutant-selective EGFR TKI lazertinib with improved dose-limiting toxicity as well as on-target potency and selectivity and serves as a noteworthy example for developing next-generation kinase inhibitors.

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Associated Content

Supporting information is available free of charge online.

Materials and methods, X-ray crystallography data and statistics, electron density maps, and LC-MS/MS images.

Abbreviations

H-bond (hydrogen bond), Epidermal growth factor receptor (EGFR), *N,N*-dimethylmethyleneamine (methyleneamine), Human epidermal growth factor receptor 2 (HER2).