ACS Medicinal Chemistry Letters

Letter

pubs.acs.org/acsmedchemlett

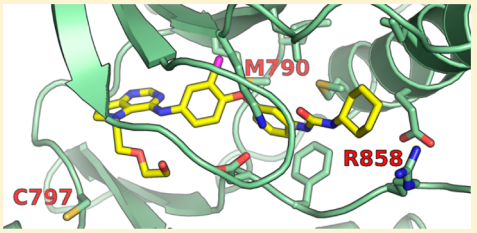
Structure-Based Approach for the Discovery of Pyrrolo[3,2-*d*]pyrimidine-Based EGFR T790M/L858R Mutant Inhibitors

Satoshi Sogabe,*[,‡] Youichi Kawakita,[‡] Shigeru Igaki,[‡] Hidehisa Iwata, Hiroshi Miki, Douglas R. Cary, Terufumi Takagi, Shinji Takagi, Yoshikazu Ohta, and Tomoyasu Ishikawa*

Pharmaceutical Research Division, Takeda Pharmaceutical Company Limited, 26-1, Muraoka-Higashi 2-chome, Fujisawa, Kanagawa 251-8555, Japan

Supporting Information

ABSTRACT: The epidermal growth factor receptor (EGFR) family plays a critical role in vital cellular processes and in various cancers. Known EGFR inhibitors exhibit distinct antitumor responses against the various EGFR mutants associated with nonsmall-cell lung cancer. The L858R mutation enhances clinical sensitivity to gefitinib and erlotinib as compared with wild type and reduces the relative sensitivity to lapatinib. In contrast, the T790M mutation confers drug resistance to gefitinib and erlotinib. We determined crystal structures of the wild-type and T790M/L858R double mutant EGFR kinases with reversible and irreversible pyrrolo[3,2-*d*]pyrimidine inhibitors based on analogues of TAK-285 and neratinib. In these structures, M790 adopts distinct conformations to accommodate different inhibitors, whereas R858 allows conformational variations of the activation loop. These results provide structural insights for understanding the structure−activity relationships that should contribute to the development of potent inhibitors against drug-sensitive or -resistant EGFR mutations.



KEYWORDS: *EGFR T790/L858 mutant inhibitors, NSCLC, drug sensitivity and resistance, crystal structure, pyrrolo[3,2-d]pyrimidine derivatives, TAK-285*

The signal transduction pathway based on binding of epidermal growth factor (EGF) to EGF receptor (EGFR) on the cell surface regulates cell proliferation, differentiation, and migration. The EGFR family plays a critical role in vital cellular processes and in various cancers and is a proven target in the treatment of cancer.[1] Several EGFR kinase inhibitors have been approved for clinical use or are currently in clinical development. These include gefitinib,[2] erlotinib,[3] lapatinib,[4] and TAK-285 (Figure 1).[5]

Several specific mutations of EGFR have been clinically identified in nonsmall-cell lung cancer (NSCLC) patients.[6] Known EGFR kinase inhibitors such as gefitinib, erlotinib, and lapatinib show distinct inhibitory profiles against these EGFR mutants. The most frequent single activation-loop mutation (L858R) increases inhibitory activity for gefitinib and erlotinib as compared with the wild-type EGFR and reduces the sensitivity to lapatinib.[2] After treatment with these inhibitors, however, emergence of an additional gate keeper mutation (T790M) has been reported as a key factor of drug resistance. The secondary T790M mutation reduces sensitivity to gefitinib and erlotinib.[7] The differential susceptibility of NSCLC to various inhibitors gave rise to a number of questions regarding the relation between EGFR mutation and clinical efficacy of EGFR inhibitors.

The development of EGFR inhibitors to target drug-resistant mutants has become a significant unmet need for the treatments of NSCLC patients. To date, several irreversible inhibitors such as neratinib (HKI-272),[8] afatinib (BIBW-2992),[9] and WZ4002[10] have been developed to target a cysteine (C797) residue located at the lip of the adenosine triphosphate (ATP)-binding cleft. These compounds were rationally designed to make a covalent bond with C797, which provides the potential to inhibit EGFR-T790M mutation.[7] However, such irreversible inhibitors have potential safety concerns due to poor selectivity with respect to other kinases as a result of their chemical reactivity with other cysteine residues.[11] Therefore, it is a worthy goal to discover novel, potent, noncovalent, reversible inhibitors against both the wild-type and/or the T790M/L858R double-mutated EGFR. Herein, we present a structural approach for the development of novel reversible pyrrolo[3,2-*d*]pyrimidine inhibitors of the EGFR double mutant.

We previously reported potent dual inhibitors of HER2/EGFR based on a pyrrolo[3,2-*d*]pyrimidine scaffold with significant in vivo antitumor efficacy.[5,12] Crystallographic analysis showed that the pyrrolo[3,2-*d*]pyrimidine compounds bind to the inactive conformation of EGFR, as has been reported for lapatinib.[13] Against the T790/L858R mutant,

Received: October 10, 2012
Accepted: December 18, 2012
Published: December 18, 2012

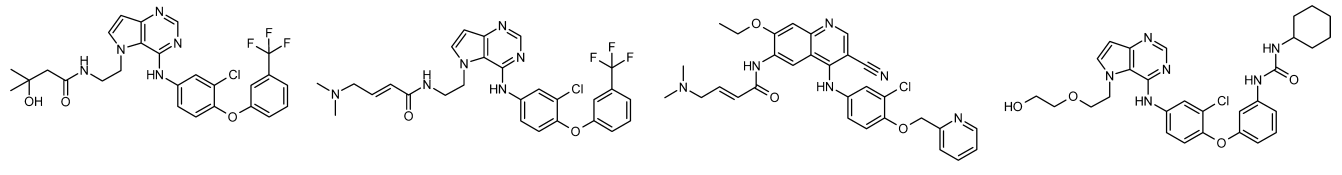


Figure 1. Structures of EGFR inhibitors.

TAK-285 (**1**) has no inhibitory activity, as shown in Table 1. We initially designed an irreversible inhibitor (**2**) based on the scaffold of compound **1** with a pendant Michael acceptor to bind covalently at C797 in an attempt to provide inhibitory activity against the mutant protein. However, compound **2** shows no significant inhibition of the T790M/L858R mutant, nor does it show any time dependency with an incubation time of up to 1 h (data not shown). We believed that structural analysis of compound **2** bound to mutant EGFR would provide valuable insights into the key interactions at the mutation sites and would help facilitate the rational design of potent EGFR mutant inhibitors. Therefore, we determined a crystal structure of the T790M/L858R double mutant in complex with compound **2** (Figure 2a).

Table 1. IC_{50} Values for Compounds in the EGFR Enzyme Assays

	EGFR IC_{50} (nM) (95% CI)[a]	
compd	wild-type	T790M/L858R
1	4.0 (2.9−5.6)	8400 (6700−11000)
2	4.6 (3.3−6.3)	8100 (6300−11000)
3	2.5 (1.4−4.3)	66 (43−100)
4	6.9 (4.6−10)	19 (12−32)

[a] IC_{50} values and 95% confidence intervals (CI) were calculated by nonlinear regression analysis of the percentage inhibitions (n = 2).

The crystal structure of T790M/L858R mutant adopts the activelike conformation, similar to that of the complex with erlotinib.[3] Compound **2** forms a covalent bond with C797, as expected, and occupies the ATP binding cleft, which is wider than that found for the erlotinib complex due to a conformational shift of the N-lobe to accommodate the bulky substituent of M790 adjacent to the regulatory αC-helix. The salt-bridge interaction between two highly conserved residues (K745 and E762), which is a canonical feature in the active conformation, is elongated due to a slight outward shift of the αC-helix (Figure 2a). The N-1 nitrogen on the pyrrolo[3,2-*d*]pyrimidine ring makes a hydrogen bond with the main-chain nitrogen of Met793 of the hinge region between the N- and the C-lobes. The trifluoromethyl-phenoxy group is positioned in a hydrophobic space, defined predominantly by I759, M766, L777, L788, and M790, in the vicinity of the αC-helix, as discussed below.

We also determined a crystal structure of the T790M/L858R mutant in complex with TAK-285 (Figure S1 in the Supporting Information). The binding conformation of TAK-285 with the T790M/L858R mutant is almost identical to that of compound **2**, suggesting that the interaction with the hinge region is independent of the covalent bond with C797. The irreversibility of the covalent bond seems not to contribute to the binding affinity against the T790M/L858R mutant for TAK-285.

On the other hand, structural comparison with the complex of TAK-285 bound to wild-type EGFR (Figure 2b) reveals that the T790M/L858R mutant adopts a different conformation than when bound to compound **2** or TAK-285. The orientation of the trifluoromethyl-phenoxy group is flipped between the wild-type and the mutant EGFR structures due to a change in the αC-helix conformation. The trifluoromethyl group is exposed to solvent region in both the mutant structures. For the T790M/L858R mutant, the pyrrolo[3,2-*d*]pyrimidine ring

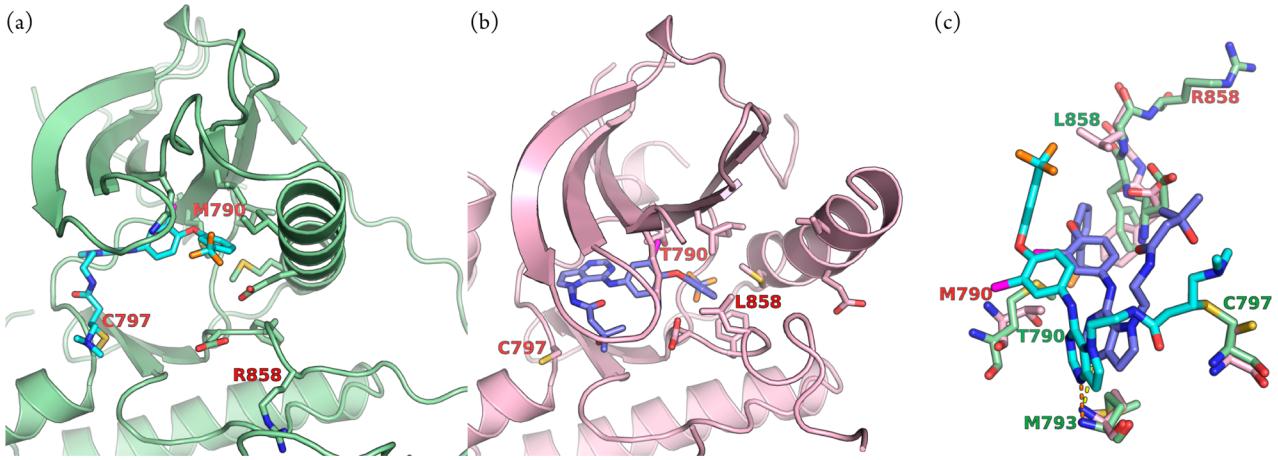


Figure 2. (a) Crystal structure of compound **2** bound to the T790M/L858R mutant, colored in green. Two mutation sites and C797 are labeled. (b) Crystal structure of TAK-285 (**1**) with wild-type EGFR (PDB code: 3POZ), colored in pink. (c) Superposition of compound **2** (cyan) with TAK-285 (blue) with key residues surrounding the binding site. Hydrogen bonds found in a and b are indicated by orange and yellow dashed lines, respectively.

dx.doi.org/10.1021/ml300327z | *ACS Med. Chem. Lett.* 2013, 4, 201−205

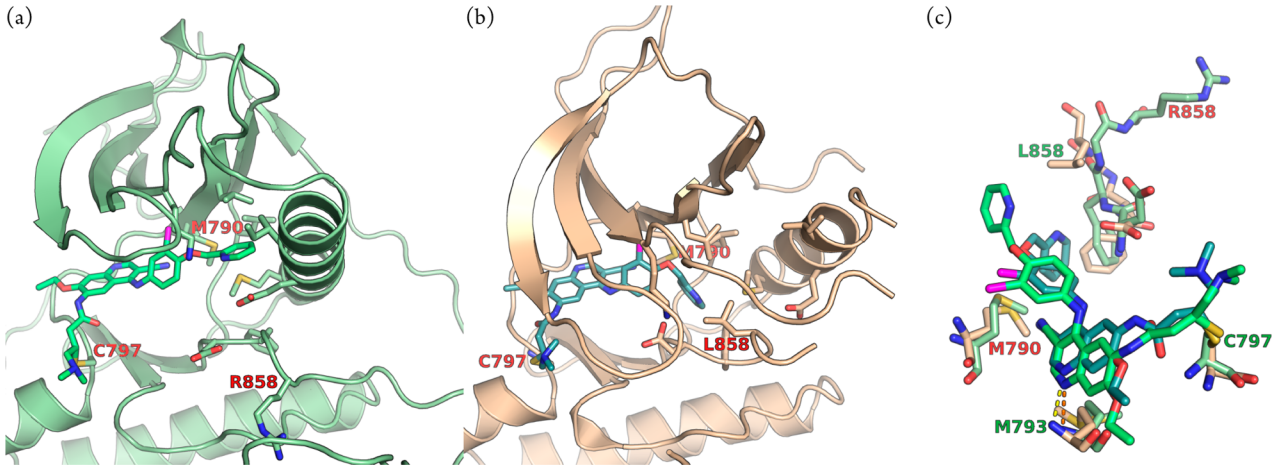


Figure 3. (a) Crystal structure of neratinib (**3**) bound to the T790M/L858R mutant, colored in green. The two mutation sites and Cys-797 are labeled. (b) Crystal structure of neratinib (**3**) bound to the T790M mutant (PDB code: 2JIV), colored in gold. (c) Overlaid structures of neratinib with key residues surrounding the binding site. Hydrogen bonds found in a and b are indicated with orange and yellow dashed lines, respectively.

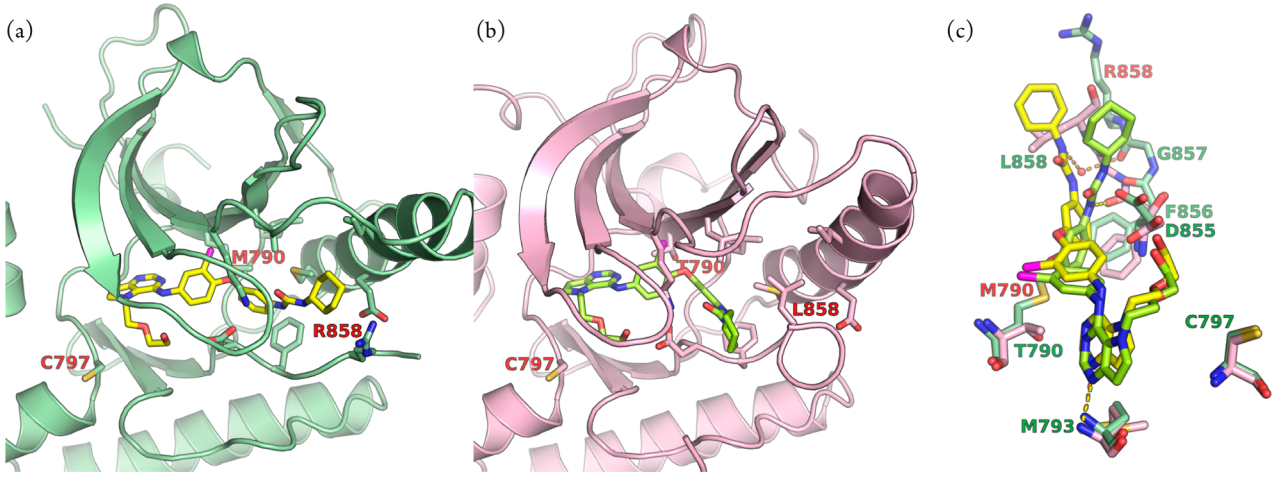


Figure 4. (a) Crystal structure of compound **4** bound to the T790M/L858R mutant, colored in green. Two mutation sites and Cys-797 are labeled. (b) Crystal structure of compound **4** bound to wild-type EGFR, colored in pink. (c) Overlaid structures of compound **4** with key residues surrounding the binding site. Hydrogen bonds found in a and b are indicated by orange and yellow dashed lines, respectively.

is shifted downward by steric hindrance from the αC-helix (Figure 2c). The hydrogen-bonding pattern with the hinge region seems not to be optimal, resulting in weak inhibitory activity against the T790M/L858R mutant. As described above, the L858R mutant confers sensitivity to inhibitors that bind to the active conformation but decreases the affinity for inhibitors that bind to the inactive conformation. Our structural data suggest that the loss of structural flexibility and stabilization of the activelike conformation would interfere with optimal binding for compound **2** or TAK-285. As a consequence, in the case of these pyrrolo[3,2-*d*]pyrimidine derivatives, the covalent bond with C797 was not effective at improving the EGFR mutant inhibitory activity. To clarify the binding mode of an irreversible inhibitor with the T790M/L858R mutant, we determined the structure of its complex with neratinib (**3**), which is an irreversible inhibitor against both the wild-type and the T790M/L858R mutant of EGFR (Table 1).

The crystal structure of the T790M/L858R EGFR complex of neratinib (**3**) revealed that neratinib binds in the ATP-binding cleft with a covalent bond to C797 in a fashion similar to that observed in the complex with compound **2** (Figure 3a). The C797 side-chain orientation is associated with the position and length of the acceptor substituent to accommodate covalent binding. The quinoline nitrogen of neratinib forms a hydrogen bond to the M793 main-chain nitrogen of the hinge region. Despite a similar binding mode found in the complexes of both compound **2** and of neratinib, a distinct conformation change for M790 was observed in the vicinity of these compounds. The side-chain rearrangement of M790 is necessary to accommodate binding of the cyano group for neratinib. The steric interaction with M790 appears to facilitate optimal interaction of the quinoline ring with the hinge region, whereas no steric effect of M790 was observed for pyrrolo[3,2-*d*]pyrimidine **2**. The terminal pyridyl group of neratinib roughly occupies the same space as the trifluoromethyl-phenoxy group of compound **2**. The pyrrolo[3,2-*d*]pyrimidine compound with the same anilino substituent of neratinib [3-chloro-4-(pyridin-2-ylmethoxy)phenyl group] shows weak inhibitory activity although the compound covalently binds in the same fashion as that of compound **2** (data not shown). Therefore, we concluded that the covalent interaction with C797 alone is insufficient to produce potent activity for the pyrrolo[3,2-*d*]pyrimidine scaffold.

dx.doi.org/10.1021/ml300327z | *ACS Med. Chem. Lett.* 2013, 4, 201−205

The crystal structure of neratinib bound to single mutant T790M EGFR has been reported.[7] Structural comparison with the single mutant structure indicates that the pyridyl-methoxy group occupies the hydrophobic pocket adjacent to the αC-helix with different binding conformations (Figure 3b,c). It was speculated that the L858R mutation prevents the kinase from adopting the inactive conformation by stabilizing the activation loop, resulting in a constitutively active form of the protein. On the other hand, in the T790M single mutant structure, the activation loop around L858 forms a short helix, and the αC-helix is oriented outward to facilitate the inactive conformation. The activation-loop conformation of the T790M/L858R mutant is very similar to that of other L858R mutants.[2,14] Although the binding conformation of neratinib with the T790M/L858R mutant may not be optimal, the covalent bond with C797 and the hydrogen-bonding interaction with the hinge region result in the retention of kinase inhibitory activity. This finding was conducive to further chemical modification of the pyrrolo[3,2-*d*]pyrimidine derivatives.

To enhance the EGFR T790M/L858R double mutant kinase inhibitory activity, we designed a back-pocket urea linker with a hydrophobic moiety to interact with the activation loop and the αC-helix, because the urea moiety should be able to engage in structural rearrangement of the DFG-motif conformation, such as that found for VEGFR2 inhibitors.[15] This approach resulted in the discovery of compound **4**, which exhibits potent kinase inhibitory activity against both the wild-type EGFR and the T790M/L858R double mutant (Table 1).

The cocrystal structures of wild-type EGFR and the T790M/L858R mutant bound to reversible pyrrolo[3,2-*d*]pyrimidine inhibitor **4** are shown in Figure 4. The structures reveal several interesting features, which may explain the drug sensitivity and resistance of the EGFR mutant. Compound **4** is bound to the ATP binding cleft, and the protein adopts the inactive conformation (Figure 4a,b). The overall fold of the protein is similar to that observed in the wild-type EGFR/lapatinib structure.[4] However, there are distinct differences in the orientation of the N- and C-lobes between the two structures with the compound **4**. These differences are caused by a conformational rearrangement of the N-lobe, resulting from the T790M mutation. The cyclohexyl-urea moiety makes a water-mediated hydrogen bond with the G857 carbonyl oxygen of the DFG motif in the case of the T790M/L858R mutant, whereas it forms direct hydrogen bonds with the F856 and G857 carbonyl oxygens of the wild type (Figure 4c). In the wild-type structure, L858 forms a short distorted helix that inserts into a hydrophobic pocket beside the αC-helix and interacts with the cyclohexyl group. On the other hand, in the mutant structure, the activation loop is elongated because R858 is solvent exposed with conformational flexibility. The binding conformation of the cyclohexyl-urea moiety allows the activation loop to occupy the hydrophobic space by displacement of the αC-helix. These findings indicate that the L858R mutation is a key factor in determining the conformation adopted by the N-terminal segment of the activation loop. Hydrophobic moieties such as a short helix for the wild-type and a bulky substituent for the double mutant are implicated in giving rise to potent inhibitory activity. At the gate keeper position, M790 of the mutant forms closer contacts with the phenoxy-chloroanilino group than those made by T790 in the wild-type protein. However, the pyrrolo[3,2-*d*]pyrimidine scaffold interacts with the hinge region in the same manner for both structures. This suggests that M790 can optimize its conformation to accommodate the bound ligand. A bulky substituent such as the cyclohexyl-urea group presumably facilitates the optimal positioning of the inhibitor in the ATP binding cleft for the T790M/L858R mutant.

In summary, structural analysis provided valuable insights into drug sensitivity (or insensitivity) caused by EGFR mutation. Irreversible covalent bond formation with the cysteine residue C797 is well-known to overcome drug resistance caused by T790M mutation. However, such covalent bond formation with C797 was ineffective for a slowly dissociating inhibitor such as TAK-285. The steric configuration between the cysteine and the gate keeper residues is critical for the interaction of inhibitors with the hinge region to give optimal binding in the ATP binding cleft. Therefore, the T790M mutation gives rise to differential binding affinity for different inhibitors based on structural rearrangement of the protein. The L858R mutation stabilizes the active conformation due to the presence of the larger side chain. Substituents such as the trifluoromethyl-phenoxy group of TAK-285 and the pyridyl group of neratinib are insufficient to exclude the αC-helix because the activation loop is incapable of forming a short helix to make a hydrophobic cluster with the N-lobe and αC-helix. Larger substituents such as the cyclohexyl-urea moiety require the short-helix position to adopt the inactive conformation. Ultimately, we discovered the novel reversible pyrrolo[3,2-*d*]pyrimidine inhibitor **4** with activity against both EGFR wild-type and T790M/L858R double mutant. Although compound **4** exhibited weak inhibition of tumor cell growth (IC_{50} = 4.8 μM in an EGFR mutant H1975 cell line), our results have important implications for further structure-guided development of more potent noncovalent reversible inhibitors of drug-resistant EGFR mutants with minimal safety concerns.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthetic procedures and characterization of synthesized compounds, protein expression and purification methods, biological enzymatic assay methods, X-ray crystallography, and Figure S1. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +81-467-32-2839. Fax: +81-466-29-4484. E-mail: satoshi.sogabe@takeda.com (S.S.). Tel: +81-466-32-1155. Fax: +81-466-29-4449. E-mail: tomoyasu.ishikawa@takeda.com (T.I.).

Author Contributions

‡These authors contributed equally.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Gyorgy Snell and Weston Lane for data collection at Advance Light Source and members of the Structural Biology Research Center for data collection at Photon Factory.

■ ABBREVIATIONS

EGFR, epidermal growth factor receptor; NSCLC, nonsmall-cell lung cancer; ATP, adenosine triphosphate

REFERENCES

(1) Hynes, N. E.; Lane, H. A. ERBB receptors and cancer: The complexity of targeted inhibitors. *Nat. Rev. Cancer* **2005**, *5*, 341−354.

(2) Yun, C.-H.; Boggon, T. J.; Li, Y.; Woo, M. S.; Greulich, H.; Meyerson, M.; Eck, M. J. Structures of lung cancer-derived EGFR mutants and inhibitor complexes: Mechanism of activation and insights into differential inhibitor sensitivity. *Cancer Cell* **2007**, *11*, 217−227.

(3) Stamos, J.; Sliwkowski, M. X.; Eigenbrot, C. Structure of the EGF receptor kinase domain alone and in complex with a 4-anilinoquinazoline inhibitor. *J. Biol. Chem.* **2002**, *277*, 46265−46272.

(4) Wood, E. R.; Truesdale, A. T.; McDonald, O. B.; Yuan, D.; Hassell, A.; Dickerson, S. H.; Ellis, B.; Pennisi, C.; Horne, E.; Lackey, K.; Alligood, K. J.; Rusnak, D. W.; Gilmer, T. M.; Shewchuk, L. A unique structure for epidermal growth factor receptor bound to GW572016 (Lapatinib): Relationships among protein conformation, inhibitor off-rate, and receptor activity in tumor cells. *Cancer Res.* **2004**, *64*, 6652−6659.

(5) Ishikawa, T.; Seto, M.; Banno, H.; Kawakita, Y.; Oorui, M.; Taniguchi, T.; Ohta, Y.; Tamura, T.; Nakayama, A.; Miki, H.; Kamiguchi, H.; Tanaka, T.; Habuka, N.; Sogabe, S.; Yano, J.; Aertgeerts, K.; Kamiyama, K. Design and Synthesis of Novel Human Epidermal Growth Factor Receptor 2 (HER2)/Epidermal Growth Factor Receptor (EGFR) Dual Inhibitors Bearing a Pyrrolo[3,2-d]pyrimidine Scaffold. *J. Med. Chem.* **2011**, *54*, 8030−8050.

(6) Paez, J. G.; Janne, P. A.; Lee, J. C.; Tracy, S.; Greulich, H.; Gabriel, S.; Herman, P.; Kaye, F. J.; Lindema,n, N.; Boggon, T. J.; Naoki, K.; Sasaki, H.; Fujii, Y.; Eck, M. J.; Sellers, W. R.; Johnson, B. E.; Meyerson, M. EGFR mutations in lung cancer: Correlation with clinical response to gefitinib therapy. *Science* **2004**, *304*, 1497−1500.

(7) Yun, C.-H.; Mengwasser, K. E.; Toms, A. V.; Woo, M. S.; Greulich, H.; Wong, K.-K.; Meyerson, M.; Eck, M. J. The T790M mutation in EGFR kinase causes drug resistance by increasing the affinity for ATP. *Proc. Natl. Acad. Sci.* **2008**, *105*, 2070−2075.

(8) Rabindran, S. K.; Discafani, C. M.; Rosfjord, E. C.; Baxter, M.; Floyd, M. B.; Golas, J.; Hallett, W. A.; Johnson, B. D.; Nilakantan, R.; Overbeek, E.; Reich, M. F.; Shen, R.; Shi, X.; Tsou, H. R.; Wang, Y. F.; Wissner, A. Antitumor activity of HKI-272, an orally active, irreversible inhibitor of the HER-2 tyrosine kinase. *Cancer Res.* **2004**, *64*, 3958−3965.

(9) Li, D.; Ambrogio, L.; Shimamura, T.; Kubo, S.; Takahashi, M.; Chirieac, L. R.; Padera, R. F.; Shapiro, G. I.; Baum, A.; Himmelsbach, F.; Rettig, W. J.; Meyerson, M.; Solca, F.; Greulich, H.; Wong, K.-K. BIBW2992, an irreversible EGFR/HER2 inhibitor highly effective in preclinical lung cancer models. *Oncogene* **2008**, *27*, 4702−4711.

(10) Zhou, W.; Ercan, D.; Chen, L.; Yun, C.-H.; Li, D.; Capelletti, M.; Cortot, A. B.; Chirieac, L.; Iacob, R. E.; Padera, R.; Engen, J. R.; Wong, K.-K.; Eck, M. J.; Gray, N. S.; Janne, P. A. Novel mutant-selective EGFR kinase inhibitors against EGFR T790M. *Nature* **2009**, *462*, 1070−1074.

(11) Carmi, C.; Galvani, E.; Vacondio, F.; Rivara, S.; Lodola, A.; Russo, S.; Aiello, S.; Bordi, F.; Costantino, G.; Cavazzoni, A.; Alfieri, R. R.; Ardizzoni, A.; Petronini, P. G.; Mor, M. Irreversible inhibition of epidermal growth factor receptor activity by 3-aminopropanamides. *J. Med. Chem.* **2012**, *55*, 2251−2264.

(12) Kawakita, Y.; Banno, H.; Ohashi, T.; Tamura, T.; Yusa, T.; Nakayama, A.; Miki, H.; Iwata, H.; Kamiguchi, H.; Tanaka, T.; Habuka, N.; Sogabe, S.; Ohta, Y.; Ishikawa, T. Design and Synthesis of Pyrrolo[3,2-d]pyrimidine Human Epidermal Growth Factor Receptor 2 (HER2)/Epidermal Growth Factor Receptor (EGFR) Dual Inhibitors: Exploration of Novel Back-Pocket Binders. *J. Med. Chem.* **2012**, *55*, 3975−3991.

(13) Aertgeerts, K.; Skene, R.; Yano, J.; Sang, B.; Zou, H.; Snell, G.; Jennings, A.; Iwamoto, K.; Habuka, N.; Hirokawa, A.; Ishikawa, T.; Tanaka, T.; Miki, H.; Ohta, Y.; Sogabe, S. Structural analysis of the mechanism of inhibition and allosteric activation of the kinase domain of HER2 protein. *J. Biol. Chem.* **2011**, *286*, 18756−18765.

(14) Yoshikawa, S.; Kukimoto-Niino, M.; Parker, L.; Handa, N.; Terada, T.; Fujimoto, T.; Terazawa, Y.; Wakiyama, M.; Sato, M.; Sano, S.; Kobayashi, T.; Tanaka, T.; Chen, L.; Liu, Z. J.; Wang, B. C.; Shirouzu, M.; Kawa, S.; Semba, K.; Yamamoto, T.; Yokoyama, S. Structural basis for the altered drug sensitivities of non-small cell lung cancer-associated mutants of human epidermal growth factor receptor. *Oncogene* **2012**, DOI: 10.1038/onc.2012.21.

(15) Iwata, H.; Imamura, S.; Hori, A.; Hixon, M. S.; Kimura, H.; Miki, H. Biochemical characterization of a novel type-II VEGFR2 kinase inhibitor: Comparison of binding to non-phosphorylated and phosphorylated VEGFR2. *Bioorg. Med. Chem.* **2011**, *19*, 5342−5351.