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An ATP-Competitive Inhibitor Modulates the Allosteric Function of the HER3 Pseudokinase

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SUMMARY

Human epidermal growth factor receptor 3 (HER3) is a receptor tyrosine kinase that lacks catalytic activity but is essential for cellular homeostasis due to its ability to allosterically activate EGFR and HER2. Although catalytically inactive, HER3 binds ATP tightly, hinting at a possible role of the nucleotide-binding pocket in modulating HER3 function. We report a structure of the HER3 pseudokinase bound to the ATP-competitive inhibitor bosutinib. Previously solved structures show that bosutinib can potently interact with multiple kinase domain conformations. In complex with HER3, bosutinib binds to yet another conformation, which is nearly identical to that observed in the HER3-ATP complex. Interestingly, occupation of the ATP-binding site by bosutinib improves the ability of HER3 to act as an allosteric activator of EGFR in vitro by increasing the affinity of the HER3-EGFR heterodimer in a membrane-dependent manner.

INTRODUCTION

Pseudokinases represent a subgroup of the kinase superfamily, whose members are catalytically inactive but retain an overall kinase domain fold. Several pseudokinases play important roles as allosteric regulators of other proteins (Boudeau et al., 2006). HER3, a member of the human epidermal growth factor receptor (HER/ErbB) family of tyrosine kinases, which also includes EGFR, HER2, and HER4, is a pseudokinase frequently deregulated in human cancers (Amin et al., 2010). HER3 is capable of signaling through ligand-induced heterodimerization with EGFR and HER2, which results in tyrosine phosphorylation of the HER3 C-terminal tail and subsequent activation of the phosphatidylinositol 3-kinase/Akt pathway. Sustained HER3 phosphorylation contributes to drug-induced resistance to HER2-targeting agents in breast cancer and EGFR-directed therapies in lung adenocarcinoma (Engelman et al., 2005; Sergina et al., 2007). These findings identify HER3 as an important target for anticancer therapies.

The pseudokinase domain of HER3 plays a vital role in the catalytic activation of HER receptors with which HER3 dimerizes

upon ligand binding. This is possible because activation of HER-family kinases requires formation of an asymmetric dimer between two kinase domains in which one kinase (the activator kinase) does not require catalytic activity but rather serves as an allosteric activator of its dimerization partner (the receiver kinase) (Zhang et al., 2006). In complex with other HER receptors, HER3 assumes the role of the activator kinase, and mutation of the HER3 activator interface, which directly contacts the receiver kinase, ablates catalytic activation of the signaling partners of HER3 (Jura et al., 2009b). The allosteric activator function of the HER3 pseudokinase domain is therefore an attractive target for HER3-directed therapies. However, selective targeting of the HER3 activator interface with small molecule inhibitors is a challenging goal because it is relatively flat, hydrophobic, and highly conserved among HER receptors.

Despite lacking catalytic activity, HER3 binds ATP tightly (Jura et al., 2009b; Shi et al., 2010). Residues important for ATP coordination, including the catalytic lysine (K723) and the aspartate residue within the aspartate-phenylalanine-glycine (DFG) motif (D833), are evolutionarily conserved in HER3. This suggests that ATP binding might be essential for HER3 function by playing a noncatalytic role, in a manner analogous to that previously described for the STRADα pseudokinase (Zeqiraj et al., 2009). As a consequence, small molecules that occupy the ATP-binding site of HER3 may regulate its ability to serve as an allosteric activator of other HER-family kinases. Although there are currently no reported ATP-competitive molecules developed specifically for HER3, an unbiased screen of 72 different ATP-competitive inhibitors against 442 human kinases identified bosutinib (SKI-606) as a high-affinity binder of HER3 (K_d 0.77 nM) (Davis et al., 2011). Bosutinib is a 4-anilinoquinoline-3-carbonitrile inhibitor and is similar in structure to gefitinib and erlotinib, Food and Drug Administration-approved inhibitors of EGFR and HER2. Using bosutinib, we addressed the intriguing possibility that binding of an ATP-competitive molecule to the HER3 pseudokinase domain could regulate its allosteric activator function.

RESULTS AND DISCUSSION

We first confirmed that bosutinib does in fact bind HER3 with high affinity. The HER3-bosutinib dissociation constant was determined to be 0.52 ± 0.06 nM, in close agreement with the previously reported value (Figure S1 available online) (Davis
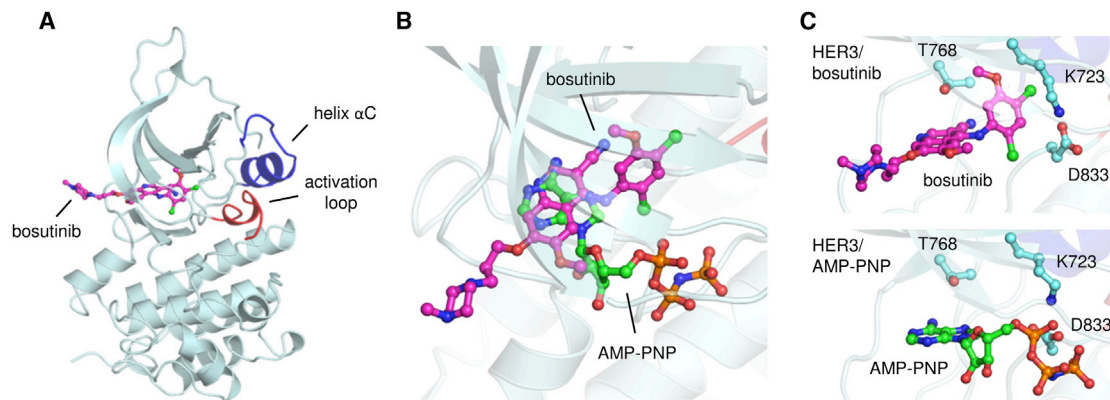
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Figure 1. Crystal Structure of the HER3 Pseudokinase Domain Bound to Bosutinib

(A) Overall structural organization of the HER3-bosutinib complex, highlighting the conformation of the αC helix (blue) and the activation loop (red).

(B) Overlay of bosutinib and AMP-PNP bound in the HER3 ATP-binding site. Carbon atoms of bosutinib are shown in magenta; those of AMP-PNP are shown in green.

(C) Close-up view of ligand interactions with selected HER3 residues involved in bosutinib and AMP-PNP binding. HER3-AMP-PNP: PDB ID code 3KEX.

et al., 2011). Bosutinib binding was abolished by mutation of the gatekeeper residue (T768I), a position that serves as a master regulator for access of small molecules to the ATP-binding site of kinases (Blencke et al., 2004; Noble et al., 2004). We then determined a crystal structure of the HER3-bosutinib complex to a resolution of 2.5 Å (Figure 1; Figure S2; Table S1). The structure contains the kinase domain of HER3 with bosutinib bound in the ATP-binding cleft in a manner similar to the previously reported complexes of bosutinib with the Abl and CamKII kinases (Chao et al., 2011; Levinson and Boxer, 2012). Despite similarities in the drug-binding mode, bosutinib-bound HER3 adopts a significantly different conformation from either Abl or CamKII in their bosutinib-bound states (Figure 2; Figure S3). In the structures of Abl and CamKII, the activation loop is in a fully extended conformation and the catalytically critical αC helix is rotated toward the active site, which is reminiscent of an active state of a kinase. However, the catalytically important DFG motif is in the inactive conformation, called DFG-out, in which the phenylalanine replaces the aspartate in the nucleotide-binding pocket. Bosutinib-bound HER3 adopts a very different conformation. Its DFG-aspartate is oriented toward the active site (DFG-in conformation), but the αC helix and the activation loop adopt an inactive position denoted as the Src/CDK-like inactive conformation. In this conformation, the αC helix is rotated away from the active site and the N-terminal portion of the activation loop forms a single-turn helix. The same conformation is adopted by the HER3 kinase domain bound to the ATP analog adenylyl-imidodiphosphate (AMP-PNP) (root-mean-square deviation 0.39 Å) (Figure S4) (Jura et al., 2009b; Shi et al., 2010). This is surprising given the significantly different binding modes employed by bosutinib and the nucleotide, which show similarity only through a partial overlay between the quinoline ring of bosutinib and the adenine ring of the nucleotide (Figure 1B). The methoxy substituent of bosutinib's quinoline ring extends in the same direction as the ribose sugar of AMP-PNP, but the 2,4-dichloro-5-methoxyaniline fragment lies 4–8 Å away from the triphosphate linkage. This region of bosutinib packs deeper into the ATP-binding cleft, where it nonetheless interacts with a

subset of HER3 residues involved in phosphate binding, including K723 and D833 (Figure 1C). Weaker electron density corresponding to the N-propoxy-N-methylpiperazine moiety indicates that this region of bosutinib does not interact strongly with HER3 (Figure S2B).

Binding of a small molecule to the nucleotide-binding pocket has recently been shown to regulate the allosteric functions of several kinases (Hatzivassiliou et al., 2010; Korennykh et al., 2011; Okuzumi et al., 2009; Poulikakos et al., 2010). Because such functionality would present a potential strategy to regulate HER3, we investigated whether bosutinib can modulate the allosteric activator function of HER3. We reconstituted the EGFR-HER3 heterodimer in vitro using purified HER3 and EGFR kinase domains and small unilamellar vesicles (Jura et al., 2009b). The EGFR kinase domain contained a V924R mutation, which prevents EGFR from functioning as an activator kinase and thus requires heterodimerization with HER3 for catalytic activation (Jura et al., 2009b). The reconstituted heterodimer was incubated with increasing concentrations of bosutinib and assayed for EGFR kinase activity. At high inhibitor concentrations, both HER3 and EGFR are bound by bosutinib, resulting in decreased catalytic activity due to EGFR inhibition (Figures 3A and 3D). However, at lower concentrations, where only HER3 should be bound by bosutinib due to a 45-fold higher binding affinity of bosutinib to HER3 than to EGFR (Davis et al., 2011), the HER3-dependent catalytic activity of EGFR-V924R was enhanced. This suggested that bosutinib binding might regulate the allosteric activator function of HER3.

To separate the effect of bosutinib on the activator function of HER3 from its direct inhibition of the EGFR kinase, we made EGFR refractory to bosutinib binding by introducing the gatekeeper mutation T766M (Davis et al., 2011). The basal catalytic activity of EGFR-V924R/T766M is increased compared to that of EGFR-V924R, but it is still highly dependent on dimerization with HER3 (Figure 3B). In contrast to EGFR-V924R, the catalytic activity of EGFR-V924R/T766M was enhanced at all bosutinib concentrations (Figures 3B and 3D). To test whether this effect is mediated by bosutinib binding to HER3, we eliminated the
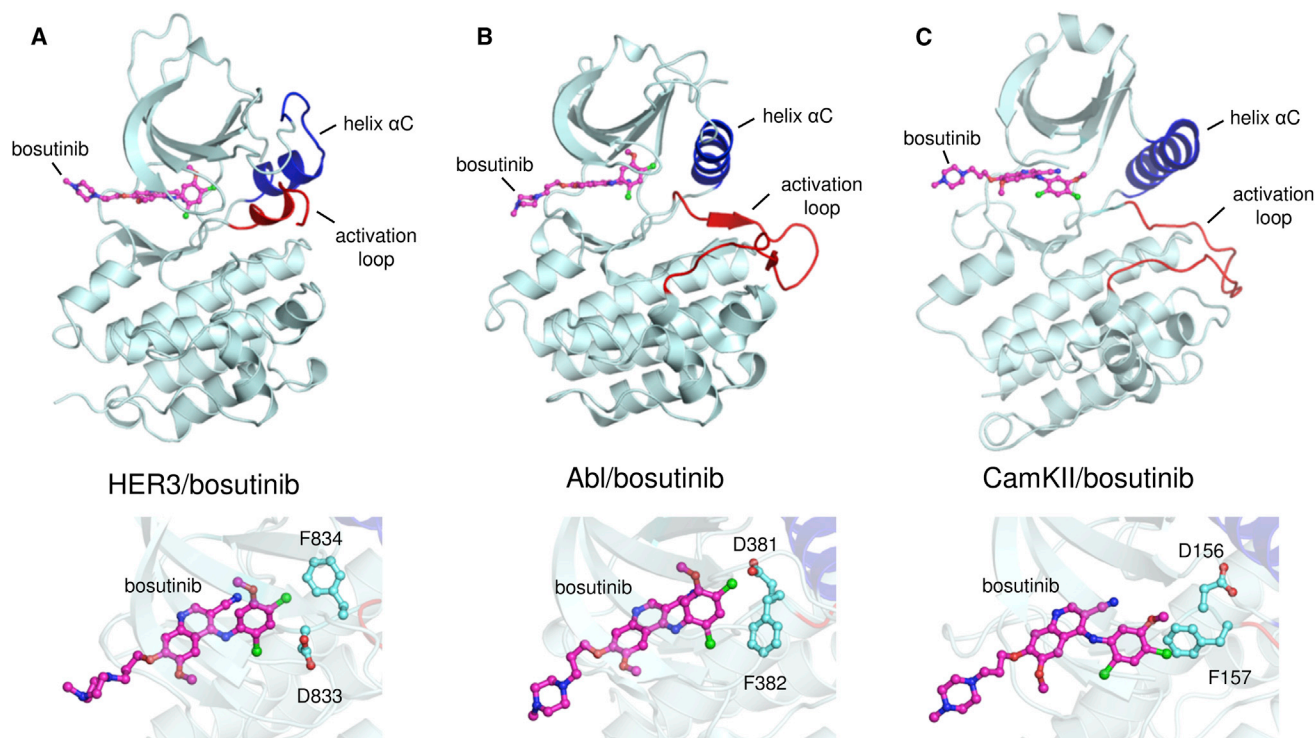
Figure 2. Comparison between HER3-Bosutinib, Abl-Bosutinib, and CamKII-Bosutinib Complexes
(A) HER3-bosutinib.
(B) Abl-bosutinib.
(C) CamKII-bosutinib.
Upper: cartoon representations highlighting the overall structural organization of the aligned kinase domains. Helix αC and the activation loop of each kinase are shown in blue and red, respectively. Carbon atoms of bosutinib are shown in magenta. Lower: close-up views of the interactions between bosutinib and the DFG motif of each kinase domain. Abl-bosutinib: PDB ID code 3UE4; CamKII-bosutinib: PDB ID code 3SOA.

ability of HER3 to bind bosutinib by introducing the gatekeeper mutation T768I. In this context, bosutinib failed to potentiate the kinase activity of EGFR-V924R/T766M (Figures 3C and 3D). Thus, binding of bosutinib to the ATP-binding site of HER3 enhances its function as an allosteric activator of the EGFR kinase.

We next sought to characterize the mechanism by which bosutinib enhances the allosteric function of HER3. A likely explanation is that bosutinib binding increases the strength of the association between the EGFR and HER3 kinase domains. If this were the case, bosutinib would not be expected to increase HER3 function under conditions in which all EGFR kinases are bound by HER3. The interaction between isolated kinase domains is very weak (Jura et al., 2009a; Zhang et al., 2006), and we were unable to saturate the EGFR-HER3 heterodimer on the surface of lipid vesicles due to limited binding capacity of the lipid membrane (Figure S5). To increase the EGFR-HER3 binding affinity, we extended the kinase domain constructs to include the juxtamembrane segments (JM-EGFR and JM-HER3), which potently increase HER receptor dimerization (Jura et al., 2009a). Using these constructs, we were able to reach near-saturating concentrations of JM-HER3 on vesicles (Figure 4A, left plot). Under these conditions, bosutinib increased EGFR activation only at low concentrations of JM-HER3. The activating effect of bosutinib in this experiment was lower than

that observed for isolated kinase domains, likely because of the already-increased dimerization affinity imposed by the juxtamembrane segments. When JM-HER3 levels approached saturation, EGFR kinase activity could no longer be elevated by bosutinib (Figure 4A, right plot). This result indicates that bosutinib binding to the HER3 pseudokinase increases the affinity of the EGFR-HER3 interaction.

Juxtamembrane segments can drive efficient dimerization of HER-family kinases in solution (Jura et al., 2009a), allowing us to determine whether bosutinib exerts its enhancing effect on EGFR-HER3 affinity in the absence of membrane. Interestingly, addition of bosutinib had minimal effect in this context (Figure 4B). This indicates that bosutinib binding to the HER3 pseudokinase domain potentiates EGFR-HER3 dimerization by modulating interactions that only occur on the surface of a lipid membrane. Although we do not currently understand the molecular requirements for this effect, increasing evidence points to an active contribution of the membrane surface in modulating protein-protein interactions. For example, interaction with the phospholipid bilayer is thought to negatively regulate the EGFR kinase (Arkhipov et al., 2013; Endres et al., 2013). If a similar mechanism were operative for HER3, bosutinib could increase the pool of HER3 available for interaction with EGFR by disrupting such inhibitory interactions. Another inhibitory mechanism thought to restrict HER3 function at the membrane involves the
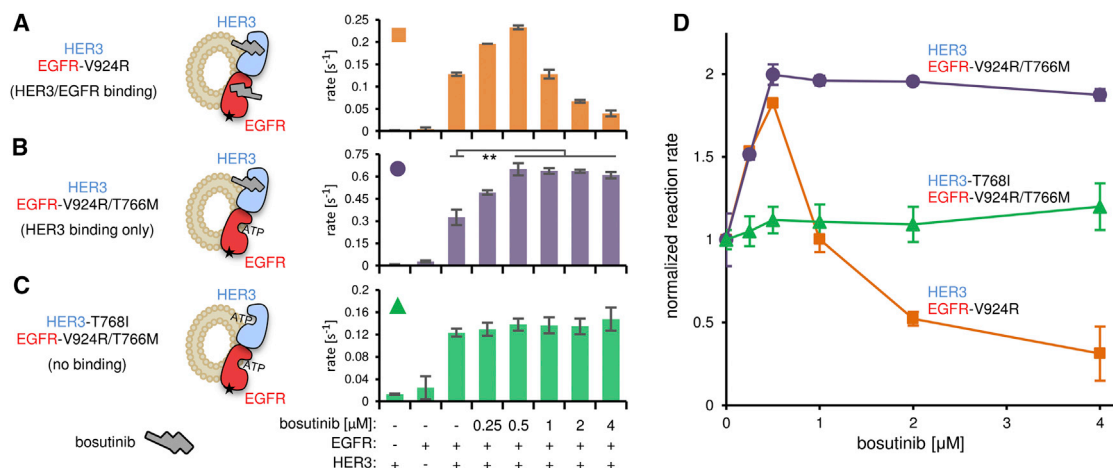
Figure 3. Effect of Bosutinib on the Allosteric Activator Function of HER3 In Vitro

(A–C) Concentration-dependent effect of bosutinib on measured kinase activity in the following bosutinib binding modalities: (A) HER3 and EGFR binding (HER3 and EGFR-V924R, orange squares); (B) HER3 binding only (HER3 and EGFR-V924R/T766M, purple circles); and (C) no binding (HER3-T768I and EGFR-V924R/T766M, green triangles). The black stars on the EGFR kinase domains represent the V924R mutation preventing EGFR self-activation through homodimerization.
(D) Normalized kinase activity in response to bosutinib titration.
Error bars in all plots represent SD of two independent measurements. **p < 0.001.

formation of inactive homo-oligomers (Kani et al., 2005; Landgraf and Eisenberg, 2000). In this scenario, the activating effect of bosutinib could arise from its ability to weaken autoinhibitory contacts between HER3 monomers.

Our results provide evidence for modulation of the allosteric function of the HER3 pseudokinase through an ATP-competitive inhibitor. HER3 therefore joins a diverse group of kinases, including the active kinases IRE1, RAF, and Akt and the pseudokinase STRADα, that can be regulated through their interactions with ATP or ATP-competitive molecules (Korennykh et al., 2011; Okuzumi et al., 2009; Poulikakos et al., 2010; Zeqiraj et al., 2009). Although the structural basis for bosutinib-mediated regulation of HER3 is currently unknown, the similarities between bosutinib-bound and AMP-PNP-bound HER3 pseudokinase structures suggest that the bosutinib-bound state mimics an ATP-bound state. Recently published work (Red Brewer et al., 2013) investigating the mechanistic basis for signaling by cancer-associated EGFR mutants shows that these mutants are impaired in their allosteric activator function (Littlefield and Jura, 2013). Because these mutations activate EGFR through destabilization of the Src/CDK-like inactive conformation (Shan et al., 2012; Zhang et al., 2006), this suggests that the Src/CDK-like conformation might be important for the allosteric activator function of HER receptors. Consequently, bosutinib likely exerts its modest yet significant activating effect on HER3 function in our assays through enhanced stabilization of the Src/CDK-like inactive conformation.

Although bosutinib binding increases HER3 function, it is likely that ATP-competitive molecules could be designed to similarly downregulate HER3 function through stabilization of an activator-incompetent state. Such molecules would be of therapeutic benefit in treating disease states in which HER3 signaling is misregulated. Our analysis of the interaction between bosutinib and the HER3 pseudokinase domain provides a fundamental proof of principle regarding the efficacy of future investigations into ATP-competitive inhibitors of HER3 function.

SIGNIFICANCE

The HER3 pseudokinase receptor exerts powerful control over signaling cascades that mediate cell survival and, consequently, HER3 is commonly deregulated in cancer. Due to the defining feature of HER3 as a catalytically inactive kinase, inhibiting HER3 function in a chemically tractable manner has remained elusive. In this study, we present a crystal structure of the kinase inhibitor bosutinib bound to the HER3 pseudokinase domain. We further explore this interaction to show that bosutinib binding modulates the capacity of HER3 to function as an allosteric activator of its coreceptor EGFR. To our knowledge, regulation of a pseudokinase function via the binding of an ATP-competitive small molecule has not been previously reported. These results highlight the possible significance of ATP binding in HER3 function. More importantly, our results provide a platform for the rational design of HER3-directed ATP-competitive small molecules that may effectively interfere with aberrant HER3 signaling in cancer.

EXPERIMENTAL PROCEDURES

Cloning and Protein Purification

The human HER3 kinase domain fragment, residues 674–1001 (numbering without the 19 amino acid signal peptide), and the EGFR kinase domain, residues 672–998 (numbering without the 24 amino acid signal peptide), were expressed in SF9 cells using the Bac-to-Bac expression system (Invitrogen) and purified as previously described (Jura et al., 2009b; Zhang et al., 2006). Mutations were introduced by QuikChange site-directed mutagenesis (Stratagene) and confirmed by DNA sequencing. Constructs including juxtamembrane segments contained HER3 residues 648–1001 and EGFR residues 645–998. All
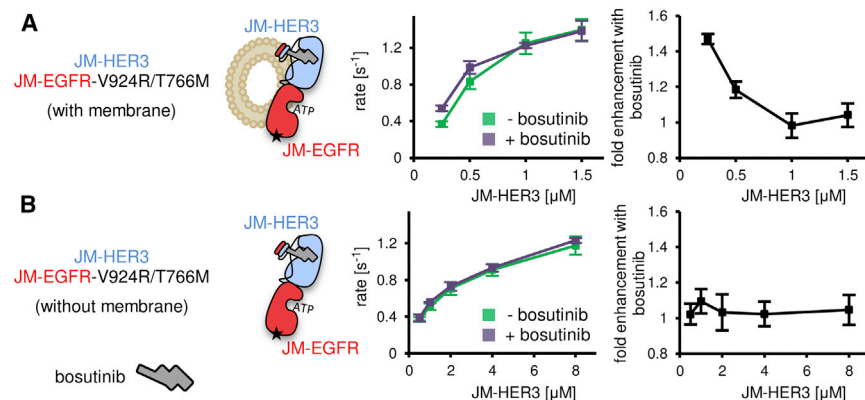
Figure 4. JM-EGFR Kinase Activity Measured under Titration with JM-HER3

(A) Kinase activity measured when JM-EGFR and JM-HER3 constructs were concentrated on a membrane surface. Left: observed reaction rates. Right: fold enhancement in kinase activity produced by the addition of bosutinib.

(B) As in (A), for kinase activity measured in the absence of lipid membranes. JM-HER3 concentrations in all panels reflect the bulk solution concentration. The concentration of JM-EGFR was held constant in all experiments. Bosutinib concentrations were equal to the listed JM-HER3 concentration plus an additional 2 μM excess. Error bars in all plots represent SD of two independent measurements.

constructs contained N-terminal polyhistidine tags, which were retained during purification.

Bosutinib Binding Assays

As described previously, bosutinib becomes fluorescent when it is bound to a kinase (Levinson and Boxer, 2012). Purified HER3 kinase domain was mixed with varying concentrations of bosutinib in 20 mM Tris (pH 8.0), 150 mM NaCl. The fluorescence emission of HER3-bound bosutinib was monitored at 463 nm with excitation at 280 nm. Background fluorescence of unbound bosutinib was subtracted before analysis of binding data. The published HER3-bosutinib Kd (0.77 nM) (Davis et al., 2011) was too strong to measure via a conventional ligand titration binding assay at a concentration of HER3 required for sufficient fluorescent signal. Therefore, 1.1 mM ATP/Mg^{2+} was included in the binding buffer in order to increase the apparent HER3-bosutinib Kd to an accurately measurable value. We used a competitive binding model

$$Kd_{app} = Kd_{bosutinib} * \left(1 + \frac{[ATP]}{Kd_{ATP}}\right)$$

to convert the observed K_d (Kd_{app}) into a standard dissociation constant using the published HER3-ATP K_d (1.1 μM) (Shi et al., 2010). Competitive binding curves were fit to a standard single-site binding model in GraphPad Prism (GraphPad Software).

Crystallography

The HER3-bosutinib complex was formed by dilution of DMSO-solubilized bosutinib in crystallization buffer (10 mM Tris [pH 8.0], 150 mM NaCl, 1 mM DTT, 1 mM Tris[2-carboxyethyl]phosphine) to a final concentration of 210 μM. HER3 kinase domain was then added to a final concentration of 6 mg/ml (160 μM). The complex was crystallized in the hanging-drop format by diluting the above solution with an equal volume of mother liquor containing 100 mM 2-(N-morpholino) ethanesulfonic acid (pH 6.7), 10% PEG 20,000. Crystals were cryoprotected by soaking in a solution containing mother liquor plus 30% glycerol, and then flash-frozen in liquid nitrogen. Diffraction data were collected on beamline 8.3.1 of the Advanced Light Source at the Lawrence Berkeley National Laboratory.

Data processing was completed using Mosflm (Leslie and Powell, 2007) and Scala (CCP4, 1994). Two data sets were merged to obtain greater completeness in the high-resolution shells. The crystal structure was determined by molecular replacement using a structure of the HER3 kinase domain bound to AMP-PNP (Protein Data Bank [PDB] ID code 3KEX) after removal of the ligand (Jura et al., 2009b). A positive electron density corresponding to bosutinib was visible in the ATP-binding site immediately after molecular replacement (Figure S2A). After two rounds of manual model building and automated refinement using Coot (Emsley et al., 2010) and PHENIX (Adams et al., 2010), respectively, bosutinib was built into the positive density and the structure was refined to completion. Detailed statistics for data collection and refinement can be found in Table S1.

In Vitro Kinase Assay

Kinase activity was measured using a continuous enzyme-coupled reaction system as previously described (Zhang et al., 2006). The reaction buffer con-

tained 20 mM Tris (pH 7.5), 10 mM MgCl$_2$, 500 μM ATP. Poly(4Glu:Tyr) peptide (Sigma) was used as the phosphorylation substrate at a concentration of 1 mg/ml. Small unilamellar vesicles were produced by extrusion through a membrane containing 100 nm pores (Whatman) using a mix of 90% 1,2-dioleoyl-sn-glycero-3-phosphocholine and 10% 1,2-dioleoyl-sn-glycero-3-([N-(5-amino-1-carboxypentyl)iminodiacetic acid]succinyl) (nickel salt) lipids (Avanti Polar Lipids).

ACCESSION NUMBERS

Coordinates and structure factors for the HER3-bosutinib complex have been deposited in the Protein Data Bank under PDB ID code 4OTW.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2014.02.011.

AUTHOR CONTRIBUTIONS

P.L. performed all experiments. P.L., M.M.M., and N.J. conceived the study. P.L. and N.J. analyzed the data and wrote the paper.

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