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Citation

Published Version
doi:10.1021/cb500674s

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DFG-out Mode of Inhibition by an Irreversible Type-1 Inhibitor Capable of Overcoming Gate-Keeper Mutations in FGF Receptors

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Supporting Information

ABSTRACT: Drug-resistance acquisition through kinase gate-keeper mutations is a major hurdle in the clinic. Here, we determined the first crystal structures of the human FGFR4 kinase domain (FGFR4K) alone and complexed with ponatinib, a promiscuous type-2 (DFG-out) kinase inhibitor, and an oncogenic FGFR4K harboring the V550L gate-keeper mutation bound to FIIN-2, a new type-1 irreversible inhibitor. Remarkably, like ponatinib, FIIN-2 also binds in the DFG-out mode despite lacking a functional group necessary to occupy the pocket vacated upon the DFG-out flip. Structural analysis reveals that the covalent bond between FIIN-2 and a cysteine, uniquely present in the glycine-rich loop of FGFR kinases, facilitates the DFG-out conformation, which together with the internal flexibility of FIIN-2 enables FIIN-2 to avoid the steric clash with the gate-keeper mutation that causes the ponatinib resistance. The structural data provide a blueprint for the development of next generation anticancer inhibitors through combining the salient inhibitory mechanisms of ponatinib and FIIN-2.

The FGF family of ligands consists of 18 structurally related polypeptides that signal in paracrine or endocrine fashion through four FGFRs (FGFR1-FGFR4) and their alternatively spliced isoforms to regulate a myriad of biological processes in human development, metabolism, and tissue homeostasis.1,2 FGFs bind and dimerize the extracellular domains of FGFRs in concert with heparan sulfate glycosaminoglycans or single-pass Klotho coreceptor proteins positioning the cytoplasmic kinase domains in proper proximity/orientation for transphosphorylation on A-loop tyrosines.3,4 This event elevates the intrinsic kinase activity of FGFRs leading to subsequent autophosphorylation on tyrosines in the flanking juxtamembrane (JM) and C-tail regions that mediate recruitment and phosphorylation of a distinct set of intracellular effector proteins by the activated FGFR evoking activation of intracellular signaling pathways.4–6

Uncontrolled activation of FGF signaling due to gain-of-function mutations in FGFRs, FGFR gene fusions involving various dimerizing partners, or overexpression/misexpression of FGFs and FGFRs contributes to a number of developmental disorders and cancer.7–11 Gain-of-function mutations in FGFRs were initially discovered in human congenital craniosynostosis and dwarfism syndromes. Later studies showed that the very same mutations occur somatically in diverse cancers, including multiple myeloma,12 bladder cancers,13 endometrial cancer,14 glioblastoma,15 lung cancer,16 adenoid cystic carcinoma,17 and benign skin cancer.18 FGFR gene fusions, originally found in the 8p11 myeloproliferative syndrome (an aggressive atypical stem cell myeloproliferative disorder),7,19 have since been extended to glioblastoma, bladder, and lung cancers.20,21 Overexpression of FGFs and FGFRs has been documented in breast, prostate, and bladder cancers.22 Single nucleotide polymorphism in FGFR2 has been linked with susceptibility to breast cancer,23 and SNP in FGFR4 has been associated with resistance to chemotherapy.24 In light of these data, FGFRs are now considered major targets for cancer drug discovery.

Indeed, several small molecule ATP-competitive inhibitors are being pursued in the clinic for FGFR-associated cancers including endometrial and prostate cancer. These include dovitinib,25 ponatinib,26,27 brivanib,28 multitargeted RTK inhibitors with coverage of FGFRs, and AZD4547,29 which has a more restricted FGFR target specificity profile. In addition, there are historical FGFR inhibitors such as PD173074,30 SU5402,31 and FIIN-132 which have been extensively used as pharmacological probes. All of these inhibitors are reversible ATP-competitive inhibitors with the exception of FIIN-1, which covalently targets an unusual
cysteine located in the glycine-rich loop of FGFR1−4. These inhibitors exhibit differential activity profiles with most acting primarily on the autoinhibited FGFRKs, while others also show activity against FGFR kinases carrying gain-of-function mutations. However, these inhibitors are ineffective against gate-keeper mutations,33,34 a mechanism that has been well documented to confer resistance in the clinic to many drugs targeting oncogenic kinases such as Bcr-Abl (T315I), EGFR (T790M), PDGFR (T674I), and c-Kit (T670I).

There is a major impetus to elucidate the structure−function relationships of FGFR kinases including the mechanisms of action of gain-of-function mutations and inhibitors as such data can provide crucial information to guide the development of inhibitors with improved selectivity and potency toward FGFR isoforms. To date, crystal structures of FGFR1−3 kinases in an autoinhibited state or in an activated state induced either by A-loop phosphorylation or by gain-of-function mutations have been determined.35−37 In addition, for FGFR1 and FGFR2 kinases, crystal structures exist of inhibitor bound forms.38−40 These structural data have guided the discovery of inhibitors with narrowed specificity toward FGFR kinases. Notably, the FGFR1K−PD173074 structure40 was used as template to develop FIIN-132 and FIIN-2, pyridopyrimidine-based irreversible inhibitors that exhibit greater specificity toward FGFRs. These inhibitors carry a reactive acrylamide group that is capable of forming a covalent bond with the thiol group of a cysteine uniquely present in the glycine-rich loop of FGFRs. Importantly, FIIN-2 shows activity against the FGFR kinase harboring gate-keeper mutation.

Rhabdomyosarcoma is the most common soft tissue sarcoma in children.8 FGFR4 activation due to overexpression or gain-of-function mutations in the FGFR4 kinase domain has been correlated with advanced-stage cancer and poor survival.8,41 FGFR4 inhibition has been shown to stop growth of rhabdomyosarcoma cell lines and cause tumor shrinkage in xenograft studies,42,43 supporting the notion that these mutations play causal roles in tumorigenesis. To facilitate the ongoing drug discovery for rhabdomyosarcoma, we solved the first crystal structures of FGFR4K alone and in complex with ponatinib and FIIN-2. These structures provide the first examples for a DFG-out mode of inhibition of FGFRK by an ATP-competitive inhibitor. Remarkably, FIIN-2 also binds in the DFG-out mode despite not conforming to the pharmacophore required for this binding mode.44 In addition, the FIIN-2 gate-keeper mutant complex demonstrates how the internal rotational flexibility allows this compound to adapt to the bulkier side chains at the gate-keeper location, thus retaining its inhibitory activity. These findings have general implications for the structure-guided design of inhibitors that can overcome the gate-keeper mutation in FGFR and likely other kinases.

■ RESULTS

Crystal Structure of Autoinhibited FGFR4 Kinase. As expected, the FGFR4 kinase domain (FGFR4K) adopts the canonical bilobate fold of protein kinases with the smaller N-

Figure 1. The crystal structure of wild-type FGFR4 kinase. (A) Ribbon diagram of the wild-type FGFR4K structure. β strands and α helices are colored cyan and green, respectively. The A-loop, catalytic loop, kinase insert, and kinase hinge are colored magenta, orange, wheat, and yellow, respectively. (B) Close-up view of the N-lobe showing formation of the catalytically critical salt bridge between K503 and E520. (C) Close-up view of the molecular brake at the kinase hinge region. (D) Close-up view of the active site of FGFR4K35 and activated FGFR2K (in slate) complexed with peptide (in yellow sticks) following superimposition of these two structures. Note that bidentate hydrogen bonds between R650 and D612 (the general base) block the access of substrate tyrosine into the active site of FGFR4K. The side chain of R650 in the FGFR4K structure occupies roughly the same space as the substrate tyrosine in the FGFR2K−peptide complex structure. In all figures, side chains of selected residues are shown as sticks, and atom colorings are as follows: oxygens in red, nitrogens in blue, and coloring of carbons follow the coloring scheme of the specific region of the kinase from which they derive. The hydrogen bonds are shown as black dashed lines.
The unphosphorylated FGFR4K is in an autoinhibited catalytically incompetent state, as evidenced by its comparison with the published crystal structures of unphosphorylated FGFR1−2 kinases, and activated FGFR1−3 kinases either by A-loop tyrosine phosphorylation or by pathogenic gain-of-function mutations (Supporting Information Figure S2). As in FGFR1−3 kinases, FGFR4K auto-inhibition is principally governed by a network of inhibitory hydrogen bonds at the kinase hinge region termed the molecular brake (Figure 1C). This network, which is mediated by a triad of residues consisting of Asn-535, Glu-551, and Lys-627 in FGFR4K, restrains the N-lobe movement toward the C-lobe that accompanies kinase activation. Reminiscent of the unphosphorylated FGFR1K structure, an additional constraint is provided by the DFGLAR motif at the beginning of the A-helix and the larger C-terminal lobe (Figure 1A). Both the A-loop and the loop connecting αD and αE helices, referred to as the kinase insert, are fully ordered. The ordering of the A-loop is due to the intramolecular contacts between the loop and the rigid body of the C-lobe. By contrast, the observed conformation of the kinase insert is solely attributable to favorable crystal lattice contacts. The kinase insert region of FGFR4K bulges out of the main body of the kinase domain and, unlike FGFR1−3 kinases, lacks tyrosine autophosphorylation sites (Supporting Information Figure S1A).

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The V550L gate-keeper mutants were resistant to inhibition by ponatinib, however (Figure 3A and Supporting Information Figure S5). By contrast, FIIN-2 effectively inhibited the substrate phosphorylation ability of all mutants including the V550L gate-keeper mutant (Figure 3B). To understand the molecular basis for the differential sensitivity of the FGFR4K gate-keeper mutations to ponatinib and FIIN-2, we decided to determine the crystal structures of wild-type FGFR4K bound to ponatinib, the FGFR4KV550L gate-keeper mutant alone, and in complex with FIIN-2.

**Ponatinib Inhibits FGFR4K by Inducing a DFG-in → DFG-out Rearrangement.** The FGFR4K–ponatinib complex crystallized under identical conditions to the free FGFR4K, yielding crystals that were isomorphous to the free FGFR4K crystals. As anticipated, ponatinib binds into the ATP binding pocket between the N and C lobes (Figure 4A). Drug binding...
does not induce any significant change in the interlobe angle. The C-alpha atoms of FGFR4K−ponatinib and apo-FGFR4K structures superimpose very well (RMSD of 0.2 Å) with the exception of the DFG motif at the beginning of the A-loop, which undergoes a dramatic DFG-in → DFG-out rearrangement in response to ponatinib binding (Figure 4A and Supporting Information Figure S6A).

Ponatinib consists of an imidazo[1,2-b]pyridazine heterocyclic scaffold that is linked via an acetylene group to a methylphenyl ring that in turn is joined via an amide bond to a trifluoromethylphenyl aromatic ring (Figure 4A). A methylpiperazine ring has been appended via methyl linkage to the trifluoromethylphenyl aromatic ring to aid in penetration of the drug into cells. Ponatinib binds into the ATP binding cleft in an extended conformation. The methylphenyl and trifluoromethylphenyl rings are almost coplanar and have a 90° interplanar angle with the (imidazo[1,2-b]pyridazine) bicyclic aromatic ring. Ponatinib engages a vast area that stretches from the kinase hinge region at the back of the kinase all the way to the catalytic pocket at the front end of the kinase.

The aromatic rings of the drug engage three sites within the ATP binding cleft (Figure 4A). At site 2, the imidazo[1,2-b]pyridazine scaffold occupies approximately the same space as the adenine ring of ATP and makes a single hydrogen bond with the backbone amide nitrogen atom of Ala-553 in the kinase hinge region (Figure 4A, middle panel). Reminiscent of ATP binding, the imidazo[1,2-b]pyridazine aromatic ring is sandwiched between hydrophobic residues from N- and C-lobes of the kinase. The rigid acetylene linkage directs the remaining portion of the drug deep toward the rear corner of the ATP binding pocket where the methylphenyl and 3-trifluoromethylphenyl aromatic rings of the inhibitor engage sites 2 and 3, respectively (Figure 4A, middle panel). Importantly, these aromatic rings induce two significant conformational changes in the ATP binding cleft including (I) displacement of the catalytic Lys-503 side chain in site 2 and (II) an outward flipping of the DFG motif in site 3 (Figure 4A, right panel). These structural rearrangements are necessary to alleviate steric conflicts with the compound ultimately optimizing binding interactions of the inhibitor.

At site 2, the methylphenyl group pushes the side chain of catalytic Lys-503, which along with Val-550, the gate-keeper residue, and Met-524 from the αC helix form a hydrophobic pocket that binds the methylphenyl aromatic ring. The displacement of Lys-503 indirectly helps the catalytic Glu-520 from the αC helix to make a direct hydrogen bond with the amide linkage between aromatic rings of the drug (Figure 4A, middle panel). At site 3, the 3-trifluoromethylphenyl moiety expels the DFG phenylalanine out of the cleft and occupies the hydrophobic pit that becomes vacant upon outward movement of the phenylalanine (Figure 4A, right panel). The displaced phenylalanine side chain is now in position to engage in
favorable hydrophobic contacts with the scaffold and the acetylene linker (Figure 4A, middle panel). As another important consequence of phenylalanine displacement, Asp-630 from DFG is also forced into a catalytically incompetent orientation where the backbone atoms of Asp-630 gain the ability to make hydrogen bonds with the amide linkage between the aromatic rings of the inhibitor. Interestingly, even the piperazine moiety of the drug contributes to drug binding affinity. The piperazine ring falls in the vicinity of the catalytic loop where it engages in hydrogen bonds with the loop (Figure 4A, middle panel). The overall binding mode of ponatinib in the FGFR4K−ponatinib structure resembles that observed in the Abl−Ponatinib complex structure where the DFG-out mode of inhibition was initially observed.46 Unlike Abl−Ponatinib, however, the glycine-rich loop of FGFR4 does not partake in ponatinib recognition.

**Gate-Keeper Mutations Confer Resistance to Ponatinib Inhibition by Introducing a Steric Clash with the Inhibitor.** As shown in Supporting Information Figure S5, ponatinib is capable of silencing all the FGFR4 pathogenic mutations with the exception of the V550L and V550E gate-keeper mutations. To understand the molecular basis for how these mutations render the kinase refractory to inhibition, we also solved the crystal structure of FGFR4K−FIIN-2 complex structure onto FGFR4KV550L revealed steric clashes between the added methyl group in Leu-550 and the imidazo[1,2-b]pyridazine scaffold of ponatinib.
which underlie the resistance of the FGFR4K<sub>V550L</sub> to ponatinib (Figure 4B).

**Crystal Structure of FGFR4K<sub>V550L</sub> Complexed with FIIN-2.** As shown in Figure 3, unlike ponatinib, FIIN-2 is capable of inhibiting the gate-keeper V550L mutant. To understand how FIIN-2 is capable of overcoming gate-keeper mutations, the crystal structure of the FGFR4K<sub>V550L</sub> mutant in complex with FIIN-2 was solved. FIIN-2 is a PD173074-based compound in which the cyclic urea N of the pyridopyrimidine scaffold has been derivatized with an acrylamidobenzyl substituent possessing a reactive acrylamide in the para position (Figure 5A and Supporting Information Figure S7A). The benzyl moiety serves as a spacer to position the acrylamide, the electrophilic center of the compound in the vicinity of the thiol group of a unique cysteine in the glycine-rich loop of FGFRs (Supporting Information Figure S1C) allowing for formation of a covalent bond via a Michael addition reaction. To this end, we first used mass spectrometry to demonstrate that FIIN-2 irreversibly reacts with Cys-477 (in the glycine-rich loop) in both FGFR4K<sub>WT</sub> and FGFR4K<sub>V550L</sub>. To do so, FGFR4K<sub>WT</sub> and FGFR4K<sub>V550L</sub> were incubated with FIIN-2 and digested with trypsin, and adduct formation between the tryptic peptide containing Cys-477 and FIIN-2 was analyzed by tandem mass spectrometry. As shown in Figure 5B, in the presence of FIIN-2, the mass of the tryptic peptide containing the Cys-477 increased by 634.3 Da, corresponding to the mass of the drug, confirming that FIIN-2 can irreversibly inhibit both FGFR4K<sub>WT</sub> and FGFR4K<sub>V550L</sub>.

The FGFR4K<sub>V550L</sub>−FIIN-2 complex structure shows that like ponatinib, FIIN-2 binding does not affect the kinase interlobe angle (Figure SC and Supporting Information Figure S7A). The C-alpha atoms of FGFR4K<sub>V550L</sub>−FIIN-2 and apo-FGFR4K<sub>V550L</sub> match closely with the exceptions of the glycine-rich loop and the DFG motif, both of which undergo major conformational rearrangements in response to drug binding (Figure SC,D,E and Supporting Information Figure S7B). Remarkably, the DFG motif undergoes an outward transition reminiscent of that seen in the FGFR4K−ponatinib structure (Figure SE). This is rather surprising because FIIN-2, like its parent molecule PD173074, lacks a functional group necessary to actively force the DFG out of the ATP binding cleft. As detailed below, this unusual property of FIIN-2, a type-I inhibitor, to bind FGFR4K like a type-II inhibitor is a direct consequence of the covalent bonding between FIIN-2 to FGFR4K.

The pyridopyrimidine scaffold of FIIN-2 occupies roughly the same space as the bicyclic ring of ponatinib (site 1) and is sandwiched by hydrophobic residues from the N and C lobes of kinase (Figure SC,D). Likewise, the dimethoxyphenyl ring, the key determinant of selectivity of PD173074 and likewise FIIN-2 for FGFRs, penetrates deep into the back pocket of the cleft engaging the same site as the methylphenyl ring of ponatinib (site 2). The acrylamidobenzyl group protrudes out of the ATP-binding pocket and places its α,β-unsaturated acrylamide, the electrophilic center of the drug, in the vicinity of nucleophilic thiol group of Cys-477, resulting in a covalent bond formation via a Michael addition reaction (Figure S). This covalent bonding pulls the glycine-rich loop toward the compound enabling additional contacts between the compound and the glycine-rich loop to form. Specifically, the phenylalanine from the glycine-rich loop makes hydrophobic contacts with the acrylamidobenzyl ring of drug and backbone amid nitrogens of the glycine-rich loop form a hydrogen bond with the carbonyl group of acrylamide of the drug (Figure SD). In addition to causing the observed conformational change of the glycine-rich loop, the covalent bond between FIIN-2 and the glycine-rich loop cysteine is ultimately responsible for the DFG-flip seen in the structure. Specifically, the altered conformation of the glycine-rich loop creates favorable π−π stacking contacts between the Phe-478 from the glycine-rich loop and the Phe-631 from the DFG motif (Figure SD), which stabilizes the DFG-out conformation enabling the Phe-631 to also contribute to inhibitor binding.

Comparison of the FGFR4K<sub>V550L</sub>−FIIN-2 and FGFR4K<sub>WT</sub>−ponatinib structures explains the molecular basis for the differential sensitivity of these two inhibitors toward the V550L gate-keeper mutation. While the added methyl group in Leu-550 introduces steric clash with the imidazo[1,2-b]pyridazine scaffold of ponatinib (Figure 4B, right panel), Leu-550 is still able to make favorable hydrophobic contacts with the dimethoxyphenyl ring of FIIN-2 (Figure SD). It is noteworthy that the rigidity of the acetylene linkage is disadvantageous as it precludes any structural adjustment of the methylphenyl moiety of ponatinib (Supporting Information Figure S3A) to alleviate this steric clash. In contrast, rotational freedom around the single bond linking the scaffold and dimethoxyphenyl of FIIN-2 (Supporting Information Figure S3B) would allow for small structural adjustments to bypass any potential steric clash with the bulkier side chain of L550. Indeed, an overlay of the crystal structure of the FGFR4K<sub>WT</sub>−FIIN-2 complex onto the FGFR4K<sub>V550L</sub>−FIIN-2 structure shows that the dimethoxyphenyl ring in the FGFR4K<sub>V550L</sub>−FIIN-2 complex undergoes slight rotation around the single bond linker to accommodate for the bulkier leucine side chain in the gate-keeper mutant (Figure SF,G).

**DISCUSSION**

In this report, we elucidated the molecular bases for FGFR4 kinase autoinhibition, inhibition by ponatinib and FIIN-2, and drug resistance caused by gate-keeper mutations. The FGFR4K−ponatinib and FGFR4K−FIIN-2, reported here, are the first examples of FGFR−inhibitor complexes featuring a DFG-out mode of inhibition. In fact, only a tiny fraction of published tyrosine kinase-inhibitor structures depict a DFG-out mode of inhibition. Among RTKs, KIT, TIE2, MET, and VEGFR2 are the only examples that have been shown so far to bind inhibitors in a DFG-out fashion. 47−49

Previously, crystal structures of FGFR1 and FGFR2 kinases in complex with inhibitors bearing oxyindole and pyridopyrimidine and quinazolinol as scaffolds have been solved. 38,39,50 In none of these structures, however, does the inhibitor penetrate deep enough to access site 3, and accordingly the DFG motif remains in its original "in" conformation. In fact, the oxyindoles do not even make use of site 2, although they can induce conformational changes in the glycine-rich loop to create hydrophobic contacts between conserved phenylalanine from the glycine-rich loop and the drug contributing to drug affinity. The FGFR4K−ponatinib (Figure 4) and FGFR4K−FIIN-2 (Figure S) complex structures elegantly demonstrate that these two inhibitors attain their superior inhibitory potency against FGFR kinases by binding the kinase via a DFG-out mechanism. In addition, the FGFR4K−FIIN-2 structure, the first structure of an FGFR kinase with a covalently acting inhibitor (Figure S), shows how this compound takes advantage of a unique cysteine in the glycine-rich loop of FGFRs to achieve FGFR target specificity.
Interestingly, in contrast to FGFRs, where the DFG-out conformation has never been visualized in the previously published apo crystal structures, there are structures of unliganded KIT and Abl that display a DFG-out conformation in the absence of an inhibitor. These data imply that in the absence of an inhibitor, these RTKs can adopt both DFG-in and DFG-out conformations. The relative distribution of the DFG-in/DFG-out states will dictate the design of more selective covalent inhibitors for FGFR4K.

### METHODS

Please refer to the Supporting Information for full details.

### Protein Expression and Purification

The human FGFR4 kinase domains FGFR3K<sup>553–573</sup> and FGFR2K<sup>577–632</sup>, including its mutated forms, and the C-terminal tail peptide of FGFR2 kinase (FGFR2K<sup>577–632</sup>) were all expressed using pET bacterial expression vectors with an N-terminal 6XHis-tag to aid in protein purification.  

### Crystallization and Structure Determination

All the crystals were grown by hanging drop vapor diffusion method either at 4 °C (FGFR4K<sup>WT</sup>, FGFR4K<sup>V550L</sup>, and FGFR4K<sup>V550L–FIIN-2</sup>) or 18 °C (FGFR4K<sup>V550L/Cys477</sup>–FIIN-2). FGFR4K<sup>WT</sup> crystallized in a buffer composed of 0.1 M MES (pH 5.5), 20% (w/v) PEG 4000, 0.2 M Li<sub>2</sub>SO<sub>4</sub> and 0.01 M taurine. Crystals of the FGFR4K<sup>V550L</sup> were obtained using a crystallization buffer composed of 0.1 M Tris (pH 7.5), 20% (w/v) PEG 1500, and 0.2 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. The FGFR4K<sup>WT–ponatinib</sup> complex was crystallized using a crystallization buffer composed of 0.1 M MES (pH 5.5), 10–12 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and 10 mM yttrium(III) chloride hexahydrate. All diffraction data were processed using the HKL2000 suite and the crystal structures were solved using maximum likelihood molecular replacement program Phaser in the PHENIX software suite. The crystal structure of wild-type FGFR2 kinase (PDB ID: 2PSQ) was used as the search model.  

### Peptide Substrate Phosphorylation Assay by Native Gel and MALDI-TOF Mass Spectrometry

Peptide substrate phosphorylation activities of wild-type and pathogenic mutated FGFR4 kinases (FGFR4K<sup>WT</sup>, FGFR4K<sup>V550L</sup>, and FGFR4K<sup>V550L–FIIN-2</sup>) and the inhibitory efficiency of ponatinib or FIIN-2 were analyzed by native gel electrophoresis and positive ion MALDI-TOF MS (Bruker Autoflex MALDI-TOF, Bruker Daltonics) in linear mode.

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**Table 1. X-ray Data Collection and Refinement Statistics**

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Numbers in parentheses refer to the highest resolution shell. R<sub>sym</sub> = ΣI − ΣI<sub>i</sub> where I is the observed intensity of a reflection, and ΣI is the average intensity of all the symmetry related reflections.
Inhibition of Kinase Autophosphorylation by Ponatinib. The autophosphorylation of the wild-type FGFR4 kinase and its pathogenic variants (FGFR4K<sup>V550K</sup>, FGFR4K<sup>V550D</sup>, FGFR4K<sup>V550E</sup>, FGFR4K<sup>V550A</sup>) inhibited by ponatinib were analyzed with native gel electrophoresis and LTQ Orbitrap (Thermo Electron) LC-MS/MS.

BaF3 Cell Viability Assay. TEL-FGFR4-transformed BaF3 cells were seeded in a 96 well plate and treated with the indicated concentration of the compounds. After 72 h, cell viability was assessed by MTS assay. The IC<sub>50</sub> values were calculated using GraphPad Prism version 5.0 (GraphPad Software Inc.). To generate the FGFR4<sup>V550K</sup> expressing BaF3 cell line, the V550L mutation was introduced into the Tel-FGFR4<sup>WT</sup> chimera, which had been subcloned into the retroviral expression vector, using site-directed mutagenesis (Agilent) and was transduced into BaF3 cell line using retroviral infection.

Analysis of Covalent Bond Formation between FIIN-2 and FGFR4K by LC-MS/MS. To test if FIIN-2 can form a covalent bond with Cys477 in the glycine-rich loops, FGFR4K<sup>WT/C477</sup> and FGFR4K<sup>V550L</sup>, FGFR4K<sup>V550E</sup>) inhibited by ponatinib were analyzed by LC-MS/MS.

ACKNOWLEDGMENTS
The authors declare no competing financial interest.

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Identification of targetable FGF receptor gene fusions in diverse cancers. 

Cancer Discovery 3, 636–647.


