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Illuminating the Molecular Mechanisms of Tyrosine Kinase Inhibitor Resistance for the FGFR1 Gatekeeper Mutation: The Achilles' Heel of Targeted Therapy

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Abstract

Human fibroblast growth factor receptors (FGFRs) 1–4 are a family of receptor tyrosine kinases that can serve as drivers of tumorigenesis. In particular, *FGFR1* gene amplification has been implicated in squamous cell lung and breast cancers. Tyrosine kinase inhibitors (TKIs) targeting FGFR1, including AZD4547 and E3810 (Lucitanib), are currently in early phase clinical trials. Unfortunately, drug resistance limits the long-term success of TKIs, with mutations at the “gatekeeper” residue leading to tumor progression. Here we show the first structural and kinetic characterization of the FGFR1 gatekeeper mutation, V561M FGFR1. The V561M mutation confers a 38-fold increase in autophosphorylation achieved at least in part by a network of interacting residues forming a hydrophobic spine to stabilize the active conformation. Moreover, kinetic assays established that the V561M mutation confers significant resistance to E3810, while retaining affinity for AZD4547. Structural analyses of these TKIs with wild type (WT) and gatekeeper mutant forms of FGFR1 offer clues to developing inhibitors that maintain potency against gatekeeper mutations. We show that AZD4547 affinity is preserved by V561M FGFR1 due to a flexible linker that allows multiple inhibitor binding modes. This is the first example of a TKI binding in distinct conformations to WT and gatekeeper mutant forms of FGFR, highlighting adaptable regions in both the inhibitor and binding pocket crucial for drug design. Exploiting inhibitor flexibility to overcome drug resistance has been a successful strategy for combatting diseases such as AIDS and may be an important approach for designing inhibitors effective against kinase gatekeeper mutations.

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Supporting Information

Supporting figures, table, and detailed methods are available free of charge via the Internet at <http://pubs.acs.org>

Accession Codes

The PDB codes are notated as follows: apo V561M FGFR1 (4RWI), WT FGFR1•AZD4547 (4RWJ), V561M FGFR1•AZD4547 (4RWK), WT FGFR1•E3810 (4RWL).

Notes

The authors declare no competing financial interest.

Fibroblast growth factor receptors (FGFR) 1–4 are a family of transmembrane receptor tyrosine kinases (RTKs) that regulate development, tissue homeostasis, and wound healing by activating signaling cascades involved in differentiation, migration, proliferation, angiogenesis, and survival.^{1,2} FGFRs activate signaling networks initiated by ligand binding of FGFs, resulting in protein dimerization and subsequent autophosphorylation. In FGFR1, activation occurs upon Y653 phosphorylation, which helps stabilize the activated conformation of the activation loop. Ordered, sequential phosphorylation of additional tyrosines located in the activation loop further stabilize the active conformation and create docking sites to initiate MAPK, PI3K/Akt, STATs, and PLC γ signaling pathways.^{3–7}

Mutation or amplification of FGFR can lead to constitutive activity, implicating this kinase family in cancers including nonsmall cell lung, breast, gastric, bladder, and endometrial as well as multiple myeloma and rhabdomyosarcoma.⁸ In particular, gene amplification of FGFR1 has been implicated in ~20% of squamous cell lung cancers and up to 27% of luminal B breast cancers, two very prevalent cancer types.^{9,10} As a result, the FGFR family, and FGFR1 amplification in particular, have been identified as important targets for anticancer drug development.

AZD4547 (Figure 1) is currently in phase II clinical trials for FGFR-dependent tumors and is selective for FGFR1–3 with low nanomolar IC₅₀ values (0.2 and 12 nM for enzyme and cells, respectively, for FGFR1).¹¹ This inhibitor contains a 3,5-dimethoxyphenyl group, a moiety commonly seen in FGFR inhibitors since it confers selectivity for the FGFR family.^{12–17} E3810 (lucitanib) is a dual FGFR-VEGFR inhibitor in phase II clinical trials for FGFR-dependent tumors, with IC₅₀ values ranging from low- to mid-nanomolar levels for VEGFR1–3 and FGFR1–3.^{18–20} While kinases have proved to be successful targets for anticancer therapy, long-term efficacy of TKIs is severely hindered by acquired resistance in a significant proportion of patients.²¹

TKI resistance can be achieved by using compensatory signaling pathways or by developing new mutations in the targeted kinase, commonly at the gatekeeper residue. This residue is located near the hinge region and helps dictate the size of the hydrophobic pocket that forms after several activation loop residues (including residues 641–645 in FGFR1) are moved

upon transition to the DFG-out conformation. Proper positioning of the DFG residues, comprising D641, F642, and G643 in FGFR1, allows nearby hydrophobic residues to align to form the “hydrophobic spine” in the DFG-in conformation that stabilizes the active conformation of the catalytic domain, while the hydrophobic spine is abolished in the DFG-out (inactive) conformation.^{21–23} The kinase can adopt the DFG-in conformation while still remaining in an inactivated state; kinase activation is achieved when the DFG-in conformation occurs in the context of tyrosine phosphorylation.²⁴ Importantly, mutations at the gatekeeper residue are a well-established barrier in achieving long-term TKI efficacy. Several well-established examples of gatekeeper mutations include T315I Bcr-Abl, which confers resistance to imatinib (gleevec), and T790M EGFR, which imparts resistance to gefitinib and erlotinib. These mutations can alter inhibitor binding by disrupting hydrogen bonding, introducing steric hindrance, or stabilizing the DFG-in conformation of the kinase domain.²⁵ Gatekeeper mutations for the FGFR family have already been observed both intrinsically in cancer patients and upon treating cells with targeted FGFR inhibitors, and are expected to appear in the clinic upon FDA approval of FGFR-targeted therapies.^{26–29}

Earlier drug development of TKIs typically addressed the issue of drug resistance due to the kinase gatekeeper mutations only after treatment failure of the first generation of inhibitors.³⁰ For example, ponatinib has been developed as a successful inhibitor of T315I Bcr-Abl, and structural data show that unlike the first generation inhibitor imatinib, structural features of ponatinib accommodate the isoleucine gatekeeper residue well.³¹ Recent structural work has also explored interactions of this inhibitor with FGFR1 and FGFR4.³² Covalent TKIs targeting T790M EGFR such as the recently FDA approved BIBW2992 (afatinib or gilotrif) and ibrutinib also offer an effective strategy for circumventing drug resistance.^{33,34} In a similar manner, new covalent TKIs have recently been developed that are effective against both the wild type (WT) and the gatekeeper mutations of FGFR1 and FGFR2, indicating an evolution of inhibitor benchmarks.¹⁷ In parallel, assessing the resistance profiles and mechanisms for FGFR inhibitors currently in clinical trials, such as AZD4547 and E3810 (Figure 1), is vital for understanding the challenges that next generation TKIs must overcome. While a crystal structure of AZD4547 in complex with WT FGFR1 has been recently solved, structural information for E3810 interactions with FGFR1 or for either inhibitor with the V561M FGFR1 gatekeeper mutation is not available, despite being essential for understanding modes of drug resistance.³²

Overcoming or delaying inhibitor resistance is not a unique problem for TKI development; similar challenges are present when designing antiretroviral inhibitors that target the viral polymerase, HIV-1 reverse transcriptase (RT), of the rapidly mutating virus. Non-nucleoside reverse transcriptase inhibitors (NNRTIs) allosterically inhibit RT with nM affinities, making them an effective component of highly active antiretroviral therapy (HAART) for patients infected with HIV. Due to the relatively low replicative fidelity of RT, NNRTI resistance is a significant problem. One very successful strategy that has been employed in overcoming NNRTI resistance is transitioning away from earlier first generation drugs that are planar, rigid inhibitors to flexible compounds that can bind to viral RT in multiple conformations.^{35,36} In this way, an inhibitor can bind in new conformations that accommodate acquisition of RT point mutation(s) in the allosteric binding pocket that would potentially impart drug resistance, thereby preserving high inhibitor affinity. Thus, efforts to

address the inevitable antiretroviral resistance begin at the drug development stage in which inhibitors are tested for efficacy against WT RT as well as a panel of known resistance mutations. This is in contrast to the current paradigm in TKI development, where new therapies effective against resistance mutations, several of which can be readily predicted, are developed only after the first-generation drug has failed.

To understand the catalytic and structural consequences of the FGFR1 gatekeeper mutation, we show that V561M FGFR1 has a significantly increased rate of autophosphorylation. We provide structural evidence that this catalytic activation is likely mediated through favoring the active conformation via stabilization of the hydrophobic spine. The V561M mutation also confers significant resistance to E3810. In contrast, AZD4547 is able to sample several binding conformations to preserve nanomolar range affinity for V561M FGFR1, analogous to NNRTI binding flexibility to viral RT. These findings provide important insights for designing novel inhibitors that can effectively target gatekeeper mutations in kinases. We propose exploiting inhibitor and binding pocket flexibility in future TKI design to prolong the effectiveness of targeted anticancer therapies.

RESULTS

The Rate of Autophosphorylation Is Faster in V561M than WT FGFR1

Here, we present the first kinetic analysis of the V561M FGFR1 gatekeeper mutation. This mutation confers a 38-fold increase in k_{cat} relative to WT FGFR1 (Supporting Information Figure S1, Table 1). Titrations with the ATP analog AMP-PNP indicated essentially no change in ATP affinity (K_d), but at physiological concentrations of ATP, k_{cat} will be the dominant parameter in driving autophosphorylation kinetics. This significant increase in rate indicates that the V561M mutation can catalytically activate this kinase.

The Structure of V561M FGFR1 Shows a Stabilized Hydrophobic Spine

To understand the catalytic activation gained by the FGFR1 gatekeeper mutation, we sought to determine the first crystal structure of V561M FGFR1.³⁷ Crystals of the V561M FGFR1 catalytic domain diffracted to amplitudes extending to 2.29 Å, and the structure was solved by molecular Fourier synthesis using PDB code 3KY2 as the start model (Figure 2). Two molecules were present in the asymmetric unit (Figure 2A), showing a varying degree of disorder in the P-loop, which is involved in ATP binding, and the activation loop, which contains the tyrosine phosphorylation sites.^{16,38} Superimposition with the previously solved structure of unphosphorylated apo WT FGFR1 in the DFG-in conformation (3KY2) indicates that the V561M mutation caused no major changes to the overall protein fold (Figure 3).³⁸ The inactive state of the kinase (i.e., unphosphorylated) is stabilized by a series of hydrogen bonds in the hinge domain termed the molecular brake.³⁹ This motif is also essentially unchanged upon introducing the V561M mutation, indicating that brake disruption is not a mechanism for catalytic activation (Supporting Information Figure S2A).³⁹

The hydrophobic spine in V561M FGFR1 is intact (Figure 2B), indicating stabilization of the DFG-in conformation. This is the first structure to date of an FGFR1 gatekeeper

mutation in this conformation. Importantly, the V561M residue closely abuts this spine motif. We show via a space filling view that in V561M FGFR1, V561M and M535 have improved hydrophobic interactions, with the M535 residue rotating to align well with V561M (Figure 2B). This is the first structural evidence that the FGFR gatekeeper mutation can stabilize the hydrophobic spine to promote catalytic activation, a mechanism predicted in a structural model of V555M FGFR3.²⁷ Since most FGFR inhibitors, and kinase inhibitors in general, target the DFG-in conformation, this structure can serve as a model for designing future inhibitors effective against FGFR gatekeeper mutations.⁴⁰

The Gatekeeper Mutation Confers Resistance to E3810 but Not AZD4547

Mutations in the gatekeeper residue are a well-established means of developing resistance to TKIs. To determine if this is an important limitation to FGFR inhibitors currently in clinical trials, we determined the changes in affinity of AZD4547 and E3810 for V561M FGFR1 using fluorescence-based titrations. Consistent with previous studies, AZD4547 and E3810 bind with K_d values of 2 nM and 8 nM, respectively, for WT FGFR1 (Figure 1).^{11,18,19} However, an increase in K_d to 40 μ M for E3810 binding to V561M FGFR1 represents a 5000-fold decrease in affinity upon introduction of the gatekeeper mutation. In contrast, the K_d for AZD4547 interaction with V561M FGFR1 is 64 nM, indicating only a 32-fold loss of affinity. These data show that introduction of the gatekeeper mutation is a probable mechanism of resistance to E3810, but less likely for AZD4547.

AZD4547 Preserves Affinity for V561M FGFR1 via Binding Flexibility

The molecular mechanisms of inhibitor interactions with V561M FGFR1 were explored using X-ray crystallography. Crystals of the WT and V561M FGFR1 catalytic domains in complex with AZD4547 diffracted to amplitudes extending to 2.49 and 2.98 Å, respectively, and both structures were solved by molecular Fourier synthesis using PDB code 4RWI (Figure 4). For both structures, two molecules were present in the asymmetric unit, with slight changes in the density present in the activation loop in the two molecules. AZD4547 binds as a type I inhibitor, consistent with a recently solved structure (Figure 5).³² Clear electron density in the omit $2mF_o - F_c$ electron density maps allows confident assignment of the orientation of AZD4547 (Figure 6).

Importantly, AZD4547 binds to each of the molecules of the asymmetric unit of the V561M FGFR1 structure in different ways, unlike WT FGFR1, which shows a single inhibitor binding conformation. In one conformation, AZD4547 binds to V561M FGFR1 in a very similar fashion to WT FGFR1 (Figure 5B). Minor adjustments are seen in the P-loop and hinge region in order to accommodate the 2.8 Å increase in length that occurs upon replacing a valine residue with a methionine in the observed rotamer. The methionine residue is accommodated by situating closer to the inhibitor, with V561M extending 0.5 Å closer to AZD4547 than V561, and by movement in one of the methoxy groups of AZD4547 (Figure 5A,B). For both structures, density exists to model in the full P-loop, although some side chain rotamers in V561M FGFR1 that could not be reliably modeled were omitted.

Significant changes are observed in the binding configuration of AZD4547 in the second molecule of the asymmetric unit of V561M FGFR1. Severe bending in the ethyl linker connecting the dimethoxyphenyl and pyrazole moieties of AZD4547 occurs to better accommodate the methionine mutation (Figure 5C). The shortest distance between the inhibitor and a residue of an adjacent symmetry molecule is 24 Å, suggesting that this alternative binding conformation is not a crystal packing artifact. Superimposition of the inhibitors for both the WT and V561M FGFR1 structures shows that this bending results in a distance change of 5.6 Å from the C4 positions of the 3,5-dimethoxyphenyl rings (Figure 5D). The methionine of the WT-like conformation of AZD4547 is thus 0.8 Å closer to the inhibitor compared to the bent conformation (Figure 5B,C). The B factor associated with the molecule displaying the bent AZD4547 conformation is lower than that for the WT-like conformation, indicating less overall protein mobility, which may be anticipated due to the improved density for the activation loop (Supporting Information Table S1).

AZD4547 makes five hydrogen bonds with the ATP binding site of WT FGFR1 (Figure 6A). Three of these hydrogen bonds, all in the hinge region, are maintained for both conformations of the V561M FGFR1 structure (Figure 6B,C), supporting the measured nanomolar affinity. The hydrogen bond with D641 is lost in the bent side configuration but maintained in the WT-like conformation, suggesting the latter may be slightly favored. In both conformations, the hydrogen bond with the backbone of G485 is lost.

Superimposition of V561M FGFR1 with apo WT FGFR1 shows that no major global changes occur upon mutation of the gatekeeper residue (Figure 3), although the largest RMSD is seen with the V561M FGFR1•AZD4547 bent conformation structure.³⁸ Both WT and V561M bind AZD4547 in the DFG-in conformation (Figure 3C). This is in contrast to the only other available ligand-bound structure of an FGFR gatekeeper mutation; FGFR4 V550L is shown in complex with the inhibitor FIIN-3 in a DFG-out conformation.¹⁷ Interestingly, most structural variation occurs in the degree of closure of the P-loop; WT FGFR1•AZD4547 shows significant P-loop closure over the inhibitor, consistent with the previously determined structure.³² Perhaps unsurprisingly, the V561M FGFR1 in the AZD4547 WT-like conformation shows almost the same degree of P-loop closure. In contrast, V561M FGFR1 in the AZD4547 bent conformation has partial P-loop closure over the inhibitor, falling in between the closed P-loop of WT FGFR1•AZD4547 and the open P-loop of apo WT and apo V561M FGFR1 structures (Figure 3B).²⁴

Few changes are observed when comparing the hydrophobic spine of the structures (Supporting Information Figure S3). Consistent with previous work, the molecular brake is engaged for the WT FGFR1•AZD4547 structure, making the same hydrogen bond contacts seen for the apo V561M FGFR1 structure (Supporting Information Figure S2).³² One of these hydrogen bond interactions is lost in the AZD4547 bent conformation of the V561M FGFR1 structure, while two are lost in the WT-like conformation, suggesting that some of the minor loss of inhibitor efficacy for the gatekeeper mutant may result from molecular brake destabilization. Overall, we show the flexibility provided by the ethyl linker in AZD4547 allows multiple binding configurations that can be sampled in order to better accommodate the V561M gatekeeper mutation. This supports our findings that AZD4547 maintains nanomolar affinity for V561M FGFR1.

Structural Data Illuminate Features of Only WT FGFR1•E3810 Complex

As the crystal structures of AZD4547 co-complexed with FGFR1 were insightful for determining mechanisms of inhibitor resistance by the gatekeeper mutation, we sought to obtain X-ray crystal structures of FGFR1 in complex with E3810. Crystals of the WT FGFR1 catalytic domain bound to E3810 diffracted to amplitudes extending to 2.19 Å, and the structure was solved by molecular Fourier synthesis using PDB code 4NKA (Figure 7).¹⁴ Density for the inhibitor-bound symmetry mate as indicated in the omit $2mF_o - F_c$ electron density map (Figure 7C) allowed high confidence in assigning E3810 orientation. Only one inhibitor was observed in the two symmetry mates making up the asymmetric unit, not an uncommon phenomenon.^{41,42} For the symmetry mate lacking the inhibitor, some density was observed in the omit $2mF_o - F_c$ electron density map at a location corresponding to where E3810 was expected to bind. This suggests the presence of E3810 binding at a lower occupancy (Supporting Information Figure S4). The P-loop is well-defined only in the molecule containing E3810. Like AZD4547, E3810 binds as a type I inhibitor, forming four hydrogen bonds (Figure 7B).

Superimposition with apo WT FGFR1 shows that like the AZD4547 structures, WT FGFR1•E3810 is in the DFG-in conformation (Figure 3).³⁸ The P-loop is partially closed, situated between the fully closed conformations seen for AZD4547 structures and the open conformations of the apo V561M structure (Figure 3B). The molecular brake is engaged, showing an additional hydrogen bond (Supporting Information Figure S2D), and minimal changes are seen in the hydrophobic spine region (Supporting Information S3).

Despite collecting data sets on three different crystals, density for E3810 was not observed in structures of V561M FGFR1. One of these crystals generated the apo V561M FGFR structure (Figure 2). Inability to cocrystallize E3810 with V561M FGFR1 is consistent with the 5000-fold increase in K_d . The valine residue is 3.6 Å from the naphthamide ring of E3810, so accommodating a 2.8 Å increase in residue length upon methionine substitution would require inhibitor rearrangement. Since the gatekeeper residue is immediately below the portion of the inhibitor making the majority of nonwater-mediated hydrogen bonds, it is quite feasible that mutation to a methionine residue would disrupt these interactions.

DISCUSSION

To our knowledge, this study provides the first direct kinetic and structural evidence for catalytic activation by the V561M FGFR1 gatekeeper mutation. Structural and computational modeling of kinases including FGFR2, SRC, and EGFR indicate that gatekeeper mutations can promote the active conformation of kinases by stabilizing the hydrophobic spine.^{22,27} Specifically, this ability of gatekeeper mutations to catalytically activate the kinase via hydrophobic spine stabilization was proposed in cellular and structural studies of c-Abl and c-Src.²² Stabilization occurs through improved packing afforded by mutation to larger gatekeeper residues.^{43,44} In particular, favorable interactions among methionines at the gatekeeper position and in the hydrophobic spine, like that shown for V561M FGFR1 (Figure 2B), are quite stabilizing.⁴⁵ Improved hydrophobic spine stabilization afforded by methionines at the gatekeeper residue and within the spine have been attributed to the alternate binding conformations of gleevec to Syk kinase, and it is

possible that a similar mechanism may be operative here.⁴⁵ These findings of catalytic activation are supported by the previous observation that cells overexpressing V561M FGFR1 have increased levels of FGFR1 phosphorylation, and cell-based resistance studies suggest a similar catalytic activation for V555M FGFR3.^{28,46}

The rapid onset of drug resistance is an important Achilles' heel for developing TKIs with long-term efficacy. One strategy of delaying resistance resulting from kinase gatekeeper mutations has been through the development of covalent inhibitors. This approach has been an effective method for targeting gatekeeper mutations of FGFR and EGFR, overcoming initial concerns of toxicity.^{17,47} We propose a complementary approach of delaying resistance that has been highly effective in treating HIV, which often relies on inhibitor binding flexibility to overcome a rapidly mutating target.^{35,36} Here, we present the first evidence that this may be an effective strategy by showing that the V561M FGFR1 gatekeeper mutation maintains nanomolar level binding affinity ($K_d = 64$ nM) for AZD4547 by allowing multiple binding conformations, both of which show full occupancy in our crystal structure indicating both are stable. However, the impact of the 32-fold lower affinity for V561M relative to WT FGFR1 in a clinical setting is unclear. Recent studies in myeloma cell-based assays have shown that the V555M FGFR3 gatekeeper mutation is acquired upon extended AZD4547 treatment.²⁸

The concept of employing a flexible linker to permit different binding configurations is also illustrated by earlier studies showing another FGFR inhibitor (in this case, an ethyl linker connecting pyrazole and phenyl moieties) that adopts two relatively similar binding configurations within the WT FGFR binding pocket rather than occurring upon mutation of the gatekeeper residue.¹⁵ Further, a review has highlighted the value of allowing ligand flexibility when exploring interactions with kinases.⁴⁸ Taken together, this study demonstrates the merits of a flexible scaffold such as an ethyl linker for inhibitor design.

In contrast to AZD4547, E3810 is far more planar, rigid, and thus less flexible. A second important difference between these two inhibitors is observed when comparing hydrogen bonds. AZD4547 makes five hydrogen bonds with the ATP binding site of WT FGFR1 (Figure 6A), all with backbone versus side chain atoms.³² For E3810, one hydrogen bond is water mediated, and one involves residue side chains versus backbone atoms. Upon the basis of these structural comparisons of the protein–ligand interactions for these two TKIs, it is not unanticipated that combining inhibitor flexibility with backbone atom-mediated hydrogen bonds as in the case of AZD4547 would likely better accommodate point mutations, while a more rigid inhibitor like E3810 that relies more on hydrogen bonds mediated by a side chain atom and a water molecule may be less able to accommodate mutations, making acquired resistance more facile to achieve.

Of course, the development of more flexible inhibitors still capable of binding their kinase target even after a gatekeeper mutation has occurred must be balanced with the other challenge of developing TKIs, namely selectivity. Since all kinases use ATP as their substrate, developing small molecule inhibitors that selectively target the active site of a particular kinase is challenging. Developing an inhibitor that can accommodate many mutations may lead to high kinase promiscuity and thus toxicity related to these off-target

interactions. However, this balance of flexibility versus toxicity has been successfully achieved; for example, second-generation inhibitors dasatinib and nilotinib targeting Bcr-Abl are effective against at least 50 clinically relevant mutations of this enzyme.⁴⁹ Additionally, it should be noted that many successful kinase inhibitors show varying degrees of promiscuity, and this in fact enhances their effectiveness. E3810 itself is being assessed as a dual FGFR/VEGF inhibitor.^{50,49}

Overall, we have determined molecular mechanisms that the FGFR1 gatekeeper mutation can employ to achieve catalytic activation, and to both hinder and to facilitate resistance to two inhibitors currently in clinical trials. Stabilization of the hydrophobic spine afforded by the valine to methionine mutation likely contributes to the faster autophosphorylation rate seen in V561M FGFR1. We show the over 150-fold greater loss of affinity for E3810 compared to AZD4547 conferred by the V561M FGFR1 mutation. This affinity loss may stem from altered binding conformations afforded by the flexible linker in AZD4547 to preserve nanomolar K_d values. This highlights the first evidence of a TKI binding in different conformations to WT and the gatekeeper mutant forms of FGFR1, revealing regions of flexibility in the inhibitor and inhibitor binding pocket that are critical for developing future FGFR inhibitors with delayed resistance profiles. Using inhibitor flexibility to combat drug resistance has been a highly effective means of managing resistance to anti-HIV inhibitors, and we propose it might be an important approach to explore when designing novel TKIs effective against kinase gatekeeper mutations.

METHODS

Please refer to the Supporting Information for full details.

Protein Expression and Purification

The WT and V561M FGFR1 full-length catalytic domain (aa 401–822) and the shortened catalytic domain (aa 458–765) with the C488A and C584S mutations both contained *N*-terminal 6× histidine tags and were heterologously expressed in *E. coli* using p-ET bacterial expression vectors. Nickel-NTA, phenyl-sepharose, and Q-sepharose resin chromatography were used for purification.

Kinetic Characterization

Steady-state parameters of the autophosphorylation of the shortened catalytic domain of WT and V561M FGFR1 (k_{cat} and K_m values) were obtained using ^{32}P - γ -ATP as the substrate. Excess substrate and radiolabeled protein were separated using polyacrylamide gel electrophoresis, and phosphorimaging was used to quantify the radiolabeled protein product. Higher concentrations of V561M FGFR1 were required to achieve saturating rates of autophosphorylation; this upper bound for autophosphorylation rate was important to determine since FGFR1 amplification leads to tumorigenesis. As a result, the concentration of ATP required to ensure saturating substrate conditions was clearly higher than the K_m since maximum rates were observed even at small excesses of ATP, which precluded obtaining a K_m value. Intrinsic protein (FGFR1) fluorescence quenching upon increasing concentrations of E3810, AZD4547, or AMP-PNP was monitored to obtain K_d

measurements. The full-length catalytic domain was used for the inhibitor titrations, and the shortened crystallographic construct was used for the AMP-PNP titrations.

Crystallization and Structure Determination

FGFR1 (2 mg mL⁻¹) was preincubated with 110 μ M inhibitor, even in the case of the V561M FGFR1 apo structure (E3810 incubation), followed by protein concentration to 10 mg mL⁻¹. The hanging drop vapor diffusion method was used to grow crystals that typically formed after 1 month under the following conditions. apo V561M: 20 °C, 0.1 M sodium cacodylate, pH 6.4, 30% PEG 8000, and 0.2 M ammonium sulfate. V561M FGFR1•AZD4547: 20 °C, 0.1 M MES, pH 6.6, 34% PEG 8000, and 0.2 M ammonium sulfate. WT FGFR1•AZD4547: 4 °C, 0.1 M sodium cacodylate, pH 6.4, 22% PEG 8000, and 0.2 M ammonium sulfate. WT FGFR1•E3810: 20 °C, 0.1 M sodium cacodylate, pH 6.6, 34% PEG 8000, and 0.2 M ammonium sulfate. Crystals were harvested and flash frozen in liquid nitrogen.

Diffraction data were collected at the National Synchrotron Light Source, Brookhaven National Laboratory, and processed using the HKL2000 Suite.⁵¹ All structures were solved using Fourier synthesis, using 3KY2 for apo V561M FGFR1, 4RWI for WT FGFR1•AZD4547 and V561M FGFR1•AZD4547, and 4NKA for WT FGFR1•E3810.^{14,38} All structures were refined using Phenix, and figures were prepared using PyMOL Molecular Graphics System, Schrödinger, LLC.⁵² X-ray data collection and refinement statistics are shown in Supporting Information Table S1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Structure and steady-state binding of FGFR inhibitors. (A) Structures of inhibitors. (B) AZD4547 binding to WT FGFR1, $K_d = 2 \pm 1$ nM (quadratic fit). (C) E3810 binding to WT FGFR1, $K_d = 8 \pm 2$ nM (quadratic fit). (D) AZD4547 binding to V561M FGFR1, $K_d = 64 \pm 11$ nM (hyperbolic fit). (E) E3810 binding to V561M FGFR1, $K_d = 40 \pm 7$ μ M (hyperbolic fit). Each titration was performed in duplicate, with fluorescence contributions from the buffer and inhibitor subtracted. The SE is deviation from the curve fits. Intrinsic fluorescence quenching upon AZD4547 or E3810 binding to FGFR1 was used to obtain K_d values.

Figure 2.

Structure of the catalytic domain of apo V561M FGFR1 (PDB code 4RWI). (A) Ribbon diagrams of the two molecules in the asymmetric unit are shown. The catalytic loop is dark blue. The P-loop is orange. The activation loop is yellow, and the hinge region is red. (B) A close-up of the hydrophobic spine and activation loop. The apo V561M FGFR1 structure is in cyan and is superimposed with apo WT FGFR1 (3KY2) in magenta (RMSD = 0.282, 3246, of 3905 atoms used from both molecules in the asymmetric unit).³⁸ The hydrophobic spine is shown in spheres for the V561M FGFR1 structure, and in stick format for both structures. The DFG region is highlighted in stick format. An inset rotated ~45° down showing the alignment of the V/M561 and M535 residues is shown. (C) The omit $2mF_o - F_c$ electron density map contoured to 1.0σ is shown in blue for key residues. The same color scheme described in A is used. The orientation is rotated ~120° down from that in B.

Figure 3.

Superimposition of the four structures with apo WT FGFR1. (A) WT FGFR1 (3KY2) is shown in yellow and used for the following superimpositions: WT FGFR1•AZD4547 in magenta (RMSD = 0.512, 1658 of 2101 atoms used), WT FGFR1•E3810 in green (RMSD = 0.410, 1699 of 2137 atoms used), V561M FGFR1 apo in cyan (RMSD = 0.489, 1658 of 2121 atoms used), and V561M FGFR1•AZD4547 in black for the bent AZD4547 conformation (RMSD = 0.613, 1680 of 2118 atoms used) and orange for the WT-like conformation (RMSD = 0.313, 1717 of 2062 atoms used).³⁸ Backbone ribbon traces are shown. (B) A zoomed-in view, highlighting the activation loop, P-loop, and α C helix. (C) The view from B rotated $\sim 90^\circ$. The V/M561 gatekeeper and D623 catalytic residues and residues making up the DFG-in orientation (D641, F642, and G643) are highlighted in stick format. The same color scheme described in A is used with structures shown in ribbon format.

a) WT FGFR1 • AZD4547

b) V561M FGFR1 • AZD4547

Figure 4.

Structure of the catalytic domain of FGFR1 in complex with AZD4547. Ribbon diagrams of the two molecules in the asymmetric unit are shown for both structures. The catalytic loop is dark blue. The P-loop is orange. The activation loop is yellow, and the hinge region is red. (A) WT FGFR1•AZD4547, PDB code 4RWJ. (B) V561M FGFR1•AZD4547, PDB code 4RWK.

Figure 5.

V561M gatekeeper mutation causes local rearrangements in the binding pocket. Selected residues from the P-loop (orange) and the hinge region (red) are shown in stick format. The shortest distances of the gatekeeper residue to AZD4547 are highlighted. (A) WT FGFR1•AZD4547. (B) V561M FGFR1•AZD4547, WT-like inhibitor conformation. (C) V561M FGFR1•AZD4547, bent inhibitor conformation. (D) Superimposition of the inhibitors from the WT FGFR1•AZD4547 complex (magenta) and V561M FGFR1•AZD4547 complex (green, bent conformation). The distance between the two 3,5-dimethoxyphenyl rings is shown (RMSD = 0.306, 1681 of 2009 atoms used).

Figure 6.

AZD4547 binding to FGFR1. The P-loop is orange. The hinge region is red, and a magnesium ion is shown as a purple sphere. Residues involved in hydrogen bonding with the inhibitor are shown in stick format with distances highlighted. The orientation is rotated $\sim 120^\circ$ up from that shown in Figure 4. The omit $2mF_o - F_c$ electron density map contoured to 1.0σ is shown in blue for key residues and AZD4547. (A) WT FGFR1•AZD4547. (B) V561M FGFR1•AZD4547, WT-like inhibitor conformation. (C) V561M FGFR1•AZD4547 bent inhibitor conformation. In B and C, lost hydrogen bonds are indicated by the absence of a dashed line, but the distance ($>3.5 \text{ \AA}$) is still noted.

Figure 7.

Structure of the catalytic domain of WT FGFR1 in complex with E3810, PDB code 4RWL. Ribbon diagrams of the two molecules in the asymmetric unit are shown for both structures. The catalytic loop is dark blue. The P-loop is orange. The activation loop is yellow. The hinge region is red, and an SO_4 ion is shown in stick format in brown. (A) WT FGFR1•E3810. (B) Hydrogen bond interactions with E3810 are highlighted including a bond mediated by a water molecule shown as a red sphere. While the distance is not indicated, V561 is $3.4 \text{ \AA}$ from the naphthamide ring of E3810. (C) The omit $2mF_o - F_c$ electron density map contoured to 1.0σ is shown in blue for key residues. The orientation is rotated $\sim 90^\circ$ down from that in B.

Table 1

Kinetic Parameters for WT and V561M FGFR1

FGFR1	$k_{\text{cat}}, \text{s}^{-1}$	$K_{\text{m}}, \mu\text{M}$	$k_{\text{cat}}/K_{\text{m}}, \mu\text{M}^{-1}/\text{s}^{-1}$	$K_{\text{d}}, \mu\text{M}, \text{AMP-PNP}^a$
WT	0.0032 ± 0.0002	140 ± 50	2.3×10^{-5}	8 ± 4
V561M	0.12 ± 0.01	ND ^b	ND ^b	14 ± 4

^a AMP-PNP is used to approximate affinity for ATP.

^b To achieve a saturating rate, [V561M FGFR1] = 200 μM , which precluded evaluating concentrations of ATP below 1000 μM to ensure saturating substrate conditions. Since k_{obs} values at this concentration of ATP were already saturating, a K_{m} could not be determined (see Supporting Information Figure S1).