Crystal Structure of the Tyrosine Kinase Domain of Colony-stimulating Factor-1 Receptor (cFMS) in Complex with Two Inhibitors*

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Carsten Schubert 1, Céline Schalk-Hihi, Geoffrey T. Struble, Hong-Chang Ma, Ioanna P. Petrounia, Benjamin Brandt, Ingrid C. Deckman, Raymond J. Patch, Mark R. Player, John C. Spurlino, and Barry A. Springer
From Structural Biology, Johnson & Johnson Pharmaceuticals Research and Development, L.L.C., Exton, Pennsylvania 19341

The cFMS proto-oncogene encodes for the colony-stimulating factor-1 receptor, a receptor-tyrosine kinase responsible for the differentiation and maturation of certain macrophages. Upon binding its ligand colony-stimulating factor-1 cFMS auto-phosphorylates, dimerizes, and induces phosphorylation of downstream targets. We report the novel crystal structure of unphosphorylated cFMS in complex with two members of different classes of drug-like protein kinase inhibitors. cFMS exhibits a typical bi-lobal kinase fold, and its activation loop and DFG motif are found to be in the canonical inactive conformation. Both ATP competitive inhibitors are bound in the active site and demonstrate a binding mode similar to that of STI-571 bound to cABL. The DFG motif is prevented from switching into the catalytically competent conformation through interactions with the inhibitors. Activation of cFMS is also inhibited by the juxtamembrane domain, which interacts with residues of the active site and prevents formation of the activated kinase. Together the structures of cFMS provide further insight into the autoinhibition of receptor-tyrosine kinases via their respective juxtamembrane domains; additionally the binding mode of two novel classes of kinase inhibitors will guide the design of novel molecules targeting macrophage-related diseases.

Protein kinases are enzymes that serve as key components of signal transduction pathways by catalyzing the transfer of the terminal phosphate from ATP to the hydroxyl group of tyrosine, serine, or threonine residues of proteins. The overexpression or inappropriate expression of normal or mutant protein kinases in mammals has been linked to the development of many diseases, including cancer, diabetes, and autoimmune diseases.

Protein kinases can be divided into two classes: those which preferentially phosphorylate tyrosine residues (protein-tyrosine kinases), and those which preferentially phosphorylate serine and/or threonine residues (protein serine/threonine kinases). Protein-tyrosine kinases perform diverse functions ranging from stimulation of cell growth and differentiation to arrest of cell proliferation. They can be classified as either receptor protein-tyrosine kinases (RTKs) or intracellular protein-tyrosine kinases. The FMS or CSF-1-R proto-oncogene encodes the receptor-tyrosine kinase cFMS (CSF-1-R), which is the cell surface receptor for the (macro) colony-stimulating factor-1 (CSF-1 or M-CSF) (1). cFMS is part of the platelet-derived growth factor (PDGF) receptor family, which includes PDGFR-α and -β, the stem cell factor receptor (cKIT), and the FMS-like tyrosine kinase 3 (FLT3) (2). This family shares a common architecture consisting of an extracellular ligand-binding domain comprised of five immunoglobulin-like domains, a single transmembrane segment, a juxtamembrane (JM) domain and a kinase domain divided by a kinase insert domain (KID).

Binding of the ligand to the ligand binding domain induces a conformational change, which leads to receptor dimerization, autophosphorylation of specific tyrosine residues in trans, and activation of the kinase. The phosphorylated tyrosines serve as binding sites for a wide variety of signaling molecules through their phosphorytyrosine binding domains (3).

CSF-1 is a polypeptide growth factor that stimulates the survival, proliferation, and differentiation of hematopoietic cells of the monocyte-macrophage series. Multiple forms of soluble CSF-1 are produced through proteolytic cleavage of membrane-bound precursors, some of which are stably expressed at the cell surface (4).

Valuable insight into the signaling role of CSF-1 and its receptor cFMS comes from a CSF-1-deficient mouse strain (Csf1or/Csf1or) (5). These mice exhibit a selective reduction of monocytes, osteoclasts, and macrophages in muscle, joint, and other tissues. Furthermore, these mice are osteopetrotic and exhibit reduced fertility, but the incapability to produce functional CSF-1 appears not to be life threatening per se; Csf1or/
CsF1<sup>−/−</sup> mice are resistant to collagen-induced arthritis and show a reduced rate of mammary tumor progression into metastasis (6). Similar, but more pronounced effects, were observed with a mouse model in which the cFMS gene had been inactivated (Csfr<sup>−/−</sup>/Csfr<sup>−/−</sup>) (7).

CSF-1 has been shown to exacerbate collagen-induced arthritis in mouse models, an effect that could be suppressed with M-CSF-blocking antibodies (8). In another study, CSF-1 and GM-CSF (granulocyte macrophage colony-stimulating factor) induced prolonged inflammation and recruitment of macrophages in an mBSA-induced arthritis model (9). These studies demonstrated that CSF-1 and GM-CSF could exacerbate and prolong the histopathology of acute inflammatory arthritis and lend support to the hypothesis that monocytes/macrophages are a driving influence in the pathogenesis of inflammatory arthritis. The data shown in these studies suggest that either CSF-1 or its cognate receptor cFMS are suitable targets for treating arthritis or other macrophage-induced inflammatory diseases.

In a recent study, expression of cFMS in various tumors was linked to poor survivability and increased tumor size (10). CSF-1 and cFMS are also expressed in some types of breast carcinomas and other epithelia of the female reproductive tract, where activation of the receptor by ligand, produced either by the tumor cells or by stromal elements, stimulates tumor cell invasion through a urokinase-dependent mechanism (11). These results support other preclinical findings that the CSF-1-signaling pathway may be involved in local invasion and metastasis, rendering cFMS a putative anti-cancer target.

In this study, we present the structures of two chimeric kinase domains of non-phosphorylated cFMS in complex with two inhibitors. The kinase domain is arranged in the bi-lobal kinase fold typical for protein kinases. Both compounds are competitive inhibitors and occupy the ATP pocket of cFMS. They form a typical kinase binding motif, in which one or more hydrogen bonds to backbone atoms of the hinge region of cFMS are formed. The compounds also prevent cFMS from switching into an active state by preventing the conserved kinase Asp<sup>-585</sup> (H9262) (12). The chimeras were created by replacing the cFMS KID with the KID sequences from the FGFR receptor and TIE2 receptor. For expression in SF9 cells, a recombinant baculovirus was generated by subcloning the cFMS chimeras into a modified Invitrogen GATEWAY pDEST8 vector. Further steps followed the protocol for baculovirus expression according to the Bac-to-Bac (Invitrogen) manual.

cFMS was purified by affinity chromatography on BD Talon resin, TEV protease cleavage to remove the histidine tag, and gel filtration chromatography. The apo form was concentrated to ~10 mg/ml and subjected to crystallization trials. For crystallization trials with inhibitors present, the inhibitors (10 mM in Me<sub>2</sub>SO) were added to a diluted solution of cFMS (1 mg/ml) and co-concentrated to 7–11 mg/ml.

**Crystal Structure of cFMS Kinase**

**Overview of the cFMS Structure**—This structure of cFMS was obtained by utilizing a construct of cFMS, which included the whole JM domain and the catalytic kinase domain minus the C-terminal tail of cFMS. Early protein constructs that represented the kinase domain, including the full KID (residues 680–751), did not yield any crystals. This is probably because the KID is mostly unstructured and interferes with crystallization. A design strategy in which the kinase insert domain was replaced with the significantly shorter KIDs of previously solved RTKs (FGFR, TIE2, and IRK) proved to be successful. We were able to obtain crystals from the cFMS-FGFR and the cFMS-TIE2 chimeras. The cFMS-IRK chimera did not express well and was not pursued further. Choice of the cFMS ligand

**Experimental Procedures**

Cloning/Protein Purification—The construct design and purification of cFMS have been described in more detail elsewhere (Schalk-Hihi et al., 33). Briefly, all constructs used in the crystallization of cFMS encompass the JM domain and the kinase domain, beginning at amino acid 538 of cFMS and ending at amino acid 922. The chimeras were created by replacing the cFMS KID with the KID sequences from the FGFR receptor and TIE2 receptor. For expression in SF9 cells, a recombinant baculovirus was generated by subcloning the cFMS chimeras into a modified Invitrogen GATEWAY pDEST8 vector. Further steps followed the protocol for baculovirus expression according to the Bac-to-Bac (Invitrogen) manual.

cFMS was purified by affinity chromatography on BD Talon resin, TEV protease cleavage to remove the histidine tag, and gel filtration chromatography. The apo form was concentrated to ~10 mg/ml and subjected to crystallization trials. For crystallization trials with inhibitors present, the inhibitors (10 mM in Me<sub>2</sub>SO) were added to a diluted solution of cFMS (1 mg/ml) and co-concentrated to 7–11 mg/ml.

**Crystallization and Structure Determination**—In a typical crystallization experiment, 1–2 μl of cFMS protein complexed with the inhibitor of interest and concentrated to 7–10 mg/ml was mixed in a 1:1 ratio with well solution (15–28% PEG3350 (Hampton Research), 100 mM sodium acetate, pH 5.0–5.6, 200 mM Li<sub>2</sub>SO<sub>4</sub>, 5 mM dithiothreitol, 0–3% glycerol) and placed on a glass coverslip. The coverslip was inverted and sealed over a reservoir of 500–1000 μl of well solution and incubated at 22°C. Crystals of the complex with compound 1 (arylamide) usually appeared spontaneously overnight; for other complexes, μ-seeding was used to induce crystallization. For data collection, the crystals were quickly transferred to cryo-solution (27% PEG 3350, 100 mM sodium acetate pH 5.5, 200 mM Li<sub>2</sub>SO<sub>4</sub>, 5 mM dithiothreitol, 15% glycerol) and frozen by immersion in liquid nitrogen. Data were collected at 100 K on a Bruker AXS MO6XCE rotating anode and a SMART 6000 CCD detector or at the IMCA-CAT ID-17 beamline at the Argonne National Laboratory. The diffraction data were processed with the Bruker Proteum suite or the HKL suite. The initial cFMS structure was solved by molecular replacement using the structure of the FGFR kinase domain (1FGK, (14)) as a search model in CNUX. Density modification as implemented in CNUX was used to reduce model bias. Structure refinement and model building were carried out according to standard protocols using CNUX (15) and O (16). In subsequent cFMS inhibitor structures, model bias was removed through simulated annealing and the inhibitor placed in the resulting 2F<sub>c</sub>−F<sub>o</sub> map (Table 1). The following residues have been omitted from the final structure because of insufficient density; for the cFMS-TIE2-Arylamide complex: 535–546, 557–558, 686–692, 922; for the cFMS-FGFR-Arylamide complex: 535–547, 558–559, 680–695, 814, 917–922; for the cFMS-FGFR-Quinolone complex: 535–543, 680–695, 814, 917–922.

**RESULTS AND DISCUSSION**

Overview of the cFMS Structure—This structure of cFMS was obtained by utilizing a construct of cFMS, which included the whole JM domain and the catalytic kinase domain minus the C-terminal tail of cFMS. Early protein constructs that represented the kinase domain, including the full KID (residues 680–751), did not yield any crystals. This is probably because the KID is mostly unstructured and interferes with crystallization. A design strategy in which the kinase insert domain was replaced with the significantly shorter KIDs of previously solved RTKs (FGFR, TIE2, and IRK) proved to be successful. We were able to obtain crystals from the cFMS-FGFR and the cFMS-TIE2 chimeras. The cFMS-IRK chimera did not express well and was not pursued further. Choice of the cFMS ligand
Crystal Structure of cFMS Kinase

TABLE 1

X-ray data collection and refinement statistics

|                      | cFMS-FGFR Arylamide | cFMS-TIE2 Arylamide | cFMS-FGFR Quinolone |
|----------------------|---------------------|---------------------|---------------------|
| Data collection      |                     |                     |                     |
| Unit cell dimensions, Å | $a = b = 81.07, c = 144.67$ | $a = b = 80.44, c = 143.76$ | $a = b = 82.19, c = 143.30$ |
| Space group          | R3 (H3)             | R3 (H3)             | R3 (H3)             |
| Resolution, Å        | 26-1.75             | 63-1.8              | 50-2.70             |
| Total reflections    | 149941 (32580)      | 95204 (30512)       | 44021 (9834)        |
| I/|Io| (final shell), % | 91.0 (27.2)         | 94.8 (90.1)         | 98.6 (99.5)         |
| $R_{merge}$           | 12.7 (2.1)          | 9.4 (1.8)           | 6.35 (1.9)          |
| $R_{merge}$" (final shell) | 6.9 (60.5)      | 5.2 (38.8)          | 11.2 (44.2)         |

Refinement

|                      |                     |                     |                     |
| Resolution, Å        | 26-1.9              | 63-1.8              | 30-2.8              |
| Reflections (Rfree set) | 27350 (2066)    | 32176 (1510)        | 8730 (895)          |
| R-factor (Rfree)     | 0.006               | 0.0054              | 0.009               |
| Bond angle, *        | 1.89                | 1.19                | 1.51                |
| Average B-factors, Å²|                     |                     |                     |
| Protein              | 37.1                | 35.6                | 39.5                |
| Ligand               | 30.2                | 28.8                | 30.8                |
| Solvent              | 38.5                | 37.2                | 25.4                |

R.m.s. deviation from ideal

|                      |                     |                     |                     |
| Bond angle, *        |                     |                     |                     |
| Protein              | 0.006               | 0.0054              | 0.009               |
| Ligand               | 1.89                | 1.19                | 1.51                |
| Solvent              | 37.1                | 35.6                | 39.5                |

$a R_{merge} = \sum_{i} \left( I_i - \langle I_i \rangle \right) / \sum I_i$, where $\langle I_i \rangle$ is the average of $I_i$ over all symmetry equivalents.

$b R-factor = \sum |F_o - F_c| / \sum F_o$, where $F_o$ and $F_c$ are the observed and calculated structure factor amplitudes, respectively.

FIGURE 1. A, overview of the cFMS structure (TIE2-KID) with bound inhibitor 1. Structural elements are color-coded: blue, (N)nucleotide binding loop (P-loop); red, activation loop; green, catalytic loop; salmon, hinge region; cyan, KID; yellow, JM domain. B, structures of the inhibitors used in this study, 1 aryldine series inhibitor, 2 quinolone series inhibitor.

also proved to be critical for crystallization, because neither apo-cFMS nor nucleotide-complexed cFMS yielded usable crystals. Co-crystallization with the aryldine series inhibitor proved to be a reliable tool for obtaining diffraction quality crystals.

The structure of the cFMS kinase domain closely resembles other receptor-tyrosine kinase domain structures in the inactive form determined so far (12, 13). The protein is organized in a two-lobe structure (Fig. 1A), whereby the N-lobe, comprised of five twisted β-sheets and a single α-helix, is connected to the mostly α-helical C-lobe by a hinge region. The N-lobe and hinge regions are mainly responsible for nucleotide or inhibitor binding and provide part of the catalytic residues, whereas the C-lobe is responsible for substrate binding and catalysis.

Nucleotide or inhibitor binding takes place in a deep cleft between the N- and C-lobe. Further important structural motifs are the glycine-rich nucleotide binding loop or P-loop (residues 590–594), the activation loop (residues 796–825), and the catalytic loop (residues 776–783). The native cFMS kinase insert domain (residues 680–751), which has been replaced by the TIE2-KID or FGFR-KID, is located between α-helix-D and α-helix-E and is mostly disordered. The juxtamembrane domain (residues 538–581) loops from the cleft between the N- and C-lobe over the α-helix C to the N-lobe portion of the molecule.

Activation Loop—The activation loop is an essential element for the regulation of kinase activity. In RTKs the activation loop is ~22 amino acids long, begins with a conserved Asp-Phe-Gly (DFG) motif, and ends with a Pro that is conserved among tyrosine kinases (17). Autophosphorylation of tyrosines present in the activation loop has been shown to be essential for stimulation of activity for RTKs. In the absence of phosphorylation, the activation loop is not properly positioned for catalysis and prevents binding of ATP and/or substrate. Phosphorylation events in the activation loop cause a significant structural change and stabilize a conformation in which the active site is accessible to substrates, and residues important for catalysis are positioned properly.

The cFMS activation loop is present in the canonical inactive or closed conformation, examples of which can also be found in FLT3 (PDBID 1RJB) or IRK (PDBID 1IRK). A typical short anti-parallel β-strand, observed in many inactive RTKs, is also present in the structure.

Tyr809 is the single tyrosine in the cFMS activation loop and is one of several tyrosines that are phosphorylated in response to ligand binding. Tyr809 is bound in the active site in a manner very similar to that of Tyr1162 of the inactive form of IRK (18) and Tyr842 of FLT3 (13). The phenol group of Tyr809 forms hydrogen bonding interactions with Asp778.
and Arg$^{782}$ of the catalytic loop, which stabilize the inactive conformation of the activation loop.

The effect of phosphorylation on this critical residue in the activation loop has been established by several mutagenesis studies. For instance, a Y809F mutation prevents differentiation of CSF-1-dependent bone marrow macrophages into osteoclasts (19). In a rat cell line model, Y809G abolished kinase activity and Y809F reduced kinase activity by 40–60% (20). In a previous study, the same Y809F mutation was shown to retain activity as a tyrosine kinase in vitro and in vivo, was able to undergo CSF-1-dependent association with a phosphatidylinositol 3-kinase, and induced expression of the proto-oncogenes c-Fos and JunB, underscoring its ability to trigger some of the known cellular responses to CSF-1 (21). On the other hand, the mutated receptor failed to induce mitogenesis.

The DFG motif in cFMS (Asp$^{796}$, Phe$^{797}$, Gly$^{798}$) signifies the beginning of the activation loop. Asp$^{796}$ is conserved in kinases, and serves as the catalytic base in the phosphotransfer reaction. Depending on the activation state of the kinase, the DFG motif can be present in two distinct conformations. Activated kinases in a catalytically competent state display the so-called “DFG-in” conformation, in which the aspartic acid points toward the β-phosphate of the bound ATP and helps to coordinate a catalytically important Mg$^{2+}$ ion. The phenylalanine points away from the ATP and is tucked away under α-helix C. In the “DFG-out” conformation, which is exhibited by cFMS, the aspartic acid and phenylalanine switch sides, i.e. the aspartic acid is pointing away from the location where ATP would be bound. This conformational change renders the kinase inactive.

**Juxtamembrane Domain**—JM domains in RTKs constitute important regulatory elements. The cFMS JM domain corresponds to residues 538–581$^3$ and contains two tyrosines, which upon phosphorylation mediate association of cFMS with partner proteins. Tyr$^{546}$ was shown to be a major autophosphorylation site and binds to a yet unidentified 55-kDa phosphoprotein (22). A phosphopeptide modeled on the sequence of Tyr$^{561}$ and surrounding residues competed with the association of Fyn with cFMS (23). Furthermore, mutational analysis demonstrated that this and other sequences were required for the efficient association of Src family kinases with activated cFMS in vivo. In addition to their role as substrates for phosphorylation, the JM domains also serve as autoinhibitory elements for RTKs. Mutations in the JM domain of cKIT and FLT3, for instance, are associated with loss of autoinhibitory function and lead to tumorigenesis. Details of this function were revealed by the recent structures of cKIT and FLT3 (12, 13), which belong to the same family of type III receptor-tyrosine kinases. In both structures, aromatic residues of the JM domain protrude into the interface between the N- and C-lobe of the kinase and prevent the DFG motif and the activation loop from achieving a catalytically active conformation. This mode of inhibition differs from the one observed in the case of EphB2 (24), where the JM domain forms an α-helix, which distorts the conformation of the catalytically important α-helix C, and prevents activation of the enzyme.

Structurally, the cFMS JM domain adopts a very similar arrangement as the JM domains observed in the autoinhibited FLT3 and cKIT kinase structures (Fig. 2). It forms a hairpin-like loop, interrupted in part by disordered residues, and is tightly connected to the interface between the N- and C-lobe. In all complex structures of cFMS with various inhibitors, twelve residues N-terminal to Gln$^{547}$ do not show any electron density and are disordered. In some of the structures presented in this report, intermediate parts of the JM domain are also disordered; this is in contrast to the FLT3 and cKIT structures in which the corresponding residues could be traced without interruptions.

It is unlikely that the individual lengths of the JM domains are responsible for the observed difference in structural order between the JM domains, because all of them share a similar length. The cFMS JM domain is 44 amino acids long, whereas the FLT3 JM domain (residues 564–609) and cKIT JM domain (residues 547–588) are 46 and 42 amino acids long, respectively. More likely in the case of cFMS, the difference in order can be explained by slight variations of crystal packing from structure to structure. On the other hand, we cannot exclude the possibility that the variations of structural order are of biological significance. For instance, one could speculate that the more ordered JM domains bind tighter to the respective kinases and therefore have higher autoinhibitory ability.

Residues 548–552 are wedged between the catalytically important α-helix C and a β-sheet like loop region (residues 772–776) just preceding the catalytic loop. Trp$^{550}$ serves as the main anchor for the JM domain, and its indole sidechain is

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$^3$ In this article, we refer to the JM domain as the sequence from the first residue past the transmembrane segment to the last residue before β-sheet 1 in the structures. The resulting residue ranges are: cFMS-(538–581), cKIT-(547–588), and FLT3-(564–609).
inserted deep into a cleft under α-helix C. The residue is located in a hydrophobic pocket formed by (Ile^636, Met^637, Leu^640, Ile^646, Leu^649, Cys^774, and Ile^794). The indole ring of Trp^550 forms a π-face-to-edge interaction with His^779. The backbone participates in hydrogen bonding interactions with the sidechains of Arg^777 and Asp^796. Asp^796 is part of the DFG motif and signifies the start of the activation loop. The DFG motif is present in the catalytically inactive DFG-out conformation, in which the aspartate points away from the active site and is unable to coordinate the catalytically important Mg^{2+} ion. The backbone interaction of the carbonyl group of Asp^796 with Trp^550 stabilizes the DFG-out conformation of the activation loop, and prevents cFMS from obtaining a catalytically competent conformation.

Superposition of cFMS onto the structure of the auto-inhibited form of cKIT (PDB-ID 1T45) shows that these structural motifs are closely shared between these two proteins, and to a lesser extent with the homologous FLT3 (Fig. 2). By comparing the autoinhibited form of cKIT with the activated form of cKIT (12), the authors were able to show that the equivalent residue to Trp^550 in cKIT (Trp^557) prevents the phenylalanine of the DFG motif to switch into a catalytically active conformation by occluding the necessary space. A similar structural comparison for c-FMS is not possible, since no structure of cFMS in the activated form has been reported. On the other hand, based on the close structural similarities between cKIT and cFMS, one can reasonably assume that Trp^550 plays the same role as its counterpart in cKIT, and is therefore a crucial element in the autoinhibitory mechanism of cFMS by ultimately preventing cFMS from switching into the active conformation (Fig. 3).

Kinase Insert Domain—The kinase insert domain is an additional loop region found in a subset of RTKs, which is located between α-helix D and α-helix E. It can vary in length from a dozen to almost 80 residues. The cFMS KID (residues 680–751) contains several phosphorylation sites (Tyr^699, Tyr^708, and Tyr^723), which have been implicated in mediating protein-protein interactions and downstream signaling upon phosphorylation. The analysis of these results is complicated by the fact that the outcome of similar experiments is dependent on the context of the cell lines used. Deletion of the entire KID completely abrogated signal transduction by murine cFMS expressed in Rat-2 fibroblasts (25, 26). On the other hand, expression of a triple mutant (Y697F, Y706F, Y721F) of murine cFMS in FDC-P1 cells slightly increased proliferation (27). Specific interactions have been mapped to individual tyrosines. For instance, Grb2 associates with Tyr^699 upon receptor stimulation with CSF-1 in Rat-2 fibroblasts, whereas Tyr^723 was found to be a binding site for the p85 subunit of PI3-kinase (26). Another study in FDC-P1 cells identified Tyr^706 as a binding site for the transcription factor STAT1 (28). Mutation of either Tyr^697 or Tyr^721 (Tyr^699 and Tyr^723 in human cFMS) compromises signal transduction by cFMS, and the receptor lost all ability to induce changes in morphology or to increase cell growth rate in response to CSF-1.

With ~73 residues, cFMS possesses one of the larger KIDs in the RTK superfamily. Early cFMS constructs comprising the kinase domain including the full-length KID did not express well and did not yield crystals. This is probably because the KID is mostly unstructured and tends to interfere with crystallization. Instead of testing out various deletion mutants, our design strategy for new constructs focused on replacement of the KID with KIDs from previously determined receptor-tyrosine kinases. We chose to replace the cFMS KID with the corresponding counterparts from the FGF receptor, TIE2 receptor and IRK, because these are considerably shorter (between 13 and 20 residues) and were at least partially ordered in their respective crystal structures. Both FGFR and TIE2 chimeras yielded crystals, whereas the IRK chimera did not express well and was not further pursued.

Ligands of cFMS—In this report, we used two compounds to study the specific interactions between cFMS and inhibitory molecules. Our study focused on two series of inhibitors, arylamides and quinolones, which were obtained through optimization of initial leads identified via the proprietary ThermoFluor® assay (29). The arylamides (Compound 1, Fig. 1B) are derived from ortho-piperidin-1-yl-phenylamines that have been capped with 5-membered heteroaromatic carboxylic acids via amide linkages. The quinolones (Compound 2, Fig. 1B) are bicyclic heteroaromatic systems, comprised of 6-substituted-2-quinolone core scaffolds that are further substituted at the 3- and 4-positions with heterocyclic and carbocyclic rings, respectively. Both compounds examined in this study are potent inhibitors of cFMS.
inhibitors of cFMS. The arylamide inhibits cFMS with an IC\textsubscript{50} of 24 nM, whereas the quinolone exhibits an IC\textsubscript{50} of 160 nM.

Almost all ATP competitive kinase inhibitors for which x-ray structures have been published mimic in part the binding of ATP. The adenine ring of ATP is involved in a tridentate hydrogen-bonding interaction with the backbone of the hinge region, which connects the N- and C-lobe of the protein. N1 of the purine ring acts as a hydrogen bond-acceptor, whereas N6 and C2 function as donors, the later as an example of an unconventional CH•••OH-bond (30). This H-bonding pattern is at the heart of the interaction of ATP with kinases, and has been used extensively to design and optimize ATP competitive inhibitors.

Inhibitors of the arylamide series mimic this interaction in part (Fig. 4A). The bridging amide carbonyl accepts a hydrogen bond from the backbone nitrogen of Cys\textsuperscript{666}. The five-membered ring together with the cyano group occupies the adenine pocket. A π-π stacking interaction is formed with Phe\textsuperscript{797} of the DFG motif. Other van der Waals interactions are mediated by the surrounding hydrophobic pocket formed by Val\textsuperscript{596}, Ala\textsuperscript{614}, Lys\textsuperscript{616}, Val\textsuperscript{647}, Thr\textsuperscript{663}, Leu\textsuperscript{785}, and Ala\textsuperscript{800}. The piperazine part of STI-571 occupies the same space that Phe\textsuperscript{382} would if the DFG motif were to switch into the catalytically active DFG-in state. Tyr\textsuperscript{393}, which becomes phosphorylated on activation of cABL, mimics a bound substrate and thereby prevents access to the binding site. In the PD173955 complex (Fig. 5D), the activation loop adopts the active conformation seen in many examples of activated kinases, where the activation loop is folded outwards and permits access to the binding site. The DFG motif, however, is still in the DFG-out position, which is why the conformation of the activation loop is more correctly described as being pseudo-active. The binding mode of STI-571 is very interesting, because the inhibitor not only prevents ATP from entering the active site, but it also locks the activation loop into an inactive conformation. STI-571 accomplishes this fact by two different means. First, the inhibitor clamps down onto Phe\textsuperscript{382} of the DFG motif and forms a hydrogen-bond to the backbone of the corresponding Asp\textsuperscript{581}, thus effectively preventing the DFG motif from shifting and the activation loop from adopting an active conformation. Second, the phenyl-piperazine part of STI-571 occupies the same space that Phe\textsuperscript{382} would if the DFG motif were to switch into the catalytically active DFG-in state. This property of the inhibitor also blocks the activation loop from switching into the active conformation.

During the binding of nucleotides to kinases, the activation loop is displaced from its canonical inactive conformation to the open conformation. This conformational rearrangement has to take place, because the activation loop is occupying and blocking parts of the nucleotide binding site. In contrast, small molecule inhibitors can accommodate various conformations of the activation loop. This has been illustrated in the case of cABL kinase with the inhibitors STI-571 and PD173955 (31, 32). The inhibitor STI-571 binds to the inactive form of cABL (Fig. 5C); the activation loop is in the inactive conformation with the DFG motif in the catalytically incompetent DFG-out state. Tyr\textsuperscript{393}, which becomes phosphorylated on activation of cABL, mimics a bound substrate and thereby prevents access to the binding site. In the PD173955 complex (Fig. 5D), the activation loop adopts the active conformation seen in many examples of activated kinases, where the activation loop is folded outwards and permits access to the binding site. The DFG motif, however, is still in the DFG-out position, which is why the conformation of the activation loop is more correctly described as being pseudo-active. The binding mode of STI-571 is very interesting, because the inhibitor not only prevents ATP from entering the active site, but it also locks the activation loop into an inactive conformation. STI-571 accomplishes this fact by two different means. First, the inhibitor clamps down onto Phe\textsuperscript{382} of the DFG motif and forms a hydrogen-bond to the backbone of the corresponding Asp\textsuperscript{581}, thus effectively preventing the DFG motif from shifting and the activation loop from adopting an active conformation. Second, the phenyl-piperazine part of STI-571 occupies the same space that Phe\textsuperscript{382} would if the DFG motif were to switch into the catalytically active DFG-in state. This property of the inhibitor also blocks the activation loop from switching into the active conformation.

The binding mode of our inhibitors is similar with respect to the clamping effect on the DFG motif (Fig. 5, A and B). In both series, the inhibitor shields the corresponding Phe\textsuperscript{382} almost completely from the solvent. The accessible surface area for
Phe797 in the arylamide complex is reduced from 31.5 Å² to 4 Å² (7.8-fold reduction), whereas in the quinolone complex, the reduction from 26.7 Å² to 0.9 Å² (29.6-fold) is even more profound. This clamping effect exerted by the inhibitors blocks the movement of Phe797, and locks cFMS into the DFG-out state; therefore, the activation loop cannot adopt a catalytically competent conformation while the inhibitors are bound.

Our two series of compounds, however, do not extend as far into the back pocket of cFMS as STI-571 does. We were unable to exploit that part of the binding pocket, because functional groups projecting into that branch of the molecule drastically reduced the potency of the inhibitors. The reason for this is probably the energetic penalty imposed by the disruption of the critical salt bridge between Lys616 and Glu633, which would occur if the inhibitor projected into that space.

Our crystal structures of cFMS in complex with two inhibitors reveal insight into the structural organization of this receptor-tyrosine kinase and highlight the binding mode of two classes of inhibitors. The inhibitors were shown to bind in the ATP pocket and form a typical kinase binding motif, by participating in hydrogen bonds to the backbone of the hinge region. Aside from being ATP-competitive, the inhibitors also lock the conserved DFG motif of cFMS into the catalytically inactive DFG-out conformation, and in turn prevent the activation loop from obtaining a catalytically competent conformation. Additionally, based on structural analysis and homology with other RTKs, we present a model for the partial autoinhibition of cFMS by its JM domain. The JM domain is anchored in the cleft between the N- and C-lobe, mainly through the interaction of Trp550 with a hydrophobic region. Based on homology with activated cKIT we established that the JM domain occludes the same part of cFMS that would be occupied by an activation loop in an open conformation, thus the JM domain prevents the activation loop to switch from the closed to the catalytically competent open conformation. Additionally, Trp550 blocks the DFG motif from switching from the DFG-out into the DFG-in conformation by occluding the necessary space needed by Phe797 to perform the conformational change. Our results provide insight into the molecular basis for cFMS’ autoinhibition, and the binding mode of two series of potent inhibitors. This will guide the development of improved inhibitors to active pharmaceuticals for the treatment of cFMS-related illnesses.

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