Proline-rich tyrosine kinase 2 (PYK2) is a cytoplasmic, non-receptor tyrosine kinase implicated in multiple signaling pathways. It is a negative regulator of osteogenesis and considered a viable drug target for osteoporosis treatment. The high-resolution structures of the human PYK2 kinase domain with different inhibitor complexes establish the conventional bilobal kinase architecture and show the conformational variability of the DFG loop. The basis for the lack of selectivity for the classical kinase inhibitor, PF-431396, within the FAK family is explained by our structural analyses. Importantly, the novel DFG-out conformation with two diarylurea inhibitors (BIRB796, PF-4618433) reveals a distinct subclass of non-receptor tyrosine kinases identifiable by the gatekeeper Met-502 and the unique hinge loop conformation of Leu-504. This is the first example of a leucine residue in the hinge loop that blocks the ATP binding site in the DFG-out conformation. Our structural, biophysical, and pharmacological studies suggest that the unique features of the DFG motif, including Leu-504 hinge-loop variability, can be exploited for the development of selective protein kinase inhibitors.

Proline-rich tyrosine kinase 2 (PYK2) and focal adhesion kinase (FAK) comprise the focal adhesion kinase subfamily of non-receptor tyrosine kinases. PYK2 and FAK are large multidomain proteins containing an N-terminal FERM domain, a central catalytic domain, and a C-terminal segment containing dual proline rich (PR) subdomains and a focal adhesion targeting (FAT) region (1, 2). While FAK is widely expressed, PYK2 expression is relatively restricted with highest levels in brain and the hematopoietic system. Unlike FAK, optimal PYK2 activation is dependent on Ca2+ mobilization. PYK2 (−/−) animals have been described previously, and develop normally (3, 4). Characterization of the immune system of PYK2(−/−) animals revealed the absence of marginal zone B-cells along with abnormal T-cell independent type II responses (4), and altered macrophage morphology, migration and signaling in response to cell attachment or chemokine treatment (3). These studies strengthen the link between PYK2 and signaling through chemokine and integrin receptors. In addition, PYK2(−/−) mice were shown to have increased susceptibility to diet-induced obesity and diabetes (5).

Recently, the characterization of PYK2(−/−) mice showed a high bone mass phenotype resulting from increased osteogenesis and osteoblast activity. Using PYK2(−/−) mouse bone marrow cultures and hMSCs expressing a PYK2 shRNA, elimination or reduction of PYK2 protein levels resulted in significantly enhanced osteogenesis. Importantly, the daily administration of a pyrimidine-based PYK2 inhibitor, PF-431396, increased bone formation, and protected against bone loss in ovariectomized rats (6). PYK2(−/−) mice showed mild osteopetrosis which was attributed to the impairment in osteoclast function (7). Therefore, the high bone mass phenotype may result from both enhanced osteoblast and impaired osteoclast elements.

PYK2 is one member of a family of over 500 evolutionarily conserved enzymes with high amino acid and structural conservation within the catalytic ATP binding pocket. Classical kinase inhibitors bind to the ATP site and compete for substrate binding. Thus, while classical inhibitors based on ATP binding analogs have been readily identified, the inherent promiscuity of action for this class has presented significant challenges to drug design (8). With the exception of cancer therapeutics, where additional therapeutic benefits may be gained by the inhibition of multiple kinase targets (e.g. Sutent, Sorafenib), minimizing off-target activity is most often desired. Therefore, there is great interest in identifying unique allosteric regulatory domains for specific kinase targets. Despite intense effort, small molecule inhibitors exploiting extra-catalytic allosteric sites have been limited to a few examples including IKK (9) and MEK (10). Alternatively, bipartite inhibitors have been developed that stabilize an inactive conformation of the protein kinase, the prototypical example being BIRB796 binding to p38 and Gleevec binding to Abl. Such compounds make contact with both the conserved ATP site and less conserved regions of the activation loop, thus offering the potential for improved selectivity (11). The N terminus of the activation loop contains an invariant Asp-Phe-Gly (DFG) motif, and is an important determinant of enzyme activity. In the active or “DFG-in” conformation, these amino acids are involved in the coordination
of ATP. Conversely, the “DFG-out” state does not bind ATP and the kinase is inactive. While a handful of kinases are known to adopt a DFG-out conformation (e.g., p38, Abl, etc.), it remains to be determined how general this strategy might be in the design of selective kinase inhibitors.

To help elucidate the molecular mechanism of PYK2 and its substrate specificity, we used biophysical methods and determined multiple x-ray structures of the PYK2 kinase domain. High-resolution structures of apo and ATPγS-bound forms were obtained as well as a complex with PF-431396, a “classical” kinase inhibitor. Empirical screening identified BIRB796 as a weak PYK2 kinase inhibitor. Surface plasmon resonance (SPR) and NMR studies indicated that PYK2 could adopt a “DFG-out” conformation. Despite the low affinity, a 1.75-Å co-crystal structure was obtained with BIRB796 revealing a novel binding mode. Our biophysical and structural results provide insight into the enzyme-substrate complex and allowed us to advance the rational design of a selective DFG-out inhibitor with improved PYK2 selectivity and potency. The compound, PF-4618433, showed robust osteogenic activity in hMSC cultures.

**EXPERIMENTAL PROCEDURES**

**Protein Cloning, Expression, and Purification**—A modified pFastBac vector was constructed to have an N-terminal His6 tag followed by a thrombin cleavage site. PfTurbo (from Strategene) was used to amplify the catalytic domain of PYK2 from a full-length clone. The 854-bp DNA fragment was digested with BamHI and XhoI and cloned into the modified pFastBac vector with and without 13C decoupling were collected (data not shown). To assign the Phe of the DFG loop, a mutated sample of [15N]Phe-labeled PYK2 was collected at 600 MHz on a Bruker DRX600 spectrometer equipped with cryoprobe. The concentration of the PYK2 protein was 100 μM in 50 mM deuterated Tris buffer, 100 mM NaCl, and pH 7.5. Ligands were added from deuterated DMSO stock solution to yield final ligand concentration of 150 μM. To assign the Phe of the DFG loop, a mutated sample of [13C]-Asp-[15N]-Phe-labeled PYK2 was also made, and high resolution TROSY spectra of the protein with and without [13C] decoupling were collected (data not shown). Because the DFG loop contains the only Asp-Phe pattern in the PYK2 kinase sequence, the Phe of the DFG loop was identified by the change in 13C-coupling pattern in the TROSY spectra.

**Crystallization**—Crystallization of apo-PYK2, with the BIRB796, with PF-431396, and with the PF-4618433 inhibitor was achieved using a 5 mg/ml protein stock. For co-crystallization experiments, inhibitor (from a DMSO stock of 30 mM) was used to give a final concentration between 0.5–1 mM. Hanging drops of 2 μl + 2 μl were set up over 750 μl of well solution in standard Linbro plates and incubated at 22 °C. Initial screening using Hampton screens produced a condition, 0.1 mM Bis-Tris: pH 6.0–7.0, 0.2 mM MgCl2, 20–27% PEG 3350, 1 mM TCEP, which gave needle crystals that were optimized by refinement of the conditions as well as multiple rounds of streak and microseeding. For the inhibitor co-crystal crystallization, the optimized crystals were further soaked overnight in 3 mM inhibitor to increase the occupancy of the inhibitor.

Crystallization of PYK2 with ATPγS was achieved using a protein solution at 15 mg/ml with 1–2 mM ATPγS added, 2 + 2 μl hanging drops were set up over 750 μl of well solutions con-
taining 0.1 M citrate; pH 5.0–6.0, 0.5–1.5 M lithium sulfate, and 0.2–0.4 M ammonium sulfate. Plates were incubated at 22 °C, not to completely scan wells devoid of nodules. Objects with the scan for that well was aborted. This criterion was set so as more than 10 fields were assayed with 5 or less objects per field, eliminating 0.1 m citrate; pH 5.0–6.0, 0.5–1.5 m lithium sulfate, and 0.2–0.4 m ammonium sulfate. Plates were incubated at 22 °C, and crystals resembling plate clusters were obtained after 3–5 days.

Structure Determination—Diffraction data were collected from flash-frozen crystals at 100 K at beamline 17-ID at Advanced Photon Source of Argonne National Laboratory. Data were processed using the HKL2000 suite of software (12). Data collection statistics are summarized in Table 1.

The structure of PYK2-apo was solved by molecular replacement methods with the CCP4 version of PHASER (13), using the FAK structure (PDB code 1MP8) as a search model. (During the manuscript preparation, a PYK2-apo structure was deposited with PDB code of 3CC6). After molecular replacement, the FAK structure (PDB code 1MP8) was added to the models of PYK2-apo and other complexes were performed using the lsq options in O (15). Figures were prepared with PYMOL (Delano Scientific).

Osteogenesis Assays—hMSC cultures, alkaline phosphatase assays, and mineralization analysis were performed as described previously (6), except that for mineralization analysis, blue calcein was added on day 7, cells fixed on day 14, and analyzed for total intensity.

Image Analysis of Calcein-labeled Bone Nodules—For hMSC cultures, osteoblast nodule formation was quantified by application of the Cellomics Spot Detector Bio Application. Images were taken at a fixed exposure and the blue fluorescence of calcein-labeled nodules was captured with a 12-bit camera and a 10× objective. One hundred fields were collected per well. If more than 10 fields were assayed with 5 or less objects per field, the scan for that well was aborted. This criterion was set so as not to completely scan wells devoid of nodules. Objects with fluorescence above a minimum threshold were evaluated for size, shape, and average/total intensity. The object selection criteria were applied uniformly to all wells, and designed to eliminate fluorescent objects that did not resemble osteoblast nodules. All analyzed fields were also manually examined for image processing artifacts (bubble, flare, or dust), and all fields containing artifacts were excluded from the analysis.

Kinase Assays and Selectivity Profiling—Kinase assays for PYK2 and FAK were as described (6) except that unactivated full-length, C-terminal his-tagged human proteins were used in place of the Src-activated kinase domain protein and that test compounds were preincubated with enzyme for 1 h prior to the addition of 500 μM ATP. IC50 determinations were generated from 8 point dose response curves. Selectivity profiling against a diverse panel of kinases was performed by a commercial vendor (Invitrogen) and reported as average percent inhibition of duplicate tests.

RESULTS AND DISCUSSION

Overall Structures of PYK2—Five crystal structures were determined for unphosphorylated human PYK2 kinase domain, all of which were refined to acceptable R-values and geometry (Table 1). The overall structure is very similar to other kinase catalytic domains, displaying a bilobal architecture. The smaller N-terminal lobe contains a five-stranded anti-parallel β-sheet and a single α-helix (αC). The larger C-terminal lobe is mostly helical and contains the activation loop residues beyond DFG within the ATP pocket. The activation loop residues beyond DFG motif are partially disordered in all five structures and are not visible in the electron density maps; residues 577–586 (Apo),

| Table 1: Data collection and refinement statistics |
|-------------------------------------------------|
| Complex                                         |
| PYK2-apo                                        |
| PYK2:ATPγS                                      |
| PYK2: PF-431396                                  |
| PYK2: BIRB796                                    |
| PYK2: PF-4618433                                 |
| **Data collection**                              |
| PDB code                                        |
| 3FZO                                             |
| 3FZP                                             |
| 3FZR                                             |
| 3FS                                             |
| 3FZT                                             |
| **Space group**                                 |
| P21                                              |
| P2                                              |
| P42                                              |
| P2,2,2,2,2                                      |
| **Cell parameters (Å)**                          |
| a = 37.3, b = 97.0, c = 43.1                    |
| a = 36.9, b = 92.7, c = 42.7                    |
| a = 107.3, b = 81.7, c = 75.8                   |
| a = 37.5, b = 81.7, c = 87.3                    |
| a = 37.7, b = 82.9, c = 86.4                    |
| **Resolution (Å)**                              |
| 2.2 (2.28-2.23)                                 |
| 2.1 (2.18-2.11)                                 |
| 2.7 (2.86-2.79)                                 |
| 1.75 (1.86-1.75)                                |
| 1.95 (2.02-1.95)                                |
| **Unique observations**                         |
| 15582 (1553)                                    |
| 15837 (1527)                                    |
| 12497 (1836)                                    |
| 27824 (2932)                                    |
| 19176 (1525)                                    |
| **Completeness (%)**                            |
| 99.9 (99.9)                                     |
| 94.1 (90.5)                                     |
| 99.4 (97.5)                                     |
| 93.0 (90.3)                                     |
| 93.3 (73.8)                                     |
| **Redundancy**                                  |
| 3.4 (3.1)                                       |
| 2.3 (2.2)                                       |
| 12.1 (6.8)                                      |
| 4.4 (3.0)                                       |
| 7.3 (7.0)                                       |
| **Reflections (Rmerge)**                         |
| 14747 (778)                                     |
| 14989 (802)                                     |
| 12412 (601)                                     |
| 25335 (1327)                                    |
| 18100 (979)                                     |
| **Rmerge/Rfree (%)**                            |
| 14747 (778)                                     |
| 14989 (802)                                     |
| 12412 (601)                                     |
| 25335 (1327)                                    |
| 18100 (979)                                     |
| **Ramachandran plot (%)**                       |
| Most favorable region (outlier)                 |
| 92.7 (0)                                        |
| 92.6 (0.4)                                      |
| 83.7 (1.7)                                      |
| 95.6 (0)                                        |
| 92.5 (0)                                        |

* Values in brackets represent statistics for highest-resolution shells.
residues 570–584 (ATPγS-bound form), residues 573–584 (PF-431396 complex), residues 570–586 (BIRB796 complex), and residues 573–586 (PF-4618433 complex).

The catalytic domains of PYK2 and FAK share about 60% sequence identity. Least-squares Cα superposition of the PYK2 (ATPγS-bound form) and FAK (ADP-bound form, PDB:1MP8) kinase domain reveals this similarity and yields an overall R.M.S.D. value of 1.2 Å for 244 Cα atom pairs. Secondary structure elements of the PYK2 are represented by noodles (α-helices) and arrows (β-strands). They are colored cyan for helices and orange for strands. Identical residues are highlighted in magenta. The DFG motif is boxed in red. The conserved residues involved in forming the back pocket are shown as ◆ below the sequences. The gatekeeper residue is shown as an asterisk below sequences. The Leu-504, which undergoes significant conformational changes with BIRB796, is shown as ◆. The two conserved cysteine residues that form a disulfide bond in FAK are shown as ◆.

Crystal Structure of PYK2

structures of the catalytic domains of PYK2 and FAK are the most common targets for the design of small molecule inhibitors. Discovery and optimization of ATP-competitive inhibitors had been perceived as a difficult obstacle to overcome due to the highly conserved



FIGURE 1. Three-dimensional structure of PYK2:PF-4618433 complex from human. A, secondary structure elements are shown in cyan (α-helices), orange (β-strands), and gray (loops). The bound PF-4618433 is shown as sticks. The Fo-Fc omit map contoured at 3.0 σ (magenta) is shown for the PF-4618433. B, chemical structures of three PYK2 inhibitors used for co-crystallization. C, structure-based sequence alignment of the PYK2 with FAK kinase domains. Secondary structure elements of the PYK2 are represented by noodles (α-helices) and arrows (β-strands). They are colored cyan for helices and orange for strands. Identical residues are highlighted in magenta. The conserved residues involved in forming the back pocket are shown as ◆ below the sequences. The gatekeeper residue is shown as an asterisk below sequences. The Leu-504, which undergoes significant conformational changes with BIRB796, is shown as ◆. The two conserved cysteine residues that form a disulfide bond in FAK are shown as ◆.
nature of the ATP binding site among kinase domains. However, small structural differences and plasticity between the ATP binding sites of even closely homologous kinases has been successfully exploited to achieve selectivity and potency (18, 19). In the PYK2-ATP \( \gamma S \) complex, the adenine base inserts into a hydrophobic pocket formed by the side chains of Leu-431, Ala-455, Val-487, Leu-504, and Leu-556. The N1 nitrogen and the amino group of the adenine ring hydrogen bond to the main chain of Tyr-505 and Glu-503, respectively. This H-bond pattern is at the heart of the interaction of ATP with kinases, and has been used extensively to design and optimize ATP competitive inhibitors (Fig. 2A).

Inhibitors of the trifluoromethyl pyrimidine series (PF-431396) occupy the nucleotide binding pocket, with the pyrimidine ring located in the adenine pocket (Fig. 2B). Both the N1 nitrogen of pyrimidine ring and amide nitrogen of the 5-amino oxindole form hydrogen-bonding interactions with the carbonyl and amide backbone of Tyr-505. The oxindole ring is further stabilized by water-mediated hydrogen bonding interaction with the side chain of Glu-509. The trifluoromethyl group is involved in hydrophobic interactions with Val-487, Met-502, and Ala-455. The hydrophobic phenyl ring occupies the ribose binding pocket and is perpendicular to the pyrimidine ring. The amine group connecting pyrimidine and phenyl ring forms a water-mediated hydrogen bond interaction with Asp-567 of the DFG motif. The methyl group attached to the sulfonamide is wedged into the glycine-rich loop by hydrophobic interactions with Leu-431, Gly-432, and Val-439. PF-431396 exhibits strong dual inhibitory activity for both PYK2 and FAK with an IC50 of 11 and 2 nM, respectively. Molecular modeling of PF-431396 predicts the same binding mode in FAK with small conformational changes in the glycine-rich loop.

The structural analysis of PF-431396 offered an explanation for the lack of compound selectivity between these two FAK family enzymes. In fact, more than half of the kinases tested with PF-431396 in a diverse subset panel at 10 \( \mu M \) showed >50% inhibition (Fig. 3), and greater than 90% inhibition was observed with this compound for JAK3, TrkA, and Aur2 even at 1 \( \mu M \) (data not shown). Thus, while PF-431396 shows an overall preference for the FAK family, it is not absolutely selective. Off-target cross activity may be related to the modest activity of bone efficacy in a cell culture model (see Fig. 7, below). Therefore, we sought to determine whether the PYK2 kinase might be a candidate for a DFG-out inhibitor approach, a strategy that has led to compounds with improved selectivity against other kinases. The prototype DFG-out p38 kinase inhibitor, BIRB796, was profiled in a PYK2 enzyme assay and was active with an IC50 of 1.5 \( \mu M \) (Fig. 3).

**Surface Plasmon Resonance (SPR)—**Classical kinase inhibitors bind catalytic sites at rates that approach diffusion limits (20, 21, 22), whereas DFG-out inhibitors of p38 MAP kinase have been shown to bind two to three orders of magnitude slower (23, 24). Results of SPR kinetic analysis show that this is also the case for PYK2 (Fig. 4). As expected, the classical inhibitor, PF-431396 binds with an on-rate constant of \( 10^{6}/\text{Ms} \), whereas non-classical inhibitors, BIRB796 and PF-4618433 bind with on-rate con-
constants of $10^3$/Ms. It is of interest to note that BIRB796 binds to both activated and non-activated PYK2 catalytic domains with similar on-rate constants (Fig. 4) as well as the activated and non-activated full-length enzyme. Similar effects were also observed with DFG-out inhibitors of the p38 MAP kinase catalytic domain.\(^3\) These data may have implications about the solution structure of activated forms and the mechanism of DFG-out binding. It is often assumed, based on crystal structures that the activated kinase resides primarily in the DFG-in conformation. If this were indeed the case, one might expect that the on-constants for the active kinase would be much slower than for non-activated kinase as the latter exhibits more conformational heterogeneity based on NMR. The fact that these constants are essentially the same suggests that either equivalent sampling of the appropriate DFG-out conformation occurs or that “DFG-out compounds” can induce the conformational change regardless of activation state.

Another point of interest is that despite considerable efforts to develop more potent DFG-out inhibitors of PYK2, significantly faster on-rate constants were not achieved. This may imply that PYK2 only infrequently samples the particular DFG-out conformation required for binding resulting in only a small fraction of the total population as competent for binding. This upper limit on the rate of association may represent an insurmountable energy barrier and has implications for the successful development of DFG-out PYK2 inhibitors, necessitating a significant decrease in off-rate as observed in the p38-BIRB796 interaction.

**NMR Spectroscopy**—It has been demonstrated for p38 kinase that binding of DFG-out inhibitors to P38 kinase causes sharpening of TROSY spectra due to their ability to stabilize the DFG-out conformation (25). Using the same approach, BIRB796 was assessed as a DFG-out inhibitor for PYK2. All the phenylalanine residues of the PYK2 kinase domain were selectively labeled with $^{15}$N, and TROSY spectra were collected. In the apo state, the TROSY

---

\(^3\) Non-published results for BIRB796 and Pfizer inhibitors.
spectrum of $^{15}$N-Phe labeled PYK2 showed only a few phenylalanine peaks, with the rest of the peaks missing (Fig. 5). The missing peaks are the result of extreme line broadening caused by conformational exchange in the intermediate exchange regime of the NMR time scale (millisecond). Tight binding of a classical ATP site inhibitor of the trifluoromethyl pyrimidine series does not change the characteristic of the TROSY spectra, indicating that the DFG-loop is still flexible. However, the addition of BIRB796 caused a dramatic change in the TROSY spectra. The linewidth of all the peaks became uniform with the peaks in the rigid region of the structure, and the missing Phe in the DFG loop was clearly visible. These data indicate that BIRB796 was able to freeze the DFG loop motion, consistent with it being a DFG-out binder as seen in P38 kinase. The millisecond exchange rate observed by NMR line broadening is consistent with the slow $10^{3}$/Ms on-rate of DFG out inhibitors as measured by SPR. This suggests that the slow on-rate of DFG-out inhibitors is limited by the intrinsic rate of opening of the DFG-out conformation. Therefore, further optimization of DFG-out inhibitors will likely result from improvement in the off-rates instead of the on-rates.

**DFG-out Conformation of PYK2 upon BIRB796 Binding**—In the PYK2-BIRB796 complex, the activation loop forms an inactive conformation with the DFG motif in a catalytically incompetent DFG-out state. The Phe-568 side chain in the DFG motif moves by $\pm1$ Å and sandwiches the naphthyl moiety of the BIRB796 with the gatekeeper residue, Met-502 (Fig. 6A). Interestingly, the naphthyl moiety of BIRB796 in the PYK2 structure is perpendicular to that of BIRB796 in the P38α structure (23) (Fig. 6B). Similar to the P38-BIRB796 structures, several distinct inhibitors such as imatinib, sorafenib and AAL993, which have been crystallographically proven to stabilize the DFG-out conformation (26, 27, 28), show significantly different orientation of the phenyl moieties in the crystal structures of ABL, B-RAF, and VEGFR (Fig. 6C). The different binding mode of the naphthyl moiety seen in PYK2 compared with the corresponding moieties in P38α, ABL, B-RAF, and VEGFR is in part due to the difference in the gatekeeper residue. In the PYK2-BIRB796 complex, a conserved gatekeeper residue, Met502, connecting
the N-terminal domain and the hinge loop (Glu-503–Gly-508) plays an important structural role and is involved in forming an additional lipophilic pocket with Met-478, Leu-489, and Ile-500. In contrast, the crystal structures of P38α-BIRB796, ABL-imatinib, B-RAF-sorafenib, and VEGFR-AAL993 all contain smaller threonine or valine side chains as the gatekeeper residue. As a result, the rotation of the naphthyl ring to 90° will lead to steric clashes with Met-506 in PYK2.

The tolyl group on the pyrazole ring interacts with the hydrophobic portion of the side chain of the conserved Glu-474, which makes hydrogen bonds with both NH groups of the urea moiety. The t-butyl group is buried in a hydrophobic pocket composed of Leu-481, Ile-486, Leu-540, and Leu-565. The DFG-out conformation is stabilized by H-bond interactions of the urea carbonyl group with the amide backbone of Asp-567 of the DFG-motif and the amide backbone of Phe-568 with the Asn-554 side chain. The Phe side chain is further stabilized by hydrophobic interactions with Leu-556, Val-487 and the naphthyl group of BIRB796.

Importantly, the morpholino substituent in PYK2-BIRB796 complex does not form a hydrogen bond at the hinge loop. In the P38α-BIRB796 structure, the ethoxymorpholino substituent on the naphthyl ring enhanced the affinity by ~11 fold (23) probably due to a hydrogen bond with the backbone amide of residue 109, which is equivalent to the H-bond made by the N1 atom of the adenine ring of ATPγS. Upon BIRB796 binding in PYK2, the β-turn in the hinge loop formed by the carbonyl and amide backbones of Tyr-505 and Gly-508 vanishes due to hinge movement of the N-terminal lobe and the Leu-504 side chain moves ~2.5 Å to block the ATP-binding pocket to form a hydrophobic pocket with Leu-431, Ala-455, Val-487, Met-502, Leu-556, and Phe-568 (Fig. 6, A and D). This movement of the leucine side chain prevents hydrogen bond interactions with backbone residues in the hinge loop and changes the shape and nature of the ATP binding pocket. Hence, the DFG-out conformation with Leu-504 movement is incompatible with ATP binding because of steric overlap of both Phe-568 and Leu-504 with the phosphate and adenine groups of ATP. To our knowledge, this is the first example that a leucine residue in the hinge loop blocks the ATP binding site in the DFG-out conformation.

**Conformation of PYK2 in Complex with PF-4618433 and Its Pharmacological Activity**—With a definitive crystal structure in hand demonstrating a DFG-out binding mode for PYK2, we undertook efforts to modify BIRB796 to improve potency and selectivity for PYK2. Initial efforts were focused on modification of the tolyl-tert-butylpyrazole moiety. Modification of this group generally decreased potency, which is surprising given that this group is frequently viewed as a selectivity element. Conversely, modification of the naphthyl group led to modest improvements in potency. Through a combination of library and singleton chemistry efforts, we identified PF-4618433 wherein the N-morpholinylethoxynaphthalene of BIRB796 has been replaced with a pyridinoloxymethylpyrazole. This modification gave rise to a PYK2 inhibitor with an IC_{50} of 637 nM.

In contrast to the increased osteogenesis observed with shPYK2 or expression of DN PYK2 in hMSC cultures previously reported (6), PF-431396 treatment led to a modest albeit significant increase in day 7 alkaline phosphatase activity, but only when dosing was restricted to days 4–7 (Fig. 7A). Off-target activity may be obscuring the osteogenic action of PF-431396, as the compound-treated cells appeared to be stressed and the cultures did not mineralize (data not shown). While kinase selectivity profiling indicated that the two DFG-out compounds have a more restricted spectrum of activity, PF-4618433 showed improved PYK2 potency, reduced p38 activity, and superior overall selectivity relative to the prototype BIRB796 (Fig. 3). Thus, PF-4618433 was tested for pharmacological activity in the functional osteogenesis assay. Cultures treated with increasing concentrations of PF-4618433 starting at day 1 and maintained throughout showed a dose-dependent increase in both alkaline phosphatase activity (Fig. 7B) and mineralization (Fig. 7, C and D). The improved osteogenic activity of the DFG-out PYK2 inhibitor PF-4618433 was revealed across a series of DFG-out analogs (LB, PB, and AB, data not shown),
and correlates with the enhanced kinase selectivity profile when compared with classical inhibitors such as PF-431396.

In conclusion, the crystal structures of PYK2 with three different inhibitor complexes show the conformational variability of the DFG motif. The DFG-out conformation in two diarylurea inhibitors reveals a distinct subclass of non-receptor tyrosine kinases identifiable in part by gatekeeper Met-502 and the unique hinge loop conformation of Leu-504. Our structural and solution studies suggest that the important features of the DFG motif including Leu-504 variability in the hinge loop can be utilized in the design and development of new selective inhibitors against protein kinases.

Acknowledgments—We thank Molly Wythes for quantitative analysis of mineral in hMSC cultures.

REFERENCES

1. Avraham, A., Park, S. Y., Shinkmann, K., and Avraham, S. (2000) Cell. Sig. 12, 123–133
2. Hanks, S. K. (2004) Encyclopedia of Biol. Chem. 2, 80–84
3. Okigaki, M., Davis, C., Falasca, M., Harroch, S., Felsenfeld, D. P., Sheetz, M. P., and Schlessinger, J. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 10740–10745
4. Guinanard, R., Okigaki, M., Schlessinger, J., and Ravetch, J. V. (2000) Nat. Immun. 1, 31–36
5. Yu, Y., Ross, S. A., Halseth, A. E., Hollenbach, P. W., Hill, R. J., Gulve, E. A., and Bond, B. R. (2005) Biochem. Biophys. Res. Commun. 334, 1085–1091
6. Buckbinder, L., Crawford, D. T., Qi, H., Ke, H. Z., Olson, L. M., Long, K. R., Bonnette, P. C., Baumann, A. P., Hambor, J. E., Grasser, III, W. A., Pan, L. C., Owen, T. A., Luzzio, M. J., Hulford, C. A., Gebhard, D. F., Paralkar, V. M., Simmons, H. A., Kath, J. C., Roberts, W. G., Smock, S. L., Guzman-Perez, A., Brown, T. A., and Li, M. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 10619–10624
7. Gil-Henn, H., Destaing, O., Sims, N. A., Aoki, K., Alles, N., Neff, L., Sanjay, A., Bruzzaniti, A., De Camilli, P., Baron, R., and Schlessinger, J. (2007) J. Cell Biol. 178, 1053–1064
8. Traxler, P., and Furet, P. (1999) Pharmacol. Ther. 82, 195–206
9. Burke, J. R., Pattoli, M. A., Gregor, K. R., Grassil, P. J., MacMaster, J. F., McIntrye, K. W., Yang, X., Iotova, S. A., Clarke, W., Strnad, J., Qiu, Y., and Zusi, F. C. (2003) J. Biol. Chem. 278, 1450–1456
10. Ohren, J. F., Chen, H., Pavlovsky, A., Whitehead, C., Zhang, E., Kuffa, P., Yan, C., McConnell, P., Spessard, C., Banotai, C., Mueller, W. T., Delaney, A., Omer, C., Sebolt-Leopold, J., Dudley, D. T., Leung, I. K., Flamee, C., Warmus, J., Kaufiman, M., Barrett, S., Teale, H., and Hasemann, C. A. (2004) Nat. Struct. Mol. Biol. 11, 1192–1197
11. Liu, Y., and Gray, N. S. (2006) Nat. Chem. Biol. 2, 358–364
12. Otwinowski, Z., and Minor, W. (1997) Methods Enzymol. 276, 307–326
13. McCoy, A. J., Groske-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C., and Read, R. J. (2007) J. Appl. Crystallogr. 40, 658–674
14. Murshudov, G. N., Vagin, A., and Dodson, E. J. (1997) Acta Crystallogr. Sect D 53, 240–255
15. Jones, T. A., Zou, S. W., Cowan, S. W., and Kjeldgaard, M. (1991) Acta Crystallogr. Sect A 47, 110–119
16. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) J. Appl. Crystallogr. 24, 946–950
17. Nowakowski, J., Cronin, C. N., McRee, D. E., Knuth, M. W., Nelson, C. G., Sang, B.-C., Scheibe, D. N., Swanson, R. V., and Thompson, D. A. (2002) Structure 10, 1659–1667
18. Wang, Z., Canagarajah, B. J., Boehm, J. C., Kassisa, S., Cobb, M. H., Young, P. R., Abdel-Meguid, S., Adams, J. L., and Goldsmith, E. J. (1998) Structure 6, 1117–1128
19. Noble, M. E., and Endicott, J. A. (1999) Pharmacol. Ther. 82, 269–278
20. Regan, J., Brefeldtfer, S., Cirillo, P., Gilmore, T., Graham, A. G., Hickey, E., Klaus, B., Madwed, J., Morak, M., Moss, N., Pargellis, C., Pav, S., Proto, A., Swinamer, A., Tong, L., and Torcellini, C. (2002) J. Med. Chem. 45, 2994–3008
21. Casper, D., Bukhtiyarova, M., and Springerman, E. B. (2004) Anal. Biochem. 325, 126–136
22. Nordin, H., Jungmelius, M., Karlsson, R., and Karlsson, O. (2005) Anal. Biochem. 340, 359–368
23. Pargellis, C., Tong, L., Churchill, L., Cirillo, P. F., Gilmore, T., Graham, A. G., Grob, P. M., Hickey, E. R., Moss, N., Pav, S., and Regan, J. (2002) Nat. Struct. Biol. 9, 268–272
24. Regan, J., Pargellis, C. A., Cirillo, P. F., Gilmore, T., Hickey, E. R., Peet, G. W., Proto, A., Swinamer, A., and Moss, N. (2003) Bioorg. Med. Chem. Lett. 13, 3101–3104
25. Vogtherr, M., Saxena, K., Hoelder, S., Grimme, S., Betz, M., Schieborr, U., Pescatore, B., Robin, M., Delarbre, L., Langer, T., Wendt, K. U., and Schwalbe, H. (2006) Angew. Chem. Int. Ed. 45, 993–997
26. Manley, P. W., Bold, G., Brüggen, I., Fendrich, G., Furet, P., Mestan, J., Schnell, C., Stolz, B., Meyer, T., Meyhak, B., Stark, W., Strauss, A., and Wood, J. (2004) Biochim. Biophys. Acta 1697, 17–27
27. Schindler, T., Bornmann, W., Pellicena, P., Miller, W. T., Clarkson, B., and Kuriyan, J. (2000) Science 289, 1938–1942
28. Wan, P. T., Garnett, M. J., Roe, S. M., Lee, S., Niculescu-Duvaz, D., Good, V. M., Jones, C. M., Marshall, C. J., Springer, C. J., Barford, D., and Marais, R. (2004) Cell 116, 855–867

FIGURE 8. Active site structure of PYK2 in complex with PF-4618433. The Fo-Fc omit map contoured at 3.0 σ (magenta) is shown for the PF-4618433. Activation loop containing the DFG-motif is shown in pink, and two residues (Asp-576 and Glu-577) involved in crystal packing in yellow.