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Identification of small molecule inhibitors of proline-rich tyrosine kinase 2 (Pyk2) with osteogenic activity in osteoblast cells

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ABSTRACT

A screening campaign of a diverse collection of ~250,000 small molecule compounds was performed to identify inhibitors of proline-rich tyrosine kinase 2 (Pyk2) with potential osteogenic activity in osteoblast cells. Compounds were prioritized based on selectivity following a counter-screen against focal adhesion kinase (FAK), a closely related kinase. 4-Amino and 5-aryl substituted pyridinone series were identified that showed strong biochemical potency against Pyk2 and up to 3700-fold selectivity over FAK. Modeling analysis suggested that structural differences in the substrate binding cleft could explain the high selectivity of these chemical series against FAK. Representative compounds from each series showed inhibition of Pyk2 autophosphorylation in 293T cells (IC_{50} ~0.11 μM), complete inhibition of endogenous Pyk2 in A7r5 cells and increased levels of osteogenic markers in MC3T3 osteoblast cells (EC_{50}'s ~0.01 μM). These results revealed a new class of compounds with osteogenic-inducing activity in osteoblast cells and a starting point for the development of more potent and selective Pyk2 inhibitors.

Bone diseases associated with loss of bone such as osteoporosis represent an imbalance between the rate of bone formation and the rate of bone resorption. During normal remodeling of the skeleton, osteoclast cells digest mineralized bone and osteoblast cells follow with the replacement of new bone in a 'coupling' process. Failure of osteoblasts to counter the osteoclast resorptive effects can lead to the gradual net loss of bone that may be associated with decreases in bone mineral density and strength, and increased risk of fracture. Currently recombinant human parathyroid hormone (rhPTH, 1–34; teriparatide), that functions through the parathyroid hormone 1 receptor (PTH1R), is the only FDA approved marketed therapeutic with bone anabolic activity.[1,2]

The non-receptor protein tyrosine kinase Pyk2 was originally identified as an enzyme abundantly expressed in the brain and regulated by various extracellular signals that increased intracellular calcium.[3] Pyk2 plays a role as a mediator of intracellular signaling where Pyk2 phosphorylation leads to activation of the ERK pathway via recruitment of Src and also activation of the JNK pathway.[4] Recent evidence suggests an important role for Pyk2 in both resorptive and anabolic effects in bone. Pyk2 is structurally related to focal adhesion kinase (FAK) sharing 76% homology in the kinase domain and 88% identity in the active site region that is defined by a canonical set of residues whose side chains are directed into the ATP-binding site.

Previously it was shown that Pyk2 was required for the bone resorptive function of osteoclast cells.[5–10] In these studies, Pyk2 was expressed in osteoclasts and found to be present in the sealing zone. The phosphorylation of Pyk2 correlated with formation of the actin-rich sealing zone and the spreading of osteoclasts.[5] Adenovirus overexpression of Pyk2 antisense inhibited osteoclast function by preventing cell spreading and bone resorption in vitro.[7] Pyk2 activity was dependent on binding to Src and Cbl, where the deletion of both is known to decrease osteoclast cell migration and lead to osteopetrosis.[6,8,9] Recently it was demonstrated that $Pyk2^{-/-}$ mice had osteopetrosis of bone and impaired osteoclast function.[11] Partial rescue of the osteoclast-dependent phenotype was observed with a kinase-negative Pyk2 mutant, suggesting that Pyk2 function may include recruitment of signaling molecules as well as kinase activity.

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Pyk2 has also been implicated in osteoblast biology.[12–14] Recently it was shown that Pyk2$^{-/-}$ mice had greater than normal bone mass that was apparently driven by increases in osteoblast number and activity.[15] In the same study, a small molecule Pyk2/FAK dual inhibitor, PF-431396 (Fig. 1) prevented the loss of bone in ovariectomized rats. Furthermore, overexpression of a catalytically inactive Pyk2 mutant K457A was shown to increase mineralization in human mesenchymal stem cell (MSC) cultures suggesting a non-enzymatic Pyk2 function. Taken together, these findings supported a role for Pyk2 in the regulation of bone mass and suggested that Pyk2 inhibition could potentially have anabolic effects in bone.

The kinase domain of human Pyk2 (amino acids 404–708) was used in a homogeneous time-resolved fluorescence (HTRF)-based biochemical assay[16] to screen ~250,000 compounds from a diverse chemical collection. Compounds that were found to inhibit Pyk2 were counter-screened in a similar HTRF-based assay for FAK, in order to facilitate identification of selective compounds at an early stage of the screening process. Here we report the structure–activity relationship (SAR) trends in potency and selectivity of two related series of pyridinone-based Pyk2 inhibitors. Representative compounds **1** and **2** (Fig. 1) demonstrated potent inhibition of Pyk2 autophosphorylation in cellular assays and showed osteogenic-inducing activity in mouse MC3T3 osteoblast cells.

4-Amino and 5-aryl substituted pyridinones had been prepared as previously reported[17] and SAR data are shown in Table 1. Both series provided potent compounds with R^2 = methylamino (**1**) or R^3 = thiophen-2-yl (**2**), as well as excellent FAK selectivity (12 to >3700-fold). In the 4-amino series, a tertiary amine resulted in reduced Pyk2 inhibition (**3** vs **1**). Larger 2-methoxy-ethylamino (**4**) or tetrahydro-2*H*-pyran-4-ylamino (**5**) groups were tolerated by Pyk2, but additional favorable contacts with FAK may have been made as selectivity dropped to 30-fold in **5**. Interestingly, FAK selectivity with the 2-methoxy-ethylamino substituent could be restored by deleting the quinoline 6-methoxy group (**8**), without significant loss of Pyk2 potency. The 4-methylpiperazin-1-yl compound **6** was less active on both Pyk2 (IC_{50} = 2.8 μM) and FAK (IC_{50} >125 μM) than **4** or **5**. Phenylamino substituted compound **7** was about 40-fold less potent on Pyk2 than compound **1**. Compound **9** indicated that the 1-benzyl group was less favorable than 1-phenyl (**9**: Pyk2 IC_{50} = 0.17 μM, vs **1**: IC_{50} = 0.035 μM).

In the pyridinone 5-aryl series, heteroaryl substituents thiophen-2-yl (**2**) and pyridin-3-yl (**10**) provided potent compounds (Pyk2 IC_{50} = 0.053 and 0.033 μM, respectively). Compound **11** bearing a benzyl substituent was also potent against Pyk2 (IC_{50} = 0.049 μM), but was much less selective (37-fold versus FAK) than other compounds in this series. Deletion of the quinoline 6-methoxy in pyridine-3-yl compound **10** resulted in a compound that maintained FAK selectivity (**12**: IC_{50} > 125 μM), but showed a 10-fold loss in Pyk2 potency (**12** vs **10**). Pyrazin-2-yl **13** also had poor Pyk2 potency. 6-Desmethoxy quinoline may have been better tolerated in the 4-amino pyridinones.

A previously described hierarchical molecular modeling method[18] was used to dock compound **1** into both Pyk2 and FAK. Publicly available crystal structures for unphosphorylated Pyk2[19] (apo) and phosphorylated FAK[20] (co-crystal with adenylyl-imidodiphosphate, AMPPNP) showed no significant differences in the adenosine binding pocket. The overall predicted binding modes for compound **1** in Pyk2 and FAK were very similar, with a shape consisting of the quinoline ring on the left creating one plane and the three rings on the right creating a second plane that extended into the substrate pocket (Fig. 2). However, modeling analysis suggested that differences in the substrate binding cleft could contribute to the selectivity associated with compounds from the pyridinone series in Table 1. This cleft appeared to be significantly narrower in FAK (Fig. 2A), resulting in the pyridinone *N*-phenyl group sitting unfavorably against the polar face of FAK's Arg550-Asp546 salt-bridge, according to the most energetically favorable predicted binding mode. In contrast, in Pyk2's wider substrate binding cleft, the Arg553-Asp549 ionic interaction in Pyk2 was predicted to exhibit a very different behavior, with the hydrophobic face of the salt-bridge lying parallel to the face of the *N*-phenyl ring.

The R^2 methylamino group of compound **1** contained an important hydrogen bond donor that was predicted to contribute to the planarity of *N*-pyridinyl-3-amido-2-pyridinone ring system via an intramolecular hydrogen bond with the amide oxygen. R^2 groups lacking this NH, such as compounds **3** and **6** (Table 1) were unable to maintain the second plane and showed a resultant loss of potency toward both Pyk2 and FAK. Exaggerated effects on the latter may have been due to an already tenuous *N*-phenyl-Arg553-Asp549 interaction.

Two key residue differences between Pyk2 and FAK (Gly511/Arg508 and Gln624/Lys621) may rationalize the structure–activity relationship at the R^3 position (Fig. 2B). According to the docked models, in Pyk2, the Gly511 and Gln624 are both >10 Å from the pyridinone R^3 branch point, whereas in FAK the corresponding residues Arg508 and Lys621 are roughly 7 Å away which would place hydrophobic aromatic R^3 substituents (compounds **2**, **10**, **12** and **13**) directly in contact with the charged side chains of these residues. Large, hydrophobic substituents at the R^3 position would thus be predicted to interfere with FAK binding, leading to superior selectivity against FAK. In compound **11**, however, the phenyl ring of the benzyl group appeared to exhibit sufficient conformational flexibility to rotate this hydrophobic substituent away from the basic Arg508 and Lys621, thus restoring some binding to FAK. Overall Pyk2 appeared to show much less sensitivity to R^3 substituents, consistent with the predicted binding modes that suggested the region occupied by R^3 in Pyk2 to be much more open to bulk solvent than it was in FAK.

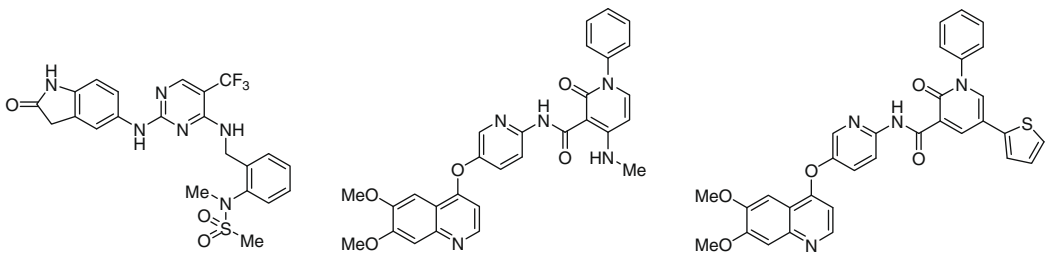
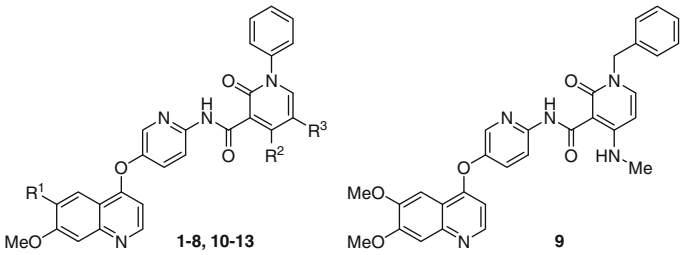


Figure 1. Reported Pfizer Pyk2 inhibitor PF-431396[15] and representative lead screening compounds from the 4-amino (**1**) and 5-aryl substituted (**2**) pyridinone series.

Table 1
Pyk2 and FAK biochemical IC_{50} data for 4-amino and 5-aryl substituted pyridinone analogs



Compound	R^1	R^2	R^3	Pyk2 IC_{50}[a] (μM)	FAK IC_{50}[a] (μM)
1	OMe	Methylamino	H	0.035	17
3	OMe	Dimethylamino	H	0.20	>125
4	OMe	2-Methoxy-ethylamino	H	0.050	1.8
5	OMe	Pyran-4-ylamino	H	0.044	1.3
6	OMe	4-Methylpiperazin-1-yl	H	2.8	>125
7	OMe	Phenylamino	H	1.5	18
8	H	2-Methoxy-ethylamino	H	0.12	>125
9				0.17	>125
2	OMe	H	Thiophen-2-yl	0.053	>125
10	OMe	H	Pyridin-3-yl	0.033	>125
11	OMe	H	Benzyl	0.049	1.8
12	H	H	Pyridin-3-yl	0.43	>125
13	H	H	Pyrazin-2-yl	1.0	>125

[a] Values were the means of at least two experiments.

Recently, the co-crystal structures of unphosphorylated Pyk2 in a DFG-out conformation were released suggesting a new approach for the design of more selective Pyk2 inhibitors.[21] Interestingly, the chemical structures of the current pyridinone series also appeared to contain key features known to elicit DFG-out inhibition, an extended conformation with two H-bond acceptors separated by ~9 Å that are essential for satisfying the backbone NH groups of both the linker residue (Tyr505) and the DFG motif's Asp (Asp567). However, upon examination of compound **1** docked into the Pyk2 DFG-out structure (PDB entry 3FZT),[21] we found the resulting binding mode to be unfavorable, primarily due to the pyridinone carbonyl oxygen being forced to reside unfavorably close to the Glu474 carboxylate group, in order for the rest of the compound to be accommodated by the DFG-out protein conformation. In addition, the DFG-out binding mode was not compatible with the Pyk2 SAR shown for the pyridinone R^2 and R^3 groups, in that the aryl substitutions led to steric overlap with Ile486 & Leu540, respectively when modeled in the DFG-out mode. In contrast to the DFG-out modeling predictions, these substitutions did not result in biochemical loss of Pyk2 inhibition. Furthermore, according to sequence alignment of FAK and Pyk2 in the region of the protein exclusively associated with DFG-out inhibition, no residue differences exist to explain the dramatic target selectivity over FAK observed by the pyridinone series described here. While we cannot rule out the possibility that the pyridinone series bind to the inactive DFG-out conformation of Pyk2, our analysis sug-

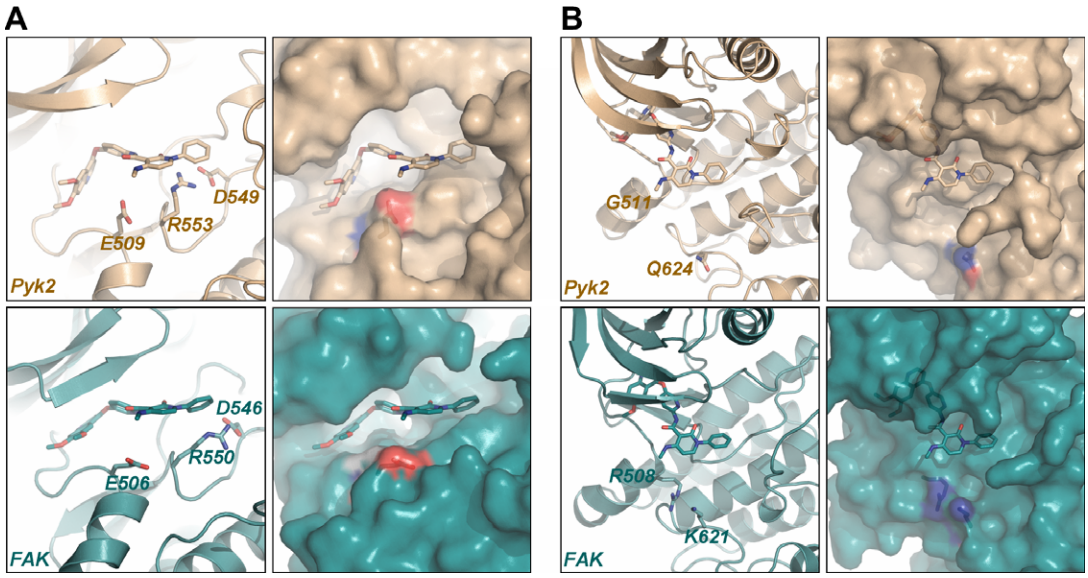


Figure 2. Compound **1** docked into the apo crystal structure of Pyk2 (PDB entry 3CC6), depicted in brown and into the protein structure of FAK obtained from a co-crystal in complex with the non-hydrolyzable ATP analogue AMPPNP (PDB entry 2J0L) depicted in green. A. The substrate binding cleft of Pyk2 was observed to be much wider than that of FAK, with the resulting Arg-Asp ionic interaction presenting a more favorable face to compound **1** in Pyk2 than in FAK. B. Key G511R/R508 and Q624/K621 differences between Pyk2 and FAK along the substrate binding cleft may contribute to the selectivity differences observed for the 4-amino and 5-aryl pyridinone chemical series.

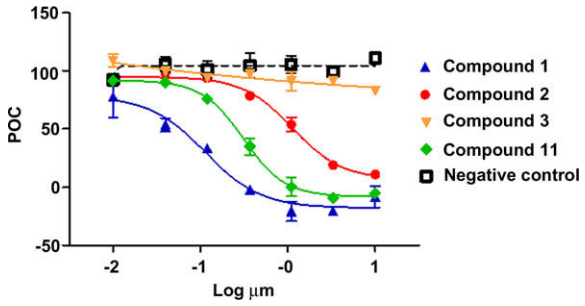


Figure 3. Compound inhibition of Pyk2 Y402 autophosphorylation in stable 293T cells conditionally expressing human Pyk2. Dose–response curves showed most potent inhibition by compound **1** (POC = percent of control). Negative control shown represents a compound with both Pyk2 and FAK biochemical activity >125 μM.

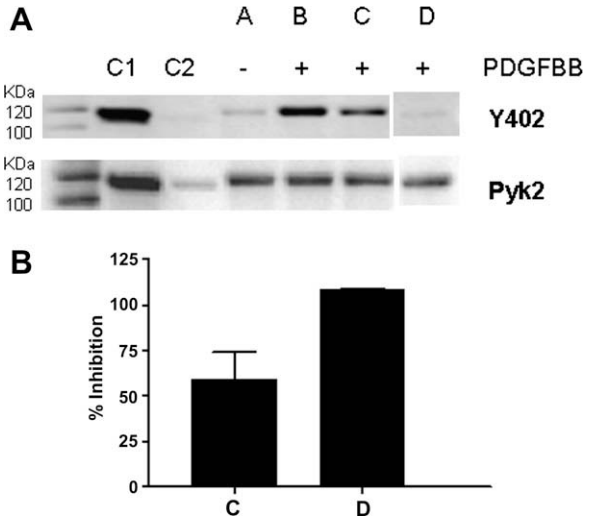


Figure 4. Inhibition of PDGFBB-dependent Pyk2 Y402 autophosphorylation in A7r5 cells. Cells were cultured with 25 ng/ml PDGFBB for 5 minutes and treated with compounds at a single dose of 2.5 μM. (A) Western blotting showing positive control C1 (tetracycline-induced Pyk2 phosphorylation from 293T cells) and negative control C2 (un-induced 293T cells) for Y402 phosphorylation. A, B, cells cultured without or with PDGFBB; C, PF-431396; D, compound **1**. (B) Quantitation of ratio of Y402 phosphorylation to total Pyk2 showing complete inhibition by compound **1** (D).

gested instead that the majority of the inhibitory activity resulted from binding to the activated DFG-in Pyk2 conformation.

Lead screening compounds **1** and **2** with Pyk2 inhibitory activity and FAK selectivity were assessed for cellular activity in three independent assays. First, compounds were tested for their ability to inhibit Pyk2 Y402 autophosphorylation in stably transfected 293T cells capable of conditionally overexpressing Pyk2. Thus cells cultured in the presence of tetracycline were induced to express human Pyk2 which was associated with increased Y402 autophosphorylation.[22] When cells were cultured in the presence of Pyk2 inhibitors, cellular immunofluorescence assays demonstrated a dose-dependent inhibition of Pyk2 Y402 autophosphorylation (Fig. 3). The IC_{50} for compound **1** was ~0.11 μM whereas compounds **11** (~0.31 μM) and **2** (~1.12 μM) were less potent. There was very little cellular activity observed with compound **3** in this phosphorylation assay which appeared to correlate with its lower biochemical potency shown in Table 1.

Compound **1** was next tested in a cellular assay expressing endogenous Pyk2. We confirmed induction of Pyk2 Y402 autophosphorylation by platelet derived growth factor beta (PDGFBB) in rat aortic smooth muscle cells (A7r5) as shown previously.[23,24] Stimulation of A7r5 cells with 25 ng/ml PDGFBB for 5 minutes led to a ~9-fold induction in Pyk2 Y402 autophosphorylation. When cells were treated in the presence of compound **1**, Western blotting showed a decrease in PDGFBB-dependent Y402 autophosphorylation (Fig. 4A). Quantitation of Western blotting results showed complete inhibition of PDGFBB-induced phosphorylation by compound **1** (Fig. 4B).[25]

Following confirmation that the 4-alkylamino pyridinone series inhibited Pyk2 Y402 autophosphorylation in vitro, the compounds were next tested in an osteoblast functional assay. It was hypothesized that if Pyk2 played a role in osteoblast function, inhibition of Pyk2 would result in induction of an osteogenic response. Previously it was shown that the MC3T3 osteoblast cell line could be used to assess the activity of osteogenic small molecules.[26] Expression of Pyk2 in MC3T3 cells was confirmed to be present at a low level when detected by mRNA analysis and Western blotting (data not shown). When MC3T3 cells were cultured in the presence of increasing concentrations of Pyk2 inhibitor compounds **1** and **2** for up to 3 weeks, a consistent, dose-dependent increase in the production of the osteogenic markers alkaline phosphatase (ALP), osteocalcin (OCL) and calcium was observed (Fig. 5). The EC_{50}'s for both compounds **1** and **2** were similar to each other and similar for all three biomarkers at approximately 0.01 μM. These results suggested that the potent osteogenic effects observed in MC3T3 cells were mediated by inhibition of Pyk2 phosphorylation.

The cellular activity of compound **1** showing inhibition of Pyk2 Y402 phosphorylation and enhanced osteogenic activity was consistent with its biochemical profile. The high degree of biochemical selectivity of compound **1** over FAK suggested that inhibition of FAK was not required for stimulation of osteoblast activity. However, this would require confirmation of FAK inhibition in a FAK

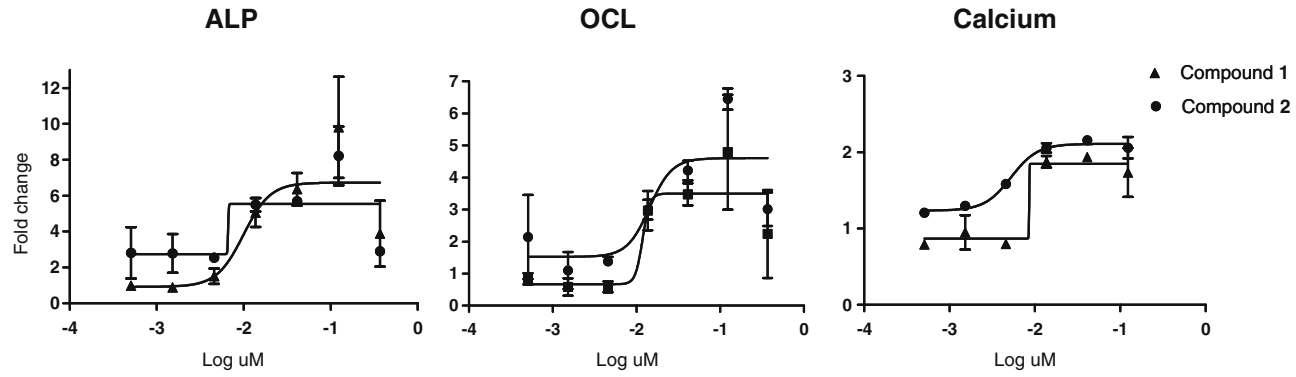


Figure 5. Induction of osteogenic biomarkers by Pyk2 inhibitors in MC3T3 osteoblast cells after three weeks in culture. ALP was measured at additional time points and showed increases in activity at two weeks (data not shown). Conditions for cell culture and compound dosing were as described previously.[26]

cell-based assay. Compound **1** also demonstrated selectivity (IC50 >1 μM) over a limited number of other kinases that were counter-screened including Jak1, Jak2, Jak3, Tyk2, PEK, PKR, and Aurora-2, which ranged in Pyk2 active site homology from 73% to 84% and in Pyk2 kinase domain homology from 39% to 57%.[27] We cannot rule out the possibility, however, that non-selective effects via inhibition of other potential kinases accounted for part of the osteogenic response.

Along these lines, although compound PF-431396 increased bone formation in vivo,[15] it was also reported to inhibit FAK activity[28] and to show >50% inhibitory activity at 10 μM on more than half of a panel of 35 diverse kinases outside of the FAK family,[21] thus complicating interpretation of Pyk2's role in osteogenesis. However, the same group more recently reported[21] on the DFG-out binder PF-4618433, that demonstrated weaker Pyk2 potency compared to PF-431396 but improved selectivity over a panel of other kinases. In addition, PF-4618433 was reported to show enhanced osteogenic activity in human MSC cultures[21] thereby providing additional evidence for a role of Pyk2 in osteoblast biology. With significant FAK selectivity, the 4-amino and 5-aryl substituted pyridinone series presented in this work represent a unique starting point for further optimization of compounds for bone anabolic effects.

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References and Notes

1. Lane, N. E.; Kelman, A. *Arthritis Res. Ther.* **2003**, *5*, 214.
2. Khosla, S.; Westendorf, J. J.; Oursler, M. J. *J. Clin. Invest.* **2008**, *118*, 421.
3. Lev, S.; Moreno, H.; Martinez, R.; Canoli, P.; Peles, E.; Musacchio, J. M.; Plowman, G. D.; Rudy, B.; Schlessinger, J. *Nature* **1995**, *376*, 737.
4. Avraham, H.; Park, S.-Y.; Schinkmann, K.; Avraham, S. *Cell. Signal.* **2000**, *12*, 123.
5. Duong, L. T.; Lakkakorpi, P. T.; Nakamura, I.; Machwate, M.; Nagy, R. M.; Rodan, G. A. *J. Clin. Invest.* **1998**, *102*, 881.
6. Sanjay, A.; Houghton, A.; Neff, L.; DiDomenico, E.; Bardelay, C.; Antoine, E.; Levy, J.; Gailit, J.; Bowtell, D.; Horne, W. C.; Baron, R. *J. Cell Biol.* **2001**, *152*, 181.
7. Duong, L. T.; Nakamura, I.; Lakkakorpi, P. T.; Lipfert, L.; Bett, A. J.; Rodan, G. A. *J. Biol. Chem.* **2001**, *276*, 7484.
8. Lakkakorpi, P. T.; Bett, A. J.; Lipfert, L.; Rodan, G. A.; Duong, L. T. *J. Biol. Chem.* **2003**, *278*, 11502.
9. Miyazaki, T.; Sanjay, A.; Neff, L.; Tanaka, S.; Horne, W. C.; Baron, R. *J. Biol. Chem.* **2004**, *279*, 17660.
10. Park, S.-Y.; Avraham, H. K.; Avraham, S. *J. Biol. Chem.* **2004**, *279*, 33315.
11. Gil-Henn, H.; Destaing, O.; Sims, N. A.; Aoki, K.; Alles, N.; Neff, L.; Sanjay, A.; Bruzzaniti, A.; De Camilli, P.; Baron, R.; Schlessinger, J. *J. Cell Biol.* **2007**, *178*, 1053.
12. Jeschke, M.; Standke, G. J. R.; Susa, M. *J. Biol. Chem.* **1998**, *273*, 11354.
13. Boutahar, N.; Guignandon, A.; Vico, L.; Lafage-Proust, M.-H. *J. Biol. Chem.* **2004**, *279*, 30588.
14. Kim, J.-B.; Leucht, P.; Luppen, C. A.; Park, Y. J.; Beggs, H. E.; Damsky, C. H.; Helms, J. A. *Bone* **2007**, *41*, 39.
15. Buckbinder, L.; Crawford, D. T.; Qi, H.; Ke, H. K.; Olson, L. M.; Long, K. R.; Bonnette, P. C.; Baumann, A. P.; Hambor, J. E.; Grasser, 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.; Li, M. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 10619.
16. HTRF assay conditions: N-terminal GST fusion kinase domain of human Pyk2 (amino acids 404–708), with 5 μM ATP and 1 μM biotinylated-EEEEAYGWLDF; detection by homogeneous time-resolved fluorescence.
17. Kim, T.-S.; Bauer, D.; Bellon, S.; Boezio, A.; Booker, S.; Choquette, D.; D'Amico, D. C.; D'Angelo, N.; Dominguez, C.; Fellows, I. M.; Germain, J.; Graceffa, R.; Harmange, J.-C.; Hirai, S.; La, D.; Lee, M.; Liu, L.; Norman, M. H.; Potashman, M.; Roveto, P.; Siegmund, A. C.; Xi, N.; Yang, K. US Patent Application 20080312232A1, 2008. Experimental procedures and cMet data for some of these compounds will be published in due course.
18. Lee, M. R.; Sun, Y. J. *Chem. Theo. Comp.* **2007**, *3*, 1106.
19. PDB entry 3CC6 (1.6 Å): Busam, R. D.; Lehtio, L.; Karlberg, T.; Arrowsmith, C. H.; Bountra, C.; Collins, R.; Dahlgren, L. G.; Edwards, A. M.; Flodin, S.; Flores, A.; Graslund, S.; Hammarstrom, M.; Helleday, T.; Herman, M. D.; Johansson, A.; Johansson, I.; Kallas, A.; Kotenyova, T.; Moche, M.; Nilsson, M. E.; Nordlund, P.; Nyman, T.; Persson, C.; Sagemark, J.; Svensson, L.; Thorsell, A. G.; Tresaugues, L.; Van den Berg, S.; Weigelt, J.; Welin, M.; Berglund, H.
20. PDB entry 2J0L (2.3 Å): Lietha, D.; Cai, X.; Ceccarelli, D. F. J.; Li, Y.; Schaller, M. D.; Eck, M. J. *Cell* **2007**, *129*, 1177.
21. Han, S.; Mistry, A.; Chang, J. S.; Cunningham, D.; Giffor, M.; Bonnette, P. C.; Wang, H.; Chrunyk, B. A.; Aspnes, G. E.; Walker, D. P.; Brosius, A. D.; Buckbinder, L. *J. Biol. Chem.* **2009**, *284*, 13193.
22. 293T cells were stably transfected with plasmids expressing a tetracycline repressor protein and a human Pyk2 cDNA regulated by a tetracycline operator sequence (T-REx™ System, Invitrogen, Carlsbad CA). In the absence of tetracycline basal Pyk2 expression was very low; in the presence of 100 ng/ml tetracycline Pyk2 expression was induced and was associated with increased Y402 autophosphorylation. Cellular immunofluorescence using antibodies for total Pyk2 and Y402 phosphorylation was quantitated in 96-well format using an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln NE).
23. Zheng, C.; Xing, Z.; Bian, C.; Guo, C.; Akbay, A.; Warner, L.; Guan, J.-L. *J. Biol. Chem.* **1998**, *273*, 2384.
24. Ueda, H.; Abbi, S.; Zheng, C.; Guan, J.-L. *J. Cell Biol.* **2000**, *149*, 423.
25. Pyk2 inhibitors from different chemical series also showed variable activity in this assay (data not shown).
26. Han, C.-Y. E.; Wang, Y.; Yu, L.; Powers, D.; Xiong, X.; Yu, V.; Nguyen, Y., St; Jean, D. J.; Babij, P. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 1442.
27. Selectivity profiling was not run on compound **2** for as many targets as compound **1** but Jak3, Tyk2 and Aurora-2 were all >1 μM.
28. Walker, D. P.; Bi, F. C.; Kalgutkar, A. S.; Bauman, J. N.; Zhao, S. X.; Soglia, J. R.; Aspnes, G. E.; Kung, D. W.; Klug-McLeod, J.; Zawistoski, M. P.; McGlynn, M. A.; Oliver, R.; Dunn, M.; Li, J.-C.; Richter, D. T.; Cooper, B. A.; Kath, J. C.; Hulford, C. A.; Autry, C. L.; Luzzio, M. J.; Ung, E. J.; Roberts, W. G.; Bonnette, P. C.; Buckbinder, L.; Mistry, A.; Griffor, M. C.; Han, S.; Guzman-Perez, A. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 6071.