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Leveraging the Pre-DFG Residue Thr-406 To Obtain High Kinase Selectivity in an Aminopyrazole-Type PAK1 Inhibitor Series

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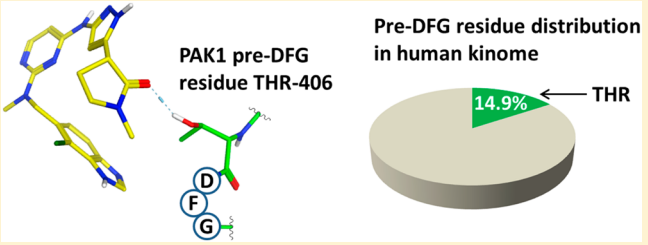
Supporting Information

ABSTRACT: To increase kinase selectivity in an aminopyrazole-based PAK1 inhibitor series, analogues were designed to interact with the PAK1 deep-front pocket pre-DFG residue Thr-406, a residue that is hydrophobic in most kinases. This goal was achieved by installing lactam head groups to the aminopyrazole hinge binding moiety. The corresponding analogues represent the most kinase selective ATP-competitive Group I PAK inhibitors described to date. Hydrogen bonding with the Thr-406 side chain was demonstrated by X-ray crystallography, and inhibitory activities, particularly against kinases with hydrophobic pre-DFG residues, were mitigated. Leveraging hydrogen bonding side chain interactions with polar pre-DFG residues is unprecedented, and similar strategies should be applicable to other appropriate kinases.



KEYWORDS: *Pre-DFG residue, PAK1 kinase, aminopyrazole*

The p21-activated kinases (PAKs) are Ser/Thr kinases in the STE20 kinase family that have generated significant interest as therapeutic targets in cancer and other therapeutic areas.[1−3] PAKs were found to have key roles as regulators of cytoskeletal organization, cell migration, and nuclear signaling, and additionally have been implicated in growth factor pathways, cell proliferation, and pro-survival signaling.

The PAK group comprises six members and is subdivided into two subfamilies, Group I (PAK1, 2, and 3) and II (PAK4, 5, and 6), based on sequence and structural homology. We earlier reported prevalence of increased PAK1 DNA copy number and expression in breast cancer.[4] These findings were supported by subsequent studies, and we now know that focal amplification of PAK1 occurs in approximately 7% of breast cancer and is enriched in the poor prognosis, luminal B subtype.[5,6] PAK1 dysregulation has also been observed in other tumor types, non-small cell lung cancer, ovarian cancer, and melanoma, suggesting a broader role of PAK1 as a driver of tumorigenesis.

No PAK inhibitors have yet been advanced to enable proof-of-concept studies in patients,[7] and the identification of such compounds has proven challenging, presumably due to the large ATP binding pocket and high conformational flexibility of the individual PAK members.[8] Attaining satisfactory kinase selectivity is an additional hurdle; while we recently succeeded in identifying a highly selective PAK4 inhibitor series,[9] no selective ATP-competitive PAK1 inhibitor series has been reported to date.

We recently identified a series of PAK1 inhibitors containing a pyrimidine core combined with an aminopyrazole hinge binding template, a compound series derived from an internal Aurora kinase program.[10] Exemplified compounds demonstrated high PAK1 potency and PAK1 vs PAK4 selectivity (e.g., compound **1**, Table 1); however, selectivity against the broader kinome, including Aurora A, was poor.

As one of the strategies to address this shortcoming we sought opportunities for specific ligand interactions with side chains in the ATP binding site that are unique to PAK1. Focusing initially on Aurora A as a surrogate off-target kinase, we noticed that the residue immediately upstream of the DFG motif ("pre-DFG"), in proximity to the Met gatekeeper residue and in possible reach for ligand contacts, is a threonine residue in PAK1 (Thr-406) but an alanine in Aurora A. In most human kinases this residue is hydrophobic (mostly alanine; see details in the Supporting Information). Ligand van der Waals contacts with hydrophobic pre-DFG residues have been documented.[11] However, to the best of our knowledge, attempts for directed ligand hydrogen bonding interactions with polar pre-DFG

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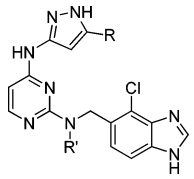
Table 1. Head Group SAR for Compound 1 and Structural Analogues Designed to Reach Thr-406 via Hydrogen Bond Interaction[a]



Number	R	R'	PAK1 Ki [μM]	PAK4 Ki [μM]	Aurora A Ki [μM]
1		Me	0.005	0.272 (51x)	0.003 (0.5x)
2[b]		H	0.025	1.3 (52x)	0.025 (1x)
3[b]		Me	0.016	0.629 (39x)	0.017 (1x)
4[c]		Me	0.029	0.617 (21x)	0.131 (4.5x)
5		Me	0.373	>2.9 (>8x)	0.893 (2.4x)
6		Me	0.552	>2.9 (>5x)	>5.4 (>10x)
7		Me	0.018	0.950 (53x)	>5.4 (>300x)

[a]Unless otherwise noted, data represent an average of ≥2 separate determinations. [b]Racemate. [c]Eutomer. Absolute configuration unknown.

residues, with the aim of driving kinase selectivity, have not been reported in the literature to date. A number of available in-house PAK1 crystal structures showed that the amine backbone of Asp-407 consistently engaged in a hydrogen bond to the Thr-406 hydroxyl group; hence, we expected that this residue would act exclusively as an H-bond donor with the potential to interact with an H-bond acceptor function provided by an appropriately substituted ligand (Figure 1).

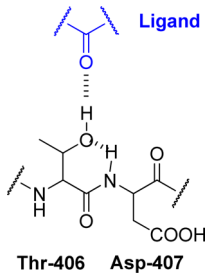


Figure 1. Anticipated hydrogen bonding network between the side chain of the pre-DFG residue Thr-406 with the neighboring Asp-407 amino backbone residue and an appropriately substituted ligand. Stereobonds omitted for simplification.

The best opportunity for incorporating an H-bond acceptor group to our inhibitor molecules appeared to be the headgroup; hence, we set out to explore SAR in this area. The synthesis of these compounds is illustrated in Scheme 1.

As shown in Table 1, the majority of the explored headgroups equipped with oxygen H-bond acceptor functions (either ether or carbonyl) did not impart a selectivity gain over Aurora A, presumably because of imperfect orientation relative to the Thr-406 side chain. For example, in the case of the tetrahydrofuran analogue **2** we believe the oxygen atom is too remote from the Thr-406 hydroxyl group, while in the case of **3** the geometry for forming a hydrogen bond between the carbonyl group and the Thr hydroxyl group is likely not ideal, in addition to a potential for competing H-bond formation with the conserved catalytic lysine residue. The *N*-methyl-pyrrolidone headgroup analogue **7**, however, marked a breakthrough, entirely abolishing Aurora activity with only little loss against PAK1 compared to **1**. Interestingly, the opposite enantiomer **6** ((*S*)-enantiomer) was considerably less active against PAK1 (31-fold), suggesting a tight fit of this headgroup in the binding pocket.

To validate our selectivity hypothesis, we determined the PAK1 ligand cocrystal structure of **7** and compared it with the cocrystal structure of parent compound **8** (analogue of **1** with R′ = H). Gratifyingly, the pyrrolidone carbonyl group of **7** exhibited the desired hydrogen bond contact with the Thr-406 side chain (d(O···O) = 2.8 Å), while otherwise both ligand and protein showed a close overlap with the cocrystal structure of **8** (Supporting Information).

The pyrrolidone ring of **7** in the crystal structure is flanked by the gatekeeper residue Met-344 and fits tightly into the deep front pocket;[13] in fact some expansion is necessary to accommodate this group, mainly through side chain displacement of Met-344 itself and Lys-299 (Supporting Information). We speculate that the pyrrolidone carbonyl group would abrogate interaction with kinases possessing hydrophobic residues at the equivalent position, such as Aurora A. To validate this claim, we tested **7** in an expanded kinase panel (66 kinases) and compared it with the kinase selectivity panel of **1**. As shown in Figure 3, **7** indeed exhibited considerably better kinase selectivity in this panel than its counterpart (detailed numbers provided in Supporting Information).

Consistent with the selectivity hypothesis, activities against other kinases that possess a Thr pre-DFG residue, such as PAK1−3, were overall less diminished than activities against kinases that possess pre-DFG residues different from threonine (Figure 3).[14]

In an effort to increase kinase selectivity further we substituted the (4-chloro-1*H*-benzimidazol-5-yl)methyl tail group of **7** with another moiety identified in earlier SAR studies, 4-aminocyclohexylmethyl. While PAK1 vs PAK4 selectivity has usually been lower with this group, broad kinome selectivity has typically been found to improve. This is likely a result of effectively leveraging electrostatic interactions with acidic residues abundant in the ribose pocket of PAK1.

As expected, PAK1 vs PAK4 selectivity with these analogues decreased (Table 2), but high selectivity against Aurora A was maintained. Most importantly, when tested against a larger kinase panel, analogue **11** displayed exquisite kinase selectivity superior to the earlier analogue **7** (Figure 3). Analogue **11** possesses an ethyl group at the tail linker nitrogen atom. Earlier SAR studies had demonstrated a gain in kinase selectivity of ethyl compared to methyl analogues. This is presumably due to lower energy of the water molecules replaced in this area in PAK1 compared to corresponding water molecules in other kinases. Analogue **11** was tested in an expanded panel of 232 kinases, and besides PAK1, 2, and 3, only two other kinases, CDK2 and PKCθ, were inhibited to a degree of >75% @ 1 μM (see Supporting Information). To the best of our knowledge,

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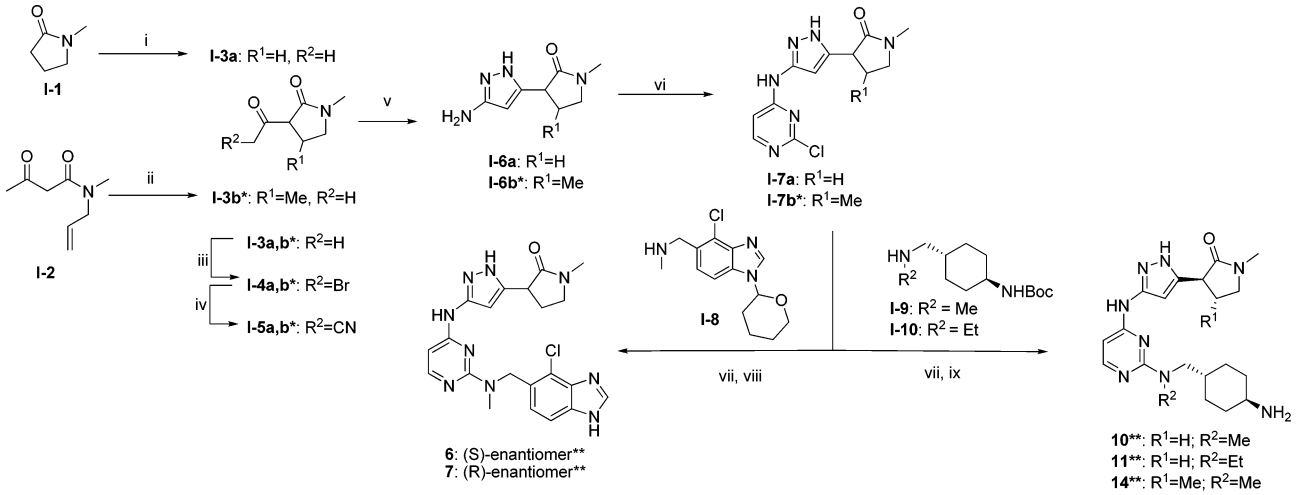
Scheme 1. Preparation of Aminopyrazoles 6, 7, 10, 11, and 14[a]



[a]Reagents and conditions: (i) acetylacetate, LDA, THF, −78 °C. (ii) Au(JohnPhos)Cl (5 mol %), AgOTf (5 mol %), toluene, sealed tube, 90 °C, 24 h.[12] (iii) Br_2, $CHCl_3$, 0 °C−r.t., 4−10 h. (iv) NaCN, EtOH, H_2O, 0.5 h. (v) Hydrazine, EtOH, 75 °C, 3−10 h. (vi) 2,4-Dichloropyrimidine, DIPEA, DMSO, 60 °C, 16−18 h. (vii) Huenig's base, sealed tube, 110−120 °C, 16−18 h. (viii) TsOH, MeOH, H_2O, 60 °C, 5 h. (ix) TFA, DCM, rt. *Racemate of *trans*-diastereomer. **Single enantiomers obtained after final chiral HPLC separation. The absolute stereochemistry was determined via X-ray crystallography.

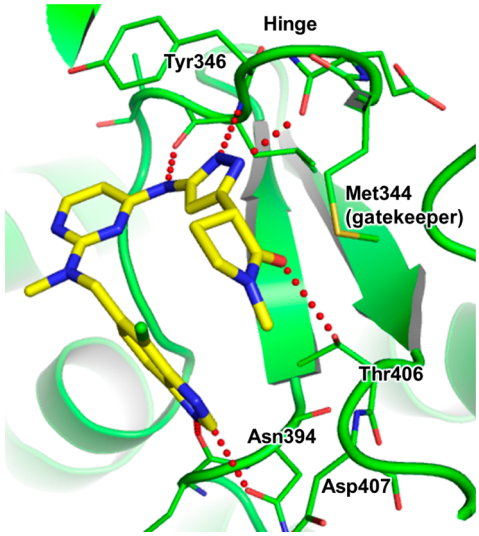


Figure 2. Cocrystal structure of compound 7 (rendered in yellow) and PAK1 (resolution: 2.8 Å). Water molecules are omitted. Hydrogen bonds are depicted as dotted red lines. The shown interaction between the hydrogen at the 2-position of the benzimidazole group and the Asn394 represents a nonclassical hydrogen bond.

no ATP-competitive group 1 PAK inhibitor with such exquisite kinase selectivity has been described to date.

Apart from analogues with *N*-alkylated linkers (**10**−**15**), we also prepared one example with R′ = H (**9**). Consistent with previous SARs, this led to a loss of PAK1 potency likely as a consequence of diminished contact with hydrophobic P-loop residues. Expansion of the lactam ring by one carbon (**12**) decreased potency, reflecting again the tight fit of the headgroup in the deep front pocket. However, as predicted by the crystal structure of **7** bound to PAK1 (Figure 2) the vector that the *N*-alkyl substituent is pointing to offers room for expansion, and ethyl substitution (**13**) imparted increased potency and selectivity over both PAK4 and Aurora A. Furthermore, the crystal structure suggested room for introduction of a *trans*-oriented methyl group at the pyrrolidinone 4-position. Indeed this modification was tolerated (**14**) but did not impart any gain in PAK1 potency, albeit further gain in kinase selectivity was observed. PAK4 and Aurora A selectivity were improved; in addition, in an abbreviated kinase panel that included CDK2 and PKCθ, no kinases were inhibited to a degree of >75% besides PAK1 (PAK2 and PAK3 not tested).

The crystal structure of **14** bound to PAK1 indeed shows a close fit of the methyl group in the available pocket, with an

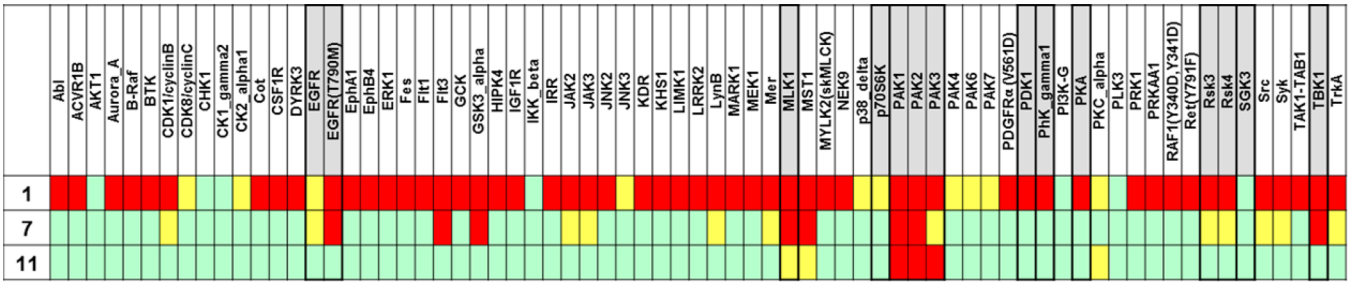


Figure 3. Kinase activity profile of compounds **1**, **7**, and **11** tested in a panel of 66 kinases. Red fields denote >75%, yellow fields between 50 and 75%, and green fields <50% inhibition at a compound concentration of 1 μM. Kinases with a threonine pre-DFG residue are rendered in gray.

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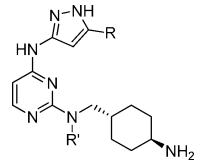
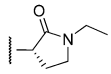
Table 2. SARs of Lactam Head Group Analogues[a]



Number	R	R'	PAK1 Ki [μM]	PAK4 Ki [μM]	Aurora A Ki [μM]
9		H	0.111	1.27 (11x)	>5.4 (>48x)
10		Me	0.037	0.344 (9x)	3.5 (95x)
11		Et	0.049	0.621 (13x)	>5.4 (>110x)
12[b]		Me	0.362	2.49 (7x)	>5.4 (>15x)
13		Me	0.018	0.817 (45x)	>5.4 (>300x)
14		Me	0.046	1.02 (22x)	>5.4 (>117x)
15*		Me	>4.5	>2.9	>5.4

[a]Unless otherwise noted, data represent an average of ≥2 separate determinations. [b]Racemate.

unchanged orientation of the pyrrolidone group and maintained hydrogen bond to Thr-406 (Figure 4). Installation of a second methyl group (gem-dimethyl analogue **15**) completely abrogated PAK1 activity, consistent with the spacial constraints predicted by the crystal structure. Another feature of the PAK1 cocrystal structure with **14** worth pointing out is the interaction

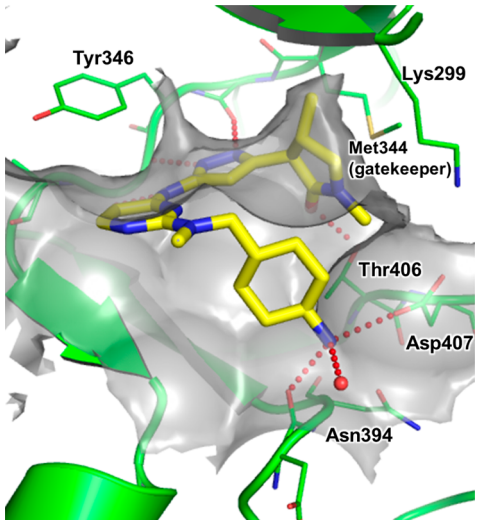


Figure 4. Cocrystal structure of compound **14** (rendered in yellow) and PAK1 (resolution: 2.25 Å). Hydrogen bonds are depicted as dotted red lines. A selected water molecule engaging in a hydrogen bonding interaction with the terminal amino group of **14** is shown as a red sphere. All other water molecules are omitted.

of the amino group at the tail portion with residues in the ribose pocket of the binding site. Similar to the benzimidazole NH portion of compound **7** bound to PAK1 (Figure 2), the amino group of **15** extends a hydrogen bond to the carbonyl backbone of Asp-393; additionally, hydrogen bonds to a neighboring water molecule, and the DFG−aspartate locks the tail portion of **14** into place.

While achieving such kinase selectivity with the described compounds marked progress in our search for PAK1 tool compounds, the feature installed to impart selectivity, lactam head groups, came at the price of increased polarity.

Compounds **7** and especially the more basic and polar analogues **10** and **11** have a low experimental logD and, as a result, are poorly permeable. Likely as a consequence of the low permeability, all three compounds lacked cell potency and had poor oral bioavailability (<1%), as determined in mouse pharmacokinetic studies and hence were not developed further (Table 3). Decreasing polarity at the tail group, hinge binding

Table 3. LogD, Cell Potency, Permeability, and Mouse PK Data of the Selective PAK Inhibitors 7, 10, and 11

	7	10	11
$LogD_{7.4}$[a]	1.8	0.96	0.12
tPSA	118	116	116
MDCK P_{app} A to B [10×10^{-06} cm/s][b]	0.5	0	0
phospho-MEK IC_{50} [μM][c]	7.8	>10	>10
mouse iv PK, CL [mL/min/kg][d]	7	n.d.	33
mouse po PK, AUC [μM·h][e]	5.2	n.d.	BLQ

[a]Experimental logD determined at pH 7.4. [b]Apparent permeability (P_{app}) in the apical to basal A−B and basal to apical B−A directions, determined in MDCK cells. [c]Cellular assay to determine the inhibition of the phosphorylation of residue S298 of MEK in EBC1 cells. Data represent an average of ≥2 separate determinations. [d]Intravenously administered bolus of a 0.3 mg/kg dose of drug, using a formulation of 35% PEG400/65% water. [e]Orally administered dose of 25 mg/kg of drug using a formulation of 65% PEG400/35% water.

moiety, or the selectivity-imparting headgroup itself, with the aim of increasing permeability, was necessary, and efforts in this direction will be described in future publications.

In summary, we were able to significantly increase kinase selectivity in an aminopyrazole-based PAK1 inhibitor series by leveraging a specific residue in PAK1, the deep front pocket pre-DFG residue Thr-406. In most human kinases, this residue is hydrophobic (Ala in >50% of the cases). Incorporation of a pyrrolidone moiety linked to the pyrazole hinge binder moiety allows for a hydrogen bond of the pyrrolidone carbonyl group to the Thr-406 hydroxy group, and the same carbonyl group abrogates interaction in cases of hydrophobic pre-DFG residues. Formation of this hydrogen bond was demonstrated in several PAK1 ligand−cocrystal structures, and consistent with the selectivity hypothesis, activities against other kinases that possess a Thr pre-DFG residue like PAK1−3 were overall less diminished than against kinases with a non-threonine pre-DFG residue. While the compounds presented in this Letter are poorly permeable and hence not usable for in vivo studies, we believe that the concept of leveraging a specific polar pre-DFG residue for obtaining kinase selectivity presented here is unprecedented, and similar strategies should be applicable to other suitable kinases.

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■ ASSOCIATED CONTENT

Supporting Information

Synthetic procedures of key compounds. Cocrystal structure of **8** bound to PAK1 and overlay of this X-ray structure with the cocrystal structure of **7** bound to PAK1. Crystallographic parameters of all X-ray structures. Detailed kinase selectivity data of key compounds. Chart of pre-DFG residues in the human kinome. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.5b00151.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

BLQ, below the limit of quantification; CL, clearance; d, distance; DIPEA, diisopropylethylamine; LDA, lithium diisopropylamide; MDCK, Madin−Darby canine kidney; n.d., not determined; PAK, p21-activated kinase; PK, pharmacokinetics; SAR, structure−activity relationship; TsOH, toluenesulfonic acid; tPSA, topological polar surface area

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(14) Ten of the kinases reported in Figure 3 possess a serine as pre-DFG residue (BTK, CHK1, EpA1, EphB4, Fes, PAK4−6, Ret, and Syk). A high degree of selectivity against these kinases was observed with compounds **7** and **11**, suggesting that the way these compounds achieve kinase selectivity is threonine-specific.

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