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Optimization of highly kinase selective bis-anilino pyrimidine PAK1 inhibitors

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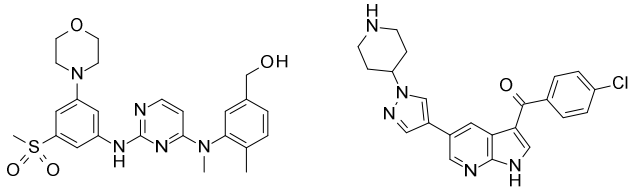
KEYWORDS: PAK, LLE, kinase selectivity, probe, bis-anilino pyrimidine.

ABSTRACT: Group I p21-activated kinase (PAK) inhibitors are indicated as important in cancer progression but achieving high kinase selectivity has been challenging. A bis-anilino pyrimidine PAK1 inhibitor was identified and optimized through structure-based drug design to improve PAK1 potency and achieve high kinase selectivity, giving *in vitro* probe compound **AZ13705339** (**18**). Reduction of lipophilicity to lower clearance afforded **AZ13711265** (**14**) as an *in vivo* probe compound with oral exposure in mouse. Such probes will allow further investigation of PAK1 biology.

The p21-activated kinases (PAKs) are a family of six serine/threonine-specific intracellular protein kinases which are positioned at the intersection of multiple signaling pathways of importance in cancer progression.[1,2] The PAK family is comprised of two subgroups based on sequence homology: group I (PAKs 1-3) and group II (PAKs 4-6). Group I PAKs have high sequence identity in the kinase domain and possess an auto-inhibitory domain which is relieved by binding of the GTP-binding proteins Rac or Cdc42, whilst group II PAKs have lower kinase domain homology and are not activated by Rho GTPases.[3] Group I PAKs are overexpressed in a wide variety of cancers and PAK1 is commonly overexpressed in breast tumours with poor prognosis.[4] In ovarian cancer cells characterized by PAK1 amplification, treatment with a PAK1 inhibitor decreased proliferation and migration.[5] In tumours characterised by neurofibromatosis type 2 (NF2) inactivation, group I PAKs have been shown to be hyperactivated.[6] Consequently, there is increasing interest in identifying potent and selective small molecule inhibitors of PAK1 for therapeutic use in tumours characterised by PAK activation.[7]

There are relatively few chemotypes described in the literature for PAK1 inhibitors[8] and only pan-PAK inhibitor PF-3578309[9] progressed into clinical trials but is now stopped. Ourselves[10] and others[11, 12] have recently disclosed PAK1 inhibitors but achieving high kinase selectivity with a chemotype amenable to achieving oral exposure has been challenging. Without high kinase selectivity it is difficult to discern whether PAK1 is driving efficacy thus new alternative chemotypes for selective PAK1 inhibition are required.

Following a kinase-focused subset screen (120k compounds) of the AstraZeneca compound collection, bis-anilino pyrimidine **1** was identified as a modestly potent PAK1 inhibitor (IC_{50} = 100 nM) with 11-fold selectivity over PAK4 (Table 1). While selectivity within the group I PAKs was not expected, PAK4 was utilized to monitor selectivity against group II PAKs. Selectivity against some key kinase anti-targets, KDR and FGFR1,[13] was modest. Since **1** originated from an EphB4 kinase project,[14] a member of the Src kinase family, high activity for Src kinase was expected. Src family kinase activity was of less concern to us but was monitored as a measure of overall kinase selectivity. A crystal structure of **1** bound to PAK1 was obtained and compared with a crystal structure of a 7-azaindole PAK1 inhibitor **2** (PAK1 IC_{50} = 260 nM) previously reported by us.[10] Overlay of these different chemotypes allowed us to visualize four areas of structure **1** to be explored to optimize PAK1 binding.



1 **2**

Figure 1. PAK1 inhibitors use to guide structure-based drug design

Firstly, the R4 position of the pyrimidine was unsubstituted but a small space was available as occupied by the 7-azaindole of **2** but not the pyrimidine of **1**. Consequently, addition of a fluorine at R4 in **3** increased potency 7-fold and this potency gain more than offset the higher lipophilicity (increased LLE[15]).

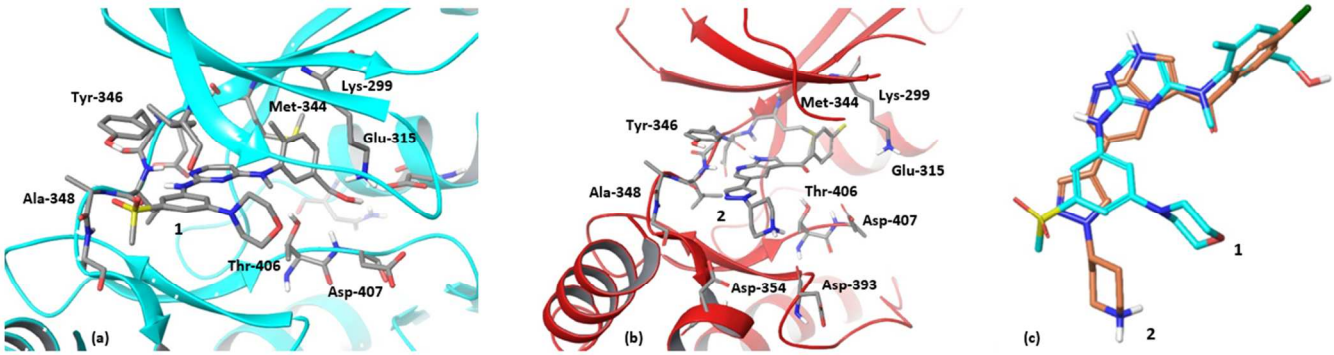
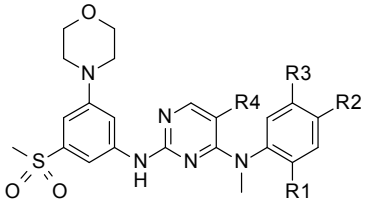


Figure 1. X-ray crystallography of compounds in complex with PAK1: (a) 1 (PDB 5KBQ); (b) 2 (PDB 5KBR); (c) overlay of 1 & 2.

Table 1. "Head-group" SAR around initial screening hit



Cpd	R1	R2	R3	R4	PAK1 IC_{50} (nM)[a]	pPAK1 IC_{50} (μM)[a]	PAK4 ratio[b]	KDR ratio[b]	FGFR1 ratio[b]	Src ratio[b]	log$D_{7.4}$	LLE[c]
1	Me	H	CH_2OH	H	100	3.1	11	2.5	7.0	0.0021	2.9	4.1
3	Me	H	CH_2OH	F	14	0.86	14	25	81	0.034	3.3	4.6
4	H	H	CH_2OH	F	45	14	93	2.0	43	0.11	3	4.4
5	H	Me	CH_2OH	F	19	0.42	21	0.52	1.4	0.069	3.3	4.4
6	Me	H	CH_2OMe	F	189	3.3[d]	26	1.7	1.7	0.0093	3.7	3
7	Cl	H	CH_2OH	F	9.6	0.48	9.5	18	63	0.14	3.7	4.3
8	Me	Me	CH_2OH	F	3.0	0.22	24	22	66	0.17	3.8	4.7
FRAX-597					2.8	0.088	260	1.2	1.7	6.2	>4	<4.6

[a]Geometric mean of at least two experiments unless otherwise stated. IC_{50} values were determined at ATP concentrations within 2-fold of the measured K_M ATP for their respective kinases; [b]selectivity calculated from ratio of IC_{50} values; [c]LLE = pIC_{50} (PAK1) - log$D_{7.4}$; [d]n=1

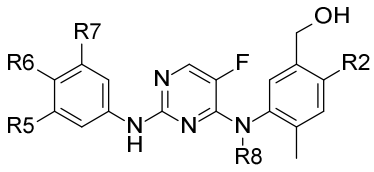
This was viewed as a significant improvement and F at R4 was utilized in all subsequent compounds (Table 1).

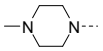
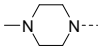
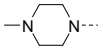
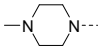
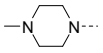
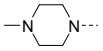
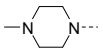
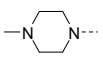
Secondly, the benzylic alcohol "head group" formed some beneficial interactions in the kinase selectivity pocket with Me at R1 occupying a lipophilic cavity and methanol at R3 realizing a polar interaction with Glu315 Based on the overlay, the unsubstituted R2 position appears most likely to tolerate further modification into the same region as the chlorine atom in **2**. Consequently we explored some destructive SAR plus R2 exploration and the key findings are shown in Table 1. Removal of the Me at R1 resulted in **4** with 3-fold reduced PAK1 potency and reduced KDR selectivity. Moving the Me group from R1 to R2 in **5** maintained PAK1 potency but eroded all KDR and FGFR1 selectivity, indicating the need for an R1 substituent for improved selectivity. Methylation of the R3 alcohol in **6** reduced PAK1 potency 14-fold confirming the importance of the alcohol to Glu315 interaction. Switching Me for Cl at R1 in **7** maintained PAK1 potency and selectivity against the other kinases indicating that either of these groups was beneficial. The di-Me combination compound **8** improved PAK1 potency to 3 nM with a similar selectivity profile to mono-Me **3**. It is notable that using LLE as a measure of compound quality that **3** and **8** contain the optimal head groups from this SAR exploration. Furthermore, **8** already compared favorably in terms of PAK1 potency and selectivity with FRAX-597,[16] the best external benchmarking PAK1 inhibitor available at the time (Table 1). Also, **8** already has improved selectivity against our key kinase anti-targets KDR and FGFR1 although FRAX-597 was 10-fold more selective against PAK4.

The third area suggested from the overlay was in the solvent accessible channel region of PAK1. The piperidine of **2** bisected the two aryl substituent of **1**, suggesting that a 6 membered cyclic base would be tolerated para to the aniline of **1**. Thus para-piperazine **9** was prepared which exhibited encouraging PAK1 potency of 33 nM and increased selectivity of 150-fold over PAK4 (Table 2). In our 7-azaindole series we had achieved kinase selectivity improvements in the solvent channel area[10] thus we expected similar selectivity gains could be achieved by further optimization. The methanesul-

fonyl group was tolerated in combination with piperazine and **10** exhibited significantly improved selectivity against KDR and FGFR1 while maintaining potency relative to matched pair morpholine compound **3**. Increasing the bulk of the sulfone to ethyl in **11** gave a 4-fold PAK1 potency increase and in excess of 100-fold selectivity against the three main kinase anti-targets. The alternative di-Me head group compound **12** again gave similar LLE in line with a lipophilicity driven 4-fold PAK1 potency increase. The matched pair morpholine compound **13** was informative in that it was isolipophilic with **12** but it was less potent against PAK1 and has lower selectivity against all the other kinases measured. Lipophilicity could be lowered without loss of PAK1 potency or kinase selectivity by utilizing a bridged piperazine as in compound **14**.

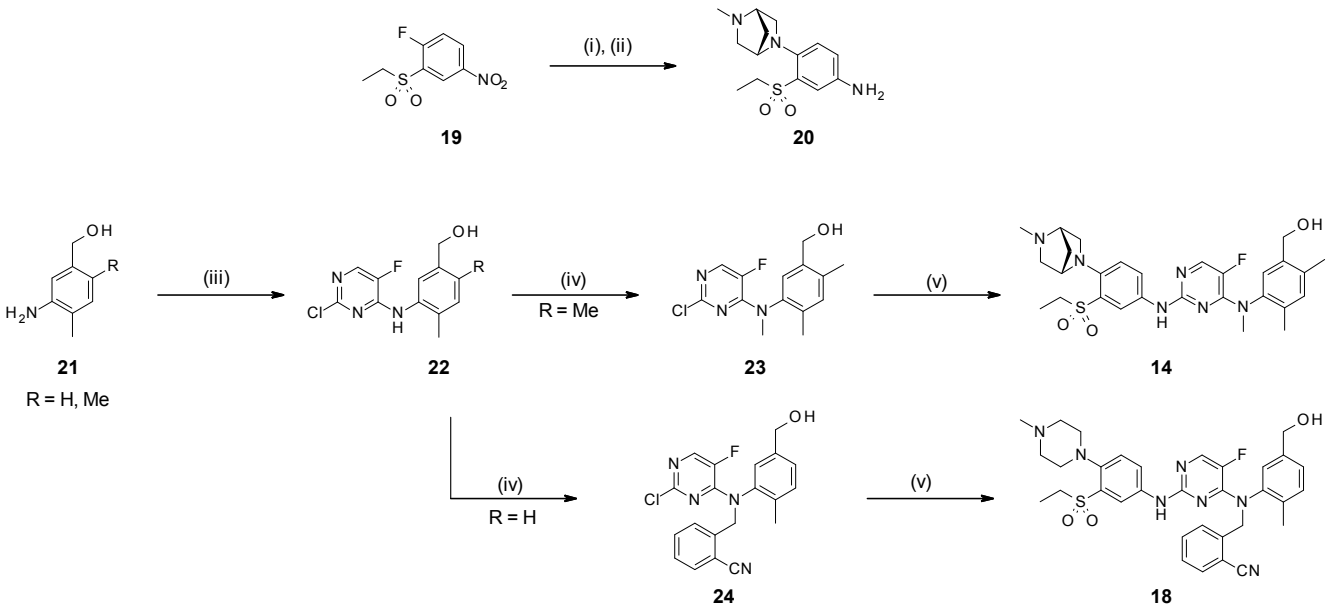
Table 2. "Solvent tail" & p-loop SAR



Cpd	R2	R5	R6	R7	R8	PAK1 IC_{50} (nM)[a]	pPAK1 IC_{50} (μM)[a]	PAK4 / KDR / FGFR1 / Src ratio[b]	log$D_{7.4}$	LLE[c]
9	Me	H		H	Me	33	2.1	150 / 8.9 / 13 / 0.72	3	4.5
10	H	$MeSO_2$		H	Me	8.7	0.81	80 / 500/ 570 / 1.5	2.6	5.5
11	H	$EtSO_2$		H	Me	2.1	0.49	100 / 760 / 1400 / 2.2	3	5.7
12	Me	$EtSO_2$		H	Me	0.45	0.20	160 / 330 / 1300 / 3.3	3.8	5.6
13	Me	$EtSO_2$	H		Me	2.8	0.27	17 / 8.2 / 41 / 0.12	3.8	4.7
14	Me	$EtSO_2$		H	Me	0.58[d]	0.11	90 / 2100 / 880 / 0.96	2.5	6.7
15	H	$EtSO_2$		H	Et	2.0	0.53	200 / 24 / 620 / 2.3	3.5	5.2
16	H	$EtSO_2$		H	Bn	1.5	0.23	1500 / 420 / 1600 / 11	2.8	6.0
17	H	$EtSO_2$		H		0.35[d]	0.13	3800 / 950 / 1700 / 9.0	3.3	6.2
18	H	$EtSO_2$		H		0.33	0.059	2600 / 4100 / 470 / 14	3.7	5.7

[a]Geometric mean of at least two experiments unless otherwise stated. IC_{50} values were determined at ATP concentrations within 2-fold of the measured K_M ATP for their respective kinases; [b]selectivity calculated from ratio of IC_{50} values; [c]LLE = pIC_{50} (PAK1) - log$D_{7.4}$; [d]n=1.

Scheme 1. Synthesis of 14 and 18[a]



[a]Reagents and conditions: (i) K_2CO_3, DMSO, 55 °C, 42%; (ii) Pd/C, H_2, EtOAc, EtOH, 93%; (iii) *n*BuOH, 2,4-diCl,5-F-pyrimidine, (*i*Pr)$_2$NEt, 100 °C, R=Me 77%; R=H, 67%; (iv) R=Me, MeI, DMF 100%, R=H, 2-CN-BnCl, DMF 79%; (v) HCl, TsOH, *i*PrOH, R=Me; **20** 30%; R=H, 3-EtSO_2-4-(1-methylpiperazine)-aniline 47%.

The fourth and final area for optimization suggested from crystal structure analysis was increasing the size of substituent attached to the aniline beyond the current methyl substitution. The NMe of **1** has a similar directionality to the ketone of **2**, with both being directed into an area of space not occupied by the protein. We reasoned that increasing bulk could reach towards the p-loop and potentially further improve potency and selectivity. While ethyl substitution (compare **15** with **11**) gave no advantage, benzyl compound **16** improved PAK4 selectivity 15-fold. Addition of F (**17**) or CN (**18**) to the *ortho* position of the benzyl group gave a 4-fold PAK1 potency increase with improved selectivity against all anti-target kinases measured, including Src.

A representative synthesis of this series of compounds is shown in Scheme 1. Reaction of commercially available 3-(ethanesulfonyl)-4-fluoroaniline **19** with either 1-methylpiperazine or (1*S*,4*S*)-2-methyl-2,5-diazabicyclo[2.2.1]heptane followed by hydrogenation gave the desired anilines for solvent tail exploration. 2,4-Dichloro-5-fluoropyrimidine underwent an S_NAr reaction with either 3-amino-4-methylbenzyl alcohol **21** (R=H) or (5-amino-2,4-dimethylphenyl)methanol **21** (R=Me) in 67% and 61% yields respectively. *N*-Alkylation followed by a final S_NAr reaction with the appropriate aniline provides the desired compounds **14** and **18**.

Throughout the optimization process, cellular pPAK1 inhibition was measured in an MCF10A cell line.[10] This cell potency was found to correlate well with the PAK1 enzyme activity, with a shift as expected based on different ATP concentrations in the two assays (see Supporting Information). A cross check with a similar assay measuring pPAK1 inhibition in the PAK1 amplified cell line OVCAR3 for **11** showed comparable inhibition (IC_{50} = 0.54 vs 0.49 μM for OVCAR3 and MCF10A respectively).

AZ13705339 (**18**) had 59 nM cellular pPAK1 potency, high selectivity against PAK4, KDR and FGFR1, and even 14-fold selectivity against Src so was considered to be an excellent *in vitro* probe compound. To further evaluate **18**, screening was conducted against a panel of 125 kinases (screened at 100 nM concentration at Millipore, equal to 303-fold greater than PAK1 IC_{50}). High kinase selectivity was observed for **18**, with only 8 kinases giving greater than 80% inhibition (Figure 3). This included PAK1 and PAK2 and predominantly a few Src-family kinases. The IC_{50} values for these kinases were determined as shown in Table 3. PAK1 was measured as out of range which is consistent with our internal potency assay and selectivity clearly exists for PAK1 over all kinases tested. Interestingly, this includes PAK2, albeit PAK2 potency is still high at 6 nM. Selectivity between PAK1 and PAK2 was not expected but this can be attributed to differences in K_M for ATP (71 μM for PAK2 and 17 μM for PAK1). PAK2 is known to lead to embryo lethality thus a compound with PAK1 over PAK2 selectivity could be of value.[17] **18** was screened in an alternative binding assay format at DiscoveRx and the PAK1 and PAK2 affinities were measured at K_d of 0.28 and 0.32 nM respectively, indicating that **18** does equally bind to PAK1 and PAK2.

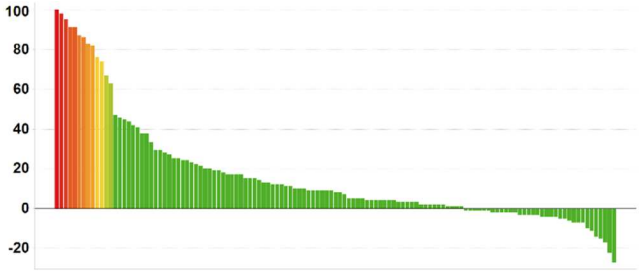


Figure 3. Kinase inhibition for **18** (125 kinases @ 100 nM)

Table 3. Kinase inhibition for 18 (>80% @ 100 nM)[a]

kinase	% inhibition @ 100 nM	IC_{50} (nM)
PAK1	100	< 1
Yes	98	4
PAK2	95	6
Lyn	91	31
PKCtheta	91	7
Fyn	87	45
Src	86	93
Lck	83	36

[a]Data was obtained using Millipore KinaseProfiler service

Furthermore, in vitro pharmacological profiling[18] of **18** against a panel of over 80 diverse targets was conducted and exhibited good selectivity (see Supporting Information). No targets showed activity below 100 nM and 10 targets showed activity below 1 μM (M1, M2, M5, D3, $5HT_{2B}$, $5HT_{2C}$, DT, CaV-L and ARa_{1B}, ARa_{2C})

While **18** is an excellent *in vitro* tool compound for group I PAK inhibition, it does not have optimal properties for *in vivo* exposure, mainly due to moderate clearance (CL_{int} = 30 μL/min/10^6 in rat hepatocytes) likely driven by high lipophilicity. The lower lipophilicity analogue **AZ13711265** (**14)** displayed lower clearance (CL_{int} = 11 μL/min/10^6) and was profiled in mouse pharmacokinetics (Table 4). Oral exposure was achieved for **14** with moderate clearance *in vivo* and an oral C_{max} of 7.7 μM from a 100 mg/Kg dose. Correcting for plasma protein binding this translates into 2-fold excess free cover at C_{max} of the cellular IC_{50} for pPAK1. A recent publication[19] has linked free cover above a cellular PAK1 endpoint with acute toxicity, thus it is worth noting that we did not observe any visible toxicity in this experiment. However, this was not a maximum tolerated dose experiment and **14** achieved free cover for about 4 h above the pPAK1 IC_{50} which may not be exactly comparable with the cell assay used to calculate acute toxicity in the publication,[19] thus we cannot rule out the possibility of toxicity at higher doses.

Table 4. Mouse pharmacokinetic parameters for 14[a]

parameter	**14**
IV CL (mL/min/Kg)	22
V_{dss} (L/Kg)	1.4
IV half-life (h)	1.0
po C_{max} (μM)	7.7
PPB (%free)	2.9

[a]**18** was dosed IV at 0.5 mg/Kg (n=2) in 5% DMSO: 95% SBE-β-CD (30% w/v) in water, and po at 100 mg/Kg (n=2) in 10% DMSO: 90% kleptose (45% w/v) in water.

In conclusion, bis-anilino pyrimidine inhibitors of PAK1 were obtained from a kinase subset screen. Using an overlay of two chemotypes bound to PAK1, we were able to optimize potency against PAK1 by maximizing the efficiency of interactions at four positions around the molecule. Increasing kinase selectivity was incorporated into this optimization, particularly against key anti-targets, resulting in a highly selective, cellular active *in vitro* probe **18**. Compound quality was monitored throughout the process and this enabled *in vivo* probe **14** to also be identified from this series. Such probes will be valuable tools for those looking to understand PAK1 biology and our own further investigations will be the published in due course.

ASSOCIATED CONTENT

Supporting Information

Procedures for synthesis and characterization of compounds; kinase selectivity data for compound **18**; correlation of pPAK1 cell assay with PAK1 enzyme assay; effect of **18** in in vitro radioligand binding, enzyme and functional assays; protein expression, purification, crystallization and structure determination for **1** and **2** in PAK1 (PDF). The Supporting Information is available free of charge on the ACS Publications website.

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

Notes

The authors declare no competing financial interest

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ABBREVIATIONS

ATP, adenosine triphosphate; PAK, p21-activated kinase; LLE, ligand lipophilicity efficiency; PPB, plasma protein binding.

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