

A Chemical Probe Targeting AAK1 and BMP2K

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ABSTRACT: Inhibitors based on a 3-acylaminoindazole scaffold were synthesized to yield potent dual AAK1/BMP2K inhibitors. Optimization furnished a small molecule chemical probe (SGC-AAK1-1, **25**) that is potent and selective for AAK1/BMP2K over other NAK family members, demonstrates narrow activity in a kinome-wide screen, and is functionally active in cells. This inhibitor represents one of the best available small molecule tools to study the functions of AAK1 and BMP2K.

Human protein Ser/Thr kinases Adaptor protein 2-Associated Kinase 1 (AAK1) and BMP-2-Inducible Kinase (BMP2K/BIKE) play critical roles in mediating endocytosis and other key signaling pathways. Both are broadly expressed members of the NAK family of human kinases, which also includes Cyclin G-Associated Kinase (GAK) and Myristoylated and Palmitoylated Serine/Threonine Kinase 1 (MPSK1/STK16). The family shares little homology outside of their kinase domains (KDs).¹ AAK1 and BMP2K are the most closely related, with overall sequence identity of 50% and KD sequence identity of 74%.² A key function of AAK1 is regulation of receptor-mediated endocytosis via binding directly to clathrin and phosphorylating the medium subunit of AP2 (adaptor protein 2), which stimulates binding to cargo proteins.³ AAK1 also modulates the Notch pathway, partially through its phosphorylation of Numb.^{4,5} BMP2K plays a role in osteoblast

differentiation, is a clathrin-coated vesicle-associated protein, and, like AAK1, also associates with Numb.^{6,7}

Due to their many functions, AAK1 and BMP2K have been implicated as potential drug targets for diverse conditions. AAK1 has been linked to diseases affecting the brain such as schizophrenia, Parkinson's disease and amyotrophic lateral sclerosis as well as implicated as a potential anti-viral target for treatment of hepatitis C.^{8,9} BMP2K has been associated with myopia and evaluated as a potential treatment for HIV.^{10,11} Figure S1 shows the current inhibitor landscape and how their pharmacophores bind to AAK1/BMP2K. Patented AAK1 inhibitors were recently reviewed.¹²

X-ray crystal structures for the KDs of all NAK family members have been solved and reported.^{2,13} Published and novel high-resolution crystal structures of AAK1 and BMP2K reveal target-specific structural features that enabled our design of specific chemical probes and allowed further interrogation of

the roles that these kinases play.² Screening a library of kinase inhibitors (Published Kinase Inhibitor Set, PKIS) via differential scanning fluorimetry (DSF), revealed a chemical starting point showing strong AAK1 binding.¹⁴ Structure–activity relationships (SAR) were initially established by examining the 3-acylaminoindazole analogs profiled within PKIS (Figures 1 and S2). Next, more than 200 analogs were prepared via the route shown in Scheme S1. A summary of key analogs prepared to arrive at **14** is included in Table 1. Compounds were profiled using the AAK1 split luciferase assay developed by Luceome.¹⁵ Potent inhibition of BMP2K was not observed in this assay format.

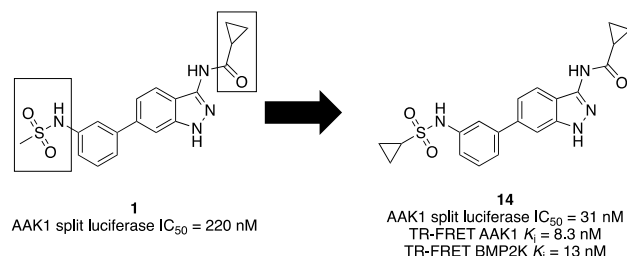


Figure 1. Structures of PKIS hit (**1**) and key analog **14** with parts of the molecule modified during SAR exploration boxed.

The effects of aryl substitution on the distal aryl ring were explored, including 3- and 4-position substituents of variable size, electronics, and hydrogen-bonding capacity (Table 1). A sulfonamide was found to be optimal for AAK1 inhibition but only when appended at the 3-position of the aryl ring (**1** versus **2**). Sulfonamides with small saturated rings (<6 atoms) and short alkyl chains or alkyl amines were the most potent (**1**, **11**, **13**, **14**). This part of the binding pocket was not tolerant to incorporation of large groups. 3,5-disubstitution and 3,6-disubstitution was disfavored, supporting a small binding pocket for this aryl ring that is sensitive to even minor changes, such as incorporation of a fluorine in place of a hydrogen (data not shown). Finally, activity of **1** and **8** suggests the presence of a hydrogen-bonding network within the ATP-binding pocket that can be accessed by 3-position groups capable of donating a hydrogen. Interestingly, methylation of the aryl 3-nitrogen (**11**) or insertion of a methylene spacer between the sulfonamide and aryl ring (**12**) increased potency for AAK1. These results motivated design of a range of sulfonamides to further explore the SAR of this part of the molecule.

The portion of the ATP-binding pocket accessed by the 3-position of the indazole was explored through substitution with a variety of groups (Table 2). Compounds in Table 2 were prepared using the route in Scheme S1, but the step order was reversed. The indazole 3-position substituent appears to form a polar interaction with the kinase hinge region and is sensitive to structural modifications. The alkyl group was varied from cyclopropyl to methyl, isopropyl, cyclobutyl, phenyl, and cyclopropylmethyl to explore the steric bulk tolerated by the pocket. Alternatively, a urea or sulfonamide was incorporated in place of the acylated amine (**20**, **21**, **22**) but resulted in loss of AAK1 affinity. The cyclopropanecarboxamide was found to be optimal (**14**). Minor changes, such as ring opening of the cyclopropane (**16**) or expansion to a cyclobutane (**17**) were found to result in modest loss of AAK1 affinity.

Based on the initial SAR, two additional 3-acylaminoindazoles (**23** and **24**) were prepared (Table 3) incorporating

the aryl 3-cyclopropanecarboxamide that was found to yield potent inhibitors of AAK1. The analogs were designed to probe

Table 1. SAR of 3-acylaminoindazole aryl ring.






Cmpd	R	AAK1 IC_{50} (nM) ²²
1	3-NHSO ₂ Me	220
2	4-NHSO ₂ Me	1200
3	3-CONH ₂	3800
4	4-CONH ₂	910
5	3-NHAc	1100
6	3-CO ₂ H	18000
7	4-CO ₂ H	2800
8	3-OH	350
9	4-OH	280
10	3-NH ₂	800
11	3-N(CH ₃)SO ₂ Me	120
12	3-CH ₂ NHSO ₂ Me	71
13	3-NHSO ₂ (isopropyl)	54
14	3-NHSO ₂ (cyclopropyl)	31

whether an alkyl amine or longer chain hydrocarbon could be tolerated at the indazole 3-position. Like lead compound **14**, these analogs demonstrated promising inhibition of AAK1 in the split luciferase assay (93%I and 94%I of AAK1 at 1 μ M for **23** and **24**, respectively). TR-FRET binding-displacement assays were employed to further interrogate the biochemical selectivity of these promising 3-aminoacylindazoles across the NAK family (Table 3).²¹ **23** and **24** were found to be dual AAK1/BMP2K inhibitors with more than 5-fold and 50-fold selectivity over STK16 and GAK, respectively.

To understand the selectivity of **23** and **24** across NAK family kinases, co-crystal structures were solved with the BMP2K KD (Figure 2, Table S1). Attempts to obtain co-crystals of acylaminoindazoles bound to the AAK1 KD were not successful. The BMP2K co-crystal structures were solved to ~2.4 Å resolution and showed that the core indazole formed the critical interaction with the hinge region of the KD.

Table 2. SAR of 3-acylaminoindazole carboxamide.

Cmpd	R	AAK1 IC_{50} (nM) ²²
14		31
15		3500
16		18000
17		370

18		560
19		2200
20		>50000
21		>50000
22		7700

Given the high structural and sequence conservation between the ATP-binding sites of the BMP2K and AAK1 KDs (Figures 2 and S3), it is likely that both kinases bind to the indazole inhibitors using common interactions and thus unlikely, using this chemotype, we can design inhibitors that target only AAK1 or BMP2K. The pendant aryl ring is accommodated by the hydrophobic region adjacent to the gatekeeper residue (residue Met130/126, BMP2K/AAK1 numbering) within the kinase ATP-binding site. These co-crystal structures suggest that large groups attached to the 4-position of the aryl ring (**2** and **4**) are likely not tolerated due to steric hinderance. Smaller groups at the 3- and 4-positions that can act as hydrogen-bond donors (**8** and **9**) can make favorable interactions with the DFG motif aspartate residue (Asp198/194, BMP2K/AAK1 numbering), whereas small acidic groups at these positions (**6** and **7**) are not favored due to charge repulsion. The structures also reveal that a sulfonamide at the 3-position can form hydrogen-bonds with multiple polar residues within or adjacent to the protein ATP-binding pocket (Gln137/133, Asn185/181 and Asp198/194). Groups attached to this sulfonamide are accommodated by a small cavity within the protein P-loop. When considering substituents attached to the indazole ring, poor binding is observed for compounds bearing sulfonamide groups in place of an amide (**21** and **22**). This finding is due to steric clash and electronic effects caused by the proximity of the sulfonamide oxygen atoms to nearby protein side chains. Compound **20** is proposed to adopt a planar conformation that introduces steric clash not observed with smaller groups and/or those that are not flat (**14** and **17**). Co-crystal structures also reveal that groups attached to the 3-position amide are solvent exposed, an observation that helps rationalize the moderate tolerance of AAK1 for larger and bulkier substituents at this position (**15**, **17** and **19**).

Guided by co-crystal structures, analogs were designed focusing on modifications to the sulfonamide portion of the molecule. Sulfonamides with pendant alkyl chains, alkyl amines, fluorinated alkyl chains, small hydrocarbon-based rings, and fluorinated aryl rings were synthesized. Table 4 includes some

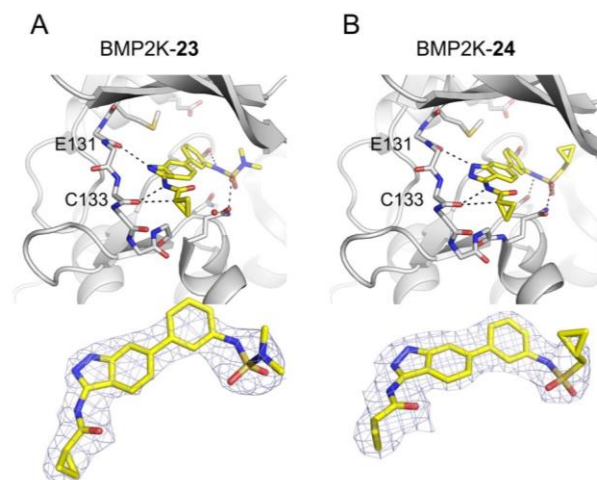


Figure 2. Co-crystal structures of **23** (A) and **24** (B) bound to the BMP2K-KD. Main-chain atoms for residues (131–137) within the BMP2K-KD hinge region, side-chain atoms for BMP2K-KD gatekeeper residue (Met130) and those taking part in polar interactions with the ligands are shown as sticks. Black dashed lines depict possible hydrogen-bonds between protein and ligand atoms. Red spheres denote the position of crystallographic water molecules. The 2Fo-Fc electron density maps (contoured at 1.0 σ) are shown below.

of our most promising analogs, the design of which was informed by more than 200 compounds prepared before them, some of which are included herein. Our years of effort on this scaffold enabled us to produce many highly potent compounds. Compounds built on Scaffold B were prepared via Scheme S1. Those built on Scaffold A were prepared using a slightly modified route (Scheme S2). Analogs (**25**–**39**) were evaluated for potency and selectivity across the NAK family TR-FRET binding assays. Furthermore, family cellular target engagement was determined via NanoBRET (NB) assays, where the respective kinases were fused to 19-kDa luciferase (NLuc) and transiently expressed in HEK293 cells, and then incubated with a cell-permeable fluorescent energy transfer probe (tracer).¹⁶ Using increasing concentrations of test compounds dose-dependent displacement of tracer was observed, allowing calculation of the IC₅₀ values in Table 4. These NB assays enabled measurement of potency and selectivity of the compounds across the NAK family in living cells (Figure S4).

All analogs (**25**–**39**) were found to be dual AAK1/BMP2K inhibitors. Insertion of the methylene spacer between the aryl ring and sulfonamide (scaffold B) reduced potency for AAK1/BMP2K. Within the scaffold B group of molecules, incorporation of an alkyl chain capped with fluorines (**33**, **34**, **35**) increased the *in vitro* biochemical affinity for GAK, but not sufficiently to demonstrate GAK activity in cells (Table S3). In previous work, we had also observed that only low nanomolar

Table 3. NAK family binding-displacement assay selectivity analysis.

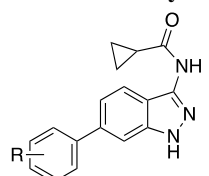
	Cmpd	R	AAK1 <i>K_i</i> (nM) ²³	BMP2K <i>K_i</i> (nM) ²³	GAK <i>K_i</i> (nM) ²³	STK16 <i>K_i</i> (nM) ²³
	14	3-NHSO ₂ (cyclopropyl)	8.3	13	1600	330
	23	3-NHSO ₂ N(CH ₃) ₂	10	19	2600	350
	24	3-NHSO ₂ CH ₂ (cyclopropyl)	6.3	17	870	91

Table 4. *In vitro* and cellular SAR of 3-acylaminoindazole probe candidates.

A **B**

Cmpd	Scaffold	R	AAK1 (nM)		BMP2K (nM)		GAK (nM)		STK16 (nM)		S ₁₀ (1 μM) ²⁶
			K _i ²³	NB IC ₅₀ ²⁴	K _i ²³	NB IC ₅₀ ²⁴	K _i ²³	NB IC ₅₀ ²⁵	K _i ²³	NB IC ₅₀ ²⁵	
14	A	cyclopropyl	8.3	641	13	1420	1600	>10000	330	>10000	0.005
25	A	N(CH ₂ CH ₃) ₂	9.1	770	17	2800	1700	>10000	270	>10000	0.02
26	A	N(CH ₂ CH ₃)(CH ₃)	8.2	375	20	1890	1700	>10000	310	>10000	0.017
27	A	CH ₂ CH(CH ₃) ₂	6.2	490	17	1490	1300	>10000	78	>10000	0.002
28	A	CH ₂ CH ₂ CH ₃	12	655	30	2180	1100	9250	110	>10000	0.01
29	A	4-F-benzyl	59	1020	120	2950	7000	9240	430	>10000	0.007
30	A	2-F-benzyl	22	528	51	1620	4600	>10000	470	>10000	0.022
31	A	3-F-benzyl	24	379	53	1190	4600	>10000	510	>10000	0.017
32	A	cyclobutyl	8.5	192	17	707	2900	>10000	240	>10000	0.007
33	B	CH ₂ CH ₂ CF ₃	32	2710	27	1890	510	>10000	180	>10000	0.022
34	B	N(CH ₂ CF ₃)(CH ₃)	44	3400	42	3030	780	>10000	290	>10000	
35	B	CH ₂ CF ₃	39	2350	33	1620	580	>10000	180	>10000	0.005
36	B	CH ₂ CH(CH ₃) ₂	30	1530	59	6550	3900	>10000	450	>10000	0.002
37	B	N(CH ₂ CH ₃) ₂	33	1470	74	3830	3200	>10000	220	>10000	
38	B	CH ₂ (cyclopropyl)	22	1260	37	2740	3200	>10000	150	>10000	0.01
39	B	N(CH ₂ CH ₃)(CH ₃)	20	1790	36	2960	360	>10000	86	>10000	

inhibitors of GAK possessed cellular activity.¹⁷ Nearly all were also devoid of activity in the STK16 NB assays. Several single-digit nanomolar biochemical inhibitors of AAK1 were prepared based upon scaffold A that also demonstrated target engagement with IC₅₀ values between 190–1020 nM. Incorporation of fluorinated aryl rings was tolerated by AAK1 and BMP2K but not by GAK or STK16 (**29**, **30**, **31**).

3-fluoroaryl analog (**31**) was one of the most potent compounds in the NB assays for AAK1 and BMP2K, while the 4-fluoro analog (**29**) was 3-fold less potent on both kinases. Compounds bearing small hydrocarbon chains and rings or alkyl amines on the sulfonamide proved to be the best dual AAK1/BMP2K inhibitors biochemically (**14**, **25**, **26**, **27**, **28**, **32**) and demonstrated at least 35-fold selectivity for BMP2K over GAK. The biochemical selectivity for BMP2K over STK16 was more variable (4–16-fold). When considering the cell-based activity, **14**, **26**, **27**, and **30–32** were the best performers in both the AAK1 and BMP2K NB, while **25** and **28** exhibited a slight drop in potency when transitioning from the biochemical to cell-based assays. Confirmation of cell penetrance and target engagement enables the facile translation of these compounds to *in vivo* studies. Furthermore, LipE was calculated for all compounds in Table 4 to estimate their quality as drug candidates (Figure S5).

All compounds from Table 4 built upon Scaffold A and a few representative compounds from Scaffold B (**25–33**, **35**, **36** and **38**) were evaluated for selectivity by KINOMEScan (DiscoverX) against 403 wildtype human protein kinases at 1 μM (Table 4).¹⁸ The compounds demonstrated remarkable selectivity for AAK1/BMP2K across the 403 human kinases. All proved to be narrow spectrum inhibitors of AAK1/BMP2K with >90% inhibition of only 1–9 kinases (S₁₀(1 μM) = 0.005–0.022, Figure S6).

To explore the phenotypic effect of AAK1/BMP2K inhibition, lead compound **14** and inactive analog **16** were tested in several cell lines. Surprisingly, we observed that **14** had cytostatic effects on cells after 24h of continuous treatment. Further

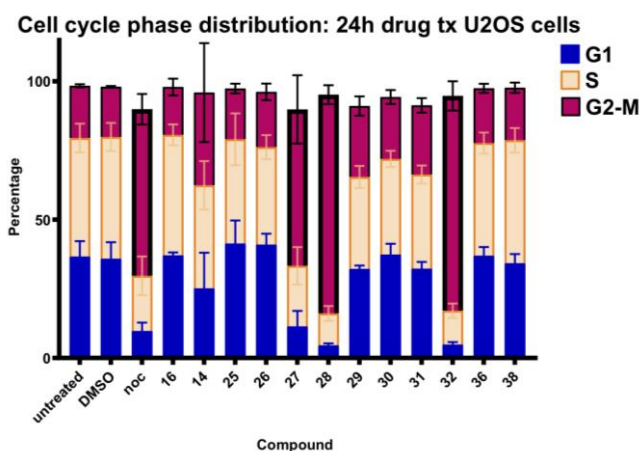


Figure 3. Cell cycle distributions. Cell cycle phases were determined by analytical flow cytometry of DNA content and DNA synthesis in U2OS cells after 24h treatment with 5 μM of the indicated compounds (n=3).

study in U2OS human osteosarcoma cells showed that **14** elicited cell cycle arrest in G2/M phase at 5–10 μM, which was not captured at the 5 μM dose (Figure S7). Although the molecular mechanism of the cell cycle arrest proved to be elusive, we employed U2OS cells as a screen to remove analogs that displayed this phenotype for consideration as chemical probes. A total of 12 analogs were screened for their effect on G2/M arrest (Figure 3). Nocodazole treatment was used as a positive control for a strong arrest in mitosis. Only analogs **27**, **28**, and **32** demonstrated the collateral G2 or M phase arrest at 5 μM, while the remaining analogs were devoid of this phenotype (Figures 3 and S8). Potent inhibition of AAK1/BMP2K by arrestors and non-arrestors alike indicated that G2 or M phase arrest was likely due to inhibition of an off-target kinase.

Compounds **25** and **26** emerged as probe candidates based on their potency on AAK1 and BMP2K, kinome-wide selectivity, lack of a G2/M arresting phenotype, and LipE values. Following KINOMEScan of compounds **25** (SGC-AAK1-1) and **26**, the binding affinities (K_D) of all kinases inhibited >80% at 1 μ M plus AAK1 and a few kinases inhibited by structurally related analogs were determined (Table S4). Only 3 kinases were bound by **25** within 30-fold of the potency of **25** for AAK1: RIOK1 ($K_D = 72$), RIOK3 ($K_D = 290$) and PIP5K1C ($K_D = 260$).¹⁹ Compound **26** bound 8 kinases within 30-fold of the potency of AAK1 (Table S4). Most compounds in Table 4 demonstrate potent affinity for RIOK1 and RIOK3 in the corresponding DiscoverX binding assays. Given that RIOK1 and RIOK3 are ATPases, an assay that has ATP present would be much more relevant but has not yet been developed.²⁰ In accordance with our previously described probe criteria,²¹ SGC-AAK1-1 (**25**) is a high quality dual AAK1/BMP2K chemical probe. Compound **16** (SGC-AAK1-1N) is the complementary negative control analog. The probe set has been made commercially available, making SGC-AAK1-1 the only AAK1-targeting chemical probe that is available alongside its negative control.

We have described the design, synthesis and biological evaluation of a series of acylaminoindazoles as selective dual inhibitors of AAK1/BMP2K. Compound **25** emerged as our best probe candidate. X-ray co-crystal structures of selected acylaminoindazoles bound to BMP2K revealed key interactions within the ATP binding pocket that explain the high affinity of this series for AAK1 and BMP2K. The effect of **25** on WNT signaling was recently reported.¹⁹ Further investigations into effects of selective pharmacological inhibition of AAK1 are ongoing.

ASSOCIATED CONTENT

Supporting Information

Supplemental material is available free of charge at <http://pubs.acs.org>.

Protein crystallization conditions, NB and cell cycle assay details, experimental methods, synthesis and characterization of compounds **25**, **26**, **27**, and **28**.

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Notes

The authors declare no competing financial interest.

Author Contributions

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

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ABBREVIATIONS

AAK1, AP2-associated protein kinase 1; BMP2K/BIKE, BMP2-inducible kinase; STK16, serine/threonine kinase 16; GAK, cyclin-G-associated kinase; NAK, numb-associated kinase; AP2, adaptor protein 2; TR-FRET, time-resolved fluorescence resonance energy transfer; PKIS, published kinase inhibitor set; DSF, differential scanning fluorimetry; SAR, structure-activity relationship; HEK, human embryonic kidney; NB, NanoBRET; U2OS, U2 osteosarcoma; KD, kinase domain; NLuc, Nanoluciferase; tx, treatment; LipE, lipophilic efficiency.

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22. Each test compound was screened in AAK1 split luciferase assay in duplicate (n=2) against AAK1 in dose-response (8-pt curve).
23. Each test compound was screened in duplicate (n=2) in TR-FRET binding-displacement assay in dose-response (16-pt curve). To allow comparison between difference kinases, IC₅₀ values (Table S2) were converted to K_i values using the Cheng-Prusoff equation and the concentration and K_D values for the tracer.
24. AAK1 and BMP2K NB cellular assays were performed in triplicate (n=3) to provide IC₅₀ values (11-pt curve).
25. STK16 and GAK NB cellular assays performed in singlicate (n=1) based on demonstration of poor activity.
26. Calculation of S₁₀ discussed in Figure S6.
27. PDB codes: compound **23**, 5I3O; compound **24**, 5I3R.