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SGC-GAK-1: a chemical probe for cyclin G associated kinase (GAK)

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Abstract

We describe SGC-GAK-1 (**11**), a potent, selective, and cell-active inhibitor of cyclin G-associated kinase (GAK), together with a structurally-related negative control SGC-GAK-1N (**14**). **11** was

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Author Contributions

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Supporting Information

Compound synthesis, assay protocols, tabulated assay output, dose-response curves, and supportin figures and tables (PDF)

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highly selective in an in vitro kinome-wide screen, but cellular engagement assays defined RIPK2 as a collateral target. We identified **18** as a potent RIPK2 inhibitor lacking GAK activity. Together, this chemical probe set can be used to interrogate GAK cellular biology.

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INTRODUCTION

Cyclin G-associated kinase (GAK) is a 160 kDa serine/threonine kinase originally identified as a direct association partner of cyclin G.¹ GAK is a member of the numb-associated kinase (NAK) family, which includes AAK1 (adaptor protein 2-associated kinase), STK16/MPSK1 (serine/threonine kinase 16/myristoylated and palmitoylated serine/threonine kinase 1), and BMP2K/BIKE (BMP-2 inducible kinase).² In addition to its kinase domain, the C-terminus of GAK protein has high homology with auxilin and tensin.³ GAK is ubiquitously expressed and within the cell localizes to the Golgi complex, cytoplasm, and nucleus.⁴

Genetic studies have implicated GAK in several diverse biological processes. Genome-wide association studies have identified single nucleotide polymorphisms in *gak* associated with susceptibility to Parkinson's disease.⁵ GAK, like other members of the NAK family, is involved in membrane trafficking and sorting of proteins and as an essential cofactor for HSC70-dependent uncoating of clathrin-coated vesicles in the cytoplasm.^{6, 7} GAK is also required for maintenance of centrosome maturation and progression through mitosis.⁸ GAK is over-expressed in osteosarcoma cell lines and tissues where it contributes to proliferation and survival.⁹ Notably, GAK expression increases during prostate cancer progression to androgen independence and is positively correlated with Gleason score in resections from prostate cancer patients.^{10, 11}

We have a program to develop small molecule chemical probes to elucidate the biological role of kinases such as GAK that belong the lesser-studied portion of the kinome.¹² To be useful research reagents, these chemical probes must be potent, selective, and cell-active.¹³ Specifically the aspirational criteria we follow are (i) in vitro biochemical $IC_{50} < 50$ nM; (ii)

30-fold selective relative to other kinases in a large assay panel such as DiscoverX KINOMEScan; and (iii) cellular activity or target engagement $IC_{50} < 1 \mu M$. In addition, the probe should be accompanied by a structurally related negative control that is at least several orders of magnitude less potent at the primary kinase target.¹³

A number of quinazoline-based kinase inhibitors show cross-reactivity with GAK, including the approved drugs gefitinib and erlotinib (Figure 1).¹⁴ These drugs were designed as

inhibitors of either epidermal growth factor receptor (EGFR) or SRC kinase and show similar or higher affinity for several other kinases, making them ineffective tools for studying GAK biology. Thus, it is not known whether the efficacy or adverse events observed with these kinase inhibitor drugs are associated with their cross-activity on GAK. Notably, it has been proposed that GAK inhibition causes toxicity due to pulmonary alveolar dysfunction. This hypothesis is based in part on the phenotype observed in transgenic mice expressing catalytically inactive GAK but has not yet been tested with a selective small molecule GAK inhibitor.¹⁵

Isothiazolo[5,4-b]pyridine **1** has been described as a selective inhibitor of GAK but shows cross-reactivity with other kinases, including KIT, PDGFRB, FLT3, and MEK5,¹⁶ any of which could lead to confounding biology in cells. The availability of a GAK chemical probe with improved selectivity would be a valuable tool to probe GAK biology. We previously described 4-anilinoquinoline inhibitors of GAK, exemplified by **2**.¹⁷ The optimization of this series to a GAK chemical probe is described.

RESULTS

Quinoline **2** was profiled at 1 μ M across a panel of over 400 wild-type human kinases using heterogeneous assays employing displacement of kinase protein from an immobilized inhibitor. Subsequent KD determination for those kinases with > 50% binding at 1 μ M identified three kinases (receptor-interacting protein kinase 2, RIPK2; AarF domain containing kinase 3, ADCK3; and nemo-like kinase, NLK) with K_D values within 30-fold of that of GAK (Table S1). While little is known about the activity of quinazolines on ADCK3 and NLK, a prior study of quinazoline inhibitors of RIPK2 showed H-bonding between Ser25 of RIPK2 near the solvent-exposed portion of the ATP-binding pocket.¹⁸ Since GAK has Ala47 as the corresponding residue, we hypothesized that small, nonpolar substituents at the quinoline 6- and 7-positions might show improved selectivity over RIPK2.

To investigate this hypothesis and define the SAR of ADCK3 and NLK, we profiled several quinolines prepared in our previous study against the three collateral targets (Table 1).¹⁷ 6,7-Dimethoxyquinoline **3** had the highest GAK affinity but also showed increased binding affinity to RIPK2 relative to **2**. Replacement of either methoxy group with a hydrogen, as in **4** and **5**, led to reduced affinity on all four kinases. Notably, the unsubstituted quinoline **6** showed an improved selectivity profile, although it had the poorest affinity for GAK among the analogs. The nitrile isomers **7** and **8** both retained single digit nanomolar affinity for GAK yet had different effects on selectivity profile: the 6-isomer **7** had an improved selectivity profile while the 7-isomer **8** showed binding to all three off-targets within ten-fold. A tert-butyl group in the 6-position (**9**) led to improvement of the selectivity profile against RIPK2 and ADCK3 with a decrease in selectivity over NLK compared with **2**.

Comparison of the two matched molecular pairs with methoxy and nitrile (**4** and **8**; **5** and **7** respectively) suggested that electron withdrawing substituents at the quinoline 6-position showed improved selectivity and warranted further investigation. Quinolines **10-12** were prepared by nucleophilic substitution of 4-chloroquinolines with 3,4,5-trimethoxyaniline.¹⁷ The halogen-substituted analogs **10-12** showed high GAK affinity, all with $K_D < 10$ nM

(Table 2). **11** also displayed remarkable selectivity, with the closest off-target kinase (RIPK2) having > 50-fold weaker affinity.

To confirm the selectivity profiles across the NAK subfamily, the affinity of **11** was evaluated in homogeneous time-resolved fluorescence resonance energy transfer (TR-FRET)-based ligand binding displacement assays (Figure 2A). **11** displayed an impressive profile of over 16000-fold selectivity relative to the other three NAK family members. In addition, a differential scanning fluorimetry (DSF) assay¹⁹ and isothermal titration calorimetry (ITC) were employed to confirm direct interaction with GAK (Figure 2B and 2C). **11** increased the thermal stability of the isolated GAK kinase domain, suggesting strong binding of the inhibitor. ITC revealed potent affinity for GAK ($K_D = 4.5$ nM). We assessed the kinome selectivity of **11** in the KINOMEScan assay panel (Figure 2D and Table S2). K_D values were measured for kinases with binding greater than 50% at 1 μ M (Table S3). **11** showed > 50-fold selectivity relative to all 400 kinases. The closest off-targets were RIPK2, ADCK3, NLK and ACVR1.

An established best practice for the use of chemical probes in biological assays is to test in parallel a compound closely related in structure to the probe but inactive for the target protein.¹³ Demonstration of divergent results between the chemical probe and its negative control increases confidence that the engagement of the target protein is responsible for the observed biology. We designed and prepared two potential negative controls that were anticipated to lack GAK activity. First, **13** incorporated a 2-methylquinoline that would directly hinder hydrogen bonding to the hinge region of the kinase. Second, the *N*-methyl **14** was expected to disrupt a water network necessary for favorable compound binding (Asquith, C. R. M. and Laitinen, T., unpublished results). Neither compound induced thermal stabilization of GAK protein, and both **13** and **14** demonstrated >10,000-fold weaker GAK binding affinity in the TR-FRET assay (Table 3). KINOMEScan and subsequent K_D determinations of **13** and **14** identified only a single kinase with < 1 μ M affinity (14: interleukin 1 receptor associated kinase 3, IRAK3 $K_D = 740$ nM) (Tables S2 and S4). Both compounds were suitable as GAK negative controls, and we selected **14** based on (a) its higher selectivity in the NAK family ligand binding displacement assay and (b) the structural modification leading to loss of GAK activity was not expected to directly interfere with kinase hinge binding.

Having confirmed potent GAK binding and selectivity relative to other NAK family members, we next sought to demonstrate that activity and selectivity of **11** in live cells using a NanoBRET target engagement assay.²⁰ **11** and **14** were evaluated for their ability to displace a fluorescent tracer molecule from the ATP binding site of a transiently expressed full length GAK protein *N*-terminally fused with NanoLuc luciferase (Nluc) in HEK293T cells. In the absence of compound, the tracer molecule and fusion protein were in proximity and able to generate an observable bioluminescence resonance energy transfer (BRET) signal. Increasing concentrations of compound displaced the tracer, causing a loss of BRET signal. **11** had high affinity for GAK in cells ($IC_{50} = 120$ nM), although the measured IC_{50} was 63-fold higher than the in vitro K_D value. We interpret the difference as most likely due to competition with ATP in cells. The negative control **14** showed no cellular binding to GAK up to 5 μ M.

Notably, the previously reported GAK inhibitor **1** had micromolar GAK cellular potency, approximately ten-fold weaker than **11**. We also evaluated **11** and **14** in a RIPK2 NanoBRET assay as this kinase was the closest off-target. We were surprised that **11** had only three-fold selectivity for GAK over RIPK2 in cells (Figure 3, GAK IC₅₀ = 120 ± 50 nM; RIPK2 IC₅₀ = 360 ± 190 nM). The negative control **14** and the previously described GAK inhibitor **1** showed no affinity for RIPK2 up to 10 μM.

Given the observed cross-activity of **11** with RIPK2 in cells, we sought to identify an additional control with RIPK2 but not GAK activity. Surveying known RIPK2 inhibitors (Table 4 and Figure S1), we first evaluated OD36 (**15**), since it had previously been characterized as selective for RIPK2 across a panel of over 400 wild type human kinase assays (although this panel did not include GAK).²¹ We also expected the distinct macrocyclic chemotype of **15** might have a lower propensity for binding GAK relative to a quinoline and were surprised to observe that **15** was nearly equipotent with RIPK2 and GAK in cells. Affinity of **15** for GAK was confirmed in an in vitro binding assay. Next, we selected three quinazoline-based RIPK2 inhibitors (**16–18**) reported by GlaxoSmithKline.^{18,22} **16** and **17** displayed high GAK affinity and were not subsequently evaluated for cellular target engagement. **18** had poor affinity for GAK, both biochemically and in cells (Figure 3).²³ **18** demonstrated high RIPK2 cellular affinity and was selected as RIPK2 control.

We propose that SGC-GAK-1 (**11**) and the controls SGC-GAK-1N (**14**) and **18** can be used in parallel as a probe set to study GAK biology in cells. As an illustrative example, we used the probe set to explore the potential role of GAK in prostate cancer.¹¹ The probe set was tested for effects on the viability of five well-characterized prostate cancer cell lines (PC3, DU145, LNCaP, VCaP, and 22Rv1 cells). Western blotting experiments confirmed that GAK was expressed at similar levels in these cell lines (Figure S2). In an initial experiment, the cell lines were incubated with **11**, **14**, and **18** at three different concentrations (0.1, 1, and 10 μM), and viability was measured at 48 h or 72 h. No effect was observed with the controls **14** or **18** in any of the cell lines at these concentrations. However, SGC-GAK-1 (**11**) showed strong growth inhibition in LNCaP, VCaP, and 22Rv1 cells at 10 μM, but minimal effect in PC3 and DU145 cells. In dose response **11** showed potent antiproliferative activity in LNCaP and 22Rv1 cells at a similar dose range that it engages GAK (Table 5). However, variable results were observed in the slow growing VCaP cells, preventing the determination of an accurate IC₅₀ value. Overall, these results demonstrate robust effects of **11** in a subset of prostate cancer cell lines.

Previous reports have demonstrated that GAK is required for proper mitotic progression.²⁴ Specifically, siRNA knockdown of GAK in HeLa cells induced cell cycle arrest at the metaphase and increase levels of phosphorylated histone H3 Ser10.²⁴ Prolonged metaphase arrest is known to lead to activation of apoptotic pathways.²⁵ We observed that treatment of 22Rv1 cells with **11** led to PARP cleavage, a marker of cells undergoing apoptosis (Figure S3), and an increase in phosphorylated histone H3 Ser10, as had been observed in with GAK targeting siRNA.²⁴ Thus, our probe set demonstrated that pharmacological inhibition of the GAK kinase domain can phenocopy the effect on cell growth shown by depletion of GAK protein.

DISCUSSION

We describe the identification of a potent, selective, and cell-active GAK probe, SGC-GAK-1 (**11**), its negative control SGC-GAK-1N (**14**), and the RIPK2 control **18**. **11** was characterized for selectivity across a broad panel of kinase assays. The use of controls **14** and **18** can further increase confidence in GAK inhibition as the mediator of phenotypes induced by **11** in cells. SGC-GAK-1 (**11**) has several advantages over the previously reported isothiazolo[4,3-b]pyridine GAK inhibitor **1**, including superior cellular potency and improved kinase selectivity. The GAK probe set (**11**, **14**, **18**) has been made openly available (see <https://www.thesgc.org/chemical-probes/SGC-GAK-1>). We expect it will find use in elucidating GAK biology.

The identification of RIPK2 as a target of **11** is surprising as GAK and RIPK2 belong to distal branches of the kinome and have divergent ATP-binding pockets—of the 28 residues lining the pockets, only 14 are identical or similar. The propensity of **11** to bind both kinases results from higher order interactions not presently understood.

The narrowed GAK/RIPK2 selectivity window in cells highlights the importance of profiling in relevant assay systems. **11** demonstrated a > 50-fold difference in affinity between GAK and RIPK2 in an in vitro binding assay, whereas it was within 4-fold affinity on the two kinases in live cell target engagement assays involving full length protein, physiological levels of ATP, and endogenous protein partners.²⁰ We identified the quinazoline **18** as a RIPK2 inhibitor that lacks affinity for GAK as a means to control for this off-target in cells. There remains a finite possibility that cellular screening would reveal additional potent kinase targets of **11**, but the KINOMEScan data suggest this is highly unlikely. Protein kinase inhibitors such as **11** also have the potential to interact with non-kinase targets.²⁶ The use of structurally related **14** and **18** is intended to control for this possibility. For these reasons **11**, **14**, and **18** should be used in parallel to support robust interpretation of the biology of GAK inhibition.

The observation that GAK expression is associated with prostate cancer progression prompted us to explore the effect of GAK inhibition across a range of cell lines. To date studies of the role of GAK in prostate cancer have utilized knock-down of the whole protein.²⁷ We now show that inhibition of the GAK kinase domain can block the growth of prostate cancer cells. Our results demonstrate that the kinase and not the auxilin-homology domain is critical for this phenotype in cells. SGC-GAK-1 (**11**), but not the controls **14** and **18**, robustly blocked growth of 22Rv1 and LNCaP cell lines. DU145 or PC3 cell lines were less responsive. Since neither DU145 nor PC3 express the AR, their proliferation is not expected to be AR-dependent. In contrast LNCaP and 22Rv1 are AR positive and are cell models for androgen-dependent growth. Interestingly, the most potently inhibited cell line (22Rv1) expresses the AR-V7 and other splice variants often increased in patients who become resistant to androgen deprivation.²⁸ Why the AR-V7 expressing lines are more sensitive and what are the mechanisms by which GAK inhibitors target a subset of AR expressing prostate cancer cells are the subjects of ongoing investigation.

In summary, we have generated the first chemical probe set for the poorly studied kinase GAK. SGC-GAK-1 (**11**), when used in parallel with **14** and **18**, can be used to study the cell biology of GAK. We demonstrate that the GAK kinase domain is a potential target for inhibition of prostate cancer growth in cells that express AR splice variants associated with poor clinical prognosis.

EXPERIMENTAL

2-14 were synthesized as previously reported, and **2-9** were consistent with previous reports.¹⁷ General procedures and characterization of **10**, **12**, and **13** are included in the SI. All compounds were > 98 % pure by ¹H/¹³C NMR and LCMS.

General procedure for the synthesis of 4-anilinoquinolines: 6-Bromo-4-chloroquinoline (1.0 eq.), 3,4,5-trimethoxyaniline (1.1 eq.), and i-Pr₂NEt (2.5 eq.) were suspended in ethanol (30 mL) and refluxed for 18 h. The crude mixture was purified by flash chromatography using EtOAc:hexane followed by 1–5 % methanol in EtOAc. After solvent removal under reduced pressure, the product was obtained.

6-Bromo-*N*-(3,4,5-trimethoxyphenyl)quinolin-4-amine (**11**) was obtained as a mustard solid (310 mg, 0.79 mmol, 64%) MP 178–180 °C; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.06 (s, 1H), 9.19 (d, *J* = 1.7 Hz, 1H), 8.48 (d, *J* = 6.9 Hz, 1H), 8.32 – 7.82 (m, 2H), 6.92 (d, *J* = 6.9 Hz, 1H), 6.82 (s, 2H), 3.80 (s, 6H), 3.72 (s, 3H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 154.0, 153.6 (s, 2C), 143.0, 137.6, 136.5, 136.3, 132.8, 126.2, 122.7, 119.7, 118.5, 103.0 (s, 2C), 100.9, 60.2, 56.1 (s, 2C). HRMS-ESI (*m/z*): [M+H]⁺ calcd for C₁₈H₁₈N₂O₃Br: 389.0500, found 389.0490; LC - T_r = 3.60 min, purity > 98 %.

2-Methyl-6-(trifluoromethyl)-*N*-(3,4,5-trimethoxyphenyl)-quinolin-4-amine (**14**) was obtained as a yellow solid (330 mg, 0.84 mmol, 69%) MP 240 °C decomp.; ¹H NMR (400 MHz, DMSO-*d*₆) δ 10.87 (s, 1H), 9.20 (s, 1H), 8.23 (s, 2H), 6.87 (s, 1H), 6.79 (s, 2H), 3.81 (s, 6H), 3.73 (s, 3H), 2.65 (s, 3H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 157.3, 154.7, 154.1 (s, 2C), 136.9, 133.2, 129.2–129.1 (m, 1C), 126.7, 126.7, 126.4, 125.7, 123.0, 122.4 (d, *J* = 3.8 Hz), 116.2, 103.4, 102.0, 60.6, 56.6 (s, 2C), 20.9. HRMS-ESI (*m/z*): [M+H]⁺ calcd for C₂₀H₁₉F₃N₂O₃: 393.1426, found 393.1413; LC - T_r = 4.02 min, purity > 98 %.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED

DSF	differential scanning fluorimetry
GAK	cyclin G associated kinase
ITC	isothermal titration calorimetry

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Gefitinib, GAK K_D = 13 nM

Erlotinib, GAK K_D = 3.1 nM

1, GAK K_D = 8.9 nM

2, GAK K_D = 31 nM

Figure 1.
Previously described inhibitors of GAK.

Figure 2.

(A) Binding affinity of **11** for NAK family kinases. ^aTemperature shift of GAK denaturation ($n = 4$); ^bTR-FRET-based ligand binding displacement assay ($n = 1$). (B) DSF curve of GAK and **11**; (C) ITC of GAK and **11**; (D) KINOMEScan results.

Figure 3.
Cellular target engagement assays. a. GAK, b. RIPK2. Cntrl: DMSO + tracer. Background: DMSO, no tracer.

Table 1.

Kinase affinity of 4-anilinoquinolines.

Ar =
	R ¹	R ²	GAK ^a	RIPK2 ^a	ADCK3 ^a	NLK ^a
2	CF ₃	H	31	180	220	790
3	OMe	OMe	2.9	42	250	410
4	H	OMe	17	200	1200	700
5	OMe	H	21	170	2000	2600
6	H	H	59	2900	4100	6200
7	CN	H	3.9	88	170	29
8	H	CN	9.7	96	66	28
9	t-Bu	H	25	670	740	350

^a K_D (nM) in competition binding assay (DiscoverX, n = 2).

Table 2.

Kinase binding of halogenated quiniolines.

Ar =
	R	GAK ^a	RIPK2 ^a	ADCK3 ^a	NLK ^a
10	Cl	6.7	85	170	650
11	Br	1.9	110	190	520
12	I	7.9	69	190	660

^a *K*_D (nM), competition binding assay (DiscoverX, n = 2).

Table 3.

NAK family binding of 13 and 14.

13		14		Ar =
	GAK ^a	AAK1 ^b	BMP2K ^b	GAK ^b	STK16 ^b
13	−0.52	>100	>100	19	>100
14	−0.30	78	95	2.5	15

^a T_m (°C) measured by DSF;^b K_i (μM) in TR-FRET-based ligand binding displacement assay (n = 1).

Table 4.

Evaluation of potential RIPK2 controls.

Compound	GAK ^a	GAK ^b	RIPK2 ^b
15 (OD36)	0.0038	0.060	0.011
16 (GSK583)	0.22	nt	nt
17	0.049	nt	nt
18	8.2	> 10	0.0020

^aTR-FRET ligand binding displacement assay K_i (μ M) (n = 2);^bNanoBRET assay IC₅₀ (nM) (n = 2, nt = not tested).

Table 5.

Prostate cancer cell line viability at 72 h.

Compound	LNCaPa	22Rv1a
11	0.65 ± 0.15	0.17 ± 0.05
14	> 100	>100
18	99 ± 26	> 100

^aIC₅₀ ± SEM (μM), (n = 3).