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Selective Inhibitors of Cyclin G Associated Kinase (GAK) as Anti-Hepatitis C Agents

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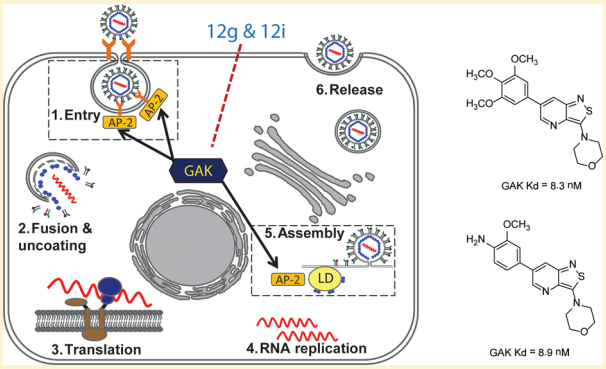
Supporting Information

ABSTRACT: Cyclin G associated kinase (GAK) emerged as a promising drug target for the treatment of viral infections. However, no potent and selective GAK inhibitors have been reported in the literature to date. This paper describes the discovery of isothiazolo[5,4-*b*]pyridines as selective GAK inhibitors, with the most potent congeners displaying low nanomolar binding affinity for GAK. Cocrystallization experiments revealed that these compounds behaved as classic type I ATP-competitive kinase inhibitors. In addition, we have demonstrated that these compounds exhibit a potent activity against hepatitis C virus (HCV) by inhibiting two temporally distinct steps in the HCV life cycle (i.e., viral entry and assembly). Hence, these GAK inhibitors represent chemical probes to study GAK function in different disease areas where GAK has been implicated (including viral infection, cancer, and Parkinson's disease).



INTRODUCTION

Cyclin G associated kinase (GAK) was first identified in experiments investigating proteins associated with cyclin G, a protein involved in cell cycle regulation.[1] GAK (also known as auxillin 2) is a 160 kDa serine/threonine protein kinase that belongs to the numb-associated kinase (NAK) family, which also includes STK16/MPSK1 (serine/threonine kinase 16/myristoylated and palmitoylated serine/threonine kinase 1), AAK1 (adaptor-associated kinase), and BIKE (BMP-2 inducible kinase).[2]

GAK is expressed ubiquitously and bears a strong homology (43%) to the neuronal-specific protein auxilin, a heat shock cognate 70 (Hsc70) cochaperone with a role in uncoating clathrin vesicles. GAK is a key regulator of clathrin-mediated trafficking in both the endocytic and secretory pathways. It recruits clathrin and clathrin adaptor protein complex 2 (AP-2) to the plasma membrane[3] and phosphorylates a T156 residue within AP2M1, the μ subunit of AP-2, thereby stimulating its binding to cargo proteins and enhancing cargo recruitment, vesicle assembly, and efficient internalization.[3−6] Moreover, GAK regulates endocytosis of receptors mediated by alternate clathrin adaptors[3] and is implicated in later steps of endocytosis, including regulation of clathrin-coated vesicles (CCVs) uncoating, which enables recycling of clathrin back to the cell surface.[3,5] GAK is an important regulator of epidermal growth factor receptor (EGFR); it is known to promote EGF uptake[3] and may also function in receptor signaling.[7] Last, GAK also plays an important role in regulating clathrin-mediated sorting events in the trans-Golgi network.[3,5]

Interestingly, GAK-dependent phosphorylation of clathrin adaptor proteins has been implicated in the regulation of viruses. AP2M1 was shown to be recruited to the surface of lipid droplets by the HCV capsid protein, core.[8] The interaction between HCV core and AP2M1 was shown to be critical for HCV assembly.[8] Notably, either overexpression of an AP2M1 phosphorylation-site mutant or suppression of GAK expression disrupted core-AP2M1 binding and HCV assembly.[8] More recently, GAK was shown to regulate HCV entry independently of its effect on HCV assembly, in part by activating AP2M1.[11] Hence, GAK represents

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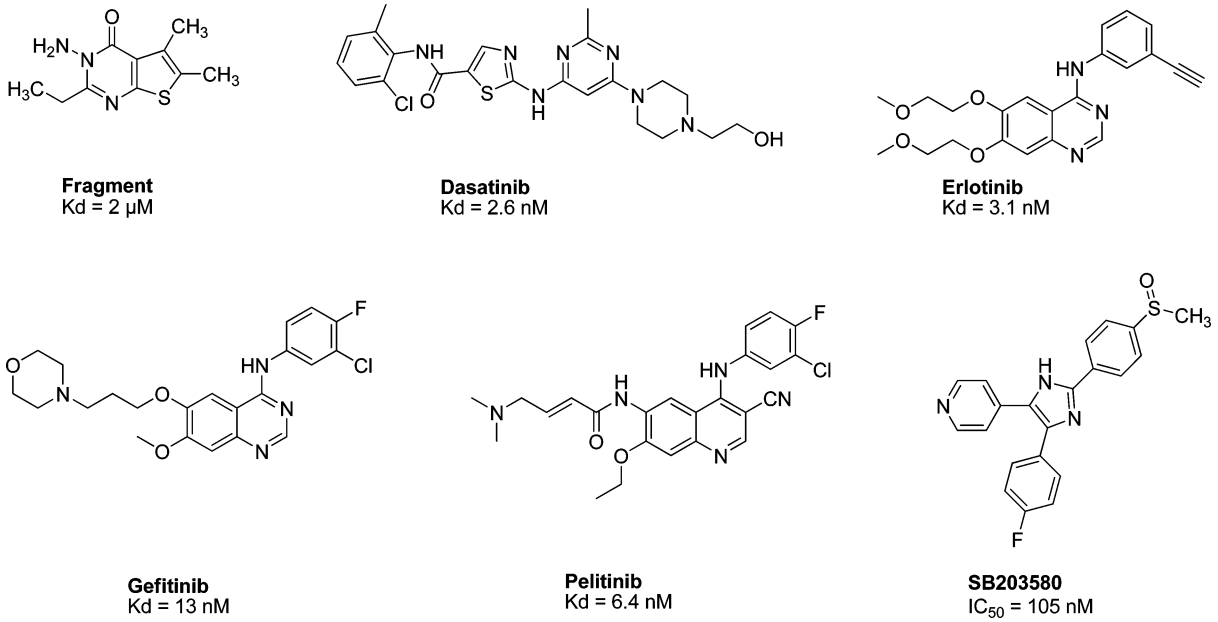
Chart 1. Known GAK Inhibitors



a cellular host factor essential for regulation of HCV entry and assembly and a potential target for antiviral strategies. Indeed, erlotinib, an approved anticancer drug that potently inhibits GAK (in addition to its known cancer target, EGFR[9,10]), inhibits HCV entry as well as core-AP2M1 binding, thereby also disrupting HCV assembly but not HCV RNA replication.[8,11]

To the best of our knowledge, no potent and selective GAK inhibitors have been reported in the literature to date. Like erlotinib, other approved kinase inhibitors, such as dasatinib, gefitinib, and pelitinib, display a high affinity for GAK with K_d values in the low nanomolar range (Chart 1).[12] Similarly, pyridinylimidazoles, such as SB203580 and SB201290 that have been developed as p38 inhibitors, potently inhibit GAK.[13] Nevertheless, since all these compounds were designed to target other kinases, their inhibitory effect on GAK represents an off-target effect, and their use is limited by significant toxicities resulting from lack of selectivity. Moreover, while several compounds that bind GAK with an excellent ligand efficiency (LE) of 0.51 kcal/mol (Figure 1) were discovered by a fragment-based screening using weak affinity chromatography, their binding affinity was low (K_d value of 2 μM).[14]

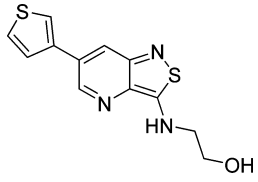


Figure 1. Hit compound.

Because of the potential for GAK to serve as an antiviral drug target and the lack of selective small-molecule GAK inhibitors, we embarked on the synthesis and biological evaluation of a novel series of GAK inhibitors. In addition to their potential as lead molecules for the development of a novel antiviral strategy, these compounds represent useful chemical probes to further investigate the function of GAK in aspects of general cell biology and other disease conditions, such as cancer[15] and Parkinson's disease,[16] where GAK plays an important role.

SCREENING: HIT DISCOVERY

In order to discover novel GAK inhibitors, a druglike compound library of 150 analogues was screened to identify potential ligands of GAK. This compound collection was made as part of a program to synthesize novel compound libraries based on original and patentable chemistry, which has not been explored before in drug discovery and hence represent unexplored chemical space. This library consists mainly of compounds based on a bicyclic, heteroaromatic flat scaffold. The different scaffolds are unrelated to each other, and in addition, they differ in their substitution pattern (the nature, as well as the spatial orientation of the substituents). These flat core structures are typical for kinase inhibitors, since they function as a central scaffold that binds to the ATP-binding site of the enzyme and is therefore predicted to yield a high hit rate in kinase assays. We opted for the KINOMEscan screening platform that employs an active site-directed competition binding assay to quantitatively measure interactions between a test compound and a given kinase.[12] Compounds that bind the kinase active site and either directly (sterically) or indirectly (allosterically) prevent kinase binding to the immobilized ligand reduce the amount of kinase captured on the solid support. Conversely, test molecules that do not bind the kinase have no effect on the amount of kinase captured on the solid support. Hits are identified by measuring the amount of kinase captured in test versus control samples using a quantitative, precise, and sensitive qPCR method that detects the associated DNA label. In the first round of screening, the compound library was tested at a single concentration of 10 μM. The results are reported as the percentage of kinase/phage remaining bound to the ligands/beads, relative to a control. High affinity compounds have % of control values close to zero, while weaker binders have higher % control values. For the most promising compounds, dose−response curves were generated to determine binding constant (K_d) values. This initial screening led to the discovery of a promising hit (Figure 1), with a good potency (%Ctrl = 0.3 ; K_d = 0.3 μM), moderate lipophilicity

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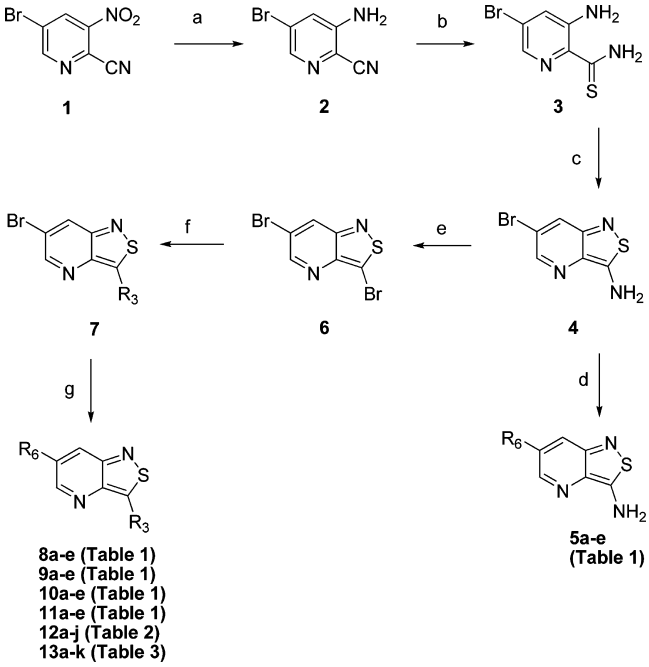
(cLogP = 2.44), and a favorable ligand efficiency (LE) of 0.494 kcal/mol. Overall, these parameters qualified this hit compound as an optimal starting point for the discovery of novel GAK inhibitors. Notably, this hit compound is based on an isothiazolo[4,3-*b*]pyridine scaffold, a skeleton that is unexplored in organic chemistry. This is in contrast to the bicyclic heteroaromatics (such as purines, quinazolines, benzimidazoles, and indoles), which are considered "privileged structures" in kinase drug discovery and are widely described in scientific and patent literature, making novelty difficult to achieve. Indeed, SciFinder searches revealed only 19 known isothiazolo[4,3-*b*]pyridine derivatives,[17] and hence, further elaboration of this scaffold gives access to a largely unexplored chemical space.

CHEMISTRY

The general approach for the synthesis of the isothiazolo[4,3-*b*]pyridine derivatives is shown in Scheme 1. The synthesis started from 3-nitro-5-bromopyridine-2-carbonitrile **1**. Reduction of the nitro group to the corresponding amino group was achieved by treatment with iron under acidic conditions.[18] The major compound was the desired compound **2**; however, it was always accompanied by formation of the corresponding carboxamide, resulting from acidic hydrolysis of the cyano group. This mixture was used as such in the subsequent reaction. The aromatic thioamide **3** was obtained by treatment of the mixture with phosphorus pentasulfide as thionation reagent.[19] An oxidative ring closure using hydrogen peroxide yielded the 3-amino-6-bromoisothiazolo[4,3-*b*]pyridine **4**.[20] Subsequent palladium-catalyzed Suzuki coupling[21] with appropriate arylboronic acids afforded a series of 3-amino-6-aryl-isothiazolo[4,3-*b*]-pyridines **5a**–**e**. Alternatively, diazotation of the exocyclic amino group with sodium nitrite, hydrogen bromide, and CuBr furnished the 3,6-dibromoisothiazolo[4,3-*b*]pyridine **6**.[20] Treatment of **6** with sodium methoxide or nitrogen-containing nucleophiles yielded a series of 3-substituted-6-bromo-isothiazolo[4,3-*b*]pyridine analogues **7**. Finally, reaction of **7** with a number of arylboronic acids then yielded target compounds **8**–**13**. These Suzuki reactions were conducted in a mixture of dioxane/water, using sodium carbonate as base and $Pd(dppf)Cl_2$ as a catalyst, or alternatively in a mixture of dimethoxyethane/water with potassium carbonate as a base and $Pd(PPh_3)_4$ as a catalyst.

Scheme 1. Synthesis of Isothiazolo[4,3-*b*]pyridine Analogues[a]



[a]Reagents and conditions: (a) Fe, CH_3COOH, 0 °C to rt; (b) P_2S_5, EtOH, 75 °C; (c) 30% aq H_2O_2, CH_3OH, rt; (d) $R_6B(OH)_2$, Na_2CO_3, $Pd(dppf)Cl_2$, dioxane/water, 100 °C; (e) CuBr, HBr, $NaNO_2$,H_2O, 0 °C to rt; (f) R_3H, EtOH, 75 °C; (g) $R_6B(OH)_2$, Na_2CO_3, $Pd(dppf)Cl_2$, dioxane/water, 100 °C or $R_6B(OH)_2$, K_2CO_3, $Pd(PPh_3)_4$, H_2O, DME.

STRUCTURE–ACTIVITY RELATIONSHIP STUDIES

The isothiazolo[4,3-*b*]pyridine scaffold offers two sites for structural variation. Therefore, the structure–activity relationship (SAR) study started with the synthesis of a small library matrix of isothiazolo[4,3-*b*]pyridines with structural variation at positions 3 (amino, methoxy, ethanolamine, morpholine, and *N*-Me-piperazine) and 6 (substituted aryl and heteroaryl groups). Similar to our primary screening strategy, all compounds were tested at a 10 μM concentration and the most promising ones were then subjected to determination of K_d values. The matrix depicted in Table 1 demonstrates that compounds **9c** and **9d**, resulting from the combination of a 3,4-dimethoxyphenyl or a 3-thienyl substituent at position 6 with an ethanolamino or a morpholino moiety at position 3, have a strong affinity for GAK (K_d values of 52 and 42 nM, respectively).

A dual strategy was then used for further optimization. To probe the optimal substitution pattern at position 6, the morpholino substituent at position 3 was fixed, and a wide range of substituted aryl or heteroaryl groups was evaluated. As it became clear that a 3,4-dimethoxyphenyl or 3-thienyl substituents were optimal for GAK binding, the SAR focused on closely related derivatives of both of these aryl groups (Table 2).

Replacing the 3-thienyl group of compound **9d** by a 2-thienyl group (compound **12a**) led to equipotent compounds. On the other hand, the presence of a 2-furanyl moiety led to a 5-fold decrease in GAK affinity, yielding compound **12b** with a K_d value of 0.2 μM. The SAR of the dimethoxyphenyl ring was investigated by the synthesis of a number of regioisomeric dimethoxyphenyl derivatives. Compared to the original 3,4-dimethoxyphenyl analogue **9c** (K_d = 0.052 μM), the 2,4-dimethoxyphenyl analogue (compound **12c**) lacks any affinity for the GAK enzyme, the 2,5-dimethoxyphenyl derivative **12d** shows intermediate affinity (K_d = 0.13 μM), whereas the 3,5-dimethoxyphenyl derivative **12e** demonstrates a similar GAK affinity (K_d = 0.072 μM). The dioxolane analogue **12f** (a ring-closed analogue of the 3,4-dimethoxyphenyl moiety) is completely inactive. The insertion of an additional methoxy group yielded the 3,4,5-trimethoxyphenyl analogue **12g**, displaying very strong binding affinity for the GAK enzyme (K_d = 0.0083 μM). In another round of SAR, the 3-methoxy group was kept intact and the 4-methoxy moiety was replaced by an ester moiety (compound **12h**), an amino group (compound **12i**), and a hydroxyl function (compound **12j**), generating compounds with a strong affinity for the GAK enzyme, displaying K_d values of 0.018, 0.0089, and 0.018 μM, respectively.

Since the first library screening demonstrated that a 3,4-dimethoxyphenyl moiety at position 6 was favorable for GAK binding, this substituent was kept intact, and a variety of amines were introduced at position 3 (Table 3). Although a free amino

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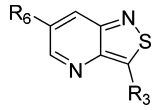
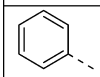
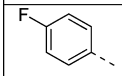
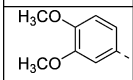
Table 1. GAK Affinity Data of an Isothiazolo[4,3-*b*]pyridine Library[a]



R_3 / R_6	---NH_2	---NH–OH	N-morpholine	N-methylpiperazine	--OCH_3
	**5a;** %Ctrl:68	**8a**; %Ctrl:18	**9a;** %Ctrl:0,8; Kd = 0,5 μM	**10a**; %Ctrl:76	**11a**; %Ctrl:18
	**5b**; %Ctrl:55	**8b**; %Ctrl:2.2	**9b**; %Ctrl:11	**10b**; %Ctrl:72	**11b**; %Ctrl:37
	**5c**; %Ctrl:70	**8c**; %Ctrl:0.4; Kd = 0,12 μM	**9c**; %Ctrl:0; Kd= 0,052 μM	**10c**; % Ctrl:19	**11c**; %Ctrl:19
	**5d**; %Ctrl:61	**8d**; % Ctrl 0,3; Kd = 0,3 μM	**9d**; %Ctrl:0.1; Kd= 0,042 μM	**10d;** %Ctrl:7.4	**11d**; %Ctrl:22
	**5e**; %Ctrl:80	**8e**; % Ctrl:45	**9e**; %Ctrl:25	**10e**; %Ctrl:88	**11e**; %Ctrl: 93

[a]Values represent the average of two independent experiments.

group at position 3 of the isothiazolo[5,4-*b*]pyridine scaffold is not tolerated for GAK binding (Table 1, compound **5c**), the presence of small aliphatic amines (primary as well as secondary), such as dimethylamino (compound **13a**), methoxyethylamino (compound **13b**), diethanolamino (**13c**), gives rise to compounds displaying K_d values in the range of 0.14−0.3 μM. Cycloaliphatic amines, such as a pyrrolidino (compound **13d**) and a cyclopropylmethylamino (compound **13e**), yielded compounds with a higher potency, with K_d values of 100 and 27 nM for compounds **13d** and **13e**, respectively.

The isosteric replacement of the oxygen of the morpholine moiety by a carbon (resulting in compound **13f**) or a sulfur (resulting in compound **13g**) leads to a 5-fold drop in GAK affinity when compared to the corresponding morpholine analogue **9c**. To better mimic the morpholine substituent, a 4-hydroxypiperidino and a 4-aminopyrane moiety were introduced, giving rise to isothiazolo[4,3-*b*]pyridines **13i** and **13j**, both exhibiting a strong affinity for the GAK enzyme (K_d values of 63 and 88 nM, respectively). Overall, the data in Table 3 indicate quite high tolerance for structural variation at position 3, with only an aromatic phenethylamino group (compound **13k**) completely lacking GAK affinity.

Although improving binding affinity is important in an optimization campaign, an overemphasis on potency has often resulted in molecules with unsuitable physicochemical properties for further development for in vivo use. We therefore complemented our lead optimization by monitoring a number of in silico parameters (Table 4). The optimized compounds had a low molecular weight, which in combination with their potent binding affinity yielded very "efficient" LE values exceeding 0.4 kcal/mol. Moreover, the optimization campaign did not result in any gain in lipophilicity, as the most potent GAK ligands, **12g** and **12i**, displayed cLogP values of 2.49 and 2.28, respectively.

SELECTIVITY PROFILING

Most kinase inhibitors are ATP-competitive and bind to the ATP domain, which is highly conserved across the kinome. Selectivity screens usually involve close analogues of the target kinase harboring similar ATP binding sites. However, we opted for a kinome-wide selectivity screen, using a panel of 456 kinases available at DiscoverX. As a representative example, **12g**, the most potent congener, was selected for the selectivity profiling at a single concentration of 10 μM. Remarkably, no affinity of **12g** was observed for any of the other members of the NAK family (AAK1, 90%; STK16, 92%; BIKE, 92% compared to the DMSO control) (Figure 2). Moreover, only seven additional kinases appeared to interact with **12g**, with a binding activity of less than 10%, when compared to DMSO control. The exact K_d values for these kinases were determined (Table 5). This large scale kinase profiling confirms that the identified lead compound is selective. Compound **12g** displays the strongest affinity for GAK (K_d = 8.3 nM). A 3-fold level of selectivity is observed when compared with the affinity for KIT (K_d = 29 nM), whereas for the other kinases (CLK2, CSF1R, FLT3, MEK5, PDGFRA, and PDGFRB), at least a 8-fold level of selectivity is achieved.

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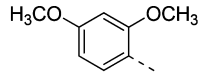
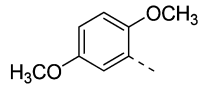
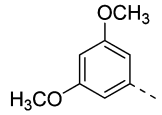
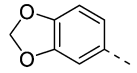
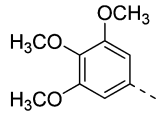
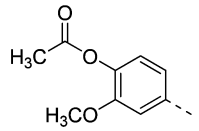
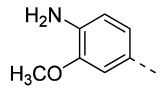
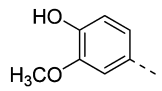
Table 2. SAR of the Aryl Moiety

Cpmd #	Structure	% Ctrl @10 μM[a]	Kd (μM)[a]
12a		0.05	0.047
12b		0.15	0.2
12c		6.3	ND
12d		0.7	0.13
12e		0.2	0.072
12f		51	ND
12g		0	0.0083
12h		0	0.018
12i		0	0.0089
12j		0	0.018

[a]Values represent the average of two independent experiments. ND = not determined.

X-RAY CRYSTALLOGRAPHY

To shed a light on the binding mode of the compounds to GAK, cocrystallization experiments were initiated. We have recently solved the structure of GAK in complex with a single chain antibody (nanobody), which resulted in reproducible crystallization conditions for the GAK kinase domain.[22] The nanobody bound distal to the ATP binding site interacting with the lower kinase lobe (Figure 3). The lead compound **12i** was cocrystallized and bound as expected to the ATP binding site of GAK. The binding mode of the inhibitor was well-defined by electron density, and the structure was refined to 2.1 Å resolution. Data collection and refinement statistics are provided in supplemental Table 2 (see Supporting Information). Two kinase domain:nanobody complexes were present in the asymmetric unit that interacted via the extended activation loops of GAK as described previously.[22] The upper lobe of the kinase domain was flexible as indicated by high *B*-factors in particular in the N-terminal region (Supporting Information Figure 1). The helix αC and the DFG motive were in an active conformation compatible with the type I

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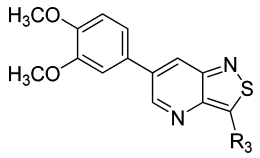
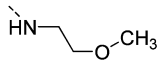
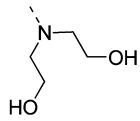
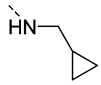
Table 3. SAR of the Amine Moiety



Cmpd#	R_3	% Ctrl (10 μM)[a]	Kd (μM)[a]
13a		0.1	0.19
13b		0.1	0.14
13c		4	0.32
13d		0	0.1
13e		0	0.027
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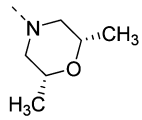
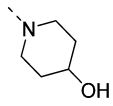
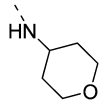
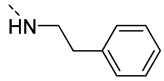
13g		0.8	0.23
13h		0.1	0.11
13i		0.1	0.063
13j		0.1	0.088
13k		81	ND

[a]Values represent the average of two independent experiments. ND = not determined.

binding mode of the inhibitor (Figure 3). The nitrogen of the isothiazolo moiety of the inhibitor formed a hydrogen bond with the backbone amide of Cys126. Interactions with small gatekeepers, which are found in only a small subset of kinases,

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Table 4. In Silico Parameters of Hit and Optimized Compounds

compd	MW	K_d (μM)	LE (kcal/mol)	cLogP
6d (hit)	277.37	0.3	0.494	2.44
12g	387.45	0.0083	0.408	2.49
12i	342.42	0.0089	0.458	2.28

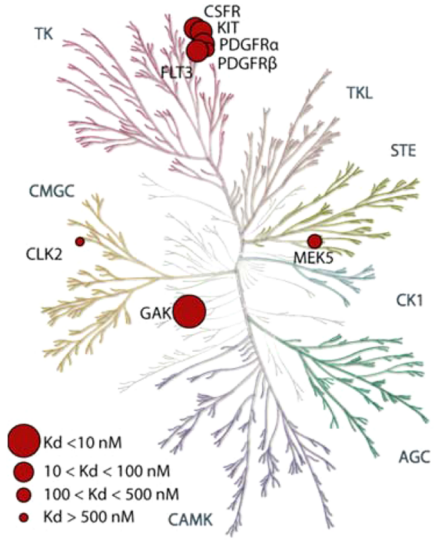

Figure 2. Specificity profile of compound **12g**.

Table 5. K_d Values for Off-Target Kinases Affected by 12g

kinase	K_d (nM)[a]
GAK	8.3
CLK2	710
CSF1R	320
FLT3	110
KIT	29
MEK5	150
PDGFRA	220
PDGFRB	70

[a]Values represent the average of two independent experiments.

have been explored for the development of selective kinase inhibitors.[23] Therefore, our observation of this contact in the case of **12i** may explain its excellent selectivity for GAK. The 4-amino-3-methoxyphenyl substituent of **12i** was oriented toward the solvent exposed area forming a number of hydrophobic contacts, in particular with Leu46 and a long-range polar interaction with Arg44. Overall, **12i** exhibits a good shape complementarity with the GAK ATP binding site.

ANTIVIRAL ACTIVITY

We have recently shown that GAK is a regulator of HCV entry and assembly and therefore represents a potential target for anti-HCV treatment. Moreover, erlotinib, an approved anticancer drug known to target GAK significantly inhibited binding of HCV core to AP2M1, HCV entry, and assembly.[8,11] Nevertheless, while erlotinib binds GAK with a high affinity (K_d = 3.4 nM), it binds EGFR, its primary anticancer target, with a comparable affinity (K_d = 1 nM) and several other kinases (albeit at a lower affinity).[9,10] The use of erlotinib as a chemical tool to probe the role of GAK in HCV infection is therefore somewhat limited, particularly since EGFR has also been recognized as an essential host factor for HCV infection.[24] Moreover, the limited selectivity contributes to erlotinib's side effects, which may reduce its potential as an antiviral agent, particularly in long duration regimens as those required for the treatment of HCV. We therefore hypothesized that since the isothiazolo[4,3-*b*]pyridines **12g** and **12i** are structurally unrelated to erlotinib and lack anti-EGFR activity, they represent attractive chemical tools to study the role of GAK in HCV infection and that their strong potency, promising selectivity profile, and favorable drug-like properties make them a potential novel class of antiviral agents.

To further validate GAK as an antiviral target and determine the antiviral effect of these compounds on HCV infection, Huh-7.5 (human hepatoma) cells were infected with cell culture grown J6/JFH(p7-Rluc2A) HCV (HCVcc), a *Renilla* luciferase-containing reporter virus that replicates and produces high viral titers in Huh-7.5 cells.[25] Infected cells were treated with various concentrations of **12g**, **12i**, or DMSO. Drug-containing medium was replenished every 24 h. Antiviral activity and cellular viability were measured by luciferase and alamarBlue-based assays, respectively, 72 h postinfection. As shown in Figure 4A, treatment with either **12g** or **12i** resulted in a dose-dependent inhibition of viral replication. Half maximal effective concentration (EC_{50}) values were 2.55 ± 0.43 μM (p = 0.0002) and 2.81 ± 0.8 μM (p = 0.009) for **12g** and **12i**, respectively. The half maximal cytotoxic concentration (CC_{50}) values were 23.27 ± 3.4

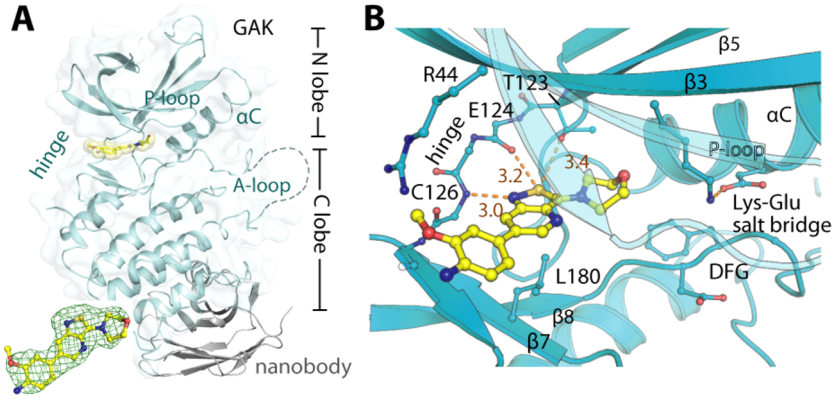

Figure 3. Structure of GAK in complex with **12i**. (A) Overview of GAK:NbGAK_4 complex with inhibitor **12i** bound. The inset shows $|F_O| - |F_C|$ omitted map contoured at 3σ for the bound inhibitor. (B) Detailed interactions of **12i** within the GAK ATP binding site.

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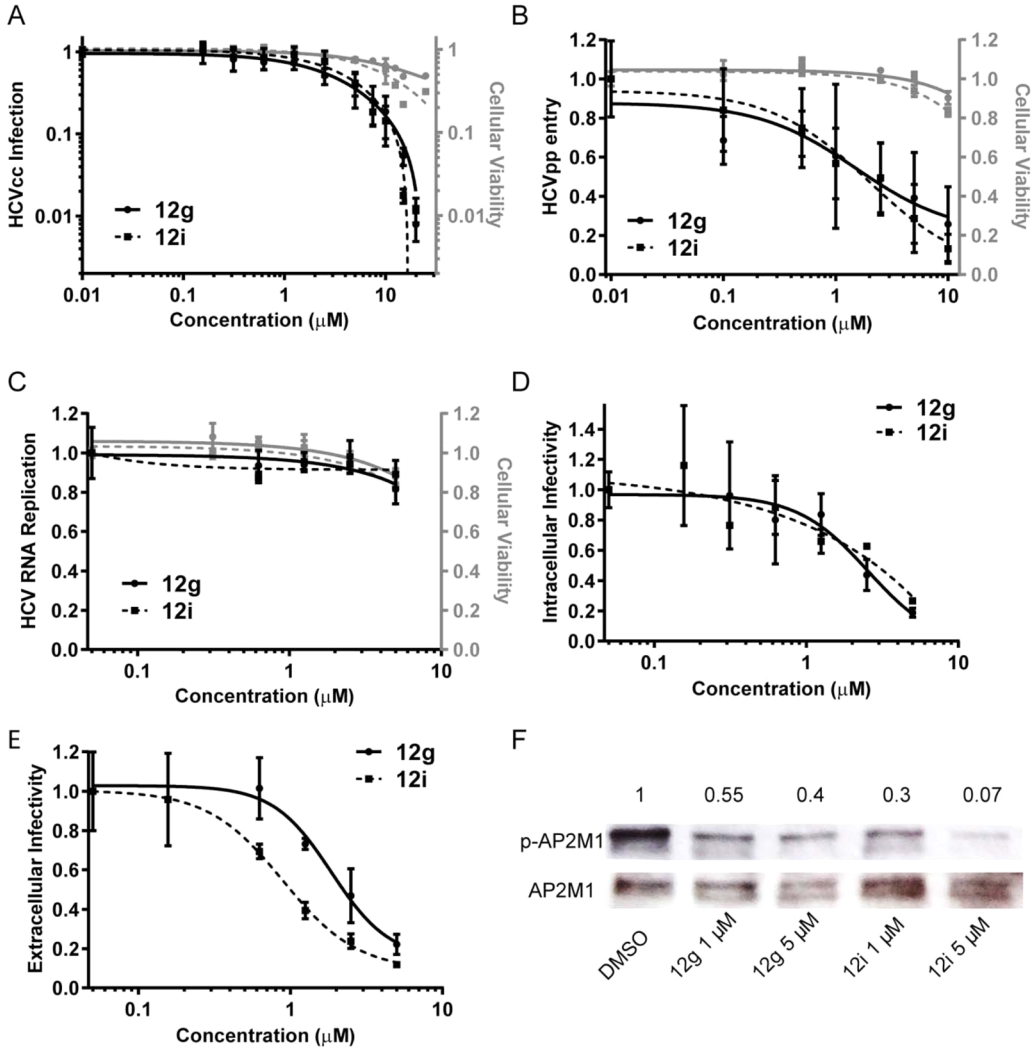

Figure 4. Selective GAK inhibitors inhibit HCV infection and AP2M1 phosphorylation: Dose−response curves of **12g** and **12i** effects on infection of Huh-7.5 cells with cell culture grown HCV (HCVcc) (A), entry of pseudoparticles of HCV (HCVpp) (B), HCV RNA replication 72 h postelectroporation with J6/JFH(p7-Rluc2A) RNA (C), and intra- (D) and extracellular (E) infectivity in naive cells inoculated with cell lysates or supernatants derived from the electroporated cells, respectively. Plotted in black (left *y*-axes) are relative luciferase values normalized to DMSO treated controls. Corresponding cellular viability, as measured by alamarBlue-based assays, are plotted in gray (right *y*-axes). Data reflect mean values and sd (error bars). (F) Effect of the inhibitors on AP2M1 phosphorylation by Western analysis in lysates derived from Huh-7.5 cells. A representative membrane blotted with antiphospho-AP2M1 (p-AP2M1) and anti-AP2M1 antibodies is shown. Numbers represent relative p-AP2M1/total AP2M1 protein ratio normalized to DMSO controls.

μM (p = 0.000 012) for **12g** and 8.92 ± 1.43 μM (p = 0.000 044) for **12i**.

Next, we sought to pinpoint the step of the viral life cycle that is disrupted by these GAK inhibitors. To study the effect of the GAK inhibitors on HCV entry, we measured the entry of pseudoparticles of HCV (HCVpp) (lentiviral vectors that incorporate the HCV glycoproteins on the viral envelope[26]) upon a 4 h treatment with various concentrations of **12g** and **12i** using luciferase reporter-based assays. Huh-7.5 cells were infected with HCVpp for 1 h on ice followed by a temperature shift to 37 °C, 4 h treatment with various concentrations of the compounds or DMSO, and medium replacement for removal of residual drugs and unbound virus. These compounds inhibited HCVpp entry in a dose-dependent manner, with half maximal effective concentrations (EC_{50} values) of 3.6 ± 1.2 μM (p = 0.03) and 2.05 ± 0.36 μM (p = 0.0025), respectively, and a minimal effect on cellular viability (Figure 4B).

To determine the effect of the compounds on later steps of the HCV life cycle and distinguish between a defect in viral RNA replication, assembly and/or release, Huh-7.5 cells were electroporated with in vitro transcribed luciferase reporter J6/JFH(p7-Rluc2A) HCV RNA[24] and treated daily for 72 h with **12g**, **12i**, or DMSO. HCV RNA replication was measured by luciferase assays 72 h postelectroporation and intra- and extracellular infectivity was measured by luciferase assays in naive cells inoculated with either clarified cell lysates or supernatants derived from the electroporated cells, respectively. Treatment with **12g** and **12i** did not affect HCV RNA replication (Figure 4C). Nevertheless, **12g** and **12i** demonstrated a dose-dependent effect on intracellular infectivity (Figure 4D), with EC_{50} values of 1.64 ± 0.272 μM (p = 0.009) and 2.43 ± 0.72 μM (p = 0.0281), respectively. Moreover, **12g** and **12i** significantly inhibited extracellular infectivity (Figure 4E), with EC_{50} values of 2.13 ± 0.72 μM (p = 0.04) and 2.47 ± 0.9 μM (p = 0.04), respectively. These data indicate that the two selective GAK

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inhibitors tested disrupt HCV assembly and infectious virus production without affecting HCV RNA replication.

Similar to small interfering RNAs (siRNAs) that target GAK as well as erlotinib,[8,11] these compounds thus inhibit two temporally distinct steps in the HCV life cycle: entry and assembly. The EC_{50} values for the antiviral effect of these compounds range from ~1.5−3 μM, depending on the assay used. These EC_{50} values are slightly higher or comparable to those of erlotinib (0.5−1.5 μM),[8,11] likely reflecting the absence of EGFR inhibition exhibited by erlotinib.

Taken together, these results provide a pharmacological validation of the requirement for GAK in the regulation of HCV entry and assembly. Moreover, the selective GAK inhibitors represent candidate compounds to target two steps of the HCV life cycle: viral entry and assembly (Figure 5).

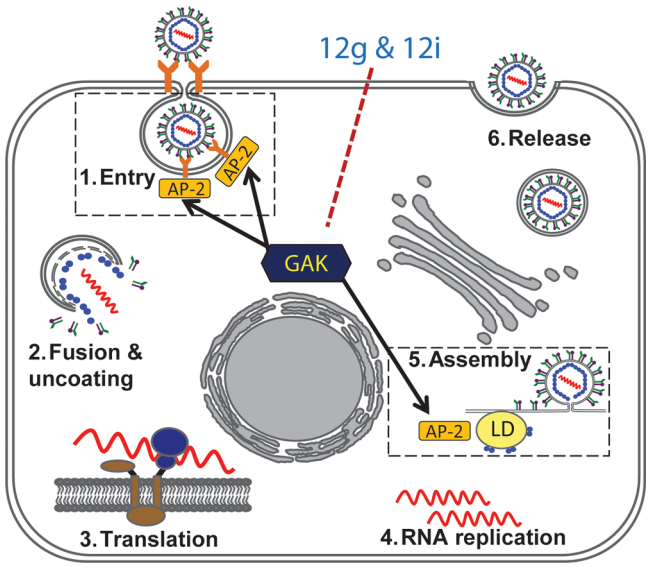

Figure 5. Proposed model for the mechanism by which the selective GAK inhibitors, **12g** and **12i**, inhibit HCV infection. By directly inhibiting GAK kinase activity, **12g** and **12i** interfere with clathrin/AP-2-mediated endocytosis of HCV (step 1) as well as AP-2 binding to core in HCV assembly (step 5).

To validate the mechanism of action of these compounds, we studied their effect on AP2M1 phosphorylation. Huh-7.5 cells were treated with various concentrations of the compounds or DMSO. Since AP2M1 phosphorylation is transient (because of the activity of the phosphatase PP2A),[27] to allow capturing of the phosphorylated AP2M1 state, these cells were incubated for 1 h in the presence of the PP2A inhibitor, calyculin A, prior to lysis. The ratio of phosphorylated AP2M1 (p-AP2M1) to total AP2M1 was measured by quantitating Western blot band intensity. p-AP2M1 to AP2M1 ratios were reduced by either **12g** or **12i** in a dose-dependent manner and were significantly lower than the ratios measured in the DMSO control (Figure 4F). These results indicate that **12g** and **12i** modulate AP2M1 phosphorylation, as predicted for compounds that target GAK.

CONCLUSION

We have previously reported that GAK is an essential host factor for HCV entry and assembly and that erlotinib, an approved anticancer drug with inhibitory activity against GAK, effectively disrupts HCV entry and assembly.[8,11] Here, for the first time, we report on a specific optimization campaign toward the identification of novel GAK inhibitors. This endeavor resulted in the identification of novel and highly selective GAK inhibitors, based on an isothiazolo[4,3-*b*]pyridine scaffold, an unexplored chemical space. Their druglike properties, as well as their selectivity profile, make these compounds an attractive tool to study regulatory mechanisms of intracellular traffic in cell biology and disease conditions, where GAK is known to be implicated. We used these compounds to better understand the role of GAK in HCV infection and further validate this host factor as an antiviral target. We have demonstrated that compounds **12g** and **12i** exhibit a potent in vitro anti-HCV activity and that their antiviral effect correlates with a decrease in AP2M1 phosphorylation. Although to the best of our knowledge, KIT has not been reported to have a role in HCV infection, it is bound by these compounds, albeit at a lower affinity (~30 nM). We therefore cannot exclude the possibility that inhibition of KIT or other cellular kinases, in addition to GAK, contributes to the antiviral effect of these compounds.

Unlike approved direct acting antivirals (DAA), which target HCV RNA replication, the developed compounds inhibit two distinct steps of the HCV life cycle: entry and assembly. By displaying limited off-target binding to other kinases, these more selective GAK inhibitors have the potential to reduce drug toxicity. Moreover, inhibiting a host rather than a viral target is more likely to provide a higher genetic barrier for drug resistance than most DAAs and a comprehensive protection against all HCV genotypes. Hence, selective GAK inhibitors may potentially represent a novel class of antivirals for inclusion in future combination drug regimens for treating HCV. Last, since multiple other viruses hijack clathrin adaptor proteins-mediated pathways for either viral entry or late steps of their viral life cycle,[28−30] we predict that the requirement for GAK is broadly conserved among RNA viruses and therefore that selective GAK inhibitors may display a broad-spectrum antiviral activity.

EXPERIMENTAL SECTION

General. For all reactions, analytical grade solvents were used. All moisture-sensitive reactions were carried out in oven-dried glassware (135 °C). ^{1}H and ^{13}C NMR spectra: Bruker Avance 300 MHz instrument (^{1}H NMR, 300 MHz; ^{13}C NMR, 75 MHz), 500 MHz instrument (^{1}H NMR, 500 MHz; ^{13}C NMR, 125 MHz), and 600 MHz instrument (^{1}H NMR, 600 MHz; ^{13}C NMR, 150 MHz), using tetramethylsilane as internal standard for ^{1}H NMR spectra and DMSO-d_6 (39.5 ppm) or $CDCl_3$ (77.2 ppm) and CD_3OD (49.0 ppm) for ^{13}C NMR spectra. Abbreviations used are s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. Coupling constants are expressed in Hz. Mass spectra are obtained with a Finnigan LCQ Advantage Max (ion trap) mass spectrometer from Thermo Finnigan, San Jose, CA, USA. High resolution mass spectra were acquired on a quadrupole orthogonal acceleration time-of-flight mass spectrometer (Synapt G2 HDMS, Waters, Milford, MA). Samples were infused at 3 μL/min, and spectra were obtained in positive (or negative) ionization mode with a resolution of 15 000 (fwhm) using leucine enkephalin as lock mass. Precoated aluminum sheets (Fluka silica gel/TLC-cards, 254 nm) were used for TLC. Column chromatography (CC) was performed on ICN silica gel 63−200, 60 Å. Purity of final compounds was determined by analytical RP-HPLC analysis on a XBridge column (C-18, 5 μm, 4.6 mm × 150 mm) in combination with a Waters 600 HPLC system and a Waters 2996 photodiode array detector from Waters, Milford, MA, USA. Elution was done using a gradient mixture of H_2O containing 0.2% (vol) of TFA (A) and acetonitrile (B) (Supporting Information). All compounds were at least 95% pure, with the exception of compound **11b** (90.32% purity).

3-Amino-5-bromopyridine-2-carbonitrile (2). A solution of iron powder (3.36 g, 60 mmol) in acetic acid (15 mL) was stirred at 0 °C. To this solution was added dropwise a solution of 3-nitro-5-bromopyridine-

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2-carbonitrile (2.51 g, 11 mmol) in acetic acid (15 mL). The reaction mixture was stirred at room temperature for 2 h. Then, ethyl acetate (300 mL) was added and the mixture was filtered (paper filter). The filter cake was washed with ethyl acetate. The filtrate was evaporated and partitioned between ethyl acetate (500 mL) and water (250 mL). The organic phase was washed with a 1 N NaOH solution (~200 mL). The combined organic phases were dried and evaporated in vacuo, yielding a mixture of two compounds, i.e., 3-amino-5-bromopyridine-2-carbonitrile (major compound) and 3-amino-5-bromopyridine-2-carboxamide (minor compound). This mixture was used as such in the next reaction.

3-Amino-5-bromo-2-pyridinecarbothioamide (3). To a solution of a mixture of 3-amino-5-bromopyridine-2-carbonitrile and 3-amino-5-bromopyridine-2-carboxamide (crude) in ethanol (25 mL) was added phosphorus pentasulfide (2 equiv; 4.84 g). The mixture was heated overnight at 75 °C. The solvents were evaporated and the crude residue was purified by flash chromatography on silica, using a mixture of cyclohexane/ethyl acetate (in a ratio of 7:1) as mobile phase, yielding the title compound (3.36 g crude). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.95 (bs, 2H, NH_2), 7.27 (d, J = 1.95 Hz, 1H, arom H), 7.89 (d, J = 1.95 Hz, 1H, arom H), 9.37 (bs, 2H, NH_2) ppm.

3-Amino-6-bromoisothiazolo[4,3-*b*]pyridine (4). To a solution of 3-amino-5-bromo-2-pyridinecarbothioamide in methanol (50 mL) was added dropwise a 30% H_2O_2 solution in water (3.5 mL) at 0 °C. The reaction mixture was stirred overnight at room temperature and then cooled again to 0 °C. The crystals were filtered off and washed with cold methanol, yielding the title compound (1.6 g, 63%). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 8.03 (d, J = 2.01 Hz, 1H, arom H), 8.08 (bs, 2H, NH_2), 8.29 (d, J = 2.01 Hz, 1H, arom H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ = 120.56 (C_q), 129.39 (CH), 133.23 (C_q), 143.69 (C_q), 153.58 (CH), 173.34 (C_q) ppm.

Synthesis of 3-Amino-6-arylisothiazolo[4,3-*b*]pyridines (5a–e). *General Procedure.* To a solution of 3-amino-6-bromo-isothiazolo-[4,3-*b*]pyridine (166 mg, 0.72 mmol) in a mixture of dioxane (10 mL) and water (1.5 mL) were added an appropriate boronic acid (2 equiv), sodium carbonate (2 equiv, 153 mg), and Pd(dppf)Cl_2 (0.1 equiv, 59 mg) The reaction mixture was stirred overnight at 100 °C. The reaction mixture was cooled to room temperature and the reaction was partitioned between ethyl acetate (60 mL) and brine (30 mL). The aqueous phase was then extracted with ethyl acetate (40 mL). The combined organic phases were dried over $MgSO_4$ and evaporated in vacuo. The residue was purified by flash chromatography on silica gel, yielding the pure title compounds.

The following compounds were made according to this procedure.

3-Amino-6-phenylisothiazolo[4,3-*b*]pyridine (5a). This compound was obtained using phenylboronic acid and the crude residue was purified by flash chromatography using a mixture of cyclohexane and ethyl acetate (in a ratio of 4:1) as mobile phase, affording the title compound in 77% yield (125 mg, 0.55 mmol). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.84 (s, 2H, NH_2), 7.52 (m, 3H, arom H), 7.66 (m, 2H, arom H), 7.95 (d, J = 1.98 Hz, 1H, arom H), 8.68 (d, J = 1.95 Hz, 1H, arom H) ppm. ^{13}C NMR (150 MHz, DMSO-d_6): δ = 124.37 (CH), 127.38 (CH), 128.40 (CH), 129.18 (CH), 134.06 (C_q), 135.37 (C_q), 137.25 (C_q), 143.27 (CH), 153.67 (C_q), 172.38 (C_q) ppm. HR-MS $[M + H]^+$ found 228.0594, calculated 228.0551.

3-Amino-6-(4-fluorophenyl)isothiazolo[4,3-*b*]pyridine (5b). This compound was obtained using 4-fluorophenylboronic acid and the crude residue was purified by flash chromatography using a mixture of cyclohexane and ethyl acetate (in a ratio of 4:1) as mobile phase, affording the title compound in 76% yield (134 mg, 0.54 mmol). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 6.36 (br s, 2H, NH_2), 7.34 (m, 2H, arom H), 7.38 (d, J = 3.1 Hz, 1H, arom H), 7.71 (m, 2H, arom H), 8.17 (d, J = 1.92 Hz, 1H, arom H) ppm. ^{13}C NMR (150 MHz, DMSO-d_6): δ = 115.94 (CH), 116.23 (CH), 124.43 (CH), 129.53 (CH), 129.65 (CH), 133.76 (C_q), 134.43 (C_q), 143.19 (CH), 153.65 (C_q), 160.88 (C_q), 164.24 (C_q), 172.49 (C_q) ppm. HR-MS $[M + H]^+$ found 246.0497, calculated 243.0457.

3-Amino-6-(3,4-dimethoxyphenyl)isothiazolo[4,3-*b*]pyridine (5c). This compound was obtained using 3,4-dimethoxyphenylboronic acid and the crude residue was purified by flash chromatography using a mixture of methanol and dichloromethane (in a ratio of 1:40) as mobile phase, affording the title compound in 57% yield (117 mg, 0.41 mmol). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.81 (s, 3H, OCH_3), 3.88 (s, 3H, OCH_3), 7.07 (d, J = 8.89 Hz, 1H, arom H), 7.35 (m, 2H, arom H), 7.84 (br s, 2H, NH_2), 7.89 (d, J = 1.92 Hz, 1H, arom H), 8.63 (d, J = 1.95 Hz, 1H, arom H) ppm. ^{13}C NMR (150 MHz, DMSO-d_6): δ = 55.76 (CH_3), 55.82 (CH_3), 111.00 (CH), 112.34 (CH), 119.76 (CH), 123.56 (CH), 129.82 (C_q), 133.77 (C_q), 135.31 (C_q), 143.53 (CH), 149.38 (2 × C_q), 153.96 (C_q), 172.20 (C_q) ppm. HR-MS $[M + H]^+$ found 288.0800, calculated 288.0762.

3-Amino-6-(3-thienyl)isothiazolo[4,3-*b*]pyridine (5d). This compound was obtained using 3-thienylboronic acid and the crude residue was purified by flash chromatography using a mixture of cyclohexane and ethyl acetate (in a ratio of 3:2) as mobile phase, affording the title compound in 69% yield (115 mg, 0.49 mmol). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 7.70 (m, 2H, arom H), 7.84 (br s, 2H, NH_2), 7.98 (d, J = 1.89 Hz, 1H, arom H), 8.17 (q, J = 1.32 Hz, 1H, arom H), 8.74 (d, J = 1.95 Hz, 1H, arom H) ppm. ^{13}C NMR (150 MHz, DMSO-d_6): δ = 122.99 (CH), 123.20 (CH), 126.60 (CH), 127.65 (CH), 130.35 (C_q), 133.71 (C_q), 138.38 (C_q), 143.17 (CH), 153.91 (C_q), 172.22 (C_q) ppm. HR-MS $[M + H]^+$ found 243.0157, calculated 243.0115.

3-Amino-6-(3-pyridyl)isothiazolo[4,3-*b*]pyridine (5e). This compound was obtained using 3-pyridylboronic acid and the crude residue was purified by flash chromatography using a mixture of methanol and dichloromethane (in a ratio of 1:20) as mobile phase, affording the title compound in 75% yield (123 mg, 0.54 mmol). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 7.52 (m, 1H, arom H), 7.95 (br s, 2H, NH_2), 8.02 (d, J = 1.98 Hz, 1H, arom H), 8.24 (m, 1H, arom H), 8.66 (m, 2H, arom H), 9.03 (d, J = 1.98 Hz, 1H, arom H) ppm. ^{13}C NMR (150 MHz, DMSO-d_6): δ = 124.06 (CH), 125.16 (CH), 132.54 (C_q), 133.01 (C_q), 134.92 (CH), 142.85 (CH), 148.26 (CH), 149.37 (CH), 153.46 (C_q), 173.63 (C_q) ppm. HR-MS $[M + H]^+$ found 229.0537, calculated 229.0503.

3,6-Dibromoisothiazolo[4,3-*b*]pyridine (6). A solution of 3-amino-6-bromoisothiazolo[4,3-*b*]pyridine (1.1 g, 4.78 mmol) in HBr (50 mL) was stirred for 10 min at room temperature, and then CuBr was added in one portion (1.37 g, 9.56 mmol, 2.0 equiv). The resulting mixture was cooled to 0 °C. A solution of sodium nitrite (0.99 g, 14.34 mmol, 3.0 equiv) in H_2O (15 mL) was added dropwise to the mixture over a period of 30 min. The reaction mixture was stirred for 2 h at 0 °C and then overnight at room temperature. Then, the mixture was cooled to 0 °C and carefully neutralized with solid potassium carbonate and extracted with ethyl acetate (2 × 50 mL). The combined organic phases were dried over $MgSO_4$ and evaporated in vacuo. The crude residue was purified by silica gel flash chromatography, using the mixture of cyclohexane and ethyl acetate (in a ratio of 95:5) as mobile phase. The title compound was obtained in 78% yield (1.1 g, 3.75 mmol). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 8.70 (d, J = 2.01 Hz, 1H, arom H), 8.92 (d, J = 2.04 Hz, 1H, arom H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ = 121.12 (C_q), 131.29 (CH), 137.79 (C_q), 144.54 (C_q), 153.31 (CH), 154.58 (C_q) ppm.

6-Bromo-3-substituted-isothiazolo[4,3-*b*]pyridines (7a–c). *General Procedure.* To a solution of 3,6-dibromoisothiazolo[4,3-*b*]pyridine (150 mg, 0.51 mmol) in ethanol (10 mL) was added an appropriate nitrogen nucleophile (3 equiv). The reaction was stirred overnight at 75 °C. The solvent was evaporated in vacuo and the crude residue was purified by silica gel flash chromatography, the mobile phase being a mixture of cyclohexane and ethyl acetate (in a ratio of 4:1), yielding the pure title compounds.

The following compounds were made according to this procedure.

6-Bromo-3-morpholinoisothiazolo[4,3-*b*]pyridine (7a). This compound was made using morpholine as nucleophile and the crude residue was purified using a mixture of cyclohexane and ethyl acetate (in a ratio of 4:1) as mobile phase, affording the title compound in 92% yield (140 mg, 0.46 mmol). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.84 (br s, 4H, 2 × NCH_2), 3.87 (br s, 4 H, 2 × OCH_2), 8.15 (s, 1H, arom H), 8.37 (s, 1H, arom H) ppm.

6-Bromo-6-ethanolaminoisothiazolo[4,3-*b*]pyridine (7b). This compound was made using ethanolamine as nucleophile and the crude residue was purified using a mixture of cyclohexane and ethyl

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acetate (in a ratio gradually ranging from 1:1 to 1:4) as mobile phase, affording the title compound in 94% yield (131 mg, 0.47 mmol). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.39 (q, J = 5.75 Hz, 2H, NCH_2), 3.68 (q, J = 5.68 Hz, 2H, OCH_2), 4.91 (t, J = 5.57 Hz, 1H), 8.04 (d, J = 2.01 Hz, 1H, arom H), 8.29 (d, J = 1.98 Hz, 1H, arom H), 8.66 (t, J = 5.78 Hz, 1H) ppm.

6-Bromo-3-(*N*-methylpiperazino)isothiazolo[4,3-*b*]pyridine (7c). This compound was made using *N*-methylpiperazine as nucleophile and the crude residue was purified using a mixture of methanol and dichloromethane (in a ratio of 3:100) as mobile phase, affording the title compound in 92% yield (146 mg, 0.46 mmol). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.24 (s, 3H, NCH_3), 2.52 (m, 4H, 2 × NCH_2), 3.88 (t, J = Hz, 4 H, 2 × NCH_2), 8.12 (d, J = 2.01 Hz, 1H, arom H), 8.36 (d, J = 1.23 Hz, 1H, arom H) ppm.

Synthesis of 3-Ethanolamino-6-arylisothiazolo[4,3-*b*]-pyridines (8a–e). *General Procedure.* To a solution of 3-ethanolamino-6-bromoisothiazolo[4,3-*b*]pyridine (0.37 mmol, 101 mg) in dioxane (5 mL) and water (1 mL) were added an appropriate boronic acid (2 equiv), sodium carbonate (2 equiv, 78 mg) and Pd(dppf)Cl_2 (0.01 equiv, 30 mg). The reaction was stirred overnight at 75 °C. The reaction mixture was diluted with ethyl acetate (50 mL) and brine (30 mL). The aqueous phase was extracted with ethyl acetate (50 mL). The organic phases were dried and evaporated. The crude residue was purified by silica gel flash chromatography yielding the pure title compounds.

The following compounds were made according to this procedure.

3-Ethanolamino-6-phenylisothiazolo[4,3-*b*]pyridine (8a). This compound was prepared using phenylboronic acid and was purified using a mixture of dichloromethane and methanol (in a ratio 20:1), affording the title compound in 99% yield (99 mg, 0.36 mmol). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.41 (q, J = 5.80 Hz, 2H, NCH_2), 3.71 (q, J = 5.61 Hz, 2H, OCH_2), 4.95 (t, J = 5.52 Hz, 1H), 7.49 (m, 5H, arom H), 7.80 (br d, J = 7.23 Hz, 1H, arom H), 7.91 (s, 1H, arom H), 8.50 (br t, 1H), 8.63 (d, J = 1.59 Hz, 1H, arom H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$): δ = 50.09 (CH_2), 59.84 (CH_2), 125.97 (CH), 127.42 (CH), 128.47 (CH), 129.14 (CH), 132.86 (C_q), 136.81 (C_q), 137.46 (C_q), 144.10 (CH), 154.71 (C_q), 172.35 (C_q) ppm. HR-MS $[M + H]^+$ found 272.0852, calculated 272.0552.

3-Ethanolamino-6-(3-(4-fluorophenyl))isothiazolo[4,3-*b*]-pyridine (8b). This compound was prepared using 4-fluorophenylboronic acid and was purified using a mixture of dichloromethane and methanol (in a ratio 20:1), affording the title compound in 81% yield (86 mg, 0.29 mmol). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.30 (2H, NCH_2, hidden under H_2O peak), 3.71 (q, J = 5.61 Hz, 2H, OCH_2), 4.96 (t, J = 5.45 Hz, 1H), 7.35 (t, J = 8.64 Hz, 1H, arom H), 7.88 (m, 3H, arom H), 8.47 (br t, 1H), 8.62 (br s, 1H, arom H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$): δ = 50.06 (CH_2), 60.28 (CH_2), 116.10 (CH), 116.25 (CH), 125.57 (CH), 129.14 (CH), 129.19 (CH), 133.43 (C_q), 133.74 (C_q), 135.94 (C_q), 144.19 (CH), 153.53 (C_q), 162.26 (C_q), 163.90 (C_q), 172.30 (C_q) ppm. HR-MS $[M + H]^+$ found 290.0765, calculated 290.0719.

3-Ethanolamino-6-(3,4-dimethoxyphenyl)isothiazolo[4,3-*b*]-pyridine (8c). This compound was prepared using 3,4-dimethoxyphenylboronic acid and was purified using a mixture of dichloromethane and methanol (in a ratio 30:1), affording the title compound in 95% yield (116 mg, 0.35 mmol). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.42 (q, J = 5.62 Hz, 2H, NCH_2), 3.70 (q, J = 5.42 Hz, 2H, OCH_2), 3.81 (s, 3H, OCH_3), 3.88 (s, 3H, OCH_3), 4.93 (t, J = 5.67 Hz, 1H), 7.07 (d, J = 8.34 Hz, 1H, arom H), 7.36 (m, 2H, arom H), 7.92 (s, 1H, arom H), 8.40 (t, J = 5.55 Hz, 1 arom H), 8.64 (br s, 1H, arom H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$): δ = 50.06 (CH_2), 56.01 (CH_3), 56.05 (CH_3), 59.75 (CH_2), 110.40 (CH), 111.63 (CH), 119.93 (CH), 125.18 (CH), 130.13 (C_q), 132.53 (C_q), 136.55 (C_q), 144.01 (CH), 149.50 (C_q), 149.49 (C_q), 154.82 (C_q), 172.29 (C_q) ppm. HR-MS $[M + H]^+$ found 332.1067, calculated 332.1063.

3-Ethanolamino-6-(3-thienyl)isothiazolo[4,3-*b*]pyridine (8d). This compound was prepared using 3-thienylboronic acid and was purified using a mixture of dichloromethane and methanol (in a ratio 30:1), affording the title compound in 90% yield (92 mg, 0.33 mmol). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.40 (q, J = 5.58 Hz, 2H, NCH_2), 3.70 (q, J = 5.64 Hz, 2H, OCH_2), 4.93 (t, J = 5.49 Hz, 1H), 7.74 (m, 2H, arom H), 8.01 (br s, 1H, arom H), 8.19 (br s, 1 arom H), 8.42 (br t, 1H), 8.75 (br s, 1H, arom H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$): δ = 50.08 (CH_2), 60.18 (CH_2), 122.30 (CH), 124.47 (CH), 126.15 (CH), 127.13 (CH), 131.51 (C_q), 134.20 (C_q), 138.54 (C_q), 143.86 (CH), 154.73 (C_q), 172.20 (C_q) ppm. HR-MS $[M + H]^+$ found 278.1415, calculated 278.0416.

3-Ethanolamino-6-(3-pyridyl)isothiazolo[4,3-*b*]pyridine (8e). This compound was prepared using 3-pyridylboronic acid and was purified using a mixture of dichloromethane and methanol (in a ratio gradually ranging from 20:1 to 10:1), affording the title compound in 90% yield (90 mg, 0.33 mmol). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.42 (q, J = 5.67 Hz, 2H, NCH_2), 3.71 (t, J = 5.64 Hz, 2H, OCH_2), 4.95 (br s, 1H), 7.54 (m, 1H, arom H), 8.05 (br s, 1H, arom H), 8.25 (br d, J = 7.95 Hz, 1H, arom H), 8.52 (br t, 1H), 8.61 (m, 2H, arom H), 9.04 (d, J = 2.13 Hz 1H, arom H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ = 50.70 (CH_2), 59.16 (CH_2), 124.08 (CH), 125.05 (CH), 132.76 (C_q), 132.97 (C_q), 133.90 (C_q), 134.98 (CH), 142.52 (CH), 148.22 (CH), 149.34 (CH), 153.81 (C_q), 173.35 (C_q) ppm. HR-MS $[M + H]^+$ found 273.0807, calculated 273.0804.

Synthesis of 3-Morpholino-6-arylisothiazolo[4,3-*b*]pyridines (9a–e). *General Procedure.* To a solution of 3-morpholino-6-bromoisothiazolo[4,3-*b*]pyridine (0.37 mmol, 111 mg) in DME (5 mL) and water (1 mL) were added an appropriate boronic acid (2 equiv), sodium carbonate (2 equiv, 78 mg), and Pd(dppf)Cl_2 (0.01 equiv, 30 mg). The reaction was heated overnight at 75 °C. The reaction mixture was diluted with ethyl acetate (50 mL) and brine (30 mL). The aqueous phase was extracted with ethyl acetate (50 mL). The organic phases were dried and evaporated. The crude residue was purified by silica gel flash chromatography, yielding the pure title compounds.

The following compounds were made according to this procedure.

3-Morpholino-6-phenylisothiazolo[4,3-*b*]pyridine (9a). This compound was prepared using phenylboronic acid and was purified using a mixture of cyclohexane/ethyl acetate in a ratio of 2:1, affording the title compound in 78% yield (85 mg. 0.28 mmol). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.86 (br s, 4 H, 2 × NCH_2), 3.90 (br s, 4H, 2 × OCH_2), 7.50 (m, 3H, arom H), 7.82 (m, 2H, arom H), 8.01 (d, J = 2.1 Hz, 1H, arom H), 8.72 (d, J = 2.1 Hz, 1H, arom H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$): δ = 50.40 (CH_2), 66.20 (CH_2), 125.69 (CH), 127.38 (CH), 128.39 (CH), 129.13 (CH), 134.35 (C_q), 135.81 (C_q), 137.56 (C_q), 144.54 (CH), 156.23 (C_q), 173.32 (C_q) ppm. HR-MS $[M + H]^+$ found 298.1010, calculated 298.1008.

3-Morpholino-6-(4-fluorophenyl)isothiazolo[4,3-*b*]pyridine (9b). This compound was prepared using 4-fluorophenylboronic acid and was purified using a mixture of cyclohexane/ethyl acetate in a ratio of 3:1, affording the title compound in 95% yield (110 mg, 0.35 mmol). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.85 (br s, 4H, 2 × NCH_2), 3.89 (br s, 4H, 2 × OCH_2), 7.34 (t, J = 8.82 Hz, 2H, arom H), 7.87 (m, 2H, arom H), 8.00 (d, J = 2.04 Hz, 1H, arom H), 8.69 (d, J = 2.04 Hz, 1H, arom H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$): δ = 50.38 (CH_2), 66.18 (CH_2), 116.08 (CH), 116.23 (CH), 125.52 (CH), 129.03 (CH), 129.09 (CH), 133.65 (C_q), 134.31 (C_q), 144.17 (CH), 156.05 (C_q), 162.22 (C_q), 163.86 (C_q), 173.38 (C_q) ppm. HR-MS $[M + H]^+$ found 316.0912, calculated 316.0914.

3-Morpholino-6-(3,4-dimethoxyphenyl)isothiazolo[4,3-*b*]-pyridine (9c). This compound was prepared using 3,4-dimethoxyphenylboronic acid and was purified using a mixture of cyclohexane/ethyl acetate in a ratio of 2:1, affording the title compound in 72% yield (95 mg, 0.26 mmol). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.82 (s, 3H, OCH_3), 3.89 (br s, 11H, 2 × NCH_2, 2 × OCH_2 and OCH_3), 7.09 (d, J = 8.04 Hz, 1H, arom H), 7.39 (m, 2H, arom H), 8.02 (d, J = 2.01 Hz, 1 H, arom H), 8.75 (d, J = 1.95 Hz, 1H, arom H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$): δ = 50.37 (CH_2), 55.97 (CH_3), 56.00 (CH_3), 66.18 (CH_2), 110.33 (CH), 111.64 (CH), 119.84 (CH), 124.80 (CH), 130.23 (C_q), 134.06 (C_q), 135.55 (C_q), 144.51 (CH), 149.47 (C_q), 149.53 (C_q), 156.31 (C_q), 173.21 (C_q) ppm. HR-MS $[M + H]^+$ found 358.1223, calculated 358.1219.

3-Morpholino-6-(3-thienyl)isothiazolo[4,3-*b*]pyridine (9d). This compound was prepared using 3-thienylboronic acid and was purified using a mixture of cyclohexane/ethyl acetate in a ratio of 2:1,

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affording the title compound in 71% yield (79 mg, 0.26 mmol). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.86 (br s, 4 H, 2 × NCH_2), 3.88 (br s, 4H, 2 × OCH_2), 7.73 (m, 1H, arom H), 7.77 (m, 1H, arom H), 8.09 (d, J = 2.01 Hz, 1H, arom H), 8.21 (m, 1H, arom H), 8.84 (d, J = 2.04 Hz, 1H, arom H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$): δ = 50.36 (CH_2), 66.19 (CH_2), 122.22 (CH), 124.29 (CH), 126.08 (CH), 127.07 (CH), 130.46 (C_q), 134.06 (C_q), 138.54 (C_q), 143.99 (CH), 156.24 (C_q), 173.23 (C_q) ppm. HR-MS $[M + H]^+$ found 304.0575, calculated 304.0572.

3-Morpholino-6-(3-pyridyl))isothiazolo[4,3-*b*]pyridine (9e). This compound was prepared using 3-pyridinylboronic acid and was purified using a mixture of methanol/dichloromethane in a ratio of 1:25, affording the title compound in 80% yield (88 mg, 0.29 mmol). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.86 (br s, 4H, 2 × NCH_2), 3.91 (br s, 4H, 2 × OCH_2), 7.55 (m, 1H, arom H), 8.15 (d, J = 1.95 Hz, 2H, arom H), 8.26 (br d, 1 H, arom H), 8.65 (br d, 1H, arom H), 8.76 (d, J = 1.92 Hz, 1H, arom H), 9.05 (br s, 1H, arom H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$): δ = 50.37 (CH_2), 66.15 (CH_2), 123.82 (CH), 126.22 (CH), 132.59 (C_q), 133.33 (C_q), 134.60 (CH), 134.71 (C_q), 143.39 (CH), 148.36 (CH), 149.50 (CH), 155.70 (C_q), 173.56 (C_q) ppm. HR-MS $[M + H]^+$ found 299.0959, calculated 299.0961.

Synthesis of 3-(*N*-Me-piperazinyl)-6-arylisothiazolo[4,3-*b*]-pyridines 10a–e. *General Procedure.* To a solution of 3-(*N*-methylpiperazino)-6-bromoisothiazolo[4,3-*b*]pyridine (100 mg, 0.32 mmol) in dioxane (6 mL) and water (2.5 mL) were added an appropriate boronic acid (2 equiv), sodium carbonate (2 equiv, 68 mg), and Pd(dppf)Cl_2 (0.01 equiv, 26 mg). The reaction mixture was heated at 95 °C overnight. The reaction mixture was diluted with ethyl acetate (50 mL) and brine (30 mL). The aqueous phase was extracted with ethyl acetate (50 mL). The organic phases were dried and evaporated. The crude residue was purified by silica gel flash chromatography, yielding the pure title compounds

The following compounds were made according to this procedure:

3-(*N*-Me-piperazinyl)-6-phenylisothiazolo[4,3-*b*]pyridine (10a). This compound was prepared using phenylboronic acid and the crude residue was purified using a mixture of methanol and dichloromethane (in a ratio of 3:100), affording the title compound in 91% yield (90 mg, 0.29 mmol). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.39 (s, 3H, NCH_3), 2.67 (t, J = 5.01 Hz, 4H, 2 × NCH_2), 4.00 (t, J = 4.99 Hz, 4H, 2 × NCH_2), 7.47 (m, 3H, arom H), 7.65 (m, 2H, arom H), 7.89 (d, J = 1.83 Hz, 1H, arom H), 8.63 (d, J = 1.83 Hz, 1H, arom H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$): δ = 46.19 (CH_3), 50.25 (CH_2), 54.21 (CH_2), 125.61 (CH), 127.35 (CH), 128.32 (CH), 129.09 (CH), 134.19 (C_q), 135.64 (C_q), 137.64 (C_q), 144.13 (CH), 156.20 (C_q), 173.21 (C_q) ppm. HR-MS $[M + H]^+$ found 311.1330, calculated 311.1324.

3-(*N*-Me-piperazinyl)-6-(4-fluorophenyl)isothiazolo[4,3-*b*]-pyridine (10b). This compound was prepared using 4-fluorophenylboronic acid and the crude residue was purified using a mixture of methanol and dichloromethane (in a ratio of 3:100), affording the title compound in 96% yield (100 mg, 0.30 mmol). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.40 (s, 3H, NCH_3), 2.68 (br s, 4H, 2 × NCH_2), 4.00 (br s, 4H, 2 × NCH_2), 7.19 (t, J = 8.61 Hz, 1H, arom H), 7.59 (m, 2H, arom H), 7.84 (d, J = 2.01 Hz, 1H, arom H), 8.57 (d, J = 2.01 Hz, arom H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$): δ = 46.18 (CH_3), 50.24 (CH_2), 54.20 (CH_2), 116.05 (CH), 116.19 (CH), 125.46 (CH), 129.01 (CH), 129.06 (CH), 133.76 (C_q), 134.68 (C_q), 143.78 (CH), 156.03 (C_q), 162.18 (C_q), 163.83 (C_q), 173.26 (C_q) ppm. HR-MS $[M + H]^+$ found 329.1231, calculated 329.1230.

3-(*N*-Me-piperazinyl)-6-(3,4-dimethoxyphenyl)isothiazolo-[4,3-*b*]pyridine (10c). This compound was prepared using 3,4-dimethoxyphenylboronic acid and the crude residue was purified using a mixture of methanol and dichloromethane (in a ratio of 1:30), affording the title compound in 66% yield (78 mg, 0.21 mmol). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.27 (s, 3H, NCH_3), 2.56 (t, J = 4.91 Hz, 4H, 2 × NCH_2), 3.82 (s, 3H, OCH_3), 3.88 (s, 3H, OCH_3), 3.92 (t, J = 4.88 Hz, 4H, 2 × NCH_2), 7.09 (d, J = 8.43 Hz, 1H, arom H), 7.38 (m, 2H, arom H), 7.99 (d, J = 1.98 Hz, 1H, arom H), 8.75 (d, J = 2.01 Hz, 1H, arom H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$): δ = 46.20 (CH_3), 50.24 (CH_2), 54.22 (CH_2), 55.96 (CH_3), 55.99 (CH_3), 110.33 (CH), 111.64 (CH), 119.81 (CH), 124.76 (CH), 130.34 (C_q), 133.93 (C_q), 135.39 (C_q), 144.11 (CH), 149.45 (C_q), 149.47 (C_q), 156.30 (C_q), 173.12 (C_q) ppm. HR-MS $[M + H]^+$ found 371.1532, calculated 371.1536.

3-(*N*-Me-piperazinyl)-6-(3-thienyl)isothiazolo[4,3-*b*]pyridine (10d). This compound was prepared using 3-thienylboronic acid and the crude residue was purified using a mixture of methanol and dichloromethane (in a ratio of 3:100), affording the title compound in 84% yield (84 mg, 0.26 mmol). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.26 (s, 3H, NCH_3), 2.55 (t, J = 4.91 Hz, 4H, 2 × NCH_2), 3.91 (t, J = 4.88 Hz, 4H, 2 × NCH_2), 7.72 (m, 2H, arom H), 8.07 (br s, 1H, arom H), 8.07 (br s, 1H, arom H), 8.19 (br s, 1H, arom H), 8.83 (d, J = 1.98 Hz, 1H, arom H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$): δ = 46.18 (CH_3), 50.20 (CH_2), 54.20 (CH_2), 122.12 (CH), 124.24 (CH), 126.09 (CH), 127.00 (CH), 130.31 (C_q), 133.91 (C_q), 138.62 (C_q), 143.59 (CH), 156.21 (C_q), 173.10 (C_q) ppm. HR-MS $[M + H]^+$ found 317.0893, calculated 317.0850.

3-(*N*-Me-piperazinyl)-6-(3-pyridyl)isothiazolo[4,3-*b*]pyridine (10e). This compound was prepared using 3-pyridylboronic acid and the crude residue was purified using a mixture of methanol and dichloromethane (in a ratio of 1:20), affording the title compound in 83% yield (82 mg, 0.26 mmol). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.26 (s, 3H, NCH_3), 2.55 (t, J = 4.85 Hz, 4H, 2 × NCH_2), 3.92 (t, J = 4.80 Hz, 4H, 2 × NCH_2), 7.54 (m, 1H, arom H), 8.12 (d, J = 1.98 Hz, 1H, arom H), 8.24 (d, J = 7.86 Hz, 1H, arom H), 8.65 (d, J = 4.74 Hz, 1H, arom H), 8.75 (d, J = 1.92 Hz, 1H, arom H), 9.04 (d, J = 1.95 Hz, 1H, arom H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$): δ = 46.17 (CH_3), 50.24 (CH_2), 54.17 (CH_2), 123.79 (CH), 126.15 (CH), 132.44 (C_q), 133.41 (C_q), 134.58 (CH, C_q), 142.99 (CH), 148.36 (CH), 149.45 (CH), 155.68 (C_q), 173.43 (C_q) ppm. HR-MS $[M + H]^+$ found 312.1277, calculated 312.1277.

3-Methoxy-6-bromoisothiazolo[4,3-*b*]pyridine (7d). To a solution of 3,6-dibromoisothiazolo[4,3-*b*]pyridine (700 mg, 2.38 mmol) in absolute methanol (50 mL) was added carefully at 0 °C sodium methoxide (2.5 equiv, 322 mg) in small portions. The resulting reaction mixture was stirred overnight at room temperature and then heated at 55 °C for 8 h. The reaction was cooled to room temperature, neutralized with a 5% HCl solution, and evaporated in vacuo. The residue was divided between ethyl acetate (250 mL) and water (150 mL). The organic phase was dried and evaporated. The crude residue was purified by silica gel flash chromatography, the mobile phase being a mixture of cyclohexane and ethyl acetate (in a ratio gradually ranging from 5:1 to 4:1), yielding the pure title compound in 96% yield (558 mg, 2.27 mmol). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 4.35 (s, 3H, OCH_3), 8.37 (d, J = 2.01 Hz, 1H, arom H), 8.60 (d, J = 1.98 Hz, 1H, arom H) ppm.

3-Methoxy-6-arylisothiazolo[4,3-*b*]pyridines (11a–e). *General Procedure.* To a solution of 3-methoxy-6-bromoisothiazolo[4,3-*b*]pyridine (100 mg, 0.41 mmol) in dioxane (5 mL) and water (1 mL) were added an appropriate boronic acid (2 equiv), sodium carbonate (2 equiv, 87 mg) and Pd(dppf)Cl_2 (0.01 equiv, 33 mg). The reaction mixture was heated at 95 °C overnight. The reaction mixture was diluted with ethyl acetate (50 mL) and brine (30 mL). The aqueous phase was extracted with ethyl acetate (50 mL). The organic phases were dried and evaporated. The crude residue was purified by silica gel flash chromatography, yielding the pure title compounds.

The following compounds were made according to this procedure.

3-Methoxy-6-phenylisothiazolo[4,3-*b*]pyridine (11a). This compound was prepared using phenylboronic acid and the crude residue was purified using a mixture of cyclohexane and ethyl acetate (in a ratio of 2:1), affording the title compound in 87% yield (86 mg, 0.35 mmol). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.37 (s, 3H, OCH3), 7.50 (m, 3H, arom H), 7.68 (m, 2H, arom H), 8.00 (d, J = 2.01 Hz, 1H, arom H), 8.86 (d, J = 1.98 Hz, 1H, arom H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$): δ = 62.37 (CH_3), 125.55 (CH), 127.15 (CH), 128.28 (CH), 128.84 (CH), 133.08 (C_q), 136.47 (C_q), 136.85 (C_q), 148.37 (CH), 154.54 (C_q), 182.58 (C_q) ppm. HR-MS $[M + H]^+$ found 243.0584, calculated 243.0547.

3-Methoxy-6-(4-fluorophenyl)isothiazolo[4,3-*b*]pyridine (11b). This compound was prepared using 4-fluorophenylboronic acid and the crude residue was purified using a mixture of cyclohexane and ethyl acetate (in a ratio of 2:1), affording the title compound in 76%

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yield (81 mg, 0.31 mmol). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 4.36 (s, 3H, OCH_3), 7.37 (t, J = 8.79, 3H, arom H), 7.93 (m, 2H, arom H), 8.18 (d, J = 1.98 Hz, 1H, arom H), 8.93 (d, J = 1.92 Hz, 1H, arom H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$): δ = 62.75 (CH_3), 116.18 (CH), 116.33 (CH), 125.77 (CH), 129.21 (CH), 129.27 (CH), 133.31 (C_q), 135.83 (C_q), 148.39 (CH), 154.72 (C_q), 162.35 (C_q), 164.00 (C_q), 183.04 (C_q) ppm. HR-MS $[M + H]^+$ found 261.0503, calculated 261.0453.

3-Methoxy-6-(3,4-dimethoxyphenyl)isothiazolo[4,3-*b*]-pyridine (11c). This compound was prepared using 3,4-dimethoxyphenylboronic acid and the crude residue was purified using a mixture of cyclohexane and ethyl acetate (in a ratio of 1:1), affording the title compound in 74% yield (91 mg, 0.30 mmol). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.82 (s, 3H, OCH_3), 3.89 (s, 3H, OCH_3), 4.35 (s, 3H, OCH_3), 7.09 (d, J = 9 Hz, 1H, arom H), 7.42 (m, 2H, arom H), 8.16 (d, J = 1.92 Hz, 1H, arom H), 8.97 (d, J = 1.92 Hz, 1H, arom H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$): δ = 56.02 (CH_3), 56.04 (CH_3), 62.69 (CH_2), 110.42 (CH), 111.67 (CH), 120.11 (CH), 125.02 (CH), 129.90 (C_q), 133.18 (C_q), 136.58 (C_q), 148.71 (CH), 149.55 (C_q), 149.74 (C_q), 155.01 (C_q), 182.83 (C_q) ppm. HR-MS $[M + H]^+$ found 303.0796, calculated 303.0797.

3-Methoxy-6-(3-thienyl)isothiazolo[4,3-*b*]pyridine (11d). This compound was prepared using 3-thienyllboronic acid and the crude residue was purified using a mixture of cyclohexane and ethylacetae (in a ratio of 2:1), affording the title compound in 75% yield (74 mg, 0.30 mmol). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 4.35 (s, 3H, OCH_3), 7.74 (m, 1H, arom H), 7.82 (m, 1H, arom H), 8.25 (d, J = 1.98 Hz, 1H, arom H), 8.29 (m, 1H, arom H), 9.06 (d, J = 1.95 Hz, 1H, arom H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$): δ = 62.70 (CH_3), 122.73 (CH), 124.44 (CH), 126.08 (CH), 127.26 (CH), 131.43 (C_q), 133.17 (C_q), 138.19 (C_q), 148.18 (CH), 154.92 (C_q), 182.84 (C_q) ppm. HR-MS $[M + H]^+$ found 249.0156, calculated 249.0150.

3-Methoxy-6-(3-pyridyl)isothiazolo[4,3-*b*]pyridine (11e). This compound was prepared using 3-pyridylboronic acid and the crude residue was purified using a mixture of methanol and dichloromethane (in a ratio of 1:30), affording the title compound in 55% yield (54 mg, 0.22 mmol). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 4.40 (s, 3H, OCH_3), 7.57 (m, 1H, arom H), 8.31 (m, 2H, arom H), 8.67 (d, J = 4.23 Hz, 1H, arom H), 8.99 (d, J = 1.71 Hz, 1H, arom H), 9.09 (d, J = 1.89 Hz, 1H, arom H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$): δ = 62.87 (CH_2), 123.86 (CH), 126.60 (CH), 133.05 (C_q), 133.64 (C_q), 133.87 (C_q), 134.77 (CH), 147.75 (CH), 148.49 (CH), 149.78 (CH), 154.39 (C_q), 183.38 (C_q) ppm. HR-MS $[M + H]^+$ found 244.0542, calculated 244.0500.

Synthesis of 3-Morpholino-6-arylisothiazolo[4,3-*b*]pyridines (12a–j). *General Procedure.* To a solution of 3-morpholino-6-bromo-isothiazolo[4,3-*b*]pyridine in DME (2 mL) were added an appropriate boronic acid (2 equiv or 1.5 equiv) and potassium carbonate (2 equiv, 1 M solution in H_2O). Mixture was degassed, and $Pd(PPh_3)_4$ (10 mol %) was added. The reaction was heated at 80 °C. After the completion of reaction, solvents were evaporated. The crude residue was purified by silica gel flash chromatography, yielding the pure title compounds.

The following compounds were made according to this procedure.

4-(6-(Thiophen-2-yl)isothiazolo[4,3-*b*]pyridin-3-yl)-morpholine (12a). This compound was prepared from 3-morpholino-6-bromoisothiazolo[4,3-*b*]pyridine (90 mg, 0.3 mmol) using thiophene-2-boronic acid (0.6 mmol, 76 mg), 1 M K_2CO_3 (0.6 mL), and $Pd(PPh_3)_4$ (0.03 mmol, 34 mg). The crude product was purified using a mixture of cyclohexane/ethyl acetate in a ratio of 1:1, affording the title compound in 78% yield (71 mg, 0.234 mmol). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.96 (m, 8H, 4 × CH_2), 7.16 (dd, J = 3.66 Hz, J = 5.10 Hz, 1H, arom H), 7.42 (dd, J = 1.08 Hz, J = 5.10 Hz, 1H, arom H), 7.49 (dd, J = 3.66 Hz, J = 1.08 Hz, 1H, arom H), 7.93 (d, J = 2.07 Hz, 1H, arom H), 8.69 (d, J = 2.10 Hz, 1H, arom H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$): δ = 50.35 (CH_2), 66.17 (CH_2), 123.49 (CH), 124.99 (CH), 126.56 (CH), 128.39 (CH), 129.36 (C_q), 134.08 (C_q), 140.42 (C_q), 143.12 (CH), 155.91 (C_q), 173.24 (C_q) ppm. HR-MS $[M + H]^+$ found 304.0577, calculated 304.0572.

4-(6-(Furan-3-yl)isothiazolo[4,3-*b*]pyridin-3-yl)morpholine (12b). This compound was prepared from 3-morpholino-6-bromo-isothiazolo[4,3-*b*]pyridine (40 mg, 0.133 mmol) using furan-3-boronic acid (0.2 mmol, 23 mg), 1 M K_2CO_3 (0.26 mL), and $Pd(PPh_3)_4$ (0.013 mmol, 36 mg). The product was purified using a mixture of cyclohexane/ethyl acetate in a ratio of 2:1, affording the title compound in 51% yield (19 mg, 0.067 mmol). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.96 (m, 8H, 4 × CH_2), 6.79 (s, 1H, arom H), 7.56 (m, 1H, arom H), 7.81 (d, J = 2.01 Hz, 1H, arom H), 7.90 (s, 1H, arom H), 8.56 (d, J = 2.01 Hz, 1H, arom H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$): δ = 50.37 (CH_2), 66.19 (CH_2), 108.55 (CH), 123.25 (C_q), 123.59 (CH), 127.58 (C_q), 133.94 (C_q), 139.58 (CH), 143.40 (CH), 144.26 (CH), 156.19 (C_q), 173.23 (C_q) ppm. HR-MS $[M + H]^+$ found 288.0803, calculated 288.0801.

4-(6-(2,4-Dimethoxyphenyl)isothiazolo[4,3-*b*]pyridin-3-yl)-morpholine (12c). This compound was prepared from 3-morpholino-6-bromoisothiazolo[4,3-*b*]pyridine (90 mg, 0.3 mmol) using 2,4-dimethoxyphenylboronic acid (0.6 mmol, 109 mg), 1 M K_2CO_3 (0.6 mL), and $Pd(PPh_3)_4$ (0.03 mmol, 34 mg). The crude product was purified using a mixture of cyclohexane/ethyl acetate in a ratio of 2:1, affording the title compound in 73% yield (79 mg, 0.221 mmol). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.84 (s, 3H, OCH_3), 3.88 (s, 3H, OCH_3), 3.96 (m, 8H, 4 × CH_2), 6.60–6.64 (m, 2H, arom H), 7.33 (d, J = 8.19 Hz, 1H, arom H), 7.84 (d, J = 1.95 Hz, 1H, arom H), 8.57 (d, J = 1.92 Hz, 1H, arom H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$): δ = 50.14 (CH_2), 55.17 (CH_3), 55.22 (CH_3), 65.92 (CH_2), 98.76 (CH), 104.77 (CH), 119.29 (C_q), 127.22 (CH), 131.03 (CH), 133.16 (C_q), 133.52 (C_q), 146.66 (CH), 156.22 (C_q), 157.56 (C_q), 161.00 (C_q), 172.70 (C_q) ppm. HR-MS $[M + H]^+$ found 358.1224, calculated 358.1219.

4-(6-(2,5-Dimethoxyphenyl)isothiazolo[4,3-*b*]pyridin-3-yl)-morpholine (12d). This compound was prepared from 3-morpholino-6-bromoisothiazolo[4,3-*b*]pyridine (90 mg, 0.3 mmol) using 2,5-dimethoxyphenylboronic acid (0.6 mmol, 109 mg), 1 M K_2CO_3 (0.6 mL), and $Pd(PPh_3)_4$ (0.03 mmol, 34 mg). The product was purified using a mixture of cyclohexane/ethyl acetate in a ratio of 2:1, affording the title compound in 69% yield (74 mg, 0.207 mmol). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.80 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3), 3.97 (m, 8H, 4 × CH_2), 6.95–6.97 (m, 3H, arom H), 7.89 (d, J = 1.95 Hz, 1H, arom H), 8.58 (d, J = 1.92 Hz, 1H, arom H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$): δ = 50.13 (CH_2), 55.51 (CH_3), 55.89 (CH_3), 65.92 (CH_2), 112.35 (CH), 114.15 (CH), 116.19 (CH), 123.33 (C_q), 127.36 (C_q), 127.77 (CH), 133.55 (C_q), 146.23 (CH), 150.71 (C_q), 153.60 (C_q), 155.98 (C_q), 172.84 (C_q) ppm. HR-MS $[M + H]^+$ found 358.1221, calculated 358.1219.

4-(6-(3,5-Dimethoxyphenyl)isothiazolo[4,3-*b*]pyridin-3-yl)-morpholine (12e). This compound was prepared from 3-morpholino-6-bromoisothiazolo[4,3-*b*]pyridine (60 mg, 0.2 mmol) using 3,5-dimethoxyphenylboronic acid (0.4 mmol, 72 mg), 1 M K_2CO_3 (0.4 mL), and $Pd(PPh_3)_4$ (0.02 mmol, 23 mg). The crude product was purified using a mixture of cyclohexane/ethyl acetate in a ratio of 2:1, affording the title compound in 70% yield (50 mg, 0.140 mmol). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.88 (s, 6H, 2 × OCH_3), 3.98 (m, 8H, 4 × CH_2), 6.55 (m, 1H, arom H), 6.80 (d, J = 2.19 Hz, 2H, arom H), 7.92 (d, J = 1.98 Hz, 1H, arom H), 8.63 (d, J = 2.01 Hz, 1H, arom H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$): δ = 50.09 (CH_2), 55.16 (CH_3), 65.88 (CH_2), 99.97 (CH), 105.32 (CH), 125.46 (CH), 134.18 (C_q), 135.48 (C_q), 139.32 (C_q), 144.14 (CH), 155.83 (C_q), 160.99 (C_q), 173.01 (C_q) ppm. HR-MS $[M + H]^+$ found 358.1218, calculated 358.1219.

4-(6-(Benzo[*d*][1,3]dioxol-5-yl)isothiazolo[4,3-*b*]pyridin-3-yl)morpholine (12f). This compound was prepared from 3-morpholino-6-bromoisothiazolo[4,3-*b*]pyridine (60 mg, 0.2 mmol) using 3,4-methylenedioxyphenylboronic acid (0.4 mmol, 66 mg), 1 M K_2CO_3 (0.4 mL), and $Pd(PPh_3)_4$ (0.02 mmol, 23 mg). Product was purified using a mixture of cyclohexane/ethyl acetate in a ratio of 2:1, affording the title compound in 64% yield (43 mg, 0.128 mmol). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.96 (m, 8H, 4 × CH_2), 6.05 (s, 2H, CH_2), 6.94 (d, J = 7.86 Hz, 1H, arom H), 7.15 (m, 2H, arom H), 7.83 (d, J = 2.01 Hz, 1H, arom H), 8.58 (d, J = 2.04 Hz, 1H, arom H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$): δ = 50.38 (CH_2), 66.19 (CH_2), 101.41 (CH_2), 107.66 (CH), 108.94 (CH), 121.23 (CH), 124.98 (CH), 131.65 (C_q), 134.11 (C_q), 135.55 (C_q), 144.47 (CH), 148.05 (C_q), 148.45 (C_q), 156.22 (C_q), 173.24 (C_q). HR-MS $[M + H]^+$ found 342.0905, calculated 342.0906.

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4-(6-(3,4,5-Trimethoxyphenyl)isothiazolo[4,3-*b*]pyridin-3-yl)morpholine (12g). This compound was prepared from 3-morpholino-6-bromoisothiazolo[4,3-*b*]pyridine (76 mg, 0.25 mmol) using 3,4,5-trimethoxyphenylboronic acid (0.5 mmol, 107 mg), 1 M K_2CO_3 (0.5 mL), and $Pd(PPh_3)_4$ (0.025 mmol, 29 mg). The crude product was purified using a mixture of cyclohexane/ethyl acetate in a ratio of 2:1, affording the title compound in 56% yield (54 mg, 0.141 mmol). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.72 (s, 3H, OCH_3), 3.86 (s, 6H, 2 × OCH_3), 3.90 (m, 8H, 4 × CH_2), 7.10 (s, 2H, arom H), 8.08 (d, J = 2.01 Hz, 1H, arom H), 8.78 (d, J = 1.98 Hz, 1H, arom H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ = 50.15 (CH_2), 56.24 (OCH_3), 60.21 (OCH_3), 65.52 (CH_2), 104.97 (CH), 124.83 (CH), 132.39 (C), 133.59 (C), 135.00 (C), 138.11 (C), 144.37 (CH), 153.53 (C), 156.68 (C), 172.18 (C) ppm. HR-MS [M + H]$^+$ found 388.1323, calculated 388.1325.

2-Methoxy-4-(3-morpholinoisothiazolo[4,3-*b*]pyridin-6-yl)-phenyl Acetate (12h). This compound was prepared from 3-morpholino-6-bromoisothiazolo[4,3-*b*]pyridine (60 mg, 0.2 mmol) using 4-acetoxy-3-methoxyphenylboronic acid pinacol ester (0.4 mmol, 116 mg), 1 M K_2CO_3 (0.4 mL), and $Pd(PPh_3)_4$ (0.02 mmol, 23 mg). Product was purified using a mixture of cyclohexane/ethyl acetate in a ratio of 2:1, affording the title compound in 58% yield (45 mg, 0.116 mmol). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.37 (s, 3H, CH_3(acetyl)), 3.93 (s, 3H, OCH_3), 3.98 (m, 8H, 4 × CH_2), 7.16−7.28 (m, 3H, arom H), 7.91 (d, J = 1.98 Hz, 1H, arom H), 8.53 (d, J = 2.01 Hz, 1H, arom H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$): δ = 20.67 (CH_3), 50.38 (CH_2), 55.97 (CH_3), 66.18 (CH_2), 111.51 (CH), 119.80 (CH), 123.44 (CH), 125.71 (CH), 134.40 (C_q), 135.32 (C_q), 136.58 (C_q), 140.06 (C_q), 144.27 (CH), 151.53 (C_q), 156.06 (C_q), 169.00 (CO), 173.36 (C_q) ppm. HR-MS [M + H]$^+$ found 386.1165, calculated 386.1168.

2-Methoxy-4-(3-morpholinoisothiazolo[4,3-*b*]pyridin-6-yl)-aniline (12i). This compound was prepared from 3-morpholino-6-bromoisothiazolo[4,3-*b*]pyridine (60 mg, 0.2 mmol) using 4-amino-3-methoxyphenylboronic acid pinacol ester (0.4 mmol, 99 mg), 1 M K_2CO_3 (0.4 mL), and $Pd(PPh_3)_4$ (0.02 mmol, 23 mg). The crude product was purified using a mixture of cyclohexane/ethyl acetate in a ratio of 2:1, affording the title compound in 58% yield (40 mg, 0.116 mmol). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.89 (m, 11H, 4 × CH_2 and OCH_3), 5.09 (br s, 2H, NH_2), 6.75 (d, J = 8.1 Hz, 1H, arom H), 7.22 (m, 2H, arom H), 7.90 (d, J = 1.86 Hz, 1H, arom H), 8.74 (d, J = 1.86 Hz, 1H, arom H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ = 50.12 (CH_2), 55.63 (CH_3), 65.54 (CH_2), 109.43 (CH), 113.91 (CH), 120.21 (CH), 122.21 (CH), 124.09 (CH), 132.86 (C_q), 135.49 (C_q), 138.89 (C_q), 144.44 (CH), 146.85 (C_q), 156.18 (C_q), 172.53 (C_q) ppm. HR-MS [M + H]$^+$ found 343.1222, calculated 343.1223.

2-Methoxy-4-(3-morpholinoisothiazolo[4,3-*b*]pyridin-6-yl)-phenol (12j). This compound was prepared from 3-morpholino-6-bromoisothiazolo[4,3-*b*]pyridine (60 mg, 0.2 mmol) using methoxy-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenol (0.3 mmol, 75 mg), 1 M K_2CO_3 (0.4 mL), and $Pd(PPh_3)_4$ (0.02 mmol, 23 mg). The crude product was purified using a mixture of cyclohexane/ethyl acetate in a ratio of 3:2, affording the title compound in 44% yield (30 mg, 0.087 mmol). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.97 (m, 11H, 4 × CH_2, CH_3), 5.93 (s, 1H, OH), 7.04 (d, J = 8.16 Hz, 1H, arom H), 7.14 (d, J = 1.95 Hz, 1H, arom H), 7.19 (dd, J = 2.04 Hz, J = 8.16 Hz, 1H, arom H), 7.87 (d, J = 2.07 Hz, 1H, arom H), 8.64 (d, J = 2.07 Hz, 1H, arom H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ = 50.43 (CH_2), 56.05 (OCH3), 66.23 (CH_2), 109.80 (CH), 115.14 (CH), 120.61 (CH), 124.74 (CH), 129.75 (C), 132.12 (C), 135.78 (C), 144.62 (CH), 146.37 (C), 147.15 (C), 156.37 (C), 173.25 (C) ppm. HR-MS [M + H]$^+$ found 344.1067, calculated 344.1063.

Synthesis of 3-Substituted-6-(3,4-dimethoxyphenyl)-isothiazolo[4,3-*b*]pyridine (13a−k). *General Procedure A.* To a solution of 3,6-dibromoisothiazolo[4,3-*b*]pyridine in ethanol was added an appropriate nitrogen nucleophile (3 equiv). The reaction was stirred at 75 °C. After reaction finished, solvent was evaporated in vacuo and the crude residue was purified by silica gel flash chromatography yielding the 3-substituted-6-bromoisothiazolo[4,3-*b*]pyridine derivatives. To a solution of 3-substituted-6-bromoisothiazolo[4,3-*b*]pyridine in DME (2 mL) were added 3,4-dimethoxyphenylboronic acid (1.5 equiv) and potassium carbonate (2 equiv, 1 M solution in H_2O). The mixture was degassed and $Pd(PPh_3)_4$ (10 mol %) was added. The reaction was heated at 80 °C. After the completion of reaction, solvents were evaporated. The crude residue was purified by silica gel flash chromatography, yielding the pure title compounds.

The following compounds were made according to this procedure: **13b**, **13c**, **13d**, **13e**, **13i**, **13j**, **13k**.

General Procedure B. To a solution of 3,6-dibromoisothiazolo[4,3-*b*]pyridine in ethanol was added an appropriate nitrogen nucleophile (3 equiv). The reaction was stirred at 70 °C. After reaction finished, solvent was evaporated in vacuo and the crude residue was purified by silica gel flash chromatography, yielding the 3-substituted-6-bromoisothiazolo-[4,3-*b*]pyridine derivatives. To a solution of 3-substituted-6-bromo-isothiazolo[4,3-*b*]pyridine in dioxane (2 mL) were added 3,4-dimethoxyphenylboronic acid (1.5 equiv) and potassium carbonate (3 equiv). The mixture was degassed, and $Pd(dppf)Cl_2$ (5 mol %) was added. The reaction was heated at 100 °C. After the completion of reaction, solvents were evaporated. The crude residue was purified by silica gel flash chromatography, yielding the pure title compounds.

The following compounds were made according to this procedure: **13a**, **13f**, **13g**, and **13h**.

6-(3,4-Dimethoxyphenyl)-*N*,*N*-dimethylisothiazolo[4,3-*b*]-pyridin-3-amine (13a). 6-Bromo-*N*,*N*-dimethylisothiazolo[4,3-*b*]-pyridin-3-amine was prepared from 3,6-di-bromoisothiazolo[4,3-*b*]-pyridine (0.5 mmol, 146 mg) and dimethylamine (44 μL, 1.5 mmol) in EtOH (10 mL). The product was purified using a mixture of cyclohexane/ethyl acetate in a ratio of 4:1, affording the 6-bromo-*N*,*N*-dimethylisothiazolo[4,3-*b*]pyridin-3-amine in 98 % yield (379 mg, 0.49 mmol). The title compound was prepared from 6-bromo-*N*,*N*-dimethylisothiazolo[4,3-*b*]pyridin-3-amine (52 mg, 0.2 mmol) using 3,4-dimethoxyphenylboronic acid (0.3 mmol, 54.5 mg), K_2CO_3 (83 mg, 0.6 mmol), and $Pd(dppf)Cl_2$ (0.01 mmol, 7.4 mg). The crude product was purified using a mixture of cyclohexane/ethyl acetate in a ratio of 1:1, affording the title compound in 72% yield (46.6 mg, 1.5 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 3.49 (s, 6H, 2 × CH_3), 3.92 (s, 3H, OCH_3), 3.94 (s, 3H, OCH_3), 6.97 (d, J = 8.3 Hz, 1H, arom H), 7.15 (s, 1H, arom H), 7.21 (dd, J = 2.1 Hz, J = 8.3 Hz, 1H arom H), 7.80 (d, J = 2.0 Hz, 1H, arom H), 8.56 (d, J = 2.0 Hz, 1H, arom H) ppm. ^{13}C NMR (300 MHz, $CDCl_3$), δ = 43.0 (CH_3), 56.1 (OCH_3), 56.1 (OCH_3), 110.5 (CH), 111.8 (CH), 119.9 (CH), 124.6 (CH), 130.6 (C), 133.5 (C), 135.3 (C), 143.4 (CH), 149.5 (C), 156.2 (C), 173.2 (C) ppm. HR-MS: [M + H]$^+$ found 316.1113, calculated 316.1114.

6-(3,4-Dimethoxyphenyl)-*N*-(2-methoxyethyl)isothiazolo-[4,3-*b*]pyridin-3-amine (13b). 6-Bromo-*N*-(2-methoxyethyl)-isothiazolo[4,3-*b*]pyridin-3-amine was prepared from 3,6-di-bromo-isothiazolo[4,3-*b*]pyridine (0.5 mmol, 146 mg) and 2-methoxyethyl-amine (0.13 mL, 1.5 mmol) in EtOH (10 mL). The crude product was purified using a mixture of cyclohexane/ethyl acetate in a ratio of 4:1, affording the 6-bromo-*N*-(2-methoxyethyl)isothiazolo[4,3-*b*]pyridin-3-amine in 83% yield (120 mg, 0.416 mmol). The title compound was prepared from 6-bromo-*N*-(2-methoxyethyl)isothiazolo[4,3-*b*]pyridin-3-amine (57 mg, 0.2 mmol) using 3,4-dimethoxyphenylboronic acid (0.3 mmol, 54.5 mg), 1 M K_2CO_3 (0.40 mL), and $Pd(PPh_3)_4$ (0.02 mmol, 23 mg). The product was purified using a mixture of cyclohexane/ethyl acetate in a ratio of 1:1, affording the title compound in 22% yield (15 mg, 0.043 mmol). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.40 (s, 3H, OCH_3), 3.55 (m, 2H, CH_2), 3.58 (m, 2H, CH_2), 3.95 (s, 3H, OCH_3), 3.97 (s, 3H, OCH_3), 6.53 (m, 1H, NH), 6.99 (d, J = 8.31 Hz, 1H, arom H), 7.15 (d, 1H, J = 1.98 Hz, arom H), 7.21 (dd, J = 1.98 Hz, J = 8.22 Hz, 1H, arom H), 7.87 (d, J = 1.80 Hz, 1H, arom H), 8.60 (d, J = 1.80 Hz, 1H, arom H) ppm. ^{13}C NMR (125 MHz, DMSO-d_6): δ = 47.33 (CH_2), 55.70 (OCH_3), 58.67 (OCH_3), 69.76 (CH2), 110.24 (CH), 111.38 (CH), 119.64 (CH), 124.25 (CH), 130.24 (C), 133.24 (C), 136.37 (C), 144.33 (CH), 149.20 (C), 149.23 (C), 154.40 (C), 171.72 (C) ppm. HR-MS [M + H]$^+$ found 346.1223, calculated 346.1219.

2,2′-(6-(3,4-Dimethoxyphenyl)isothiazolo[4,3-*b*]pyridin-3-ylazanediyl)diethanol (13c). 2,2′-(6-Bromoisothiazolo[4,3-*b*]-pyridin-3-ylazanediyl)diethanol was prepared from 3,6-dibromo-isothiazolo[4,3-*b*]pyridine (0.5 mmol, 146 mg) and diethanolamine (0.145 mL, 1.5 mmol) in EtOH (10 mL). The crude product was

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purified using a mixture of DCM/MeOH in a ratio of 95:5, affording 2,2′-(6-bromoisothiazolo[4,3-*b*]pyridin-3-ylazanediyl)diethanol in 81% yield (129 mg, 0.405 mmol). The title compound was prepared from 2,2′-(6-bromoisothiazolo[4,3-*b*]pyridin-3-ylazanediyl)diethanol (63.6 mg, 0.2 mmol) using 3,4-dimethoxyphenylboronic acid (0.3 mmol, 54.5 mg), 1 M K_2CO_3 (0.4 mL), and $Pd(PPh_3)_4$ (0.02 mmol, 23 mg). The crude product was purified using a mixture of DCM/MeOH in a ratio of 100:1, affording the title compound in 60% yield (45 mg, 0.12 mmol). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.96 (s, 3H, OCH_3), 3.98 (s, 3H, OCH_3), 4.05 (m, 8H, 4 × CH_2), 4.20 (m, 2H, 2 × OH), 7.02 (d, J = 8.31 Hz, 1H, arom H), 7.15 (d, J = 2.04 Hz, 1H, arom H), 7.24 (dd, J = 8.28, J = 2.13 Hz, 1H, arom H), 7.86 (d, J = 2.04 Hz, 1H, arom H), 8.56 (d, J = 2.04 Hz, 1H, arom H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ = 55.70 (OCH_3), 57.80 (CH_2), 59.36 (CH2), 109.98 (CH), 111.38 (CH), 119.52 (CH), 124.54 (CH), 129.60 (C), 132.80 (C), 135.39 (C), 143.58 (CH), 149.21 (C), 149.33 (C), 155.62 (C), 172.05 (C) ppm. HR-MS [M + H]$^+$ found 376.1322, calculated 376.1252.

6-(3,4-Dimethoxyphenyl)-3-(pyrrolidin-1-yl)isothiazolo[4,3-*b*]pyridine (13d). 6-Bromo-3-(pyrrolidin-1-yl)isothiazolo[4,3-*b*]-pyridine was prepared from 3,6-di-bromoisothiazolo[4,3-*b*]pyridine (0.5 mmol, 146 mg) and pyrrolidine (0.04 mL, 1.5 mmol) in EtOH (10 mL). The crude product was purified using a mixture of cyclohexane/ethyl acetate in a ratio of 4:1, affording the 6-bromo-3-(pyrrolidin-1-yl)isothiazolo[4,3-*b*]pyridine in 90% yield (128 mg, 0.450 mmol). The title compound was prepared from 6-bromo-3-(pyrrolidin-1-yl)-isothiazolo[4,3-*b*]pyridine (71 mg, 0.25 mmol) using 3,4-dimethoxyphenylboronic acid (0.375 mmol, 68 mg), 1 M K_2CO_3 (0.50 mL), and $Pd(PPh_3)_4$ (0.025 mmol, 29 mg). The crude product was purified using a mixture of cyclohexane/ethyl acetate in a ratio of 3:2, affording the title compound in 63% yield (54 mg, 0.158 mmol). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.17 (m, 4H, 2 × CH_2), 3.87 (m, 4H, 2 × CH_2), 3.96 (s, 3H, OCH_3), 3.98 (s, 3H, OCH_3), 7.00 (d, J = 8.28 Hz, 1H, arom H), 7.18 (d, 1H, J = 2.01 Hz, arom H), 7.26 (dd, J = 8.52, J = 2.28 Hz, 1H, arom H), 7.82 (d, J = 1.95 Hz, 1H, arom H), 8.58 (d, J = 1.95 Hz, 1H, arom H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ = 25.37 (CH_2), 51.68 (CH_2), 55.63 (OCH3), 55.67 (OCH_3), 110.12 (CH), 111.36 (CH), 119.45 (CH), 123.73 (CH), 130.89 (C), 133.44 (C), 135.20 (C), 142.98 (CH), 149.06 (C), 149.11 (C), 155.50 (C), 169.25 (C) ppm. HR-MS [M + H]$^+$ found 342.1273, calculated 342.1270.

***N*-(Cyclopropylmethyl)-6-(3,4-dimethoxyphenyl)isothiazolo-[4,3-*b*]pyridin-3-amine (13e).** 6-Bromo-*N*-(cyclopropylmethyl)-isothiazolo[4,3-*b*]pyridin-3-amine was prepared from 3,6-dibromo-isothiazolo[4,3-*b*]pyridine (0.5 mmol, 146 mg) and aminomethyl-cyclopropane (0.086 mL, 3.0 mmol) in EtOH (10 mL). The crude product was purified using a mixture of cyclohexane/ethyl acetate in a ratio of 9:1, affording the 6-bromo-*N*-(cyclopropylmethyl)isothiazolo-[4,3-*b*]pyridin-3-amine in 84 % yield (120 mg, 0.422 mmol). The title compound was prepared from 6-bromo-*N*-(cyclopropylmethyl)-isothiazolo[4,3-*b*]pyridin-3-amine (85 mg, 0.3 mmol) using 3,4-dimethoxyphenylboronic acid (0.45 mmol, 82 mg), 1 M K_2CO_3 (0.60 mL), and $Pd(PPh_3)_4$ (0.03 mmol, 34 mg). The crude residue was purified using a mixture of cyclohexane/ethyl acetate in a ratio of 4:1, affording the title compound in 73% yield (75 mg, 0.210 mmol). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.67 (q, 2H, CH_2), 0.69 (q, 2H, CH_2), 1.25 (m, 1H, CH), 3.24 (q, 2H, NCH_2), 3.96 (s, 3H, OCH_3), 3.98 (s, 3H, OCH_3), 6.36 (t, 1H, NH), 7.02 (d, J = 8.34 Hz, 1H, arom H), 7.17 (d, J = 2.07 Hz, 1H, arom H), 7.25 (dd, J = 8.28, J = 2.1 Hz, 1H, arom H), 7.88 (d, J = 1.92 Hz, 1H, arom H), 8.59 (d, J = 1.95 Hz, 1H, arom H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ = 3.93 (CH_2), 10.42 (CH), 53.11 (CH_2), 56.07 (OCH_3), 110.58 (CH), 111.43 (CH), 120.00 (CH), 124.71 (CH), 130.58 (C), 132.14 (C), 136.74 (C), 144.39 (CH), 149.59 (C), 149.65 (C), 154.71 (C), 171.77 (C) ppm. HR-MS [M + H]$^+$ found 342.1269, calculated 342.1270.

6-(3,4-Dimethoxyphenyl)-3-(piperidin-1-yl)isothiazolo[4,3-*b*]pyridine (13f). 6-Bromo-3-(piperidin-1-yl)isothiazolo[4,3-*b*]-pyridine was prepared from 3,6-dibromoisothiazolo[4,3-*b*]pyridine (0.5 mmol, 146 mg) and piperidine (148 μL, 1.5 mmol) in EtOH (10 mL). The crude product was purified using a mixture of cyclohexane/ethyl acetate in a ratio of 4:1, affording 6-bromo-*N*,*N*-dimethyl-isothiazolo[4,3-*b*]pyridin-3-amine in 84 % yield (125 mg, 0.42 mmol). The title compound was prepared from 6-bromo-3-(piperidin-1-yl)isothiazolo[4,3-*b*]pyridine (60 mg, 0.2 mmol) using 3,4-dimethoxyphenylboronic acid (0.3 mmol, 54.5 mg), K_2CO_3 (83 mg, 0.6 mmol), and $Pd(dppf)Cl_2$ (0.01 mmol, 7.4 mg). The crude product was purified using a mixture of cyclohexane/ethyl acetate in a ratio of 1:1, affording the title compound in 62% yield (43 mg, 0.12 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 1.74 (m, 6H, 3 × CH_2), 3.88 (br s, 4H, 2 × NCH_2), 3.91 (s, 3H, OCH_3), 3.90 (s, 3H, OCH_3), 6.95 (d, J = 8.31 Hz, 1H, arom H), 7.13 (s, 1H, arom H), 7.19 (dd, J = 1.93 Hz, J = 8.26 Hz, 1H arom H), 7.80 (d, J = 1.98 Hz, 1H, arom H), 8.57 (d, J = 1.98 Hz, 1H, arom H) ppm. ^{13}C NMR (300 MHz, $CDCl_3$), δ = 24.0 (CH_2), 25.3 (CH_2), 51.8 (CH_2), 56.0 (CH_3), 56.0 (CH_3), 110.4 (CH), 111.7 (CH), 119.8 (CH), 124.6 (CH), 130.5 (C), 133.7 (C), 135.3 (C), 143.5 (CH), 149.5 (C), 156.3 (C), 173.5 (C) ppm. HR-MS [M + H]$^+$ found 356.1426, calculated 356.1427.

6-(3,4-Dimethoxyphenyl)-3-thiomorpholinoisothiazolo[4,3-*b*]pyridine (13g). 6-Bromo-3-thiomorpholinoisothiazolo[4,3-*b*]-pyridine was prepared from 3,6-dibromoisothiazolo[4,3-*b*]pyridine (0.5 mmol, 146 mg) and thiomorpholine (151 μL, 1.5 mmol) in EtOH (10 mL). The product was purified using a mixture of cyclohexane/ethyl acetate in a ratio of 4:1, affording the 6-bromo-3-thiomorpholinoisothiazolo[4,3-*b*]pyridine in 85 % yield (134.5 mg, 0.425 mmol). The title compound was prepared from 6-bromo-3-thiomorpholinoisothiazolo[4,3-*b*]pyridine (63 mg, 0.2 mmol) using 3,4-dimethoxyphenylboronic acid (0.3 mmol, 54.5 mg), K_2CO_3 (83 mg, 0.6 mmol), and $Pd(dppf)Cl_2$ (0.01 mmol, 7.4 mg). The crude residue was purified using a mixture of cyclohexane/ethyl acetate in a ratio of 1:1, affording the title compound in 51% yield (38 mg, 0.102 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 2.85 (t, J = 4.97 Hz 4H, 2 × SCH_2), 3.92 (s, 3H, OCH_3), 3.94 (s, 3H, OCH_3), 4.29 (t, J = 4.99 Hz 4H, 2 × NCH_2), 6.96 (d, J = 8.25 Hz, 1H, arom H), 7.13 (s, 1H, arom H), 7.20 (dd, J = 1.45, J = 8.26 Hz, 1H, arom H), 7.81 (d, J = 1.95 Hz, 1H, arom H), 8.58 (d, J = 1.41 Hz, 1H, arom H) ppm. ^{13}C NMR (300 MHz, $CDCl_3$), δ = 26.5 (CH_2), 53.2 (CH_2), 56.0 (CH_3), 56.0 (CH_3), 110.4 (CH), 111.7 (CH), 119.9 (CH), 124.8 (CH), 130.3 (C), 133.7 (C), 135.6 (C), 144.2 (CH), 149.5 (C), 149.6 (C), 156.6 (C), 172.3 (C) ppm. HR-MS [M + H]$^+$ found 374.0987, calculated 374.0991.

(2*R*,6*S*)-4-(6-(3,4-Dimethoxyphenyl)isothiazolo[4,3-*b*]-pyridin-3-yl)-2,6-dimethylmorpholine (13h). (2*R*,6*S*)-4-(6-Bromoisothiazolo[4,3-*b*]pyridin-3-yl)-2,6-dimethylmorpholine was prepared from 3,6-dibromoisothiazolo[4,3-*b*]pyridine (0.5 mmol, 146 mg) and *cis*-2,6-dimethylmopholine (185 μL, 1.5 mmol) in EtOH (10 mL). The crude product was purified using a mixture of cyclohexane/ethyl acetate in a ratio of 4:1, affording the (2*R*,6*S*)-4-(6-bromoisothiazolo[4,3-*b*]pyridin-3-yl)-2,6-dimethylmorpholine in 74 % yield (121.4 mg, 0.37 mmol). The title compound was prepared from (2*R*,6*S*)-4-(6-bromoisothiazolo[4,3-*b*]pyridin-3-yl)-2,6-dimethyl-morpholine (65 mg, 0.2 mmol) using 3,4-dimethoxyphenylboronic acid (0.3 mmol, 54.5 mg), K_2CO_3 (83 mg, 0.6 mmol), and $Pd(dppf)Cl_2$ (0.01 mmol, 7.4 mg). The crude product was purified using a mixture of cyclohexane/ethyl acetate in a ratio of 1:1, affording the title compound in 40% yield (31 mg, 0.08 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 1.31 (d, J = 6.3 Hz, 6H, 2 × CH_3), 2.90 (t, J = 11.7 Hz, 2H, 2 × NCH), 3.94 (m, 8H, 2 × OCH_3, 2 × OCH), 4.50, (d, J = 12.6 Hz, 2 × NCH), 6.98 (d, J = 8.4 Hz, 1H, arom H), 7.15 (d, J = 2.1 Hz, 1H, arom H), 7.23 (dd, J = 2.2 Hz, J = 8.3 Hz, 1H arom H), 7.84 (d, J = 2.1 Hz, 1H, arom H), 8.62 (d, J = 2.1 Hz, 1H, arom H) ppm. ^{13}C NMR (300 MHz, $CDCl_3$), δ = 18.9 (CH_3), 55.5 (CH_2), 56.1 (CH_3), 56.1 (CH_3), 71.3 (CH), 110.5 (CH), 111.8 (CH), 119.9 (CH), 124.8 (CH), 130.4 (C), 134.0 (C), 135.6 (C), 144.4 (CH), 149.6 (C), 156.5 (C), 172.9 (C) ppm. HR-MS [M + H]$^+$ found 386.1523, calculated 386.1533.

1-(6-(3,4-Dimethoxyphenyl)isothiazolo[4,3-*b*]pyridin-3-yl)-piperidin-4-ol (13i). 1-(6-Bromoisothiazolo[4,3-*b*]pyridin-3-yl)-piperidin-4-ol was prepared from 3,6-dibromoisothiazolo[4,3-*b*]-pyridine (0.5 mmol, 146 mg) and 4-hydroxypiperidine (1.5 mmol, 151 mg) in EtOH (10 mL). The product was purified using a mixture of DCM/MeOH in a ratio of 95:5, affording 1-(6-bromoisothiazolo[4,3-*b*]pyridin-3-yl)piperidin-4-ol in 82 % yield (130 mg, 0.414 mmol). The title compound was prepared from 1-(6-bromoisothiazolo[4,3-*b*]-pyridin-3-yl)piperidin-4-ol (63 mg, 0.2 mmol) using 3,4-

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dimethoxyphenylboronic acid (0.3 mmol, 54.5 mg), 1 M K_2CO_3 (0.4 mL), and $Pd(PPh_3)_4$ (0.02 mmol, 23 mg). The product was purified using a mixture of cyclohexane/ethyl acetate in a ratio of 1:1, affording the title compound in 57% yield (42.8 mg, 0.115 mmol). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.83 (m, 2H, CH_2), 2.09 (m, 2H, CH_2), 3.70 (m, 2H, CH_2), 3.94 (s, 3H, OCH_3), 3.96 (s, 3H, OCH_3), 4.06 (m, 1H, CH), 4.35 (m, 2H, CH_2), 6.97 (d, J = 8.34 Hz, 1H, arom H), 7.17 (d, J = 2.07 Hz, 1H, arom H), 7.24 (dd, J = 8.28 Hz, J = 2.07 Hz, 1H, arom H), 7.86 (d, J = 2.01 Hz, 1H, arom H), 8.61 (d, J = 2.04 Hz, 1H, arom H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ = 33.06 (CH_2), 47.76 (CH_2), 55.69 (OCH_3), 66.07 (CH) 110.11 (CH), 111.40 (CH), 119.56 (CH), 124.22 (CH), 129.97 (C), 133.34 (C), 135.22 (C), 143.58 (CH), 149.18 (C), 149.24 (C), 155.84 (C), 172.69 (C) ppm. HR-MS $[M + H]^+$ found 372.1378, calculated 372.1376.

6-(3,4-Dimethoxyphenyl)-*N*-(tetrahydro-2*H*-pyran-4-yl)-isothiazolo[4,3-*b*]pyridin-3-amine (13j). 6-Bromo-*N*-(tetrahydro-2*H*-pyran-4-yl)isothiazolo[4,3-*b*]pyridin-3-amine was prepared from 3,6-dibromoisothiazolo[4,3-*b*]pyridine (0.5 mmol, 146 mg) and 4-aminopyrane (0.151 mL, 1.5 mmol) in EtOH (10 mL). The crude product was purified using a mixture of cyclohexane/ethyl acetate in a ratio of 3:1, affording 6-bromo-*N*-(tetrahydro-2*H*-pyran-4-yl)-isothiazolo[4,3-*b*]pyridin-3-amine in 40% yield (64 mg, 0.203 mmol). The title compound was prepared from 6-bromo-*N*-(tetrahydro-2*H*-pyran-4-yl)isothiazolo[4,3-*b*]pyridin-3-amine (64 mg, 0.203 mmol) using 3,4-dimethoxyphenylboronic acid (0.304 mmol, 55.3 mg), 1 M K_2CO_3 (0.406 mL), and $Pd(PPh_3)_4$ (0.02 mmol, 23 mg). The crude product was purified using a mixture of cyclohexane/ethyl acetate in a ratio of 1:1, affording the title compound in 16% yield (5.5 mg, 0.015 mmol). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.72 (m, 2H, 2 × CH_2), 2.25 (m, 2H, CH_2), 3.54 (m, 3H, CH, CH_2) 3.95 (s, 3H, OCH_3), 3.97 (s, 3H, OCH_3), 4.12 (m, 2H, CH_2), 6.26 (d, J = 7.59, 1H, NH) 6.99 (d, J = 8.34 Hz, 1H, arom H), 7.16 (d, J = 2.04 Hz, 1H, arom H), 7.24 (dd, J = 8.28 Hz, J = 2.04 Hz, 1H, arom H), 7.89 (d, J = 1.89 Hz, 1H, arom H), 8.59 (d, J = 1.89 Hz, 1H, arom H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 31.90 (CH_2), 54.18 (CH), 55.73 (CH_3), 66.06 (CH_2), 110.20 (CH), 111.38 (CH), 119.66 (CH), 124.33 (CH), 130.02 (C_q), 133.15 (C_q), 136.63 (C_q), 144.33 (CH), 149.23 (C_q), 149.32 (C_q), 154.19 (C_q), 169.57 (C_q) ppm. HR-MS $[M + H]^+$ found 372.1377, calculated 372.1376.

6-(3,4-Dimethoxyphenyl)-*N*-phenethylisothiazolo[4,3-*b*]-pyridin-3-amine (13k). 6-Bromo-*N*-phenethylisothiazolo[4,3-*b*]-pyridin-3-amine was prepared from 3,6-dibromoisothiazolo[4,3-*b*]-pyridine (0.5 mmol, 146 mg) and phenethylamine (0.188 mL, 1.5 mmol) in EtOH (10 mL). The crude product was purified using a mixture of cyclohexane/ethyl acetate in a ratio of 4:1, affording the 6-bromo-*N*-phenethylisothiazolo[4,3-*b*]pyridin-3-amine in 77 % yield (130 mg, 0.389 mmol). The title compound was prepared from 6-bromo-*N*-phenethylisothiazolo[4,3-*b*]pyridin-3-amine (130 mg, 0.389 mmol) using 3,4-dimethoxyphenylboronic acid (0.583 mmol, 106 mg), 1 M K_2CO_3 (0.78 mL), and $Pd(PPh_3)_4$ (0.038 mmol, 45 mg). The crude residue was purified using a mixture of cyclohexane/ethyl acetate in a ratio of 9:1, affording the title compound in 25% yield (39 mg, 0.099 mmol). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.11 (t, J = 7.05 Hz, J = 6.99 Hz, 1H, CH_2), 3.66 (m, 2H, CH_2), 3.95 (s, 3H, OCH_3), 3.97 (s, 3H, OCH_3), 6.36 (m, 1H, NH) 7.01 (d, J = 8.31 Hz, 1H, arom H), 7.15 (d, 1H, J = 1.89 Hz, arom H), 7.21−7.37 (m, 6H, arom H), 7.87 (d, J = 1.86 Hz, 1H, arom H), 8.66 (d, J = 1.83 Hz, 1H, arom H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ = 34.60 (CH_2), 49.04 (CH_2), 55.70 (OCH_3), 110.23 (CH), 111.38 (CH), 119.64 (CH), 124.22 (CH), 126.64 (CH), 128.38 (CH), 128.56 (CH), 130.21 (C), 133.15 (C), 136.42 (C), 137.43 (C), 144.30 (CH), 149.20 (C), 149.24 (C), 154.33 (C), 171.39 (C) ppm. HR-MS $[M + H]^+$ found 392.1422, calculated 392.1427.

Structure Determination. Recombinant GAK (aa 14−351) was mixed with the GAK-specific NbGAK_4 nanobody (a gift from Serge Muyldermans, Vrije Universiteit Brussel), and the complex was purified as previously described.[22] The GAK:NbGAK_4 complex was preincubated with 1 mM compound **12i** and crystallized using the sitting drop vapor diffusion method at 20 °C and the reservoir solution containing 9% broad-molecular-weight PEG smears (mixture of PEGs with their molecular weight ranging from 400 to 10 000 Da), 0.1 M MES, pH 6.2, and 0.15 M $CaCl_2$. Viable crystals were cryoprotected with mother liquor supplemented with 22% ethylene glycol and flash-frozen in liquid nitrogen. Diffraction data collected at Diamond Light Source, beamline I04-1, were processed and scaled with XDS[31] and SCALA from the CCP4 suite,[32] respectively. Initial structure solution was solved by molecular replacement using PHASER[33] and the coordinate of the GAK:NbGAK_4 complex.[22] Manual model building in COOT[34] alternated with refinement in REFMAC[35] was performed, and the geometric correctness of the complete model was verified with MolProbity.[36] Data collection and refinement statistics are summarized in Table 2 of the Supporting Information.

Plasmids. pFL-J6/JFH(p7-Rluc2A) was a gift from Dr. C. M. Rice.[24] Plasmids used in the HCVpp entry assays (pNL4-3.Luc.R-E, pcDM8, and pcDM8-E1E2) were a gift from Dr. Shoshana Levy.

Cells. Huh-7.5 cells and 293T cells were grown in Dulbecco's modified Eagle medium (DMEM; Mediatech) supplemented with 10% fetal bovine serum (Omega Scientific), nonessential amino acids (Gibco), 1% L-glutamine (Gibco), and 1% penicillin−streptomycin (Gibco) and maintained in 5% CO_2 at 37 °C.

In Vitro Transcription of Viral RNA, Transfection, and HCVcc Generation. HCV RNA was generated and delivered into Huh-7.5 cells, as previously described.[37] Briefly, RNA was reverse transcribed from XbaI-linearized J6/JFH(p7-Rluc2A) template using the T7 MEGAscript kit according to the manufacturer's instructions (Ambion). Viral RNA was purified using the RNeasy kit (Qiagen). 6 ×10^6 Huh7.5 cells were washed three times with ice-cold RNase-free PBS (BioWhittaker) and electroporated (0.82 kV, five 99 μs pulses) with 2 μg of viral RNA in a 2 mm gap cuvette (BTX) using a BTX-830 electroporator. After a 15 min recovery at room temperature, cells were resuspended in prewarmed growth medium and plated into 96 or 6-well culture plates. For HCVcc generation, cells were diluted in 30 mL of prewarmed growth medium and plated. Viral supernatants were collected daily from up to five passages of the electroporated cells, filtered through a 0.22 μm cellulose nitrate filter, and kept at −80 °C. Viral titers were determined by limiting dilution and immunohistochemical staining using an antibody directed to core. 50% tissue culture infectious dose ($TCID_{50}$) was calculated, as described.[36] Results are expressed as $TCID_{50}$/mL.

HCVcc Infection. 6 ×10^3 Huh-7.5 cells seeded in 96-well plates were infected in triplicate with HCVcc J6/JFH(p7-Rluc2A) at MOI (multiplicity of infection) of 0.1 in the presence of serial dilutions of the compounds. Culture medium was replaced daily with medium containing serial dilutions of the inhibitors. HCVcc infection was measured by standard luciferase assays at 72 h postinfection, using a *Renilla* luciferase substrate and a Tecan luminometer (Tecan) according to the manufacturers' protocols. Alternatively, 4 h after infection, cells were washed and medium was replaced, followed by standard luciferase assays at 24 h after infection.

HCV RNA Replication by Luciferase Assays. HCV RNA replication was measured at 72 h postelectroporation, as described.[8] Electroporated cells plated in quadruplicates in 96-well plates were washed twice with PBS and lysed with 30 μL of *Renilla* lysis buffer (Promega). Following 15 min shaking at room temperature, luciferase activity was quantified by standard luciferase assays.

Extracellular and Intracellular Infectivity. Huh-7.5 cells electroporated with J6/JFH(p7-Rluc2A) RNA and plated in six-well dishes were treated every 24 h with GAK inhibitors for a total of 72 h. For measurements of extracellular infectivity, supernatants were harvested, filtered using a 0.22 μm pore size filter and used to infect naïve Huh-7.5 cells in triplicate. For intracellular infectivity measurements, electroporated cells were trypsinized, collected by centrifugation, resuspended in 500 μL medium, lysed by four freeze−thaw cycles, and pelleted at 3650*g*. Clarified supernatants were diluted in complete medium and used to inoculate naive Huh-7.5 cells in triplicate. At 72 h postinfection cells were lysed and luciferase activity quantified as above.

HCVpp Production and Entry Assays. HCVpp (H77c strain, genotype 1a) was generated as described previously.[26] Briefly, 293T cells were transfected with a 1:1 ratio of plasmids encoding HIV provirus expressing luciferase and HCV E1E2 envelope glycoproteins. Supernatants were harvested 48 h posttransfection and filtered. Huh-7.5 cells were infected with HCVpp and 8 mg/mL Polybrene (Sigma) for 4 h.

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Cell lysates were collected at 48 h after HCVpp infection, and firefly luciferase (Promega) activity was measured using a Tecan luminometer (Tecan).

Viability Assay. Following treatment with GAK inhibitors, Huh-7.5 cells either infected with HCVcc and HCVpp or electroporated with HCV RNA were incubated for 2−4 h with medium supplemented with 10% alamarBlue reagent (TREK Diagnostic Systems) at 37 °C. Fluorescence at 560 nm was measured via FLEXstation II 384 (Molecular Devices, Inc.) as readout of cellular metabolic activity.

Effect of the Compounds on AP2M1 Phosphorylation. Huh-7.5 cells were treated with various concentrations of the compounds or DMSO in serum free medium. To allow capturing of the phosphorylated AP2M1 state, 100 nM PP2A inhibitor calyculin A was added to all wells 30 min after drug administration. One hour later cells were lysed and samples subjected to SDS−PAGE and blotting with antibodies targeting AP2M1 (Santa Cruz Biotechnology) and p-AP2M1 (Cell Signaling).

■ ASSOCIATED CONTENT

Supporting Information

NMR spectra of representative compounds, HPLC purity data of final compounds, and data collection and refinement statistics for the X-ray crystallography. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

The coordinates have been deposited in the PDB with accession code 4Y8D.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

AAK1, adaptor-associated kinase; AcOH, acetic acid; AP-2, adaptor protein complex 2; ATP, adenosine triphosphate; BIKE, BMP-2 inducible kinase; CCV, clathrin-coated vesicle; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; EGFR, epidermal growth factor receptor; GAK, cyclin G associated kinase; HCV, hepatitis C virus; K_d, binding affinity constant; LE, ligand efficiency; NAK, numb-associated kinase; ND, not determined; rt, room temperature; SAR, structure−activity relationship; THF, tetrahydrofuran; STK16/MPSK1, serine/threonine kinase 16/myristoylated and palmitoylated serine/threonine kinase 1

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