Bioorganic & Medicinal Chemistry xxx (xxxx) xxxx

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Structure-activity relationship study of the pyridine moiety of isothiazolo[4,3-*b*]pyridines as antiviral agents targeting cyclin G-associated kinase

Belén Martinez-Gualda[a], Szu-Yuan Pu[b], Mathy Froeyen[a], Piet Herdewijn[a], Shirit Einav[b], Steven De Jonghe[c,*]

[a] KU Leuven, Rega Institute for Medical Research, Laboratory of Medicinal Chemistry, Herestraat 49, 3000 Leuven, Belgium
[b] Department of Medicine, Division of Infectious Diseases and Geographic Medicine, and Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA 94305, USA
[c] KU Leuven, Department of Microbiology, Immunology and Transplantation, Rega Institute for Medical Research, Laboratory of Virology and Chemotherapy, Herestraat 49, 3000 Leuven, Belgium

ARTICLE INFO

Keywords:
Cyclin G-associated kinase
Isothiazolo[4,3-*b*]pyridine
Dengue virus
Kinase inhibitor
Antiviral drugs

ABSTRACT

Previously, we reported the discovery of 3,6-disubstituted isothiazolo[4,3-*b*]pyridines as potent and selective cyclin G-associated kinase (GAK) inhibitors with promising antiviral activity. In this manuscript, the structure-activity relationship study was expanded to synthesis of isothiazolo[4,3-*b*]pyridines with modifications of the pyridine moiety. This effort led to the discovery of an isothiazolo[4,3-*b*]pyridine derivative with a 3,4-dimethoxyphenyl residue at position 5 that displayed low nanomolar GAK binding affinity and antiviral activity against dengue virus.

1. Introduction

Cyclin G-associated kinase (GAK), also known as auxilin 2, is a cellular serine/threonine kinase that belongs to the numb-associated kinases (NAK). This family also includes adaptor-associated kinase 1 (AAK1), BMP-2 inducible kinase (BIKE/BMP2K) and myristoylated and palmitoylated serine/threonine kinase 1 (MPSK1, also known as serine/threonine kinase 16 or STK16).[1] GAK, ubiquitously expressed within the cell in the Golgi apparatus, cytoplasm and nucleus, regulates the clathrin-mediated intracellular trafficking of cellular cargo proteins.[2] Interactions between clathrin- associated adaptor protein (AP) complexes and transmembrane cargo play an important role in membrane trafficking, as these AP complexes orchestrate the formation of vesicles destined for transport in distinct intracellular pathways.[3,4] Phosphorylation of the μ subunits of AP1 and AP2 by GAK leads to a conformational change that enhances their binding to sorting motifs within cargo proteins.[5,6] GAK regulates the recruitment of clathrin and AP-2 to the plasma membrane and of AP-1 to the *trans*-Golgi network (TGN).[3,7,8] GAK also controls the uncoating of clathrin coated vesicles (CCVs) for recycling clathrin back to the cell surface.[3,5]

Viruses hijack these intracellular membrane trafficking machineries, as indicated by the important roles played by various AP complexes in the life cycle of multiple unrelated viruses.[7,9,10] It has been demonstrated that GAK regulates early and late stages of the viral lifecycle and hence, functions as a master regulator of viral infection. GAK is therefore a promising target for the discovery of novel antiviral agents. Such a host-targeted approach offers advantages, including a high barrier to resistance and an opportunity to develop broad-spectrum antiviral agents when focusing on host factors, such as GAK, that are essential for the life cycle of multiple viruses[11].

Erlotinib (Fig. 1), an approved anticancer drug with potent GAK affinity (dissociation constant value or K_D = 3.1 nM), has shown antiviral activity against an array of unrelated viruses including hepatitis C virus (HCV), dengue virus (DENV), and Zika virus (ZIKV) from the Flaviviridae family, the filovirus Ebola (EBOV) and the alpha virus Chikungunya (CHIKV).[9] However, since erlotinib targets EGFR with a comparable affinity to GAK and several other kinases at a lower affinity, it is not an ideal chemical tool to study the role of GAK in viral infection. Several medicinal chemists were thus inspired to develop potent, drug-like and selective GAK inhibitors. Exploring the chemistry of 4-anilino-quinazolines and –quinolines[12] led to the discovery of SGC-GAK-1 (Fig. 1) that was endowed with very strong GAK affinity (K_D = 1.9 nM) and displayed an excellent kinase selectivity profile. This compound was not investigated for its antiviral activity, yet it showed cytotoxic activity against selected prostate cancer cell lines.[13]

Our group reported the discovery of 3,6-disubstituted isothiazolo[4,3-*b*]pyridines as potent and selective GAK inhibitors. The original series of compounds, as represented by compound **2** (Fig. 1), carried a

* Corresponding author.
E-mail address: steven.dejonghe@kuleuven.be (S. De Jonghe).

https://doi.org/10.1016/j.bmc.2019.115188
Received 28 August 2019; Received in revised form 21 October 2019; Accepted 25 October 2019

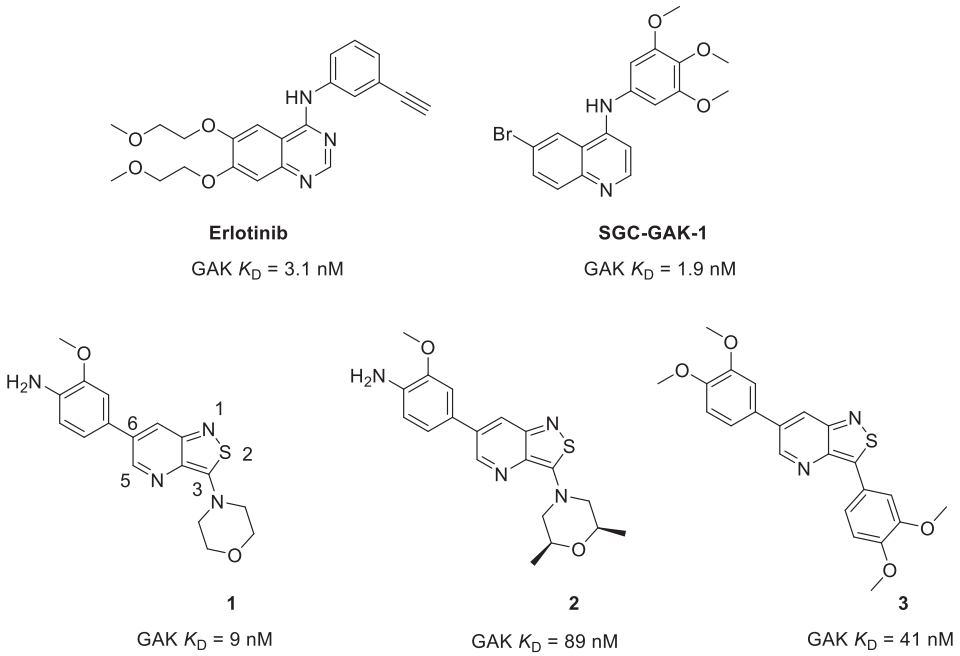


Fig. 1. Previously studied GAK inhibitors.

morpholino residue at position 3 and was endowed with high affinity for GAK (K_D = 9 nM), but displayed only low μM antiviral activity against HCV and DENV.[14] Subsequent optimization revealed that a *cis*-2,6-dimethylmorpholine residue at this position (compound **2**, Fig. 1) had a detrimental effect on the GAK affinity (K_D = 89 nM), but a favourable effect on the activity against DENV, EBOV and CHIKV.[15] The incorporation of other amines, alkoxides, and carboxamides at position 3 yielded compounds that were much less active as GAK ligands and/or showed weaker antiviral activity.[16] To increase the structural variety at this position, palladium-catalyzed cross couplings (such as Suzuki and Sonogashira reactions) have been performed, leading to the discovery of a congener (compound **3**, Fig. 1) with high GAK affinity (K_D = 41 nM) and a moderate antiviral activity against DENV.[17] More recently, the importance of the isothiazolo[4,3-*b*]pyridine core has been evaluated by a scaffold hopping approach, yielding a series of novel scaffolds that displayed a reduced GAK affinity relative to the original scaffold.[18]

Although a broad structural variety has been introduced at position 3 of the isothiazolo[4,3-*b*]pyridine scaffold, and a wide range of heterocyclic core structures have been prepared, the substitution pattern on the pyridine moiety of the isothiazolo[4,3-*b*]pyridine scaffold has not been previously studied in detail. In our prior medicinal chemistry optimization campaigns,[14–18] an aryl group was always present at position 6 of the isothiazolo[4,3-*b*]pyridine scaffold, with a 3,4-dimethoxyphenyl, 3,4,6-trimethoxyphenyl and 3-methoxy-4-aminophenyl being optimal for GAK affinity (Fig. 2). In the present work, we describe our efforts to introduce structural modifications on the pyridine ring of the isothiazolo[4,3-*b*]pyridine core, keeping the *cis*-2,6-dimethylmorpholine substituent fixed. Several variations were introduced. Besides additional Suzuki couplings, several linkers (amino, alkenyl and alkynyl) were inserted between the phenyl ring at position 6 and the isothiazolo[4,3-*b*]pyridine scaffold. In addition, a number of previously unknown 5-aryl-isothiazolo[4,3-*b*]pyridines were prepared. All compounds were evaluated for their GAK affinity and the most potent GAK ligands were also assessed for anti-DENV activity.

2. Chemistry

For the synthesis of the 6-substituted isothiazolo[4,3-*b*]pyridines, the known 6-bromo-3-(*cis*-2,6-dimethylmorpholino)isothiazolo[4,3-*b*]pyridine **4** was selected as the key intermediate (Scheme 1).[15] Aryl groups were conveniently introduced by Suzuki cross-couplings using classical reaction circumstances: an appropriate arylboronic acid, Pd$(PPh_3)_4$ as catalyst and potassium carbonate as base in a mixture of dioxane/water. This procedure allowed to assemble a focused library of 6-aryl-isothiazolo[4,3-*b*]pyridines **5a-f** in yields ranging from 78% to 91%. The insertion of an ethenyl linker between the central core structure and the phenyl ring was performed by a palladium-catalyzed cross-coupling between styrene and isothiazolo[4,3-*b*]pyridine **4** applying Heck reaction conditions, using $Pd(OAc)_2$ as catalyst, *N*-phenylurea as ligand and potassium carbonate as base (step b, Scheme 1).[19] This allowed the isolation of the desired compound **5 g** in high yield (88%). Coupling of different anilines at position 6 of the isothiazolo[4,3-*b*]pyridine scaffold was achieved via a Buchwald reaction using Pd

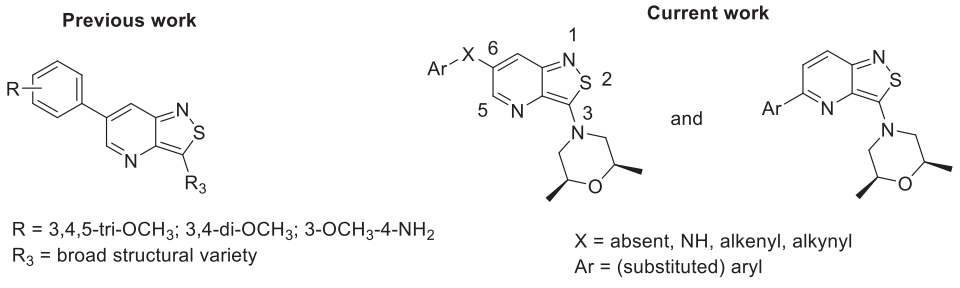
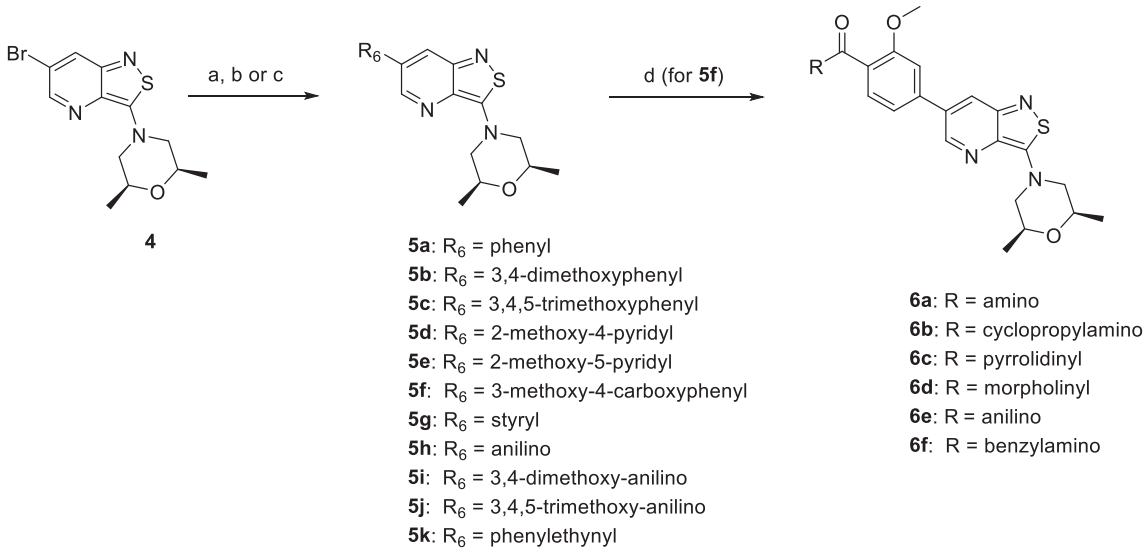


Fig. 2. Medicinal chemistry strategy.

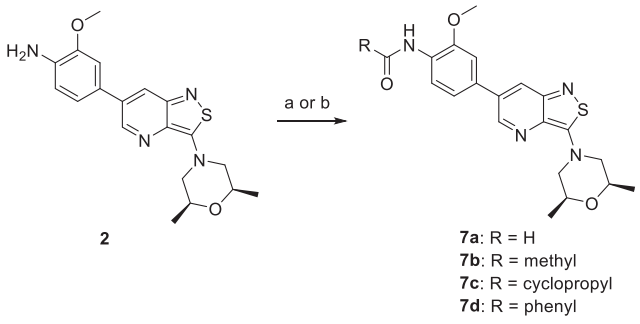


Scheme 1. *Reagents and conditions.* (a) $R_6B(OH)_2$, $Pd(PPh_3)_4$, K_2CO_3, dioxane/water, 90 °C; (b) styrene, *N*-phenylurea, $Pd(OAc)_2$, K_2CO_3, DMF, 120 °C, overnight; (c) aniline or phenylacetylene, $Pd(dba)_2$, SPhos, *t*BuOK, toluene, 90 °C; (d) (i) $SOCl_2$, DCM, 40 °C, 3 h; (ii) amine, Et_3N, rt, 3 h.

$(dba)_2$ as catalyst, SPhos as ligand, potassium *tert*-butoxide as base and toluene as solvent, yielding compounds **5 h-j** in good yields ranging from 73% to 85%.[20] For the insertion of a phenylethynyl residue at position 6 of isothiazolo[4,3-*b*]pyridine scaffold, classical Sonogashira reaction conditions (using ethynylbenzene, $Pd(PPh_3)_2Cl_2$ and copper(I) iodide) were initially applied, but failed. However, when the same Buchwald conditions, previously employed for the coupling of anilines, were used for the coupling of phenylacetylene (step c, Scheme 1), compound **5k** was obtained in good yield (87%). Prior to the addition of the catalyst, it was necessary to pass a continuous flow of argon for 5–10 min through the reaction mixture, as this led to increased reaction yields in all these palladium-catalyzed cross-couplings.

The carboxylic acid moiety of compound **5f** was converted to several amides.[21] The acid chloride was generated *in situ* by reaction with thionyl chloride, followed by reaction with a number of amines generating target compounds **6a-f** in yields varying from 68% to 85%. Similarly, the amino group of compound **2** was also used to prepare a number of amides (Scheme 2). The formylamino congener **7a** was prepared using MnO_2 as catalyst and formamide as solvent.[22] The reaction of compound **2** with acetic anhydride, cyclopropanecarbonyl chloride and benzoyl chloride provided compounds **7b**, **7c** and **7d**, respectively.

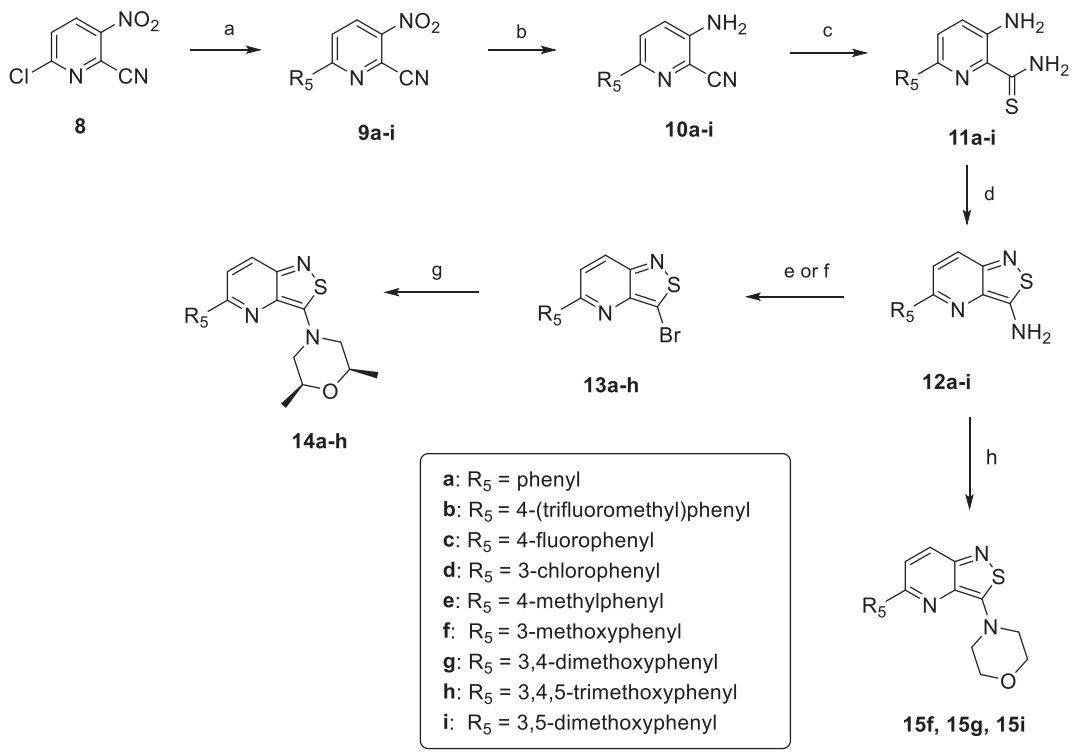
For the synthesis of 5-substituted isothiazolo[4,3-*b*]pyridines, an alternative synthetic pathway was followed. Initial attempts were focused on the synthesis of an intermediate (i.e. 3-bromo-5-chloro-isothiazolo[4,3-*b*]pyridine) that could serve as key synthon from which structural variety at two different positions of the scaffold could be



Scheme 2. *Reagents and conditions.* (a) formamide, MnO_2, 150 °C, 5 h; (b) acetic anhydride or RCOCl, Et_3N, DCM, rt, overnight.

easily introduced. Commercially available 6-chloro-2-cyano-3-nitropyridine **8** was selected as starting material. Reduction of the nitro group to the corresponding amino group led to a concomitant complete hydrolysis of the cyano moiety affording 3-amino-6-chloropicolinic acid as the sole product. As an alternative, we introduced the corresponding aryl group via a Suzuki cross-coupling reaction in the first step of the synthesis (Scheme 3). These reactions were carried out in toluene as solvent (except for compound **9 h** bearing a 3,4,5-trimethoxyphenyl group) instead of the dioxane/water mixture that was commonly applied for the preparation of 6-substituted derivatives. Thus, several aryl groups were introduced at position 6 of compound **8** generating derivatives **9a-i** in yields ranging from 76% to 96%. The nitro group of compounds **9a-i** was reduced by treatment with iron under acidic conditions (acetic acid at 60 °C) affording 3-amino-6-aryl-picolinonitriles **10a-i**. Inevitably, small amounts of the corresponding 3-amino-6-phenylpicolinamides were formed, resulting from hydrolysis of the cyano group. These mixtures were used as such for the next step. Thionation of the nitrile functionality using phosphorus pentasulfide yielded thioamides **11a-i**. The isothiazole moiety was then constructed by an oxidative ring closure using hydrogen peroxide in methanol. As this reaction proceeded smoothly and cleanly, purification of the crude residue was not necessary, and the corresponding 5-aryl-3-amino-isothiazolo[4,3-*b*]pyridines **12a-i** were isolated in excellent yields. The conversion of the amino group to a bromine was achieved via a Sandmeyer reaction. Applying the classical reaction conditions ($NaNO_2$, HBr, CuBr, water) was successful for compounds **13a-d** and **13 h**. However, for the analogues (compounds **12e-g**), in which the 5-phenyl moiety carried one or two electron-donating substituents (methyl or methoxy groups), none to very low yields the most were obtained. This result was attributed to multiple brominations at different positions of the 5-phenyl ring. To minimise these aromatic bromination side reactions, milder conditions (*t*BuONO, $CuBr_2$ and dry acetonitrile) were used.[23] This slightly improved the yields of compounds **13e-g** (34–51%), but not of compound **13i**. Finally, *cis*-2,6-dimethylmorpholine was introduced at position 3 of the isothiazolo[4,3-*b*]pyridine scaffold, affording the desired compounds **14a-h** in yields ranging from 55% to 91%.

To circumvent the Sandmeyer reaction, a 3-*N*-morpholinyl moiety was directly synthesized from the corresponding 3-amino congeners. Reaction of compounds **12f**, **12 g** and **12i** with 2-bromoethyl ether and potassium carbonate as a base generated the desired compounds **15f**, **15 g** and **15i** in decent yields (71–77%).



Scheme 3. *Reagents and conditions*. (a) $R_3B(OH)_2$, $Pd(PPh_3)_4$, K_2CO_3, toluene or dioxane/H_2O, 95 °C; (b) Fe, CH_3COOH, 60 °C; (c) P_2S_5, EtOH, 75 °C; (d) 35% aq·H_2O_2, MeOH, rt; (e) $NaNO_2$, HBr, CuBr, H_2O, 0 °C to rt; (f) *t*BuONO, $CuBr_2$, dry CH_3CN, −10 °C to rt; (g) 2,6-dimethylmorpholine, EtOH, reflux, overnight; (h) 2-bromoethyl ether, K_2CO_3, DMF, 100 °C, 4–5 h.

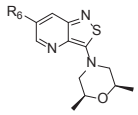
3. GAK binding affinity

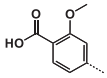
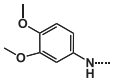
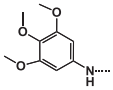
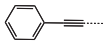
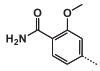
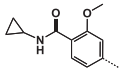
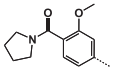
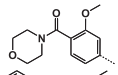
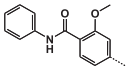
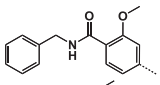
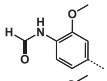
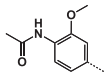
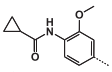
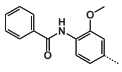
The binding affinity of compounds **5a-k**, **6a-f**, **7a-d**, **14a-h**, **15f**, **15 g** and **15i** for GAK was evaluated via a commercially available LanthaScreen® Europium kinase binding assay.[24] In this assay format, an Alexa Fluor 647-labeled conjugate or tracer competes with the GAK ligand for the ATP binding site. Signal detection is dependent on the time-resolved fluorescence resonance energy transfer (TR-FRET) signal resulting from the antibody and tracer binding. When the ATP site is occupied by the tracer, there is a high TR-FRET signal. When the tracer is displaced by the GAK inhibitor, a reduction in TR-FRET signal is observed. The GAK binding affinity of reference compound **2** was measured via a LanthaScreen assay with an IC_{50} value of 0.0394 μM, which is in agreement with the K_D of 0.089 μM previously measured via the DiscoverX screening assay.[15] To drive the subsequent structure-activity relationship (SAR) study, the GAK binding affinity of all compounds was evaluated in the Lanthascreen assay (Tables 1 and 2).

Prior SAR studies of 3-*N*-morpholino-isothiazolo[4,3-*b*]pyridines demonstrated that electron-donating substituents (e.g. methoxy, amino) at the 6-phenyl moiety are optimal for GAK binding. Yet, combination of a *cis*-2,6-dimethylmorpholinyl substituent at position 3, which is known to confer potent antiviral activity,[15] with structural variation of the 6-phenyl moiety has not been previously explored. Based on the beneficial effect of methoxy groups on GAK binding,[14] the 6-(3,4-dimethoxy)- and 6-(3,4,5-trimethoxyphenyl) derivatives were synthesized (Table 1). The presence of a 3,4-dimethoxyphenyl residue (compound **5b**) caused a 3-fold drop in GAK affinity (IC_{50} = 0.136 μM) relative to compound **2**. Conversely, the 3,4,5-trimethoxyphenyl congener (compound **5c**) showed a slightly improved GAK affinity (IC_{50} = 0.071 μM). Removal of the methoxy groups furnished compound **5a** which shows an IC_{50} of 0.661 μM, which is about 10-fold less potent than the trimethoxyphenyl congener **5c**. Replacement of one of the methoxy groups by an endocyclic pyridine nitrogen gave rise to compounds **5d** and **5e** that display IC_{50} values of 0.545 μM and > 3.33 μM, respectively. This indicated that the methoxy group at position 3 is more important for GAK binding than the one at position 4. Therefore, keeping the 3-methoxy group fixed, a number of derivatives were prepared with alternative functional groups at position 4 of the aromatic ring. The presence of a 4-carboxylic acid resulted in compound **5f** that showed very potent GAK inhibition (IC_{50} = 0.042 μM). The carboxylic acid was used as a chemical handle to make a small set of amides, including a simple carboxamide analogue (compound **6a**), cycloaliphatic amides (compounds **6b-d**) and aromatic amides (**6e-f**). Based on the X-ray crystallographic data that we previously reported,[14] this region (i.e. the 6-phenyl moiety) is oriented towards the solvent and therefore tolerates structural variety. This is in agreement with the experimentally determined IC_{50} values, as several of these compounds, and particularly the carboxamide congener **6a** (IC_{50} = 0.022 μM), show good GAK affinity. Because of the good affinity of these amides, a number of reverse amides (compounds **7a-d**) with aliphatic and aromatic side chains were prepared, displaying GAK IC_{50} values in the range of 0.1 to 0.4 μM. To further explore the SAR, the effect of various linkers between the isothiazolo[4,3-*b*]pyridine scaffold and the 6-phenyl moiety on GAK affinity were investigated. An alkenyl (compound **5 g**) or alkynyl (compound **5 k**) spacer gave compounds that were completely devoid of GAK affinity. Albeit weak, an anilino substituent at position 6 (compound **5 h**) gave rise to GAK affinity with an IC_{50} of 1.63 μM. In an effort to restore GAK affinity of the 6-anilino congener, the 3,4-dimethoxy- (compound **5i**) and 3,4,5-trimethoxy-anilino (compound **5j**) congeners were prepared. Consistent with the 6-phenyl series, the 3,4,5-trimethoxyanilino derivative **5j** showed the most potent GAK affinity, with an IC_{50} of 0.458 μM.

To expand the SAR, a series of regioisomeric 5-aryl-isothiazolo[4,3-*b*]pyridines (compounds **14a-i**) was synthesized (Table 2). Because of synthetic difficulties (*vide supra*), a number of derivatives (compounds **15f**, **15 g** and **15i**) carrying a morpholine instead of a *cis*-2,6-dimethylmorpholino residue were synthesized. An unsubstituted phenyl (compound **14a**), halogen substituted phenyls (compounds **14c-d**) and

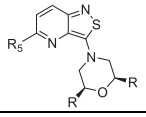
Table 1
GAK binding affinity of 6-substituted isothiazolo[4,3-*b*]pyridines.



Cmpd	R_6	GAK IC_{50} (μM)
2		0.0394
5a		0.661
5b		0.136
5c		0.071
5d		0.545
5e		> 3.33
5f		0.042
5g		> 3.33
5h		1.63
5i		1.29
5j		0.458
5k		> 10
6a		0.022
6b		0.118
6c		0.083
6d		0.101
6e		> 0.37
6f		0.073
7a		0.113
7b		0.132
7c		0.173
7d		0.37

p-tolyl (compound **14e**) at position 5 of the isothiazolo[4,3-*b*]pyridine scaffold yielded compounds that displayed GAK IC_{50} values in the 0.1–0.3 μM range. Only the 4-trifluoromethylphenyl analogue (compound **14b**) was less active as GAK ligand. As methoxy substitution of

Table 2
GAK binding affinity of 5-aryl-isothiazolo[4,3-*b*]pyridines.



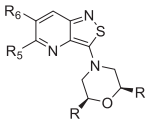
Cmpd	R	R_5	GAK IC_{50} (μM)
14a	CH_3		0.364
14b	CH_3		> 0.37
14c	CH_3		0.11
14d	CH_3		0.262
14e	CH_3		0.214
14f	CH_3		0.099
14g	CH_3		0.024
14h	CH_3		0.422
15f	H		0.136
15g	H		0.124
15i	H		0.126

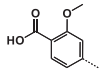
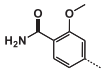
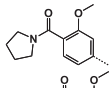
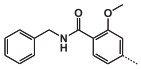
the 6-phenyl ring is known to be advantageous for GAK affinity,[14] the phenyl at position 5 was also substituted with one, two or three methoxy groups affording compounds **14f-h**, **15f-g** and **15i**. This series displayed GAK IC_{50} values in the 0.1–0.5 μM range, with compound **14 g** (carrying a 3,4-dimethoxyphenyl at position 5 and a *cis*-2,6-dimethylmorpholinyl group at position 3) having the most potent GAK affinity (IC_{50} = 0.024 μM). In contrast, the corresponding 3-*N*-morpholinyl derivative (compound **15 g**) is 5-fold less active as a GAK ligand (IC_{50} = 0.124 μM).

4. Antiviral activity against DENV

The compounds with the strongest GAK binding affinity (IC_{50} < 0.1 μM) were assessed for antiviral activity in human hepatoma (Huh7) cells infected with DENV2 expressing a luciferase reporter (Table 3). Their effect on DENV2 infection was measured at 48 h post-infection via luciferase assays, and the half-maximal effective concentration (EC_{50} value) was calculated. In parallel, the cytotoxicity of the compounds in the infected cells (expressed as the half-maximal cytotoxic concentration or CC_{50} value) was measured via an Alamar-Blue assay. Compound **2** was included as a reference compound, as it was previously shown to have a promising anti-DENV2 activity, with an EC_{50} of 0.82 μM and no apparent cytotoxicity.[9] The 6-(3,4,5-trimethoxyphenyl) derivative **5c** reduces the anti-DENV activity by 3 folds relative to compound **2**. This is in line with its slightly reduced GAK affinity. Although compound **5f** is a very potent GAK ligand (IC_{50} = 0.042 μM), it lacks antiviral activity. This might be due to impaired cellular permeability resulting from the presence of a polar carboxylic acid group. The corresponding amides (compounds **6a** and **6c**) and particularly compound **6f** (EC_{50} = 5.83 μM) show an improved antiviral activity when compared to the carboxylic acid derivative **5f**. These findings suggest that this site of the molecule tolerates some structural variation with respect to GAK affinity, and that this position can therefore be used to tune physicochemical properties, such as

Table 3
Antiviral activity against DENV2.



Cmpd	R	R_5	R_6	DENV EC_{50} (μM)[a]	DENV CC_{50} (μM)[b]
2	CH_3	H		0.82	> 25
5c	CH_3	H		2.79	> 10
5f	CH_3	H		> 10	> 10
6a	CH_3	H		10.05	> 10
6c	CH_3	H		8.85	> 10
6f	CH_3	H		5.83	> 10
14f	CH_3		H	> 10	> 10
14g	CH_3		H	1.049	> 10
Erlotinib	–	–	–	1.22	> 10

[a] EC_{50} = half-maximal effective concentration.
[b] CC_{50} = half-maximal cytotoxic concentration.

aqueous solubility and cellular permeability. Within the 5-aryl-isothiazolo[4,3-*b*]pyridines, two compounds were investigated for antiviral activity. Whereas compound **14f** did not show antiviral activity, the 5-(3,4-dimethoxyphenyl) derivative **14g** was a potent GAK inhibitor (IC_{50} = 0.024 μM) demonstrating potent antiviral activity (EC_{50} and CC_{50} values of 1.049 μM and > 10 μM, respectively). Despite the fact that compound **14g** is less active as GAK ligand when compared to erlotinib (K_D = 3.1 nM), both compounds are equally active as anti-DENV agents. As erlotinib is known to affect multiple other kinases besides GAK, compound **14g** might be a better lead for the discovery of GAK inhibitors as antiviral drugs.

5. Molecular docking of compound 14g into GAK

To rationalize the potent GAK affinity of compound **14g**, molecular docking was initiated starting from the co-crystal X-ray structure of GAK and compound **1** (PDB 4Y8D),[14] with a docking protocol as described before,[17] using the software packages Autodocktools and Autodock vina.[25,26] Compound **14g** has a slightly different substituent at position 3 of the isothiazolo[4,3-*b*]pyridine scaffold from the original crystallized GAK inhibitor **1**. More specifically, compound **14g** bears a *cis*-2,6-dimethylmorpholino ring of which the intrinsic torsion angles are not flexible during docking. However, since the choice of the ring conformation might be important for the final docking orientation and since morpholine can exist in different conformations in crystallized structures,[27] we used different conformations of the morpholino ring of compound **14g** for docking. This analysis revealed that a chair conformation with both methyl groups in an equatorial orientation was optimal. The nitrogen has more a sp^2 character and lays in the same plane as the aromatic ring system. Compound **14g** docked in a favourable way in the ATP binding site. Primarily hydrophobic interactions were observed between GAK and compound **14g,** similarly to compound **1** (Fig. 3). One hydrogen bond with Thr123.B was observed.

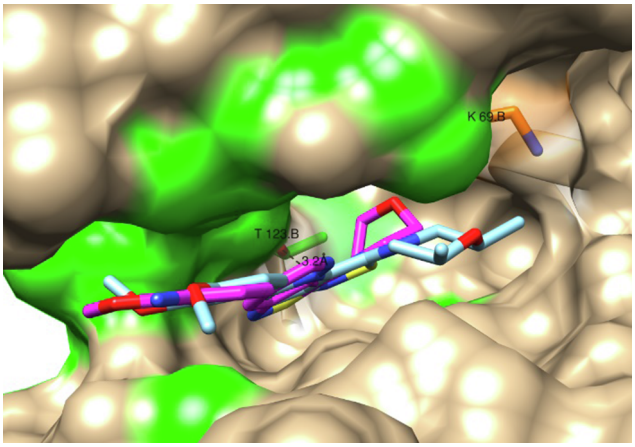


Fig. 3. Autodock Vina docking of compound **14 g** (cyan carbons) superimposed on compound **1** (magenta carbons). Amino acids having van der Waals interactions are coloured as green surface.

During this docking process, a rigid enzyme conformation and a flexible ligand by variable torsion angles was applied. Taking into account induced fit effects, the observed interactions may in fact be more favourable. For instance, the Lys69.B side chain is within interaction distance with the oxygen atom of the morpholino ring, possibly forming a hydrogen bond, that is currently not detected.

6. Conclusion

Isothiazolo[4,3-*b*]pyridines are well known as potent and selective GAK inhibitors with antiviral activity. Prior research has focused on structural modifications of the scaffold and the substituent at position 3. In this manuscript, the SAR of the pyridine moiety of the isothiazolo [4,3-*b*]pyridine core structure was studied in detail. The insertion of various linkers between the 6-phenyl ring and the central scaffold generated compounds with decreased GAK affinity. However, position 4 of the 6-phenyl moiety tolerates some structural variety with respect to GAK affinity, and structural variation at this site can be used to modulate the antiviral activity. Furthermore, a novel class of 5-substituted isothiazolo[4,3-*b*]pyridines was synthesized, yielding selected congeners with potent GAK affinity and anti-DENV activity.

7. Experimental section

7.1. Chemistry

For all reactions, analytical grade solvents were used. Argon was used to carry out reactions under an inert atmosphere. Melting points were recorded with a Stuart SMP20 melting point apparatus. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance 300 MHz instrument (^1H NMR, 300 MHz; ^{13}C NMR, 75 MHz), 500 MHz instrument (^1H NMR, 500 MHz; ^{13}C NMR, 125 MHz) or a 600 MHz instrument (^1H NMR, 600 MHz; ^{13}C NMR, 150 MHz, ^{19}F NMR, 471 MHz), using tetramethylsilane as internal standard for ^1H NMR spectra and DMSO-d_6 (39.5 ppm) or $CDCl_3$ (77.2 ppm) for ^{13}C NMR spectra. Abbreviations used are s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, b = broad. Coupling constants are expressed in Hz. 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 mL/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 was performed on silica gel 0.060–0.200 mm, 60 (Acros Organics). Purity of final compounds was

verified to be > 95% by HPLC analysis. HPLC conditions to assess purity were as follows: Shimadzu HPLC equipped with a LC-20AT pump, DGU-20A5 degasser, and a SPD-20A UV–VIS detector; Symmetry C18 column (5 μm, 4.6 mm × 150 mm); gradient elution of H_2O/CH_3CN from 95/5 or 70/30 to 5/95 over 25 min; flow rate 1 mL/min; wavelength, UV 254 nm. Preparative HPLC purifications were performed using a Phenomenex Gemini 110A column (C18, 10 μM, 21.2 mm × 250 mm).

7.1.1. Synthesis of 6-aryl-3-(cis-2,6-dimethylmorpholino)isothiazolo[4,3-b]pyridines

General procedure. A solution of 6-bromo-3-(*cis*-2,6-dimethylmorpholino) isothiazolo[4,3-*b*]pyridine (1 equiv) in a mixture of dioxane/water (ratio 4:1) was degassed with argon and subsequently, were added the corresponding boronic acid (1.2 equiv), $Pd(PPh_3)_4$ (0.02 equiv) and K_2CO_3 (2 equiv). The mixture was degassed a second time, filled with argon and stirred at 90 °C overnight. After completion of the reaction as monitored by TLC, the volatiles were evaporated to dryness and the crude residue was diluted with EtOAc (20 mL) and washed with water (2 × 20 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The resulting residue was purified by silica gel flash chromatography, yielding the corresponding 6-substituted-3-(*cis*-2,6-dimethylmorpholino)isothiazolo[4,3-*b*]pyridines. The following compounds were made according to this general procedure.

*7.1.1.1. 3-(cis-2,6-Dimethylmorpholino)-6-(phenyl)isothiazolo[4,3-b] pyridine (**5a**)*. This compound was obtained using phenylboronic acid. The crude residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 4:1) as mobile phase, affording the title compound as an amorphous yellow solid in 89% yield (88.3 mg, 0.27 mmol). Mp 141.5–142.3 °C. ^{1}H NMR (500 MHz, $CDCl_3$) *δ*: 1.31 (d, *J* = 6.2, Hz, 6H, 2 × CH_3), 2.91 (dd, *J* = 12.6, 10.7 Hz, 2H, 2 × NCH), 3.91–3.99 (m, 2H, 2 × OCH), 4.50–4.54 (m, 2H, 2 × NCH), 7.41–7.45 (m, 1H, arom H), 7.48–7.52 (m, 2H, arom H), 7.62–7.68 (m, 2H, arom H), 7.90 (d, *J* = 2.1 Hz, 1H, arom H), 8.63 (d, *J* = 2.1 Hz, 1H, arom H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$) *δ:* 18.8 (CH_3), 55.4 (CH_2), 71.2 (CH), 125.6 (CH), 127.4 (CH), 128.4 (CH), 129.1 (CH), 134.2 (C), 135.8 (C), 137.6 (C), 144.3 (CH), 156.3 (C), 172.9 (C) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{18}H_{19}N_3OS$: 326.1322, found 326.1319.

*7.1.1.2. 6-(3,4-Dimethoxyphenyl)-3-(cis-2,6-dimethylmorpholino) isothiazolo[4,3-b]pyridine (**5b**)*. This compound was obtained using 3,4-dimethoxyphenylboronic acid. The crude residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 7:3) as mobile phase, affording the title compound as an amorphous yellow solid in 90% yield (52.7 mg, 0.13 mmol). Mp 156.2–157.9 °C. ^{1}H NMR (600 MHz, $CDCl_3$) *δ*: 1.32 (d, *J* = 6.3 Hz, 6H, 2 × CH_3), 2.91 (dd, *J* = 12.5, 10.7 Hz, 2H, 2 × NCH), 3.92–3.96 (m, 5H, OCH_3, 2 × OCH), 3.96 (s, 3H, OCH_3), 4.51 (dd, *J* = 12.7, 1.9 Hz, 2H, 2 × NCH), 7.00 (d, *J* = 8.3 Hz, 1H, arom H), 7.16 (d, *J* = 2.1 Hz, 1H, arom H), 7.24 (dd, *J* = 8.3, 2.1 Hz, 1H, arom H), 7.85 (d, *J* = 2.1 Hz, 1H, arom H), 8.63 (d, *J* = 2.1 Hz, 1H, arom H) ppm. ^{13}C NMR (151 MHz, $CDCl_3$) *δ*: 18.8 (CH_3), 55.4 (CH_2), 56.0 (OCH_3), 56.0 (OCH_3), 71.2 (CH), 110.3 (CH), 111.6 (CH), 119.8 (CH), 124.8 (CH), 130.3 (C), 133.9 (C), 135.5 (C), 144.3 (CH), 149.5 (C), 149.5 (C), 156.4 (C), 172.9 (C) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{20}H_{23}N_3O_3S$: 386.1532, found 386.1527.

*7.1.1.3. 6-(3,4,5-Trimethoxyphenyl)-3-(cis-2,6-dimethylmorpholino) isothiazolo[4,3-b]pyridine (**5c**)*. This compound was obtained using 3,4,5-trimethoxyphenylboronic acid. The crude residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 7:3) as mobile phase, affording the title compound as an amorphous yellow solid in 91% yield (57.7 mg, 0.14 mmol). Mp 154.1–155.5 °C. ^{1}H NMR (500 MHz, $CDCl_3$) *δ*: 1.32 (d, *J* = 6.3 Hz, 6H, 2 × CH_3), 2.92 (dd, *J* = 12.5, 10.7 Hz, 2H, 2 × NCH), 3.91 (s, 3H, OCH_3), 3.93–3.97 (m, 8H, 2 × OCH, 2 × OCH_3), 4.52 (dd, *J* = 12.7, 1.9 Hz, 2H, 2 × NCH), 6.85 (s, 2H, arom H), 7.87 (d, *J* = 2.1 Hz, 1H, arom H), 8.62 (d, *J* = 2.1 Hz, 1H, arom H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$) *δ*: 18.8 (CH_3), 55.4 (CH_2), 56.2 (OCH_3), 61.0 (OCH_3), 71.2 (CH), 104.6 (CH), 125.3 (CH), 133.3 (C), 134.1 (C), 135.8 (C), 138.5 (C), 144.1 (CH), 153.7 (C), 156.2 (C), 172.9 (C) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{21}H_{25}N_3O_4S$: 416.1638, found 416.1629.

*7.1.1.4. 6-(2-Methoxy-4-pyridyl)-3-(cis-2,6-dimethylmorpholino) isothiazolo[4,3-b]pyridine (**5d**)*. This compound was obtained using 2-methoxypyridine-4-boronic acid. The crude residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 7:3) as mobile phase, affording the title compound as an amorphous yellow solid in 87% yield (47.2 mg, 0.13 mmol). Mp 160.5–162.1 °C. ^{1}H NMR (600 MHz, $CDCl_3$) *δ:* 1.32 (d, *J* = 6.3 Hz, 6H, 2 × CH_3), 2.93 (dd, *J* = 12.6, 10.7 Hz, 2H, 2 × NCH), 3.91–3.97 (m, 2H, 2 × OCH), 4.01 (s, 3H, OCH_3), 4.52 (dd, *J* = 12.7, 1.9 Hz, 2H, 2 × NCH), 7.01–7.02 (m, 1H, arom H), 7.15 (dd, *J* = 5.3, 1.5 Hz, 1H, arom H), 7.94 (d, *J* = 2.1 Hz, 1H, arom H), 8.27–8.29 (m, 1H, arom H), 8.59 (d, *J* = 2.1 Hz, 1H, arom H) ppm. ^{13}C NMR (151 MHz, $CDCl_3$) *δ:* 18.8 (CH_3), 53.6 (CH), 55.4 (CH_2), 71.2 (OCH_3), 108.9 (CH), 115.2 (CH), 126.4 (CH), 133.0 (C), 135.0 (C), 142.9 (CH), 147.7 (CH), 147.8 (C), 155.7 (C), 164.9 (C), 173.2 (C) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{18}H_{20}N_4O_2S$: 357.1380, found 357.1374.

*7.1.1.5. 6-(2-Methoxy-5-pyridyl)-3-(cis-2,6-dimethylmorpholino) isothiazolo[4,3-b]pyridine (**5e**)*. This compound was obtained using 2-methoxy-5-pyridineboronic acid. The crude residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 7:3) as mobile phase, affording the title compound as an amorphous yellow solid in 88% yield (47.7 mg, 0.13 mmol). Mp 160.2–162.1 °C. ^{1}H NMR (600 MHz, $CDCl_3$) *δ*: 1.32 (d, *J* = 6.3 Hz, 6H, 2 × CH_3), 2.92 (dd, *J* = 12.5, 10.7 Hz, 2H, 2 × NCH), 3.91–3.97 (m, 2H, 2 × OCH), 4.00 (s, 3H, OCH_3), 4.51 (dd, *J* = 12.7, 1.9 Hz, 2H, 2 × NCH), 6.88 (dd, *J* = 8.6, 0.6 Hz, 1H, arom H), 7.82–7.87 (m, 2H, arom H), 8.47 (dd, *J* = 2.5, 0.6 Hz, 1H, arom H), 8.57 (d, *J* = 2.1 Hz, 1H, arom H) ppm. ^{13}C NMR (151 MHz, $CDCl_3$) *δ*: 18.8 (CH_3), 53.7 (OCH_3), 55.4 (CH_2), 71.2 (CH), 111.4 (CH), 125.0 (CH), 126.5 (C), 132.6 (C), 134.1 (C), 137.4 (CH), 143.4 (CH), 145.4 (CH), 156.0 (C), 164.2 (C), 173.0 (C) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{18}H_{20}N_4O_2S$: 357.1380, found 357.1374.

*7.1.1.6. 6-(3-Methoxy-4-carboxyphenyl)-3-(cis-2,6-dimethylmorpholino) isothiazolo[4,3-b]pyridine (**5f**)*. This compound was obtained using 3-methoxy-4-carboxyphenylboronic acid. After completion of the reaction, a solution of HCl (1 M) was added until reach pH acid. The crude residue was purified by silica gel flash chromatography using a mixture of dichloromethane and methanol (in a ratio of 20:1) as mobile phase, affording the title compound as an amorphous yellow solid in 78% yield (47.4 mg, 0.12 mmol). Mp 251.3–253.1 °C. ^{1}H NMR (600 MHz, DMSO) *δ*: 1.20 (d, *J* = 6.2 Hz, 6H, 2 × CH_3), 2.91 (dd, *J* = 12.1, 11.0 Hz, 2H, 2 × NCH), 3.83–3.89 (m, 2H, 2 × OCH), 3.94 (s, 3H, OCH_3), 4.52 (d, *J* = 12.0 Hz, 2H, 2 × NCH), 7.45 (dd, *J* = 8.0, 1.4 Hz, 1H, arom H), 7.52 (d, *J* = 1.2 Hz, 1H, arom H), 7.75 (d, *J* = 7.9 Hz, 1H, arom H), 8.15 (d, *J* = 2.0 Hz, 1H, arom H), 8.81 (d, *J* = 2.0 Hz, 1H, arom H) ppm. ^{13}C NMR (151 MHz, DMSO) *δ*: 18.6 (CH_3), 54.8 (CH_2), 56.1 (OCH_3), 70.6 (CH), 111.5 (CH), 118.9 (CH), 121.1 (C), 125.7 (CH), 131.5 (CH), 133.8 (C), 134.0 (C), 141.4 (C), 143.8 (CH), 155.4 (C), 158.7 (C), 167.1 (C), 172.5 (C) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{20}H_{21}N_3O_4S$: 400.1325, found 400.1324.

*7.1.1.7. 6-(trans-Styryl)-3-(cis-2,6-dimethylmorpholino)isothiazolo[4,3-b] pyridine (**5g**)*. A solution of 6-bromo-3-(*cis*-2,6-dimethylmorpholino) isothiazolo[4,3-*b*]pyridine (1 equiv) in DMF was degassed with argon. Styrene (1.5 equiv), *N*-phenylurea (0.02 equiv), $Pd(OAc)_2$ (0.01 equiv)

and K_2CO_3 (2 equiv) were added. The resulting mixture was degassed a second time, filled with argon and stirred at 120 °C overnight. After completion of the reaction as monitored by TLC, the volatiles were evaporated to dryness and the crude residue was diluted with EtOAc (20 mL) and washed with a 0.5 M HCl solution (2 × 10 mL) and water (20 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The resulting residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 4:1) as mobile phase, affording the title compound as an amorphous yellow solid in 88% yield (47 mg, 0.13 mmol). Mp 177.4–178.5 °C. ^{1}H NMR (600 MHz, $CDCl_3$) *δ:* 1.31 (d, *J* = 6.3 Hz, 6H, 2 × CH_3), 2.89 (dd, *J* = 12.2, 10.9 Hz, 2H, 2 × NCH), 3.90–3.97 (m, 2H, 2 × OCH), 4.48 (dd, *J* = 12.7, 1.6 Hz, 2H, NCH), 7.12 (d, *J* = 16.4 Hz, 1H, double bond CH), 7.28 (d, *J* = 16.4 Hz, 1H, double bond CH), 7.32 (t, *J* = 7.3 Hz, 1H, arom H), 7.40 (t, *J* = 7.7 Hz, 2H, arom H), 7.56 (d, *J* = 7.5 Hz, 2H, arom H), 7.74 (d, *J* = 1.8 Hz, 1H, arom H), 8.61 (d, *J* = 1.9 Hz, 1H, arom H) ppm. ^{13}C NMR (151 MHz, $CDCl_3$) *δ*: 18.8 (CH_3), 55.4 (CH_2), 71.2 (CH), 124.9 (CH), 125.3 (CH), 126.8 (CH), 128.4 (CH), 128.8 (CH), 131.7 (CH), 132.2 (C), 134.0 (C), 136.5 (C), 143.4 (CH), 156.4 (C), 172.7 (C) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{20}H_{21}N_3OS$: 352.1478, found 352.1473.

7.1.2. Synthesis of 6-anilino-3-(cis-2,6-dimethylmorpholino)isothiazolo [4,3-b]pyridines

General procedure. $Pd(dba)_2$ (0.03 equiv) and SPhos (0.06 equiv) were added to a degassed solution of toluene (15 mL). The resulting mixture was stirred under argon for 5–10 min. Subsequently, 6-bromo-3-(*cis*-2,6-dimethylmorpholino)isothiazolo[4,3-*b*]pyridine (1 equiv), the appropriate aniline or ethynylbenzene (1.5 equiv) and *t*-BuOK (1.5 equiv) were added and the mixture was degassed a second time. The reaction was filled with argon and stirred at 90 °C for 4–5 h. After completion of the reaction as monitored by TLC, the volatiles were evaporated to dryness. The crude residue was diluted with EtOAc (20 mL) and washed twice with a 0.5 M HCl solution (2 × 10 mL) and brine (20 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The resulting residue was purified by silica gel flash chromatography, yielding the corresponding 6-substituted-3-(*cis*-2,6-dimethylmorpholino)isothiazolo[4,*3-b*]pyridines. The following compounds were made according to this procedure.

*7.1.2.1. 3-(cis-2,6-Dimethylmorpholino)-6-(phenylamino)isothiazolo[4,3-b]pyridine (**5h**).*

This compound was obtained using aniline. The crude residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 7:3) as mobile phase, affording the title compound as an amorphous yellow solid in 85% yield (44 mg, 0.13 mmol). Mp 186.1–187.5 °C. ^{1}H NMR (600 MHz, $CDCl_3$) *δ:* 1.29 (d, *J* = 6.3 Hz, 6H, 2 × CH_3), 2.83–2.89 (m, 2H, 2 × NCH), 3.87–3.95 (m, 2H, 2 × OCH), 4.41 (d, *J* = 12.3 Hz, 2H, 2 × NCH), 6.04 (bs, 1H, NH), 7.04 (t, *J* = 7.3 Hz, 1H, arom H), 7.17 (d, *J* = 7.7 Hz, 2H, arom H), 7.26 (s, 1H, arom H), 7.32 (t, *J* = 7.8 Hz, 2H, arom H), 8.10 (d, *J* = 2.4 Hz, 1H, arom H) ppm. ^{13}C NMR (151 MHz, $CDCl_3$) *δ:* 18.8 (CH_3), 55.3 (CH_2), 71.2 (CH), 106.7 (CH), 119.6 (CH), 122.9 (CH), 129.5 (CH), 129.9 (C), 139.2 (C), 140.1 (CH), 140.8 (C), 157.2 (C), 172.0 (C) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{18}H_{20}N_4OS$: 341.1431, found 341.1431.

*7.1.2.2. 6-(3,4-Dimethoxyphenylamino)-3-(cis-2,6-dimethylmorpholino) isothiazolo[4,3-b]pyridine (**5i**).*

This compound was obtained using 3,4-dimethoxyaniline. The crude residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 3:2) as mobile phase, affording the title compound as an amorphous yellow solid in 79% yield (48 mg, 0.12 mmol). Mp 207.1–208.9 °C. ^{1}H NMR (600 MHz, $CDCl_3$) *δ:* 1.29 (d, *J* = 6.3 Hz, 6H, 2 × CH_3). 2.86 (dd, *J* = 12.5, 10.7 Hz, 2H, 2 × NCH), 3.86 (s, 3H, OCH_3), 3.89 (s, 3H, OCH_3), 3.90–3.94 (m, 2H, 2 × OCH), 4.40 (dd, *J* = 12.6, 1.6 Hz, 2H, 2 × NCH), 5.78 (bs, 1H, NH), 6.75–6.79 (m, 2H, arom H), 6.85 (d, *J* = 8.2 Hz, 1H, arom H), 7.06 (d, *J* = 2.4 Hz, 1H, arom H), 8.05 (d, *J* = 2.5 Hz, 1H, arom H) ppm. ^{13}C NMR (151 MHz, $CDCl_3$) *δ*: 18.9 (CH_3), 55.5 (CH_2), 56.1 (OCH_3), 56.4 (OCH_3), 71.4 (CH), 105.1, (CH), 106.5 (CH), 112.2 (CH), 113.9 (CH), 129.7 (C), 133.9 (C), 139.8 (CH), 140.9 (C), 146.1 (C), 149.9 (C), 157.5 (C), 172.1 (C) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{20}H_{24}N_4O_3S$: 401,1642, found 401,1638.

*7.1.2.3. 6-(3,4,5-Trimethoxyanilino)-3-(cis-2,6-dimethylmorpholino) isothiazolo[4,3-b]pyridine (**5j**).*

This compound was obtained using 3,4,5-trimethoxyaniline. The crude residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 3:2) as mobile phase, affording the title compound as an amorphous yellow solid in 73% yield (47.8 mg, 0.11 mmol). Mp 252.2–253.5 °C. ^{1}H NMR (300 MHz, $CDCl_3$) *δ*: 1.29 (d, *J* = 6.3 Hz, 6H, 2 × CH_3), 2.86 (dd, *J* = 12.5, 10.7 Hz, 2H, 2x NCH), 3.83 (s, 6H, 2 × OCH_3), 3.83 (s, 3H, OCH_3), 3.86–3.98 (m, 2H, 2 × OCH), 4.41 (dd, *J* = 12.8, 2.0 Hz, 2H, 2 × NCH), 5.88 (bs, 1H, NH), 6.42 (s, 2H, arom H), 7.18 (d, *J* = 2.5 Hz, 1H, arom H), 8.08 (d, *J* = 2.5 Hz, 1H, arom H) ppm. ^{13}C NMR (151 MHz, $CDCl_3$) *δ*: 18.8 (CH_3), 55.3 (CH_2), 56.2 (OCH_3), 61.0 (OCH_3), 71.2 (CH), 98.2 (CH), 106.4 (CH), 129.8 (C), 134.3 (C), 136.8 (C), 139.7 (C), 139.8 (CH), 154.0 (C), 157.2 (C), 172.1 (C) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{21}H_{26}N_4O_4S$: 431.1747, found 431.1745.

*7.1.2.4. 3-(cis-2,6-Dimethylmorpholino)-6-(phenylethynyl)isothiazolo [4,3-b]pyridine (**5k**).*

This compound was obtained using ethynylbenzene. The crude residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 4:1) as mobile phase, affording the title compound as an amorphous yellow solid in 87% yield (46.3 mg, 0.13 mmol). Mp 162.4–163.8 °C. ^{1}H NMR (500 MHz, $CDCl_3$) *δ*: 1.31 (d, *J* = 6.1 Hz, 6H, 2 × CH_3), 2.90 (t, *J* = 11.5 Hz, 2H, 2 × NCH), 3.90–3.98 (m, 2H, 2 × OCH), 4.47 (d, *J* = 12.5 Hz, 2H, 2 × NCH), 7.35–7.41 (m, 3H, arom H), 7.56–7.61 (m, 2H, arom H), 7.89 (s, 1H, arom H), 8.39 (s, 1H, arom H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$) *δ*: 18.8 (CH_3), 55.4 (CH_2), 71.2 (CH), 86.6 (C), 93.4 (C), 119.4 (C), 122.5 (C), 128.5 (CH), 128.9 (CH), 130.9 (CH), 131.8 (CH), 133.6 (C), 145.5 (CH), 155.0 (C), 173.0 (C) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{20}H_{19}N_3OS$: 350.1322, found 350.1315.

7.1.3. Synthesis of 6-(benzamide-4-yl)-3-(cis-2,6-dimethylmorpholino) isothiazolo[4,3-b]pyridines

General procedure. To a solution of compound **5f** (6-(3-methoxy-4-carboxyphenyl)-3-(*cis*-2,6-dimethylmorpholino)isothiazolo[4,3-*b*]pyridine) (1 equiv) in dry dichloromethane (15 mL) was added thionyl chloride ($SOCl_2$) (2 equiv). The mixture was stirred at 40 °C for 3 h. Then, triethylamine (2 equiv) and the appropriate amine (2 equiv) were added and the resulting mixture was stirred at room temperature overnight. After completion of the reaction as monitored by TLC, the residue was diluted with dichloromethane (20 mL) and washed with 0.5 M HCl solution (2 × 10 mL) and water (20 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The resulting residue was purified by silica gel flash chromatography, yielding the title compound. The following compounds were made according to this procedure.

*7.1.3.1. 6-(2-Methoxybenzamide-4-yl)-3-(cis-2,6-dimethylmorpholino) isothiazolo[4,3-b]pyridine (**6a**).*

This compound was obtained using aqueous ammonia (25%) (2 mL). After completion of the reaction as monitored by TLC, water (15 mL) was added, followed by extraction with dichloromethane (2 × 20 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The resulting residue was purified by silica gel flash chromatography, using a mixture of dichloromethane and methanol (20:0.5) as mobile phase, yielding the title compound as an amorphous yellow solid in 67% yield (33.4 mg, 0.08 mmol). Mp 270.6–272.2 °C. ^{1}H NMR (300 MHz, DMSO) *δ:* 1.20 (d, *J* = 6.2 Hz, 6H, 2 × CH_3), 2.90 (dd, *J* = 12.2, 11.0 Hz, 2H, 2 × NCH),

3.87 (dd, J = 12.3, 4.3 Hz, 2H, 2 × OCH), 4.03 (s, 3H, OCH_3), 4.51 (d, J = 12.3 Hz, 2H, 2 × NCH), 7.48 (dd, J = 8.1, 1.5 Hz, 1H, arom H), 7.53 (s, 1H, arom H), 7.62 (bs, 1H, NH), 7.70 (bs, 1H, NH), 7.91 (d, J = 8.0 Hz, 1H, arom H), 8.15 (d, J = 2.1 Hz, 1H, arom H), 8.81 (d, J = 2.1 Hz, 1H, arom H) ppm. ^{13}C NMR (75 MHz, DMSO) δ: 18.7 (CH_3), 55.0 (CH_2), 56.3 (OCH_3), 70.7 (CH), 111.1 (CH), 119.4 (CH), 122.7 (C), 125.6 (CH), 131.6 (CH), 133.8 (C), 134.1 (C), 140.9 (C), 143.9 (CH), 155.5 (C), 157.9 (C), 166.0 (C), 172.5 (C) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{20}H_{22}N_4O_3S$: 399.1485, found 399.1483.

7.1.3.2. 6-(N-Cyclopropyl-2-methoxybenzamide-4-yl)-3-(cis-2,6-dimethylmorpholino) isothiazolo[4,3-b]pyridine ***(6b)***. This compound was obtained using cyclopropylamine. The crude residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 3:7) as mobile phase, affording the title compound as an amorphous yellow solid in 70% yield (38 mg, 0.09 mmol). Mp 195.1–196.8 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.61–0.64 (m, 2H, CH_2), 0.86–0.91 (m, 2H, CH_2), 1.33 (d, J = 6.3 Hz, 6H, 2 × CH_3), 2.91–2.99 (m, 3H, CH, 2 × NCH), 3.92–3.99 (m, 2H, 2 × OCH), 4.03 (s, 3H, OCH_3), 4.53 (dd, J = 12.7, 1.8 Hz, 2H, 2 × NCH), 7.20 (d, J = 1.6 Hz, 1H, arom H), 7.38 (dd, J = 8.1, 1.6 Hz, 1H, arom H), 7.91 (d, J = 2.8 Hz, 1H, NH), 7.93 (d, J = 2.1 Hz, 1H, arom H), 8.35 (d, J = 8.1 Hz, 1H, arom H), 8.63 (d, J = 2.1 Hz, 1H, arom H) ppm. ^{13}C NMR (151 MHz, $CDCl_3$) δ: 6.9 (CH_2), 18.8 (CH_3), 22.9 (CH), 55.4 (CH_2), 56.1 (OCH_3), 71.2 (CH), 110.3 (CH), 120.3 (CH), 121.3 (C), 126.1 (CH), 133.1 (CH), 134.5 (C), 134.8 (C), 142.1 (C), 143.6 (CH), 155.9 (C), 157.8 (C), 166.1 (C), 173.1 (C) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{23}H_{26}N_4O_3S$: 439.1798, found 439.1798.

7.1.3.3. (4-(3-(cis-2,6-Dimethylmorpholino)isothiazolo[4,3-b]pyridin-6-yl)-2-methoxyphenyl)(pyrrolidin-1-yl)methanone ***(6c)***. This compound was obtained using pyrrolidine. The crude residue was purified by silica gel flash chromatography using a mixture of dichloromethane and methanol (in a ratio of 10:0.1) as mobile phase, affording the title compound as an amorphous yellow solid in 65% yield (36.6 mg, 0.08 mmol). Mp 173.1–174.9 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 1.32 (d, J = 6.3 Hz, 6H, 2 × CH_3), 1.86–1.92 (m, 2H, CH_2), 1.94–2.01 (m, 2H, CH_2), 2.93 (dd, J = 12.5, 10.7 Hz, 2H, 2 × NCH), 3.30 (t, J = 6.7 Hz, 2H, CH_2), 3.68 (t, J = 7.0 Hz, 2H, CH_2), 3.92 (s, 3H, OCH_3), 3.93–3.98 (m, 2H, 2 × OCH), 4.53 (dd, J = 12.7, 1.8 Hz, 2H, 2 × NCH), 7.16 (d, J = 1.4 Hz, 1H, arom H), 7.27 (dd, J = 7.7, 1.5 Hz, 1H, arom H), 7.40 (d, J = 7.7 Hz, 1H, arom H), 7.90 (d, J = 2.1 Hz, 1H, arom H), 8.61 (d, J = 2.1 Hz, 1H, arom H) ppm. ^{13}C NMR (151 MHz, $CDCl_3$) δ: 18.8 (CH_3), 24.6 (CH_2), 25.8 (CH_2), 45.6 (CH_2), 47.7 (CH_2), 55.4 (CH_2), 55.7 (OCH_3), 71.2 (CH), 110.2 (CH), 119.9 (CH), 125.8 (CH), 127.5 (C), 128.6 (CH), 134.3 (C), 135.3 (C), 139.9 (C), 143.9 (CH), 155.8 (C), 156.0 (C), 167.2 (C), 173.0 (C) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{24}H_{28}N_4O_3S$: 453.1955, found 453.1945.

7.1.3.4. (4-(3-cis-2,6-Dimethylmorpholino)isothiazolo[4,3-b]pyridin-6-yl)-2-methoxyphenyl)(morpholino)methanone ***(6d)***. This compound was obtained using morpholine. The crude residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 3:7) as mobile phase, affording the title compound as an amorphous yellow solid in 68% yield (39 mg, 0.08 mmol). Mp 134.2–135.9 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 1.32 (d, J = 6.3 Hz, 6H, 2 × CH_3), 2.90–2.97 (m, 2H, 2 × NCH), 3.27–3.40 (m, 2H, CH_2), 3.58–3.71 (m, 2H, CH_2), 3.76–3.89 (m, 4H, 2 × CH_2), 3.93 (s, 3H, OCH_3), 3.93–3.98 (m, 2H, 2 × OCH), 4.53 (d, J = 11.3 Hz, 2H, 2 × NCH), 7.15 (d, J = 1.2 Hz, 1H, arom H), 7.29 (dd, J = 7.7, 1.4 Hz, 1H, arom H), 7.39 (d, J = 7.7 Hz, 1H, arom H), 7.90 (d, J = 1.7 Hz, 1H, arom H), 8.60 (d, J = 1.9 Hz, 1H, arom H) ppm. ^{13}C NMR (151 MHz, $CDCl_3$) δ: 18.8 (CH_3), 42.2 (CH_2), 47.4 (CH_2), 55.4 (CH_2), 55.7 (OCH_3), 66.8 (CH_2), 67.0 (CH_2), 71.2 (CH), 110.0 (CH), 120.2 (CH), 125.3 (C), 125.9 (CH), 128.9 (CH), 135.1 (C), 140.3 (C), 143.7 (CH), 155.8 (C), 167.3 (C), 173.1 (C) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{24}H_{28}N_4O_4S$: 469.1904, found 469.1898.

7.1.3.5. 3-(cis-2,6-Dimethylmorpholino)-6-(N-phenyl-2-methoxybenzamide-4-yl)isothiazolo[4,3-b]pyridine ***(6e)***. This compound was obtained using aniline. The crude residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 3:2) as mobile phase, affording the title compound as an amorphous yellow solid in 76% yield (45 mg, 0.09 mmol). Mp 251.1–252.5 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 1.33 (d, J = 6.3, 6H, 2 × CH_3), 2.94 (dd, J = 12.6, 10.7 Hz, 2H, 2 × NCH), 3.92–3.98 (m, 2H, 2 × OCH), 4.14 (s, 3H, OCH_3), 4.53 (dd, J = 12.6, 1.7 Hz, 2H, 2 × NCH), 7.13–7.16 (m, 1H, arom H), 7.27 (d, J = 1.5 Hz, 1H, arom H), 7.36–7.40 (m, 2H, arom H), 7.42 (dd, J = 8.1, 1.6 Hz, 1H, arom H), 7.69–7.71 (dd, J = 8.5, 1.0 Hz, 2H, arom H), 7.95 (d, J = 2.1 Hz, 1H, arom H), 8.40 (d, J = 8.1 Hz, 1H, arom H), 8.64 (d, J = 2.1 Hz, 1H, arom H), 9.79 (s, 1H, NH) ppm. ^{13}C NMR (151 MHz, $CDCl_3$) δ: 18.8 (CH_3), 55.4 (CH_2), 56.4 (OCH_3), 71.2 (CH), 110.5 (CH), 120.4 (CH), 120.6 (CH), 121.6 (C), 124.3 (CH), 126.1 (CH), 129.0 (CH), 133.4 (CH), 134.5 (C), 138.3 (C), 142.6 (C), 143.5 (CH), 155.8 (C), 157.6 (C), 162.7 (C), 173.1 (C) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{26}H_{26}N_4O_3S$ 475.1798, found 475.1799.

7.1.3.6. 6-(N-Benzyl-2-methoxybenzamide-4-yl)-3-(cis-2,6-dimethylmorpholino) isothiazolo[4,3-b]pyridine ***(6f)***. This compound was obtained using benzylamine. The crude residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 1:1) as mobile phase, affording the title compound as an amorphous yellow solid in 85% yield (51.2 mg, 0.11 mmol). Mp 172.2–173.4 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 1.32 (d, J = 6.2 Hz, 6H, 2 × CH_3), 2.93 (dd, J = 12.2, 11.0 Hz, 2H, 2 × NCH), 3.91–3.98 (m, 2H, 2 × OCH), 4.00 (s, 3H, OCH_3), 4.53 (d, J = 11.6 Hz, 2H, 2 × NCH), 4.72 (d, J = 5.6 Hz, 2H, CH_2), 7.21 (d, J = 0.8 Hz, 1H, arom H), 7.27–7.31 (m, 1H, arom H), 7.33–7.42 (m, 5H, arom H), 7.93 (d, J = 1.9 Hz, 1H, arom H), 8.20 (m, 1H, NH), 8.38 (d, J = 8.1 Hz, 1H, arom H), 8.63 (d, J = 1.9 Hz, 1H, arom H) ppm.^{13}C NMR (151 MHz, $CDCl_3$) δ: 18.8 (CH_3), 43.8 (CH_2), 55.4 (CH_2), 56.1 (OCH_3), 71.2 (CH), 110.3 (CH), 120.3 (CH), 121.3 (C), 126.1 (CH), 127.3 (CH), 127.5 (CH), 128.7 (CH), 133.3 (CH), 134.5 (C), 134.7 (C), 138.7 (C), 142.2 (C), 143.6 (CH), 155.9 (C), 157.9 (C), 164.8 (C), 173.1 (C) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{27}H_{28}N_4O_3S$: 489.1955, found 489.1946.

7.1.3.7. 6-(3-Methoxy-4-formamidophenyl)-3-(cis-2,6-dimethylmorpholino)isothiazolo[4,3-b]pyridine ***(7a)***. To a solution of 6-(4-amino-3-methoxyphenyl)-3-(*cis*-2,6-dimethylmorpholino)isothiazolo [4,3-*b*]pyridine (1 equiv) in formamide (10 mL) was added MnO_2 (5% mol) in one portion. The reaction mixture was stirred at 150 °C for 5 h. After completion of the reaction as monitored by TLC, the volatiles were evaporated to dryness and the residue was purified by silica gel flash chromatography, using a mixture of hexane and acetyl acetate, yielding the title compound as an amorphous yellow solid in 73% yield (39.2 mg, 0.10 mmol). Mp 234.3–235.7 °C. Doubling of signals for the protons of the amine and the formyl group in the ^{1}H NMR spectrum at 25 °C was observed, due to restricted rotation around the C—N amide bond. Based on the integration of the signals in ^{1}H NMR spectrum, the ratio of the isomers was determined to be 95:5 (*cis*:*trans*).[28] When running the ^{1}H NMR spectrum at 50 °C, coalescence of both signals is observed (see Supporting Information). ^{1}H NMR (600 MHz, DMSO) δ: 1.19 (d, J = 6.2 Hz, 6H, 2 × CH_3), 2.89 (t, J = 11.5 Hz, 2H, 2 × NCH), 3.83–3.89 (m, 2H, 2 × OCH), 3.98 (s, 3H, OCH_3), 4.51 (d, J = 12.0 Hz, 1H, 2 × NCH), 7.42 (dd, J = 8.3, 1.3 Hz, 1H, arom H), 7.50 (d, J = 1.4 Hz, 1H, arom H), 8.06 (d, J = 1.8 Hz, 1H, arom H), 8.31 (d, J = 8.3 Hz, 1H, arom H), 8.35 (d, J = 1.3 Hz, 1H, HCO), 8.80 (d, J = 1.8 Hz, 1H, arom H), 9.80 (s, 1H, NH) ppm. ^{13}C NMR (151 MHz, DMSO) δ: 18.6 (CH_3), 54.8 (CH_2), 56.2 (OCH_3), 70.6 (CH), 110.0 (CH), 119.5 (CH), 120.5 (CH), 124.3 (CH), 127.5 (C), 132.3 (C), 133.3 (C), 134.5 (C), 144.0 (CH), 149.0 (C), 155.7 (C), 160.3 (HCO), 172.3 (C)

ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{20}H_{22}N_4O_3S$: 399.1485, found 399.1485.

7.1.4. Synthesis of 6-(4-aminyl-3-methoxyphenyl)-3-(cis-2,6-dimethylmorpholino) isothiazolo[4,3-b]pyridine

General procedure. To a solution of the precursor (6-(4-amino-3-methoxyphenyl)-3-(*cis*-2,6-dimethylmorpholino)isothiazolo[4,3-*b*]pyridine) (1 equiv) in dry dichloromethane (15 mL), was added trimethylamine (2 equiv). The reaction mixture was stirred under argon for 10 min and cooled to 0 °C. Then, the appropriate acid chloride (1.5 equiv) or acetic anhydride (2 equiv) was added and the resulting mixture was stirred at room temperature overnight. After completion of the reaction as monitored by TLC, dichloromethane was added (10 mL) and the reaction mixture was washed with 0.5 M HCl solution (2 × 10 mL) and water (20 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The resulting residue was purified by silica gel flash chromatography, yielding the title compound. The following compounds were made according to this procedure.

*7.1.4.1. 6-(3-Methoxy-4-acetamidophenyl)-3-(cis-2,6-dimethylmorpholino) isothiazolo[4,3-b]pyridine (**7b**)*. This compound was obtained using acetic anhydride. The crude residue was purified by silica gel flash chromatography using a mixture of dichloromethane and acetone (in a ratio of 95:5) as mobile phase, affording the title compound as an amorphous yellow solid in 73% yield (32.5 mg, 0.08 mmol). Mp 255.1–256–4 °C. ^{1}H NMR (600 MHz, $CDCl_3$) *δ*: 1.32 (d, *J* = 6.3 Hz, 6H, 2 × CH_3), 2.24 (s, 3H, CH_3), 2.92 (dd, *J* = 12.6, 10.7 Hz, 2H, 2 × NCH), 3.92–3.98 (m, 5H, 2 × OCH, OCH_3), 4.52 (dd, *J* = 12.8, 1.9 Hz, 2H, 2 × NCH), 7.14 (d, *J* = 1.9 Hz, 1H, arom H), 7.28 (dd, *J* = 8.3, 1.9 Hz, 1H, arom H), 7.82 (s, 1H, NH), 7.86 (d, *J* = 2.1 Hz, 1H, arom H), 8.49 (d, *J* = 8.4 Hz, 1H, arom H), 8.63 (d, *J* = 2.1 Hz, 1H, arom H) ppm. ^{13}C NMR (151 MHz, $CDCl_3$) *δ*: 18.8 (CH_3), 25.0 (CH_3), 55.4 (CH_2), 55.8 (OCH_3), 71.2 (CH), 108.7 (CH), 120.1 (CH), 120.2 (CH), 125.0 (CH), 128.1 (C), 132.9 (C), 134.0 (C), 135.4 (C), 144.1 (CH), 148.1 (C), 156.3 (C), 168.2 (C), 172.9 (C) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{21}H_{24}N_4O_3S$: 413.1642, found 413.1638.

*7.1.4.2. 6-(3-Methoxy-4-cyclopropylamidophenyl)-3-(cis-2,6-dimethylmorpholino) isothiazolo[4,3-b]pyridine (**7c**)*. This compound was obtained using cyclopropanecarbonyl chloride. The crude residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 6:4) as mobile phase, affording the title compound as an amorphous yellow solid in 79% yield (37 mg, 0.08 mmol). Mp 256.4–257.8 °C. ^{1}H NMR (500 MHz, $CDCl_3$) *δ*: 0.85–0.91 (m, 2H, CH_2), 1.10–1.14 (m, 2H, CH_2), 1.32 (d, *J* = 6.3 Hz, 6H, 2 × CH_3), 1.57–1.64 (m, 1H, CH), 2.92 (dd, *J* = 12.5, 10.7 Hz, 2H, 2 × NCH), 3.94 (m, 2H, 2 × OCH), 3.98 (s, 3H, OCH_3), 4.51 (dd, *J* = 12.6, 1.7 Hz, 2H, 2 × NCH), 7.15 (d, *J* = 1.9 Hz, 1H, arom H), 7.26–7.28 (m, 1H, arom H), 7.86 (d, *J* = 2.1 Hz, 1H, arom H), 8.05 (bs, 1H, NH), 8.48 (d, *J* = 8.2 Hz, 1H, arom H), 8.64 (d, *J* = 2.1 Hz, 1H, arom H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$) *δ*: 8.1 (CH_2), 18.8 (CH_3), 55.4 (CH_2), 55.8 (CH_3), 71.2 (CH), 108.8 (CH), 120.0 (CH), 120.2 (CH), 124.9 (CH), 128.4 (C), 132.6 (C), 134.0 (C), 135.5 (C), 144.2 (CH), 148.0 (C), 156.3 (C), 171.8 (C), 172.9 (C) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{23}H_{26}N_4O_3S$: 439.1798, found 439.1794.

*7.1.4.3. 6-(3-Methoxy-4-phenylamidophenyl)-3-(cis-2,6-dimethylmopholino) isothiazolo[4,3-b]pyridine (**7d**)*. This compound was obtained using benzoyl chloride. The crude residue was purified by silica gel flash chromatography using a mixture of dichloromethane and acetone (in a ratio of 95:5) as mobile phase, affording the title compound as an amorphous yellow solid in 81% yield (41.1 mg, 0.09 mmol). Mp 195.4–196.3 °C. ^{1}H NMR (600 MHz, $CDCl_3$) *δ*: 1.32 (d, *J* = 6.3 Hz, 6H, 2 × CH_3), 2.93 (dd, *J* = 12.5, 10.7 Hz, 2H, 2 × NCH), 3.93–3.98 (m, 2H, 2 × OCH), 4.02 (s, 3H, OCH_3), 4.53 (dd, *J* = 12.7, 1.8 Hz, 2H, 2 × NCH), 7.20 (d, *J* = 1.9 Hz, 1H, arom H), 7.35 (dd, *J* = 8.3, 1.9 Hz, 1H, arom H), 7.50–7.55 (m, 2H, arom H), 7.56–7.60 (m, 1H, arom H), 7.90 (d, *J* = 2.1 Hz, 1H, arom H), 7.91–7.95 (m, 2H, arom H), 8.62 (bs, *J* = 8.2 Hz, 1H, NH), 8.66–8.69 (m, 2H, arom H) ppm. ^{13}C NMR (151 MHz, $CDCl_3$) *δ*: 18.8 (CH_3), 55.4 (CH_2), 56.0 (OCH_3), 71.2 (CH), 108.8 (CH), 120.2 (CH), 120.3 (CH), 125.0 (CH), 127.1 (CH), 128.2 (C), 128.8 (CH), 131.9 (CH), 133.2 (C), 134.1 (C), 135.1 (C), 135.4 (C), 144.2 (CH), 148.6 (C), 156.3 (C), 165.3 (C), 172.9 (C) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{26}H_{26}N_4O_3S$: 475.1798, found 475.1794.

*7.1.4.4. Synthesis of 6-aryl-3-nitropyridine-2-carbonitriles (**9a-i**). General procedure*. A solution of 6-chloro-2-cyano-3-nitropyridine (1 equiv) in toluene or dioxane/water (see compound description) was degassed with argon and subsequently, the corresponding aryl boronic acid (1.2 equiv), $Pd(PPh_3)_4$ (0.02 equiv) and K_2CO_3 (2 equiv) were added. The mixture was degassed a second time, filled with argon and stirred at 95 °C overnight. After completion of the reaction as monitored by TLC, the volatiles were evaporated to dryness and the crude residue was diluted with EtOAc (20 mL) and washed with water (2 × 20 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The resulting residue was purified by silica gel flash chromatography, yielding the corresponding 6-aryl-3-nitropyridine-2-carbonitrile. The following compounds were made according to this procedure.

*7.1.4.5. 3-Nitro-6-phenylpyridine-2-carbonitrile (**9a**)*. This compound was obtained using phenyl boronic acid and toluene as solvent. The crude residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 8:2) as mobile phase, affording the title compound as an amorphous light yellow solid in 81% yield (397.6 mg, 1.8 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.65 (d, *J* = 8.9 Hz, 1H, arom H), 8.11–8.19 (m, 3H, arom H), 7.53–7.62 (m, 3H, arom H) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{12}H_7N_3O_2$: 226.0611, found 226.0611.

*7.1.4.6. 6-(4-(Trifluoromethyl)phenyl)-3-nitropyridine-2-carbonitrile (**9b**)*. This compound was obtained using 4-(trifluoromethyl)phenyl boronic acid and toluene as solvent. The crude residue was purified by silica gel flash chromatography using a mixture of hexane and dichloromethane (in a ratio of 4:6) as mobile phase, affording the title compound as an amorphous light yellow solid in 85% yield (543.3 mg, 1.85 mmol). ^{1}H NMR (600 MHz, $CDCl_3$) *δ*: 7.84 (dd, *J* = 8.7, 0.5 Hz, 2H, arom H), 8.22 (d, *J* = 8.8 Hz, 1H, arom H), 8.27 (dd, *J* = 8.8, 0.7 Hz, 2H, arom H), 8.72 (d, *J* = 8.8 Hz, 1H, arom H) ppm. HR-MS *m/z* $[M+Na]^+$ calcd for $C_{13}H_6F_3N_3O_2$: 316.0305, found 316.0312.

*7.1.4.7. 6-(4-Fluorophenyl)-3-nitropyridine-2-carbonitrile (**9c**)*. This compound was obtained using 4-fluorophenylboronic acid and toluene as solvent. The crude residue was purified by silica gel flash chromatography using a mixture of hexane and dichloromethane (in a ratio of 4:6) as mobile phase, affording the title compound as an amorphous light yellow solid in 94% yield (498.3 mg, 2.05 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) *δ*: 7.21–7.29 (m, 2H, arom H), 8.11 (d, *J* = 8.9 Hz, 1H, arom H), 8.14–8.20 (m, 2H, arom H), 8.65 (d, *J* = 8.9 Hz, 1H, arom H) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{12}H_6FN_3O_2$: 244.0517, found 244.0521.

*7.1.4.8. 6-(3-Chlorophenyl)-3-nitropyridine-2-carbonitrile (**9d**)*. This compound was obtained using 3-chlorophenylboronic acid and toluene as solvent. The crude residue was purified by silica gel flash chromatography using a mixture of hexane and dichloromethane (in a ratio of 4:6) as mobile phase, affording the title compound as an amorphous white solid in 98% yield (545.2 mg, 2.1 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) *δ*: 7.47–7.60 (m, 2H, arom H), 8.01 (dt, *J* = 7.3, 1.6 Hz, 1H, arom H), 8.12–8.17 (m, 2H, arom H), 8.67 (d, *J* = 8.8 Hz,

1H, arom H) ppm. HR-MS *m/z* $[M+Na]^+$ calcd for $C_{12}H_6ClN_3O_2$: 282.0041found 282.0044.

7.1.4.9. 6-(4-Methylphenyl)-3-nitropyridine-2-carbonitrile (9e). This compound was obtained using 4-methylphenylboronic acid and toluene as solvent. The crude residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 7:3) as mobile phase, affording the title compound as an amorphous light yellow solid in 93% yield (485 mg, 2.03 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) *δ*: 2.46 (s, 3H, CH_3), 7.36 (d, *J* = 8.1 Hz, 2H, arom H), 8.04 (d, *J* = 8.3 Hz, 2H, arom H), 8.10 (d, *J* = 8.9 Hz, 1H, arom H), 8.60 (d, *J* = 8.9 Hz, 1H, arom H) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{13}H_9N_3O_2$: 240.0767, found 240.0775.

7.1.4.10. 6-(3-Methoxyphenyl)-3-nitropyridine-2-carbonitrile (9f). This compound was obtained using 3-methoxyphenylboronic acid and toluene as solvent. The crude residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 7:3) as mobile phase, affording the title compound as an amorphous yellow solid in 94% yield (523 mg, 2.05 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) *δ*: 3.92 (s, 3H, OCH_3), 7.09–7.15 (m, 1H, arom H), 7.47 (t, *J* = 8.0 Hz, 1H, arom H), 7.63–7.68 (m, 1H, arom H), 7.68–7.72 (m, 1H, arom H), 8.13 (d, *J* = 8.9 Hz, 1H, arom H), 8.63 (d, *J* = 8.9 Hz, 1H, arom H) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{13}H_9N_3O_3$: 256.0717, found 256.0715.

7.1.4.11. 6-(3,4-Dimethoxyphenyl)-3-nitropyridine-2-carbonitrile (9g). This compound was obtained using 3,4-dimethoxyphenylboronic acid and toluene as solvent. The crude residue was purified by silica gel flash chromatography using a mixture of hexane and acetone (in a ratio of 7:3) as mobile phase, affording the title compound as an amorphous orange solid in 78% yield (485.1 mg, 1.7 mmol). ^{1}H NMR (300 MHz, DMSO) δ: 3.87 (s, 3H, OCH_3), 3.90 (s, 3H, OCH_3), 7.17 (d, *J* = 8.6 Hz, 1H, arom H), 7.77 (d, *J* = 2.0 Hz, 1H, arom H), 7.87 (dd, *J* = 8.5, 2.1 Hz, 1H, arom H), 8.54 (d, *J* = 9.0 Hz, 1H, arom H), 8.75 (d, *J* = 9.0 Hz, 1H, arom H) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{14}H_{11}N_3O_4$: 286.0822, found 286.0822.

7.1.4.12. 6-(3,4,5-Trimethoxyphenyl)-3-nitropyridine-2-carbonitrile (9h). This compound was obtained using 3,4,5-trimethoxyphenylboronic acid and dioxane/water (in ratio 4:1) as solvent. The crude residue was purified by silica gel flash chromatography using a mixture of hexane and acetone (in a ratio of 7:3) as mobile phase, affording the title compound as an amorphous orange solid in 96% yield (659.8 mg, 2.1 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) *δ*: 3.94 (s, 3H, OCH_3), 4.00 (s, 6H, 2 × OCH_3), 7.37 (s, 2H, arom H), 8.09 (d, *J* = 8.9 Hz, 1H, arom H), 8.61 (d, *J* = 8.9 Hz, 1H, arom H) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{15}H_{13}N_3O_5$: 316.0928, found 316.0931.

7.1.4.13. 6-(3,5-Dimethoxyphenyl)-3-nitropyridine-2-carbonitrile (9i). This compound was obtained using 3,5-dimethoxyphenylboronic acid and toluene as solvent. The crude residue was purified by silica gel flash chromatography using a mixture of hexane and acetone (in a ratio of 7:3) as mobile phase, affording the title compound as an amorphous orange solid in 81% yield (503.5 mg, 2.1 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) *δ*: 3.91 (s, 6H, 2 × OCH_3), 6.66 (t, *J* = 2.2 Hz, 1H, arom H), 7.25 (d, *J* = 2.2 Hz, 2H, arom H), 8.10 (d, *J* = 8.9 Hz, 1H, arom H), 8.62 (d, *J* = 8.9 Hz, 1H, arom H) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{14}H_{11}N_3O_4$: 286.0822, found 286.0818.

7.1.4.14. Synthesis of 3-amino-6-aryl-pyridine-2-carbonitriles (10a-i). General procedure. To a stirred suspension of iron powder (3 equiv) in acetic acid (40 mL) at 60 °C, the corresponding 6-aryl-3-nitropyridine-2-carbonitrile (1 equiv) was added. The reaction mixture was stirred at 60 °C until disappearance of the starting material as monitored by TLC (3–6 h). After cooling, the volatiles were evaporated to dryness and the crude was diluted with EtOAc (50 mL) and filtered through a paper filter. The filter cake was washed repeated times with ethyl acetate. The organic phase was washed with aqueous solution 1 N NaOH (3 × 30 mL), dried over anhydrous Na_2SO_4 and evaporated to dryness. This mixture of two compounds (3-amino-6-aryl-pyridine-2-carbonitrile as the major and 3-amino-6-aryl-pyridine-2-carboxamide as the minor compound) was used as such in the next reaction.

7.1.4.15. Synthesis of 3-amino-6-aryl-pyridine-2-carbothioamides (11a-i). General procedure. To a solution of the crude mixture of 3-amino-6-aryl-pyridine-2-carbonitrile and 3-amino-6-aryl-pyridine-2-carboxamide in absolute ethanol (40 mL), was added phosphorus pentasulfide (1.2 equiv). The corresponding solution was then heated overnight at 75 °C overnight. After the completion of the reaction as monitored by TLC, the solvent was evaporated to dryness and the residue was washed with an aqueous 1 N NaOH (2 × 30 mL) solution, dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue was purified by silica gel flash chromatography yielding the 3-amino-6-aryl-pyridine-2-carbothioamide. The following compounds were made according to this procedure.

7.1.4.15.1. 3-Amino-6-phenylpyridine-2-carbothioamide (11a). This compound was obtained from a crude mixture of 3-amino-6-phenylpyridine-2-carbonitrile and 3-amino-6-phenylpyridine-2-carboxamide. The residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 8:2) as mobile phase, affording the title compound as an amorphous yellow solid in 71% yield (166.8 mg, 0.73 mmol). ^{1}H NMR (300 MHz, DMSO) *δ:* 7.35 (m, 2H, arom H), 7.43 (m, 2H, arom H), 7.84 (bs, 2H, NH_2), 7.90 (d, *J* = 8.7 Hz, 1H, arom H), 8.08 (d, *J* = 7.4 Hz, 2H, arom H), 9.66 (bs, 1H, NH), 9.74 (bs, 1H, NH) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{12}H_{11}N_3S$: 230.0746, found 230.0743.

7.1.4.15.2. 3-Amino-6-(4-trifluoromethylphenyl)pyridine-2-carbothioamide (11b). This compound was obtained from a crude mixture of 3-amino-6-(4-trifluoromethylphenyl)pyridine-2-carbonitrile and 3-amino-6-(4-trifluoromethylphenyl)pyridine-2-carboxamide. The residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 7:3) as mobile phase, affording the title compound as an amorphous yellow solid in 81% yield (183 mg, 0.62 mmol). ^{1}H NMR (300 MHz, DMSO) *δ:* 7.37 (d, *J* = 8.8 Hz, 1H, arom H), 7.75 (d, *J* = 8.4 Hz, 2H, arom H), 7.99 (d, *J* = 8.8 Hz, 1H, arom H), 7.93 (bs, 2H, NH_2), 8.32 (d, *J* = 8.2 Hz, 2H, arom H), 9.74 (s, 1H, NH), 9.78 (s, 1H, NH) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{13}H_{10}F_3N_3S$: 298.0620, found 298.0614.

7.1.4.15.3. 3-Amino-6-(4-fluorophenyl)pyridine-2-carbothioamide (11c). This compound was obtained from a crude mixture of 3-amino-6-(4-fluorophenyl)pyridine-2-carbonitrile and 3-amino-6-(4-fluorophenyl)pyridine-2-carboxamide. The residue was purified by flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 8:2) as mobile phase, affording the title compound as an amorphous yellow solid in 79% yield (160.4 mg, 0.65 mmol). ^{1}H NMR (300 MHz, DMSO) *δ*: 7.23 (t, *J* = 8.8 Hz, 2H, arom H), 7.34 (d, *J* = 8.8 Hz, 1H, arom H), 7.83 (s, 2H, NH_2), 7.88 (d, *J* = 8.8 Hz, 1H, arom H), 8.15 (dd, *J* = 8.7, 5.6 Hz, 2H, arom H), 9.75 (bs, 1H, NH), 9.66 (bs, 1H, NH) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{12}H_{10}FN_3S$: 248.0652, found 248.0651.

7.1.4.15.4. 3-Amino-6-(3-chlorophenyl)pyridine-2-carbothioamide (11d). This compound was obtained from a crude mixture of 3-amino-6-(3-chlorophenyl)pyridine-2-carbonitrile and 3-amino-6-(3-chlorophenyl) pyridine-2-carboxamide. The residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 8:2) as mobile phase, affording the title compound as an amorphous yellow solid in 71% yield (173 mg, 0.62 mmol). ^{1}H NMR (300 MHz, DMSO) *δ:* 7.31–7.48 (m, 3H, arom H), 7.86 (bs, 2H, NH_2), 7.94 (d, *J* = 8.8 Hz, 1H, arom H), 8.04 (d, *J* = 7.6 Hz, 1H, arom H), 8.19 (t, *J* = 1.6 Hz 1H, arom H), 9.68 (bs, 1H, NH), 9.79 (bs, 1H, NH) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{12}H_{10}ClN_3S$: 264.0357, found 264.0353.

7.1.4.15.5. 3-Amino-6-(4-methylphenyl)pyridine-2-carbothioamide

(11e). This compound was obtained from a crude mixture of 3-amino-6-(4-methylphenyl)pyridine-2-carbonitrile and 3-amino-6-(4-methylphenyl) pyridine-2-carboxamide. The residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 8:2) as mobile phase, affording the title compound as an amorphous yellow solid in 83% yield (242.3 mg, 1.0 mmol). ^{1}H NMR (300 MHz, DMSO) *δ*: 2.34 (s, 3H, CH_3), 7.23 (d, *J* = 8.0 Hz, 2H, arom H), 7.32 (d, *J* = 8.8 Hz, 1H, arom H), 7.80 (bs, 2H, NH_2), 7.86 (d, *J* = 8.8 Hz, 1H, arom H), 7.97 (d, *J* = 8.2 Hz, 2H, arom H), 9.63 (bs, 1H, NH), 9.72 (bs, 1H, NH) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{13}H_{13}N_3S$: 244.0903, found 244.0916.

7.1.4.15.6. 3-Amino-6-(3-methoxyphenyl)pyridine-2-carbothioamide ***(11f)***. This compound was obtained from a crude mixture of 3-amino-6-(3-methoxyphenyl)pyridine-2-carbonitrile and 3-amino-6-(4-methoxyphenyl) pyridine-2-carboxamide. The residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 8:2) as mobile phase, affording the title compound as an amorphous yellow solid in 72% yield (261.4 mg, 1.0 mmol). ^{1}H NMR (300 MHz, DMSO) *δ*: 3.83 (s, 3H, OCH_3), 6.91 (dd, *J* = 8.1, 2.5 Hz, 1H, arom H), 7.30–7.38 (m, 2H, arom H), 7.57–7.66 (m, 2H, arom H), 7.89 (d, *J* = 8.8 Hz, 1H, arom H), 9.66 (bs, 1H, NH), 9.72 (bs, 1H, NH) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{13}H_{13}N_3OS$: 260.0852, found 260.0848.

7.1.4.15.7. 3-Amino-6-(3,4-dimethylphenyl)pyridine-2-carbothioamide ***(11g)***. This compound was obtained from a crude mixture of 3-amino-6-(3,4-dimethylphenyl)pyridine-2-carbonitrile and 3-amino-6-(3,4-dimethylphenyl)pyridine-2-carboxamide. The residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 7:3) as mobile phase, affording the title compound as an amorphous yellow solid in 73% yield (165.4 mg, 0.57 mmol). ^{1}H NMR (300 MHz, DMSO) *δ:* 3.79 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 6.99 (d, *J* = 8.4 Hz, 1H, arom H), 7.32 (d, *J* = 8.8 Hz, 1H, arom H), 7.55–7.60 (m, 1H, arom H), 7.62 (d, *J* = 1.9 Hz, 1H, arom H), 7.76 (bs, 2H, NH_2), 7.86 (d, *J* = 8.8 Hz, 1H, arom H), 9.62 (bs, 1H, NH), 9.71 (bs, 1H, NH) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{14}H_{15}N_3O_2S$: 290.0958, found 290.0941.

7.1.4.15.8. 3-Amino-6-(3,4,5-trimethoxyphenyl)pyridine-2-carbothioamide ***(11h)***. This compound was obtained from a crude mixture of 3-amino-6-(3,4,5-trimethoxyphenyl)pyridine-2-carbonitrile and 3-amino-6-(3,4,5-trimethoxyphenyl)pyridine-carboxamide. The residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 6:4) as mobile phase, affording the title compound as an amorphous yellow solid in 76% yield (170.1 mg, 0.53 mmol). ^{1}H NMR (600 MHz, $CDCl_3$) *δ*: 3.90 (s, 3H, OCH_3), 3.95 (s, 6H, 2 × OCH_3), 6.94 (bs, 2H, NH_2), 7.05 (s, 2H, arom H), 7.15–7.18 (m, 1H, arom H), 7.30 (bs, 1H, NH), 7.62–7.64 (m, 1H, arom H), 9.64 (bs, 1H, NH) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{15}H_{17}N_3O_3S$: 320.1063, found 320.1072.

7.1.4.15.9. 3-Amino-6-(3,5-dimethylphenyl)pyridine-2-carbothioamide ***(11i)***. This compound was obtained from a crude mixture of 3-amino-6-(3,5-dimethylphenyl)pyridine-2-carbonitrile and 3-amino-6-(3,5-dimethylphenyl)pyridine-2-carboxamide. The residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 7:3) as mobile phase, affording the title compound as an amorphous yellow solid in 76% yield (258.3 mg, 1.17 mmol). ^{1}H NMR (300 MHz, DMSO) *δ:* 3.81 (s, 6H, 2 × OCH_3), 6.48 (t, *J* = 2.1 Hz, 1H, arom H), 7.18 (d, *J* = 2.2 Hz, 2H, arom H), 7.32 (d, *J* = 8.8 Hz, 1H, arom H), 7.81 (bs, 2H, NH_2), 7.88 (d, *J* = 8.8 Hz, 1H, arom H), 9.65 (bs, 1H, NH), 9.70 (bs, 1H, NH) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{14}H_{15}N_3O_2S$: 290.0958, found 290.0963.

7.1.5. Synthesis of 3-amino-5-(aryl)isothiazolo[4,3-b]pyridines ***(12a-i)***

General procedure. To a solution of a 3-amino-6-(aryl)pyridine-2-carbothioamides (1 equiv) in methanol (50 mL) was added dropwise a 35% H_2O_2 (2.5 equiv) solution in water at 0 °C. The reaction mixture was stirred overnight at room temperature. After disappearance of the starting material as monitored by TLC, the solvent was evaporated to dryness affording the desired 3-amino-5-aryl-isothiazolo[4,3-*b*]pyridine, that was used as such in the next reaction without any further purification. The following compounds were made according to this procedure.

7.1.5.1. 3-Amino-5-(phenyl)isothiazolo[4,3-b]pyridine ***(12a)***. This compound was prepared from 3-amino-6-phenylpyridine-2-carbothioamide affording the title compound as an amorphous yellow solid in 95% yield (89.4 mg, 0.39 mmol). ^{1}H NMR (600 MHz, DMSO) *δ*: 7.41–7.46 (m, 1H, arom H), 7.48–7.53 (m, 2H, arom H), 7.81 (d, *J* = 9.2, 1H, arom H), 7.96 (d, *J* = 9.3 Hz, 1H, arom H), 8.20–8.24 (m, 2H, arom H) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{12}H_9N_3S$: 228.0590, found 228.0583.

7.1.5.2. 3-Amino-5-(4-trifluoromethylphenyl)isothiazolo[4,3-b]pyridine ***(12b)***. This compound was prepared from 3-amino-6-(4-fluorophenyl) pyridine-2-carbothioamide affording the title compound as an amorphous yellow solid in 97% yield (120.3 mg, 0.41 mmol). ^{1}H NMR (300 MHz, DMSO) *δ:* 7.79–7.89 (m, 3H, arom H), 8.01 (d, *J* = 9.4 Hz, 1H, arom H), 8.10 (bs, 2H, NH_2), 8.44 (d, *J* = 8.1 Hz, 2H, arom H) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{13}H_8F_3N_3S$: 296.0464, found 296.0471.

7.1.5.3. 3-Amino-5-(4-fluorophenyl)isothiazolo[4,3-b]pyridine ***(12c)***. This compound was prepared from 3-amino-6-(4-fluorophenyl) pyridine-2-carbothioamide affording the title compound as an amorphous yellow solid in 98% yield (106.9 mg, 0.44 mmol). ^{1}H NMR (600 MHz, DMSO) *δ*: 7.30–7.36 (m, 2H, arom H), 7.79 (d, *J* = 9.3 Hz, 1H, arom H), 7.90 (bs, 2H, NH_2), 7.91 (d, *J* = 9.4 Hz, 1H, arom H), 8.25–8.30 (m, 2H, arom H) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{12}H_8FN_3S$: 246.0496, found 246.0506.

7.1.5.4. 3-Amino-5-(3-chlorophenyl)isothiazolo[4,3-b]pyridine ***(12d)***. This compound was prepared from 3-amino-6-(3-chlorophenyl) pyridine-2-carbothioamide affording the title compound as an amorphous yellow solid in 97% yield (192.9 mg, 0.74 mmol). ^{1}H NMR (300 MHz, DMSO) *δ:* 7.45–7.57 (m, 2H, arom H), 7.79 (d, *J* = 9.4 Hz, 1H, arom H), 7.97 (d, *J* = 9.4 Hz, 1H, arom H), 8.07 (bs, 2H, NH_2), 8.15 (d, *J* = 7.4 Hz, 1H, arom H), 8.36 (s, 1H, arom H) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{12}H_8ClN_3S$: 262.0200, found 262.0196.

7.1.5.5. 3-Amino-5-(4-methylphenyl)isothiazolo[4,3-b]pyridine ***(12e)***. This compound was prepared from 3-amino-6-(4-methylphenyl) pyridine-2-carbothioamide affording the title compound as an amorphous yellow solid in 98% yield (194.0 mg, 0.8 mmol). ^{1}H NMR (300 MHz, DMSO) *δ*: 2.37 (s, 3H, CH_3), 7.31 (d, *J* = 8.1 Hz, 2H, arom H), 7.78 (d, *J* = 9.4 Hz, 1H, arom H), 7.93 (d, *J* = 9.3 Hz, 1H, arom H), 7.96 (bs, 2H, NH_2), 8.13 (d, *J* = 8.1 Hz, 2H, arom H) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{13}H_{11}N_3S$: 242.0746, found 242.0750.

7.1.5.6. 3-Amino-5-(3-methoxyphenyl)isothiazolo[4,3-b]pyridine ***(12f)***. This compound was prepared from 3-amino-6-(3-methoxyphenyl) pyridine-2-carbothioamide affording the title compound as an amorphous yellow solid in 91% yield (117.4 mg, 0.46 mmol). ^{1}H NMR (600 MHz, DMSO) *δ:* 3.86 (s, OCH_3), 7.02 (dd, *J* = 8.1, 2.2 Hz, 1H, arom H), 7.41 (t, *J* = 7.9 Hz, 1H, arom H), 7.78 (d, *J* = 7.7 Hz, 1H, arom H), 7.81–7.85 (m, 2H, arom H), 8.01 (d, *J* = 9.0 Hz, 2H, arom H) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{13}H_{11}N_3OS$: 258.0696, found 258.0688.

7.1.5.7. 3-Amino-5-(3,4-dimethoxyphenyl)isothiazolo[4,3-b]pyridine ***(12g)***. This compound was prepared from 3-amino-6-(3,4-dimethylphenyl)pyridine-2-carbothioamide affording the title compound as an amorphous yellow solid in 92% yield (79.3 mg, 0.28 mmol). ^{1}H NMR (600 MHz, DMSO) *δ*: 3.83 (s, 3H, OCH_3), 3.90 (s, 3H, OCH_3), 7.05

(d, J = 8.5 Hz, 1H, arom H), 7.72 (dd, J = 8.4, 2.1 Hz, 1H, arom H), 7.76 (d, J = 9.4 Hz, 1H, arom H), 7.91 (d, J = 2.0 Hz, 1H, arom H), 7.94 (d, J = 9.4 Hz, 1H, arom H) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{14}H_{13}N_3O_2S$: 288.0801, found 288.0798.

*7.1.5.8. 3-Amino-5-(3,4,5-trimethoxyphenyl)isothiazolo[4,3-b]pyridine **(12h)***. This compound was prepared from 3-amino-6-(3,4,5-trimethylphenyl)pyridine-2-carbothioamide affording the title compound as an amorphous orange solid in 94% yield (112.1 mg, 0.35 mmol). ^{1}H NMR (300 MHz, DMSO) δ: 3.73 (s, 3H, OCH_3), 3.92 (s, 6H, 2 × OCH_3), 7.54 (s, 2H, arom H), 7.78 (d, J = 9.4 Hz, 1H, arom H), 8.02 (d, J = 9.4 Hz, 1H, arom H) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{15}H_{15}N_3O_3S$: 318.0907, found 318.0904.

*7.1.5.9. 3-Amino-5-(3,5-dimethoxyphenyl)isothiazolo[4,3-b]pyridine **(12i)***. This compound was prepared from 3-amino-6-(3,5-dimethylphenyl)pyridine-2-carbothioamide affording the title compound as an amorphous yellow solid in 93% yield (92.2 mg, 0.35 mmol). ^{1}H NMR (300 MHz, DMSO) δ: 3.85 (s, 6H, 2 × OCH_3), 6.57 (bs, 1H, arom H), 7.40 (s, 2H, arom H), 7.77 (d, J = 9.4 Hz, 1H, arom H), 7.95 (d, J = 9.4 Hz, 1H, arom H), 8.05 (bs, 2H, NH_2) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{14}H_{13}N_3O_2S$: 288.0801, found 288.0803.

*7.1.6. Synthesis of 5-aryl-3-bromoisothiazolo[4,3-b]pyridines **(13a-h)***

General procedure A. A solution of 5-aryl-3-aminoisothiazolo[4,3-*b*] pyridine (1 equiv) in HBr (30 mL) was stirred for 10 min at room temperature, and then CuBr was added (2 equiv). The resulting mixture was cooled at 0 °C. A solution of sodium nitrite (3 equiv) in H_2O (15 mL) was added dropwise over a period of 30 min. The reaction mixture was stirred for 2 h at 0 °C and overnight at room temperature. The mixture was cooled at 0 °C and carefully neutralized with a 2 N NaOH solution. The mixture was extracted with ethyl acetate (3 × 30 mL) and the combined organic layers were dried over anhydrous Na_2SO_4 and evaporated to dryness. The crude residue was purified by silica gel flash chromatography (using a mixture of hexane/ethyl acetate as mobile phase), yielding the corresponding 5-aryl-3-bromoisothiazolo[4,3-*b*]pyridine.

General procedure B. To a stirred solution of $CuBr_2$ (1.2 equiv) and 5-aryl-3-aminoisothiazolo[4,3-*b*]pyridine (1 equiv) in dry acetonitrile (15 mL) under argon atmosphere at −10 °C, was slowly added *tert*-butylnitrite (1.3 equiv). The resulting mixture was stirred at 0 °C for 2 h and additional 3 h at room temperature. After disappearance of the starting material as monitored by TLC, the volatiles were evaporated and the crude residue was purified by silica gel flash column chromatography (using a mixture of hexane/ethyl acetate as eluent) yielding the corresponding 5-aryl-3-bromoisothiazolo[4,3-*b*]pyridine.

The following compounds were made according to the general procedure A:

*7.1.6.1. 3-Bromo-5-(phenyl)isothiazolo[4,3-b]pyridine **(13a)***. This compound was prepared from 3-amino-5-phenylisothiazolo[4,3-*b*] pyridine and the residue was purified by flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 9:1) as mobile phase, affording the title compound as an amorphous white solid in 61% yield (39 mg, 0.13 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.48–7.58 (m, 3H, arom H), 7.90 (d, J = 9.3 Hz, 1H, arom H), 8.11–8.22 (m, 3H, arom H) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{12}H_7BrN_2S$: 290.9587, found 290.9590.

*7.1.6.2. 3-Bromo-5-(4-trifluoromethylphenyl)isothiazolo[4,3-b]pyridine **(13b)***. This compound was prepared from 3-amino-5-(4-trifluoromethylphenyl)isothiazolo[4,3-*b*]pyridine and the residue was purified by flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 9:1) as mobile phase, affording the title compound as an amorphous white solid in 57% yield (48.3 mg, 0.13 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.79 (d, J = 8.4 Hz, 2H, arom H), 7.91 (d, J = 9.3 Hz, 1H, arom H), 8.20 (d, J = 9.3 Hz, 1H, arom H), 8.30 (d, J = 8.4 Hz, 2H, arom H) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{13}H_6BrF_3N_2S$: 358,9460, found 358,9464.

*7.1.6.3. 3-Bromo-5-(4-fluorophenyl)isothiazolo[4,3-b]pyridine **(13c)***. This compound was prepared from 3-amino-5-(4-fluorophenyl) isothiazolo[4,3-*b*]pyridine and the residue was purified by flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 9:1) as mobile phase, affording the title compound as an amorphous white solid in 60% yield (105 mg, 0.34 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.21 (m, 2H, arom H), 7.86 (d, J = 9.4 Hz, 1H, arom H), 8.14 (d, J = 9.4 Hz, 1H, arom H), 8.17–8.23 (m, 2H, arom H) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{12}H_6BrFN_2S$: 308,9492, found 308,9503.

*7.1.6.4. 3-Bromo-5-(3-chlorophenyl)isothiazolo[4,3-b]pyridine **(13d)***. This compound was prepared from 3-amino-5-(3-chlorophenyl) isothiazolo[4,3-*b*]pyridine and the residue was purified by flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 8:2) as mobile phase, affording the title compound as an amorphous white solid in 51% yield (25.4 mg, 0.08 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.41–7.45 (m, 2H, arom H), 7.87 (d, J = 9.4 Hz, 1H, arom H), 8.02–8.09 (m, 1H, arom H), 8.17 (d, J = 9.4 Hz, 1H, arom H), 8.21 (s, 1H, arom H) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{12}H_6BrClN_2S$: 324.9197, found 324.9191.

The following compounds were made according to the general procedure B:

*7.1.6.5. 3-Bromo-5-(4-methylphenyl)isothiazolo[4,3-b]pyridine **(13e)***. This compound was prepared from 3-amino-5-(4-methylphenyl) isothiazolo[4,3-*b*]pyridine and the residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 9:1) as mobile phase, affording the title compound as an amorphous white solid in 51% yield (25.5 mg, 0.08 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.44 (s, 3H, CH_3), 7.34 (d, J = 8.1 Hz, 2H, arom H), 7.89 (d, J = 9.4 Hz, 1H, arom H), 8.07–8.15 (m, 3H, arom H) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{13}H_9BrN_2S$: 304.9743, found 304.9748.

*7.1.6.6. 3-Bromo-5-(3-methoxyphenyl)isothiazolo[4,3-b]pyridine **(13f)***. This compound was prepared from 3-amino-5-(3-methoxyphenyl) isothiazolo[4,3-*b*]pyridine and the residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 9:1) as mobile phase, affording the title compound as an amorphous light yellow solid in 34% yield (62 mg, 0.19 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 3.93 (s, 3H, OCH_3), 7.06 (dd, J = 8.0, 2.3 Hz, 1H, arom H), 7.44 (t, J = 8.0 Hz, 1H, arom H), 7.72 (d, J = 7.8 Hz, 1H, arom H), 7.77–7.81 (m, 1H, arom H), 7.89 (d, J = 9.4 Hz, 1H, arom H), 8.14 (d, J = 9.4 Hz, 1H, arom H) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{13}H_9BrN_2S$: 320.9692, found 320.9693.

*7.1.6.7. 3-Bromo-5-(3,4-dimethoxyphenyl)isothiazolo[4,3-b]pyridine **(13g)***. This compound was prepared from 3-amino-5-(3,4-dimethoxyphenyl)isothiazolo[4,3-*b*]pyridine and the residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 8:2) as mobile phase, affording the title compound as an amorphous light yellow solid in 56% yield (58.2 mg, 0.17 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 3.97 (s, 3H, OCH_3), 4.04 (s, 3H, OCH_3), 6.98 (d, J = 8.4 Hz, 1H, arom H), 7.68 (dd, J = 8.4, 2.1 Hz, 1H, arom H), 7.89 (dd, J = 6.6, 6.0 Hz, 2H, arom H), 8.09 (d, J = 9.4 Hz, 1H, arom H) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{14}H_{11}BrN_2O_2S$: 350.9798, found 350.9796.

*7.1.6.8. 3-Bromo-5-(3,4,5-trimethoxyphenyl)isothiazolo[4,3-b]pyridine **(13h)***. This compound was prepared from 3-amino-5-(3,4,5-trimethoxyphenyl)isothiazolo[4,3-*b*]pyridine and the residue was

purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 7:3) as mobile phase, affording the title compound as an amorphous light yellow solid in 35% yield (19.7 mg, 0.05 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 3.93 (s, 3H, OCH_3), 4.01 (s, 6H, 2 × OCH_3), 7.42 (s, 2H, arom H), 7.87 (d, J = 9.4 Hz, 1H, arom H), 8.14 (d, J = 9.3 Hz, 1H, arom H) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{15}H_{13}BrN_2O_3S$: 380.9903, found 380.9888.

*7.1.7. Synthesis of 5-aryl-3-(cis-2,6-dimethylmorpholino)isothiazolo[4,3-b]pyridines (**14a-h**)*

General procedure. To a solution of a 5-substituted-3-bromoisothiazolo[4,3-*b*]pyridine (1 equiv) in absolute ethanol (20 mL) was added *cis*-2,6-dimethylmorpholine (3 equiv) and the mixture was stirred at reflux overnight. After disappearance of the starting material as monitored by TLC, the volatiles were evaporated to dryness and the residue was purified by silica gel flash column chromatography yielding the 5-substituted-3-(*cis*-2,6-dimethylmorpholino)isothiazolo[4,3-*b*]pyridine. The following compounds were made according to this procedure:

*7.1.7.1. 3-(cis-2,6-Dimethylmorpholino)-5-phenylisothiazolo[4,3-b] pyridine (**14a**).* This compound was prepared from 3-bromo-5-phenylisothiazolo[4,3-*b*]pyridine. The residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 4:1) as mobile phase, affording the title compound as an amorphous yellow solid in 91% yield (40.5 mg, 0.12 mmol). Mp 154.1–155.8 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 1.32 (d, J = 6.3 Hz, 6H, 2 × CH_3), 2.96 (dd, J = 12.5, 10.8 Hz, 2H, 2 × NCH), 3.92–4.00 (m, 2H, 2 × OCH), 4.66 (d, J = 11.6 Hz, 2H, 2 × NCH), 7.40–7.44 (m, 1H, arom H), 7.47–7.50 (m, 2H, arom H), 7.72 (d, J = 9.4 Hz, 1H, arom H), 7.83 (d, J = 9.4 Hz, 1H, arom H), 7.97–8.01 (m, 2H, arom H) ppm. ^{13}C NMR (151 MHz, $CDCl_3$) δ: 18.8 (CH_3), 55.6 (CH_2), 71.2 (CH), 121.2 (CH), 126.7 (CH), 128.8 (CH), 128.9 (CH), 129.5 (CH), 134.8 (C), 139.1, (C), 150.6 (C), 155.5 (C), 172.5 (C) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{18}H_{19}N_3OS$: 326.1322, found 326.1321.

*7.1.7.2. 5-(4-Trifluoromethylphenyl)-3-(cis-2,6-dimethylmorpholino) isothiazolo[4,3-b]pyridine (**14b**).* This compound was prepared from 3-bromo-5-(4-trifluoromethylphenyl)isothiazolo[4,3-*b*]pyridine. The residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 7:3) as mobile phase, affording the title compound as an amorphous yellow solid in 85% yield (37 mg, 0.09 mmol). Mp 238.2–239.7 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 1.33 (d, J = 6.3 Hz, 6H, 2 × CH_3), 2.99 (dd, J = 12.6, 10.7 Hz, 2H, 2 × NCH), 3.93–3.99 (m, 2H, 2 × OCH), 4.64 (d, J = 11.8 Hz, 2H, 2 × NCH), 7.72 (d, J = 9.4 Hz, 1H, arom H), 7.74 (d, J = 8.2 Hz, 2H, arom H), 7.87 (d, J = 9.4 Hz, 1H, arom H), 8.09 (d, J = 8.1 Hz, 2H, arom H) ppm. ^{13}C NMR (151 MHz, $CDCl_3$) δ: 18.9 (CH_3), 55.6 (CH_2), 71.2 (CH), 121.0 (CH), 124.2 (q, J = 272.0 Hz, C), 125.7 (bq, J = 3.2 Hz, CH), 126.9 (CH), 129.8 (CH), 130.6 (q, J = 32.4 Hz, C), 135.0 (C), 142.5 (C), 148.9 (C), 155.3 (C), 173.0 (C) ppm. ^{19}F NMR (471 MHz, $CDCl_3$) δ: −62.23 ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{19}H_{18}F_3N_3OS$: 394.1195, found 394.1200.

*7.1.7.3. 5-(4-Fluorophenyl)-3-(cis-2,6-dimethylmorpholino)isothiazolo [4,3-b]pyridine (**14c**).* This compound was prepared from 3-bromo-5-(4-fluorophenyl)isothiazolo[4,3-*b*]pyridine. The residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 7:3) as mobile phase, affording the title compound as an amorphous yellow solid in 91% yield (40 mg, 0.12 mmol). Mp 165.5–166.9 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 1.32 (d, J = 6.3 Hz, 6H, 2 × CH_3), 2.95 (dd, J = 12.5, 10.7 Hz, 2H, 2 × NCH), 3.92–3.99 (m, 2H, 2 × OCH), 4.62 (dd, J = 12.5, 1.3 Hz, 1H, 2 × NCH), 7.13–7.20 (m, 2H, arom H), 7.66 (d, J = 9.4 Hz, 1H, arom H), 7.83 (d, J = 9.4 Hz, 1H, arom H), 7.94–7.98 (m, 2H, arom H) ppm. ^{13}C NMR (151 MHz, $CDCl_3$) δ: 18.8 (CH_3), 55.6 (CH_2), 71.2 (C), 115.70 (d, J = 21.6 Hz, CH), 120.9 (CH), 128.43 (d, J = 8.2 Hz, CH), 129.7 (C), 134.7 (C), 135.3 (C), 149.6 (C), 155.3 (C), 163.4 (d, J = 248.9 Hz, C), 172.50 (C) ppm. ^{19}F NMR (471 MHz, $CDCl_3$) δ: −111.93 ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{18}H_{18}FN_3OS$: 344.1227, found 344.1235.

*7.1.7.4. 5-(3-Chlorophenyl)-3-(cis-2,6-dimethylmorpholino)isothiazolo [4,3-b]pyridine (**14d**).* This compound was prepared from 3-bromo-5-(3-chlorophenyl)isothiazolo[4,3-*b*]pyridine and the residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 4:1) as mobile phase, affording the title compound as an amorphous yellow solid in 78% yield (34.5 mg, 0.09 mmol). Mp 160.3–161.9 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 1.33 (d, J = 6.3 Hz, 6H, 2 × CH_3), 2.95–3.01 (m, 2H, 2 × NCH), 3.93–4.00 (m, 2H, 2 × OCH), 4.65 (d, J = 12.5 Hz, 2H, 2 × NCH), 7.36–7.43 (m, 2H, arom H), 7.67 (d, J = 9.4 Hz, 1H, arom H), 7.82–7.85 (m, 2H, arom H), 8.00–8.02 (m, 1H, arom H) ppm. ^{13}C NMR (151 MHz, $CDCl_3$) δ: 18.8 (CH_3), 55.6 (CH_2), 71.3 (CH), 120.7 (CH), 124.6 (CH), 126.9 (CH), 128.8 (CH), 129.7 (CH), 130.0 (CH), 134.8 (C), 134.9 (C), 140.8 (C), 148.8 (C), 155.4 (C), 172.7 (C) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{18}H_{18}ClN_3OS$: 360.0932, found 360.0932.

*7.1.7.5. 3-(cis-2,6-Dimethylmorpholino)-5-(4-methylphenyl)isothiazolo [4,3-b]pyridine (**14e**).* This compound was prepared from 3-bromo-5-(4-methylphenyl)isothiazolo[4,3-*b*]pyridine. The residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 4:1) as mobile phase, affording the title compound as an amorphous orange solid in 88% yield (39.1 mg, 0.12 mmol). Mp 198.1–199.8 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 1.32 (d, J = 6.3 Hz, 6H, 2 × CH_3), 2.42 (s, 3H, CH_3), 2.95 (dd, J = 12.6, 10.7 Hz, 2H, 2 × NCH), 3.93–4.00 (m, 2H, 2 × OCH), 4.66 (d, J = 11.2 Hz, 2H, 2 × NCH), 7.30 (d, J = 7.9 Hz, 2H, arom H), 7.70 (d, J = 9.4 Hz, 1H, arom H), 7.82 (d, J = 9.4 Hz, 1H, arom H), 7.89 (d, J = 8.2 Hz, 2H, arom H) ppm. ^{13}C NMR (151 MHz, $CDCl_3$) δ: 18.8 (CH_3), 21.3 (CH_3), 55.6 (CH_2), 71.2 (CH), 121.1 (CH), 126.6 (CH), 129.4 (CH), 129.5 (CH), 134.7 (C), 136.4 (C), 139.0 (C), 150.7 (C), 155.5 (C), 172.3 (C) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{19}H_{21}N_3OS$: 340.1478, found 340.1473.

*7.1.7.6. 3-(cis-2,6-Dimethylmorpholino)-5-(3-methoxyphenyl)isothiazolo [4,3-b]pyridine (**14f**).* This compound was prepared from 3-bromo-5-(3-methoxyphenyl)isothiazolo[4,3-*b*]pyridine and the residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 7:3) as mobile phase, affording the title compound as an amorphous yellow solid in 91% yield (40 mg, 0.11 mmol). Mp 146.2–147.9 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 1.32 (d, J = 6.3 Hz, 6H, 2 × CH_3), 2.97 (dd, J = 12.5, 10.8 Hz, 2H, 2 × NCH), 3.89 (s, 3H, OCH_3), 3.93–4.00 (m, 2H, 2 × OCH), 4.67 (d, J = 11.6 Hz, 2H, 2 × NCH), 6.98 (ddd, J = 8.2, 2.6, 0.7 Hz, 1H, arom H), 7.40 (t, J = 7.9 Hz, 1H, arom H), 7.55–7.57 (m, 1H, arom H), 7.58–7.61 (m, 1H, arom H), 7.71 (d, J = 9.4 Hz, 1H, arom H), 7.83 (d, J = 9.4 Hz, 1H, arom H) ppm. ^{13}C NMR (151 MHz, $CDCl_3$) δ: 18.8 (CH_3), 55.2 (OCH_3), 55.5 (CH_2), 71.2 (CH), 111.9 (CH), 114.8 (CH), 119.2 (CH), 121.2 (CH), 129.5 (CH), 129.8 (CH), 134.7 (C), 140.5 (C), 150.3 (C), 155.5 (C), 160.0 (C), 172.5 (C) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{19}H_{21}N_3O_2S$: 356.1427, found 356.1427.

*7.1.7.7. 3-(cis-2,6-Dimethylmorpholino)-5-(3,4-dimethoxyphenyl) isothiazolo[4,3-b]pyridine (**14g**).* This compound was prepared from 3-bromo-5-(3,4-dimethoxyphenyl)isothiazolo[4,3-*b*]pyridine. The residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 7:3) as mobile phase, affording the title compound as an amorphous yellow solid in 55% yield (24 mg, 0.06 mmol). Mp 200.4–201.7 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 1.31 (d, J = 6.3 Hz, 6H, 2 × CH_3), 2.95 (dd, J = 12.3, 10.8 Hz, 2H, 2 × NCH), 3.93–4.00 (m, 8H, 2 × OCH_3, 2 × OCH), 4.64 (d, J = 11.4 Hz, 2H, 2 × NCH), 6.96 (d, J = 8.3, 1H, arom H), 7.52 (dd, J = 8.4, 2.0 Hz, 1H, arom H), 7.68 (d, J = 2.0 Hz, 1H, arom H), 7.69–7.72 (m, 1H, arom H),

7.81 (d, J = 9.4 Hz, 1H, arom H) ppm. ^{13}C NMR (151 MHz, $CDCl_3$) δ: 18.8 (CH_3), 55.5 (CH_2), 55.6 (OCH_3), 56.0 (OCH_3), 71.2 (CH), 109.4 (CH), 111.0 (CH), 119.4 (CH), 120.8 (CH), 129.5 (CH), 131.9 (C), 149.2 (C), 150.1 (C), 150.2 (C), 155.5 (C), 172.1 (C) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{20}H_{23}N_3O_3S$: 386.1533, found 386.1526.

*7.1.7.8. 3-(cis-2,6-Dimethylmorpholino)-5-(3,4,5-trimethoxyphenyl) isothiazolo[4,3-b]pyridine (**14h**).* This compound was prepared from 3-bromo-5-(3,4,5-trimethoxyphenyl)isothiazolo[4,3-*b*]pyridine. The residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 3:2) as mobile phase, affording the title compound as an amorphous yellow solid in 80% yield (34.8 mg, 0.08 mmol). Mp 196.3–198.1 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 1.31 (d, J = 6.3 Hz, 6H, 2 × CH_3), 2.94–3.00 (m, 2H, 2 × NCH), 3.92 (s, J = 2.5 Hz, 3H, OCH_3), 3.95–3.99 (m, 9H, 2 × OCH, 2 × OCH_3), 4.64–4.68 (m, 2H, 2 × NCH), 7.27 (s, 2H, arom H), 7.69 (d, J = 9.4 Hz, 1H, arom H), 7.84 (d, J = 9.4 Hz, 1H, arom H) ppm. ^{13}C NMR (151 MHz, $CDCl_3$) δ: 18.8 (CH_3), 55.5 (CH_2), 56.1 (OCH_3), 61.0 (OCH_3), 71.2 (CH), 104.0 (CH), 121.0 (CH), 129.6 (CH), 134.7 (C), 139.2 (C), 150.2 (C), 153.5 (C), 155.4 (C), 172.3 (C) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{21}H_{25}N_3O_4S$: 416.16384, found 416.1636.

7.1.8. Synthesis of 5-aryl-3-(N-morpholino)isothiazolo[4,3-b]pyridines

General procedure. To a solution of 3-amino-5-arylisothiazolo[4,3-*b*] pyridine (1 equiv) in DMF (15 mL), was added K_2CO_3 (2 equiv) and 2-bromoethyl ether (0.7 equiv). The reaction mixture was stirred at 100 °C for 4–5 h. After disappearance of the starting material as monitored by TLC, the volatiles were evaporated to dryness and the residue was purified by silica gel flash chromatography, yielding the corresponding 5-aryl-3-(*N*-morpholino)isothiazolo[4,3-*b*]pyridines. The following compounds were made according to this procedure:

*7.1.8.1. 5-(3-Methoxyphenyl)-3-(N-morpholino)isothiazolo[4,3-b] pyridine (**15f**).* This compound was prepared from 3-amino-5-(3-methoxyphenyl)isothiazolo[4,3-*b*]pyridine. The residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 7:3) as mobile phase, affording the title compound as an amorphous yellow solid in 77% yield (24.5 mg, 0.08 mmol). Mp 169.1–170.6 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 3.89 (s, 3H, OCH_3), 3.97–4.00 (m, 4H, 2 × OCH_2), 4.02–4.04 (m, 4H, 2 × NCH_2), 6.98 (ddd, J = 8.2, 2.5, 0.9 Hz, 1H, arom H), 7.40 (t, J = 7.9 Hz, 1H, arom H), 7.55–7.58 (m, 2H, arom H), 7.71 (d, J = 9.4 Hz, 1H, arom H), 7.85 (d, J = 9.4 Hz, 1H, arom H) ppm. ^{13}C NMR (151 MHz, $CDCl_3$) δ: 50.5 (CH_2) 55.3 (OCH_3), 66.2 (CH_2), 112.6 (CH), 114.3 (CH), 119.4 (CH), 121.4 (CH), 129.6 (CH), 129.8 (CH), 135.0 (C), 140.6 (C), 150.8 (C), 155.5 (C), 160.0 (C), 173.0 (C) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{17}H_{17}N_3O_2S$: 328.1114, found 328.1111.

*7.1.8.2. 5-(3,4-Dimethoxyphenyl)-3-(N-morpholino)isothiazolo[4,3-b] pyridine (**15g**).* This compound was prepared from 3-amino-5-(3,4-dimethoxyphenyl)isothiazolo[4,3-*b*]pyridine. The residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 3:2) as mobile phase, affording the title compound as an amorphous yellow solid in 73% yield (18.1 mg, 0.05 mmol). Mp 151.2–152.9 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 3.95 (s, 3H, OCH_3), 3.97–4.00 (m, 7H, OCH_3, 2 × OCH_2), 4.01–4.04 (m, 4H, 2 × NCH_2), 6.98 (d, J = 8.3 Hz, 1H, arom H), 7.55 (dd, J = 8.3, 2.1 Hz, 1H, arom H), 7.62 (d, J = 2.1 Hz, 1H, arom H), 7.70 (d, J = 9.4 Hz, 1H, arom H), 7.84 (d, J = 9.4 Hz, 1H, arom H) ppm. ^{13}C NMR (151 MHz, $CDCl_3$) δ: 50.6 (CH_2), 55.8 (OCH_3), 56.0 (OCH_3), 66.2 (CH_2), 109.8 (CH), 111.1 (CH), 119.7 (CH), 121.1 (CH), 129.6 (CH), 132.1 (C), 135.0 (C), 149.2 (C), 150.2 (C), 150.8 (C), 155.4 (C), 172.6 (C) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{18}H_{19}N_3O_3S$: 358.1220, found 358.1220.

*7.1.8.3. 5-(3,5-Dimethoxyphenyl)-3-(N-morpholino)isothiazolo[4,3-b] pyridine (**15i**).* This compound was prepared from 3-amino-5-(3,5-dimethoxyphenyl)isothiazolo[4,3-*b*]pyridine. The residue was purified by silica gel flash chromatography using a mixture of hexane and ethyl acetate (in a ratio of 3:2) as mobile phase, affording the title compound as an amorphous yellow solid in 71% yield (17.5 mg, 0.05 mmol). Mp 149.2–150.8 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 3.87 (s, 6H, 2 × OCH_3), 3.96–3.99 (m, 4H, 2 × OCH_2), 4.01–4.04 (m, 4H, 2 × NCH_2), 6.54 (t, J = 2.3 Hz, 1H, arom H), 7.14 (d, J = 2.3 Hz, 2H, arom H), 7.67 (d, J = 9.4 Hz, 1H, arom H), 7.84 (d, J = 9.4 Hz, 1H, arom H) ppm. ^{13}C NMR (151 MHz, $CDCl_3$) δ: 50.5 (CH_2), 55.4 (OCH_3), 66.2 (CH_2), 100.7 (CH), 105.2 (CH), 121.5 (CH), 129.5 (CH), 134.9 (C), 141.3 (C), 150.7 (C), 155.5 (C), 161.1 (C), 173.0 (C) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{18}H_{19}N_3O_3S$: 358.1220, found 358.1221.

7.2. GAK LanthaScreen™ Eu binding assay

The compounds were subjected to a LanthaScreen™ binding assay in which 10 titrations of dissolved test compound in DMSO are transferred to a 384-well plate. Sequential addition of the kinase buffer (50 mM HEPES pH 7.5, 0.01% BRIJ-35, 10 mM MgCl and 1 mM EGTA), the 2X kinase antibody (Eu Anti GST) mixture and the 4X Tracer 222 solution was performed. After shaking for 30 s and a one hour incubation period at room temperature, the plate was read on a fluorescence plate reader. When the bound tracer in the active site was displaced by the test compound, fluorescence was not observed. The collected data were then compared to a 0% displacement control with pure DMSO and a 100% displacement control with staurosporine, a known inhibitor of GAK and plotted against the logarithmic concentration parameter. The IC_{50} was subsequently extracted.

7.3. Antiviral assays

Virus construct. DENV2 (New Guinea C strain)[29,30] Renilla reporter plasmid used for the antiviral assays was a gift from Pei-Yong Shi (The University of Texas Medical Branch).

Cells. Huh7 (Apath LLC) cells were grown in DMEM (Mediatech) supplemented with 10% FBS (Omega Scientific), nonessential aminoacids, 1% L-glutamine, and 1% penicillin-streptomycin (Thermo-Fisher Scientific) and maintained in a humidified incubator with 5% CO_2 at 37 °C.

Virus Production. DENV2 RNA was transcribed *in vitro* using mMessage/mMachine (Ambion) kits. DENV was produced by electroporating RNA into BHK-21 cells, harvesting supernatants on day 10 and titering via standard plaque assays on BHK-21 cells. Virus titers were determined via standard plaque assay on Vero76 cells.

Infection assays. Huh7 cells were infected with DENV in replicates (n = 3–10) for 4 h at MOI of 0.05. Overall infection was measured at 48 h by standard luciferase assays.

Viability assays. Viability was assessed using AlamarBlue® reagent (Invitrogen) according to manufacturer's protocol. Fluorescence was detected at 560 nm on InfiniteM1000 plate reader.

Declaration of Competing Interest

None.

Acknowledgments

This work was supported by award number W81XWH-16-1-0691 from the Department of Defense (DoD), Congressionally Directed Medical Research Programs (CDMRP) to S.E, P.H; Grant 12393481 from the Defense Threat Reduction Agency (DTRA), Fundamental Research to Counter Weapons of Mass Destruction to S.E. and P.H.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmc.2019.115188.

References

1. Sorrell FJ, Szklarz M, Abdul Azeez KR, Elkins JM, Knapp S. Family-wide structural analysis of human Numb-associated protein kinases. *Structure.* 2016;24:401–411.
2. Edeling MA, Smith C, Owen D. Life of a clathrin coat: insights from clathrin and AP structures. *Nat Rev Mol Cell Biol.* 2006;7:32–44.
3. Zhang CX, Engqvist-Goldstein AEY, Carreno S, Owen DJ, Smythe E, Drubin DG. Multiple roles for cyclin G-associated kinase in clathrin-mediated sorting events. *Traffic.* 2005;6:1103–1113.
4. Korolchuk VI, Banting G. CK2 and GAK/auxilin2 are major protein kinases in clathrin-coated vesicles. *Traffic.* 2002;3:428–439.
5. Ghosh P, Kornfeld S. AP-1 binding to sorting signals and release from clathrin-coated vesicles is regulated by phosphorylation. *J Cell Biol.* 2003;160:699–708.
6. Umeda A, Meyerholz A, Ungewickell E. Identification of the universal cofactor (auxilin 2) in clathrin coat dissociation. *Eur J Cell Biol.* 2000;79:336–342.
7. Neveu G, Barouch-Bentov R, Ziv-Av A, Gerber D, Jacob Y, Einav S. Identification and targeting of an interaction between a tyrosine motif within hepatitis C virus core protein and AP2M1 essential for viral assembly. *PLoS Pathog.* 2012;8:e1002845.
8. McMahon HT, Boucrot E. Molecular mechanism and physiological functions of clathrin-mediated endocytosis. *Nat Rev Mol Cell Biol.* 2011;12:517–533.
9. Bekerman E, Neveu G, Shulla A, et al. Anticancer kinase inhibitors impair intracellular viral trafficking and exert broad-spectrum antiviral effects. *J Clin Invest.* 2017;127:1338–1352.
10. Neveu G, Ziv-Av A, Barouch-Bentov R, Berkerman E, Mulholland J, Einav S. AP-2-associated protein kinase 1 and cyclin G-associated kinase regulate hepatitis C virus entry and are potential drug targets. *J Virol.* 2015;8:4387–4404.
11. Bekerman E, Einav S. Infectious disease. Combating emerging viral threats. *Science.* 2015;348:282–283.
12. Asquith CRM, Laitinen T, Bennett JM, et al. Identification and optimization of 4-anilinoquinolines as inhibitors of cyclin G associated kinase. *ChemMedChem.* 2018;13:48–66.
13. Asquith CRM, Berger BT, Wan J, et al. SGC-GAK-1: a chemical probe for cyclin G associated kinase (GAK). *J Med Chem.* 2019;62:2830–2836.
14. Kovackova S, Chang L, Bekerman E, et al. Selective inhibitors of cyclin G associated kinase (GAK) as anti-hepatitis C agents. *J Med Chem.* 2015;58:3393–3410.
15. Pu SY, Wouters R, Schor S, Rozenski J, Barouch-Bentov R, Prugar LI, O'Brien CM, Brannan JM, et al. *J Med Chem.* 2018;61:6178–6192.
16. Li J, Kovackova S, Pu SY, et al. Isothiazolo[4,3-b]pyridines as inhibitors of cyclin G associated kinase: synthesis, structure–activity relationship studies and antiviral activity. *Med Chem Comm.* 2015;6:1666–1672.
17. Wouters R, Pu SY, Froeyen M, et al. Cyclin G-associated kinase (GAK) affinity and antiviral activity studies of a series of 3-C-substituted isothiazolo[4,3-b]pyridines. *Eur J Med Chem.* 2019;163:256–265.
18. Wouters R, Tian J, Herdewijn P, De Jonghe S. A scaffold-hopping strategy toward the identification of inhibitors of cyclin G associated kinase. *ChemMedChem.* 2018;13:1–19.
19. Cui X, Zhou Y, Wang N, Liu L, Guo QX. *N*-Phenylurea as an inexpensive and efficient ligand for Pd-catalyzed Heck and room-temperature Suzuki reactions. *Tetrahedron Lett.* 2007;48:163–167.
20. Hanthorn JJ, Valgimigli L, Pratt DA. Preparation of highly reactive pyridine- and pyrimidine-containing diarylamine antioxidants. *J Org Chem.* 2012;77:6908–6916.
21. Zhongzhong Y, Aiping L, Mingzhi H, et al. Design, synthesis, DFT study and antifungal activity of the derivatives of pyrazolecarboxamide containing thiazole or oxazole ring. *Eur J Med Chem.* 2018;149:170–181.
22. Subhash LY, Denvert SD, Bhalchandra MB. MnO_2 catalyzed formylation of amines and transamidation of amides under solvent-free conditions. *RSC Adv.* 2015;5:80441–80449.
23. Striela R, Urbelis G, Sudzius J, Stoncius S, Sadzeviciene R, Labanauskas L. Synthesis of bromocyclopropylpyridines via the Sandmeyer reaction. *Tetrahedron Lett.* 2017;58:1681–1683.
24. Lebakken CS, Riddle SM, Singh U, Frazee WJ, Eliason HC, et al. Development and applications of a broad-coverage, TR-FRET-based kinase binding assay platform. *J Biomol Screening.* 2009;14:924–935.
25. Morris GM, Huey R, Lindstrom W, et al. Autodock4 and AutoDockTools4: automated docking with selective receptor flexibility. *J Comput Chem.* 2009;16:2785–2791.
26. Trott O, Olson AJ. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization and multithreading. *J Comput Chem.* 2010;31:455–461.
27. Bandaru SSM, Kapdi AR, Schulzkea C. Crystal structure of 4-(pyrazin-2-yl)morpholine. *Acta Crystallogr E Crystallogr Commun.* 2018;74:137–140.
28. Quintanilla-Licea R, Colunga-Valladares JF, Caballero-Quintero A, et al. NMR Detection of isomers arising from restricted rotation of the C-N amide bond of N-formyl-o-toluidine and N,N'-bis-formyl-o-tolidine. *Molecules.* 2002;7:662–673.
29. Xie X, Gayen S, Kang C, Yuan Z, Shi P-Y. Membrane topology and function of dengue virus NS2A protein. *J Virol.* 2013;87:4609–4622.
30. Zou G, Xu HY, Qing M, Wang QY, Shi PY. Development and characterization of a stable luciferase dengue virus for high-throughput screening. *Antivir Res.* 2011;91:11–19.