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Article

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Fragment-based discovery of dual JC virus and BK virus helicase inhibitors

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KEYWORDS: JCV, BKV, helicase, ATP-competitive, fragment based drug design

ABSTRACT

There are currently no treatment for life-threatening infections caused by human polyomaviruses JCV and BKV. We therefore report herein the first crystal structure of the hexameric helicase of

JCV large T antigen (apo) and its use to drive the structure-based design of dual JCV and BKV ATP-competitive inhibitors. The crystal structures obtained by soaking our early inhibitors into the JCV helicase allowed us to rapidly improve the biochemical activity of our inhibitors from 18 μM for the early 6-(2-methoxyphenyl)- and the 6-(2-ethoxyphenyl)-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole hits **1a** and **1b** to 0.6 μM for triazolopyridine **12i**. In addition, we were able to demonstrate measurable antiviral activity in Vero cells for our thiazolopyridine series in the absence of marked cytotoxicity, thus confirming the usefulness of this approach.

1. INTRODUCTION

Human polyomaviruses JC (JCV) and BK (BKV) infect a large fraction of the world's population (>50 %),[1,2] leading to asymptomatic persistent infections in healthy individuals but causing severe diseases in immunocompromised patients. JCV leads to progressive multifocal leukoencephalopathy (PML), a rapidly progressing and fatal central nervous system condition,[3] while reactivation of BKV triggers polyomavirus-associated nephropathy (PVN) in kidney transplant patients and hemorrhagic cystitis (HC) in cancer patients undergoing a bone marrow transplant.[4,5] There are currently no antiviral therapies available to treat these life-threatening infections.

JCV and BKV are members of the polyomavirus family of small double-stranded DNA (dsDNA) viruses. Polyomaviruses possess a viral genome that is divided into three main regions: two coding and one noncoding. Early in the infection cycle, the *Polyomaviridae* encode the small tumor antigen (tAg) and the large tumor antigen (LTAg). Later in the infection cycle, they

encode for the viral capsid proteins VP1, VP2, and VP3, as well as the viral nonstructural protein called agnoprotein. The LTAgs encoded by the early coding region are essential for viral DNA replication. They contain a SF3 hexameric helicase domain that uses energy derived from ATP hydrolysis to unwind duplex DNA. Because LTags are solely viral proteins with no human counterpart, they represent attractive targets for therapeutic intervention. [6,7] As a result, early proof of concept screens for the modulation of LTags ATPase activity have recently been reported for polyomaviruses BKV,[8,9] JCV[10] and SV40.[9] To the best of our knowledge, these efforts have not been pursued past the stage of hit identification.

2. RESULTS AND DISCUSSION

We report herein the first structure-based approach to the rational design of dual JCV and BKV helicase inhibitors. The helicase ATP-binding pockets of JCV and BKV are highly conserved, with only four divergent residues (I395/L396; A552/I553; S559/Q560 and C560/N561),[8] and we hypothesized that dual JCV/BKV inhibitors could be identified. As a result, we simultaneously initiated a high throughput screen of approximately 350,000 compounds using a JCV DNA-unwinding assay as primary readout and a broad crystallization effort towards the elucidation of the structure of the JCV LTAg helicase.

Crystallization of the JCV LTAg. The JCV LTAg is a 688 residue protein consisting of a DNA-J domain at the amino-terminus, an origin binding domain and a helicase domain. In order to unwind dsDNA, the LTAg assembles into a donut-shaped hexamer that allows one DNA strand to pass through the small central hole, while the other strand is guided along the outside of the hexamer. The separation of the two DNA strands occurs by steric occlusion as the enzyme pulls the DNA strand through the central hole. This separation requires ATP hydrolysis and is

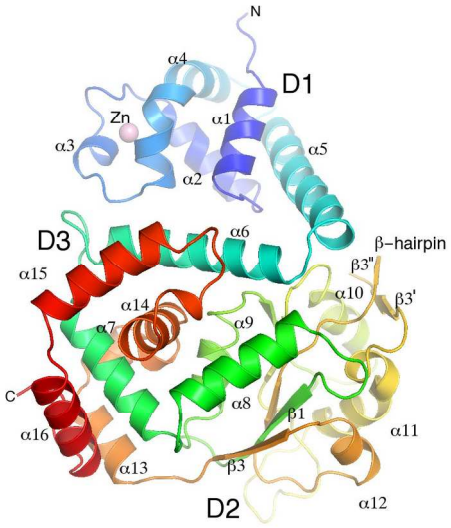
believed to occur with a rotary mechanism where each of the six LTAg molecules cycles through an ATP-, ADP-bound and nucleotide free states.[11]

The JCV helicase domain stretches from residue ~260 to 630 and contains three subdomains. We expressed and purified the helicase domain containing residues 261 to 628 from E. coli. Initial crystals were obtained with 25-30% Peg400, 0.1 M Li_2SO_4, 0.1M HEPES (pH 7.0), 0.2 M NaCl. The diffraction pattern showed high mosaicity and low resolution preventing us from solving the structure. After analyzing the crystal contacts from the previously published SV40 LTAg,[12] we decided to introduce the following eleven mutations in the JCV LTAg: E280D, D295N, N299A, Q301A, Q302A, K304A, K305A, E307A, K308A, K309A, R624A. These mutated residues are high entropy residues located at the surface of the protein and away from the ATP binding site.

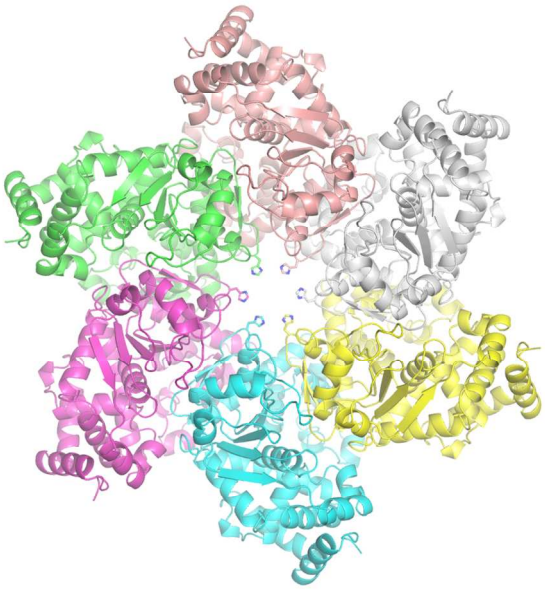
The introduction of these nine mutations resulted in better diffracting crystals that allowed us to solve the crystal structure using 2.2Å data. The protein crystals belonged to space group P6, with one molecule in the asymmetric unit. The donut-shaped helicase complex was assembled by applying the 6-fold symmetry of the space group, forcing all six molecules to be in an identical conformation (Figure 1).

The JCV helicase sequence is 75% identical with SV40 LTAg [12, 13] and is structurally very similar. The RMSD between the two molecules is 0.692 (with 322 Cα superimposed). At the N-terminus is a Zn-binding domain (residues 267 to 331), followed by domains 2 and 3. The Zn-binding domain is made from four helices (Figure 1b α5). The Zn-ion is held in place by C303, C306, H314 and H318. The Zn binding domain at the amino-terminus is connected with domain 3 through a long helical linker. Although Zn-fingers are often involved in DNA binding, in the

case of JCV (and SV40) the Zn ion is distant from the central channel and its purpose is structural support to accommodate homo-hexamerization with other JCV LTAgs. Domain 2 is inserted in between Domain 3 and has the typical AAA+ fold of a 5 stranded beta-sheet surrounded by four alpha-helices. Helices 10-12 are on one side of the beta-sheet while helix α9 is on the other side. Between beta-strand 1 and helix 9 is the Walker A motif (or P-loop) a conserved motif involved in ATP's triphosphate binding. Domain 2 also has a beta-hairpin inserted after beta-strand 3, beta-strands 3' and 3", with the conserved H514 and K513 responsible for pulling the DNA strand through the pore of the hexamer. Domain 3 contains 7 alpha-helices. Helices α6 through α8 are followed by Domain 2, followed by alpha-helices α13 through α16.



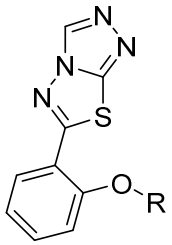
a



b

Figure 1. a) Structure of the JCV LTAg monomer; b) Structure of the JCV LTAg hexamer along the 6-fold axis

Inhibitor design. Following the high-throughput screen, the hits underwent a rigorous triage that included i) removal of all hits with low binding efficiency (BEI < 15)[14]; ii) mechanistic evaluation to identify ATP-competitive inhibitors that lacked DNA-binding ability. This process allowed us to identify two closely related, equipotent, ATP-competitive triazolothiadiazole inhibitors of JCV DNA unwinding activity, the 6-(2-methoxyphenyl)- and the 6-(2-ethoxyphenyl)-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole **1a** and **1b** (Figure 2, Table 1).



1a : R = Me, JCV helicase IC_{50} : 18 μM

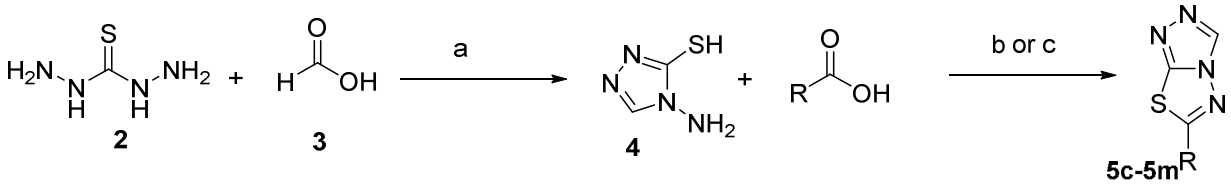
1b : R = Et, JCV helicase IC_{50} : 19.4 μM

Figure 2. Fragment hits **1a**,**b**

To follow up on the hits, we purchased commercially available **5a** and **5b,** and initiated the synthesis of additional closely related analogs.

Initially, analogous triazolothiadiazoles were synthesized as shown in Scheme 1. The 4-amino-4*H*-1,2,4-triazole-3-thiol **4**, obtained through the neat condensation of diaminothiourea with formic acid, was condensed with benzoic acids, under microwave conditions to give products **5c-5m**.

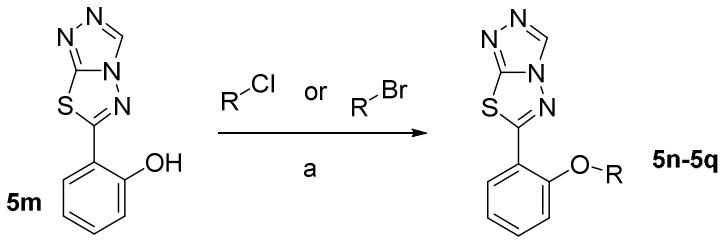
Scheme 1. Condensation of carboxylic acids with the 4-amino-4*H*-1,2,4-triazole-3-thiol



Conditions: (a) reflux, neat, overnight, 42%; (b) $POCl_3$, 110-120˚C, 30-90 min, microwave, or (c) T_3P, DIPEA, EtOAc, 140˚C, 20-120 min, microwave

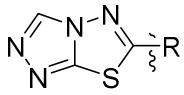
To probe the space for potential interactions with the helicase, while keeping an emphasis on introducing sp^3 carbon rich chains, additional ether analogs were synthesized via alkylation of **5m** with alkylchlorides or alkylbromides (**5n-q)** (Scheme 2).

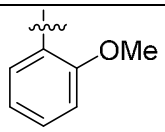
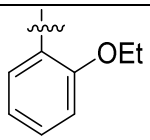
Scheme 2: Ether synthesis through alkylation of phenol **5m**

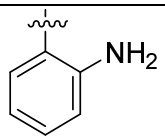
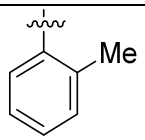
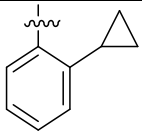
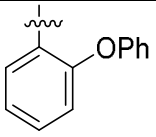
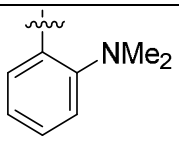
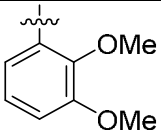
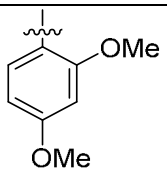
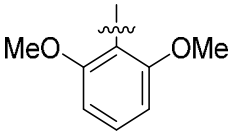


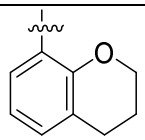
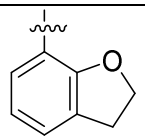
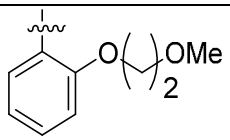
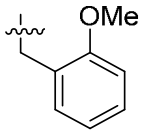
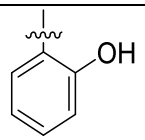
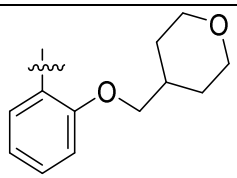
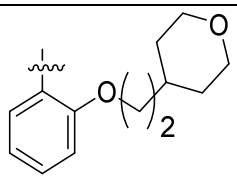
Conditions: (a) K_2CO_3 CH_3CN, 100-130˚C, 30 min, microwave

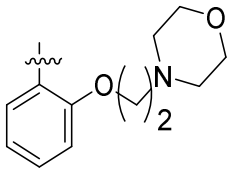
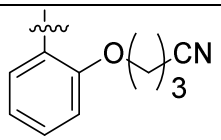
Table 1. Biochemical activity of early triazolo[3,4-*b*][1,3,4]thiadiazoles (TT) (**5a-5q**).



R	Compound	JCV helicase IC_{50} (μM)[a]	BKV helicase IC_{50} (μM)[a]
	**1a**	19.4	29.0
	**1b**	18.0	N/A

	**5a**	>80	>80
	**5b**	>80	>80
	**5c**	>80	>80
	**5d**	4.9	3.7
	**5e**	>80	>80
	**5f**	62	>80
	**5g**	18	24
	**5h**	>80	>80

	**5i**	15.5	24
	**5j**	13.5	21
	**5k**	28.5	63
	**5l**	>80	>80
	**5m**	>80	>80
	**5n**	6.3	17
	**5o**	5.8	8.4

	**5p**	52	73
	**5q**	25	42

[a] Minimum Significant Ratio (MSR)[15] = 3.2; i.e., compound that have a difference in IC_{50} of at least a factor of 3.2 are considered significantly different.

This effort rapidly established the importance of the ether at the 2-position of the phenyl ring since the 2-hydroxyphenyl analog **5m**, the 2-amino- and 2-dimethylamino-analog **5a** and **5e,** as well as the 2-methyl and 2-cyclopropyl analogs **5b** and **5c** lost all activity against both enzymes compared to the original hits **1a** and **1b**. The direct linkage of the phenyl ring to the thiadiazole was also found to be of importance since the benzylic analog of **1a** (**5l**) was inactive.

Even though a wide variety of ethers were introduced at the 2-position of the phenyl ring, improving upon the activity of **1a**,**b** proved to be difficult. Only the phenoxy-analog **5d** displayed slightly improved IC_{50s} on both JCV and BKV around 5 μM. The THP ether analogs **5n** and **5o** were also well tolerated whereas switching from THP (**5o**) to morpholine (**5p**) resulted in a ten-fold decrease in potency.

Our first X-ray crystal structure of a triazolo[3,4-*b*][1,3,4]thiazole in JCV was obtained by soaking **5d** into apo JCV crystals (Figure 3). This structure demonstrated that **5d** makes a single interaction with the backbone NH of K551 in the pseudo-hinge region while the phenoxy group is engaged in π-stacking with R555, possibly accounting for the slight improvement in potency

compared to methoxy and ethoxy hits **1a**,**b** from 18 μM to 4.5 μM. It is worth noting that **5d** interacts solely with one of the JCV monomers that constitute the ATP pocket and does not engage any of the residues of the neighboring JCV monomer, as shown in Figure 3.

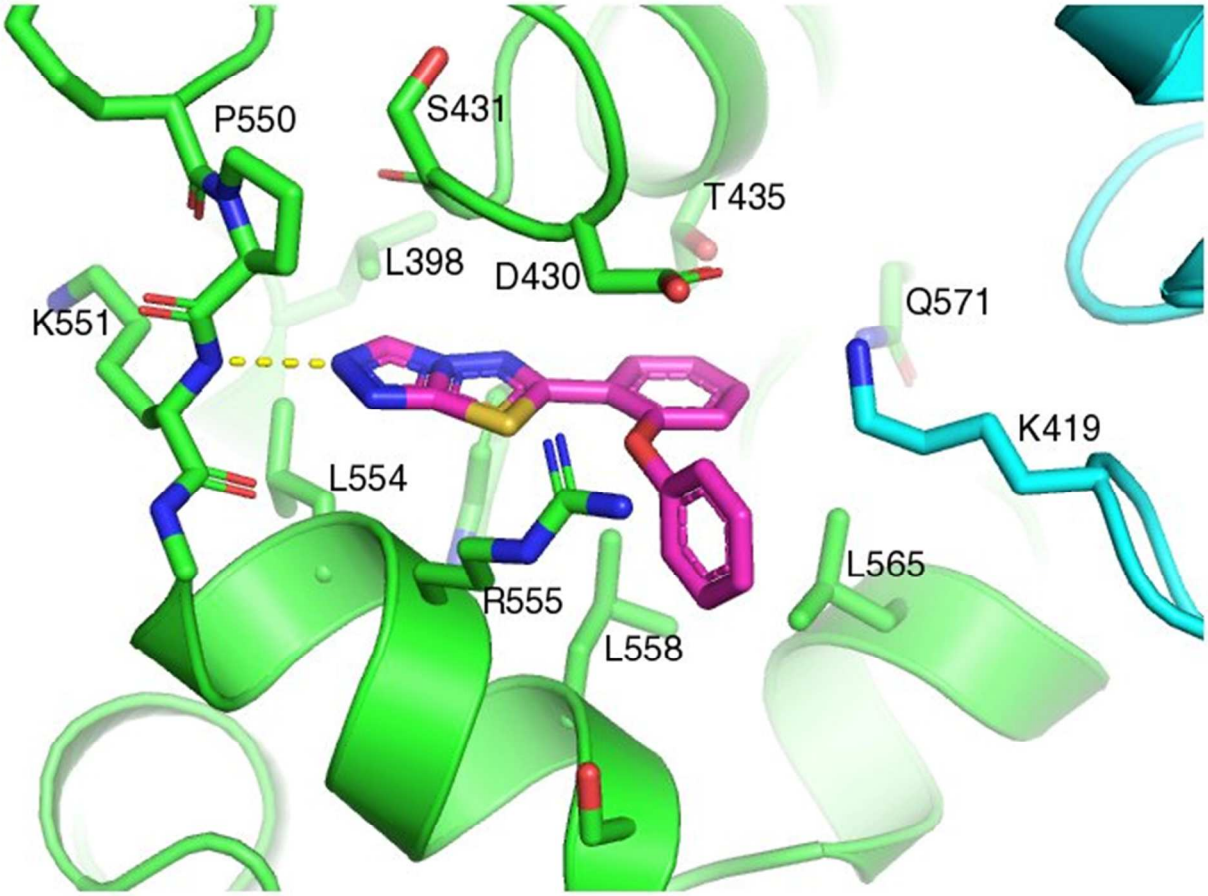


Figure 3. Crystal structure of **5d** in the JCV ATP pocket constituted of two JCV monomers, one shown in green and the other in blue.

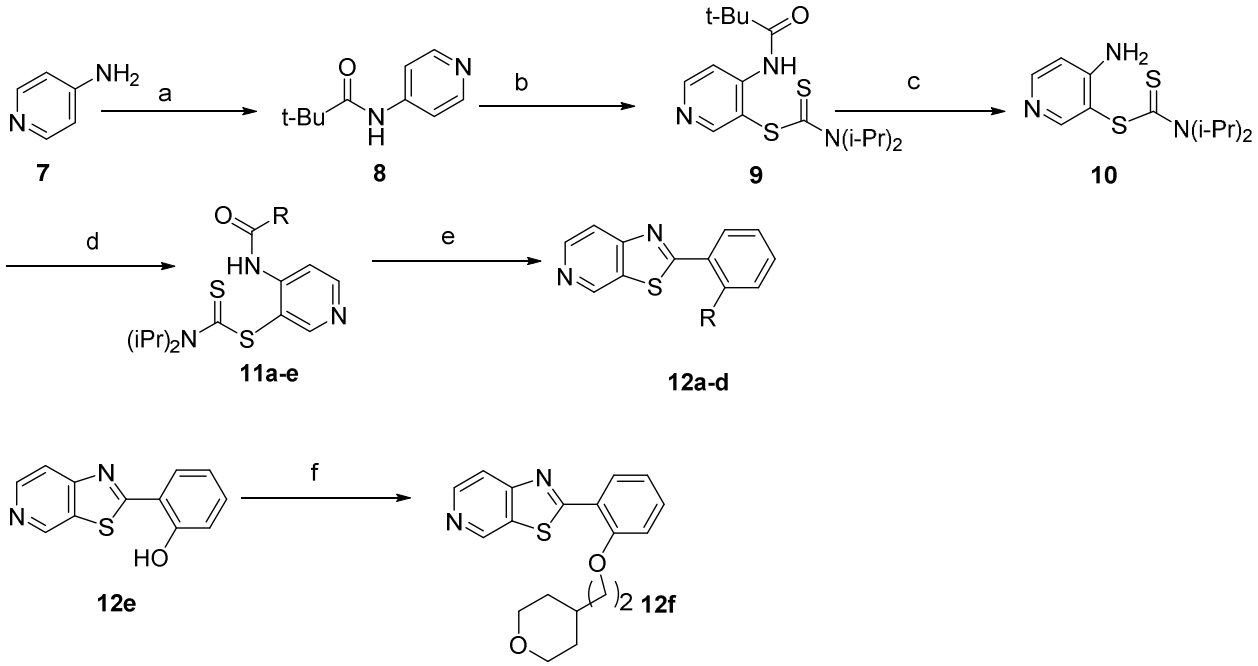
Based on the crystal structure of **5d** in JCV, we identified the closest residues, within 4 Å, that would be available for additional interactions with our scaffold. These residues are i) the carbonyls of S431 and K551 in the hinge region as well as the side chain OH of T435 and the

side chain amino group of Gln571 from the first JCV monomer, ii) the terminal amino group K419 from the second JCV monomer.

In order to optimally target K551 and S431 in the pseudo-hinge region, we morphed our 5,5-membered ring triazolothiadiazole core into a 6,5-membered ring thiazolopyridine core. The two regioisomeric thiazolopyridines corresponding to original hit **1**, the thiazolo[4,5-*c*]pyridine (**12a**), and the thiazolo[5,4-*c*]pyridine (**15**) were synthesized according to Schemes 3 and 4.

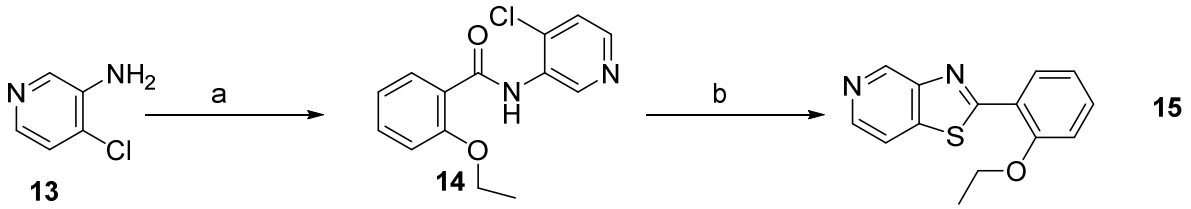
As expected from the binding mode of **5d** in JCV, thiazolopyridines **12a-b** were found to inhibit JCV's DNA unwinding ability in a comparable manner to triazolothiadiazoles **1a-b**, with an IC_{50} of 5.0 and 9.1 uM respectively while **12b**'s regioisomeric thiazolopyridine **19** was inactive in these assays with IC_{50s} > 80 μM. Furthermore, thiazolo[4,5-*c*]pyridines **12a-f** were found to be equipotent to their triazolothiadiazole analogs **1a-b**, **5d**, **5j**, **5m,** , and **5o**, allowing us to transfer existing triazolothiadiazole SAR to the new series (Table 2).

Scheme 3. Synthesis of thiazolo[4,5-*c*]pyridines (**12a-12f**)



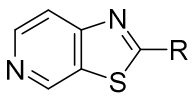
Conditions: (a) t-BuCOCl, Et_3N, DCM 0˚C to RT, 78%; (b) n-BuLi, tetraisopropylthiuram disulfide, THF, -78˚C to RT, 74%; (c) NaOH, MeOH , RT , 1 hr, 88%; (d) RCOCl, Et_3N, DCM, RT, 10 min, 62%; (e) aq HCl (5M), 80˚C, 2 hrs, 45%; (f) 4-(2-bromoethyl)tetrahydropyran, K_2CO_3, CH_3CN, 80˚C, 3 hrs, 87%

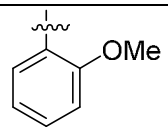
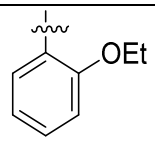
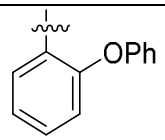
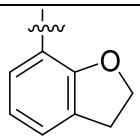
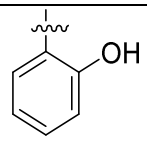
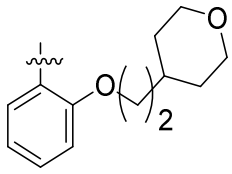
Scheme 4. Synthesis of the 2-(2-ethoxyphenyl)thiazolo[4,5-*c*]pyridine (**15**).



Conditions: (a) 2-Ethoxybenzoyl chloride, K_2CO_3, THF, RT, overnight, 11 %; (b) Lawesson's reagent, toluene, reflux, overnight, 43%

Table 2. Biochemical activity of early thiazolo[4,5-*c*]pyridines

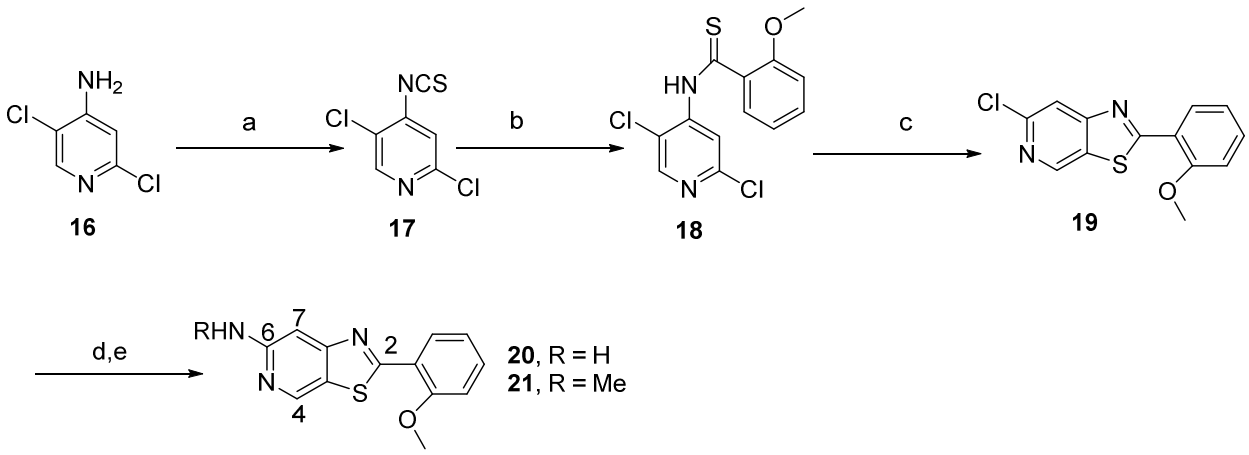


R		JCV helicase IC_{50} (μM)	BKV helicase IC_{50} (μM)
	**12a**	5.0	10.7
	**12b**	9.1	8.0
	**12c**	10.5	10.2
	**12d**	8.7	15
	**12e**	37	53
	**12f**	9.6	11

Probing for interactions with K551 and S431 required the introduction of an amino group at position 6 (**20**, **21** Scheme 5) and/or position 4 (**26,** Scheme 5) of the thiazolopyridine core. Interestingly, the presence of the amino group was tolerated at position 6 (**20**) and detrimental at

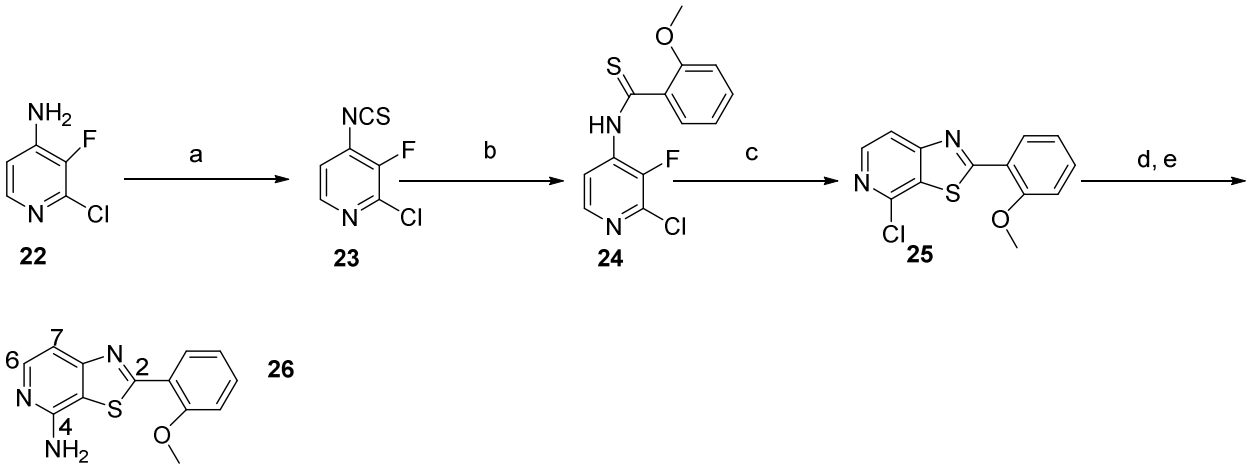
position 4 (**30**). However, the additional hydrogen-bond donor at position 6 did not lead to an increase in activity as **20** was found to be equipotent with its unsubstituted analog **12a**. This is all the more surprising, as subsequent monomethylation of the 6-amino group in **20** led to a complete loss of activity (**21**, Table 3).

Scheme 5: Synthesis of the 2-(2-methoxyphenyl)thiazolo[5,4-*c*] pyridin-6-amine (**20**) and 2-(2-methoxyphenyl)-*N*-methylthiazolo[5,4-*c*]pyridin-6-amine (**21**).



Conditions: (a) Thiocarbonyl dichloride, Na_2CO_3,DCM, RT, 4 days, 57%; (b) 1-bromo-2-methoxybenzene, n-BuLi, THF, DMF, -78˚C, 20 min (assumed quantitative); (c) Na_2CO_3, DMF, 120˚C, 3hrs, 26%; for **20** (d) *tert*-butyl carbamate, NaO*t*-Bu, XPhos palladacycle, dioxane, 120˚C, 20 min, microwave; (e) TFA, THF, 41 %; for **21** (d) methylamine, NaO*t*-Bu, BrettPhos palladacycle, dioxane, 135˚C, 30 min, microwave, 42 %

Scheme 6: Synthesis of the 2-(2-methoxyphenyl)thiazolo[5,4-*c*]pyridin-4-amine (**26**)



Conditions: (a) Thiocarbonyl dichloride, Na_2CO_3, $CSCl_2$, DCM, RT, 24 h, 51%; (b) 1-bromo-2-methoxybenzene, n-BuLi, THF, -78˚C, 20 min; (c) Na_2CO_3, 120˚C, 12 hr ,40%; (d) *tert*-butyl carbamate, NaO*t*-Bu, XPhos palladacycle, dioxane, 135˚C, 30 min, microwave; (e) TFA, THF, 44%

Table 3: Biochemical activity of thiazolo[5,4-*c*]pyridinamines

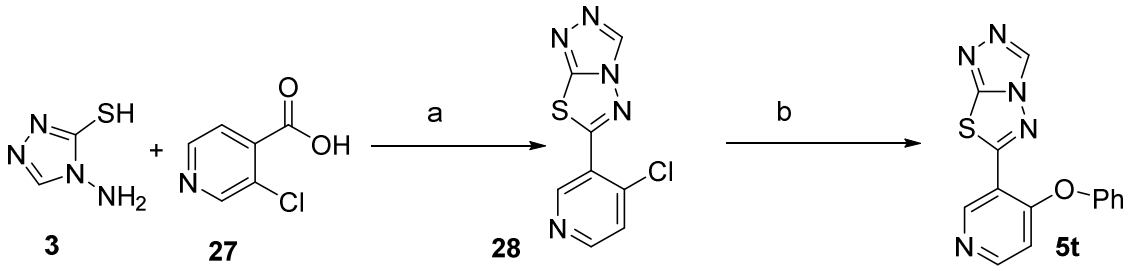
Inhibitor	JCV helicase IC_{50} (μM)	BKV helicase IC_{50} (μM)
20	4.3	8.7
21	>80	>80
26	>80	>80

As the addition of a donor-acceptor motif targeting the hinge region of the 1st JCV monomer did not result in increased activity, our efforts shifted towards T435 and K419, the neighboring residues of the second JCV monomer (Figure 3). In an attempt to engage these residues, we

initially evaluated the replacement of the 2-phenoxyphenyl group with a 2-phenoxypyridine or 2-phenoxypyrimidine groups.

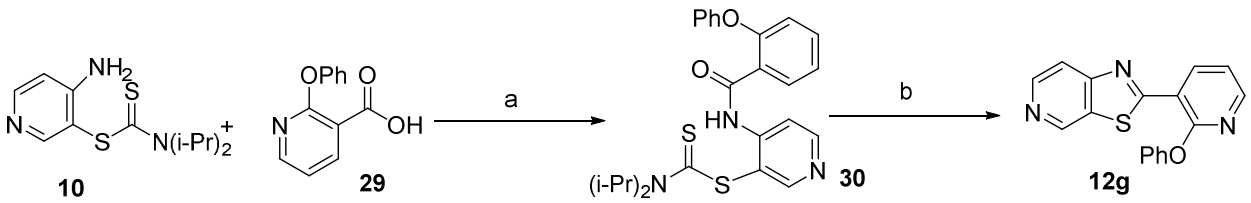
Triazolothiazoles **5r-s** were obtained from the 2-phenoxypyridine-3-carboxylic acid and the 4-phenoxypyrimidine-5-carboxylic acid respectively, using the reaction conditions depicted in Scheme 1 (**5r** conditions (a;c); **5s** conditions (a;b)). Triazolothiazole **5t** was synthesized from the 6-(4-chloropyridin-3-yl)-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole as shown in Scheme 7. Finally, the thiazolopyridine analog of **5r** (**12g**) was obtained in 41% yield through the acid promoted intramolecular cyclization of intermediate **30** as depicted in Scheme 8.

Scheme 7 : Synthesis of 6-(4-phenoxypyridin-3-yl)-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole **5t**.



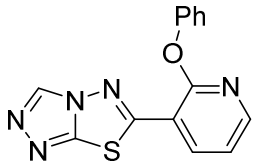
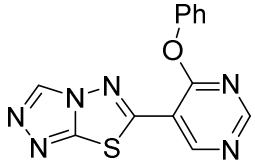
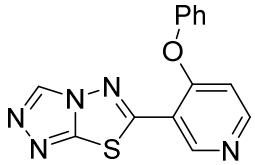
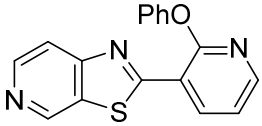
Conditions: (a) $POCl_3$, 100˚C, 35 min, microwave, 43%; b) phenol, K_2CO_3, DMF, 130˚C, 60 min, microwave, 3%

Scheme 8: Synthesis of the 2-(2-phenoxypyridin-3-yl)thiazolo[5,4-*c*]pyridine (**12g**).



Conditions: (a) HATU, DIPEA, DMF; b) 6M HCl, 80˚C, 5hrs, 41%.

Table 4: Activity of phenoxypyridines and phenoxypyrimidines.

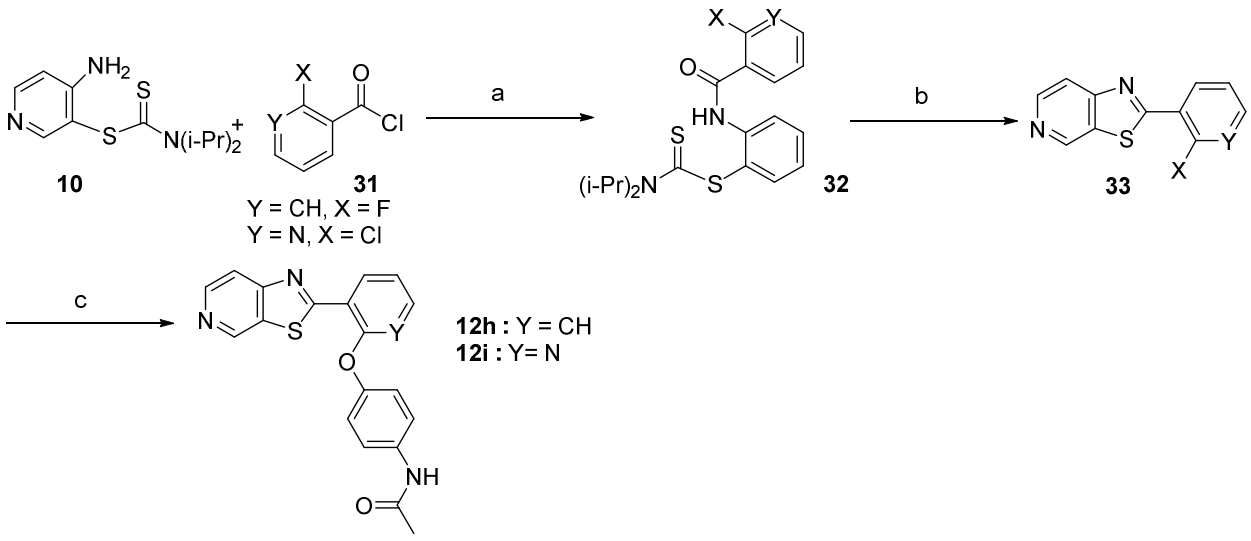
R	Inhibitor	JCV helicase IC_{50} (μM)	BKV helicase IC_{50} (μM)
	**5r**	8.8	11.0
	**5s**	>80	>80
	**5t**	5.5	7.3
	**12g**	1.4	1.3

The introduction of a 2-phenoxypyridinyl group was well tolerated in the thiazolotriazole scaffold (**5r**, **5s**), but did not lead to an increase in activity compared to 2-phenoxyphenyl analog **5d**. The corresponding 2-phenoxypyrimidinyl analog **5s** lost activity. In contrast, **12g** showed a 10-fold increase in activity on both viruses, with IC_{50s} of 1.4 μM (JCV) and 1.3 μM (BKV) compared to 2-phenoxyanalog **12c** with IC_{50s} of 10.5 μM (JCV) and 10.2 μM (BKV). This increase in activity could be attributed to the formation of a new productive interaction between the phenoxypyridine nitrogen of **12g** and K 419 of the second JCV monomer as shown in the

crystal structure obtained by soaking **12g** into JCV apo (Figure 4a). It is worth noting that K419 in JCV corresponds to K420 in BKV, a key residue of the ATP pocket found to be critical for viral replication using an in vitro directed mutagenesis approach.[8]

As we continued to look for additional interactions with the second JCV monomer, we discovered that another lysine, K420, could effectively form a hydrogen bond with our thiazolopyridines when an acetamide was introduced at the *p*-position of the phenoxy group of **12c** (**12h**, Scheme 9). In this case, K420 was found to produce a productive interaction with the acetamide carbonyl of **12h** located at 2.8 Å, leading to a 10-fold increase in potency with IC_{50} values of 0.9 μM and 1.6 μM on JCV and BKV respectively (Figure 4b).

Scheme 9: Synthesis of the N-(4-(2-(thiazolo[5,4-c]pyridin-2-yl)phenoxy)phenyl)acetamide (**12h**) and the N-(4-((3-(thiazolo[5,4-*c*]pyridin-2-yl)pyridin-2-yl)oxy)phenyl)acetamide (**12i**)



Conditions: (a) Et_3N, CH_2Cl_2, RT, 11-18 hrs; (b) 6M HCl, RT or 80˚C, 30 min; (c) *N*-(4-hydroxyphenyl)acetamide,CS_2CO_3, or K_2CO_3, DMSO, 150˚C, 4 hrs to overnight

Since the introduction of a 2-phenoxypyridine (**12g**) and that of an acetamide at the *p*-position of the phenoxy group (**12h**) both led to a profound increase in activity on both viruses, these two key structural features were combined within a single compound, thiazolopyridine **12i** (Scheme 9).

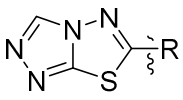
To our surprise, **12i** was found to be equipotent to **12h** with IC_{50s} of 0.6 μM of both JCV and BKV. This finding can be rationalized with the crystal structures obtained with **12g** and **12h** (Figure 4). In these structures, significant movements were observed for both K419 and K420. As K420 moves in 2.5Å to bind to the acetamide carbonyl of **12h**, K419 moves 3.4Å away from the pyridine nitrogen of **12g**, only allowing for a single productive interaction to be formed between our inhibitors and the second JCV monomer.

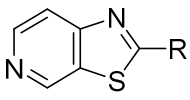
The selectivity of two of our inhibitors **5d** and **12h** was evaluated against a subset of 66 kinases of the Eurofin Kinaseprofiler™ panel. Both compounds displayed an exquisite selectivity with no inhibition greater than 35% at 10 μM.[16]

While improving the activity by increasing the number of interactions between the inhibitor and the residues of the ATP pocket proved to be challenging, we believed that the level of activity achieved with some of our most potent analogs would allow us to demonstrate a measurable effect on viral replication in a cellular assay. We therefore developed a branched DNA (bDNA) assay that measured the production levels of the late viral protein and major capsid protein VP1. The data summarized in Table 5, showed that the triazolopyridine were generally better tolerated than their thiazolotriazoles analogs with cell viability windows (CC_{50}/EC_{50}) > 5 with the single

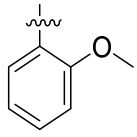
exception of phenoxy analogs **5d** and **12c**. In spite of their lack of general cytotoxicity, the antiviral activity of the thiazolopyrines did not correlate to their intrinsic ability to inhibit DNA unwinding. This is clearly demonstrated with the most efficacious thiazolopyridine (**12d**) with an EC_{50} of 0.26 μM in the bDNA assay and an IC_{50} of 8.7 μM in the unwinding assay. This phenomenon can potentially be explained by the fact that the bDNA assay monitors late capsid protein VP1 and therefore provides a read on the entire viral life cycle that not only involves DNA-unwinding, but a variety of host proteins. An orthogonal assay with a readout that would be more proximal to the DNA-unwinding step could potentially provide a better alignment. The antiviral activity of the thiazolopyridines may also be the result of a mechanism of action far more complex than DNA unwinding inhibition and possibly involving more than one target. The success of such a multi-target inhibitory profile has been demonstrated for Herpes virus helicase(SF1)-primase inhibitor amenamevir (ASP2151) which was found to be a more potent primase inhibitor than DNA unwinding inhibitor.[17]

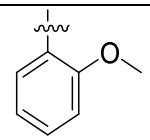
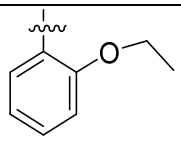
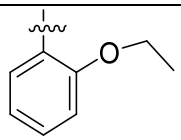
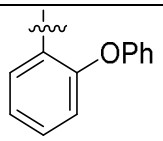
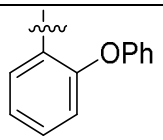
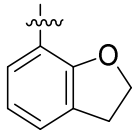
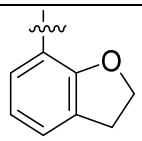
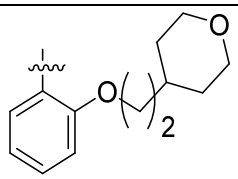
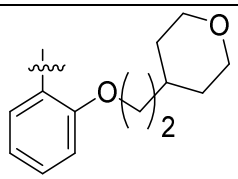
Table 5. Anti-viral activity in Vero cells

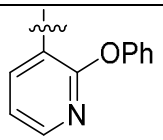
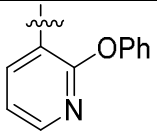
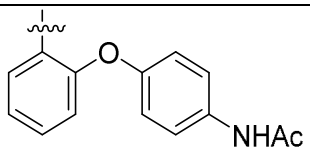
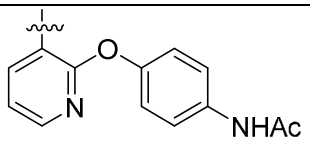


(TT) vs


(TP)

Inhibitor	Core	R	JCV bDNA[a] EC_{50} (μM)	JCV bDNA CC_{50} (μM)	CC_{50}/EC_{50}
1a	TT		>60	>80	ND

12a	TP		1.5	>50	>33
1b	TT		44	>65	ND
12b	TP		7.4	>50	**>6**
5d	TT		34	>50	ND
12c	TP		36	>50	ND
5j	TT		>16	>50	ND
12d	TP		0.26	>50	>166
5o	TT		15	>50	ND
12f	TP		7.7	>50	**>6**

5r	TT		76	>50	ND
12g	TP		3.8	>50	> 13
12h	TP		48	>50	ND
12i	TP		40	>50	ND

[a] Minimum Significant Ratio (MSR)[15] = 2.6; i.e., compound that have a difference in EC_{50} of at least a factor of 2.6 are considered significantly different

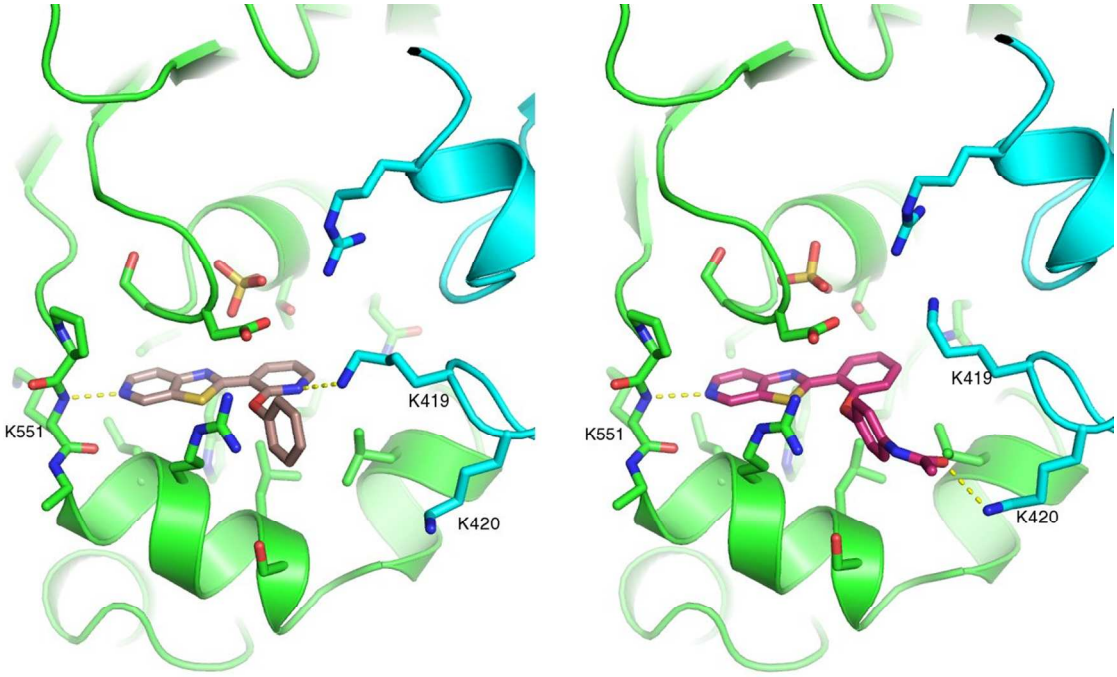


Figure 4. Crystal structure of (a) **12g** and **(**b) **12h** bound to JCV LTAg

CONCLUSION

In summary, we have described our path towards the structure-based design of dual JCV and BKV ATP-competitive inhibitors. To our knowledge, this is the first report of a structure based approach targeting a hexameric helicase of the SF3 family. We reported the first crystal structure of the hexameric helicase of JCV LTag (apo) which was used for soaking our fragment inhibitors and for driving fragment design. In agreement with our hypothesis, we demonstrated that the activity of our ATP-competitive inhibitors of DNA-unwinding tracked well between the two viruses. Based on structural information, we rapidly increased the activity of our scaffolds 30-fold from 18 μM for the original hits **1a,b** to 0.6 μM for triazolopyridine **12i**. Capitalizing further on our structural findings, was however hindered by the complexity of the ATP pocket in combination with the mobility of two of its key

residues, K419 and K420, thus only allowing for the formation of two productive interactions between the inhibitor and the helicase. In spite of this limitation, it is encouraging to note that we were able to demonstrate measurable antiviral activity for our thiazolopyridines in the absence of marked cytotoxicity.

EXPERIMENTAL SECTION

JCV large T antigen purification. JCV LTag (E264-D628) N299A, Q301A, Q302A, K304A, K305A, E307A, K308A, K309A, R624A was cloned in pET28b.1 plasmid and expressed at 15˚C in E. coli for 28 hours. All purification steps were performed at 4˚C or on ice. Cell paste was resuspended in 10 volumes of buffer A (50 mM HEPES [pH 7.8], 10% glycerol, 400 mM NaCl, 5 mM β-mercaptoethanol, 5 mM imidazole, 0.2% Tween-20, 2.5 g/ml leupeptin, 2.0 μg/ml E-64, 10 μM DFP) containing benzonase™ nuclease (Novagen) and lysed by one pass through a microfluidizer (Microfluidics Corporation, Newton, MA). The lysate was clarified by centrifugation at 54,000 *x g* for 1 hour and the supernatant was batch absorbed for 2 hours with nickel-nitrilotriacetic acid (NTA) agarose resin (Sigma-Aldrich). The resin was poured into a gravity flow column, washed with 10 volumes of buffer B (buffer A containing 10 mM imidazole) and eluted with 5 volumes of buffer C (50 mM HEPES [pH 7.8], 10% glycerol, 400 mM NaCl, 5 mM β-mercaptoethanol, 0.2% Tween-20, 350 mM imidazole). Fractions containing JCV protein were pooled, concentrated and loaded onto a HiPrep 26/60 Sephacryl S-300 HR column (GE Healthcare Life Sciences) equilibrated in buffer D (25 mM HEPES [pH 7.2], 10% glycerol, 300 mM NaCl, 2 mM dithiothreitol). Fractions containing JCV protein were pooled, concentrated and the polyhistidine tag was removed by incubation with bovine thrombin (Calbiochem). After tag

removal was complete, the protein was loaded onto a Superdex 200 10/300 gl column (GE Healthcare Life Sciences) equilibrated in buffer E (25 mM HEPES [pH 7.5], 250 mM NaCl, 2 mM dithiothreitol). Fractions containing JCV were pooled and concentrated to 25-30 mg/ml.

BKV large T antigen purification. BKV LTag (E265-D629) was cloned in pET28b.1 plasmid and expressed at 15˚C in E. coli for 28 hours. All purification steps were performed at 4˚C or on ice. Cell paste was resuspended in 10 volumes of buffer F (50 mM HEPES [pH 7.8], 10% glycerol, 500 mM NaCl, 5 mM β-mercaptoethanol, 20 mM imidazole, 0.2% Tween-20, 2.5 g/ml leupeptin, 2.0 μg/ml E-64, 10 μM DFP) containing benzonase™ nuclease (Novagen) and lysed by one pass through a microfluidizer (Microfluidics Corporation, Newton, MA). The lysate was clarified by centrifugation at 54,000 *x g* for 1 hour and the supernatant was batch absorbed overnight with nickel-nitrilotriacetic acid (NTA) agarose resin (Qiagen 304430). The resin was poured into a gravity flow column, washed with 20 volumes of buffer G (50 mM HEPES [pH 7.8], 10% glycerol, 500 mM NaCl, 5 mM β-mercaptoethanol, 20 mM imidazole) and eluted with 3 volumes of buffer H (50 mM HEPES [pH 7.8], 10% glycerol, 400 mM NaCl, 5 mM β-mercaptoethanol, 350 mM imidazole). The eluate was immediately diluted 3-fold with buffer I (25 mM HEPES [pH 7.0], 10% Glycerol, 400 mM NaCl, 2 mM dithiothreitol) and incubated overnight with bovine thrombin (Calbiochem) to remove the polyhsitidine tag. The cleaved protein was diluted with 5 volumes of buffer J (25 mM HEPES [pH 7.0], 10% Glycerol, 2 mM dithiothreitol) and loaded onto a HiTrap™ SP FF column (GE Healthcare Life Sciences 17-5157-01) equilibrated in buffer K (buffer J containing 70 mM NaCl). The column was eluted with a 30 column

volume linear gradient from 70 mM NaCl to 500 mM NaCl in buffer J. Fractions containing BKV protein were pooled and stored at minus 70˚C.

JCV DNA unwinding assay

The assay was run in 50 mM HEPES pH 7.5, 100 mM NaCl, 7 mM $MgCl_2$, 0.002% Brij-35, 1 mM DTT, 2% DMSO, 360 nM of JCV LTAg, 15nM Cy5/BHQ3 dsDNA, 15 nM of unlabeled dsDNA and 30u M ATP. The ATP was regenerated with the PK/LDH coupling

All assay components were assembled and incubated at room temp for 20 min to initiate the reaction. 20 uL of the reaction mixture was added to assay ready plates and incubated 120-180 minutes at room temperature. Fluorescence intensity was read throughout incubation and slope of RFU v, then time analyzed.

BKV DNA unwinding assay

The assay was run in 50 mM HEPES pH 7.5, 100 mM NaCl, 7 mM $MgCl_2$, 0.002% Brij-35, 1mM DTT, 2% DMSO, 540 nM BK LTAg, 15 nM Cy5/BHQ3 dsDNA, 15nM unlabeled dsDNA and 30uM ATP. The ATP was regenerated with the PK/LDH coupling system.

All assay components were assembled and incubated at room temperature for 20 mn to initiate the reaction. 20 uL of the reaction mixture were added to assay ready plates and incubated 120-180 mn at room temperature. The fluorescence intensity was read throughout incubation and slope of RFU v then time analyzed.

DNA binding assay.

10 mM DMSO stocks of compounds were added at 500 uM final concentration (5% DMSO) to a solution of 4 uM salmon sperm DNA (Aldrich) dissolved in 50 mM potassium

phosphate pH 7, 50 mM Na_2EDTA, D_2O. NMR experiments were performed at 295 K on a 500 MHz Bruker Avance III NMR spectrometer equipped with a 5 mm triple resonance inverse (TXI) cryoprobe. One dimensional ^1H NMR spectra were collected with WATERGATE 3919 water suppression to identify insoluble or impure compounds. Saturation transfer difference (STD) spectra were collected using a 2 sec train of 50 ms Gaussian pulses for selective saturation at 5.8 ppm (H5',5" region), with 32 interleaved sets of 8 on- and off-resonance scans. Compounds giving STD signals with signal-to-noise > 10 were classified as DNA binders. 500μ ethidium bromide was used as a positive control.

DNA bDNA assay: JC virus VP1 specific DNA bDNA probes were requested by Vertex and synthesized by Affymatrix, based on Affymatrix v2.0 kit. Cultured Vero cells were lysed by adding 1/3 volume of lysis mixture from Quantigene v2.0 kit. 20 μl of the completely mixed lysate were mixed with 20 mM EDTA, 10 μL 2.5N NaOH and 60 μL dH_2O, incubated at 52˚C for 30 min to denature the DNA and remove the RNA. 50 μl of 2.0 M HEPES was added, mixed, and then transferred 20 μl of mixture into bDNA capture plates containing 80 μl of JC VP1 specific DNA bDNA probes (50 μl of lysis mixture, 28.7 μl of H_2O, 1 μl of blocking solution, and 0.3 μl of probe stock). Purified DNA is applied to the assay once the DNA was suspended into lysis buffer (1:2 diluted lysis mixture).

EC_{50} determination of Antiviral Agents. 7.5xE6 G144 Vero cells were plated in a T-150 flask pre-coated with poly-Orinithine (0.005%) and Laminie (10 μg/ml) in the growth medium for 3 hr to overnight. The cells were infected with MAD4 virus at MOI = 1 $TCID_{50}$ for 24 hr. For the BKV assay, 10xE6 Vero cells were plated in a T-150 flask in the growth medium for overnight. The cells were infected with BKV virus at MOI = 0.1 $TCID_{50}$ for 24

hr. The JCV infected cells were replated into 96 well plates pre-coated with poly-Orinithine (0.005%) and Laminie (10 μg/ml) at 5,000 cells/well in 100 μl of differentiation medium. Compounds diluted into DMSO were used to treat the infected cells at final concentration of 0.5% DMSO. After 72 hr incubation, the viral RNA and DNA levels were determined as described in the study. The EC_{50} values of the antiviral agents were calculated using a four-parameter curve fitting method in the Softmax Pro program (Molecular Devices Corporation, Sunnyvale, CA).

Compound Preparation and Characterization.

All commercially available reagents and anhydrous solvents were used without further purification. Unless specified otherwise, all compounds were>95% purity. Mass samples were analyzed on a Micro Mass ZQ, ZMD, Quattro LC, or Quatro II mass spectrometer operated in a single MS mode with electrospray ionization. Samples were introduced into the mass spectrometer using flow injection (FIA) or chromatography. The mobile phase for all mass analysis consisted of acetonitrile to water mixtures with either 0.2% formic acid or ammoniumformate. ^{1}H NMR spectra were recorded either using a Bruker Avance 400 (400 MHz) or a Bruker Avance II300 (300 MHz) instrument. Column chromatography was performed using RediSep®Rf Gold Normal Phase silica flash columns using a Teledyne ISCO Combiflash Companion or Combiflash Rf purification system. Preparative reversed phase chromatography was carried out using a Gilson 215 liquid handler coupled to a UV2VIS 156 Gilson detector, an Agilent Zorbax SB2C18 column, 21.2 mm × 100 mm, a linear gradient from 10 to 90% CH_3CN in H_2O over 10 min (0.1% trifluoroacetic acid); the flow rate was 20 mL/min. High resolution mass spectrometry data was collected on a

Thermo Scientfic QExactive mass spectrometer coupled to a Waters Acquity UPLC system. Samples were analyzed from a 100 μM DMSO solution with 3 μL injection volumes. The chromatographic column was a Waters Acquity CSH C18, 2.1 × 50 mm, 1.7 μm particle size. Gradient elution was employed using 0.1% formic acid in water as mobile phase A and 0.1% formic acid as mobile phase B. The gradient began at 10% B, increased to 60% B over 0.8 minutes, to 100% B over the next 0.2 minutes, and was followed by a 0.5 minute reequilibration at initial conditions. The mass spectrometer was run in full MS mode, positive polarity, with resolution set to 35,000. A heated electrospray source was used with settings of 3.5 kV and 400 °C.

4-amino-1,2,4-triazole-3-thiol (4). 1,3-Diaminothiourea (30g, 282.6 mmol) and formic acid (30 mL, 795.2 mmol) were refluxed for 12h, then cooled to room temperature leading to the formation of a crystalline material which was filtered and triturated with Et_2O. The solid thus obtained was crystallized from EtOH to give **3** (14g, 42%). ^{1}HNMR (DMSO-d_6) δ 13.65 (s, 1H), 8.46 (s, 1H), 5.69 (s, 2H).

Preparation of the thiazolotriazoles (5c-5l) by condensation with the 4-amino-4*H*-1,2,4-triazole-3-thiol

General procedure A

The 4-amino-1,2,4-triazole-3-thiol (**4**) (0.43 mmol) and a carboxylic acid (0.47 mmol) were combined in $POCl_3$ (0.5 mL) in a microwave vial and heated to 110-120˚C for 30 minutes to 90 minutes and the reaction monitored by LC/MS. The reaction mixture was cooled using an ice bath. A 2M aqueous solution of NaOH was added until precipitation occurred. The solids were filtered off, washed with H_2O then Et_2O and were dried under vacuum.

6-(2-Cyclopropylphenyl)-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole (**5c**). 1.4% (1.3 mg) ; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.67 (d, J = 4.7 Hz, 1H), 7.73 (dq, J = 11.8, 6.0, 4.1 Hz, 1H), 7.56 (t, J = 7.6 Hz, 1H), 7.43 (dt, J = 21.6, 7.5 Hz, 1H), 7.23 (d, J = 7.9 Hz, 1H), 2.33 (ddd, J = 13.6, 8.4, 5.3 Hz, 1H), 1.12 – 0.90 (m, 2H), 0.88 – 0.65 (m, 2H); LCMS $[M+ H]^+$ = 243.42, rt = 2.67 min (5-95% CH_3CN in water with 0.1% TFA); HRMS $[M+ H]^+$ calculated for ($C_{12}H_{10}N_4S+ H^+$): 243.06989, found: 243.06936

6-(2-Phenoxyphenyl)-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole (**5d**). 32 % (5.1 mg); ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.65 (s, 1H), 8.27 (d, J = 7.8 Hz, 1H), 7.74 – 6.94 (m, 8H); LCMS $[M+ H]^+$ = 295.39, rt = 0.83 min (5-95% CH_3CN in water with 0.1% TFA); HRMS $[M+ H]^+$ calculated for ($C_{15}H_{10}N_4OS+ H^+$): 295.06481, found: 295.06493

2-([1,2,4]Triazolo[3,4-*b*][1,3,4]thiadiazol-6-yl)-*N*,*N*-dimethylaniline (**5e**). 89% (92 mg); ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.53 (s, 1H), 8.26 – 7.97 (m, 1H), 7.71 – 7.54 (m, 2H), 7.40 (ddd, J = 8.3, 6.0, 2.5 Hz, 1H), 2.69 (s, 7H); CMS $[M+ H]^+$ = 246.46, rt = 2.52 min (5-95% CH_3CN in water with 0.1% TFA); HRMS $[M+ H]^+$ calculated for ($C_{11}H_{11}N_5S+ H^+$): 246.08079, found: 246.08021

6-(2,3-Dimethoxyphenyl)-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole (**5f**). 4.4% (12.7 mg); ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.61 (s, 1H), 7.71 (dd, J = 7.8, 1.7 Hz, 1H), 7.50 – 7.09 (m, 2H), 3.98 (s, 3H), 3.92 (s, 3H); LCMS $[M+ H]^+$ = 263.33, rt = 0.76 min (5-95% CH_3CN in water with 0.1% TFA);HRMS $[M+ H]^+$ calculated for ($C_{11}H_{10}N_4O_2S+ H^+$): 263.05972, found: 263.05913

6-(2,4-Dimethoxyphenyl)-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole (**5g**). 10% (5.6mg); ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.52 (s, 1H), 8.08 (d, J = 8.9 Hz, 1H), 6.95 – 6.63 (m, 2H), 4.06 (s, 3H), 3.89 (s, 3H); LCMS $[M+ H]^+$ = 263.06, rt = 0.85 min (10-60% CH_3CN in water

with 0.1% TFA); HRMS $[M+ H]^+$ calculated for ($C_{11}H_{10}N_4O_2S + H^+$): 263.05972, found: 263.05911

6-(2,6-Dimethoxyphenyl)-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole (5h). 64.2% (25 mg); ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.63 (s, 1H), 7.59 (t, *J* = 8.5 Hz, 1H), 6.88 (d, *J* = 8.5 Hz, 2H), 3.83 (s, 6H);); LCMS $[M+ H]^+$ = 263.42, rt = 2.29 min (5-95% CH_3CN in water with 0.1% TFA); HRMS $[M+ H]^+$ calculated for ($C_{11}H_{10}N_4O_2S + H^+$): 263.05972, found: 263.05923

6-(Chroman-8-yl)-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole (**5i**). 82% (71.6 mg); ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.60 (s, 1H), 7.96 (dd, *J* = 8.0, 1.6 Hz, 1H), 7.63 – 7.15 (m, 1H), 7.06 (t, *J* = 7.7 Hz, 1H), 4.59 – 4.09 (m, 2H), 2.86 (t, *J* = 6.4 Hz, 2H), 2.04 (dt, *J* = 11.7, 6.3 Hz, 2H); LCMS $[M+ H]^+$ = 259.4, rt = 2.71 min (5-95% CH_3CN in water with 0.1% TFA); HRMS $[M+ H]^+$ calculated for ($C_{12}H_{10}N_4OS + H^+$): 259.06481, found 259.06421

6-(2,3-Dihydrobenzofuran-7-yl)-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole (**5j**). 15% (19 mg); ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.61 (s, 1H), 8.06 – 7.71 (m, 1H), 7.53 (dd, *J* = 7.2, 1.2 Hz, 1H), 7.24 – 6.69 (m, 1H), 4.84 (t, *J* = 8.7 Hz, 2H), 3.33 (s, 2H); LCMS $[M+ H]^+$ = 245.4, rt = 2.48 min (5-95% CH_3CN in water with 0.1% TFA); HRMS $[M+ H]^+$ calculated for ($C_{11}H_8N_4OS + H^+$): 245.04916, found 245.0486

6-(2-(2-Methoxyethoxy)phenyl)-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole (**5k**). 24 % (29 mg); ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.59 (s, 1H), 8.28 – 8.00 (m, 1H), 7.77 – 7.56 (m, 1H), 7.36 (d, *J* = 8.1 Hz, 1H), 7.20 (t, *J* = 7.1 Hz, 1H), 4.63 – 4.27 (m, 2H), 4.04 – 3.81 (m, 2H), 3.40 (s, 3H) ; LCMS $[M+ H]^+$ = 277.42, rt = 2.46 min (5-95% CH_3CN in water with 0.1% TFA); HRMS $[M+ H]^+$ calculated for ($C_{12}H_{12}N_4O_2S + H^+$): 277.07537, found 277.07481

General procedure B

Diisopropylethylamine (0.86 mmol) was added to a suspension of 4-amino-1,2,4-triazole-3-thiol (0.43 mmol) and a carboxylic acid (0.47 mmol) in a 50% solution of T3P in EtOAc (0.86 mmol). The reaction mixture was heated in a microwave vial at 140˚C for 20 min-2 hrs and monitored by LC/MS.

6-(2-Methoxybenzyl)-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole (**5l**). The reaction mixture was poured into water. The resulting white solid was filtered and washed with Et_2O (41% , 46 mg); ^{1}HNMR (400 MHz, $CDCl_3$) δ 8.74 (s, 1H), 7.26-7.31 (m,1H), 7.23 – 7.13 (m,1H), 6.86-6.93 (m, 2H), 4.22 (s, 2H), 3.81 (s, 3H); LCMS $[M+ H]^+$ = 248.08, rt = 2.37 min (10-90% CH_3CN in water with 0.1% formic acid); HRMS [M+ H]+ calculated for ($C_{11}H_{10}N_4OS+ H^+$): 247.06481, found: 247.06424

2-([1,2,4]Triazolo[3,4-b][1,3,4]thiadiazol-6-yl)phenol (**5m**). The reaction mixture was poured into water. The resulting white solid was filtered and washed with Et_2O (61%, 532 mg) ^{1} H NMR (300 MHz, DMSO-d_6) δ 11.73 (s, 1H), 9.56 (s, 1H), 8.08 (dd, J = 8.0, 1.6 Hz, 1H), 7.53 - 7.44 (m, 1H), 7.10-7.02 (m, 2H); LCMS $[M+ H]^+$ = 219.07; rt = 0.53 min (5-95% CH_3CN in water with 0.1% NH_4OH); HRMS $[M+ H]^+$ calculated for ($C_9H_6N_4OS+ H^+$): 219.03351, found: 219.03353

General method for the reparation of the thiazolotriazoles (5n-5q) via alkylation of the 2-([1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazol-6-yl)phenol (5m)

The 2-([1,2,4]triazolo[3,4-b][1,3,4]thiadiazol-6-yl)phenol (**5m**) (0.14 mmol), an alkyl chloride or alkyl bromide (0.14 mmol) and K_2CO_3 (0.14 mmol) were taken in CH_3CN (2 mL) and the reaction mixture was heated in a microwave vial at 100-130˚C for 30 min. The

volatiles were evaporated. The residue was dissolved in DM (1 mL), filtered and the filtrate was purified by reverse phase preparative HPLC.

6-(2-((Tetrahydro-2H-pyran-4-yl)methoxy)phenyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole (5n). 35% (15 mg) ; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.60 (s, 1H), 8.17 (d, *J* = 7.0 Hz, 1H), 7.64 (t, *J* = 7.8 Hz, 1H), 7.36 (d, *J* = 8.5 Hz, 1H), 7.19 (t, *J* = 7.6 Hz, 1H), 4.16 (d, *J* = 6.3 Hz, 2H), 3.92 (dd, *J* = 11.1, 3.4 Hz, 3H), 3.40 (q, *J* = 13.6, 12.1 Hz, 2H), 2.31 – 2.18 (m, 1H), 1.82 (d, *J* = 11.6 Hz, 2H), 1.43 (qd, *J* = 12.5, 4.7 Hz, 2H); HRMS [M+ H]+ calculated for ($C_{15}H_{16}N_4O_2S + H^+$):317.10667, found: 317.106

6-(2-(2-(Tetrahydro-2H-pyran-4-yl)ethoxy)phenyl)-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole (**5o**). 35% (13.2 mg); ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.96 (s, 1H), 8.27 (dd, *J* = 8.0, 1.7 Hz, 1H), 7.57 (ddd, *J* = 8.5, 7.4, 1.7 Hz, 1H), 7.23 – 6.92 (m, 2H), 4.31 (t, *J* = 6.3 Hz, 2H), 4.14 – 3.70 (m, 2H), 3.43 (td, *J* = 11.9, 2.0 Hz, 2H), 1.99 (q, *J* = 6.7, 6.2 Hz, 3H), 1.74 (d, *J* = 13.0 Hz, 2H), 1.44 (qd, *J* = 13.0, 12.5, 4.5 Hz, 2H); [M+ H]$^+$ calculated for ($C_{16}H_{18}N_4O_2S + H^+$): 331.12232, found 331.12238

4-(2-(2-([1,2,4]Triazolo[3,4-*b*][1,3,4]thiadiazol-6-yl)phenoxy)ethyl)morpholine (**5p**). 41% (14.3 mg); ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.62 (s, 1H), 8.18 (d, *J* = 8.1 Hz, 1H), 7.40 (d, *J* = 8.0 Hz, 1H), 7.27 (t, *J* = 7.2 Hz, 1H), 4.67 (s, 2H), 3.80 (s, 2H), 3.27 (s, 2H); HRMS [M+ H]$^+$ calculated for ($C_{15}H_{17}N_5O_2S + H^+$): 332.11757, found 332.11784

4-(2-([1,2,4]Triazolo[3,4-*b*][1,3,4]thiadiazol-6-yl)phenoxy)butanenitrile (5q). 15% (5.3 mg); ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.60 (s, 1H), 8.30 – 8.10 (m, 1H), 7.65 (d, *J* = 8.9 Hz, 1H), 7.36 (d, *J* = 8.7 Hz, 1H), 7.22 (t, *J* = 7.6 Hz, 1H), 4.36 (t, *J* = 6.0 Hz, 2H), 2.80 (t, *J* = 7.3 Hz, 2H), 2.25 (t, *J* = 6.1 Hz, 2H); LCMS [M+ H]$^+$ = 286.06; rt = 0.81 min (10-60% CH_3CN in

water with 0.1% TFA); HRMS [M+ H]$^+$ calculated for (C13H11N5OS+ H$^+$): 286.07571, found 286.07508

6-(2-Phenoxypyridin-3-yl)-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole (**5r**). 13.3% (16 mg); ^{1}HNMR (400 MHz, $CDCl_3$) δ 8.91 (s, 1H), 8.57 (dd, *J* = 7.8, 1.9 Hz, 1H), 8.29 (dd, *J* = 4.8, 1.9Hz, 1H), 7.42 (m, 2H), 7.26 (dd, *J* = 10.7, 4.2 Hz, 1H), 7.21 - 7.03 (m, 4H); LCMS [M+ H]$^+$ = 296.08; rt = 2.48 min (10-90% CH_3CN in water with 0.1% TFA); HRMS [M+ H]+ calculated for ($C_{14}H_9N_5OS$ + H$^+$):296.06006, found: 296.05985

6-(4-Phenoxypyrimidin-5-yl)-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole (**5s**). 32 % (11.4 mg); ^{1}H NMR (300 MHz, Methanol- d_4): 9.49 (d, *J* = 0.7 Hz, 2H), 8.85 (s, 1H), 7.78 - 7.14 (m, 5H); LCMS [M+ H]$^+$ = 297.15, rt= 3.07min (2-98% CH_3CN in water with 0.1% TFA); HRMS [M+ H]$^+$ calculated for ($C_{13}H_8N_6OS$): 297.05531, found: 297.05536

6-(4-phenoxypyridin-3-yl)-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole (**5t**). The 6-(4-chloro-3-pyridyl)-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole (50 mg, 0.21 mmol), phenol (19.8 mg, 0.21 mmol) and K_2CO_3 (29 mg, 0.21 mmol) were taken DMF (3.0 mL). The reaction mixture was heated in a microwave at 130˚C for an hour. The mixture was filtered and the filtrate was purified by reverse phase chromatography (10-100% CH_3CN/ water with 1%TFA) to get 1.9 mg (3.3%) of the desired product. ^{1}H NMR (300 MHz, Methanol- d_4): 9.46 (s, 1H), 9.39 (s, 1H), 8.57 (d, *J* = 6.0 Hz, 1H), 7.74 - 7.51 (m, 2H), 7.52 - 7.22 (m, 3H), 6.95 (d, *J* = 6.0 Hz, 1H); LCMS [M+ H]$^+$ = 296.11; rt = 2.5 min (2-98% CH_3CN in water with 0.1% TFA); HRMS [M+ H]$^+$ calculated for ($C_{14}H_9N_5OS$+ H$^+$): 296.06006, found: 296.06014

N-(Pyridin-4-yl)pivalamide (**8**). A solution of 2,2-dimethylpropanoyl chloride (21.43 g, 21.87 mL, 177.7 mmol) in DCM (50 mL) was slowly added to a cooled (0 °C) solution of pyridin-4-amine (15.2 g, 161.5 mmol) and triethylamine (45.0 mL, 323.0 mmol) in CH_2Cl_2(100 mL) . After addition was complete, the ice bath was removed and the resulting mixture was stirred at room temperature overnight. The reaction mixture was poured into water and extracted with CH_2Cl_2. The organic extracts were dried over $MgSO_4$, and concentrated under reduced pressure. The residue was triturated with DCM/Et_2O: 1/10 to give 21g of a slightly tan solid that was collected by filtration (66%); ^{1}H NMR (300 MHz, CDCl $_3$) δ 8.51 (dd, J = 5.0, 1.4 Hz, 2H), 7.60 -7.42 (m, 3H), 1.35 (s, 9H);); LCMS $[M+ H]^+$ = 178.74; rt = 0.61 min (5-95% CH_3CN in water with 0.1% NH_4OH).

4-Pivalamidopyridin-3-yl diisopropylcarbamodithioate (**9**). n-Butyl lithium (25.5 mL of 2.5 M, 63.8 mmol) was added to 2,2-dimethyl-N-(4-pyridyl)propanamide (**8**) (4.55 g, 25.53 mmol) in THF (100 mL) a t - 78°C. After the addition was complete, the dry ice acetone bath was removed, replaced with an ice bath and the mixture stirred at 0°C for 4hrs. The resulting mixture was cooled to -78 °C again and a solution of tetraisopropylthiuram disulfide (9.0g, 25.53 mmol) in anhydrous THF (20 mL) was slowly added. After addition, the mixture was allowed to warm to room temperature and stirred for1hr. Water (200 mL) and EtOAc (200 mL) were added sequentially. The organic layer was separated, dried over $MgSO_4$, and concentrated under reduced pressure. The resulting residue was purified by silica gel column chromatograph, eluting with hexane / EtOAc : 100/0-50/50 to give 5.68g of the desired material (73%). ^{1}H NMR (300 MHz, DMSO) δ 8.59 (d, J = 5.5 Hz, 1H), 8.48 (s, 1H), 8.46 (s, 1H), 8.18 (d, J = 5.5 Hz, 1H), 1.42 (s, 12H), 1.21 (s, 9H); LCMS $[M+ H]^+$ = 354.18; rt = 0.91 min (5-95% CH_3CN in water with 0.1% NH_4OH).

(4-Amino-3-pyridyl) *N,N*-diisopropylcarbamodithioate (**10**). A mixture of 4-pivalamidopyridin-3-yl diisopropylcarbamodithioate (**9**) (5.43 g, 13.8 mmol) and sodium hydroxide (1.10 g, 27.6 mmol) was reacted at room temperature in MeOH (100 mL) overnight. The mixture was concentrated. And the resulting solid (1.6g) was filtered off and used without further purification (38%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.01 (d, J = 5.7 Hz, 1H), 7.90 (s, 1H), 6.62 (d, J = 5.7 Hz, 1H), 6.14 (s, 2H), 1.42 (s, 12H).

2-(2-Methoxyphenyl)thiazolo[5,4-*c*]pyridine (**12a**). A solution of (4-amino-3-pyridyl) *N,N*-diisopropylcarbamodithioate (**10**) (50 mg, 0.19 mmol), 3-methoxybenzoic acid (34 mg, 0.22 mmol), HATU (96.3 mg, 0.25 mmol) and DIPEA 0.65 mL, 0.37 mmol) in DMF (5 mL) was stirred at room temperature for 18 h and the solution was diluted with water (10 mL). The aqueous layer was extracted with EtOAc (3x5 mL), dried over Na_2SO_4 and concentrated under reduced pressure to give **11a** as an off white solid (48 mg) as off white solid. A solution of **11a** (48 m g) in 6M HCl (5 mL) was heated at 80 ^{o}C for 30 min and the solution was cooled to RT. The solvent was removed under reduced pressure and basified with Sat $NaHCO_3$. The aqueous layer was extracted with EtOAc (3x10 mL), dried over Na_2SO_4 and concentrated under reduced pressure. The crude material was purified by reverse phase column chromatography (C18aq, 0-100% ACN/water, 0.1% TFA, 20 min) to afford **12a** (28 mg, 44%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.16 (s, 1H), 8.57 (d, J = 5.6 Hz, 1H), 8.53 - 8.36 (m, 1H),7.87 (dd, J = 5.6, 0.8 Hz, 1H), 7.56 - 7.30 (m, 1H), 7.12 - 7.07 (m, 1H), 7.04 (d, J = 8.4Hz, 1H), 4.04 (s, 3H). LCMS $[M+ H]^+$ = 243.44; rt = 0.63 min (10-60% CH_3CN in water with 0.1% TFA); HRMS $[M+ H]^+$ calculated for ($C_{13}H_{10}N_2OS+ H^+$): 243.05860, found: 243.05888

2-(2-Ethoxyphenyl)thiazolo[5,4-c]pyridine (12b). The 2-ethoxybenzoyl chloride (48 mg, 0.26 mmol) was added to a solution of (4-amino-3-pyridyl) *N,N*-diisopropy-carbamodithioate (**10**) (70 mg, 0.26 mmol) and triethylamine (52.6 mg, 0.52 mmol) in CH_2CL_2 (4 mL) . The reaction mixture was stirred at room temperature for 10 min. The reaction mixture was extracted with CH_2Cl_2 and water. The organic layer was dried over $MgSO_4$, filtered and evaporated. The crude material was purified by chromatography on silicagel eluting with Hexanes/EtOAc: 100/0-30/70 to afford 73 mg of 4-(2-ethoxybenzamido)pyridin-3-yl diisopropylcarbamodithioate as a clear colorless oil (19%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.53 (s, 1H), 8.70 (d, J = 5.9 Hz, 1H), 8.61 (d, J = 5.9 Hz, 1H), 8.53 (s, 1H), 8.21 (dd, J = 7.9, 1.8 Hz, 1H), 7.56 -7.45 (m, 1H), 7.20 - 7.10 (m, 1H), 7.04 (d, J = 8.4 Hz, 1H), 4.36 (q, J = 7.0 Hz, 2H), 1.49 (m, 15H); LCMS $[M+ H]^+$ = 418.2; rt = 0.92 min (5-95% CH_3CN in water with 0.1% NH_4OH).

A solution of [4-[(2-ethoxybenzoyl)amino]-3-pyridyl] N,N-diisopropylcarbamodithioate (73 mg, 0.17 mmol) in in 5 M aqueous HCL (10 mL) was stirred at 80 °C for 2 hours. The reaction mixture was cooled to room temperature leading to the formation of a precipitate. The pH of the mixture was adjusted to 10 with a 2N aqueous sodium hydroxide solution and the aqueous phase extracted with EtOAc. The organic extract was dried over $MgSO_4$ and evaporated. The resulting material was purified by chromatography on silicagel eluting with Hexane/EtOAc : 100/0-4 0/60 to give 22mg of a white solid (42%). ^{1}H NMR (300 MHz, DMSO) δ 9.41 (s, 1H), 8.63 (d, J = 5.6 Hz, 1H), 8.51 (dd, J = 7.9, 1.7 Hz, 1H), 8.01 (dd, J = 5.6, 0.9 Hz, 1H), 7.67- 7.57 (m, 1H), 7.34 (d, J = 8.4 Hz, 1H), 7.25 -7.14 (m, 1H), 4.40 (q, J = 7.0 Hz, 2H), 1.59 (t, J = 7.0 Hz, 3H); LCMS $[M+ H]^+$ = 257.39; rt = 0.84 min (5-95%

CH_3CN in water with 0.1% NH_4OH); HRMS $[M+ H]^+$ calculated for ($C_{14}H_{12}N_2OS+ H^+$): 257.07431, found: 257.07447

2-(2-Methoxyphenyl)thiazolo[5,4-*c*]pyridine (**12a**). A solution of (4-amino-3-pyridyl) *N,N*-diisopropylcarbamodithioate (**10**) (50 mg, 0.19 mmol), 3-methoxybenzoic acid (34 mg, 0.22 mmol), HATU (96.3 mg, 0.25 mmol) and DIPEA 0.65 mL, 0.37 mmol) in DMF (5 mL) was stirred at room temperature for 18 h and the solution was diluted with water (10 mL). The aqueous layer was extracted with EtOAc (3x5 mL), dried over Na_2SO_4 and concentrated under reduced pressure to give **13a** as an off white solid (48 mg) as off white solid. A solution of **13a** (48 m g) in 6M HCl (5 mL) was heated at 80 ^oC for 30 min and the solution was cooled to RT. The solvent was removed under reduced pressure and basified with Sat $NaHCO_3$. The aqueous layer was extracted with EtOAc (3x10 mL), dried over Na_2SO_4 and concentrated under reduced pressure. The crude material was purified by reverse phase column chromatography (C18aq, 0-100% ACN/water, 0.1% TFA, 20 min) to afford **12a** (28 mg, 44%) as a white solid. ^1H NMR (400 MHz, $CDCl_3$) δ 9.16 (s, 1H), 8.57 (d, J = 5.6 Hz, 1H), 8.53 - 8.36 (m, 1H),7.87 (dd, J = 5.6, 0.8 Hz, 1H), 7.56 - 7.30 (m, 1H), 7.12 - 7.07 (m, 1H), 7.04 (d, J = 8.4Hz, 1H), 4.04 (s, 3H). LCMS $[M+ H]^+$ = 243.44; rt = 0.63 min (10-60% CH_3CN in water with 0.1% TFA); HRMS $[M+ H]^+$ calculated for ($C_{13}H_{10}N_2OS+ H^+$): 243.05860, found: 243.05888

2-(2-Phenoxyphenyl)thiazolo[5,4-*c*]pyridine (12c). The 2-phenoxybenzoyl chloride (64.8 mg, 0.20 mmol) was added to a solution of (4-amino-3-pyridyl) *N,N*-diisopropy-carbamodithioate (**10**) (73 mg, 0.20 mmol) and triethylamine (40.8 mg, 0.40 mmol) in CH_2Cl_2 (3 mL). The reaction mixture was stirred at room temperature for 10 min. The

reaction mixture was extracted with CH_2Cl_2 and water. The organic layer was dried over $MgSO_4$, filtered and evaporated. The crude material was purified by chromatography on silicagel eluting with Hexanes/EtOAc: 100/0-20/80 to afford 93mg of [4-[(2-phenoxybenzoyl)amino]-3-pyridyl] N,N-diisopropylcarbamodithioate as a white foam (87%). ^{1}H NMR (300 MHz, DMSO) δ 10.49 (s, 1H), 8.60 (d, J = 5.7 Hz, 1H), 8.49 (d, J = 5.7 Hz, 1H), 8.43 (s, 1H), 8.11 (dd, J = 8.0, 1.5 Hz, 1H), 7.59 - 7.52 (m, 1H), 7.48-7.45 (m, 2H), 7.30 (dd, J = 10.8, 4.3 Hz, 2H), 7.23 - 7.14 (m, 2H), 6.78 (d, J = 8.0 Hz, 1H), 1.26 (s, 12H);); LCMS $[M+ H]^+$ = 466.18; rt = 0.98 min (5-95% CH_3CN in water with 0.1% NH_4OH).

A solution of [4-[(2-phenoxybenzoyl)amino]-3-pyridyl] N,N-diisopropylcarbamodithioate (93 mg, 0.17 mmol) in 5 M HCL (10 mL) was stirred at 80 °C overnight. The reaction mixture was cooled to room temperature leading to the formation of a solid. The pH of the mixture was adjusted to 10 with a 2N aqueous sodium hydroxide solution. The aqueous phase extracted with EtOAc. The organic phase was dried over $MgSO_4$ and evaporated. The resulting solid was purified by chromatography on silicagel eluting with Hexane/EtOAc: 100/0-4 0/60 to give 25mg of a white solid (41%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.39 (d, J = 0.8 Hz, 1H), 8.64 (d, J = 5.6 Hz, 1H), 8.59 (dd, J = 8.2, 1.7 Hz, 1H), 8.06 (dd, J = 5.6, 0.8 Hz, 1H), 7.69 - 7.59 (m, 1H), 7.53 - 7.37 (m, 3H), 7.30 - 7.16 (m, 3H), 7.11 (dd, J = 8.2, 0.8 Hz, 1H); LCMS $[M+ H]^+$ = 305.43; rt = 0.91 min (5-95% CH_3CN in water with 0.1% NH_4OH); HRMS $[M+ H]^+$ calculated for ($C_{18}H_{12}N_2OS+ H^+$): 305.07431, found: 305.07438

2-(2,3-Dihydrobenzofuran-7-yl)thiazolo[5,4-*c*]pyridine (**12d**). The 4-amino-3-pyridyl) *N,N*-diisopropylcarbamodithioate (**10**) (50 mg, 0.13 mmol), 2,3-dihydrobenzofuran-7-

carboxylic acid (22 mg, 0.13 mmol) and HATU (50.7 mg, 0.13 mmol) were taken in DCM (5 mL). To this mixture was added triethylamine (27 mg, 0.27 mmol). The reaction mixture was stirred for 30 min at room temperature. The reaction mixture was concentrated and taken up in ethylacetae/ water. The organic layer was separated, dried over $MgSO_4$, filtered and concentrated. The residue was taken up in 6N HCl (3ml) and stirred at room temperature for 30 min. The reaction mixture was neutralized with 2N aqueous NaOH, extracted with EtOAc. The organic layer was separated, dried over $MgSO_4$, filtered and concentrated. The crude material was purified by reverse phase chromatography using an ISCO (10-100% CH_3CN/ water, 1%TFA) to get 7.9 mg (12%) of the desired compound as a white solid. ^1H NMR (300 MHz, Methanol-d_4) δ 9.57 (t, J = 0.8 Hz, 1H), 8.86 - 8.63 (m, 1H), 8.48 - 8.16 (m, 2H), 7.55 (dq, J = 7.2, 1.2 Hz, 1H), 7.09 (dd, J = 8.4, 7.2 Hz, 1H), 4.95 (d, J = 8.4 Hz, 2H), 3.50-3.34 (m, 2H); LCMS $[M+ H]^+$ =255.32; rt = 0.61min (2-98% CH_3CN in water with 0.1% TFA); HRMS $[M+ H]^+$ calculated for ($C_{14}H_{10}N_2OS + H^+$): 255.05866, found: 255.05804

2-(Thiazolo[5,4-*c*]pyridin-2-yl)phenol (**12e**). The (2-chlorocarbonylphenyl) acetate (221.0 mg, 1.11 mmol) was added to a solution of 4-amino-3-pyridyl) N,N-diisopropylcarbamodithioate (**10**) (300 mg, 1.11 mmol) and triethylamine (225.0 mg, 2.22 mmol) in CH_2Cl_2 (6 mL) . The reaction mixture was stirred at room temperature for 10 min, partitioned between water and CH_2Cl_2. The organic layer was dried over $MgSO_4$, filtered and concentrated. The resulting material was purified by chromatography on silicagel eluting with Hexanes/EtOAc: 100/0-30/70 to give 190 mg of [2-[[3-(diisopropylcarbamothioylsulfanyl)-4-pyridyl]carbamoyl]phenyl] acetate as a colorless oil

(31%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.45 (s, 1H), 8.63 (d, J = 5.3 Hz, 1H), 8.48 (s, 1H), 8.17 (d, J = 5.3 Hz, 1H), 7.75 - 7.60 (m, 2H), 7.40 (td, J = 7.6, 1.0 Hz, 1H), 7.30 (dd, J = 8.1, 1.0 Hz, 1H), 3.33 (s, 3H), 1.43 (s, broad,12H); LCMS [M+ H]$^+$ = 432.49; rt = 0.85 min (5-95% CH_3CN in water with 0.1% NH_4OH).

A solution of [2-[[3-(diisopropylcarbamothioylsulfanyl)-4-pyridyl]carbamoyl]phenyl] acetate (190 mg, 0.44 mmol) in 5 M HCL (10 mL) was stirred at 80 °C for 1 hr. The resulting pale yellow solid was filtered and washed with water. The solid was dissolved in DCM/MeOH, evaporated and triturated with Et_2O to give 90mg of 2-(thiazolo[5,4-c]pyridin-2-yl)phenol (85%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.09 (s, 1H), 9.68 (s, 1H), 8.80 (d, J = 6.3 Hz, 1H), 8.44 (dd, J = 8.3, 1.6 Hz, 1H), 8.38 (d, J = 6.3 Hz, 1H), 7.56 (ddd, J = 8.3, 7.5, 1.6 Hz, 1H), 7.20 (d, J = 7.5 Hz, 1H), 7.14 - 7.03 (m, 1H); LCMS [M+ H]$^+$ = 229.38; rt = 0.75 min (5-95% CH_3CN in water with 0.1% NH_4OH); HRMS [M+ H]$^+$ calculated for ($C_{12}H_8N_2OS$+ H$^+$): 229.04301, found: 229.04317

2-(2-(2-(Tetrahydro-2*H*-pyran-4-yl)ethoxy)phenyl)thiazolo[5,4-*c*]pyridine (**12f**). The 2-thiazolo[5,4-*c*]pyridin-2-ylphenol (30 mg, 0.1314 mmol) , 4-(2-bromoethyl)tetrahydropyran (**12f**) (25.4 mg, 0.13 mmol) was reacted in CH_3CN (0.5 mL) at 80˚C for 3hrs in the presence of $_{K2CO3\ (}$90.8 mg, 0.66 mmol). After cooling to room temperature, the reaction mixture was poured into water and extracted with EtOAc. The organic phase was dried over $MgSO_4$, filtered and evaporated. The resulting material was purified by reverse phase preparative HPLC to give 16.3mg of a white solid (36%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.61 (s, 1H), 8.74 (d, J = 5.9 Hz, 1H), 8.54 (dd, J = 8.1, 1.6 Hz, 1H), 8.24 (d, J = 5.9 Hz, 1H), 7.72 - 7.62 (m, 1H), 7.42 (d, J = 8.1 Hz, 1H), 7.22 (t, J = 7.6 Hz, 1H), 4.42 (t, J = 6.7 Hz, 2H), 3.86

(dd, *J* = 10.7, 3.2 Hz, 2H), 3.30 (td, *J* = 11.9, 2.0 Hz, 2H), 2.0-1.86 (m, 2H), 1.91 -1.79 (m, 1H), 1.73 (d, *J* = 12.7 Hz, 2H), 1.36-1.24 (m, 2H); LCMS $[M+ H]^+$ =341.44; rt = 0.85 min (5-95% CH_3CN in water with 0.1% NH_4OH); HRMS $[M+ H]^+$ calculated for ($C_{19}H_{20}N_2O_2S+ H^+$): 341.131831, found: 341.13212

2-(2-Phenoxypyridin-3-yl)thiazolo[5,4-*c*]pyridine (**12g**). Compound **12g** was synthesized adapting the procedure described for compound **12b** using 2-phenyxoypyridine-3-carboxylic acid with 43% yield. ^1H NMR (400 MHz, $CDCl_3$) δ 9.18 (s, 1H), 8.91 (dd, *J* = 7.7, 2.0 Hz, 1H), 8.62 (d, *J* = 5.7Hz, 1H), 8.26 (dd, *J* = 4.8, 2.0 Hz, 1H), 7.92 (d, *J* = 5.4 Hz, 1H), 7.61 - 7.36 (m, 2H), 7.32- 6.90 (m, 4H); LCMS $[M+ H]^+$ = 306.4; rt = 0.70 min (10-90% CH_3CN in water with 0.1% TFA); HRMS $[M+ H]^+$ calculated for ($C_{17}H_{11}N_3OS+ H^+$): 306.06956, found: 306.06966

***N*-(4-(2-(Thiazolo[5,4-*c*]pyridin-2-yl)phenoxy)phenyl)acetamide** (**12h**). A solution of 2-(2-fluorophenyl)thiazolo[5,4-c]pyridine (prepared adapting the procedure described for compound **12f**), N-(4-hydroxyphenyl)acetamide (0.028 g, 0.19 mmol) and CS_2CO_3 (0.08 g, 0.25 mmol) in DMSO (2 mL) was heated in a pressure vial (Q-tube) at 150 ^oC for 4 hrs and the reaction mixture was cooled to room temperature and the inorganic solid was filtered. The crude product was purified by reverse phase column chromatography (C18 aq, 0-80% ACN/water, 0.1% TFA, 15 min) to afford title compound **12h** (0.02 g, 45%) as pale brown solid.; ^1H NMR (400 MHz, $CDCl_3$) δ 9.11 (s, 1H), 8.58 (d, *J* = 5.4 Hz, 2H), 7.89 (d, *J* = 5.5 Hz, 1H), 7.58 - 7.37 (m, 3H), 7.39 (d, *J* = 7.7 Hz, 1H), 7.29-7.24 (m, 2H), 7.04 (d, J = 8.6 Hz, 2H), 6.94 (d, *J* = 8.2 Hz, 1H), 2.13(s, 3H); LCMS $[M+ H]^+$ = 362.38; rt = 0.66 min (10-

60% CH_3CN in water with 0.1% TFA); HRMS $[M+ H]^+$ calculated for ($C_{20}H_{15}N_3O_2S+ H^+$): 362.09577, found: 362.09509

N-(4-((3-(thiazolo[5,4-c]pyridin-2-yl)pyridin-2-yl)oxy)phenyl)acetamide (**12i**). The 2-(2-chloro-3-pyridyl)thiazolo[5,4-*c*]pyridine (70 mg, 0.10 mmol), *N*-(4-hydroxyphenyl) acetamide (15.9 mg, 0.10 mmol) were taken in DMF (3 mL). K_2CO_3 (14.6 mg, 0.10 mmol) was added and the resulting reaction mixture was heated at 100°C overnight. The reaction mixture was diluted with EtOAc/ water. The organic layer was extracted, dried over $MgSO_4$, filtered, evaporated and purified by reverse phase HPLC using an ISCO (10-100% CH_3CN/ water, 1% TFA) to get 25mg (65%) of the desired compound as a white solid. ^{1}HNMR (300 MHz, DMSO-d_6) δ 10.07 (s, 1H), 9.58 (d, *J* = 0.9 Hz, 1H), 8.96 (dd, *J* = 7.7, 1.9 Hz, 1H), 8.76 (d, *J* = 5.9 Hz, 1H), 8.41 (dd, *J* = 4.8, 1.9 Hz, 1H), 8.26 (dd, *J* = 5.9, 0.9 Hz, 1H), 7.83-7.59 (m, 2H), 7.44 (dd, *J* = 7.7, 4.8 Hz, 1H), 7.40 - 7.12 (m, 2H), 2.07 (s, 3H); LCMS $[M+ H]^+$ = 363.03; rt =0.54 min; HRMS $[M+ H]^+$ calculated for ($C_{19}H_{14}N4O_2S + H^+$): 363.09102, found: 393.09121

2-(2-Ethoxyphenyl)thiazolo[4,5-c]pyridine (15). The 2-ethoxybenzoyl chloride (287.3 mg, 1.56 mmol) was added to a suspension of 4-chloropyridin-3-amine (200 mg, 1.56 mmol) and K_2CO_3 (215 mg, 1.56 mmol) in THF (4 mL). DMF (2 mL) was added to help with the solubility of the 4-chloropyridin-3-amine and the mixture was stirred over night at rt. The reaction mixture was partitioned between water and EtOAc. The organic layer was collected, dried over $MgSO_4$, filtered and evaporated. The crude material was purified by chromatography on silicagel eluting with heptane/EtOAc: 100/0-30/70 to afford 50.6 mg (11%) of the *N*-(4-chloropyridin-3-yl)-2-ethoxybenzamide (**14**). ^{1}H NMR (300 MHz, $CDCl_3$)

δ 10.37 (s, 1H), 9.90-9.83 (m, 1H), 8.39-8.28 (m, 2H), 7.55 (ddd, *J* = 8.2, 7.3, 1.9 Hz, 1H), 7.47 -7.38 (m, 1H), 7.17 (ddd, *J* = 8.2, 7.3, 1.1 Hz, 1H), 7.12-7.04 (m, 1H), 4.39 (q, J = 7.0 Hz, 2H), 1.60 (t, J = 7.0 Hz, 3H).

The *N*-(4-chloro-3-pyridyl)-2-ethoxy-benzamide (**14**) (50 mg, 0.180 mmol) and the Lawesson's reagent (51.2 mg, 0.126 mmol) were refluxed in toluene (2 mL) over night. The reaction mixture was cooled to rt. Water was added and the layers separated. The aqueous layer was extracted with DCM. The combined organic phases were washed with a saturated aqueous solution of Na_2CO_3, dried over $MgSO_4$, filtered and evaporated. The crude material was purified by chromatography on silicagel Hexanes/EtOAC : 100/-40/60 to give the desired product as a white solid (22 mg , 43%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.32 (d, *J* = 1.0 Hz, 1H), 8.54 - 8.43 (m, 2H), 8.22 (dd, *J* = 5.4, 1.0 Hz, 1H), 7.59 (ddd, *J* = 8.5, 7.2, 1.8 Hz, 1H), 7.37 - 7.28 (m, 1H), 7.19 (ddd, *J* = 8.5, 7.2, 1.0 Hz, 1H), 4.38 (q, *J* = 7.0 Hz, 2H), 1.58 (t, *J* = 7.0 Hz, 3H); LCMS $[M+ H]^+$ = 257.35 rt = 0.85 min (5-95% CH_3CN in water with 0.1% NH_4OAc); HRMS $[M+ H]^+$ calculated for ($C_{14}H_{12}N_2OS+ H^+$): 257.07431, found: 257.07441

2-(2-Methoxyphenyl)thiazolo[5,4-*c*]pyridin-6-amine (**20**). Thiocarbonyl dichloride (4.10 g, 2.7 mL, 35.58 mmol) was added to a stirred solution of 2,5-dichloropyridin-4-amine (2.9 g, 17.8 mmol) and Na_2CO_3 (7.80 g, 73.59 mmol) dry dichloromethane (20 mL) in a pressure bottle. The reaction was stirred at room temperature for 4 days and the reaction mixture was filtered and solid was washed with dichloromethane (10 mL). The combined filtrate was evaporated to give a red color oil. The crude product was purified by silica gel chromatography (0-30 % EtOAc/hex) to afford compound **17** (2.1 g, 57%) as crystalline orange color solid. ^{1}H NMR (400 MHz,

$CDCl_3$); δ 8.32(s, 1H), 7.08(s, 1H), LCMS $[M+ H]^+$ = 205.06 rt = 1.01 min (5-95% CH_3CN in water with 0.1% TFA).

n-Butyl lithium (1.83 mL of 1.6 M, 2.93 mmol) was added to a stirred solution of 1-bromo-2-methoxy-benzene (0.45 g, 2.44 mmol) in THF (5 mL) under N_2 at -78 ^oC and the solution was stirred for 20 min at -78 ^oC. Compound **17** (0.5 g, 2.45 mmol) in THF (3 mL) was added and the solution was stirred at -78 ^oC for 20 min. The dark red solution was quenched with saturated NH_4Cl (5 mL) and the product was extracted with EtOAc (3x10 mL). The combined organic layers were dried over Na_2SO_4 and the solvent was removed under reduced pressure to afford compound **18** (0.76 g, 99%) as a yellow solid; LCMS $[M+ H]^+$ = 313.28 rt = 0.97 min (5-95% CH_3CN in water with 0.1% TFA)

A solution of compound **18** (0.76 g, 2.45 mmol) and Na_2CO_3 (0.38 g, 3.65 mmol) in DMF (5 mL) was heated at 120 ^oC for 3 hr and the reaction mixture was cooled to room temperature. Water (20 mL) was added and the aqueous layer was extracted with EtOAc (3x10 mL). The combined organic layers were dried over Na_2SO_4 and the solvent was removed under reduced pressure. The crude product was purified by silica gel chromatography (0-30% EtOAc/hex) to afford the 6-chloro-2-(2-methoxyphenyl)thiazolo[5,4-*c*]pyridine (**20**, 0.18 g 26%) as a pale brown solid;^{1}H NMR (400 MHz, $CDCl_3$) δ 8.90 (d, *J* = 0.8 Hz, 1H), 8.50 (dd, *J* = 7.9, 1.7 Hz, 1H), 7.88 (d, *J* = 0.8 Hz, 1H), 7.48 (m, 1H), 7.10 (dd, *J* = 11.1, 4.1 Hz, 1H), 7.04 (d, *J* = 8.4 Hz, 1H), 4.04(s,3H); LCMS $[M+ H]^+$ = 277.64 rt = 0.96 min (5-95% CH_3CN in water with 0.1% TFA).

2-(2-Methoxyphenyl)-N-methylthiazolo[5,4-*c*]pyridin-6-amine (**21**). XPhos Palladacylcle (9.4 mg, 0.013 mmol) was added to a solution of of *tert*-butyl carbamate (22.3 mg, 0.19

mmol) , 6-chloro-2-(2-methoxyphenyl)thiazolo[5,4-c]pyridine (**19**) (37 mg, 0.127 mmol) and sodium *tert*-butoxide (18.3 mg, 0.190 mmol) in dioxane (2mL) previously purged with nitrogen. The reaction mixture was heated to 135˚C in amicrowave for 30 min. The reaction mixture was poured into water (5 mL) and extracted with EtOAc (3 x 5 mL). The organic phase was dried other $MgSO_4$, filtered and evaporated. The resulting residue was dissolved in DCM (1 mL) and TFA (3 mL) and was stirred at room temperature for 2h. The mixture was concentrated and purified by reversed phase chromatography (C18Aq (0-80%ACN/ water, 0.1% TFA, 30 min)) to give the 2-(2-methoxyphenyl)thiazolo[5,4-*c*]pyridin-6-amine (14 mg, 41%) as off white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.60 (s, 1H), 8.43 (d, *J* = 8.1 Hz, 1H), 7.41 (s, 1H), 6.98-7.04 (m,3H), 4.37 (brs, 2H), 3.98 (s, 3H); LCMS $[M+ H]^+$ = 259.43; rt = 0.65 min (10-90% CH_3CN in water with 0.1% TFA); HRMS $[M+ H]^+$ calculated for ($C_{13}H_{11}N_3OS+ H^+$): 258.06956, found: 258.06966 with a deviation of only 0.391 ppm.

2-(2-Methoxyphenyl)thiazolo[5,4-*c*]pyridin-4-amine (**26)**. Thiocarbonyl dichloride (0.55 g, 4.78 mmol) was added to a stirred solution of 2-chloro-3-fluoro-pyridin-4-amine (0.50 g, 3.41 mmol) and Na_2CO_3 (0.94 g, 8.87mmol) in dry DCM (3 mL) was stirred at room temperature for 18 hr. The reaction mixture was filtered and solid was washed with dichloromethane (10 mL). The combined filtrate was evaporated to give a red color The crude product was purified by silica gel chromatography (0-60% EtOAc/hex) to afford compound **23** (0.37 g, 57%) as a light yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$ δ 8.09 (dd, *J* = 5.2, 0.5 Hz, 1H), 6.96 (t, *J* = 5.1 Hz, 1H); LCMS $[M+ H]^+$ = 188.64 rt = 1.01 min (10-60% CH_3CN in water with 0.1% TFA).

n-Butyl lithium (1.07 mL of 1.6 M, 1.72 mmol) was added to a stirred solution of 1-bromo-2-methoxy-benzene (0.27 g, 1.43mmol) in THF (2 mL) under N_2 at -78 ^{o}C and the solution was

stirred for 20 min. Compound **23** (0.27 g, 1.43 mmol) in THF (2 mL) was added and the solution was stirred at -78 ^{o}C for 20 min. The dark red solution was quenched with saturated NH_4Cl (5 mL). The product was extracted with EtOAc (3x10 mL), dried over Na_2SO_4 and the solvent was removed under reduced pressure to afford compound **24** (0.34 g, 78%) as a yellow solid. LCMS $[M+ H]^+$ = 297.35 rt = 0.89min (5-95% CH_3CN in water with 0.1% TFA).

A solution of compound **24** (0.334 g, 1.12 mmol) and Na_2CO_3 (0.23 g) in DMF (5 mL) was heated at 120^{o}C for 12 h and the reaction mixture was cooled to room temperature. Water (20 mL) was added and the aqueous layer was extracted with EtOAc (3x10 mL). The organic layers were dried over Na_2SO_4 and the solvent was removed under reduced pressure. The crude product was purified by silica gel chromatography (0-30% EtOAc/hex) to afford compound **25** (0.17 g 54%) as a yellow solid.; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.50 (dd, *J* = 7.9, 1.7 Hz, 1H), 8.34 (d, *J* = 5.6 Hz, 1H), 7.77 (d, *J* = 5.6 Hz, 1H), 7.48 (ddd, *J* = 8.4, 7.3, 1.8 Hz, 1H), 7.10-7.04 (m, 2H), 4.07 (s, 3H).; LCMS $[M+ H]^+$ = 277.37 rt = 0.97 min (5-95% CH_3CN in water with 0.1% TFA).

XPhos Palladacycle (0.013 g, 0.017mmol) was added to a nitrogen purged solution of tert-butyl carbamate (0.030 g, 0.26 mmol), compound **25** (0.05 g, 0.17 mmol in dioxane (2 mL) in a microwave vial. And the solution was heated in a Biotage microwave oven at 135 ^{o}C for 30 min. The solution was poured into water (5 mL) and extracted withe EtOAc (2x 5 mL). The organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by reversed phase column chromatography (C18Aq (0-80%ACN/ water, 0.1% TFA, 30 min) to afford title compound **26** (0. 012 g, 44%) as off white solid.; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.62 (s, 1H), 8.32 (t, *J* = 7.0 Hz, 1H), 7.43 (d, *J* = 6.8 Hz, 1H),

7.33 - 7.07 (m, 2H), 7.05 (s, 1H), 4.43 (brs, 2H).; LCMS $[M+ H]^+$ = 258.43 rt = 0.65 min (10-60% CH_3CN in water with 0.1% TFA). HRMS $[M+ H]^+$ calculated for ($C_{13}H_{11}N_3OS+ H^+$): 258.06956, found: 258.06909

ASSOCIATED CONTENT

Supporting Information includes PDB ID codes for all reported X-ray crystal structures Codes along with the atomic coordinates

PDB CODES for the crystal structures in Figure 1, 3 and 4. Accession Codes, coordinates, anisotropic temperature factors, bond lengths, and bond angles have been deposited at the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ,United Kingdom, http://www.ccdc.cam.ac.uk, under the following deposition numbers:

PDB code for the crystal structure depicted in Figure 1 (JCV apo) is: 5J40

PDB code for the crystal structure depicted in Figure 3 with **5d** is: 5J47

PDB code for the crystal structure depicted in Figure 4 are: 5J4V with **12g** and 5J4Y with **12h**

Authors will release the atomic coordinates and experimental data upon article publication

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NOTES

The authors declare no competing financial interest

ABBREVIATION

N/A- not available; RMSD- Root Mean Square Deviation, T_3P- propylphosphonic anhydride; DIPEA- di(isopropyl)ethylamine; XPhos palladacycle- (2-dicyclohexylphosphino-2′,4′,6′-triisopropyl-1,1′-biphenyl)[2-(2-amino-ethyl)phenyl)]palladium(II); BrettPhos palladacycle-chloro[2-(dicyclohexylphosphino)-3,6-dimethoxy-2′,4′, 6′-triisopropyl-1,1′-biphenyl][2-(2-aminoethyl)phenyl]palladium(II); HEPES- HEPES buffer- **4**-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid buffer.

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