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# Potent, Specific CDK8 Kinase Inhibitors Lack CDK8-dependent Antiproliferative Activity in HCT-116 Colon Cancer Cell Line

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**KEYWORDS** CDK8, Cyclin C, kinase inhibitor

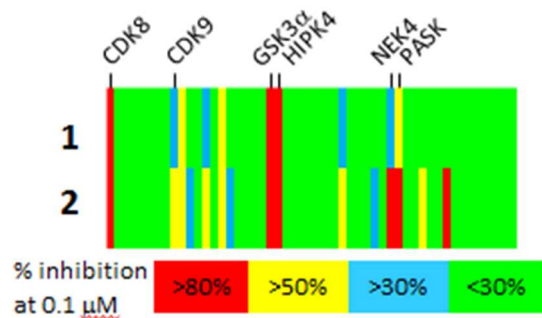
**ABSTRACT:** Beginning with promiscuous COT inhibitors, which were found to inhibit CDK8, a series of 6-aza-benzothiophene containing compounds were developed into potent, selective CDK8 inhibitors. When co-crystallized with CDK8 and cyclin C, these compounds exhibit an unusual binding mode, making a single hydrogen bond to the hinge residue A100, a second to K252 and a key cation- $\pi$  interaction with R356. Structure based drug design resulted in tool compounds **13** and **32**, which are highly potent, kinase selective, permeable compounds with a free fraction >2% and no measurable efflux. Despite these attractive properties, these compounds exhibit weak antiproliferative activity in the HCT-116 colon cancer cell line. Further examination of the activity of **32** in this cell line revealed that the compound reduced phosphorylation of the known CDK8 substrate STAT1 in a manner identical to a CDK8 knockout clone, illustrating the complex effects of inhibition of CDK8 kinase activity in proliferation in these cells.

CDK8 is a cyclin-dependent kinase that forms part of the Mediator complex, which itself regulates the transcriptional activity of RNA polymerase II. A number of studies have shown that CDK8 modulates the transcriptional output from distinct transcription factors involved in oncogenic control.<sup>1</sup> These factors include the Wnt/ $\beta$ -catenin pathway, Notch, p53 and TGF- $\beta$ .<sup>2,3</sup>

While the means by which CDK8 activity accomplishes the regulation of these various pathways remains an active area of investigation, CDK8 has been found to be amplified and overexpressed in colon cancer. In this context, CDK8 has been reported to act as a colon cancer oncogene<sup>4</sup>, driving Wnt pathway activity by mediating  $\beta$ -catenin transcriptional output.<sup>5</sup> Suppression of CDK8 expression in colon cancer cell lines expressing high levels of both CDK8 and  $\beta$ -catenin led to reduced proliferation of these cell lines and induced differentiation.<sup>6</sup> Furthermore, in a survey of colorectal cancer tissue samples, CDK8 was associated with  $\beta$ -catenin activation and with reduced rates of survival.<sup>7</sup> More recently, it has been shown that suppression of CDK8 expression in melanoma cells reduces proliferation.<sup>8</sup> Interestingly, the reverse has also been reported; mutations in MED12 which disrupt the association of CDK8 with the mediator complex are associated with uterine leiomyomas.<sup>9</sup>

Additionally, the conditional knockout of Cdk8 in young adult mice produced no gross abnormalities, while Cdk8 deletion in the Apc<sup>Min</sup> tumor model reduced survival and increased tumor burden.<sup>10</sup>

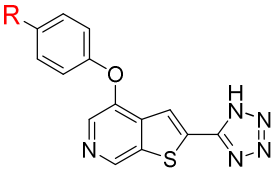
To date, the investigations of the role of CDK8 in both cellular signaling and colon cancer have relied upon RNAi mediated suppression of CDK8<sup>5</sup> and on the use of a kinase dead mutant CDK8<sup>4</sup>. In order to more fully

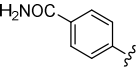


**Figure 1.** Kinase selectivity for compounds **1** and **2** measured at a concentration of 0.1  $\mu$ M in a panel of 52 kinases. Each vertical line represents a single kinase and is colored by the

percent inhibition seen. Selected potentially inhibited kinases are highlighted. Complete data is in the supplementary material.

**Table 1. Potency, permeability and plasma protein binding data for 2-tetrazolyl-thieno[2,3-c]pyridines 1-10**



	R	CDK8 IC <sub>50</sub> (nM)	MDCK permeability AB x 10 <sup>-6</sup> cm/sec	TPSA (Å <sup>2</sup> )
1	I	0.7	24	76
2		3.0	1.9	119
3	H	8.3	4.1	76
4	F	5.5	9.1	76
5	Cl	1.1	11	76
6	CH <sub>3</sub>	1.6	12	76
7	OCF <sub>3</sub>	1.1	24	85
8	CN	1.0	2.5	100
9	NHAc	2.0	0.8	105
10	CONH <sub>2</sub>	4.4	0.7	119

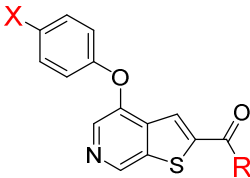
investigate the role of CDK8 in colon cancer, we aimed to develop a potent and selective small molecule inhibitor of CDK8. In order for such a molecule to serve as a useful tool compound in understanding CDK8 activity in colon cancer cell lines, we required that it also be cell permeable with minimal efflux, and have a high free fraction in plasma.

The two small molecule inhibitors of CDK8 reported at the start of our investigations, sorafenib<sup>11</sup> and Senexin A<sup>12</sup> exhibited insufficient kinase selectivity and potency, respectively, to serve our purposes. We therefore examined our in-house database of kinase inhibition data and looked for compounds which might have been prepared for a different target, but serendipitously inhibited CDK8.

We were pleased to find that compound **2**, which has been reported to be a COT inhibitor<sup>13,14</sup>, potently inhibited CDK8. As part of our standard follow-up for a lead, we resynthesized the material and assayed intermediates in the synthesis in a fluorescence polarization binding assay. We were surprised to discover that compound **1** was a still more potent inhibitor of CDK8 than our initial lead, and exhibited superior selectivity as well (Figure 1 & Table 1).

Having determined that the second aryl ring in the biphenyl ether provided no advantage, we prepared and evaluated compounds **2-10** in an effort to understand the limitations of this chemotype. Despite the presence of the highly polar tetrazole group, compounds with a topological polar surface area (TPSA)

**Table 2. Alternatives to the tetrazole<sup>a</sup>**



	X	R	CDK8 IC <sub>50</sub> (nM)	PPB % bound (H/R/M)
11	Cl	OH	2.8	99.8/99.7/99.7
12	Cl	OMe	62	n/d
13	Cl	NH <sub>2</sub>	6.2	98.0/97.0/98.0
14	Cl	NHMe	8.2	98.5/99.0/97.7
15	Cl	NMe <sub>2</sub>	75	n/d
16	Cl	NHEt	16	99.3/99.6/98.8
17	Cl	NHC <sub>3</sub> H <sub>5</sub>	24	99.2/99.3/99.1
18	Cl	NHiPr	100	n/d
19	Cl	Me	32	99.7/99.8/99.7
20	Cl	Et	29	>99.9/>99.9/>99.9
21	Cl	C <sub>3</sub> H <sub>5</sub>	59	n/d
22	I	NHMe	5.3	99.8/99.9/99.8

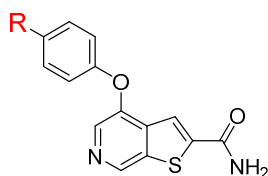
<sup>a</sup> PPB figures in human, rat and mouse plasma (H/R/M)

lower than approximately 100Å<sup>2</sup> were permeable as measured in a standard MDCK assay. None of the compounds examined exhibited any significant level of efflux.

As might be expected from a set of lipophilic compounds containing a tetrazole, which would be negatively charged at physiological pH, the compounds were found to be highly protein bound, with all compounds >99.7% bound in human, rat and mouse serum. In an effort to reduce plasma protein binding, polar functionality was introduced at the 4-position of our aryl ether. These modifications did not adversely affect the potency of these compounds for CDK8, but as the TPSA went above 100Å<sup>2</sup>, the MDCK permeability dropped dramatically, making them unlikely to be active in a cellular context. We therefore initiated an examination of what functional groups would be tolerated in place of the tetrazole.

We began our exploration of substitution at the 2-position of the thieno[2,3-c]pyridines by examining the carboxylate **11** (Table 2).<sup>15</sup> As expected, the activity of the compound was only slightly diminished, and the plasma protein binding was reduced only very slightly. To determine if an anion was required at physiological pH, we prepared inactive ester **12** and amides **13-18**. With the smallest amide substituents, the inhibition of CDK8 remained potent, but alkyl groups larger than methyl and particularly disubstitution of the amide

**Table 3. Incorporation of polar functional groups on aryl ether.<sup>a</sup>**



	R	CDK8 IC <sub>50</sub> (nM)	PPB % bound (H/R/M)	kinetic solubility (μM)
<b>23</b>	Me	6.0	97.2/95.9/96.1	<1
<b>24</b>	OMe	25	93.6/94.0/90.2	10
<b>25</b>	OCF <sub>3</sub>	12	97.9/96.8/97.2	<1
<b>26</b>	CN	5.1	84.7/87.3/89.1	8
<b>27</b>	CONH <sub>2</sub>	21	77.3/76.8/78.3	7
<b>28</b>	NHAc	29	91.0/84.8/79.6	18

<sup>a</sup> PPB figures in human, rat and mouse plasma (H/R/M)

resulted in 10- to 100-fold losses in activity. More promisingly, the plasma protein binding was seen to diminish in both the carboxamide **13** and N-methyl carboxamide **14**.

Finally, we examined the necessity of having a hydrogen bond donor as part of the 2-substituent and prepared compounds **19-21**. As with the amides, the smaller substituents were more potent, but we observed a 5-fold or greater loss in affinity relative to the carboxamide, and plasma protein binding was greater than 99% for all the ketones examined.

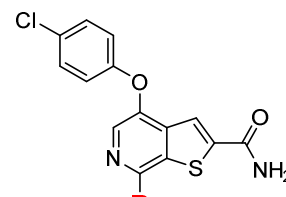
With the knowledge that carboxamides combined the best potency and the lowest PPB yet obtained, we prepared another series of analogs to determine whether we could continue to reduce PPB while maintaining potency and permeability by incorporating polar functional groups at the 4-position of our aryl ether (Table 3).

In preparing these analogs we discovered that alkyl ethers at this position led to decreases in potency, as seen in compounds **24** and **25**, perhaps due to the incorporation of the polar oxygen atom, which can be partially compensated for by the increased lipophilicity of the trifluoromethyl group in **25**. Addition of a further heteroatom in amide **27** and reverse amide **28** led to potency very similar to that of the methyl ether. Nitrile **26** pushes its heteroatom further away from the

ring and offered good potency, reduced PPB and moderate kinetic solubility.

Having generated SAR data at both of the two most easily modified regions of our molecules, we determined the 2.2 Å resolution crystal structure (PDB: 5CEI and Supporting Information) of the CDK8/cyclin C

**Table 4. 7-substituted thieno[2,3-c]pyridines.<sup>a</sup>**



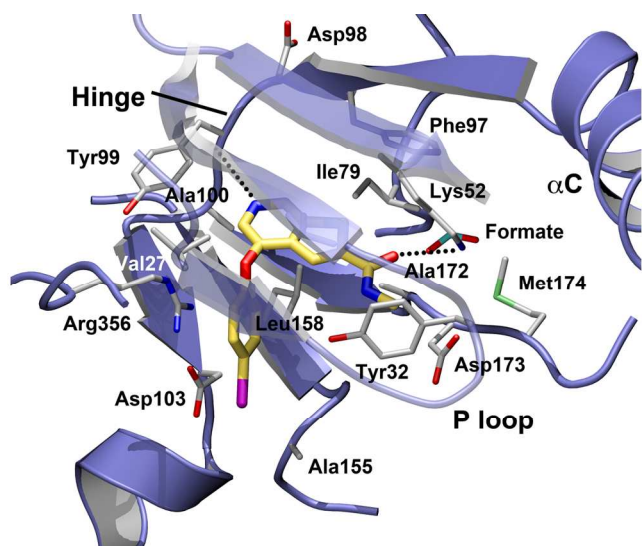
	R	CDK8 IC <sub>50</sub> (nM)	PPB % bound (H/R/M)	kinetic solubility (μM)
<b>13</b>	H	6.2	98.0/97.0/98.0	6.3
<b>29</b>	F	16	n/d	<1
<b>30</b>	Cl	11	n/d	<1
<b>31</b>	Me	8	99.3/99.2/98.8	10
<b>32</b>	NH <sub>2</sub>	1.5	98.13/97.7/95.9	5.5

<sup>a</sup> PPB figures in human, rat and mouse plasma (H/R/M)

complex with **22** bound to facilitate design. The structure contains a single monomer of the CDK8/cyclin C complex in the asymmetric unit, with CDK8 stabilized in the “active” conformation (helix C “in” and the sidechain of Glu66 of the helix coordinated to the active site Lys252). Prior structures of sorafenib and other inhibitors bound the kinase with both DMG-in and DMG-out binding modes.<sup>11,16</sup> The complex with **22** elicited the DMG-in conformation in the enzyme and interactions between the protein and **22** resemble those proposed for molecules of this type binding to COT (Figure 2). A cation-π interaction is seen in the close approach of Arg356 to the iodophenyl ring, stabilizing a 65° rotation of the iodophenyl ring out of the plane of the heterocycle. Hydrogen bonds are formed between the pyridyl nitrogen of **22** and the backbone nitrogen of Ala100 of the hinge as well as from the sidechain of Lys52 to the amide carbonyl. While the binding mode of the compound is clear from the electron density (Table S1), the temperature factors of the ligand suggest its presence at 50-75% occupancy; however, the intermediate resolution of the data precluded definitive refinement of that parameter. Interestingly, a formate ion is observed at partial occupancy in the region underneath the P loop, adjacent to the inhibitor, raising the possibility of building into that region to increase the buried surface area. Examination of the structure prompted the design of a few targeted analogs in an effort to maximize potency.

Our first analogs were targeted at probing the region in the vicinity of the hinge binding nitrogen, which accepts a hydrogen bond from the NH of Ala100. We noted that there was space available to make a contact with the main chain of Asp98 from the 7-position of our heterocycle, and we made a series of analogs to access this region (Table 4). Interestingly, hydrophobic analogs **29-31** retained much of their CDK8 binding



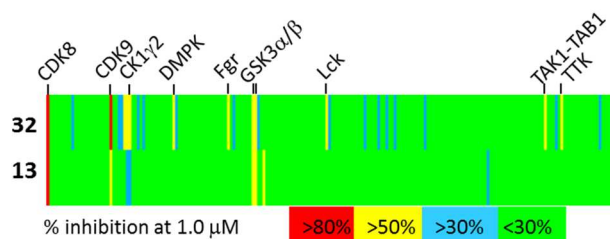


**Figure 2.** Structure of compound **22**. Hydrogen bonding interactions with Ala100 and Lys52 are indicated with dashed lines.

activity, with **29** losing less than 3-fold, and the others less than 2-fold. Compound **32** was found to bind 4 fold more potently than its parent compound **13**, which confirmed our modeling of the compound which indicates that the NH2 can readily make a hydrogen bond to the backbone carbonyl of Asp98.

Having generated extremely potent binders of CDK8, it was important to examine the selectivity of these compounds relative to other kinases. We focused especially on ensuring that there was minimal activity against CDKs 1 and 4, as these would be expected to have antiproliferative effects via disruption of the cell cycle, confounding our ability to assess the effects of inhibiting CDK8. We therefore submitted compounds **13** and **32** for profiling against a panel of 209 kinases. We were pleased to find that both compounds exhibit excellent selectivity for CDK8 over every other kinase examined (Figure 3) even when tested at concentrations 160- and 670-fold greater than the measured  $IC_{50}$ , respectively.

Having identified CDK9/cyclin T1 as our most active off-target, we therefore determined the  $IC_{50}$  values for **13** and **32** in a fluorescence polarization activity assay



**Figure 3.** Kinase selectivity for compounds **13** and **32** measured at a concentration of 1  $\mu$ M in a panel of 209 kinases. Each vertical line represents a single kinase and is colored by the percent inhibition seen. Selected potentially inhibited kinases are highlighted. Complete data is in the supplementary material.

**Table 6.** Antiproliferative activity of compounds **13**, **31** and **32** on HCT-116 cells. Compounds **5-8**, **11** and **25** were also evaluated, but had  $EC_{50}$  values  $>10$   $\mu$ M under both high and low serum conditions.

compound	HCT116 $EC_{50}$ 10% FBS ( $\mu$ M)	HCT116 $EC_{50}$ 0.625% FBS ( $\mu$ M)
<b>13</b>	$>10$	8.8
<b>31</b>	$>10$	8.7
<b>32</b>	6.7	5.4
flavopiridol	0.034	0.059

Compound **13** had an  $IC_{50}$  value of 2.2  $\mu$ M for CDK9/cyclin T1, and therefore a selectivity of 350-fold for CDK8/cyclin C. Compound **32** had an  $IC_{50}$  value of 1.1  $\mu$ M, and a selectivity of 730-fold. The overall profile of these two molecules now met our goal of producing potent, selective CDK8 inhibitors with solubility, plasma protein binding, permeability and low efflux (Table S2). We therefore evaluated the antiproliferative effects of our compounds in the colorectal carcinoma HCT-116 cell line, which had previously been shown to exhibit sharply reduced proliferation in response to CDK8 protein depletion using targeted shRNA.<sup>4</sup> Cells were treated with compounds for 72h in media containing fetal bovine serum (FBS) at a concentration of either 0.625% or 10%. The lower concentration of FBS was used to mitigate the effects of the low free fraction of some of our compounds. Throughout the course of the experiment, the cells were in growth phase, and yet we did not observe significant growth inhibition (Table 6). Flavopiridol, a potent inhibitor of multiple CDKs<sup>17</sup> with known antiproliferative properties<sup>18</sup> was used as a positive control. In each case, the activity of the compounds appeared at approximately 1000-fold higher concentration than the  $IC_{50}$  value for the purified enzyme. Concerned that the compounds examined were failing to act on CDK8 in the cellular context, we examined phosphorylation of Ser727 of STAT1, a substrate of CDK8<sup>19</sup> as a biomarker.

In order to validate STAT1 Ser727 as a substrate in HCT116 cells, CRISPR guide RNAs were created to target CDK8 for genomic deletion.<sup>20-23</sup> Three separate clones showed complete ablation of CDK8 protein while its close homolog CDK19<sup>24</sup> and actin expression remained unchanged (Figure 4A). Clone 1 was then used to generate three CDK8/CDK19 double knockouts, for which all three generated clones showed the complete absence of both proteins. Interestingly, cyclin C levels were greatly reduced in the absence of CDK8 alone, and the protein was undetectable in the double knockout, likely due to instability when unable to associate with the other components of the mediator complex, and highlighting the possibility of secondary effects when protein expression is altered using siRNA or CRISPR.

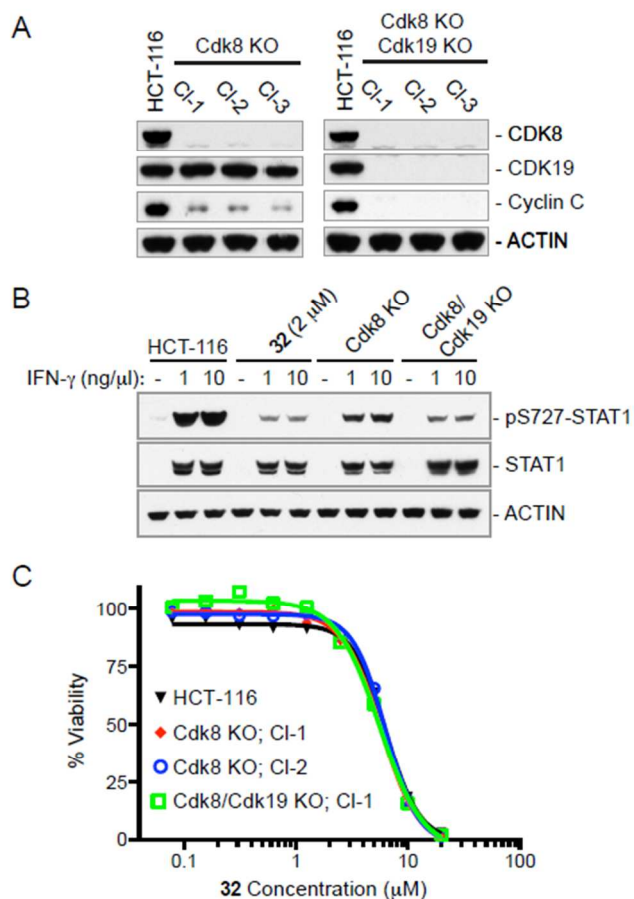
HCT-116 cells along with their respective CDK8 knockout and CDK8/CDK19 double knockout clones were then probed with an antibody specific for phosphorylated Ser727 on STAT1. As had been previously reported in bone marrow derived macrophages<sup>19</sup> and peripheral blood

mononuclear cells<sup>25</sup>, stimulation of HCT-116 cells with interferon- $\gamma$  resulted in increased expression of STAT1 and a large increase in the amount of phosphorylated Ser727 (Figure 4B). This phosphorylation was reduced in the CDK8 knockout, and further reduced in the CDK8/CDK19 double knockout clones stimulated in the same manner while the total amount of STAT1 expressed remained relatively constant. Encouragingly, cells treated with 2  $\mu$ M compound **32** showed levels of phosphorylated Ser727 nearly identical to that of the double knockout cell line, clearly demonstrating that this molecule is able to down-regulate signaling in cultured cells.

Having demonstrated that **32** was in fact inhibiting CDK8 function in HCT-116 cells, we used the CDK8 and CDK8/19 knockout cell lines created to demonstrate that the observed antiproliferative effects of **32** were independent of their inhibition of the targeted protein. Wild type HCT-116 cells, along with the CDK8 and CDK8/19 deletion clones derived from them, were incubated with varying concentrations of **32**. As can be seen in Figure 4C, each of these cell lines showed identical growth inhibition curves, indicating that growth inhibition in these cells is an off-target effect.

During the preparation of this manuscript, additional selective CDK8 inhibitors have been reported, both of which have been shown to sharply reduce the presence in cells of STAT1-pS727 analogously to **32**. Cortistatin A is reported<sup>26</sup> to have mixed antiproliferative effects on cell lines with sensitivity driven by the set of genes upregulated upon treatment, with HCT-116 cells among the insensitive lines. The compound CCT251545, identified through its inhibition of the WNT pathway in cells, binds to and inhibits CDK8 and CDK19,<sup>27</sup> and inhibits proliferation of SW620 colon cancer cells after 96h in culture.

Our development of potent, highly specific CDK8 inhibitors and their lack of CDK8-dependent antiproliferative activity on the HCT-116 colon cancer cell line has therefore served to substantiate the emerging, complex picture of the role of CDK8 in cancer. STAT1 pSer727 has been potentially downregulated by inhibition of CDK8/19 by different compounds, and appears to be a useful biomarker of inhibition. The impact of CDK8 kinase inhibition in proliferation is cell line dependent, and possibly condition dependent, and appears to diverge from the scaffolding functions of CDK8 as evidenced by the differing antiproliferative effects of CDK8 knockdown and kinase inhibition in HCT-116 cells. Further investigation of these differences is clearly important to understanding the roles of CDK8.



**Figure 4.** A. Immunoblot analysis of Cdk8 knockout and Cdk/CDK19 double knockout cells. Three independent clones of each mutant are shown. B. Pharmacological inhibition of Cdk8 kinase activity reduces STAT1 Ser727 phosphorylation following stimulation with IFN $\gamma$ . Cells were treated for 24h in the presence or absence of 2  $\mu$ M **32**. C. Cell viability in the presence of **32** at 72h.

## ASSOCIATED CONTENT

**Supporting Information.** A description of the preparation of **1-32** are provided along with kinase selectivity and experimental procedures used in the determination of the co-crystal structure of **22** with CDK8 (PDB: 5CEI). Additionally, the preparation and characterization of our genetically altered HCT-116 cell line is presented. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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## ACKNOWLEDGMENT

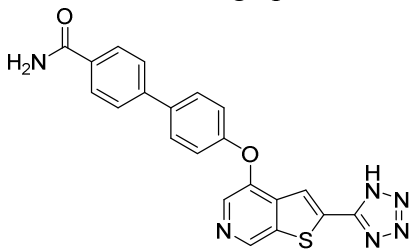
We thank members of the DMPK and purification groups within Genentech Small Molecule Drug Discovery for purification and analytical support.

The crystallographic experiments were performed on the X06SA beamline at the Swiss Light Source, Paul Scherrer Institut, Villigen, Switzerland.

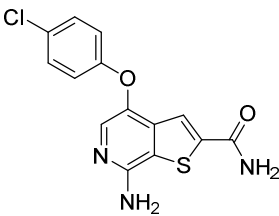
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Table of contents graphic:



Compound 2:  
CDK8 IC<sub>50</sub> = 3 nM  
Inhibits 12/51 kinases >50%  
at 0.1 μM



Compound 32:  
CDK8 IC<sub>50</sub> = 1.5 nM  
Inhibits 11/209 kinases >50%  
at 1 μM