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Inhibition of eEF2-K by thieno[2,3-*b*]pyridine analogues

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ABSTRACT

Several series of thieno[2-3-*b*]pyridine analogues were synthesized and screened for inhibitory activity against eukaryotic elongation factor-2 kinase (eEF2-K). Modifications around several regions of the lead molecules were made, with a ring fusion adjacent to the nitrogen on the thienopyridine core being critical for activity. The most active compound **34** shows an IC_{50} of 170 nM against eEF2-K in vitro.

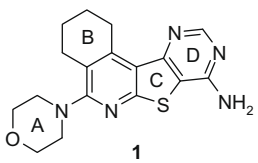
Eukaryotic elongation factor-2 kinase (eEF2-K; EC 2.7.11.20), also called Ca^{2+}/calmodulin-dependent protein kinase III, is a member of the alpha kinase family of atypical kinases.[1,2] Phosphorylation of elongation factor eEF-2 at T56 and T58, catalyzed by eEF2-K, leads to decreased translocation of tRNA and mRNA within the ribosome and transient inhibition of protein synthesis.[3,4] The activity of eEF2-K increases significantly during mitosis, correlating well with decreased protein synthesis during cell division.[5]

eEF2-K activity has been shown to be upregulated in numerous malignant cell lines with a high level of activity seen in MCF7 cells derived from breast carcinoma.[6] Increased activity was also demonstrated in breast carcinoma tissue with little or no activity observed in normal breast tissue. Growth factors including IGF-1 and EGF stimulate the activity of eEF2-K, and cell proliferation induced by these growth factors can be mitigated by eEF2-K inhibition.

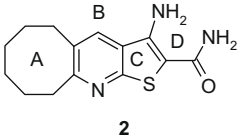
The natural product rottlerin has shown a growth inhibitory effect and cytotoxicity in glioma cell lines at a concentration coincident with its inhibition of eEF2-K.[7] Rottlerin is known to inhibit other kinases, notably PKCδ, but its cytotoxic timecourse is consistent with its antiproliferative effects being due to inhibition of eEF2-K and not PKCδ. Inhibition of eEF2-K has also been correlated with antiproliferative effects using a series of 2-methylimidazolium derivatives.[8] NH125, the most active of these compounds, showed IC_{50} = 60 nM in vitro against eEF2-K and cytotoxicity against a panel of cancer cell lines with IC_{50} values of 700 nM to 3.6 μM. This compound was also assayed in a P388 xenograft model, where at 1 mg/kg the mean survival was increased 29% over vehicle. A series of 1,3-selenazine derivatives have also been synthesized and screened against eEF2-K.[9] The most potent of these compounds (TS-2) showed IC_{50} = 360 nM and was selective for eEF2-K over a panel of similar kinases.

A high-throughput screen for eEF2-K inhibition was undertaken using a fluorometric, coupled-enzyme assay that monitored ADP release. An alternate assay based on polarization of a fluorescently labeled peptide substrate was used to validate primary screening hits.[10] These assays led to the identification of one structural class of inhibitors: thieno[2,3-*b*]pyridine-containing heterocycles exemplified by **1** and **2** (Scheme 1). Similar compounds have been shown to possess antianaphylatic properties,[11] neurotropic activity,[12] and activity against IκB kinase-β.[13] Compounds **1** and **2** showed shifts in their IC_{50} against eEF2-K as the ATP concentration in the in vitro assay was increased from 2- to 50-times K_M, suggesting that both were ATP-competitive inhibitors of eEF2-K.

The thieno[2,3-*b*]pyridine cores were divided into two sub-classes depending on the position of the fused ring around the pyridine ring: compounds with the fused ring at the 3- and 4-positions relative to the pyridyl nitrogen such as **1** and those such as **2** with the



1



2

Scheme 1. Lead molecules. **1**, IC_{50} = 0.22 μM; **2**, IC_{50} = 2.5 μM.

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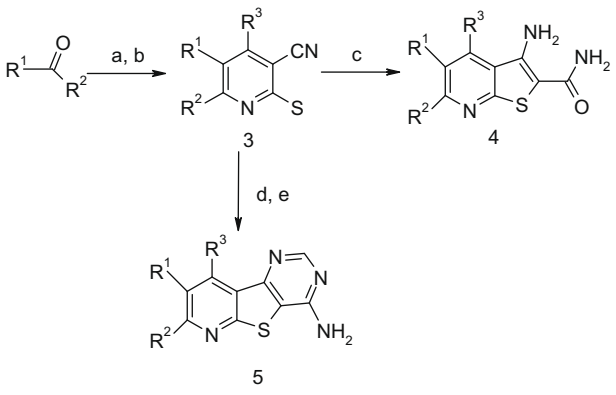
fused ring at the 2- and 3-positions. Both sub-classes were further divided into four zones (A, B, C, and D) and an SAR was developed around each zone.

Analogues inspired by **1** were synthesized using variations on a variety of known routes.[14–16] Compounds related to **2** were generated using a one-pot aldol-heterocyclization approach (Scheme 2).[17] Treatment of a ketone with KOH in methanol was followed by slow addition of an aldehyde, leading to an α–β-unsaturated ketone in situ that was cyclized with 2-cyanothioacetamide in the presence of NaOMe to generate 2-mercaptopyridine intermediate **3**. To generate D-region amino amides **4**, **3** was treated with 2-bromoacetamide and NaOMe.[18] Reaction of **3** with chloroacetonitrile followed by condensation with formamide yielded D-region 4-aminopyrimidines **5**.[19]

The fluorometric assay used in the HTS screen[10] was utilized for subsequent assay of designed compounds. None of the variants of compound **1** exhibited potent in vitro inhibition of eEF2-K. Even small changes were not tolerated. Morpholine to thiomorpholine or *N*-methyl piperazine in zone A, cyclohexyl to cycloheptyl or cyclopentyl in zone B, conversion of zone C to an indole or furan, and all secondary or tertiary amides or N-alkylated pyrimidines in zone D led to compounds with greater than 10 μM activity.

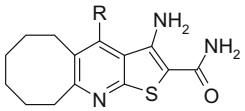
Modifications in the B-region of **2** produced many potent compounds. Increasing the size of the B-region group with an aliphatic chain appeared to increase potency, but the effect tapered off with chain size and branching (Table 1). Very large groups were not tolerated, especially when highly flexible (e.g., **9**). Substituted phenyl rings gave similar results to midsized alkyl substitution. The parent phenyl substitution (e.g., **12**) abrogated activity but all other groups tested gave moderate activity, approximately 1 μM, without regard to electronics.

B-region heterocycles were then synthesized and screened (Table 2). Most of the compounds tested showed activities within a narrow threefold range, but a subtle dependence on B-region substituent size was again noted. Five-membered heterocycles with substitution directed away from the thienopyridine core exhibited the best activity. This preference was not electronic in nature, as furans containing electron-poor groups such as chloro (**25**) and trifluoromethyl (**26**) showed similar activities to those with similarly sized electron-rich ethyl (**28**) and hydroxymethyl (**29**) substitution. The effect also appears to have a conformational component, as phenethyl derivative **24** is inactive while alkenylfuran **17** is among the most potent compounds tested. Further work is needed to explain why methyl substitution is favored in a phenyl system (**15**: IC_{50} = 1.2 μM, **12**: IC_{50} >20 μM) while the reverse is seen with pyridyl substitution (**19**: IC_{50} = 0.94 μM, **23**: IC_{50} >20 μM).



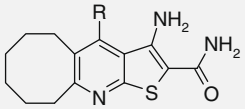
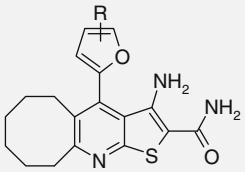
Scheme 2. Reagents and conditions: (a) R_3CHO, KOH, MeOH; (b) 2-cyanothioacetamide; (c) 2-bromoacetamide, NaOMe, MeOH; (d) chloroacetonitrile, NaOMe, MeOH; (e) formamide.

Table 1
In vitro activities of compounds **2**–**15**: zone B aliphatic and phenyl substitution



Compound	R	IC_{50} (μM)
2	H	2.5
6	Me	1.1
7	*i*-Bu	0.86
8	*Cy*-Pr	2
9	*rac*-CH(CH_3)CH_2CH_2C=CMe_2	10
10	3,4-Cl_2-Ph	0.99
11	4-Cl-Ph	1.1
12	Ph	>20
13	4-COOH-Ph	0.86
14	2-F, 5-OMe-Ph	1.3
15	3-Me-Ph	1.2

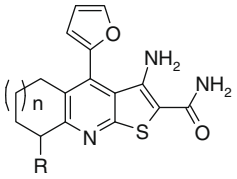
Table 2
In vitro activities of compounds **16**–**31**: further B-region modification

Compound	R	IC_{50} (μM)
		
16	Furan-2-yl	0.64
17	*E*-CH=CH-furan-2-yl	0.32
18	3-Me-thiophen-2-yl	0.46
19	Pyrid-3-yl	0.94
20	1-Me-pyrrol-2-yl	0.99
21	Furan-3-yl	1.1
22	Thiazol-2-yl	1.4
23	3-Me-pyrid-2-yl	>20
24	CH_2CH_2Ph	>20
		
25	5-Cl	0.28
26	5-CF_3	0.30
27	Benzofuran-2-yl	0.33
28	5-Et	0.66
29	CH_2OH	0.80
30	4,5-Me_2	1.1
31	4-Me,5-COOMe	>20

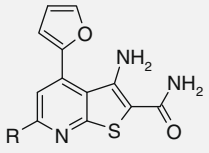
In vitro eEF2-K activity of the analogues of **2** showed a clear dependence on the size of the A-region (Table 3). The effect was similar to that observed in the B-region but more dramatic: moderate bulk was optimal while larger or smaller groups led to a drop-off in activity. When the B-region was occupied by a furan-2-yl group, the potency of the compounds related to the size of the A-region ring: $9 > 8 > 7 \gg 6 \approx 10$. Further, compounds with non-cyclic A-regions lost all potency. Compound **34** was the most potent compound tested in vitro.

As the lead compounds included both amino amides and pyrimidines in zone D, both sets of compounds were made with several analogues of **2**. The potency of the compounds varied considerably when the amino amide was replaced with the aminopyrimidine, but the effect was not unidirectional (Table 4). A significant

Table 3
In vitro activities of compounds **32**–**38**: zone A modifications



Compound	*n*	R	IC_{50} (μM)
32	1	*rac*-Me	>20
33	2	H	1.1
16	3	H	0.640
34	4	H	0.170
35	5	H	>20



R	
36	2,4-$(MeO)_2$Ph	>10
37	CH_2-[2,4-$(MeO)_2$Ph]	>10
38	$CH_2C_6H_{11}$	>10

improvement in activity was seen upon cyclization with the change of **11** to **42**, but a greater decrease in potency was seen when **27** was changed to yield **41**. Additionally, for **21**/**39**, **16/40**, and **33/43** the effect of modifying zone D was negligible.

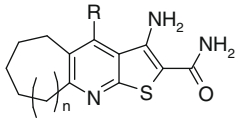
Selected compounds were tested in cellular assays to determine both eEF2-K inhibition and cytotoxicity. In an ELISA assay that monitored the levels of phospho-EF2, the lead compound **1** exhibited an IC_{50} that ranged from 2 to 10 μM. Of the synthesized analogues tested, only compound **34** showed any cellular kinase activity when tested at 100 μM.

Selected compounds were screened for 72 h cytotoxicity in both HCT-116 and A549 cell lines (Table 5). Compounds showed cytotoxicity at 50–400-fold greater concentrations than their in vitro inhibition of eEF2-K. This is the expected cell shift for these compounds, as they are ATP competitive and the in vitro assays were run at 2.5 μM ATP, approximately twice substrate K_M, while cellular ATP levels are 1–10 mM. The best cytotoxicity in HCT-116 cells was observed with compounds **34** (EC_{50} = 17 μM) and **17** (EC_{50} = 19 μM).

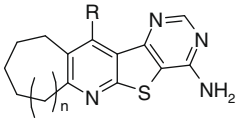
The observed cytotoxicity generally tracked with in vitro eEF2-K inhibition, although **25** was 4–5-fold less potent than other compounds with similar in vitro IC_{50} values. Compound **2** exhibited considerably more cytotoxicity than would be predicted by its in vitro activity, suggesting off-target effects in cells. Selectivity is an important element of kinase-based drug design, and we planned on screening compounds with IC_{50} <100 nM against a panel of phenotypically relevant kinases. No compounds tested, however, met this threshold and consequently no such analysis was performed.

Table 4
In vitro activities of compounds **39**–**43**: zone D modifications

a



b



Compound		*n*	R	IC_{50} (μM)	
a	b			a	b
2	**39**	2	H	2.5	3.7
16	**40**	2	2-Furanyl	0.64	0.35
27	**41**	2	2-Benzofuranyl	0.33	>20
11	**42**	2	4-Cl-phenyl	1.1	0.11
33	**43**	1	2-Furanyl	1.1	4.7

Table 5
Cytotoxicity of selected compounds[a]

Compound	IC_{50} (μM)	Cytotoxicity EC_{50} (μM)	
		HCT-116	A549
1	0.22	29	6.7
2	2.5	38	38
17	0.32	19	15
25	0.28	100	64
34	0.17	17	n/d
43	4.7	>100	17

[a] n/d = not done.

In conclusion, a series of thieno[2,3-*b*]pyridine-containing compounds was synthesized and screened against eEF2-K. Chemistry was developed that allowed for modification of the core structure in all regions of the lead molecule. A D-region amino amide or 4-aminopyrimidine was critical to in vitro potency. Both the A- and B-regions showed a preference for substituents of a certain size, with fused cyclooctyl and cyclononyl rings preferred in the A-region and five-membered heterocycles substituted away from the central thienopyridyl core in the B-region. These compounds were found to be ATP competitive and the best show sub-micromolar in vitro inhibition of eEF2-K.

Selected compounds were screened for cytotoxicity in both HVT-116 and A549 cell lines. Cytotoxicities were similar in the two cell lines, and showed correlation with in vitro potency. The best cytotoxicity in HCT-116 cells was observed with compounds **34** (EC_{50} = 17 μM) and **17** (EC_{50} = 19 μM). These compounds adhere to each of Lipinski's rule-of-five,[20] and consequently are the first true drug-like inhibitors of eEF2-K described.

Acknowledgments

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10. Full-length GST fusion eEF2-K produced in the *E. coli* system was used for in vitro inhibition assays including HTS. Its calmodulin-stimulated kinase activity towards myelin basic protein (MBP) was monitored using a previously developed fluorometric method (Ostanin, K.; Hunsaker, T. US7338775). In order to eliminate false positives, all compounds were also subjected to a

counterassay which was conducted as described above except for replacement of eEF2-K with 1 μM ADP. Inhibitory activities of the most potent compounds were confirmed in FP-based assay format using HitHunter-PKC assay kit purchased from DiscoveRx.
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17. All synthesized compounds were characterized by reverse phase-HPLC/MS and ^{1}H NMR.
18. *Compound* **16** *is typical:* Cyclooctanone (1 equiv), 3-methylbutanal (1 equiv), and KOH (2 equiv) are stirred in MeOH at rt for 3 h. 2-Cyanothioacetamide (1 equiv) and NaOMe (3 equiv) are added and the solution heated at reflux overnight. 2-Bromoacetamide (1 equiv) and NaOMe (1 equiv) are added and the reaction mixture heated at reflux until product is observed by LC/MS. The solution is concentrated and purified by RP-HPLC.
19. *Compound* **42** *is typical:* Cyclooctanone (1 equiv), furan-2-carbaldehyde (1 equiv), and KOH (2 equiv) are stirred in MeOH at rt for 3 h. 2-Cyanothioacetamide (1 equiv) and NaOMe (3 equiv) are added and the solution heated at reflux overnight. Chloroacetonitrile (5 equiv) and NaOMe (1 equiv) are added and the reaction mixture heated at reflux for 3 h. The mixture is cooled, diluted with DCM, run through a silica plug, and concentrated. Formamide (6 mL/mmol) is added and the solution heated at reflux for 90 min. The mixture is concentrated and purified by RP-MPLC.
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