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Discovery of thienopyrimidine-based FLT3 inhibitors from the structural modification of known IKKβ inhibitors

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ABSTRACT

Inactivation of the NF-κB signaling pathway by inhibition of IKKβ is a well-known approach to treat inflammatory diseases such as rheumatoid arthritis and cancer. Thienopyrimidine-based analogues were designed through modification of the known IKKβ inhibitor, **SPC-839**, and then biologically evaluated. The resulting analogues had good inhibitory activity against both nitric oxide and TNF-α, which are well-known inflammatory responses generated by activated NF-κB. However, no inhibitory activity against IKKβ was observed with these compounds. The thienopyrimidine-based analogues were subsequently screened for a target kinase, and FLT3, which is a potential target for acute myeloid leukemia (AML), was identified. Thienopyrimidine-based FLT3 inhibitors showed good inhibition profiles against FLT3 under 1 μM. Overall, these compounds represent a promising family of inhibitors for future development of a treatment for AML.

FLT3 (Fms-like tyrosine kinase 3), a receptor tyrosine kinase, is a member of receptor III tyrosine kinase family including PDGFR, KIT and FMS.[1] FLT3 is normally expressed on the surface of hematopoietic progenitor cells by immature hematopoietic cell and it has important roles in normal stem cell development and the immune system.[2,3] Abnormal overexpression and mutation of FLT3 is often observed in patients with leukemia. Specifically, diverse mutations of FLT3, such as D835V, D835Y, and ITD (internal tandem duplication), were reported in AML.[4] AML cells induce molecular defects, which promote leukemic proliferation, defect in differentiation and resistance to apoptosis.[5] Increased transcriptional level of FLT3 is observed in AML, and then this increase of expression level may influence to phosphorylation of FLT3.[6] The phosphorylation and activation of FLT3 receptor may cause the activation of a down-stream kinase pathway, such as Ras/Mitogen-activated protein kinase (MAPK), which can induce an abnormal cell growth and gene regulation.[3,7,8] For these reasons, FLT3 is recently regarded as a one of important target for treatment of AML patients.

Our primary research purpose was to discover the IKKβ inhibitors for the treatment of inflammatory diseases. IκB kinase β (IKKβ) is considered as a promising target for diverse diseases related with NF-κB pathway including inflammatory diseases and autoimmune diseases such as rheumatoid arthritis (RA).[9–14] Recently, new structural and functional data for IKKα and IKKβ have revealed that IKKβ is a useful target of the NF-κB activation pathway for the development of anti-inflammatory and anti-cancer therapeutic agents.[15–17] We have designed and synthesized thienopyrimidine analogues as potent IKKβ inhibitors, however those analogues did not have IKKβ inhibitory activities. From an assay, FLT3 has been discovered as a new target for thienopyrimidine analogues. Herein, we describe this novel series of thienopyrimidine-based FLT3 inhibitors made through the modification of IKKβ inhibitors.

Toward the discovery of small molecule inhibitors of IKKβ, we have performed fragment-based virtual screening from known

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0960-894X/

IKKβ active compounds libraries[22,23] and the thienopyrimidine core has been discovered. We have designed and synthesized a novel series of thienopyrimidine derivatives through structural modifications of the thienopyrimidine core and the quinazoline analogue **SPC-839**, and then biologically evaluated these derivatives (Fig. 1). We introduced thienopyrimidine as a core instead of quinazoline and diversified its functional groups, using alkyl, substituted aromatic, and heterocyclic groups at positions 2, 4 and 5 of the thienopyrimidine core.

Early attempts to synthesize thienopyrimidine-based analogues 1 began with modifications of the 3-methyl-1*H*-pyrrole-2,5-dione moiety at C_4 position, followed by substitutions of moderate sizes at the C_2 and C_5 positions. The synthetic routes for the newly synthesized thienopyrimidine derivatives are outlined in Schemes 1–3.

Compounds **8a**–**e** in Table 1 were synthesized from 2-acetylthiophene according to Scheme 1. Knoevenagel condensation of 2-acetylthiophene with malononitrile in the presence of NH_4OAc and AcOH yielded 2-[1-(thiophen-2-yl)ethylidene]malononitrile **2**,[18] which was treated with elemental sulfur and piperidine to produce thiophene **3a**,[19] Compound **3a** was heated with formamide at 180 °C to produce thienopyrimidine **4a**, which was converted to chloride **5a** by Sandmeyer reaction under $CuCl_2$ and *t*-BuONO.[20,21] 5-(Thiophen-2-yl)thieno[2,3-*d*]pyrimidin-4-amine derivatives **8a**–**e** was then prepared in a stepwise fashion by first treating **5a** with hydrazine hydrate in tetrahydrofuran followed by reacting with the appropriate furans **7a**–**e**.

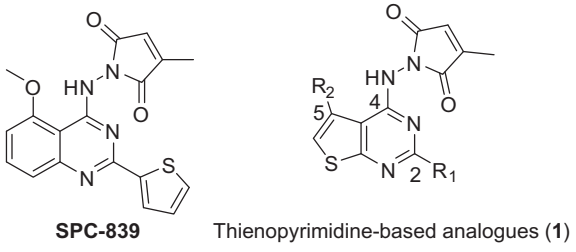
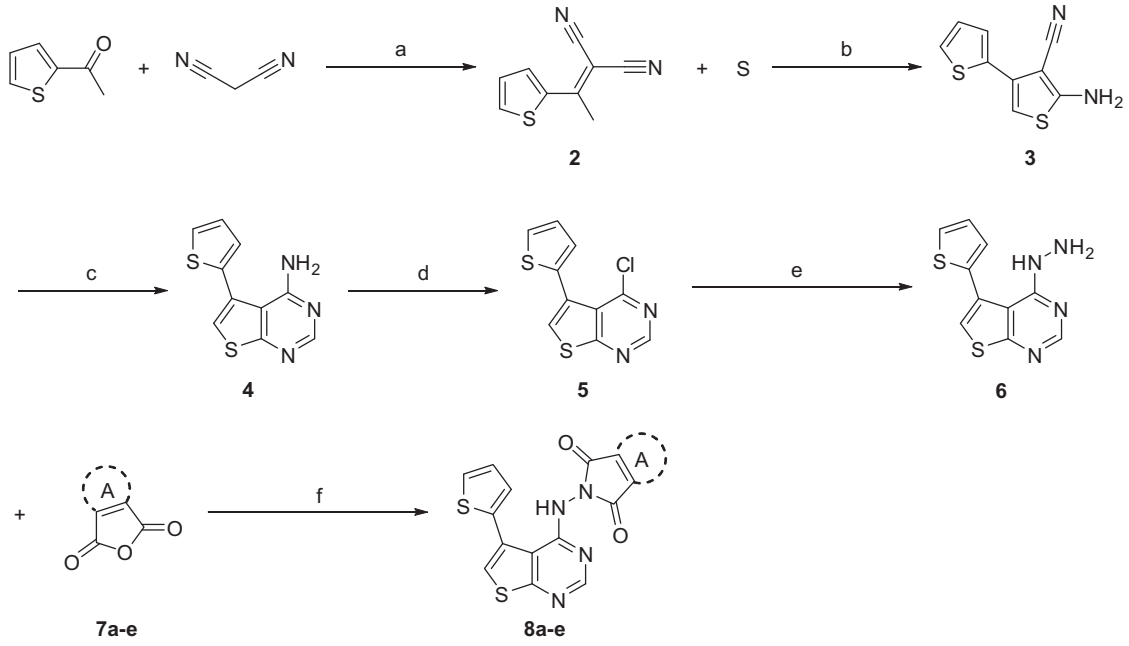


Figure 1. Known IKKβ inhibitor, **SPC-839** and thienopyrimidine-based analogues **1**.

The analogues **13a**–**m** were prepared from two synthetic methods. Compounds **3b**–**e** were heated with commercially available substituted cyanide under acidic condition at 110 °C to produce thienopyrimidine **4b**–**e**, which were converted to chloride **5b**–**e** by the Sandmeyer reaction under $CuCl_2$ and *t*-BuONO according to method 1 of Scheme 2. Substituted 2-aminothiophen-3-carboxylate compounds **10a**–**i** were obtained by the Gewald reaction as depicted in method 2 of Scheme 2. Compounds **11a**–**i** were conveniently synthesized by heating carboxylates **10a**–**i** with an appropriate carbonitrile under acidic conditions, which were chlorinated using $POCl_3$ at 100 °C to produce 4-chloro-thieno[2,3-*d*]pyrimidine **12a**–**i**. Thieno[2,3-*d*]pyrimidin-4-amine derivatives **13a**–**m** were then prepared in a stepwise fashion by first treating **5a**–**d** or **12a**–**i** with hydrazine hydrate in tetrahydrofuran, followed by reacting with 3-methylfuran-2,5-dione.

Synthesis of analogues **17a**–**d**, containing a phenol group substituted with terminal amine units at the C_5-position of thieno[2,3-*d*]pyrimidine, is described in Scheme 3. Compounds **16a**–**d** were synthesized from chlorides **14a**–**d**, which were obtained from phenols **12h** and **12i** through the Mitsunobu reaction according to the same method described above for the preparation of 3-methyl-1*H*-pyrrole-2,5-dione derivatives **13a**–**m**. Finally, the N-substituted amine salt compounds **17a**–**d** were prepared using 4 M HCl in 1,4-dioxane to enhance water solubility.

Our initial studies investigated structure–activity relationships that optimized inhibitory activity against production of nitric oxide (NO) and TNF-α, two well-known inflammatory responses caused by activated NF-κB in LPS-treated RAW264.7 cells.[24] Table 1 shows a summary of results of NO and TNF-α inhibition assay by the thienopyrimidine derivatives with various pyrrole substituents at the C_4 position. Among those analogues, we found that thienopyrimidine **8b**, with 3-methyl-1*H*-pyrrole-2,5-dione moiety, displayed significant activity in the NO inhibition assay (IC_{50} = 2.49 μM), but did not show subsequent activity in the TNF-α inhibition assay. Except for compound **8b**, most of C_4-substituted analogues, such as **8a** with 1*H*-pyrrole-2,5-dione and **8c** with 3,4-dimethyl-1*H*-pyrrole-2,5-dione,



Scheme 1. (a) NH_4OAc, AcOH, toluene, reflux; (b) piperidine, EtOH, 80 °C; (c) $HCONH_2$, 180 °C; (d) $CuCl_2$, *t*-BuONO, THF/MeCN, 70 °C; (e) H_2N-NH_2·H_2O, THF, 80 °C; (f) $CHCl_3$, 80 °C.

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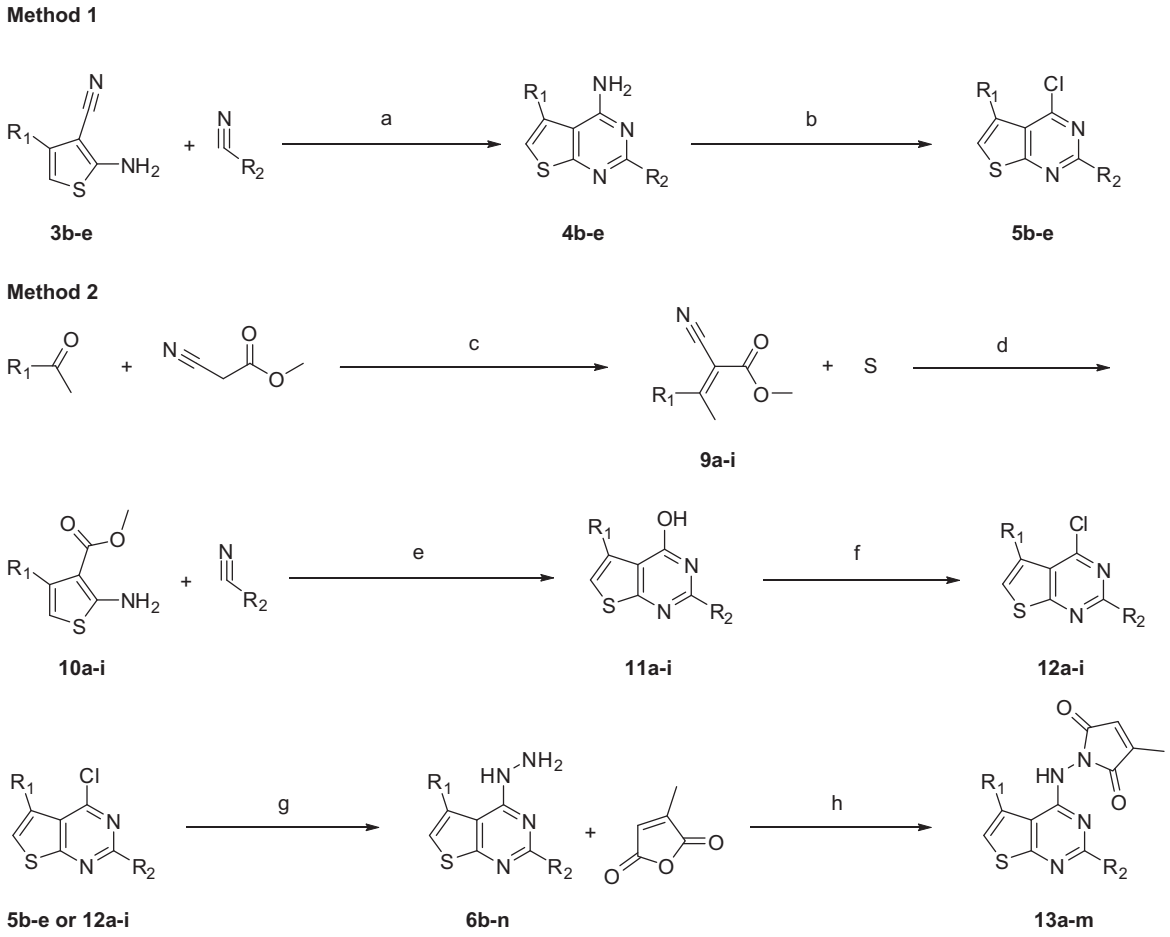
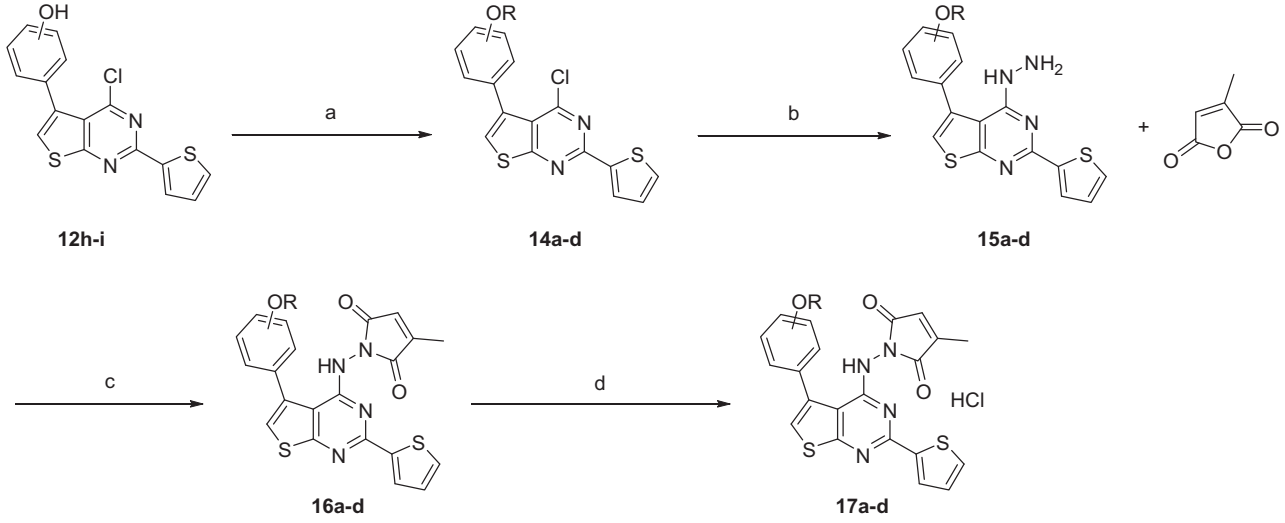


Scheme 2. (a) 4 M HCl, 1,4-dioxane, 110 °C; (b) $CuCl_2$, *t*-BuONO, THF/MeCN, 70 °C; (c) NH_4OAc, AcOH, toluene, reflux; (d) piperidine, EtOH, 80 °C; (e) 4 M HCl, 1,4-dioxane, 110 °C; (f) $POCl_3$, 100 °C; (g) $H_2N\text{-}NH_2\cdot H_2O$, THF, 80 °C; (h) $CHCl_3$, 80 °C.

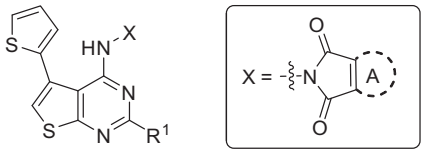


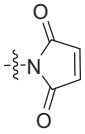
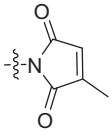
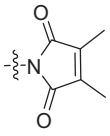
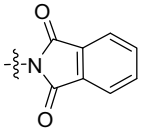
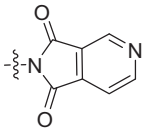
Scheme 3. (a) R-OH, PPh_3, DIAD, THF; (b) $H_2N\text{-}NH_2\cdot H_2O$, THF, 80 °C; (c) $CHCl_3$, 80 °C; (d) 4 M HCl, 1,4-dioxane.

did not exhibit NO inhibitory activity. To maintain NO inhibitory activity in downstream reactions, we fixed 3-methyl-1*H*-pyrrole-2,5-dione moiety in the C_4 position, and then proceeded with the additional steps.

Next, we modified the C_2 and C_5 positions of thienopyrimidine analogues based on their inhibitory activity of cellular NO and TNF-α. We introduced various substituents, (methyl, monocyclic aromatic, phenyl and heterocyclic, such as furan, thiophene and pyridine) at C_2 and C_5 position of thienopyrimidine core structure. Table 2 summarizes the inhibitory activity against IKKβ of the tested compounds. Most of thienopyrimidine analogues showed good inhibition profile against NO and TNF-α (average = 3.13 and 3.36 μM, respectively). **SPC-839**, a well-known IKKβ inhibitor, gave an inferior inhibition profile (4.41 and 3.32 μM, respectively)

Table 1
Structure–activity-relationship of compounds varied at the C_4-position



Compound	R_1	X	Cell-based assay[a]		IKKβ kinase assay	
			NO IC_{50} (μM)	TNF-α IC_{50} (μM)	Lance ULight system (μM)	Radioisotope system (μM)
8a	H		>10	NA	NT	NT
8b	H		2.49	>10	NT	NT
8c	H		>10	NA	NT	NT
8d	H		>10	NA	NT	NT
8e	H		>10	NA	NT	NT
SPC-839	—	—	4.41	3.32	0.062[b]	NT

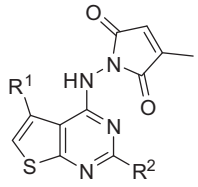
NT: Not tested, NA: Not active.
[a] Values are means of three experiments.
[b] The value was reported from Celgene.

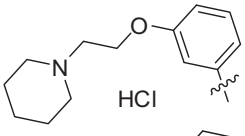
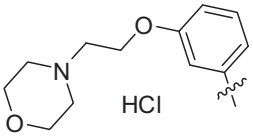
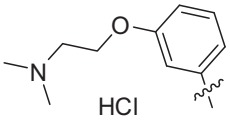
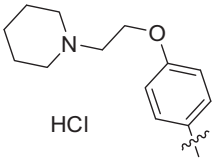
compared to most of the thienopyrimidine analogues. In particular, **13k**, 2-thiophene at C_2 position and phenyl at C_5 position showed excellent NO and TNF-α inhibitory activity (0.75 and 1.29 μM, respectively). However, most of the analogues did not demonstrate significant inhibition of IKKβ (IC_{50} = 59.45 ~ 365.9 μM), while these analogues showed good inhibitory activities against NO and TNF-α. For example, compound **17c**, with relatively poor NO and TNF-α inhibitory activity (7.54 and 9.88 μM, respectively), showed very low IKKβ inhibitory activity about 365.9 μM. In contrast, compound **13k**, with potent inhibition against NO and TNF-α (0.75 and 1.29 μM, respectively), also showed poor IKKβ inhibitory activity over 50 μM. Overall, we found that these derived compounds demonstrated poor IKKβ inhibition regardless of their ability to inhibit NO and TNF-α. To confirm the IKKβ inhibition profile of thienopyrimidine analogues, we performed a second IKKβ kinase assay using a radioisotope system (Merck Millipore). However, most analogues showed no IKKβ inhibitory activity by this second assay, and a few analogues showed very low inhibitory activity, with IC_{50} >50 μM.

Even though thienopyrimidine-based analogues showed no IKKβ inhibitory activity, they could inhibit NO and TNF-α. These results imply that thienopyrimidine analogues may target another kinase in the signaling cascade. To identify the target kinase of the thienopyrimidine-based analogues, we selected 22 kinases, which are implicated in the inflammatory response, including IKKβ, and performed a kinase panel assay (KinaseProfiler™, Merck Millipore, UK) with compounds **13k** and **17a**. As shown in Table 3, representative thienopyrimidine analogues **13k** and **17a** showed no inhibition profile against IKKβ (116% and 84% remaining activities with 10 μM treatment). However, these analogues have significant inhibitory activity against FLT3 (8% and 4% remaining activities in 10 μM, respectively).

Once we identified FLT3 as a possible target kinase for the thienopyrimidine-based analogues, we selected 10 analogues with good NO and TNF-α inhibition to test FLT3 kinase inhibition with a FLT3-specific assay. As shown Table 4, most of thienopyrimidine analogues showed good inhibition profiles against FLT3 (IC_{50} = 0.065 ~ 0.750 μM). These analogues showed a similar range of inhibitory profiles with well-known FLT3 inhibitors, **AC220** and **MLN518** (IC_{50} = 0.120 and 0.102 μM, respectively). Among those tested compounds, **13i**[25] with methyl group at C_5 position showed the best FLT3 inhibitory activity (IC_{50} = 0.065 μM). **13h** with hydrogen at C_5 position showed less inhibitory activity (IC_{50} = 0.175 μM) than **13i**. Compounds with the hydroxyl group at the *meta*- or *para*-position relative to the phenyl group (**13l** and **13m**) at C_5 position (0.488 and 0.750 μM, respectively) showed less inhibitory activity than the unsubstituted phenyl group (**13k**, 0.208 μM). However, appropriate bulky groups such as a piperidinylethoxy group on

Table 2
Structure–activity-relationships of compounds varied at the C_2- and C_5-positions



Compound	R_1	R_2	Cell-based assay[a]		IKKβ kinase assay	
			NO IC_{50} (μM)	TNF-α IC_{50} (μM)	Lance ULight system (μM)	Radioisotope system (μM)
13a	Thiophen-2-yl	Thiophen-2-yl	2.19	0.71	NT	NT
13b	Thiophen-2-yl	Phenyl	3.13	1.13	NT	NT
13c	Thiophen-2-yl	4-Pyridinyl	2.11	>10	NT	NT
13d	Thiophen-2-yl	Cyclopropanyl	3.12	4.35	NT	NT
13e	Thiophen-2-yl	Furan-2-yl	5.25	3.96	NT	NT
13f	Thiophen-2-yl	3-Methoxy phenyl	3.47	1.24	NT	NT
13g	Thiophen-2-yl	4-Methoxy phenyl	2.79	1.39	NT	NT
13h	H	Thiophen-2-yl	6.98	8.95	>50	NA
13i	Methyl	Thiophen-2-yl	1.92	4.22	>50	NA
13j	CH_2COOEt	Thiophen-2-yl	5.07	5.23	>50	NA
13k	Phenyl	Thiophen-2-yl	0.75	1.29	>50	NA
13l	3-Hydroxy-phenyl	Thiophen-2-yl	1.23	2.67	NT	NA
13m	4-Hydroxy-phenyl	Thiophen-2-yl	0.82	1.32	NT	NA
17a		Thiophen-2-yl	2.15	2.92	NT	NT
17b		Thiophen-2-yl	3.26	2.38	NT	>50
17c		Thiophen-2-yl	7.54	9.88	>50	>50
17d		Thiophen-2-yl	1.68	2.14	NT	NA
SPC-839	–	–	4.41	3.32	0.062[b]	NT
Average (**13a**–**17d**)	–	–	3.14	3.36	–	–

NT: Not tested, NA: Not active.
[a] Values are means of three experiments.
[b] The value was reported from Celgene.

Table 3
Remaining kinase activity profiles of kinases by **13k** and **17a** (%)

Kinase	Compound (10 μM)[a]		Kinase	Compound (10 μM)	
	13k	**17a**		**13k**	**17a**
BTK(h)	76	100	MKK4(m)	113	112
FLT3(h)	8	4	MKK6(h)	99	96
IKKα(h)	119	98	MKK7β(h)	122	108
IKKβ(h)	116	84	MSK1(h)	96	102
JAK1(h)	103	111	PKA(h)	125	126
JNK1α1(h)	87	93	ROCK-II(h)	99	96
JNK2α2(h)	94	117	SAPK2a(h)	92	96
JNK3(h)	121	112	SAPK2b(h)	98	103
Lck(h)	86	74	SAPK3(h)	109	99
Lyn(h)	92	81	SAPK4(h)	90	100
MAPKAP-K2(h)	102	94	TYK2(h)	86	87

[a] Remaining was determined by using the KinaseProfiler service at Millipore.

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Table 4
FLT3 kinase assay of thienopyrimidine-based analogues

Compound	FLT3 kinase assay IC_{50}[a] (μM)	Compound	FLT3 kinase assay IC_{50} (μM)
13h	0.175 ± 0.229	**13m**	0.750 ± 0.109
13i	0.065 ± 0.125	**17a**	0.133 ± 0.084
13j	0.673 ± 0.113	**17b**	0.540 ± 0.098
13k	0.208 ± 0.161	**17c**	0.175 ± 0.056
13l	0.488 ± 0.214	**17d**	0.085 ± 0.190
AC220	0.120 ± 0.030	**MLN518**	0.102 ± 0.303

[a] IC_{50} was determined by using the KinaseProfiler service at Millipore. Values are means of duplicates.

Table 5
Remaining kinase activity and tumor growth inhibition profiles of **13i**

Compound	Kinase[a] (%)			GI_{50}[b] (μM)		
	FLT3(h)	FLT3(D835Y)(h)	K562	MV4-11	HL60	THP1
13i	5 ± 1	2 ± 0	0.257 ± 0.1189	0.003 ± 0.0298	0.264 ± 0.1459	0.111 ± 0.2463

[a] Remaining activity% in 10 μM. Values are means of duplicates.
[b] Growth inhibition was measured by XTT assay. Values are means of four experiments.

the *meta*- or *para*-positions (**17a** and **17d**) relative to the phenyl group at the C_5 position (0.133 and 0.085 μM, respectively) showed better FLT3 inhibitory activities.

The most active compound **13i** has been selected to evaluate the inhibitory profiles against wild type and mutant FLT3. As shown in Table 5, **13i** showed excellent inhibitory activity against wild type FLT3 with about 5% remaining activity at 10 μM, as well as 2% remaining activity at 10 μM against D835Y mutant FLT3. In addition, **13i** was evaluated for cell growth inhibition activities using four leukemia cell lines: MV4-11, THP1, HL60 and K562. Among these 4 different leukemia cell lines, **13i** showed better inhibitory activities against MV4-11 and THP1 with wild type and mutant FLT3 (IC_{50} = under 0.1 and 0.111 μM, respectively) than the inhibitory activities against FLT-ITD negative K562 and HL60 cell lines (0.257 and 0.264 μM, respectively).

Thienopyrimidine-based FLT3 inhibitors were designed from the well-known IKKβ inhibitor, **SPC-839**. However, the resulting analogues appear to target a different kinase, FLT3. We evaluated thienopyrimidine analogues with good NO and TNF-α inhibition profiles, well-known inflammatory responses that indicate activated NF-κB. However, thienopyrimidine analogues exhibited no IKKβ inhibitory activity. Instead, we have identified several thienopyrimidine analogues that act to inhibit FLT3. Furthermore, these analogues demonstrated inhibitory activities against mutant FLT3 as well. The most active compound, **13i** had good inhibitory profiles on wild and mutant FLT3, and it also had potent inhibitory activities against AML cell lines with/without FLT3-ITD mutation. Thus, these analogues could represent a promising class of drugs for AML. Future studies will focus on optimizing these thienopyrimidine analogues.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.04.058. These data include MOL files and InChiKeys of the most important compounds described in this article.

References and notes

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25. 3-Methyl-1-[5-methyl-2-(thiophen-2-yl)thieno[2,3-*d*]pyrimidin-4-ylamino]-1*H*-pyrrole-2,5-dione (**13i**): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (d, 1H, J = 3.6 Hz), 7.40 (s, 1H), 7.37 (d, 1H, J = 5.2 Hz), 7.07 (t, 1H, J = 4.4 Hz), 6.87 (s, 1H), 6.62 (s, 1H), 2.58 (s, 3H), 2.26 (s, 3H); ESI (m/z) 357 (MH^+); HRMS (ESI) calcd for $C_{16}H_{12}N_4O_2S_2$ [MH^+]: 357.0480, found: 357.0464.