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Original article

Synthesis and biological evaluation of novel thieno[2,3-*d*]pyrimidine-based FLT3 inhibitors as anti-leukemic agents

Jee Sun Yang [a,1], Chun-Ho Park [b,1], Chulho Lee [a], Hwan Kim [a], Changmok Oh [a], Yejoo Choi [a], Jong Soon Kang [c], Jieun Yun [c], Jin-Hyun Jeong [d], Myung-Hwa Kim [e], Gyoonhee Han [a,f,*]

[a] *Translational Research Center for Protein Function Control, Department of Biotechnology, Yonsei University, Seoul 120-749, Republic of Korea*
[b] *Graduate Program in Biomaterials Science & Engineering, Yonsei University, Seoul 120-749, Republic of Korea*
[c] *Bioevaluation Center, Korea Research Institute of Bioscience and Biotechnology, Ochang, Cheongwon, Chungbuk 363-883, Republic of Korea*
[d] *College of Pharmacy and Yonsei Institute of Pharmaceutical Sciences, Yonsei University, Yeonsu-gu, Incheon 406-840, Republic of Korea*
[e] *Korea Drug Development Fund, 14th Fl, KT&G SeodaemunTower, 21-1, Migeun-dong, Seodaemun-gu, Seoul, Republic of Korea*
[f] *Department of Integrated OMICS for Biomedical Sciences (WCU Program), Yonsei University, Seoul 120-749, Republic of Korea*

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The most common mutations in acute myeloid leukemia (AML) are those that cause the activation of FMS-like tyrosine kinase 3 (FLT3). Therefore, FLT3 is regarded as a potential target for the treatment of AML. A novel series of thieno[2,3-*d*]pyrimidine-based analogs was designed and synthesized as FLT3 inhibitors. All synthesized compounds were assayed for the tyrosine kinase activity of FLT3 and growth inhibitory activity in four human leukemia cell lines (THP1, MV4-11, K562, and HL-60). Among these compounds, compound **17a**, which possesses relatively short and simple substituents at the C_6 position of thieno[2,3-*d*]pyrimidine, emerged as the most promising anti-leukemic agent. Compound **17a** exhibited potent inhibition of FLT3-positive leukemic cell growth and of the FLT3 D835Y kinase; such inhibition is required for the successful treatment of AML. The data supports the further investigation of this class of compounds as potential anti-leukemic agents.

1. Introduction

Acute myeloid leukemia (AML) is a clonal hematopoietic stem cell disorder characterized by the abnormal proliferation and differentiation of immature blast cells in the bone marrow and peripheral blood [1]. Several studies have explored abnormalities in the FMS-like tyrosine kinase 3 (*FLT3*), *RAS*, and *p53* genes in search of clues relating to the pathogenesis of AML [2–7]. The *FLT3* gene is found to have mutations in 30% of adult AML cases, whereas approximately 20% and 5% of adult AML cases reported mutations in *RAS* and *p53*, respectively. The most common molecular abnormalities in AML are activating mutations of *FLT3*, which are linked with poor patient prognosis [8]. FLT3-activating mutations are divided into two broad categories: internal tandem duplications (ITDs) within the juxtamembrane (JM) domain and point mutations within the tyrosine kinase domain (TKD). Approximately 23% of patients with *de novo* AML harbor FLT3/ITD mutations, which represent the most frequent activating mutation. This portends a poor prognosis and these patients experience a high relapse rate [8–13]. The other major FLT3-activating mutations are FLT3 TKD mutations comprising about 7% of *de novo* AML cases. Point mutations in which the aspartate residue at position 835 (D835) is replaced by various amino acids occur most commonly, but with lower frequency than the ITD mutations [14,15]. Another method by which FLT3 is constitutively activated in AML is the overexpression of wild-type FLT3 proteins. Although overexpression of wt-FLT3 is relatively uncommon in AML cases, it is reported that it could function as an unfavorable prognostic factor in cases that do not exhibit the FLT3/ITD mutation [7]. Since FLT3 activating mutations are the most common mutations in AML, inhibition of FLT3 has proved to be a therapeutic target in AML. In particular, because FLT3/ITD mutations have emerged as a negative prognostic factor in AML, FLT3/ITD has become an attractive target for the treatment of AML.

* Corresponding author. Translational Research Center for Protein Function Control, Department of Biotechnology, Yonsei University, Seoul 120-749, Republic of Korea.
E-mail addresses: gyoonhee@yonsei.ac.kr, gyoonhee@gmail.com (G. Han).
[1] These authors contributed equally to this work.

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Several small-molecule inhibitors of FLT3 tyrosine kinase are under development, and are being evaluated in early-phase clinical trials (Fig. 1). The first-generation of FLT3 inhibitors, such as sorafenib (BAY-43-9006) [16], lestaurtinib (CEP-701) [17], sunitinib (SU11248) [18], and tandutinib (MLN518) [19], were discovered during studies of other receptor tyrosine kinase inhibitors. Initially, these compounds were not developed or optimized for FLT3 inhibition; thus, they inhibited not only FLT3 but also other multiple kinases, resulting in off-target effects. To evaluate more focused and potent FLT3 inhibitors for AML therapy, a second-generation inhibitor, quizartinib (**AC220**), was specifically designed [20]. All of these reported drugs efficaciously inhibited FLT3 in cellular assays and *in vivo* models of FLT3-ITD AML. Clinical activity was also seen in all of them, but only a few patients had complete remission in the trials due to insufficient efficacy of FLT3 inhibition and/or several mutations on FLT3 kinase that impart resistance. Therefore, resistance to therapy has become a significant barrier to the development of successful FLT3 inhibitors [21].

Thieno[2,3-*d*]pyrimidine derivatives are a novel class of FLT3 inhibitors for the treatment of AML. A series of compounds were incidentally found to have selective activity against FLT3 while developing IKKβ inhibitors from the quinazoline analog **SPC-839**, as reported in our previous study [22]. Herein, we report the synthesis and biological activities of a novel series of thieno[2,3-*d*]pyrimidine derivatives based on our previous work. We demonstrate that novel thieno[2,3-*d*]pyrimidine-based compounds inhibit FLT3 with great potency, and also have potent inhibitory activities against AML cell lines with FLT3/ITD mutations compared with **AC220** and **MLN518**. We further demonstrate that the high potency exhibited by this series of compounds on FLT3/ITD and FLT3 D835Y (the most common kinase domain mutant in AML [23]) indicates the feasibility of developing successful FLT3 inhibitors by reducing resistance. Our results suggest that novel thieno[2,3-*d*]pyrimidine derivatives represent promising chemotherapeutic agents for the treatment of AML.

2. Chemistry

Based on the thieno[2,3-*d*]pyrimidine structures of the compounds described in our previous work, we designed and synthesized 22 analogs with diverse substitutions on the thieno[2,3-*d*]pyrimidine moiety at the C_2, C_4, and C_6 positions. **5a** and **12b** were prepared according to literature precedent [22]. As illustrated in Schemes 1–3, the final compounds were synthesized from various 2-aminothiophenes by three or four-step procedures. Thienopyrimidine derivatives (**2**, **9**, and **14**) were conveniently synthesized by heating various 2-aminothiophenes (**1**, **8**, and **13**) with the appropriate carbonitrile under acidic conditions. Compound **2** was converted to chloride (**3**) by a Sandmeyer reaction, while compounds **9** and **14** were chlorinated using $POCl_3$ at 100 °C to generate 4-chlorothieno[2,3-*d*]pyrimidine **10** and **15**. Thieno[2,3-*d*]pyrimidin-4-amine derivatives **5**, **12**, and **17** were then prepared in a stepwise fashion by first treating **10** or **15** with hydrazine hydrate in tetrahydrofuran, followed by treatment with 3-methylfuran-2,5-dione. The structures of the newly synthesized compounds were characterized by ^{1}H and ^{13}C nuclear magnetic resonance (NMR), low-resolution mass spectroscopy (LRMS), and high-resolution mass spectroscopy (HRMS). The compounds were screened for their *in vitro* biological activities.

3. Results and discussion

3.1. Biological evaluation of final compounds

All synthesized compounds were assayed for tyrosine kinase activity of FLT3 and growth inhibitory activity in four human leukemia cell lines that harbored either wt-FLT3 (THP1), a mutated FLT3 kinase (MV4-11), or were FLT3-null (K562 and HL-60). The results are tabulated as IC_{50} and GI_{50} values in the micromolar range (Table 1). **AC220** and **MLN518** were used as positive references for the comparison of *in vitro* activities.

As shown in Table 1, the primary *in vitro* kinase assay results revealed that five compounds exhibited moderate tyrosine kinase inhibitory activities against human FLT3. In particular, compounds **5a** and **17a** showed superior inhibitory activities (0.069 and 0.055 μM, respectively) compared with the positive controls, **AC220** and **MLN518** (0.12 and 0.102 μM, respectively). Most of the evaluated compounds also showed favorable growth inhibitory activities against the MV4-11 and THP1 cell lines, which represent FLT3/ITD mutations and wt-FLT3 in AML, respectively. Notably, compounds **17b**, **17c**, and **17d** exhibited excellent inhibitory activities in the MV4-11 cell line, with GI_{50} values of 86, 91, and 92 nM, respectively. As indicated in Table 1, most of the synthesized compounds

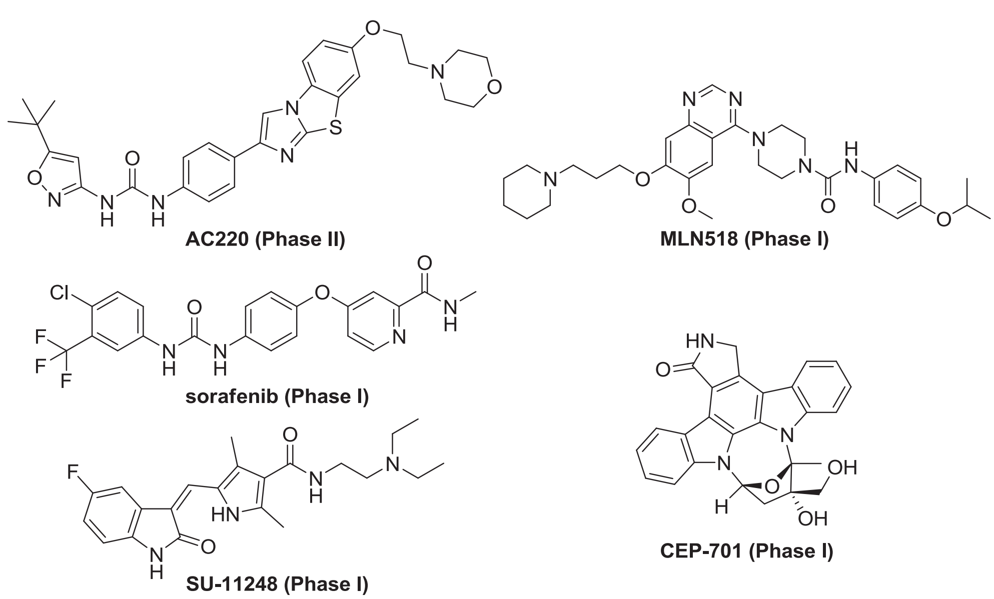
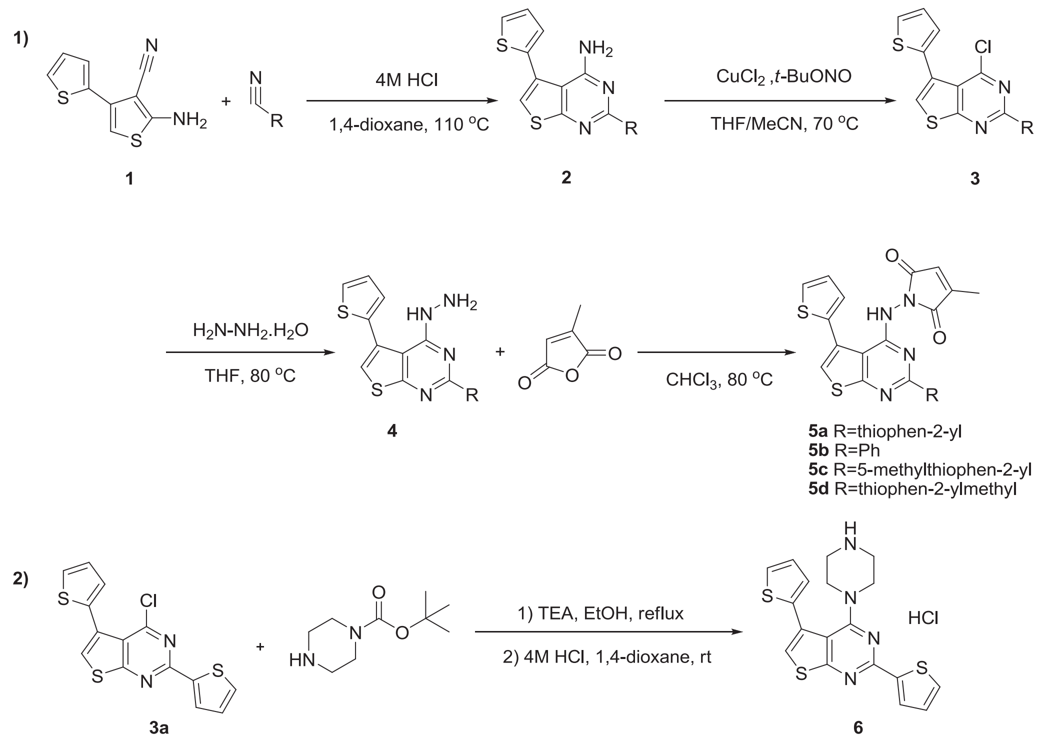
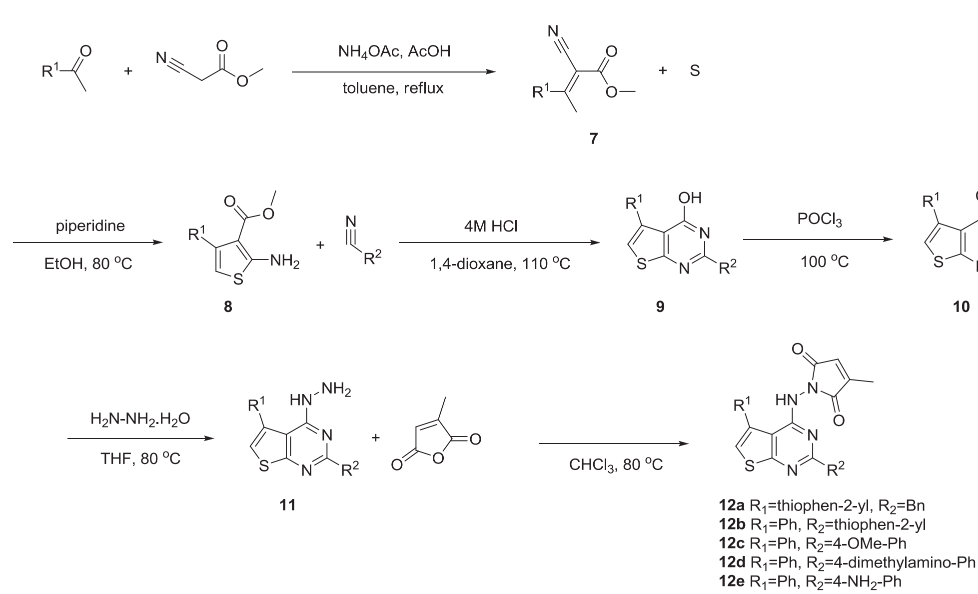


Fig. 1. Chemical structures of FLT3 inhibitors.



Scheme 1. 1) Schematic for the synthesis of final compounds **5a**–**5d** and 2) Schematic for the synthesis of final compound **6**.



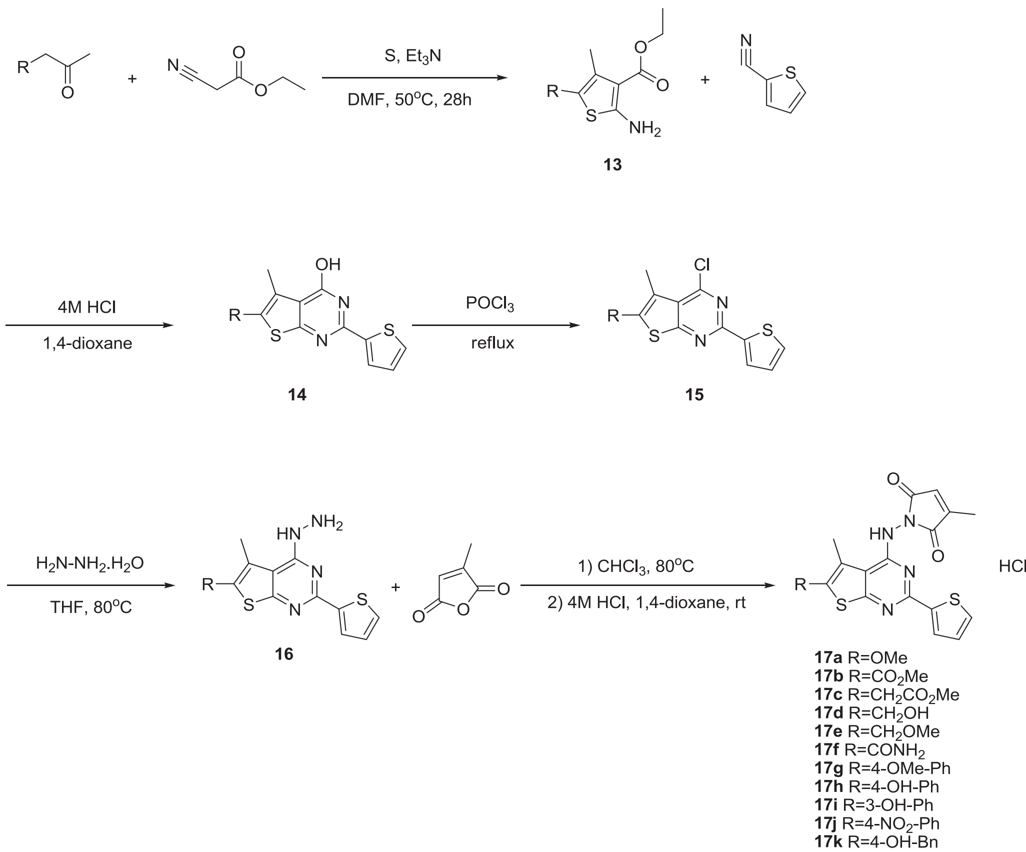
Scheme 2. Schematic for the synthesis of final compounds **12a**–**12e**.

inhibited FLT3/ITD and wt-FLT3 more effectively than the known selective inhibitor, **AC220**.

3.2. SAR study

Initially, in order to assess the effects of different substituents at the C_4 position on thieno[2,3-*d*]pyrimidine, we synthesized three compounds, **4a**, **5a**, and **6**. The 2-thienyl group at the C_2 and C_5 position of the compounds was fixed and various substituents, such as 3-methyl-1H-pyrrole-2,5-dione, hydrazine, or piperazine, were introduced at the C_4 position of thienopyrimidine. These newly synthesized compounds inhibited the tyrosine kinase activity of FLT3 with IC_{50} values of 0.47, 0.069, and 0.521 μM, respectively. Among these three compounds, only **5a** inhibited FLT3 kinase activity and the growth of MV4-11 cells significantly better than did **AC220**.

To evaluate the effect of different substituents at the C_2 position of the compounds, nine compounds were prepared containing 3-methyl-1H-pyrrole-2,5-dione at the C_4 position of the thienopyrimidine core, since this moiety was the only active compound in the MV4-11 cell line. The nine compounds were divided into two groups, one containing a phenyl group (**12b**–**12e**) and the other



Scheme 3. Schematic for the synthesis of final compounds **17a**–**17k**.

Table 1
Biological activities of thieno[2,3-*d*]pyrimidine derivatives.

Cpd	FLT3 kinase assay IC_{50} (μM)[a]	SD	GI_{50} (μM)[b]			
			K562	MV4-11	HL60	THP1
AC220	0.12	0.030	>10	3.310	8.014	5.574
MLN518	0.102	0.303	>10	8.756	>10	>10
4a	0.47	0.111	>10	>10	>10	>10
5a	0.069	0.208	5.551	0.320	0.746	2.673
5b	1.603	0.124	6.302	0.836	0.862	3.728
5c	0.611	0.085	1.916	0.775	0.403	2.805
5d	NA[c]	–	>10	2.009	0.725	6.261
6	0.521	0.367	>10	>10	>10	>10
12a	NA	–	3.517	3.829	1.517	6.658
12b	0.208	0.161	0.074	0.233	0.758	0.155
12c	NA	–	0.703	0.407	1.313	2.205
12d	NA	–	1.742	0.755	2.188	1.628
12e	0.546	0.201	8.159	2.100	3.714	4.112
17a	0.055	0.073	1.457	0.100	0.240	0.906
17b	0.787	0.079	0.030	0.086	0.136	0.172
17c	0.866	0.063	0.483	0.091	0.656	2.902
17d	0.175	0.167	3.843	0.092	0.712	2.595
17e	0.282	0.031	1.256	0.115	0.266	1.781
17f	0.131	0.064	2.777	0.193	7.802	2.686
17g	0.846	0.040	0.154	0.135	0.624	0.902
17h	0.721	0.061	0.145	0.131	0.221	0.600
17i	0.633	0.040	0.003	0.123	0.383	0.107
17j	0.301	0.145	1.882	0.112	0.418	0.846
17k	3.286	0.416	0.880	0.103	0.213	0.948

[a] IC_{50} was determined by using the KinaseProfiler service at Millipore (average of duplicates).
[b] Growth inhibition was measured by XTT assay (average of four replicates).
[c] NA) not active.

containing a 2-thienyl group (**5a**–**5d** and **12a**) at the C_5 position of thieno[2,3-*d*]pyrimidine with diverse C_2-substituents. All of these compounds, except **12a**, showed good growth inhibitory activity superior to the positive control **AC220** (3.310 μM) on the MV4-11 cell line, while they did not exhibit favorable FLT3 kinase activity as much as **AC220**. However, these series of compounds, except **5a** and **12b**, showed good growth inhibition not only in FLT3-positive cell lines but also in FLT3-negative cell lines. Additionally, they displayed almost no activity on FLT3 kinase. These results might imply that these compounds inhibit the growth of AML cells by their effects on kinases other than FLT3. Therefore, compounds containing a 2-(thiophen-2-yl)thieno[2,3-d]pyrimidine moiety (**5a** and **12b**) emerged as potent compounds in this series that exhibited good activity for both wt-FLT3 and FLT3/ITD.

Based on compounds **5a** and **12b**, which showed potent antiproliferative activity in FLT3-positive AML cell lines and inhibitory activity on FLT3 kinase, eleven compounds with an array of substituents at the C_6 position of thieno[2,3-*d*]pyrimidine were also synthesized. Based on results from our previous study, we fixed a methyl group at the C_5 position, as this had exhibited excellent inhibitory activities. To assess the effects of different substituents at the C_6 position of the compounds, small substituents (**17a**–**17f**), such as methoxy, carboxylate, methanol, or carboxamide, and substituted phenyl or benzyl (**17g**–**17k**) moieties were introduced. These groups of compounds displayed outstanding GI_{50} values of 86–193 nM which are 17- to 39-fold better than **AC220** for the FLT3/ITD mutated cell line. Interestingly, the potency of the compounds with small substituents on FLT3 kinase and the MV4-11 cell line surpassed that of the phenyl- or benzyl-substituted compounds. Moreover, **17a** exhibited the most potent kinase activity ($IC_{50} = 0.055$ μM) and the kinase activity of **17d** and **17f** were also comparable to the positive controls. The relatively simple and short groups at the C_6 position of thieno[2,3-*d*]pyrimidine transpired to be the most favorable substituent for FLT3 inhibitory activity.

3.3. D835Y

Many researchers have reported that intrinsic and acquired resistance to therapy might be surmounted by targeting not only the FLT3/ITD mutations, but also the acquired TKD point mutations. Therefore, the IC_{50} values of some of the synthesized compounds on the most common kinase domain mutant, FLT3 D835Y, were also determined. Based on the results of FLT3 kinase activity and anti-proliferative activity, compounds **17a**, **17c**, **17d**, **17f**, and **17j** were selected for evaluation. Results are tabulated as IC_{50} values in the micromolar range in Table 2. All of these selected compounds showed excellent IC_{50} values of 46 nM–587 nM. Among these compounds, three compounds **17a**, **17d**, and **17f** exhibited superior activities (46, 112, and 119 nM, respectively) compared with the positive control **AC220** (137 nM). Remarkably, compound **17a** showed high potency against wt-FLT3, FLT3-ITD, and FLT3 D835Y.

We have designed and synthesized 22 thieno[2,3-*d*]pyrimidine-based analogs for the treatment of AML, based on our previous study. We then evaluated the effects of diversity at the C_2, C_4, and C_6 positions of the thieno[2,3-*d*]pyrimidine moiety on FLT3 kinase and four human leukemia cell lines. Most of the synthesized thieno[2,3-*d*]pyrimidine analogs inhibited FLT3/ITD more effectively than did **AC220**. As weak and transient inhibitions were considered major obstacles to the development of FLT3 inhibitors, this series of compounds showed potential to overcome barriers to optimal FLT3 inhibition. Moreover, three compounds (**17a**, **17d**, and **17f**) potently inhibited the TKD point mutant (D835Y) together with FLT3/ITD. In conclusion, compound **17a** emerged as the most promising anti-leukemic agent, exhibiting potent inhibition of FLT3-positive leukemic cell growth and the FLT3 D835Y kinase; such inhibition is required for the successful treatment of AML. Our results show that synthetic thieno[2,3-*d*]pyrimidine derivatives represent promising chemotherapeutic agents for the treatment of AML.

4. Experimental section

All chemicals were obtained from commercial suppliers and used without further purification. All reactions were monitored by thin-layer chromatography (TLC) on pre-coated silica gel 60 F_{254} (mesh) (Merck, Mumbai, India), and spots were visualized under UV light (254 nm). Flash column chromatography was performed with silica (Merck EM9385, 230–400 mesh). ^1H and ^{13}C NMR spectra were recorded at 400 MHz and 100 MHz on a Varian 400 Mercury plus spectrometer; chemical shifts are reported in δ (ppm) units relative to the internal reference tetramethylsilane (TMS). Mass spectra were obtained on an Applied Biosystems API 2000 mass spectrometer and an Agilent 1200 series LC system. HPLC (high performance liquid chromatography) experiments were conducted using Agilent analytic column eclipse-XDB-C18 (150*4.6 mm, 5 μm) on Shimazu HPLC 2010 instruments. Conditions and retention times are described in the Supporting Information.

Table 2
FLT3 D835Y kinase assay of thieno[2,3-*d*]pyrimidine derivatives.

Cpd	FLT3 (D835Y) activity (μM)[a]	SD
AC220	0.137	0.060
MLN518	2.444	0.099
17a	0.046	0.028
17c	0.587	0.049
17d	0.112	0.159
17f	0.119	0.042
17j	0.505	0.321

[a] IC_{50} was determined by using the KinaseProfiler service at Millipore (average of duplicates).

4.1. General procedure for the preparation of title compound 4a

According to the reported procedures [22], the key intermediate and title compound **4a** was synthesized by a three-step process, as shown in Scheme 1.

4.1.1. 2,5-Di(thiophen-2-yl)thieno[2,3-d]pyrimidin-4-amine (2a)

5′-Amino-2,3′-bithiophene-4′-carbonitrile **1** (300 mg, 1.45 mmol) and 2-thiophene carbonitrile (162 μl, 1.75 mmol) were dissolved in 4 M HCl 1,4-dioxane solution (6 ml), and the resulting mixture was stirred at 110 °C for 24 h. After cooling, the reaction mixture was poured into ice-cold water and extracted with EtOAc. The combined organic layer was washed with saturated $NaHCO_3$ aqueous solution, dried over Na_2SO_4, and concentrated under reduced pressure to afford the title compound as a brown solid (260 mg, 56%). ^1H NMR (400 MHz, $CDCl_3$): δ 7.95 (s, 1H), 7.41 (t, $J = 3.6$ Hz, 1H), 7.13 (m, 4H), 5.49 (br, 2H).

4.1.2. 4-Chloro-2,5-di(thiophen-2-yl)thieno[2,3-d]pyrimidine (3a)

$CuCl_2$ (174 mg, 1.29 mmol) and *t*-BuONO (192 μl, 1.62 mmol) were dissolved in MeCN (5 ml), and the resulting mixture was stirred at 70 °C for 30 min. Into this mixture, 2,5-di(thiophen-2-yl)thieno[2,3-*d*]pyrimidin-4-amine **2a** (340 mg, 1.08 mmol) in tetrahydrofuran (THF; 2 ml) was added dropwise and the reaction mixture was stirred at the same temperature for 3 h. The cooled mixture was poured into ice-cold water and extracted with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated to dryness. The residue was then purified by flash column chromatography (n-Hx:EtOAc = 3:1) to afford the title compound **3a** as a yellow oil (75 mg, 21%). ^1H NMR (400 MHz, $CDCl_3$): δ 8.06 (d, $J = 4.0$ Hz, 1H), 7.50 (d, $J = 5.6$ Hz, 1H), 7.46 (s, 1H), 7.40 (d, $J = 5.6$ Hz, 1H), 7.16 (m, 2H), 7.12 (dd, 1H, $J = 3.2$ Hz, $J = 5.2$ Hz).

4.1.3. 4-Hydrazinyl-2,5-di(thiophen-2-yl)thieno[2,3-d]pyrimidine hydrochloride (4a)

To a stirred solution of **3** (0.22 mmol) in THF (3 ml), hydrazine monohydrate (0.67 mmol) was added dropwise, and the mixture was stirred at 80 °C for 4 h. After cooling, the title compound **4a** was obtained by removing solvent under reduced pressure and concentrating *in vacuo*. Yellow solid (76%); ^1H NMR (400 MHz, DMSO-*d6*): δ 9.04 (s, 1H), 8.25 (d, $J = 3.6$ Hz, 1H), 7.81 (d, $J = 5.2$ Hz, 1H), 7.77 (s, 1H), 7.69 (d, $J = 5.2$ Hz, 1H), 7.29–7.18 (m, 3H); ^{13}C NMR (100 MHz, DMSO-*d6*): δ 155.68, 155.05, 152.17, 141.80, 135.32, 131.26, 130.79, 128.52, 128.29, 128.14, 127.24, 126.92, 124.54, 111.73 ppm; ESI (*m*/*z*) 331 (MH^+); HRMS (ESI) calculated for $C_{14}H_{10}N_4S_3$ [MH^+]: 331.0146, found: 331.0138.

4.2. General procedure for the preparation of title compounds 5a–5d

To a stirred solution of **3** (0.22 mmol) in THF (3 ml) hydrazine monohydrate (0.67 mmol) was added dropwise, and the mixture was stirred at 80 °C for 4 h. After cooling, the solvent was removed under reduced pressure and concentrated *in vacuo*. The residue **4** was then dissolved in $CHCl_3$ (3 ml), and citraconic anhydride (0.67 mmol) was slowly added. The resulting mixture was heated to 80 °C and stirred for 18 h. The reaction mixture was cooled to room temperature and washed with water. The organic layer was dried over Na_2SO_4 and concentrated to dryness. The residue was then purified by flash column chromatography (n-Hx:EtOAc = 2:1) to afford the title compound **5**.

4.2.1. 1-[2,5-Di(thiophen-2-yl)thieno[2,3-d]pyrimidin-4-ylamino]-3-methyl-1H-pyrrole-2,5-dione (*5a*)

Pale yellow solid (48%); ^{1}H NMR (400 MHz, DMSO-*d6*): 8.68 (s, 1H), 7.75 (s, 1H), 7.69 (m, 3H), 7.30 (d, J = 3.6 Hz, 1H), 7.20 (t, J = 4.0 Hz, 1H), 7.14 (t, J = 4.8 Hz, 1H), 6.93 (s, 1H), 2.12 (s, 3H); ^{13}C NMR (100 MHz, DMSO-*d6*): δ 169.92, 169.10, 168.84, 155.91, 155.53, 145.40, 142.68, 135.57, 131.28, 129.03, 128.97, 128.76, 128.48, 127.67, 127.28, 127.05, 124.73, 111.30, 11.64 ppm; ESI (*m/z*) 425 (MH$^+$); HRMS (ESI) calculated for $C_{19}H_{12}N_4O_2S_3$ [MH$^+$]: 425.0201, found: 425.0189; HPLC (t^R : purity = 8.11 min, 93.26%).

4.2.2. 3-Methyl-1-[2-phenyl-5-(thiophen-2-yl)thieno[2,3-d]pyrimidin-4-ylamino]-1H-pyrrole-2,5-dione (*5b*)

White solid (96%); ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.23 (d, J = 7.2 Hz, 2H), 7.45 (d, J = 4.4 Hz, 1H), 7.41–7.38 (m, 4H), 7.34 (s, 1H), 7.20–7.17 (m, 2H), 6.59 (s, 1H), 2.22 (s, 3H); ^{13}C NMR (100 MHz, DMSO-*d6*): δ 169.50, 169.01, 168.40, 158.21, 155.80, 145.03, 136.49, 135.23, 130.81, 128.66 (2), 128.36, 128.09, 127.60 (2), 127.27, 126.91, 126.49, 124.80, 111.23, 11.18 ppm; ESI (*m/z*) 419 (MH$^+$); HRMS (ESI) calculated for $C_{21}H_{14}N_4O_2S_2$ [MH$^+$]: 419.0636, found: 419.0627; HPLC (t^R : purity = 6.15 min, 94.48%).

4.2.3. 3-Methyl-1-[2-(5-methylthiophen-2-yl)-5-(thiophen-2-yl)thieno[2,3-d]pyrimidin-4-ylamino]-1H-pyrrole-2,5-dione (*5c*)

Pale yellow solid (73%); ^{1}H NMR (400 MHz, DMSO-*d6*): δ 8.63 (s, 1H), 7.74 (s, 1H), 7.68 (d, J = 4.8 Hz, 1H), 7.55 (d, J = 3.2 Hz, 1H), 7.30 (d, J = 3.2 Hz, 1H), 7.21 (m, 1H), 6.94 (s, 1H), 6.86 (d, J = 3.6 Hz, 1H), 2.47 (s, 3H), 2.14 (s, 3H); ^{13}C NMR (100 MHz, DMSO-*d6*): δ 169.52, 168.66, 168.43, 155.50, 155.14, 144.98, 144.82, 139.85, 135.24, 128.94, 128.36, 128.06, 127.26, 127.14, 126.90, 126.64, 124.01, 110.66, 15.45, 11.23 ppm; ESI (*m/z*) 439 (MH$^+$); HRMS (ESI) calculated for $C_{20}H_{14}N_4O_2S_3$ [MH$^+$]: 439.0357, found: 439.0351; HPLC (t^R : purity = 6.17 min, 95.66%).

4.2.4. 3-Methyl-1-[5-(thiophen-2-yl)-2-(thiophen-2-ylmethyl)thieno[2,3-d]pyrimidin-4-ylamino]-1H-pyrrole-2,5-dione (*5d*)

Brown solid (71%); ^{1}H NMR (400 MHz, DMSO-*d6*): δ 8.87 (s, 1H), 8.17 (s, 1H), 8.09 (d, J = 4.8 Hz, 1H), 7.75 (d, J = 4.8 Hz, 1H), 7.69–7.68 (m, 1H), 7.61 (t, J = 4.2 Hz, 1H), 7.33–7.31 (m, 1H), 7.28 (br s, 1H), 7.22 (s, 1H), 4.62 (s, 2H), 2.49 (s, 3H); ESI (*m/z*) 433 (MH$^+$); HRMS (ESI) calculated for $C_{22}H_{16}N_4O_2S_2$ [MH$^+$]: 433.0793, found: 433.0788.

4.3. Procedure for the preparation of the title compound 4-(piperazin-1-yl)-2,5-di(thiophen-2-yl)thieno[2,3-d]pyrimidine hydrochloride (*6*)

To a stirred solution of 4-chloro-2,5-di(thiophen-2-yl)thieno[2,3-d]pyrimidine **3a** (50 mg, 0.15 mmol) in EtOH (5 ml), TEA (31 µl, 0.22 mmol) and 1-Boc-piperazine (56 mg, 0.30 mmol) were sequentially added at 0 °C. The resulting mixture was refluxed for 1 day. After cooling, the solvent was removed under reduced pressure and the residue was extracted with CH_2Cl_2. The combined organic layer was dried over Na_2SO_4 and concentrated to dryness. The residue was then purified by flash column chromatography (n-Hx:EtOAc = 2:3) to afford *t*-butyl 4-[2,5-di(thiophen-2-yl)thieno[2,3-*d*]pyrimidin-4-yl]piperazine-1-carboxylate (40 mg, 55%).

4 M HCl 1,4-dioxane solution (0.5 ml) was added to a stirred solution of 4-(piperazin-1-yl)-2,5-di(thiophen-2-yl)thieno[2,3-*d*]pyrimidine in 1,4-dioxane (3 ml) at 0 °C. After stirring for 1 h, the reaction mixture was concentrated *in vacuo* to afford the title compound **6** as a white solid (40 mg, 87%). ^{1}H NMR (400 MHz, DMSO-*d6*): δ 10.66 (br s, 1H), 8.01 (d, J = 3.6 Hz, 1H), 7.81 (s, 1H), 7.78 (d, J = 5.2 Hz, 1H), 7.68 (d, J = 4.8 Hz, 1H), 7.28–7.27 (m, 1H), 7.24–7.21 (m, 2H), 3.97–3.93 (m, 2H), 3.28–3.15 (m, 4H), 2.86–2.78 (m, 2H), 2.69–2.68 (m, 2H); ESI (*m/z*) 385 (MH$^+$); HRMS (ESI) calculated for $C_{18}H_{16}N_4S_3$ [MH$^+$]: 385.0615, found: 385.0612; HPLC (t^R : purity = 4.39 min, 99.82%).

4.4. Procedure for the preparation of intermediate compound *10*

According to the following procedures, the key intermediate compound **10** was synthesized by a four-step process, as shown in Scheme 2.

4.4.1. Methyl 2-cyano-3-(thiophen-2-yl)but-2-enoate (*7a*)

To a stirred solution of 2-acetylthiophene (1.0 g, 7.92 mmol) in toluene (20 ml), methyl cyanoacetate (0.84 ml, 9.51 mmol), ammonium acetate (1.80 g, 23.77 mmol), and acetic acid (1.36 ml, 23.77 mmol) were added. After refluxing for 16 h, the reaction mixture was poured into ice-cold water and extracted with EtOAc. The combined organic layer was washed with 1 N HCl aqueous solution, dried over Na_2SO_4, and concentrated to dryness. The residue was then purified by flash column chromatography (n-Hx:EtOAc = 5:1) to afford the title compound **7a** as a yellow oil (810 mg, 53%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.03 (d, J = 4.0 Hz, 1H), 7.80 (d, J = 4.8 Hz, 1H), 7.78 (t, J = 4.4 Hz, 1H), 3.88 (s, 3H) 2.71 (s, 3H).

4.4.2. Methyl 5′-amino-2,3′-bithiophene-4′-carboxylate (*8a*)

To a stirred solution of methyl 2-cyano-3-(thiophen-2-yl)but-2-enoate **7a** (250 mg, 1.21 mmol) and sulfur (46 mg, 1.45 mmol) in EtOH (10 ml) was added piperidine (143 µl, 1.45 mmol), and the resulting mixture was stirred at 80 °C for 18 h. After cooling to room temperature, the reaction mixture was poured into ice-water and extracted with EtOAc. The combined organic layer was washed with saturated NH_4Cl aqueous solution, dried over Na_2SO_4, and concentrated to dryness. The residue was then purified by flash column chromatography (n-Hx:EtOAc = 5:1) to afford the title compound **8a** as a yellow solid (88 mg, 31%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.25 (d, J = 3.6 Hz, 1H), 7.01 (m, 2H), 6.22 (s, 1H), 6.11 (s, 2H), 3.67 (s, 3H).

4.4.3. 2-Benzyl-5-(thiophen-2-yl)thieno[2,3-d]pyrimidin-4-ol (*9a*)

Methyl 5′-amino-2,3′-bithiophene-4′-carboxylate **8a** (100 mg, 0.41 mmol) and phenylacetonitrile (140 µl, 1.25 mmol) were dissolved in 4 M HCl 1,4-dioxane solution (3 ml), and the resulting mixture was stirred at 100 °C for 1 day. After cooling, the reaction mixture was poured into ice-cold water and extracted with EtOAc. The combined organic layer was washed with saturated $NaHCO_3$ aqueous solution, dried over Na_2SO_4, and concentrated under reduced pressure to afford the title compound **9a** as a yellow oil (110 mg, 83%).

4.4.4. 2-Benzyl-4-chloro-5-(thiophen-2-yl)thieno[2,3-d]pyrimidine (*10a*)

2-Benzyl-5-(thiophen-2-yl)thieno[2,3-d]pyrimidin-4-ol **9a** (110 mg, 0.33 mmol) was dissolved in $POCl_3$ (1 ml) and the resulting mixture was heated for 3 h at 110 °C. After cooling to room temperature, the reaction mixture was poured into ice-cold water and extracted with $CHCl_3$. The combined organic layer was washed with saturated $NaHCO_3$ aqueous solution, dried over Na_2SO_4 and concentrated to dryness. The residue was then purified by flash column chromatography (n-Hx:EtOAc = 4:1) to afford the title compound **10a** as a white solid (26 mg, 22%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.50 (s, 1H), 7.44–7.40 (m, 3H), 7.33–7.21 (m, 3H), 7.13–7.08 (m, 2H), 4.36 (s, 2H).

4.5. General procedure for the preparation of title compounds **12a–12e**

To a stirred solution of **10** (0.06 mmol) in THF (3 ml), hydrazine monohydrate (0.19 mmol) was added dropwise, and the mixture was stirred at 80 °C for 7 h. After cooling, the solvent was removed under reduced pressure, and the residue **11** was then dissolved in $CHCl_3$ (5 ml). Citraconic anhydride (0.19 mmol) was then slowly added and the resulting mixture was heated to 80 °C for 2 h. The reaction mixture was cooled to room temperature and washed with water. The organic layer was then dried over Na_2SO_4, concentrated to dryness, and purified by flash column chromatography (n-Hx:EtOAc = 2:1) to afford the title compound **12**.

4.5.1. 1-[2-Benzyl-5-(thiophen-2-yl)thieno[2,3-d]pyrimidin-4-ylamino]-3-methyl-1H-pyrrole-2,5-dione (**12a**)

Yellow solid (79%); ^{1}H NMR (400 MHz, DMSO-*d6*): δ 8.42 (s, 1H), 7.73 (s, 1H), 7.67–7.65 (m, 1H), 7.27–7.16 (m, 8H), 6.78 (s, 1H), 3.98 (s, 2H), 2.05 (s, 3H); ESI (*m*/*z*) 433 (MH^+); HRMS (ESI) calculated for $C_{22}H_{16}N_4O_2S_2$ [MH^+]: 433.0793, found: 433.0788; HPLC (t^R : purity = 7.09 min, 94.62%).

4.5.2. 3-Methyl-1-[5-phenyl-2-(thiophen-2-yl)thieno[2,3-d]pyrimidin-4-ylamino]-1H-pyrrole-2,5-dione (**12b**)

Pale yellow solid (64%); ^{1}H NMR (400 MHz, DMSO-*d6*): δ 8.41 (s, 1H), 7.72 (br, 3H), 7.51 (m, 5H), 7.16 (s, 1H), 6.93 (s, 1H), 2.12 (s, 3H); ^{13}C NMR (100 MHz, DMSO-*d6*): δ 169.48, 168.98, 168.39, 155.40, 154.89, 146.28, 144.90, 142.36, 134.67, 134.49, 130.69, 128.92, 128.63, 128.49, 128.41, 128.30, 127.39, 126.81, 123.17, 110.90, 11.18 ppm; ESI (*m*/*z*) 419 (MH^+); HRMS (ESI) calculated for $C_{21}H_{14}N_4O_2S_2$ [MH^+]: 419.0636, found: 419.0615; HPLC (t^R : purity = 8.39 min, 93.71%).

4.5.3. 1-[2-(4-Methoxyphenyl)-5-phenylthieno[2,3-d]pyrimidin-4-ylamino]-3-methyl-1H-pyrrole-2,5-dione (**12c**)

Pale yellow solid (61%); ^{1}H NMR (400 MHz, DMSO-*d6*): δ 8.28 (br, 1H), 8.10 (d, *J* = 8.8 Hz, 2H), 7.66 (s, 1H), 7.58 (d, *J* = 7.7 Hz, 2H), 7.52 (t, *J* = 7.5 Hz, 2H), 7.47–7.44 (m, 1H), 7.02 (d, *J* = 8.4 Hz, 2H), 6.94 (s, 1H), 3.81 (s, 3H), 2.13 (s, 3H); ^{13}C NMR (100 MHz, DMSO-*d6*): δ 169.93, 169.66, 168.83, 161.89, 158.33, 156.06, 145.39, 135.26, 134.79, 129.70 (2), 129.37 (2), 129.33, 129.07 (2), 128.71, 127.27, 123.36, 114.45 (2), 111.22, 55.77, 11.59 ppm; ESI (*m*/*z*) 443 (MH^+); HRMS (ESI) calculated for $C_{24}H_{18}N_4O_3S$ [MH^+]: 443.1178, found: 443.1168; HPLC (t^R : purity = 9.76 min, 99.09%).

4.5.4. 1-{2-[4-(Dimethylamino)phenyl]-5-phenylthieno[2,3-d]pyrimidin-4-ylamino}-3-methyl-1H-pyrrole-2,5-dione (**12d**)

Yellow solid (94%); ^{1}H NMR (400 MHz, DMSO-*d6*): δ 8.21 (s, 1H), 8.06 (d, *J* = 8.8 Hz, 2H), 7.60–7.58 (m, 3H), 7.53–7.45 (m, 3H), 6.93 (s, 3H), 3.01 (s, 6H), 2.14 (s, 3H); ESI (*m*/*z*) 456 (MH^+); HRMS (ESI) calculated for $C_{25}H_{21}N_5O_2S$ [MH^+]: 456.1494, found: 456.1474; HPLC (t^R : purity = 10.86 min, 94.24%).

4.5.5. 1-[2-(4-Aminophenyl)-5-phenylthieno[2,3-d]pyrimidin-4-ylamino]-3-methyl-1H-pyrrole-2,5-dione (**12e**)

Yellow solid (79%); ^{1}H NMR (400 MHz, DMSO-*d6*): δ 8.35 (s, 1H), 8.16 (d, *J* = 8.4 Hz, 2H), 7.71 (s, 1H), 7.59 (d, *J* = 7.2 Hz, 2H), 7.52 (t, *J* = 7.2 Hz, 2H), 7.47 (d, *J* = 6.4 Hz, 1H), 7.26 (d, *J* = 8.0 Hz, 2H), 6.94 (s, 1H), 2.13 (s, 3H); ^{13}C NMR (100 MHz, DMSO-*d6*): δ 169.52, 169.15, 168.38, 157.30, 155.78, 145.01, 134.73, 134.42, 128.98 (4), 128.92 (2), 128.70 (2), 128.36, 126.92, 123.66, 121.79, 111.22, 11.21 ppm; ESI (*m*/*z*) 428 (MH^+); HRMS (ESI) calculated for $C_{23}H_{17}N_5O_2S$ [MH^+]: 428.1181, found: 428.1163; HPLC (t^R : purity = 5.16 min, 96.53%).

4.6. Procedure for the preparation of intermediate compounds **15**

According to the following procedures, the key intermediate compound **10** was synthesized by a three-step process, as shown in Scheme 3.

4.6.1. Ethyl 2-amino-5-(4-methoxyphenyl)-4-methylthiophene-3-carboxylate (**13g**)

To a stirred solution of 4-methoxyphenylacetone (3.08 ml, 20 mmol), ethyl cyanoacetate (2.13 ml, 22 mmol) and sulfur (704 mg, 20 mmol) in EtOH (15 ml), diethylamine (2.07 ml, 20 mmol) was added dropwise, and the resulting was stirred at room temperature for 36 h. The reaction mixture was poured into ice-cold water and extracted with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated to dryness. The residue was then purified by flash column chromatography (n-Hx:EtOAc = 10:1) to afford the title compound **13g** as a pale yellow solid (3.79 g, 65%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.39 (br, 2H), 7.23 (d, *J* = 8.8 Hz, 2H), 6.96 (d, *J* = 8.8 Hz, 2H), 4.19 (qt, *J* = 6.8 Hz, 2H), 3.76 (s, 3H), 2.19 (s, 3H), 1.26 (t, *J* = 6.8 Hz, 3H).

4.6.2. 6-(4-Methoxyphenyl)-5-methyl-2-(thiophen-2-yl)thieno[2,3-d]pyrimidin-4-ol (**14g**)

Ethyl 2-amino-5-(4-methoxyphenyl)-4-methylthiophene-3-carboxylate **13g** (874 mg, 3.0 mmol) and 2-thiophene carbonitrile (420 μl, 4.50 mmol) were dissolved in 4 M HCl 1,4-dioxane solution (10 ml), and the resulting mixture was stirred at 110 °C for 36 h. After cooling, the reaction mixture was poured into saturated $NaHCO_3$ aqueous solution and the resulting solid was filtered. The filter cake was then washed with H_2O and EtOAc to afford the title compound **14g** as a yellow solid (950 mg, 93%). ^{1}H NMR (400 MHz, DMSO-*d6*): δ 8.25 (d, *J* = 3.6 Hz, 1H), 7.89 (d, *J* = 4.8 Hz, 1H), 7.46 (d, *J* = 8.8 Hz, 2H), 7.25 (t, *J* = 4.8 Hz, 1H), 7.07 (d, *J* = 8.8 Hz, 2H), 3.81 (s, 3H), 2.54 (s, 3H).

4.6.3. 4-Chloro-6-(4-methoxyphenyl)-5-methyl-2-(thiophen-2-yl)thieno[2,3-d]pyrimidine (**15g**)

6-(4-Methoxyphenyl)-5-methyl-2-(thiophen-2-yl)thieno[2,3-d]pyrimidin-4-ol **14g** (950 mg, 2.80 mmol) was dissolved in $POCl_3$ (5 ml) and the resulting mixture was heated for 3 h at 100 °C. After cooling to room temperature, the reaction mixture was poured into saturated $NaHCO_3$ aqueous solution and the resulting solid was filtered. The filter cake was then washed with H_2O and n-hexane to afford the title compound **15g** as a yellow solid (950 mg, 91%).

4.7. General procedure for the preparation of title compounds **17a–17k**

To a stirred solution of **15** (0.15 mmol) in THF (5 ml) hydrazine monohydrate (0.44 mmol) was added dropwise, and the resulting mixture was stirred at 80 °C for 10 h. After cooling to room temperature, the solvent was removed under reduced pressure and concentrated *in vacuo*. The residue **16** was then dissolved in $CHCl_3$ (5 ml), and citraconic anhydride (0.44 mmol) was slowly added. The mixture was heated to 80 °C and stirred for 20 h. After cooling to room temperature, the reaction mixture was extracted with $CHCl_3$ and the combined organic layer was washed with brine. After drying over Na_2SO_4, the mixture was filtered and the filtrate was concentrated under reduced pressure. The residue was then purified by flash column chromatography (n-Hx:EtOAc = 1:2) to afford the title compound **17**.

4.7.1. 1-[6-Methoxy-5-methyl-2-(thiophen-2-yl)thieno[2,3-d]pyrimidin-4-ylamino]-3-methyl-1H-pyrrole-2,5-dione (***17a***)

Yellow solid (73%); ^{1}H NMR (400 MHz, DMSO-*d*6): δ 9.43 (s, 1H), 7.65–7.61 (m, 2H), 7.12 (t, J = 6.0 Hz, 1H), 6.98 (d, J = 1.7 Hz, 1H), 4.00 (s, 3H), 2.42 (s, 3H), 2.17 (s, 3H); ^{13}C NMR (100 MHz, DMSO-*d*6): δ 170.51, 169.46, 160.29, 155.80, 154.77, 153.78, 145.45, 143.14, 130.22, 128.78, 127.86, 127.38, 113.97, 108.12, 62.93, 11.72, 11.66 ppm; ESI (*m*/*z*) 387 (MH$^+$); HRMS (ESI) calculated for $C_{17}H_{14}N_4O_3S_2$ [MH$^+$]: 387.0586, found: 387.0565; HPLC (t^R : purity = 5.61 min, 94.37%).

4.7.2. Methyl 5-methyl-4-(3-methyl-2,5-dioxo-2,5-dihydro-1H-pyrrol-1-ylamino)-2-(thiophen-2-yl)thieno[2,3-d]pyrimidine-6-carboxylate (***17b***)

White solid (58%); ^{1}H NMR (400 MHz, DMSO-*d*6): δ 9.96 (s, 1H), 7.75 (m, 2H), 7.16 (t, J = 4.8 Hz, 1H), 7.02 (s, 1H), 3.87 (s, 3H), 2.98 (s, 3H), 2.18 (s, 3H); ^{13}C NMR (100 MHz, DMSO-*d*6): δ 170.13, 169.06, 168.46, 162.78, 157.57, 157.30, 145.64, 142.32, 139.64, 132.14, 129.79, 129.12, 127.51, 122.20, 115.19, 53.00, 16.10, 11.70 ppm; ESI (*m*/*z*) 415 (MH$^+$); HRMS (ESI) calculated for $C_{18}H_{14}N_4O_4S_2$ [MH$^+$]: 415.0535, found: 415.0513; HPLC (t^R : purity = 6.04 min, 99.43%).

4.7.3. Methyl 2-[5-methyl-4-(3-methyl-2,5-dioxo-2,5-dihydro-1H-pyrrol-1-ylamino)-2-(thiophen-2-yl)thieno[2,3-d]pyrimidin-6-yl]acetate (***17c***)

Yellow solid (82%); ^{1}H NMR (400 MHz, DMSO-*d*6): δ 9.58 (s, 1H), 7.67 (d, J = 5.0 Hz, 2H), 7.13 (t, J = 4.0 Hz, 1H), 7.00 (s, 1H), 4.06 (s, 2H), 3.68 (s, 3H), 2.52 (s, 3H), 2.18 (s, 3H); ^{13}C NMR (100 MHz, DMSO-*d*6): δ 170.11, 170.02, 168.97, 166.95, 155.32, 154.43, 145.11, 142.52, 130.41, 128.45, 128.13, 127.33, 127.01, 126.87, 114.27, 52.18, 32.71, 14.29, 11.26 ppm; ESI (*m*/*z*) 429 (MH$^+$); HRMS (ESI) calculated for $C_{19}H_{16}N_4O_4S_2$ [MH$^+$]: 429.0691, found: 429.0667.

4.7.4. 1-[6-(Hydroxymethyl)-5-methyl-2-(thiophen-2-yl)thieno[2,3-d]pyrimidin-4-ylamino]-3-methyl-1H-pyrrole-2,5-dione (***17d***)

Orange solid (100%); ^{1}H NMR (400 MHz, DMSO-*d*6): δ 9.54 (s, 1H), 7.66 (m, 2H), 7.13 (m, 1H), 7.00 (s, 1H), 4.73 (s, 2H), 2.45 (s, 3H), 2.17 (s, 3H); ^{13}C NMR (100 MHz, DMSO-*d*6): δ 170.11, 169.05, 166.50, 155.54, 153.96, 145.18, 142.49, 138.88, 130.54, 128.57, 128.24, 127.11, 123.21, 114.96, 56.79, 14.33, 11.36 ppm; ESI (*m*/*z*) 387 (MH$^+$); HRMS (ESI) calculated for $C_{17}H_{14}N_4O_3S_2$ [MH$^+$]: 387.0586, found: 387.0563; HPLC (t^R : purity = 10.19 min, 99.05%).

4.7.5. 1-[6-(Methoxymethyl)-5-methyl-2-(thiophen-2-yl)thieno[2,3-d]pyrimidin-4-ylamino]-3-methyl-1H-pyrrole-2,5-dione (***17e***)

Orange solid; ^{1}H NMR (400 MHz, DMSO-*d*6): δ 9.68 (br, 1H), 7.45 (t, J = 8.0 Hz, 1H), 7.16 (m, 1H), 7.06 (d, J = 8.0 Hz, 1H), 7.01 (s, 1H), 3.69 (s, 2H), 3.31 (s, 3H), 2.65 (s, 3H), 2.19 (s, 3H); ESI (*m*/*z*) 401 (MH$^+$); HRMS (ESI) calculated for $C_{18}H_{16}N_4O_3S_2$ [MH$^+$]: 401.0742, found: 401.0741; HPLC (t^R : purity = 10.19 min, 100%).

4.7.6. 5-Methyl-4-(3-methyl-2,5-dioxo-2,5-dihydro-1H-pyrrol-1-ylamino)-2-(thiophen-2-yl)thieno[2,3-d]pyrimidine-6-carboxamide (***17f***)

Pale yellow solid (49%); ^{1}H NMR (400 MHz, DMSO-*d*6): δ 10.09 (s, 1H), 7.76 (m, 2H), 7.50 (br, 2H), 7.17 (d, J = 4.0 Hz, 1H), 7.03 (s, 1H), 2.81 (s, 3H), 2.18 (s, 3H); ^{13}C NMR (100 MHz, DMSO-*d*6): δ 169.98, 169.78, 168.90, 157.98, 157.17, 145.70, 144.16, 141.91, 132.63, 130.22, 129.21, 127.53, 114.34, 112.74, 101.26, 17.57, 11.72 ppm; ESI (*m*/*z*) 400 (MH$^+$); HRMS (ESI) calculated for $C_{17}H_{13}N_5O_3S_2$ [MH$^+$]: 400.0538, found: 400.0515; HPLC (t^R : purity = 5.72 min, 97.66%).

4.7.7. 1-[6-(4-Methoxyphenyl)-5-methyl-2-(thiophen-2-yl)thieno[2,3-d]pyrimidin-4-ylamino]-3-methyl-1H-pyrrole-2,5-dione (***17g***)

Yellow solid (70%); ^{1}H NMR (400 MHz, DMSO-*d*6): δ 9.66 (s, 1H), 7.70–7.68 (m, 2H), 7.49 (d, J = 8.8 Hz, 2H), 7.16–7.09 (m, 3H), 7.01 (d, J = 2.0 Hz, 1H), 3.82 (s, 3H), 2.62 (s, 3H), 2.18 (d, J = 2.0 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-*d*6): δ 170.42, 169.36, 166.90, 159.89, 156.07, 154.83, 145.52, 142.94, 134.65, 131.40 (2), 130.90, 128.89, 128.60, 127.44, 125.22, 124.45, 115.67, 114.89 (2), 55.74, 15.73, 11.68 ppm; ESI (*m*/*z*) 463 (MH$^+$); HRMS (ESI) calculated for $C_{23}H_{18}N_4O_3S_2$ [MH$^+$]: 463.0899, found: 463.0883; HPLC (t^R : purity = 10.06 min, 100%).

4.7.8. 1-(6-(4-Hydroxyphenyl)-5-methyl-2-(thiophen-2-yl)thieno[2,3-d]pyrimidin-4-ylamino)-3-methyl-1H-pyrrole-2,5-dione (***17h***)

Yellow solid (72%); ^{1}H NMR (400 MHz, DMSO-*d*6): δ 9.64 (s, 1H), 7.69–7.67 (m, 2H), 7.38 (d, J = 8.4 Hz, 2H), 7.14 (t, J = 4.4 Hz, 1H), 7.01 (d, J = 2.0 Hz, 1H), 6.92 (d, J = 8.4 Hz, 2H), 2.61 (s, 3H), 2.18 (s, 3H); ESI (*m*/*z*) 449 (MH$^+$); HRMS (ESI) calculated for $C_{22}H_{16}N_4O_3S_2$ [MH$^+$]: 449.0742, found: 447.0722; HPLC (t^R : purity = 4.93 min, 99.37%).

4.7.9. 1-[6-(3-Hydroxyphenyl)-5-methyl-2-(thiophen-2-yl)thieno[2,3-d]pyrimidin-4-ylamino]-3-methyl-1H-pyrrole-2,5-dione (***17i***)

Pale yellow solid; ^{1}H NMR (400 MHz, DMSO-*d*6): δ 9.79 (s, 1H), 9.66 (s, 1H), 7.70 (d, J = 4.2 Hz, 2H), 7.34 (t, J = 7.9 Hz, 1H), 7.17–7.13 (m, 1H), 7.03–6.97 (m, 2H), 6.95 (s, 1H), 6.87 (d, J = 8.1 Hz, 1H), 2.65 (s, 3H), 2.19 (s, 3H); ^{13}C NMR (100 MHz, DMSO-*d*6): δ 170.00, 168.95, 166.73, 157.70, 155.80, 154.62, 145.14, 142.50, 134.31, 133.80, 130.58, 130.16, 128.51, 128.27, 127.04, 124.64, 120.35, 116.27, 115.69, 115.22, 15.42, 11.28 ppm; ESI (*m*/*z*) 449 (MH$^+$).

4.7.10. 3-Methyl-1-[5-methyl-6-(4-nitrophenyl)-2-(thiophen-2-yl)thieno[2,3-d]pyrimidin-4-ylamino]-1H-pyrrole-2,5-dione (***17j***)

Yellow solid; ^{1}H NMR (400 MHz, DMSO-*d*6): δ 9.81 (s, 1H), 8.36 (d, J = 7.2 Hz, 2H), 7.86 (d, J = 7.6 Hz, 2H), 7.72 (d, J = 3.6 Hz, 2H), 7.16 (m, 1H), 7.03 (m, 1H), 2.69 (s, 3H), 2.20 (s, 3H); ^{13}C NMR (100 MHz, DMSO-*d*6): δ 170.34, 169.27, 167.79, 156.54, 155.57, 147.35, 145.60, 142.67, 139.89, 131.88, 131.38, 131.27 (2), 129.06, 128.97, 127.89, 127.49, 124.53 (2), 115.55, 16.05, 11.70 ppm; ESI (*m*/*z*) 478 (MH$^+$); HPLC (t^R : purity = 9.44 min, 99.70%).

4.7.11. 1-[6-(4-Hydroxybenzyl)-5-methyl-2-(thiophen-2-yl)thieno[2,3-d]pyrimidin-4-ylamino]-3-methyl-1H-pyrrole-2,5-dione (***17k***)

Brown solid; ^{1}H NMR (400 MHz, DMSO-*d*6): δ 9.42 (s, 1H), 7.66–7.63 (m, 2H), 7.12 (t, J = 4.0 Hz, 1H), 7.08 (d, J = 8.0 Hz, 2H), 7.00 (s, 1H), 6.72 (d, J = 8.0 Hz, 2H), 4.10 (s, 2H), 2.59 (s, 3H), 2.18 (s, 3H); ^{13}C NMR (100 MHz, DMSO-*d*6): δ 170.07, 169.02, 166.59, 156.05, 155.16, 154.06, 145.08, 142.62, 136.39, 130.23 (2), 129.44, 128.41, 127.93, 127.00, 124.25, 115.41 (2), 114.73, 32.31, 30.72, 14.25, 11.27 ppm; ESI (*m*/*z*) 463 (MH$^+$).

4.8. Kinase inhibition analysis

Kinase assays were conducted using Millipore's KinaseProfiler according to the protocols detailed at http://www.millipore.com/drugdiscovery/dd3/KinaseProfiler. The title compounds were tested against human FLT3 and FLT3 (D835Y) at five decreasing concentrations (e.g., 10, 1, 0.1, 0.01, and 0.01 μM) with 10 μM ATP according to the Millipore protocol, and their corresponding IC_{50} values were obtained.

4.9. Proliferation assays

Human leukemia cell lines MV4-11, THP-1, K562 and HL-60 were purchased from American Type Culture Collection (Manassas, VA).

The cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). Cell cultures were maintained at 37 °C under a humidified atmosphere of 5% CO_2.

Cell proliferation assays were performed using the Cell Proliferation Kit II (XTT) assay (Roche, Indianapolis, IN) as described by the manufacturer. Briefly, 6000 cells were seeded in 96-well plates. The next day, the cells were treated with the testing compounds. After 48 h, 50 μl of XTT labeling mixture, which was prepared by mixing 50 volumes of 1 mg/ml sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate with 1 volume of 0.383 mg/ml of N-methyldibenzopyrazine methyl sulfate, was added to each well and incubated for 2 h at 37 °C. Absorbance was measured at 490 nm with a reference wavelength of 655 nm using an ELISA plate reader (Molecular Devices, Sunnyvale, CA).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.08.001.

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