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Research paper

Structural modifications at the 6-position of thieno[2,3-*d*]pyrimidines and their effects on potency at FLT3 for treatment of acute myeloid leukemia

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ABSTRACT

Fms-like tyrosine kinase 3 (FLT3) is a well-known and important target for the treatment of acute myeloid leukemia (AML). A series of thieno[2,3-*d*]pyrimidine derivatives from a modification at the 6-position were synthesized to identify effective FLT3 inhibitors. Although compounds **1** and **2** emerged as promising FLT3 inhibitors among the synthesized compounds, both compounds exhibited poor metabolic stability in human and rat liver microsomes. Hence, further optimization was required for the discovery of FLT3 inhibitors, with a focus on improving metabolic stability. Compound **16d**, which had structural modifications of the methyl group at the 5-position and the 4-(2-methylaminoethoxy) phenyl group at the 6-position, exhibited good inhibitory activity against FLT3 and showed effective anti-proliferative activity against four leukemia cell lines, including MV4-11. Moreover, compound **16d** displayed enhanced metabolic stability. The results of this study indicated that **16d** could be a promising compound for further optimization and development as a potent FLT3 inhibitor.

1. Introduction

Acute myeloid leukemia (AML) is a heterogeneous disorder of the blood and bone marrow that is characterized by an increased number of immature myeloid cells in the bone marrow and a loss of normal hematopoietic function [1,2]. AML develops following the genetic damage of cell-surface receptors such as Ras, or of receptor tyrosine kinases such as Fms-like tyrosine kinase 3 (FLT3) and c-KIT [3]; however, FLT3 damage is a frequent genetic event in AML [4,5].

FLT3 is a member of the class III receptor tyrosine kinase family that is mainly expressed by immature hematopoietic cells and is important for hematopoiesis [6]. Further, mutations in the FLT3 gene are present in approximately 30% of AML patients [7,8]. The predominant FLT3 mutation is an internal tandem duplication mutation (FLT3-ITD) within the juxtamembrane domain that occurs in approximately 25% of AML patients. The second most common FLT3 mutation is a point mutation within the tyrosine kinase domain (FLT3-TKD) most often involving aspartic acid 835 (D835) that occurs in approximately 7% of AML patients. Both mutations result in constitutive ligand-independent active conformations that promote the activation of downstream signaling pathways [9] and result in a growth advantage for leukemia cells [10]. In addition, the overexpression of FLT3 is an unfavorable prognostic factor for overall survival in AML. In particular, FLT3-ITD causes significantly higher rates of relapse in AML patients after remission [11,12]. Therefore, the importance of FLT3 in the pathogenesis and prognosis of AML patients is both evident and profound.

Due to the importance of FLT3 in the treatment of AML, much attention has been focused on the development of FLT3 inhibitors [13]. Currently, several FLT3 inhibitors including Sorafenib [14], Tandutinib [15], Midostaurin [16], and Quizartinib [17], have

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0223-5234/

demonstrated effective inhibition of FLT3 and have advanced to clinical trials. Despite their promising results, these inhibitors also display unwanted side effects, such as overexpression of the FLT3 receptor, FLT3 ligand stimulation, and secondary mutations [18,19]. As a result, recent studies have centered in the investigation of highly potent and more selective FLT3 inhibitors with a focus on overcoming drug resistance.

We recently identified a series of thieno[2,3-*d*]pyrimidines as a new class of FLT3 inhibitors that carried a chemical modification of SPC-839, a well-known IκB kinase β (IKKβ) inhibitor, by the introduction of thiophene instead of a phenyl ring on the quinazoline structure [20]. The structure-based efforts resulted in thieno[2,3-*d*] pyrimidine derivatives **1** and **2** (Fig. 1), both of which are potent inhibitors of FLT3 with significant antiproliferative activity against MV4-11 cells, which harbor FLT3-ITD [20,21]. Unfortunately, their poor metabolic stability precluded any further evaluation (**1** and **2** showed 17.2% and 6.68% remaining after 30 min incubation with rat liver microsomes, respectively). However, compound **2** improved the inhibition of FLT3-D835Y and maintained inhibitory activity against wild type FLT3 (FLT3-wt) through the introduction of a methoxy group at the 6-position of the structure of compound **1**, which suggested that thieno[2,3-*d*] pyrimidine might require further optimization. Therefore, compound **1** served as the starting point for optimization to improve a number of properties, including potency and metabolic stability.

In this study, we describe the synthesis of thieno[2,3-*d*]pyrimidine derivatives and their biological activities. Our results indicate the identification of an optimized substitution pattern of thieno [2,3-*d*]pyrimidine for the future discovery of potent FLT3 inhibitors.

2. Chemistry

The synthetic methods for the preparation of the thieno[2,3-*d*] pyrimidine derivatives are illustrated below (Schemes 1–5). As detailed in Scheme 1, 2-aminothiophenes **3a**–**3i** were synthesized from the corresponding ketone or aldehyde, along with ethyl cyanoacetate, sulfur, and the appropriate base using the Gewald reaction [22,23]. The synthesis of the thieno[2,3-*d*]pyrimidine derivatives **8a**–**8l** was previously described (Scheme 2) [20,21]. In addition, 4-chloro-6-(4-hydroxyphenyl)thieno[2,3-*d*]pyrimidines **6a** and **6b** were synthesized by demethylation using boron tribromide (Scheme 3).

Compound **11** with an amide group at the 6-position of thieno [2,3-*d*]pyrimidine was synthesized according to Scheme 4. Ethyl thieno[2,3-*d*]pyrimidine-6-carboxylate **4d** was converted to thieno [2,3-*d*]pyrimidine-6-carboxylic acid **9** by hydrolysis using sodium hydroxide. The 4-chlorothieno[2,3-*d*]pyrimidine compound **10** was synthesized by heating thieno[2,3-*d*]pyrimidine-6-carboxylic acid **9** with phosphorous oxychloride. The amide coupling of 4-chlorothieno[2,3-*d*]pyrimidine compound **10** with dimethylethylenediamine using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) resulted in compound **11**. Furthermore, diverse alkoxy groups at the 4-position of the phenyl group at the 6-position of the thieno[2,3-*d*]pyrimidine were introduced using

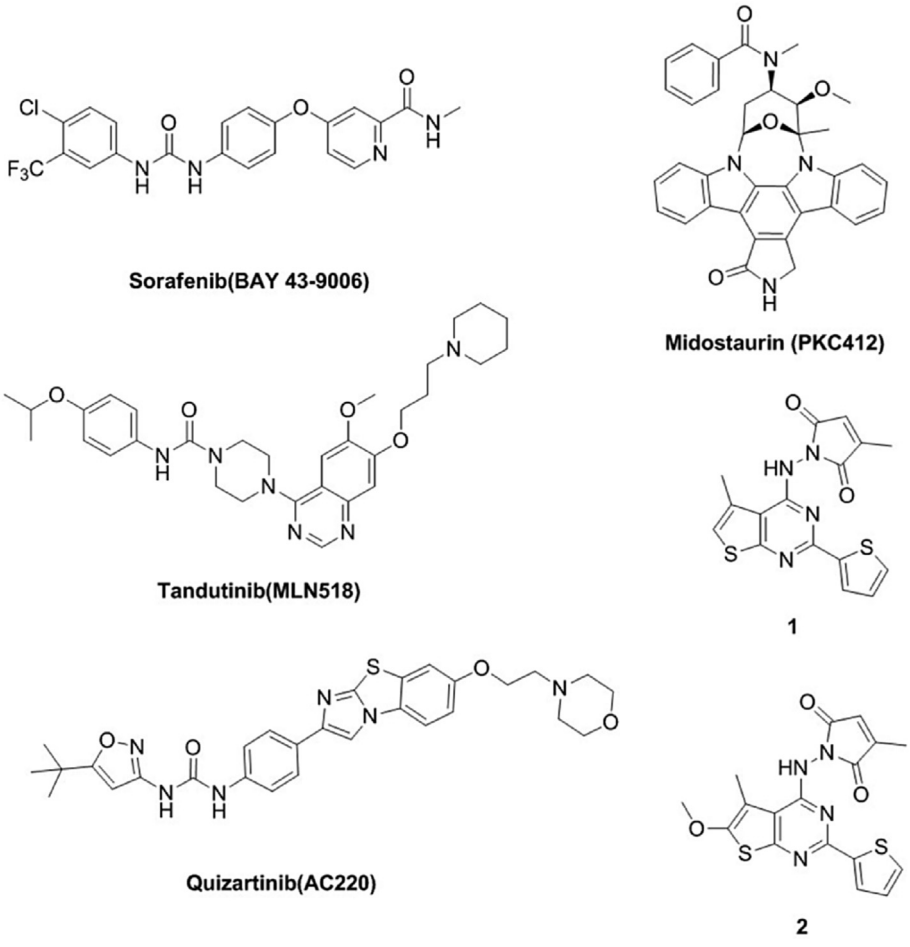
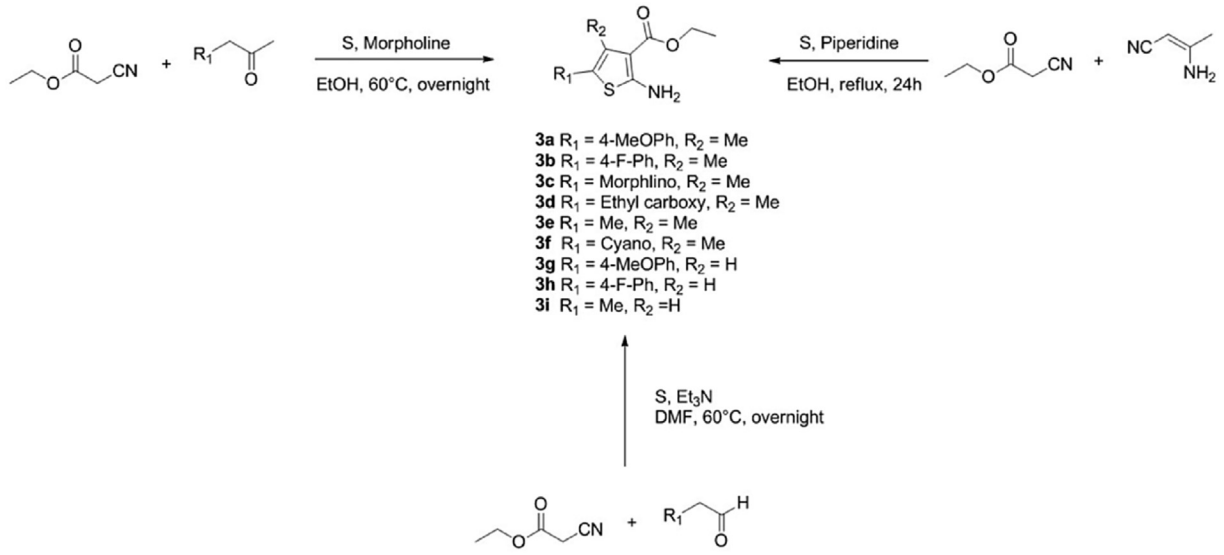
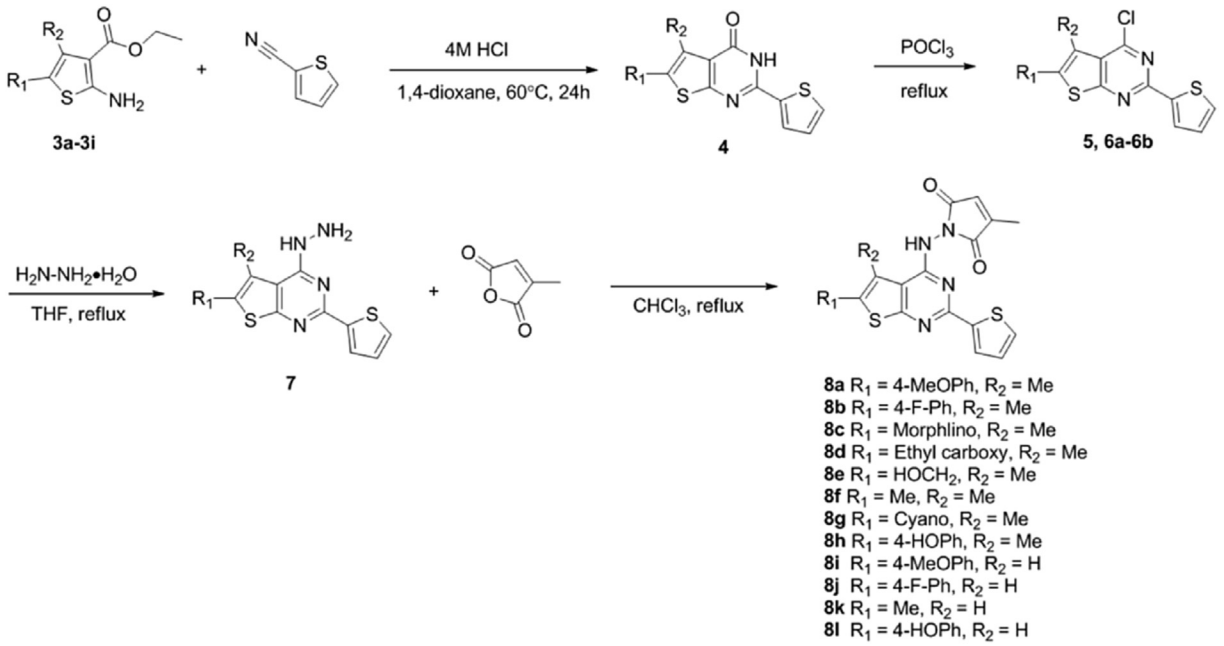


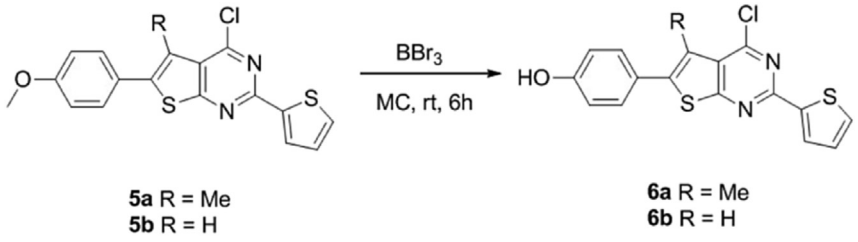
Fig. 1. Chemical structures of FLT3 inhibitors.



Scheme 1. The synthesis of 2-aminothiophene intermediates **3a**–**3i**.



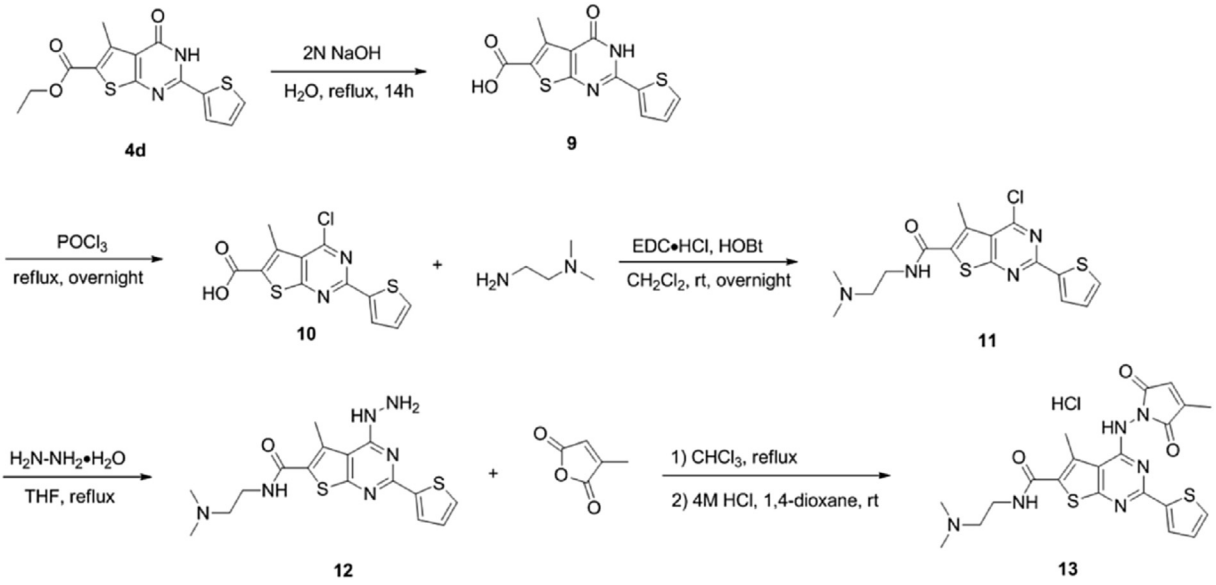
Scheme 2. The synthesis of thieno[2,3-*d*]pyrimidine derivatives **8a**–**8l**.



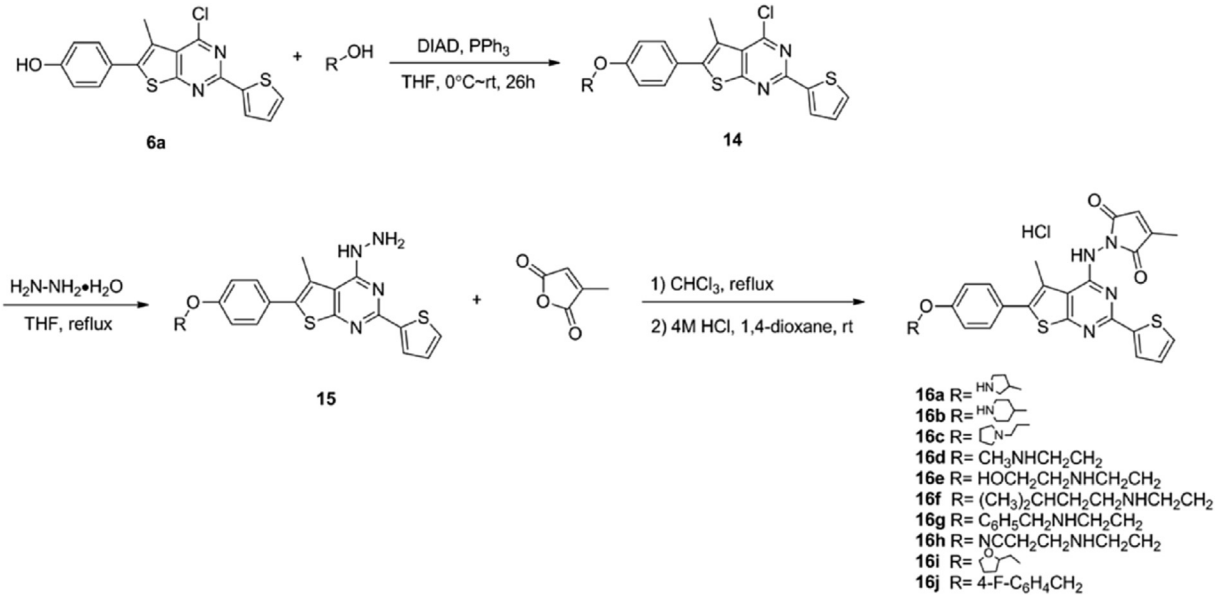
Scheme 3. The synthesis of 6-(4-Hydroxyphenyl) thieno[2,3-*d*]pyrimidine intermediates **6a** and **6b**.

the Mitsunobu reaction of 4-chlorothieno[2,3-*d*]pyrimidine **6a** with various alcohols as shown in Scheme 5.

Next, the 4-hydrazino thieno[2,3-*d*]pyrimidines **12** and **15** were obtained by refluxing 4-chlorothieno[2,3-*d*]pyrimidines **11** or **14** with hydrazine hydrate. Finally, the treatment of 4-hydrazino thieno[2,3-*d*]pyrimidines **12** or **15** with 3-methylfuran-2, 5-dione



Scheme 4. The synthesis of thieno[2,3-*d*]pyrimidine derivative **13**.



Scheme 5. The synthesis of thieno[2,3-*d*]pyrimidine derivative **16a**–**16j**.

yielded thieno[2,3-*d*]pyrimidine derivatives **13** or **16a**–**16j**.

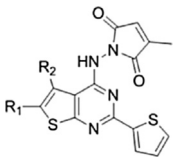
3. Results and discussion

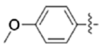
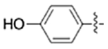
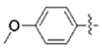
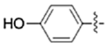
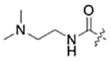
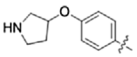
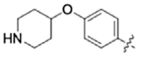
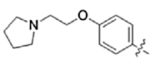
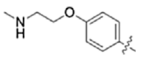
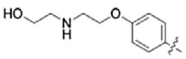
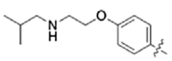
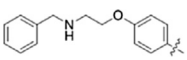
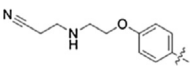
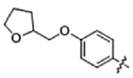
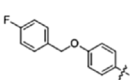
It has been previously reported that the thiophene at the 2-position and the 2, 5-dioxo-4-methylpyrroloamino group at the 4-position are important for inhibitory activity against FLT3-wt [20,21]. Therefore, our initial efforts focused on introducing diverse substituents at the 6-position of thieno[2,3-*d*]pyrimidine to improve inhibitory activity and metabolic stability.

The percentage of remaining activity of FLT3-wt at 1 μM and the inhibitory activity (IC_{50}) of the prepared compounds against FLT3-wt and FLT3-D835Y were evaluated using the KinaseProfiler™ service. Cell proliferation against four representative human leukemia cell lines, including non-FLT3 expressing cells (K562, which contained BCR-ABL expression), FLT3-wt expressing cells (THP-1 and HL-60), and FLT3-ITD harboring cells (MV4-11), was measured using an XTT assay. The known FLT3 inhibitor **AC220** (**Quizartinib**) was used as a positive control.

As shown in Table 1, the remaining activity of FLT3-wt at 1 μM of the prepared compound was evaluated for inhibitory activity. As indicated, most compounds with low remaining activity exhibited good inhibitory activity against FLT3-wt. The compounds with a small groups at the 6-position (**8f** and **8g)** exhibited < 20% remaining activity. The compounds with a relatively large group at the 6-position (**8c** and **13)** exhibited high remaining activity. However, the phenyl groups with the substituent in the para position at the 6-position (**8a**, **8b**, **8h**, and **16a**–**16i**) exhibited < 50% remaining activity. In particular, the compound with the cycloaminoalkoxy or elongated aminoethoxy group in the para position

Table 1
The remaining activity of FLT3-wt at 1 μM and inhibitory activities of thieno[2,3-*d*]pyrimidine derivatives against FLT3-wt and FLT3-D835Y.



Cpd	Structure		Remaining activity (%)[a]	IC_{50}(nM)[b]	
	R_1	R_2		FLT3-wt	FLT3-D835Y
8a		Me	49	NT[c]	NT[c]
8b		Me	41	NT[c]	NT[c]
8c		Me	50	NT[c]	NT[c]
8d		Me	52	NT[c]	NT[c]
8e		Me	20	156.0 ± 1.408	91.23 ± 3.191
8f	Me	Me	7	11.38 ± 0.849	13.68 ± 0.684
8g		Me	16	118.2 ± 1.337	74.97 ± 1.758
8h		Me	45	NT[c]	NT[c]
8i		H	7	68.64 ± 1.392	47.08 ± 1.393
8j		H	13	124.0 ± 1.145	54.37 ± 1.396
8k	Me	H	4	16.78 ± 0.882	14.66 ± 1.016
8l		H	11	75.51 ± 1.510	78.71 ± 1.114
13		Me	61	NT[c]	NT[c]
16a		Me	5	3.769 ± 2.089	29.32 ± 1.091
16b		Me	4	31.44 ± 0.664	27.20 ± 1.579
16c		Me	9	6.427 ± 0.807	16.52 ± 0.825
16d		Me	9	8.026 ± 0.594	10.15 ± 0.652
16e		Me	8	5.314 ± 0.643	0.334 ± 26.19
16f		Me	7	2.495 ± 3.621	35.86 ± 1.116
16g		Me	13	71.87 ± 1.629	9.478 ± 2.514
16h		Me	17	191.4 ± 32.82	29.41 ± 1.038
16i		Me	32	NT[c]	NT[c]
16j		Me	82	NT[c]	NT[c]
AC220			–	26.08 ± 1.378	288.7 ± 3.758

[a] The percentage of remaining activity was evaluated by the KinaseProfiler™ service at Eurofins Pharma Discovery Services. (average of duplicates).
[b] IC_{50} values were determined by the remaining activity of nine concentrations (1, 3, 10, 30, 100, 300, 1000, 3000, and 10,000 nM).
[c] NT: not tested.

of the phenyl group on the 6-position (**16a**–**16h**) exhibited < 20% remaining activity. The compounds without a methyl group at the 5-position (**8i**–**8l)** were also evaluated at 1 μM for their remaining activity against FLT3-wt. Although no significant effect on FLT3-wt inhibition was anticipated from these derivatives, the result was notably different from expected, as these derivatives exhibited <20% remaining activity. The IC_{50} against both FLT3-wt and FLT3-D835Y of the compounds with <30% remaining activity were evaluated. The data in Table 1 demonstrates that a methyl group (**8f** and **8k**) and a cycloaminoalkoxy or elongated aminoethoxy group in the para position of the phenyl group at the 6-position (**16a**–**16f**) provided effective inhibitory activities against FLT3-wt. In addition, the absence of substituents at the 5-position (**8i**–**8l**) displayed enhanced inhibitory activity against FLT3-wt. As a result, the compounds with <10% remaining activities of FLT3-wt (**8f**, **8k**, **16a**, and **16c**–**16f**) exhibited higher inhibitory activities against FLT3-wt than **AC220** (26 nM), with the exception of **16b** (31 nM).

The inhibitory activities against FLT3-D835Y of the compounds with <20% remaining activities of FLT3-wt (**8e**–**8g**, **8i**–**8l**, **16a**–**16h**) were confirmed to be higher than with **AC220** (288 nM). Unlike the inhibitory activities against FLT3-wt, a hydroxymethyl (**8e**), cyano (**8g**), 4-(piperidine-4-yl)phenyl (**16b**), 4-{2-(*N*-benzyl)amino-ethoxy}phenyl (**16g**), or 4-{2-(*N*-cyanoethyl)aminoethoxy}phenyl group (**16h**) at the 6-position exhibited good inhibitory activity against FLT3-D835Y with IC_{50} values < 100 nM. Moreover, when comparing the IC_{50} values between FLT3-wt and FLT3-D835Y, these derivatives were more active against FLT3-D835Y than FLT3-wt. Aside from this finding, the structure-activity relationships following the evaluation of the prepared compounds against FLT3-D835Y were similar to the inhibitory activity against FLT3-wt. As a result, the compounds with <10% remaining activity of FLT3-wt (**8f**, **8k**, and **16a**–**16f**) showed strong inhibitory activity against FLT3-wt, as well as its drug-resistant mutant FLT3-D835Y, with IC_{50} between 0.3 and 36 nM. Among them, the compound with a methyl group at the 5-position and a 2-(*N*-hydroxyethyl)aminoethoxy group at the 6-position (**16e**) displayed significant inhibitory activities against both FLT3-wt and FLT3-D835Y (5 and 0.3 nM, respectively).

The antiproliferative activities (GI_{50}) of the compounds that displayed the inhibitory activity against FLT3-wt and had IC_{50} values < 100 nM (**8f**, **8i**, **8k**, **8l**, and **16a**–**16f**) against the four leukemia cell lines are presented in Table 2. Most of the prepared derivatives exhibited higher antiproliferative activity against FLT3-wt expressing cells (THP-1 and HL-60) and FLT3-ITD harboring cells (MV4-11) than those treated with **AC220** (2.125, 1.488, and 1.103 μM, respectively), except compounds **8i** and **16e**. Although compound **16e** demonstrated significant inhibitory activities against FLT3-wt and FLT3-D835Y, it did not exhibit remarkable antiproliferative activity against the leukemia cells. Furthermore, the compounds without a methyl group at the 5-position (**8i**, **8k**, and **8l**) did not provide significantly improved antiproliferative activity. Thus, the methyl group at the 5-position could provide effective antiproliferative activity against the leukemia cells. The compound with a methyl group at the 6-position (**8f**) had the highest antiproliferative activity against most leukemia cells, including THP-1, HL-60, and MV4-11 cells (0.222, 0.127, and 0.191 μM, respectively). Moreover, the compound with the cycloaminoalkoxy or elongated aminoethoxy group in the para position of the phenyl group at the 6-position (**16a**–**16d** and **16f**) demonstrated effective antiproliferative activity against MV4-11 cells with GI_{50} values < 0.600 μM.

However, the compounds with small or simple groups at the 6-positions typically showed low metabolic stability (Table 3). Compound **8f** exhibited the highest antiproliferative activity against FLT3-ITD harboring cells (MV4-11) but showed a low percentage of remaining human and rat liver microsomes (32.4% and 22.7% remaining after the 30 min incubation, respectively). Fortunately, the compounds with a cycloaminoalkoxy or elongated aminoethoxy group in the para position of the phenyl group at the 6-position (**16a**–**16f**) exhibited enhanced metabolic stabilities in human and rat liver microsomes. As shown in Table 3, most of the compounds with a para-substituted phenyl group at the 6-position (**8b**, **8h**, **8j**, **8l**, and **16a**–**16i**) displayed slightly enhanced metabolic stability with a remaining percentage of >40% after the 30 min incubation with human liver microsomes. This result suggested that the presence of a para-substituted phenyl group at the 6-position partially blocked any metabolic activity. Among them, the compound with a methyl group on the 5-position and a 4-(2-methylaminoethoxy) phenyl group at the 6-postion (**16d**) exhibited relatively high metabolic stability (79.8% and 67.1% remaining after the 30 min incubation in the human and rat liver microsomes, respectively). Despite a methyl group at the 6-position, which contributed to effective FLT3 inhibition and antiproliferative

Table 2
Cell proliferation assay of thieno[2,3-*d*]pyrimidine derivatives.

Cpd	GI_{50}(μM)[a]			
	K562[b]	THP-1[c]	HL-60[d]	MV4-11[e]
8f	0.105	0.222	0.127	0.191
8i	1.090	1.271	0.682	1.703
8k	1.005	1.236	0.422	1.055
8l	0.506	0.765	0.336	0.927
16a	0.315	0.227	0.214	0.366
16b	0.425	0.46	0.377	0.58
16c	1.045	0.636	1.262	0.585
16d	0.564	0.559	0.25	0.540
16e	1.413	0.843	1.345	1.132
16f	0.489	0.449	0.598	0.278
AC220	2.638	2.125	1.488	1.103

[a] Growth inhibition was measured by the XTT assay (average of four replicates).
[b] Non-FLT3 expressing cells.
[c] FLT3-wt expressing cells.
[d] FLT3-wt expressing cells.
[e] FLT3-ITD harboring cells.

Table 3
The *in vitro* metabolic stability of thieno[2,3-*d*]pyrimidine derivatives.

Cpd	*In vitro* metabolic stability(%)[a]	
	HLM[b]	RLM[c]
8b	39.52	54.46
8e	24.62	20.45
8f	32.37	22.69
8g	52.66	26.08
8h	71.84	28.84
8j	61.04	26.91
8k	58.82	32.63
8l	74.14	28.21
16a	50.79	34.21
16b	52.35	47.84
16c	72.67	41.52
16d	79.79	67.09
16e	91.29	ND[d]
16f	66.15	39.68
16g	86.23	30.41
16h	52.04	32.13
16i	41.73	41.03
16j	66.86	35.65

[a] Remaining percentage of the compound after the 30 min incubation (average of triplicate).
[b] Human liver microsomes.
[c] Rat liver microsomes.
[d] ND: Remaining compound was not detected after the 30 min incubation.

activities against the leukemia cells, this compound showed poor metabolic stability. However, the cycloaminoalkoxy or elongated aminoethoxy group in the para position of the phenyl group at the 6-position displayed enhanced metabolic stability but no significantly improved antiproliferative activities against the leukemia cells. Compound **16d** exhibited higher inhibitory activities against FLT3-wt and FLT3-D835Y (8 and 10 nM, respectively) and effective antiproliferative activity against MV4-11 cells (0.540 μM).

Interestingly, all of the prepared compounds exhibited higher antiproliferative activity against non-FLT3 expressing cells (K562) than against AC220 (2.638 μM). In particular, compounds **8f**, **8l**, **16a**, **16b**, **16d**, and **16f** exhibited good antiproliferative activities against K562 cells with GI_{50} values ranging from 105 to 564 nM. The K562 cells express high BCR-ABL, as well as Abl1 kinase levels. Accordingly, compound **16d** was tested against the remaining activities of 56 kinases at a 10 μM to determine if **16d** inhibited any other kinases (Fig. 2). In accordance with the remaining activities of the 56 kinases, FLT3-wt and FLT3-D835Y were identified as the primary target of compound **16d**. Moreover, as shown in Table 4, compound **16d** had a moderate effect on Abl1 and Tie2 kinases with IC_{50} values of 2.88 μM and 9.75 μM, respectively. These results indicated that thieno[2,3-*d*]pyrimidine derivatives did not selectively inhibit FLT3 kinases or Abl1 kinase activity, but showed good antiproliferative activity against K562 cells. Furthermore, AC220 inhibited effectively proliferation of K562 and HL60 cells unlike the previous results, which showed no antiproliferative activity [24]. It is thought to be arisen from the differences of the initial density of cell seeding in the proliferation assays.

The compound with the highest metabolic stability (**16d**) was further evaluated in a rat pharmacokinetic study (Table 5). Compound **16d** demonstrated acceptable solubility in phosphate buffer solution (2.96 μM) and relatively favorable oral bioavailability (17.04%). Based on these results, **16d** appeared to be promising lead compound for further optimization and potential development as a

Table 4
The inhibitory activities and remaining activities against Abl1 and Tie2 kinases of **8f**, **16a**, and **16d**.

Compound	IC_{50}(μM)[b]	
	Abl1	Tie2
8f	29% at 10 μM[a]	32% at 10 μM[a]
16a	26% at 10 μM[a]	25% at 10 μM[a]
16d	2.877 ± 0.501	9.750 ± 12.50

[a] The percentage of remaining activity was evaluated by the KinaseProfiler™ service at Eurofins Pharma Discovery Services. (average of duplicates).
[b] IC_{50} values were determined by the remaining activity of nine concentrations (1, 3, 10, 30, 100, 300, 1000, 3000, and 10,000 nM).

Table 5
Rat pharmacokinetic study and solubility of **16d**.

	IV[a] (n = 4, 5 mg/kg)	PO[b] (n = 4, 30 mg/kg)
T_{max}[c] (hr)	–	0.25
C_{max}[d] (μg/mL)	–	0.42
$AUC_{0\text{-inf}}$[e] (hr·μg/mL)	2.125	2.173
CL[f] (L/hr/kg)	2.197	–
V_{ss}[g] (L/kg)	8.217	–
$t_{1/2}$[h] (hr)	7.610	15.15
F[i] (%)	–	17.04
Solubility[j] (μM)	2.96	

[a] Intravenous.
[b] Oral administration.
[c] Time to maximum plasma concentration.
[d] Maximum plasma concentration after oral administration.
[e] Area under the curve.
[f] Clearance.
[g] Volume of distribution at steady state.
[h] Elimination half life.
[i] Bioavailability.
[j] The solubility was tested in 100 mM pH 7.4 phosphate buffer solution.

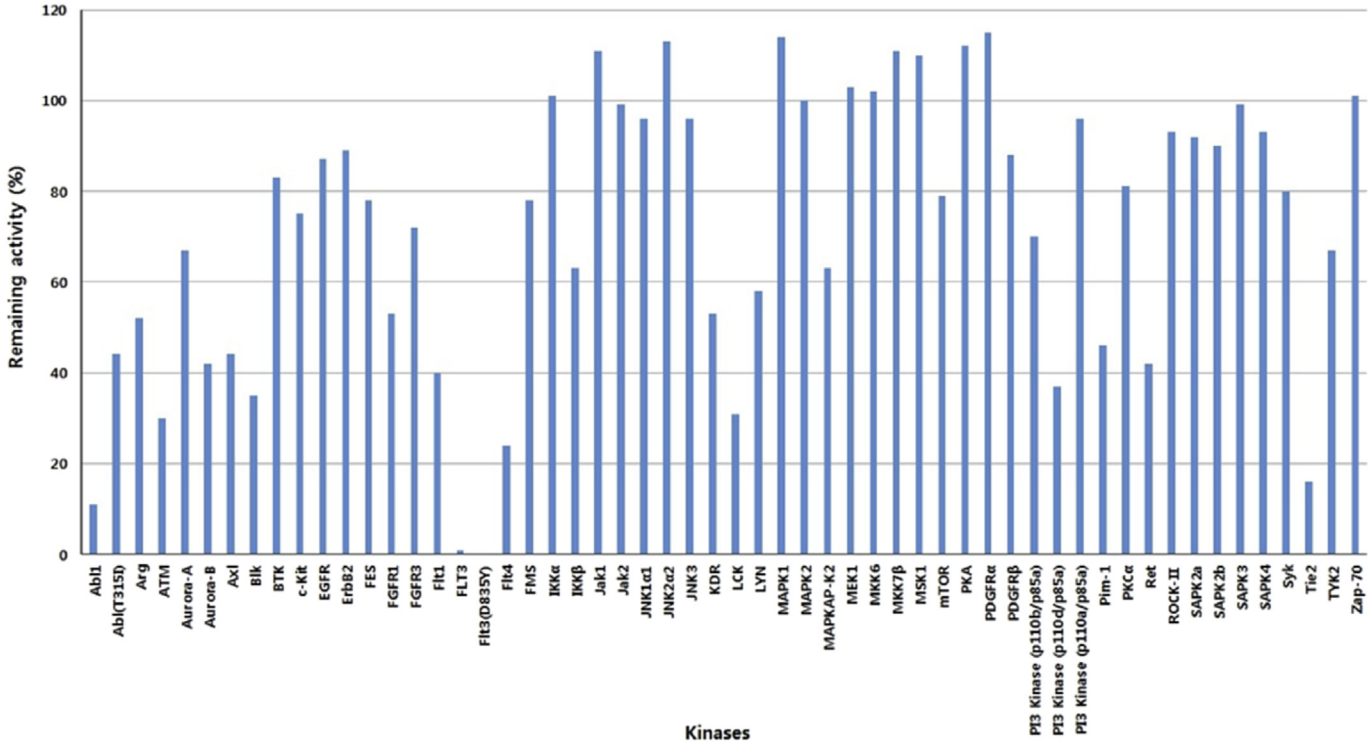


Fig. 2. Percentage of **16d** kinase activities against 56 kinases at 10 μM.

FLT3 inhibitor. Taken together, structural modifications at the 6-position led to improved potency and metabolic stability, which provided a good opportunity for further optimization of the PK profiles.

4. Conclusions

We prepared thieno[2,3-*d*]pyrimidine derivatives to serve as potential FLT3 inhibitors for the treatment of AML. The structural modification of thieno[2,3-*d*]pyrimidines was determined by the results of kinase and cell proliferation assays; however, metabolic stability was also considered. As a result, a methyl group at the 5-position and a 4-(2-methylaminoethoxy) phenyl group at the 6-position were associated with effective inhibition of FLT3 kinase activity, as well as inhibition of the growth of several leukemia cells with a relatively high metabolic stability. On the basis of the inhibitory activity, antiproliferative activity and *in vitro* metabolic stability, compound **16d** we have generated appears to be a well-balanced potential FLT3 inhibitor. We expect that **16d** will provide the structural basis for further optimization and could prove to be a promising therapeutic agent for the treatment of AML.

5. Experimental

5.1. Chemistry

Reagents and solvents were purchased and used without further purification. The reaction progress was determined by thin-layer chromatography (TLC) on silica gel 60G F_{254} plates (Merck, Mumbai, India). Flash column chromatography was performed on silica (Merck EM9385, 230–400 mesh). The spots were visualized using UV light (254 nm). ^{1}H NMR spectra were recorded at 400 MHz on a Varian 400 Mercury Plus spectrometer (Varian, Palo Alto, CA, USA). Chemical shifts are reported in ppm (δ) relative to tetramethylsilane (TMS), which was used as an internal standard. Multiplicities are given as s (singlet), d (doublet), dd (double-doublet), t (triplet), q (quadruplet), m (multiplet), and br s (broad signal). The synthesis of **8a**, **8e**, and **8h** were previously reported [20,21]. Low-resolution mass spectra and high-resolution spectra were obtained on a Shimadzu LCMS-2010EV system (Kyoto, Japan) and an Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) liquid chromatography-mass spectrometry (LC/MS) system (Santa Clara, CA, USA) with electrospray ionization (ESI).

5.2. General procedure for the synthesis of 2-aminothiophene intermediate ***3a*–*3e***

The synthesis of 2-aminothiophenes **3a**–**3e** was previously reported [20,21].

5.2.1. Ethyl 2-amino-5-morpholino-4-methylthiophene-3-carboxylate (***3c***)

A mixture of ethyl cyanoacetate (1.54 g, 13.6 mmol), acetone (0.79 g, 13.6 mmol), and sulfur (3.5 g, 13.6 mmol) in ethanol (15 mL) was stirred at 60 °C. Morpholine (2.37 g, 27.2 mmol) was added dropwise over 10 min. The mixture was stirred at 60 °C overnight and filtered. The filtrate was cooled and evaporated under reduced pressure. The title compound **3c** (yellow solid, 120 mg, 3.3%) was obtained by column chromatography (EtOAc: hexane = 1: 10), ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.23 (s, 2H), 4.11 (q, J = 6.8 Hz, 2H), 3.62 (t, J = 4.4 Hz, 4H), 2.60 (t, J = 4.4 Hz, 4H), 2.08 (s, 3H), 1.20 (t, J = 7.0 Hz, 3H).

5.2.2. Ethyl 2-amino-5-cyano-4-methylthiophene-3-carboxylate (***3f***)

A mixture of ethyl cyanoacetate (1.25 g, 11.0 mmol), 3-aminocrotonitrile (0.91 g, 11.0 mmol), sulfur (1.4 g, 11.0 mmol), and piperidine (0.94 g, 11.0 mmol) in ethanol (15 mL) was refluxed for 24 h then cooled and concentrated under reduced pressure. Compound **3d** (pale yellow, 0.53 g, 22.9%) was obtained by column chromatography (EtOAc: hexane = 1: 7), ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.10 (s, 2H), 4.18 (q, = 7.2 Hz, 2H), 2.33 (s, 3H), 1.24 (t, J = 7.0 Hz, 3H).

5.3. General procedure for the synthesis of 2-aminothiophenes ***3g*–*3i***

A mixture of ethyl cyanoacetate (13.6 mmol), sulfur (13.6 mmol) and triethylamine (13.6 mmol) in dimethylformamide (DMF; 15 mL) was stirred at 50 °C. A solution of the corresponding aldehyde (13.6 mmol) in DMF (10 mL) was added dropwise over 20 min. The mixture then was stirred at 50 °C overnight. The reaction mixture was cooled, poured into water, and the aqueous layer was extracted with ethyl acetate. The organic layer was separated, washed with brine, and dried over Na_2SO_4 then concentrated under reduced pressure. The title compound was obtained by column chromatography (EtOAc: hexane = 1: 15).

5.3.1. Ethyl 2-amino-5-(4-methoxyphenyl)lthiophene-3-carboxylate(***3g***)

The parameters for **3g** were: pale yellow solid, 60% yield, ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.37 (d, J = 8.8 Hz, 2H), 7.01 (s, 1H), 6.87 (d, J = 8.8 Hz, 2H), 5.95 (br, 2H), 4.29 (q, J = 6.8 Hz, 2H), 3.81 (s, 3H), 1.37 (t, J = 6.8 Hz, 3H).

5.3.2. Ethyl 2-amino-5-(4-fluorophenyl)lthiophene-3-carboxylate (***3h***)

The parameters for **3h** were: yellow solid, 26% yield, ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.45–7.42 (m, 4H), 7.15 (s, 1H), 7.12 (t, J = 8.8 Hz, 2H), 4.17 (q, J = 6.8 Hz, 2H), 1.24 (t, J = 7.2 Hz, 3H).

5.3.3. Ethyl 2-amino-5-methylthiophene-3-carboxylate (***3i***)

The parameters for **3i** were: yellow solid, 17% yield, ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.06 (s, 2H), 6.45 (s, 1H), 4.10 (q, J = 7.2 Hz, 2H), 1.19 (t, J = 7.0 Hz, 3H).

5.4. General procedure for the synthesis of 4-chlorothieno[2,3-d] pyrimidine intermediate ***5***

A mixture of the corresponding ethyl 2-aminothiophene **3** (0.48 mmol) and thiophene-2-carbonitrile (0.62 mmol) in 4M HCl 1,4-dioxane solution (6 ml) was stirred at 60 °C under argon gas overnight. The reaction mixture was then cooled and concentrated under reduced pressure and the resulting residue was triturated with diethyl ether. The resulting thienopyrimidin-4-one **4** was dried under vacuum and used directly in the next step. A mixture of the corresponding thienopyrimidin-4-one **4** in phosphoryl chloride (4 ml) was refluxed for 12 h then the reaction mixture was cooled and concentrated under reduced pressure. The resulting residue was diluted with CH_2Cl_2/saturated aqueous $NaHCO_3$. The organic layer was separated, washed with brine, dried over Na_2SO_4 and concentrated under reduced pressure. The title compound **5** was then obtained by column chromatography (EtOAc: hexane = 1: 20).

5.4.1. 4-Chloro-5-methyl-6-morpholino-2-(thiophen-2-yl)thieno[2,3-d]pyrimidine (***5c***)

The parameters for **5c** were: yellow solid, 32% yield, ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.90 (d, J = 3.6 Hz, 1H), 7.76 (d, J = 4.8 Hz,

1H), 7.18 (m, 1H), 3.75 (t, J = 4.4 Hz, 4H), 2.97 (t, J = 4.4 Hz, 4H), 2.44(s, 3H).

*5.4.2. 4-Chloro-6-cyano-5-methyl-2-(thiophen-2-yl)thieno[2,3-d] pyrimidine (**5f**)*

The parameters for **5f** were: white solid, 37% yield, ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.05 (d, J = 3.6 Hz, 1H), 7.92 (d, J = 5.2 Hz, 1H), 7.25 (m, 1H), 2.75 (s, 3H).

*5.4.3. 4-Chloro-6-(4-methoxyphenyl)-2-(thiophen-2-yl)thieno[2,3-d]pyrimidine (**5g**)*

The parameters for **5g** were: yellow solid, 92% yield, ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.06 (dd, J = 3.6, 1.2 Hz, 1H), 7.65 (d, J = 8.8 Hz, 2H), 7.50 (dd, J = 5.2, 1.2 Hz, 1H), 7.41 (s, 1H), 7.16 (dd, J = 5.2, 3.6 Hz, 1H), 6.98 (d, J = 8.8 Hz, 2H), 3.87 (s, 3H).

*5.5. General procedure for the synthesis of 4-Chloro-6-(4-hydroxy) phenylthieno[2,3-d]pyrimidine intermediate (**6a** and **6b**)*

A solution of the corresponding 4-chlorothienopyrimidine **5a** and **5b** (1.32 mmol) in CH_2Cl_2 was stirred at room temp. A 1M boron tribromide hexane solution (8 ml) was added dropwise to the reaction mixture followed by stirring at room temperature for 6 h. The reaction mixture was diluted with ethyl acetate/saturated aqueous NH_4Cl. The organic layer was separated, washed with brine, dried over Na_2SO_4 and concentrated under reduced pressure. The title compound **6** was then obtained by column chromatography (EtOAc: hexane = 1: 3).

*5.5.1. 4-Chloro-6-(4-hydroxyphenyl)-5-methyl-2-(thiophen-2-yl) thieno[2,3-d]pyrimidine (**6a**)*

The parameters for **6a** were: yellow solid, 81% yield, ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.93 (s, 1H), 7.88–7.85 (m, 1H), 7.74–7.73 (m, 1H), 7.29 (d, J = 8.4 Hz, 2H), 7.14–7.12 (m, 1H), 6.87 (d, J = 8.4 Hz, 2H), 2.43 (s, 3H).

*5.5.2. 4-Chloro-6-(4-hydroxyphenyl)-2-(thiophen-2-yl)thieno[2,3-d]pyrimidine (**6b**)*

The parameters for **6b** were: yellow solid, 92% yield, ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.0 (s, 1H), 7.87 (m, 1H), 7.74–7.73 (m, 1H), 7.61 (d, J = 8.0 Hz, 2H), 7.54 (s, 1H), 7.14 (m, 1H), 6.83 (d, J = 8.4 Hz, 2H).

*5.6. General procedure for the synthesis of thieno[2,3-d]pyrimidine derivatives **8a**–**8k***

A solution of the corresponding 4-chlorothienopyrimidine **5, 6a**–**6b** (0.22 mmol) in tetrahydrofuran (THF) was stirred at room temperature. Hydrazine hydrate (0.67 mmol) was added dropwise and the reaction mixture was refluxed overnight. The reaction mixture was then cooled and concentrated under reduced pressure and the resulting solid compound **7** was used directly in the next step. A mixture of the corresponding hydrazino-intermediate **7** and citraconic anhydride (0.67 mmol) was refluxed overnight. The reaction mixture was cooled and concentrated under reduced pressure. The title compound **8** was then obtained by column chromatography (EtOAc: hexane = 1: 2).

*5.6.1. 1-[6-(4-Fluorophenyl)-5-methyl-2-(thiophen-2-yl)thieno [2,3-d]pyrimidin-4-ylamino]-3-methyl-1H-pyrrole-2,5-dione (**8b**)*

The parameters for **8b** were: yellow solid, ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.65 (s, 1H), 7.67–7.66 (m, 2H), 7.61–7.58 (m, 2H), 7.36 (t, J = 8.4 Hz, 2H), 7.12 (m, 1H), 6.98 (m, 1H), 2.59 (s, 3H), 2.16 (s, 3H); ESI (m/z) 451 (MH^+); HRMS (ESI) calculated for $C_{22}H_{15}FN_4O_2S_2$ [MH^+]: 451.0693, detected: 451.0685.

*5.6.2. 1-[5-Methyl-6-morpholino-2-(thiophen-2-yl)thieno[2,3-d] pyrimidin-4-ylamino]-3-methyl-1H-pyrrole-2,5-dione (**8c**)*

The parameters for **8c** were: yellow solid, ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.43 (s, NH), 7.61–7.60 (m, 2H), 7.08–7.07 (m, 1H), 6.95 (m, 1H), 3.74–3.73 (m, 4H), 2.88 (m, 4H), 2.48 (s, 3H), 2.14 (s, 3H); ESI (m/z) 442 (MH^+); HRMS (ESI) calculated for $C_{20}H_{19}N_5O_3S_2$ [MH^+]: 442.1002, detected: 442.0999.

*5.6.3. Ethyl 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 (**8d**)*

The parameters for **8d** were: pale yellow solid, ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.87 (s, 1H), 7.72–7.71 (m, 2H), 7.14–7.11 (m, 1H), 6.99–6.98 (m, 1H), 4.31 (q, J = 6.8 Hz, 2H), 2.94 (s, 3H), 2.15 (s, 3H), 1.29 (t, J = 7.2 Hz, 3H); ESI (m/z) 429 (MH^+); HRMS (ESI) calculated for $C_{19}H_{16}N_4O_4S_2$ [MH^+]: 429.0686, detected: 442.0682.

*5.6.4. 1-[5,6-Dimethyl-2-(thiophen-2-yl)thieno[2,3-d]pyrimidin-4-ylamino]-3-methyl-1H-pyrrole-2,5-dione (**8f**)*

The parameters for **8f** were: pale yellow solid, ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.46 (s, 1H), 7.62 (d, J = 4.4 Hz, 2H), 7.10–7.07 (m, 1H), 6.95 (m, 1H), 2.48 (s, 3H), 2.41 (s, 3H), 2.14 (s, 3H); ESI (m/z) 371 (MH^+); HRMS (ESI) calculated for $C_{17}H_{14}N_4O_2S_2$ [MH^+]: 371.0631, detected: 371.0623.

*5.6.5. 1-[6-Cyano-5-methyl-2-(thiophen-2-yl)thieno[2,3-d] pyrimidin-4-ylamino]-3-methyl-1H-pyrrole-2,5-dione (**8g**)*

The parameters for **8g** were: brown solid, ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.00 (s, 1H), 7.76–7.73 (m, 2H), 7.15–7.13 (m, 1H), 6.99 (m, 1H), 2.78 (s, 3H), 2.15 (s, 3H); ESI (m/z) 382 (MH^+); HRMS (ESI) calculated for $C_{17}H_{11}N_5O_2S_2$ [MH^+]: 382.0427, detected: 371.0418.

*5.6.6. 1-[6-(4-Methoxyphenyl)-2-(thiophen-2-yl)thieno[2,3-d] pyrimidin-4-ylamino]-3-methyl-1H-pyrrole-2,5-dione (**8i**)*

The parameters for **8i** were: yellow solid, ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.6 (s, 1H), 7.82 (s, 1H), 7.66–7.61 (m, 4H), 7.10 (m, 1H), 7.06 (d, J = 4.4 Hz, 2H), 6.97 (m, 1H), 3.70 (s, 3H), 2.15 (s, 3H); ESI (m/z) 449 (MH^+); HRMS (ESI) calculated for $C_{22}H_{16}N_4O_3S_2$ [MH^+]: 449.0737, detected: 449.0734.

*5.6.7. 1-[6-(4-Fluorophenyl)-2-(thiophen-2-yl)thieno[2,3-d] pyrimidin-4-ylamino]-3-methyl-1H-pyrrole-2,5-dione (**8j**)*

The parameters for **8j** were: yellow solid, ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.7(s, 1H), 7.91(s, 1H), 7.73(dd, J = 5.2, 4.2 Hz, 2H), 7.67–7.65(m, 2H), 7.34 (t, J = 8.6 Hz, 2H), 7.12–7.09 (m, 1H), 6.98–6.97 (m, 1H), 2.15 (s, 3H); ESI (m/z) 437 (MH^+); HRMS (ESI) calculated for $C_{21}H_{13}FN_4O_2S_2$ [MH^+]: 437.0537, detected: 437.0534.

*5.6.8. 1-[6-Methyl-2-(thiophen-2-yl)thieno[2,3-d]pyrimidin-4-ylamino]-3-methyl-1H-pyrrole-2,5-dione (**8k**)*

The parameters for **8k** were: pale yellow solid, ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.5 (s, 1H), 7.62–7.61 (m, 2H), 7.28 (s, 1H), 7.09–7.07 (m, 1H), 6.95 (m, 1H), 2.56 (s, 3H), 2.13 (s, 3H); ESI (m/z) 357 (MH^+); HRMS (ESI) calculated for $C_{16}H_{12}N_4O_2S_2$ [MH^+]: 357.0474, detected: 357.0471.

*5.6.9. 1-[6-(4-Hydroxyphenyl)-2-(thiophen-2-yl)thieno[2,3-d] pyrimidin-4-ylamino]-3-methyl-1H-pyrrole-2,5-dione (**8l**)*

The parameters for **8l** were: yellow solid, ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.73 (br, 1H), 7.84 (s, 1H), 7.69–7.67 (m, 2H), 7.56 (dd, J = 8.8 Hz, 2.0 Hz, 2H), 7.16–7.12 (m, 1H), 7.01 (d, J = 2.0 Hz, 1H), 6.93 (dd, J = 8.8, 2.0 Hz, 2H), 2.18 (d, J = 2.0 Hz, 3H); ESI (m/z) 435 (MH^+); HRMS (ESI) calculated for $C_{21}H_{14}N_4O_3S_2$ [MH^+]: 435.0580, detected: 435.0560.

5.7. 5-Methyl-4-oxo-2-(thiophen-2-yl)-3,4-dihydrothieno[2,3-d] pyrimidine-6-carboxylic acid (**9**)

A solution of the thieno[2,3-*d*]pyrimidine-4-one **4d** (0.48 g, 1.5 mmol) in 2 N aqueous NaOH (30 ml) was refluxed for 14 h. The reaction mixture was cooled and washed with ethyl acetate and the aqueous layer was neutralized with 6 N aqueous HCl. The precipitates were collected by filtration and washed with water, which yielded the title compound **9** (white solid, 0.41 g, 94%), ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.28 (d, J = 4.0 Hz, 1H), 7.94 (d, J = 4.8 Hz, 1H), 7.26–7.24 (m, 1H), 2.82 (s, 3H).

5.7.1. 4-Chloro-5-Methyl-2-(thiophen-2-yl)-3,4-dihydrothieno[2,3-d]pyrimidine-6-carboxylic acid-{(2-dimethyl-amino)ethyl}amide (**11**)

A mixture of the 4-oxo-thieno[2,3-*d*]pyrimidine-6-carboxylic acid **9** (350 mg, 1.2 mmol) in phosphoryl chloride was refluxed overnight and the reaction mixture was cooled and concentrated under reduced pressure. The resulting residue was diluted with CH_2Cl_2/saturated aqueous $NaHCO_3$. The organic layer was separated, washed with brine, dried over Na_2SO_4 and concentrated under reduced pressure. The resulting 4-chloro-thienopyrimidin-6-carboxylic acid **10** was dried under vacuum and used directly in the next step. A mixture of 4-chloro-thienopyrimidin-6-carboxylic acid **10** (60 mg, 0.20 mmol), 1-(3-methylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl, 48 mg, 0.26 mmol), 1-hydroxybenzotriazole hydrate (2 mg, 0.02 mmol) in CH_2Cl_2 was stirred at room temperature. *N, N*-dimethylethylenediamine (24 μL, 0.22 mmol) was added slowly dropwise and the reaction mixture was stirred for 2 h then concentrated under reduced pressure. The title compound **11** (yellow solid, 60 mg, 80%) was obtained by column chromatography (CH_2Cl_2: MeOH = 9: 1), ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.10 (d, J = 4.0 Hz, 1H), 7.55 (d, J = 4.8 Hz, 1H), 7.19–7.17 (m, 1H), 6.84 (br, 1H), 3.54 (q, J = 5.6 Hz, 2H), 2.91 (s, 3H), 2.55 (t, J = 5.6 Hz, 2H), 2.29 (s, 6H).

5.8. General procedure for the synthesis of 4-Chlorothieno[2,3-d] pyrimidine derivatives **14**

A mixture of the 4-chlorothieno[2,3-*d*]pyrimidine **6a** (0.42 mmol), triphenylphosphine (PPh_3; 0.84 mmol) and the corresponding alcohol intermediate (0.42 mmol) in THF was stirred at 0 °C. Diisopropyl azodicarboxylate (0.84 mmol) was added dropwise over 20 min and the reaction mixture was slowly warmed to room temperature, stirred overnight and concentrated under reduced pressure. The title compound **14** was then obtained by column chromatography (EtOAc: hexane = 1: 7).

5.8.1. The tert-butyl [2-[4-(4-chloro-5-methyl-2-(thiophen-2-yl) thieno[2,3-d]pyrimidine-6-yl)phenoxy]ethyl]methylcarbamate (**14d**)

The parameters for **14d** were: yellow solid, ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.88 (d, J = 3.2 Hz, 1H), 7.76 (d, J = 4.8 Hz, 1H), 7.38 (d, J = 8.4 Hz, 2H), 7.15 (t, J = 4.4 Hz, 1H), 7.03 (d, J = 8.4 Hz, 2H), 4.11 (m, 2H), 3.52 (m, 2H), 2.83 (m, 3H), 2.45 (s, 3H), 1.30 (s, 9H).

5.9. General procedure for the synthesis of thieno[2,3-d]pyrimidine derivatives **13**, **16a**–**16h**

A solution of the corresponding 4-chlorothienopyrimidine **11** or **14** in THF was stirred at room temperature. Hydrazine hydrate was added dropwise and the reaction mixture was refluxed overnight then cooled and concentrated under reduced pressure. The resulting residue **12** or **15** was used directly in the next step. A mixture of the corresponding hydrazino-intermediate **12** or **15** and citraconic anhydride was refluxed overnight then the reaction mixture was cooled and concentrated under reduced pressure. The resulting residue was purified by column chromatography (EtOAc: hexane = 1: 1) and dissolved in 4M HCl 1,4-dioxane solution, then concentrated under reduced pressure. The crude residue was recrystallized from diethyl ether to obtain the title compound **13** or **16**.

5.9.1. N-[2-(dimethylamino)ethyl]-5-methyl-4-[(3-methyl-2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)amino]-2-thiophen-2-yl)thieno [2,3-d]pyrimidine-6-carboxamide hydrochloride (**13**)

The parameters for **13** were: yellow solid, ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.34 (br, 1H), 9.55 (br, 1H), 8.78 (t, J = 5.6 Hz, 1H), 7.84 (d, J = 3.6 Hz, 1H), 7.47 (d, J = 4.8 Hz, 1H), 7.10 (t, J = 3.6 Hz, 1H), 6.69 (d, J = 1.2 Hz, 1H), 3.86–3.82 (m, 2H), 3.43–3.39 (m, 2H), 2.97 (s, 3H), 2.94 (d, J = 4.8 Hz, 6H), 2.24 (d, J = 1.2 Hz, 3H); ESI (m/z) 471 (MH^+); HRMS (ESI) calculated for $C_{21}H_{22}N_6O_3S_2$ [MH^+]: 471.1268, detected: 471.1254.

5.9.2. 3-Methyl-1-{[5-methyl-6-(4-(pyrrolidin-3-yloxy)phenyl)-2-(thiophen-2-yl)thieno[2,3-d]pyrimidin-4-yl]amino}-1H-pyrrole-2,5-dione hydrochloride (**16a**)

The parameters for **16a** were: yellow solid, ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.78 (br, 1H), 9.63 (s, 1H), 9.56 (br, 1H), 7.65 (d, J = 4.0 Hz, 2H), 7.49 (d, J = 8.4 Hz, 2H), 7.11–7.09 (m, 3H), 6.97 (s, 1H), 3.49–3.45 (m, 1H), 3.34–3.2 (m, 3H), 2.59 (s, 3H), 2.15 (s, 3H); ESI (m/z) 518 (MH^+); HRMS (ESI) calculated for $C_{26}H_{23}N_5O_3S_2$ [MNa^+]: 540.1135, detected: 540.1133.

5.9.3. 3-Methyl-1-{[5-methyl-6-(4-(piperidin-4-yloxy)phenyl)-2-(thiophen-2-yl)thieno[2,3-d]pyrimidin-4-yl]amino}-1H-pyrrole-2,5-dione hydrochloride (**16b**)

The parameters for **16b** were: yellow solid, ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.65 (s, 1H), 8.91 (br, 2H), 7.69 (m, 2H), 7.52 (d, J = 8.8 Hz, 2H), 7.18–7.02 (m, 3H), 7.01 (s, 1H), 4.77–4.74 (m, 1H), 3.24 (br, 2H), 3.09 (br, 2H), 2.62 (s, 3H), 2.19 (s, 3H), 2.14–2.08 (m, 2H), 1.91–1.87 (m, 2H); ESI (m/z) 532 (MH^+); HRMS (ESI) calculated for $C_{27}H_{25}N_5O_3S_2$ [MH^+]: 532.1472, detected: 532.1464.

5.9.4. 3-Methyl-1-{[5-methyl-6-(4-(2-(pyrrolidin-1-yl)ethoxy) phenyl)-2-(thiophen-2-yl)thieno[2,3-d]pyrimidin-4-yl]amino}-1H-pyrrole-2,5-dione hydrochloride (**16c**)

The parameters for **16c** were: yellow solid, ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.68 (s, 1H), 7.69 (m, 2H), 7.55 (d, J = 8.4 Hz, 2H), 7.15 (m, 3H), 7.02 (d, J = 2.0 Hz, 1H), 4.45 (t, J = 4.8 Hz, 2H), 3.58 (m, 4H), 3.10 (m, 2H), 2.62 (s, 3H), 2.19 (d, J = 2.0 Hz, 3H), 2.00 (m, 2H), 1.89 (m, 2H); ESI(m/z) 546 (MH^+); HRMS (ESI) calculated for $C_{28}H_{27}N_5O_3S_2$ [MH^+]: 546.1628, detected: 546.1622.

5.9.5. 3-Methyl-1-{[5-methyl-6-[4-(2-(methylamino)ethoxy) phenyl]-2-(thiophen-2-yl)thieno[2,3-d]pyrimidin-4-yl]amino}-1H-pyrrole-2,5-dione hydrochloride (**16d**)

The parameters for **16d** were: yellow solid, ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.68 (s, 1H), 9.24 (s, 2H), 7.70–7.68 (m, 2H), 7.54 (d, J = 8.8 Hz, 2H), 7.17–7.13 (m, 3H), 7.02 (s, 1H), 4.35 (t, J = 4.8 Hz, 2H), 3.36–3.32 (m, 2H), 2.62 (s, 6H), 2.19 (s, 3H); ESI (m/z) 506 (MH^+); HRMS (ESI) calculated for $C_{25}H_{23}N_5O_3S_2$ [MH^+]: 506.1315, detected: 506.1311.

5.9.6. 1-{[6-(4-(2-((2-Hydroxyethyl)amino)ethoxy)phenyl)-5-methyl-2-(thiophen-2-yl)thieno[2,3-d]pyrimidin-4-yl)]amino}-3-methyl-1H-pyrrole-2,5-dione hydrochloride (**16e**)

The parameters for **16e** were: yellow solid, ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.67(s, 1H), 8.93(s, 2H), 7.70–7.68 (m, 2H), 7.55 (d, J = 8.4 Hz, 2H), 7.18–7.14 (m, 3H), 7.02 (s, 1H), 4.36 (t, J = 4.8 Hz, 2H),

3.72–3.70 (m, 2H), 3.42–3.40 (m, 2H), 3.13–3.10 (m, 2H), 2.62 (s, 3H), 2.19 (s, 3H); ESI (*m*/*z*) 536 (MH^+); HRMS (ESI) calculated for $C_{26}H_{25}N_5O_4S_2$ [MH^+]: 536.1421, detected: 536.1407.

*5.9.7. 1-{[6-(4-(2-(Isopentylamino)ethoxy)phenyl)-5-methyl-2-(thiophen-2-yl)thieno[2,3-d]pyrimidin-4-yl]amino}-3-methyl-1H-pyrrole-2,5-dione hydrochloride (**16f**)*

The parameters for **16f** were: yellow solid, ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.67 (s, 1H), 9.10 (s, 2H), 7.70–7.69 (m, 2H), 7.55 (d, J = 7.6 Hz, 2H), 7.17–7.14 (m, 3H), 7.02 (s, 1H), 4.37 (t, J = 4.8 Hz, 2H), 3.37 (m, 2H), 3.00 (m, 2H), 2.63 (s, 3H), 2.19 (s, 3H), 1.68–1.63 (m, 1H), 1.62–1.54 (m, 2H), 0.91 (s, 3H), 0.90 (s, 3H); ESI (*m*/*z*) 562 (MH^+); HRMS (ESI) calculated for $C_{29}H_{31}N_5O_3S_2$ [MNa^+]: 584.1761, detected: 584.1746.

*5.9.8. 1-{[6-(4-(2-(Benzylamino)ethoxy)phenyl)-5-methyl-2-(thiophen-2-yl)thieno[2,3-d]pyrimidin-4-yl]amino}-3-methyl-1H-pyrrole-2,5-dione hydrochloride (**16g**)*

The parameters for **16g** were: yellow solid, ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.66 (s, 1H), 9.50 (br, 2H), 7.69 (m, 2H), 7.59 (m, 2H), 7.54 (m, 2H), 7.48–7.43 (m, 3H), 7.17–7.13 (m, 3H), 7.02 (s, 1H), 4.38 (t, J = 4.7 Hz, 2H), 3.37 (m, 2H), 4.26 (t, J = 5.1 Hz, 2H), 3.36 (br, 2H), 2.62 (s, 3H), 2.19 (s, 3H); ESI (*m*/*z*) 582 (MH^+); HRMS (ESI) calculated for $C_{31}H_{27}N_5O_3S_2$ [MNa^+]: 604.1448, detected: 604.1439.

*5.9.9. 3-{[2-(4-(5-Methyl-4-((3-methyl-2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)amino)-2-(thiophen-2-yl)thieno[2,3-d]pyrimidin-6-yl)phenoxy)ethyl]amino}propanenitrile hydrochloride (**16h**)*

The parameters for **16h** were: yellow solid, ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.67 (s, 1H), 9.58 (br, 2H), 7.69 (m, 2H), 7.56 (d, J = 8.4 Hz, 2H), 7.18–7.13 (m, 3H), 7.02 (s, 1H), 4.36 (t, J = 4.6 Hz, 2H), 3.42–3.36 (m, 4H), 3.06 (t, J = 7.1 Hz, 2H), 2.62 (s, 3H), 2.19 (s, 3H); ESI (*m*/*z*) 545 (MH^+); HRMS (ESI) calculated for $C_{27}H_{24}N_6O_3S_2$ [MH^+]: 545.1424, detected: 545.1413.

*5.9.10. 3-Methyl-1-[{5-methyl-6-(4-((tetrahydrofuran-2-yl)methoxy)phenyl)-2-(thiophen-2-yl)thieno[2,3-d]pyrimidin-4-yl}amino]-1H-pyrrole-2,5-dione (**16i**)*

The parameters for **16i** were: pale yellow solid, ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.56 (s, 1H), 7.65 (m, 2H), 7.45 (d, J = 8.4 Hz, 2H), 7.11 (t, J = 4.4 Hz, 1H), 7.07 (d, J = 8.8 Hz, 2H), 6.97 (m, 1H), 4.18–4.12 (m, 1H), 4.02–3.93 (m, 2H), 3.80–3.74 (m, 1H), 3.68–3.63 (m, 1H), 2.58 (s, 3H), 2.16 (s, 3H), 2.03–1.95 (m, 1H), 1.92–1.75 (m, 2H), 1.70–1.60 (m, 1H); ESI (*m*/*z*) 533 (MH^+); HRMS (ESI) calculated for $C_{27}H_{24}N_4O_4S_2$ [MH^+]: 533.1312, detected: 533.1274.

*5.9.11. 1-[{6-(4-((4-Fluorobenzyl)oxy)phenyl)-5-methyl-2-(thiophen-2-yl)thieno[2,3-d]pyrimidin-4-yl}amino]-3-methyl-1H-pyrrole-2,5-dione (**16j**)*

The parameters for **16j** were: pale yellow solid, ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.61 (s, 1H), 7.66–7.65 (m, 2H), 7.52–7.46 (m, 4H), 7.21 (t, J = 8.8 Hz, 2H), 7.15–7.10 (m, 3H), 6.97 (m, 1H), 5.13 (s, 2H), 2.58 (s, 3H), 2.16 (s, 3H); ESI (*m*/*z*) 557 (MH^+); HRMS (ESI) calculated for $C_{29}H_{21}FN_4O_3S_2$ [MH^+]: 557.1112, detected: 557.1073.

5.10. Liver microsomal stability

The compounds (1 μM) were incubated with human liver microsomal (1 mg/mL) protein in the presence of nicotinamide adenine dinucleotide phosphate (NADPH; 1 mM) in phosphate-buffered saline (PBS; 0.1 mM) at 37 °C for 0 and 30 min. The microsomal reaction was terminated by adding the same volume of acetonitrile containing carbamazepine (IS). The resulting mixture was vortexed for 5 min and centrifuged at 7000 rpm for 15 min at 4 °C. Supernatants were analyzed by LC/MS using an Agilent 1260 HPLC and an Agilent 6490 triple quadrupole LC/MS with an ESI source. The percentage of the compound remaining was determined by calculating the ratio between peak areas of the compounds after incubation for 0 min and peak area at 30 min.

5.11. The remaining activity and kinase inhibition analysis

The remaining activity and kinase inhibition were determined using the KinaseProfiler™ assay protocols (Eurofins Pharma Discovery Services, Dundee, UK). The IC_{50} values of FLT3 and FLT3-D835Y were determined by evaluating the remaining activity at 10,000, 3000, 1000, 300, 100, 30, 10, 3, and 1 nM with 10 μM ATP according to the KinaseProfiler™ assay protocol.

5.12. Cell proliferation assays

The human leukemia cell lines K562, HL-60, THP-1, and MV4-11 were purchased from American Type Culture Collection (Manassas, VA, USA) and 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) (Roche, Indianapolis, IN, USA) according to the manufacturer's protocol. Briefly, 6000–8000 cells were seeded on 96-well plates and treated the folloing day with the test compounds. After 48 h, 50 μL of XTT labeling mixture, which was prepared by mixing 50 vol 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. The absorbance was measured at 490 nm with a reference wavelength of 655 nm using an ELISA plate reader (Molecular Devices, Sunnyvale, CA, USA).

5.13. Solubility test

The hydrochloride salt of the appropriate compound was used in the study. Compound **16d** was suspended in pH 7.4 PBS. The samples were gently shaken for 1 h at 37 °C, centrifuged for 5 min, and filtered over 0.2 μm filters. An amount equal to 100 μL of each solution was added to 100 μL of acetonitrile and analyzed by the Agilent 1260 HPLC and 6490 triple quadruple LC/MS with an ESI source. The solubility was determined from a standard curve sing standards that were prepared in acetonitrile/PBS (1:1).

5.14. Pharmacokinetics study

The hydrochloride salt of the appropriate compound was used in the study. Male Sprague Dawley (SD) rats (8 weeks of age) were acclimated to the testing facility under controlled temperature and humidity conditions for approximately 1 week prior to the study. A jugular vein catheter (Braintree Scientific, Inc., Braintree, MA, USA) was surgically implanted into the right jugular vein under ketamine-xylazine anesthesia. Compound **16d** was dissolved in saline and administered orally (30 mg/kg dosing level) and intravenously (iv; 5 mg/kg dosing level was) to four male rats per time point. Blood samples were collected in heparinized micro-tubes at 0.08, 0.25, 0.5, 1, 2, 3, 4, 6, 8, and 24 h after oral administration, and at 0.05, 0.25, 0.5, 1, 3, 5, 8, and 24 h after iv administration. Blood samples were centrifuged at 3000 rpm for 10 min to collect plasma and stored in a freezer until analyzed. Plasma samples were prepared for analysis by the following protein precipitation method. The plasma samples (100 μL) were transferred to a micro-tube, three volumes of acetonitrile containing carbamazepine (IS) were added, and the resulting mixture was vortexed for 5 min. The

micro-tube was then centrifuged at 12,000 rpm for 10 min and the supernatant was analyzed using the API2000 LC/MS/MS system. Pharmacokinetic parameters were obtained by non-compartmental analysis of the plasma concentration-time profiles, using PK Solution 2.0 (Summit Research Services, Montrose, CO, USA).

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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.2016.05.022.

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