Article

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Identification of Pyrrolo[2,3-*d*]pyrimidine-based Derivatives as Potent and Orally Effective Fms-like Tyrosine Receptor Kinase 3 (FLT3) Inhibitors for Treating Acute Myelogenous Leukemia

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Abstract

A series of pyrrolo[2,3-*d*]pyrimidine derivatives were prepared and optimized for cytotoxic activities against FLT3-ITD mutants cancer cells. Among them, compound **9u** possessed of nanomolar FLT3 inhibitory activity and subnanomolar inhibitory activities against MV4-11 and Molm-13 cells. It also showed excellent inhibitory activities in FLT3-ITD-D835V and FLT3-ITD-F691L cells which were resistant to quizartinib. Furthermore, **9u** exhibited over 40-fold selectivity toward FLT3 relative to c-Kit kinase, which might reduce myelosuppression toxicity. Cellular assays demonstrated that **9u** inhibited phosphorylated FLT3 and downstream signaling factors and also induced cell cycle arrest in G_0/G_1 stage and apoptosis in MV4-11 and Molm-13 cells. Oral administration of **9u** at 10 mg/kg could achieve rapid tumor

extinction in MV4-11 xenograft model and significantly inhibited the tumor growth in

MOLM-13 xenograft model with TGI of 96% without obvious toxicity. Additionally,

9u demonstrated high bioavailability (F=59.5%) and suitable eliminated half-life time

($T_{1/2}$=2.06 h), suggesting that **9u** may be a potent candidate for treating AML.

Introduction

Acute myelogenous leukemia (AML) belongs to an invasive hematologic disorder

characterized by blocked differentiation and deregulated proliferation of

hematopoietic precursor cells[1, 2]. Fms-like tyrosine receptor kinase 3 (FLT3) plays an

essential role in hematopoiesis and leukemogenesis. The most frequent mutations in

the FLT3 receptor gene are internal tandem duplications (FLT3-ITD), which account

for approximately 25% in AML patients. Mutations in tyrosine kinase domain

(FLT3-TKD) occupy close to 5%, mostly at the activation loop residue D835[3].

FLT3-ITD and FLT3-TKD mutations are activated, causing ligand-independent FLT3

receptor signaling transduction, and facilitating survival and proliferation of

cytokine-independent AML cell, resulting in poor prognosis, higher recurrence, and

low overall survival rate[4-8].

Recently decades, plenty of FLT3 inhibitors have been studied and applied in clinical

trials. The first-generation FLT3 inhibitors are multiple tyrosine receptor kinase

inhibitors (RTKs), such as lestaurtinib[9], midostaurin (PKC-412)[10], and sorafenib[11],

and could enhance anti-leukemia efficacy but lack specificity for FLT3, which may

cause off-target toxicities. Midostaurin (PKC-412) blocks FLT3 autophosphorylation

and induces growth arrest and apoptosis which has been approved by FDA for

treating newly diagnosed AML patients[12-14]. Quizartinib (AC220)[3], as a second-generation specific FLT3 inhibitor, is in phase III clinical trial as monotherapy in refractory or relapsed AML patients (NCT02039726). However, the occurance of FLT3-TKD mutations, most commonly at D835 and F691, is a mechanism of acquired resistance to quizartinib[15, 16]. To overcome drug-resistance mutations, a novel, selective, and irreversible FLT3 inhibitor, FF-10101, which is in phase I clinical trial in US in refractory or relapsed AML patients (NCT03194685), has favorable therapeutic effects against AML cells with FLT3 mutations[17]. Although the first- and second-generation inhibitors achieve potent efficacy in AML, off-target toxicities such as myelosuppression caused by simultaneous inhibition of FLT3 and c-Kit[18-20], and FLT3-TKD mutations remain a big challenge for treating AML. For example, the selectivity margin of FLT3 vs c-Kit is only 10-fold, 3-fold and 20-fold for FF-10101, quizartinib and midostaurin respectively, which is in low scope of therapeutic window[17, 21]. Thereby, improvement of the selectivity of FLT3 vs c-Kit could be a good strategy to decrease potential myelosuppression with maintance of potent FLT3 inhibition effects.
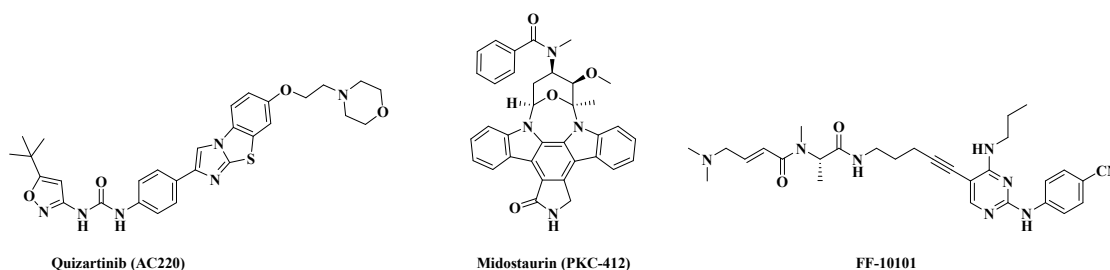

Figure 1. Illustration of representative FLT3 inhibitors.

Qingsong Liu group made great efforts focused on the development of FLT3 inhibitors in treating AML[19, 22, 23]. They developed one FLT3 inhibitor

(CHMFL-FLT3-213) using pyrazolo[3,4-*d*]pyrimidine as core pharmacophore using a structure-guided drug design methodology. CHMFL-FLT3-213 potently interfered FLT3-ITD mediated signal pathways and showed highly potent anti-tumor effect in FLT3-ITD positive AML[16]. However, the low selectivity scope of FLT3 vs c-Kit (0.9-fold) and relatively low bioavailability may affect effectiveness in clinical trial. On the basis of their studies, we further studied the binding mode of CHMFL-FLT3-213 with FLT3 kinase, and found that pyrazolo[3,4-*d*]pyrimidine of CHMFL-FLT3-213 can be replaced by pyrrolo[2,3-*d*]pyrimidine. The positions of urea-linked benzene ring and ethylmorpholine can be exchanged while the binding capacity with FLT3 kinase can be retained. Hence, we adopted pyrrolo[2,3-*d*]pyrimidine as the core skeleton and simplified functional group with retained key functional group and tried to improve the selectivity of FLT3 vs c-Kit and bioavailability. Numerous compounds were designed, prepared, and evaluated for biochemical effects. Among them, **9u** showed efficient inhibitory effects in FLT3-ITD positive cancer cells and two secondary mutation transformed BaF3 cells including FLT3-ITD-D835V and FLT3-ITD-F691L (Figure 2). **9u** had higher selectivity toward FLT3 relative to c-Kit than quizartinib and CHMFL-FLT3-213. More importantly, **9u** possessed good pharmacokinetics and favorable safety profiles. **9u** could achieve complete tumor remission at low dosage without obvious observed toxicities. All the results indicated that **9u** was an excellent drug candidate for treating AML.
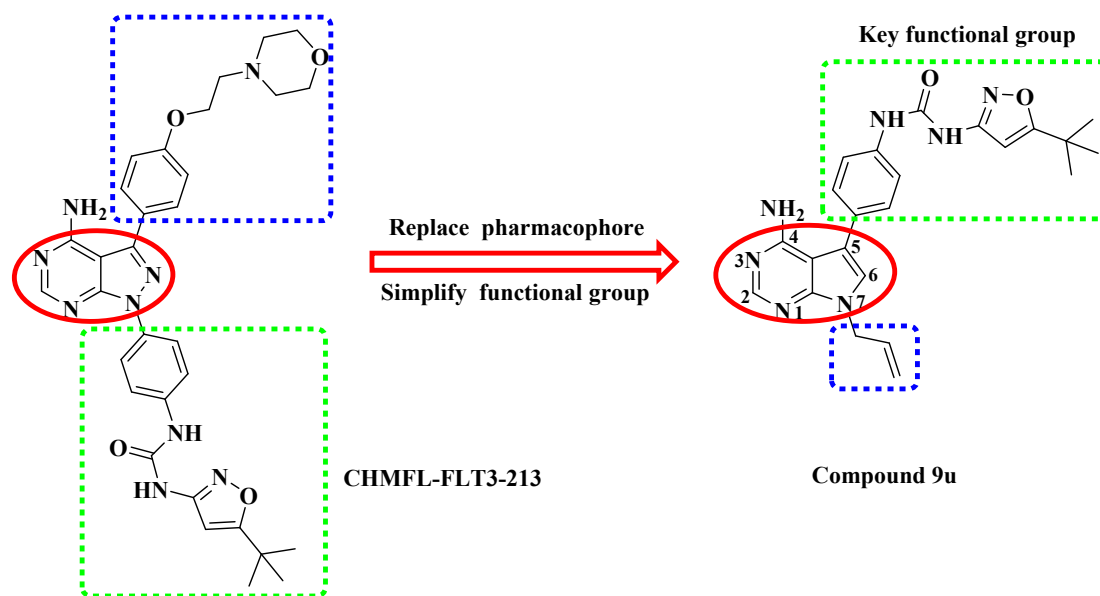
Figure 2. Structural explaination of development of compound **9u**.

Results and Discussions

Rational Strategy Based on Computer Aided Design

We first investigated the similarities and differences of binding mode between compound **9a** with pyrrolo[2,3-*d*]pyrimidine and reference compound CHMFL-FLT3-213 with FLT3 kinase. As illustrated in Figure 3A, CHMFL-FLT3-213 formed three canonical hydrogen bonds between the urea moiety with Glu661 and Asp829 residues. Two hydrogen bonds formed between the amino and pyrimidine with Cys694 and Cys695, and pyrazol didn't form hydrogen bonds with ambient amino acid residues. The hydrophobic pocket was occupied by the tert-butyl substituted isoxazole. When urea-linked benzene ring moiety was moved to C-5 position and ethylmorpholine was moved to N-7 position of pyrrole, compound **9a** adopted a similar docking mode with FLT3 kinase (Figure 3B). **9a** still formed three canonical hydrogen bonds by the urea moiety with the residues of Glu661 and Asp829. Amino of pyrimidine formed hydrogen with Glu692 and π-π stacking was

formed between the linking phenyl with Phe691 and Phe830 residues. When

CHMFL-FLT3-213 and **9a** were placed together, they adopted highly similar

alignment and were well embedded in the pocket (Figure 3C). Ispired by the

encouraging design outcomes, we continued to design and synthesize a series of

compounds and evaluate their biochemical activities in vitro and in vivo.
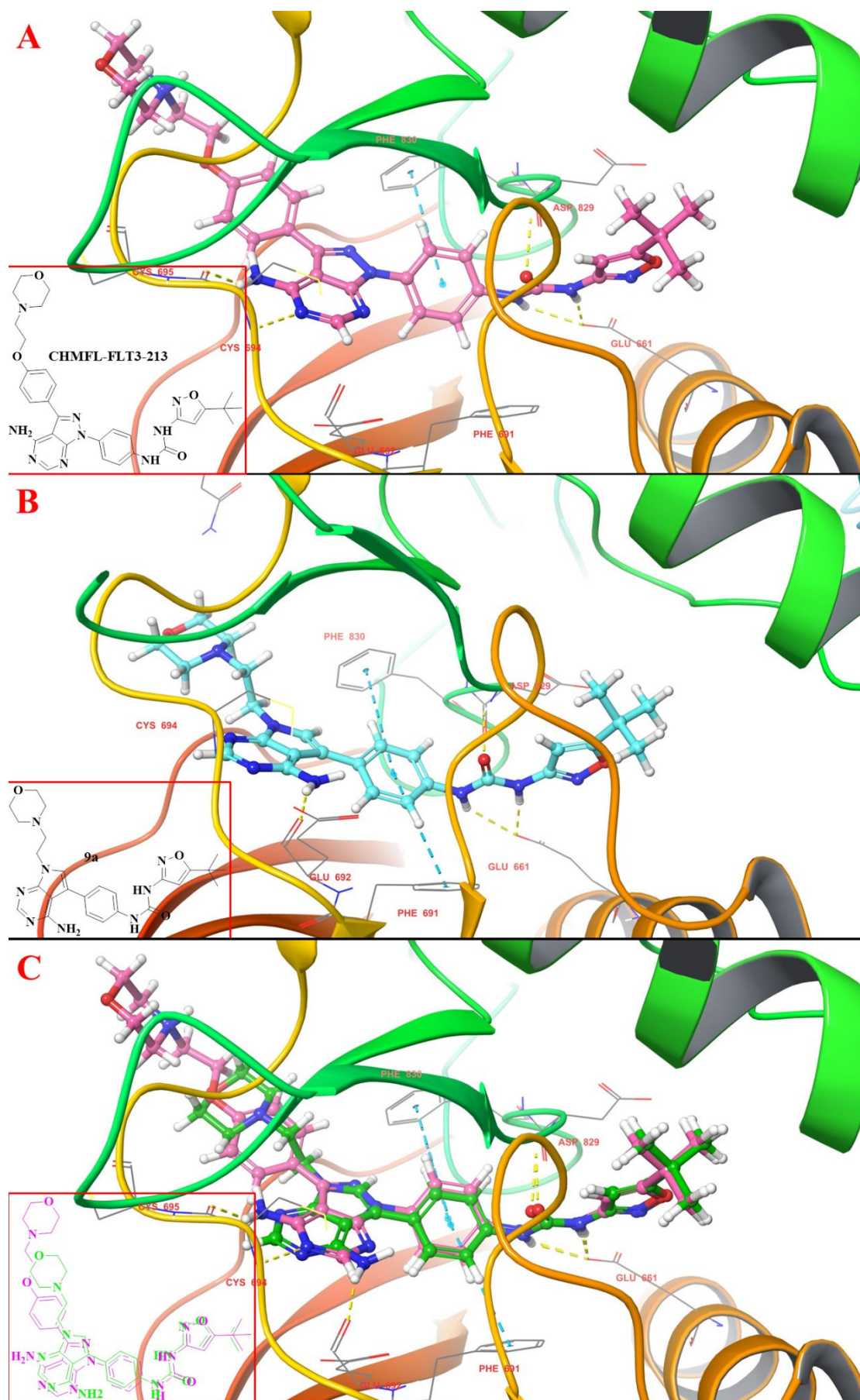
Figure 3. Docking model using FLT3 kinase (PDB code: 4XUF). (A) CHMFL-FLT3-213 was docked into the FLT3 kinase. (B) **9a** was docked into the FLT3 kinase. (C) Alignment of CHMFL-FLT3-213 and **9a** into the FLT3 kinase.

In Vitro Cell Growth Inhibitory Effects and Enzymatic Inhibitory Activities against FLT3 Kinase. The introduction of 3-amino-5-tert-butylisoxazole group to aniline could improve the potency for FLT3[3, 16, 24]. Thus we attempted to keep the key functional group and compounds **9a-ae** were synthesized. MV4-11 and Molm-13 cells are both FLT3-ITD positive cells, which can reflect the antitumor activities of compounds against FLT3 mutant cells. Thereby we primarily evaluated the inhibitory activities of compounds **9a-ae** in MV4-11 and Molm-13 cells. As expected, all the compounds showed potent inhibitory activities against MV4-11 and Molm-13 cells and the IC_{50} values were below 10 nM (Table 1). **9a** with ethylmorpholine showed comparable inhibitory activity to quizartinib. **9f** with extended carbon chain of morpholine enhanced the inhibitory activity with IC_{50} values of 0.069 nM and 0.193nM in MV4-11 and Molm-13 cells respectively. Introduction with large substituents at N-7 postion such as benzyl (**9o**), cyclopentane (**9n**) and 1-ethyl-4-(methylsulfonyl)piperazine (**9r**) significantly decreased the inhibitory effects. Interestingly, compounds with short nonsubstitutive aliphatic chains, such as **9g-k**, **9p**, **9s-v**, exhibited stronger inhibitory activities at the subnamolar level or even the picomole level. Surprisingly, **9b** without substitution at N-7 positon showed the strongest inhibitory effect with IC_{50} < 1 pM against Molm-13. These compounds were further evaluated IC_{50} values against FLT3 kinase. Several compounds showed potent

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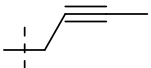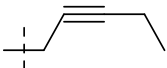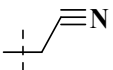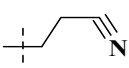
inhibitory activities against FLT3 with average IC_{50} below 10 nM and displayed

comparable effects with quizartinib (IC_{50} = 7.4 nM)[25]. These results were in

accordance with our previous rational drug design and these potent compounds should

be further evaluated to obtain the optimal drug candidate.

Table 1. IC_{50} Values of Compounds **9a-ae** in MV4-11, Molm-13, and FLT3 Kinase

and Quizartinib as Positive Control
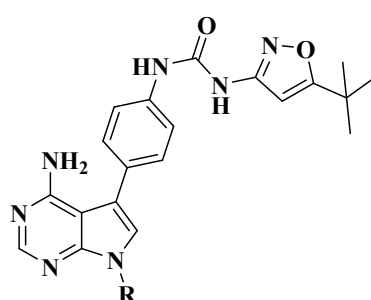
Compd	R	$IC_{50} \pm SD^a$, nM		IC_{50}^b, nM
		MV4-11	Molm-13	FLT3
9a		0.329 ± 0.035	1.153 ± 0.096	20
9b	H	0.043 ± 0.007	< 0.001	6
9c	Boc	0.107 ± 0.010	0.081 ± 0.025	25
9d		2.536 ± 0.193	4.435 ± 0.364	ND^c
9e		1.751 ± 0.010	2.56 ± 0.513	9
9f		0.069 ± 0.015	0.193 ± 0.027	9
9g		0.152 ± 0.010	0.134 ± 0.016	17
9h	Methyl	0.311 ± 0.084	0.014 ± 0.003	7

9i	Ethyl	0.476 ± 0.057	0.144 ± 0.017	7
9j	Isopropyl	0.100 ± 0.045	0.971 ± 0.121	20
9k	Propyl	0.403 ± 0.091	0.736 ± 0.010	15
9l	n-butyl	2.179 ± 0.281	1.425 ± 0.306	34
9m	n-hexyl	5.133 ± 2.010	3.771 ± 0.468	105
9n		2.160 ± 0.063	1.511 ± 0.038	32
9o		1.756 ± 0.198	2.495 ± 0.225	86
9p		0.876 ± 0.131	0.140 ± 0.001	16
9q		1.734 ± 0.049	1.946 ± 0.052	31
9r		14.173 ± 0.620	10.601 ± 0.110	26
9s		0.149 ± 0.042	0.049 ± 0.010	21
9t		1.468 ± 0.022	0.117 ± 0.010	37
9u		0.089 ± 0.001	0.022 ± 0.003	7
9v		1.753 ± 0.134	1.061 ± 0.055	38
9w		10.325 ± 1.255	2.767 ± 0.116	113
9x		1.746 ± 0.219	1.025 ± 0.097	30
9y		2.940 ± 0.282	2.222 ± 0.184	69
9z		1.788 ± 0.615	2.855 ± 0.016	70
9aa		0.602 ± 0.068	0.262 ± 0.041	13

9ab		3.923 ± 0.384	1.145 ± 0.072	26
9ac		5.842 ± 0.221	2.046 ± 0.210	64
9ad		0.032 ± 0.007	0.038 ± 0.010	9
9ae		0.376 ± 0.041	0.520 ± 0.030	6
quizartinib		0.452 ± 0.177	1.885 ± 0.220	7.4

[a] IC_{50}, compound concentration required to inhibit tumor cell proliferation by 50%, data are expressed as the mean $\pm$ SD from the dose-response curves of at least three independent experiments. [b] IC_{50} values for enzymatic inhibition of FLT3 kinase; data are expressed from the dose-response curves of at least two independent experiments. [c] ND, not detected.
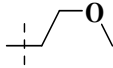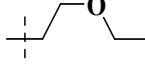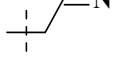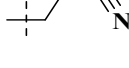
Although compound **9b** showed potent anti-proliferative activity against Molm-13 with $IC_{50} < 1$ pM, which was more than 100-times active than quizartinib. However, **9b** showed unsatisfactory bioavailability and anti-tumor activity in vivo (data was not shown). The bioavailability and anti-tumor activity of other two promising compounds **9a** and **9f** in vivo were also unsatisfactory (data was not shown). Very luckly, **9u** with allylic substitution obtained satisfactory bioavailability and anti-proliferation effect both in MV4-11 and Molm-13 cells (Table 1). It is well known that introduction of key functional groups (such as halogen atom) is an important structural modification strategy to improve metabolic stability and bioavailability of lead compounds[26]. We attempted to introduce F atom to C-3 position of phenyl in selected coumpounds and **13a-h** were synthesized and evaluated for inhibitory activities in MV4-11 and Molm-13 cells. The data were illustrated in

Table 2. Although F-substitued analogues maintained potent inhibitory activities in MV4-11 and Molm-13 cells, they decreased efficacy in comparision with corresponding analogues without F atom. Worsemore, F-substitued analogues decreased inhibitory activities against FLT3 kinase except for **13a** with IC_{50} of 4 nM. Since wild-type FLT3 is essential for the proliferation of normal primitive hematopoietic cells[27], all the compounds were further evaluated the inhibitory activities in RS4;11 (acute lymphoblastic leukemia, FLT3 wt) and HL-60 (promyelocytic leukemia cell, FLT3 wt). These compounds showed very weak inhibitory activities against RS4;11 and HL-60, and the IC_{50} values were > 1000 nM (Table S2 and S3), indicating that these compounds possessed of excellent selectivity between FLT3 wt and FLT3-ITD mutants (MV4-11, Molm-13).

Table 2. IC_{50} Values of Compounds **13a-h** in MV4-11, Molm-13, and FLT3 Kinase and Quizartinib as Positive Control
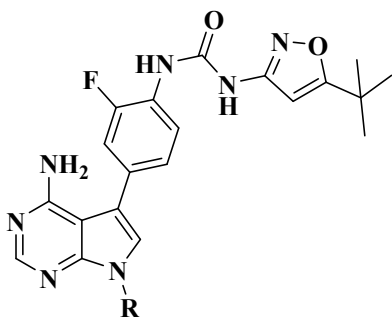
Compd	R	$IC_{50} \pm SD^a$, nM		IC_{50}^b, nM
		MV4-11	**Molm-13**	**FLT3**
13a	H	0.131 ± 0.035	0.007 ± 0.001	4
13b		2.336 ± 0.022	0.650 ± 0.038	17
13c		3.223 ± 0.067	1.450 ± 0.050	51

13d		0.434 ± 0.084	0.389 ± 0.047	46
13e		1.977 ± 0.100	0.580 ± 0.010	32
13f		1.343 ± 0.157	1.550 ± 0.180	49
13g		0.422 ± 0.057	0.730 ± 0.120	19
13h		2.208 ± 0.298	2.330 ± 0.190	22
quizartinib		0.452 ± 0.177	1.885 ± 0.220	7.4

[a] IC$_{50}$, compound concentration required to inhibit tumor cell proliferation by 50%, data are expressed as the mean $\pm$ SD from the dose-response curves of at least three independent experiments. [b] IC$_{50}$ values for enzymatic inhibition of FLT3 kinase; data are expressed from the dose-response curves of at least two independent experiments.

Patients with FLT3-ITD mutations which relapsed while treated with quizartinib usually had point mutations in activation loop (most frequently at D835) or "gatekeeper" domain[28]. F691L is a hotspot for drug resistance to FLT3 inhibitors which situated at the "gatekeeper" location in FLT3 kinase[16, 29]. These secondary mutations conferring resistance in kinase domain will pose a significant clinical challenge, which prompted us to identify new FLT3 inhibitors against FLT3 resistant variants[30]. FLT3-ITD, FLT3-ITD-F691L and D835V transformed BaF3 cells were established to evaluate inhibitory activities of all the compounds. On the basis of the characteristics of inhibitory activities in MV4-11 and Molm-13 cells and IC$_{50}$ values of FLT3 kinase of the above compounds, all the compounds were further evaluated the inhibitory activities against BaF3-FLT3-ITD cell. As shown in Table 3, all the

compounds strongly inhibited proliferation of BaF3-FLT3-ITD with IC_{50} values at the nanomolar level, but showed great difference between BaF3-FLT3-ITD-F691L and BaF3-FLT3-ITD-D835V. Most compounds still exhibited potent inhibitory effects against these two mutational cells which were resistant to quizartinib and a majority of these compounds were below 100 nM (IC_{50}). **9b**, **9j**, **9p**, **9u**, **9aa**, **9ad**, and **13a** showed considerable and equivalent potent effects against FLT3-ITD-F691L and FLT3-ITD-D835V cells. Conclusion can be drawn that these compounds could overcome two crucial point mutations which conferred resistance to quizartinib. Considering of cytotoxicity and FLT3 kinase assay, **9b**, **9j**, **9p**, **9u**, **9aa**, **9ad**, and **13a** were further selected out and evaluated their bioavailability in SD rats.

Table 3. IC_{50} Values of Compounds **9a-ae** and **13a-h** against FLT3 Mutants Transformed BaF3 Cells and Quizartinib as Positive Control

Compd	$IC_{50} \pm SD^a$, nM		
	BaF3-ITD	**BaF3-ITD-D835V**	**BaF3-ITD-F691L**
9a	1.07 ± 0.05	52.38 ± 1.66	34.81 ± 6.75
9b	0.16 ± 0.04	4.83 ± 0.25	2.35 ± 0.40
9c	1.28 ± 0.19	12.39 ± 1.26	8.92 ± 1.18
9d	8.95 ± 0.81	385.4 ± 82.2	> 500
9e	8.67 ± 0.32	155.67 ± 9.54	> 200
9f	0.99 ± 0.10	61.84 ± 8.11	32.12 ± 3.52
9g	1.62 ± 0.15	170.23 ± 12.40	85.02 ± 8.00
9h	1.44 ± 0.09	46.25 ± 3.05	27.92 ± 0.71
9i	0.92 ± 0.13	23.09 ± 2.31	28.53 ± 3.93
9j	5.30 ± 0.57	12.19 ± 1.86	12.56 ± 0.52
9k	2.72 ± 0.24	41.49 ± 2.69	72.09 ± 3.96

9l	5.69 ± 0.80	111.5 ± 0.2	208.7 ± 31.7
9m	20.71 ± 1.98	187.0 ± 11.9	252.7 ± 27.8
9n	7.25 ± 0.79	36.80 ± 5.57	90.87 ± 0.83
9o	8.45 ± 0.07	95.40 ± 6.43	133.5 ± 22.3
9p	1.73 ± 0.03	14.74 ± 0.91	13.63 ± 1.82
9q	3.10 ± 0.47	28.50 ± 1.86	28.23 ± 4.31
9r	3.59 ± 0.24	166.5 ± 4.4	85.99 ± 13.22
9s	0.95 ± 0.12	50.14 ± 1.89	28.65 ± 0.70
9t	3.21 ± 0.78	173.0 ± 5.2	98.6 ± 12.1
9u	0.92 ± 0.01	20.71 ± 2.32	12.99 ± 0.87
9v	4.92 ± 1.04	175.6 ± 11.4	91.8 ± 0.7
9w	8.67 ± 0.29	305.3 ± 31.7	216.5 ± 6.0
9x	5.09 ± 0.96	93.7 ± 9.3	57.79 ± 8.30
9y	5.95 ± 0.18	139.8 ± 10.2	200.3 ± 5.7
9z	6.06 ± 0.72	185.3 ± 8.5	228.1 ± 16.9
9aa	1.66 ± 0.04	21.15 ± 1.09	13.88 ± 0.77
9ab	6.18 ± 0.69	86.43 ± 5.68	51.17 ± 6.26
9ac	8.65 ± 0.02	361.1 ± 21.3	147.9 ± 6.8
9ad	0.97 ± 0.07	9.17 ± 0.48	3.61 ± 0.01
9ae	1.60 ± 0.06	24.64 ± 0.44	19.60 ± 1.33
13a	0.25 ± 0.04	18.01 ± 0.87	10.71 ± 1.48
13b	3.04 ± 0.26	51.85 ± 5.17	82.43 ± 4.92
13c	5.41 ± 0.53	84.41 ± 4.24	50.25 ± 2.16
13d	2.48 ± 0.16	102.5 ± 5.7	16.08 ± 0.86
13e	5.57 ± 0.27	69.70 ± 5.97	39.39 ± 2.57
13f	4.60 ± 0.33	55.37 ± 4.21	42.66 ± 2.76
13g	1.27 ± 0.09	86.69 ± 1.24	9.84 ± 0.58
13h	4.26 ± 0.33	111.5 ± 10.9	31.46 ± 1.58
quizartinib	4.94 ± 0.20	> 1000	> 1000

[a] IC_{50}, compound concentration required to inhibit tumor cell proliferation by 50%, data are expressed as the mean ± SD from the dose-response curves of at least three independent experiments.

As shown in Table 4, **13a** with F-substitution showed poor pharmacokinetic (PK) properties than **9b**, suggesting that halogen contributed worse effect to PK properties. **9p** with hydroxyl group significantly promoted the clearance of drug. Compound **9ad** wasn't further explored due to its high clearance rate and low bioavailability. Among them, compound **9u** demonstrated excellent PK properties with low clearance (1.22 L/h/kg), reasonable half-life time (2.06 h), and highest oral exposure (59.5%). These PK properties showed strong correlation with bioavailability predicted by swissADME tool (Figure S1 and S2)[31].

Table 4. Oral Pharmacokinetic Study of the Selected Compounds in SD Rats[a]

Parameter	9 b	9j	9p	9u	9aa	9ad	13a
T_{max} (h)	0.28	0.50	0.31	0.75	0.83	0.44	0.88
$T_{1/2}$ (h)	6.22	4.22	2.77	2.06	2.78	3.7	2.54
CL (L/h/kg)	0.89	15.31	48.07	1.22	1.70	9.17	3.38
F (%)	22.5	12.1	9.8	59.5	40.0	11.2	25.6

[a] Dosage, 5 mg/kg po; T_{max}, time for peak concentration; $T_{1/2}$, elimination half-life period; Vss, distribution volume; CL, plasma clearance; F (%), percent oral bioavailability.

Kinase Selectivity Profiling of Compound 9u. To further understand the selectivity of compound **9u**, we then evaluated its wide kinome selectivity profile. The results

indicated that compound **9u** possessed a relatively good selectivity in a panel of more than 400 kinases and mutants at 100 nM (Table S1). The **S** (10) selectivity score, which is calculated by dividing the number of nonmutant kinases which are ≤ 10% of control can be determined by the total number of nonmutant kinases[32]. From the nonmutant kinases there were 38 hits, giving the selectivity of 0.17 (Figure 4). Because receptor tyrosine kinase inhibitors usually target many other targets which share homologous sequences. Thereby the inhibitory activities of **9u** against hit targets were further evaluated the IC_{50} values and the data were shown in Table S4. Most importantly, **9u** showed good selectivity of FLT3 to c-Kit, and its selectivity range of FLT3 vs c-Kit was > 40-fold (FLT3, IC_{50} = 7 nM; c-Kit, IC_{50} > 300 nM), much better than quizartinib and CHMFL-FLT3-213, indicating that **9u** possessed of better safety window. Additionally, Hck, Ret, Lyn, Lck, Mer, Met, and Tie2 kinases have been proved as promising targets for treating leukemia[33-39]. Fibroblast growth factor receptor (FGFR) is also overexpressed in leukemia cells and could be one promising druggable target for treating leukemia[40-42]. **9u** also strongly inhibited PDGFRα mutants at D842V and V561D and Abl (T315I), which might overcome imatinib-resistant mutants[43, 44].
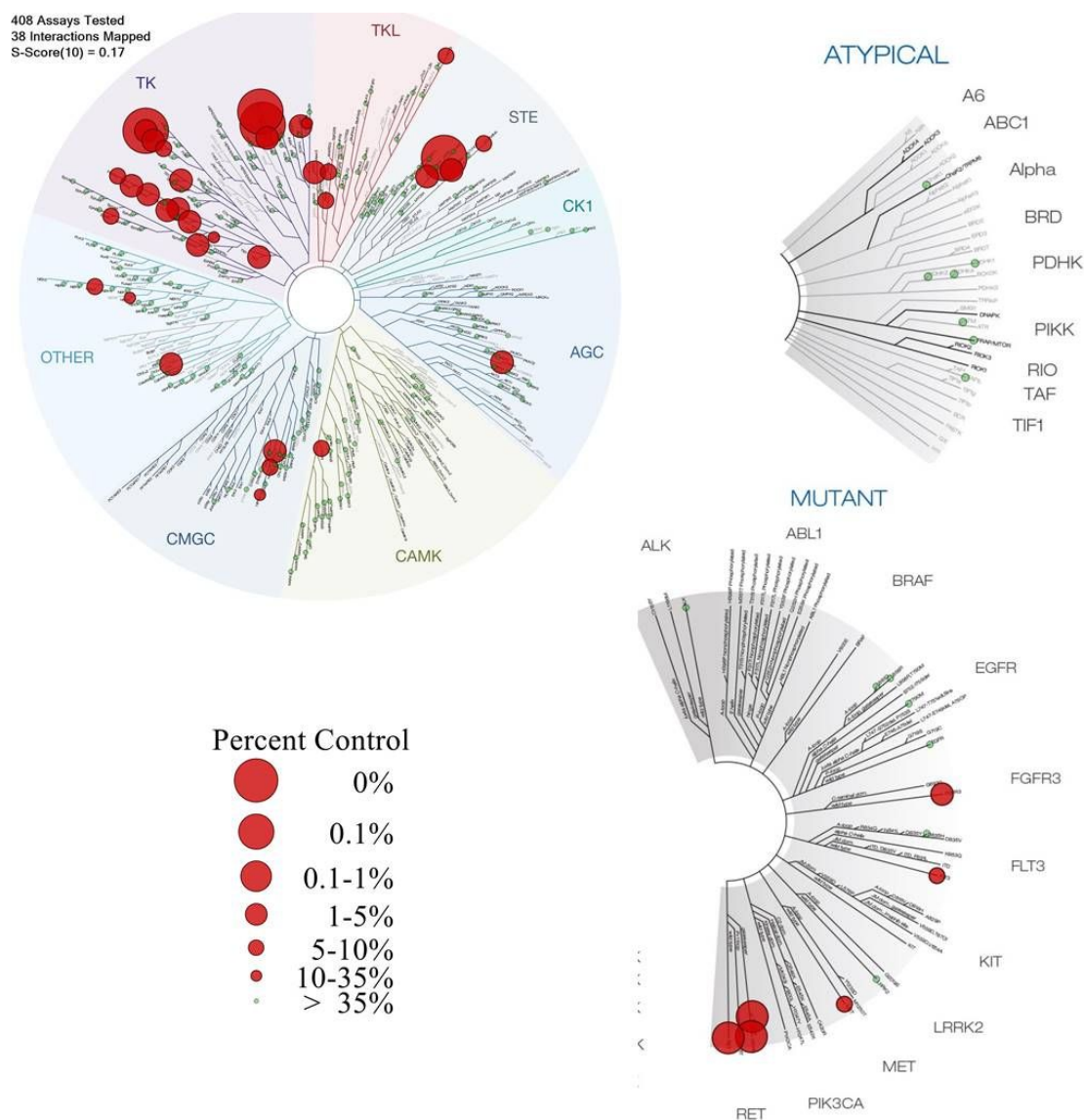
Figure 4. Wide kinome selectivity profiling of compound **9u** with Eurofins KinaseProfile platform. Tests were conducted at 0.1 μM of compound **9u**. Treespot image was mapped by TREEspot[TM] software tool provided by DiscoverX corporation.

Clinically relevant FLT3-resisitant mutants indicated poor prognosis and the dissociation constants (K_d) of **9u** against a panel of FLT3-ITD/TKD mutations were evaluated by DiscoverX Kinomescan technology. As displayed in Table 5, **9u** binds with high affinity to wt FLT3 and FLT3 mutants. In particular, **9u** displayed nice

binding affinity with drug-resistance mutants such as D835 mutants. As for the secondary ITD-TKD mutants, **9u** also showed considerable binding affinity for FLT3-ITD-D835V and FLT3-ITD-F691L mutants (K_d: 41.26 nM, 48.47 nM, respectively). The high affinity for those mutants confering dramatical resistance to FLT3 inhibitory monotherapy made **9u** very promising for clinical applications.

Table 5. Dissociation Constants of Compound **9u** against Wt and Mutant FLT3 Kinases[a]

Mutant type	K_d, nM
FLT3 wt	4.37
FLT3(D835V)	7.04
FLT3(ITD)	20.52
FLT3(ITD, D835V)	41.26
FLT3(ITD, F691L)	48.47
FLT3(N841I)	2.37
FLT3(R834Q)	10.15
FLT3(D835H)	5.14
FLT3(D835Y)	7.78
FLT3(K663Q)	2.48

[a] The biochemical tests were provided by DiscoverX. All the data were obtained by duplicated test.

Molecular Docking of Compounds into FLT3 kinase. In order to further understand the different activities of compounds **9b**, **9u**, and **13a** against FLT3 kinase, we performed the molecular docking into FLT3. The active pocket of FLT3 kinase is narrow and long with a small opening at the bottom of the pocket to be communicated with the outside (PDB code 4XUF). Docking of **9b** into the FLT3 kinase (DFG-out

inactive conformation) indicated that compound **9b** was well embedded in the pocket, and the nitrogen atom of pyrimidine formed hydrogen bond with Cys694 residue (Figure 5C). Amino of pyrimidine formed one hydrogen bond with carbonyl of Glu692 residue. Additionally, the π-π stacking was formed between the linking phenyl with Phe691 and Phe830 residues. Canonical hydrogen bonds were formed between Glu661 and Asp829 by urea linkage. The hydrophobic pocket was occupied by the tert-butyl substituted isoxazole. Through these actions, **9b** occupied the active pocket well and restricted conformational changes of the FLT3 active pocket, thereby inhibiting FLT3 kinase activity. The docking pattern of compound **9u** was highly similar to **9b**, with one hydrogen bond loss between N at pyrimidine with Cys694, which led to slight decline of inhibitory activities against FLT3 and tumor cells (Figure 5A and 5B). However, the introduction of F atom to benzene ring (**13a**) blocked the hydrogen bond action between N-H of urea linkage and Glu661, which caused slight efficacy decrease in comparison to **9b** (Figure 5D).
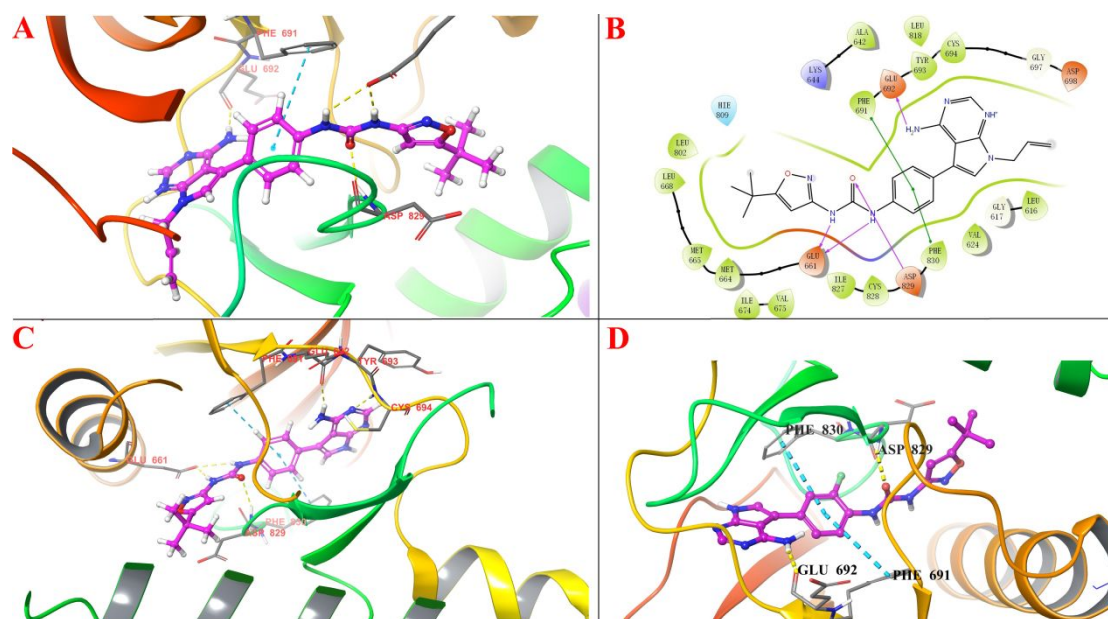
Figure 5. (A, B): Mimetic binding mode of compound **9u** (purple stick) within FLT3

protein; (C): Mimetic binding mode of compound **9b** (purple stick) within FLT3

protein; (D): Mimetic binding mode of compound **13a** (purple stick) within FLT3

protein. For clarity, nitrogen, oxygen and fluorine atoms are dispalyed in blue, red,

and green, respectively.

Cell Cycle Arrest Assays and Apoptosis Assays by Flow Cytometry. MAPK and

STAT pathways are crucial regulators of cell proliferation and their inhibition induce

cell cycle arrest in the G_1/G_0 phase[45]. Cell cycle analyses in MV4-11 and Molm-13

cells treated with compound **9u** were conducted. As shown in Figure 6A, **9u** strongly

induced cell cycle arrest with G_1/G_0 percentage of 53% even at 1 nM compared to

vehicle in Molm-13 cell. The percentage of G_1/G_0 cells populations increased from

53% to 94% after treatment with **9u** at 1 nM, 3 nM, and 10 nM. As expected, similar

results were observed at the same concentration in MV4-11 cells. We next conducted

apoptosis assays to confirm that compound **9u** could induce cell death. As exhibited

in Figure 6C, the total apoptosis accounted for 34% when Molm-13 cells were treated

with **9u** at 10 nM. Moreover, the apoptosis effects were strengthening as the dose

increased. As expected, similar results were observed at the same concentration in

MV4-11 cells. Obviously, compound **9u** blocked the AML cells in G_1 stage, which

led to decreased S-phase and G_2-phase percentage and also induced AML cells

apoptosis.
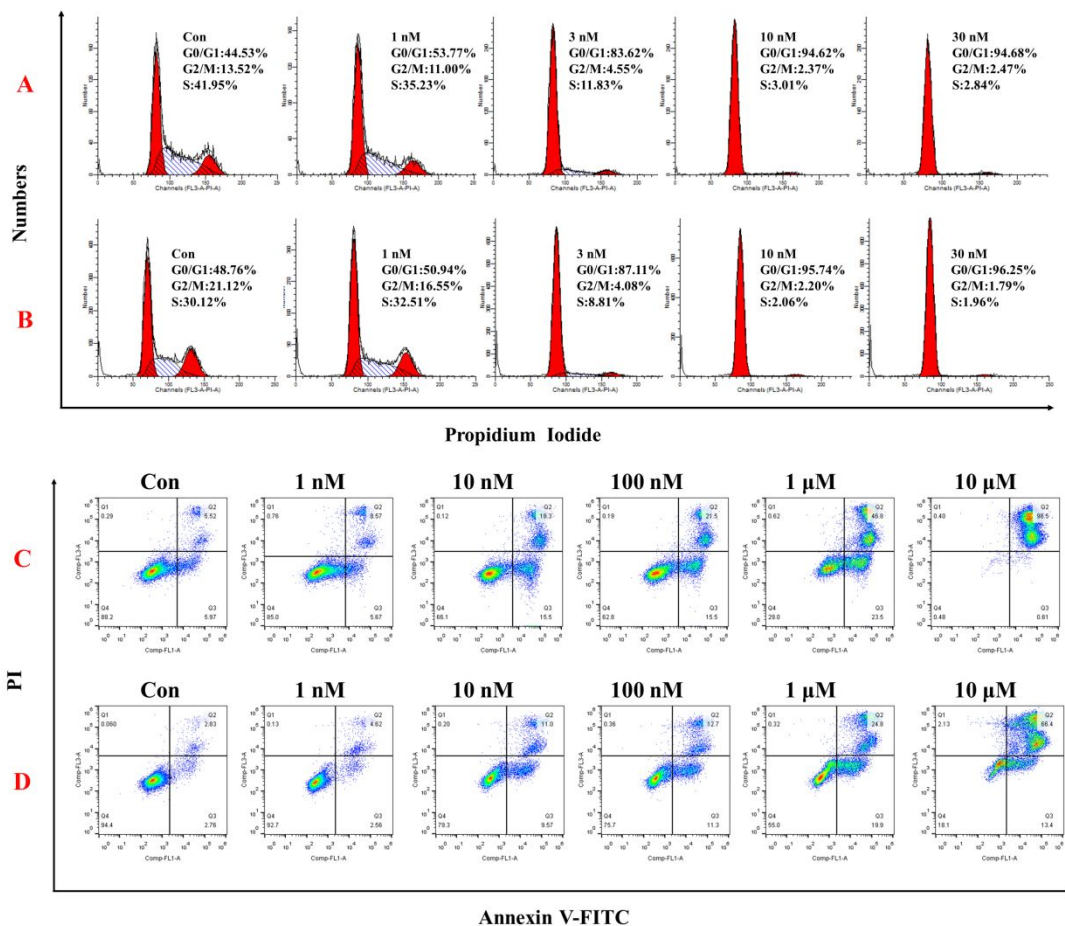
Figure 6. (A, B): Molm-13 and MV4-11 cells were incubated with compound **9u**, respectively. (C, D): Molm-13 and MV4-11 cells were incubated with compound **9u**, respectively.

Western Blot of Signaling Pathways for FLT3 Kinase. To further investigate whether the anti-tumor activities were relevant to inhibition of FLT3 and its downstream signal factors, we then examined the signaling proteins in Molm-13 and MV4-11 cells treated with compound **9u** by western blot and conducted densitometric quantification by ImageJ software. As the results showed (Figure 7), compound **9u** blocked FLT3 autophosphorylation at Tyr591 at 10 nM completely, which exhibited comparable inhibition effects with quizartinib. Notably, **9u** at 3 nM abolished phosphorylation of STAT5 at Y694, which was a direct substrate of the oncogenic

FLT3-ITD variant. Phosphorylation ERK at T202/Y204, one key component of MAPK signaling pathway, was obviously reduced upon treatment with compound **9u**. Similar results were obtained in MV4-11 cells when treated with compound **9u** (Figure 7).

MV4-11		9u					AC220			
	Con	1	3	10	30	100	1	3	10	(nM)
p-FLT3										
FLT3										
p-STAT5										
STAT5										
p-ERK										
ERK										
β-Actin										
p-FLT3/β-Actin	1	0.46	0.19	0	0	0	0.15	0.02	0	
p-STAT5/β-Actin	1	0.54	0.39	0	0	0	0.46	0.30	0	
p-ERK/β-Actin	1	1.07	0.41	0	0	0	1.1	0.22	0	

Molm-13		9u					AC220			
	Con	1	3	10	30	100	1	3	10	(nM)
p-FLT3										
FLT3										
p-STAT5										
STAT5										
p-ERK										
ERK										
β-Actin										
p-FLT3/β-Actin	1	0.88	0.53	0	0	0	1.02	0.13	0	
p-STAT5/β-Actin	1	0.14	0	0	0	0	0.26	0	0	
p-ERK/β-Actin	1	0.48	0.15	0.07	0.10	0.12	0.46	0.31	0.11	
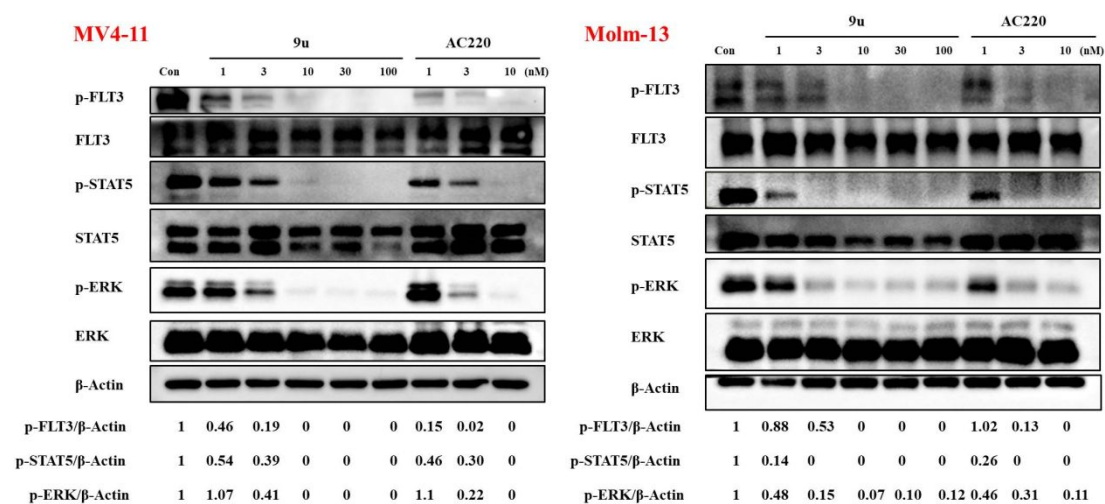
Figure 7. Compound **9u** inhibited phosphorylation of FLT3 and downstream signaling proteins STAT5 and ERK after treatment for 2 h.

Anti-tumor Efficacy in AML Xenograft Models. We then evaluated the anti-tumor efficacy of compound **9u** in Molm-13 and MV4-11 cells inoculated xenograft mouse models (Table 7). As shown in Figure 8A and 8B, **9u** significantly and dose-dependent inhibited the tumor growth both in Molm-13 and MV4-11 cells inoculated xenograft models. Oral administration of **9u** at 1 mg/kg showed potent antitumor effect and tumor growth inhibitory rate (TGI) was 77.9% in Molm-13 bearing mice model. Moreover, **9u** exhibited comparable antitumor activity with quizartinib (AC220) at dosage of 3 mg/kg. No obvious toxicity was observed during the treatment stage in all the experiment groups. Not surprisingly, **9u** also showed excellent anti-tumor activities in MV4-11 xenograft model (Table 6 and Figure 8B).

Treatment with **9u** at 3 mg/kg achieved one-third tumor regression on day 10. Both **9u** and quizartinib treatment groups achieved rapid and complete tumor extinction on day 8. Mice from control, 1 mg/kg, and 3 mg/kg groups were sacrificed on day 14 and tumors were extracted out and photographed subsequently. **9u** and quizartinib at 10 mg/kg groups continued to investigate the recurrence of tumor. After continuous treatment for prolonged 4 days, both **9u** and quizartinib groups were stopped treatment on day 18. However, two groups both recurred quickly at day 24 and 26, respectively. These results suggested that leukemic stem cells may lead to tumor recurrence due to the complex regulatory mechanisms, such as incomplete and and discontinuous inhibiton of FLT3[17, 46]. We next determined to evaluate higher dosage for delaying or preventing tumor recurrence.

Table 6. Tumor Growth Inhibition of **9u** in Vivo.

Tumor models	Cmpd	Treatment			Survivors (day)	Tumor mass change(%)
		Schedule[a]	Dosage (mg/kg)	Route		
Molm-13	**9u**	QD×10	1	po	6/6	77.9
	9u	QD×10	3	po	6/6	94.5
	9u	QD×10	10	po	6/6	96.0
	quizartinib	QD×10	3	po	6/6	98.2
MV4-11	**9u**	QD×14	1	po	6/6	70.5
	9u	QD×14	3	po	6/6	98.2
	9u	QD×18	10	po	6/6	100
	quizartinib	QD×18	3	po	6/6	100

[a] QD, per day.
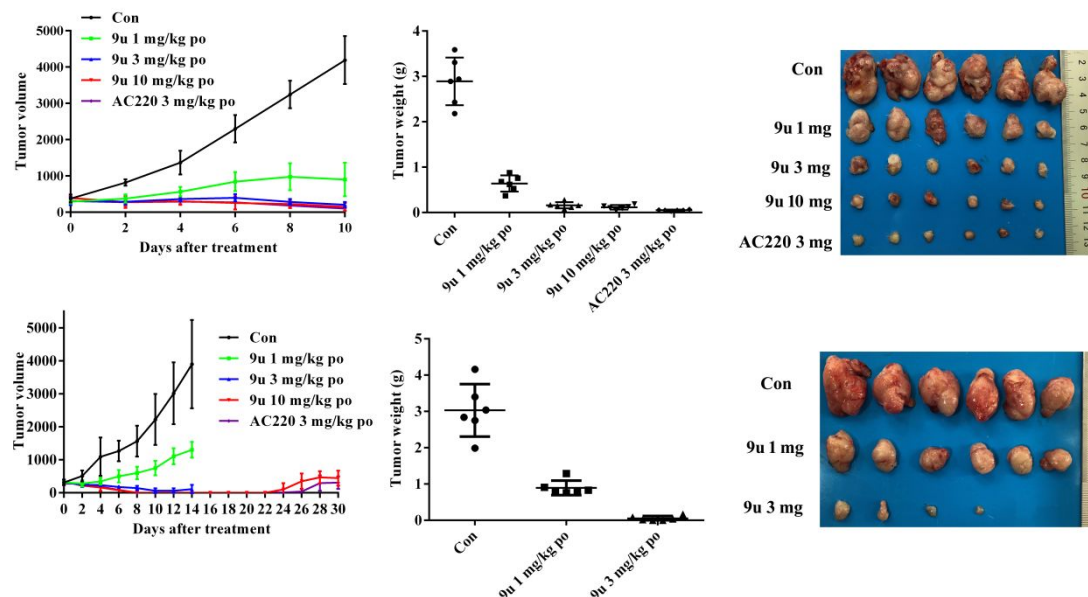
Figure 8. Anti-tumor activities of **9u** in Molm-13 and MV4-11 xenograft models.

Conclusion

FLT3-ITD/TKD resistant mutants conferred poor prognosis and higher relapsed rate

after treatment with FLT3 inhibitors. Overcoming FLT3 mutants made it a promising

drug design strategy for treating AML. Combination of advantages and disadvantages

of clinical trials and applications of FLT3 inhibitors, we designed and synthesized a

series of novel FLT3 kinase inhibitors. Compound **9u** possessed low nanomolar FLT3

kinase inhibitory activities and lower-dose anti-AML activities. It showed relatively

good kinases selectivity and high binding affinity to FLT3 mutants. Compound **9u**

also showed potent antiproliferation activities against MV4-11 and Molm-13 cells. It

also showed excellent potent antiproliferation activities against FLT3-ITD-D835V

and F691L cells which were resistant to quizartinib. Furthermore, **9u** had higher

selectivity toward FLT3 relative to c-Kit than quizartinib, which might lower

myelosuppression toxicity. Cellular assays demonstrated that **9u** inhibited

phosphorylated FLT3 and downstream signaling factors and also induced cell cycle

arrest in G_0/G_1 stage and apoptosis in MV4-11 and Molm-13 cells. Compound **9u**, with high bioavailability, strongly suppressed tumor growth in Molm-13 model and achieved rapid tumor regression at 10 mg/kg in MV4-11 model via oral administration. All the reported results suggested that **9u** may be a potent candidate for treating AML.

Experimental Section

Chemistry. All the chemical solvents and reagents, which were analytically pure without further purification, were commercially available. TLC was performed on 0.20 mm Silica Gel 60 F_{254} plates (Qingdao Haiyang Chemical, China). ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker Avance 400 spectrometer (Bruker Company, Germany), using TMS as an internal standard. Chemical shifts were given in ppm (parts per million). Mass spectra were recorded on Q-TOF Priemier mass spectrometer (Micromass, Manchester, UK). The purity of each compound (> 95%) was determined on an Waters e2695 series LC system (column, Xtimate C18, 4.6 mm ×150 mm, 5 μm; mobile phase, methanol (70%)/H_2O (30%); low rate, 1.0 mL/min; UV wavelength, 254 - 400 nM; temperature, 25 °C; injection volume, 10 μL).

The general procedure to prepare compounds **9a-ae** has been shown in Scheme 1 and 2. 4-chloro-*7H*-pyrrolo[2,3-*d*]pyrimidine (**1**) was aminated with ammonium hydroxide to obtain **2**. Iodine was added to C-5 position of compound **2** by NIS to obtain **3**. Then N-H of **3** was protected by Ts group to give **4**. By Suzuki coupling reaction with 4-aminophenylboronic acid, **4** was converted to **5**. Under strong alkali condition, Ts group was removed from **5** to give **6**. 3-amino-5-tert-butylisoxazole was acylated by triphosgene with itself to give the key intermediate **8**. N-H of compound **6** was substituted by appropriate halide to give **7a-ae**. Then via ammonolysis **7a-ae** were converted to target molecules **9a-ae**.

Scheme 1. Synthesis of Intermediate **8**[a]
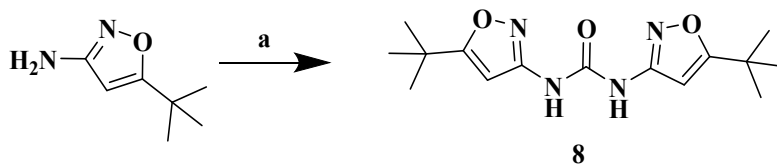
Scheme 2. Syntheses of Compounds **9a-ae**[a]
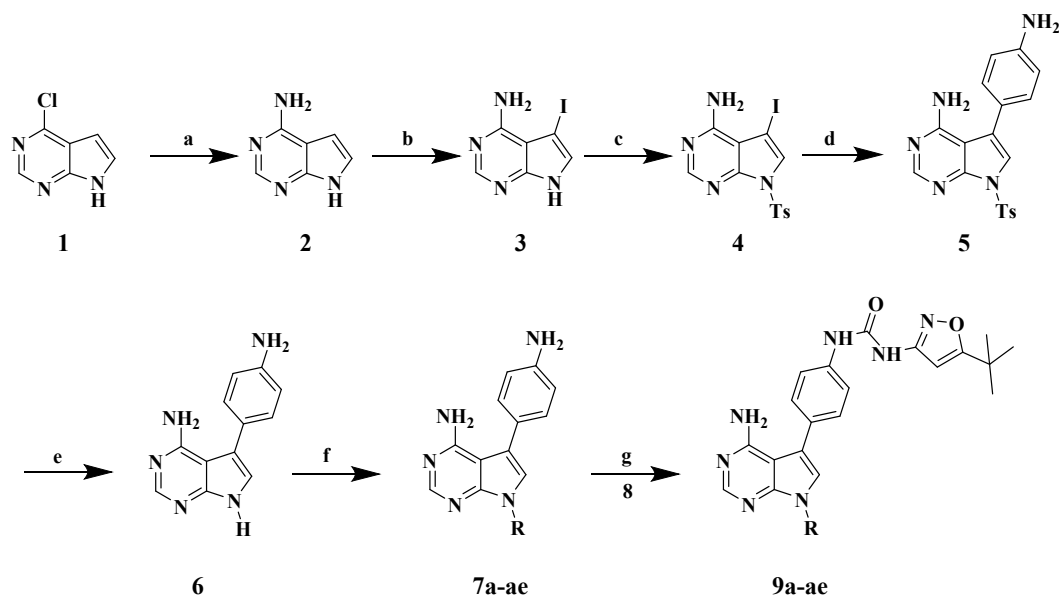
[a] Reagents and conditions: (a) NH₃·H₂O, high pressure, 130 °C, 4 h; (b) NIS, THF, rt,

1 h; (c) TsCl, DMAP, NEt₃, CH₃CN, 80 °C, 2 h; (d) 4-aminophenylboronic acid,

(dppf)PdCl₂, K₂CO₃, dioxane/EtOH/H₂O = 7/3/4(v/v/v), 80 °C, 2 h; (e) KOH, H₂O, rt,

1 h; (f) Appropriate halide, Cs₂CO₃, 80 °C, 1 h; (g) Intermediate **8**, CH₃CN, 80 °C, 1

h.

Scheme 3. Syntheses of Compounds **13a-h**[a]
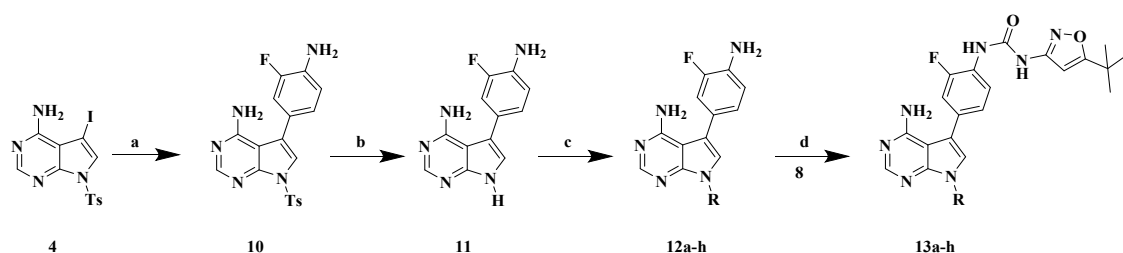
[a] Reagents and conditions: (a) 4-amino-3-fluorobenzeneboronic acid pinacol ester,

(dppf)PdCl$_2$, K$_2$CO$_3$, dioxane/EtOH/H$_2$O = 7/3/4(v/v/v), 80 ^{o}C, 2 h; (b) KOH, H$_2$O, rt, 1 h; (c) Appropriate halide, Cs$_2$CO$_3$, 80 ^{o}C, 1 h; (d) Intermediate **8**, CH$_3$CN, 80 ^{o}C, 1 h.

General Procedures of Method A for the Syntheses of Compounds 9a-ae

7*H*-pyrrolo[2,3-*d*]pyrimidin-4-amine (2). Commercially available **1** (20 g, 50 mmol, 1 equiv) was added to ammonia solution (150 ml, 25-28% NH$_3$ in water) and the mixture reacted in high-pressure autoclave. After reaction for 4 h at 130 ^{o}C, the mixture was cooled to room tempreture (rt). After filtration, the residue was washed with diethyl ether and dried at 80 ^{o}C for 2 h. The crude wasn't purified further. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.45 (s, 1H), 8.03 (s, 1H), 7.06 (d, *J* = 3.2 Hz, 1H), 6.88 (s, 2H), 6.51 (d, *J* = 3.3 Hz, 1H).

5-iodo-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-amine (3). To a solution of **2** (16 g, 112 mmol, 1 equiv) in tetrahydrofuran (THF) (200 ml) was added *N*-iodosuccinimide (NIS) (37.8 g, 168 mmol, 1.5 equiv) by proportion. After 1 h, the solvent was removed under reduced pressure to get the residue. The residue was dispersed in water (300 mL) and Na$_2$S$_2$O$_3$ • 5H$_2$O (13.9 g, 56 mmol, 0.5 equiv) was added whereafter. After stirring for 0.5 h, the mixture was filtered. The residue was washed with diethyl ether and dried at 80 ^{o}C for 2 h. The crude wasn't purified further. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.97 (s, 1H), 8.07 (s, 1H), 7.36 (s, 1H), 6.56 (s, 2H).

5-iodo-7-tosyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-amine (4). To the mixture of **3** (26 g, 100 mmol, 1 equiv) in CH$_3$CN (500 ml) was added 4-dimethylaminopyridine (DMAP) (14.6 g, 120 mmol, 1.2 equiv) and triethylamine (NEt$_3$) (26 ml, 200 mmol, 2

equiv). *p*-Toluenesulfonyl chloride (TsCl) (28.6 g, 150 mmol, 1.5 equiv) was dissolved in CH_3CN (100 mL) and added to the mixture by proportion. After 2 h at 80 °C, the solvent was removed under reduced pressure to get the residue. The residue was dispersed in CH_3CN (300 mL) and stirred for 1 h. The residue was filtered and washed with diethyl ether and dried at 80 °C for 2 h. The crude wasn't purified further. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.20 (s, 1H), 8.00 (d, J = 8.4 Hz, 2H), 7.82 (s, 1H), 7.45 (d, J = 8.2 Hz, 2H), 6.94 (s, 2H), 2.37 (s, 3H).

5-(4-aminophenyl)-7-tosyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-amine (**5**). **4** (10.7 g, 25.8 mmol, 1 equiv) was dissolved in 1,4-dioxane/ethanol/water (v:v:v, 7:3:4, 200 ml) and treated with 4-aminophenylboronic acid (4.9 g, 28.4 mmol, 1.1 equiv), $PdCl_2$(dppf), and K_2CO_3 (7.1 g, 51.6 mmol, 2 equiv). The vial was sealed and heated with stirring at 80 °C for 2 h. The solvent was removed under reduced pressure to get the residue without purification.

5-(4-aminophenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-amine (**6**). The crude production **5** was dissolved in water (100 mL) and KOH solid was added by proportion until pH was up to 14. After 1 h, the Ts group was removed completely. The mixture was quenched with water and extracted with CH_2Cl_2. The organic layer was separated and washed with saturated brine. After dried over Na_2SO_4, the solvent was removed under reduced pressure to afford the residue. The residue was dispersed in diethyl ether (50 mL) and stirred for 1 h. The residue was filtered and washed with diethyl ether to get **6** as gray solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.57 (s, 1H), 8.07 (s, 1H), 7.12 (d, J = 8.3 Hz, 2H), 7.03 (s, 1H), 6.66 (d, J = 8.3 Hz, 2H), 5.94 (s, 2H), 5.16 (s, 2H).

To a mixture of **6** (225 mg, 1 mmol, 1 equiv) in CH_3CN was added Cs_2CO_3 (650 mg, 2 mmol, 2 equiv), followed by appropriate halide (1.2 mmol, 1.2 equiv). After reacting at 80 °C for 1 h, the mixture was filtered to remove salts and the filtrate was concentrated under reduced pressure to afford the crude **7a-ae** without purification.

1,3-bis(5-(tert-butyl)isoxazol-3-yl)urea (8). To a solution of triphosgene (47.07 g, 156.9 mmol, 1.1 equiv) in THF (500 ml) was added 3-amino-5-tert-butylisoxazole (20 g, 142.6 mmol, 1 equiv) by proportion. Then NEt_3 (39.8 mL, 285.3 mmol, 2 equiv) was added drop by drop. After reaction at 65 °C for 5 h, the mixture was filtered and the solvent was removed under reduced pressure to get the residue. The crude wasn't purified further as pale yellow powder. ^1H NMR (400 MHz, DMSO-d_6) δ: 6.71 (s, 1H), 6.35 (s, 1H), 4.90 (s, 2H), 1.36 (s, 9H), 1.35 (s, 9H). MS (ESI), m/z: 307.37 [M+H$^+$].

To a solution of intermediate **7a-ae** (1 mmol, 1 equiv) in CH_3CN (20 mL) was added **8** (306 mg, 1 mmol, 1 equiv). After reaction at 80 °C for 1 h, a large amount of solid formed and the solid was obtained by filter and washed by diethyl ether. The corresponding crude **9a-ae** wasn't purified further as off-white or pale yellow powder.

1-(4-(4-amino-7-(2-morpholinoethyl)-7H-pyrrolo[2,3-d]pyrimidin-5-yl)phenyl)-3-(5-(tert-butyl)isoxazol-3-yl)urea (9a). ^1H NMR (400 MHz, DMSO-d_6) δ: 9.53 (s, 1H), 8.91 (s, 1H), 8.14 (s, 1H), 7.56 (d, J = 8.3 Hz, 2H), 7.40 (d, J = 8.2 Hz, 2H), 7.33 (s, 1H), 6.52 (s, 1H), 6.06 (s, 2H), 4.29 (t, J = 6.3 Hz, 2H), 3.54 (s, 4H), 2.71 (t, J = 6.4 Hz, 2H), 2.46 (s, 4H), 1.31 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 180.67, 158.86, 157.71, 151.95, 151.84, 150.71, 138.09, 129.48, 129.32, 123.90, 119.49,

115.16, 100.36, 92.92, 66.66, 58.15, 53.65, 41.24, 32.95, 28.83. HRMS (ESI), m/z: 505.2672 [M+H$^+$].

1-(4-(4-amino-7H-pyrrolo[2,3-d]pyrimidin-5-yl)phenyl)-3-(5-(tert-butyl)isoxazol-3-yl)urea (9b). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.74 (s, 1H), 9.53 (s, 1H), 8.93 (s, 1H), 8.11 (s, 1H), 7.55 (d, J = 8.5 Hz, 2H), 7.40 (d, J = 8.5 Hz, 2H), 7.19 (d, J = 2.3 Hz, 1H), 6.52 (s, 1H), 5.99 (s, 2H), 1.31 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 180.65, 158.86, 157.59, 152.01, 151.88, 151.79, 138.09, 129.78, 129.44, 120.45, 119.40, 115.90, 100.36, 92.94, 32.94, 28.83. HRMS (ESI), m/z: 392.1831 [M+H$^+$].

tert-butyl-4-amino-5-(4-(3-(5-(tert-butyl)isoxazol-3-yl)ureido)phenyl)-7H-pyrrolo[2,3-d]pyrimidine-7-carboxylate (9c). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.57 (s, 1H), 9.02 (s, 1H), 8.28 (s, 1H), 7.59 (d, J = 8.6 Hz, 2H), 7.47 (s, 1H), 7.44 (d, J = 8.5 Hz, 2H), 6.52 (s, 1H), 6.23 (s, 2H), 1.61 (s, 9H), 1.31 (s, 9H). HRMS (ESI), m/z: 592.2353 [M+H$^+$].

tert-butyl-4-(2-(4-amino-5-(4-(3-(5-(tert-butyl)isoxazol-3-yl)ureido)phenyl)-7H-pyrrolo[2,3-d]pyrimidin-7-yl)ethyl)piperazine-1-carboxylate (9d). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.53 (s, 1H), 8.91 (s, 1H), 8.14 (s, 1H), 7.56 (d, J = 8.5 Hz, 2H), 7.39 (d, J = 8.5 Hz, 2H), 7.33 (s, 1H), 6.52 (s, 1H), 6.06 (s, 2H), 4.29 (t, J = 6.5 Hz, 2H), 3.28 (s, 4H), 2.73 (t, J = 6.5 Hz, 2H), 2.46 – 2.37 (m, 4H), 1.39 (s, 9H), 1.31 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 180.67, 158.87, 157.70, 154.30, 151.95, 151.83, 150.70, 138.09, 129.47, 129.33, 123.89, 119.48, 115.17, 100.37, 92.92, 79.20, 57.66, 52.84, 41.47, 32.95, 28.83, 28.52. HRMS (ESI), m/z: 604.3353 [M+H$^+$].

1-(4-(4-amino-7-(2-(piperazin-1-yl)ethyl)-7H-pyrrolo[2,3-d]pyrimidin-5-yl)pheny

l)-3-(5-(tert-butyl)isoxazol-3-yl)urea (**9e**). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.58 (s, 1H), 9.01 (s, 1H), 8.14 (s, 1H), 7.57 (d, J = 8.5 Hz, 2H), 7.39 (d, J = 8.5 Hz, 2H), 7.33 (s, 1H), 6.52 (s, 1H), 6.05 (s, 2H), 4.27 (t, J = 6.6 Hz, 2H), 2.75 – 2.63 (m, 6H), 2.41 (s, 4H), 1.31 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 180.62, 158.86, 157.70, 151.92, 150.69, 138.20, 129.39, 129.30, 123.90, 119.41, 115.16, 100.36, 92.94, 58.14, 53.12, 45.28, 41.38, 32.94, 28.84. HRMS (ESI), m/z: 504.2829 [M+H$^+$].

1-(4-(4-amino-7-(3-morpholinopropyl)-7H-pyrrolo[2,3-d]pyrimidin-5-yl)phenyl)-3-(5-(tert-butyl)isoxazol-3-yl)urea (**9f**). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.53 (s, 1H), 8.90 (s, 1H), 8.14 (s, 1H), 7.56 (d, J = 8.6 Hz, 2H), 7.40 (d, J = 8.6 Hz, 2H), 7.31 (s, 1H), 6.52 (s, 1H), 6.05 (s, 2H), 4.20 (t, J = 7.0 Hz, 2H), 3.59 – 3.51 (m, 4H), 2.29 (dd, J = 14.3, 7.0 Hz, 6H), 2.02 – 1.91 (m, 2H), 1.31 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 180.68, 158.86, 157.71, 151.96, 151.83, 150.66, 138.07, 129.50, 129.34, 123.68, 119.48, 115.18, 100.45, 92.92, 66.65, 55.80, 53.71, 42.57, 32.95, 28.83, 27.07. HRMS (ESI), m/z: 519.2825 [M+H$^+$].

1-(4-(4-amino-7-(2-(dimethylamino)ethyl)-7H-pyrrolo[2,3-d]pyrimidin-5-yl)phenyl)-3-(5-(tert-butyl)isoxazol-3-yl)urea (**9g**). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.54 (s, 1H), 8.93 (s, 1H), 8.14 (s, 1H), 7.56 (d, J = 8.3 Hz, 2H), 7.39 (d, J = 8.3 Hz, 2H), 7.33 (s, 1H), 6.52 (s, 1H), 6.05 (s, 2H), 4.26 (t, J = 6.6 Hz, 2H), 2.67 (t, J = 6.5 Hz, 2H), 2.20 (s, 6H), 1.31 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 180.65, 158.87, 157.70, 151.93, 151.85, 150.71, 138.11, 129.47, 129.31, 123.73, 119.46, 115.17, 100.36, 92.93, 58.93, 45.57, 42.00, 32.94, 28.83. HRMS (ESI), m/z: 463.2563 [M+H$^+$].

1-(4-(4-amino-7-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)phenyl)-3-(5-(tert-butyl)isoxazol-3-yl)urea (9h). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.79 (s, 1H), 9.57 (s, 1H), 8.49 (s, 1H), 7.62 (s, 1H), 7.41 (d, J = 8.6 Hz, 2H), 7.11 (d, J = 8.5 Hz, 2H), 6.53 (s, 1H), 3.85 (s, 3H), 1.31 (s, 9H).^{13}C NMR (101 MHz, DMSO-d_6) δ: 180.56, 158.74, 152.05, 151.72, 147.94, 142.85, 139.28, 129.35, 127.76, 126.63, 119.29, 118.32, 98.85, 92.97, 32.93, 32.06, 28.85. HRMS (ESI), m/z: 406.1992 [M+H^{+}].

1-(4-(4-amino-7-ethyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)phenyl)-3-(5-(tert-butyl)is oxazol-3-yl)urea (9i). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.78 (s, 1H), 9.61 (s, 1H), 8.48 (s, 1H), 7.69 (s, 1H), 7.61 (d, J = 8.6 Hz, 2H), 7.42 (d, J = 8.5 Hz, 2H), 6.53 (s, 1H), 4.30 (q, J = 7.2 Hz, 2H), 1.43 (t, J = 7.2 Hz, 3H), 1.31 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 180.52, 158.74, 152.08, 151.82, 147.40, 142.80, 139.30, 129.36, 126.68, 126.30, 119.22, 118.52, 98.91, 92.97, 32.93, 28.85, 15.84. HRMS (ESI), m/z: 420.2142 [M+H^{+}].

1-(4-(4-amino-7-isopropyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)phenyl)-3-(5-(tert-bu tyl)isoxazol-3-yl)urea (9j). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.55 (s, 1H), 8.97 (s, 1H), 8.33 (s, 1H), 7.65 (s, 1H), 7.59 (d, J = 8.6 Hz, 2H), 7.43 (d, J = 8.5 Hz, 2H), 6.52 (s, 1H), 5.10 – 4.93 (m, 1H), 1.49 (d, J = 6.8 Hz, 6H), 1.31 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 180.71, 171.97, 158.82, 153.98, 151.81, 148.27, 146.51, 138.59, 129.46, 128.10, 122.43, 119.47, 117.44, 99.57, 92.92, 32.95, 28.83, 22.97, 22.76. HRMS (ESI), m/z: 434.2299 [M+H^{+}].

1-(4-(4-amino-7-propyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)phenyl)-3-(5-(tert-butyl)isoxazol-3-yl)urea (9k). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.80 (s, 1H), 9.66 (s,

1H), 8.48 (s, 1H), 7.68 (s, 1H), 7.61 (d, J = 8.5 Hz, 2H), 7.41 (d, J = 8.5 Hz, 2H), 6.53

(s, 1H), 4.23 (t, J = 7.0 Hz, 2H), 1.90 – 1.77 (m, 2H), 1.31 (s, 9H), 0.87 (t, J = 7.4 Hz,

3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 180.55, 158.75, 152.04, 151.80, 147.79,

142.88, 139.27, 129.40, 126.76, 126.69, 119.27, 118.44, 98.84, 92.97, 46.64, 32.93,

28.85, 23.50, 11.44. HRMS (ESI), m/z: 434.2303 [M+H$^+$].

1-(4-(4-amino-7-butyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)phenyl)-3-(5-(tert-butyl)i

soxazol-3-yl)urea (9l). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.78 (s, 1H), 9.61 (s, 1H),

8.48 (s, 1H), 7.68 (s, 1H), 7.61 (d, J = 8.6 Hz, 2H), 7.41 (d, J = 8.5 Hz, 2H), 6.53 (s,

1H), 4.26 (t, J = 7.1 Hz, 2H), 1.87 – 1.75 (m, 2H), 1.31 (s, 9H), 1.26 (dd, J = 14.9, 7.5

Hz, 2H), 0.91 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 180.55, 158.75,

152.02, 151.68, 147.70, 142.76, 139.26, 129.39, 126.76, 126.66, 119.28, 118.53,

98.80, 92.96, 44.78, 32.92, 32.13, 28.84, 19.75, 13.88. HRMS (ESI), m/z: 448.2460

[M+H$^+$].

1-(4-(4-amino-7-hexyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)phenyl)-3-(5-(tert-butyl)i

soxazol-3-yl)urea (9m). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.71 (s, 1H), 9.40 (s, 1H),

8.46 (s, 1H), 7.67 (s, 1H), 7.61 (d, J = 8.6 Hz, 2H), 7.41 (d, J = 8.5 Hz, 2H), 6.52 (s,

1H), 4.25 (t, J = 7.1 Hz, 2H), 1.88 – 1.77 (m, 2H), 1.31 (s, 9H), 1.28 (m, 6H), 0.86 (t,

J = 6.8 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 180.54, 158.75, 152.02, 151.68,

147.69, 142.75, 139.27, 129.38, 126.75, 126.66, 119.28, 118.52, 98.79, 92.96, 45.06,

32.93, 31.13, 30.03, 28.84, 26.11, 22.38, 14.31. HRMS (ESI), m/z: 476.2770 [M+H$^+$].

1-(4-(4-amino-7-cyclopentyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)phenyl)-3-(5-(tert-

butyl)isoxazol-3-yl)urea (9n). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.78 (s, 1H), 9.62

(s, 1H), 8.47 (s, 1H), 7.73 (s, 1H), 7.61 (d, J = 8.6 Hz, 2H), 7.44 (d, J = 8.5 Hz, 2H),

6.53 (s, 1H), 5.22 – 5.08 (m, 1H), 2.16 (d, J = 7.9 Hz, 2H), 2.02 – 1.83 (m, 4H), 1.71

(d, J = 7.0 Hz, 2H), 1.31 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 180.51, 158.73,

152.08, 151.80, 147.66, 142.61, 139.32, 129.47, 126.69, 124.20, 119.15, 118.90,

99.03, 92.98, 56.11, 32.92, 32.79, 28.85, 24.21. HRMS (ESI), m/z: 460.2457 [M+H$^+$].

1-(4-(4-amino-7-benzyl-7H-pyrrolo[2,3-d]pyrimidin-5-yl)phenyl)-3-(5-(tert-butyl)

isoxazol-3-yl)urea (9o). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.71 (s, 1H), 8.49 (s, 1H),

7.74 (s, 1H), 7.60 (d, J = 8.6 Hz, 2H), 7.41 (d, J = 8.5 Hz, 2H), 7.38 – 7.27 (m, 5H),

6.52 (s, 1H), 5.49 (s, 2H), 1.31 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 180.59,

158.75, 151.98, 151.79, 147.78, 143.31, 139.29, 137.27, 129.42, 129.21, 128.36,

128.16, 126.70, 126.55, 119.31, 118.90, 99.09, 92.97, 48.46, 32.93, 28.84. HRMS

(ESI), m/z: 482.2303 [M+H$^+$].

1-(4-(4-amino-7-(4-hydroxybutyl)-7H-pyrrolo[2,3-d]pyrimidin-5-yl)phenyl)-3-(5-

(tert-butyl)isoxazol-3-yl)urea (9p). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.53 (s, 1H),

8.91 (s, 1H), 8.14 (s, 1H), 7.56 (d, J = 8.4 Hz, 2H), 7.40 (d, J = 8.4 Hz, 2H), 7.32 (s,

1H), 6.52 (s, 1H), 6.05 (s, 2H), 4.41 (t, J = 5.2 Hz, 1H), 4.17 (t, J = 7.0 Hz, 2H), 4.09

(q, J = 5.2 Hz, 1H), 3.41 (dd, J = 11.8, 6.2 Hz, 2H), 3.18 (d, J = 5.2 Hz, 2H), 1.88 –

1.76 (m, 2H), 1.46 – 1.36 (m, 2H), 1.31 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ:

180.68, 158.86, 157.71, 151.95, 151.83, 150.64, 138.08, 129.48, 129.36, 123.53,

119.46, 115.23, 100.37, 92.93, 60.75, 44.09, 32.95, 30.16, 28.83, 27.05. HRMS (ESI),

m/z: 464.2409 [M+H$^+$].

4-amino-5-(4-(3-(5-(tert-butyl)isoxazol-3-yl)ureido)phenyl)-N,N-diethyl-7H-pyrr

olo[2,3-*d*]pyrimidine-7-carboxamide (**9q**). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.55 (s, 1H), 8.96 (s, 1H), 8.20 (s, 1H), 7.58 (d, *J* = 8.6 Hz, 2H), 7.44 (d, *J* = 8.5 Hz, 2H), 7.41 (s, 1H), 6.53 (s, 1H), 6.25 (s, 2H), 3.39 (m, 4H), 1.31 (s, 9H), 1.21 − 1.06 (m, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 180.69, 158.84, 158.04, 153.19, 151.83, 151.53, 150.56, 138.71, 129.57, 128.19, 121.42, 119.42, 117.82, 100.52, 92.93, 55.37, 32.95, 28.83. HRMS (ESI), m/z: 491.2493 [M+H^{+}].

1-(4-(4-amino-7-(2-(4-(methylsulfonyl)piperazin-1-yl)ethyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)phenyl)-3-(5-(tert-butyl)isoxazol-3-yl)urea (**9r**). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.53 (s, 1H), 8.92 (s, 1H), 8.15 (s, 1H), 7.56 (d, *J* = 8.6 Hz, 2H), 7.40 (d, *J* = 8.5 Hz, 2H), 7.34 (s, 1H), 6.52 (s, 1H), 6.05 (s, 2H), 4.30 (t, *J* = 6.4 Hz, 2H), 3.12 − 3.03 (m, 4H), 2.85 (s, 3H), 2.79 (t, *J* = 6.5 Hz, 2H), 2.62 − 2.54 (m, 4H), 1.31 (s, 9H). HRMS (ESI), m/z: 582.2610 [M+H^{+}].

1-(4-(4-amino-7-(2-methoxyethyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)phenyl)-3-(5-(tert-butyl)isoxazol-3-yl)urea (**9s**). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.56 (s, 1H), 8.95 (s, 1H), 8.15 (s, 1H), 7.56 (d, *J* = 8.4 Hz, 2H), 7.39 (d, *J* = 8.4 Hz, 2H), 7.29 (s, 1H), 6.53 (s, 1H), 6.09 (s, 2H), 4.33 (t, *J* = 5.3 Hz, 2H), 3.71 (t, *J* = 5.4 Hz, 2H), 3.26 (s, 3H), 1.31 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 180.68, 158.86, 157.70, 151.95, 151.84, 150.74, 138.13, 129.37, 129.33, 123.87, 119.47, 115.23, 100.41, 92.93, 70.96, 58.40, 43.72, 32.95, 28.84. HRMS (ESI), m/z: 450.2249 [M+H^{+}].

1-(4-(4-amino-7-(2-ethoxyethyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)phenyl)-3-(5-(tert-butyl)isoxazol-3-yl)urea (**9t**). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.53 (s, 1H), 8.92 (s, 1H), 8.15 (s, 1H), 7.56 (d, *J* = 8.4 Hz, 2H), 7.39 (d, *J* = 8.4 Hz, 2H), 7.29 (s,

1H), 6.52 (s, 1H), 6.06 (s, 2H), 4.32 (t, J = 5.4 Hz, 2H), 3.74 (t, J = 5.5 Hz, 2H), 3.46 (q, J = 6.9 Hz, 2H), 1.31 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 180.56, 158.75, 152.02, 151.77, 147.78, 142.95, 139.26, 135.00, 129.37, 126.69, 119.29, 118.38, 118.02, 98.85, 92.96, 44.33, 34.25, 32.93, 28.84. HRMS (ESI), m/z: 464.2406 [M+H$^+$].

1-(4-(7-allyl-4-amino-7H-pyrrolo[2,3-d]pyrimidin-5-yl)phenyl)-3-(5-(tert-butyl)isoxazol-3-yl)urea (9u). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.53 (s, 1H), 8.91 (s, 1H), 8.15 (s, 1H), 7.56 (d, J = 8.5 Hz, 2H), 7.40 (d, J = 8.4 Hz, 2H), 7.25 (s, 1H), 6.52 (s, 1H), 6.06 (m, 3H), 5.18 (d, J = 10.1 Hz, 1H), 5.09 (d, J = 17.1 Hz, 1H), 4.81 (d, J = 5.4 Hz, 2H), 1.31 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 180.64, 158.77, 152.59, 151.93, 148.08, 144.53, 139.08, 133.75, 129.43, 127.04, 126.11, 119.39, 118.36, 118.26, 99.20, 92.95, 47.13, 32.95, 28.84. HRMS (ESI), m/z: 432.2149 [M+H$^+$].

1-(4-(4-amino-7-(but-3-en-1-yl)-7H-pyrrolo[2,3-d]pyrimidin-5-yl)phenyl)-3-(5-(tert-butyl)isoxazol-3-yl)urea (9v). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.57 (s, 1H), 8.99 (s, 1H), 8.15 (s, 1H), 7.56 (d, J = 8.4 Hz, 2H), 7.39 (d, J = 8.3 Hz, 2H), 7.32 (s, 1H), 6.52 (s, 1H), 6.05 (s, 2H), 5.82 (ddd, J = 23.8, 10.3, 6.7 Hz, 1H), 5.14 – 4.93 (m, 2H), 4.24 (t, J = 7.0 Hz, 2H), 2.56 (dd, J = 13.3, 6.5 Hz, 2H), 1.31 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 180.67, 158.86, 157.71, 151.97, 151.85, 150.63, 138.13, 135.58, 129.43, 129.34, 123.50, 119.44, 117.54, 115.21, 100.41, 92.93, 43.48, 34.42, 32.95, 28.83. HRMS (ESI), m/z: 446.2302 [M+H$^+$].

1-(4-(4-amino-7-(3-methylbut-2-en-1-yl)-7H-pyrrolo[2,3-d]pyrimidin-5-yl)phenyl)-3-(5-(tert-butyl)isoxazol-3-yl)urea (9w). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.53

(s, 1H), 8.92 (s, 1H), 8.15 (s, 1H), 7.56 (d, J = 8.4 Hz, 2H), 7.39 (d, J = 8.4 Hz, 2H),

7.23 (s, 1H), 6.52 (s, 1H), 6.05 (s, 2H), 5.41 (t, J = 6.8 Hz, 1H), 4.76 (d, J = 7.0 Hz,

2H), 1.81 (s, 3H), 1.72 (s, 3H), 1.31 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ:

180.66, 158.86, 157.70, 152.04, 151.85, 150.32, 138.13, 136.47, 129.40, 129.35,

122.91, 120.60, 119.42, 115.49, 100.39, 92.93, 41.77, 32.95, 28.83, 25.81, 18.32.

HRMS (ESI), m/z: 460.2457 [M+H$^+$].

1-(4-(4-amino-7-(2-methylallyl)-7H-pyrrolo[2,3-*d*]pyrimidin-5-yl)phenyl)-3-(5-(te

rt-butyl)isoxazol-3-yl)urea (**9x**). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.53 (s, 1H),

8.92 (s, 1H), 8.15 (s, 1H), 7.56 (d, J = 8.6 Hz, 2H), 7.40 (d, J = 8.6 Hz, 2H), 7.20 (s,

1H), 6.52 (s, 1H), 6.09 (s, 2H), 4.87 (s, 1H), 4.73 (s, 2H), 4.61 (s, 1H), 1.68 (s, 3H),

1.31 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 180.66, 158.86, 157.78, 152.18,

151.83, 150.88, 142.16, 138.19, 129.39, 129.28, 123.45, 119.45, 115.69, 112.35,

100.25, 92.93, 49.50, 32.94, 28.82, 20.37. HRMS (ESI), m/z: 446.2298 [M+H$^+$].

1-(4-(4-amino-7-(pent-4-en-1-yl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)phenyl)-3-(5-(t

ert-butyl)isoxazol-3-yl)urea (**9y**). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.77 (s, 1H),

9.58 (s, 1H), 8.48 (s, 1H), 7.68 (s, 1H), 7.61 (d, J = 8.6 Hz, 2H), 7.42 (d, J = 8.6 Hz,

2H), 6.53 (s, 1H), 5.83 (ddt, J = 16.8, 10.2, 6.4 Hz, 1H), 5.09 – 4.95 (m, 2H), 4.27 (t,

J = 7.0 Hz, 2H), 2.05 (dd, J = 13.7, 6.7 Hz, 2H), 1.99 – 1.87 (m, 2H), 1.31 (s, 9H). ^{13}C

NMR (101 MHz, DMSO-d_6) δ: 180.55, 158.75, 152.03, 151.74, 147.76, 142.84,

139.27, 137.85, 129.39, 126.73, 126.67, 119.28, 118.55, 115.96, 98.88, 92.96, 44.63,

32.93, 30.65, 29.20, 28.84. HRMS (ESI), m/z: 460.2458 [M+H$^+$].

1-(4-(4-amino-7-(hex-5-en-1-yl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)phenyl)-3-(5-(te

rt-butyl)isoxazol-3-yl)urea (**9z**). [^1]H NMR (400 MHz, DMSO-d_6) δ: 9.78 (s, 1H), 9.60 (s, 1H), 8.48 (s, 1H), 7.68 (s, 1H), 7.61 (d, J = 8.6 Hz, 2H), 7.41 (d, J = 8.6 Hz, 2H), 6.53 (s, 1H), 5.78 (ddt, J = 16.9, 10.2, 6.7 Hz, 1H), 5.05 – 4.90 (m, 2H), 4.27 (t, J = 7.0 Hz, 2H), 2.06 (q, J = 7.2 Hz, 2H), 1.83 (dt, J = 14.9, 7.2 Hz, 2H), 1.41 – 1.31 (m, 2H), 1.31 (s, 9H). [^13]C NMR (101 MHz, DMSO-d_6) δ: 180.55, 158.75, 152.03, 151.76, 147.73, 142.86, 139.27, 138.75, 129.39, 126.72, 126.67, 119.27, 118.52, 115.55, 98.83, 92.96, 44.90, 33.03, 32.93, 29.58, 28.84, 25.73. HRMS (ESI), m/z: 474.2618 [M+H$^+$].

1-(4-(4-amino-7-(prop-2-yn-1-yl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)phenyl)-3-(5-(tert-butyl)isoxazol-3-yl)urea (9aa). [^1]H NMR (400 MHz, DMSO-d_6) δ: 9.66 (s, 1H), 9.28 (s, 1H), 8.49 (s, 1H), 7.66 (s, 1H), 7.61 (d, J = 8.5 Hz, 2H), 7.43 (d, J = 8.5 Hz, 2H), 6.52 (s, 1H), 5.15 (d, J = 2.3 Hz, 2H), 3.52 (t, J = 2.4 Hz, 2H), 1.31 (s, 9H). [^13]C NMR (101 MHz, DMSO-d_6) δ: 180.63, 158.75, 151.95, 151.84, 147.54, 143.60, 139.33, 129.45, 126.41, 125.92, 119.38, 119.06, 99.28, 92.96, 78.54, 76.82, 34.59, 32.94, 28.84. HRMS (ESI), m/z: 430.1985 [M+H$^+$].

1-(4-(4-amino-7-(but-2-yn-1-yl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)phenyl)-3-(5-(tert-butyl)isoxazol-3-yl)urea (9ab). [^1]H NMR (400 MHz, DMSO-d_6) δ: 9.55 (s, 1H), 8.97 (s, 1H), 8.17 (s, 1H), 7.57 (d, J = 8.5 Hz, 2H), 7.41 (d, J = 8.4 Hz, 2H), 7.32 (s, 1H), 6.53 (s, 1H), 6.12 (s, 1H), 4.97 (d, J = 2.3 Hz, 2H), 1.81 (dd, J = 5.3, 3.1 Hz, 3H), 1.31 (s, 9H). [^13]C NMR (101 MHz, DMSO-d_6) δ: 180.68, 158.86, 157.79, 152.28, 151.85, 150.27, 138.28, 129.38, 129.10, 122.67, 119.47, 115.99, 100.45, 92.93, 80.96, 74.98, 33.79, 32.95, 28.83, 3.54. HRMS (ESI), m/z: 444.2147 [M+H$^+$].

1-(4-(4-amino-7-(pent-2-yn-1-yl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)phenyl)-3-(5-(tert-butyl)isoxazol-3-yl)urea (9ac). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.53 (s, 1H), 8.93 (s, 1H), 8.16 (s, 1H), 7.56 (d, J = 8.6 Hz, 2H), 7.40 (d, J = 8.5 Hz, 2H), 7.31 (s, 1H), 6.52 (s, 1H), 6.12 (s, 2H), 4.98 (t, J = 2.1 Hz, 2H), 2.25 – 2.16 (m, 2H), 1.30 (s, 9H), 1.06 (t, J = 7.5 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 180.68, 158.86, 157.79, 152.28, 151.84, 150.27, 138.28, 129.38, 129.10, 122.58, 119.49, 116.00, 100.44, 92.93, 86.47, 75.10, 33.76, 32.95, 28.83, 14.02, 12.10. HRMS (ESI), m/z: 458.2299 [M+H$^+$].

1-(4-(4-amino-7-(cyanomethyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)phenyl)-3-(5-(tert-butyl)isoxazol-3-yl)urea (9ad). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.54 (s, 1H), 8.94 (s, 1H), 8.24 (s, 1H), 7.58 (d, J = 7.4 Hz, 2H), 7.42 (d, J = 7.6 Hz, 2H), 7.38 (s, 1H), 6.53 (s, 1H), 6.26 (s, 2H), 5.41 (s, 2H), 1.31 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 180.69, 158.85, 157.90, 152.72, 151.83, 150.70, 138.56, 129.41, 128.50, 122.66, 119.51, 117.08, 116.78, 100.62, 92.93, 32.94, 32.38, 28.82. HRMS (ESI), m/z: 431.1937 [M+H$^+$].

1-(4-(4-amino-7-(2-cyanoethyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)phenyl)-3-(5-(tert-butyl)isoxazol-3-yl)urea (9ae). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.71 (s, 1H), 9.42 (s, 1H), 8.50 (s, 1H), 7.70 (s, 1H), 7.63 (d, J = 8.6 Hz, 2H), 7.42 (d, J = 8.5 Hz, 2H), 6.53 (s, 1H), 4.56 (t, J = 6.4 Hz, 2H), 3.19 (t, J = 6.4 Hz, 2H), 1.31 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 180.57, 158.74, 152.03, 151.91, 148.02, 143.34, 139.41, 129.35, 126.41, 126.28, 119.33, 118.91, 118.78, 99.15, 92.97, 40.77, 32.94, 28.85, 18.82. HRMS (ESI), m/z: 445.2097 [M+H$^+$].

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General Procedures of Method B for the Syntheses of Compounds 13a-h

5-(4-amino-3-fluorophenyl)-7-tosyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-amine (**10**). **4**

(10.7 g, 25.8 mmol, 1 equiv) was dissolved in 1,4-dioxane/ethanol/water (v:v:v, 7:3:4,

200 ml) and treated with 4-amino-3-fluorobenzeneboronic acid pinacol ester (6.7 g,

28.4 mmol, 1.1 equiv), $PdCl_2$(dppf), and K_2CO_3 (7.1 g, 51.6 mmol, 2 equiv). The vial

was sealed and heated with stirring at 80 ^{o}C for 2 h. The solvent was removed under

reduced pressure to get the residue without purification.

5-(4-amino-3-fluorophenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-amine (**11**). The crude

production **10** was dissolved in water (100 mL) and KOH solid was added by

proportion until pH was up to 14. After 1 h, the Ts group was removed completely.

The mixture was quenched with water and extracted with CH_2Cl_2. The organic layer

was separated and washed with saturated brine. After dried over Na_2SO_4, the solvent

was removed under reduced pressure to afford the residue. The residue was dispersed

in diethyl ether (50 mL) and stirred for 1 h. The residue was filtered and washed with

diethyl ether to get **11** as gray solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.66 (s, 1H),

8.08 (s, 1H), 7.11 (d, J = 2.1 Hz, 1H), 7.07 (dd, J = 12.5, 1.8 Hz, 1H), 6.98 (dd, J =

8.1, 1.8 Hz, 1H), 6.85 (dd, J = 9.5, 8.2 Hz, 1H), 5.99 (s, 2H), 5.21 (s, 2H).

To a mixture of **11** (243 mg, 1 mmol, 1 equiv) in CH_3CN was added Cs_2CO_3 (650 mg,

2 mmol, 2 equiv), followed by appropriate halide (1.2 mmol, 1.2 equiv). After

reacting at 80 ^{o}C for 1 h, the mixture was filtered to remove salts and the filtrate was

concentrated under reduced pressure to afford the crude **12a-h** without purification.

To a solution of intermediate **12a-h** (1 mmol, 1 equiv) in CH_3CN (20 mL) was added **8** (306 mg, 1 mmol, 1 equiv). After reaction at 80 °C for 1 h, a large amount of solid formed and the solid was obtained by filter and washed by diethyl ether. The corresponding crude **13a-h** wasn't purified further as off-white or pale yellow powder.

1-(4-(4-amino-7H-pyrrolo[2,3-d]pyrimidin-5-yl)-2-fluorophenyl)-3-(5-(tert-butyl) isoxazol-3-yl)urea (**13a**) ^{1}H NMR (400 MHz, DMSO-d_6) δ: 13.04 (s, 1H), 10.13 (s, 1H), 9.20 (s, 1H), 8.46 (s, 1H), 8.23 (t, J = 8.5 Hz, 1H), 7.61 (d, J = 2.3 Hz, 1H), 7.37 (dd, J = 12.0, 1.7 Hz, 1H), 7.27 (d, J = 8.4 Hz, 1H), 6.51 (s, 2H), 1.31 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 180.78, 158.65, 153.91, 151.95, 151.74, 149.06, 142.92, 128.17, 126.74, 125.07, 124.38, 121.59, 118.03, 115.82, 98.84, 92.90, 32.96, 28.82. HRMS (ESI), m/z: 410.1734 [M+H$^+$].

1-(4-(7-allyl-4-amino-7H-pyrrolo[2,3-d]pyrimidin-5-yl)-2-fluorophenyl)-3-(5-(tert -butyl)isoxazol-3-yl)urea (**13b**) ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.86 (s, 1H), 8.88 (s, 1H), 8.20 (t, J = 8.5 Hz, 1H), 8.16 (s, 1H), 7.33 (s, 1H), 7.33 (dd, J = 12.1, 1.8 Hz, 2H), 7.26 (dd, J = 8.5, 1.3 Hz, 1H), 6.51 (s, 1H), 6.19 (s, 2H), 6.05 (ddd, J = 22.5, 10.6, 5.5 Hz, 1H), 5.18 (dd, J = 10.2, 1.3 Hz, 1H), 5.09 (dd, J = 17.1, 1.4 Hz, 1H), 4.80 (d, J = 5.5 Hz, 2H), 1.31 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 180.84, 158.69, 152.21, 151.59, 150.71, 134.51, 130.37, 125.86, 125.76, 124.83, 123.86, 121.48, 117.68, 115.50, 115.30, 114.70, 100.21, 92.83, 46.41, 32.95, 28.79. HRMS (ESI), m/z: 450.2042 [M+H$^+$].

1-(4-(4-amino-7-(but-3-en-1-yl)-7H-pyrrolo[2,3-d]pyrimidin-5-yl)-2-fluorophenyl

)-3-(5-(tert-butyl)isoxazol-3-yl)urea (**13c**) ^{1}H NMR (400 MHz, DMSO-d_6) δ: 10.05

(s, 1H), 9.12 (s, 1H), 8.49 (s, 1H), 8.25 (t, J = 8.5 Hz, 1H), 7.73 (s, 1H), 7.36 (dd, J =

12.0, 1.7 Hz, 1H), 7.26 (d, J = 8.4 Hz, 1H), 6.51 (s, 1H), 5.80 (ddt, J = 17.0, 10.3, 6.7

Hz, 1H), 5.10 – 4.99 (m, 2H), 4.34 (t, J = 7.0 Hz, 2H), 2.61 (q, J = 6.8 Hz, 2H), 1.31

(s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 180.76, 158.64, 153.93, 151.84, 151.51,

147.91, 143.06, 134.96, 127.77, 127.26, 126.75, 124.97, 121.66, 118.06, 117.28,

115.74, 98.85, 92.91, 44.38, 34.21, 32.95, 28.83. HRMS (ESI), m/z:

464.2212[M+H^{+}].

1-(4-(4-amino-7-(prop-2-yn-1-yl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)-2-fluorophen

yl)-3-(5-(tert-butyl)isoxazol-3-yl)urea (**13d**) ^{1}H NMR (400 MHz, DMSO-d_6) δ:

10.01 (s, 1H), 9.08 (s, 1H), 8.52 (s, 1H), 8.26 (t, J = 8.5 Hz, 1H), 7.73 (s, 1H), 7.39

(dd, J = 12.0, 1.8 Hz, 1H), 7.30 – 7.25 (m, 1H), 6.51 (s, 1H), 5.16 (d, J = 2.4 Hz, 2H),

1.31 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 180.79, 158.62, 153.89, 151.89,

151.72, 151.47, 147.62, 143.65, 139.19, 126.44, 125.07, 121.60, 117.95, 115.84,

99.28, 92.90, 78.45, 76.90, 34.64, 32.95, 28.82. HRMS (ESI), m/z: 448.1891 [M+H^{+}].

1-(4-(4-amino-7-(2-methoxyethyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)-2-fluorophe

nyl)-3-(5-(tert-butyl)isoxazol-3-yl)urea (**13e**) ^{1}H NMR (400 MHz, DMSO-d_6) δ:

10.10 (s, 1H), 9.16 (s, 1H), 8.51 (s, 1H), 8.25 (t, J = 8.5 Hz, 1H), 7.70 (s, 1H), 7.43 –

7.33 (m, 1H), 7.26 (m , 1H), 6.51 (s, 1H), 4.43 (t, J = 5.1 Hz, 2H), 3.76 (t, J = 5.3 Hz,

2H), 1.31 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 180.76, 158.64, 151.78, 148.04,

142.86, 129.36, 128.67, 127.52, 124.98, 121.68, 117.36, 115.77, 115.58, 98.89, 92.91,

70.54, 58.44, 45.73, 32.96, 28.83, 8.89. HRMS (ESI), m/z: 468.2159 [M+H^{+}].

1-(4-(4-amino-7-(2-methoxyethyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)-2-fluorophe

nyl)-3-(5-(tert-butyl)isoxazol-3-yl)urea (**13f**) ^{1}H NMR (400 MHz, DMSO-d_6) δ:

10.02 (s, 1H), 9.09 (s, 1H), 8.48 (s, 1H), 8.26 (t, *J* = 8.5 Hz, 1H), 7.68 (s, 1H), 7.37

(dd, *J* = 12.0, 1.8 Hz, 1H), 7.27 (d, *J* = 8.5 Hz, 1H), 6.51 (s, 1H), 4.42 (t, *J* = 5.3 Hz,

2H), 3.78 (t, *J* = 5.3 Hz, 2H), 3.46 (dd, *J* = 14.0, 7.0 Hz, 2H), 1.31 (s, 9H), 1.06 (t, *J* =

7.0 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 180.77, 158.64, 153.93, 151.51,

148.10, 143.07, 127.49, 126.87, 124.98, 121.66, 117.27, 115.61, 98.92, 92.90, 68.42,

65.83, 44.91, 32.96, 28.82, 15.42. HRMS (ESI), m/z: 482.2313 [M+H^{+}].

1-(4-(4-amino-7-(cyanomethyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)-2-fluorophenyl)

-3-(5-(tert-butyl)isoxazol-3-yl)urea (**13g**) ^{1}H NMR (400 MHz, DMSO-d_6) δ: 10.10

(s, 1H), 9.16 (s, 1H), 8.57 (s, 1H), 8.26 (t, *J* = 8.5 Hz, 1H), 7.72 (s, 1H), 7.39 (dd, *J* =

11.9, 1.8 Hz, 1H), 7.28 (d, *J* = 8.4 Hz, 1H), 6.51 (s, 1H), 5.55 (s, 2H), 1.31 (s, 9H).

^{13}C NMR (101 MHz, DMSO-d_6) δ: 180.77, 158.63, 153.91, 152.52, 148.27, 144.75,

127.23, 127.18, 127.07, 126.24, 125.07, 121.67, 118.30, 116.12, 115.87, 115.67,

99.82, 92.91, 32.96, 28.83. HRMS (ESI), m/z: 449.1848 [M+H^{+}].

1-(4-(4-amino-7-(2-cyanoethyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)-2-fluorophenyl)

-3-(5-(tert-butyl)isoxazol-3-yl)urea (**13h**) ^{1}H NMR (400 MHz, DMSO-d_6) δ: 10.05

(s, 1H), 9.12 (s, 1H), 8.54 (s, 1H), 8.28 (t, *J* = 8.5 Hz, 1H), 7.79 (s, 1H), 7.65 (s, 1H),

7.37 (dd, *J* = 11.9, 1.8 Hz, 1H), 7.28 (d, *J* = 8.5 Hz, 1H), 6.51 (s, 1H), 4.60 – 4.51 (t, *J*

= 6.5 Hz, 2H), 3.19 (t, *J* = 6.6 Hz, 2H), 1.31 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6)

δ: 180.72, 158.64, 153.97, 151.86, 151.55, 148.04, 143.14, 142.99, 127.38, 126.92,

126.48, 125.25, 121.76, 118.76, 117.87, 115.75, 99.12, 92.94, 32.95, 28.83, 18.81.

HRMS (ESI), m/z: 463.2004 [M+H$^+$].

Associated Contents

Supporting Information

Table S1 listing binding affinities of **9u** with more than 400 protein kinases; Table S2 listing IC$_{50}$ values of compounds **9a-ae** and **13a-h** in RS4;11 cell; Table S3 listing inhibitory rate of compounds **9a-ae** and **13a-h** in HL-60 Cell; Table S4 listing IC$_{50}$ values against hit target kinases of **9u**; Figure S1 listing the predicted bioavailability radar chart of the selected compounds; Figure S2 listing predicted boiled-egg plot of the selected compounds.

Biological assay methods: 1): Anti-proliferative assays; 2): Kinase profile assay and IC$_{50}$ test; 3): Dissociation constants (K$_d$) assay for FLT3 mutants; 4): Western blotting of signaling pathways; 5): Cell cycle progression experiment; 6): Annexin V-FITC/PI apoptosis assay; 7): Pharmacokinetic study; 8): Molecular docking study; 9): Animal tumor models and treatment.

Molecular formula strings (CSV)

PDB code 4XUF was used for modeling docking in FLT3 of compounds CHMFL-FLT3-213, **9a**, **9b**, **9u**, and **13a**. Authors will release the Atomic Coordinates and experimental data upon article publication.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. X.Y., Y.C., W.H.Z., and J.H. contributed equally.

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Abbreviations Used:

FLT3, Fms-like tyrosine receptor kinase 3; ITD, internal tandem duplications; TKD, tyrosine kinase domain; RTK, receptor tyrosine kinases; K_d, dissociation constant; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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Table of Contents Graphic

FLT3 IC_{50}: 7 nM
FLT3 vs c-Kit: > 40-fold
MV4-11 IC_{50}: 0.089 nM
Molm-13 IC_{50}: 0.022 nM
F% : 59.5%
TGI: (Molm-13, 3 mg/kg po) 94.5%
TGI: (MV4-11, 10 mg/kg po) 100%

Compound 9u

FLT3