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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Table S1. Kinome Wide Selectivity Profiling of Compound **9u**

Kinase	Kinase family	Percent control (%) @0.1 μM	Kinase	Kinase family	Percent control (%) @ 0.1 μM
AAK1(h)	MISC	96	Aurora-B(h)	MISC	4
Abl(h)	TK	35	Aurora-C(h)	MISC	50
Abl(H396P)(h)	TK	37	Axl(h)	TK	12
Abl(M351T)(h)	TK	46	BIKe(h)	MISC	95
Abl(Q252H)(h)	TK	15	Blk(h)	TK	18
Abl(T315I)(h)	TK	9	Blk(m)	TK	9
Abl(Y253F)(h)	TK	16	BMPR2(h)	TKL	89
ACK1(h)	TK	70	Bmx(h)	TK	26
ACTR2(h)	TKL	108	B-Raf(h)	TKL	33
ALK(h)	TK	14	B-Raf(V599E)(h)	TKL	22
ALK1(h)	TKL	100	BRK(h)	TK	57
ALK2(h)	TKL	101	BrSK1(h)	CAMK	89
ALK4(h)	TKL	104	BrSK2(h)	CAMK	89
ALK6(h)	TKL	103	BTK(h)	TK	45
AMPKα1(h)	CAMK	95	BTK(R28H)(h)	TK	91
AMPKα2(h)	CAMK	85	CaMKI(h)	CAMK	74
A-Raf(h)	TKL	47	CaMKIb(h)	CAMK	68
Arg(h)	TK	18	CaMKIIα(h)	CAMK	97
Arg(m)	TK	20	CaMKIIβ(h)	CAMK	103
ARK5(h)	CAMK	113	CaMKIIγ(h)	CAMK	74
ASK1(h)	STE	104	CaMKIIδ(h)	CAMK	100
ATM(h)	Lipid/Atypical	92	CaMKIV(h)	CAMK	77
ATR/ATRIP(h)	Lipid/Atypical	98	CaMKIγ(h)	CAMK	91
Aurora-A(h)	MISC	15	CaMKIδ(h)	CAMK	70

Table S1. Continued.

Kinase	Kinase family	@0.1 μM	Kinase	Kinase family	@0.1 μM
CaMKK1(h)	MISC	51	CHK1(h)	CAMK	105
CaMKK2(h)	MISC	84	CHK2(h)	CAMK	60
Cdc7/cyclinB1(h)	MISC	65	CHK2(I157T)(h)	CAMK	60
CDK1/cyclinB(h)	CMGC	91	CHK2(R145W)(h)	CAMK	70
CDK12/cyclinK(h)	CMGC	97	CK1(y)	CK1	101
CDK13/cyclinK(h)	CMGC	106	CK1γ1(h)	CK1	109
CDK14/cyclinY(h)	CMGC	21	CK1γ2(h)	CK1	100
CDK16/cyclinY(h)	CMGC	98	CK1γ3(h)	CK1	108
CDK17/cyclinY(h)	CMGC	25	CK1δ(h)	CK1	92
CDK18/cyclinY(h)	CMGC	62	CK1ε(h)	CK1	93
CDK2/cyclinA(h)	CMGC	91	CK2(h)	CMGC	106
CDK2/cyclinE(h)	CMGC	95	CK2α1(h)	CMGC	122
CDK3/cyclinE(h)	CMGC	97	CK2α2(h)	CMGC	107
CDK4/cyclinD3(h)	CMGC	93	c-Kit (V654A)(h)	TK	44
CDK5/p25(h)	CMGC	109	c-Kit(D816H)(h)	TK	95
CDK5/p35(h)	CMGC	97	c-Kit(D816V)(h)	TK	38
CDK6/cyclinD3(h)	CMGC	92	c-Kit(h)	TK	50
CDK7/cyclinH/MAT1(h)	CMGC	104	c-Kit(V560G)(h)	TK	8
CDK9/cyclinT1(h)	CMGC	72	CLIK1(h)	MISC	61
CDKL1(h)	CMGC	52	CLK1(h)	CMGC	74
CDKL2(h)	CMGC	61	CLK2(h)	CMGC	29
CDKL3(h)	CMGC	89	CLK3(h)	CMGC	64
CDKL4(h)	CMGC	100	CLK4(h)	CMGC	81
ChaK1(h)	MISC	102	c-RAF(h)	TKL	29

Table S1. Continued.

Kinase	Kinase family	@0.1 μM	Kinase	Kinase family	@0.1 μM
CRIK(h)	AGC	109	EphA2(h)	TK	2
CSK(h)	TK	72	EphA3(h)	TK	20
cSRC(h)	TK	15	EphA4(h)	TK	41
DAPK1(h)	CAMK	87	EphA5(h)	TK	19
DAPK2(h)	CAMK	125	EphA7(h)	TK	50
DCAMKL2(h)	CAMK	86	EphA8(h)	TK	14
DCAMKL3(h)	CAMK	75	EphB1(h)	TK	9
DDR1(h)	TK	17	EphB2(h)	TK	39
DDR2(h)	TK	33	EphB3(h)	TK	79
DMPK(h)	AGC	105	EphB4(h)	TK	22
DNA-PK(h)	Lipid/Atypical	76	ErbB2(h)	TK	87
DRAK1(h)	CAMK	88	ErbB4(h)	TK	101
DRAK2(h)	CAMK	92	FAK(h)	TK	38
DYRK1A(h)	CMGC	103	Fer(h)	TK	63
DYRK1B(h)	CMGC	95	Fes(h)	TK	41
DYRK2(h)	CMGC	99	FGFR1(h)	TK	1
DYRK3(h)	CMGC	59	FGFR1(V561M)(h)	TK	2
eEF-2K(h)	MISC	116	FGFR2(h)	TK	3
EGFR(h)	TK	107	FGFR2(N549H)(h)	TK	1
EGFR(L858R)(h)	TK	75	FGFR3(h)	TK	6
EGFR(L861Q)(h)	TK	82	FGFR4(h)	TK	22
EGFR(T790M)(h)	TK	41	Fgr(h)	TK	20
EGFR(T790M, L858R)(h)	TK	34	Flt1(h)	TK	1
EphA1(h)	TK	34	Flt3(D835Y)(h)	TK	22

Table S1. Continued.

Kinase	Kinase family	@0.1 μM	Kinase	Kinase family	@0.1 μM
Flt3(h)	TK	10	ICK(h)	CMGC	105
Flt4(h)	TK	1	IGF-1R activated(h)	TK	101
Fms(h)	TK	21	IGF-1R(h)	TK	62
Fms(Y969C)(h)	TK	29	IKKα(h)	MISC	90
Fyn(h)	TK	42	IKKβ(h)	MISC	90
GCK(h)	STE	32	IKKε(h)	MISC	87
GCN2(h)	MISC	86	IR activated(h)	TK	72
GRK1(h)	AGC	97	IR(h)	TK	61
GRK2(h)	AGC	98	IRAK1(h)	TKL	4
GRK3(h)	AGC	109	IRAK4(h)	TKL	49
GRK5(h)	AGC	96	IRE1(h)	MISC	58
GRK6(h)	AGC	93	IRR(h)	TK	72
GRK7(h)	AGC	93	Itk(h)	TK	4
GSK3α(h)	CMGC	85	JAK1(h)	TK	61
GSK3β(h)	CMGC	78	JAK2(h)	TK	95
haspin(h)	MISC	110	JAK3(h)	TK	78
Hck activated(h)	TK	16	JNK1α1(h)	CMGC	98
Hck(h)	TK	3	JNK2α2(h)	CMGC	59
HIPK1(h)	CMGC	16	JNK3(h)	CMGC	78
HIPK2(h)	CMGC	10	KDR(h)	TK	18
HIPK3(h)	CMGC	7	Lck activated(h)	TK	5
HIPK4(h)	CMGC	3	Lck(h)	TK	6
HPK1(h)	STE	4	LIMK1(h)	TKL	73
HRI(h)	MISC	72	LIMK2(h)	TKL	32

Table S1. Continued.

Kinase	Kinase family	@0.1 μM	Kinase	Kinase family	@0.1 μM
LKB1(h)	CAMK	103	Met(h)	TK	10
LOK(h)	STE	15	Met(M1268T)(h)	TK	8
LRRK2(h)	TKL	33	Met(Y1248C)(h)	TK	1
LTK(h)	TK	40	Met(Y1248D)(h)	TK	0
Lyn(h)	TK	1	Met(Y1248H)(h)	TK	23
MAK(h)	CMGC	109	MINK(h)	STE	5
MAP4K3(h)	STE	16	MKK3(h)	STE	72
MAP4K4(h)	STE	23	MKK6(h)	STE	92
MAP4K5(h)	STE	0	MLCK(h)	CAMK	95
MAPK1(h)	CMGC	118	MLK1(h)	TKL	45
MAPK2(h)	CMGC	99	MLK2(h)	TKL	25
MAPKAP-K2(h)	CAMK	98	MLK3(h)	TKL	9
MAPKAP-K3(h)	CAMK	99	Mnk2(h)	CAMK	26
MARK1(h)	CAMK	104	MOK(h)	CMGC	107
MARK3(h)	CAMK	103	MRCKα(h)	AGC	101
MARK4(h)	CAMK	103	MRCKβ(h)	AGC	99
MEK1 (h)	STE	86	MRCKγ(h)	AGC	110
MEK2(h)	STE	23	MSK1(h)	AGC	50
MEKK2(h)	STE	47	MSK2(h)	AGC	22
MEKK3(h)	STE	71	MSSK1(h)	CMGC	131
MELK(h)	CAMK	9	MST1(h)	STE	42
Mer(h)	TK	1	MST2(h)	STE	39
Met(D1246H)(h)	TK	23	MST3(h)	STE	81
Met(D1246N)(h)	TK	29	MST4(h)	STE	45

Table S1. Continued.

Kinase	Kinase family	@0.1 μM	Kinase	Kinase family	@0.1 μM
mTOR(h)	Lipid/Atypical	91	PAR-1Bα(h)	CAMK	99
mTOR/FKBP12(h)	Lipid/Atypical	94	PASK(h)	CAMK	102
MuSK(h)	TK	82	PDGFRα(D842V)(h)	TK	3
MYLK2(h)	CAMK	58	PDGFRα(h)	TK	65
MYO3B(h)	STE	36	PDGFRα(V561D)(h)	TK	1
NDR2(h)	AGC	70	PDGFRβ(h)	TK	89
NEK1(h)	MISC	60	PDHK2(h)	MISC	91
NEK11(h)	MISC	63	PDHK4(h)	MISC	117
NEK2(h)	MISC	83	PDK1(h)	AGC	74
NEK3(h)	MISC	94	PEK(h)	MISC	41
NEK4(h)	MISC	6	PhKγ1(h)	CAMK	72
NEK6(h)	MISC	99	PhKγ2(h)	CAMK	90
NEK7(h)	MISC	86	PI3 Kinase(p110α(E542K/p85α)(h)	Lipid/Atypical	89
NEK9(h)	MISC	10	PI3 Kinase(p110α(E545K)/p85α)(h)	Lipid/Atypical	97
NIM1(h)	CAMK	107	PI3 Kinase(p110α/p85α)(h)	Lipid/Atypical	102
NLK(h)	CMGC	82	PI3 Kinase(p110β/p85α)(h)	Lipid/Atypical	97
NUAK2(h)	CAMK	107	PI3 Kinase(p110δ/p85α)(h)	Lipid/Atypical	99
p70S6K(h)	AGC	3	PI3 Kinase(p120γ)(h)	Lipid/Atypical	79
PAK1(h)	STE	92	PI3KC2α(h)	Lipid/Atypical	100
PAK2(h)	STE	104	PI3KC2γ(h)	Lipid/Atypical	98
PAK3(h)	STE	101	Pim-1(h)	CAMK	25
PAK4(h)	STE	109	Pim-2(h)	CAMK	82
PAK5(h)	STE	91	Pim-3(h)	CAMK	112
PAK6(h)	STE	126	PIP4K2α(h)	Lipid/Atypical	100

Table S1. Continued.

Kinase	Kinase family	@0.1 μM	Kinase	Kinase family	@0.1 μM
PIP5K1α(h)	Lipid/Atypical	100	Plk3(h)	MISC	79
PIP5K1γ(h)	Lipid/Atypical	98	Plk4(h)	MISC	91
PKA(h)	AGC	86	PRAK(h)	CAMK	121
PKAcb(h)	AGC	81	PRK1(h)	AGC	102
PKBα(h)	AGC	97	PRK2(h)	AGC	93
PKBβ(h)	AGC	62	PRKG2(h)	AGC	85
PKBγ(h)	AGC	115	PrKX(h)	AGC	92
PKCα(h)	AGC	107	PRP4(h)	CMGC	89
PKCβI(h)	AGC	95	PTK5(h)	TK	1
PKCβII(h)	AGC	101	Pyk2(h)	TK	52
PKCγ(h)	AGC	99	Ret(h)	TK	1
PKCδ(h)	AGC	111	Ret(V804L)(h)	TK	4
PKCε(h)	AGC	106	Ret(V804M)(h)	TK	2
PKCζ(h)	AGC	116	RIPK1(h)	TKL	7
PKCη(h)	AGC	113	RIPK2(h)	TKL	9
PKCθ(h)	AGC	98	ROCK-I(h)	AGC	81
PKCι(h)	AGC	111	ROCK-II(h)	AGC	49
PKCμ(h)	AGC	89	Ron(h)	TK	23
PKD2(h)	CAMK	101	Ros(h)	TK	31
PKD3(h)	CAMK	85	Rse(h)	TK	9
PKG1α(h)	AGC	109	Rsk1(h)	AGC	4
PKG1β(h)	AGC	94	Rsk2(h)	AGC	19
PKR(h)	MISC	100	Rsk3(h)	AGC	18
Plk1(h)	MISC	113	Rsk4(h)	AGC	16

Table S1. Continued.

Kinase	Kinase family	@0.1 μM	Kinase	Kinase family	@0.1 μM
SAPK2a(h)	CMGC	62	STK32C(h)	MISC	106
SAPK2a(T106M)(h)	CMGC	30	STK33(h)	CAMK	36
SAPK2b(h)	CMGC	18	Syk(h)	TK	100
SAPK3(h)	CMGC	21	TAF1L(h)	MISC	93
SAPK4(h)	CMGC	5	TAK1(h)	TKL	11
SBK1(h)	MISC	111	TAO1(h)	STE	29
SGK(h)	AGC	108	TAO2(h)	STE	14
SGK2(h)	AGC	85	TAO3(h)	STE	32
SGK3(h)	AGC	95	TBK1(h)	MISC	82
SIK(h)	CAMK	78	Tec activated(h)	TK	102
SIK2(h)	CAMK	107	TGFBR1(h)	TKL	115
SIK3(h)	CAMK	106	TGFBR2(h)	TKL	97
SLK(h)	STE	21	Tie2(h)	TK	4
Snk(h)	MISC	57	Tie2(R849W)(h)	TK	1
SNRK(h)	CAMK	94	Tie2(Y897S)(h)	TK	-1
Src(1-530)(h)	TK	18	TLK1(h)	MISC	103
Src(T341M)(h)	TK	19	TLK2(h)	MISC	95
SRMS(h)	TK	51	TNIK(h)	STE	2
SRPK1(h)	CMGC	100	TRB2(h)	CAMK	58
SRPK2(h)	CMGC	115	TrkA(h)	TK	1
STK16(h)	MISC	94	TrkB(h)	TK	-3
STK25(h)	STE	25	TrkC(h)	TK	0
STK32A(h)	MISC	102	TSSK1(h)	CAMK	92
STK32B(h)	MISC	101	TSSK2(h)	CAMK	107

Table S1. Continued.

Kinase	Kinase family	@0.1 μM	Kinase	Kinase family	@0.1 μM
TSSK3(h)	CAMK	103	VRK2(h)	CK1	90
TSSK4(h)	CAMK	105	Wee1(h)	MISC	95
TTBK1(h)	CK1	98	Wee1B(h)	MISC	99
TTBK2(h)	CK1	97	WNK1(h)	MISC	93
TTK(h)	MISC	1	WNK2(h)	MISC	76
Txk(h)	TK	85	WNK3(h)	MISC	81
TYK2(h)	TK	93	WNK4(h)	MISC	93
ULK1(h)	MISC	99	Yes(h)	TK	13
ULK2(h)	MISC	103	ZAK(h)	TKL	14
ULK3(h)	MISC	35	ZAP-70(h)	TK	90
VRK1(h)	CK1	88			

Table S2. IC_{50} Values of Compounds **9a-ae** and **13a-h** in RS4;11 Cell

Compd	**IC_{50}, nM**	**Compd**	**IC_{50}, nM**
9a	> 1000	**9u**	> 1000
9b	> 1000	**9v**	> 1000
9c	> 1000	**9w**	> 1000
9d	> 1000	**9x**	> 1000
9e	> 1000	**9y**	> 1000
9f	> 1000	**9z**	> 1000
9g	> 1000	**9aa**	> 1000
9h	> 1000	**9ab**	> 1000
9i	> 1000	**9ac**	> 1000
9j	> 1000	**9ad**	> 1000

9k	> 1000	**9ae**	> 1000
9l	> 1000	**13a**	> 1000
9m	> 1000	**13b**	> 1000
9n	> 1000	**13c**	> 1000
9o	> 1000	**13d**	> 1000
9p	> 1000	**13e**	> 1000
9q	> 1000	**13f**	> 1000
9r	> 1000	**13g**	> 1000
9s	> 1000	**13h**	> 1000
9t	> 1000		

Table S3. Inhibitory Rate of Compounds **9a-ae** and **13a-h** in HL-60 Cell

Compd	**Inhibitory rate (%)**	**Compd**	**Inhibitory rate (%)**
9a	9.6	**9u**	-2.4
9b	12.8	**9v**	-9.0
9c	8.7	**9w**	-10.4
9d	-1.1	**9x**	12.2
9e	4.0	**9y**	-10.6
9f	8.8	**9z**	-8.7
9g	-12.6	**9aa**	0.9
9h	3.5	**9ab**	3.1
9i	4.2	**9ac**	-3.4

9j	8.4	**9ad**	-9.8
9k	4.4	**9ae**	-2.9
9l	-17.7	**13a**	7.6
9m	-8.5	**13b**	7.3
9n	14.8	**13c**	5.8
9o	12.3	**13d**	-0.9
9p	12.1	**13e**	-10.6
9q	5.3	**13f**	2.6
9r	-1.8	**13g**	0.4
9s	10.6	**13h**	-2.4
9t	-7.4		

Table S4. IC_{50} Values against Hit Target Kinases of **9u**[a]

Target	**IC_{50}, nM**	**Target**	**IC_{50}, nM**
Abl(T315I)	3	**LOK**	4
Aurora-B	6	**Lyn**	1
c-Kit	> 300	**Mer**	0.7
FGFR1	3	**Met**	15
FGFR2	4	**PDGFRα(D842V)**	10
FGFR3	13	**PDGFRα(V561D)**	2
FGFR4	46	**Ret**	6
Flt1(h)	5	**Rsk1**	4
Flt3(h)	7	**Tie2**	15
Flt4(h)	4	**Lck**	12
HCK	4		

[a] IC_{50} values for enzymatic inhibition of FLT3 kinase; data are expressed from the

dose-response curves of at least two independent experiments.

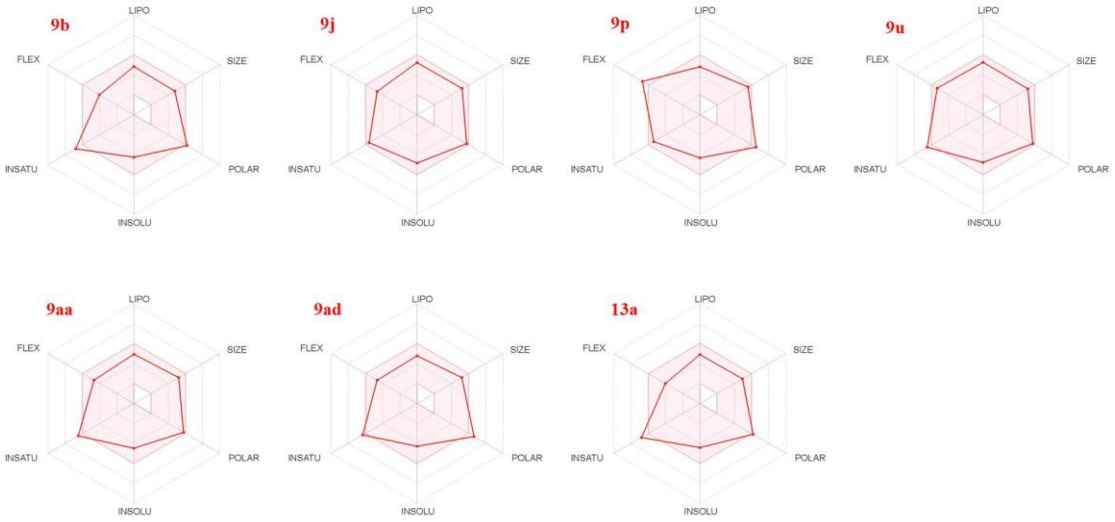

Figure S1. Bioavailability radar chart from *swiss*ADME online web tool for tested compounds. The pink area represents the range of the optimal property values for oral bioavailability and the red line is tested compounds predicted properties.

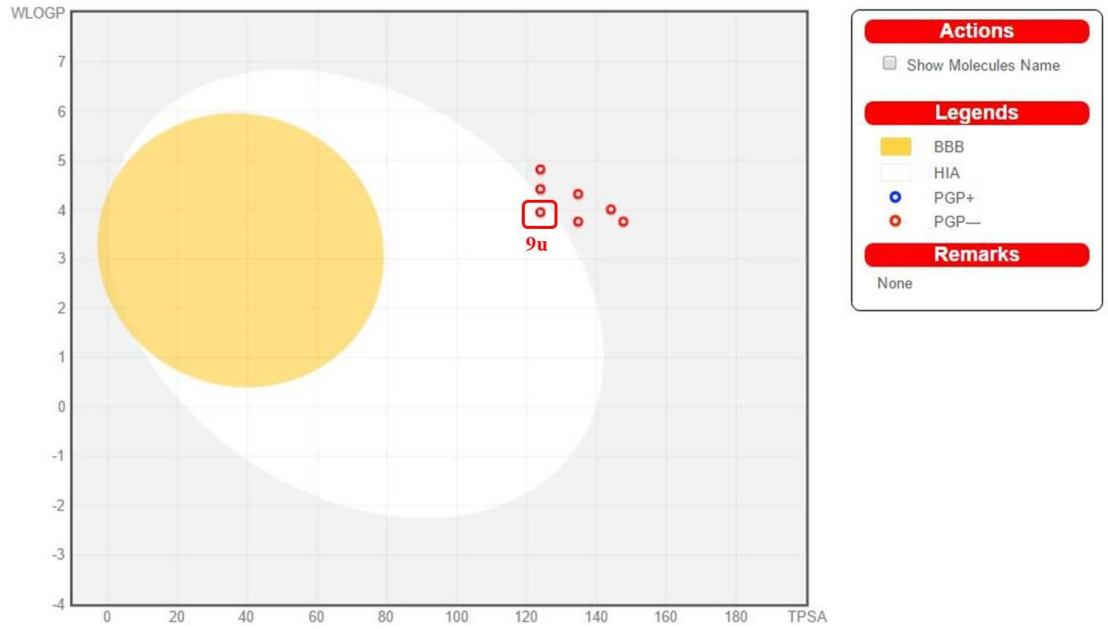

Figure S2. Predicted Boiled-Egg plot from *swiss*ADME online web tool for tested compounds (**9b**, **9j**, **9p**, **9u**, **9aa**, **9ad**, **13a**).

Biological Assay Methods

Anti-proliferative Assays. MV4-11 and Molm-13 cells were cultured in IMDM (Gibco, Milano, Italy) contained 10% fetal bovine serum (FBS) (Invitrogen, Milano, Italy). BaF3-ITD, BaF3-ITD-D835V, BaF3-ITD-F691L were cultured in RPMI (Gibco, Milano, Italy) contained 10% fetal bovine serum (FBS) (Invitrogen, Milano, Italy) and 800 μg/mL neomycin sulfate. All media contained 100 units/mL penicillin (Gibco, Milano, Italy), and 100 μg/mL streptomycin (Gibco, Milano, Italy). Cells were incubated at 37 ^{o}C in a humidified atmosphere of 5% CO_2. Cells in logarithmic phase were seeded into 96-well culture plates at densities of (10000-20000) cells per well. Then cells were treated with various concentrations of compounds for 72 h in final volumes of 200 μL. Upon end point, 20 μL MTT (5 mg/mL) was added to each well, and the cells were incubated for an additional 1-4 h. After treatment with 20% SDS overnight, absorbance values at a wavelength of 570 nM were taken on a spectrophotometer (Molecular Devices, Sunnyvale, USA). IC_{50} values were calculated using percentage of growth versus untreated control. The data were finally fitted in GraphPad Prism V6.0 software to obtain IC_{50} values using equation (Y=Bottom + (Top-Bottom)/(1+10^((LogIC50-X)*Hill Slope), Y is %inhibition and X is compound concentration.).

Kinase Profile Assay and IC_{50} Test. Kinase profiling was carried out by Eurofins Discovery Pharma Services (UK) according to the published protocols. The kinases activity of 0.1 μM **9u** on 422 kinases involved in tumor regulation in vitro were measured by radiometric assays. Briefly, each kinase was incubated with 0.1 μM **9u**

in indicated reaction solutions contained [γ-^{33}P-ATP] and other reagents such as MOPS, EDTA, EAIYAAPFAKKK, Magnesium acetate and so on (different pH, concentrations and activities according to the specific needs of different kinases). The reaction is initiated by the addition of the Mg(n)/ATP mix. After incubation for a while (specific time as required) at room temperature, the reaction is stopped by the addition of phosphoric acid to a concentration of 0.5%. 10 μL of the stopped reaction is spotted onto a P30 filtermat and washed four times for 4 minutes in 0.425% phosphoric acid and once in methanol prior to drying and scintillation counting. The in vitro kinase enzymatic inhibition assays were carried out by the Kinase Profiling Services provided by Eurofins (UK). The detailed protocol descriptions can be provided at the website (https://www.eurofinsdiscoveryservices.com).

Dissociation Constants (K_d) Assay for FLT3 Mutants. The kinase binding constants assay was conducted using the Kinomescan platform. Kinase-tagged T7 phage or DNA was expressed in *E. coli* host or HEK-293 cells. Binding reactions were assembled by kinases, ligand affinity beads, and test compounds with shaking for 1 h at room temperature, then measured by qPCR. Kinome profile assays were shown as percent of control, and K_d values were determined by 6-point 10-fold serial dilution of each test compound in this method. The detailed protocol description can be provided at the website (https://www.discoverx.com).

Western Blotting of Signaling Pathways. The cells were treated with the compounds at the indicated concentrations. Then the cells were collected and total proteins were extracted with RIPA Lysis Buffer (beyotime Co. P0013B, components: 50 mM Tris,

pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, sodium fluoride, EDTA and leupeptin). The protein concentration was measured by the BCA Protein assay (ThermoScientific, USA). Equivalent samples (30 μg of protein) were subjected to SDS-PAGE, and then the proteins were transferred onto PVDF membranes (Millipore, USA). After blocking by 5% non-fat milk for 2 h at room temperature, the membranes were incubated with the indicated primary antibodies at 4 ºC overnight and subsequently probed by the appropriate secondary antibodies conjugated to horseradish peroxidase for 1 h. Immunoreactive bands were visualized using enhanced chemiluminescence (MiniChemi, Sagecreation, Beijing). The molecular sizes of the proteins detected were determined by comparison with pertained protein markers (ThermoScientific, USA). Image J v1.8.0 software was used to conduct gray analysis and according to the protocol provided by corporation.

Cell Cycle Progression Experiment. Six-well plates were used for MV4-11 and Molm-13 cells culture. All cells were treated with increasing concentrations of the indicated compound. Cells were harvested after 24 h post-treatment, washed in phosphate buffered saline (PBS), and fixed in ice cold 75% ethanol for at least 24 h. The fixed cells were then washed with PBS and stained with propidium iodide (50 μg/mL) in the presence of 1 mg/mL RNase A, 0.5% Triton X-100 for 15 min at room temperature. The stained cells were then analyzed using a FACScan (BD Biosciences) and the resulting data analyzed with cell cycle analysis software (Modfit, BD).

Annexin V-FITC/PI Apoptosis Assay. Six-well plates were used for cells culture. MV4-11 and Molm-13 cells were treated with compound **9u** at gradient increase from 1 to 1000 nM for 48 h. Cells were washed with PBS for twice and collected to stain with an Annexin V/PI Apoptosis Detection kit (Invitrogen) according to the manufacturer's instructions. Finally, the stained cells were subjected to flow cytometry for analysis in 15 min, and 20,000 cells for each sample were examined (Attune NxT, AFC2, Life Technologies Corporation).

Pharmacokinetic Study. A 1 mg/mL dosing solution of **9b**, **9p**, **9j**, **9u**, **9aa**, **9ad**, and **13a** was respectively prepared by dissolving in physiological saline containing 2% DMSO, 2% PEG-400, 10% cyclodextrin with the pH adjusted to 5~6 for po or iv administration. Six SD rats, weighing 200−250 g each, were obtained from Beijing HFK Bioscience Co. Ltd. Each tested compound was separately administered intravenously (5 mg/kg dose) or orally (5 mg/kg) to a group of six rats per time. At time points 0 (prior to dosing), 5 min, 15 min, 30 min, 45 min, 1 h, 2 h, 4 h, 6 h, 8 h, 10 h, 12 h, and 24 h after dosing, a blood sample was collected from each animal via cardiac puncture and stored in ice (0−4 ºC). Plasma was separated from the blood by centrifugation (4000 g for 15 min at 4 ºC) and stored in a freezer at − 80 ºC. All samples were analyzed for the tested compound by LC−MS/MS (Waters Acquity UPLC system; Waters Quattro Premier XE). Data were acquired via monitoring of multiple reactions. Plasma concentration data were analyzed by a standard noncompartmental method.

Molecular Docking Study. The molecular structures of compounds were constructed

using Chemoffice software and saved as SDF format files. Then the molecular structures of compounds were added hydrogen, adjusted pH state, generated isomers and loaded OPLS3 force field to generate 3D conformation by using the Ligparep module in Schrodinger software package. The receptor protein FLT3 (PDB: 4XUF) was pretreated with the protein preparation wizard module of the Schrodinger software package, including adding hydrogen, repairation of missing residues, equilibrium charge, adjustment of unreasonable atomic overlap, and so on. And then the compound molecule was docked into the active site of FLT3 protein, and the best conformation and pose were remained.

Animal Tumor Models and Treatment. To establish the MV4-11 and Molm-13 xenograft models, MV4-11 or Molm-13 cells (10^7 cells in 100 μL serum-free IMDM) were injected subcutaneously into the right flanks of 5-6 weeks old female NOD/SCID mice. When the size of the formed xenografts reached 300 - 500 mm^3, the mice were randomly divided (6 mice per group). In these two models, the mice in the experimental group received po administration (1, 3, 10 mg/kg, dissolved in physiological saline containing 2% DMSO, 2% PEG-400, 10% cyclodextrin with the pH adjusted to 5~6) of **9u** per day. Those in the quizartinib group (positive control) received po treatment (3 mg/kg, dissolved in physiological saline containing 2% DMSO, 2% PEG-400, 10% cyclodextrin with the pH adjusted to 5~6) per day. Tumor burden was measured every 2 days by a caliper. Tumor volume (TV) was calculated using the following formula: TV =length × $width^2$ × 0.5. At the end of the experiment, mice were sacrificed and tumors were collected and weighed. The animal studies were

conducted in conformity with institutional guide for the care and use of laboratory animals, and all mouse protocols were approved by the Animal Care and Use Committee of Sichuan University (Chengdu, Sichuan, China).