Selectively Targeting FLT3-ITD Mutants over FLT3-wt by a Novel Inhibitor for Acute Myeloid Leukemia

Running title: FLT3-ITD Mutants Selective Inhibitor

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Supplementary Materials

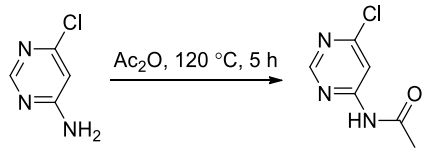
Chemicals

midostaurin

Midostaurin was purchased from Haoyuan Chemexpress Inc (Shanghai, P.R.China).

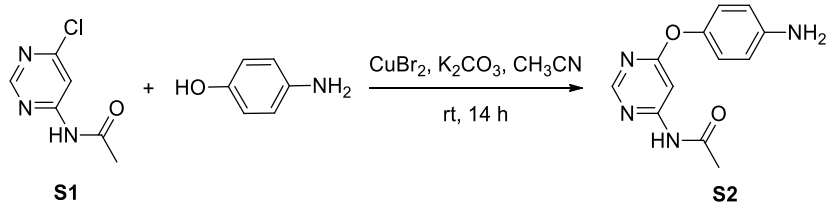
Synthesis of CHMFL-FLT3-362

General methods. All solvents and reagents were used as obtained. ^{1}H NMR spectra and ^{13}C NMR spectra were recorded with a Bruker 400 NMR or 850 NMR spectrometer and referenced to deuterium dimethyl sulfoxide (DMSO-d_6). Chemical shifts are expressed in ppm. In the NMR tabulation, s indicates singlet; d, doublet; t, triplet; q, quartet; m, multiplet; and br, broad peak. Mass spectra were measured with Agilent 6224 TOF using an ESI source coupled to an Agilent 1260 Infinity HPLC system operating in reverse mode with an Agilent Eclipse Plus C18 1.8μm 3.0 X 50 mm column. Purification of final compounds were performed with Agilent 218 preparative system (Eclipse XDB-C18 column, 9.4 X 50 mm, 5 μM) using a gradient of 5-95% acetonitrile in water containing 0.05% trifluoacetic acid (TFA) over 8 min (10 min run time) at a flow rate of 2 mL/min. The purity of all compounds were above 95% purity as determined by an Agilent 1260 Infinity HPLC with UV detection at 254 nm.

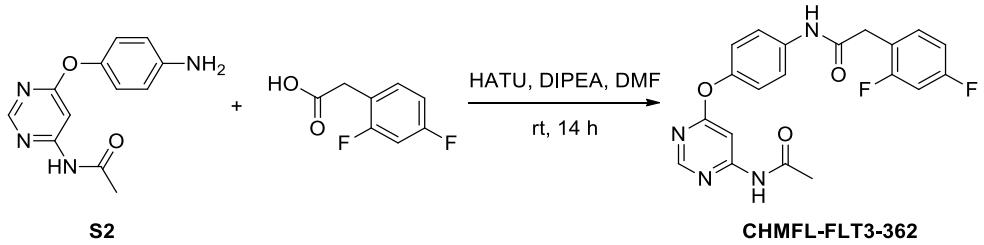


S1

N-(6-chloropyrimidin-4-yl)acetamide (**S1**). To a solution of Ac_2O (50 mL) was added 6-chloropyrimidin-4-amine (5.0 g, 68.6 mmol, 1.00 equiv.) at room temperature under argon. The reaction mixture was heated to 120 ^{o}C for 5 h. The resulting mixture was concentrated to dryness. The residue was washed with methanol to give **S1** as a white solid. Yield = 76%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.20 (s, 1H), 8.70 (s, 1H), 8.05 (s, 1H), 2.14 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.46, 161.04, 159.42, 158.89, 108.99, 24.55. LC-MS (ESI, m/z): 172.0245 $[M+H]^+$.



N-(6-(4-aminophenoxy)pyrimidin-4-yl)acetamide (**S2**). To a solution of **S1** (2.0 g, 11.7 mmol, 1.00 equiv) in CH_3CN (50 mL) was added 4-aminophenol (1.27 g, 11.7 mmol, 1.00 equiv), K_2CO_3 (1.94g, 14.04 mmol, 2.00 equiv.) and $CuBr_2$ (10 mg, 0.045 mmol, 0.04 equiv) at room temperature under argon. The reaction mixture was stirred at room temperature for 14 h. The resulting mixture was concentrated to dryness. The residue was diluted with water and extracted with EtOAc. The combined organic layers were washed with water, brine and dried over anhydrous Na_2SO_4. Evaporation of the solvent provided the crude product, which was purified by flash chromatography with MeOH in DCM (0-10%) to give **S2** as a yellow solid. Yield = 28%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.87 (s, 1H), 8.48 (s, 1H), 7.44 (s, 1H), 6.61 (d, J = 6.1 Hz, 2H), 6.48 (s, 2H), 5.09 (s, 2H), 2.10 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 171.68, 170.90, 159.94, 158.38, 147.15, 142.62, 122.15, 115.86, 93.82, 24.04. LC-MS (ESI, m/z): 245.1004 $[M+H]^+$.



N-(4-((6-acetamidopyrimidin-4-yl)oxy)phenyl)-2-(2,4-difluorophenyl)acetamide (**CHMFL-FLT3-362**). To a solution of **S2** (50 mg, 0.2 mmol, 1.00 equiv.) in anhydrous DMF (1 mL) was added 2-(2,4-difluorophenyl)acetic acid (87 mg, 0.4 mmol, 2.00 equiv), HATU (152 mg, 0.4 mmol, 2.00 equiv.) and DIPEA (0.2 mL, 1.0 mmol, 5.0 equiv) at room temperature under argon. The reaction mixture was stirred at room temperature for 14 h. The resulting mixture was concentrated to provide the crude product, which was purified by flash chromatography (eluting with MeOH in DCM 0-10%) to give compound CHMFL-FLT3-362 as a white solid. Yield = 81%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.95 (s, 1H), 10.34 (s, 1H), 8.49 (s, 1H), 7.66 (s, 2H), 7.51 (s, 1H), 7.48 – 7.41 (m, 1H), 7.23 (s, 1H),

7.15 (s, 2H), 7.08 (s, 1H), 3.74 (s, 2H), 2.11 (s, 3H). ^{13}C NMR (214 MHz, DMSO-d_6) δ 171.20, 170.98, 168.47, 161.65, 161.24, 160.08, 158.34, 147.90, 137.01, 133.34, 122.31, 120.74, 119.77, 111.57, 103.85, 94.11, 36.10, 24.51. LC-MS (ESI, m/z): 399.1231 $[M+H]^+$.

Ba/F3 isogenic cell line generation

Functional BaF3 cell lines were generated as described previously [1]. Briefly, the wt-FLT3, mutant-FLT3, cKIT, CSF1R, PDGFR, FLT1 and VEGFR2 genes were cloned into the pMSCVpuro retroviral vector for virus production in HEK-293T cells in combination with two helper plasmids. Virus-containing supernatant was used to infect BaF3 cells followed by puromycin selection and IL-3 withdrawal to obtain the stable wt-FLT3, mutant-FLT3, cKIT, CSF1R, PDGFR, FLT1 and VEGFR2 overexpressing BaF3 cells that are independent of IL-3 for cell survival and proliferation.

Cell lines and cell culture

The FLT3-ITD expressing lines MV4-11, MOLM-13 and MOLM14 were provided by Dr. Scott Armstrong, Dana Farber Cancer Institute (DFCI), Boston, MA, USA. The FLT3-wt cell lines U937, OCI-AML-2, OCI-AML-3, HL-60, CMK, M-07e and NOMO-1 were purchased from Cobioer Biosciences CO., Ltd. (Nanjing, China). MOLM-13, MOLM-14, HL-60, U937, CMK , NOMO-1 and isogenic BaF3 cells lines were cultured in RPMI 1640 media (Corning, USA) with 10% fetal bovine serum (FBS) and supplemented with 2% L-glutamine 1% penicillin/streptomycin. Parental BaF3 cells were cultured in RPMI 1640 media (Corning, USA) with 10% fetal bovine serum (FBS), supplemented with supplemented with 2% L-glutamine, 1% penicillin/streptomycin and 1 ng/mL IL-3. MV4-11 was cultured in IMDM media (Cornig, USA) with 10% FBS and supplemented with 2% L-glutamine and 1% pen/strep. M-07e was cultured in RPMI 1640 media (Corning, USA) with 10% fetal bovine serum (FBS), 10ng/ml GM-CSF and supplemented with 2% L-glutamine, 1% penicillin/streptomycin. OCI-AML-2 and OCI-AML-3 were cultured in α-MEM media (Corning, USA) with 20% FBS and supplemented with 2% L-glutamine and 1% pen/strep. All cell lines were maintained in culture media at 37 ºC with 5% CO_2.

Antibodies and immunoblotting

The following antibodies were purchased from Cell Signaling Technology (Danvers, MA): FLT3 (8F2) Rabbit mAb (#3462), Phospho-FLT3 (Tyr589/591) (30D4) Rabbit mAb (#3464), NF-κB p65 (D14E12) XP® Rabbit mAb (#8242), Phospho-NF-κB p65 (Ser536) (93H1) Rabbit mAb (#3033), Stat5 Antibody (#9363), Phospho-Stat5 (Tyr694) (C71E5) Rabbit mAb (#9314), c-Myc (D84C12) XP® Rabbit mAb(#5605), Akt (pan) (C67E7) Rabbit mAb (#4691), Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb (#4060), Phospho-Akt (Thr308) (244F9) Rabbit mAb (#4056), p44/42 MAPK (Erk1/2) (137F5) Rabbit mAb (#4695),Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb (#4370), GAPDH (D16H11) XP® Rabbit mAb. Antibodies were used at 1:1000. Cells were lysed for 30 min in lysis buffer supplemented with protease/phosphatase inhibitor cocktail (Cell Signaling Technology). Lysates were cleared by centrifugation at 13,000 g at 4 ºC for 10 min, and protein concentrations were determined by BCA. Lysates were subjected to electrophoresis through 10% or 15% gel and immobilized on the nitrocellulose membranes.

FLT3-wt

FLT3 wt and FLT3-ITD protein purification

FLT3-wt

FLT3 wt cytoplasmic fragment 564-993 with his tag was cloned into baculovirus expression vector pFASTHT-A. The recombinant bacmid was transfected into SF9 by Cellfectin (Invitrogen). High titer viral stocks were obtained by two rounds of amplification of the virus. The protein was expressed by infecting SF9 cells with high titer viral stocks for 48 h. Cells were harvested and re-suspended in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, and 1 mM PMSF). The cells were lysed by ultrasonication and the cell debris was removed by ultracentrifugation. The supernatant was incubated with Ni-affinity beads (GE). The beads were then washed by lysis buffer containing 50-250 mM imidazole. The elute was loaded to sephedex 75. The protein was concentrated to 1 mg/ml and aliquots were frozen and stored at -80 °C. The protein was used for ADP-Glo assay.

FLT3-ITD sequence was amplified from the cDNA of MV4-11 cell line and cloned into baculovirus expression vector pFASTHT-A. The recombinant bacmid was transfected into SF9 by Cellfectin (Invitrogen). High titer viral stocks were obtained by two rounds amplification of the virus. The protein was expressed by infecting SF9 cells with high titer viral stocks for 48h. Cells were harvested and resuspended in lysis buffer (50 mM Tris pH

7.5, 150 mM NaCl, and 1 mM PMSF). The cells were lysed by ultrasonication and the cell debris was removed by ultracentrifugation. The supernatant was incubated with Ni-affinity beads (GE). The beads were then washed by lysis buffer containing 50-250 mM imidazole. The elute was loaded to sephedex 75. The protein was concentrated to 1 mg/ml and aliquots were frozen and stored at -80 °C. The protein was used for ADP-Glo assay.

Biochemical kinase assay

The biochemical tests of FLT3, FLT3-ITD, KIT, PDGFRα, PDGFRβ, CSF1R, FLT1 and VEGFR2 were provided by Invitrogen (Carlsbad, CA, USA).

ATP competitive assay

ADP-Glo™ assay kits from Promega Corporation were used according to instructions. CHMFL-FLT3-362 was generally prepared with 1:3 serial dilutions for 4 concentrations (300 nM, 100 nM, 30 nM, and 10 nM). 8 concentrations were used (1 mM to 5 μM) for ATP competition experiments. 2.5 μL FLT3 (10 ng/μL) or FLT3-ITD (10 ng/μL) was incubated with CHMFL-FLT3-362 for 60 min at room temperature in reaction buffer followed by addition of 2.5 μL ATP/substrate mixture. The assay was conducted for 1 h at 37 °C. After the plate cooled for 5 min at room temperature, 5 μL of ADP-Glo reagent was added into each well to stop the reaction and consume the remaining ADP within 40 min. At the end, 10 μL kinase detection reagent was added and incubated for 30 min at room temperature. The luminescence signal was read with an envision Perkin Elmer plate reader (Envision, PE, USA).

Proliferation studies

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Cells were grown in 96-well culture plates (2000-3000/well). CHMFL-FLT3-362 and Midostaurin at 10 different final concentrations descending 3-fold from 10 μmol/L were added into the plates. DMSO concentrations were kept constant and did not exceed 0.1% of the total volume. Cell proliferation was determined after treatment with compounds for 72 h. Cell viability was measured using the CellTiter–Glo assay (Promega, USA) according to the manufacturer's instructions, and absorbance was measured in a Bio-red Microplate reader (Bio-Red, USA). Data were normalized to control groups (DMSO) and represented

by the mean of three independent measurements with standard error < 20%. GI_{50} values were calculated using Prism 7.0 (GraphPad Software, San Diego, CA).

Cell cycle analysis

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MOLM13, MOLM14, MV4-11, OCI-AML-3 and NOMO-1 cells were treated with serially diluted CHMFL-FLT3-362 or Midostaurin for 12-24 h. The cells were fixed in 70% cold ethanol and incubated at −20 °C overnight then stained with PI/RNase staining buffer (BD Pharmingen). Flow cytometry was performed using a FACS Calibur (BD), and the results were analyzed by ModFit software.

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Signaling pathway effect examination

MOLM13, MOLM14, MV4-11, OCI-AML-3 and NOMO-1 cells were treated with DMSO, serially diluted CHMFL-FLT3-362 or Midostaurin for 4 h. The cells were then washed in PBS and lysed in cell lysis buffer. FLT3, Phospho-FLT3 (Tyr589/591), AKT, Phospho-AKT Ser473, Phospho-AKT Thr308, STAT5, Phospho-STAT5 (Tyr694), NF-KB-P65, Phospho-NF-KB-P65 (Ser536), ERK, Phospho-p44/42MAPK (Erk1/2) (Thr202/Tyr204), c-Myc and GAPDH antibody (Cell signaling Technology) were used for immunoblotting.

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Apoptosis effect examination

MOLM13, MOLM14, MV4-11, OCI-AML-3 and NOMO-1 cells were treated with serially diluted CHMFL-FLT3-362 or Midostaurin for 12-24 h. The cells were then washed in PBS and lysed in cell lysis buffer. PARP, Caspase-3, GAPDH antibody (Cell signaling Technology) were used for immunoblotting.

Human AML primary cells

Bone marrow samples were obtained from patients with newly diagnosed or recurrent AML and the peripheral blood samples were obtained from healthy person. Mononuclear cells were isolated by the Ficoll-Paque method from bone marrow/blood samples. PBMC and wt-FLT3 AMLcells were tested in liquid culture (RPMI1640 medium, supplemented with 20% FBS), FLT3-ITD+ AML cells were tested in liquid culture (RPMI1640 medium, supplemented with 20% FBS, and 10 ng/ml FLT3 ligand) in the presence of different concentrations of inhibitors. All studies performed with human specimens were done with

approval from the First Hospital of Anhui Medical University. Ethical approval and informed consent was obtained for the use of human samples.

In Vivo Pharmacokinetics Studies

The pharmacokinetics study protocol was approved by the animal ethics committee of Hefei Institutes of Physical Science, Chinese Academy of Sciences (Hefei, China). The male Sprague- Dawley rats, KM mice, and Beagle dog were provided by the laboratory animal center of Anhui Medical University (Hefei, China). The animals were housed in an air-conditioned animal room at a temperature of 23 ± 2°C and a relative humidity of 50 ± 10% and allowed free access to tap water and lab. CHMFL-FLT3-362 was administered by oral gavage and plasma samples collected 0.033, 0.083, 0.25, 0.5, 1, 2, 4, 6, 9 and 12 hours after dosing. Hundred microliters of plasma was harvested by centrifuging the blood sample at 4 °C and 5000 rpm for 3 min, and then stored at −80 °C until analysis. An aliquot of 100 μL of each plasma sample was mixed with 20 μL of internal standard working solution (200 ng/mL caffeine). Methanol (400 μL) was then added for precipitation. After vortexing for 5 min and centrifuging at 14 000 rpm for 10 min, 5 μL of the supernatant was injected for LC−tandem mass spectrometry analysis. Pharmacokinetic parameters were obtained by fitting the normalized liquid chromatography tandem mass spectrometry peak areas to a noncompartmental model using the linear trapezoidal estimation method in the WinNonlin software package (Pharsight Corporation, Mountain View). The oral bioavailability (F) is calculated according to the following equation: $F = AUC_{0-\infty}$ (oral)/$AUC_{0-\infty}$ (i.v.) × dose (i.v.)/dose (oral) × 100%.

Zebrafish maintenance and embryo collection

Wild-type (WT) zebrafish were maintained under standard conditions, and embryos were staged as described[10,12]. The study was approved by the YSY Biotech (NanJing, China).

Mouse 14-day sub-drug test

CHMFL-FLT3-362

The subacute toxicity study of CHMFL-362 was conducted on 8 female and 8 male ICR mice, weighing 25-30g, five week in age. All mice were purchased from Gempharmatech

Co., Ltd (Nanjing, China). All animals were housed in a specific pathogen-free facility and used according to the animal care regulations of Hefei Institutes of Physical Science Chinese Academy of Sciences. Animals were then randomized into treatment groups of 4 mice each (2 female and 2 male) for subacute toxicity studies. CHMFL-FLT3-362 was delivered daily in a HKI solution (0.5% Methocellulose/0.4% Tween 80 in ddH2O) by orally gavage. A range of doses of CHMFL-FLT3-362 or its vehicle were administered, as indicated in figure legends. Body weight were measured daily after CHMFL-FLT3-362 treatment. Bone marrow smears were prepared and stained with modified Wright-Giemsa stain for morphological evaluation after 14 days.

MV4-11 cell subcutaneous Xenograft tumor model

Four-week old female nu/nu mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). All animals were housed in a specific pathogen-free facility and used according to the animal care regulations of Hefei Institutes of Physical Science, Chinese Academy of Sciences. Prior to implantation, the cells were harvested during exponential growth. Ten million MV4-11cells in IDMEM medium were formulated as a 1:1 mixture with Matrigel (BD Biosciences) and injected into the subcutaneous space on the right flank of nu/nu mice. Daily oral administration was initiated when tumors had reached a size of 150 to 200 mm^3. Animals were then randomized into treatment groups of 5 mice each for efficacy studies. CHMFL-FLT3-362 was delivered daily in a HKI solution (0.5% Methocellulose/0.4% Tween 80 in ddH_2O) by orally gavage. A range of doses of CHMFL-FLT3-362 or its vehicle were administered, as indicated in figure legends. Body weight and tumor growth were measured daily after CHMFL-FLT3-362 treatment. Tumor volumes were calculated as follows: tumor volume (mm^3)＝[(W^2 × L)/2] in which width (W) is defined as the smaller of the two measurements and length (L) is defined as the larger of the two measurements.

MV4-11 and MOLM-13 cells bone marrow engrafted mouse model

Five-week old female NOD-SCID mice were purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). All animals were housed in a specific pathogen free facility and used according to the animal care regulations of Hefei Institutes of Physical Science, Chinese Academy of Sciences. NOD-SCID mice were

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intraperitoneal injection cyclophosphamide (CTX) 50 mg/kg daily for two days. 8 million MV-4-11 and MOLM-13 cells in 0.3 mL IDMEM medium were injected by intravenous after 24 h. CHMFL-FLT3-362 or Midostaurin were administered daily by *p.o.* Mice were monitored daily and were euthanized when moribund or at early signs of hind limb paralysis.

FACS detection of drug efficacy

Single cell suspensions were prepared from bone marrow of MV4-11 engrafted NOD-SCID mice, and red blood cells were lysed. Single-cell suspensions were blocked and incubated with PE conjugated HLA-ABC (G46e2.6). Samples were acquired by a BECKMAN COUTER (CytoFLEX, USA) and analyzed by FlowJo software. Appropriate isotype-matched, irrelevant control mAbs were used to determine the level of background staining. The antibodies were purchased from BD Biosciences (San Diego, USA).

Supplementary Table S1. Anti-proliferative effect of CHMFL-FLT3-362 against FLT3 wt/mutants engineered BaF3 cells and FLT3ITD/wt cancer cell lines[a]

Cell	CHMFL-FLT3-362 (GI_{50}: μM)
TEL-wt-FLT3	0.75 ± 0.095
FLT3/ITD (6)	0.074 ± 0.021
FLT3/ITD (8)	0.095 ± 0.026
FLT3/ITD (10)	0.03 ± 0.007
FLT3/ITD (22)	0.026 ± 0.0085
FLT3/ITD (33)	0.065 ± 0.0085
FLT3/K663Q	>10
FLT3/D835V	>10
FLT3/D835H	>10
FLT3/D835N	>10
FLT3/D835Y	>10
FLT3-ITD/G697R	0.43 ± 0.017
FLT3-ITD/D835N	1.65 ± 0.02
FLT3-ITD/D835V	>10
FLT3-ITD/D835A	1.11 ± 0.8
FLT3-ITD/D835del	>10
FLT3-ITD/D835I	>10
FLT3-ITD/D835H	>10

FLT3-ITD/Y842R	>10
FLT3-ITD/Y842H	>10
FLT3-ITD/F691L	>10
FLT3-ITD/N676D	>10
BaF3	>10
MOLM-14 (FLT3-ITD)	0.17 ± 0.015
MV4-11 (FLT3-ITD)	0.14 ± 0.01
MOLM13 (FLT3-ITD)	0.27 ± 0.005
HL-60 (FLT3-wt)	>10
OCI-AML-2 (FLT3-wt)	>10
CMK (FLT3-wt)	>10
U937 (FLT3-wt)	>10

[a]All data were obtained through triplet testing (maximum testing concentration: 10 μM)

Supplementary Table S2. Kinome wide selectivity profiling of CHMFL-FLT3-362 with DiscoveRx KinomeScan^{TM} assay (See separate Supplementary-2)

Supplementary Table S3. Information related to primary cells of patients

Patient	Age (year)	Gender	%blasts	Pathology	Cytogenetics
AML1	73	F	82.5	AML-M5	62.7K WBC count; previous therapy:idarubicin, high dose cytarabline, mitoxantrone; cytogenetics: normal; mutations: dupMLL+; FLT3-ITD (17 aa).
AML2	4	M	90.6	AML-M5	Previous therapy:daunorubicin, cytarabline,homoharringtonine; cytogenetics:46, XY[3]; mutations: DEK-CAN+; FLT3-ITD (10 aa) .
AML3	19	F	30.0	AML-M5	218.5K WBC count; previous therapy:idarubicin, cytarabline; cytogenetics: normal; gene information: normal.
AML4	62	F	84.2	AML-M2a	23.9K WBC count; Previous therapy: idarubicin, cytarabline and anti-infective therapy; cytogenetics: normal.

Supplementary Table S4. Anti-proliferation effect of CHMFL-FLT3-362 against patients' primary cells information lines with Cell Titer-Glo assay[a]

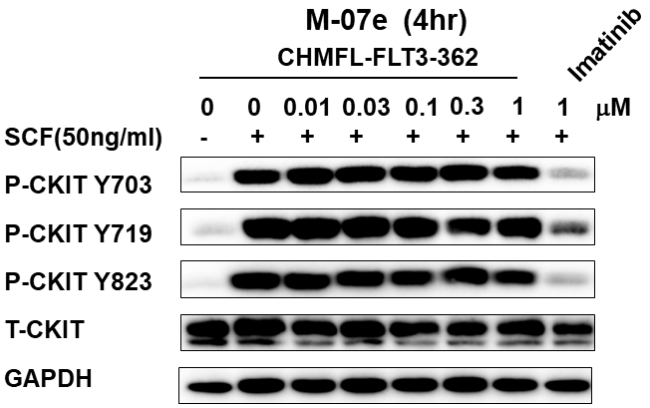
CHMFL-FLT3-362	AML1	AML2	AML3	AML4	PBMC1	PBMC2
GI_{50}: μM	0.013 ± 0.007	0.45 ± 0.012	6.8 ± 0.78	>10	>10	>10

[a]All data were obtained through triplet testing (maximum testing concentration: 10 μM)

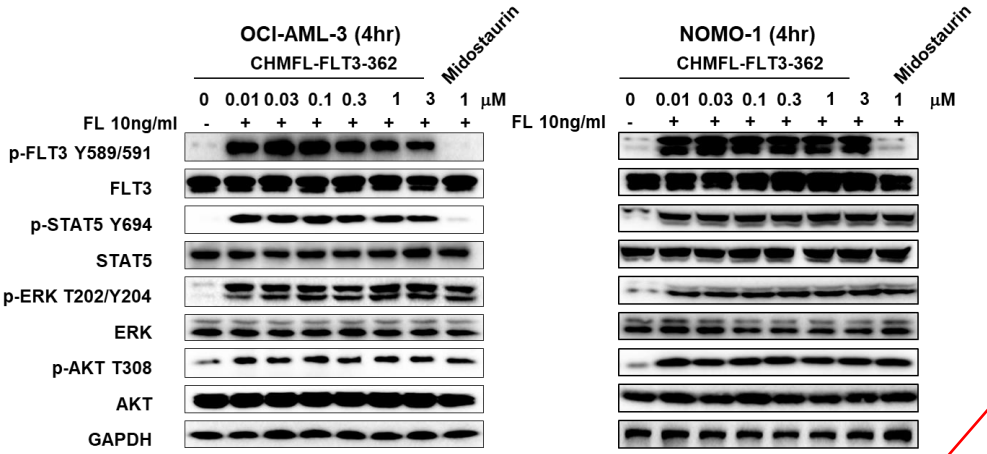
Supplementary Table S5. Pharmacokinetics properties of CHMFL-FLT3-362 in different species

	Mice		Rats		Beagle dog	
	I.V. (1mg/kg)	P.O. (10mg/kg)	I.V. (1mg/kg)	P.O. (10mg/kg)	I.V. (1mg/kg)	P.O. (5mg/kg)
$AUC_{(0-t)}$ (ng/mL·h)	4059	33722	5351.164	46742.798	2063	6648
$AUC_{(0-\infty)}$ (ng/mL·h)	4147	37753	5381.185	46960.11	2098	6648
C_{max} (ng/mL·h)	1903	6290	1749.177	3659.255	2043	3277
T_{max} (h)	0.083	0.25	0.039	6	0.033	0.50
$T_{1/2}$(h)	2.17	3.83	3.472	2.825	1.07	1.29
$MRT_{(0-\infty)}$ (h)	2.81	5.58	3.522	6.789	1.536	1.782
F (%)	/	91.0%	/	87.35	/	63.4%

Supplementary Figure S1. The phosphorylation levels of CKIT (Tyr 703/719/823) was detected by western blot in M-07e cell line. The cell were incubated with the indicated concentrations of CHMFL-FLT3-362 for 4 hours upon SCF (50 ng/ml) stimulation.



Supplementary Figure S2. The phosphorylation levels of FLT3 (Tyr 589/591), STAT5 (Tyr 694), ERK1/2 (Tyr 202/204), and AKT (Ser 308) were detected by western blot in OCI-AML-3 and NOMO-1 cell lines. These cells were incubated with the indicated concentrations of CHMFL-FLT3-362 for 4 hours before lysiscell lines upon FLT3 ligand (10 ng/ml) stimulation.



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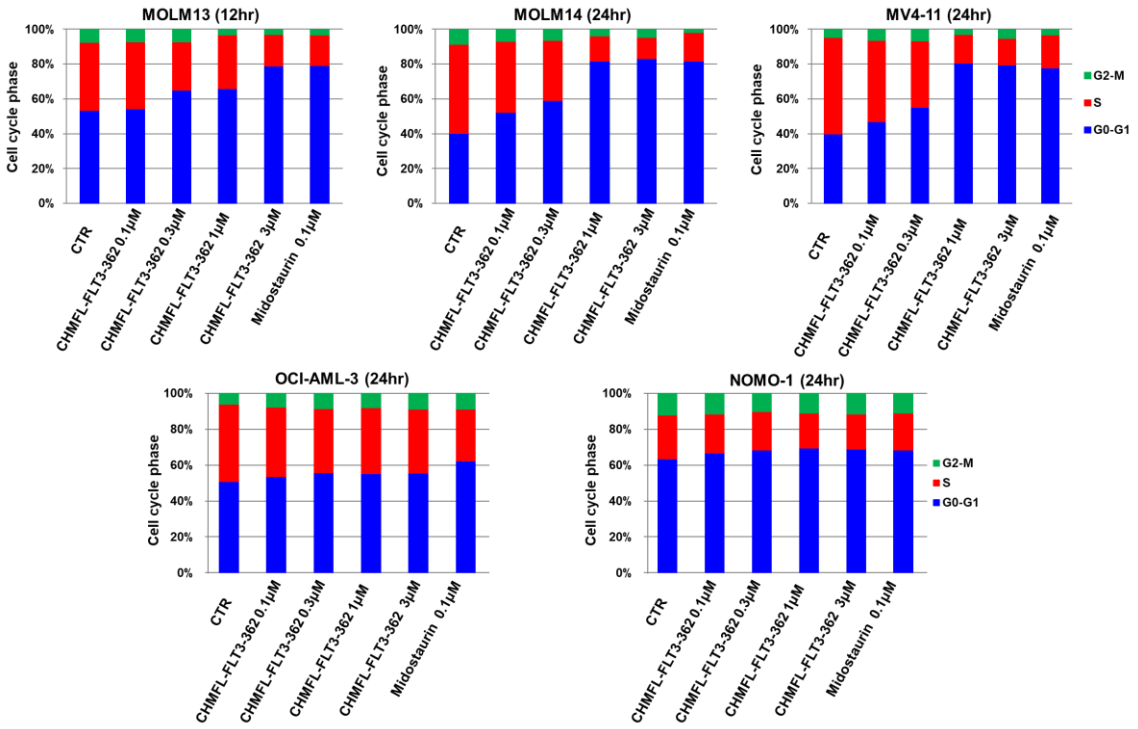
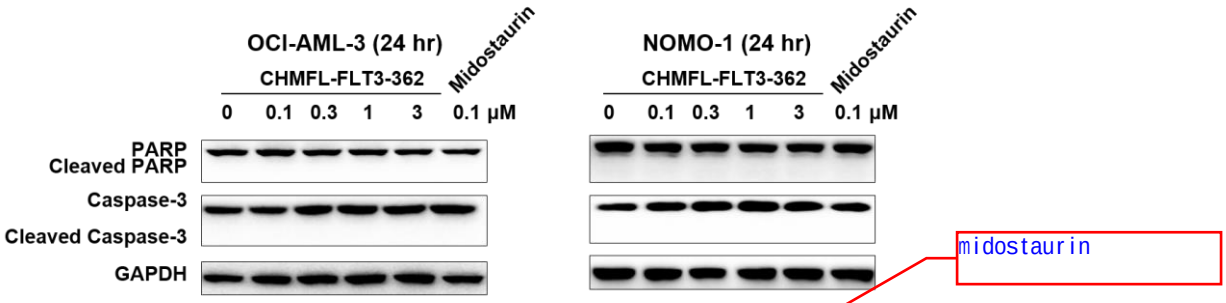
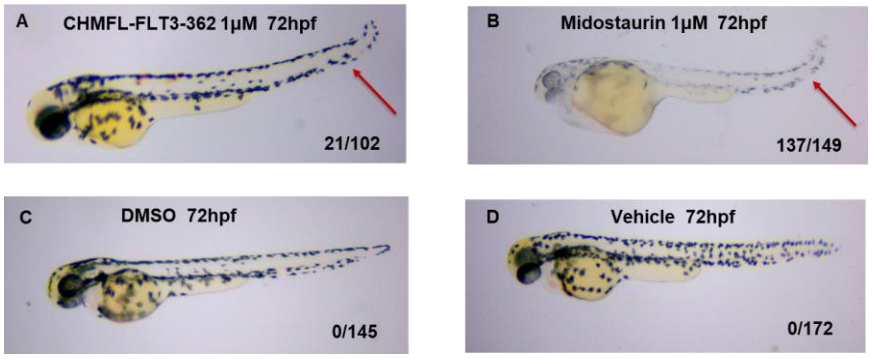
Supplementary Figure S3. MOLM13, MOLM14, MV4-11, OCI-AML-3 and NOMO-1 cells were treated with serially diluted CHMFL-FLT3-362 or Midostaurin for 12-24 h. Flow cytometry was performed using a FACS Calibur, and the results were analyzed by ModFit software. The panels demonstrate quantification of the percentage of cells in each cell cycle phase.



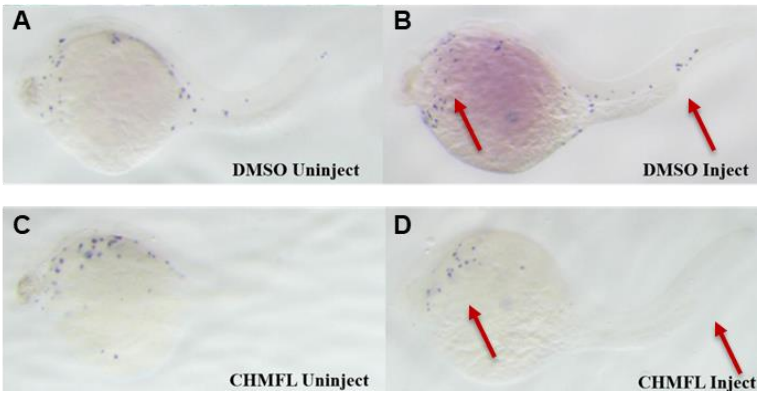
Supplementary Figure S4. Western blot analysis for the expression of PARP, cleaved PARP, caspase-3, and cleaved caspase-3 in OCI-AML-3 and NOMO-1 cells treated with serially diluted CHMFL-FLT3-362 or Midostaurin for 24 h.



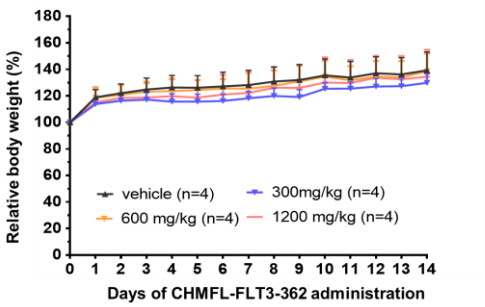
Supplementary Figure S5. Effects of Midostaurin and CHMFL-FLT3-362 on zebrafish embryonic morphological. (A) Effect of CHMFL-FLT3-362 on the development of 72hpf zebrafish embryos at 1μM. (B) Effect of Midostaurin on the development of 72hpf zebrafish embryos at 1μM. (C) Effect of DMSO on the development of 72hpf zebrafish embryos. (D)The development of 72hpf zebrafish embryos.



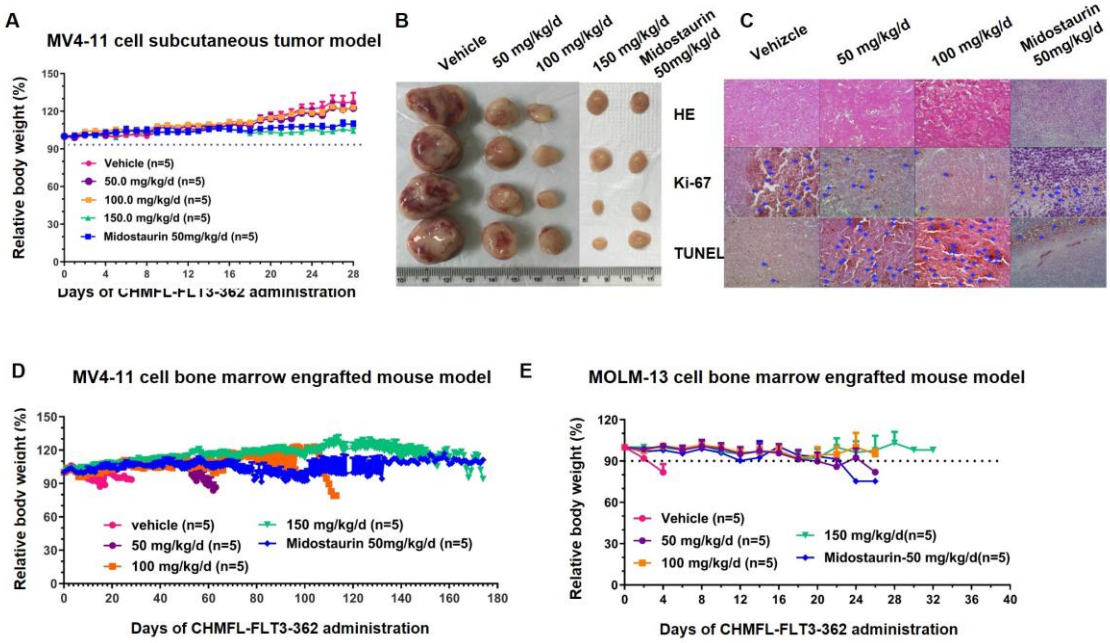
Supplementary Figure S6. Whole in situ hybridization of mpx expressed in embryos at 30 hpf. The effect of DMSO (A) and CHMFL-FLT3-362 (C) in uninjected control. The effect of DMSO (B) and CHMFL-FLT3-362 in injection of FLT3-ITD gene mRNA during the fertilized egg period, showing CHMFL-FLT3-362 inhibition the spread of FLT3-ITD-injected leukemic blasts at 1μM.



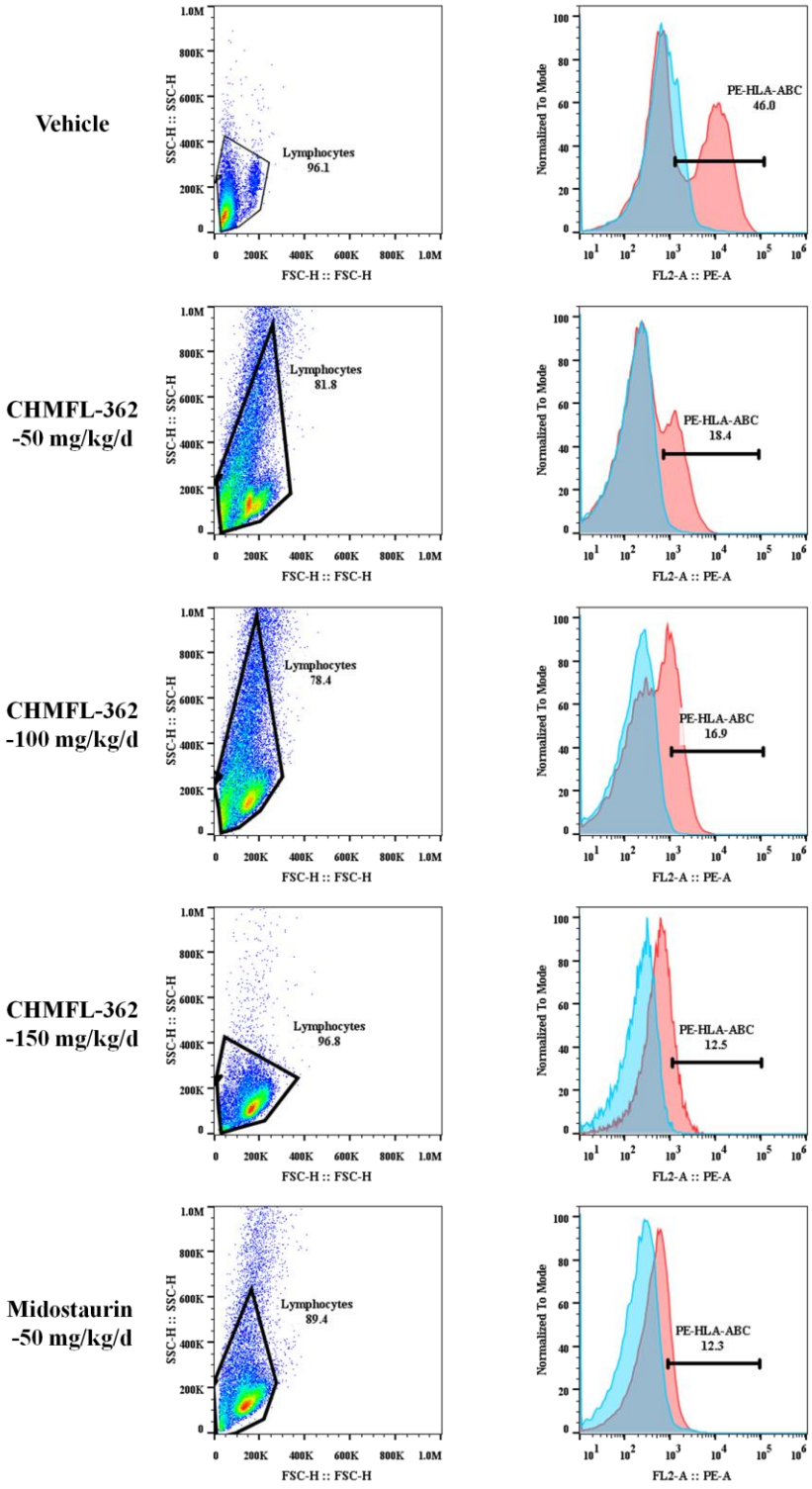
Supplementary Figure S7. Effect of CHMFL-FLT3-362 on body weight of mice with once daily treatment for 14 days.



Supplementary Figure S8. Characterization of CHMFL-FLT3-362 in vivo in preclinical AML models with FLT3-ITD mutants. (A) Body weight monitoring of CHMFL-FLT3-362 in the MV4-11 cell subcutaneous tumor model (B) Representative photographs of tumors in each group after 50, 100, 150 mg/kg/d CHMFL-FLT3-362 or vehicle treatment. (C) Immunohistochemistry stain of CHMFL-FLT3-362 in MV4-11 cell subcutaneous inoculated mouse xenograft model. (D) Body weight monitoring of CHMFL-FLT3-362 in MV4-11 cell bone marrow engrafted mouse model. (E) Body weight monitoring of CHMFL-FLT3-362 in MOLM-13 cell bone marrow engrafted mouse model.



Supplementary Figure S9. Effect of CHMFL-FLT3-362 on bone marrow in MV4-11 cell bone marrow engrafted mouse model. CHMFL-FLT3-362 significantly reduce the MV4-11 cells in the bone marrow at the dose of 50, 100 and 150 mg/kg dosages.



1. Wang Q, Liu FY, Wang BL, et al.Discovery of 4-methyl-N-(4-((4-methylpiperazin-1-yl) methyl)-3-(trifluoromethyl)phenyl)-3((1-nicotinoylpiperidin-4-yl)oxy)benzamide (CHMFL-ABL/KIT-155) as a novel highly potent type II ABL/KIT dual kinase inhibitor with a distinct hinge binding, *J. Med. Chem.* 2017; 60: 273–289.