

## Selectively targeting FLT3-ITD mutants over FLT3-wt by a novel inhibitor for acute myeloid leukemia

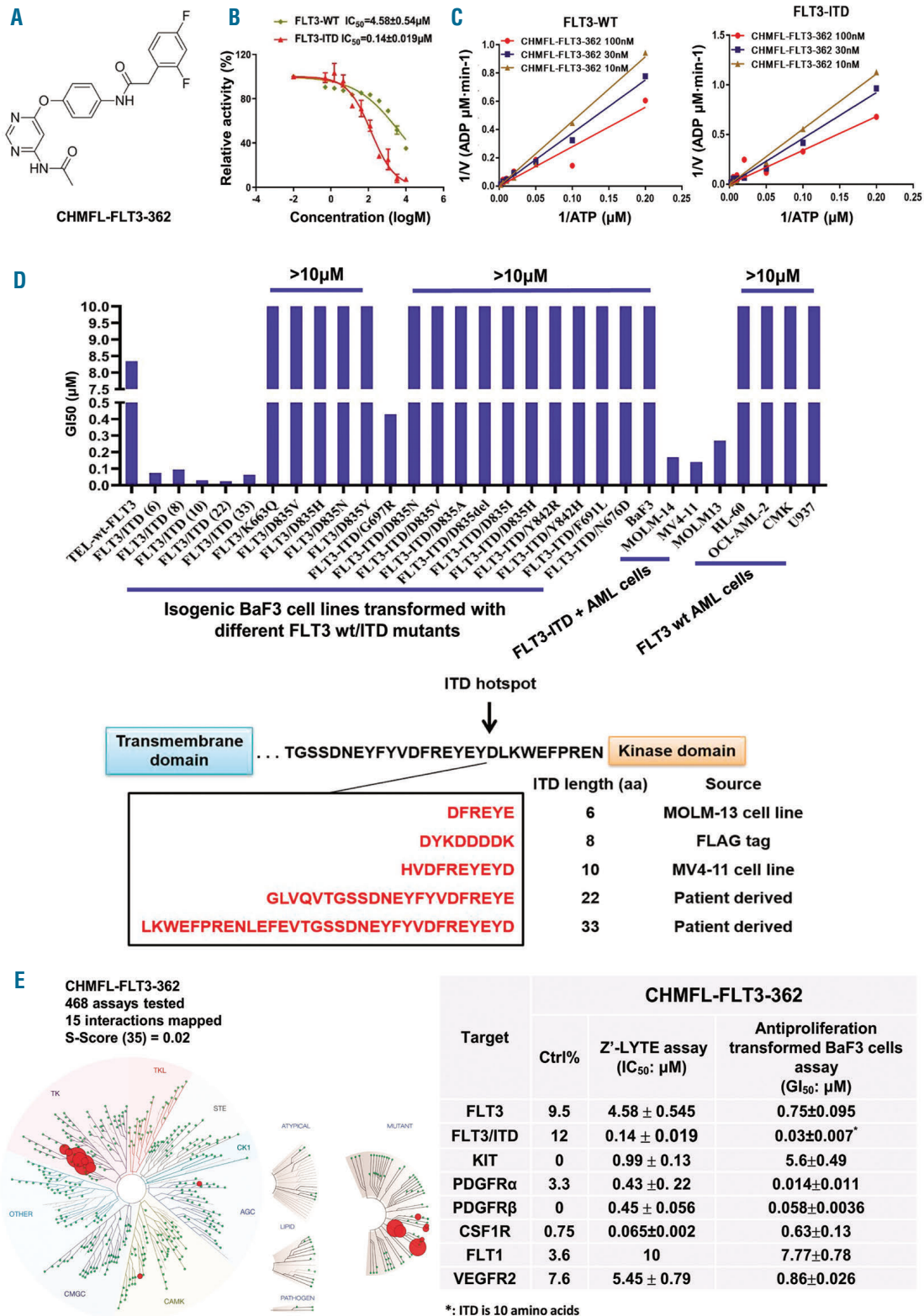
Wild-type FLT3 (FLT3-wt) kinase is expressed in immature hematopoietic cells, placenta, gonads, and brain.<sup>1</sup> It plays important roles in the differentiation and survival of hematopoietic stem cells in bone marrow.<sup>2</sup> In a normal hematopoietic environment, FLT3 is predominantly expressed in CD34 positive cells and integrally involved in early hematopoiesis, reconstitution of multilineage myeloid precursors,<sup>3</sup> and dendritic cell maturation.<sup>4,5</sup> In acute myeloid leukemia (AML), the internal tandem duplications in the juxtamembrane domain of FLT3 kinase (FLT3-ITD), which displays heterogeneity in amino acid sequences in different patients, is the most prevalent mutation of FLT3 kinase and observed in approximately 30-40% of patients. A number of FLT3 kinase inhibitors have been investigated in clinical trials, such as gilteritinib,<sup>6</sup> crenolanib,<sup>7</sup> quizartinib<sup>8</sup> and midostaurin,<sup>9</sup> etc. However, most of the current FLT3 kinase inhibitors cannot distinguish the structurally similar cKIT kinase and FLT3-wt kinase, which might lead to myelosuppression toxicity.<sup>10</sup> Here, we report the discovery of a novel FLT3-ITD mutant selective inhibitor, CHMFL-FLT3-362 (abbreviated as compound 362), which achieves high selectivity over both FLT3-wt and cKIT kinases. It also displays impressive *in vitro* and *in vivo* efficacies against the preclinical models of FLT3-ITD<sup>+</sup> AML.

We first examined the activities of compound 362 against FLT3-ITD and FLT3-wt using the Z'-LYTE (Invitrogen) biochemical assay with purified FLT3 wt/ITD mutant proteins. The data showed that compound 362 (see Figure 1A for chemical structure) achieved over 30-fold selectivity between FLT3-ITD and FLT3-wt (Figure 1B). Kinetic study of the binding mode revealed that compound 362 was an ATP competitive inhibitor (Figure 1C). We then tested the anti-proliferative effects of compound 362 with a panel of engineered BaF3 cells which were transformed with different FLT3 wt/ITD mutants (Figure 1D and *Online Supplementary Table S1*). Interestingly, the compound displayed potent inhibitory activity against all ITD mutants with different ITD lengths ranging from 6 to 33 amino acids and achieved 7~30-fold selectivity over FLT3-wt. However, it was much less potent against drug resistant mutants of FLT3-ITD including FLT3-ITD-G697R/D835(del/I/V)/Y824(R/H), and primary gain-of-function mutations including FLT3-D835V/H/N/Y and FLT3-K663Q. All these data suggested that compound 362 was a FLT3-ITD mutant selective inhibitor. As expected, this selectivity was recapitulated in leukemia cell lines evidenced by the selective inhibition against FLT3-ITD-dependent AML cells (MV4-11, MOLM-13, and MOLM-14) versus FLT3 wt-expressing cells (U937, CMK, OCI-AML-2, and HL-60) (Figure 1D and *Online Supplementary Table S1*). To further show the kinome-wide selectivity of compound 362, we examined it with the DiscoverX's KINOMEScan™ technology at the concentration of 1 μM. The results demonstrated that compound 362 exhibited a good selectivity profile (S score = 0.02). Besides FLT3, compound 362 also displayed strong binding against cKIT, CSF1R, FLT1, VEGFR2, PDGFRα and PDGFRβ kinases (Figure 1E and *Online Supplementary Table S2*). Since the KINOMEScan™ is a binding-based assay and may not really reflect the actual inhibitory activity of the kinase, we then examined compound 362 against these targets with the Z'-LYTE

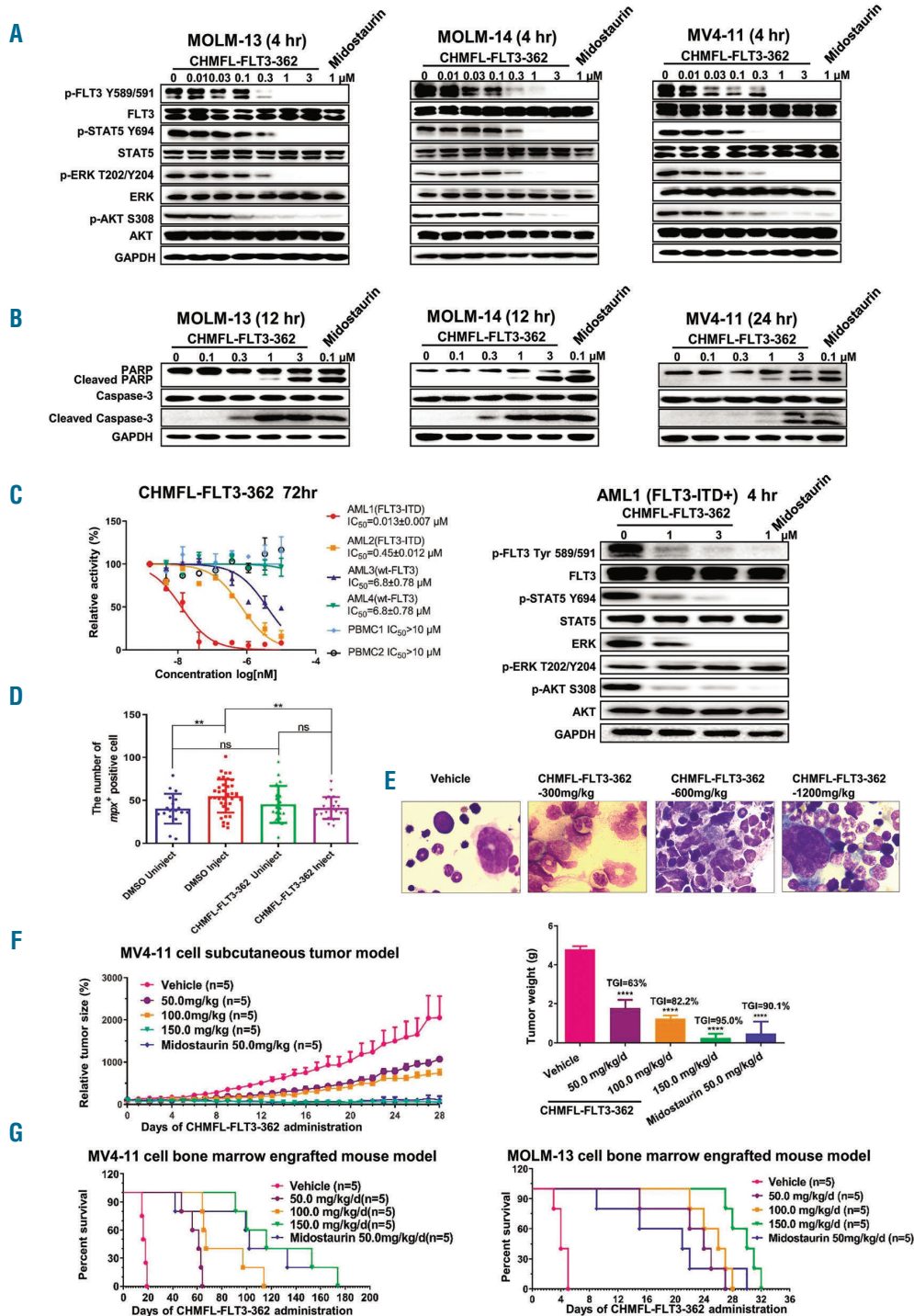
biochemical assay. The results showed that it potently inhibited CSF1R, moderately inhibited PDGFRα and PDGFRβ, but much less potently inhibited cKIT, VEGFR2 and FLT1. This is not surprising since FLT3, CSF1R, and PDGFR kinases all belong to the type III receptor tyrosine kinase family and their ATP binding pockets are structurally highly similar. In addition, since cKIT kinase is off-target of most FLT3 kinase inhibitors and it is directly correlated with myelosuppression toxicity, we further tested compound 362 in the TEL-cKIT transformed BaF3 cells; the  $GI_{50}$  value was 5.6 μM which indicated a much weaker inhibition to cKIT kinase. This result was further verified in a more physiologically relevant M-07e cell line which overexpresses cKIT wt kinase.<sup>11</sup> Again, compound 362 did not affect the phosphorylation of cKIT kinase in this cell line up to 1 μM (*Online Supplementary Figure S1*).

We then investigated the effects of compound 362 on the signaling pathways mediated by FLT3 kinase. The results displayed that the FLT3 autophosphorylation in FLT3-ITD-dependent cell lines (MV4-11, MOLM-13, and MOLM-14) were significantly decreased and the phosphorylation of downstream signaling mediators including STAT5, AKT, and ERK were almost completely inhibited between 0.1 and 0.3 μM (Figure 2A). However, in FLT3-wt-expressing cell lines (OCI-AML-3, NOMO-1), FLT3 autophosphorylation and downstream signaling were not affected up to 3 μM of compound 362, while midostaurin could potently inhibit the FLT3 auto-phosphorylation (*Online Supplementary Figure S2*). These data further confirmed the selectivity of compound 362 between FLT3-ITD mutants and FLT3-wt. We next evaluated the apoptosis-related proteins in FLT3-ITD<sup>+</sup> cell lines upon compound 362 treatment. Dose-dependent increase of the expression of cleaved caspase-3 and cleaved PARP were observed in these cell lines, which indicated apoptotic cell death (Figure 2B). In addition, dose-dependent cell cycle arrest was observed in FLT3-ITD positive cell lines but not FLT3-wt cell lines (*Online Supplementary Figures S3 and S4*). Furthermore, compound 362 exhibited remarkable anti-proliferative effects against FLT3-ITD positive AML patient primary cells but not FLT3-wt primary cells or peripheral blood mononuclear cells (PBMC) (Figure 2C and *Online Supplementary Tables S3 and S4*). Dose-dependent inhibition of the phosphorylation of FLT3 and downstream signaling mediators STAT5, ERK and AKT in these primary cells confirmed the on-target inhibition effects of compound 362 (Figure 2C).

We next evaluated the pharmacokinetics properties of compound 362 in rats, mice, and Beagle dogs following intravenous (1 mg/kg) and oral (5-10 mg/kg) administration (*Online Supplementary Table S5*). The data revealed a good drug-like PK profile including acceptable bioavailability (> 60%), suitable half-life ( $T_{1/2}$  = 1-4 hours [h]), and good  $C_{max}$  (>3,000 ng/mL). We then used the zebrafish model<sup>10,12</sup> to examine the safety of compound 362 by testing the effects at 72 h post fertilization (hpf) of embryos. The tail curvature caused by compound 362 was much less than that caused by midostaurin at 1 μM (*Online Supplementary Figure S5*), indicating a relatively lower toxicity of compound 362. In order to examine the myelosuppression effects, we further examined the effect of compound 362 on WT granulopoiesis using myeloperoxidase (mpx)<sup>+</sup> positive cell numbers as a readout of cKIT-related myelopoiesis/myelosuppression. As expected, compound 362 showed no apparent difference of cell num-



**Figure 1. Characterization of CHMFL-FLT3-362 as a highly selective FLT3-ITD mutants kinase inhibitor.** (A) Chemical structure of CHMFL-FLT3-362. (B) Invitrogen (Madison, WI, USA) Z'LYTE biochemical assay determination of the IC<sub>50</sub>s of CHMFL-FLT3-362 against FLT3 wild-type (wt)/ITD kinases. Error bars, mean ± standard error of mean (SEM), n=3. (C) Kinetic study of CHMFL-FLT3-362 against FLT3-wt/ITD kinase with varied concentrations (10 nM, 30 nM and 100 nM, respectively) of ATP. (D) Determination of the growth inhibition effects of CHMFL-FLT3-362 against a panel of 22 BaF3 engineered cell lines (including different ITD sequences and mutants), three FLT3-ITD<sup>+</sup> human leukemia cell lines and three FLT3-wt human leukemia cell lines. Cells were treated with CHMFL-FLT3-362 (maximum concentration 10 μM) for 72 hours (h), and cell viability was measured using the Cell Titer-Glo assay. Error bars, mean ± SEM, n=3. (E) (Left) Kinome-wide selectivity profiling of CHMFL-FLT3-362 with DiscoverX's KINOMEScan™ technology (<http://www.kinomescan.com>). Red circles indicate kinases bound, and circle size indicates relative binding affinity compared to DMSO (Ctrl%). The complete dataset is shown in *Online Supplementary Table S2*. (Right) Inhibition effects of CHMFL-FLT3-362 against FLT3, FLT3/ITD, KIT, PDGFRα, PDGFRβ, CSF1R, FLT1 and VEGFR2. *In vitro* kinase activity was measured by Invitrogen Z'-LYTE assay (IC<sub>50</sub>: μM, mean ± SEM, n=3) and engineered BaF3 cell lines (GI<sub>50</sub>: μM, mean ± SEM, n=3).



**Figure 2. Characterization of CHMFL-FLT3-362 in cell and *in vivo* in preclinical acute myeloid leukemia (AML) models with FLT3-ITD mutants.** (A) 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 MOLM13, MOLM14 and MV4-11 cell lines. These cells were incubated with the indicated concentrations of CHMFL-FLT3-362 for 4 hours (h) before lysis. (B) Western blot analysis for the expression of PARP, cleaved PARP, caspase-3, and cleaved caspase-3 in MOLM-13, MOLM-14, and MV4-11 cells treated with CHMFL-FLT3-362 for 24 h. (C) Growth inhibition effects of CHMFL-FLT3-362 against FLT3-ITD<sup>+</sup> AML patient primary cells, FLT3-wild-type (wt) AML patient primary cells, and peripheral blood mononuclear cells using cell Titer-Glo assay after 72 h drug treatment (maximum concentration 10 μM, mean±SEM, n=3). (D) Effects of CHMFL-FLT3-362 on zebrafish normal myelopoiesis and the FLT3/ITD-induced myeloid cells expansion. WT granulopoiesis were measured by the number of myeloperoxidase positive cells using *in situ* hybridization assay with mpx specific RNA probe. Inject-the injection of FLT3-ITD gene mRNA during the fertilized egg period, uninject-the FLT3-ITD gene mRNA was not injected during the fertilized egg period; DMSO uninject vs. CHMFL-FLT3-362 uninject,  $P=0.4165$ ; DMSO uninject vs. DMSO inject,  $P=0.0067$ ; CHMFL-FLT3-362 uninject vs. CHMFL-FLT3-362 inject,  $P=0.4385$ ; DMSO inject vs. CHMFL-FLT3-362 inject,  $P=0.0039$ ,  $**P<0.01$ , ns: not significant. (E) Bone marrow (BM) smear of mouse 14-day sub-drug test. (F) Anti-leukemia effects of CHMFL-FLT3-362 with once daily (QD) dosing at 50, 100, and 150 mg/kg. Total study length was 28 days. Each treatment group=5 animals. (Left) Representative graphs of relative tumor size are shown. (Right) Representative graphs of tumor weight are shown of different groups. Error bars, mean±SEM, n=5.  $*P<0.05$ ;  $**P<0.01$ ;  $***P<0.001$ ;  $****P<0.0001$ . (G) Anti-tumor efficacy of CHMFL-FLT3-362 in a BM engrafted mouse model. Kaplan-Meier plots of survival. (Left) The disseminated NOD/SCID mice were intravenously inoculated with MV4-11 cells. Mice received daily oral administration of CHMFL-FLT3-362 dosing at 50, 100, and 150 mg/kg. Total study length was 174 days. Each treatment group = 5 animals. (Right) Disseminated NOD/SCID mice were intravenously inoculated with MOLM-13 cells. Mice received daily oral administration of CHMFL-FLT3-362 dosing at 50, 100, and 150 mg/kg. Total study length was 32 days. Each treatment group=5 animals.

ber at 1  $\mu\text{M}$  after treatments at 30 hpf (DMSO Uninject vs. CHMFL-FLT3-362 Uninject) (Figure 2D), indicating no apparent myelosuppression toxicity. In FLT3-ITD-transduced embryos, compound 362 effectively rescued the abnormal proliferation of  $\text{mpx}^+$  myeloid cells caused by overexpression of the FLT3-ITD gene (DMSO Inject vs. CHMFL-FLT3-362 Inject) (Figure 2D) and inhibited the spread of FLT3-ITD-injected leukemic blasts at 1  $\mu\text{M}$  (Online Supplementary Figure S6) indicating that compound 362 exerted its effects through FLT3-ITD on-target inhibition. In addition, oral administration of 300, 600, and 1,200 mg/kg/day dosages for 14 days did not result in significant toxicity and weight loss in the mice (Online Supplementary Figure S7). Furthermore, bone marrow (BM) smear analysis also showed that compound 362 had no effect on the proliferation and activity of mouse BM cells (Figure 2E).

To examine the inhibitory effects of compound 362 on tumor growth, different dosages of compound 362 were administered orally every day for 28 days in the subcutaneous MV4-11 cells xenograft mice model. It displayed dose-dependent anti-tumor efficacy and achieved the tumor growth inhibition of 95% at a dosage of 150 mg/kg/day (Figure 2F). No weight loss or any other obvious signs of toxicity was observed. Immunohistochemistry staining of the tumor tissue also confirmed that the cell proliferation was inhibited (Ki-67 staining) and the apoptosis was induced (TUNEL staining) (Online Supplementary Figure S8A-C). We then further confirmed the anti-tumor efficacy of compound 362 in an orthotopic model of BM engraftment using MV4-11 and MOLM-13 cells, which physiologically differs from the subcutaneous MV4-11 xenograft model. Compound 362 dose-dependently extended the survival of mice at 50, 100, and 150 mg/kg/day dosages with no apparent weight loss at all dosages (Figure 2G and Online Supplementary Figure S8D and E). Flow cytometry analysis revealed significant reduction of the MV4-11 cells in the BM in this *in vivo* model (Online Supplementary Figure S9).

In this study, we describe a novel FLT3-ITD mutant selective inhibitor CHMFL-FLT3-362, which achieved 30-fold selectivity between FLT3-ITD mutants and FLT3-wt in biochemical assays and 10-fold selectivity in cellular context. Considering that FLT3-wt is essential for the proliferation of normal primitive hematopoietic cells, this selectivity indicates that it might provide better safety profiles. In addition, compound 362 was potent against different ITD mutants which are more relevant to the clinically observed heterogeneity. Furthermore, it also achieved great selectivity over cKIT kinase which would help to avoid the myeloid suppression toxicity due to the FLT3/cKIT dual inhibition. The unique selectivity profile combined with acceptable *in vivo* PK/PD properties in the preclinical models makes compound 362 a valuable research tool for FLT3 mediated pathological study as well as a novel potential anti-FLT3-ITD<sup>+</sup> AML drug candidate.

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Disclosures: no conflicts of interests to disclose.

Contributions: ALW, CH, CC, XFL, and BLW designed and performed the experiments; FMZ, KLY, FL, QWL, ZPQ, JJW, WWW, and LW acquired the data; ELW provided materials and reviewed the data. WCV reviewed the data and revised the manuscript. LLL, JG, and RXX collected the patient samples. JL and QSL designed the project and wrote the manuscript.

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