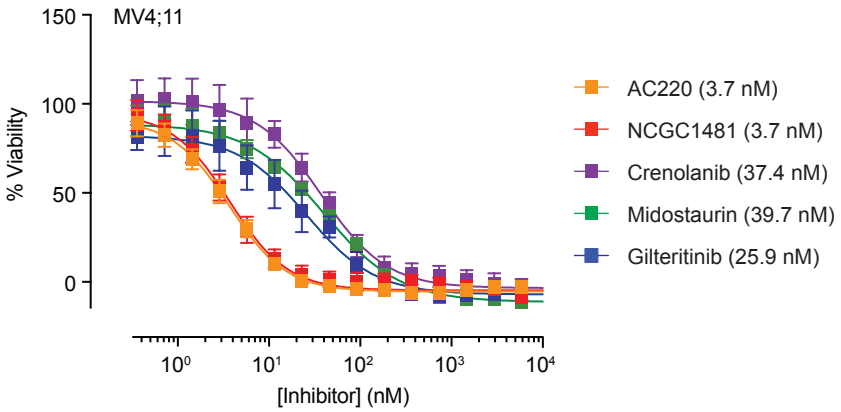
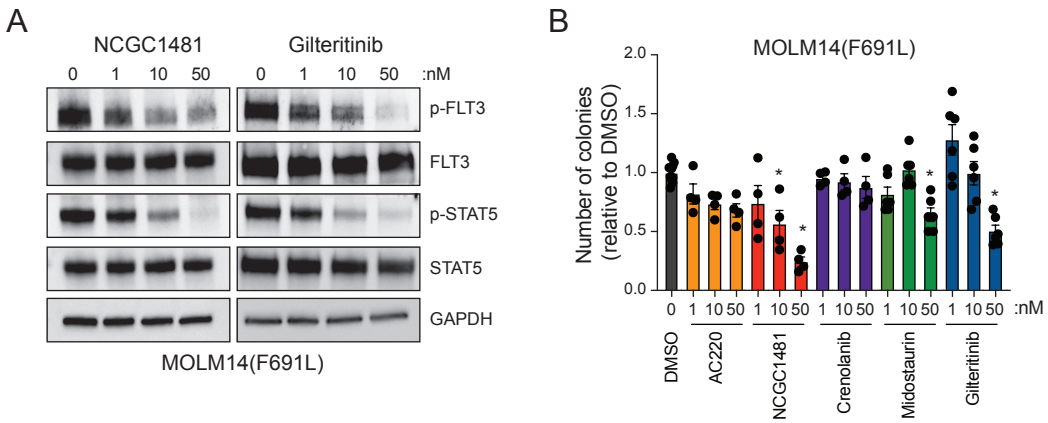
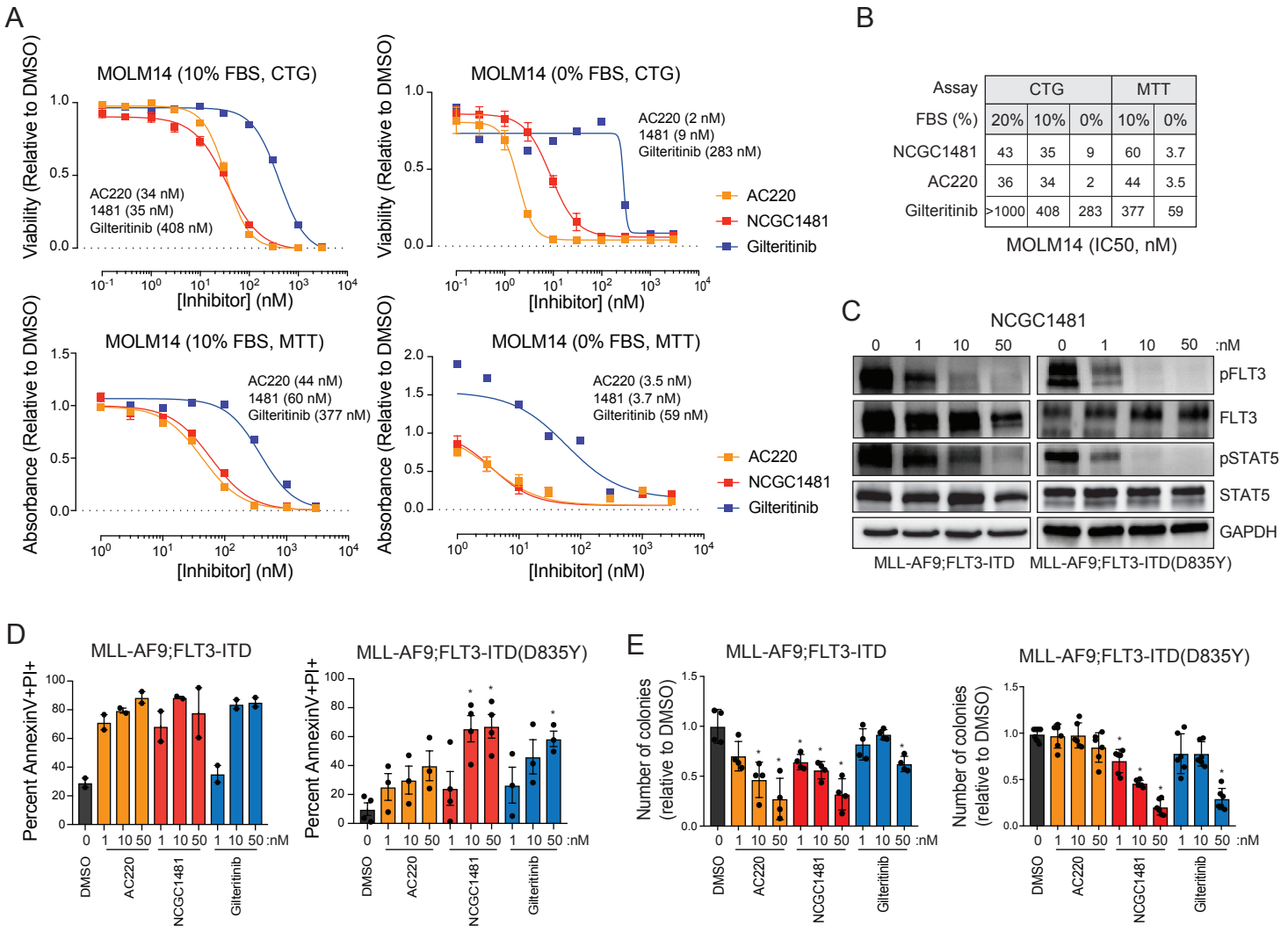


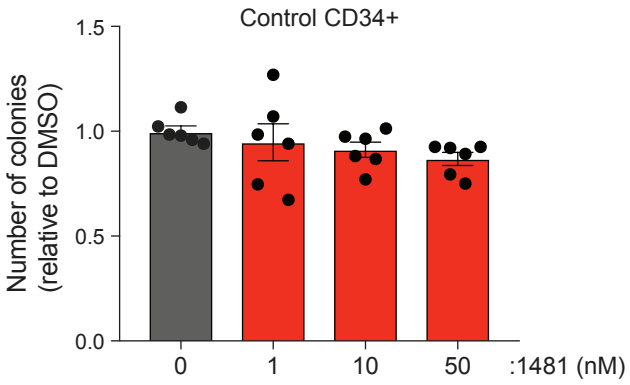
Supplemental Figure 1. Effects of NCGC1481 on MV4;11 cell viability. CellTiterGlo results from MV4;11 cells treated with the indicated inhibitors for 48 hours in 10% fetal bovine serum. Values are expressed as means +/- s.d. from biological triplicate samples. Numbers in parentheses indicate IC50 values.



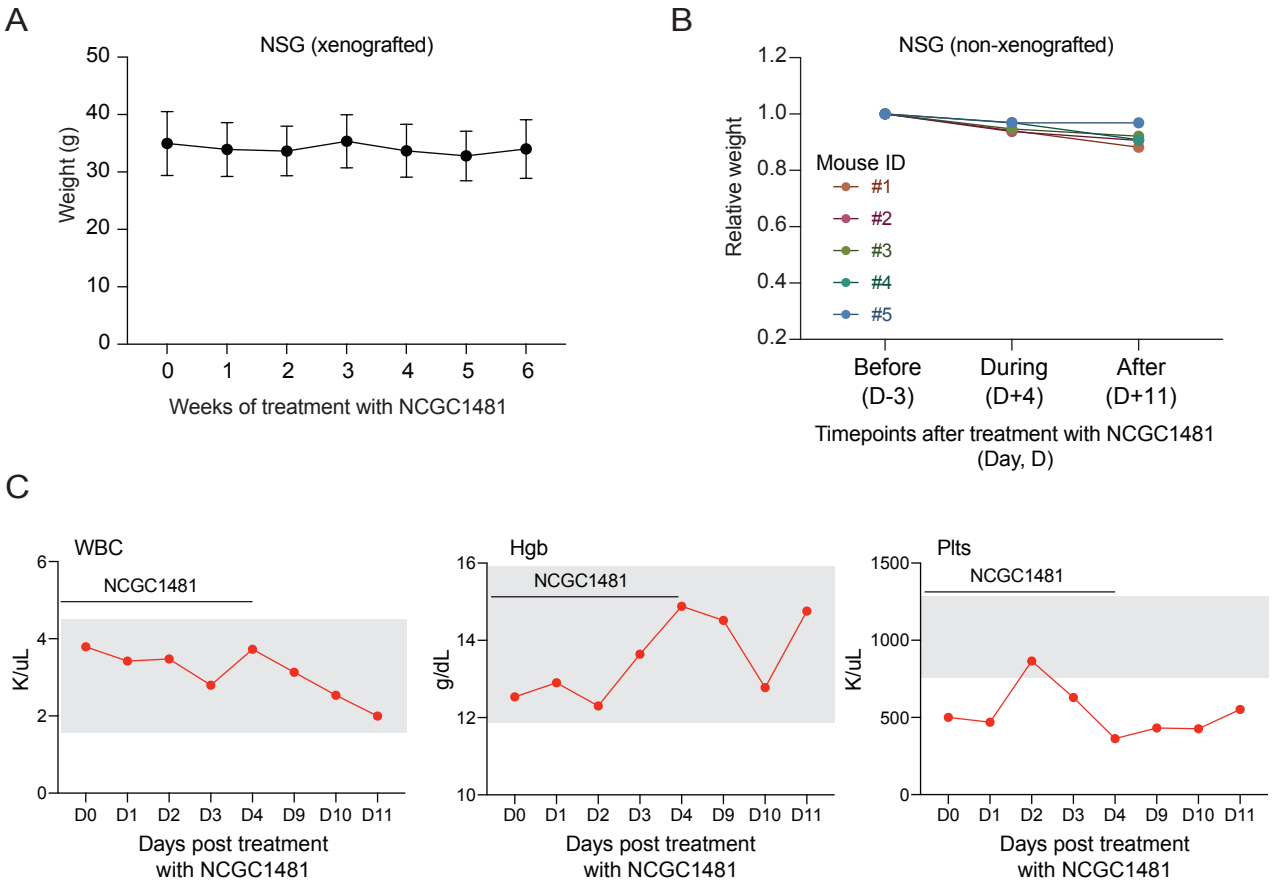
Supplemental Figure 2. Effects of NCGC1481 and gilteritinib on FLT3(F691L) AML. (**A**) Immunobloting of MOLM14;FLT3-ITD(F691L) treated with the indicated inhibitors for 90 mins. (**B**) Leukemic colony formation of MOLM14;FLT3-ITD(F691L) treated with the indicated inhibitors. Colony formation was determined after 7 days. Values are expressed as means +/- s.d from biological triplicate samples. *, $P < 0.05$ (relative to DMSO). *, $P < 0.01$ (Dunnett' multiple corrections test).



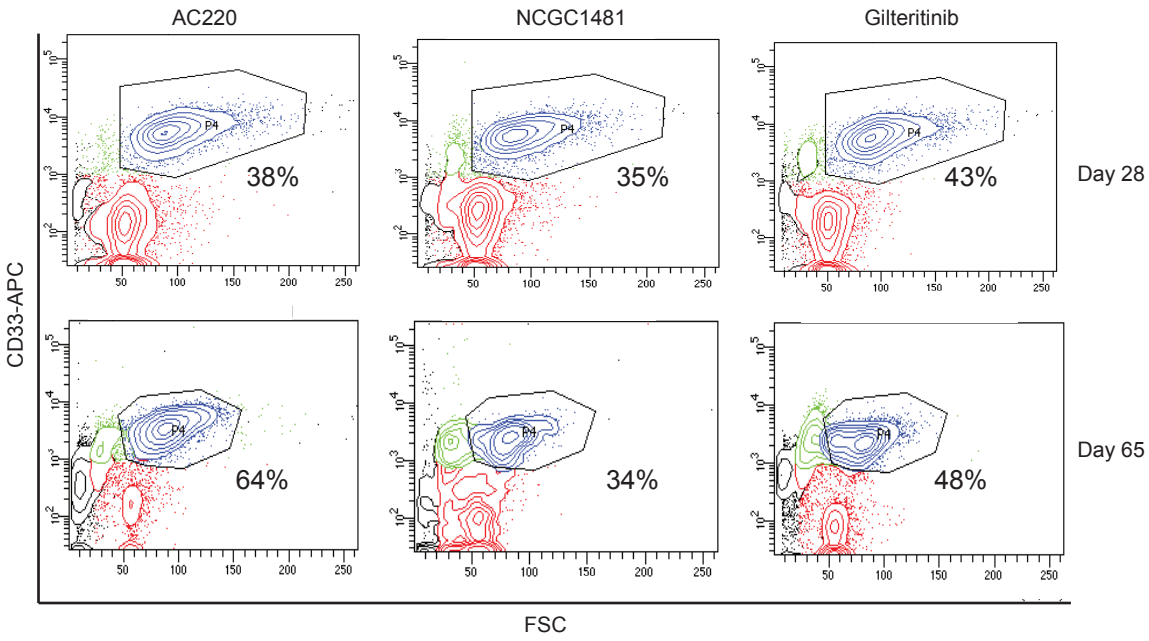
Supplemental Figure 3. Effects of NCGC1481 and FLT3 inhibitors on FLT3-mutant AML. (**A**) Viability of MOLM14 cells treated with the indicated inhibitors for 48 hours in 10% and 0% fetal bovine serum (FBS) was measured by CellTiterGlo (CTG) and MTT cell proliferation (MTT) assays. Values are expressed as means +/- s.d. from biological triplicate samples. (**B**) Summary of MOLM14 cell viability cultured in the indicated concentrations of fetal bovine serum (FBS) and evalauted by CellTiterGlo (CTG) or MTT cell proliferation (MTT) assay after treatment with the indicated inhibitors. (**C**) Immunoblotting of MLL-AF9;FLT3-ITD and MLL-AF9;FLT3-ITD(D835Y) cells treated with the indicated inhibitors for 90 mins. (**D**) AnnexinV/PI staining of MLL-AF9;FLT3-ITD and MLL-AF9;FLT3-ITD(D835Y) cells treated with the indicated inhibitors for 48 hours. Values are expressed as means +/- s.e.m. from independent duplicate or triplicate samples. (**E**) Leukemic colony formation of MLL-AF9;FLT3-ITD and MLL-AF9;FLT3-ITD(D835Y) cells treated with the indicated inhibitors. Colony formation was determined after 7 days. Values are expressed as means +/- s.e.m. from 4-6 independent samples. *, $P < 0.05$ (Dunnett' multiple corrections test).



Supplemental Figure 4. Effects of NCGC1481 on normal hematopoietic cells. Colony formation of healthy cordblood-derived CD34+ cells treated with the indicated concentrations of NCGC1481. Colony formation was determined after 7 days. Values are expressed as means +/- s.d. from two donors performed in triplicate.



Supplemental Figure 5. Effects of chronic treatment of mice with NCGC1481. (**A**) Body weight measurements of NSG mice xenografted with MOLM14(D835Y) and treated with 30 mg/kg of NCGC1481 for the indicated number of week. Data shown as s.d (n = 5 mice per time point). (**B**) Body weight measurements of wild-type (non-xenografted) NSG mice treated with 30 mg/kg of NCGC1481 during the indicated timepoints. (**C**) Peripheral blood counts of wild-type (non-xenografted) NSG mice treated with NCGC1481 (30 mg/kg). Mice were treated with NCGC1481 from Day 0 to Day 4. Grey shading indicates normal blood count range for NSG mice.



Supplemental Figure 6. Leukemic burden is stabilized with treatment of NCGC1481. (A) FACS analysis of AC220-, NCGC1481-, and Gilteritinib-treated mouse bone marrow. Top panel represents baseline (day 28) and bottom panel represents day 65 (time of death in the case of AC220). P4 gate represents cells that are positive for human CD33-APC.

Supplemental Methods

FLT3 Inhibitory Profiles

The kinase profiling (inhibitory potential) data for FLT3 and all listed FLT3 mutants was generated using KINOMEscan® (see https://www.discoverx.com/services/drug-discovery-development-services/kinase-profiling). The technology utilizes a ligand binding technology that allow active compounds to block the binding of a target kinases to an immobilized ligand. Binding is determined by ultra-sensitive qPCR method that detects an associated DNA label. This assay technology provides binding (Kd) data rather than functional (IC_{50}) data.

Cell lines

MOLM14, MOLM14-D835Y, and MOLM14-F691L cells were gifted by the Neil Shah laboratory (UCSF, San Francisco, CA). They were maintained in culture using RPMI 1640 plus 20% fetal bovine serum (FBS) and penicillin/streptomycin (P/S) at 37°C with 5% CO_2. MLL-AF9;FLT3-ITD and MLL-AF9;FLT3-ITD(D835Y) cell lines were generated by first transducing cord blood CD34+ cells with MLL-AF9 and then with FLT3-ITD or FLT3-ITD(D835Y) as previously reported (1). They were cultured in Iscove's Dulbecco's Modified Eagle's Medium (IMDM) (Corning Cell Grow, Cat#10-016-CV) with 20% fetal bovine serum (FBS) (Atlanta Biologicals, Cat#S11550) and 1% penicillin-streptomycin (P/S) (HyClone, Cat#SV30010) at 37°C with 5% CO_2. MV4;11 cells were purchased from ATCC (CRL-9591). They were maintained in culture using IMDM with 10% FBS and P/S at 37°C with 5% CO_2. Analysis of STR loci was performed on all cell lines when received and after experimentation was complete. All cell lines are routinely tested and are confirmed to be negative for mycoplasma.

Patient samples

Human CD34+ umbilical cord blood, human CD34+ bone marrow cells were maintained in StemSpan Serum-Free Expansion Media (Stemcell Techologies, Cat#09650) supplemented with 10 ng/mL of recombinant human stem cell factor (SCF) (PeproTech, Cat#300-07-50UG), recombinant human thrombopoietin (TPO) (PeproTech, Cat#300-18-50UG), recombinant human FLT3 ligand (FLT3L) (PeproTech, Cat#300-19-50UG), recombinant human interleukin-3 (IL-3) (PeproTech, Cat#200-03-50UG), and recombinant human interleukin-6 (IL-6) (PeproTech, Cat#200-06-50UG). AML primary

patient samples were obtained with written informed consent and approved by the institutional review board of Cincinnati Children's Hospital Medical Center. Sample AML-5 was purchased from the Public Repository of Xenografts (PRoXe) (Cat#DFAM-16835-V1).

Cellular viability analysis

Exponentially growing cells ($1x10^4$ cells per well) were plated in a 96-well plate with 0.2 mL of RPMI 1640 plus 20% fetal bovine serum containing 0 to 3,000 nM concentrations of NCGC1481 (synthesized as described in ref 15), AC220 (Selleckchem, Houston, TX), crenolanib (Selleckchem, Houston, TX) midostaurin (Selleckchem, Houston, TX), and gilteritinib (Indianapolis, IN). The cells were plated in triplicate, treated for 72 hours, and viability was assessed by performing CellTiterGlo™. Fifty percent inhibitory concentration (IC_{50}) were then generated using a nonlinear best-fit regression analysis in Prism 7 software (GraphPad, San Diego, CA), and the reported values are the average of 2 or more experiments.

Colony assays

Clonogenic assays were performed using cells in MethoCult H4434 as previously described(2, 3). Cells were prepared at a density of 100-200 cells/mL of methylcellulose. Colonies were plated in duplicate or triplicate for seven days, then counted, with reported values reflecting the average of multiple experiments.

Flow cytometry

After being treated for 48 hours, cells were washed with phosphate buffered saline (PBS) and/or annexin V binding buffer, then stained with Annexin V-FITC for 10 minutes, without light exposure, and finally stained with propidium iodide. Cells were then analyzed using BD-FACSCanto flow cytometer (BD Biosciences) and FACSDiva software. Reported values are representative of multiple experiments.

Immunoblot analysis

Exponentially growing cells were plated in RPMI 1640 plus 20% fetal bovine serum containing the described concentration of AC220, NCGC1481, crenolanib, midostaurin, and gilteritinib. After a ninety-minute incubation period, the cells were washed with cold PBS, then lysed with radioimmunoprecipitation assay (RIPA) lysis buffer for 30 minutes at 4°C. Once protein was isolated, lysates (50µg) were separated by 10% SDS-PAGE,

and gels were transferred to polyvinylidene fluoride microporous membrane. Thereafter, immunoblotting was performed using anti-phospho-FLT3 (#3461), anti-phospho-STAT5 (#9351), anti FLT3 (#3462), anti-STAT5 (#94205), anti-pan-actin (# 4968), and anti-GAPDH (#5174) (Cell Signaling, Beverly, MA).

Leukemia xenograft model

NOD-scid IL2Rγ$^{-/-}$ (NSG) and NOD/LtSz-scid IL2RG–SGM3 (NSGS) mice were bred at Cincinnati Children's Hospital Research Foundation, by the Comprehensive Mouse Core. For cell line derived xenografts, NSG mice (conditioned with 30 mg/kg Busulfan, B2635; Sigma) were injected with 5 x 10^4 MOLM14(D835Y) cells in 200 µL of PBS by tail vein injection as previously described(4). For patient derived xenografts, NSGS mice were injected with 1.3 x 10^6 DFAM-16835-V1 (AML-5) cells (obtained from Public Repository of Xenografts, PRoXe)(5). in Iscove's Modified Dulbecco's Medium (IMDM) with 2% FBS and pen-strep antibiotics. Mice were given intraperitoneal (IP) injections of PBS (vehicle), AC220 (10 mg/kg), NCGC1481 (30 mg/kg), and gilteritinib (30 mg/kg) at the indicated times. All animal procedures were performed in concordance with institutional guidelines. To demonstrate on target effects of NCGC1481, we injected NSG mice with MOLM14-D835Y cells, and treated them with PBS and NCGC1481 beginning 3 weeks after transplantation. The mice were then sacrificed on the following day, cells were collected from bone marrow, then sorted for human CD33. Once human CD33 cells were isolated, protein was isolated from them as described above; and immunoblotting was performed using anti-phospho FLT3, anti-phospho-STAT5, anti-FLT3, anti-STAT5, anti-GAPDH, and anti-pan actin.

Protein expression, purification and crystallization

Human FLT3 was expressed in Sf9 insect cells using a recombinant baculorvirus encoding FLT3 as previously described(6). In brief, the kinase domain of FLT3 (residues 564–958) was cloned into the pFastbac1 (Thermo Fisher), a flexible loop (residues 711–761) was deleted, and a 6XHis-tag with a TEV protease cleavage site was added to the N-terminus. The resulting baculovirus was used for expression in insect cells. Suspension cultures of Sf9 were grown to a density of 10^6 cells/mL at 27°C and infected. Cells were harvested 72 hr post infection and washed in a phosphate-buffered saline solution then stored at -80°C until thawed for purification. Thawed cell pellets were re-suspended in

lysis buffer (50 mM Tris, pH 8.0; 500 mM NaCl, 5% glycerol). Lysates were cleared by centrifugation at 15,000 rpm for 60 minutes. The supernatant was incubated with Ni-NTA beads (Qiagen) pre-equilibrated with lysis buffer at 4°C for 2 hrs, then the beads were transferred to a gravity column and washed with lysis buffer for 50 CVs. FLT3 protein was eluted with lysis buffer containing 50 mM imidazole. Eluted FLT3 protein was pooled, TEV was added to the protein solution and dialyze to lysis buffer at 4°C overnight. The sample was. then passed through a Ni-NTA column to remove the TEV protease, flow through fraction was collected and concentrated and further purified by a Superdex 200 increase 10/300 column (GE Healthcare Life Sciences) in 20 mM Tris, 150mM NaCl, 5%Glycerol, pH 8.0. According to the SDS-PAGE, fractions containing FLT3 were pooled and concentrated to 7.1 mg/ml, frozen by liquid nitrogen and stored at -80°C. Crystallization of apo FLT3 was carried out in hanging drop by vapor diffusion method at 18°C. Crystallization drops contained 1μl FLT3 protein and 1μl of reservoir buffer containing 0.1M CAPS pH10.5, 0.2M Lithium Sulfate, 1.2M Sodium dihydrogen phosphate, 0.8M Di-potassium hydrogen phosphate. Crystals were transferred to reservoir solutions containing 30% (v/v) glycerol, then flash frozen in liquid nitrogen and stored in liquid nitrogen.

Crystallization, data processing, and structure determination

The diffraction data on the crystal of FLT3/NCGC1481 was collected at the Shanghai Synchrotron Radiation Facility (SSRF) on the 18U1 beam line and processed to 2.5 Angstrom resolution in space group $P4_32_12$ using HKL3000. This structure was solved by molecular replacement using Phaser with a known FLT3 structure (PDB: 1RJB) as a search model. After initial refinement using program Refmac5, the density of NCGC1481 (excluding the pyrrolidin-3-amine tail) was observed and fitted as the compound. Then, further cycles of model building and refinement were carried out using Coot and Refmac, respectively, to complete the structure determination of FLT3/NCGC1481. See **Supplemental Table 3** for the statistical parameters for protein crystallization.

Statistical Analysis

For the in vitro experiments, values are expressed as mean ± standard deviation or standard error of the mean. For the in vivo experiments, values are expressed as mean

± standard error of the mean. Statistical values for the in vivo experiments were calculated utilizing unpaired two-tailed t-tests. A P value of < 0.05 was considered statistically significant. GraphPad Prism 7 software (GraphPad, San Diego, CA) and Microsoft Excel (Microsoft, Redmond, WA) were used for data processing.

Supplemental Methods

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