Concomitant targeting of FLT3 and BTK overcomes FLT3 inhibitor resistance in acute myeloid leukemia through the inhibition of autophagy

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Supplementary Methods and data:

Methods

Leukemia cell lines

The murine leukemia cell lines harboring *FLT3* ITD mutations (Ba/F3-*FLT3*-ITD), TKD mutation (Ba/F3-*FLT3*-D835Y) and ITD/TKD double mutations ((Ba/F3-ITD/D835Y, Ba/F3-ITD/F691L, Ba/F3-ITD/Y842D) and their parent cells Ba/F3-*FLT3*-WT were kindly provided as gift or generated by us as described previously [1]. The human *FLT3* ITD mutated AML cell lines MOLM14 from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) and MV4-11 from ATCC (Manassas, VA). The human *FLT3* WT AML cell line OCI/AML3 cell line from Dr. M. Minden (Princess Margaret Hospital, Toronto, Ontario, Canada), and THP-1 cell line from ATCC (Manassas, VA). Cells were maintained in RPMI medium supplemented with 10% fetal bovine serum (FBS) at either 21% oxygen (normoxia) or 1% oxygen (hypoxia) condition, and IL-3 dependent murine Baf3/*FLT3*-WT cells were maintained in the presence of 2 ng/mL of IL-3. Mesenchymal stem cells (MSCs), obtained from normal bone marrows (BM) following Institutional guidelines, were cultured at a density of 5,000 cells/cm2 in α-MEM supplemented with 20% FBS, 1% L-glutamine, and 1% penicillin-streptomycin. The MSCs were used for co-culture experiments after passage four.

Antibodies

The antibodies against human phosphorylated (p)-p44/42 ERK(Thr202/Tyr204), p-AKT(Ser473), p-FLT3(Tyr589/591), p-S6K(Ser240/244), p-BTK(Tyr223), p-ULK(Ser555), p-mTOR(Ser2448), p-aurora, p-AMPK(Thr172), p-Histone H3(Ser10), p-cdc25c(Thr48), p-CDC2, AKT, S6K, ATF, Beclin-1, LC3, aurora, BTK, c-Myc, Atg7, PLK1cyclin B1, cyclin G1 and cleaved-caspase-3 were purchased from Cell Signaling Technology (Danvers, MA), against ERK2, FLT3 and p53 from Santa Cruz Biotechnology (Santa Cruz, CA), against p21 from CalBiochem (San Diego, CA), against HIF1α from BD Biosciences (San Diego, CA). Interleukin-3 (IL-3) was purchased from PEPROTECH (Rocky Hill, NJ).

Cell cycle analysis

Leukemia cells were treated for 24 h with indicated drugs. BrdU was added to culture medium at a final concentration of 10 μM for an additional 30 min. The cells were harvested and fixed in 70% ethanol at 4°C for at least 24 h and washed twice in PBS. Cells were treated with 2 M HCl plus 0.5% Triton X-100 for 30 min and neutralized with 0.1 M Na2B4O7, pH 8.5 for 2 min at RT. Anti-BrdU-FITC antibody for 45 min at room temperature. Wash the cells with PBS and resuspended to PBS with propidium iodide (Invitrogen) for another 30 min on ice. The flow cytometric DNA histogram was plotted to disseminate the cell cycle was measured with FACSCalibur (Becton Dickinson, Franklin Lakes, NJ) and data interpretation and graph illustration with CellQuest and FlowJo software.

Immunofluorescence and confocal microscopy

THP-1 and OCI/AML3 cells were exposed to an indicated concentration of CG-806 for 24 h. The cells were harvested and fixed on slides for 5 min at 800 RPM, fixed directly with 90% ice-cold methanol for 10 min, and permeabilized with cooled acetone for 1 minute at –20 °C. Fixed cells were blocked with 2% bovine serum albumin (BSA) for 30 min and incubated with anti-α-tubulin polyclonal antibody and anti-γ-tubulin monoclonal antibody for 2 h at 4 °C, and followed by tetramethyl rhodamine isothiocyanate (TRITC) conjugated anti-rabbit secondary antibody (Jackson Laboratories, USA) and by Alexa Fluor 488 conjugated phalloidin anti-mouse secondary antibody for 30 min (Molecular Probes, Invitrogen, USA). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Sigma) for 30 min. Images were acquired with a confocal laser-scanning microscope (Olympus FV500).

Immunoblot assays

Immunoblotting was performed as previously described [2]. Briefly, treated cells were collected in lysis buffer (100 mM HEPES, pH = 7.5, 50 mM NaCl, 0.1% Triton X-100, 5 mM EDTA, 0.125 M EGTA). Protease and phosphatase inhibitors (0.2 mg/mL Leupeptin, 2 mg/mL, Aprotinin, 1mM PMSF and 0.1mM Na3VO4) were added prior to lysis. Cell lysates were resolved by electrophoresis on 12% precast sodium dodecyl sulfate-polyacrylamide gels, and transferred to Hybond-P membranes. After immunoblotting with antibodies, signals were detected by using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA), and a semi-quantitative assessment was determined using

the Scion Imaging system and software (beta version 4.03; Scion, Frederick, MD). All immunoblotting data from in vitro cell lines studies shown in the figures were representative of at least 2-3 repeats of the same experiments.

BTK and ATG7 knocking down

For knockdown of BTK or ATG7 proteins, either BTK or ATG7 siRNA and mock control (scramble) siRNA were purchased from Dharmacon Research, Inc. The siRNA were electroporation transfection into MOLM14 or MV4-11 using the Nucleofection system (L-solution, Q-001 for MV4-11 and V-solution, O-017 for MOLM14; Amaxa), following the manufacturer's instructions. The final concentration of siRNA was 300 nM. After 24 h of transfection, the indicated concentrations of drugs were added to the cells for an additional 24 h of culturing. Apoptosis induction was determined by measuring the percentage of annexin V–positive cells via flow cytometry, and the expression level of the relative proteins was analyzed by immunoblotting.

ATG7 knockdown in OCL-AML3 cells was generated by lentiviral transduction with ATG7 shRNA vectors into OCI-AML3 cells and non-silencing GIPZ lenti shRNA vectors were a control, which has been described in a previous publication [3]. Decreased expression of ATG7 was verified by immunoblot analysis as shown in Fig. S7.

Transmission electron microscopy

For transmission electron microscopy (TEM) analysis, MOLM14 cells were co-cultured with MSCs in an insert well for 24 h. Cells were collected and fixed in 4% cacodylate-buffered glutaraldehyde (pH 7.4) at room temperature, and post-fixed in 1% OsO4 (Electron Microscopy Sciences, Foster City, CA, USA), dehydrated with graded alcohol series, immersed in propylene oxide and embedded in Epon 812 resin. Semithin sections (2 μm thick) were obtained with an RMC MT-X ultra-microtome (EMME3, Milan, Italy) and stained with an alcoholic solution of uranyl acetate, followed by an alkaline bismuth sub nitrate solution. Ultrathin sections were observed with Leica Ultracut microtome (Leica, Deerfield, IL), stained with uranyl acetate and lead citrate in a Leica EM Stainer, and examined in a JEM 1010

transmission electron microscope (JEOL, USA, Inc., Peabody, MA) at an accelerating voltage of 80 kV. Digital images were obtained using AMT Imaging System (Advanced Microscopy Techniques Corp, Danvers, MA).

Supplementary Tables:

Table S1. Comparison of autophagy levels and resistance [a] to quizartinib in murine Ba/F3 leukemia cells harboring *FLT3* WT and different *FLT3* mutational status

	***FLT3* WT**	***FLT3* ITD**	***FLT3* D835Y**	***FLT3* ITD+D835Y**	***FLT3* ITD+F691L**
Autophagy [b]	1.0	1.5	3.0	2.8	2.8
Quizartinib	1956.0	2.2	2089.0	246.4	115.3

[a] The resistance to quizartinb was presented as IC50s which were generated from assays by exposing the cells to quizartinib for 48 h and measuring cell proliferation (nM, n = 3).

[b] Autophagy level was determined by semi-quantity the ratios of LC3-II/I based on Western blot data in Fig 1A. The autophagy level

Table S2. Characterization of AML Patient Samples

Patients[a]	FLT3 Status	Age	Time Points[a]	BM Blast (%)	PB Blast (%)	WBC ($x10^9$/L)	Clinical Response
Case 1	ITD	81	pre-dose	14	14	6.7	No response
			after-dose	12	3	6.7	
Case 2	D835	59	pre-dose	46	70	6.3	Inevaluable, died within 30 days
			after-dose	N/A	91	58.5	
Case 3	ITD+D835	58	pre-dose	81	89	21	No response
			after-dose	58	67	1.7	

[a]The patient samples were collected from a phase I clinical trial of sorafenib monotherapy.

Table S3. Measurement of cell-free enzymatic activity of CG-806

Kinase	IC_{50} (nM)	% Enzymatic Activity*		
		0.01 μM	0.1 μM	1.0 μM
Aurora A	0.38	20.1	-0.4	-4.0
FMS	0.62	23.7	5.9	3.7
BLK	0.74	22.4	5.3	0.5
FLT3(ITD)	0.82	NA	6.2	1.6
BTK(C481S)	2.52	NA	15.1	1.8
Aurora C	2.95	27.0	15.7	13.6
Aurora B	3.70	46.7	4.1	2.6
BTK	5.00	68.7	19.4	3.3

*Inhibitory activity of CG-806 was evaluated against 216 kinases. Each enzyme was incubated with different concentrations of CG-806 in the presence of ATP at 28°C for 90 min. Percent inhibition of enzyme activity was determined, and IC_{50} values were calculated by the manufacturer's protocol. Kinases with IC50 values of less than 5 nM are listed.

Table S4. IC_{50}s comparison of CG-806 and other FLT3 inhibitors in FLT3 WT and mutated cells.

FLT3 inhibitor	IC_{50} in Transfected Ba/F3 Cells (nM, n = 3)				
	FLT3 WT [a]	FLT3 ITD	FLT3 D835Y	FLT3 ITD+D835Y	FLT3 ITD+F691L
CG-806	**11.3**	**0.5**	**8.8**	**19.3**	**10.0**
Quizartinib	1956.0	2.2	2089.0	246.4	115.3
Gilteritinib	500.3	26.5	472.5	6.8	98.4
Crenolanib	2617.0	35.0	888.9	31.7	257.6
FF-10101 [b]	2300.0	1.9	0.32	0.81	10.0

[a] FLT3 WT cells are IL3-dependent and presented during the inhibitor treatment.

[b] The data were cited from Yamaura et al. (*Blood* . 2018; 131:426-438). 32D-transfectant cell lines bearing diferent FLT3 statuses were treated with FF-10101 for 48h and mean GI_{50} values were determined by CellTiter 96® Aqueous One Solution Proliferation Assay.

Supplemental Figures:

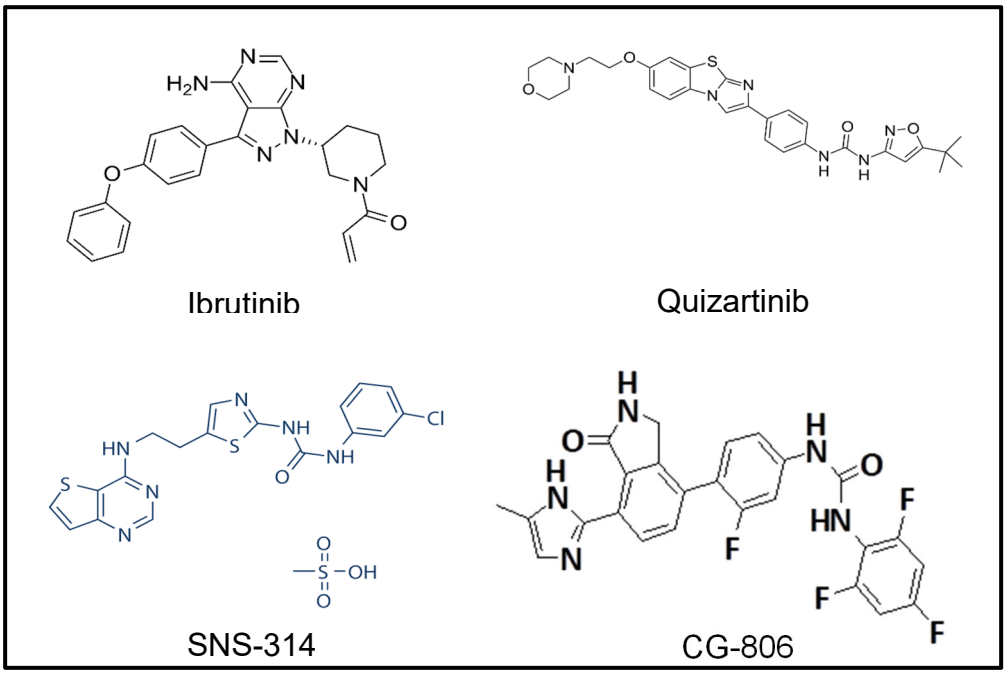

Fig. S1. Chemical structures of the kinase inhibitors used in this study.

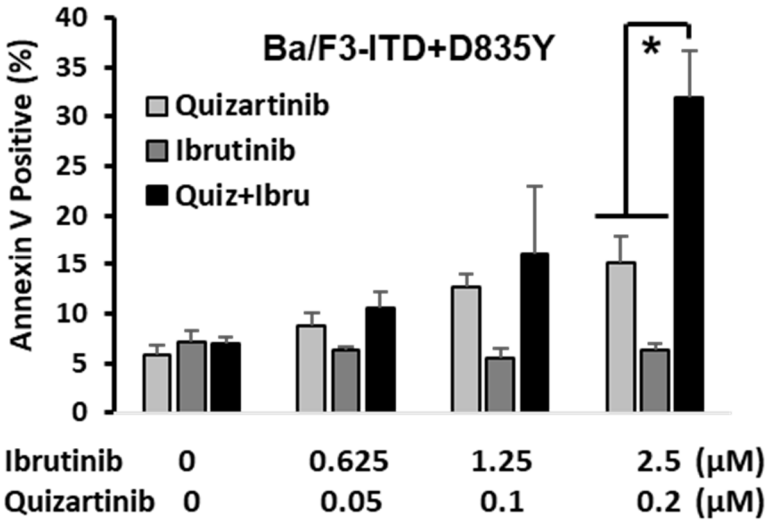

Fig. S2. Ba/F-ITD/D835Y cells were exposed to either ibrutinib (Ibru) or quizartinib (Quiz) or the combo for 48 h and apoptosis induction was assessed by measuring annexin V positivity with flow cytometry.

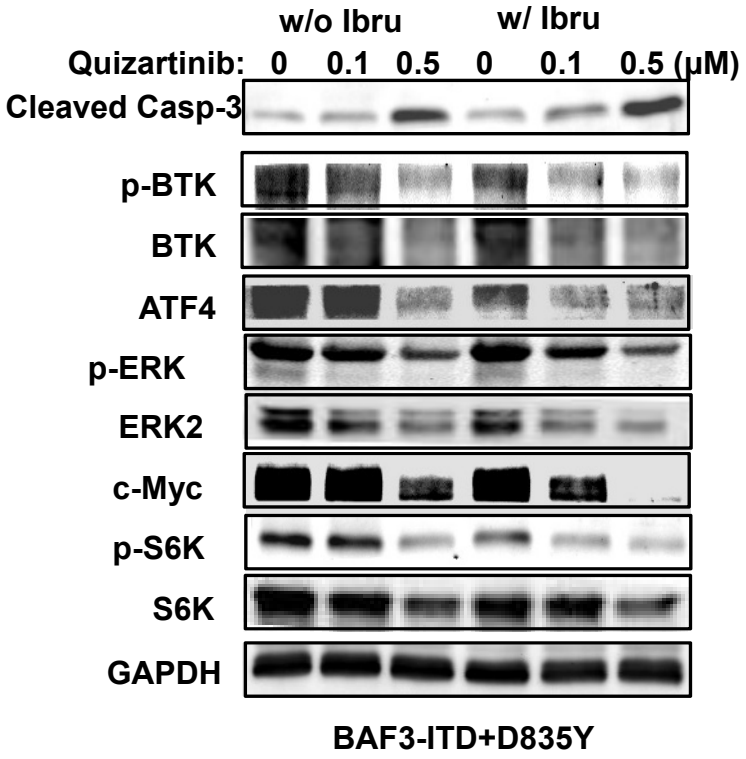

Fig. S3. Baf3-ITD+D835Y cells were treated with quizartinib in the presence/absence of ibrutinib (Ibru) for 24 h, and proteins were determined using immunoblotting.

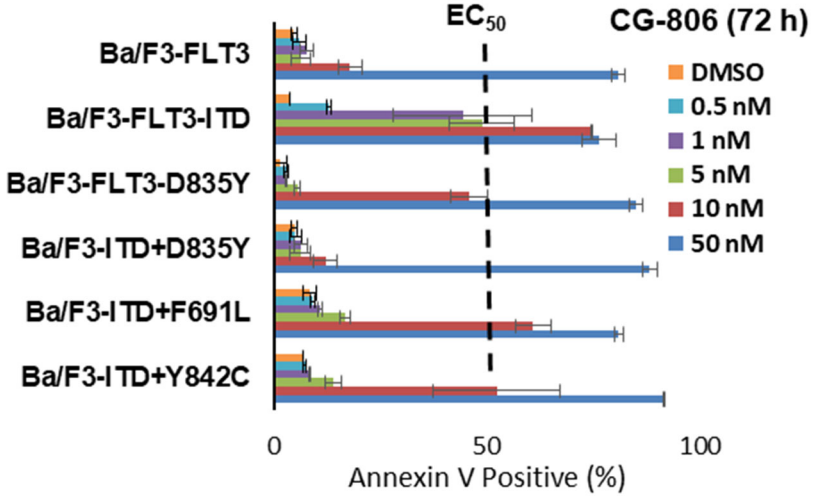

Fig. S4. Murine leukemia cells harboring different *FLT3* mutations and their parental cells Baf3/*FLT3* cells were exposed in CG-806 for 72 h, and apoptosis induction was assessed by measuring annexin V positivity with flow cytometry. Error bars were generated from three separate experiments. The dotted line indicates EC_{50}.

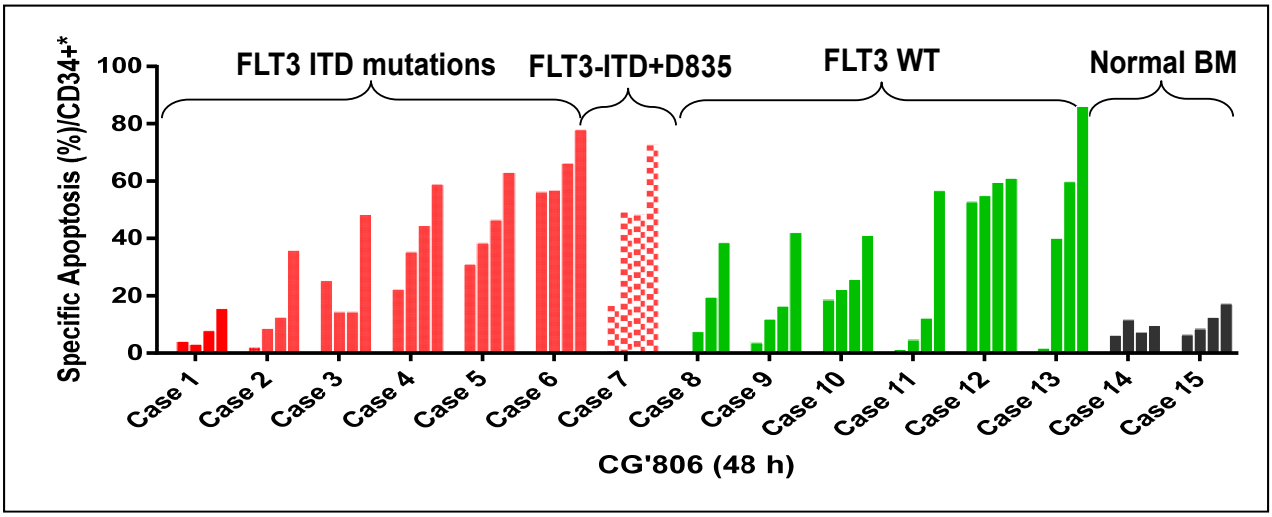

Fig.S4. AML patient samples harboring various *FLT3* mutations were exposed to different concentrations (0, 0.1, 0.5, 1 and 3 μM) CG-806 for 48 h, and the induction of specific apoptosis was determined by annexin V staining via flow cytometry

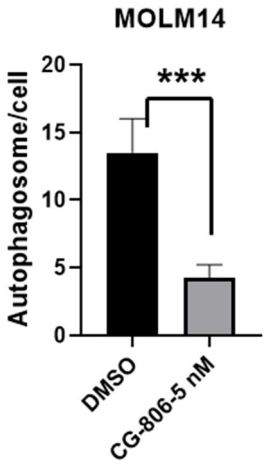

Fig.S5. A statistical analysis of autophagosome based on the TEM observation of at least 20 cells per sample.

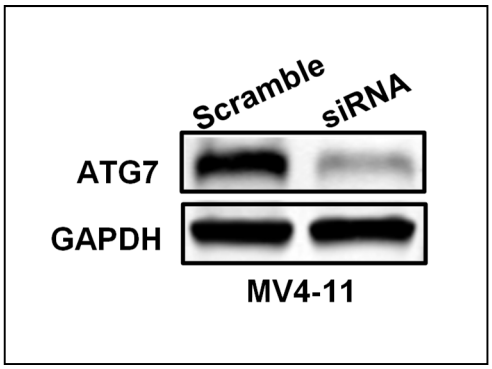

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Fig. S6. MV4-11 leukemia cells were transfected with ATG7 siRNA and the scramble siRNA for 48 h. ATG7 levels were determined by immunoblotting.

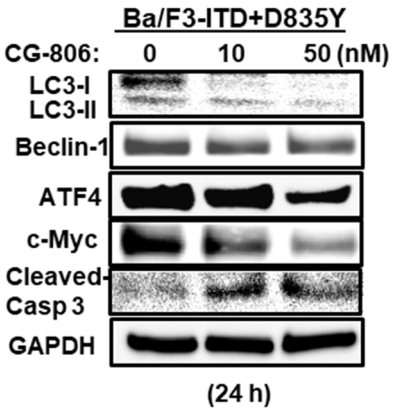

Fig. S7. Baf3-ITD/D835Y cells were exposed in CG-806 for 24 h and followed by immunoblot analysis of cellular proteins.

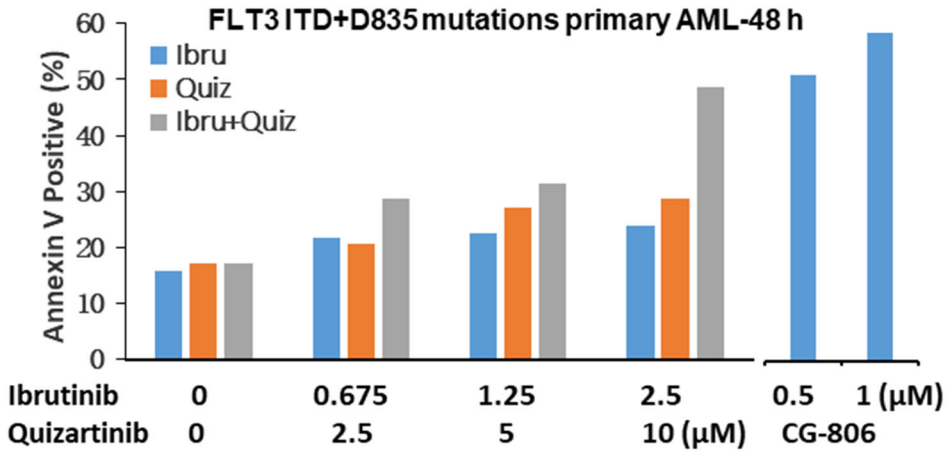

Fig. S8. *FLT3*-ITD-D835 mutated primary AML blasts were exposed in either CG-806 or quizartinib *ex vivo* for 48 h. Apoptosis induction was assessed by measuring annexin V positivity with flow cytometry.

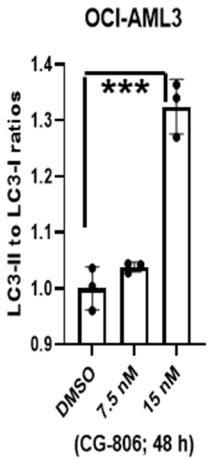

Fig. S9. Statistical analysis was performed based on a semi-quantitative analysis of LC3-II to I ratios obtained from triplicated Western blot data in Fig. 5A. *** = $p < 0.001$. Error bars of the semi-quantitative data represent the standard deviation from three independent experiments.

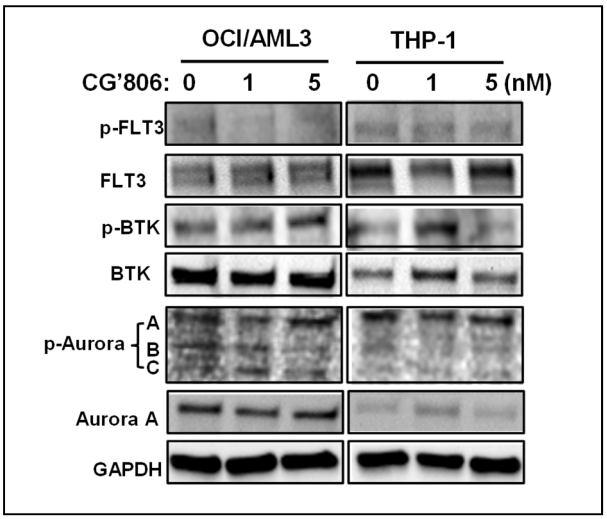

Fig. S10. OCI/AML3 and THP-1 leukemia cells were exposed to CG-806 for 4 h, the cell proteins were harvested, and the levels of the indicated proteins were determined by immunoblotting.

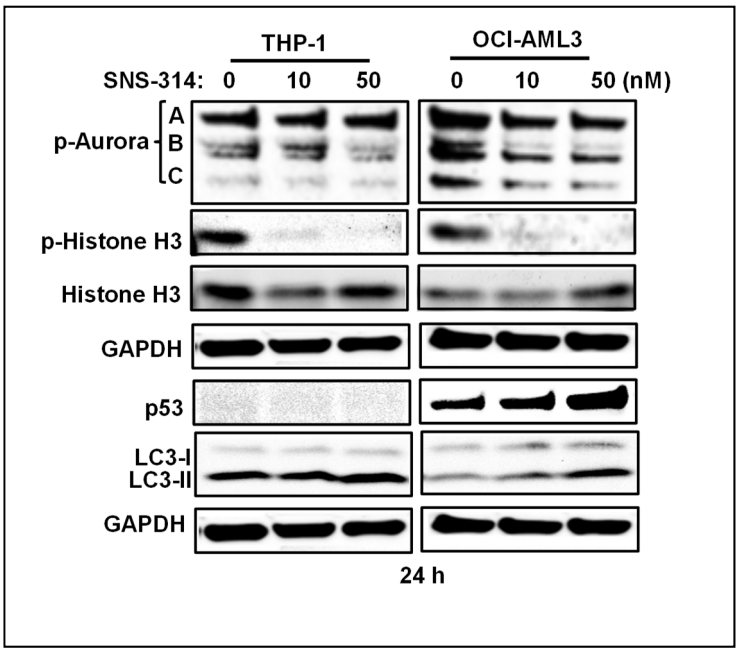

Fig. S11. THP-1 and OCI/AML3 leukemia cells were exposed to SNS-314 for 24 h, proteins were harvested, and the levels of the indicated proteins were determined by immunoblotting.

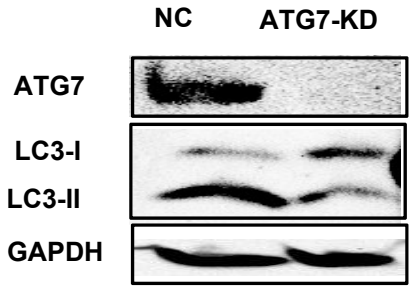

Fig. S12. ATG7 shRNA vectors were transfected into OCI/AML3 leukemia cells. ATG7 levels were determined by immunoblotting. OCI/AML3-parental (NC) and knockdown (KD) cell proteins.

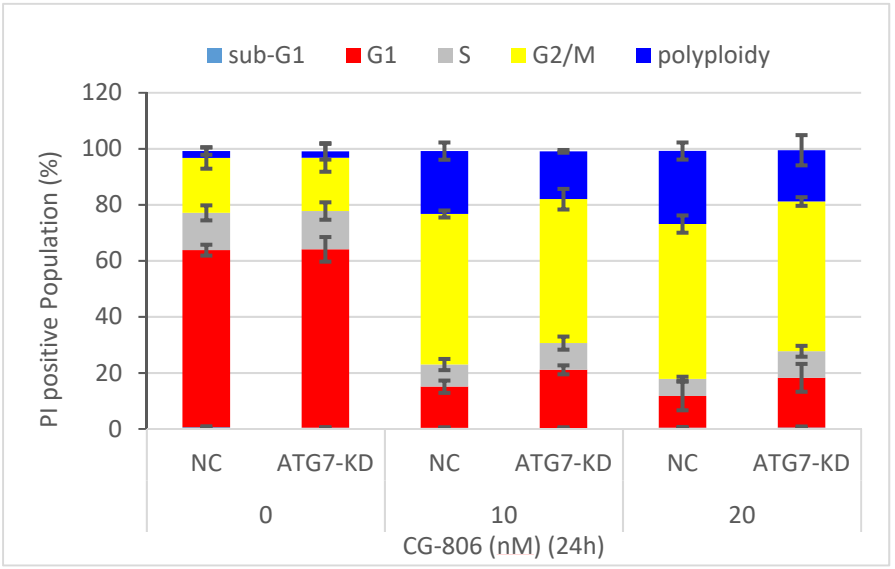

Fig. S13. OCI/AML3-Atg7-KD and parental control (NC) cells were exposed to CG-806 for 24 h and BrdU for an additional 30 min. Cells were collected and stained with anti-BrdU Ab and Propidium Iodide (PI). DNA content was measured by flow cytometry.

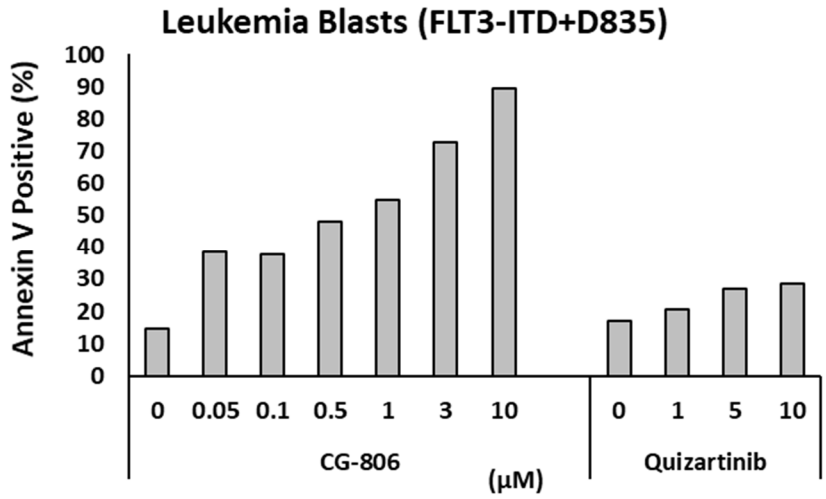

Fig. S14. Leukemia cells (hCD45+/mCD45- population) were isolated from a PDX leukemia mouse model engrafted with *FLT3*-ITD/D835-mutated primary AML blasts and then were exposed in either CG-806 or quizartinib ex vivo for 48 h. Apoptosis induction was assessed by measuring annexin V positivity with flow cytometry.

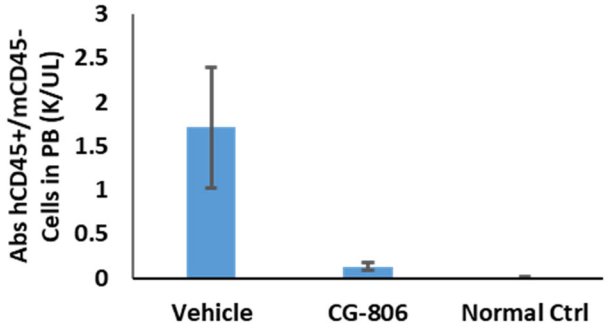

Fig. S15. Absolute number (Abs) of hCD45+/mCD45- cells was calculated by measuring WBC and leukemia cell engraftment in peripheral blood.

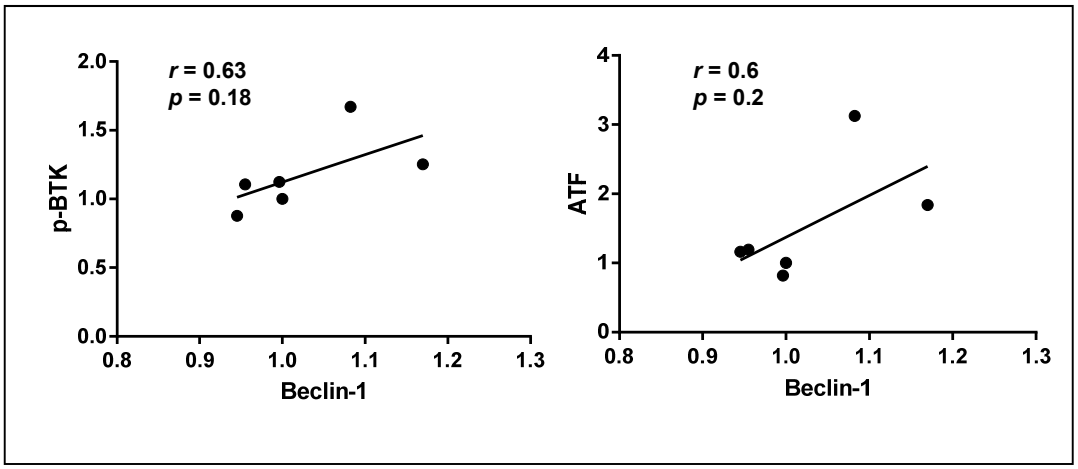

Fig. S16. Ba/F3 *FLT3* WT and mutated cell lines were collected and cell lysates were prepared for determination of basal protein levels of Beclin-1, phospho-BTK, and ATF. The correlation coefficient (r) was determined using GraphPad Prism (version 7.03) software.

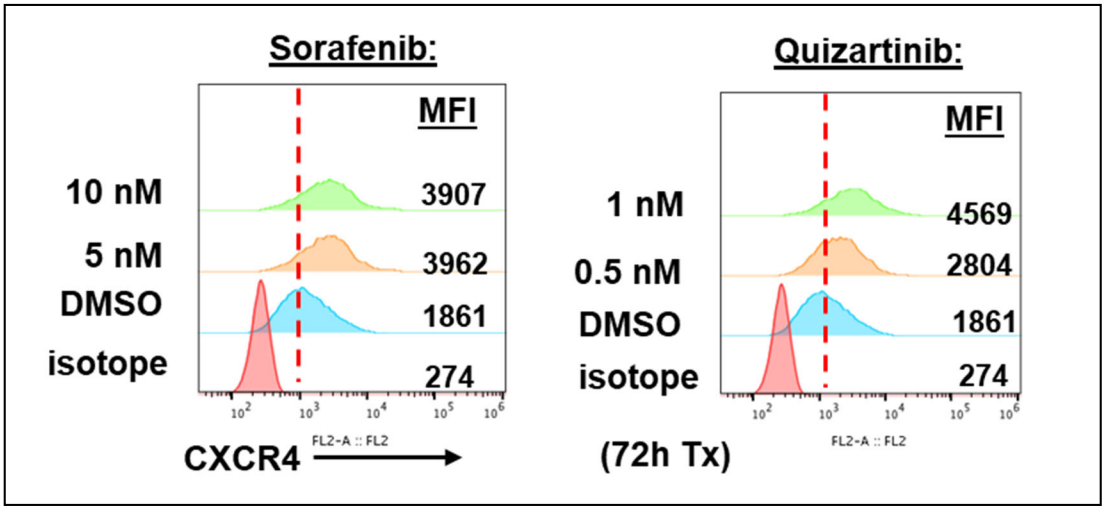

Fig. S17. MOLM14 leukemia cells were treated with FLT3 inhibitors for 72 h. CXCR4 expression was determined by flow cytometry after staining with an anti-CXCR4-PE antibody and an isotype control. MFI = Mean Fluorescent Intensity.

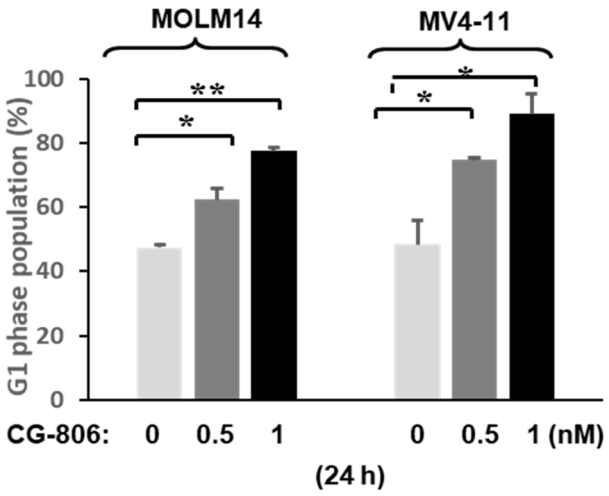

Fig. S18. *FLT3* mutated MOLM14 and MV4-11 leukemia cells were treated with CG-806 for 24 h. The G1 phase distribution was summarized from 3 independent experiments. Data are presented as the means ± standard deviations. *p ⩽ 0.05, **p ⩽ 0.01; Student t-test.

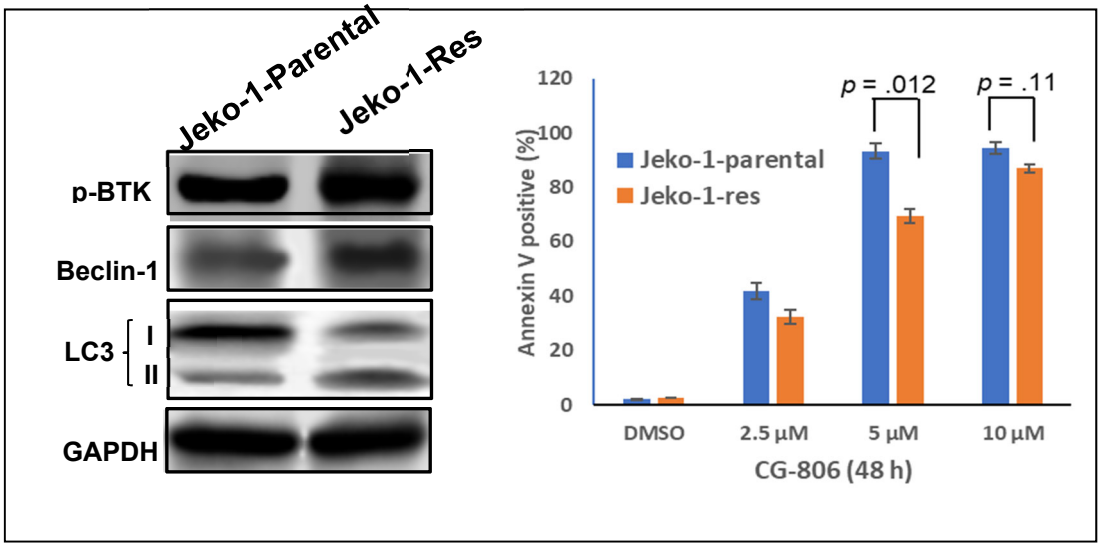

Fig. S19. Ibrutinib resistant lymphoma cell line Jeko-1-Res and the parental cells (Jeko-1-parental) were treated with CG-806 for 48 h. Apoptosis induction was assessed by measuring annexin V positivity with flow cytometry.

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2. Zhang W, Konopleva M, Shi YX, et al. Mutant FLT3: a direct target of sorafenib in acute myelogenous leukemia. JNatlCancer Inst. 2008;100(3):184-198.
3. Piya S, Kornblau SM, Ruvolo VR, et al. Atg7 suppression enhances chemotherapeutic agent sensitivity and overcomes stroma-mediated chemoresistance in acute myeloid leukemia. Blood. 2016;128(9):1260-1269.