SUPPLEMENTARY APPENDIX

Anexelekto /MER tyrosine kinase inhibitor ONO-7475 arrests growth and kills FMS-like tyrosine kinase 3-internal tandem duplication mutant acute myeloid leukemia cells by diverse mechanisms

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Supplemental Materials

Supplemental Methods

Cell culture - Cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) (Gemini Bio-Products, Woodland, CA, USA), 1 mM L-glutamine, and 50 μg/mL penicillin/streptomycin (Gibco Laboratories, Grand Island, NY, USA). Cells were grown at 37^0C with 5% CO_2. For co-culture experiments, BM-derived mesenchymal stromal cells (MSCs) were isolated and cultured as previously described.[22] On day 1, BM MSCs were plated in their growth medium at 2.5 x 10^4 cells per well in 24-well plates. After 24 h, the medium was removed and replaced with 1.0 x 10^5 AML cells in their growth medium (for a ratio of 4 AML cells to 1 MSC). After co-culture for 24 h, the medium was removed and replaced with growth medium containing drug as indicated. The cells were co-cultured for 48 h and then stained and analyzed by FACS. Human CD90-APC (BD Biosciences) was used to distinguish BM MSCs (CD90+) from AML cells (CD90-).

Cell based kinase assay - Recombinant Kinase inhibition assays were performed at Ono Pharmaceutical Company Ltd. (Osaka, Japan). Assays were performed using 100 nM of ONO-7475 against 275 kinases and 21 kinases showed potent inhibition (data not shown). For experiments of Cell-based tyrosine kinase inhibition, recombinant kinase expressing Ba/F3 cells were treated with ONO-7475 for 48 hr. Relative light units were measured with the Cell Titer-Glo Luminescent Cell Viability Assay. IC_{50} denotes half-maximal inhibitory concentration.

RPPA - Cellular proteins were denatured by 1% SDS (with beta-mercaptoethanol) and diluted in five 2-fold serial dilutions in dilution buffer (lysis buffer containing 1% SDS). Serial diluted lysates were arrayed on nitrocellulose-coated slides (Grace Biolab) by Aushon 2470 Arrayer

(Aushon BioSystems). Total 5808 array spots were arranged on each slide including the spots corresponding to positive and negative controls prepared from mixed cell lysates or dilution buffer, respectively. Each slide was probed with a validated primary antibody plus a biotin-conjugated secondary antibody. Only antibodies with a Pearson correlation coefficient between RPPA and western blotting of greater than 0.7 were used in reverse phase protein array study. Antibodies with a single or dominant band on western blotting were further assessed by direct comparison to RPPA using cell lines with differential protein expression or modulated with ligands/inhibitors or siRNA for phospho- or structural proteins, respectively. The signal obtained was amplified using a Dako Cytomation–catalyzed system (Dako) and visualized by DAB colorimetric reaction. The slides were scanned, analyzed, and quantified using a customized-software Microvigene (VigeneTech Inc.) to generate spot intensity. Each dilution curve was fitted with a logistic model ("Supercurve Fitting" developed by the Department of Bioinformatics and Computational Biology in MD Anderson Cancer Center, "http://bioinformatics.mdanderson.org/OOMPA"). This fits a single curve using all the samples (*i.e*. dilution series) on a slide with the signal intensity as the response variable and the dilution steps are independent variable. The fitted curve is plotted with the signal intensities – both observed and fitted - on the y-axis and the log2-concentration of proteins on the x-axis for diagnostic purposes. The protein concentrations of each set of slides were then normalized by median polish, which was corrected across samples by the linear expression values using the median expression levels of all antibody experiments to calculate a loading correction factor for each sample.

TaqMan Gene Expression Assays used: TaqMan assays (Life Technologies) were used as directed by the manufacturer: AXL (Hs01064444_m1), B2M (Hs99999907_m1), CDK1 (Hs00938777_m1), Cyclin B1 (Hs01030099_m1), and PLK1 (Hs00983229_m1).

Antibodies used for immunoblot analysis - AXL (Santa Cruz Biotechnology), phospho-ERK (Y202/Y204; Cell Signaling, Beverly, MA), phospho-AKT (S473; Cell Signaling, Beverly, MA), phospho-MEK (S217/S221; Cell Signaling, Beverly, MA), phospho-FLT3 (Y842; Cell Signaling, Beverly, MA), FLT3 (Cell Signaling, Beverly, MA), CDK1 (Cell Signaling, Beverly, MA), Cyclin B1 (Cell Signaling, Beverly, MA), MCL-1 (Santa Cruz Biotechnology), p53 (Santa Cruz Biotechnology), BCL2 (Dako, Carpinteria, CA), MERTK (Santa Cruz Biotechnology), and Tubulin (Sigma Aldrich, St. Louis, MO).

Human AML murine xenograft model – The animal study was reviewed and approved by The Institutional Animal Care and Use Committee. Four-week old female NSG mice (MD Anderson Animal Facility) were injected intravenously with MOLM13 cells stably expressing a dual firefly luciferase-GFP reporter. One million cells were used for low dose drug study while 0.6 million cells were used for the high dose study. Mice were tail-marked by Sharpie pen at time of leukemia transplantation and monitored once per week for engraftment and tumor growth. At 4 days after cell injection, mice were randomized into two treatment groups receiving either control feed or feed containing 0.004% ONO-7475 or 0.013% ONO-7475. The manufacturer estimates 0.004% is roughly 6 mg/kg daily consumption of drug while 0.013% is roughly 20 mg/kg daily consumption of drug (T. Yasuhiro and T. Yoshizawa, Ono Pharmaceutical Co. LTD, personal communication). For the experiment with low dose of the drug, leukemia burden was

monitored by weekly noninvasive imaging of isoflurane-anesthetized mice injected intraperitoneally with luciferin in the In Vivo Imaging System (Xenogen/Caliper Life Sciences, Hopkinton, MA) with total imaging time of 1 minute. Before imaging, mice were placed in an acrylic chamber, anesthetized with 1.5% isofluorane–air mixture, and injected intraperitoneally with 15 mg/mL of luciferin potassium salt in PBS at a dose of 150 mg/kg body weight. A digital gray scale image of each mouse was acquired, followed by acquisition and overlay of a pseudocolor image representing the spatial distribution of detected photons emerging from active luciferase within the mouse. Mice were sacrificed when they became moribund or unable to obtain food or water. When possible, tissues including sternum or long bone for BM, spleen, and liver were collected from sacrificed mice. Survival was estimated using Kaplan Meir curves and statistics and hazard ratios determined using GraphPad software.

Supplemental Table 1 Cell-based Tyrosine Kinase Inhibition Assays

Kinase	IC_{50} (nM)	Axl Fold Selectivity
AXL	0.7	1
MER	1.0	1
TYRO3	8.7	12
TRKB	15.8	23
PDGFR alpha	28.9	41
TRKA	35.7	51
FLT3	147	210
ABL	>100	>100
BLK	>100	>100
DDR2	>100	>100
EPHA5	>100	>100
FGR	>100	>100
FRK	>100	>100
FYN	>100	>100
HCK	>100	>100
LCK	>100	>100
LYN	>100	>100
MET	>100	>100
RON	>100	>100
SRC	>100	>100
TRKC	>100	>100

Supplemental Table 2: List of proteins in RPPA analysis. Proteins recognized by rabbit (R), murine (M), goat (G), or rat (T) antibodies are listed.	
14-3-3-beta (R)	JNK_pT183_Y185 (R)
14-3-3-epsilon (M)	JNK2 (R)
14-3-3-zeta (R)	LC3A-B (R)
4E-BP1 (R)	Lck (R)
4E-BP1_pS65 (R)	LDHA (R)
4E-BP1_pT37_T46 (R)	MAPK_pT202_Y204 (R)
53BP1 (R)	Mcl-1 (R)
A-Raf (R)	MCT4 (R)
ACC_pS79 (R)	MDM2_pS166 (R)
ACC1 (R)	MEK1 (R)
ACVRL1 (R)	MEK1_pS217_S221 (R)
ADAR1 (M)	MEK2 (R)
Akt (R)	Merlin (R)
Akt_pS473 (R)	MIF (R)
Akt_pT308 (R)	MIG6 (M)
AMPKa (R)	MTCO2 (M)
AMPKa_pT172 (R)	MMP2 (R)
Annexin-I (M)	MSH2 (M)
Annexin-VII (M)	MSH6 (R)
AR (R)	mTOR(R)
ARHI (M)	mTOR_pS2448 (R)
ARID1A (R)	Myosin-11 (R)
Atg3 (R)	Myosin-IIa_pS1943 (R)
Atg7 (R)	N-Cadherin (R)
ATM (R)	N-Ras (M)
ATM_pS1981 (R)	NAPSIN-A (R)
ATP5A (M)	NDRG1_pT346 (R)
Aurora-B (R)	NDUFB4 (M)
Axl (R)	NF-kB-p65_pS536 (R)
b-Actin (R)	Notch1 (R)
b-Catenin (R)	Notch3 (R)
b-Catenin_pT41_S45 (R)	P-Cadherin (R)
B-Raf (R)	p16INK4a (R)
B-Raf_pS445 (R)	p21 (R)
B7-H3 (R)	p27-Kip-1 (R)
B7-H4 (R)	p27_pT157 (R)

Bad_pS112 (R)	p27_pT198 (R)
Bak (R)	p38 (R)
BAP1 (M)	p38_pT180_Y182 (R)
Bax (R)	p53 (R)
Bcl-xL (R)	p70-S6K_pT389 (R)
Bcl2 (M)	p70-S6K1 (R)
Bcl2A1 (R)	p90RSK_pT573 (R)
Beclin (G)	PAI-1 (M)
Bid (R)	PAR (R)
Bim (R)	PARP1 (R)
BRD4 (R)	Paxillin (R)
c-Abl (R)	PCNA (M)
c-Jun_pS73 (R)	PD-L1 (R)
c-Kit (R)	Pdcd-1L1 (G)
c-Met (M)	Pdcd4 (R)
c-Met_pY1234_Y1235 (R)	PDGFR-b (R)
c-Myc (R)	PDGFR-b_pY579 (R)
C-Raf (R)	PDGFR-b_pY751 (R)
C-Raf_pS338 (R)	PDGFR-b_pY857 (R)
Caspase-3 (R)	PDGRF-b_pY1009 (R)
Caspase-7-cleaved (R)	PDGRF-b_pY1021 (R)
Caspase-8 (M)	PDGRF-b_pY716 (R)
Caveolin-1 (R)	PDGRF-b_pY740 (R)
CD171 (M)	PDGRF-b_pY771 (R)
CD31 (M)	PDK1 (R)
CD44 (M)	PDK1_pS241 (R)
CD49b (M)	PEA-15 (R)
CDK1 (R)	PEA-15_pS116 (R)
CD26 (R)	PI3K-p110-a (R)
CD29 (M)	PI3K-p110-b (M)
Chk1 (M)	PI3K-p85 (R)
Chk1_pS296 (R)	PKA-a (R)
Chk1_pS345 (R)	PKC-a (M)
Chk2 (M)	PKC-a_pS657 (R)
Chk2_pT68 (R)	PKC-b-II_pS660 (R)
Claudin-7 (R)	PKC-delta_pS664 (R)
COG3 (R)	PKM2 (R)
Collagen-VI (R)	PLC-gamma2_pY759 (R)
Complex-II-Subunit SDHB (M)	PLK1(R)

Connexin-43 (R)	PMS2 (R)
NR2F2 (R)	Porin (M)
Cox-IV (M)	PR (R)
Cox2 (R)	PRAS40 (M)
CXCR4 (R)	PRAS40_pT246 (R)
Cyclin-B1 (R)	PREX1 (R)
Cyclin-D1 (R)	PTEN (R)
Cyclin-E1 (M)	Puma (R)
Cyclophilin-F (M)	PYGM (M)
D-a-Tubulin (R)	Rab11 (R)
DJ1 (R)	Rab25 (R)
DM-Histone-H3 (R)	Rad50 (M)
DM-K9-Histone-H3 (R)	Rad51 (R)
DUSP4 (R)	Raptor (R)
Dvl3 (R)	Rb (M)
E-Cadherin (R)	Rb_pS807_S811 (R)
E2F1 (M)	RBM15(R)
eEF2 (R)	Rheb (M)
eEF2K (R)	Rictor (R)
EGFR (R)	Rictor_pT1135 (R)
EGFR_pY1068 (R)	Rock-1 (R)
EGFR_pY1173 (R)	RPA32 (T)
eIF4E (R)	RPA32_pS4_S8 (R)
eIF4G (R)	RSK (R)
Elk1_pS383 (R)	S6_pS235_S236 (R)
EMA (M)	S6_pS240_S244 (R)
ER (R)	SCD (M)
ERCC1 (M)	SDHA (R)
ERCC5 (R)	SF2 (M)
Ets-1 (R)	Shc_pY317 (R)
FAK (R)	SHP-2_pY542 (R)
FAK_pY397 (R)	SLC1A5 (R)
FASN (R)	Smac (M)
Fibronectin (R)	Smad1 (R)
FoxM1 (R)	Smad3 (R)
FoxO3a (R)	Smad4 (M)
FoxO3a_pS318_S321 (R)	Snail (M)
FRA-1 (R)	SOD2 (R)
G6PD (M)	Sox2 (R)

Gab2 (R)	Src (M)
GAPDH (M)	Src_pY416 (R)
GATA3 (M)	Src_pY527 (R)
GCN5L2 (R)	Stat3 (R)
Glutamate-D1-2 (R)	Stat3_pY705 (R)
Glutaminase (R)	Stat5a (R)
GPBB (R)	Stathmin-1 (R)
GSK-3a-b (M)	Syk (M)
GSK-3a-b_pS21_S9 (R)	Tau (M)
Gys (R)	TAZ (R)
Gys_pS641 (R)	TFAM (R)
H2AX_pS140 (M)	TFRC (R)
HER2 (M)	TIGAR (R)
HER2_pY1248 (R)	Transglutaminase (M)
HER3 (R)	TSC1 (R)
HER3_pY1289 (R)	Tuberin (R)
Heregulin (R)	Tuberin_pT1462 (R)
HES1 (R)	TWIST (M)
Hexokinase-II (R)	Tyro3 (R)
HIAP (R)	UBAC1 (R)
Hif-1-alpha (M)	Ubq-Histone-H2B (M)
Histone-H3 (R)	UGT1A (M)
HSP27 (M)	VEGFR-2 (R)
HSP27_pS82 (R)	VHL (M)
HSP70 (R)	Vimentin (M)
IGF1R_pY1135_Y1136 (R)	XBP1 (G)
IGFBP2 (R)	XIAP (R)
IGFBP5 (G)	XPA (M)
IGFRb (R)	XPF (M)
INPP4b (R)	XRCC1 (R)
IRF-1 (R)	YAP (R)
IRS1 (R)	YAP_pS127 (R)
JAB1 (M)	YB1 (R)
Jagged1 (R)	YB1_pS102 (R)
Jak2 (R)	

Supplemental Table 3: Comparison of Survival Curves for In Vivo Experiment

0.004% ONO-7475 versus control feed
0.004% ONO-7475 versus control feed
Log-rank (Mantel-Cox) test
Chi square 8.000
p value 0.0047 (**)

Gehan-Breslow-Wilcoxon test
Chi square 7.714
p value 0.0055 (**)

Median survival
Control Diet 18.00
ONO Diet (Treated) 24.00
Ratio (and its reciprocal) 0.7500 1.333
95% CI of ratio 0.2171 to 2.591 0.3860 to 4.606

Hazard Ratio (Mantel-Haenszel)	Control/0.004% ONO	0.004% ONO/Control
Ratio (and its reciprocal)	20.09	0.04979
95% CI of ratio	2.512 to 160.6	0.006227 to 0.3981

Hazard Ratio (logrank)	Control/0.004% ONO	0.004% ONO/Control
Ratio (and its reciprocal)	3.286	0.3043
95% CI of ratio	4.639 to 86.96	0.01150 to 0.2155

0.013% ONO-7475 versus control feed
Log-rank (Mantel-Cox) test
Chi square 7.484
p value 0.0062 (**)

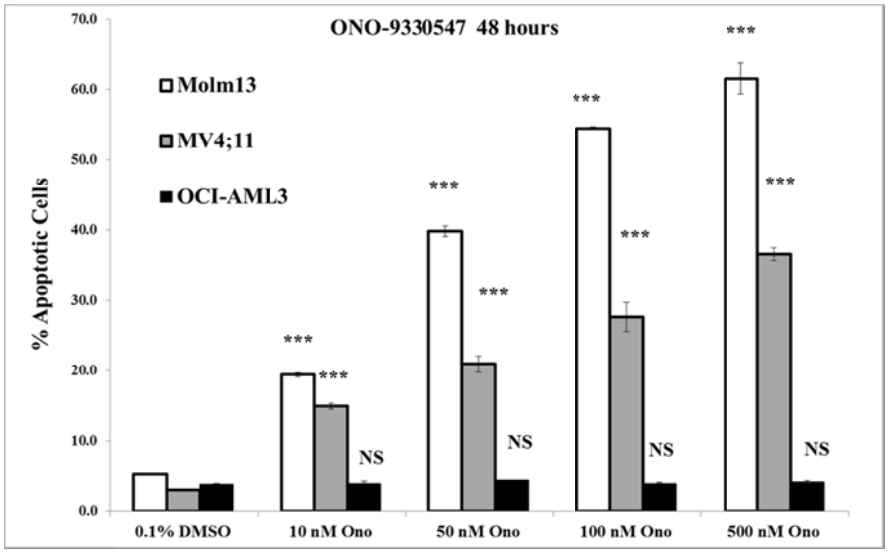
Gehan-Breslow-Wilcoxon test
Chi square 5.743
P value 0.0166 (*)

Median survival
Control Diet 26.50 days
0.013% ONO Diet (Treated) 36.00 days

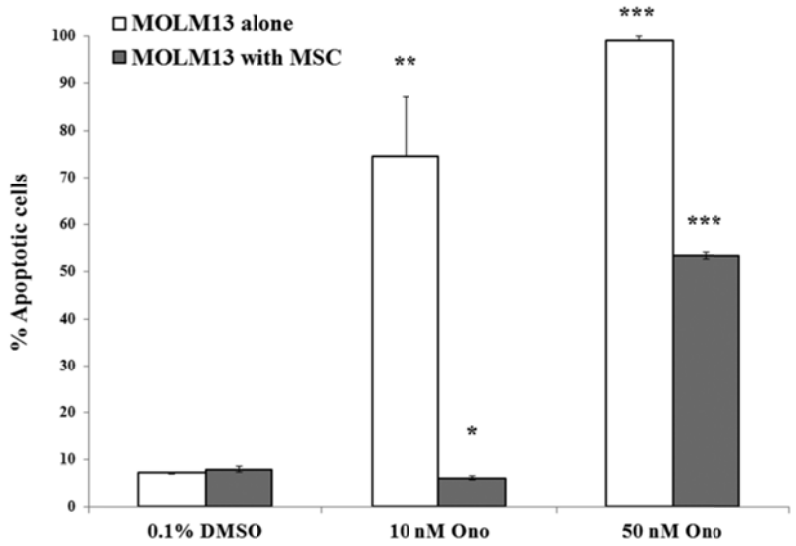
Hazard Ratio (Mantel-Haenszel)	Control/0.013% ONO	0.013% ONO/Control
Ratio (and its reciprocal)	5.530	0.1808
95% CI of ratio	1.624 to 18.83	0.05311 to 0.6158

Hazard Ratio (logrank)	Control/0.013% ONO	0.013% ONO/Control
Ratio (and its reciprocal)	3.011	0.3321
95% CI of ratio	1.900 to 16.09	0.06213 to 0.5263

Supplemental Figure 1A

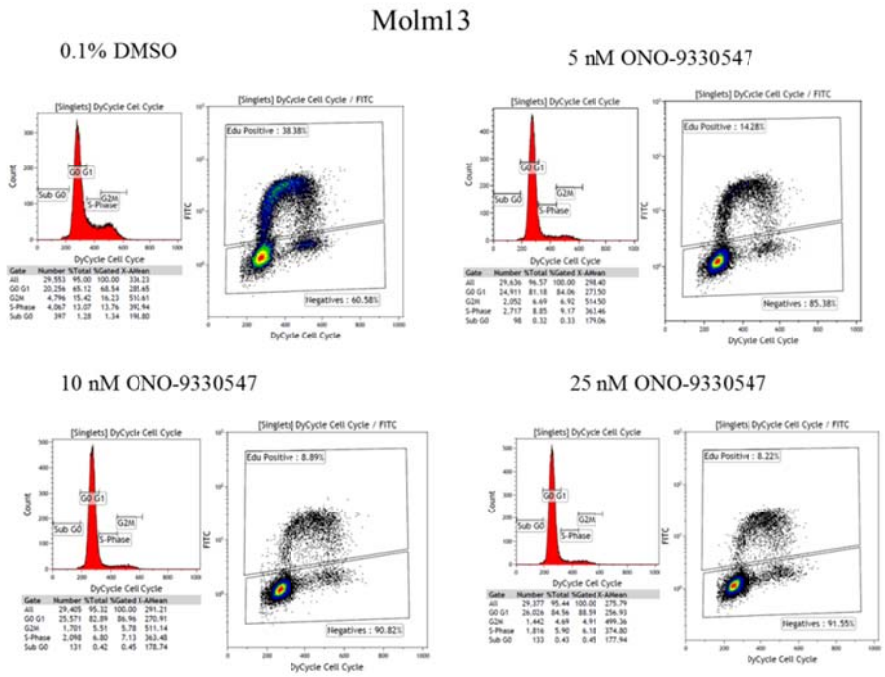


Supplemental Figure 1B

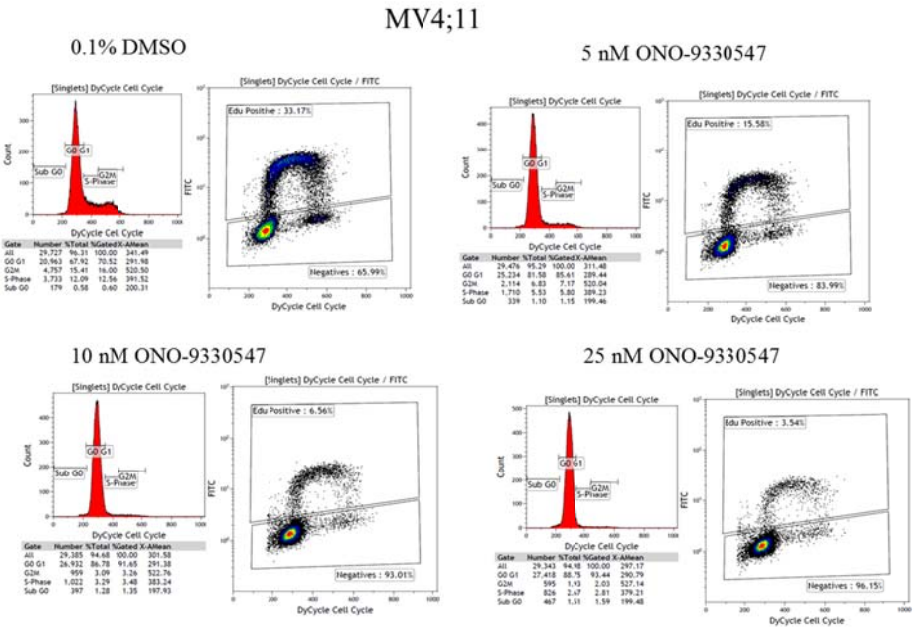


Supplemental Figure 1. ONO-7475 effectively induces apoptosis in FLT3-ITD AML cell lines even in the presence of MSC. (A) MOLM13, MV4;11, and OCI-AML3 cells were incubated with varying doses of ONO-7475 and cell viability assessed by flow cytometry. Apoptotic cells were determined using Annexin V. (B) MOLM13 cells were co-cultured with MSC as described in Methods. Cells were treated with varying doses of ONO-7475 and apoptosis assessed by flow cytometry using CD90 antibody and Annexin V. Statistical significance was determined by Student t test against vehicle treated (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Supplemental Figure 2A

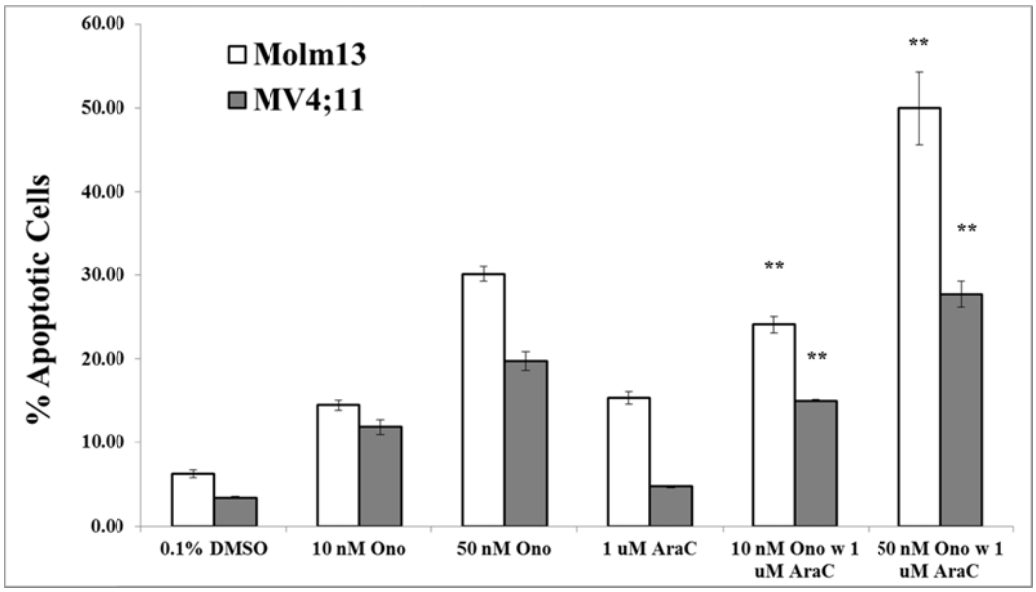


Supplemental Figure 2B

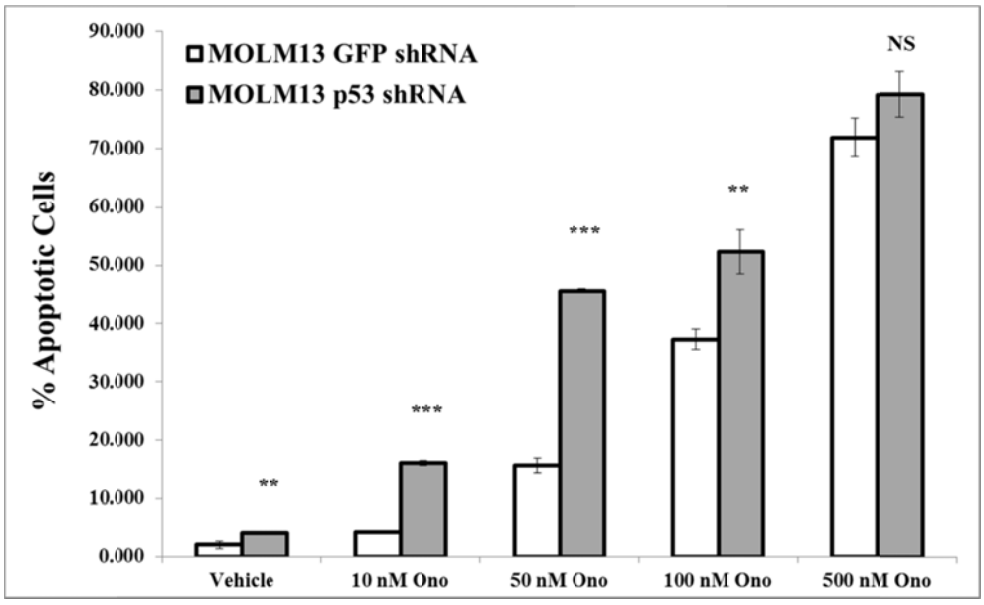


Supplemental Figure 2. ONO-7475 suppresses cell proliferation by blocking cell cycle and DNA synthesis. EdU Click-It assay and FX Violet Cycle staining was performed as described in Methods on MOLM13 cells (A) and MV4;11 cells (B).

Supplemental Figure 3A

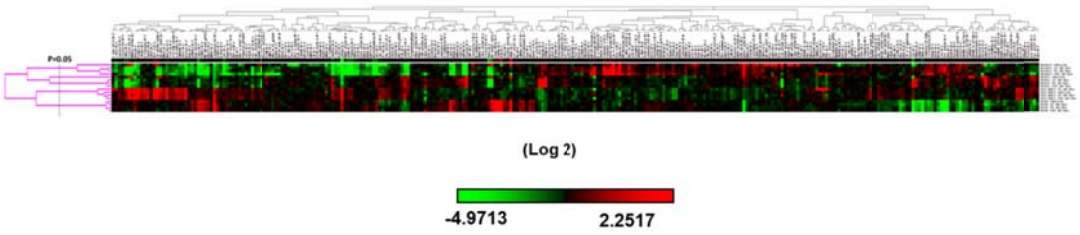


Supplemental Figure 3B

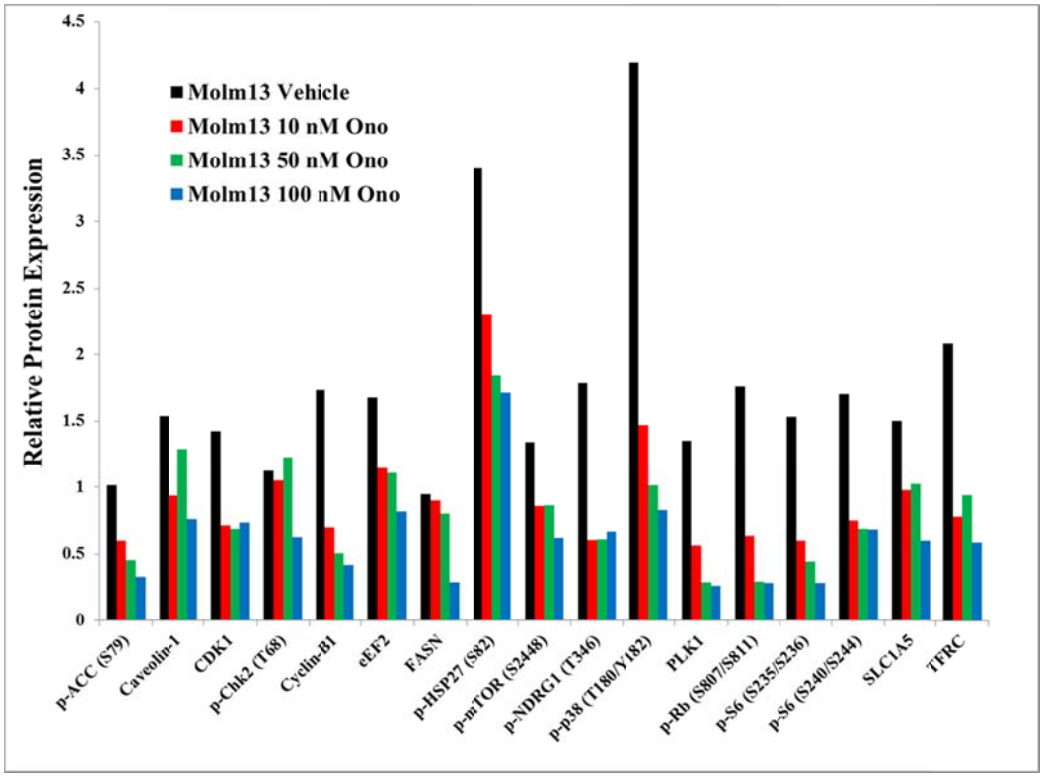


Supplemental Figure 3. ONO-7475 augments AraC-induced killing in FLT3-ITD AML cell lines though suppression of p53 sensitizes cells to the drug. (A) MOLM13 and MV4;11 were treated with varying doses of ONO-7475 and/or 1 μM AraC and then apoptosis was determined using Annexin V. (B) MOLM13 cells expressing control LKO vector or p53 shRNA were treated with varying doses of ONO-7475 and then apoptosis was determined using Annexin V. Statistical significance was determined by Student t test against AraC treated (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$) for (A). Statistical significance was determined by Student t test against GFP control (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$) for (B).

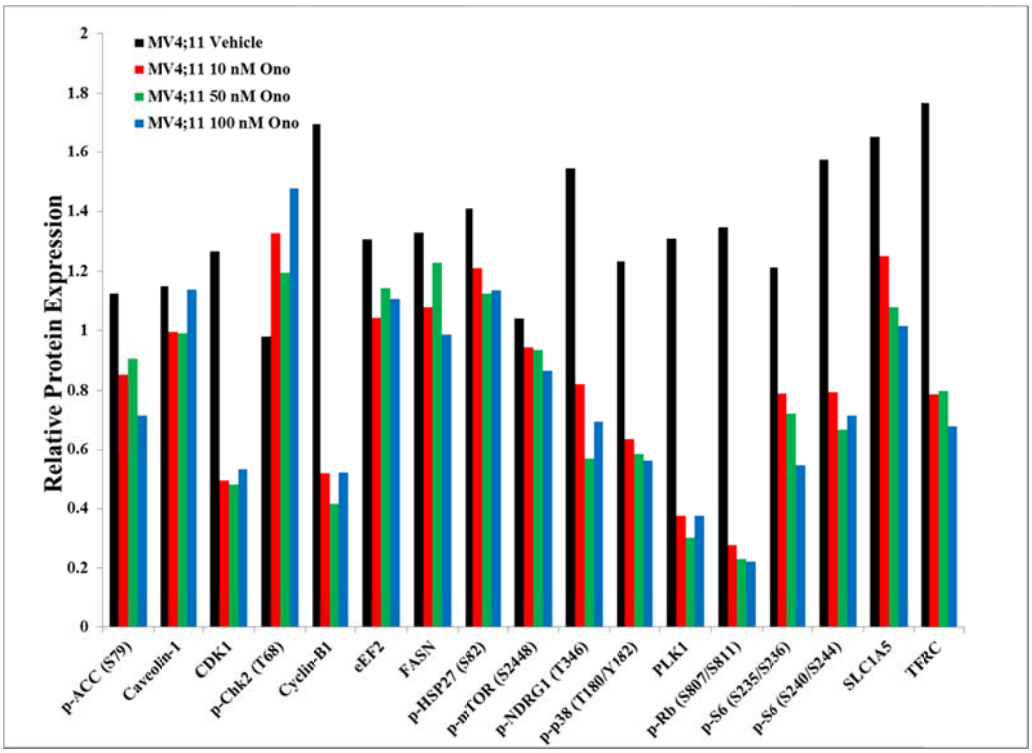
Supplemental Figure 4A



Supplemental Figure 4B

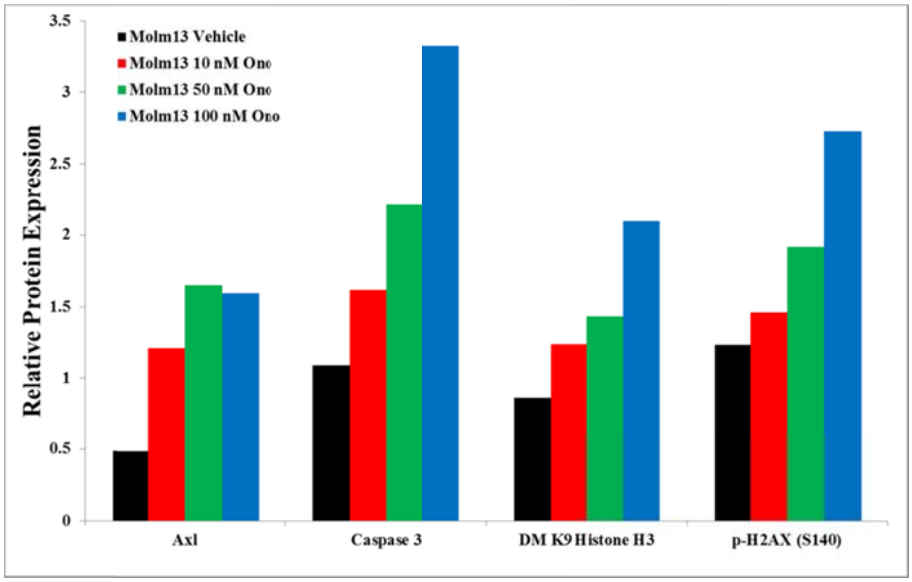


Supplemental Figure 4C

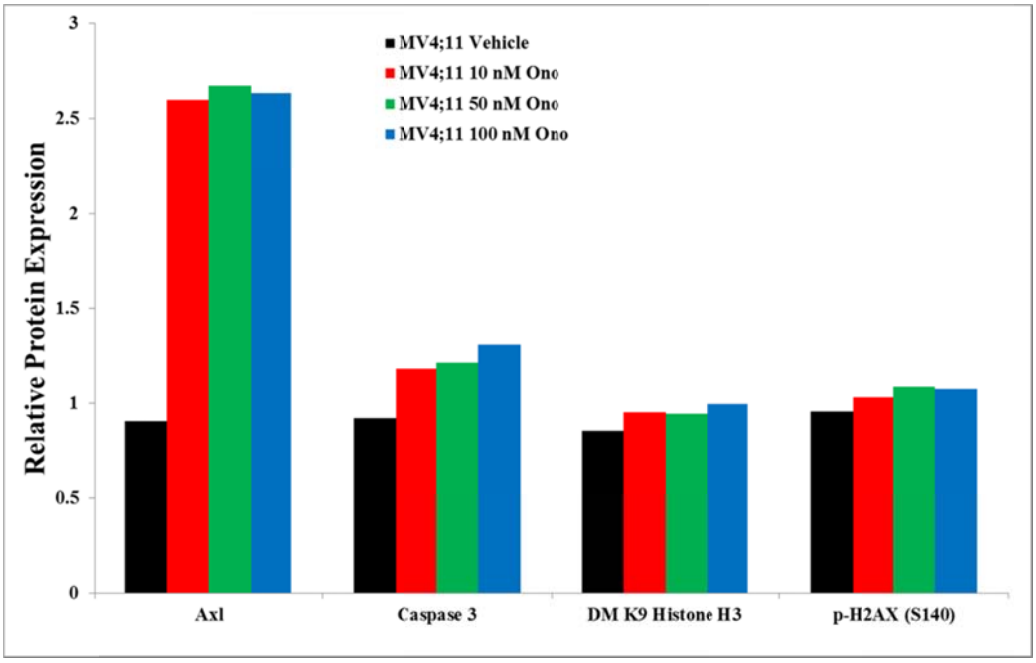


Supplemental Figure 4. RPPA reveals ONO-7475 suppresses a distinct set of proteins in MOLM13 and MV4;11 cells but not in OCI-AML3 and HL60 cells. MOLM13, MV4;11, OCI-AML3, and HL60 cells were treated with 10 nM, 50 nM, or 100 nM ONO-7475 for 24 hours, protein collected, and RPPA performed as described in Methods. (A) Heat map of changes in protein expression in all 4 cell lines. (B) Bar graph representing proteins reduced by at least 2 fold in MOLM13 cells. (C) Bar graph representing expression proteins in MV4;11 cells that were reduced by at least 2 fold in MOLM13 cells.

Supplemental Figure 5A

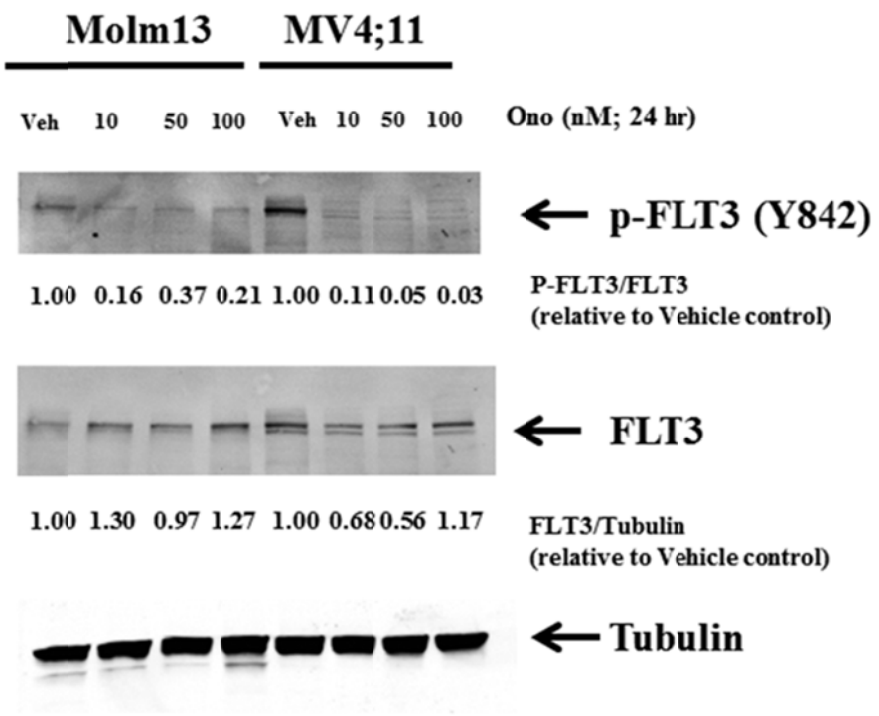


Supplemental Figure 5B



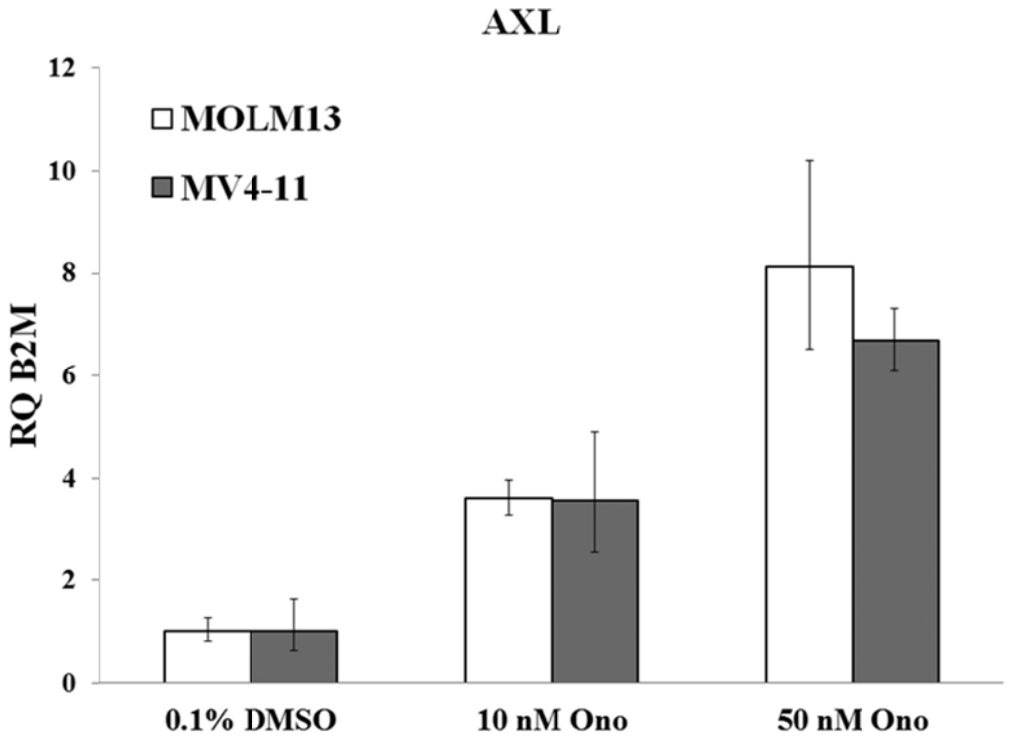
Supplemental Figure 5. RPPA reveals ONO-7475 induces AXL in MOLM13 and MV4;11 cells. MOLM13 and MV4;11 cells were treated with 10 nM, 50 nM, or 100 nM ONO-7475 for 24 hours, protein collected, and RPPA performed as described in Methods. (A) Bar graph representing proteins increased by at least 2 fold in MOLM13 cells. (B) Bar graph representing proteins increased by at least 2 fold in MV4;11 cells.

Supplemental Figure 6



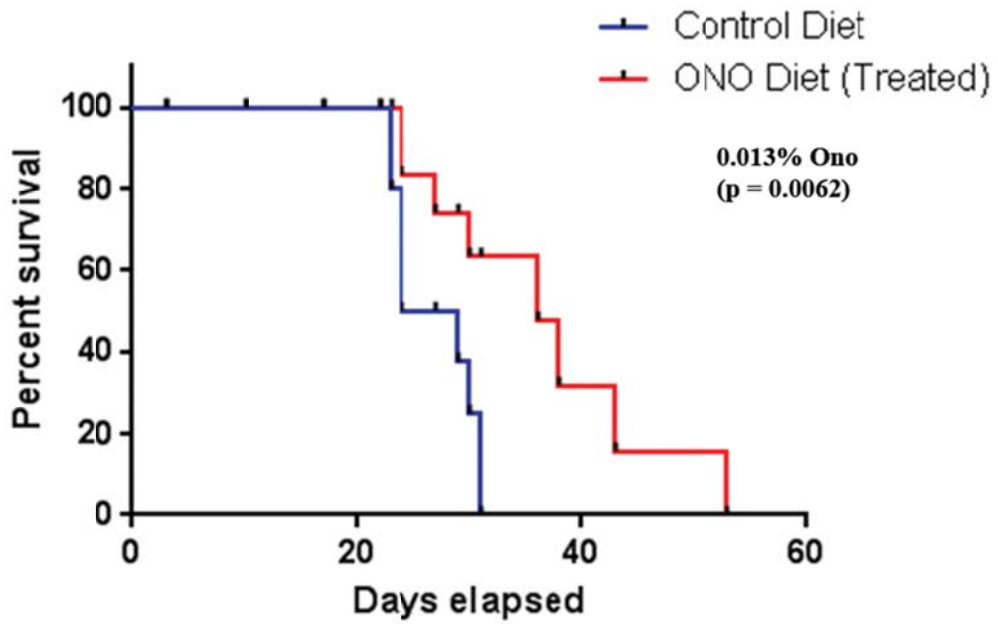
Supplemental Figure 6. ONO-7475 suppresses FLT3 phosphorylation. MOLM13 and MV4;11 were treated with varying doses of ONO-7475 for 24 hours. Immunoblot analysis was performed as described in Methods. Antibodies used were against p-FLT3 and FLT3. Antibody against Tubulin was used as a loading control. Ratio of protein expression to Tubulin loading control or to total protein for p-FLT3 was determined by densitometry using LiCor imager software.

Supplemental Figure 7



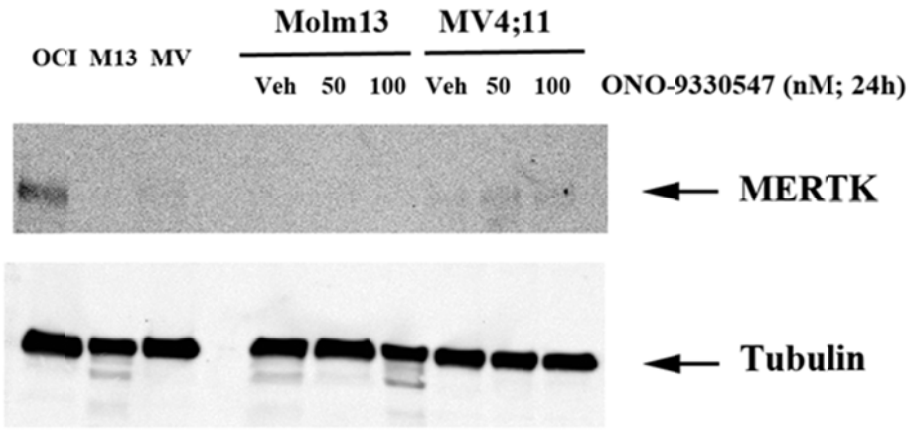
Supplemental Figure 7. ONO-7475 induces *AXL* in MOLM13 and MV4;11 cells. MOLM13 and MV4;11 were treated with varying doses of ONO-7475 for 24 hours. RNA from cells was extracted and reverse transcribed, and the abundance of transcript for AXL and B2M were determined by qRT-PCR. Gene expression levels of AXL were normalized to B2M as described in Methods.

Supplemental Figure 8



Supplemental Figure 8. 0.013% ONO-7475 diet promotes survival on mice with human AML FLT3 ITD cells. MOLM13 luc/gfp cells were introduced into NSG mice and mice were fed either control feed or feed containing 0.013% ONO-7475. Survival analysis of mice fed 0.013% drug was performed using GraphPad software. Statistical data is presented in Supplemental Table 2.

Supplemental Figure 9



Supplemental Figure 9. OCI-AML3 cells express MERTK while MOLM13 cells do not express MERTK even when treated with ONO-7475. Protein was collected from OCI-AML3 cells (OCI), MOLM13 cells (M13) and MV4;11 cells (MV). Protein was also collected from MOLM13 and MV4;11 treated with varying doses of ONO-7475 for 24 hours. Immunoblot analysis was performed as described in Methods. Antibodies used were against MERTK and Tubulin.