Supplemental Appendix

Activity of eftozanermin alfa plus venetoclax in preclinical models and patients with acute myeloid leukemia

Authors: Stephen K. Tahir[1], Emiliano Calvo[2], Benedito A. Carneiro[3], Junichiro Yuda[4], Aditya Shreenivas[5], Mojca Jongen-Lavrencic[6], Eelke Gort[7], Kenichi Ishizawa[8], Daniel Morillo[9], Carla Biesdorf[10], Morey Smith[1], Dong Cheng[1], Monica Motwani[11], David Sharon[11], Tamar Uziel[11], Dimple A. Modi[11], Greg Buchanan[1], Susan Morgan-Lappe[1], Bruno C. Medeiros[12], and Darren C. Phillips[1]

Affiliations: [1]Oncology Discovery, AbbVie Inc., North Chicago, IL, USA; [2]START Madrid-CIOCC, Centro Integral Oncológico Clara Campal, Madrid, Spain; [3]Legorreta Cancer Center at Brown University, Lifespan Cancer Institute, Providence, RI, USA; [4]Department of Hematology and Oncology, National Cancer Center Hospital East, Kashiwa, Japan; [5]Department of Medical Oncology, Medical College of Wisconsin, Wauwatosa, WI, USA; [6]Department of Hematology, Erasmus MC Cancer Institute, Rotterdam, The Netherlands; [7]Department of Medical Oncology, University Medical Center Utrecht, Utrecht, The Netherlands; [8]Department of Internal Medicine III, Division of Hematology and Cell Therapy, Yamagata University Hospital, Yamagata, Japan; [9]START Madrid-FJD, Hospital Fundación Jiménez Díaz, Madrid, Spain; [10]Clinical Pharmacology and Pharmacometrics, AbbVie Inc., North Chicago, IL, USA; [11]Precision Medicine, AbbVie Inc., North Chicago, IL, USA; [12]Oncology Early Development, AbbVie Inc., North Chicago, IL, USA.

Short Title: Active combination of eftoza + venetoclax in AML

Corresponding Author:
Darren. C. Phillips
Oncology Discovery, AbbVie Inc
1 North Waukegan Road
AP9-2, Room 02L8B
North Chicago, IL 60064
Office: 847-938-8508
Email: darren.phillips@abbvie.com

Supplemental materials and methods

Generation of engineered cells

Stable overexpression of human death receptor (DR)4 and DR5 in human erythroleukemia (HEL) cells was generated by lentiviral transduction using the lentiviral expression vector pLVX-IRES-PURO containing either full-length human DR4 or DR5 complementary DNA, respectively. Briefly, 8 mg/mL polybrene was added to HEL cells (0.2×10^6 cells) in 3 mL complete culture medium containing lentivirus. HEL cells expressing DR4 or DR5 were selected using 1 mg/mL puromycin (Gibco, Waltham, MA, USA), and receptor numbers on the cell surface were determined by quantitative flow cytometry as described below.

CRISPR-Cas9 editing to generate *BAK1/BAX*-deficient OCI-AML5 cells, *TP53*-deficient MV-4-11 cells, and *TP53*^{R248W}-overexpressing cells was performed using the IDT Alt-R™ CRISPR-Cas9 system and electroporation using the Neon™ transfection system (Thermo Fisher, Waltham, MA, USA). Briefly, the CRISPR RNAs were annealed to tracer RNA prior to addition of the Alt-R S.p. Cas9 Nuclease and Alt-R electroporation enhancer DNA according to manufacturer's specifications. For *BAK1/BAX*-deficient OCI-AML5 cells, the CRISPR RNAs (crRNAs) targeting *BAK1* (GCTCACCTGCTAGGTTGCAG) and *BAX* (TCGGAAAAAGACCTCTCGGG) were combined prior to annealing. OCI-AML5 cells were electroporated at 1 × 30-ms pulse at 1500 V. Cells were single-cell sorted, expanded, and screened for expression of BAK and BAX protein. Clones lacking protein expression were chosen and genome disruption was confirmed by Sanger sequencing.

TP53-deficient MV-4-11 cells were generated using crRNA XT targeting *TP53* (mA*mC*mCrArUrUrGrUrUrCrArArUrArUrCrGrUrCrC/CrRNA-XT/). MV-4-11 cells were electroporated at 1 × 20-ms pulse at 1700 V. Cells were cultured for a week prior to single-cell isolation through flow cytometry (BD FACSAria™ Fusion). Loss of *TP53* expression was determined by western blot analysis.

TP53^{R248W} knock-in MV-4-11 cells were generated using crRNA (AlTR1/rGrCrArUrGrGrGrCrGrGrCrArUrGrArArCrCrGrGrGrUrUrUrUrArGrArGrCrUrArUrGrCrU/AlTR2) and the donor DNA (GACTGTACCACCATCCACTACAACTACATGTGTAACAGTTCCTGCATGGGCGGCATGAACT GGAGACCCATCCTCACCATCATCACACTGGAAGACTCCAGGTCAGGAGCCACTTGCCACCCTGCA). MV-4-11 cells were electroporated at 1 × 20 ms at 1700 V. Electroporated cells were resuspended in culture medium containing Alt-R HDR (homology-directed repair) enhancer. Medium was replaced the following day and cells were further cultured for a week prior to single-cell isolation through flow cytometry (BD

FACSAria Fusion). Insertion of mutation was verified through sequencing (GENEWIZ, South Plainfield, NJ, USA).

Assessment of single or combination therapy on AML cell lines

Cell viability and caspase-3/7 activation determination

For the in vitro assays evaluating eftozanermin alfa (eftoza) alone or in combination with venetoclax, cells were treated in either 96- or 384-well plates at 10,000 or 2,000 cells/well, respectively, for 24 hours and cell viability was determined using CellTiter-Glo® (Promega, Madison, WI, USA) as described by the manufacturer's instructions. Caspase activation was determined using the Caspase-Glo® 3/7 luminescent assay as described by the manufacturer's instructions (Promega). Responses were determined as a percentage of the control treated cells and half maximal effective concentration (EC_{50}) values were determined from sigmoidal dose-response curves using GraphPad Prism (GraphPad Software, La Jolla, CA USA). For combination studies, the Bliss independence model was used to evaluate synergy in vitro.[1] The sum of the Bliss scores for each dose-combination was calculated to generate a "Bliss sum" where values >0 were considered to indicate synergy.

Kinetic live-cell imaging of caspase-3/7 activation

Cells were seeded at 35,000 cells/well in clear bottom, 96-well plates (Corning Inc., Corning, NY, USA) in medium containing the caspase-3/7 green reagent (Sartorius, Göttingen, Germany) diluted 1:1000 prior to treatment. Live-cell quantification of caspase-3/7 activation was measured by monitoring the cells every hour using an IncuCyte ZOOM® (Sartorius). Data were analyzed using the IncuCyte S3 2019 software (Sartorius) and plotted as a percentage of high caspase-positive intensity object counts (activated caspase-3/7) divided by the total object count in each well.

Quantitative flow cytometry methodology

The number of DR4 and DR5 receptors on the plasma membrane was determined using quantitative flow cytometry, as previously described.[2] Briefly, cells were stained either with anti-DR4 mouse immunoglobulin IgG1 (eBioscience, San Diego, CA, USA), anti-DR5 mouse IgG1 (eBioscience), or mouse IgG1 isotype control (eBioscience) conjugated to R-phycoerythrin (R-PE) and the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) receptor number was quantified by gating on single (forward scatter [FSC]-H vs FSC-A), live (DRAQ7™ negative; Cell Signaling Technology, Danvers, MA, USA) cells and using Quantum™ Simply Cellular ABC beads (Bangs Laboratories, Fishers, IN, USA) to generate a

standard curve as per the manufacturer's instructions. The isotype control results were subtracted from the resulting TRAIL receptor number, weighted by the percentage-positive population of cells, and the number of receptors was determined using Bangs Laboratories QuickCal® spreadsheet. The quantitation of B-cell lymphoma-2 (BCL-2) family members was determined by quantitative flow cytometry as previously described.[3]

Phase 1 trial

Treatment

Patients treated with eftoza monotherapy received doses of 1.25, 3.75, or 7.5 mg/kg by IV infusion and 3.75 or 7.5 mg/kg in combination with venetoclax on days 1, 8, and 15 of a 21-day cycle. The 7.5-mg/kg IV weekly dose of eftoza was selected as the highest administrated dose on the basis of the solid tumor optimization data.[4] Patients in the combination cohorts received 400 mg oral venetoclax daily (dose could be escalated to 600 mg) administered via a ramp-up schedule starting on cycle 1 day 2, with a 50-mg dose and increasing to 100, 200, and 400 mg on subsequent days.

Assessments

Adverse events (AEs) were recorded throughout the study and rated for severity by the investigator according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 4.03. AE relationship to study treatment was based on investigator assessment. All patients who received at least 1 dose of study drug were included in safety summaries.

Blood samples for eftoza monotherapy pharmacokinetics (PK) analysis were collected in cycles 1 and 3 on days 1 (predose and 0.25, 2, 4, and 8 hours postdose), 2, 3, 8, and 15; for cycle 2, on days 1 (predose and 0.25 hours postdose), 2, and 8. For the combination cohorts, PK samples were collected in cycles 1 and 3, on days 1 (predose and 0.25, 2, 4, and 6.5 hours postdose), 2, 3, and 8; for cycle 2, on days 1 (predose and 0.25 hours postdose) and 8. PK parameters, including maximum observed plasma concentration, the time to maximum observed plasma concentration (peak time), and area under the plasma concentration-time curve (AUC) were calculated using noncompartmental methods. Eftoza serum concentrations were determined using a validated electrochemiluminescence immunoassay. Plasma concentrations of venetoclax were determined using liquid-liquid extraction followed by liquid chromatography with tandem mass spectrometric detection.[5]

Tumor assessments for all evaluable dosed patients were performed on bone marrow aspirates and/or biopsies at baseline, on cycle 2 day 9, then every 9 weeks thereafter. Patients considered not evaluable for efficacy discontinued prior to first postbaseline disease assessment. Circulating blast counts were collected at each clinical time point and on days when bone marrow aspirates occurred. Tumor responses were assessed per modified International Working Group criteria.[6]

DR4/5 expression on leukemic cells, and immunophenotyping of peripheral blood and bone marrow aspirates by flow cytometry was instituted. DR4 and DR5 plasma membrane receptor numbers were determined by quantitative flow cytometry. TRAIL receptors on myeloblasts were identified using the TRAIL R1 PE (Clone: 69036 – R&D Systems, Minneapolis, MN, USA; catalog number FAB347P) and TRAIL R2 PE (Clone: 71908 – R&D Systems; catalog number FAB6311P) antibodies. Briefly, a white blood cell count was taken for each specimen and the cell count was adjusted to no more than 20×10^6 cells/mL with phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA). One hundred µL of sodium heparin anticoagulated whole blood or bone marrow was pipetted into the test tubes. Block solutions (Normal Mouse Serum and Fc Block) was added to all tubes. The tubes were incubated for 10 minutes at room temperature. After incubation, the appropriate antibody combinations were added to each tube. All test tubes were gently vortexed, and then incubated at room temperature in the dark for 30 minutes. The cells were washed with 2 mL of PBS-1% BSA. The red blood cells were lysed with 4 mL of warm (32–42°C) whole blood lysing solution. Test tubes were capped, inverted several times to mix, and allowed to incubate for 5 minutes at room temperature (18–22°C) in the dark. The test tubes underwent centrifugation at 400 relative centrifugal force for 5 minutes, and then the supernatant was decanted. All tubes were washed once with 2 mL PBS-1% BSA. The cell pellets were resuspended in 500 µL of PBS-1% BSA. The samples were acquired with appropriate settings on the BD SORP FACSCanto™ II until approximately 250,000 events were collected, or the maximum acquisition time of 240 seconds ended. After acquisition, the listmode files were analyzed offline using WinList™ (Verity Software House, Topsham, ME, USA) 7.0 to generate the reportables.

References

1. Borisy AA, Elliott PJ, Hurst NW, et al. Systematic discovery of multicomponent therapeutics. *Proc Natl Acad Sci U S A*. 2003;100(13):7977-7982.
2. Phillips DC, Buchanan FG, Cheng D, et al. Hexavalent TRAIL fusion protein eftozanermin alfa optimally clusters apoptosis-inducing TRAIL receptors to induce on-target antitumor activity in solid tumors. *Cancer Res*. 2021;81(12):3402-3414.
3. Smith ML, Tahir SK. Quantification of BCL-2 family members by flow cytometry. *Methods Mol Biol*. 2019;1877:163-172.
4. LoRusso P, Ratain MJ, Doi T, et al. Eftozanermin alfa (ABBV-621) monotherapy in patients with previously treated solid tumors: findings of a phase 1, first-in-human study [published online ahead of print 25 April 2022]. *Invest New Drugs*. doi:10.1007/s10637-022-01247-1.
5. Salem AH, Badawi MA, Place AE, et al. Venetoclax crosses the blood brain barrier: a pharmacokinetic analysis of the cerebrospinal fluid in pediatric leukemia patients. *Blood.*2020;136(suppl 1):30-31.
6. Cheson BD, Bennett JM, Kopecky KJ, et al. Revised recommendations of the International Working Group for Diagnosis, Standardization of Response Criteria, Treatment Outcomes, and Reporting Standards for Therapeutic Trials in Acute Myeloid Leukemia. *J Clin Oncol.* 2003;21(24):4642-4649.

Supplemental Tables

Supplemental Table 1. Commercial source of human AML cell lines	
Cell line name	**Source**
EOL-1	DSMZ*
HEL	DSMZ
HL-60	ATCC[ŧ]
Kasumi-1	ATCC
KG-1	ATCC
ML-2	DSMZ
MOLM-13	DSMZ
MOLM-14	DSMZ
MV-4-11	ATCC
NOMO-1	DSMZ
OCI-AML2	DSMZ
OCI-AML3	DSMZ
OCI-AML5	DSMZ
P31FUJ	JCRB[‡]
PL-21	DSMZ
SET-2	DSMZ
SHI-1	DSMZ
SKM-1	DSMZ
SKNO-1	DSMZ
TF-1	ATCC
THP-1	DSMZ
U-937	ATCC
UKE-1	Coriell Institute[§]

*DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkultureen, Braunschweig, Germany; [ŧ]ATCC, American Type Culture Collection, Manassas, VA, USA; [‡]JCRB, Japanese Collection of Research Bioresources Cell Bank, Osaka, Japan; [§]Coriell Institute, Camden, NJ, USA.

Supplemental Table 2. Key eligibility criteria

Inclusion

- ≥18 years of age
- AML diagnosis with histologically confirmed relapsed or refractory disease
- Eastern Cooperative Oncology Group performance score 0–2
- Received at least 1 prior systemic treatment and relapsed, progressed, or had no response to all available effective therapies
- Persistent disease after allogeneic SCT, ineligible for allogeneic SCT, or had declined this option
- Consent to provide pre- and on-treatment bone marrow aspirates for biomarker analyses
- Adequate renal and hepatic function
  - Renal function (at least 1 of the following): estimated CrCl ≥50 mL/min (Cockcroft-Gault), estimated glomerular filtration rate ≥50 mL/min/1.73 m^2 (MDRD or CKD-EPI formula), or actual CrCl as determined by 24-hour urine collection
  - Hepatic function: serum AST or ALT ≤1.5× ULN; serum AST or ALT ≤2.5× ULN in patients with known hepatic involvement of underlying malignancy; total bilirubin ≤1.5× ULN (unless bilirubin rise is due to Gilbert's syndrome or of nonhepatic origin)
- White blood cell count <25 × 10^9/L before study treatment
  - Use of hydroxyurea was permitted to achieve threshold, and could continue until completion of the first cycle of study therapy

Exclusion

- Hematologic malignancies other than AML
- Presence of primary hepatobiliary malignancy
- Major surgery <4 weeks from first study treatment
- Receipt of systemic anticancer agent ≤21 days or 3 half-lives prior to first study drug treatment
- Prior receipt of TRAIL or any TRAIL-like agonist; prior treatment with a BCL-2 inhibitor (in venetoclax combination cohorts)
- History of brain metastases without clinical/radiographic stable disease for at least 28 days, or any active central nervous system disease
- Significant uncontrolled clinical conditions

CKD-EPI, chronic kidney disease epidemiology collaboration equation; CrCl, creatinine clearance; MDRD, modification of diet in renal disease; SCT, stem cell transplant; ULN, upper limit of normal.

Supplemental Table 3. EC_{50} values, total DR4/DR5 plasma membrane levels, Bliss sum, mutations, and genetic alterations in AML cell lines used in this study

Cell line	Eftoza EC_{50} mean, nM	Apo2L EC_{50} mean (nM)	Plasma membrane receptor number (DR4 + DR5)	Eftoza-VEN Bliss sum, mean	*TP53*	P53 effect	P53 basal expression	Mutations by next-generation sequencing	*FLT3-ITD*	MLL rearrangements
SKM-1	0.004	1.9	12812	82	p. R248Q	MUT – DNA binding	Yes	*CSF3R P760T, TET2 P1440fs, FBXW7 E489D, EZH2 Y646C, TP53 R248Q, ASXL1 Y591x, STAG2 R259x*	Negative	None
ML-2	0.151	-	4192	261	WT (COSMIC)	WT (MUT clone published)	–	*NOTCH1 P2514fs*	Negative	t(6;11)(q27;q23) MLL-AF6
PL-21	0.183	0.6	7394	369	P36fs; c.107del1	MUT (hemizygous)	No	*EZH2 D730x, EZH2 R566S, TP53 P36fs, ASXL1 I597fs*	Positive	–
MOLM-14	0.304	-	5328	844	WT	–	–	–	–	–
EOL-1	0.321	-	9248	203	WT	WT	–	None	Negative	Dup(11)(q23q23) MLL PTD
U-937	0.326	-	6538	77	Splice	MUT	–	*PTPN11 G60R*	–	t(10;11)(p12;q14) PICALM/MLLT10 (AF10)
OCI-AML3	0.496	>10	6035	558	WT	WT	–	*DNMT3A R882C, NPM1 W288fs* (only known cell line), low coverage *NRAS Q61L*	Negative	None

OCI-AML2	0.799	3.1	4963	479	WT	WT	–	*DNMT3A R635W*	Negative	Possible cryptic MLL rearrangement
MOLM-13	1.241	3.2	6206	257	WT	WT	Low	None	Positive	t(9;11)(p22;q23) MLL-AF9
UKE-1	1.248	>10	1846	170	WT	WT	–	*JAK2 V617F, ETV6 K99fs, PTPN11 F71L*	Negative	–
HL-60	1.286	-	8097	-123	Null	(deletion)	–	Low coverage for *NRAS Q61L* and *CDKN2A R80X*; *TP53* no coverage (deletion)	Negative	None
NOMO-1	1.524	-	4172	515	C242fs	MUT	–	*KRAS G13D, TP53 C242fs, ASXL1 R693x*	Negative	t(9;11)(p22;q23) MLL-AF9
OCI-AML5	2.234	> 10	5832	747	WT	WT	Yes	*TET2 S846x, TET2 Y1169C, EZH2 R690H, RUNX1 T148fs, ASXL1 Y591x*	Negative	None
Kasumi-1	2.745	>10	3945	402	R248Q	MUT – DNA binding	Yes	*KIT N822K, RAD21 K330fs, TP53 R248Q*	Negative	None
SET-2	3.304	>10	3021	-120	R248W, G187 splice	MUT – DNA binding (LOF)	Yes	*DNMT3A R882H, JAK2 V617F, TP53 R248W, TP53 G187* splice	Negative	–
MV-4-11	4.738	> 10	4838	347	WT	WT	Yes	None	Positive	t(4;11)(q21;q23) /MLL-AF4
HEL	>10	>10	1706	-93	M133K	MUT – DNA binding	–	*JAK2 V617F, TP53 M133K*	Negative	Gain of MLL(11q23)

KG-1	>10	-	1039	86	E224* splice	MUT - Truncated	–	*TP53 E224* splice	Negative	None
P31/FUJ	>10	-	471	-146	p. Y236C, R196x	MUT	–	*NRAS G12C, EZH2 A736fs, PTEN R233fs, ETV6 R399C, TP53 Y236C, R196x, RUNX1 G69R*	Negative	–
SHI-1	>10	>10	320	-155	p. I195T	MUT – DNA binding	–	*KRAS Q61H, PTPN11 F71L, TP53 I195T, ZRSR2 C280W*, and *A183G*	Negative	KMT2A-AFDN (MLL-MLLT4; MLL-AF6)
SKNO-1	>10	>10	186	-78	p. R248Q	MUT – DNA binding	–	*KIT N822K, TP53 R248Q, CDKN2A P203L*	Negative	–
TF-1	>10	>10	291	-223	I251fs, splice	MUT – Frameshift	–	*TP53 I251fs, TP53* splice, *NRAS Q61P* (low coverage)	Negative	–
THP-1	>10	>10	362	-2	c.520_545del26	MUT – p.R174fs*3	–	*NRAS G12D*, no coverage for *TP53* mutation from CCLE	Negative	None

CCLE, Cancer Cell Line Encyclopedia; MLL, mixed-lineage leukemia; MUT, mutant, VEN, venetoclax; WT, wild-type.

Supplemental Table 4. BCL-2 is highly expressed as compared with BCL-X_L and MCL-1 in AML cell lines

Cell line	MESF*								
	BCL-2			BCL-X_L			MCL-1		
	Mean	SD	N	Mean	SD	N	Mean	SD	N
OCI-AML3	194766	10493	2	12298	102	2	9419	269	2
OCI-AML5	182976	4892	2	11680	638	2	3224	132	2
PL-21	360914	460	2	19527	2036	2	17911	866	2
NOMO-1	166828	6903	2	16208	3929	2	7509	716	2
MV-4-11	189696	7076	2	15553	2194	2	7729	299	2
MOLM-13	362599	613	2	11321	905	2	10214	175	2
ML-2	163655	3219	2	13570	1357	2	10775	940	2
SKM-1	115273	1040	2	18663	1365	2	10545	66	2
OCI-AML2	216780	7362	2	12178	691	2	3655	226	2
KG-1	97189	361	2	25077	2420	2	3184	173	2
UKE-1	150085	10431	2	43131	1780	2	6685	528	2
U-937	27999	235	2	23067	204	2	8325	93	2
THP-1	270238	13724	2	16624	55	2	18628	1138	2
P31/FUJ	74146	2842	2	11303	1012	2	3723	169	2
HL-60	37068	76	2	21576	5332	2	51899	1368	2
SHI-1	67134	934	2	12277	714	2	3636	102	2
SET-2	28048	1425	2	98599	11588	2	34094	441	2
Kasumi-1	186755	6183	2	37829	3467	2	6781	272	2

*The expression of BCL-2, BCL-X_L, and MCL-1 protein levels was measured by quantitative flow cytometry. AML cells were stained using directly conjugated R-phycoerythrin (R-PE)-labeled antibodies for the respective proteins and the expression was determined using molecules of equivalent soluble fluorochrome (MESF) R-PE conjugated beads.
SD, standard deviation.

Supplemental Table 5. Known treatment, karyotype, and mutational background of AML patient-derived preclinical models described in Figure 3

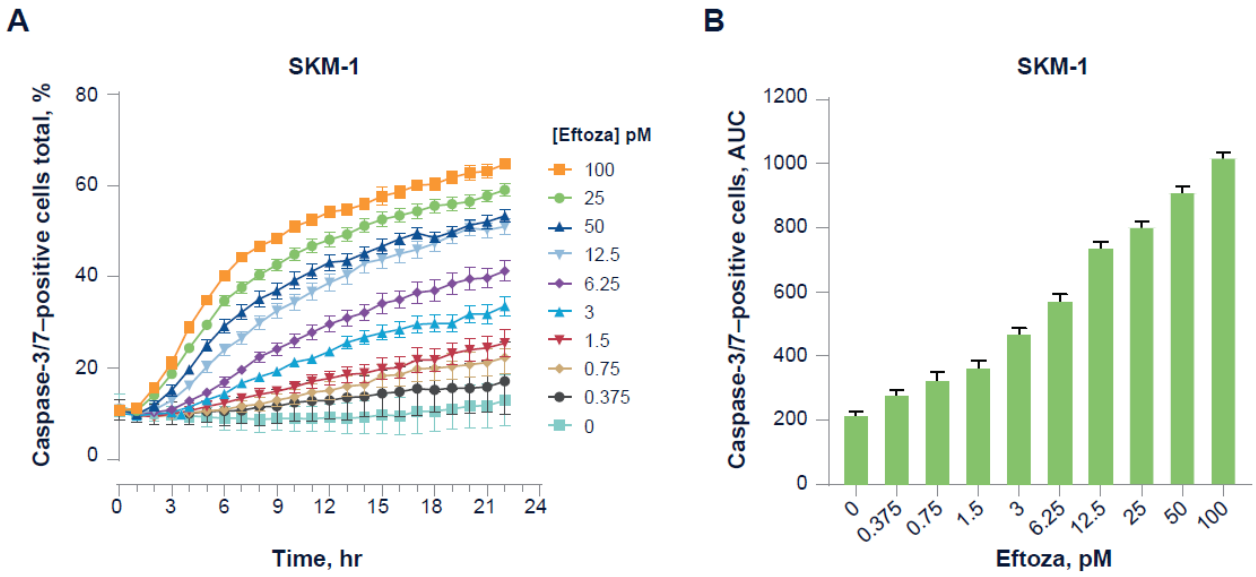
Patient	Model	Status	Karyotype/Mutations
AML-23	PDX	Newly diagnosed	+8[8]/46,XX[14]/*FLT3-ITD* low, $CEBP^{G126R}$
AML-31	PDX	Relapsed	*FLT3-ITD* low, $DNMT3^{R693C/R882C}$, *CBP* LOH, $IDH1^{R132H}$
AML-40	PDX	Recurrent	No genomic aberrations identified
AML-55	PDX	Unknown	$TP53^{P222L/R248Q}$, $NRAS^{Q61R}$, $ASXL^{X630fs}$, $CEBP^{X188fs}$
CTG-2229	Leukapheresis	Refractory	*FLT3 WT*; 46,XY,del(2)(p13p?23),t(4;13)(q31;q34),add(4)(q?25),del(6)(q13q25), t(9;22)(q34;q11.2),del(10)(q24),add(16)(q24)[20]
CTG-2226	Leukapheresis	Refractory	*FLT3 ITD*; 46,XY,add(6)(p21),del(8)(p21),add(12)(q24.1)[13]/46,XY,del(1)(q32),del(7)(q22q32), del(6;12)(q10;p10),add(22)(q?11.2),+mar[3]/45,XY,t(1;2)(p?22;q11.2), -21[1]/46,XY[3]
CTG-2238	Leukapheresis	Newly diagnosed	*FLT3-ITD*

LOH, loss of heterozygosity; PDX, patient-derived xenograft.

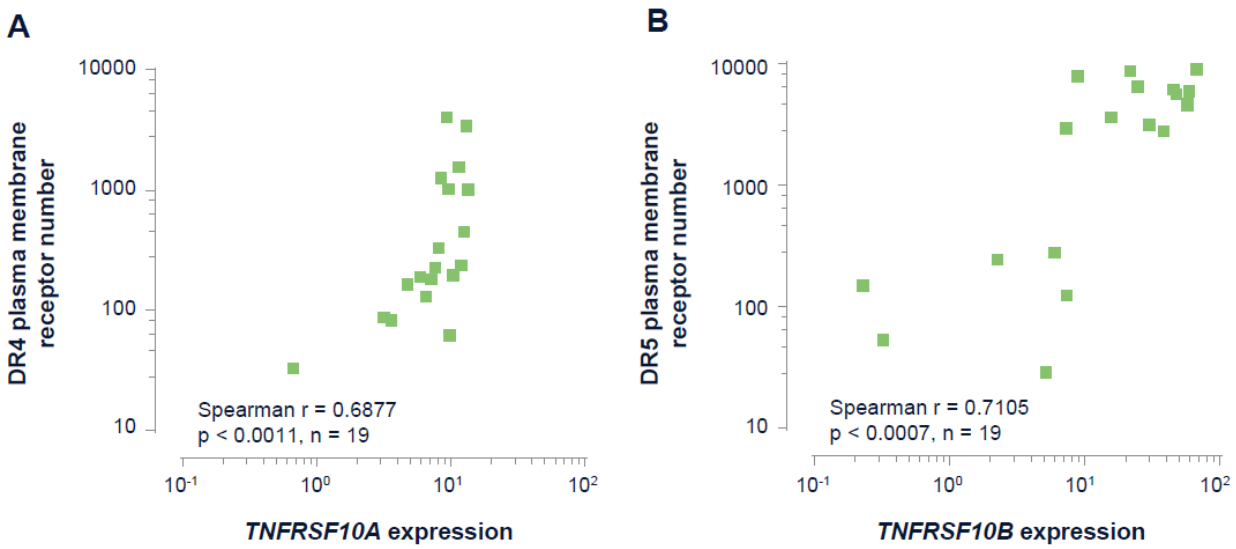
Supplemental Table 6. Median survival and statistical significance of mice inoculated with patient-derived AML PBMCs

CTG-2226					
Treatment	**Median survival, days**	**p-value (Compared to vehicle)**	**p-value (Compared to eftoza)**	**p-value (Compared to VEN)**	**p-value (Compared to eftoza + VEN)**
Vehicle	40.5	-	0.8162	<0.0001*	<0.0001*
Eftoza	37	0.8162	-	0.0006*	<0.0001*
VEN	93.5	0.0001*	0.0006*	-	0.0165*
Eftoza + VEN	105	<0.0001*	<0.0001*	0.0165*	-
CTG-2238					
Treatment	**Median survival, days**	**p-value (Compared to vehicle)**	**p-value (Compared to eftoza)**	**p-value (Compared to VEN)**	**p-value (Compared to eftoza + VEN)**
Vehicle	134	-	0.1670	0.4154	0.0322*
Eftoza	140	0.1670	-	0.0048*	0.1252
VEN	134	0.4154	0.0048*	-	0.0019*
Eftoza + VEN	155	0.00322*	0.1252	0.0019*	-
CTG-2229					
Treatment	**Median survival, days**	**p-value (Compared to vehicle)**	**p-value (Compared to eftoza)**	**p-value (Compared to VEN)**	**p-value (Compared to eftoza + VEN)**
Vehicle	17	-	0.0016*	0.3185	0.0029*
Eftoza	20	0.0016*	-	0.0068*	0.97
VEN	17	0.3185	0.0068*	-	0.013*
Eftoza + VEN	20	0.0029*	0.97	0.013*	-
		The significance difference between groups was determined using the Mantel-Cox test. *p<0.05 was considered significant. PBMC, peripheral blood mononuclear cell.			

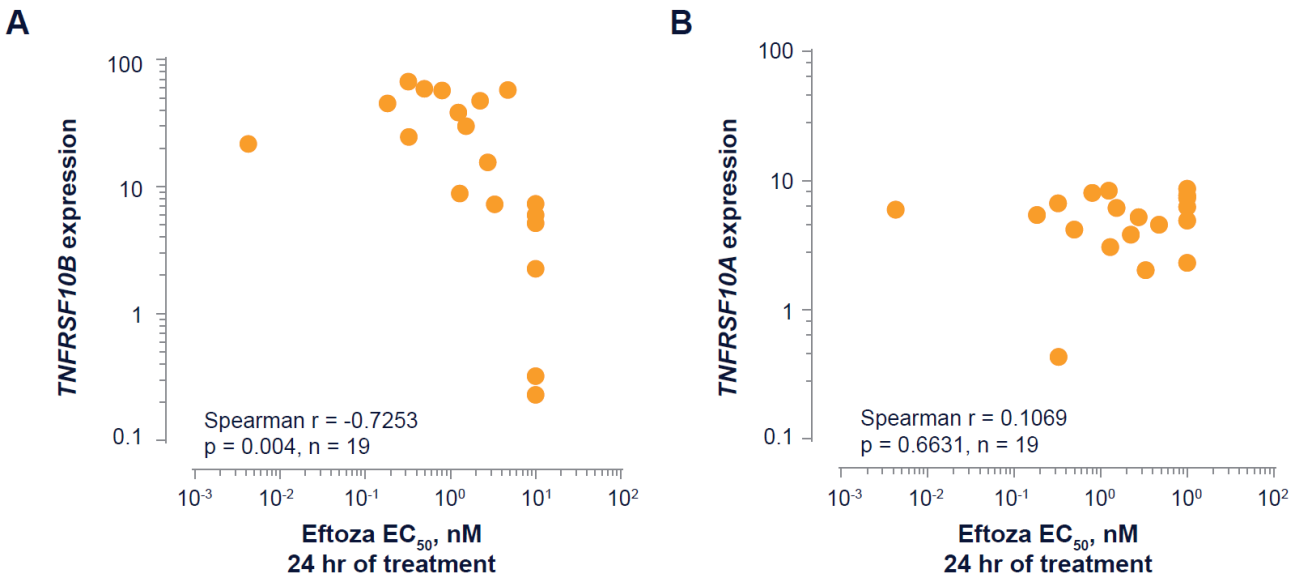
Supplemental Figures



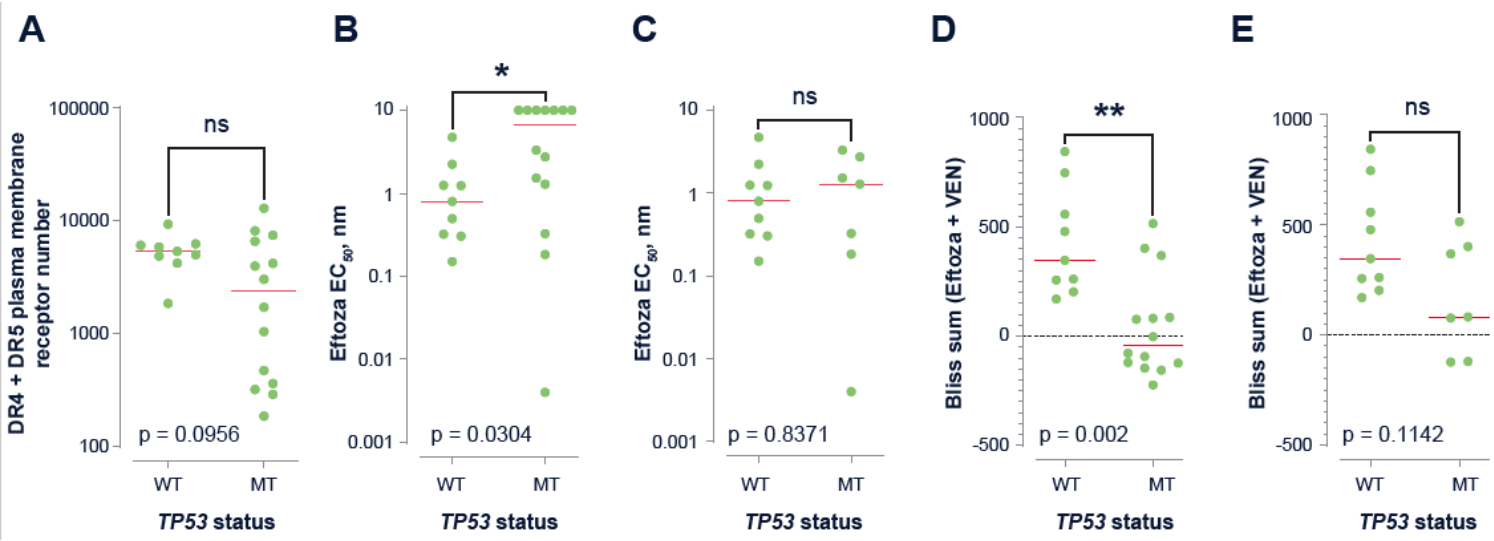
Supplemental Figure 1. Caspase-3/7 activation following eftoza treatment in SKM-1 cell line. Live-cell quantification of caspase-3/7 activation over time was measured by adding caspase-3/7 green dye at the beginning of treatment and monitoring the cells every hour using IncuCyte ZOOM. (A) Time- and dose-dependent change in caspase-3/7–positive cells as a percentage of total cells/well. Data represent mean ± SEM, n = 3). B) Caspase-3/7 activity as measured by the area under the concentration-time curve (AUC) from (A) over the 22-hour period.



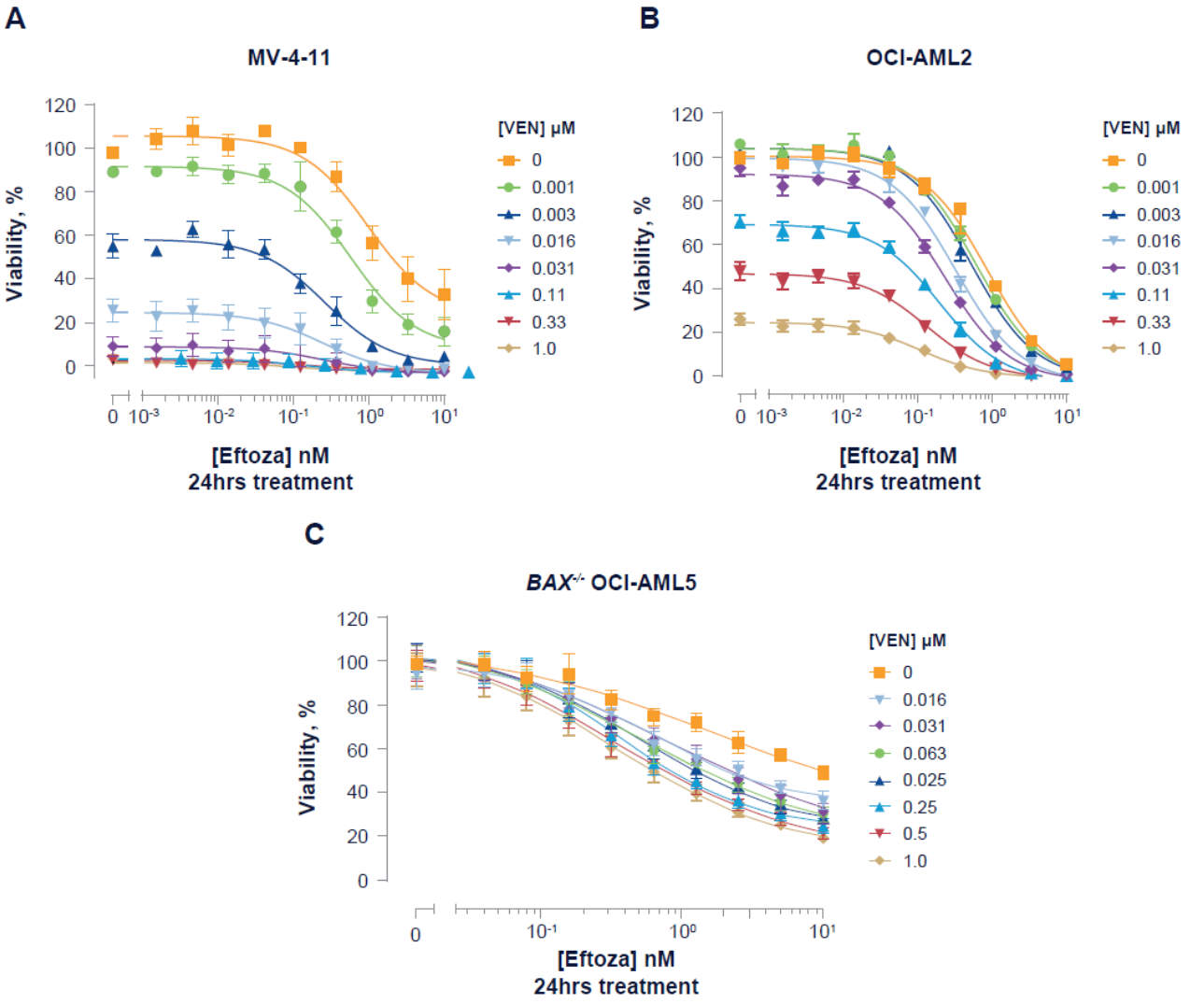
Supplemental Figure 2. DR4 and DR5 plasma membrane receptor numbers correlated with *TNFRSF10A* and *TNFRSF10B* gene expression, respectively, in AML cell lines in vitro. (A) *TNFRSF10A* and (B) *TNFRSF10B* gene expression data for a panel of AML cell lines were obtained from the CCLE and the correlation with DR4 and DR5 plasma membrane receptor numbers from **Figures 1E and 1F** were determined, respectively. $p<0.05$ was considered significant.



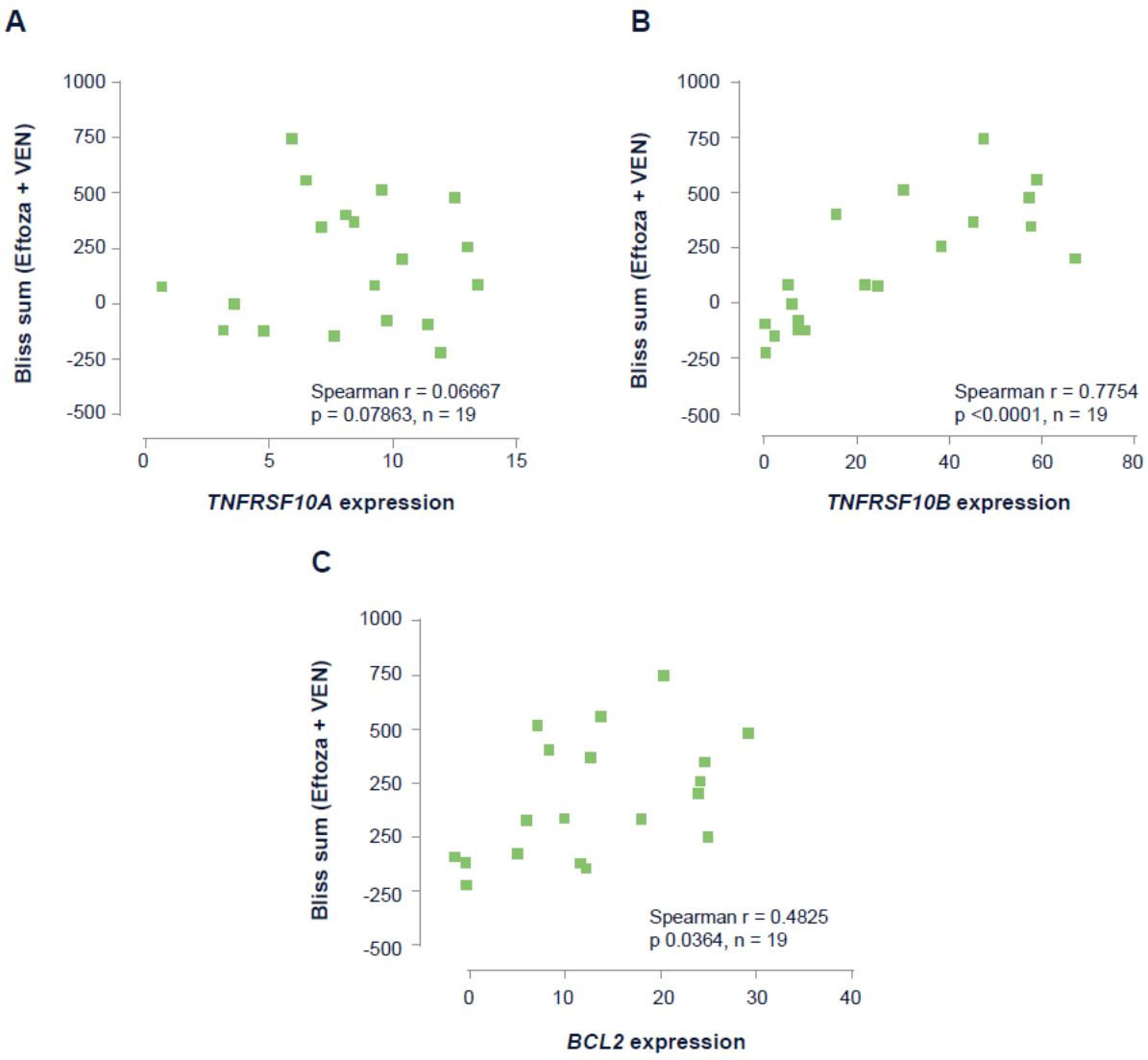
Supplemental Figure 3. *TNFRSF10B,* but not *TNFRSF10A,* expression correlated with eftoza activity in AML cell lines in vitro. (A) *TNFRSF10B* and (B) *TNFRSF10A* gene expression data for a panel of AML cell lines were obtained from CCLE and the correlation with eftoza EC_{50} values from **Figure 1A** following treatment for 24 hours were determined. $p<0.05$ was considered significant.



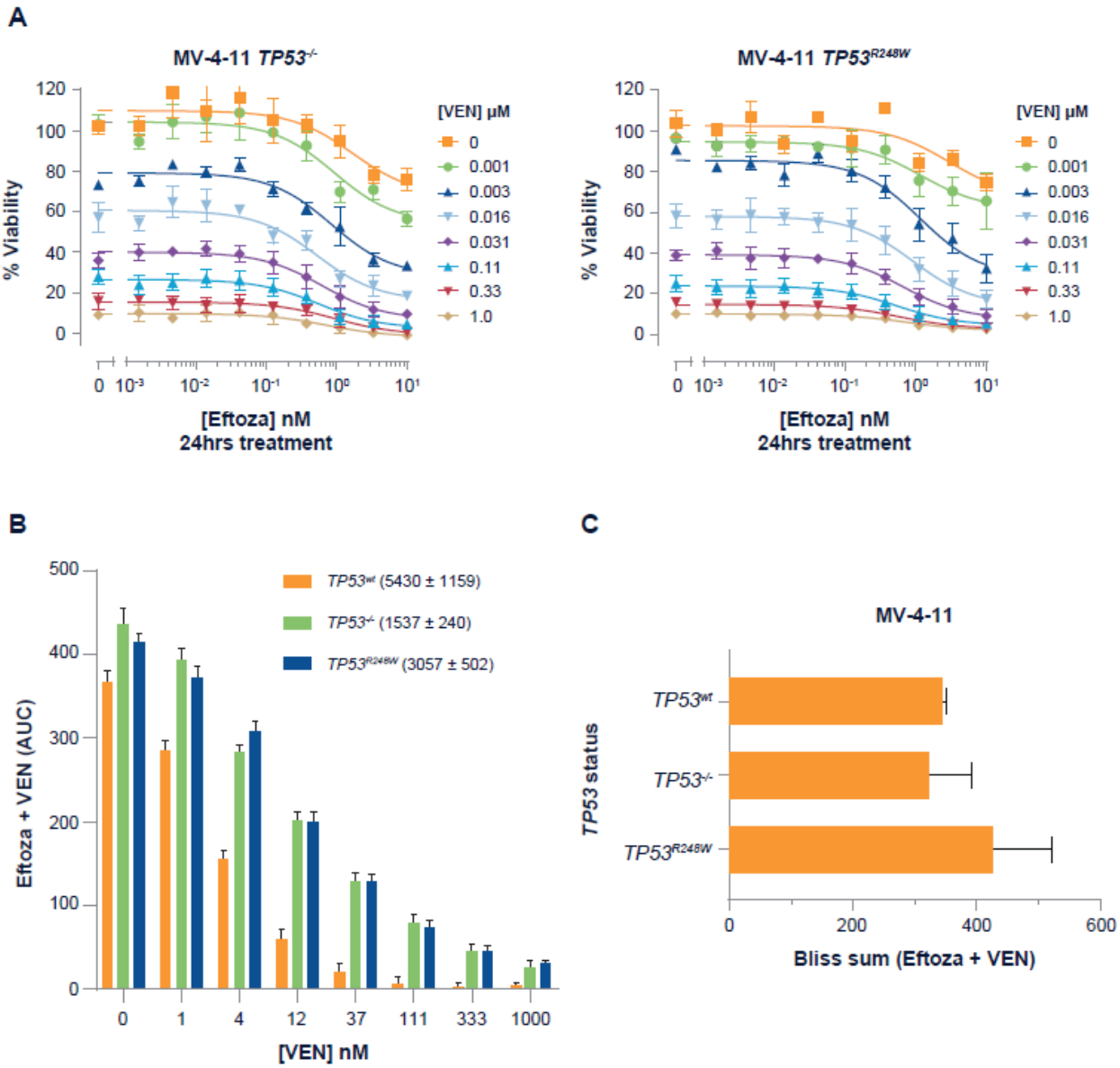
Supplemental Figure 4. Dependency of eftoza activity on *TP53* status. The *TP53* status of AML cell lines was obtained from the World Health Organization International Agency for Research on Cancer (**Supplemental Table 3**) and grouped as either *TP53* wild-type (*TP53*^{wt}) or harboring mutations affecting the expression of *TP53* (*TP53*^{mut}). (A) The expression of DR4 plus DR5 on the plasma membrane from Figure 1A of AML cell lines was determined by flow cytometry and separated according to their *TP53* mutational status. (B) AML cell lines were treated with eftoza for 24 hours and the impact on cell viability was assessed by CellTiter-Glo. EC_{50} values were determined from the resulting dose-response curves (**Supplemental Table 3**) and separated according to their *TP53* mutational status. (C) Data presented in panel B were re-evaluated in AML cell lines expressing >1750 total DR4 plus DR5 receptors on the plasma membrane. (D) AML cell lines were treated with eftoza in combination with venetoclax for 24 hours and the impact on cell viability determined by CellTiter-Glo. Synergy was subsequently assessed using the Bliss independence model and separated according to their *TP53* mutational status (**Supplemental Table 3**). (E) Data presented in panel D were re-evaluated in AML cell lines expressing >1750 total DR4 plus DR5 receptors on the plasma membrane. For all plots, data points throughout represent mean values obtained from individual AML cell lines. Red bars represent median response. Statistical analyses were conducted using the Mann-Whitney U test. $p<0.05$ (*) and $p<0.01$ (**) were considered significant; $p>0.05$ was considered not significant (ns).



Supplemental Figure 5. Enhanced activity of eftoza in combination with the BCL-2 selective inhibitor venetoclax in vitro. (A) Dose-response curves of MV-4-11, (B) OCI-AML2, and (C) *BAX*$^{-/-}$ OCI-AML5 cell lines treated alone or in combination with eftoza and venetoclax for 24 hours. Viability was determined using CellTiter-Glo (mean ± SEM, n = 3). VEN, venetoclax.



Supplemental Figure 6. Relationship between *TNFRSF10A*, *TNFRSF10B,* or *BCL2* gene expression with eftoza plus venetoclax combination activity. Gene expression of *TNFRSF10A, TNFRSF10B,* and *BCL2* were obtained from CCLE. Correlation of (A) *TNFRSF10A,* (B) *TNFRSF10B*, or (C) *BCL2* expression and Bliss sums from **Supplemental Table 3** generated in AML cell lines treated with eftoza and venetoclax in combination for 24 hours.



Supplemental Figure 7. Eftoza-venetoclax combination activity is independent of *TP53* status in MV-4-11 cells. MV-4-11 parental (*TP53* wild-type, *TP53*^{wt}), *TP53*$^{-/-}$, and *TP53*^{R248W} cells were treated with various doses of eftoza and venetoclax alone or in combination for 24 hours. (A) Viability dose-response curves of MV-4-11 *TP53*$^{-/-}$ and *TP53*^{R248W} cells. Viability was determined using CellTiter-Glo (mean ± SEM, n = 3). (B) AUC from the cell viability combination matrix of eftoza plus venetoclax in MV-4-11 *TP53*^{wt} cells (from **Supplemental Figure 5**), and *TP53*$^{-/-}$ and *TP53*^{R248W} cells from panel A (mean ± SEM, n = 3). Numbers in brackets indicate DR5 plasma membrane receptor number (mean ± SD, n = 2). (C) Bliss sum of MV-4-11 TP53^{wt}, *TP53*$^{-/-}$, and *TP53*^{R248W} cells was determined from the combination matrix following treatment with varying doses of eftoza and venetoclax for 24 hours (mean ± SEM, n = 3).