Supplementary Figure Legends

The preclinical efficacy of the novel hypomethylating agent NTX-301 as a monotherapy and in combination with venetoclax in acute myeloid leukemia

Running title: NTX-301 in acute myeloid leukemia

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Supplementary Figure S1. Summary of treatment schedules for assessing the efficacy and survival benefits of NTX-301 in four preclinical models of AML. A Schematic outline of the 3-cycle NTX-301 treatment schedule for monitoring survival and hematologic profiles in the systemic MV4-11 model. **B-E** Tables summarizing the treatment schedules for monitoring survival and hematologic profiles in the systemic MV4-11 model (**B**), for comparing the antitumor efficacy of NTX-301 with that of AZA in the systemic model bearing luciferase-labeled MV4-11 tumors (**C**), for comparing the antitumor efficacy of NTX-301 administered by different methods in the systemic model bearing luciferase-labeled MV4-11 tumors (**D**) and for evaluating the antitumor efficacy of NTX-301 in a dose-dependent manner in the subcutaneous model bearing MOLM-13 tumors (**E**). CP, cyclophosphamide; AZA, azacitidine; DAC, decitabine; NTX, NTX-301; p.o., oral administration; i.p., intraperitoneal administration; q.d., once a day.

Supplementary Figure S2. In vivo assessment of tolerability upon treatment with NTX-301, DAC, or AZA. A-B Line plots showing changes in hematologic profiles (**A**) and body weights (**B**) of female NOD/SCID mice bearing MV4-11 tumors (n=8 per group) upon treatment with NTX-301 [(1.5, 2.0, or 2.5 mg/kg (p.o.)], DAC (2.5 mg/kg (i.p.)], or AZA [5.0 mg/kg (i.p.)]. AZA, azacitidine; DAC, decitabine; NTX, NTX-301; SEM, standard error of the mean; p.o., oral administration; i.p., intraperitoneal administration.

Supplementary Figure S3. Flow cytometric analyses for the detection of

residual human CD45$^+$ MV4-11 cells in cells isolated from bone marrow. The inner boxes indicate human CD45$^+$ MV4-11 cells, and the percentages of these cells are shown on the right.

Supplementary Figure S4. Comparison of the in vivo efficacies of NTX-301 and AZA. In NOD/SCID mouse models transplanted with luciferase-labeled MV4-11 cells (n=8 per group), tumor growth was measured by the quantification of bioluminescence emission (photons/sec) upon treatment with NTX-301 (2.0 mg/kg p.o.) or AZA (5.0 mg/kg i.p.). This Supplementary Figure presents single individual values from Fig. 1C to avoid misleading by large standard deviations. *P*-values (vs. vehicle) are specified and marked as follows: *, $p<0.05$; **, $p<0.001$; NS, not significant. AZA, azacitidine; p.o., oral administration; i.p., intraperitoneal administration.

Supplementary Figure S5. Transcriptome-wide alterations induced by NTX-301 revealed MoAs of NTX-301. A GSEA plots displaying significant enrichment of a normal myeloid-like signature (left) and a leukemic stem cell signature (right) among transcriptome changes induced by NTX-301 or DAC for 48 hours in MV4-11 and HL-60 cells. **B** Heatmaps showing genesets upregulated more strongly by NTX-301 than by DAC and bar plots showing the biological pathways associated with each gene set in MV4-11, MOLM-13, and HL-60 cells. DAC, decitabine; GSEA, gene set enrichment analysis; MoA, mechanism of action; NTX, NTX-301.

Supplementary Figure S6. Transcriptome analyses implied that NTX-301 activates a p53 transcriptional program. A Heatmap showing the expression levels of p53 target genes (n=116) induced by NTX-301 or DAC for 48 hours in MV4-11 cells. Genes with marked transcriptional changes are listed. **B** Ingenuity pathway analysis (IPA) highlighting the activation of p53 as an upstream regulator of genes upregulated by DAC or NTX-301. Z-scores and *p*-values are shown. **C** GSEA plots showing significant enrichment of a p53 signature among transcriptome changes induced by NTX-301 or DAC for 48 hours in p53-proficient AML cells, MV4-11 and MOLM-13. DAC, decitabine; GSEA, gene set enrichment analysis; NTX, NTX-301.

Supplementary Figure S7. NTX-301 elicited the induction of the DDR and the p53 pathway. A Western blots showing the expression levels of proteins involved in the DDR-p53 pathway in MV4-11 cells treated with NTX-301 or DAC (60, 200, and 500 nM) for 24 hours (up). Western blot showing the protein levels of DNMT1 in MV4-11, MOLM-13, and HL-60 cells treated with NTX-301 or DAC (60, 200, and 500 nM) for 24 hours (bottom). **B** Bar plots showing the mRNA expression levels (log_2) of *TP53*, *MDM2*, and *CDKN1A* upon treatment with NTX-301 or DAC (60 nM) for 48 and 96 hours in the MV4-11 cell line. **C** Western blots showing the expression levels of p53 in parental (Con) and *TP53*-knockdown (shTP53) MV4-11 cells. DAC, decitabine; DDR, DNA damage response; NTX, NTX-301.

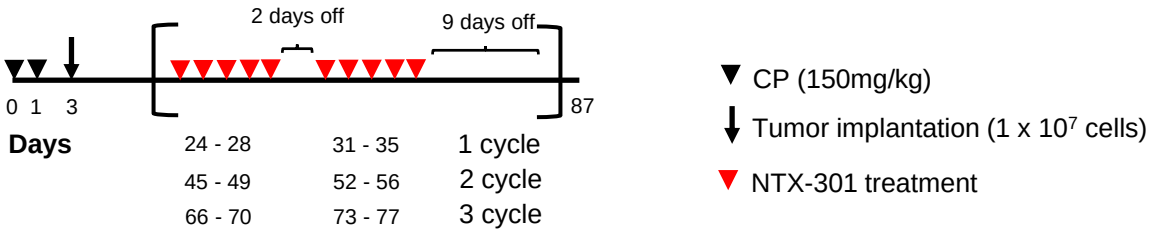
Supplementary Figure S8. Summary of the treatment schedules for assessing the efficacy of NTX-301 as a monotherapy and in combination with VCX using

two preclinical models of AML. A-B Tables summarizing the treatment schedules for comparing the combinatorial efficacies of NTX-301+VCX and AZA+VCX in the subcutaneous MV4-11 model (**A**) and for comparing the survival benefits of NTX-301+VCX and AZA+VCX in the systemic MV4-11 model (**B**). AZA, azacitidine; NTX, NTX-301; VCX, venetoclax; p.o., oral administration; i.p., intraperitoneal administration; q.d., once a day; biw, twice weekly.

Supplementary Figure S9. Assessment of mouse body weights upon treatment with NTX-301 or AZA as a monotherapy and in combination with VCX using two preclinical models of AML. A-B Line plots showing changes in the body weights of the mice described in Supplementary Fig. S8A (**A**) and Supplementary Fig. S8B (**B**). AZA, azacitidine; NTX, NTX-301; VCX, venetoclax.

Supplementary Figure S1

A



B

Group	Mice	Agent	mg/kg	Route	Schedule
1	n=8	Vehicle	---	po	qd x 5 then 2 days off, 5 days on, 9 days off for 3 cycles
2	n=8	NTX-301	1.5	po	qd x 5 then 2 days off, 5 days on, 9 days off for 3 cycles
3	n=8	NTX-301	2.0	po	qd x 5 then 2 days off, 5 days on, 9 days off for 3 cycles
4	n=8	NTX-301	2.5	po	qd x 5 then 2 days off, 5 days on, 9 days off for 3 cycles
5	n=8	DAC	2.5	ip	Three times a week for two weeks
6	n=8	AZA	5.0	ip	Twice a week for three weeks

C

Group	Mice	Agent	mg/kg	Route	Schedule
1	n=8	Vehicle	---	po	Daily on Days 21-25 and 28-32
2	n=8	NTX-301	2.0	po	Daily on Days 21-25 and 28-32
3	n=8	AZA	5.0	ip	Twice a week on days 21, 25, 28, 32, 35, and 39

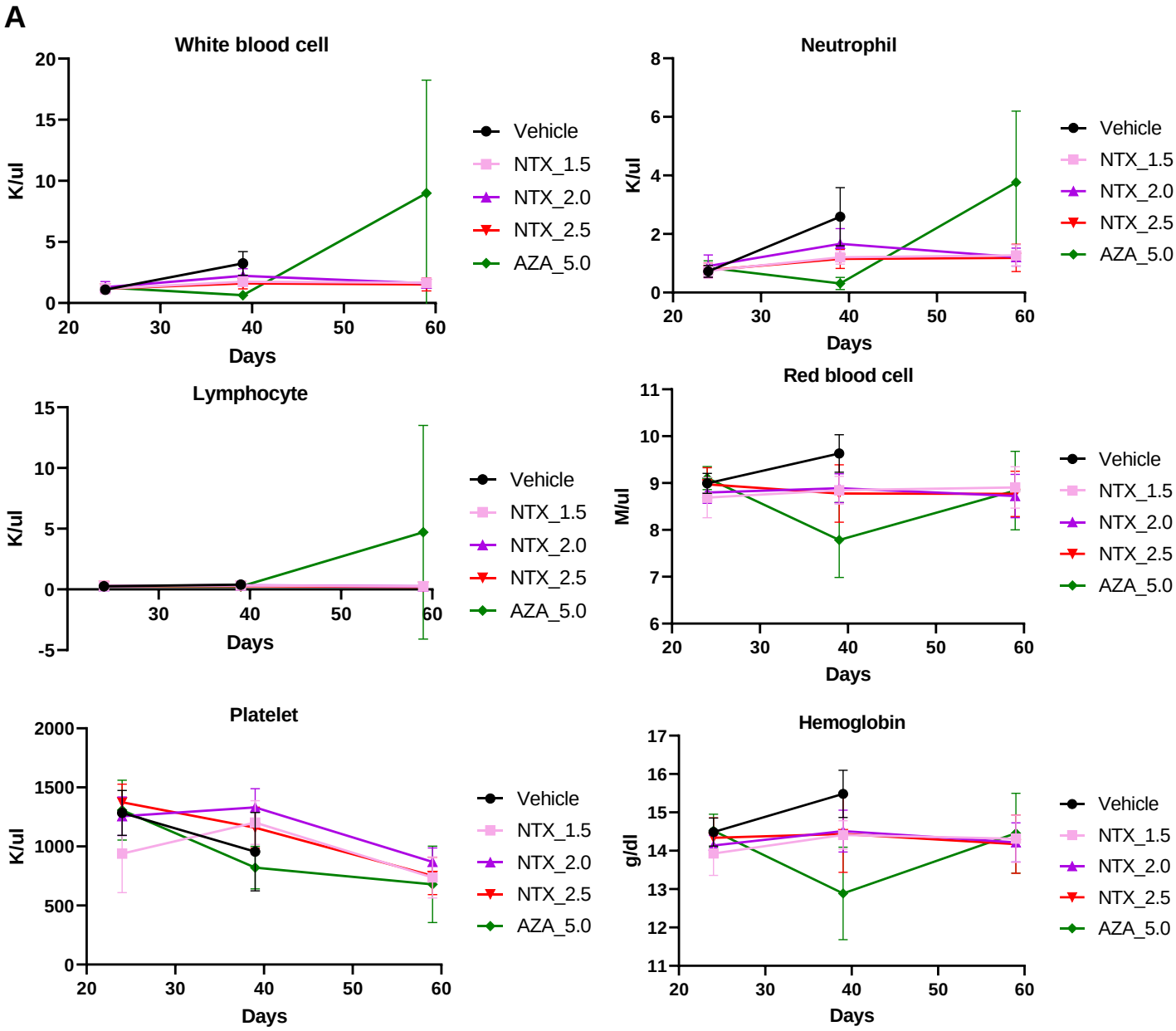
D

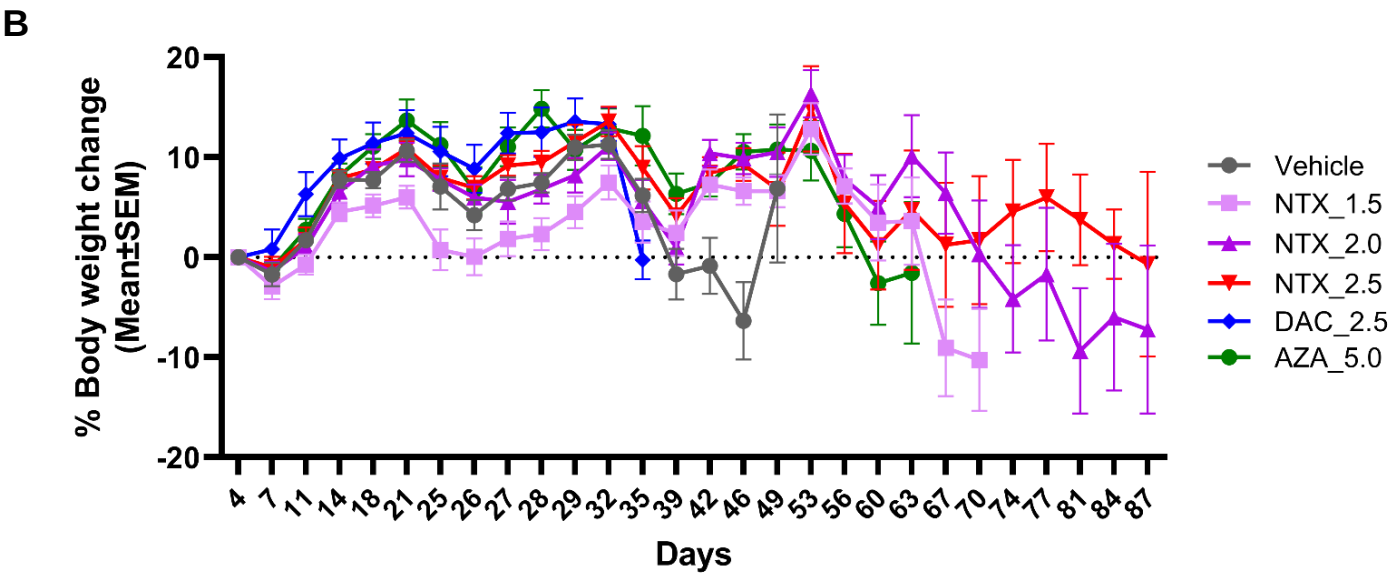
Group	Mice	Agent	Single dose (mg/kg)	Daily dose (mg/kg)	Route	Schedule
1	n=6	Vehicle	---	---	po	Daily on Days 18-22, 25-29, and 39-43
2	n=6	NTX-301	2.0	2.0	po	Daily on Days 18-22, 25-29, 39-43, and 46-48
3	n=6	NTX-301	1.0	2.0	po	2X daily on Days 18-22, 25-29, 39-43, and 46-47 and once on Day 48
4	n=6	NTX-301	1.0	1.0	po	Daily on Days 18-22, 25-29, 32-36, 39-43, and 46-489

E

Group	Mice	Agent	mg/kg	Route	Schedule
1	n=6	Vehicle	---	ip	1x daily on days 8-12 and 15-19
2	n=6	NTX-301	0.2	ip	1x daily on days 8-12 and 15-19
3	n=6	NTX-301	0.4	ip	1x daily on days 8-12 and 15-19
4	n=6	NTX-301	0.8	ip	1x daily on days 8-12 and 15-19
5	n=6	NTX-301	1.5	ip	1x daily on days 8-12 and 15-19

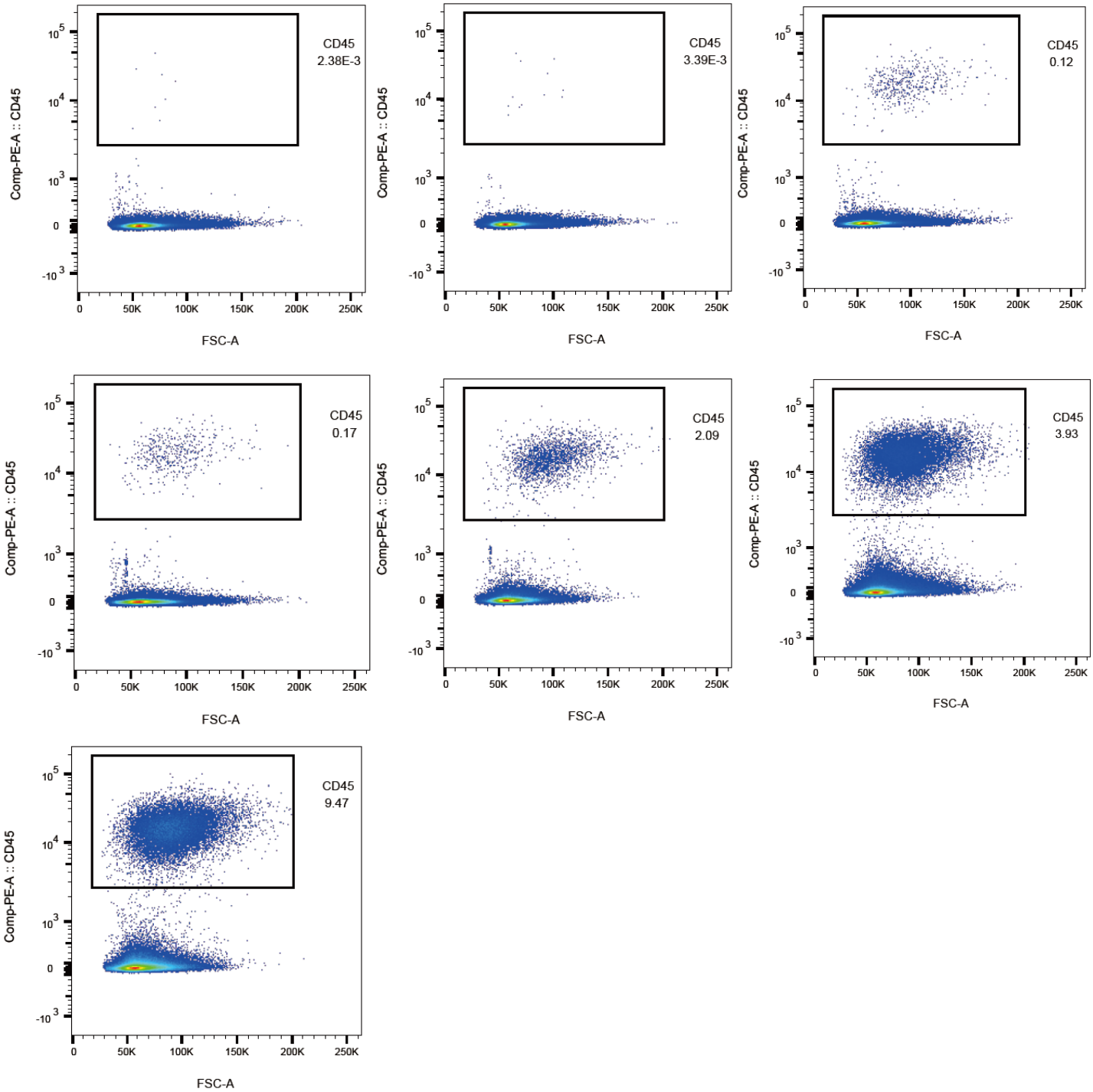
Supplementary Figure S2



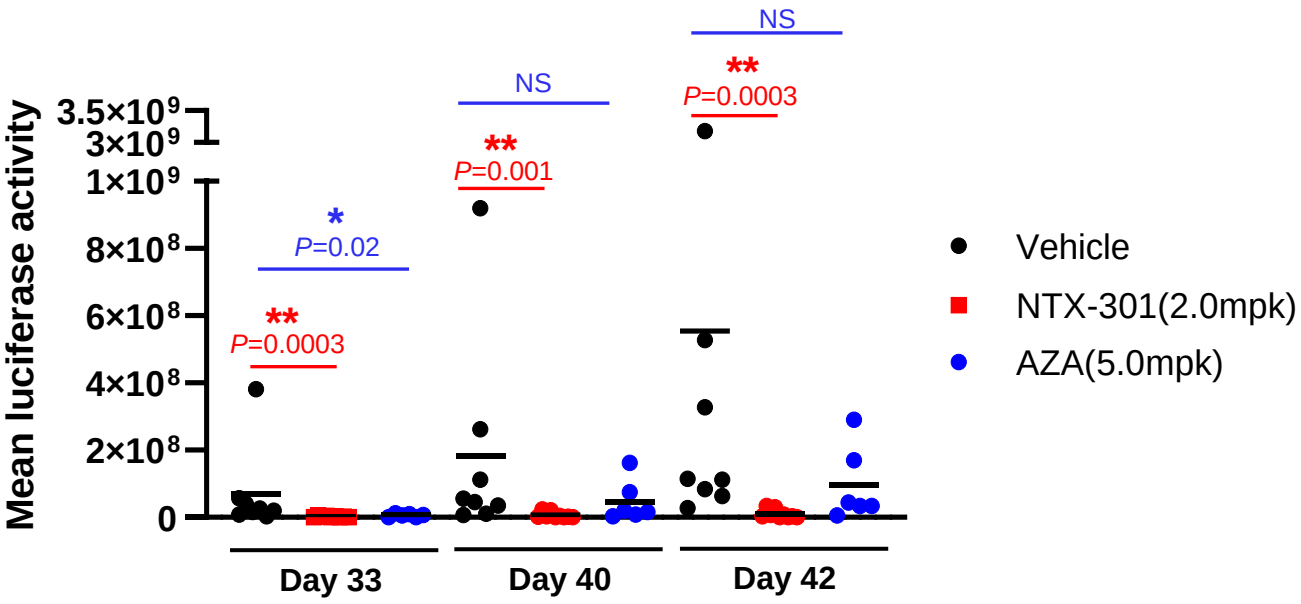


Supplementary Figure S3

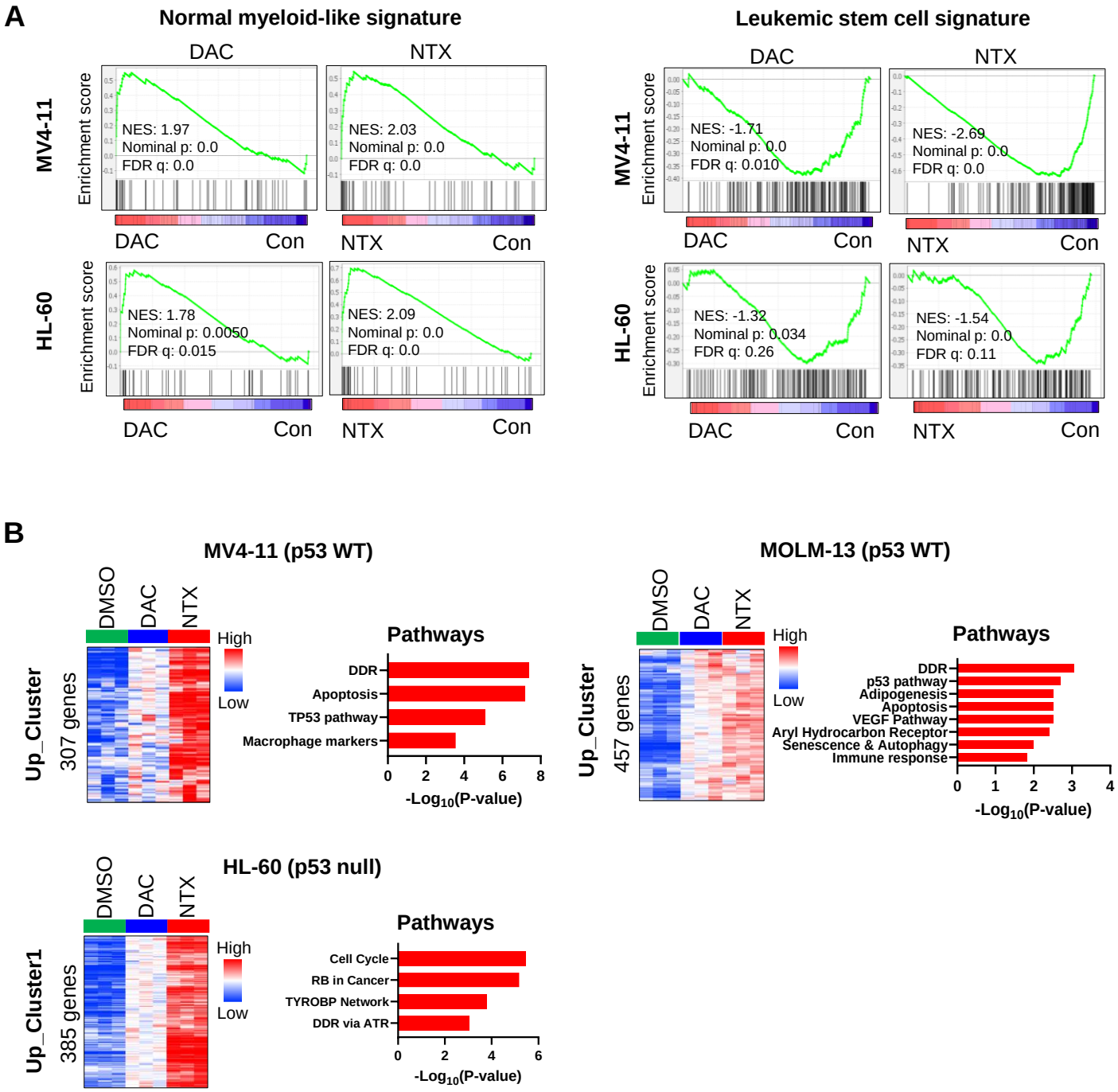
% Human CD45+ MV4-11 cells determined by FACS analysis



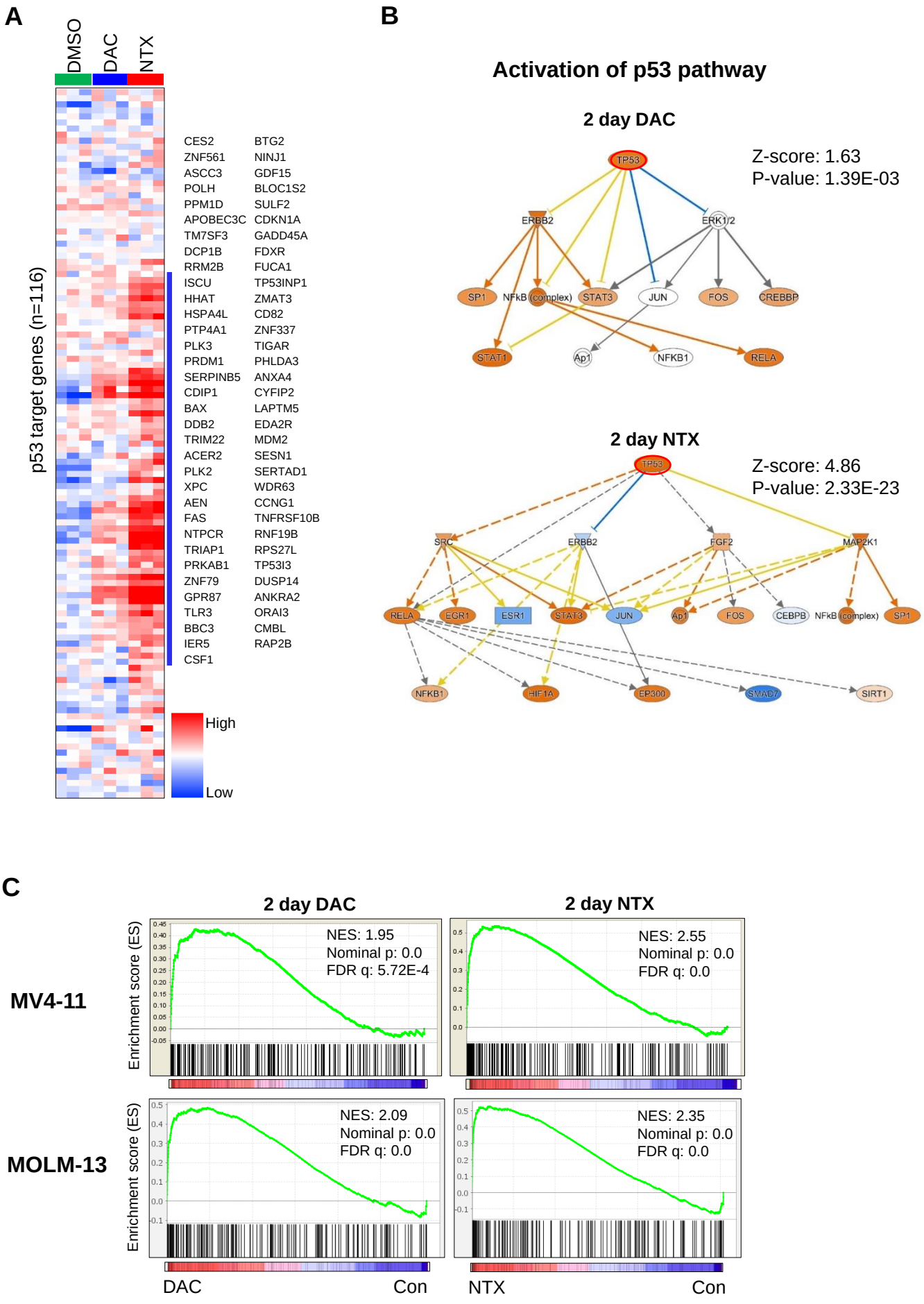
Supplementary Figure S4



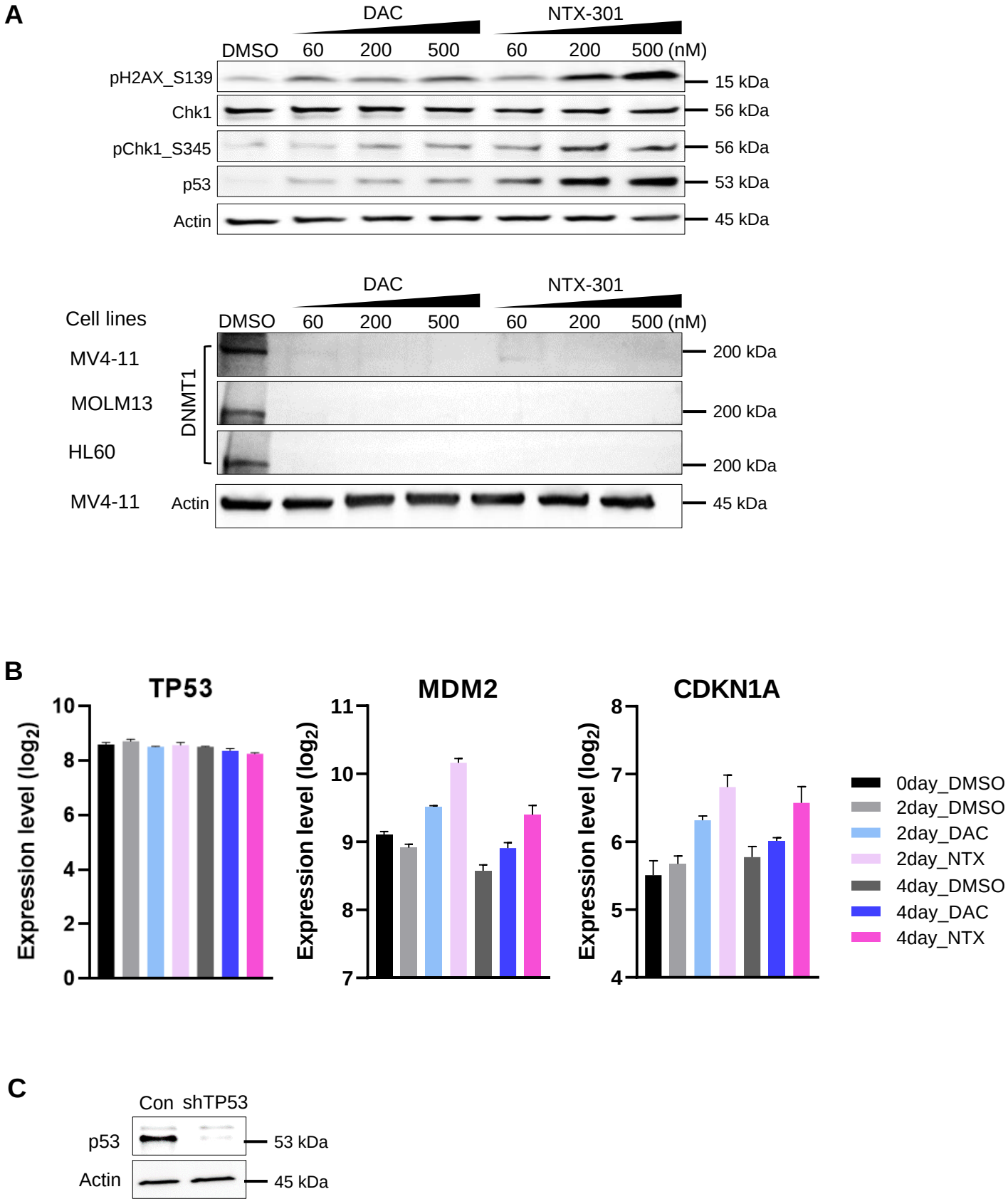
Supplementary Figure S5



Supplementary Figure S6



Supplementary Figure S7



Supplementary Figure S8

A

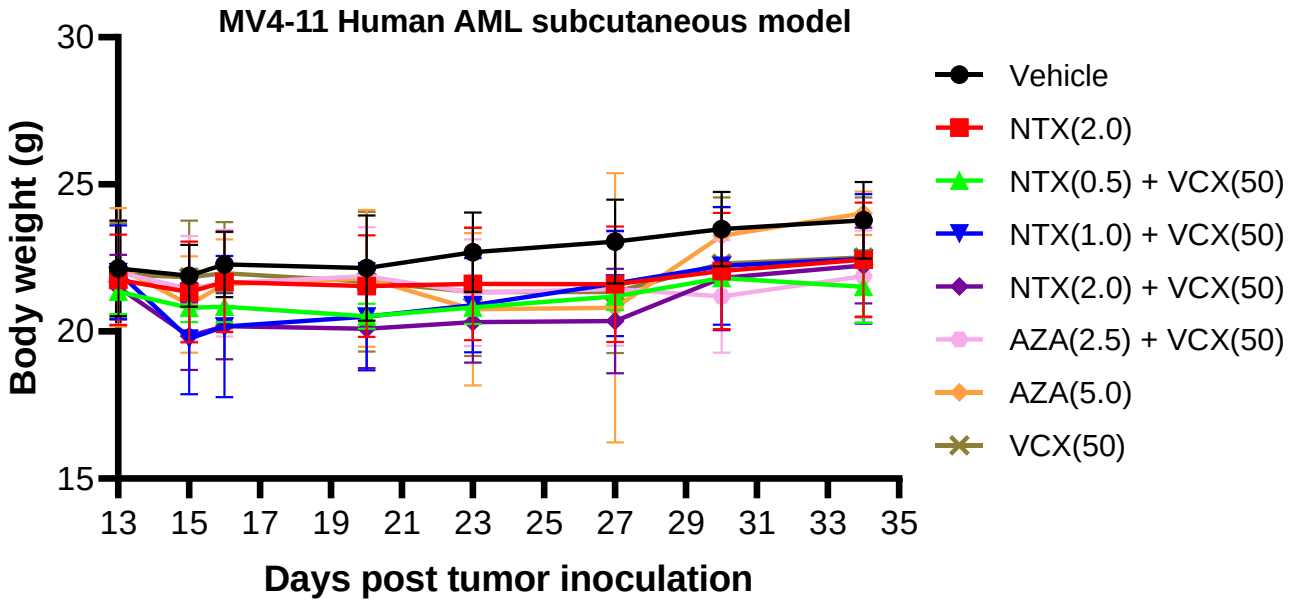
Group	Mice	Drug	Dose (mg/kg)	Route	Regimen
1	5	Vehicle	-	po	qd×5, 2 off, qd×5, 9 off for 3 cycles
2	5	NTX-301	2	po	qd×5, 2 off, qd×5, 9 off for 3 cycles
3	5	NTX+VCX	0.5 + 50	po po	qd×5, 2 off, qd×5, 9 off for 3 cycles (qd×5, 2 off) ×3wks
4	5	NTX+VCX	1 + 50	po po	qd×5, 2 off, qd×5, 9 off for 3 cycles (qd×5, 2 off) ×3wks
5	5	NTX+VCX	2 + 50	po po	qd×5, 2 off, qd×5, 9 off for 3 cycles (qd×5, 2 off) ×3wks
6	5	AZA+VCX	2.5 + 50	ip po	biw×3wks (qd×5, 2 off) ×3wks
7	5	Azacitidine	5	ip	biw×3wks
8	5	Venetoclax	50	po	(qd×5, 2 off) ×3wks

B

Group	Mice	Drug	Dose (mg/kg)	Route	Regimen
1	8	Vehicle	-	po	qd×5, 2 off, qd×5, 9 off for 3 cycles
2	8	NTX-301	2	po	qd×5, 2 off, qd×5, 9 off for 3 cycles
3	8	NTX+VCX	0.5 + 100	po po	qd×5, 2 off, qd×5, 9 off for 3 cycles (qd×5, 2 off) × 9
4	8	NTX+VCX	1 + 100	po po	qd×5, 2 off, qd×5, 9 off for 3 cycles (qd×5, 2 off) × 9
5	8	AZA+VCX	2.5 + 100	ip po	Biw × 9 (qd×5, 2 off) × 9
6	8	VCX	100	po	(qd×5, 2 off) × 9

Supplementary Figure S9

A



B