Novel L-nucleoside analog, 5-fluorotroxacitabine, displays potent efficacy against acute myeloid leukemia

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The novel L-nucleoside analogue, 5-Fluorotroxacitabine, displays potent efficacy against Acute Myeloid Leukemia

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Running Title: Anti-leukemic effects of 5FTRX.

KEYWORDS

5FTRX; CDA; nucleoside analogue; AML.

SUPPLEMENTARY INFORMATION

MATERIALS AND METHODS

Cell lines and Primary samples

OCI-AML2, MV4-11 and K562 cells were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA). KG1a and NB4 cells were cultured in RPMI 1640 medium supplemented with 10% FBS. TEX cells were cultured in IMDM augmented with 20% FBS, 2mM, L-glutamine, 2ng/mL human Interleukin-3 (IL-3), 20 ng/mL human stem cell factor (SCF) (R&D Systems). HEK293 cell lines were maintained in DMEM media. Peripheral blood was collected from consenting patients with AML. Samples with at least 80% leukemic blasts among low-density cells isolated by Ficoll density gradient centrifugation were included in this analysis. Primary AML cells were cultured in Iscove modified Dulbecco medium (IMDM) supplemented with 20% FBS, 2 mM L-glutamine, 2 ng/mL human IL-3, and 20 ng/mL human SCF. Sample collection and the use of human tissue were approved by the University Health Network institutional review. All cell lines and primary samples used in our experiments were incubated at 37°C and 5% CO2 in humidified atmosphere.

Colony forming assays

Clonogenic growth assays with primary AML cells were performed using a standard protocol as previously described.[1] AML mononuclear cells from patients with >80% blasts in their peripheral blood ($4x10^5$ cells/ml) were treated with vehicle control or increasing concentrations of 5FTRX and plated in duplicate by volume at 10^5 cells/ml per

35 mm dish (Nunclon; Rochester, USA) in MethoCult GF H4434 medium (StemCell Technologies) containing 1% methylcellulose in IMDM, 30% FBS, 1% bovine serum albumin, 3 U/ml recombinant human erythropoietin, 10-4 M 2- mercaptoethanol (2ME), 2mM L-glutamine, 50ng/ml recombinant human stem cell factor, 10ng/ml recombinant human granulocyte macrophage-colony stimulating factor and 10ng/ml recombinant human IL-3. After 7-10 days of incubation at 37°C with 5% CO_2 and 95% humidity, the numbers of colonies were counted on an inverted microscope with a cluster of 10 or more cells counted as one colony.

***In vitro* combination analysis**

Cell lines were seeded in 96-well plates and treated with ranges of concentrations of 5FTRX, Ara-C, doxorubicin and/or azacytidine. After 5 days treatment, cell viability was assessed using CCK Kit-8 (Dojindo) as described in the kit instructions. Raw data was entered into the MacSynergy II software[2] where the combined effect was calculated and plotted in 3D dose-response surface graphs. According to guidelines in the software, synergy or antagonism was defined as values greater than + /- 50 μM^2%.

Immunoblot analysis

Cells were washed in PBS and then resuspended in an equal volume of RIPA buffer (10 mM Tris (pH 7.4), 150 mM NaCl, 0.1% Triton X-100, 0.5% sodium deoxycholate, and 5 mM EDTA) containing complete protease inhibitors (Roche, Indianapolis, IN, USA). Protein concentrations were determined by the Bradford assay. Immunoblot assays were performed as described previously.[3] Briefly, equal amounts of

protein were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to nitrocellulose membranes. Membranes were incubated with monoclonal rabbit anti-CDA (1:1000 v/v dilution, ab222515, AbCAM, Cambridge, USA) and monoclonal mouse anti-β-actin (1:5,000 v/v dilution, Sigma, St Louis, MO). Secondary antibodies consisted of horseradish peroxidase (HRP)–conjugated goat anti–mouse IgG and anti–rabbit IgG (1:3000 vol/vol; Amersham, Piscataway, NJ). Detection was performed by the enhanced chemiluminescence (ECL) method (Pierce, Rockford, IL, USA). In order to detect, phospho-H2AX (pH2AX), a marker of DNA double-strand breaks and stalled DNA replication forks, cells were treated with 5FTRX using concentrations 1X, 10X and 100X the IC_{50} for each cell line. pH2AX was detected using mouse monoclonal mouse anti-phospho-histone H2AX Antibody (1:1000 v/v dilution, AbCam, ab26350, Cambridge, USA) and monoclonal mouse anti-β-actin (1:5,000 v/v dilution, Sigma, St Louis, MO) followed by HRP-conjugated goat anti–mouse IgG and anti–rabbit IgG (1:3000 vol/vol; Amersham, Piscataway, NJ).

Xenograft models of human AML

NOD/SCID mice were injected subcutaneously in the right flank with MV4-11 tumor cells (1×10^7) in mixture of 0.1 ml PBS (with Matrigel, 1:1). Treatments were started when the mean tumor size reached approximately 200 mm^3. Mice were dosed intra-peritoneally (i.p.) with 10, 30 or 100 mg/kg 5FTRX (n=10 per group) once or twice daily for up to 5 consecutive days. Ara-C was dosed at 60 mg/kg i.p. daily for 5 days, followed by 2 days off, then a second cycle of 5 days on, 2 days off. Tumor volumes were measured three times weekly in two dimensions using a caliper, and volumes expressed in mm^3 using the

formula: V = 0.5 a x b^2 where a and b are the long and short diameters of the tumor, respectively. Mice were sacrificed humanely at a maximum tumor volume of 1500mm^3.

OCI-AML2 leukemia cells (1×10^6) were injected subcutaneously into the flanks of severe combined immune deficient (SCID) mice (Ontario Cancer Institute, Toronto, ON). After the appearance of tumor of mean size 100 mm^2, the mice were treated subcutaneously with 5FTRX (synthesized by Medivir AB (Huddinge, Sweden)) (30 mg/kg and 100 mg/kg) once daily or vehicle (saline) control (n=10 per group) for 5 consecutive days. Tumor measurements were taken 3 times a week based on caliper measurements of tumor length and width (volume= tumor length × $width^2$ × 0.5236). At the end of treatment, mice were sacrificed, and tumor volumes and mass were measured from excised tumors.

To test 5FTRX's efficacy in a primary AML engraftment mouse model, a frozen aliquot of primary AML cells was thawed, counted, and re-suspended in phosphate-buffered saline (PBS). Viable trypan blue-negative cells ($2.5x10^6$) were injected into the right femur of 10-week-old female NOD/SCID mice that were sub-lethally irradiated (2Gy) and pretreated with 200 µg of anti-mouse CD122. Two weeks after injection of the primary AML cells, mice were treated once daily with 5FTRX (100 mg/kg) i.p. or vehicle (saline) control (n=10 per group) for 5 consecutive days. At the end of the experiment, mice were sacrificed, femurs flushed, and primary AML engraftment ($CD45^+CD33^+CD19^-$ cells) in the left femur was determined by flow cytometry. For secondary engraftment, 1.5x 10^6 cells harvested from the left femurs of the primary engraftment were injected into the right femurs of 10-week-old female NOD/SCID mice that were sub-lethally irradiated (2Gy) and pretreated with 200 µg of anti-mouse CD122. Six weeks after injection mice were then

sacrificed, femurs flushed and AML engraftment (CD45+CD33+CD19- cells) in the left femur was determined by flow cytometry.

In vivo studies were performed according to the regulations of the Canadian Council on Animal Care and with the approval of the Ontario Cancer Institute Animal Ethics Review board (OCI-AML2 and primary AML engraftments) or according to the regulations of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) with the review and approval by the Institutional Animal Care and Use Committee (IACUC) of CrownBio (MV4-11).

In vivo pharmacokinetic studies

NOD/SCID mice with subcutaneous MV4-11 tumor xenografts were treated with vehicle or 5FTRX twice daily for 5 days (10 administrations in total) and were terminated 2, 8 or 24 hours after the last dose. Tumors and plasma samples were collected and snap-frozen prior to bioanalysis. The concentrations of 5FTRX and its metabolites, MP, DP and TP in samples from *in vitro* incubations and *in vivo* pharmacokinetic studies were determined by liquid chromatography with tandem mass spectrometric detection (LC/MS/MS). Tumor samples were homogenized before protein precipitation.

In vivo pharmacodynamics studies

NOD/SCID mice with subcutaneous MV4-11 tumor xenografts were treated with vehicle or 5FTRX twice daily for 5 days (10 administrations in total) and were terminated 2 hours after the last dose. Two hours prior to termination, mice received 600 mg/kg BrdUrd and 60 mg/kg pimonidazole (i.p.) to label S-phase cells and hypoxic tumor

regions, respectively. Tumors were excised and snap-frozen in liquid nitrogen prior to analysis.

For CD31, pH2AX and pimonidazole staining, individual cryosections were fixed immediately in 10% NBF for 15 minutes at room temperature. Vasculature was stained using a 1:500 dilution of hamster-anti-mouse-PECAM/CD31 and 1:500 fluorescent Alexa 546 secondary (Invitrogen, Burlington, ON, CA). Anti-phospho-histone H2AX (Ser139) was detected with mouse-anti-human γH2AX (Clone JBW301, EMD Millipore) tagged with Alexa 647. Hypoxia was detected via bound pimonidazole adducts using a 1:500 mouse-anti-pimonidazole-FITC. Slides were imaged for fluorescence prior to the second staining stage. To assess BrdUrd uptake and cellularity, slides were transferred to a citrate buffer and heated to 120 C for detection of BrdUrd incorporated into DNA using a 1:500 dilution of monoclonal rat anti-BrdUrd [BU1/75 (ICR1)] (ab6326, AbCAM, Cambridge, USA) followed by 1:500 dilution of anti-mouse Alexa 750 secondary. Cellular DNA was counter-stained with Hoechst 33342 and imaged. The imaging system consisted of a proprietary robotic fluorescence microscope with a PCO Edge 4.2 camera and customized ImageJ software (public domain program developed at the U.S. National Institutes of Health, available at http://rsb.info.nih.gov/ij/) running on a Macintosh computer (Apple, Cupertino, CA, USA). The system allows tiling of adjacent microscope fields of view. Using this system, images of entire tumour cryosections 1-3 cm^2 were captured at a resolution of 1.3 μm/pixel. Image analysis Using NIH-ImageJ and user supplied algorithms, images of CD31 fluorescence and BrdUrd, pH2AX, pimonidazole & Hoechst 33342 staining from each tumour section were overlaid and areas of necrosis, acellular cavities and staining artifacts manually removed. On the fluorescence image, positive regions for each marker

were identified by selecting all pixels above tissue background levels. Analysis of whole tissue averages for each marker were determined by dividing the total number of positive pixels by the total tissue area excluding necrosis and empty regions.

Statistical analysis

All experiments were performed at least three times with technical replicates. Statistical analyses were performed using Graph Pad Prism 6.03 (La Jolla, CA, USA).

Supplementary references for Materials and methods

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2. Prichard MN, Shipman C, Jr. A three-dimensional model to analyze drug-drug interactions. Antiviral research 1990; 14(4-5): 181-205.
3. Liyanage SU, Hurren R, Voisin V, et al. Leveraging increased cytoplasmic nucleoside kinase activity to target mtDNA and oxidative phosphorylation in AML. Blood 2017; 129.19: 2657-2666

Supplementary Tables and Figures

Table S1. Patient sample characteristics

Sample #	Tissue Bank #	Age (yrs)	Gender	Diagnosis	Cytogenetics	NPM1	FLT3 TKD	FLT3 ITD
1	150375	66	M	AML with NPM1 mutation	46, XY[10]	Pos	Neg	Pos
2	120968	59	F	AML with NPM1 mutation	46, XX[20]	Pos	Neg	Neg
3	120805	29	F	AML with NPM1 mutation	46, XY[20]	Pos	Neg	Neg
4	150279	63	F	AML with NPM1 mutation	46, XY[20]	Pos	Neg	Pos
5	130854	74	M	Secondary AML (CML in blast phase)	48,XY, add(1)(q32), +del(5)(q12q33), +mar[4]/46,XY[7]	NA	NA	NA
6	248021	76	F	AML with NPM1 mutation	46,XX[20]	Pos	NA	Pos
7	140863	70	M	Secondary AML (previous MDS)	42~45, XY, -3, -5, del(7)(q21), der(12) t(12;16)(p13q11.2), -13 , -14, -16, -20, +4 mar[cp10]	NA	NA	NA
Xenograft	120860	31	F	AML with t(9;11) (p22;q23); MLLT-MLL	46, XX, t(9;11) (p22;q23)[10]	NA	NA	NA

M: Male, F: Female; AML: acute myeloid leukemia; NA: not available, Pos: positive, Neg: negative

Table S2. Next generation sequencing results on subset of patient samples

Sample #	Tissue Bank #	Gene	NM id	Variant (cDNA)	Variant (AA)	VAF (%)
1	150375	DNMT3A	NM_022552.4	c.1906G>T	p.Val636Leu	33.8
		NPM1	NM_002520.6	c.859_860insTCTG	p.Trp288CysfsTer12	31.7
4	150279	CUX1	NM_001202543.1	c.574G>A	p.Glu192Lys	45.3
		DNMT3A	NM_022552.4	c.2644C>T	p.Arg882Cys	44.7
		IDH2	NM_002168.2	c.419G>A	p.Arg140Gln	47.8
		NPM1	NM_002520.6	c.859_860insTCTG	p.Trp288CysfsTer12	42.5
		RUNX1	NM_001754.4	c.758T>C	p.Leu253Pro	5.7
6	248021	DNMT3A	NM_022552.4	c.2707G>C	p.Ala903Pro	44.8
		FLT3	NM_004119.2	c.1812_1813ins36	p.Glu604_Phe605ins12	41.5
		NPM1	NM_002520.6	c.860_863dupTCTG	p.Trp288Cysfs*12	41.2
		RUNX1	NM_001754.4	c.1155C>G	p.Tyr385*	16.6
		TET2	NM_001127208.2	c.651delC	p.Val218Trpfs*32	44.3
		TET2	NM_001127208.2	c.2662C>T	p.Gln888*	42.1

M: Male, F: Female; AML: acute myeloid leukemia; NA: not available, Pos: positive, Neg: negative, VAF: Variant allele frequency

Table S3

Exposure	5FTRX	MP	DP	TP
PBMC	8	9	33	18
MV4-11	5	15	19	19
THP-1	8	2	21	27

Intracellular 5FTRX, MP (monophosphate), DP (diphosphate) and TP (triphosphate) levels (μM) in PBMC, MV4-11 and THP1 cells after 24hrs exposure to 5FTRX at 10 μM, Data represent mean of 3 experiments.

Table S4

Primary engraftment

Control	5FTRX
45.8	3.18
41.6	2.79
46	3.31
47.1	3.15
39.3	2.99
36.1	3.24
21.4	3.46
42.7	2.86
8	3.25

Secondary engraftment

Control	5FTRX
13	9.95
27.9	7.92
14.6	8.91
55.6	8.61
30.6	9.21
39.1	8.1
56.4	6.85
74.8	6.32
48.8	2.97
19.1	8.21

Percentage of CD45+CD19-CD33+ cells in control versus 5FTRX treated mice in primary and secondary engraftment mouse models.

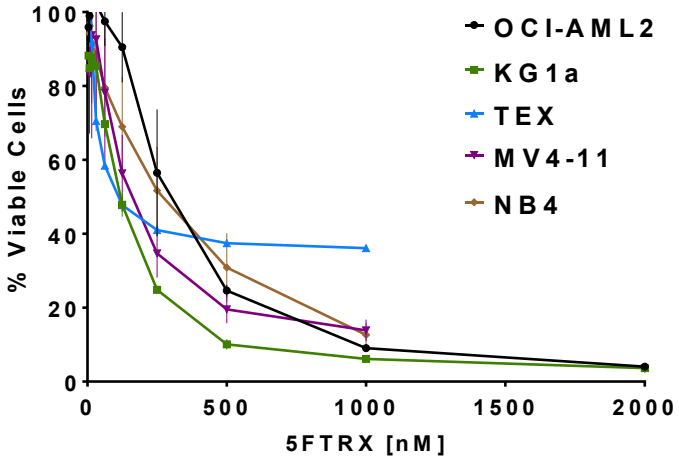
Table S5 (related to figure S6)

Combination	MV4-11			THP-1		
	Synergy volumes at 95% CI (µMol%)		Conclusion	Synergy volumes at 95% CI (µMol%)		Conclusion
	Synergy	Antagonism		Synergy	Antagonism	
5FTRX + Azacytidine	124.44 161.57	0 -0.07	Strong synergy/ insig. antagonism Strong synergy/ insig. Antagonism	209.66 249.29	-38.61 -15.66	Strong synergy/ minor antagonism Strong synergy/ insig. Antagonism
5FTRX + Ara-C	5.5 7.53	-6.48 -14.83	Insig. synergy and antagonism Insig. synergy and antagonism	61.24 118.01	-1.56 -0.62	Mod. synergy/ insig. antagonism Strong synergy / insig. Antagonism
5FTRX + Doxorubicin	54.92 79.08	-7.72 0	Mod synergy/ insig. antagonism Mod. synergy/ insig. Antagonism	144.35 57.97	-4.71 -3.33	Strong synergy/ insig. Antagonism Mod. synergy / insig. Antagonism

Mod. Moderate; Insig. insiginificant

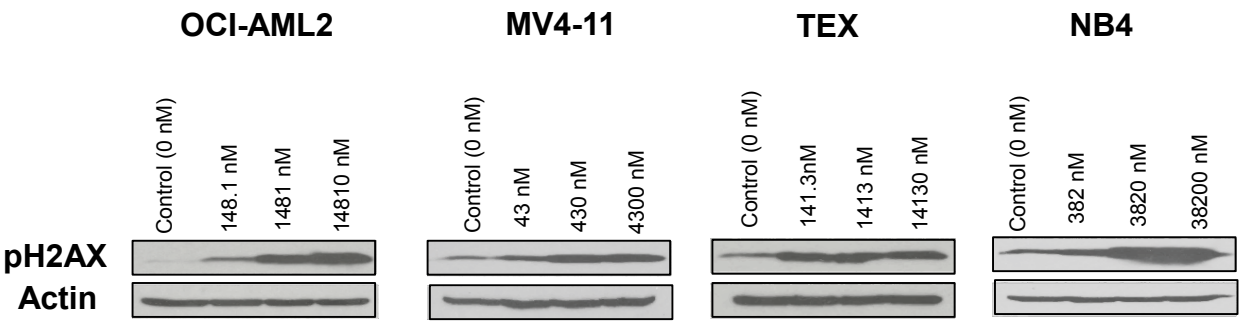
A summarized synergy score for the Bliss independent analysis (Figure S6) calculated by averaging over the whole dose-response matrix is shown.

Figure S1



Myeloid leukemia cell lines were treated with increasing concentrations of 5FTRX for 72 hours. After incubation, cell growth and viability was measured by the MTS assay. Data represent the mean $\pm$ SD IC_{50} from one of at least 3 representative experiments. Mean IC_{50} values: 297.5nM (OCI-AML2), 109.2nM (KG1a), 179nM (TEX), 160.8nM (MV4-11), 237.3nM (NB4).

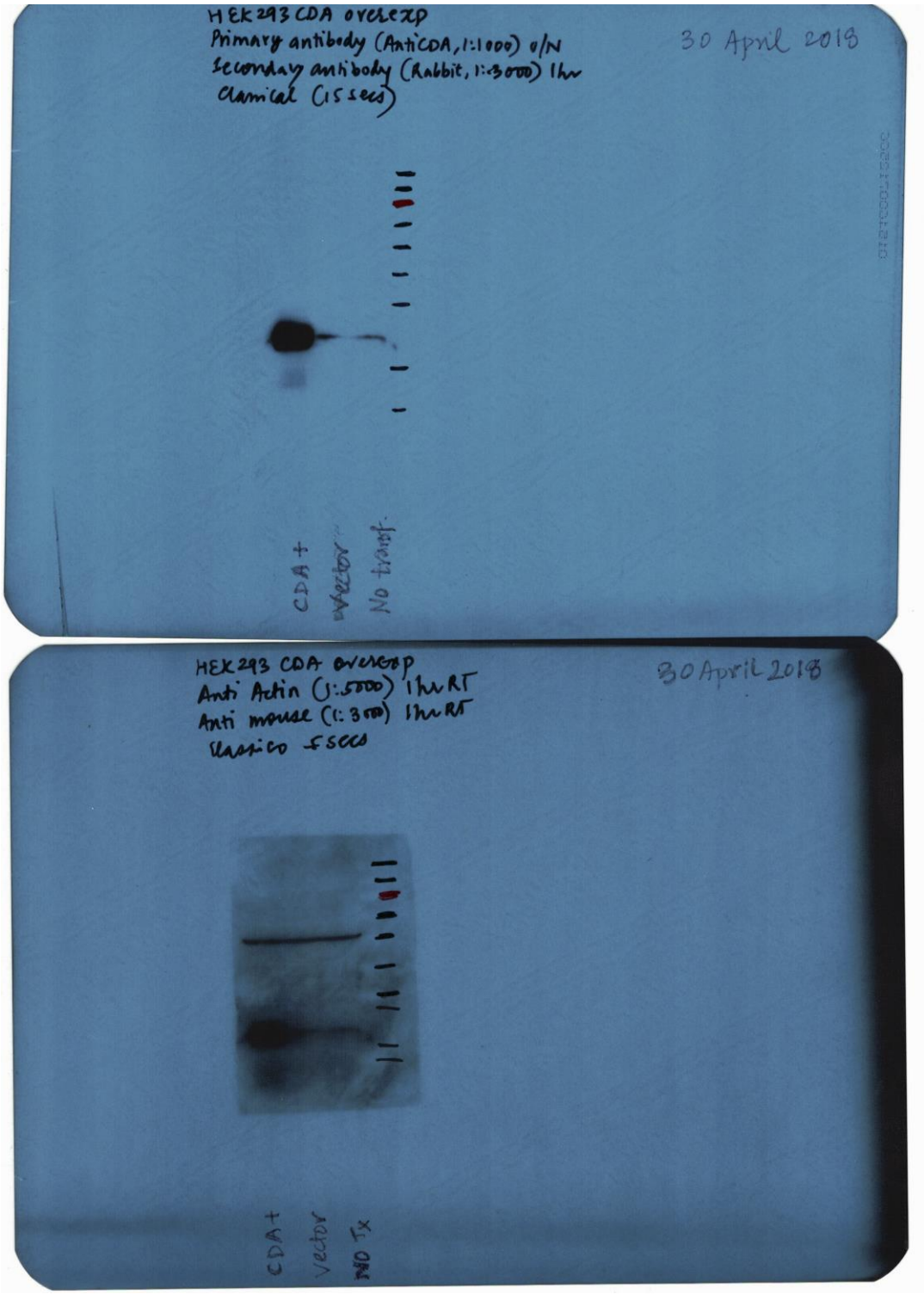
Figure S2



Myeloid leukemia cell lines were treated with increasing concentrations of 5FTRX for 24 hours. After treatment, cells were lysed and levels of phosphorylated H2AX (pH2AX) were measured by immunoblotting.

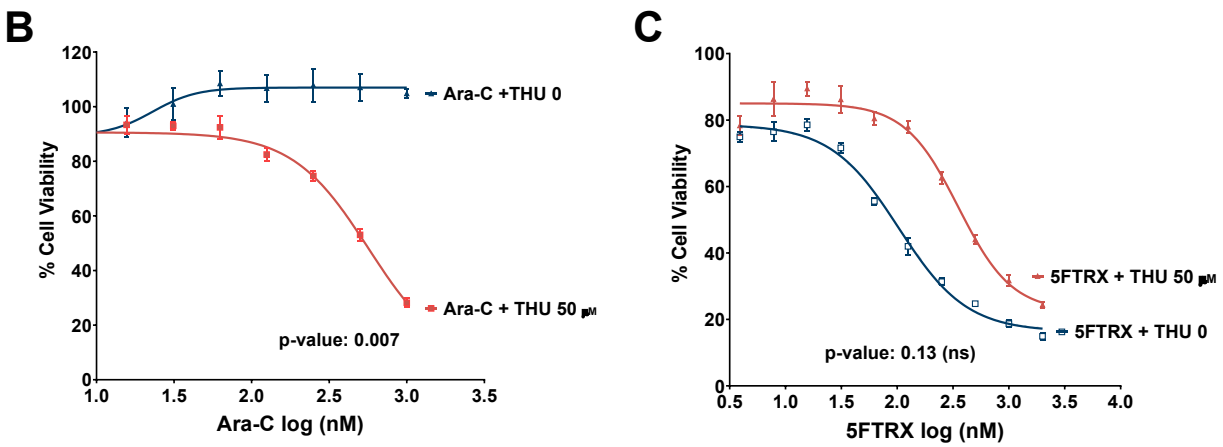
Figure S3

A



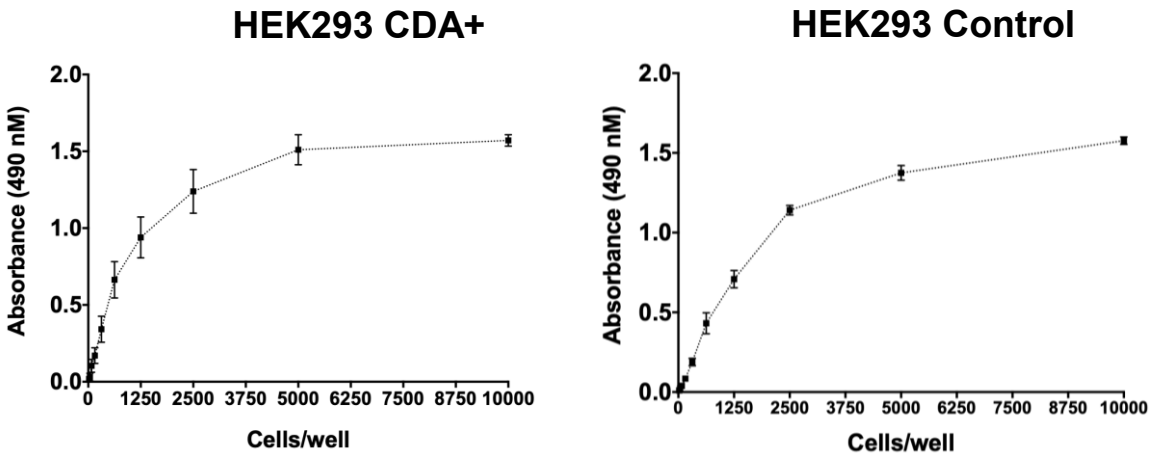
Unedited immunoblots for CDA overexpression using anti-CDA (A) primary antibody and anti-actin (B) secondary antibody.

Figure S3



HEK293 CDA+ were treated with increasing concentrations of Ara-C or 5FTRX in combination with tetrahydrouridine (THU) for 72 hours. After incubation cell growth and viability was measured by MTS assay. Data represent the mean ± SD IC_{50}. Student's *t*-test was used to calculate the p-value for significance of difference between the mean IC_{50} values.

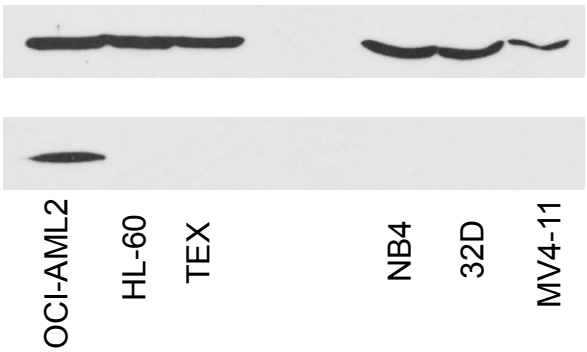
Figure S4



Increasing number of HEK293 CDA+ and control cells were cultured in a 96 well plate in a final volume of 100µl. After 96 hours incubation, growth and viability was measured using MTS assay. Each point represents mean of three replicates.

Figure S5

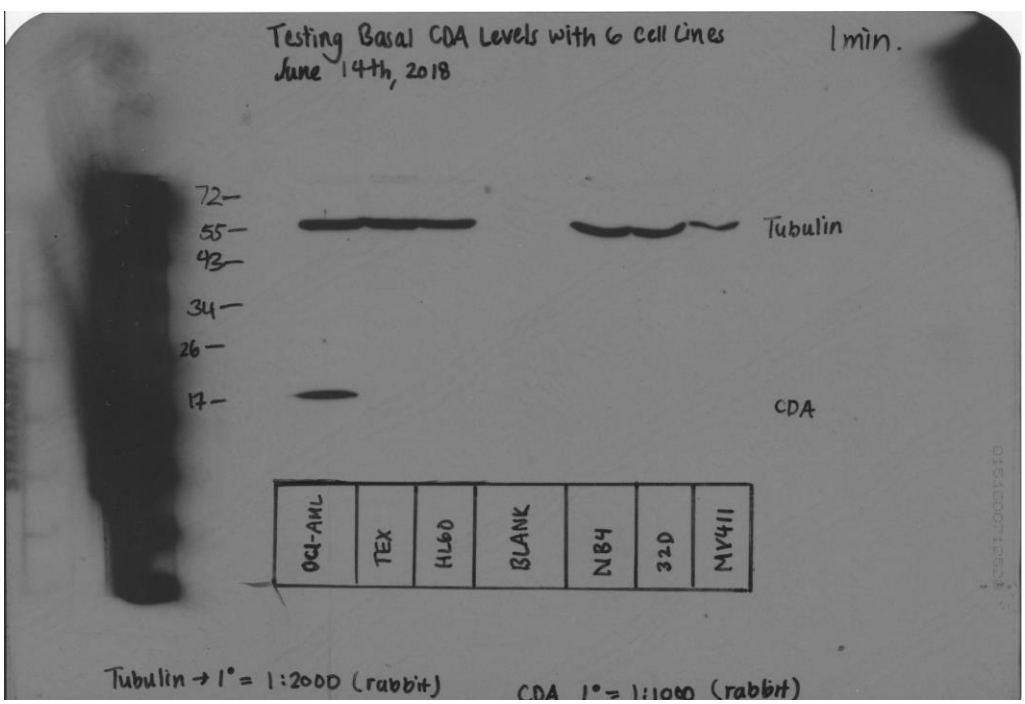
A



IC_{50}		
Cell line	**Ara-C**	**5FTRX**
OCI-AML2	306 nM	297 nM
TEX	7.990 nM	179 nM
HL60	4626 nM	2992 nM
NB4	188 nM	786 nM
32D	1694 nM	1432 nM
MV4-11	95 nM	121 nM

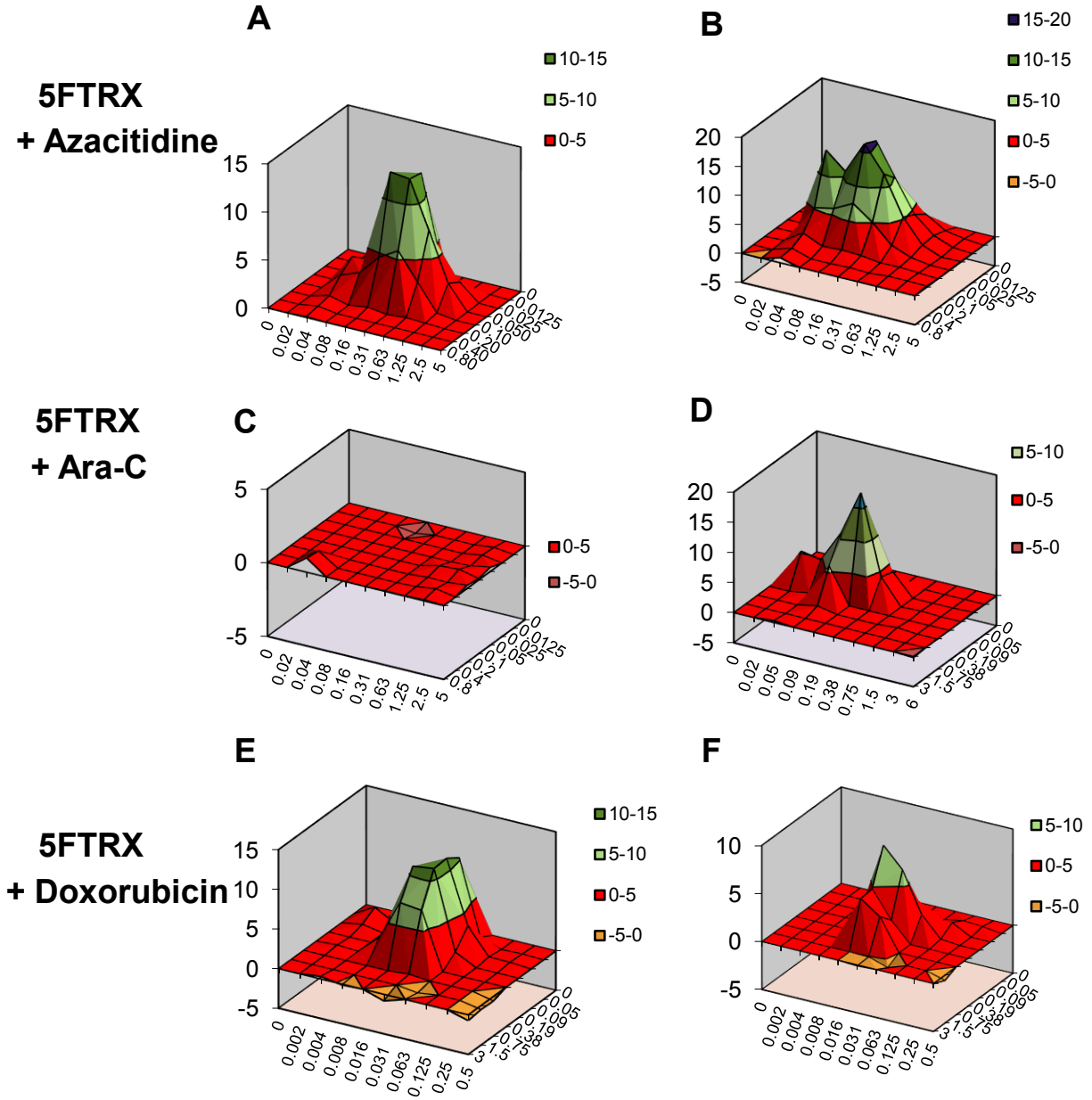
CDA expression in myeloid cell lines was measured by immunoblotting. Comparison of IC_{50} values with increasing concentrations of 5FTRX and Ara-C after incubation for 72 hours as measured using MTS assay is shown.

B



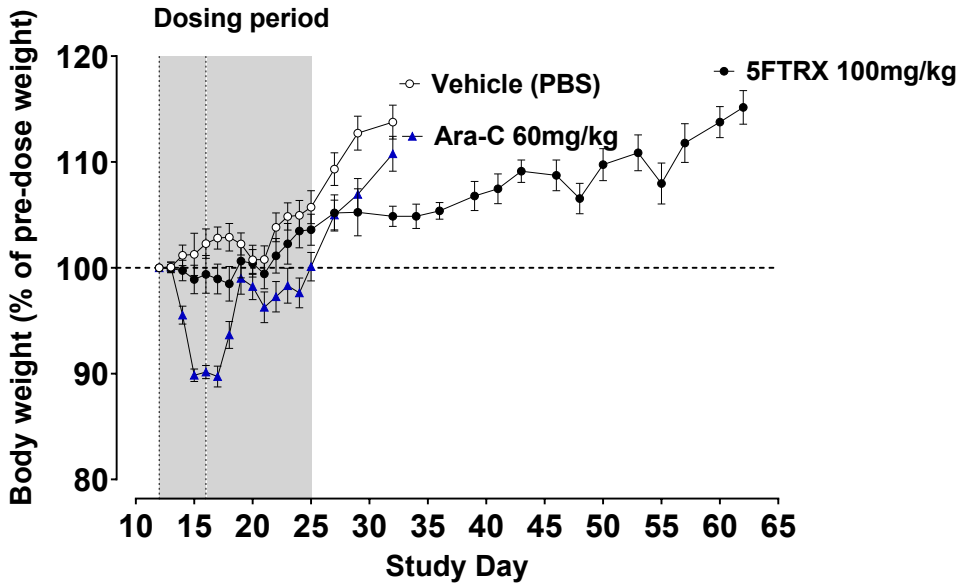
Unedited immunoblots for CDA overexpression in myeloid cell line using anti-CDA (A) primary antibody and anti-tubulin as secondary antibody.

Figure S6



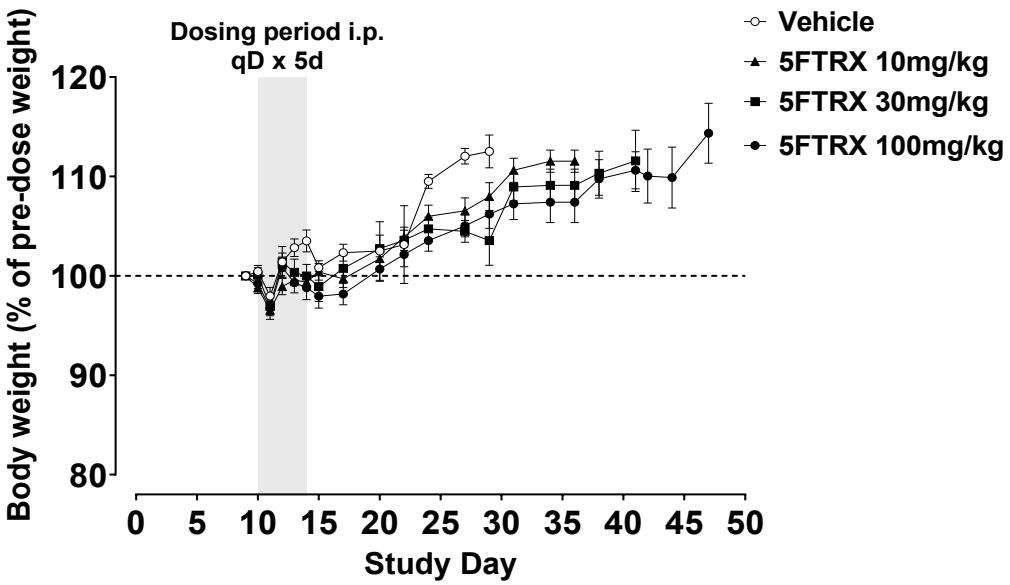
Bliss independent analysis of 5FTRX in combination with standard of care agents. The synergy scores are calculated across all the tested concentration combinations, visualized as a three-dimensional interaction surface over the dose matrix. The landscape of this interaction scoring identifies the specific dose regions where a synergistic or antagonistic drug interaction occurs. The height of the 3D drug interaction landscape is normalized as the % inhibition effect to facilitate a direct comparison of the degrees of interaction among multiple drug combinations. In addition, a summarized synergy score is provided **(Table S5)** by averaging over the whole dose-response matrix. This 3D synergy maps highlight synergistic combinations in green and additive combinations in red colors.
(A+B) MV4-11 (A) and THP1 (B) cells were treated with increasing concentrations of 5FTRX and azacytidine. (C+D) MV4-11 (C) and THP1 (D) cells were treated with increasing concentrations of 5FTRX and Ara-C (E+F) MV4-11 (E) and THP1 (F) cells were treated with increasing concentrations of 5FTRX and doxorubicin.
Synergy volumes were calculated as described in the supplement methods.

Figure S7



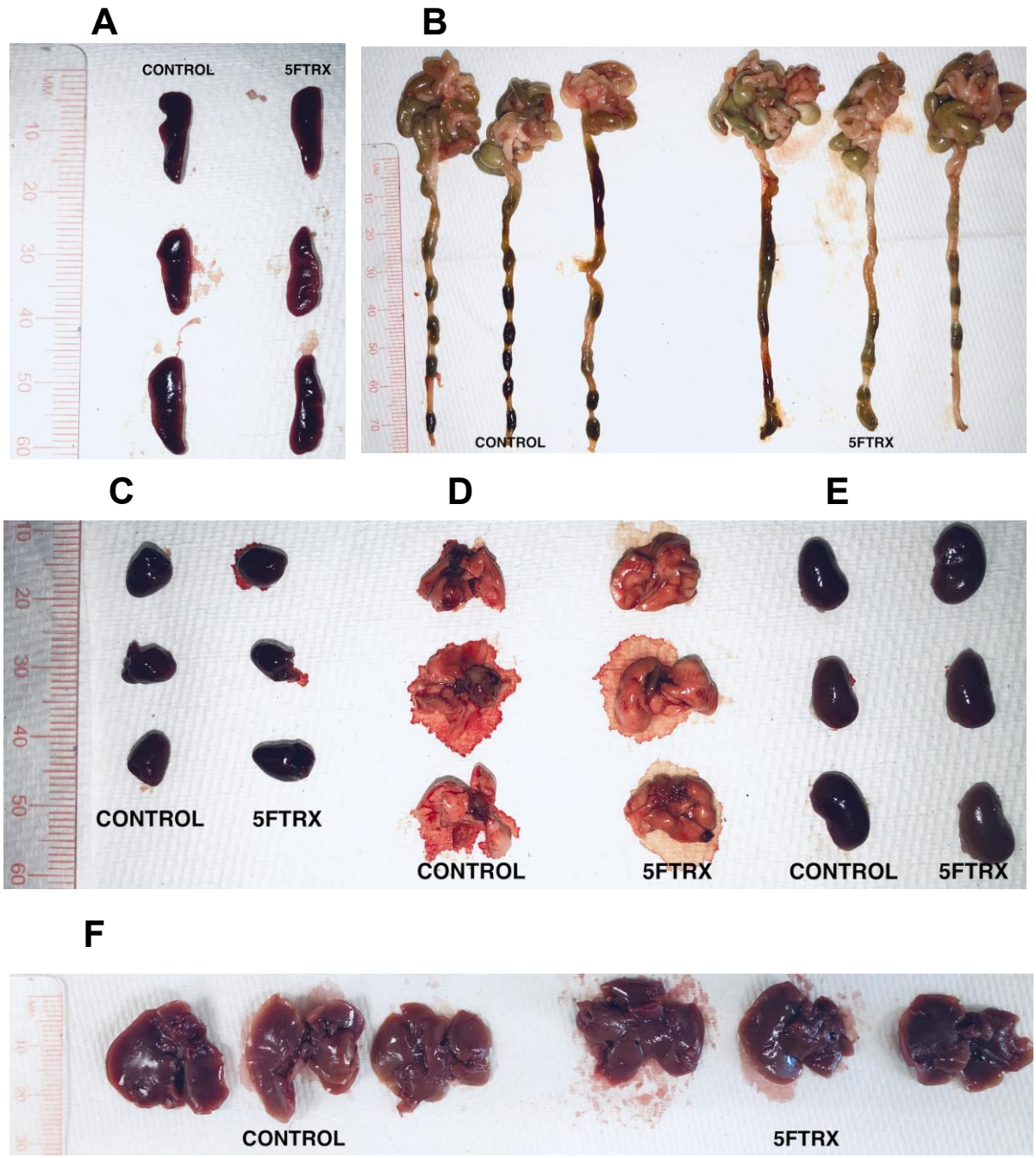
Body weight change during MV4-11 xenograft study. Dosing periods for 5FTRX (days 12-16) and Ara-C (days 12-25) are indicated in grey. No change in body weight was noted in the 5FTRX treated mice in comparison to control.

Figure S8



Change in body weight as a percentage of pre-dose body weight in MV4-11 xenografts is shown for different doses of 5FTRX and the dosing period shown indicated in grey area from d10-d14 inclusive.

Figure S9



OCI-AML2 leukemia cells (1×10^6) were injected subcutaneously into the flanks of Severe combined immune deficient (SCID) mice. After the appearance of tumor of mean size 200 mm^2, the mice were treated subcutaneously with 5FTRX 30 mg/kg once daily for 5 days or vehicle (saline) control (n=3 per group). Four days after the end of treatment, mice were sacrificed, and gross pictures of organs were obtained. (A) Kidneys (B) Colon (C) Heart (D) Lungs (E) Spleen (F) Liver.

Figure S10

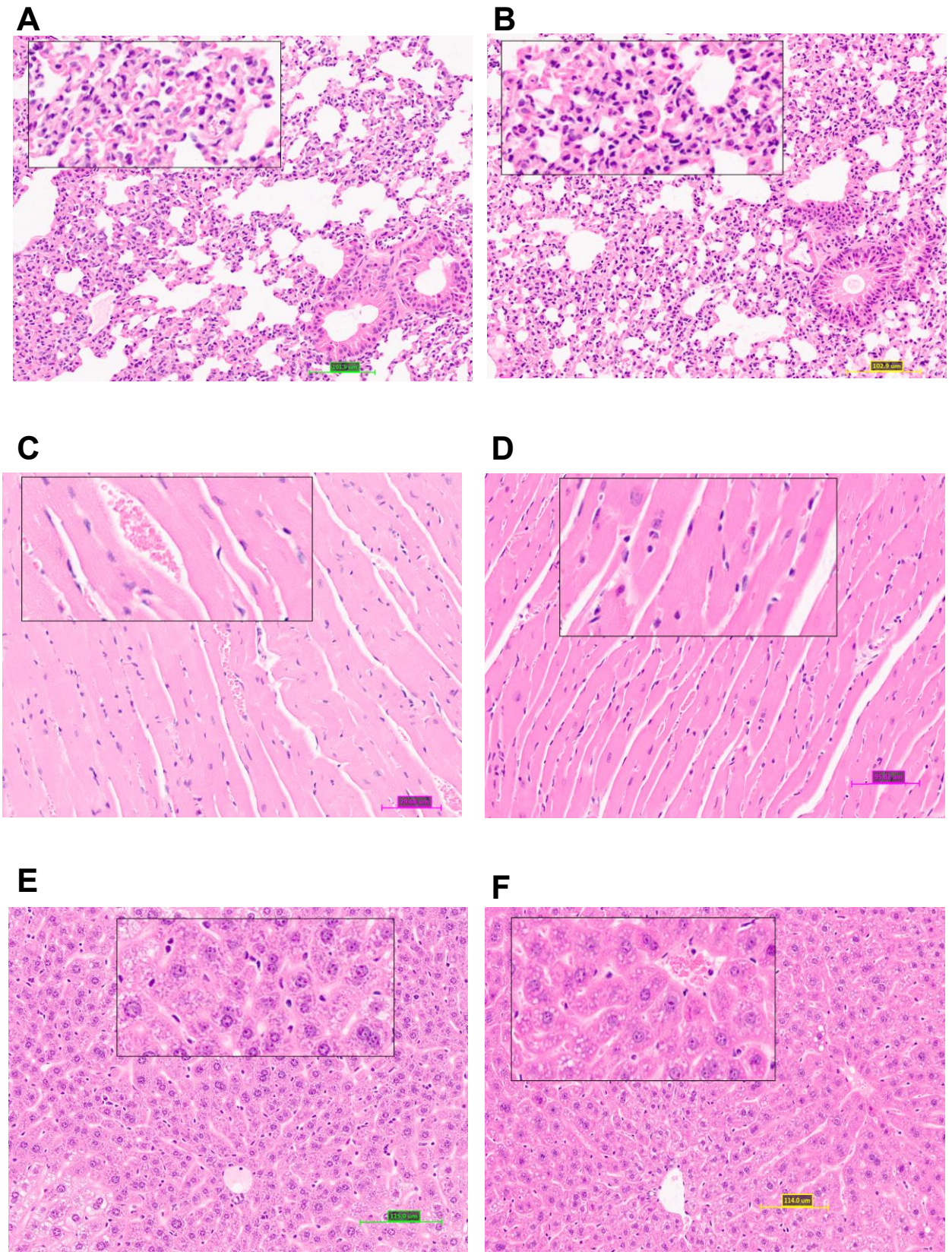
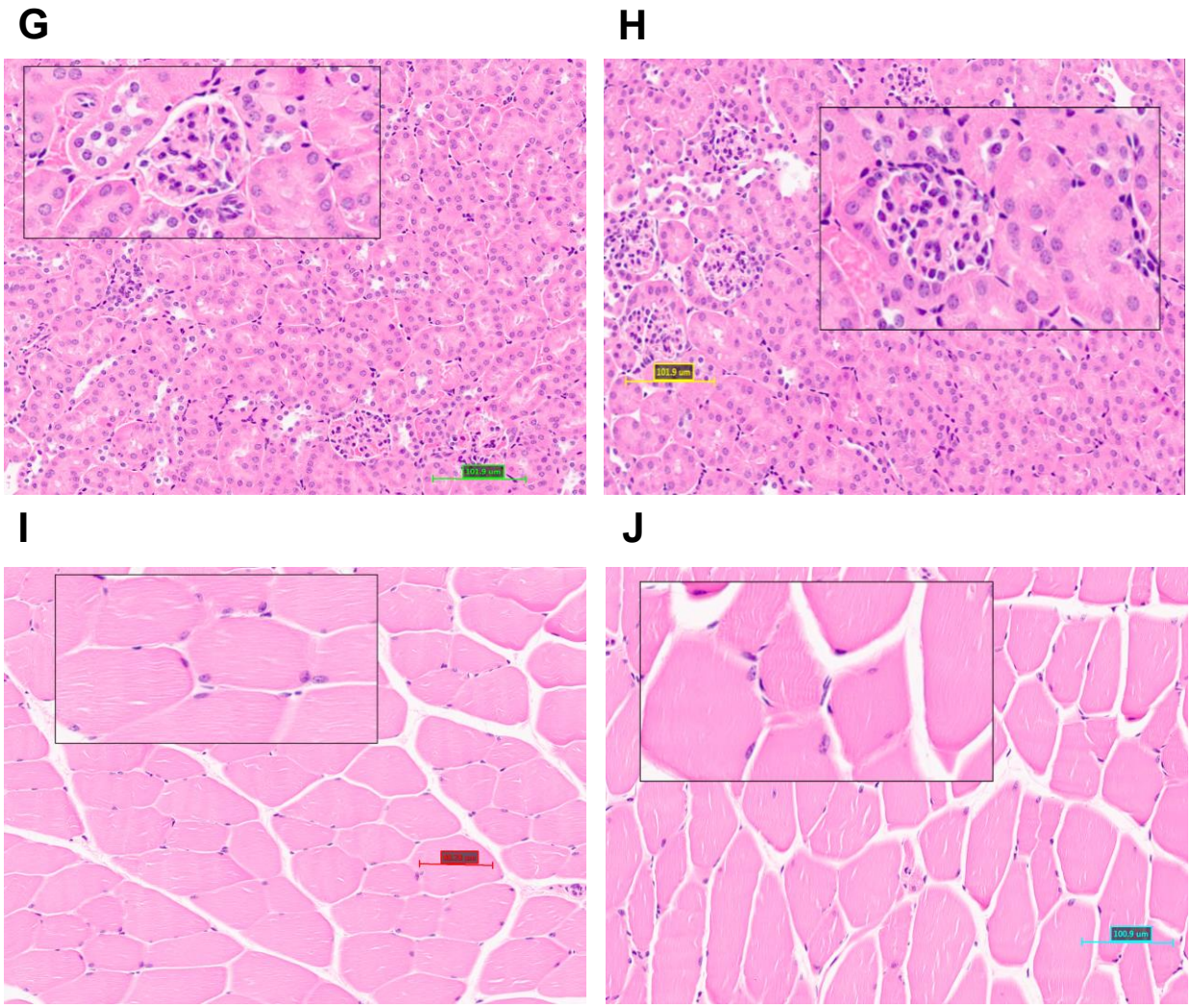


Figure S10



OCI-AML2 leukemia cells (1×10^6) were injected subcutaneously into the flanks of severe combined immune deficient (SCID) mice. After the appearance of tumor of mean size 200 mm^2, the mice were treated subcutaneously with 5FTRX 30 mg/kg once daily for 5 days or vehicle (saline) control (n=3 per group). Four days after the end of treatment, mice were sacrificed. Histopathological sections for organs prepared from control mouse for comparison with 5FTRX-treated mice (H&E stain 400x). Lung of control (A) and 5FTRX-treated (B) mouse; Cardiac muscle of a control (C) and 5FTRX treated mouse (D; Liver of control (E) and 5FTRX treated mouse (F); Kidney of control (G) and 5FTRX treated mouse (H); Skeletal muscle of control (I) and 5FTRX treated mouse (J).

Figure S11

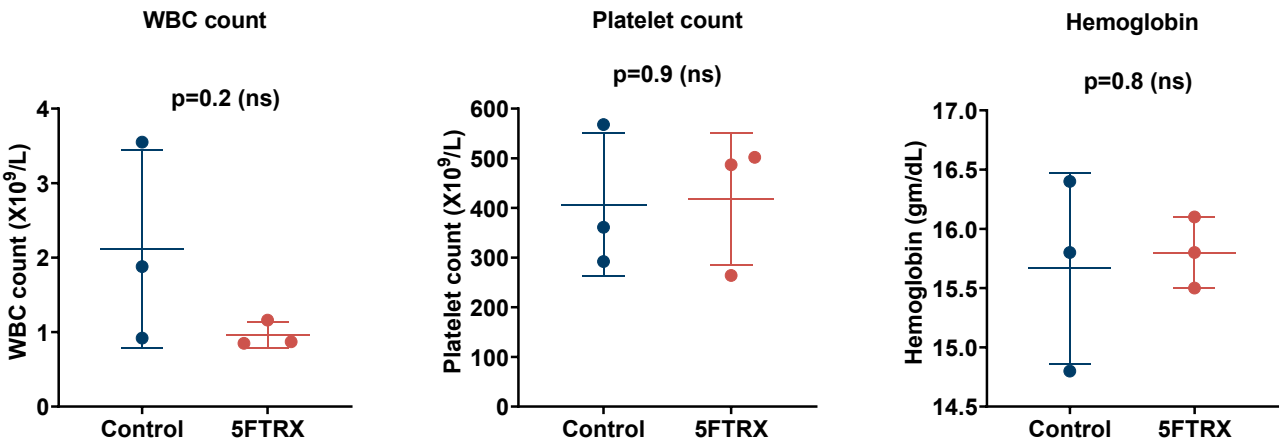


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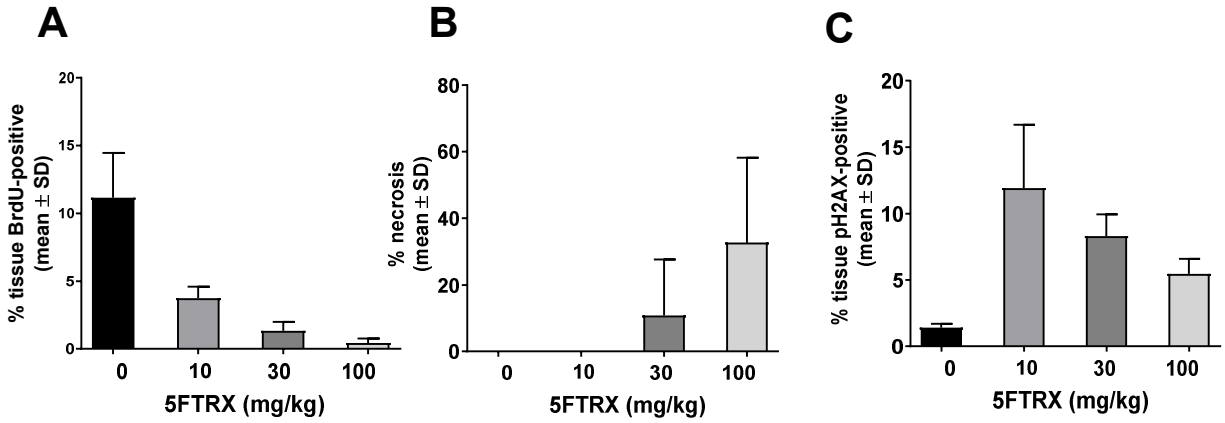
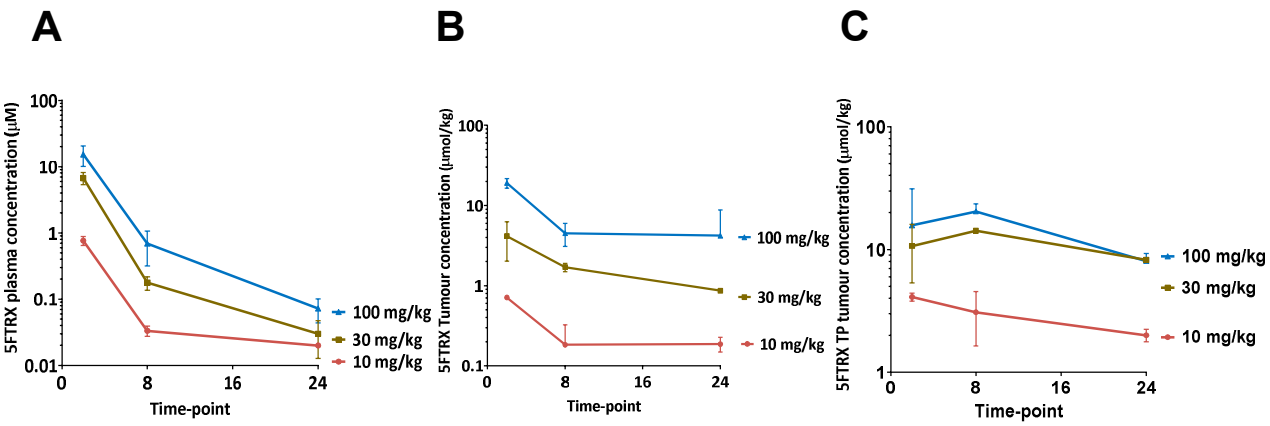
Figure S12



MV4-11 cells were xenografted into NOD/SCID mice. Once tumors were approximately 200 mm^3, mice were treated with increasing amounts of 5FTRX on days 1-5. Two hours after the last dose of 5FTRX, tumors were excised and stained for multiple immunofluorescent markers. (A) Proliferation was assessed by BrdUrd-positive staining cells. (B) Cellularity/necrosis was assessed by Hoechst-positive nuclear staining. (C) DNA damage was assessed by staining for phosphorylated histone H2AX (pH2AX). Data represent mean ± SD from 3 tumors per dose.

Figure S13



Mice with MV4-11 xenografts were treated with increasing concentrations of 5FTRX i.p. Levels of 5FTRX were measured in the plasma (A) or tumor (B) at 2h, 8h and 24h after 5FTRX treatment. (C) Levels of 5FTRX triphosphate (5FTRX TP) were measured 2h, 8h and 24h after 5FTRX treatment.