Co-targeting BCL-XL and BCL-2 by PROTAC 753B eliminates leukemia cells and enhances efficacy of chemotherapy by targeting senescent cells

Yannan Jia,[1,2] Lina Han,[1] Cassandra L. Ramage,[1] Zhe Wang,[1] Connie C. Weng,[1] Lei Yang,[1] Simona Colla,[1] Helen Ma,[1] Weiguo Zhang,[1] Michael Andreeff,[1] Naval Daver,[1] Nitin Jain,[1] Naveen Pemmaraju,[1] Kapil Bhalla,[1] Satu Mustjoki,[3,4,5] Peiyi Zhang,[6] Guangrong Zheng,[6] Daohong Zhou,[7] Qi Zhang[1] and Marina Konopleva[1]

[1]Department of Leukemia, The University of Texas MD Anderson Cancer Center, Houston, TX, USA; [2]Department of Hematology, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China; [3]Hematology Research Unit Helsinki, University of Helsinki and Helsinki University Hospital Comprehensive Cancer Center, Helsinki, Finland; [4]Translational Immunology Research Program and Department of Clinical Chemistry and Hematology, University of Helsinki, Helsinki, Finland; [5]iCAN Digital Precision Cancer Medicine Flagship, Helsinki, Finland; [6]Department of Medicinal Chemistry, College of Pharmacy, University of Florida, Gainesville, FL, USA and [7]Department of Biochemistry and Structural Biology and Center for Innovative Drug Discovery, Long School of Medicine, University of Texas Health Science Center at San Antonio, San Antonio, TX, USA

Correspondence: M. Konopleva
mkonople@mdanderson.org
Q. Zhang
qzhang9@mdanderson.org

Received: August 22, 2022.
Accepted: April 7, 2023.
Early view: April 20, 2023.

https://doi.org/10.3324/haematol.2022.281915

Supplementary methods

Classical SA-β-gal activity assay using X-Gal

SA-β-gal staining kit (Cat# 9860, Cell Signaling Technology) was used for SA-β-Gal analyses and performed according to the manufacturer's protocol.

BH3 profiling

BH3 profiling was conducted as previously reported (3). In brief, cells were treated with DMSO or 753B for 4 hours, and then permeabilized with digitonin and exposed to BH3 peptides (BIM, FS-1, MS-1, BAD, and HRK, synthesized by New England Peptide, Gardner, Massachusetts, USA) or MCL-1 inhibitor (S63845). Mitochondrial transmembrane potential loss was monitored using cytochrome C.

Colony Forming Unit (CFU) Assay

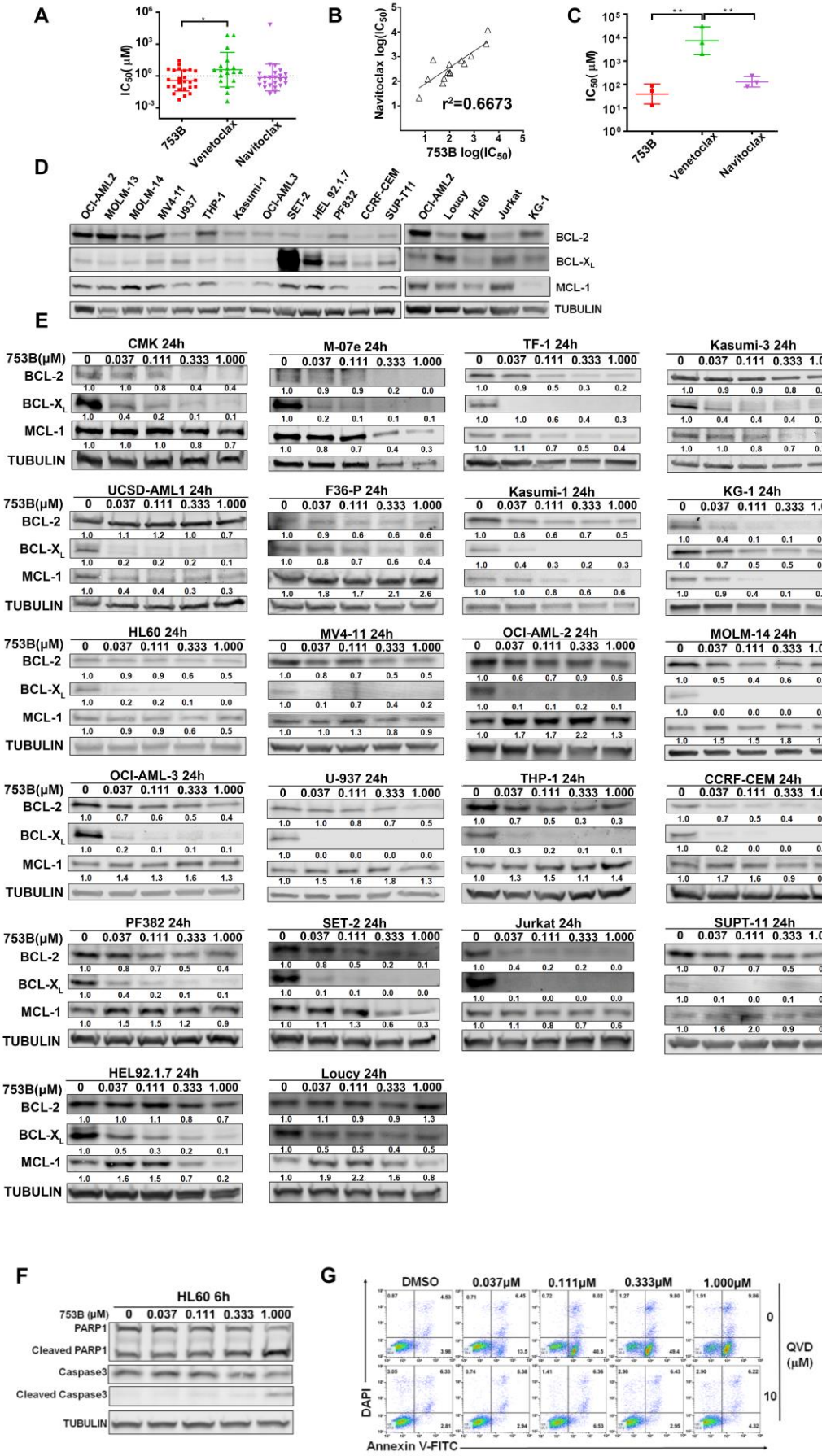
The CFU assay was performed to determine the effects of 753B on colony formation of CD34+ bone marrow cells sorted form healthy donors under different treatment conditions. CD34+ bone marrow cells were transferred to 4 ml Methocult media (Gibco) and incubated in 3 wells in the 6 well culture dish. The cells were incubated for 7 days at 37 °C, 5%CO_2. After 7 days the different hematopoietic colonies like CFU-E, CFU-G, CFU-M, CFU-GM or CFU-GEMM were counted manually under phase contrast microscope and data recorded.

Histology and immunohistochemistry

Tissues were fixed in 10% formalin overnight and embedded in paraffin. For immunohistochemistry, slides were deparaffinized in xylene and rehydrated sequentially in ethanol. For antibodies requiring antigen retrieval, antigen unmasking solution (Vector Lab, Burlingame, CA, USA) was used according to the manufacturer's instructions. Slides were quenched in hydrogen peroxide (0.3%-3%) to block endogenous peroxidase activity and then washed in automation buffer. Slides were blocked in 5% normal serum for 1 h at room temperature. Slides were incubated overnight at 4°C with primary antibody diluted

in blocking buffer. The avidinbiotin peroxidase complex method (Vector) was used, and slides were counterstained with hematoxylin. Slides were dehydrated sequentially in ethanol, cleared with xylenes, and mounted with Permount (Fisher). BCL-XL (DAKO) was used at 1:100. Biotinylated DBA lectin (Vector) was used at 1:100.

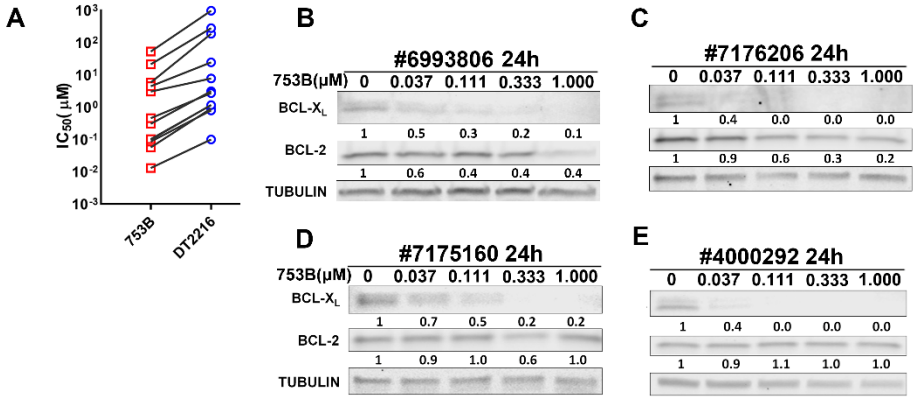
Figure S1


A
B
C
D
E
F
G
753B
Venetoclax
Navitoclax
r²=0.6673
DMSO
0.037μM
0.111μM
0.333μM
1.000μM
QVD (μM)
DAPI
Annexin V-FITC
PARP1
Cleaved PARP1
Caspase3
Cleaved Caspase3
TUBULIN
BCL-2
MCL-1
753B(μM)
CMK 24h
M-07e 24h
TF-1 24h
Kasumi-3 24h
UCSD-AML1 24h
F36-P 24h
Kasumi-1 24h
KG-1 24h
HL60 24h
MV4-11 24h
OCI-AML-2 24h
MOLM-14 24h
OCI-AML-3 24h
U-937 24h
THP-1 24h
CCRF-CEM 24h
PF382 24h
SET-2 24h
Jurkat 24h
SUPT-11 24h
HEL92.1.7 24h
Loucy 24h
HL60 6h

Supplementary Figure 1. 753B is more potent in reducing cell viability in a subset of hematologic cell lines compared with other BCL-X_L and/or BCL-2 targeting agents via degradation of BCL-X_L and BCL-2.

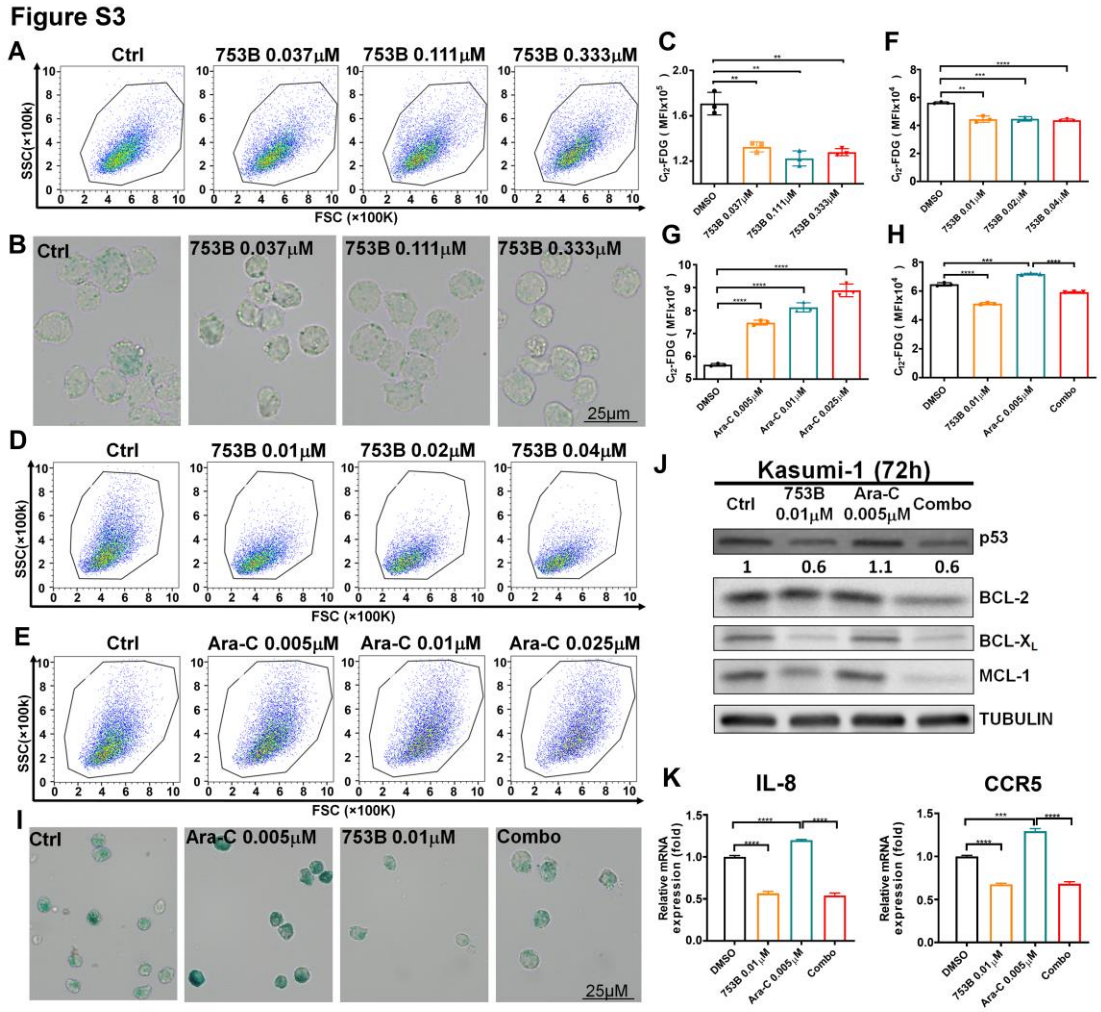
A. IC_{50} values of 753B, venetoclax, and navitoclax in leukemia cell lines treated for 24 hours. IC_{50} values were calculated based on the percentage of viable cells, normalized to control, as determined by CellTiter-Glo assay. **B**. IC50s for navitoclax (y-axis) and 753B (x-axis) in hematological cell lines listed in Figure 1B. Linear regression is solid black line. Correlation coefficient is discussed in the text. **C.** IC_{50} values of 753B, venetoclax, and navitoclax in 3 AML-EVI-1 (MECOM) rearranged cell lines (Kasumi-3, UCSD-AML1 and HNT37) treated for 24 hours. IC_{50} values were calculated based on the percentage of viable cells, normalized to control, as determined by CellTiter-Glo assay. **D.** Protein levels of BCL-2, BCL-X_L, and MCL-1 in 17 untreated leukemia cell lines detected by Western blotting. **E.** Western blotting showing protein levels of BCL-X_L, BCL-2, and MCL-1 in 22 cell lines treated with the indicated concentrations of 753B. The band intensity was quantified using Odyssey v2.0 software and was displayed numerically as a ratio of the band intensity detected in untreated cells. **F.** HL60 cells were grown in 6-well plates and treated with 753B at the indicated concentration for 24 hours, followed by Western blotting with the indicated antibodies including PARP, caspase-3 and cleaved caspase-3. **G.** Representative graphs of flow cytometry showing apoptosis of KG-1 cells treated with 753B at the indicated concentrations for 24 hours with or without the pan-caspase inhibitor QVD.

Figure S2



Supplementary Figure 2. 753B is a more potent antitumor agent than DT2216 in both leukemia cell lines and primary patient samples.

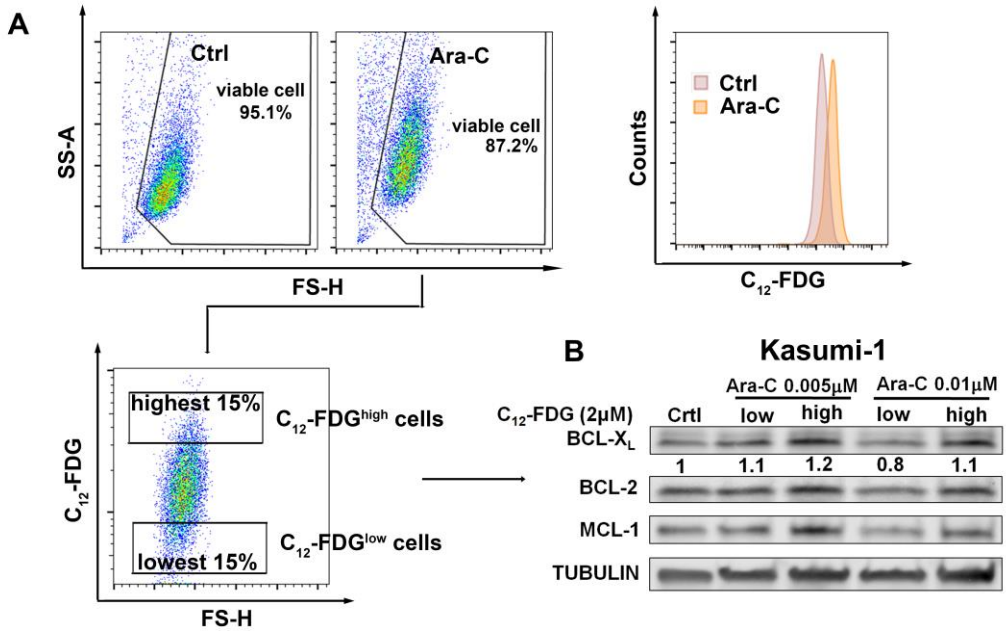
A. IC_{50} values of 753B and DT2216 in 14 BCL-X_L-dependent cell lines (CMK, Kasumi-3, M-07e, F-36P, UCSD-AML1, HNT37, TF-1, CCRF-CEM, PF832, Loucy, Jurkat, SUP-T11, HEL 92.1.7, SET-2). B-E. Western blotting analysis of BCL-2 family proteins in 4 AML primary patient samples treated with the indicated concentrations of 753B for 24 h.



Supplementary Figure 3. 753B enhances the efficacy of chemotherapy by eliminating senescent leukemia cells.

A-C. FSC vs SSC plots (A), representative images of SA-β-gal staining (B), and flow cytometry histogram (C) of MOLM-14 cells treated with increasing concentrations of 753B. **D-G.** Kasumi-1 cells were treated with increasing doses of 753B and Ara-C and stained using the fluorogenic β-galactosidase substrate C_{12}-FDG. FSC vs SSC plots (D and E) show cell size, and histograms (F and G) show senescence-associated β-galactosidase (SA-β-gal) activity detected by flow cytometry. **H** and **I**. Flow cytometry histogram (H) and SA-β-gal staining images (I) depicting SA-β-gal activity in Kasumi-1 cells, gated on viable cells ($DAPI^-$exclusion) and treated with Ara-C (0.005 μM), 753B (0.01 μM), or the combination. **J** and **K.** Protein levels of p53, BCL-X_L, BCL-2, and MCL-1 (J) and mRNA expression of senescence-associated secretion phenotype (*IL8*, *CCR5*) genes in Kasumi-1 cells exposed to Ara-C (0.005 μM), 753B (0.01 μM), or the combination (K). $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

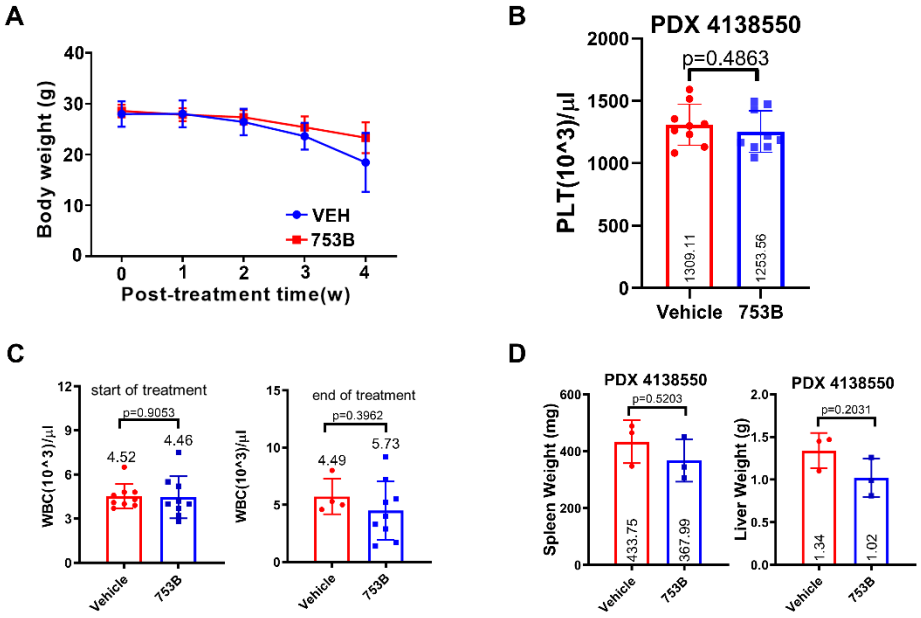
Figure S4



Supplementary Figure 4. Chemotherapy-induced senescent cells express higher levels of BCL-X_L, representing a therapeutic target for 753B.

A. Flow cytometry sorting strategy for C_{12}-FDG-low (lowest 15%) and C_{12}-FDG-high (highest 15%) viable AML cells after 3 days Ara-C treatment. **B.** Kasumi-1 cells were treated with Ara-C (0.005 μM or 0.01 μM) for 72 h. After treatment, cells were sorted based on C_{12}-FDG staining into 2 groups: C_{12}-FDG-low (lowest 15%) and C_{12}-FDG-high (highest 15%). Cells were then lysed for Western blotting showing BCL-X_L, BCL-2, and MCL-1 protein levels in these two groups compared with control cells.

Figure S5



Supplementary Figure 5. Anti-leukemia efficacy of 753B *in vivo* in AML PDX model.

A. Body weight changes in mice before and after treatment with vehicle (VEH) or 753B (5 mg/kg body weight, intraperitoneally, every 4 days). Data are presented as mean ± SEM (n = 9 mice/group at the start of treatment). **B**. Blood platelet (PLT) counts 1 day after the first treatment with vehicle or 753B. Data are presented as mean ± SEM (n = 9 mice per group). **C**. White blood cell (WBC) counts 1 day after first treatment (left panel) and 1 day after last treatment (right panel) with 753B or vehicle. Data are presented as mean ± SEM (left n = 9 mice/group; right n = 9 mice for 753B, n = 4 mice for vehicle). Each point represents data from an individual animal. Statistical significance was determined by 2-sided unpaired Student t-test. **D**. Spleen and liver weights of 3 mice euthanized at the end of treatment. Statistical significance was determined by 2-sided unpaired Student t-test.

Supplementary Table 1. Clinical characteristics of AML patients whose peripheral blood samples were used in this study.

UPIN	Blast (%)	Status	Cytogenetics	Molecular Mutations	Sample date/Ven	MK status*	SD /Ven	Clinical response to Navitoclax	IC50（nM）			
									DT2216	753B	Venetoclax	Navitoclax
4000292	91	R/R	48~50,XY,+der(1;3)(q10;q10),t(1;3)(q10;p10),+5,del(5)(q13q33)x2,-15,del(20) (q11.2q13),+21,+1~2 mar[cp20]	BCOR, BRAF, DNMT3A, IKZF1, RAD21, RUNX1, TET2, U2AF1	Rel post Rx3	rel	Post	RX3 Clad+LDAC+Ven: CR	166.3	18	14.01	9.28
7126060	29	Newly Dx	46,XY,t(6;11)(q27;q13),t(9;15)(p10;p10)[1]/46,XY,add(11)(q13)[1]/46,XY[19]	CEBPA, DNMT3A, JAK2, TET2, ZRSR2	Newly Dx/Pre Rx1	ND	Pre	Rx1 Aza+Ven+Pevo: CR	1421	267	1908	1050
7131150	40	R/R	47,XY,+8[2]/47,idem,del(10)(p11.2),add(19)(p13.3)[11]/47,idem,t(1;7)(p32;q11.2)[7]	FLT3-ITD, WT1	Pre Rx3	refr	Pre	Rx3 Aza+Ven+Gilt: NR; Rx5 Dav+Ven+Quiz: MLFS	353.2	46.1	39.11	36.87
7094156	37	R/R	45,XY,t(3;3)(q21;q26.2),-7[7]/46,idem,+mar[19]	DNMT3A, RUNX1, SF3B1, ASXL2, CBL, GATA2	Pre Rx5	refr	Post/ Pre	Rx1 Aza+Ven: CRi; Rx2 Dac+Ven: NR; Rx3 AZD5153+Ven: NR; Rx5 Aza+Ven+Gilt: NR	291	31.7	919	327.2
7136628	87	R/R	46,XY,add(1)(p13),add(3)(p13),del(12)(p12),del(13)(q12q22)[17]/47,idem,+Y[1]/46,XY[2]	ASXL2, SUZ12	Pre Rx2	rel but no prior ven	Pre	Rx 1 ALL(HCVAD>MUD1,FLAG+I>Haplo2)>AML(FLAG>MUD3). Rx2 Aza+Ven+Magro: CR	730.9	88.1	16.24	29.97
6566444	81	R/R	37~44,XX,-X,+1,add(1)(q42),del(2)(q33),der(3;7)(q10;q10),-5,add(5)(q13),-	JAK2,TP53,U2AF2,PIGA	Rel post Rx1/Pre Rx2	rel	Post/ Pre	Rx1 Dac+Ven: CR; Rx2 Palbociclib+Ven: NR	1653	243	29.97	96.43

			8,add(9)(p13),-10,-12,-14,-17,-19,-20,+22,+1~3 mar[cp12]									
6993806	30	R/R	47,XY,del(5)(q15q33),+8[20]	NRAS(2), BCOR, PTPN11, RUNX1, BCORL1 (2)	Pre Rx3	refr	Pre	Rx3 Dac+Ven: NR	4836	446	4309	726.7
6601650	ND	R/R	45,XX,inv(3)(q21q26.2),-7[19]/45, idem,t(1;10)(q21;q24)[1]	FLT3 (not ITD/D835), IKZF1, PTPN11, WT1	NR post Rx8	refr	Post	Rx4 FLAG+I+Ven: NR; Rx5 CYC065+Ven: NR	1602	175	351.5	102
4433762	87	R/R	47,XX,t(9;11)(p22;q23),+21[19]/46,XX[1]	No Mutations	Pre Rx4	refr	Post/ Pre	Rx2 Aza+Ven: NR; Rx4 Dac+ven: NR; Rx8: Clad+LDAC+Ven: NR	1742	267	10135	163
4451422	22	Newly Dx	46,XX,t(7;11)(p15;p15)[20]	KRAS, RUNX1	Newly Dx/Pre Rx1	ND	Pre	Rx1 Aza+Ven+Gilt: CR	25563	2291	469460	7075
7176206	54	Newly Dx	46,XX,del(7)(q22q34),der(16)inv(16)(p13.1q22)del(16)(q22)[18]/46,XX[2]	KRAS,FLT3-D835; CBFB-MYH11	Newly Dx	ND		No Ven Rx	1751	405	604.1	621.2
7134560	48	R/R	47,XY,add(5)(q33),del(6)(q14),+8[18]/47,idem,del(3)(p22),add(10)(q24),del(12)(p11.2p13)[1]/47,idem,t(2;7)(p10;q10)[1]	NRAS, PHF6, RUNX1, TET2, WT1 x2	NR post Rx6/Pre Rx7	refr	Post/ Pre	Rx3 Aza+Ven: NR; Rx6 CPX351+Ven: NR; Rx7 BID FA+Ven: NR	7374	1030	4450	559
7175160	38	Newly Dx	46,XY[20]	IDH2, NRAS, FLT3-ITD, IDH1, NPM1	Newly Dx	ND		No Ven Rx	979.1	213	63.21	22.44
7153558	91	R/R	46,XY,del(7)(q21)[20]	DNMT3A, IDH2, KIT, KRAS(2), NRAS, RUNX1(3), IKZF1, PHF6, PTPN11	Rel post Rx4	rel	Post	Rx4 Dac+Ven: CR	1101	145	20.27	40.6
7116584	41	R/R	46,XY,del(13)(q13q22)[12]/47,idem, +8[8]	KRAS, PTPN11, RUNX1, STAG1, TERT, TP53	Pre Rx4	refr	Post	Rx2 Dac+Ven: NR	3861	1100	4465	657.3

3761622	ND	Newly Dx	46,XX[20]	IDH1, IDH2, DNMT3A, NPM1, PTPN11	Newly Dx/Pre Rx1	ND	Pre	Rx1 Dac+Ven+Ivosidenib: CR	261.9	35.7	1360	26.04

* rel---relapse while on Ven; refr---refr to ven-based therapies; ND---newly diagnosed

Supplementary Table 2. IC_{50} (μM) and DC_{50} (nM) values (BCL-X_L and BCL-2) of 753B in 24 leukemia cell lines.

Type	Cell line	IC_{50}[1] (μM)	DC_{50}[2] (BCL-X_L, nM)	DC_{50} (BCL-2, nM)
AML	CMK	0.006	26.1	398.5
AML(EVI-1r)	Kasumi-3	0.013	13.7	>1uM
AML	M-07e	0.021	13.3	216.2
AML	F-36P	0.056	543.4	>1uM
AML(EVI-1r)	UCSD-AML1	0.053	63.2	>1uM
AML	Kasumi-1	0.060	32.3	982.2
AML(EVI-1r)	HNT37	0.086		
AML	TF-1	0.102	6.6	157.8
AML	KG-1	0.104	26.5	1057.0
AML	HL60	0.247	15.1	825.2
AML	MV4-11	0.396	42.2	611.8
AML	OCI-AML-2	0.562	10.6	>1uM
AML(EVI-1r)	MOLM-1	0.754		
AML	MOLM-14	3.186	9.4	>1uM
AML	OCI-AML-3	4.593	12.6	331.5
AML	U-937	11.938	12.6	828.7
AML	THP-1	27.352	12.6	121.5
T-ALL	CCRF-CEM	0.097	19.5	96.0
T-ALL	PF832	0.309	24.7	413.7
T-ALL	Loucy	0.454	149.1	>1uM
T-ALL	Jurkat	3.536	11.2	23.8
T-ALL	SUP-T11	3.920	12.1	496.5
MPN-AML	HEL 92.1.7	1.484	24.7	>1uM
MPN-AML	SET-2	4.449	12.0	99.9

[1]IC_{50}, the half maximal inhibitory concentration, the drug concentration causing 50% cell inhibition

[2]DC_{50}, the drug concentration causing 50% protein degradation

Supplementary Table 3. Upregulation of MCL-1 in 22 hematological cell lines with increasing concentration of 753B

		753B (μM)				
Type	Cell line	0	0.037	0.111	0.333	1
AML	CMK	1	1	1	0.8	0.7
AML	Kasumi-3	1	1	0.8	0.7	0.7
AML	M-07e	1	0.8	0.7	0.4	0.3
AML	F-36P	1	1.8	1.7	2.1	2.6
AML	UCDS-AML1	1	0.4	0.4	0.3	0.3
AML	Kasumi-1	1	1	0.8	0.6	0.6
AML	TF-1	1	1.1	0.7	0.5	0.4
AML	KG-1	1	0.9	0.4	0.1	0.1
AML	HL60	1	0.9	0.9	0.6	0.5
AML	MV4-11	1	1	1.3	0.8	0.9
AML	OCI-AML-2	1	1.7	1.7	2.2	1.3
AML	MOLM-14	1	1.5	1.5	1.8	1.6
AML	OCI-AML-3	1	1.4	1.3	1.6	1.3
AML	U-937	1	1.5	1.6	1.8	1.3
AML	THP-1	1	1.3	1.5	1.1	1.4
T-ALL	CCRF-CEM	1	1.7	1.6	0.9	0.8
T-ALL	PF832	1	1.5	1.5	1.2	0.9
T-ALL	Loucy	1	1.9	2.2	1.6	0.9
T-ALL	Jurkat	1	1.1	0.8	0.7	0.6
T-ALL	SUP-T11	1	1.6	2	0.9	0.9
MPN-AML	HEL 92.1.7	1	1.6	1.5	0.7	0.2
MPN-AML	SET-2	1	1.1	1.3	0.6	0.3