Supplementary information to

The dual BCL-2 and BCL-XL inhibitor AZD4320 acts on-target and synergizes with MCL-1 inhibition in B-cell precursor ALL

Materials and Methods:

Cell lines

Seven different BCP-ALL cell lines were used: RS4;11, KOPN-8, REH, EU-3, RCH-ACV and NALM-6 (purchased from DSMZ, Germany), UoCB6 (provided by J. Rowley, USA). Cell lines were cultured in RPMI-1640, 20% fetal bovine serum, 1% L-Glutamine and 1% Penicillin/Streptomycin. Cell lines were authenticated by short-tandem repeat (STR)-profiling (GenePrint 10 System, Promega) and tested for negativity of mycoplasma (MycoAlert Mycoplasma Detection Kit, Lonza).

Patient-derived xenograft samples

Samples were collected from patients with BCP-ALL after obtaining informed written consent in accordance with institutional ethics review board guidelines. Patient-derived xenograft (PDX) samples were established by intravenously transplanting ALL cells into female NOD/SCID mice (NOD.CB17-Prkdcscid, Charles River), following established protocols[1]. Animal experiments received approval (RP Tübingen, No. 1260). Genetic alterations of the samples were assessed by Multiplex Ligation-dependent Probe Amplification (MLPA) and RT-PCR, as previously described[2]. For *ex vivo* experiments analyzing EC_{50} values, PDX samples were cultured in AIM V medium (Gibco) without supplements.

Cell death assays

Cell death rates were assessed by propidium iodide (PI, P3566, Invitrogen) staining following exposure to AZD4320 and AZD5991 for 48h in cell lines and for 24h in PDX samples. To determine the half maximal effective concentration (EC_{50}), cells were exposed to eleven different concentrations (cell lines: 0.1, 1, 5, 10, 50, 100, 250, 500, 1000, 5000 and 10000 nM; PDX samples: 0.1, 1, 2.5, 5, 10, 25, 50, 100, 500, 1000 and 5000 nM) of each inhibitor. The EC_{50} values of venetoclax, A-1331852 and S63845 have been published previously[3]. Combination effects in cell lines were analyzed using dose-response matrix analyses upon treatment with seven different concentrations of the inhibitors (0.1, 1, 2.5, 5, 10, 100 and 500 nM) for 48 hours. Samples were measured on an Attune NxT Flow Cytometer (Thermo Fisher) using an autosampler and analyzed using the FlowJo 10.7.1 software.

ALL co-culture model with mesenchymal stroma cells (MSCs)

Primary bone marrow hTERT-immortalized mesenchymal stroma cells (MSCs)[4] were kindly provided by Dario Campana. For co-culture experiments, MSCs were seeded on day -1 at $5x10^3$ per well in 96-well plates (REF83.3924, Sarstedt) in RPMI-1640 medium supplemented with 20% fetal bovine serum, 1% L-Glutamine and 1% Penicillin/Streptomycin. After four hours, $1x10^5$ ALL PDX cells or PBMCs stained with 1 µM CellTrace Violet (C34557, Thermo Fisher) were added to the MSC cultures. On day 0, samples were exposed to increasing concentrations of the inhibitors (0.1, 1, 2.5, 5, 10, 100 and 500 nM) in a drug matrix for 72 hours. Cell death rates were assessed on an Attune NxT Flow Cytometer (Thermo Fisher) with an autosampler using propidium iodide (PI) and CellTrace Violet staining to distinguish ALL PDX cells or PBMCs from MSCs. Data analysis was performed using FlowJo 10.7.1 software.

Dynamic BH3 profiling

Dynamic BH3 profiling was carried out as previously described[5-7]. RCH-ACV cells were exposed to AZD4320 or AZD5991 for two hours. Cells were then permeabilized using digitonin, and exposed to the pro-apoptotic peptides indicated. Thereafter, cells were stained using a viability marker (1:1000 Zombie Violet; 423113, BioLegend). Cells were then fixed (formaldehyde) and neutralized in N2 buffer. Finally, cells were incubated in an intracellular staining buffer (1:1000 Alexa Fluor 647 anti-Cytochrome c antibody; 612310, BioLegend). Results were analyzed on an Attune NxT Flow Cytometer (Thermo Fisher) with an autosampler and processed with FlowJo 10.7.1.

Western blot and immunoprecipitation

Western blot analysis and immunoprecipitation were performed according to established protocols[3]. Protein extraction was performed with lysis buffer containing 30 mM Tris-HCl, 1% Triton X-100 buffer, 10% glycerol. For immunoprecipitation, cell lysates were co-incubated with BIM (C34C5) Rabbit mAb (2933, Cell Signaling) at 4°C on a rotary overnight, followed by co-incubation with Protein A Agarose Beads (9863, Cell Signaling) for 2-4 hours at 4°C. The beads were then washed five times with lysis buffer and the precipitates were subjected to western blot analysis. For immunoblotting, the following antibodies were used: Bim (C34C5) Rabbit mAb (2933, Cell Signaling), Bcl-2 (124) Mouse mAb (15071, Cell Signaling), Bcl-xL (54H6) Rabbit mAb (2764, Cell Signaling), Mcl-1 (D2W9E) Rabbit mAb (94296, Cell Signaling), α/β-Tubulin Antibody (2148, Cell Signaling) followed by mouse anti-rabbit IgG-HRP (sc-2357, Santa Cruz) or m-IgG$_k$ BP-HRP (sc-516102, Santa Cruz).

Statistics

Statistical analyses were conducted using GraphPad Prism 10 software and Microsoft Excel. Combination effects and synergy scores were analyzed using the Bliss independence model with Synergyfinder[8-10].

Supplementary References:

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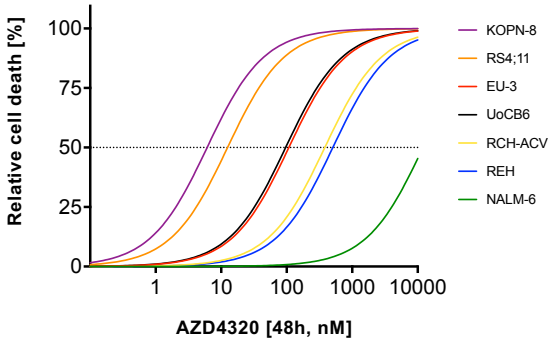
Suppl. Fig. 1

A



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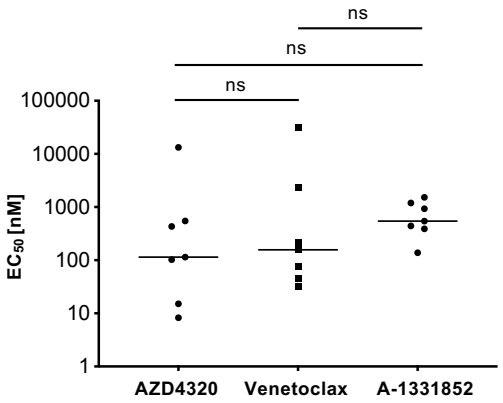

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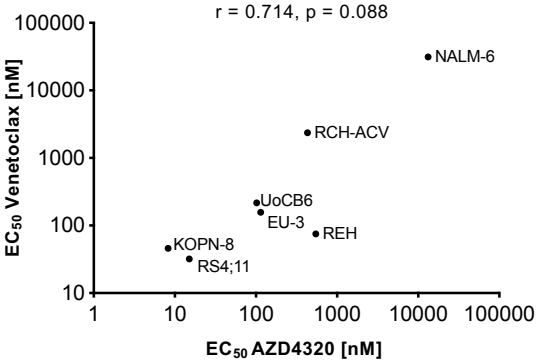

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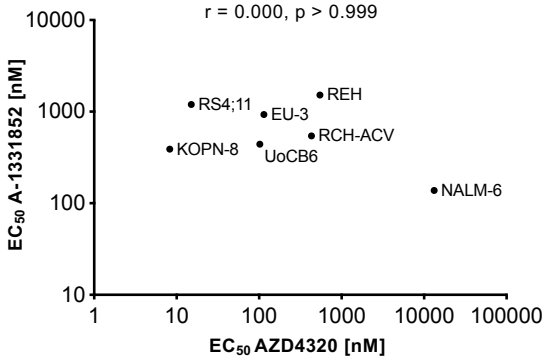

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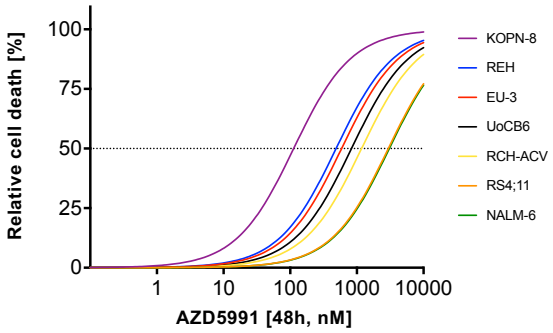

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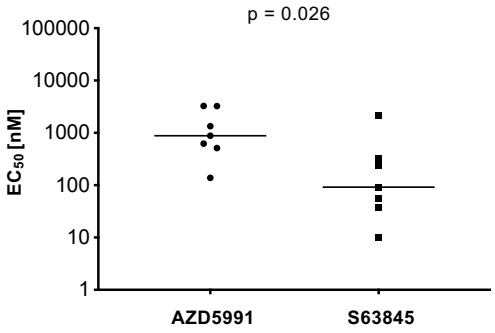

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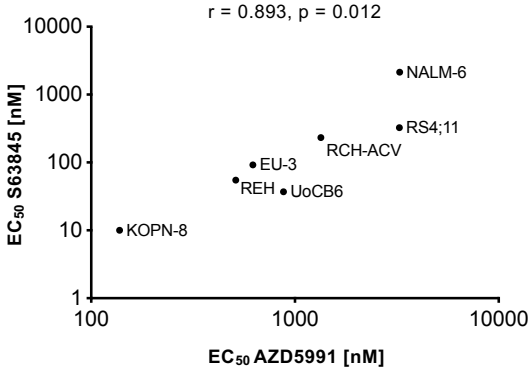

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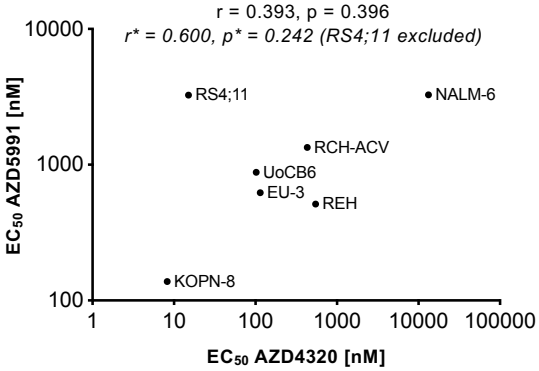
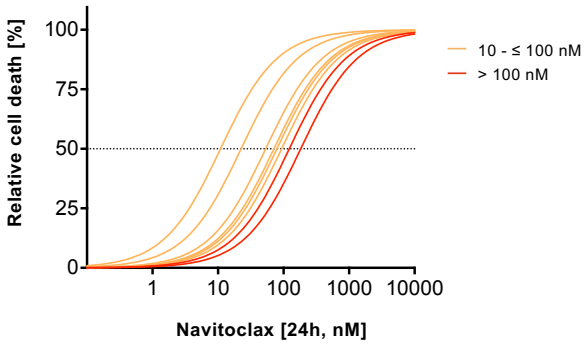


Legend to Supplementary Figure 1. Varying sensitivities towards BH3-mimetics of BCP-ALL cell lines

(A) Cell lines were exposed to eleven increasing concentrations (0.1, 1, 5, 10, 50, 100, 250, 500, 1000, 5000 and 10000 nM) of AZD4320 for 48 hours. Cell death rates were measured by propidium iodide (PI) staining. EC_{50} values were determined showing a range from 8.26 nM to 13.23 µM and a median of 114.20 nM. **(B)** The sensitivity of AZD4320 (inhibiting BCL-2/BCL-XL) was compared with venetoclax (BCL-2) and A-1331852 (BCL-XL). The scatter plot shows individual EC_{50} values and the lines show medians. Mann-Whitney U-test; p, significance. **(C)** Correlation of the sensitivities of AZD4320 with venetoclax **(D)** and of AZD4320 with A-1331852. Spearman correlation; r, correlation coefficient; p, significance. **(E)** Cell death rates (PI) were assessed upon exposure of cell lines to eleven concentrations (0.1, 1, 5, 10, 50, 100, 250, 500, 1000, 5000 and 10000 nM) of AZD5991 for 48 hours. EC_{50} values ranged between 137.8 nM and 3.27 µM with a median of 879.1 nM. **(F)** Comparison of sensitivities of two MCL-1 inhibitors. Mann-Whitney U-test; p, significance. **(G)** Spearman correlation of the EC_{50} values of both MCL-1 inhibitors **(H)** and of AZD4320 and AZD5991. Spearman correlation; r, correlation coefficient; p, significance; r* and p* indicate the correlation coefficient and significance, respectively, excluding the RS4;11 cell line. The EC_{50} values of venetoclax, A-1331852 and S63845 have been published previously (3).

A



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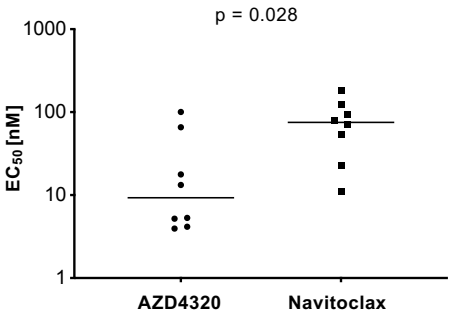

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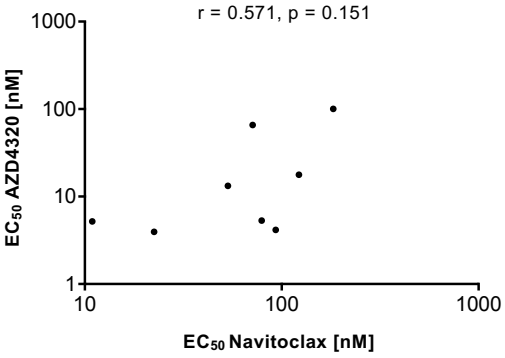

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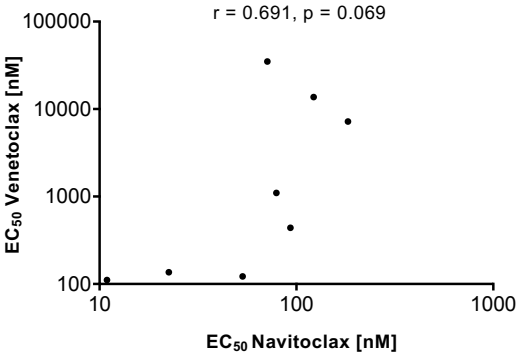

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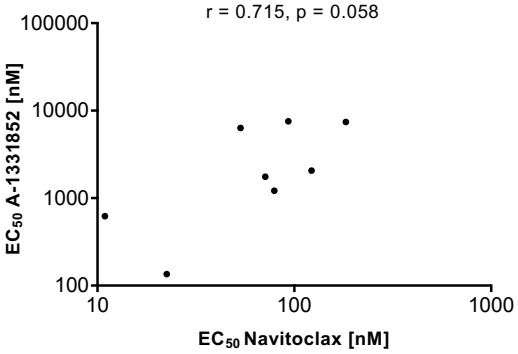
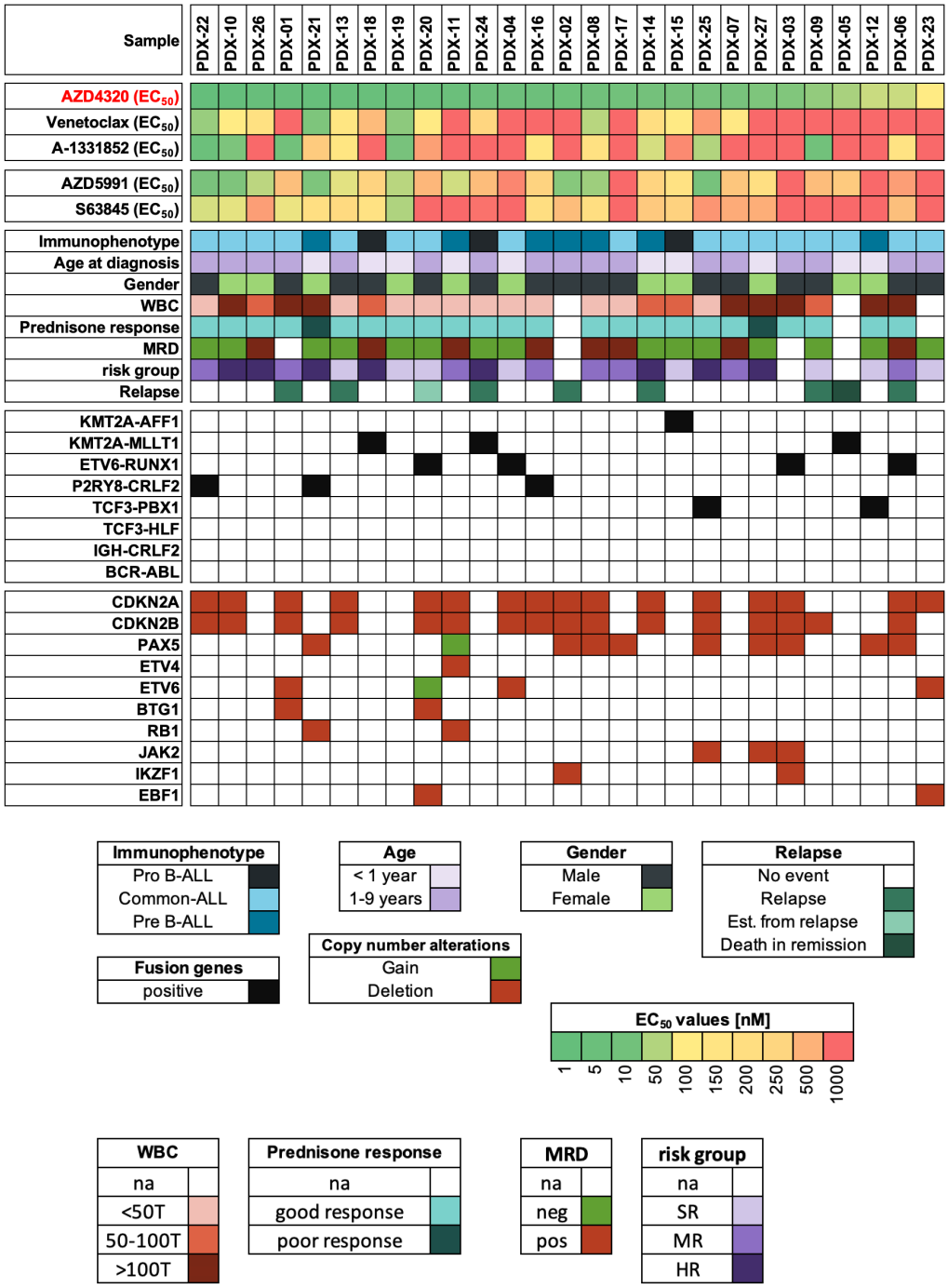


Legend to Supplementary Figure 2. Varying sensitivities towards navitoclax in BCP-ALL patient-derived xenograft samples

(A) B-cell precursor acute lymphoblastic leukemia (BCP-ALL) patient-derived xenograft (PDX) samples (N=8) were cultured for 24 hours and exposed to eleven increasing concentrations (0.1, 1, 2.5, 5, 10, 25, 50, 100, 500, 1000 and 5000 nM) of the BCL-2/BCL-XL inhibitor navitoclax. Cell death rates were measured by propidium iodide (PI) staining. EC_{50} values were determined showing a range from 10.91 to 182.9 nM and a median of 75.18 nM. **(B)** The sensitivity of navitoclax was compared with AZD4320 (both inhibiting BCL-2/BCL-XL). The scatter plot shows individual EC_{50} values and the lines show medians. Mann-Whitney U-test; p, significance. **(C-E)** Correlation of the sensitivities of navitoclax with **(C)** AZD4320, **(D)** venetoclax and **(E)** A-1331852. Spearman correlation; r, correlation coefficient; p, significance.

A

Supplementary Figure 3. Characteristics of patient-derived xenograft samples and corresponding patients. **(A)** The samples are sorted based on the EC_{50} values of AZD4320. The EC_{50} values of venetoclax, A-1331852 and S63845 have been published previously (3).



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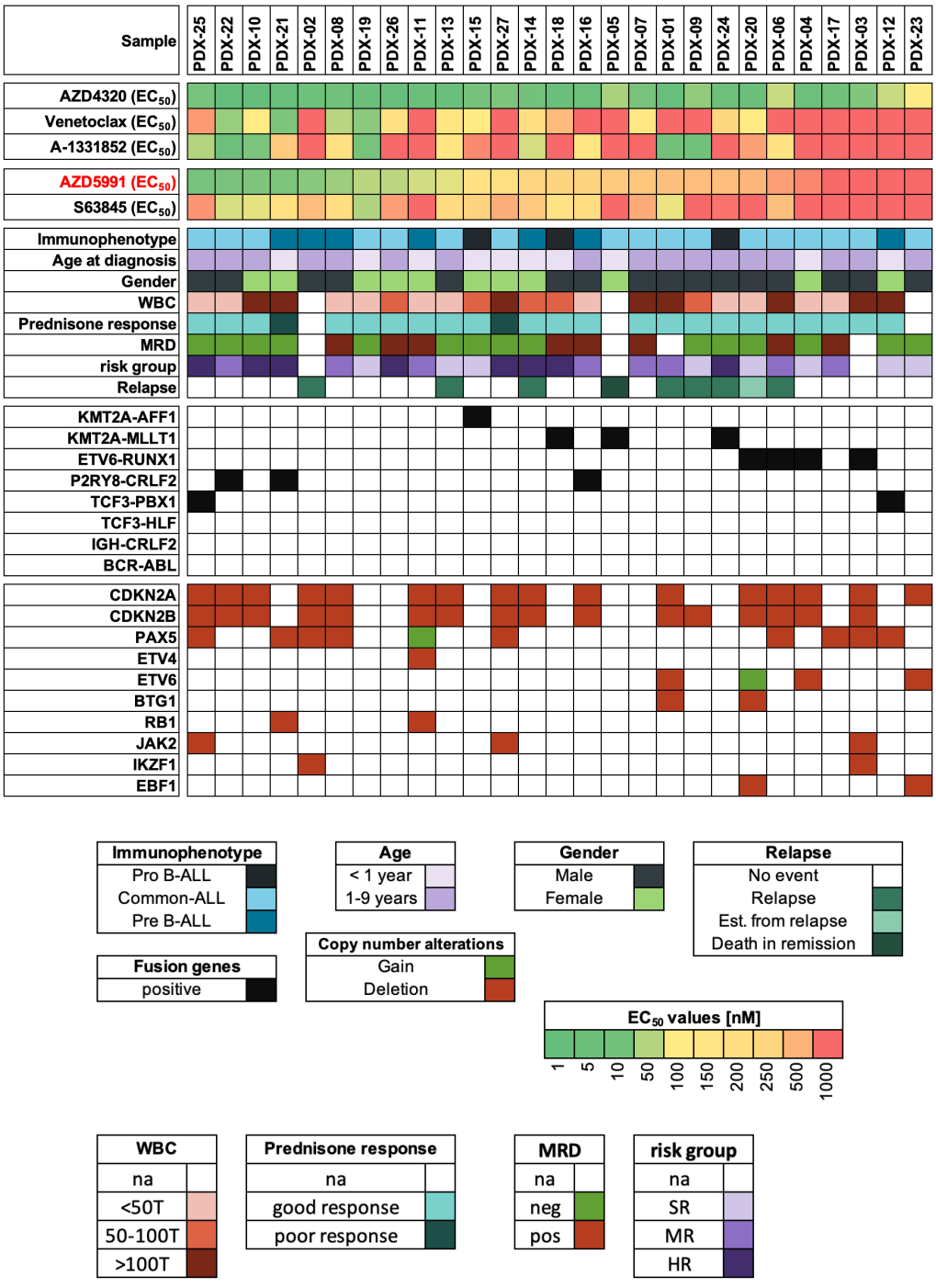
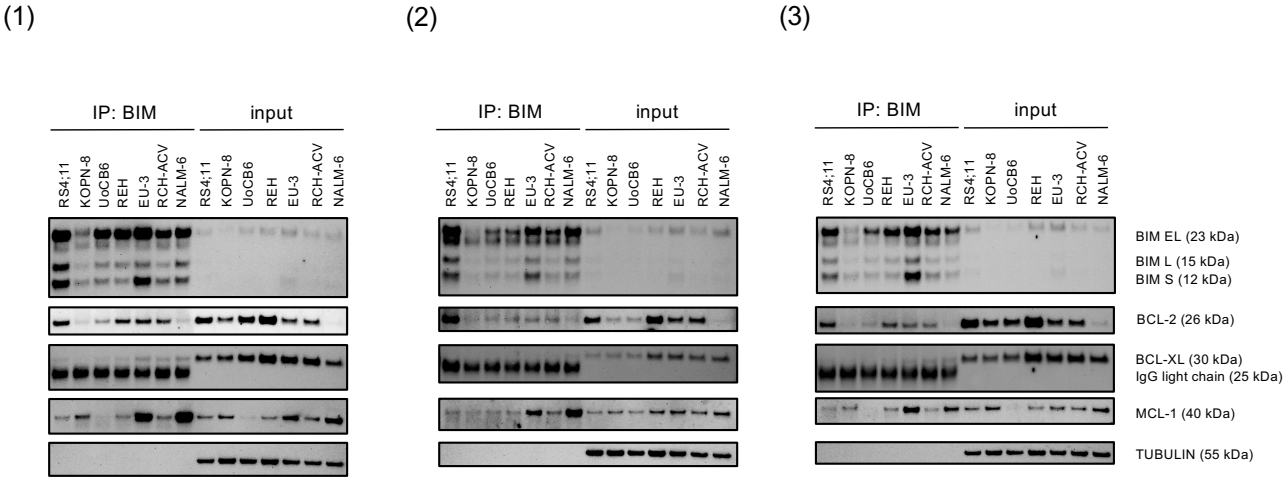
(B) The samples are sorted based on the EC_{50} values of AZD5991.



Supplementary Figure 4. Co-immunoprecipitations of BIM BCP-ALL samples.

A

(A) Basal levels of proteins and protein complexes are shown in cell lines. N=3 independent experiments are shown.



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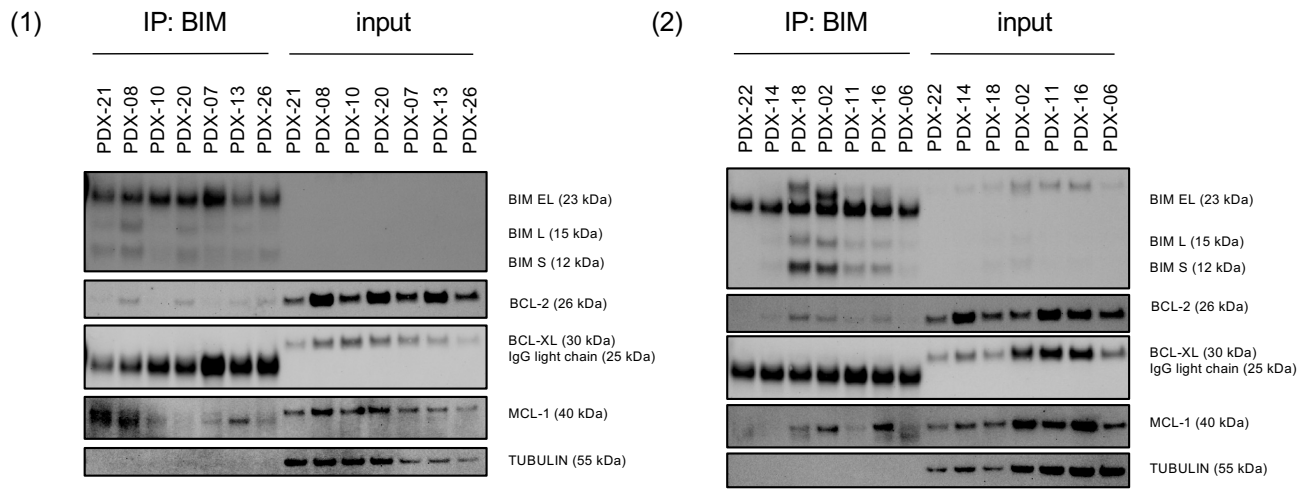
(B) Densitometric quantification of (a) protein levels (b) protein ratios and (c) protein complexes in ALL cell lines from N=3 independent experiments. Spearman correlation of proteins with the EC_{50} values of the compounds. A negative Spearman rho would indicate that sensitivity (low EC_{50}) is correlated to a high level of protein or protein complex. N=7 cell lines; p, significance; significance level (Bonferroni-adjusted): P ≤ 0.05/45 = P ≤ 0.001.

	Compounds	AZD4320		Venetoclax		A-1331852		S63845		AZD5991	
(a)	**Protein levels**	r_s	p	r_s	p	r_s	p	r_s	p	r_s	p
Input	BCL-2	0.000	1.000	-0.429	0.345	0.893	0.012	-0.036	0.963	-0.107	0.840
Input	BCL-XL	0.714	0.088	0.714	0.088	0.071	0.906	0.464	0.302	0.321	0.498
Input	MCL-1	0.393	0.396	0.464	0.302	-0.429	0.354	0.571	0.200	0.357	0.444
(b)	**Protein ratios**	r_s	p	r_s	p	r_s	p	r_s	p	r_s	p
Input	BCL-2/BCL-XL	-0.464	0.302	-0.750	0.066	0.643	0.139	-0.286	0.556	-0.214	0.662
Input	BCL-2/MCL-1	-0.143	0.783	-0.250	0.595	0.607	0.167	-0.321	0.498	-0.143	0.783
Input	BCL-2/(BCL-XL+MCL-1)	-0.429	0.354	-0.536	0.354	0.571	0.200	-0.179	0.713	0.000	1.000
(b)	**Protein complexes**	r_s	p	r_s	p	r_s	p	r_s	p	r_s	p
IP: BIM	BCL-2–BIM	-0.429	0.345	-0.857	0.024	0.714	0.088	-0.143	0.783	-0.321	0.498
IP: BIM	BCL-XL–BIM	-0.143	0.783	0.250	0.595	-0.821	0.034	-0.071	0.906	0.107	0.840
IP: BIM	MCL-1–BIM	0.071	0.906	0.143	0.783	-0.536	0.236	0.000	1.000	-0.214	0.662

C

(C) Basal levels of proteins and protein complexes are shown for PDX samples.



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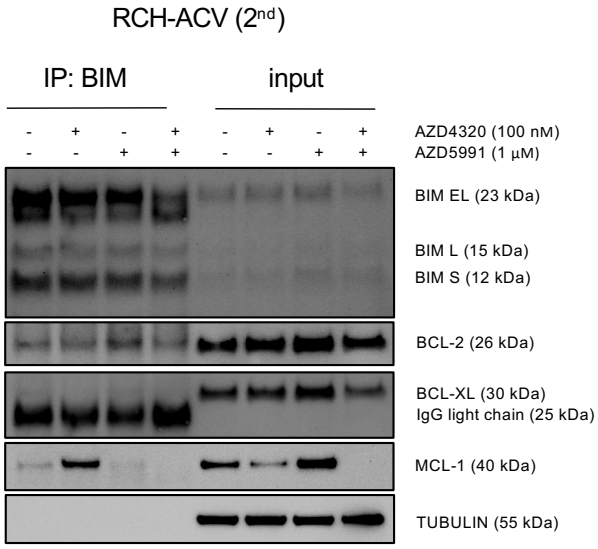
(D) Densitometric quantification of (a) protein levels, (b) protein ratios and (c) protein complexes in ALL PDX samples. Spearman correlation of proteins with the EC_{50} values of the compounds. A negative Spearman rho would indicate that sensitivity (low EC_{50}) is correlated to a high level of protein or protein complex. N=14 PDX samples; p, significance; significance level (Bonferroni-adjusted): $P \leq 0.05/45 = P \leq 0.001$.

	Compounds	AZD4320		Venetoclax		A-1331852		S63845		AZD5991	
(a)	**Protein levels**	r_s	p	r_s	p	r_s	p	r_s	p	r_s	p
Input	BCL-2	-0.024	0.940	-0.051	0.638	0.064	0.832	0.262	0.366	0.284	0.325
	BCL-XL	0.231	0.427	-0.165	0.445	0.332	0.246	0.319	0.267	0.152	0.605
	MCL-1	0.103	0.727	-0.077	0.739	0.543	0.048	0.196	0.502	0.253	0.382
(b)	**Protein ratios**	r_s	p	r_s	p	r_s	p	r_s	p	r_s	p
Input	BCL-2/BCL-XL	0.011	0.976	0.204	0.483	-0.112	0.704	0.095	0.750	0.297	0.302
	BCL-2/MCL-1	0.024	0.940	0.376	0.186	-0.376	0.616	0.244	0.400	0.288	0.318
	BCL-2/(BCL-XL+MCL-1)	-0.007	0.988	0.235	0.417	-0.152	0.605	0.130	0.659	0.288	0.318
(c)	**Protein complexes**	r_s	p	r_s	p	r_s	p	r_s	p	r_s	p
IP: BIM	BCL-2–BIM	-0.240	0.409	0.051	0.868	0.182	0.532	-0.042	0.892	-0.108	0.716
	BCL-XL–BIM	-0.323	0.260	-0.165	0.573	0.046	0.880	0.024	0.940	-0.086	0.773
	MCL-1–BIM	0.064	0.832	-0.077	0.797	-0.134	0.648	-0.402	0.155	-0.152	0.605

Supplementary Figure 5. Independent experiments of co-immunoprecipitations of BIM for **(A)** RCH-ACV corresponding to Figure 2A and for **(B)** NALM-6 corresponding to Figure 2B. Cells were exposed to the compounds at the concentrations indicated for four hours.

A



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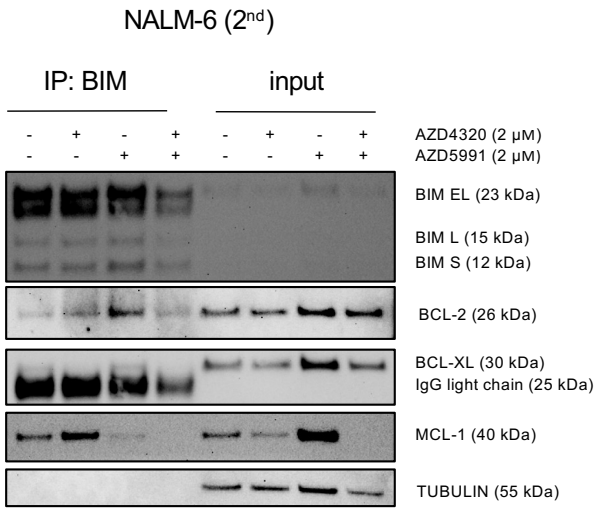

Supplementary Figure 6. Combination effects of AZD4320 and AZD5991 in BCP-ALL cell lines

Dose-response matrix analyses were conducted in three BCP-ALL cell lines that are less sensitive to AZD4320 and/or AZD5991 alone. The cell lines were exposed to seven increasing concentrations (5, 25, 50, 100, 250, 500 and 1000 nM) of AZD4320 and/or AZD5991 in a drug matrix for 48 hours. Cell death rates were determined by propidium iodide staining and flow cytometry measurement. The heatmaps show cell death rates after drug exposure. Efficacy scores were calculated as the arithmetic mean of normalized cell death rates across the matrix. Synergy effects were visualized using Synergyfinder and synergy scores were analyzed using the Bliss independence model. Dashed lines indicate the most synergistic area (MSA). Mean values of two independent experiments are shown.