SUPPLEMENTAL MATERIAL

Valosin-containing protein/p97 as a novel therapeutic target in acute lymphoblastic leukemia (Gugliotta G et al., Neoplasia 2017):

Contents

METHODS

Reagents and Vendors

CB-5083 was provided by Cleave Biosciences (Burlingame, CA, USA) dissolved in dimethyl-sulfoxide (DMSO); prednisolone, 2-Hydroxy-1-naphthaldehyde (HNA), propidium iodide (PI), polyethylenimine (PEI), 4-hydroxy tamoxifen (4-OHT), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and trypan blue solution were purchased from Sigma-Aldrich (St. Louis, MO, USA); thapsigargin from Abcam (Cambridge, MA, USA); Vincristine and Bortezomib from Selleck Chemicals (Houston, TX, USA); Roswell Park Memorial Institute medium (RPMI-1640), Iscove's Modified Dulbecco's Medium (IMDM), Fetal Bovine Serum (FBS) and 2-Mercaptoethanol (55 mM) from Fischer Scientific (Pittsburg, PA, USA); methyl-cellulose (Methocult H4230) from STEMCELL Technologies (Cambridge, MA, USA); Annexin V/PI double staining kit, BD Biosciences (San Jose, CA, USA); Total RNA extraction kit (RNAeasy) from Qiagen (Valencia, CA, USA); qScript™ cDNA synthesis kit and QPCR sybr green master mix from Quanta Biosciences (Beverly, MA, USA); RIPA Lysis Buffer, nitrocellulose (NC) and polyvinylidene difluoride (PVDF) membranes from Millipore (Billerica, MA, USA); protease inhibitor cocktail from Roche Applied Science (Indianapolis, IN); BCA assay kit and superSignal west dura chemiluminescent substrate, from Thermo Scientific (Rockford, IL); RetroNectin, from Clontech Inc (Mountain View, CA); antibodies against CHOP (#2895), GRP78 (#3177), GRP94 (#2104), Calnexin (#2679), PDI (#3501), IRE1-alpha (#3294), PERK (#5683), PARP (#9532), Cleaved-Caspase-3 (#9661), GAPDH (#2118), from Cell Signaling (Beverly, MA, USA); antibody against β-actin from Sigma-Aldrich (St Louis, MO, USA).

Cell lines

Following human B-ALL cell lines were used: BALL1, REH, NALM6, OP1, ALL-PO, 697, RS4:11, BV173, SEM, and SUPB15. OP1 cells were generously provided by Dario Campana (National University Cancer Institute, Singapore). ALL-PO cells were generously provided by Andrea Biondi (University of Milan-Bicocca, Monza, Italy). BALL1, REH, NALM6, OP1, ALL-PO, 697, RS4:11 cells were maintained in RPMI 1640 supplemented with 10% FBS. BV173 were maintained in RPMI 1640 supplemented with 20% FBS. SEM were maintained in IMDM supplemented with 10% FBS and β-Mercaptoethanol 0.05 mM. SUPB15 were maintained in IMDM supplemented with 20% FBS and β-Mercaptoethanol 0.05 mM. Murine BCR-ABL transformed B-ALL cell lines with floxed alleles (either $XBP1^{FL/FL}$,

GRP78$^{FL/FL}$, or GRP94$^{FL/FL}$) were used. These cells were maintained in IMDM supplemented with 20% FBS and β-Mercaptoethanol 0.05 mM.

Viability assay and evaluation for synergy

Cell viability was evaluated by MTT assay. For each assay, various number of cells (10,000 – 20,000 per well, depending on the cell line) were seeded in 96-well plates, followed by either vehicle (DMSO) or increasing concentrations of drug. At the desired interval (24, 48 or 72 hours), for detection of relative numbers of living cells, 10 µl of MTT (5 mg/ml) was added to each well, plates were placed in an incubator for four hours; 100 µl of SDS buffer (20% in water) was added to dissolve the crystals. Results were read on spectrophotometer machine at 570 nM wavelength. Half maximal inhibitory concentrations (IC50s) were calculated using the GraphPad Prism 6 software (GraphPad Inc., San Diego, CA). For pulse exposure assays, cells were treated with CB-5083 for the desired interval, washed three times with phosphate buffer saline (PBS), seeded with fresh media, and viability was evaluated by MTT assay as previously described.

For drug combination assays, cells were seeded in 96-well plates, followed by addition of either vehicle or increasing concentrations of CB-5083 alone, second drug (vincristine, bortezomib, HNA, or prednisolone) alone, or solutions of CB-5083 plus second drug. Viability was evaluated by MTT assay as previously described. Synergistic combination of two drugs was determined using the CompuSyn software (ComboSyn Inc, www.combosyn.com). The extent of drug interaction between the two drugs was determined using the combination index (CI) for mutually exclusive drugs. CI values were obtained when solving the equation for different concentrations of drugs. A CI of 1 indicates an additive effect, whereas a CI of <1 denotes synergy. All experiments were performed at least 3 times, in triplicates.

Cell proliferation and clonogenic assay

Cell proliferation was evaluated with trypan blue exclusion. For each experiment, 1,000 cells were seeded in 96-well plates, followed by either vehicle (DMSO) or increasing concentrations of drug. At different time points (24, 48, 72, and 96 hours), trypan blue was added to the wells, carefully mixed, and living cells were counted with a hemocytometer under an inverted microscope (final concentrations, expressed in cells/mL, were adjusted for the dilution factor). All experiments were performed three times in duplicates, and each well was counted twice. For clonogenic assay, either NALM-6 or OP1 cells were grown in methyl-cellulose (Methocult H4230, STEMCELL Technologies): 1,000 cells per well were seeded in 6-well plates with increasing concentrations of either CB-5083

or diluent control. Colonies were counted under an inverted microscope after 12 (NALM6) or 15 days (OP1). All clonogenic experiments were performed three times, in duplicates.

Apoptosis and cell cycle analysis

Cells were seeded in 12-well plates followed by culture with either diluent control (DMSO) or various concentrations of experimental drug. Cells were collected by centrifugation and washed with PBS. Apoptosis was determined by Annexin V/PI staining (BD Biosciences) according to manufacturer's instructions. Cell cycle analyses were performed by propidium iodide staining (Sigma-Aldrich) for DNA content and flow cytometric analysis. All flow cytometry data were analyzed using FlowJo software (Tree Star, Ashland, OR). All experiments were performed three times.

Western blotting

Proteins were lysed from cells using RIPA Lysis Buffer (Millipore) supplemented with protease inhibitor cocktail (Roche Applied Sciences) and phosphatase inhibitor cocktail (Roche Applied Sciences). Protein concentrations were determined by BCA assay (Thermo Scientific). Protein samples were resolved by sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) electrophoresis, transferred to either 0.45 µM NC or 0.2 µM PVDF membranes (Millipore), and membranes were blocked for 1 hr with phosphate buffer saline (PBS) containing 5% non-fat dry milk and 0.1% Tween-20 (PBST). After blocking, membranes were incubated with primary antibodies at 4°C overnight. The following day, membranes were washed with PBST and incubated with a secondary antibody-HRP conjugate at room temperature for one hour. After washing, membranes were visualized with ECL plus Substrates (Thermo Scientific). All experiments were performed at least three times.

PCR

Total RNA from cells was extracted using RNeasy isolation kit (Qiagen). cDNA was generated using qScript™ cDNA Synthesis Kit (Quanta Biosciences). Evaluation of relative expression levels of unspliced form of XBP1 / spliced XBP1 was performed by Polymerase Chain Reaction (PCR) followed by electrophoresis in 3% agarose gels. Quantitative Real-Time PCR (QRT-PCR) was performed on CFX96 qPCR System (Biorad Inc, Hercules, CA). Expression of each gene was normalized to GAPDH as a reference. The conditions for all QRT-PCR reactions are as follows: 2 minutes at 95°C followed by 15 seconds at 95°C and 30 seconds at 60°C for 40 cycles. Primers for PCR are listed in Supplemental Table 5. All experiments were performed three times, in triplicates.

Retroviral transduction and inducible knock-out

Retroviral constructs and the corresponding empty vector controls were packaged in Platinum-E (Plat-E) cells using PEI transfection method. 9 µg of plasmid (either MSCV-ER^{T2} or MSCV-Cre-ER^{T2}) was incubated with 27 µl of PEI reagent (1µg/µl) in 1000 µl Opti-MEM media (Invitrogen) for 20 min. The mixture was placed on the Plat-E cells in 10 cm culture dishes. The virus supernatants were harvested 24 h and 48 h later. Viral supernatants from 2 collections were combined, filtered through a 0.45 µm filter, loaded on RetroNectin (Clontech) coated non-tissue 6-well plates, and 2 × 10^6 cells (either BCR-ABL+ B-ALL GRP78$^{FL/FL}$, GRP94$^{FL/FL}$, or XBP1$^{FL/FL}$) per well were transduced following the manufacturer's instructions. These transduced cells were selected for 48-72 hours with puromycin (1-2 µM). CRE-mediated deletion of either GRP78, GRP94, or XBP1 was accomplished by treatment of these cells with 4-OHT (1µM) for two days.

Statistical analysis

IC50s are expressed as mean and 95% confidence intervals. All other results are expressed as mean ± SD. Statistical significance was determined by Student t test or one-way ANOVA, as appropriate. Significance of P values less than 0.05, 0.01, 0.001, and 0.0001 are shown with *, **, ***, and **** asterisks, respectively. The combined effect of CB-5083 with vincristine, prednisolone, bortezomib, or HNA was analyzed using the CompuSyn software program (ComboSyn Inc, www.combosyn.com). All other statistical analysis were performed with GraphPad Prism 6 (GraphPad Inc., San Diego, CA).

SUPPLEMENTAL TABLES

Table S1. Proliferation of BALL1, OP1, and REH cells exposed to CB-5083.

	BALL1	OP1	REH
Baseline	1	1	1
48 hours:			
Control	2.63 (± 0.05)	2.64 (± 0.17)	3.19 (± 0.07)
0.25 µM	2.19 (± 0.42)	2.1 (± 0.51)	-
0.5 µM	1.05 (± 0.17)	0.87 (± 0.17)	1.79 (± 0.23)
0.75 µM	0.26 (± 0.22)	0.47 (± 0.11)	1.23 (± 0.14)
96 hours:			
Control	5.53 (± 0.43)	7.58 (± 0.15)	8.19 (± 0.55)
0.25 µM	3.76 (± 0.49)	5.05 (± 0.61)	-
0.5 µM	0.44 (± 0.07)	0.65 (± 0.02)	4.8 (± 0.56)
0.75 µM	0.01 (± 0.02)	0.24 (± 0.08)	1.02 (± 0.17)

Fold changes (± SD) of cell numbers normalized to baseline (=1)

Table S2. Multiple comparisons test of CHOP expression in BALL1 cells treated with CB-5083

BALL1 – 4 hours				
Sidak's multiple comparisons test	**Mean Diff,**	**95% CI of diff,**	**Significant?**	**Summary**
Ctrl vs. 0.5 µM	-15,07	-27,93 to -2,205	Yes	*
Ctrl vs. 1 µM	-23,57	-36,43 to -10,71	Yes	***
Ctrl vs. 2.5 µM	-31,83	-44,69 to -18,97	Yes	****
Ctrl vs. 5 µM	-47,00	-59,86 to -34,14	Yes	****
0.5 vs. 1 µM	-8,500	-22,25 to 5,249	No	ns
0.5 vs. 2.5 µM	-16,77	-30,52 to -3,017	Yes	*
0.5 vs. 5 µM	-31,93	-45,68 to -18,18	Yes	****
1 vs. 2.5 µM	-8,267	-22,02 to 5,483	No	ns
1 vs. 5 µM	-23,43	-37,18 to -9,684	Yes	***
2.5 vs. 5 µM	-15,17	-28,92 to -1,417	Yes	*
BALL1 – 8 hours				
Sidak's multiple comparisons test	**Mean Diff,**	**95% CI of diff,**	**Significant?**	**Summary**
Ctrl vs. 0.5 µM	-12,63	-31,84 to 6,577	No	ns
Ctrl vs. 1 µM	-26,23	-45,44 to -7,023	Yes	**
Ctrl vs. 2.5 µM	-53,47	-72,68 to -34,26	Yes	****
Ctrl vs. 5 µM	-66,40	-85,61 to -47,19	Yes	****
0.5 vs. 1 µM	-13,60	-34,14 to 6,936	No	ns
0.5 vs. 2.5 µM	-40,83	-61,37 to -20,30	Yes	***
0.5 vs. 5 µM	-53,77	-74,30 to -33,23	Yes	****
1 vs. 2.5 µM	-27,23	-47,77 to -6,697	Yes	**
1 vs. 5 µM	-40,17	-60,70 to -19,63	Yes	***
2.5 vs. 5 µM	-12,93	-33,47 to 7,603	No	ns

Sidak's multiple comparisons test of CHOP expression as measured by RTQ-PCR in BALL1 cells after treatment with CB-5083 for 4 and 8 hours. Significance of P values less than 0.05, 0.01, 0.001, and 0.0001 are shown with *, **, ***, and **** asterisks, respectively; ns: not significant.

Table S3. Multiple comparisons test of CHOP expression in OP1 cells treated with CB-5083

OP1 – 4 hours				
Sidak's multiple comparisons test	**Mean Diff,**	**95% CI of diff,**	**Significant?**	**Summary**
Ctrl vs. 0.5 µM	-4,100	-13,45 to 5,251	No	ns
Ctrl vs. 1 µM	-8,133	-17,48 to 1,218	No	ns
Ctrl vs. 2.5 µM	-21,63	-30,98 to -12,28	Yes	****
Ctrl vs. 5 µM	-28,67	-38,02 to -19,32	Yes	****
0.5 vs. 1 µM	-4,033	-14,03 to 5,963	No	ns
0.5 vs. 2.5 µM	-17,53	-27,53 to -7,537	Yes	***
0.5 vs. 5 µM	-24,57	-34,56 to -14,57	Yes	****
1 vs. 2.5 µM	-13,50	-23,50 to -3,503	Yes	**
1 vs. 5 µM	-20,53	-30,53 to -10,54	Yes	***
2.5 vs. 5 µM	-7,033	-17,03 to 2,963	No	ns
OP1 – 8 hours				
Sidak's multiple comparisons test	**Mean Diff,**	**95% CI of diff,**	**Significant?**	**Summary**
Ctrl vs. 0.5 µM	-2,867	-12,70 to 6,970	No	ns
Ctrl vs. 1 µM	-18,50	-28,34 to -8,663	Yes	***
Ctrl vs. 2.5 µM	-27,03	-36,87 to -17,20	Yes	****
Ctrl vs. 5 µM	-32,67	-42,50 to -22,83	Yes	****
0.5 vs. 1 µM	-15,63	-26,15 to -5,117	Yes	**
0.5 vs. 2.5 µM	-24,17	-34,68 to -13,65	Yes	****
0.5 vs. 5 µM	-29,80	-40,32 to -19,28	Yes	****
1 vs. 2.5 µM	-8,533	-19,05 to 1,983	No	ns
1 vs. 5 µM	-14,17	-24,68 to -3,651	Yes	**
2.5 vs. 5 µM	-5,633	-16,15 to 4,883	No	ns

Sidak's multiple comparisons test of CHOP expression as measured by RTQ-PCR in OP1 cells after treatment with CB-5083 for 4 and 8 hours. Significance of P values less than 0.01, 0.001, and 0.0001 are shown with **, ***, and **** asterisks, respectively; ns: not significant.

Table S4. Multiple comparisons test of CHOP expression in REH cells treated with CB-5083

REH - 4 hours				
Sidak's multiple comparisons test	**Mean Diff,**	**95% CI of diff,**	**Significant?**	**Summary**
Ctrl vs. 0.5 µM	-5,733	-17,67 to 6,199	No	ns
Ctrl vs. 1 µM	-15,20	-27,13 to -3,268	Yes	**
Ctrl vs. 2.5 µM	-18,07	-30,00 to -6,135	Yes	**
Ctrl vs. 5 µM	-26,73	-38,67 to -14,80	Yes	****
0.5 vs. 1 µM	-9,467	-22,22 to 3,289	No	ns
0.5 vs. 2.5 µM	-12,33	-25,09 to 0,4226	No	ns
0.5 vs. 5 µM	-21,00	-33,76 to -8,244	Yes	**
1 vs. 2.5 µM	-2,867	-15,62 to 9,889	No	ns
1 vs. 5 µM	-11,53	-24,29 to 1,223	No	ns
2.5 vs. 5 µM	-8,667	-21,42 to 4,089	No	ns
REH - 8 hours				
Sidak's multiple comparisons test	**Mean Diff,**	**95% CI of diff,**	**Significant?**	**Summary**
Ctrl vs. 0.5 µM	-3,433	-24,38 to 17,51	No	ns
Ctrl vs. 1 µM	-16,87	-37,81 to 4,079	No	ns
Ctrl vs. 2.5 µM	-37,07	-58,01 to -16,12	Yes	***
Ctrl vs. 5 µM	-52,97	-73,91 to -32,02	Yes	****
0.5 vs. 1 µM	-13,43	-34,38 to 7,512	No	ns
0.5 vs. 2.5 µM	-33,63	-54,58 to -12,69	Yes	**
0.5 vs. 5 µM	-49,53	-70,48 to -28,59	Yes	****
1 vs. 2.5 µM	-20,20	-41,15 to 0,7457	No	ns
1 vs. 5 µM	-36,10	-57,05 to -15,15	Yes	**
2.5 vs. 5 µM	-15,90	-36,85 to 5,046	No	ns

Sidak's multiple comparisons test of CHOP expression as measured by RTQ-PCR in REH cells after treatment with CB-5083 for 4 and 8 hours. Significance of P values less than 0.01, 0.001, and 0.0001 are shown with **, ***, and **** asterisks, respectively; ns: not significant.

Table S5. Primer sequences for PCR

Gene name	Forward	Reverse
Xbp1 *	5′-CCTGGTTGCTGAAGAGGAGG-3′	5′-CCATGGGGAGATGTTCTGGAG-3′
CHOP	5′-CCTCCTGGAAATGAAGAGG-3′	5′-TTGTGACCTCTGCTGGTTCT-3′
GADD34	5′-ATGTATGGTGAGCGAGAGGC-3′	5′-GCAGTGTCCTTATCAGAAGGC-3′
ATF4	5′-GGGACAGATTGGATGTTGGAGA-3′	5′-ACCCAACAGGGCATCCAAGT-3′
DR5	5′-AAGACCCTTGTGCTCGTTGTC-3′	5′-GACACATTCGATGTCACTCCA-3′
CASP2	5′-AGTCACGGACTCCTGCATCG-3′	5′-GGGCAGTTGGCGTTGTCAAA-3′
GRP78	5′-TCATGACACCTCCCACAGTT-3′	5′-GTAGCGTATGGTGCTGCTGT-3′
GRP94	5′-AAGGAGAAGAACCTGCTGCATG-3′	5′-TGGGCTCCTCAACAGTTTCAGT-3′
DNAJC3	5′-GACGGAGAAGATCCTTTGGA-3′	5′-ATCTAAATGGTCCGCCTGAG-3′
DNAJB9	5′-TCATCTTTGCAATCTGCATTT-3′	5′-ACTTCATGGCCAACTTGTGA-3′
GAPDH	5′-AACATCATCCCTGCATCCA-3’	5′-CCAGTGAGCTTCCCGTTCA-3’
COX6B	5′-AACTACAAGACCGCCCCTTT-3’	5′-GCAGCCAGTTCAGATCTTCC-3’
Mouse Xbp1	5′-CCTGAGCCCGGAGGAGAA-3’	5′-CTCGAGCAGTCTGCGCTG-3’
Mouse HPRT	5′-GGGGGCTATAAGTTCTTTGC-3’	5′-TCCACCACTTCGAGAGGTCC-3’

* spliced and unspliced, for RT-PCR

SUPPLEMENTAL FIGURES LEGEND

Figure S1. Apoptosis of OP1 cells after treatment with CB-5083

Apoptosis was measured (Annexin V + Propidium iodide positivity) after treatment with CB-5083 (0.25, 0.5, and 0.75 µM) for 24, 48, and 72 hours. Lower panels: bar graph quantification of data (mean [numbers in the boxes] ± SD of three experiments; ANOVA: all $p < 0.0001$; *: $p < 0.05$; **: $p < 0.01$; ****: $p < 0.0001$)

Figure S2. Viability of BALL1, OP1, and REH cells after pulse exposure to CB-5083

After pulse exposure to either diluent or CB-5083, cells were washed with PBS and seeded in new media. After replating, viability was evaluated at 24 hours and 72 hours by MTT assays; mean (numbers in the boxes) ± SD of three experiments).

Figure S3. Apoptosis of BALL1 and OP1 cells after pulse exposure to CB-5083

After pulse exposure (8 hours) to either diluent or CB-5083, cells were washed three times with PBS and seeded in new media. After replating, apoptosis (Annexin V + Propidium iodide positivity) was evaluated at 24 hours. Lower panels: bar graph quantification of data (mean [numbers in the boxes] ± SD of three experiments; *: $p < 0.05$; **: $p < 0.01$; ****: $p < 0.0001$).

Figure S4. Protein expression of ER stress markers as determined by Western blot in BALL1 cells after treatment with CB-5083

Cells were treated for 8 or 24 hours at the indicated concentrations (Panels A and B), as well as cells treated at a fixed dose (1 µM) for the indicated time (Panel C). β-Actin was used as loading control.

Figure S5. Successful deletion of either XBP1, GRP78 or GRP94 and consequent reduction of viability

Expression of XBP1 (by QPCR), GRP78 and GRP94 (by Western blot) after treatment with 4-OHT (1 µM, 48h) of empty-vector (EV) or CRE transfected cells (Panel A). Change in viability after deletion of either XBP1, GRP78 or GRP94 (Panel B); mean (numbers in the boxes) ± SD of three experiments.

Figure S6. Combination of CB-5083 with either HNA, bortezomib, or prednisolone

Cells were treated for the indicated time with a combination of different concentrations of CB-5083 (range: 0.08 – 1.25 µM) and either HNA (range: 6.25 – 100 µM), bortezomib (range: 2.5 – 40 nM), or prednisolone (range: 6.25 – 200 µM). Viability was measured by MTT (n=3; each in triplicates). Fractional inhibition (Fa) is defined as the reduction of viability in treated cells compared to controls (Fa=0, no inhibition; Fa=1, complete inhibition). Combination index (CI) < 1 represents synergy; = 1, additive effect; > 1 antagonism. The graphs show the Fa and CI for each drug combination.

Figure S7. Induction of ER stress with thapsigargin

After treatment of 3 B-ALL cell lines (BALL1, OP1, REH) with either vehicle or high dose thapsigargin (1 µM) for either 4 or 8 hours, mRNA was extracted, and XBP1 and spliced XBP1 (sXBP1) were evaluated with RT-PCR (35 cycles) (A); and CHOP expression was measured by QRT-PCR (B). GAPDH mRNA was used as loading control (A). Quantitative gene expression data were normalized to the expression levels of GAPDH (B). Bar graphs represent mean ± SD of three experiments.

SUPPLEMENTAL FIGURES

Figure S1. Apoptosis of OP1 cells after treatment with CB-5083

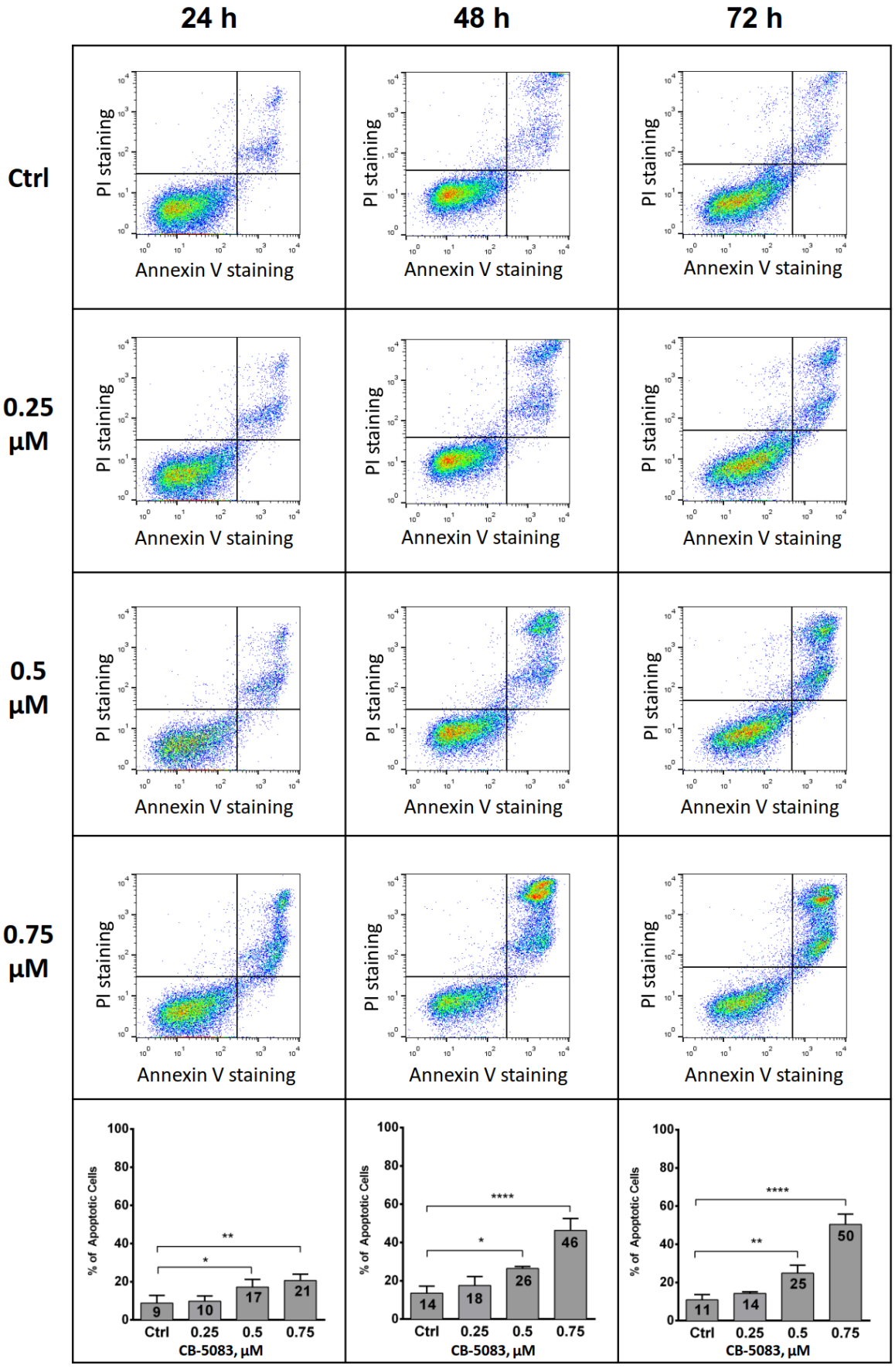

Figure S2. Viability of BALL1, OP1, and REH cells after pulse exposure to CB-5083

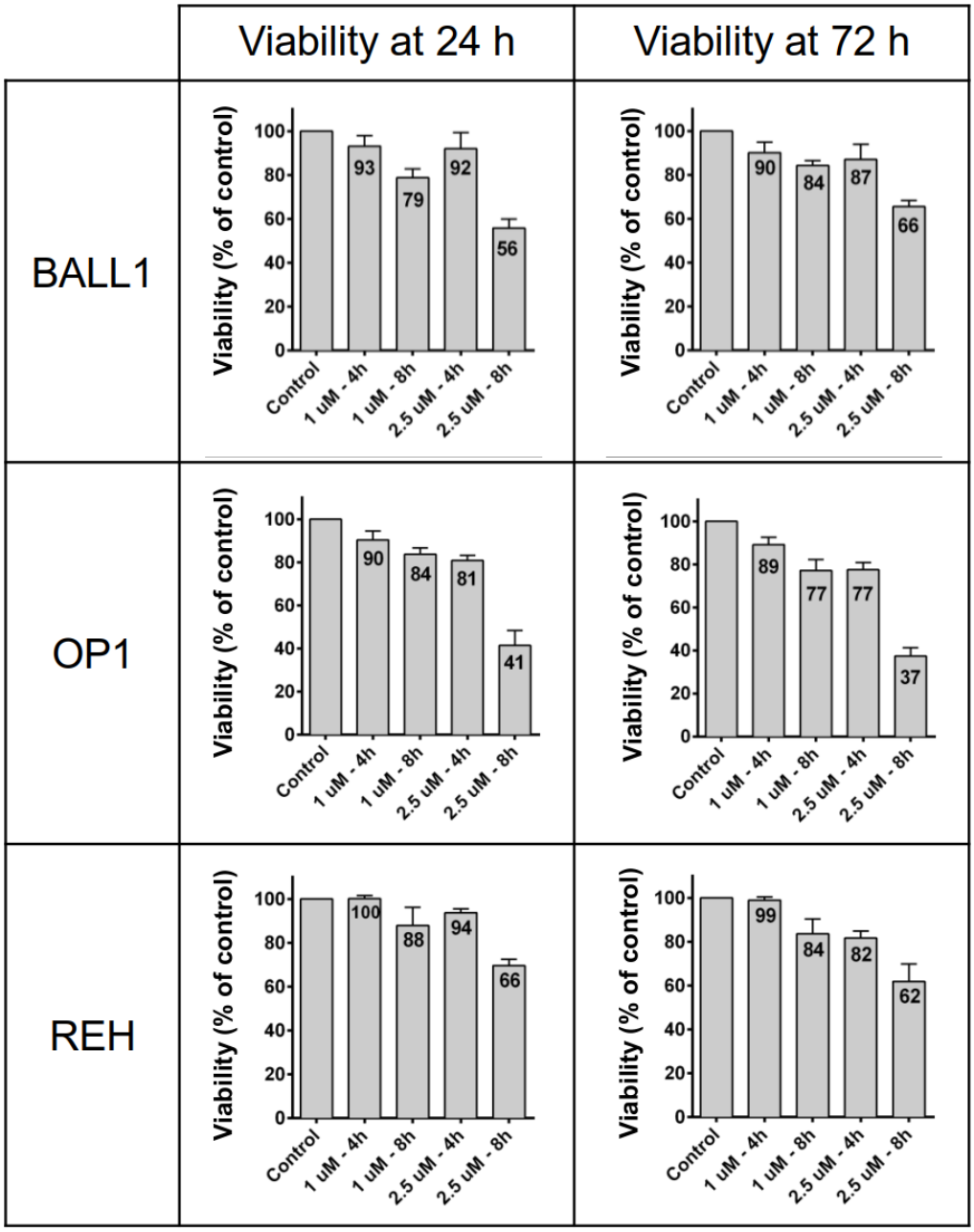

Figure S3. Apoptosis of BALL1 and OP1 cells after pulse exposure to CB-5083

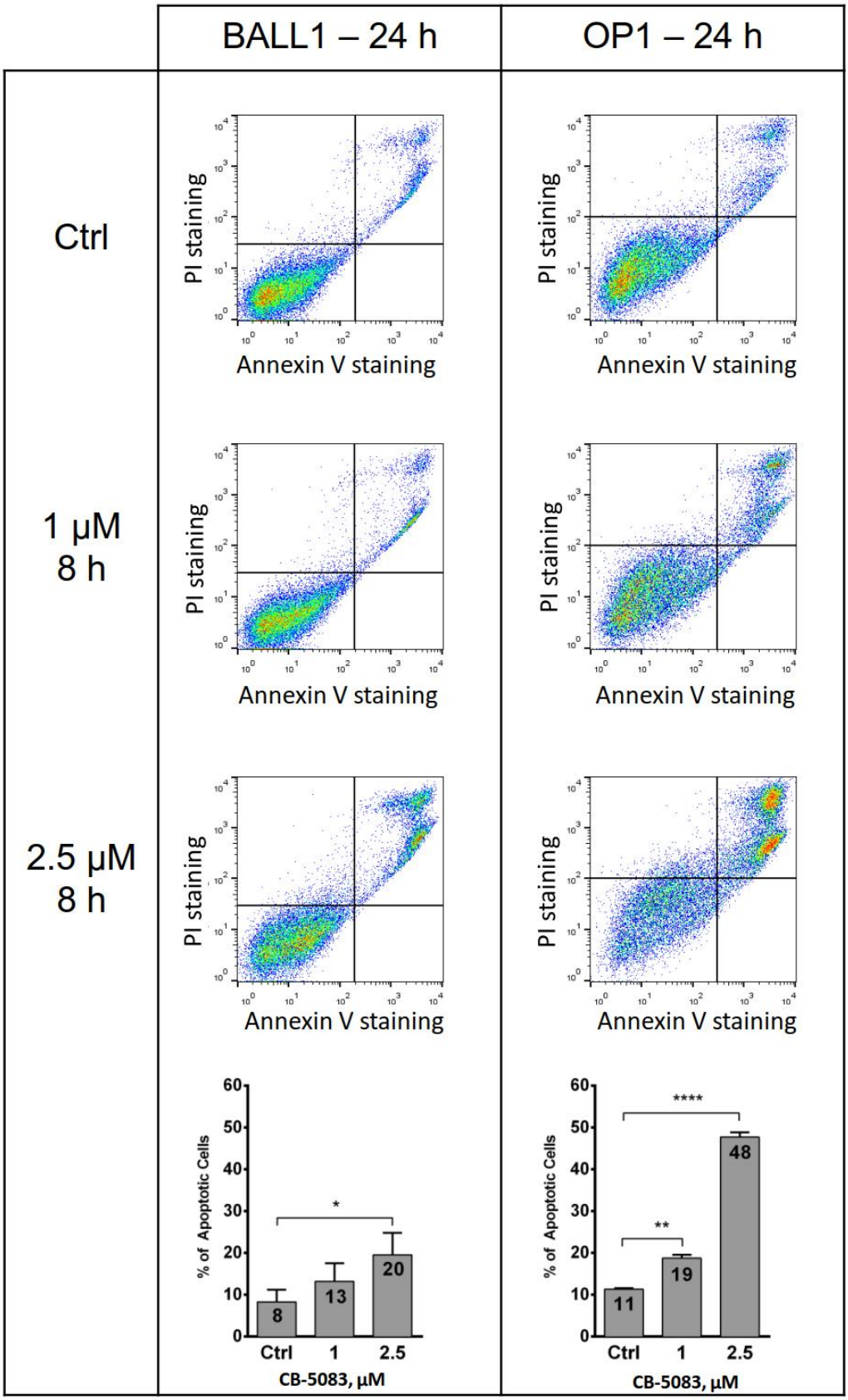

Figure S4. Protein expression of ER stress markers as determined by Western blot in BALL1 cells after treatment with CB-5083

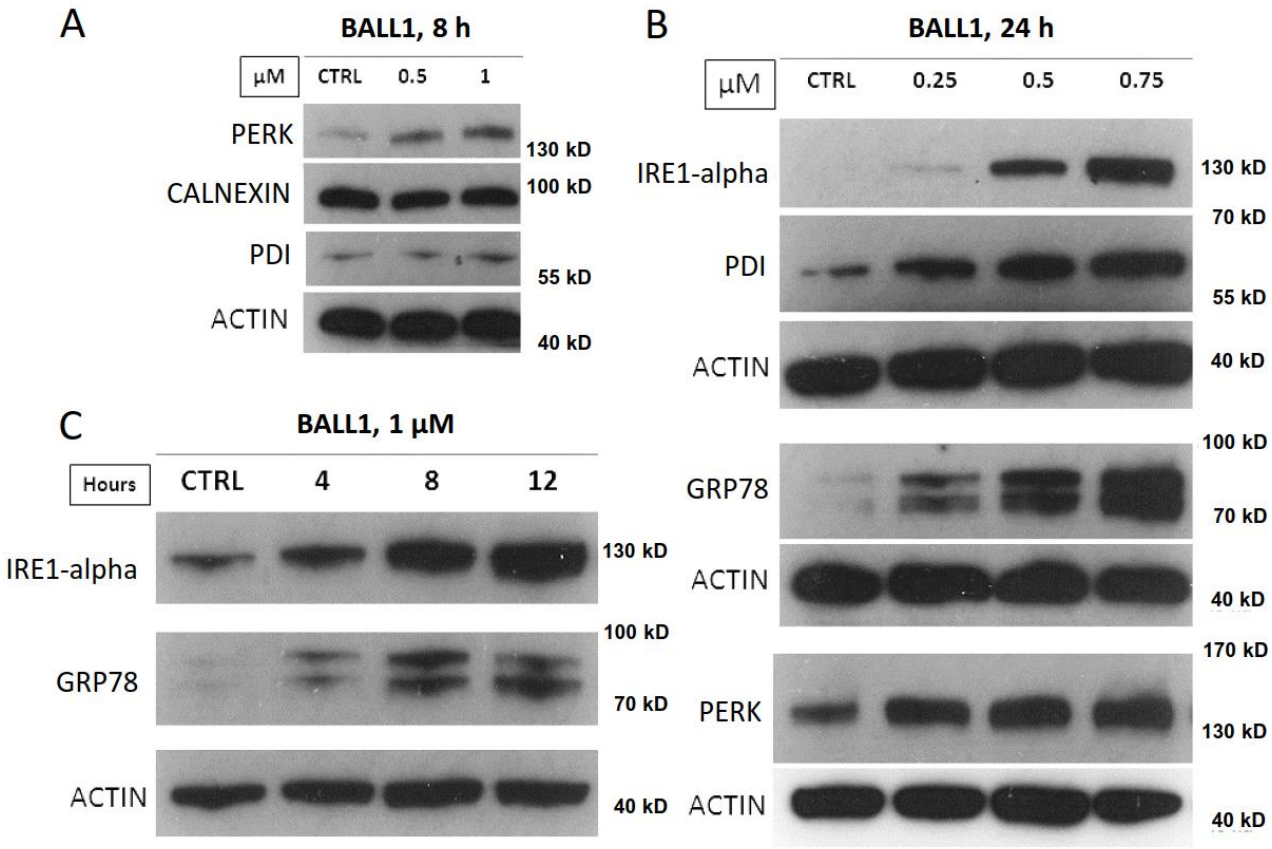

Figure S5. Successful deletion of either XBP1, GRP78 or GRP94 and consequent reduction of viability

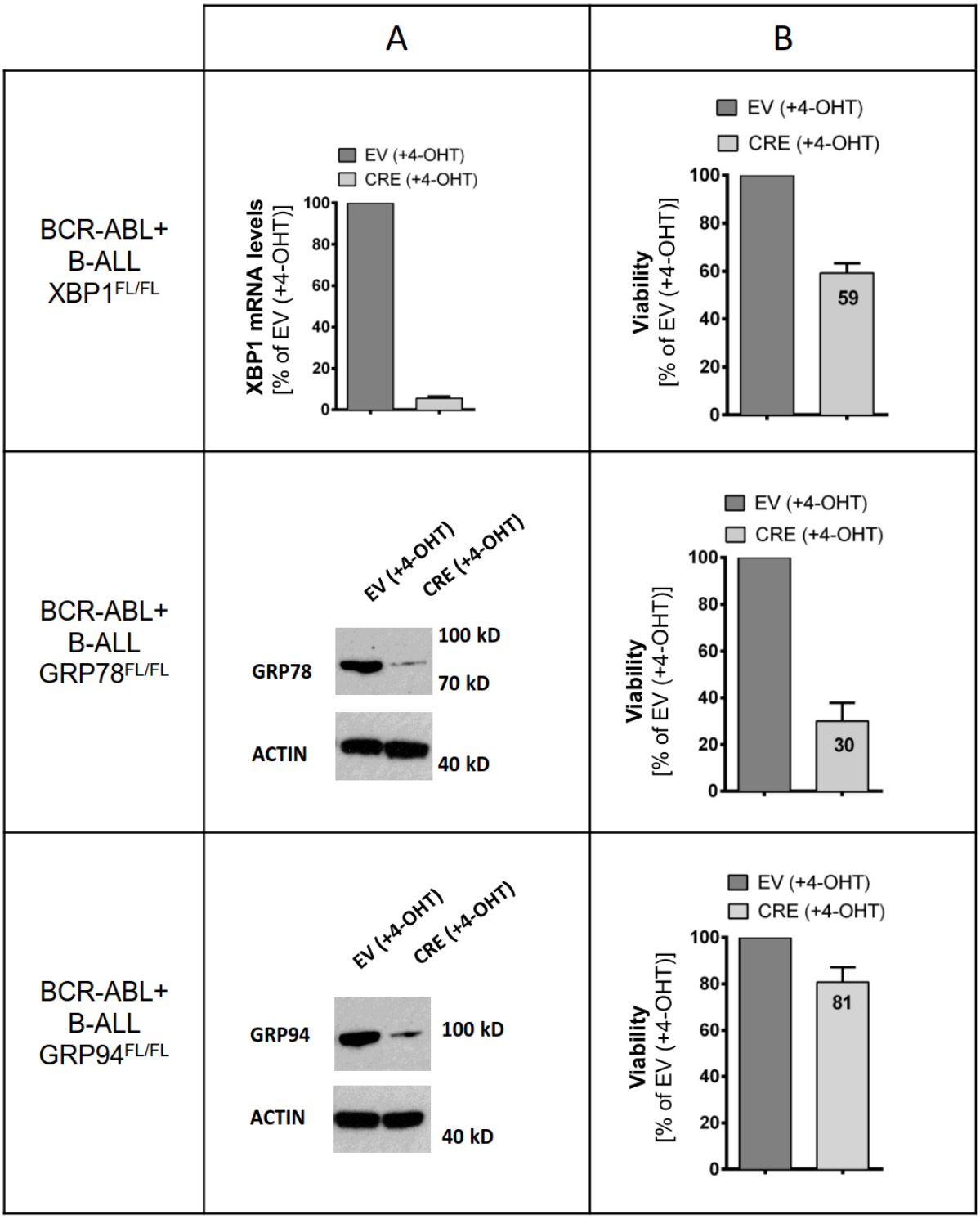

Figure S6. Combination of CB-5083 with either HNA, bortezomib, or prednisolone

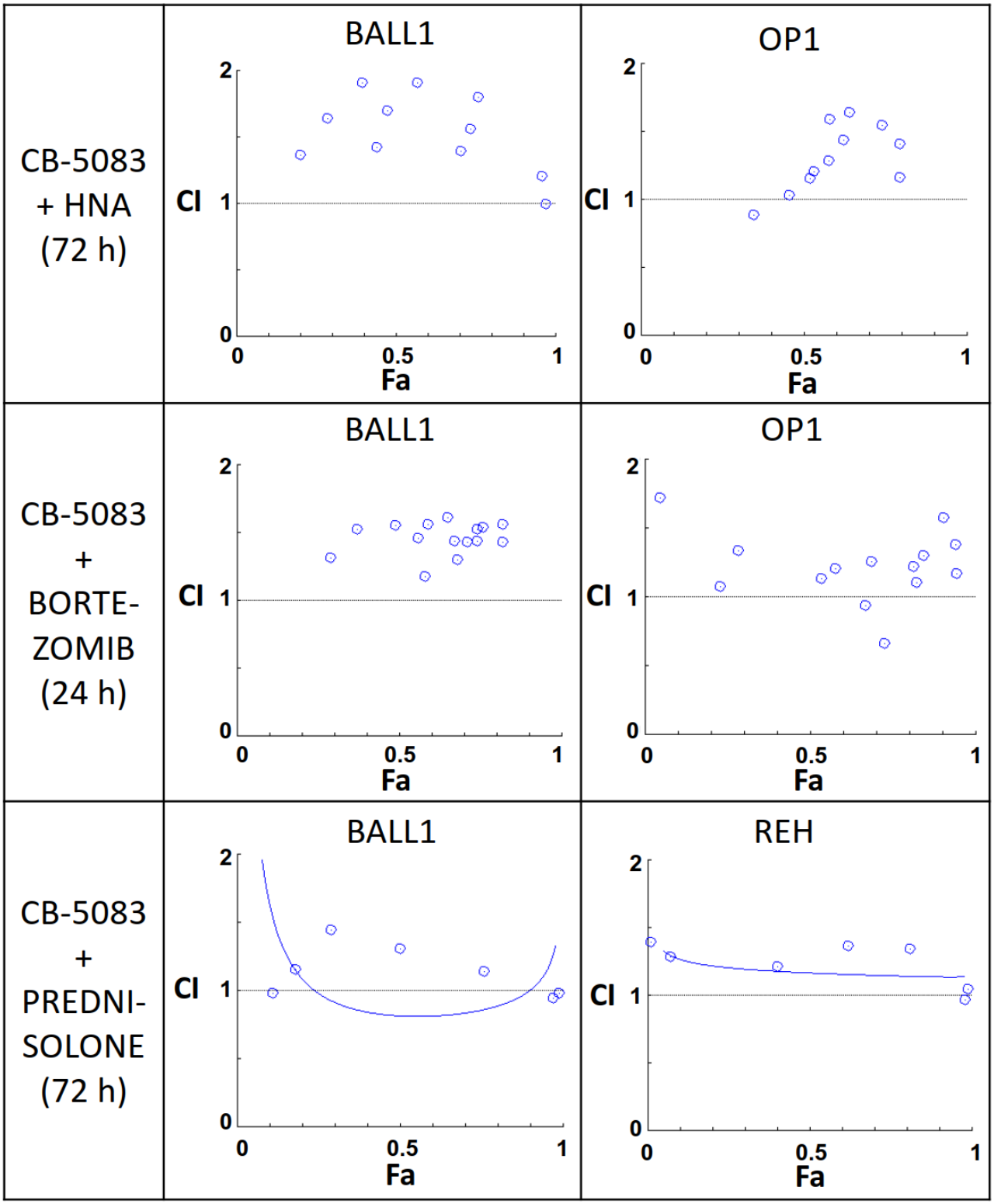

Figure S7. Induction of ER stress with thapsigargin.