Novel PI3kδ inhibitor roginolisib synergizes with venetoclax in hematologic malignancies

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Supplemental Data

1. Supplementary Materials and Methods

2. Supplementary Figures

3. Supplementary Tables

References

1. Supplementary Materials and Methods

Cell Lines and CLL Sample Culture Conditions

CLL cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 2 mM L-glutamine. Cells were periodically tested for mycoplasma negativity using the MycoAlert Mycoplasma Detection Kit (Lonza, Walkersville, MD, USA). AffiniPure F(ab')$_2$ Fragment Goat Anti-Human IgM, F(ab')$_2$ fragment specific (IgM) 10 μg/mL (Jackson ImmunoResearch, West Grove, PA, USA) was used for BCR cross-linking. The PI3Kδ inhibitors roginolisib (iOnctura SA, Geneve, Switzerland) and idelalisib, the PI3Kδ/γ inhibitor duvelisib, and the BCL-2 inhibitors venetoclax (MedChem Express, Monmouth junction, NJ, USA) were used at concentrations as indicated in figures or figure legends. Analysis of synergy was done by determining the combination index (CI) using the method of Chou and Talalay [1] and the software package CompuSyn (ComboSyn Inc., Paramus, NJ, USA).

Pharmacological Screening

A custom library based on the Cambridge Cancer Compound Library from Selleckchem was designed, including 474 compounds. Two lymphoma cell lines (SP53 and HH) with moderate sensitivity to roginolisib were used for the combination screening. Cells were seeded at 10,000 cell/well and exposed to DMSO, roginolisib (5 μM), or individual library compounds (5 μM) in single and in combination with roginolisib at the same concentrations of the single compounds. The cell culture medium was added to untreated cells, and wells containing the medium were included on each plate and used as blanks for absorbance readings. Cells were incubated for 72 hours at 37°C, 5% CO_2. The antiproliferative effect of single and combination treatments was determined by adding 20 μL MTT [3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazoliumbromide]

reagent (Sigma Aldrich, Buchs, Switzerland) to each well and the plates were incubated at 37°C for 4 hours, followed by 50 µL of sodium dodecyl sulfate (SDS) (250 µM SDS, 0.21% fuming HCl) lysis buffer. The plates were kept overnight, and then the absorbance was read at 570 nm using the Cytation 3 instrument (BioTek, Winooski, VT, USA).

Validation of Selected Inhibitor Compounds

Validation experiments were performed as previously described [2], extending the panel of lymphoma cell lines and using different compounds hitting the same targets. The lymphoma cells were exposed to increasing doses of roginolisib and the combination, either as single agents or combined, for 72 hours. Venetoclax and S55746 were used as BCL2 inhibitors, S64315 and S63845 as MCL1 targeting molecules, and belinostat and vorinostat as HDAC inhibitors. All compounds were purchased from Selleckchem (Houston, TX, USA).

Venetoclax, S55746, S64315, S63845, and belinostat were used at a maximum concentration of 10 µM with up to seven 1:4 dilutions. Roginolisib and vorinostat were used at a maximum concentration of 10µM with up to seven 1:3 dilutions. An untreated control was also included in each experiment. MTT assay was performed as described above.

Apoptosis Assay

Apoptosis analysis was performed as described elsewhere [3]. In brief, the patient PBMCs were co-cultured with HS5 fibroblast cells and treated with the drugs for 48h. The CLL cells were selected with CD19-PE and CD5-BV421 gating, and apoptotic and live cells were stained using annexin V-APC and 7AAD using the apoptosis detection kit (BioLegend, San Diego, CA, USA). The percentage of viable cells was determined by flow cytometric analysis. Apoptotic analysis for the DLBCL cell lines was done using annexin V- APC and 7AAD staining following 48h treatment

with the drugs. Cells were acquired using a Fortessa X-20 (BD Bioscience, Franklin Lakes, NJ, USA). Data were analyzed with FlowJo software v10.10.0 (RRID:SCR_008520).

Immunoblotting Analysis

Cells were washed in ice-cold PBS, pelleted, and lysed in RIPA lysis buffer containing protease and phosphatase inhibitors. The protein samples were separated by SDS-PAGE and transferred to TransBlot Turbo nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Membranes were blotted at 4°C in the presence of 5% nonfat dry milk with the following antibodies: phospho-AKT (cat. 4060, RRID:AB_2315049), phospho-ERK1/2(cat. 4370, RRID:AB_2315112), phospho-FoxO1 (cat. 2599, RRID:AB_2106814), phospho-GSK3α/β (cat. 9331, RRID:AB_329830), AKT (cat. 2920, RRID:AB_1147620), ERK1/2 (cat. 4696, RRID:AB_390780), FoxO1 (cat. 2880, RRID:AB_2106495), GSK3α/β (cat. 5676, RRID:AB_10547140), BCL_{XL} (cat. 2764, RRID:AB_2228008), BIM (cat. 2819, RRID:AB_10692515), MCL1 (cat. 94296, RRID:AB_2722740), A1/Bfl1 (cat. 14093, RRID:AB_2798390), PUMA (cat. 12450, RRID:AB_2797920), BCL2 (cat. 4223, RRID:AB_1903909), PARP (cat.9542), Caspase7 (cat. 9494) and GAPDH (cat. 97166, RRID:AB_2756824) from Cell Signaling technology, Danvers, MA, USA. Immunodetection was done on the ChemiDoc MP detection system (Bio-Rad, Hercules, CA, USA). The quantification of immunoblots was done using ImageJ software (RRID:SCR_003070).

MCL1 Knockdown and BIM Overexpression Experiments

MCL-1 knockdown experiments were performed using a dicer-substrate siRNA with the following sequence: sense strand 5’ UGGUAAGUAUUCCUUAGAUAGGUTT 3’, antisense 5’ AAACCUAUCUAAGGAA UACUUACCAAA 3’ (IDT, Newark, NJ, USA). For BIM

overexpression experiments, in vitro-transcribed capped and poly (A) tailed mRNAs were prepared from BIM_{EL}-pCMV6 and BIM_{L}-pCMV6, plasmids (Origene, Rockville, MD, USA) or empty pCDNA3 using the mMESSAGE mMACHINE T7 Ultra Kit and purified with the MEGAclear kit (Thermo Fisher Scientific, Waltham, MA, USA). Nucleofection of siRNA and mRNAs was performed with the Nucleofector system (Lonza, Walkersville, MD, USA) using Nucleofector Solution V and the C-009 program for the DLBCL cell lines or Solution V and the U-013 program for the CLL cells.

Statistical Analysis

Analysis of synergy was determined according to the Chou-Talalay Combination Index (CI) [1], calculated with the Synergy R package [4], and the HSA score [5]. The effect of the combinations was defined as synergistic ($CI < 0.9$; $HSA > 10$), additive ($0.9 < CI > 1.1$; $-10 < HSA > 10$), or antagonist ($CI > 1.1$; $HSA < -10$). Venetoclax sensitivity following MCL1 downregulation or BIM overexpression was measured by comparing the observed tumor cell survival vs expected tumor cell survival, as described elsewhere [6, 7]. In brief, observed survival corrected for apoptosis induced by control transfection was plotted against expected survival, calculated from the product of surviving cells following transfection with target siRNA or mRNA and surviving cells following venetoclax treatment of cells transfected with control siRNA or mRNA. Dots that fall on the diagonal XY line represent the situation in which observed survival equals predicted survival and indicate additive interactions. Dots beneath this XY line represent observed survival < expected survival and indicate synergistic interactions. Statistical analysis was done using One-way ANOVA and Dunnett's multiple-comparison post- test as needed using GraphPad PRISM 10 software (RRID:SCR_002798). P value ≤ 0.05 was considered significant.

2. Supplementary Figures

Supplementary Figure 1

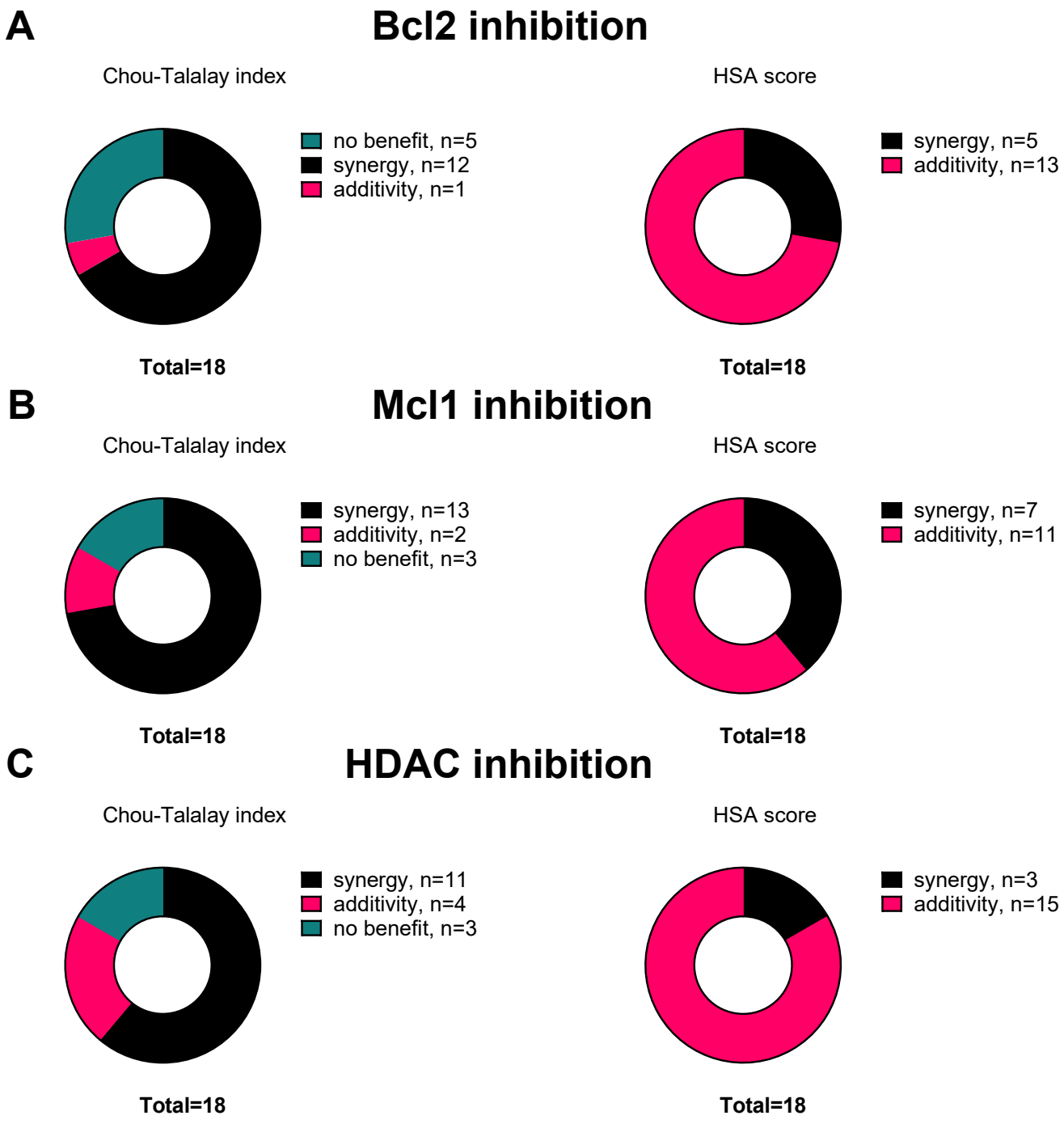

Fig. S1: Combination Index and HSA score of roginolisib with (A) Bcl2 inhibition (B) Mcl2 inhibition and (C) HDAC inhibition in lymphoma cell lines using MTT assay. Median Combination Index (Chou-Talalay index) score and HSA score for the MTT assay analysis carried out in the cell lines SU-DHL6, TMD8, FARAGE, JVM2, SP49, GRANTA519, MEC1, MJ, and YT of roginolisib in combination with the BCL2 inhibitors venetoclax and S55746 (A), with the MCL1 inhibitors S64315 (MIK665) and S63845 (B) and the HDAC inhibitors belinostat and vorinostat (C) as reported in Table S2.

Supplementary Figure 2

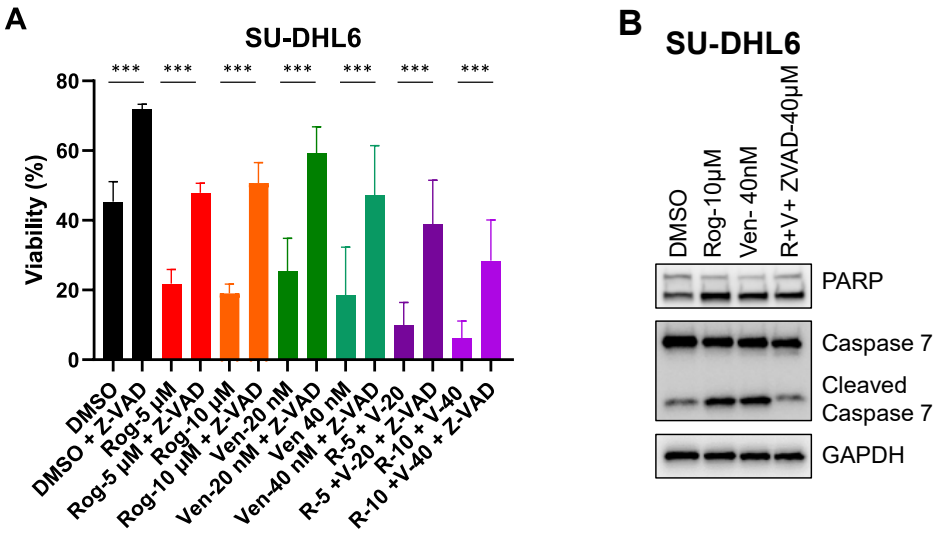

Fig. S2: Apoptosis in diffuse large B cell lymphoma (DLBCL) cell line is blocked by pan-caspase inhibitor. (A) Viability by Annexin V-APC/7AAD of cell lines SU-DHL-6 following 48h in vitro treatment with roginolisib (5 µM or 10 µM) or venetoclax (20 nM or 40 nM) alone or in combination in the presence of pan-caspase inhibitor Z-VAD (40 µM) and compared with cells treated with DMSO. The datasets are analyzed using a linear mixed models approach, with each replicate modeled as random effects. This allows us to perform 'paired tests' while using all the data to estimate variances better. **(B)** A representative Immunoblot of PARP and Caspase 7 followed by 24h treatment of roginolisib (10 µM), venetoclax (40 nM) or rogi+ven in the presence of Z-VAD in SU-DHL6 cell line.

Supplementary Figure 3

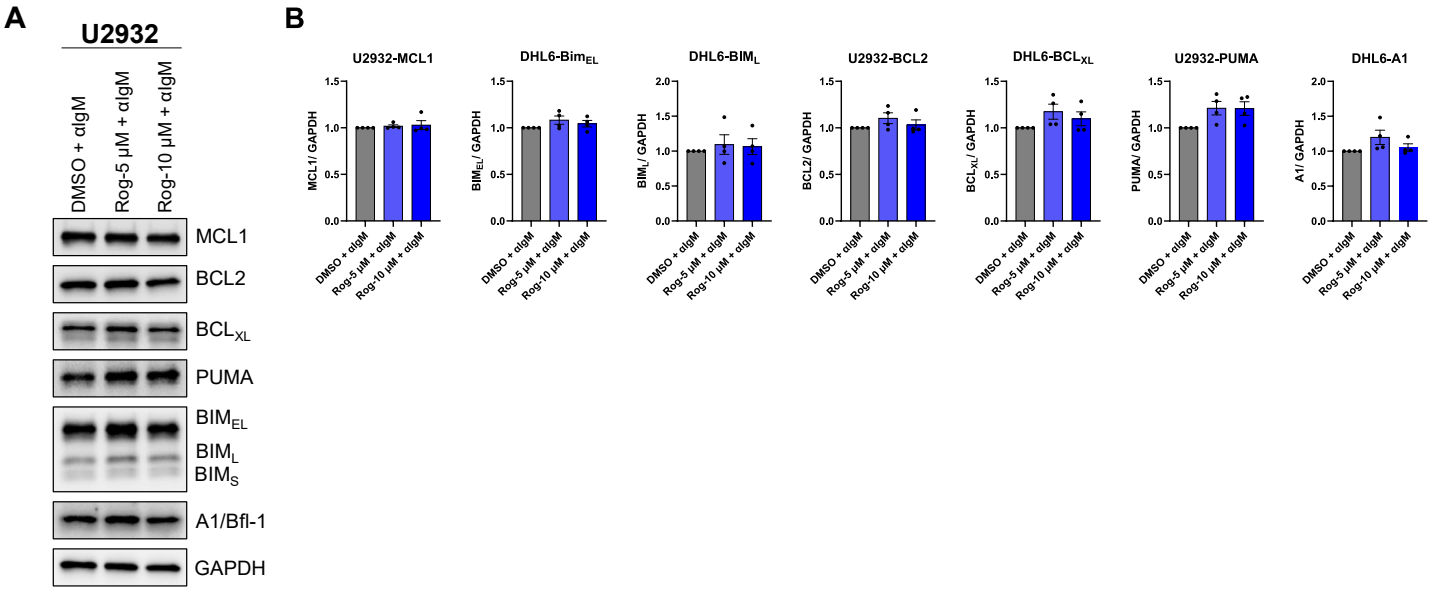

Fig. S3: Effect of roginolisib on BCL-2 family proteins in diffuse large B cell lymphoma (DLBCL) cell line U2932. **(A)** Immunoblot of Bcl-2 family proteins (B). Densitometric quantification and fold changes of MCL1, BIL$_{EL}$, BIM$_L$, BCL2, BCL$_{XL}$, PUMA and A1 following normalization with GAPDH. The levels of proteins were measured following 24h treatment with 5 μM or 10 μM of roginolisib in the presence of αIgM (10 μg/mL) and compared with cells treated with DMSO. Data shown are ± SEM from biological replicates.

Supplementary Figure 4

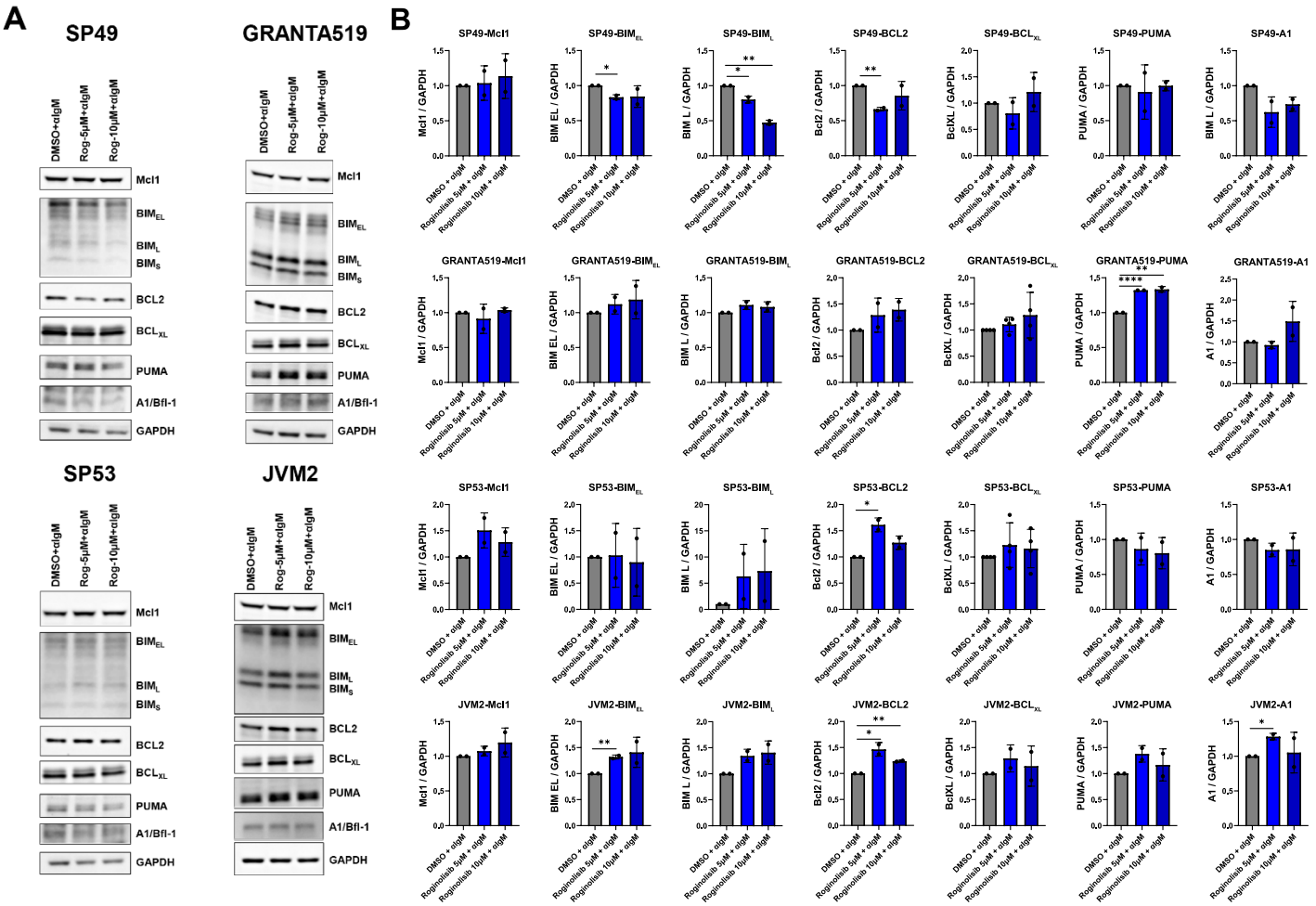

Fig. S4: Effect of roginolisib on Bcl-2 family proteins in mantle cell lymphoma (MCL) cell lines SP49, GRANTA519, SP53 and JVM2. (A) Immunoblot of Bcl-2 family proteins (B). Densitometric quantification and fold changes of MCL1, BIL_{EL}, BIM_L, BCL2, BCL_{XL}, PUMA and A1 following normalization with GAPDH. The levels of proteins were measured following 24h treatment with 5 μM or 10 μM of roginolisib in the presence of αIgM (10 μg/mL) and compared with cells treated with DMSO. Data shown are ± SEM from biological replicates.

Supplementary Figure 5

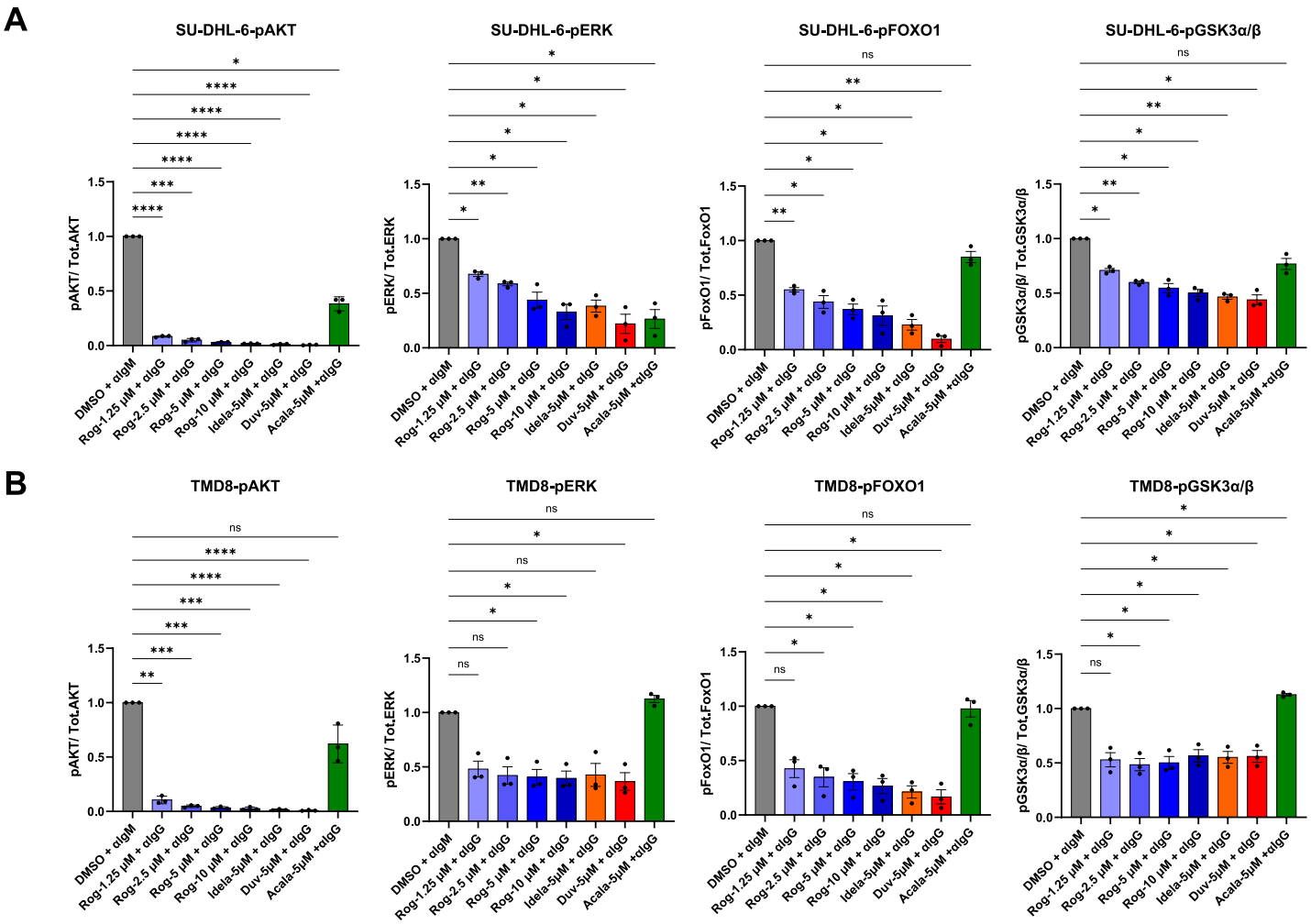

Fig. S5. Effect of roginolisib on B cell receptor (BCR) downstream kinases in diffuse large B cell lymphoma (DLBCL) cell lines SU-DHL-6 and TMD8. (A-B) Densitometry plots for phospho-AKT (S473) normalized to total AKT, phospho-ERK normalized to total ERK, phospho-FOXO1 normalized to total FOXO1 and phospho-GSK3α/β normalized to total GSK3α/β from SU-DHL-6 and TMD8. Data shown are ± SEM from biological replicates. Statistical analysis was done using one-way ANOVA and Dunnett's multiple-comparison post- test. Graphs were generated using GraphPad Prism 10.0 software. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns= non-significant.

Supplementary Figure 6

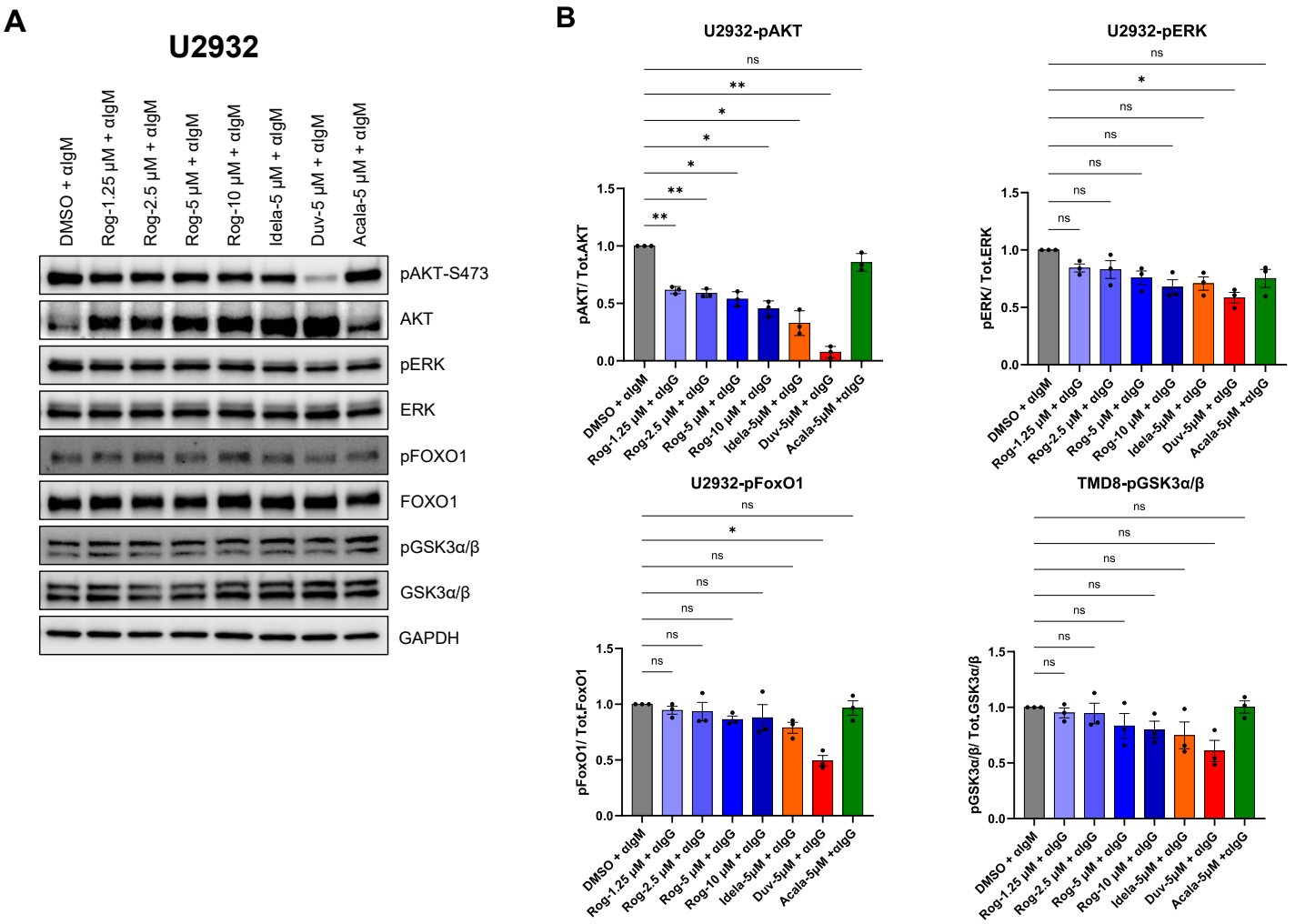

Fig. S6. Effect of roginolisib on B cell receptor (BCR) downstream kinases in diffuse large B cell lymphoma (DLBCL) cell line U2932. (A) A representative immunoblot analysis of phosphorylated AKT, ERK, FOXO1 and GSK3α/β in U2932 following 1 hour treatment with increasing concentrations of roginolisib (as indicated in the figure), or 5 µM of idelalisib, duvelisib or acalabrutinib followed by 30' stimulation with αIgM (10 µg/mL) and compared against cells treated with DMSO + αIgM control. **(B)** Densitometry plots for phospho-AKT (S473) normalized to total AKT, phospho-ERK normalized to total ERK, phospho-FOXO1 normalized to total FOXO1 and phospho-GSK3α/β normalized to total GSK3α/β from SU-DHL-6 and TMD8. Data shown are ± SEM from biological replicates. Statistical analysis was done using one-way ANOVA and Dunnett's multiple-comparison post- test. Graphs were generated using GraphPad Prism 10.0 software. *p < 0.05, **p < 0.01, ns= non-significant.

Supplementary Figure 7

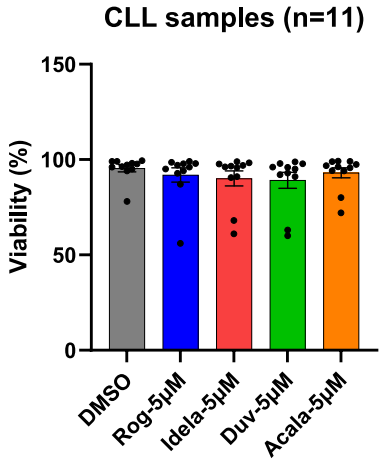

Fig. S7: Effect of BCRi on apoptosis in Chronic Lymphocytic Leukemia (CLL) patient samples. Viability by Annexin V-APC/7AAD of CLL patient PBMCs (n=11) following 48h in vitro treatment with 5 μM of roginolisib, idelalisib, duvalisib or acalabrutinib and compared with DMSO.

Supplementary Figure 8

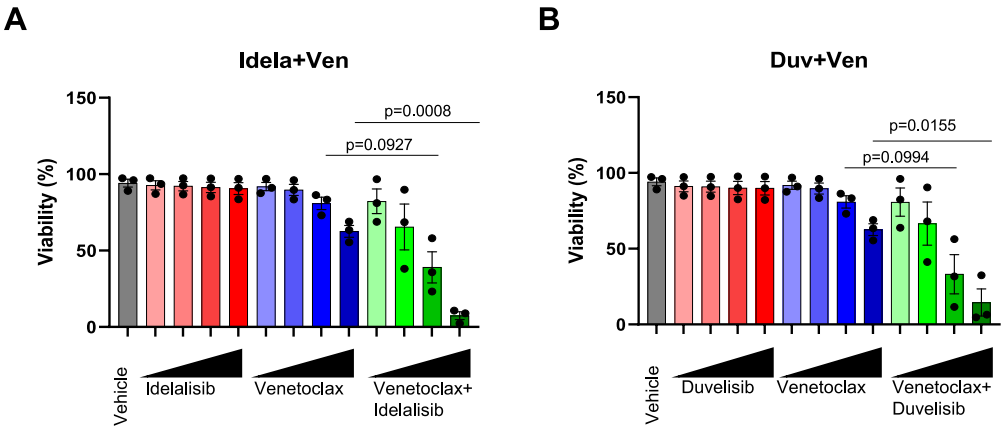

Fig. S8: PI3K inhibitors idelalisib and duvelisib show synergy with venetoclax in Chronic Lymphocytic Leukemia (CLL) patient samples. Viability by Annexin V-APC/7AAD of CLL patient PBMC following 48h in vitro treatment with increasing concentrations of idelalisib (0.625 μM, 1.25 μM, 2.55 μM or 5 μM) or duvelisib (0.625 μM, 1.25 μM, 2.55 μM or 5 μM) or venetoclax (1 nM, 2 nM, 4 nM or 8 nM) alone or in combination, in the presence of HS5 stromal cell co-culture and compared with cells treated with DMSO. Data represent ± SEM from biological replicates. Statistical analysis was done using paired t-tests.

Supplementary Figure 9

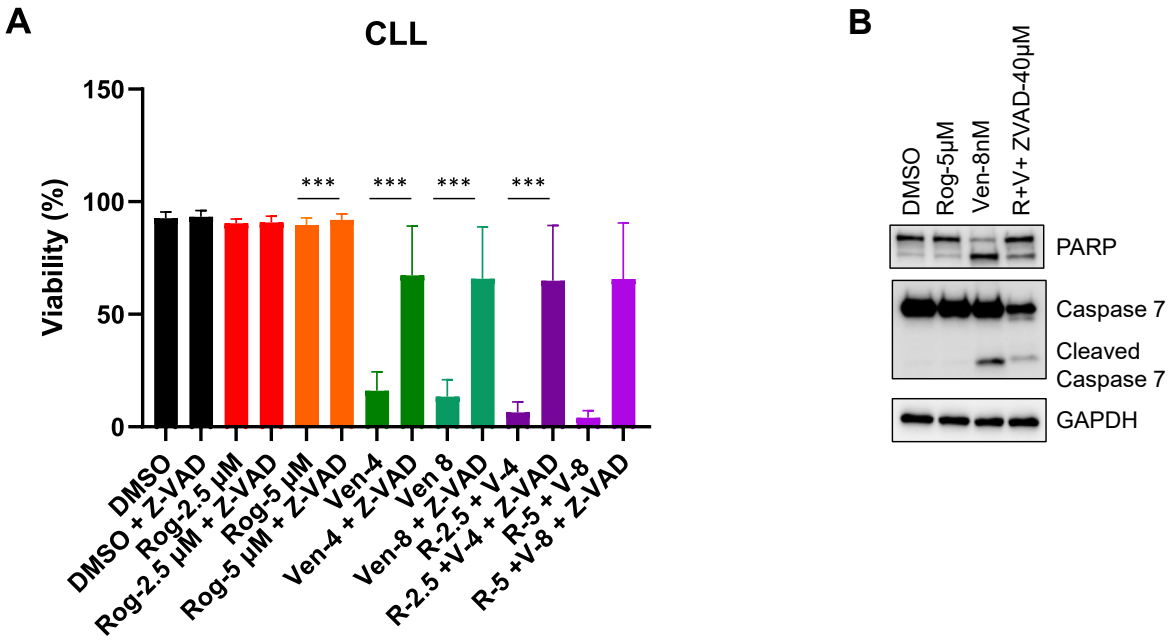

Fig. S9: Apoptosis in Chronic Lymphocytic Leukemia (CLL) cells blocked by pan-caspase inhibitor. (A) Viability by Annexin V-APC/7AAD of CLL cells following 48h in vitro treatment roginolisib (2.5 µM or 5 µM) or venetoclax (4 nM or 8 nM) alone or in combination in the presence of pan-caspase inhibitor Z-VAD (40 µM) and compared with cells treated with DMSO. The datasets are analyzed using linear mixed models' approach, with patients modeled as random effects. This allows to perform 'paired tests' while using all the data to estimate variances better. **(B)** A representative immunoblot of PARP and Caspase 7 followed by 24h treatment of roginolisib (5 µM), venetoclax (8 nM) or rogi+ven in the presence of Z-VAD in CLL cells.

Supplementary Figure 10

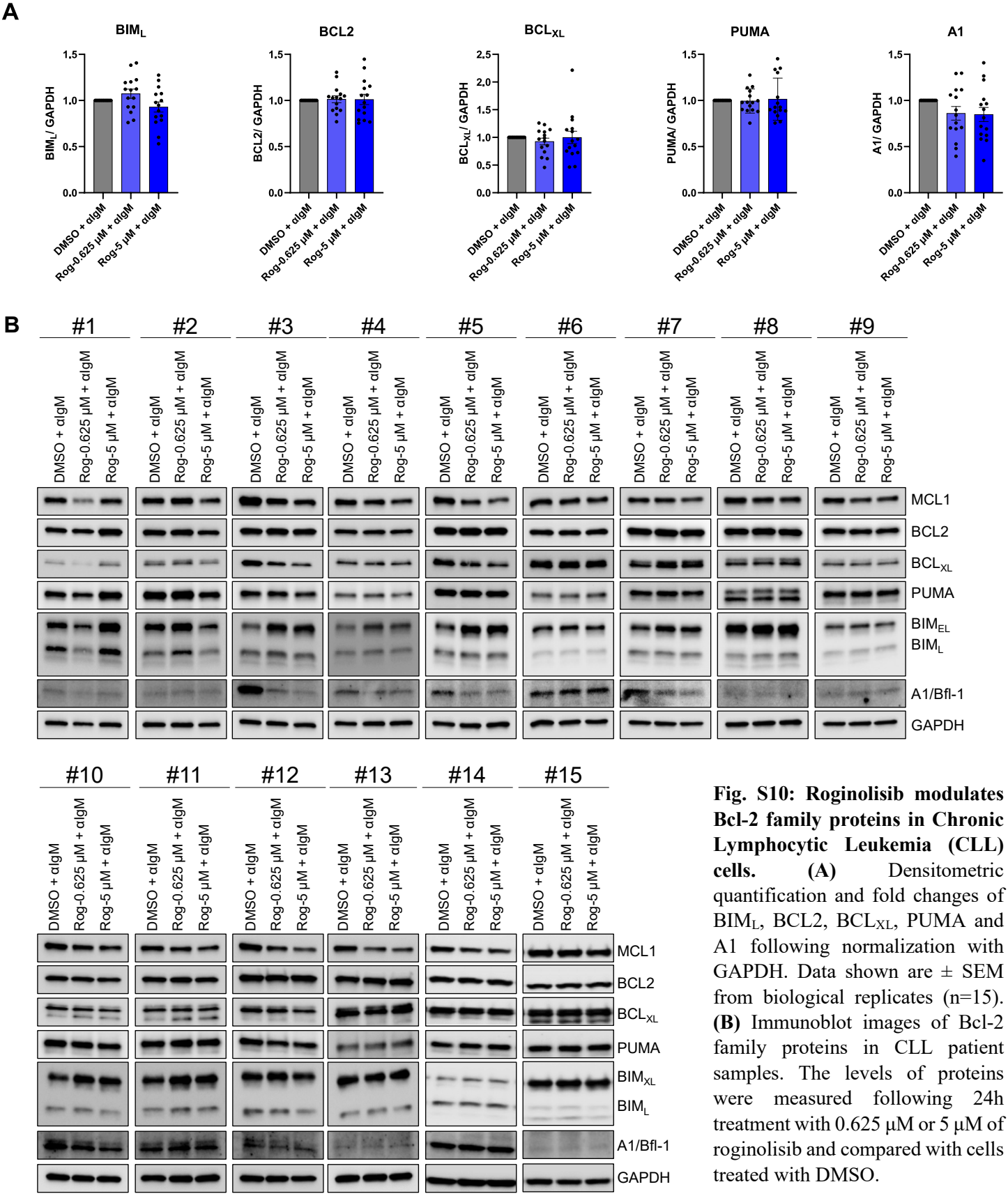

Fig. S10: Roginolisib modulates Bcl-2 family proteins in Chronic Lymphocytic Leukemia (CLL) cells. **(A)** Densitometric quantification and fold changes of BIM_L, BCL2, BCL_{XL}, PUMA and A1 following normalization with GAPDH. Data shown are ± SEM from biological replicates (n=15). **(B)** Immunoblot images of Bcl-2 family proteins in CLL patient samples. The levels of proteins were measured following 24h treatment with 0.625 μM or 5 μM of roginolisib and compared with cells treated with DMSO.

3. Supplementary Tables

Supplementary Table 1

(A) Custom library composition. (B) Proliferation percentage in SP53 and HH cell lines after 72 hours treatment with each drug in the library.

Supplementary Table 2

Combination Index (CI) score and HSA score for the MTT assay and apoptosis analysis carried out in the cell lines SU-DHL-6, TMD8, FARAGE, U2932, JVM2, SP49, SP53, GRANTA519, MEC1, MJ, and YT.

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