SUPPLEMENTAL MATERIAL

Targeting chronic lymphocytic leukemia with N-methylated peptides thrombospondin-1-derived peptides overcomes drug resistance

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SUPPLEMENTAL METHODS

Patients, B-cell purification, and culture conditions. The procedures in our manuscript were followed in accordance with the Helsinki Declaration and with the agreement of the ethical committee on human experimentation at Pitié-Salpêtrière Hospital (CPPIDF6, 05/21/2014, Paris, France). After obtaining written consent, peripheral blood was collected from 56 previously untreated CLL patients diagnosed according to classical morphological and immunophenotypic criteria[1] (Supplemental Table 1). This includes the diagnosis of the clinical Binet Staging and the assessment of the *IGHV* mutational status. Deletions of 17p13, 11q22, 13q14, trisomy 12 and 2p gain were detected by fluorescence in situ hybridization (FISH), as previously described.[2]

B-cells were positively or negatively selected by magnetic microbeads coupled either to an anti-CD19 monoclonal antibody (positive selection) or to a cocktail of biotinylated anti-CD2, CD14, CD16, CD36, CD43, and CD235a monoclonal antibodies (negative depletion) (Miltenyi Biotech). T-cells were negatively selected with a cocktail containing biotinylated antibodies against CD14, CD15, CD16, CD19, CD34, CD36, CD56, CD123 and CD235a. Natural Killers (NK) cells were negatively selected with a cocktail containing biotinylated antibodies against CD3, CD19, CD36, CD41a, CD66b, CD123, and CD235a (BD biosciences). Monocytes were negatively selected with a cocktail containing biotinylated antibodies against CD3, CD45RA, CD19, CD56 and CD235a (BD biosciences). Polynuclear neutrophiles (PNN) were isolated by dextran sedimentation from Ficoll erythrocyte-rich pellet. Mononuclear cells from bone marrow samples were purified using a standard Ficoll-Hypaque gradient. Then, hematopoietic stem cells and progenitors cells were positively selected using magnetic microbeads directly conjugated to anti-CD34 antibodies. No differences in the cell death response were encountered in positively or negatively selected cells.

Peptide synthesis. All the peptides were manually synthesized from Fmoc-protected amino acids utilizing standard solid phase peptide synthesis (SPPS) methods. The appropriate protected amino acids were sequentially coupled using PyOxim/Oxyma as coupling reagents. For N-Methylation, residues were *N*-methylated on solid-phase through Kessler's methodology: first, the free amino functionality was protected and activated with the *o*-nitrobenzenesulfonyl (*o*-NBS) group, then *N*-methylated using 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU) and dimethylsulfate (DMS), and finally deprotected (removal of *o*-NBS) by treating the resin with β-mercaptoethanol and DBU. The peptides were cleaved from the chlorotrityl or rink amide resin with the classical cleavage cocktail TFA/TIS/H_2O (95:2.5:2.5). Then, peptides were purified by reverse phase high-performance liquid chromatography (HPLC) on Waters system using preparative Macherey-Nagel column (Nucleodur HTec, C18, 250 x 16 mm id, 5 μm, 110 Å) at a flow rate of 14 mL/min with a gradient elution (0.1 % [v/v] TFA in acetonitrile) in aqueous 0.1 % [v/v] TFA. Homogeneous fractions were pooled and lyophilized after confirming purity greater than 95% by analytical HPLC. The final products were

characterized by analytical LCMS and NMR.

Flow cytometry. Tetramethylrhodamine ethyl ester (TMRE, 125 nM) was used for $\Delta\psi m$ quantification. Mitochondrial reactive oxygen species (ROS) over-generation was measured with Mitosox (5 μM) and total cellular ROS with hydroethidine (2 μM) (Invitrogen). Calreticulin (CALR) cell surface exposure was recorded with anti-calreticulin-PE antibody (Enzo Life Sciences). IgG (PE Mouse IgG1 isotype control, monoclonal antibody (MOPC-21)) was used as a negative control (Enzo Life Sciences). CD47 expression in immune cell population was measured with conjugated anti-CD47-PE (clone B6H12; BD Biosciences). IgG (PE Mouse IgG1, κ Isotype Control (MOPC-21) was used as a negative control (BD Biosciences). Activated B-cell population was assessed with conjugated CD69-FITC antibody (BD Biosciences).

To identify the different phases of the cell cycle, cells were treated with PKT16 (100 μM, 6 h) and 1 x 10^6 cells were used for bromodeoxyuridine (BrdU) assay using APC BrdU Flow kit (BD biosciences). The assay was performed according to the manufacturer's advice. After BrdU-APC (10 μM) and 7-AAD co-labelling, the percentage of cells in the different phases of the cell cycle was quantified in the flow cytometer.

Immune and hematopoietic subsets were discriminated by using combinations of fluorochrome-conjugated anti-human antibodies. CLL B-cells: CD19-PE (Miltenyi Biotech) and CD5-PE-Vio770 (Miltenyi Biotech). Normal B-cells from healthy donors CD19-PE (Miltenyi Biotech). T and NK-cells: CD19-PE, CD3-FITC and CD56-PE-Vio770 (Miltenyi Biotech). Monocytes: CD4-FITC and CD14-PE-Vio770 (Miltenyi Biotech). Hematopoietic stem cells (HSC) and common lymphoid and myeloid progenitors (CLP/CMP): CD34-PE, CD38-FITC (BD biosciences and Biolegend, respectively).

Protein extraction and immunoblotting. Cells treated or not with PKT16 (100 μM, 2 h) and pre-incubated or not with FSK (100 μM) or suramin (100 μM) were lysed with M-PER Mammalian Protein Extraction Reagent (ThermoFisher Scientific, 78501) supplemented with Halt Protease and Phosphatase Inhibitor Cocktail (ThermoFisher Scientific). The protein concentration was determined using the Bio-Rad DC kit on an Infinite 1000 PRO plate reader (TECAN). 150 μg of protein was loaded in 8 % SDS-PAGE gels. After blotting, nitrocellulose filters were probed overnight at 4°C with primary antibodies against PLCγ1 (clone D9H10; Cell Signaling Technology) and PLCγ1-Y783 (Cell Signaling Technology; 2821). Immunoreactive proteins were detected using HRP-conjugated secondary antibodies and visualized with the ECL system ThermoFisher Scientific). Immunoblot images were acquired on a Bio-Imaging System MF-ChemiBIS 4.2 (DNR Bio-Imaging Systems). PLCγ1-Y783 was quantified using the ImageJ software. The optical density was normalized to the background and was expressed relative to total PLCγ1 (set at 1.0).

DNA fragmentation. For the detection of oligonucleosomal DNA fragmentation, nuclear DNA from 2 x 10^6 lysed cells (treated with proteinase K and RNAase A according to standard protocols) was subjected to conventional horizontal agarose gel electrophoresis (1.8 %) followed by ethidium bromide staining.

Cell viability. Isolated CLL cells were treated with combinations of PKT16 at a low dose (50 μM, 6 h) and different doses of fludarabine, ibrutinib, idelalisib, or venetoclax (24 h) and cell viability was assessed using a CellTiter-Glo 2.0 Cell Viability kit (Promega), according to the manufacturer's instructions. Luminescence was measured on an Infinite M1000 PRO plate reader (TECAN).

Animals. Mice were housed at the Curie Institute animal facility (Paris, France) in strictly controlled specific-pathogen-free conditions. All experiments were done in accordance with ARRIVE ethical guidelines[3] and under the supervision of authorized investigators. The experimental protocol and animal housing followed institutional guidelines as put forth by the French Ethical Committee (Agreement number D-750602, France) and the ethics committee of the Institut Curie (Agreement number C75-05-18).

Individual tumor volume, relative tumor volume (RTV), and tumor growth inhibition were calculated according to standard methodology.[4] We also estimated the impact of the treatments on tumor progression (time for RTV x 6). This parameter indicated the probability of the tumors increasing 6 times their volume after 4NGG or PKT16 treatment. Mice were euthanized 15 days after the start of the treatments.

The hematological parameters, including Red Blood Cells (RBC), White Blood Cells (WBC), Platelet counts, Hemoglobin (Hb) levels, Hematocrit, and Mean Corpuscular Volume (MCV) were monitored in blood from either control (4NGG) or PKT16-treated mice with a Scil vet ABC analyzer (Scil veterinary excellence).

After 15 days of 4NGG or PKT16 treatment, kidneys, livers, spleens, and lymph nodes were fixed in 4 % paraformaldehyde (PFA) (ThermoFisher scientific) and embedded in paraffin with the automaton Leica ASP300. Paraffin blocks were sectioned (3 μm thickness) and deposited on slides. Slides were then deparaffinized and rehydrated for staining with hematoxylin-eosin-saffron (Autostainer DAKO 48L) and scanned with ZEISS Axio Scan Z1. Images were analyzed with Zen lite software (ZEISS).

Microscopy. OSU-CLL cells were treated during 6 h with PKHB1 or PKT16. Representative images were visualized in a Nikon Eclipse TE-2000-U fluorescent microscope and captured with a Nikon digital DXM 1200 camera.

Unless specified, reagents were from Sigma. CLL drugs and inhibitors were from SelleckChem.

SUPPLEMENTAL REFERENCES

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2. Cosson A, Chapiro E, Bougacha N, et al. Gain in the short arm of chromosome 2 (2p+) induces gene overexpression and drug resistance in chronic lymphocytic leukemia: analysis of the central role of XPO1. *Leukemia*. 2017;31(7):1625-1629.

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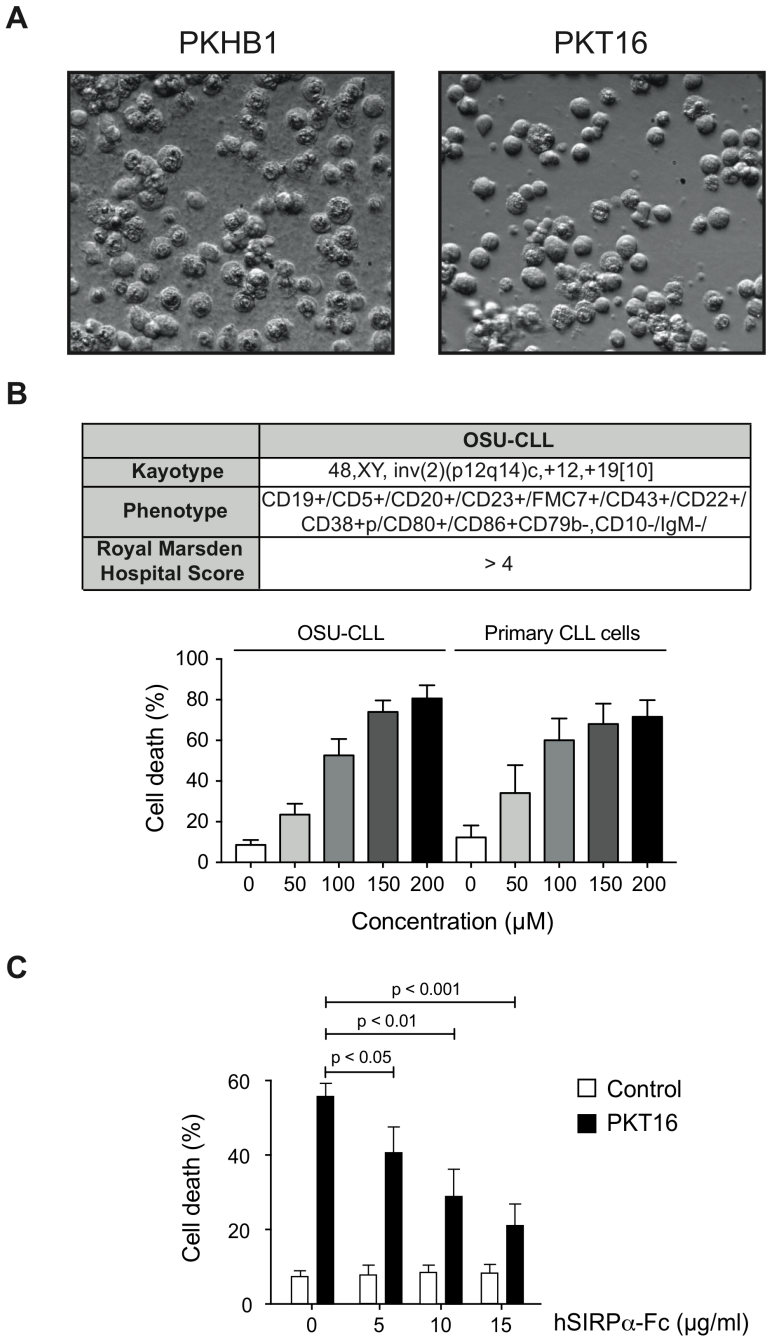
SUPPLEMENTAL TABLE 1

Patient number	Stage	*IGHV* Mutational Status	del17p	del11q	del13q	tri12	2p gain	Karyotype
Stage A								
CLL_1	A	UM	-	-	+	-	-	Normal
CLL_2	A	M	na	na	+	+	-	na
CLL_3	A	na	-	-	+	-	-	Normal
CLL_4	A	UM	-	-	-	-	-	na
CLL_5	A	UM	-	-	-	+	-	Abnormal
CLL_6	A	M	na	na	na	na	-	na
CLL_7	A	M	-	+	+	-	-	Abnormal
CLL_8	A	UM	-	-	-	-	-	Abnormal
CLL_9	A	M	-	-	-	+	-	Abnormal
CLL_10	A	M	-	-	-	-	-	na
CLL_11	A	M	-	-	+	-	-	Normal
CLL_12	A	M	-	-	+	-	-	na
CLL_13	A	M	na	na	na	na	-	na
CLL_14	A	M	-	-	-	-	-	Complex
CLL_15	A	M	-	-	+	-	-	na
CLL_16	A	M	-	-	-	-	-	Normal
CLL_17	A	UM	-	-	-	-	-	Normal
CLL_18	A	M	-	+	+	-	-	Abnormal
CLL_19	A	na	-	+	+	-	-	Abnormal
CLL_20	A	M	-	-	+	-	-	Abnormal
CLL_21	A	na	-	-	-	-	-	Abnormal
CLL_22	A	na	-	-	+	-	-	Abnormal
CLL_23	A	M	-	-	-	-	-	na
CLL_24	A	M	-	-	-	-	-	Normal
CLL_25	A	M	-	-	+	-	-	Normal
CLL_26	A	M	-	-	+	-	-	Normal
CLL_27	A	M	-	-	+	-	-	normal
CLL_28	A	UM	-	-	+	+	-	Complex
CLL_29	A	M	-	-	+	-	-	Abnormal
CLL_30	A	na	-	-	+	-	-	Normal
CLL_31	A	M	-	-	+	-	-	Normal
Stage B/C								
CLL_32	B	V3-21*	-	-	+	-	-	Abnormal
CLL_33	B	UM	+	-	-	+	+	Complex
CLL_34	B	UM	+	-	+	-	+	Complex
CLL_35	B	UM	-	-	+	-	-	Normal
CLL_36	B	UM	-	+	+	-	-	Abnormal
CLL_37	B	M	-	-	+	-	-	Abnormal
CLL_38	B	M	+	-	+	-	-	na
CLL_39	B	UM	-	+	-	-	+	Complex
CLL_40	B	UM	-	-	+	-	+	Abnormal
CLL_41	B	V3-21*	-	-	+	-	-	Abnormal
CLL_42	B	V3-21*	-	-	+	-	-	Normal
CLL_43	B	M	-	-	+	-	+	Abnormal
CLL_44	B	UM	+	-	+	-	-	Abnormal
CLL_45	B	UM	-	-	+	-	-	Normal
CLL_46	B	UM	-	+	+	-	-	Normal
CLL_47	B	UM	-	+	+	-	-	Abnormal
CLL_48	B	V3-21*	-	+	+	-	-	Abnormal
CLL_49	B	nd	+	-	+	-	-	Abnormal
CLL_50	B	UM	+	-	-	+	-	na
CLL_51	B	UM	-	+	-	-	-	Abnormal
CLL_52	C	UM	+	-	+	+	-	Complex
CLL_53	C	M	-	-	+	-	-	na
CLL_54	C	M	-	-	-	-	-	Abnormal
CLL_55	C	V3-21*	+	-	+	-	-	Complex
CLL_56	C	UM	-	-	-	-	-	Abnormal

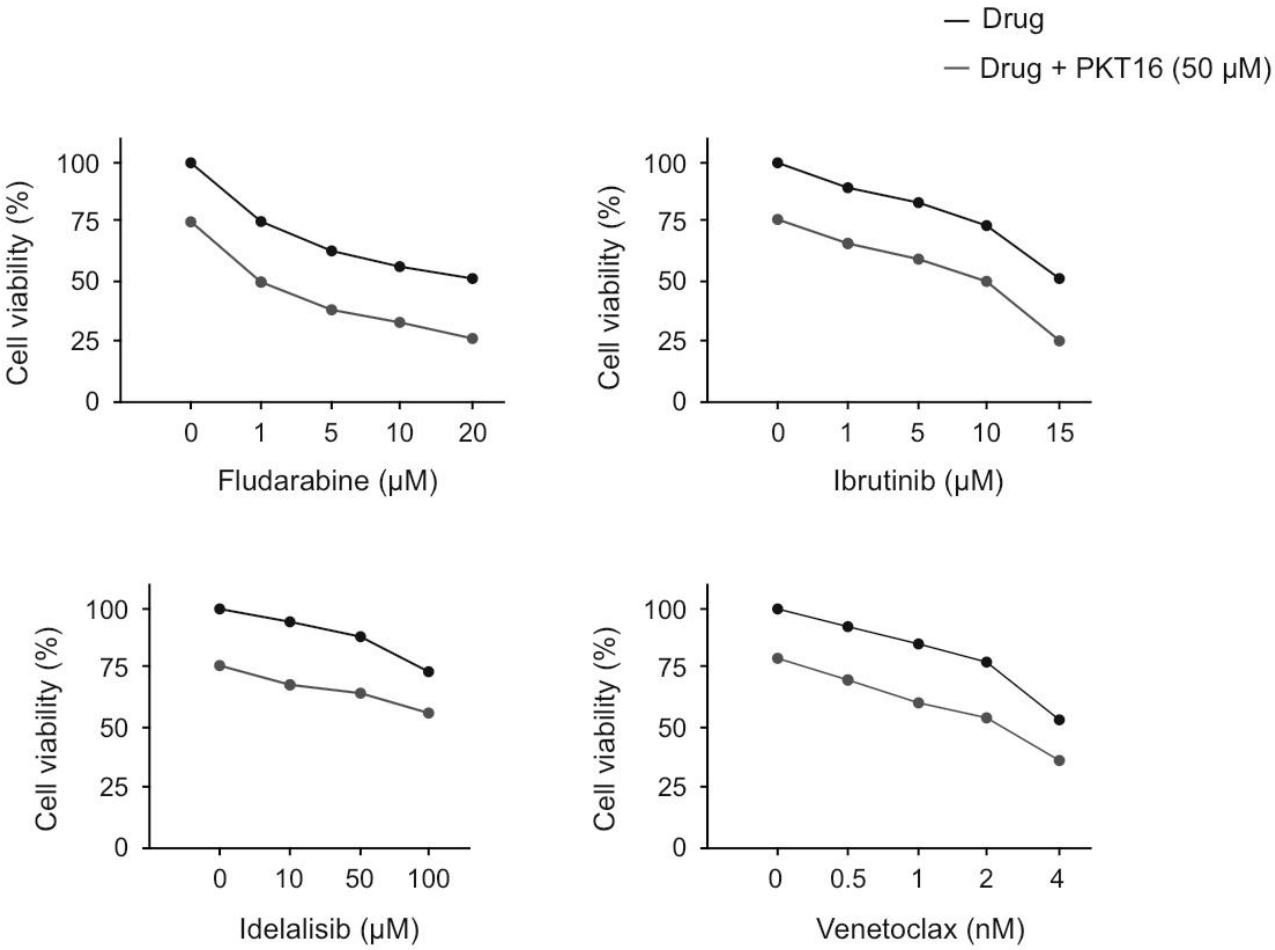
Supplemental Table 1: Features of the CLL patients included in the study. Stages A, B or C were determined according to Binet classification. *IGHV* mutational status homology ≥ 98%: M, mutated; UM, unmutated; V3-21*, subset conferring worse prognosis. Cytogenetic abnormalities were determined by karyotype and/or FISH: (-) negative; (+) positive. CLL patient karyotype was classified as: normal (no abnormalities detected), abnormal (< 3 chromosomal abnormalities), and complex (≥ 3 chromosomal abnormalities). del, deletion; na, not available.

SUPPLEMENTAL FIGURES

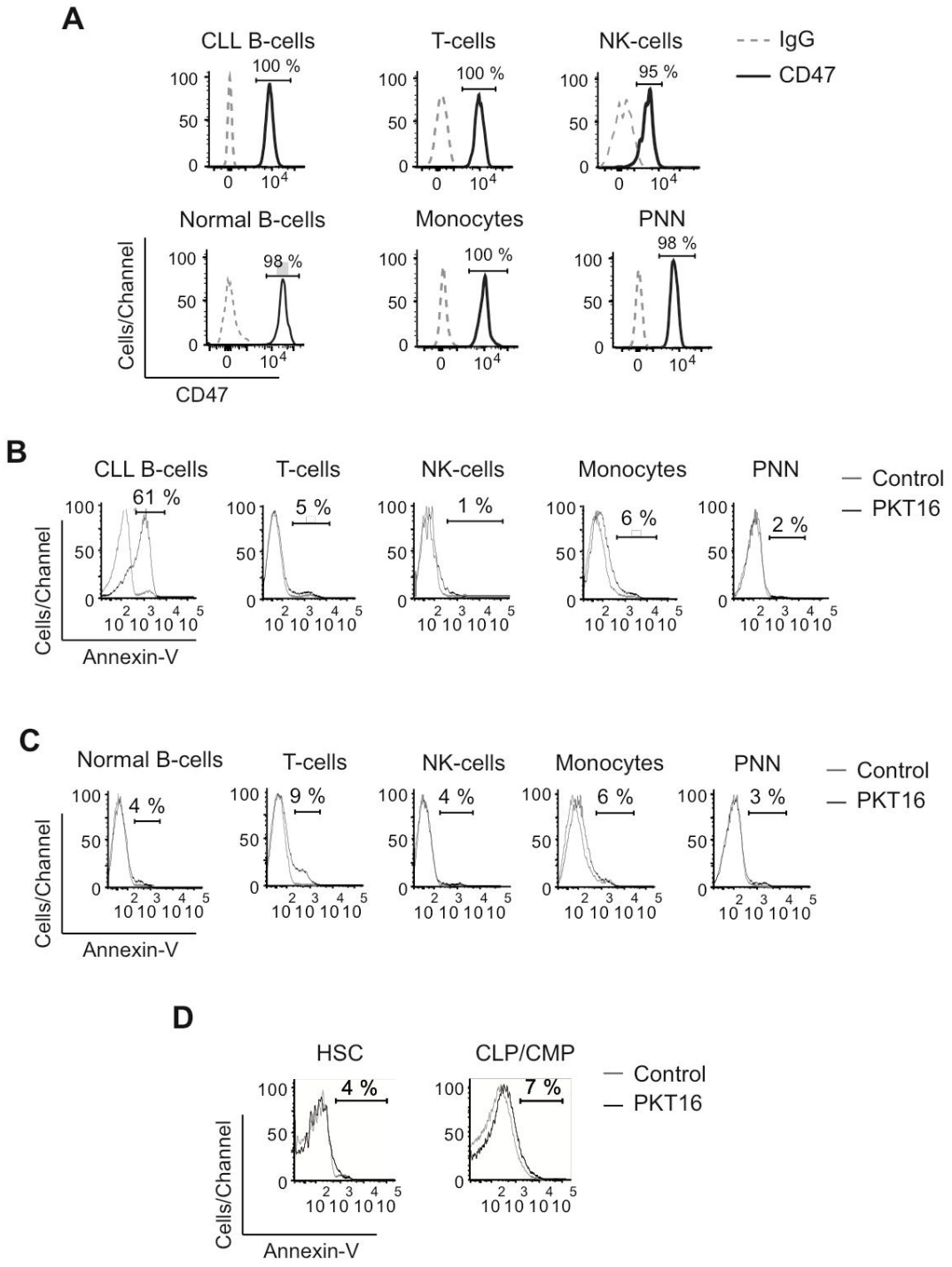


	OSU-CLL
Kayotype	48,XY, inv(2)(p12q14)c,+12,+19[10]
Phenotype	CD19+/CD5+/CD20+/CD23+/FMC7+/CD43+/CD22+/ CD38+p/CD80+/CD86+CD79b-,CD10-/IgM-/
Royal Marsden Hospital Score	> 4

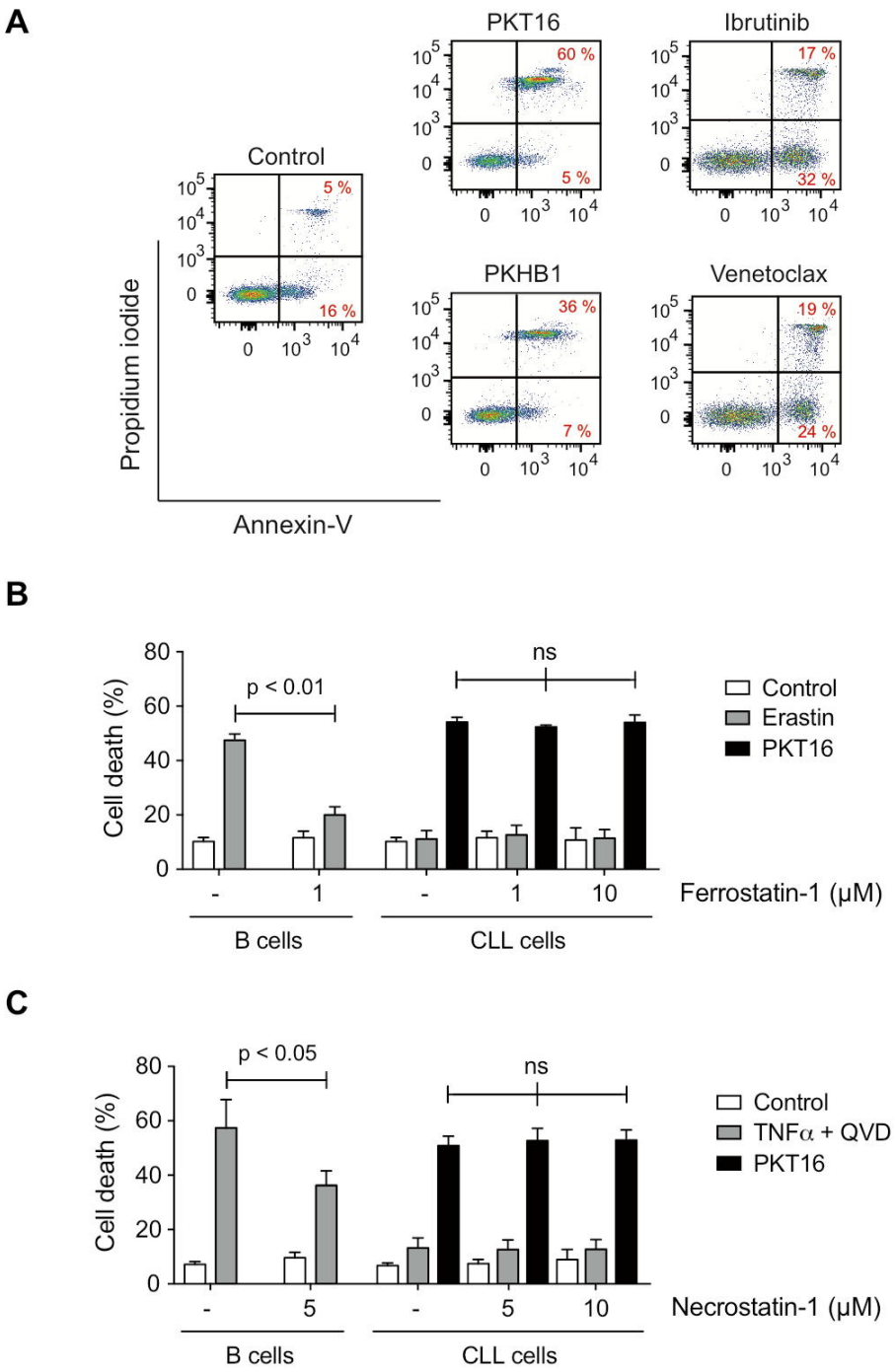
Supplemental Figure 1. PKT16, an improved TSP-1 mimetic peptide overcoming precipitation, induces PCD in primary cells and in the OSU-CLL cell line by CD47 triggering. (A) Representative images of OSU-CLL cells treated with PKHB1 or PKT16 (100 μM, 6 h). The β-sheet conformation associated to PKHB1 provokes aggregation and precipitation in culture medium (*left*). This problem was overcome in PKT16 by the introduction of the N-methyl arginine in the peptide backbone (*right*). **(B)** Table depicting the karyotype and the phenotype of the OSU-CLL cell line, an established CLL cell line that has shown a response to the TSP-1 mimetic peptides that is similar to the primary CLL cells. Cytotoxicity measured by Annexin-V/PI co-labeling in the OSU-CLL cell line and in a panel of CLL B lymphocytes treated with PKT16 (100 μM, 6 h) (n = 10). The data, referring to Annexin-V positivity, are presented in a plot as a mean ± SD. **(C)** Disruption of PKT16-CD47 interaction with the fusion protein hSIRPα-Fc inhibits PKT16-mediated PCD. Cell viability was determined in CLL cells that were pre-incubated with the indicated concentration of hSIRPα-Fc and treated with PKT16 (100 μM, 6 h). The percentages refer to the Annexin-V/PI-positive staining. The data are graphed as the mean ± SD (n = 5). Statistical significance was calculated by Mann-Whitney test.



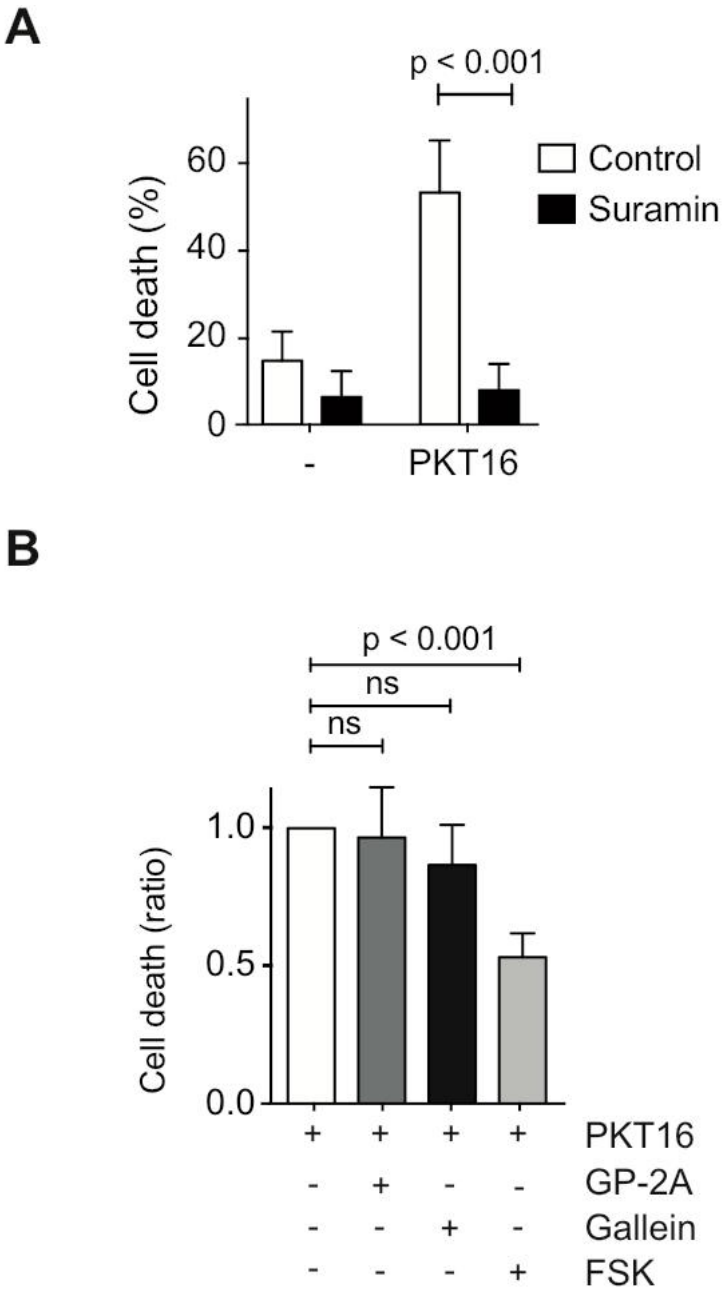
Supplemental Figure 2. Combination of PKT16 with fludarabine, ibrutinib, idelalisib, or venetoclax shows an additional cell death effect in primary CLL cells. CLL B-cells were isolated from CLL patients and incubated with combinations of PKT16 at a low dose (50 μM, 6 h) and different doses of fludarabine, ibrutinib, idelalisib, or venetoclax (24 h). Cell viability was evaluated using a CellTiter-Glo 2.0 Luminescent Cell Viability Assay kit. Luminescence was measured and graphed as a percentage of viable cells relative to drug untreated cells (100 % of viability). The results represent means for pooled triplicate values obtained in CLL cells purified from five patients.



Supplemental Figure 3. PKT16 induces PCD in the malignant CLL B-cells, sparing non-tumor immune cell subsets. (A) CD47 receptor expression was quantified by flow cytometry in different immune subsets from CLL patients (CLL B-cells, T-cells, NK-cells, Monocytes, and PNN) or in B-cells from healthy donors (Normal B-cells). A representative cytofluorometric plot for each population is shown. Note that all subsets express CD47. **(B**) CLL B-cells, T-cells, NK-cells, monocytes, and polynuclear neutrophils (PNN) were isolated from CLL patients, incubated or not (Control) with PKT16 (100 μM, 6 h), and the cytotoxicity induced was evaluated by Annexin-V labeling. A representative cytofluorometric plot for each population is shown. **(C)** B-cells, T-cells, NK-cells, monocytes, and polynuclear neutrophils (PNN) were isolated from healthy donors, incubated or not (Control) with PKT16 (100 μM, 6 h), and the cytotoxicity induced was evaluated by Annexin-V labeling. A representative cytofluorometric plot for each population is shown. **(D)** The hematopoietic stem cells (HSC) and the common lymphoid and myeloid progenitors (CLP and CMP) were purified from the bone marrow of healthy donors, incubated or not (Control) with PKT16 (100 μM, 6 h), and the induced cytotoxicity was evaluated by Annexin-V labeling. A representative cytofluorometric plot for each population is shown.

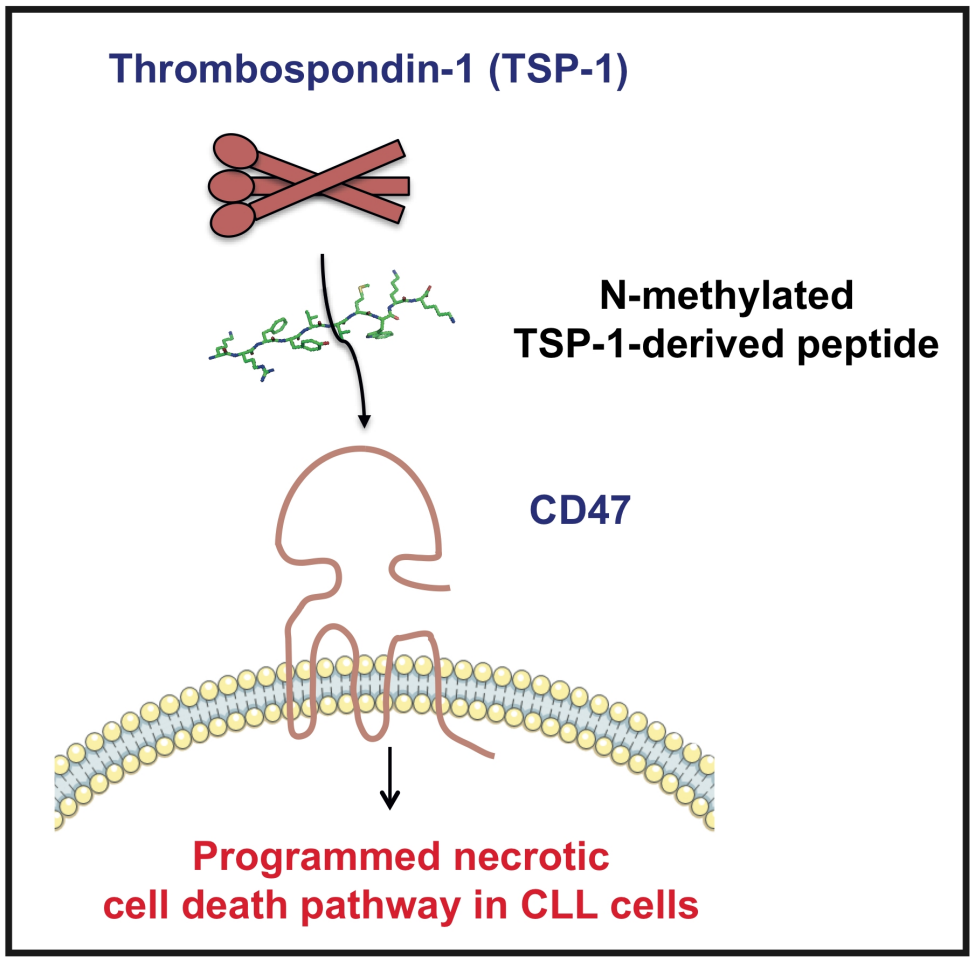


Supplemental Figure 4. The programmed necrotic cell death pathway enabled by PKT16 is not related to necroptosis or ferroptosis. (A) Cytotoxicity measured by Annexin-V/PI co-labeling in CLL B lymphocytes treated during 6 h with PKT16 or PKHB1 (100 μM) or 24 h with ibrutinib (15 μM) or venetoclax (4 nM). The percentages in red refer to Annexin-V or the Annexin-V/PI positive labeling. Representative flow cytometry plots are shown. In contrast to ibrutinib or venetoclax, the Annexin-V positive PI negative cell population is not generated after treatment with the TSP-1-mimetic peptides. **(B)** Cell death was measured in normal B cells or CLL cells pre-incubated with the vehicle (-) or the indicated concentration of the ferroptosis inhibitor ferrostatin-1 and untreated (Control) or treated with erastin (10 μM, 24 h) or PKT16 (100 μM, 6 h). After Annexin-V/PI co-staining, the percentages of Annexin-V positive cells were recorded and graphed as a mean ± SD (n = 5). **(C)** Cell death was measured in normal B cells or CLL cells pre-incubated with the vehicle (-) or the indicated concentration of the necroptosis inhibitor necrostatin-1 and untreated (Control) or treated with TNFα + QVD (30 ng/ml + 1 μM, 24 h) or PKT16 (100 μM, 6 h). After Annexin-V/PI co-staining, the percentages of Annexin-V positive cells were recorded and graphed as a mean ± SD (n = 5). Statistical significance was calculated by Mann-Whitney test.



Supplemental Figure 5. The Gαi proteins control PKT16-mediated killing. (A) Cell death was measured in the OSU-CLL cell line pre-incubated with the vehicle (Control) or the G protein inhibitor suramin (100 μM, 20 min) and untreated (-) or treated with PKT16 (100 μM, 6 h). After Annexin-V/PI co-staining, the percentages of Annexin-V positive cells were recorded and graphed as a mean ± SD (n = 10). **(B)** Cell death was measured in the OSU-CLL cell line pre-incubated with the vehicle (Control) or the GP-antagonist 2A (protein Gαq inhibitor, 30 μM, 30 min), gallein (protein G βγ dimer inhibitor, 30 μM, 30 min) or forskolin (protein Gαi inhibitor, FSK, 100 μM, 2 h) and treated with PKT16 (100 μM, 6 h). After Annexin-V/PI co-labeling and accounting for non-specific PCD, the percentages of Annexin-V positive cells were recorded, graphed, and depicted as a ratio of PCD relative to OSU-CLL cells pre-incubated with the vehicle and treated with PKT16 (considered as the maximum of PCD induced -set at 1.0-). Data are expressed as a mean ± SD (n = 12).

VISUAL ABSTRACT



CD47 ligation by N-methylated TSP-1-derived peptides enables a programmed necrotic cell death pathway in the CLL CD5^{+} B-lymphocytes.