Autologous T-cell activation fosters ABT-199 resistance in chronic lymphocytic leukemia: rationale for a combined therapy with SYK inhibitors and anti-CD20 monoclonal antibodies

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SUPPLEMENTARY DATA

SUPPLEMENTARY MATERIALS AND METHODS

Reagents and antibodies.

RPMI 1640, fetal calf serum (FCS), penicillin, trypsin and streptomycin were purchased from GIBCO (Massachusetts, USA). The Ficoll-Hypaque Plus used for cell separation was purchased from Amersham (Buckinhamshire, UK). MACS B-CLL Cell Isolation Kit (human) and CD3 MicroBeads (human) were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). BSA used for Ab staining buffer was obtained from Weiner Laboratorios (Santa Fé, Argentina). Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (Dallas, TX). Venetoclax (ABT-199 or GDC0199) and Entospletinib (GS-9973) were purchased from MedKoo Biosciences, Inc (Morrisville, NC, USA). Rituximab was obtained from Roche Diagnostics GmbH (Mannheim, Germany). FITC-, PE- -conjugated mAbs specific for CD69 (clone FN50), CD38 (clone HB7) and annexin-V FITC and propidium iodide (PI) were purchased from BD Bioscience, Pharmingen (CA, USA). FITC-, PE- or PerCP-Cy5-conjugated mAbs specific for CD3 (clone HIT3a), CD4 (clone OKT4), CD8 (clone HIT8a), CD14 (clone HCD14), CD86 (clone 2331 FUN-1), CD49d (clone 9F10) and CD25 (clone M-A251), antibodies with irrelevant specificity (isotype controls) and macrophage colony-stimulating factor (MCSF) were obtained from BioLegend (CA, USA). PC5 or PE-conjugated mAbs specific for CD56 (clone HLDA6) and CD19 (clone J3-119) and purified anti-CD3 (clone UCHT1) were obtained from Beckman Coulter (CA, USA). Carboxyfluorescein succinimidyl ester (CFSE) was purchased from Invitrogen (Massachusetts, USA). FITC anti-human IgG (H+L) and HRP-conjugated anti-rabbit IgG and HRP-conjugated anti-mouse IgG were purchased from Jackson Inmuno Research (Jennersville, PA, USA). Polyvinylidene difluoride (PVDF) membranes were purchased from Sigma-Aldrich (Dallas, TX) and the enhanced chemiluminescence (ECL) kit used for visualized protein bands was purchased from Amersham. Rabbit mAb anti-BAX (clone D2E11), rabbit mAb anti-A1/Bfl-1 (clone D1A1C), rabbit mAb anti-BCL-XL (clone 54H6) and rabbit mAb anti-MCL-1 (clone D5V5L) were purchased from Cell Signaling Tech (Massachusetts, USA). Jackson. Anti-b-actin mAb was purchased from Abgent (CA, USA).

CLL patients.

Peripheral blood samples were collected from forty-three unrelated CLL patients. All samples used in this study were obtained after informed consent in accordance with the Declaration of Helsinki

and with Institutional Review Board approval from the Academia Nacional de Medicina, Buenos Aires, Argentina. CLL was diagnosed according to standard clinical and laboratory criteria. At the time of analysis, all patients were free from clinically relevant infectious complications and were treatment naïve or had not received treatment for ≥3 months before the investigation began. The main clinical and biological characteristics of the patients enrolled in our study are summarized in **Supplementary Table S1**.

Cell separation procedures.

Mononuclear cells (PBMC) were isolated from fresh blood samples by density centrifugation over a Ficoll-Hypaque Plus gradient, washed twice with saline solution, and suspended in complete medium (RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin). Cells were used immediately or were cryopreserved in FCS 20% DMSO for further experiments.

CLL cells were purified from PBMC by negative selection using MACS B-CLL Cell Isolation Kit. The purity of B cells was checked using a FACS flow cytometer (BD Immunocytometry Systems) and anti-CD19-PC5 mAb and was always >98%. T cells ($CD3^+$) were purified from PBMC by positive selection using MACS CD3 MicroBeads. The purity of T cells was checked using a FACS flow cytometer and was always >98%. The percentage of $CD19^+$ cells and $CD3^+$ cells in each PBMC sample are shown in **Supplementary Table S1**.

Analysis of CD38 and CD49d expression by flow cytometry.

CD38 and CD49d expression on CLL cells was evaluated by flow cytometry (See **supplementary Table S1**). To this end, PBMC were stained with anti-CD38 PE mAb, anti-CD49d PE mAb or PE-conjugated isotype control Abs and PC5-conjugated mAbs specific for CD19. Patients with ≥ 7 % of CLL cells expressing CD38 were considered $CD38^+$ [1]. On the other hand patients with ≥ 30 % of CLL cells expressing CD49d were considered $CD49d^+$ [2].

Quantitation of viable cells.

Viable cells within each subpopulation was determined by Annexin V-FITC staining and PE, PC5 or PerCP-Cy5conjugated antibodies against CD19, CD3, CD56 and CD14. Briefly, cells were stained with the specific antibodies for 20 minutes at 4°C, then whased twice and incubated with annexin V-

FITC according to the manufacturer's instructions. Viable cells of each subpopulation were determined by flow cytometry as percentage of Annexin-V-negative cells. Additionally, cell viability was evaluated by flow cytometric alterations of light-scattering properties (FSC-H), where apoptotic cells could be easily distinguished from viable cells because of their lower forward light scatter, consistent with reduction of cell size and cytoplasmic volume occurring during apoptosis[3]. In these cases, cell survival was quantified as the percentage of cells within the gate of viable cells.

In vitro cultures.

ABT-199 effect on the survival of $CD19^+$, $CD3^+CD56^-$, $CD3^-CD56^+$ and $CD14^+$ cells.

PBMC from CLL patients were cultured in complete medium with the vehicle (DMSO) or ABT-199 (0.01, 0.1 and 1 μM). The viability of each subpopulation ($CD19^+$, $CD3^+$ $CD56^-$, $CD3^-$ $CD56^+$ and $CD14^+$) cells were daily evaluated by flow cytometry analysis of Annexin V staining or by flow cytometric alterations of light-scattering properties (FSC-H)

The effect of ABT-199 on the expression of CD25 and CD69 on CD3 stimulated T cells.

PBMC from CLL patients (4 x 10^6 cells/mL) were cultured in complete medium with plate-bound anti-CD3 (aCD3, 50 ng/well, 48-well plate) or the isotype control (control cultures) in the presence of DMSO or ABT-199 (0.01, 0.1 and 1 μM) for 24 hours. T-cell activation was evaluated by flow cytometry using anti-CD4-FITC, anti-CD8-Cy5, anti-CD25-PE and anti-CD69-PE.

ABT-199 effect on $CD19^+$ cells survival in anti-CD3 stimulated cultures

PBMC from CLL patients (4 x 10^6 cells/mL) were cultured in complete medium with aCD3 or the isotype control. After 48 hours, ABT-199 or DMSO were added to the cultures for another 24 hours. The viability of $CD19^+$ cells was evaluated as previously mentioned. Similar experiments were performed with purified CLL cells and purified T cells from the same patient (80% of CLL cells and 20% of T cells). CLL activation induced by autologous T cell activation was checked at 48 hours by flow cytometry using anti-CD86 and anti-CD19 mAbs.

ABT-199 effect on $CD19^+$ cells survival in anti-CD3 stimulated cultures in the presence of GS-9973.

PBMC from CLL patients (4 x 10^6 cells/mL) were cultured in complete medium with aCD3 or the isotype control with or without GS-9973 (GS, 1 μM). After 48 hours, ABT-199 or DMSO were added to the cultures for another 24 hours. The viability of $CD19^+$ cells was evaluated as previously mentioned.

Analysis of BCL-XL and MCL-1 expression by *western blot*

PBMC from eight CLL patients were cultured in complete medium with or without aCD3 for 48 hours. Then, CLL cells were purified and washed twice with cold PBS. Whole-cell lysates were obtained from 5 x 10^6 purified CLL cells using 60 μL of loading buffer 1X with 5% β-mercaptoethanol. Lysates were boiled at 99 °C for 5 min, and 30 μL of the protein extracts were separated on a standard 15% SDS-PAGE and transferred to PVDF membranes. Membranes were then blotted with antibodies against BCL-XL, MCL-1, A1/Bfl-1 and BAX followed by HRP-conjugated anti-rabbit IgG. Specific bands were developed by enhanced chemiluminescence (ECL). The same membrane was blotted with mAb anti-b-actin followed by HRP-conjugated anti-mouse IgG to compare the total amount of protein in each sample. Densitometric measurements of the specific bands were normalized to β-actin by using ImageJ (NIH).

Phagocytosis assay by flow cytometry and confocal microscopy

Macrophages were differentiated from healthy donors' monocytes by culturing them for 5 days in RPMI 1640 with 10% FCS and M-CSF (50 ng/mL). For the phagocytosis assay, CLL cells previously labeled with CFSE (1 μM) were then coated or not with rituximab (50 μg/mL) and cultured with macrophages. After 1 hour, macrophages were trypsinized and the phagocytosis was evaluated by flow cytometry, as previously described[4]. Phagocytosis was represented as the percentage of macrophages, determined by morphology in the FSC-H and SSC-H dot plot, that have taken up CLL cells as the percentage of $CFSE^+$ macrophages. As we previously reported [4, 5] phagocytosis was confirmed by confocal microscopy **(Supplementary Figure S5)**. To this aim, after the phagocytosis assay, macrophages were trypsinized, fixed with paraformaldehyde 2%, stained with anti-CD14-PE mAb and then placed onto slides and coverslips were mounted using Fluoromount-G (Sigma). Immunofluorescence images were acquired with a FluoView FV1000

confocal microscope (Olympus, Tokio, Japan) using a Plapon 60 × 1.42 NA oil immersion objective, and images were analyzed using the Olympus FV10-ASW software.

- Pre-incubation of macrophages with ABT-199 or GS-9973

In some experiments macrophages were pre-incubated with GS-9973 (1 μM) **(Figure 2 F)** or ABT-199 (1μM) (**Supplementary Figure S3**) in order to test the effect of the drugs on macrophages.

- Pre-incubation of CLL cells with ABT-199.

In this experiment CLL cells previously labeled with CFSE (1 μM) were cultured with DMSO or ABT-199 (0.1 and 1 μM) for 3 hours. Then, cells were coated or not with rituximab (50 μg/mL) and cultured with macrophages.

ABT-199 effect on Phosphatidylserine (PtSer) exposure and rituximab binding.

CLL cells were cultured with DMSO or ABT-199 (0.1 and 1 μM) for 3 hours. PtSer exposure induced by ABT-199 was evaluated by annexin-V binding using annexin-V FITC and PI. CLL cells treated or not with ABT-199 for 3 hours were then coated or not with rituximab and rituximab binding was evaluated by flow cytometry using anti-human IgG FITC.

Statistical analysis

Statistical significance was determined using the nonparametric tests: Mann-Whitney test, Column Statistics followed by Wilcoxon's signed rank test, Wilcoxon matched-pairs test, Friedman or Kruskal-Wallis, one-way ANOVA followed by Dunn's multiple comparison test, Spearman correlation test. In all cases, $p<0.05$ was considered statistically significant. Data were analyzed using the GraphPad Prism software version 7.00.

SUPPLEMENTARY TABLES

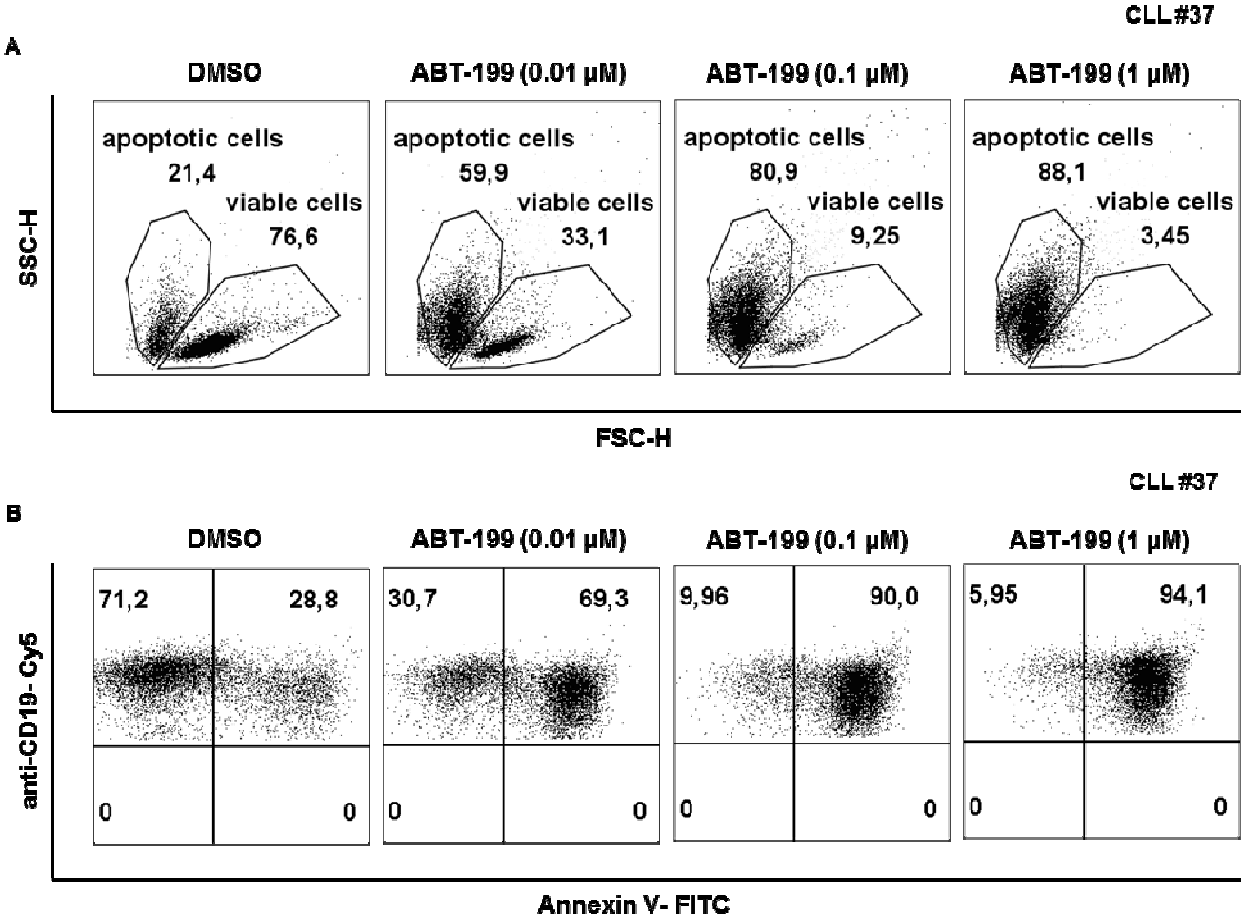
Supplementary Table S1

Clinical and biological features of CLL patients enrolled in our study														
CLL patient #	Age (years)	Gender	Binet	Leucocytes, x10^3 µL	Lymphocytes, x10^3 µL	CD3$^+$ % £	CD19$^+$ % *	CD38$^+$ % ¥	CD49d$^+$ % §	β2micro, µg/mL	LDH, U/L	HGB, g/dL	Platelets, x10^3 µL	Treatment
1	40	M	B	10	7	19	67	2	22	nd	nd	12	120	no
2	52	F	A	30	25	3	88	1	2	1.3	314	12.7	274	no
3	54	M	A	24	18	9	77	17	24	2.19	nd	12.5	201	no
4	57	M	B	75	68	6	89	1	nd	3.3	435	13.2	97	no
5	58	M	A	44	37	15	75	1.17	21	2.6	546	8	173	no
6	60	F	A	48	40	2	95	37	12	1.9	321	14.5	209	no
7	60	M	A	215	195	3	91	47	99.9	nd	nd	nd	115	no
8	61	M	C	63	58	0.6	84	0.1	1	3.1	327	14.8	80	FR
9	61	M	C	35	30	9	80	92	96	2.8	351	12.3	93	no
10	62	F	A	38	35	14	82	81	72	3.9	397	12	207	no
11	63	F	A	51	41	2	79	1	18	nd	244	14.4	148	no
12	64	F	A	21	15	2	94	2	8	nd	nd	13.7	224	no
13	67	M	C	134	122	2	93	0.3	1	3.8	498	10.1	96	RB
14	67	F	A	32	26	9	78	17	38	2.64	200	12.6	192	no
15	68	M	B	71	62	0.8	94	16	0.2	2.95	181	14.2	163	no
16	68	F	A	22	17	11	76	2.4	4.4	nd	nd	14.7	242	no
17	69	F	C	177	168	0.2	98	30	1	6.38	182	9.1	213	ChP
18	69	M	A	61	57	4	86	20	nd	nd	nd	14.8	139	no
19	70	F	C	93	65	2	92	16	0.4	5.1	511	12.3	110	BO
20	71	M	A	37	28	4	87	0.23	3	2.6	285	15	158	no
21	72	M	C	29	25	6	93	67	95	8.76	2.79	9.7	75	no
22	72	F	A	15	10	8	85	5.87	2.19	nd	nd	13.3	158	no
23	74	M	C	13	8	4	80	69	99	2.7	391	12.9	97	nd
24	74	M	B	139	130	4.2	95	2.6	40	nd	271	11.6	298	no
25	74	M	A	63	54	3.2	88	0.78	3.05	4.1	319	12.9	230	no
26	74	F	B	113	9	6.4	93	5.04	1.62	1.7	480	13.3	199	no
27	75	F	A	NA	NA	3	88	0.6	2	NA	NA	NA	NA	no
28	75	M	A	27	19	13	83	14	5	2.3	355	13.5	200	no
29	75	F	A	9	4	23	55	41	92	nd	nd	12.2	312	no
30	75	M	C	14	11	14	81	65	91	2.96	178	14.8	105	RCh
31	78	F	B	36	32	10	83	0.5	20	3.7	312	12.8	155	no
32	80	M	B	116	104	4	95	98	26	6.66	542	12.7	11	RCh
33	80	M	A	16	103	20	77	26	99	3.49	204	16	171	no
34	81	F	A	67	44	12	79	64	50	3.09	723	12.6	237	RB
35	81	F	A	35	30	9	85	0.9	100	nd	nd	14.4	188	no
36	83	M	A	27	26	2	90	0.6	2	nd	nd	11.5	116	no
37	84	M	C	650	539	3	94	7	0.5	nd	424	7.8	102	RB
38	85	M	B	44	38	12	87	0.5	nd	nd	nd	11.5	123	F
39	85	M	B	28	26	8	91	34	93	nd	275	12.4	113	no
40	86	M	A	37	29	4	84	1.6	5.53	3.8	274	12.8	180	no
41	87	F	C	40	27	4	92	3	99	nd	550	7.7	109	no
42	87	M	A	NA	NA	7	83	77	13	NA	NA	NA	NA	no
43	87	F	NA	NA	NA	6	92	0.75	1.7	NA	NA	NA	NA	NA

M indicates male; **F**, female; **nd**, not determined; **NA**, not available, **β2micro**, beta-2 microglobulin; **LDH**, lactate dehydrogenase; **HGB**, hemoglobin; **F**, fludarabine; **C**, cyclophosphamide; **R**, rituximab; **Ch**, chlorambucil; **B**, bendamustine; **O**, ofatumumab; **P**, prednisone. **£** Percentage of CD3$^+$ CD19$^-$ cells in peripheral blood lymphocytes. ***** Percentage of CD19$^+$ cells (B cells, more than 99% CLL cells) in peripheral blood lymphocytes. **¥** Percentage of CD38$^+$ cells in CD19$^+$ lymphocytes. **§** Percentage of CD49d$^+$ cells in CD19$^+$ lymphocytes

SUPPLEMENTARY FIGURES

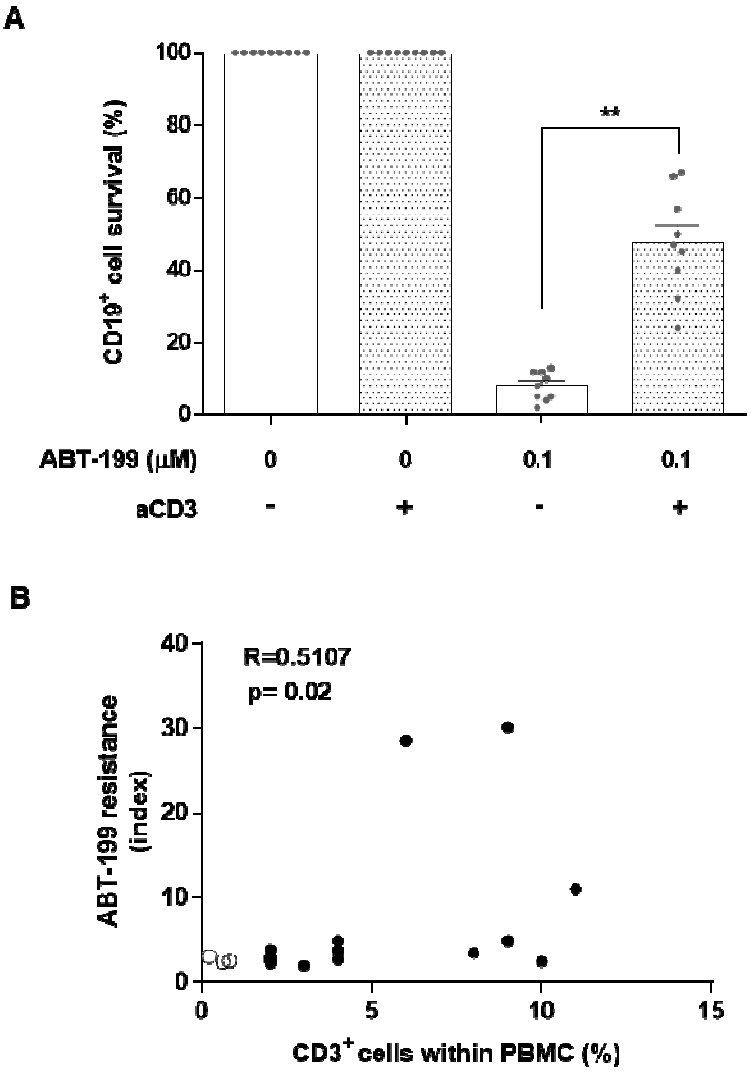
Supplementary Figure S1



Supplementary figure S1: ABT-199 induces CLL cell death in a dose dependent way.

PBMC from CLL patients (n= 30, 4 x 10^6 cells/mL) were cultured with DMSO (vehicle) or different doses of ABT-199. The survival of $CD19^+$ cells was daily evaluated by flow cytometric alterations of light-scattering properties and confirmed by Annexin V-FITC assay. **A)** The figure shows representative dot plots of control and ABT-199 cultures at 24 hours showing FSC and SSC parameters of $CD19^+$ cells. The percentage of cells in each gate is also showed. **B)** Representative dot plots of control and ABT-199 cultures at 24hs with annexin V-FITC and CD19-PC5 are shown. The percentage of cells in each quadrant is also showed. **C)** The figure shows the mean ± SEM of the percentage of cells within the gate of viable cells at 24 hours in control and ABT-199 cultures. Statistical analysis was performed using Friedman test followed by the Dunn post-test, *$p<0.05$, ** $p<0.01$ and **** $p<0.0001$

Supplementary Figure S2



Supplementary figure S2: The role of autologous T cells in ABT-199 resistance.

A) Purified CLL cells were cultured with autologous purified T cells (80% CLL, 20% T cells) with plate-bound anti-CD3 (aCD3, 50 ng/well, 48-well plate) or the isotype control (control cultures) for 48 hours. Then, ABT-199 (0.1 μM) or DMSO were added to the cultures for another 24 hours. $CD19^+$ cell survival was evaluated as mentioned above. The figure shows the mean ± SEM of the percentage of $CD19^+$ cells within the gate of viable cells in control and ABT-199 cultures. Statistical analysis was performed using Wilcoxon matched-pairs test, ** $p<0.01$. **B)** PBMC from CLL patients were cultured with or without aCD3 for 48 hours and then ABT-199 (0.1 μM) or DMSO (control)

were added to the cultures for another 24 hours. The survival of $CD19^+$ cells was evaluated as mentioned above in control, aCD3, ABT-199 and ABT-199+aCD3 cultures. These values were used to calculate the ABT-199 resistance index for each patient as follows:

ABT-199 resistance index = (ABT-199+aCD3/aCD3) x (control/ABT-199)

The graph shows the positive correlation between ABT-199 resistance index and the percentage of $CD3^+$ cells within PBMC of each patient. Open circles highlight CLL patients with less than 1% of T cells within PBMC (Patient #8, #15 and #17 of **Supplementary Table S1**). Statistical analysis was performed using Spearman rank correlation ($p=0.02$, $R=0.5183$).

Supplementary Figure S3

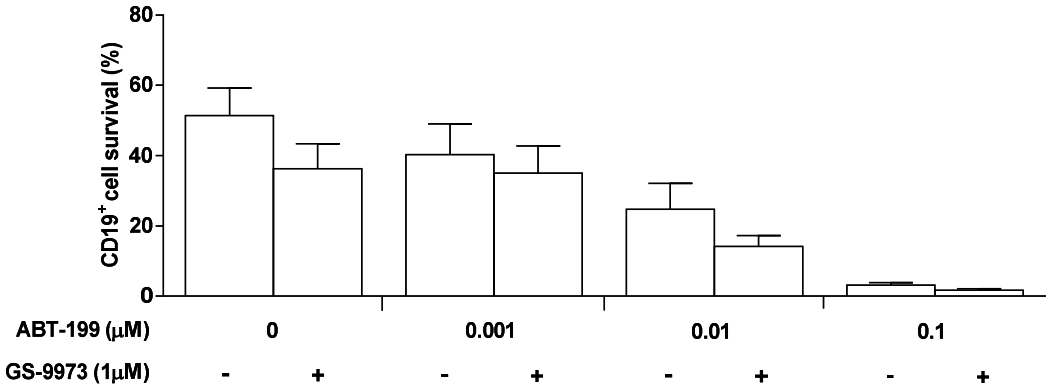
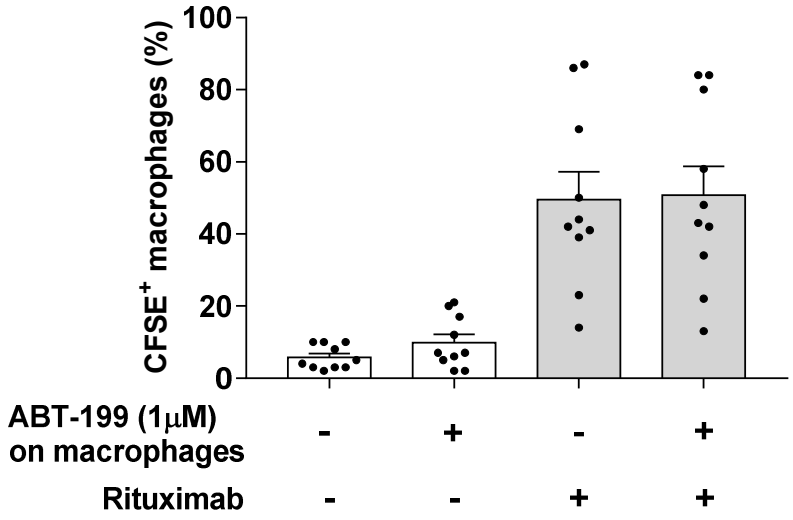


Figure S3: Effect of GS-9973 and ABT-199 on leukemic cell survival

B-CLL cells (4 x 10^6 cells/mL) were cultured with or without GS-9973 (1 μM) for 48 hours. Then, ABT-199 was added to the cultures for another 24 hours. $CD19^+$ cell survival was evaluated by flow cytometric alterations of light-scattering properties. The figure shows the mean ± SEM of the percentage of $CD19^+$ cells within the gate of viable cells. Statistical analysis was performed using Friedman test followed by Dunn's Multiple Comparison Test, n=10.

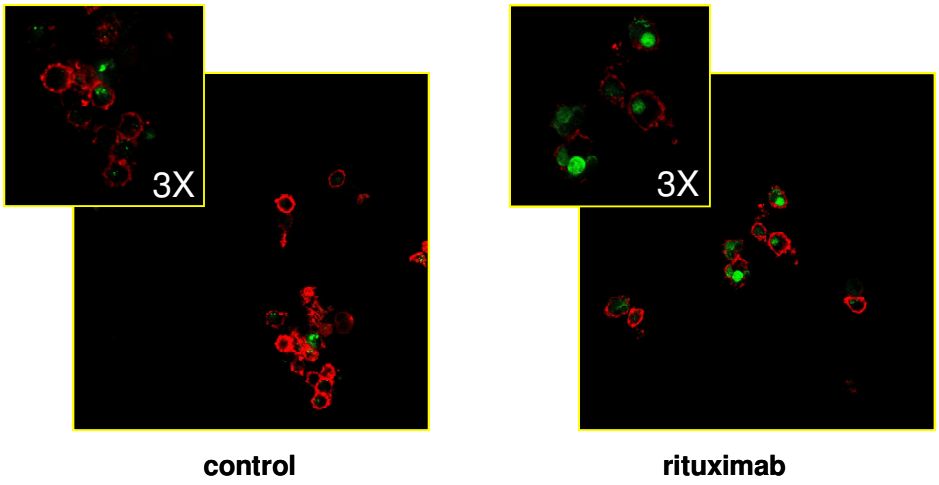
Supplementary Figure S4



Supplementary figure S4: Preincubation of macrophages with ABT-199 did not modify their capacity to uptake CLL cells

CFSE-labeled CLL cells were coated or not with Rituximab (50 µg/mL). The phagocytosis assay was performed with macrophages obtained by culturing monocytes from healthy donors PBMC in complete medium with MCSF (50 ng/ml) for 5 days. Macrophages were cultured for 3 hours with DMSO or ABT-199 (1 µM) before phagocytosis. The phagocytosis was evaluated by flow cytometry after 1 hour of culture when macrophages were tripsinized. The bars show the percentage of macrophages (determined by morphology in the FSC-H and SSC-H dot plot) that have taken up CFSE-labeled CLL cells (n=9). Statistical analysis was performed using Friedman test followed by Dunn's Multiple Comparison Test.

Supplementary Figure S5



Supplementary Figure S5: Phagocytosis assay by confocal microscopy. Phagocytosis assay was performed as described above with CFSE labeled CLL cells coated or not with rituximab. After 1 hour, macrophages were trypsinized, fixed and stained with anti-CD14-PE mAb. Phagocytosis was confirmed by confocal microscopy. Representative images are shown and 3X magnification from the original images are depicted in the insert.

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