The histone deacetylase inhibitor suberoylanilide hydroxamic acid induces apoptosis, down-regulates the CXCR4 chemokine receptor and impairs migration of chronic lymphocytic leukemia cells

Basile Stamatopoulos, Nathalie Meuleman, Cécile De Bruyn, Alain Delforge, Dominique Bron, and Laurence Lagneaux

Laboratory of Experimental Hematology, Faculty of Medicine, Institut Jules Bordet, Université Libre de Bruxelles (ULB), Brussels, Belgium

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Supplementary Appendix

Culture conditions

Cells (2×10^6 cells/mL) were immediately cultured in RPMI 1640 (Lonza Europe, Verviers, Belgium) supplemented with streptomycin, penicillin, 2 mM L-glutamine and 10% fetal bovine serum (Biochrom, Berlin, Germany) at 37°C in a humidified atmosphere containing 5% carbon dioxide. Bone marrow samples were collected from healthy donors after obtaining informed consent. Mononuclear cells isolated from normal subjects were plated in Dulbecco's modified Eagle's medium – low glucose (DMEM-LG) (Lonza Europe) supplemented with 15% fetal calf serum (Biochrom) to obtain stromal layers of mesenchymal stromal cells.

Measurement of CXCR4 mRNA expression by semi-quantitative reverse transcriptase polymerase chain reaction analysis

cDNA synthesis was performed by a standard reverse transcription reaction and subjected to PCR using the QIAGEN Multiplex PCR kit according the manufacturer's protocol (Qiagen, Hilden, Germany). The primer sequences used were as follows: β-actin forward 5'- TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA -3'; reverse 5'- CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3'; CXCR4 forward 5'- AGC TGT TGG CTG AAA AGC TGG TCT ATG-3'; reverse 5'- GCG CTT CTG GTG GCC CTT GGA GTG TG-3'. The PCR products for β-actin (610 bp) and CXCR4 (250 bp) were resolved by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. Densitometry was performed to normalize CXCR4 expression after scanning with a Fujifilm FLA-5000 Fluorescent Image Analyzer.

Chemotaxis assays and in vitro migration assay of chronic lymphocytic leukemia cells into stromal layers

Chemotaxis assays were performed using 5 μm-diameter pore filters. To examine the cell migration towards SDF-1, 100 ng/mL of SDF-1 in RPMI were added to the lower chamber of the transmigration chamber (24-well, Corning Incorporated, New York, USA). Cells were pre-incubated alone or with SAHA for 24 h before performing the migration assay. Untreated or SAHA-treated CLL cells (5×10^5) were added to the upper chamber and incubated for 3 h under culture conditions. The cells in the lower chamber were then collected, labeled with an allophycocyanin-conjugated CD19 monoclonal antibody (Miltenyi Biotec), and 100 μL of cell suspension were counted with the MACSQUANT® flow cytometer using the absolute volumetric cell counting function. The absolute number of $CD19^+$ cells was then determined. The migration index was calculated as the number of cells transmigrating in the presence of the chemoattractant divided by number of cells transmigrated in the absence of the chemoattractant.

Actin polymerization assay

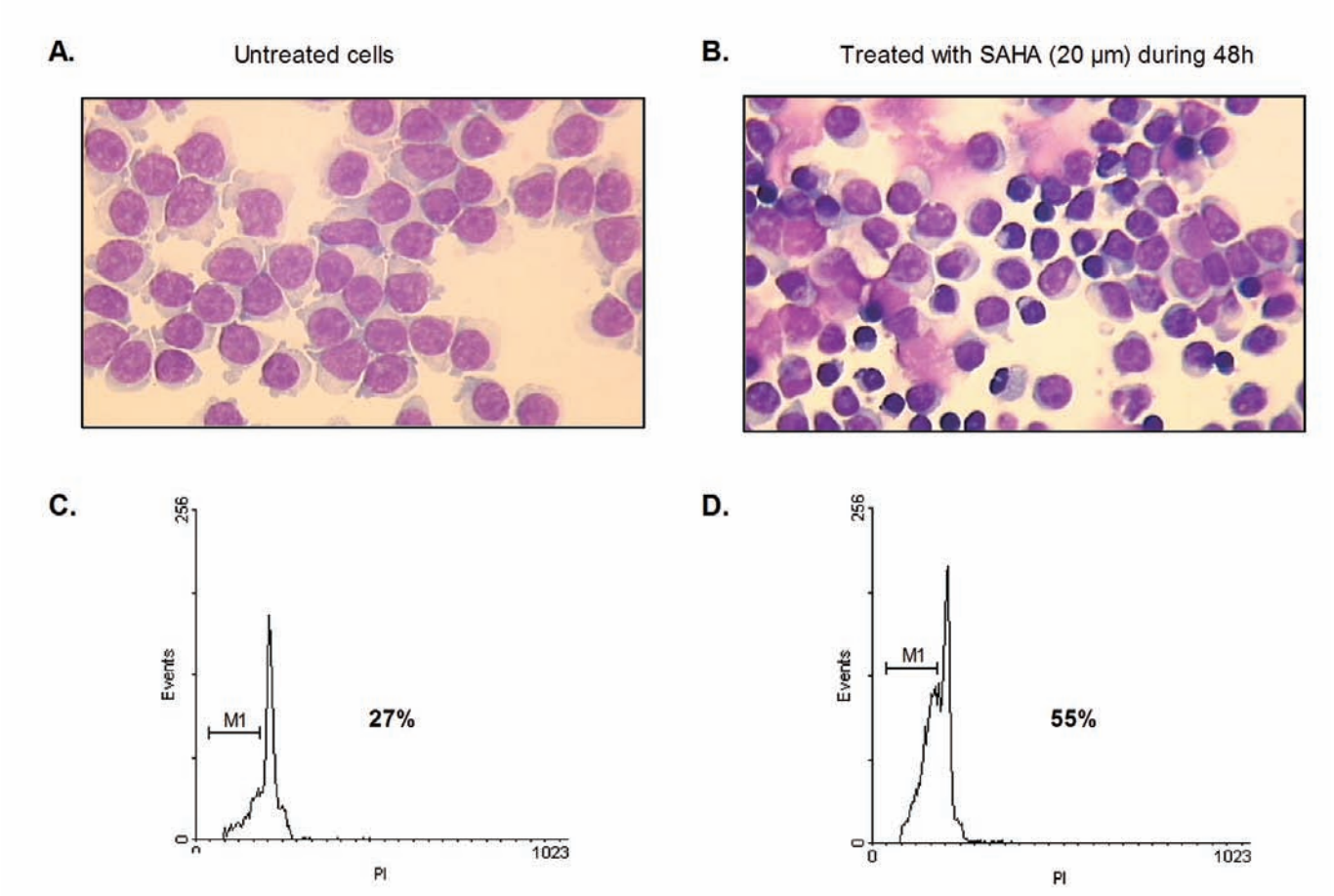
Recombinant human SDF-1 was purchased from R&D Systems (Minneapolis, MN, USA). Actin polymerization was tested as previously described. Briefly, 1.25×10^6/mL were suspended in RPMI-1640 medium with 0.5% bovine serum albumin at 37°C and incubated with 100 ng/mL SDF-1 for varying amounts of time (from 0 to 240 seconds). To determine actin polymerization in CLL cells after SAHA treatment (24 h at 20 μM), CLL cells were pre-labeled with APC-conjugated anti-CD19 monoclonal antibody (Miltenyi Biotec). Pertussis toxin (200 ng/mL), which prevents the G proteins from interacting with G protein-coupled receptors, was used as a control. At the indicated time points, 400 μL of the cell suspension were added to 100 μL of a solution containing 4×10^7 mol/L FITC-labeled phalloidin, 0.5 mg/mL 1-α-lysophosphatidylcholine (both from Sigma), and 18% formaldehyde in phosphate-buffered saline. The fixed cells were analyzed by flow cytometry and all time points are plotted relative to the mean fluorescence of the sample before addition of the chemokine.

Actin polymerization in response to 100 ng/mL SDF-1 was measured in CLL cells from six patients; the cells were cultured without any drug, with SAHA (20 μM for 24 h) or with pertussis toxin (200 ng/mL for 3 h). We detected a significant and transient increase in filamentous actin within 20-40 seconds after cell exposure to the chemokine. This increase was totally abolished after pertussis toxin treatment which was used as a control. Actin polymerization after SDF-1 stimulation was clearly inhibited by preincubation of CLL cells with SAHA: the decreases in response were about 42% after 20

seconds (P=0.0313) and 52% after 40 seconds (P=0.0469) (*Online Supplementary Figure S3*).

The effect of suberoylanilide hydroxamic acid on CD95 and TRAIL-R1 expression on chronic lymphocytic leukemia cells

CD95 was measured with FITC-conjugated anti-CD95 and PE-conjugated CD19. Since it has been recently demonstrated that activation of the FAS and TRAIL signaling pathways are critical events for apoptosis after treatment with HDAC, we investigated the expression of FAS (CD95), TRAIL-R1 (DR4) and TRAIL-R2 (DR5) on 15 different CLL cells following SAHA treatment. Only a small proportion of B-CLL cells displayed detectable expression of CD95 (12.52 ±1.45%). Twenty-four hours of treatment with SAHA resulted in an increase in CD95 expression (26.65±3.18%) compared to the untreated control (P<0.0001) (Figure 2C). However, for all patients (except three), this increase was lower than 20%. Therefore, even if statistically significant, the up-regulation of FAS probably has no biological significance. Furthermore, a FAS-blocking antibody (ZB4) did not inhibit SAHA-induced apoptosis arguing against a role of the FAS/FAS-L signaling pathway in the induction of SAHA-mediated apoptosis (*data not shown*). Using flow cytometry, we found that all the CLL samples expressed TRAIL-R1 and TRAIL-R2; however, this expression was not up-regulated upon SAHA treatment (*data not shown*). Similar results were observed for purified CLL samples (n=4) indicating that other cell types did not alter the result obtained with the unpurified population.

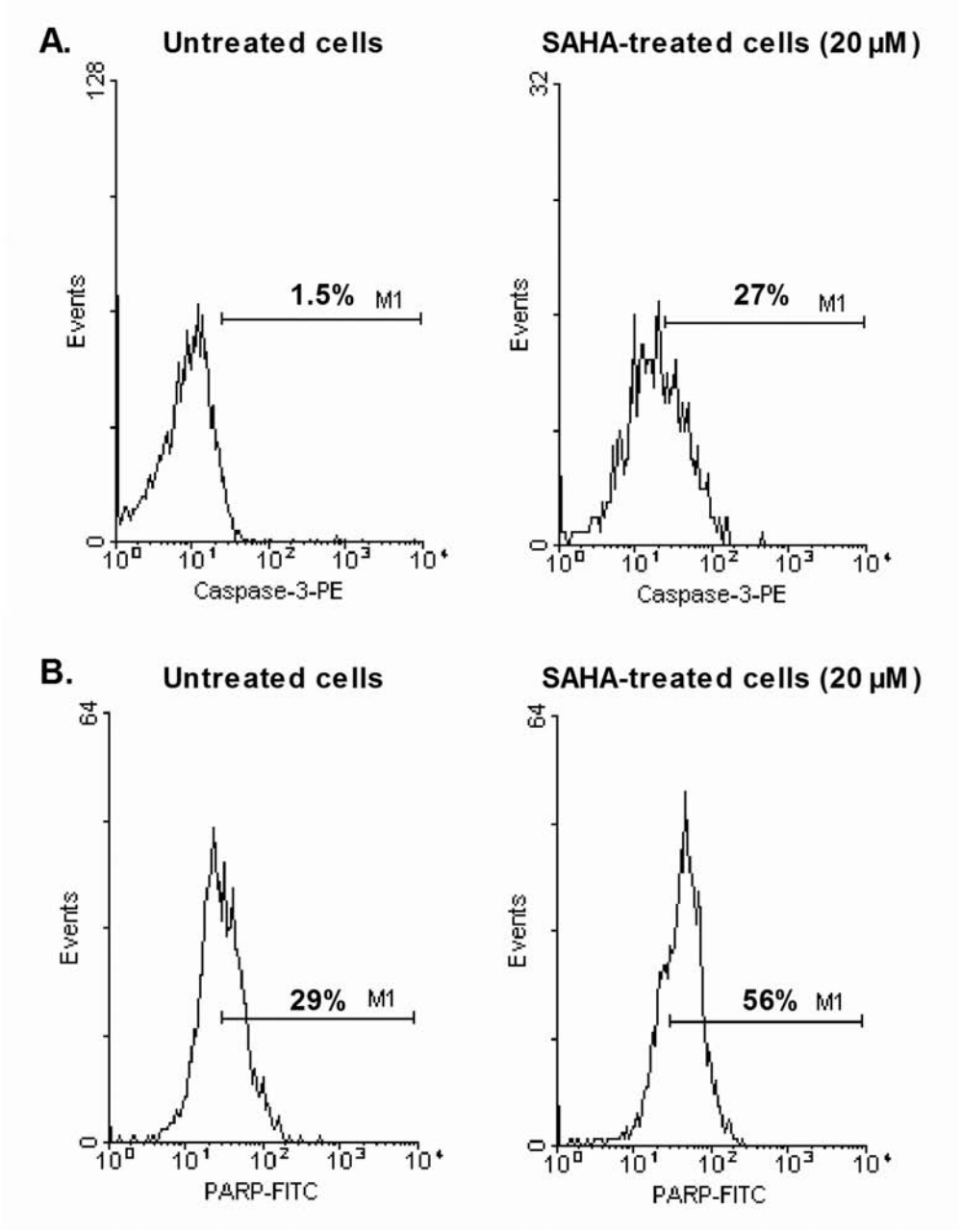


Online Supplementary Figure S1. SAHA induces an increase in the percentage of sub-diploid cells and morphological changes. Compared with the lymphocytes cultured in medium alone (A), cells treated with SAHA (B) showed nuclear condensation, cell shrinkage and the presence of apoptotic bodies. Quantification of SAHA-induced apoptosis in cells from one representative CLL patient (C) by propidium iodide staining and flow cytometry analysis after 48 h of incubation with SAHA 20 µM (D).

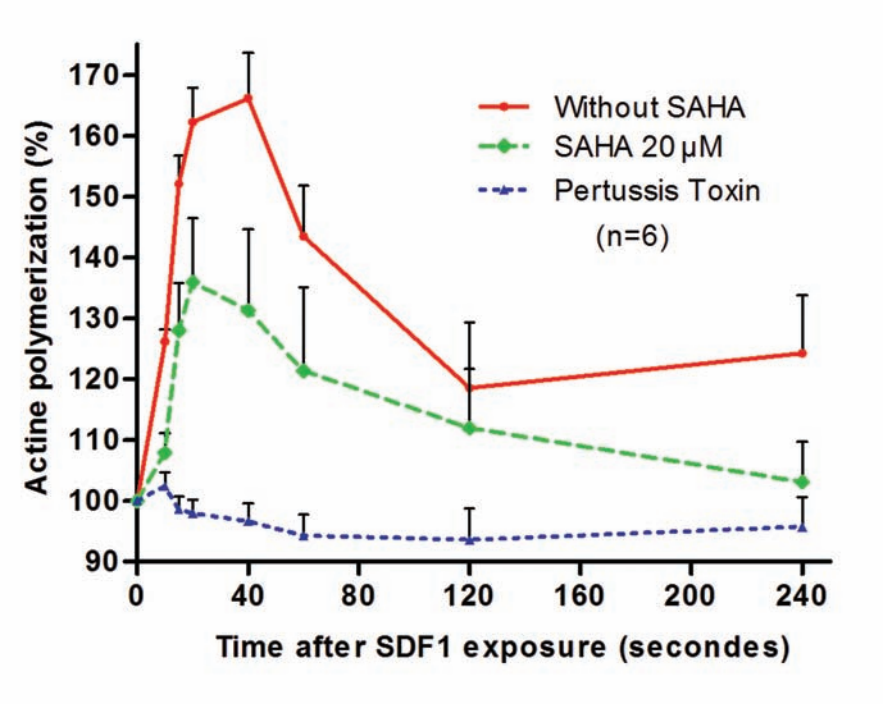
Online Supplementary Table S1. Clinical characteristics of chronic lymphocytic leukemia patients.

	Age (years)	Sex	Binet stage	White blood cell count ($x10^9$/L)	CD5/CD19 (%)	IgVH mutational status	ZAP70 expression	CD38 expression	Cytogenetic abnormalities
1	52	F	A	14.8	76	M	-	-	normal
2	52	F	A	40	79	M	-	-	ND
3	69	F	A	15.4	89	M	-	-	normal
4	67	F	A	19	56	M	-	-	normal
5	62	M	A	27.9	93	M	-	-	ND
6	70	F	A	17.7	91	ND	+	ND	ND
7	59	M	A	27.7	86	M	+	ND	normal
8	81	M	B	28.6	43	M	-	ND	del(14q)
9	75	F	A	17.3	43	UM	+	-	del(13q)
10	45	F	A	193	99	UM	+	-	del(13q)
11	67	M	B	51	85	M	-	-	del(13q)
12	56	M	A	39	15	M	-	-	normal
13	65	M	C	60.7	91	M	-	+	del(6q)
14	74	M	A	55.8	41	M	-	-	normal
15	74	M	B	71	94	M	+	+	trisomy 12
16	63	M	A	34.7	93	M	-	-	normal
17	73	F	C	37.5	89	UM	+	+	ND
18	57	F	C	38.6	95	UM	+	+	del(13q)
19	73	M	A	20.7	72	M	-	+	normal
20	78	F	A	11.6	43	M	-	+	del(13q)
21	74	M	A	16	51	ND	ND	-	ND
22	59	M	A	15.9	93	M	-	+	normal
23	69	M	B	28.5	78	UM	+	ND	normal
24	54	M	A	18.3	82	UM	+	+	normal
25	75	M	A	36.2	75	M	-	+	trisomy 12
26	61	F	A	50.3	88	UM	+	+	normal
27	64	M	B	102	92	UM	+	+	trisomy 12
28	46	F	B	78	94	UM	+	-	del(13q)
29	48	F	A	25.7	90	M	-	-	del(13q)
30	72	F	A	25.4	75	M	-	-	del(17q)
31	78	M	B	35.8	79	UM	+	+	trisomy 12
32	69	M	A	25.3	80	M	-	-	del(13q)
33	59	M	A	32.9	90	M	-	-	ND
34	62	M	A	22.1	81	M	-	-	del(16q)
35	77	M	A	34	75	M	-	+	del(13q)
36	72	F	A	71.2	56	M	-	-	normal
37	57	M	B	174	92	M	+	-	del(13q)
38	76	M	A	108.7	76	UM	+	+	ND
39	47	M	A	11.7	51	UM	+	+	normal
40	68	M	A	74.4	66	UM	+	+	del(11q)

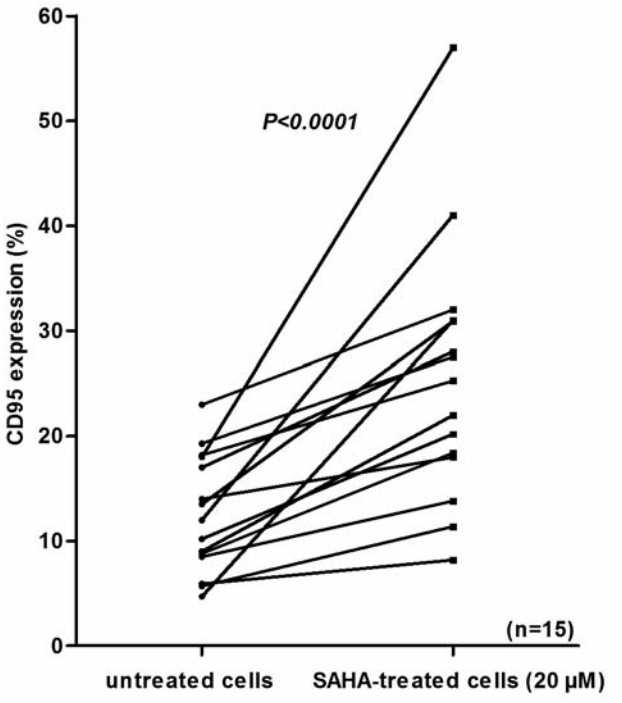
UM: unmutated; M, mutated; ND: not done. IgVH mutational status and ZAP70 are based, respectively, on 98% and 20% cut-off values (commonly accepted in the literature). Optimal cut-off for CD38, determined by ROC curve analysis, was 7%. Cytogenetic abnormalities were investigated by conventional karyotype analysis and a CLL FISH panel detecting del(17p), del(11q), del(6q), del(13q) and trisomy 12.



Online Supplementary Figure S2. SAHA induces caspase-3 activation and PARP cleavage. (A) A representative case analyzed by flow cytometry for caspase-3 activity. CLL cells were incubated in the presence or absence of SAHA (20 μM) and subsequently fixed, permeabilized and stained with anti-active caspase-3. (B) A representative case analyzed by flow cytometry for PARP cleavage after SAHA treatment (20 μM) compared to untreated CLL cells.



Online Supplementary Figure S3. SAHA reduces actin polymerization after SDF-1 stimulation. Intracellular F-actin was measured using fluorescein isothiocyanate-labeled phalloidin in CD19 pre-labeled CLL cells after addition of SDF-1 (100 ng/mL) at time 0 without drugs, in the presence of SAHA (20 µM) or in the presence of pertussis toxin (200 ng/mL) as a control.



Online Supplementary Figure S4. SAHA increases CD95 expression. CD95 expression (% of positive cells) measured by flow cytometry on CLL cells following 24 h of incubation with SAHA at 20 µM.