

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

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## ABSTRACT

### Background

Chronic lymphocytic leukemia is a neoplastic disorder that arises largely as a result of defective apoptosis leading to chemoresistance. Stromal cell-derived factor-1 and its receptor, CXCR4, have been shown to play an important role in chronic lymphocytic leukemia cell trafficking and survival.

### Design and Methods

Since histone acetylation is involved in the modulation of gene expression, we evaluated the effects of suberoylanilide hydroxamic acid, a histone deacetylase inhibitor, on chronic lymphocytic leukemia cells and in particular on cell survival, CXCR4 expression, migration, and drug sensitization.

### Results

Here, we showed that treatment with suberoylanilide hydroxamic acid (20  $\mu$ M) for 48 hours induced a decrease in chronic lymphocytic leukemia cell viability via apoptosis ( $n=20$ ,  $P=0.0032$ ). Using specific caspase inhibitors, we demonstrated the participation of caspases-3, -6 and -8, suggesting an activation of the extrinsic pathway. Additionally, suberoylanilide hydroxamic acid significantly decreased CXCR4 mRNA ( $n=10$ ,  $P=0.0010$ ) and protein expression ( $n=40$ ,  $P<0.0001$ ). As a result, chronic lymphocytic leukemia cell migration in response to stromal cell-derived factor-1 ( $n=23$ ,  $P<0.0001$ ) or through bone marrow stromal cells was dramatically impaired. Consequently, suberoylanilide hydroxamic acid reduced the protective effect of the microenvironment and thus sensitized chronic lymphocytic leukemia cells to chemotherapy such as fludarabine.

### Conclusions

In conclusion, suberoylanilide hydroxamic acid induces apoptosis in chronic lymphocytic leukemia cells via the extrinsic pathway and down-regulates CXCR4 expression leading to decreased cell migration. Suberoylanilide hydroxamic acid in combination with other drugs represents a promising therapeutic approach to inhibiting migration, chronic lymphocytic leukemia cell survival and potentially overcoming drug resistance.

Key words: suberoylanilide hydroxamic acid, chronic lymphocytic leukemia, CXCR4, migration.

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The online version of this article has a Supplementary Appendix.

## Introduction

Chronic lymphocytic leukemia (CLL) is the most common leukemia in the western world and is characterized by a gradual accumulation of leukemic cells. It is now well accepted that this accumulation could largely be explained by a defect in apoptosis.<sup>1</sup> Different factors contribute to the resistance of CLL cells to apoptosis, such as intrinsic defects in apoptotic pathways and interactions with non-leukemic cells in the microenvironment of lymph nodes, bone marrow, and other tissues.<sup>2</sup> However, recent studies suggested that CLL is not exclusively a disease of accumulation, but also one of proliferation.<sup>5</sup>

CLL has a heterogeneous clinical course which can be predicted by many classical or molecular prognostic factors.<sup>4</sup> Of these the absence of hypersomatic mutations of the immunoglobulin heavy chain region (IgVH),<sup>5</sup> zeta-associated protein 70 (ZAP70)<sup>6</sup> and CD38<sup>7</sup> expression have been abundantly described and are associated with poor outcome. Moreover, despite the introduction of novel therapeutic agents, drug resistance remains a great obstacle to the treatment of CLL and this leukemia is still considered an incurable disease justifying the need to develop alternate therapeutic approaches.

The chemokine (C-X-C motif) receptor 4 (CXCR4) is a G-protein 7-transmembrane domain receptor that is specific for the chemokine stromal cell-derived factor-1 (SDF-1), also known as CXCL12 (C-X-C motif ligand 12).<sup>8</sup> SDF-1 plays an important role in B-lymphocyte development and trafficking.<sup>9</sup> B-cell precursors deficient in the expression of the SDF-1 receptor, CXCR4, are not retained within the bone marrow microenvironment, thus demonstrating the importance of this pathway.<sup>10</sup> SDF-1 is constitutively produced by bone marrow stromal cells and acts as a chemoattractant supporting the homing of stem cells and contributing to the tropism of malignant cells for the bone marrow.<sup>11</sup>

CLL cells depend upon the supportive interaction within their microenvironment.<sup>12</sup> Several studies have demonstrated that CLL cells can interact with their microenvironment through the CXCR4/SDF-1 axis.<sup>11,13</sup> Furthermore CLL cells express a high level of CXCR4 surface receptors.<sup>11</sup> Lagneaux *et al.* showed that stromal cells could rescue CLL cells from apoptosis after they had migrated beneath a bone marrow stromal microenvironment,<sup>12</sup> while Burger *et al.* highlighted the protective function of nurse-like cells.<sup>14</sup> The CXCR4/SDF-1 axis has, therefore, been considered as a potential target for new therapeutic strategies.

Histone deacetylases (HDAC) play an important role in transcriptional regulation and the pathogenesis of cancer. HDAC inhibitors are members of a new class of agents that are capable of regulating gene expression by changing chromatin structure. By modifying the epigenetic code, this novel class of therapeutic agents may suppress aberrant gene expression or activate gene transcription to inhibit tumor growth.<sup>15</sup> Suberoylanilide hydroxamic acid (SAHA), also known as vorinostat (Zolinza, Merck, Whitehouse Station, NJ, USA), is a small molecule inhibitor of the HDAC class I and II enzymes<sup>16</sup> which can induce cell cycle arrest and apoptosis via gene expression modulation.<sup>17</sup> This drug has demonstrated activity against hematologic malignancies when used alone<sup>18,19</sup> or in combination with other chemotherapy.<sup>20</sup> Vorinostat has already been tested in clinical trials, has a known safety profile, and is effective in the treatment of cutaneous T-cell lymphoma.<sup>21,22</sup> Since the chemokine receptor CXCR4 plays

a crucial role in the survival and migration of CLL cells, we evaluated the effects of SAHA on CLL cells and, in particular, on cell survival and migration.

## Design and Methods

### Patients

This study was approved by the Bordet Institute Ethics Committee and was based on peripheral blood samples obtained with informed consent from 40 CLL patients who presented with a typical CD19<sup>+</sup>CD5<sup>+</sup>CD23<sup>+</sup> phenotype. Patients were either untreated or had received no treatment for at least 6 months before the study. A summary of the patients' characteristics is presented in *Online Supplementary Table S1*. Cytoplasmic ZAP70 expression was determined by three-color flow cytometry (CD3/ZAP70/CD19) and confirmed by quantitative real-time polymerase chain reaction (PCR) analysis.<sup>23</sup> CD38 expression, standard karyotype analysis and interphase fluorescence *in situ* hybridization screening for most common aberrations and IgVH gene mutational analysis were performed as previously described.<sup>24</sup>

### Cell culture, suberoylanilide hydroxamic acid treatment, and establishment of the bone marrow stromal layer

SAHA was obtained from Alexis Biochemicals (Lausanne, Switzerland). Mononuclear cells were isolated from peripheral blood using density gradient centrifugation (Linfocsep, Biomedics, Spain) and stromal layers of mesenchymal stromal cells were prepared as previously described.<sup>25</sup> SAHA was added at the beginning of the culture period. Culture conditions are detailed in the *Online Supplementary Appendix*.

### Viability assay, and apoptotic assays

Cell viability was determined by the trypan blue dye exclusion assay and all results were confirmed on a higher number of cells using 7AAD/CD19 staining counted by flow cytometry as previously described.<sup>26</sup> Phosphatidylserine exposure was measured by the binding of annexin V and allophycocyanin-conjugated CD19 monoclonal antibody (Miltenyi Biotec) using the protocol outlined in the ApoTarget Kit (Biosource International, Camarillo, CA, USA). DNA fragmentation was evaluated by standard propidium iodide staining as previously described.<sup>27</sup>

### Caspase inhibition, caspase-3 activity assessment and western blot

To evaluate the involvement of caspases in SAHA-induced apoptosis, patients' cells (with a purity >90% of CD19/CD5) were preincubated with the pan-caspase inhibitor ZVAD-FMK (N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone) (20 μM) (Eurobiochem, Bierges, Belgium) for 1 h prior to SAHA exposure. To examine which caspases are critical for inducing apoptosis, cells were preincubated with selective inhibitors of caspase-3, -6, -8 and -9 (Z-DEVD-FMK, Z-VEID-FMK, Z-IETD-FMK, Z-LEHD-FMK, respectively; Biosource, Nivelles, Belgium) 1 h prior to SAHA treatment. The enzymatic activity of caspases-3, -6, -8 and -9 was also determined using the ApoTarget Apoptosis colorimetric detection kit (Biosource). Caspase-3 activation was indirectly evaluated by PARP cleavage using a fluorescein isothiocyanate (FITC)-conjugated rabbit anti-PARP cleavage site antibody (Biosource). Western blot analysis was performed as previously described.<sup>24</sup> control or SAHA-treated (20 μM) cultured CLL cells were harvested after 48 h of treatment and lysed in RIPA buffer. Anti-actin (C2 – sc-8432), anti-PARP-1 (F-2 – sc-8007) and

anti-FLIP<sub>Ls</sub> (G-11 – sc-5276) were obtained from Santa Cruz (Heidelberg, Germany).

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

CLL cell total RNA was extracted with classical TriPure isolation reagent (Roche Applied Science, Indianapolis, IN, USA) in a single step extraction method.<sup>28</sup> Three CLL samples were purified using a CD19<sup>+</sup> magnetic-bead system (MidiMACS, Miltenyi Biotec, Bergish Gladbach, Germany) and their RNA was extracted to verify the influence of CD19<sup>+</sup> cells. Mean B-cell purity, as measured by flow cytometry, was greater than 99%. cDNA synthesis was performed by a standard reverse transcription reaction and subjected to PCR analysis for quantification of CXCR4 (610 bp) and  $\beta$ -actin (610 bp) mRNA. Primer sequences and additional details are provided in the *Online Supplementary Appendix*.

### Quantification of CXCR4 expression on leukemic cells

Untreated or SAHA-treated CLL cells were incubated for 30 min with FITC-conjugated anti-CD19 monoclonal antibody (Immunotech, Marseille, France) and phycoerythrin-conjugated anti-CXCR4 (BD Biosciences Pharmingen, San Diego, CA, USA). The viable cells were gated based on their forward and side scatter features in order to dissociate apoptotic and viable cells as described previously.<sup>29</sup> CXCR4 expression was evaluated in comparison with that of cells incubated with an isotype control antibody and measured on the CD19<sup>+</sup> population. CLL samples (n=4) were also purified using a CD19<sup>+</sup> magnetic-bead system (MidiMACS, Miltenyi Biotec) in order to assess the influence of other cell types on the expression of these antigens.

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

Chemotaxis assays in response to SDF-1 were performed as previously described.<sup>30</sup> Cells were pre-incubated alone or with SAHA for 24 h before performing the migration assay. Additional details are provided in the *Online Supplementary Appendix*. For migration into a stromal layer,  $5 \times 10^6$  untreated or SAHA-treated cells from six different patients were suspended in 1 mL of RPMI and added to stromal layers that were established from normal subjects. After incubation for 3 h, cells in suspension in the medium were removed. The stromal layer containing cells that had migrated was carefully washed twice with phosphate-buffered saline. Adherent CLL cells were harvested by Triple Select treatment (Gibco, Invitrogen, Merelbeke, Belgium) and labeled with allophycocyanin-conjugated CD19 monoclonal antibody (Miltenyi Biotec) and 100  $\mu$ L of cell suspension were counted with the MACSQUANT® flow cytometer using the absolute volumetric cell counting function. The absolute number of CD19<sup>+</sup> cells was then determined. In other experiments,  $2 \times 10^6$  CLL cells/mL were plated with or without SAHA (20  $\mu$ M) in the presence or absence of a stromal microenvironment. Viability and apoptosis were evaluated after 48 h.

### Drug treatment and viability assay in a chronic lymphocytic leukemia/mesenchymal stromal cell co-culture model

Fludarabine (FLUDARA®; Schering AG, Berlin, Germany) was dissolved in stock solution with water at 1 mM. Stock solutions were stored at  $-20^\circ\text{C}$ . CLL cells ( $2 \times 10^6/\text{mL}$ ) from six patients were pre-incubated for 4 h with or without a low dose of SAHA (1  $\mu$ M), and were then plated on a stromal layer with or without fludarabine (10  $\mu$ M). Viability and apoptosis were evaluated after 48 h. To evaluate the synergism between SAHA and fludarabine, a

Cooperative Index (CI) was calculated as previously described.<sup>31</sup> This index is the sum of specific apoptosis obtained by treatment with a single agent divided by the specific apoptosis obtained by combined treatment. CI values less than 1, equal to 1 and more than 1 mean that the effect is synergistic, additive or less than additive, respectively.

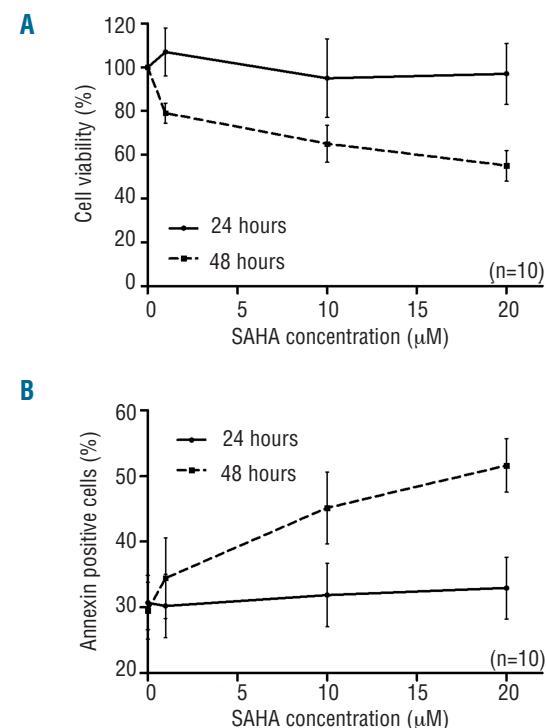
### Statistical analysis

The Wilcoxon signed ranks test was used to analyze the statistical significance of experimental results. All tests were two-sided. An effect was considered to be statistically significant when the *P* value was less than 0.05, and all analyses were performed with GraphPad Prism 5.0 software. For normalized data, the *P* values stated in this paper were obtained on primary data (before normalization) since a *P* value could not be calculated on data with the same rank.

## Results

### Suberoylanilide hydroxamic acid reduces viability and induces apoptosis in chronic lymphocytic leukemia cells after 48 hours of treatment

Mononuclear cells from ten patients were cultured in the presence or absence of different concentrations of SAHA (1, 10 and 20  $\mu$ M). After a 24-h incubation, cell viability (assessed by the trypan blue dye exclusion assay and confirmed by flow cytometry counting after 7AAD/CD19 staining) was unchanged in all patients evaluated ( $97 \pm 14\%$  of viability after treatment with SAHA 20  $\mu$ M, *P*=ns).



**Figure 1.** Viability and apoptotic effect of SAHA on CLL cells. (A) Mononuclear cells were incubated for 24 and 48 h with SAHA (1, 10 and 20  $\mu$ M). Cell viability rates stated in the figure were normalized to untreated cells. (B) Phosphatidylserine exposure after SAHA treatment. Rates stated in the figure include early and late apoptosis. Each bar represents the mean  $\pm$  SEM of 10 experiments.



However, viability was reduced after 48 h of exposure to SAHA 10 and 20  $\mu\text{M}$  ( $P < 0.05$ ) (Figure 1A). The level of apoptosis, assessed by annexin binding, increased after 48 h of exposure to SAHA and was significantly higher in SAHA-treated cells:  $45.14 \pm 5.47\%$  (at 10  $\mu\text{M}$ ,  $P < 0.001$ ) and  $51.63 \pm 4.08\%$  (20  $\mu\text{M}$ ,  $P < 0.001$ ) of apoptotic cells *versus*  $29.51 \pm 4.35\%$  for untreated cells (Figure 1B). Furthermore, we corroborated these results measuring apoptosis by propidium iodide staining to analyze DNA fragmentation, and by morphological analysis. A representative experiment is presented in *Online Supplementary Figure S1*.

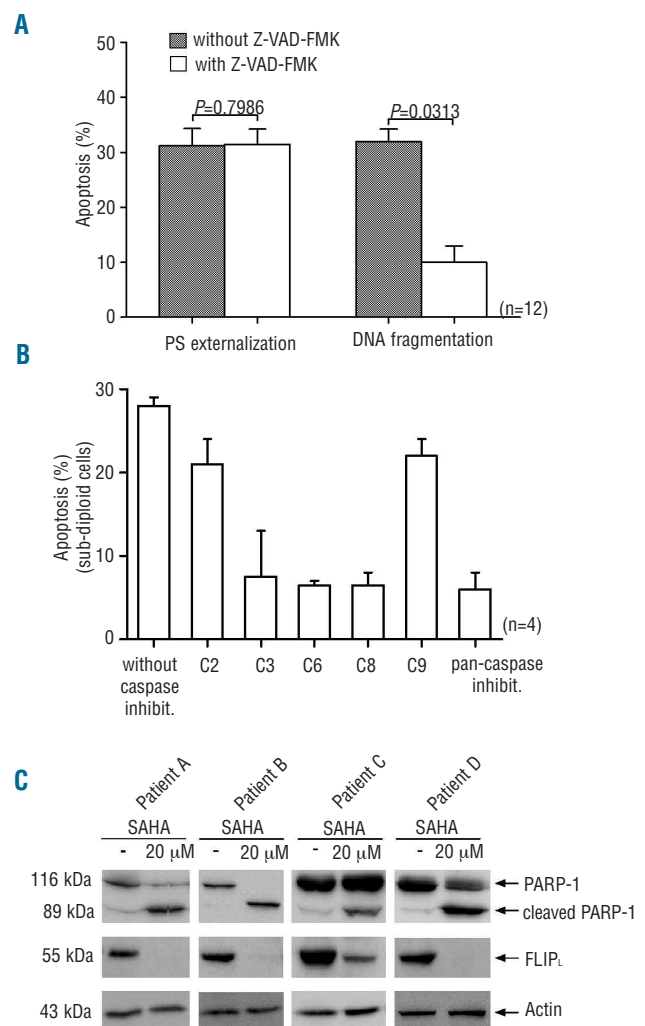
### Suberoylanilide hydroxamic acid induces caspase-dependent apoptosis

Apoptosis can occur through both caspase-dependent and -independent pathways. To investigate the requirement of caspases for SAHA cytotoxicity, CLL cells were incubated in the presence or absence of SAHA and the pan-caspase inhibitor Z-VAD-FMK. Apoptosis was measured using two different methods: annexin-V assay and propidium iodide staining. As shown in Figure 2A, pre-treatment of cells with Z-VAD before SAHA treatment had no effect on phosphatidylserine externalization. In contrast, addition of Z-VAD inhibited SAHA-induced DNA degradation. These data indicate that phosphatidylserine externalization is independent of caspases, although caspases are critical for the induction DNA fragmentation. We next examined whether the processing of DNA fragmentation could be inhibited by selective caspase inhibitors. Treatment with DEVD, VEID and IETD decreased the percentage of cells with degraded DNA demonstrating that caspases-3, -6 and -8 are critical for inducing cell apoptosis (Figure 2B). In addition, the inhibition of caspase-9, the initiator caspase in the intrinsic/mitochondrial pathway, did not influence cell apoptosis. Thus, the extrinsic pathway appears to be activated during SAHA-induced apoptosis. The enzymatic activities of caspases-3, -6 and -8 were also confirmed by a colorimetric reaction assay (*data not shown*). Flow cytometric analysis of caspase-3 activity demonstrated that SAHA activates caspase-3. In addition, cleavage of the caspase-3 substrate, PARP, was detected in all treated samples. This cleavage was confirmed by western blot analysis (Figure 2C) and flow cytometry. A representative CLL sample showing increased caspase-3 activity and PARP cleavage detected by flow cytometry is provided in *Online Supplementary Figure S2*. Finally, it was demonstrated that FLIP<sub>L</sub> decreases after SAHA treatment (Figure 2C).

### Suberoylanilide hydroxamic acid down-regulates CXCR4 mRNA and protein

CXCR4 mRNA expression was examined by semi-quantitative reverse transcriptase PCR analysis. Since tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) has been shown to induce down-regulation of CXCR4 mRNA in various cell types, TNF- $\alpha$  was used as a positive control. RNA was isolated from cells incubated in the presence or absence of SAHA and TNF- $\alpha$ . Amplified products were electrophoresed and analyzed by densitometry using the  $\beta$ -actin band as an internal control. As shown in Figure 3A, SAHA at 10 and 20  $\mu\text{M}$  induced significant changes in CXCR4 mRNA [ $32.29 \pm 12.63\%$  ( $P < 0.01$ ) and  $53.84 \pm 6.11\%$  ( $P < 0.01$ ) inhibition of control ratio, respectively], suggesting an effect at the transcriptional level. The results for one representative sample are shown in Figure 3B. The results

were similar for unpurified and purified CLL samples indicating that CD19<sup>+</sup> cells did not influence CXCR4 mRNA quantification (*data not shown*). To investigate the effect of SAHA on CXCR4 protein level, CLL cells were cultured in the presence or absence of SAHA at various concentrations for 24 h, and subsequently washed and then analyzed by flow cytometry. We observed that SAHA, even at a low concentration, significantly reduced CXCR4 levels in all patients tested ( $n = 40$ ): inhibition of control ratios were  $32.53 \pm 4.71\%$ ,  $38.98 \pm 3.49\%$  and  $42.39 \pm 3.31\%$  ( $P < 0.0001$ ) after treatment with SAHA 1  $\mu\text{M}$ , 10  $\mu\text{M}$  and 20  $\mu\text{M}$ , respectively (Figure 3C). The results for one representative sample are shown in Figure 3D. Similar results were observed on purified CLL samples ( $n = 4$ ) indicating that other cell types did not alter the results obtained with the unpurified population.



**Figure 2.** The effect of SAHA on caspase activity, phosphatidylserine (PS) externalization, DNA fragmentation and PARP and FLIP<sub>L</sub>. (A) CLL cells were preincubated for 1 h in the absence or presence of Z-VAD-FMK and then treated with 20  $\mu\text{M}$  SAHA. (B) One hour prior to exposure to SAHA (20  $\mu\text{M}$ ), cells were preincubated with selective inhibitors of caspase-3 (C3), -6 (C6), -8 (C8) and -9 (C9), and the pan-caspase inhibitor as a control. (C) Protein extract of CLL cells from four patients plated with or without 20  $\mu\text{M}$  SAHA for 48 h were analyzed by western blotting for PARP-1, FLIP<sub>L</sub> and actin expression.

### Suberoylanilide hydroxamic acid reduces migration of chronic lymphocytic leukemia cells in response to stromal-derived factor-1 and into stromal layer

We evaluated the effect of SAHA on CLL cell chemotaxis in response to SDF-1 ( $n=23$ ). As shown in Figure 4A, 10 and 20  $\mu\text{M}$  SAHA significantly decreased the migration index in response to SDF-1 [with 10  $\mu\text{M}$  of SAHA ( $P=0.0005$ ); with 20  $\mu\text{M}$  of SAHA ( $P<0.0001$ )]. These results correspond to an inhibition of  $65.35\pm 5.32\%$  (10  $\mu\text{M}$ ) and  $61.23\pm 5.84\%$  (20  $\mu\text{M}$ ) of control migration. Actin polymerization after SDF-1 stimulation was also clearly inhibited by preincubation of CLL cells with SAHA (Online Supplementary Figure S3). We also compared the migration of untreated or SAHA-treated (20  $\mu\text{M}$ ) CLL cells ( $n=6$ ) into a bone marrow stromal layer established from normal subjects: of the input CLL cells ( $5\times 10^6$ ), an average of  $11.50\pm 2.76\%$  of cells migrated into the stromal layer. Following 24 h of 20  $\mu\text{M}$  SAHA treatment, only  $57.30\pm 7.35\%$  ( $n=6$ ) of cells compared with untreated control samples (100%) had migrated after 3 h. This value was  $65.27\pm 4.77\%$  ( $n=6$ ) after pertussis toxin treatment, although this was not significantly different. In addition, viability and apoptosis were evaluated after 48 h of co-culture with a stromal layer with or without SAHA (20  $\mu\text{M}$ ). After normalization to the cells plated alone without SAHA (100% of viable cells), we observed that the decrease of viability induced by SAHA was greater in the presence of a stromal layer (Figure 4B). Indeed, SAHA induced only  $33.97\pm 4.32\%$  of cell death in the absence of a stromal layer while cell death reached  $75.86\pm 5.75\%$  in the presence of a stromal layer ( $P<0.05$ ) indicating that SAHA would be more efficient in an *in vivo* context.

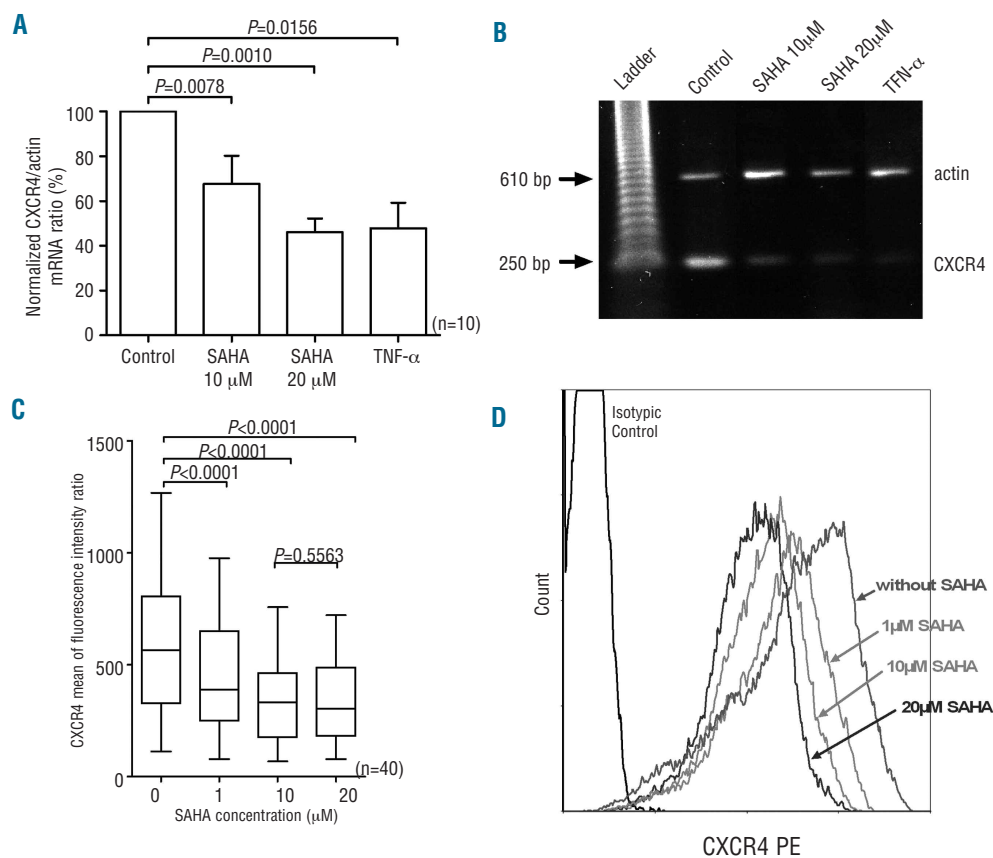
Similar results were observed with annexin staining after SAHA treatment with higher, albeit not statistically significantly so, apoptosis observed in the presence of stromal cells (Figure 4C).

### Suberoylanilide hydroxamic acid synergizes with fludarabine to induce apoptosis in a chronic lymphocytic leukemia/mesenchymal stromal cell co-culture model

As suggested previously by Kurtova *et al.*, a co-culture model with a protective microenvironment seems to be very important to study drug-induced apoptosis of CLL cells.<sup>32</sup> Indeed, such a model is essential since SAHA decreases CXCR4 expression on CLL cells. We, therefore, measured viability and apoptosis of CLL cells co-cultured with a stromal layer (composed of mesenchymal stromal cells) without drug, with a low concentration of SAHA (1  $\mu\text{M}$ ), with fludarabine (10  $\mu\text{M}$ ) or with both drugs ( $n=6$ ). After normalization to cells plated alone without SAHA (100% of viable cells), we observed that the decrease of viability induced by SAHA or fludarabine alone was relatively low but that the combination of both drugs induced a dramatic decrease of live cells ( $39.25\pm 7.03$ ) ( $P<0.05$ ) (Figure 4D). Similar results were observed for apoptosis (Figure 4E). The CI were less than 1 (mean of  $0.65\pm 0.13$ ; range, 0.20–0.97), indicating that the observed effects were synergistic.

## Discussion

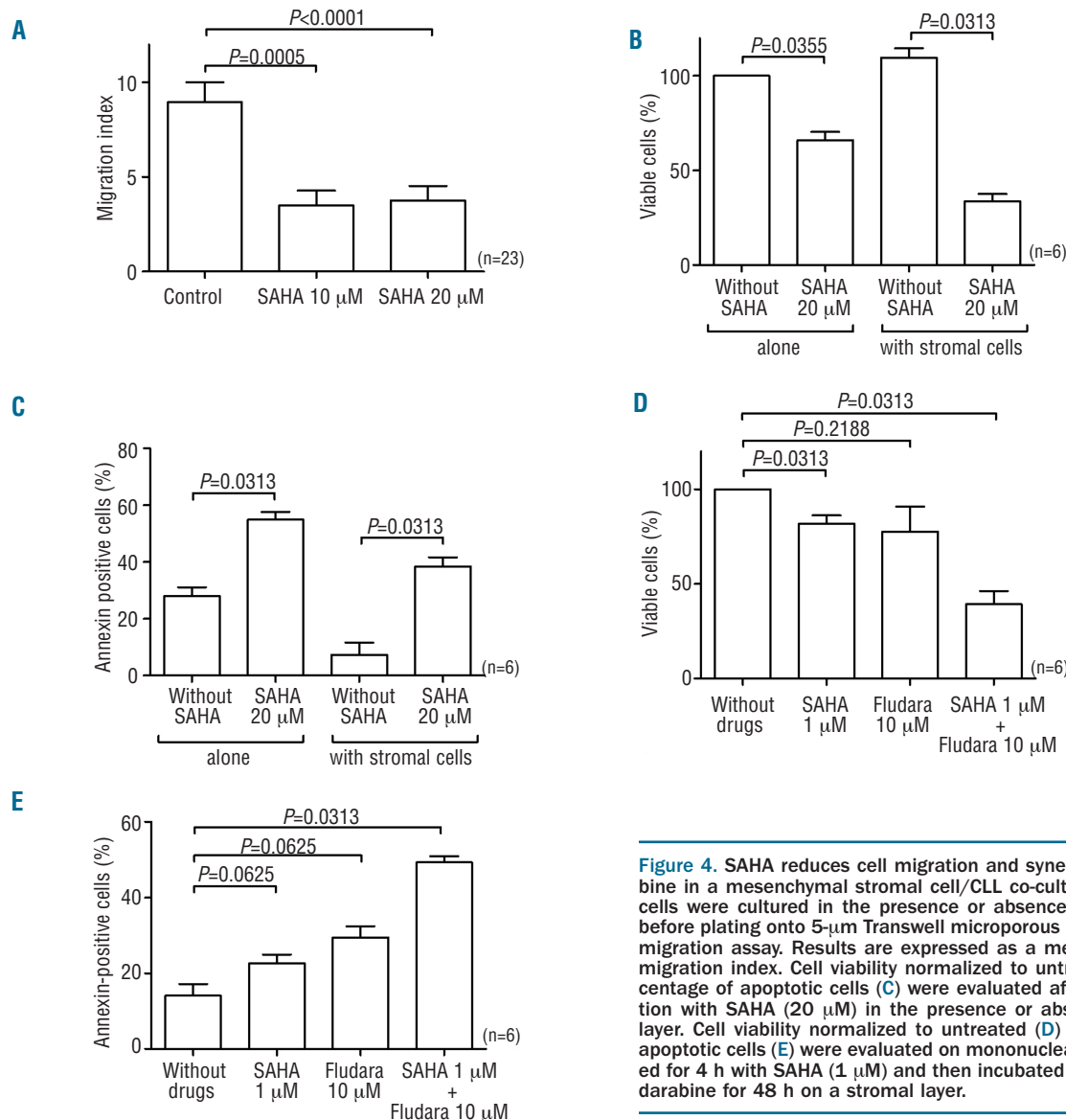
In the present study, we have demonstrated that SAHA, the prototypic member of a series of hydroxamic acid-based HDAC inhibitors, induces CLL cell death in a dose-



**Figure 3.** SAHA-induced down-regulation of CXCR4 mRNA and protein. (A) After treatment for 24 h with various concentrations of SAHA, RNA was isolated and subjected to reverse transcriptase PCR analysis. The results are expressed as mean ratios of CXCR4/ $\beta$ -actin for the ten CLL patients evaluated. (B) Electrophoresis of one representative sample. (C) Cells were cultured in medium alone or with SAHA and stained with anti-CD19 and anti-CXCR4-phycoerythrin. Results are expressed as a mean of fluorescence intensity ratio (MFIR). (D) Representative flow cytometric analysis of surface CXCR4 protein on untreated or SAHA-treated CD19 positive cells.

dependent manner after 48 h of treatment. This induction of apoptosis was demonstrated by several criteria: cell shrinkage, externalization of phosphatidylserine and DNA fragmentation. These results are consistent with previous reports demonstrating SAHA activity in various pathologies such as prostate cancer,<sup>33</sup> breast cancer,<sup>34</sup> acute T-cell leukemia, myeloma and in malignant B cells.<sup>35</sup> However, the mechanism underlying the apoptotic effect induced by SAHA has not been completely elucidated. Our results demonstrated that SAHA-mediated apoptosis in CLL cells was caspase-dependent since the pan-caspase inhibitor Z-VAD protected cells from SAHA. However, Z-VAD inhibited DNA fragmentation but not phosphatidylserine externalization suggesting that this last step is caspases-independent. Previous studies demonstrated that two different mechanisms (caspase-dependent and -independent) could exist in phosphatidylserine externalization and are dependent upon the cell type.<sup>36</sup> The existence of both mechanisms is also supported by the fact that Z-VAD inhibits phosphatidylserine externalization induced by anti-CD3 but not by glucocorticoid in T cells.<sup>37</sup>

In the caspase-dependent pathway, caspase-3 serves as an effector molecule by cleaving cellular proteins, including PARP. Here, we detected caspase-3 activation in SAHA-treated cells and demonstrated PARP cleavage by western blotting and flow cytometry. Caspase-dependent apoptosis can be initiated through a variety of signaling cascades including the TNF receptor (extrinsic pathways via caspase-8) and the mitochondria (intrinsic pathways via caspase-9) pathways of apoptosis.<sup>38</sup> Current treatments for CLL (such as fludarabine) induce apoptosis via a mitochondria-dependent pathway that involves the activation of the protease caspase-9.<sup>39</sup> Using specific caspase inhibitors, we demonstrated the role of caspases-8, -6 and -3 in the apoptotic effect of SAHA suggesting that this drug uses the cell death receptor pathway of apoptosis to activate the initiator caspase-8 leading to caspase-3 activation and finally to PARP cleavage. In the case of adult T-cell leukemia and myeloma cells, SAHA-induced cell death was caspase-independent but appeared to be mediated by transcriptional events resulting in Bid cleavage, disruption of the mitochondrial membrane and production of reactive oxygen



**Figure 4.** SAHA reduces cell migration and synergizes with fludarabine in a mesenchymal stromal cell/CLL co-culture model. (A) CLL cells were cultured in the presence or absence of SAHA for 24 h before plating onto 5-μm Transwell microporous membranes for the migration assay. Results are expressed as a mean ( $\pm$  SEM) of the migration index. Cell viability normalized to untreated (B) and percentage of apoptotic cells (C) were evaluated after 48 h of incubation with SAHA (20  $\mu$ M) in the presence or absence of a stromal layer. Cell viability normalized to untreated (D) and percentage of apoptotic cells (E) were evaluated on mononuclear cells pre-incubated for 4 h with SAHA (1  $\mu$ M) and then incubated with SAHA and fludarabine for 48 h on a stromal layer.

species.<sup>17,40</sup> In contrast, in breast cancer cells and U937 leukemia cells, caspase-3 activation and PARP degradation have been described.<sup>35</sup> Similar results have been observed for other HDAC inhibitors such as valproic acid: our group previously showed that caspase-8 activation and caspase-9 mRNA increase after valproic acid treatment,<sup>41</sup> while Bokelmann *et al.* reported that caspase-8 was the first caspase activated by valproic acid but a mitochondrial amplification loop seemed to be required.<sup>42</sup>

In CLL cells, SAHA induces caspase-dependent apoptosis via the extrinsic pathway. This alternative pathway involves caspase-8 and could be triggered by different death ligands such as FAS-L, TNF or TRAIL. However, studies have shown that this pathway is generally not functional in CLL. In our study, while we observed an increase in CD95 expression after SAHA treatment (*Online Supplementary Appendix* and *Online Supplementary Figure S4*) (probably caused by a stress-induced response activating the JNK/SAPK pathways), activation of the caspase-8 pathway did not appear to require CD95. This phenomenon has been previously described: data from experiments using FAS-blocking antibody confirmed that drug-induced apoptosis via the extrinsic pathway can indeed occur via a FAS-independent mechanism.<sup>43-45</sup> Furthermore, extrinsic pathways could be activated via TRAIL receptors (such as DR4 or DR5), as was previously reported.<sup>46,47</sup> However, in this study we did not observe a modulation of these receptors after SAHA treatment suggesting that SAHA induces activation of extrinsic pathways via an independent mechanism. Another way to induce extrinsic pathways could be down-regulation of the expression of multiple inhibitors. Indeed, we found a dramatic down-regulation of c-FLIP after SAHA treatment. This down-regulation may thus induce indirect activation of receptor aggregation.

Our data also demonstrated that SAHA down-regulated CXCR4 mRNA and protein levels in CLL cells. Our findings are in line with those of Crazzolara *et al.* who reported CXCR4 down-regulation after SAHA or butyrate treatment in acute lymphoblastic leukemia cells.<sup>48</sup> Interactions between CLL cells and accessory cells such as bone marrow stromal cells confer a growth advantage, extended cell survival and drug resistance to the leukemic cells.<sup>49</sup> In this context, the SDF-1/CXCR4 system mediates an important physical interaction between leukemic cells and the microenvironment regulating apoptosis. The SDF-1/CXCR4 pathway may, therefore, be an attractive target

for the development of novel therapeutic approaches. It has been demonstrated that SDF-1-induced migration of leukemic cells is correlated with surface CXCR4 levels and can be specifically blocked by antibodies against CXCR4 or CXCR4 antagonists.<sup>50</sup> Here, we showed that SAHA treatment induced CXCR4 down-regulation, and a reduction of actin polymerization in response to SDF-1, which is likely responsible for reduced migration of leukemic cells towards bone marrow stromal cells. Since stromal cells could protect against fludarabine-induced apoptosis, and regarding the fact that SAHA could reduce CLL/microenvironment cell interactions, we tested the combination of these two drugs and observed a clear synergistic effect in the presence of a stromal layer. These results strongly suggest that SAHA exposure could disrupt the cross-talk between CLL cells and their protective microenvironment and thus sensitize CLL cells to chemotherapy. Compared to other disrupting agents (such as CXCR4 antagonist peptide),<sup>50</sup> SAHA has an additional intrinsic pro-apoptotic activity, thus acting on different pathways to induce cell death. A small concentration of SAHA (1  $\mu$ M) in combination with other standard treatments could, therefore, be a new therapeutic strategy for the management of CLL.

In conclusion, we showed that SAHA induces apoptosis via both caspase-dependent and caspase-independent pathways in CLL cells. The mechanism of cytotoxicity involves the extrinsic pathway leading to caspase-8 activation. SAHA potently represses CXCR4 receptor expression and significantly impairs cell migration into stromal cell layers. Our results suggest that SAHA in combination with other drugs represents a promising approach to overcome drug resistance and to improve the outcome of patients with CLL.

## Authorship and Disclosures

BS performed research and the statistical analysis, analyzed data, made the figures and tables, and wrote the manuscript. AD contributed to the morphological analyses. CDB contributed to the flow cytometry experiments. NM and DB contributed patients' samples and data. LL performed, supervised and designed the research, and revised the manuscript.

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