EPIGENETICS: A NEW THERAPEUTIC APPROACH IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Abstract

Background/Aims: Nowadays, epigenetic changes have highlighted a new possible contribution to the development of Chronic Lymphocytic Leukemia B (CLL-B), namely by histone deacetylases or DNA metiltransferases. This project aims to study the potential therapeutic role of hypomethylating agents (HM) and histone deacetylase inhibitors (HDACis), in monotherapy and in association of both drugs, in a CLL-B cell line. *Materials and Methods:* A CLL-B cell line were incubated with the HM agent, decitabine (DAC), and the HDACi, trichostatin (TSA), at different concentrations; viability and proliferation studies were performed using rezasurin test; cell death was analyzed by flow cytometry and optical microscopy; methylation studies were performed using Methylation specific-PCR (MSP) and Real-Time PCR (qPCR). *Results*: Our results show a decrease in cell proliferation and viability in a dose and time dependent manner, leading to a cell death preferentially by apoptosis. Besides that, a decrease in the degree of gene methylation was also reached. *Discussion*: This study suggests that epigenetic modulation might constitute a new approach to the treatment of lymphoid malignancies, namely CLL-B.

Keywords: epigenetics, Chronic Lymphocitic Leukemia B, trichostatin A, decitabine, methylation

Introduction

Chronic Lymphocytic Leukemia B (CLL-B) is the most common leukemia in the Western World and consists on a clonal malignancy of mature neoplastic B cells, characterized by a low proliferation rate and impaired apoptosis [1,2]. These cells characteristically present low express surface immunoglobulin (slg), are monoclonal regarding to expression of either κ or λ light chains and show B-cell surface antigens, as CD19, CD20 and CD23, with the CD5 antigen, in the absence of other pan-T-cell markers [2-5].

The aetiology of CLL-B is unknown and the natural history is heterogeneous. Unlike other leukemias, in CLL-B, it is not described a causing translocation, deletion, or mutation [6]. In recent years, more interest has been focused on epigenetic changes contributing to the development of CLL-B, by an abnormal DNA methylation pattern and chromatin histones modification. Although DNA methylation is globally decreased in this disease, there is an increasing belief that regional hypermethylation of gene promoters, namely of tumour suppressor genes, leads to gene silencing, unravelling the role of epigenetic modifications in CLL-B development. So, new epigenetic drugs, such as DNA-methyltransferases inhibitors (DNMTis) and histone deacetylases inhibitors (HDACis), may attempt to correct these changes [1,6-8].

DNA methylation level in CLL-B patients is generally lower than in healthy individuals. However, regional hypermethylation of gene promoters, especially in the CpG dinucleotides, leads to gene silencing of particularly important genes in cell cycle and in apoptosis regulation. On the other hand, histones deacethylation could be associated with cancer due to the resultant chromatin compaction that blocks the access to transcription factors [6,7].

Although epigenetic modifications may play an important role in CLL-B, little is known about the extent of promoter methylation [6]. Laura *Rush* (2004) has identified many aberrantly methylated genes and suggested that a portion of these events confers a selective advantage to the malignant cell [1]. *Duhamel et al* (2008) and *Billot et al* (2011) referred that the deregulated expression of IKZF3 (Aiolos), an important transcription factor involved in the control of mature B lymphocyte differentiation and maturation, is due to epigenetic modifications, namely DNA hypomethylation and an enrichment of euchromatin associated with histone markers, such as the hypomethylation of the lysine 4 on histone H3 [9,10].

The current use of hypomethylating agents and HDACis may provide a potential mechanism to change this disordered gene expression in CLL-B. The promising preclinical activity of these drugs makes it imperative that we continue to investigate the contribution of epigenetic alterations in these haematological neoplasias, which can contribute to a better patient risk stratification and to select the subgroup of patients that will benefit from treatment with this class of drugs.

This work aimed to evaluate the potential therapeutic effect of epigenetic modulators, hypomethylating agents/DNA methyltransferase inhibitors (DNMTis), and histone deacetylase inhibitors (HDACis), in a CLL-B cell line in culture, as single agents or in combination therapy.

Materials and methods

Cell culture*:* A CLL-B cell line, the EHEB cells, isolated from the peripheral blood of a 69-year old woman, was provided by German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) [11]. The cells were maintained in culture in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 20%

fetal calf serum (Sigma-Aldrich, St. Louis, MO, USA). Cells were cultured at initial density of $0,5x10^6$ cells/ml, during 72 hours, in the absence and in the presence of increasing concentrations of epigenetic modulators, such as the hypomethylant agent 5'-Aza-2'-deoxycytidine or Decitabine (DAC) (Sigma-Aldrich, St. Louis, MO, USA) and the HDACi, Trichostatin A (TSA) (Sigma-Aldrich, St. Louis, MO, USA), alone or with each other.

Cell viability/proliferation analysis*:* Cell proliferation was accessed each 24 hours, during 72h, using the resazurin metabolic test (Sigma-Aldrich, St. Louis, MO, USA). Resazurin was prepared as a stock solution of 100 μg/ml in PBS and this stock solution was filtered with a sterile 0.20 μm-pore filter and stored at -20°C in the dark. After each 24h of treatment, a final concentration of 10 μg/ml of resazurin was added to cells and incubated at 37°C during 6 hours [12,13]. Then, absorbance was measured using a Synergy™ HT Multi-Mode Microplate Reader (BioTek Instruments). The IC_{50} was evaluated by a dose response curve.

Cell death analysis: Cell death was analysed by flow cytometry, staining the cells with annexin V-FITC (AV) and propidium iodide (PI) [14,15]. After 48h, cells, cultured in the absence or in the presence of the drugs, were collected, washed (centrifuged at 1.000 xg for 5 min) and incubated for 15 min in 100 μL AV binding buffer (Immunostep Kit, Salamanca, Spain) and 5 μl FITC-labeled AV and 5 μl PI. After incubation time, cells were diluted in 400 μL of binding buffer and analyzed by flow cytometry. The results were analysed on a flow cytometer equipped with an argon ion-laser emitting a 488 nm beam. Green fluorescence of AV was collected with a 525-nm band pass filter and red fluorescence of PI with a 610 band pass filter. The results are expressed in % of viable, early apoptotic, late apoptotic and necrotic cells.

Morphologic studies: Morphologic studies were performed in order to further characterize the results obtained by flow cytometry. Briefly, after incubation period, cells were collected and stained for 3 minutes with May-Grünwald solution (Sigma, St. Louis, MO, USA), diluted in a 1:1 ratio with distilled water, followed by staining with Giemsa solution (Sigma, St. Louis, MO, USA) diluted 1:8 in distilled water for 15 minutes. After rinsed with distilled water, smears were left to dry at room temperature and cell morphology was analyzed by light microscopy using a Nikon Eclipse 80i microscope equipped with a Nikon Digital Camera DXm 1200F.

Methylation specific PCR (MSP): One µg of genomic DNA, obtained from EHEB cells cultured in the absence and in the presence of DAC, TSA in monotherapy or with the association of both drugs for 72h, was treated with sodium bisulfite according to the EpiTect Bisulfite Kit (Qiagen, Chatsworth, USA), allowing the analysis of the methylation pattern. Methylation-specific PCR's of *p15*, *p16*, *p53*, *death-associated protein kinase* (*DAPK*) and *O^6-Methylguanine DNA methyltransferase (MGMT*) genes were carried out as previously described by others [16-18]. PCR products were resolved on 4% agarose gels, stained with ethidium bromide and visualized under UV illumination.

Real-Time PCR (qPCR): In order to quantify the amount of methylated DNA, bisulfite modified DNA (1 µL) was amplified using SsoFast™ EvaGreen® Supermix (Biorad, Mitry Mory, France) containing a final concentration of 0.25 µM of each p15 methylated primers. All PCR reactions were carried out in duplicate. The amount of methylated DNA was determined by the threshold cycle number (Ct) for each sample, compared against a standard curve generated from the dilution of DNA obtained from untreated cells (control).

Statistical analysis: Data are expressed as mean ± SD obtained from independent determinations, each one performed in duplicate or triplicate. Differences between data sets were evaluated by performing analysis of variance (ANOVA). A p value <0.05 was considered as statistically significant.

Results

Cell proliferation analysis: TSA or DAC, in monotherapy or in combination, lead to a decrease in cell proliferation in a time- and dose-dependent manner. however, TSA was more effective in monotherapy than DAC, reducing cell viability in approximately 50% at 250 nM (53% ± 9%), after 72h of incubation (Fig.1A). Even higher doses of DAC couldn't achieve the same effect. A decrease of about 40% in cell proliferation was observed in the cells treated with the highest concentration of DAC (100 μM) (Fig.1B). The combination of 5 μM of DAC with 100 nM of TSA enhanced the antiproliferative effect of these drugs at concentrations lower than those used to achieve the IC50 in monotherapy. In fact, as a single agent, and after an incubation period of 72h, 100 nM of TSA and 5 μM of DAC induced a reduction in cell proliferation of 24% and 20%, respectively. When cells were treated with the combination of drugs administered simultaneously, a decrease of 43% in cell proliferation is achieved (Fig.1C). However, the administration schedule of drugs didn't appear to influence its cytotoxic effect.

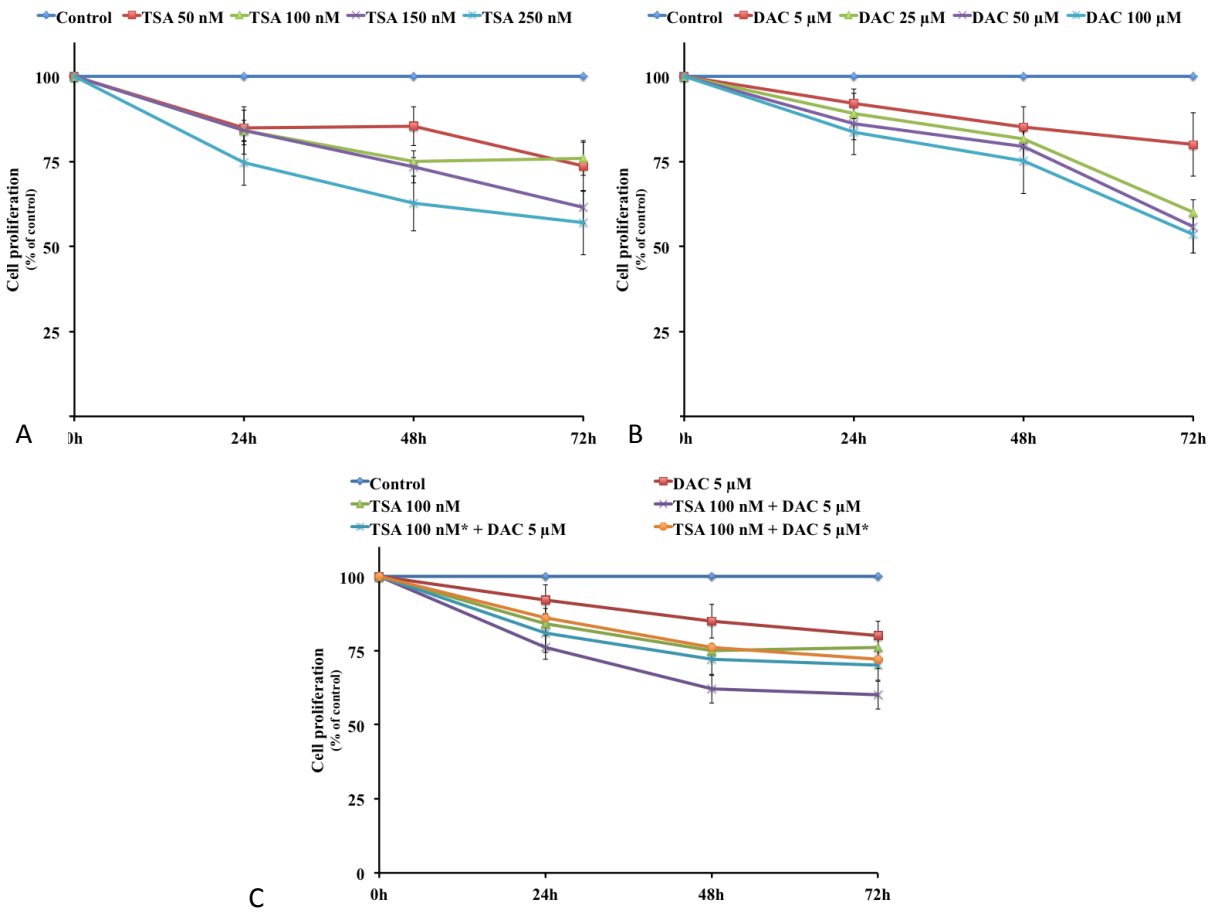

Figure 1. Dose response Curves of TSA and DAC in EHEB cells. Cell proliferation/viability was analysed each 24 h during 72 hours of incubation, with the drug concentrations represented in figure. In (A), it is represented the effect of TSA, in (B), the effect of DAC and (C) represents the effect of TSA and DAC combination therapy in EHEB cells. TSA 100 nM and DAC 5 µM were administrated, alone, simultaneously (TSA 100 nM + DAC 5 µM) or with a gap of 4 hours, TSA 100 nM firstly and 4 hours later DAC 5 µM (TSA 100 nM* + DAC 5 µM) and vice-versa (TSA 100 nM + DAC 5 µM*). The data is expressed in percentage (%) normalized to the control and represents the mean± SD of at least 3 independent experiments.

Cell death analysis: The cytotoxic effect was evaluated by flow cytometry using annexin V/PI incorporation (Fig.2A). The results show, in cells treated with 250 nM of TSA, a higher decrease in cell viability (about 60%±8%), and an increase in cell death by early apoptosis (3% ± 3%), late apoptosis/necrosis (23% ± 2%) and necrosis (14% ± 4%). However, in EHEB cells treated with 5 µM and 50 µM of DAC, only a decrease in cell viability of approximately 10% to 15%, respectively, is observed. Besides that, when drugs were administered in simultaneous combination, we observed a synergistic cytotoxic effect compared with those obtained in cells treated with each drug in monotherapy. In fact, as we can see in Fig.2A, a decrease of 35%±3% in cell viability is observed when cells are incubated with the combination of 100 nM TSA and 5 µM DAC compared with the effect obtained with the same dose of drugs administered in monotherapy (15% with TSA 100 nM and 2% with DAC 5 µM). The decrease in cell viability is accompanied by an increase in cell death mainly by apoptosis.

To confirm the cytotoxic effect observed in the previous experiments, namely the type of cell death, we analysed, by optical microscopy, the morphological characteristics of untreated (control) and treated EHEB cells. The representative cell smears, shown in Fig.2B, show that EHEB cells treated with TSA and DAC, after a treatment period of 48h, mostly display the morphological characteristics of cell death by apoptosis, such as cell contraction, nuclear fragmentation, blebbing and apoptotic bodies, although smears of cells treated with 250 nM of TSA displays also a few cell in necrosis.

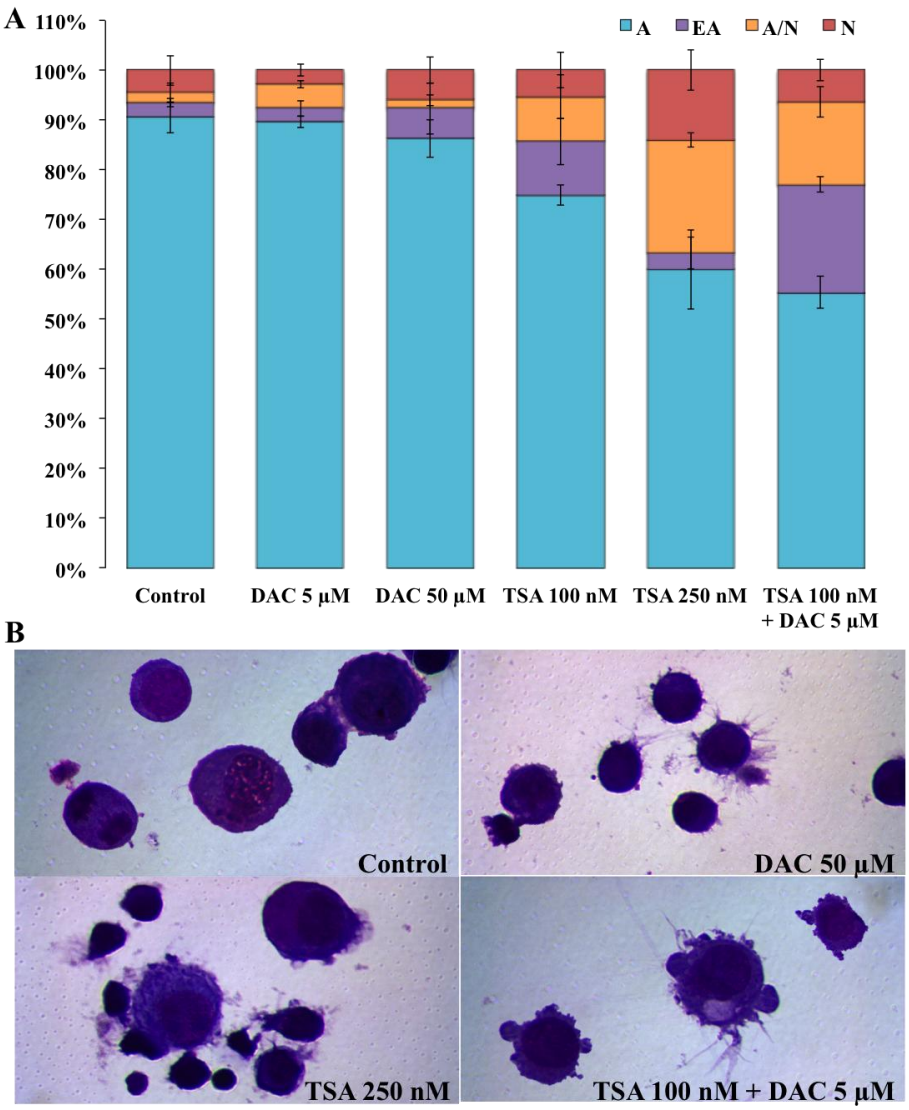

Figure 2. Cell death analysis by flow cytometry and optical morphology. In (A), it is represented the results obtained by flow cytometry when EHEB cells were incubated in the absence (control) and in the presence of drugs indicated in the figure legend. In (B), it is represented the morphological aspects of EHEB cells untreated (control) and treated with 250 nM of TSA, 50 µM of DAC and with the therapeutic combination of 100 nM of TSA plus 5 µM of DAC administered simultaneously. Cell death was detected by annexin V and propidium iodide staining and analyzed by flow cytometry and cell smears were stained with May-Grünwald-Giemsa. A - alive cells; EA - cells in early stage of apoptosis; AN - cells in late stage of apoptosis or in necrosis; N - necrotic cells. Results are expressed in % ± SD and represent the mean± SD of at least 3 independent experiments. Amplification: 500x.

Methylation status and quantification: Methylation status of *p15, p16, p53, MGMT* and *DAPK* genes was analysed in EHEB cells (Fig.3A), since epigenetic modulators induce changes in the hypermethylation of CpG islands. In this CLL-B cell line, the *p15* and *p16* tumor suppressor genes were methylated, however, *MGMT* and *DAPK* genes were demethylated. Besides that, *p53* promoter gene presented one methylated locus and the other demethylated. Since *p15* is ordinarily found methylated, and was also methylated in EHEB cells, the hypomethylating effect of both drugs was evaluated in this gene (Fig.3B). TSA, even in higher doses, induced a limited decrease in the relative *p15* methylation level, approximately 10%. However, as expected, DAC induce a higher hypomethylating effect, leading to a decrease of about 35% and 40% in the *p15* methylation, when EHEB cells were treated with 5 µM and 50 µM of DAC respectively. This demethylating effect was enhanced when cells were treated with the simultaneous association of 5 µM of DAC with 100 nM of TSA, leading to a reduction of about 70% in *p15* methylated gene.

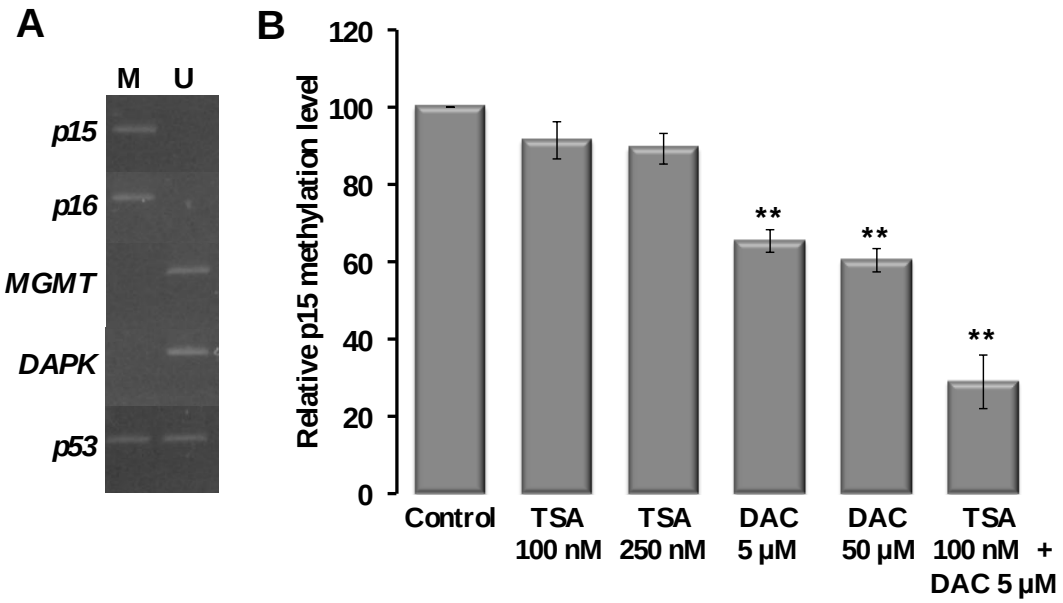

Figure 3. Analysis of gene methylation status by MSP (A) and relative p15 methylation level by qPCR (B). Methylation status was analysed before and after treatment with TSA 100nM or TSA 250nM or DAC 5µM or DAC 50µM in monotherapy or in association administered simultaneously (TSA 100nM + DAC 5µM). MC, universal methylation control (Qiagen, Chatsworth, USA); UC, universal demethylation control (Qiagen, Chatsworth, USA); M, molecular weight lane.

Discussion

"Epigenetic" is a term used to describe mitotically and meiotically heritable states of gene expression that are not due to changes in DNA sequence [19,20]. It is starting to be an issue of higher interest in this proteomic era. Today, the focus is on the expression of the genes rather than the genes themselves.

In contrast to genetic alterations, epigenetic changes in cancer are potentially reversible by pharmacological inhibition of, for example, DNA methylation and histone modification. Epigenetic therapy is a new and rapidly developing area in pharmacology. To date, most trials involving epigenetic drugs have been conducted to evaluate their effects on cancers, many of which have shown promising results [21].

In experimental settings, 5-azacytidine and DAC, in low doses, were capable of reactivating tumour suppressor genes silenced by promoter hypermethylation, in association with regional DNA hypomethylation [1].Conventional anticancer drugs are often toxic and the relapse may occur. So, epigenetic drugs may prove to be a significant advance over the conventional anticancer drugs. These drugs have been already approved as treatments for the myelodysplastic syndrome [22].

Generally, during tumorigenesis, DNA methylation accumulates in the gene promoter region, which attract methyl-binding proteins such as H3-K9 methylase SET domain bifurcated-1 (SETDB1). In addition, increased activity of histone deacetylases and histone methylases leads to loss of active markers such as H3-K4 and H4-K16 acethylation. Changes in the DNA methylation and histone tail modification lead to silencing and reduced activity of a gene, which in case of a tumour suppressor gene could be pivotal in carcinogenesis [19].

In CLL-B, DNA is globally hypomethylated, when compared with peripheral blood mononuclear cells from healthy volunteers. However, expression of tumor suppressor genes is commonly silenced by DNA hypermethylation. Another important fact is that expression of histone methyltransferases, methyl-CpG binding proteins, chromatin associated proteins and their interaction with histone acetylases and deacetylases may all contribute to CLL-B pathogenesis [6].

In our work, it was shown that either the hypomethylant agent Decitabine or the HDACi Trichostatin A induces a decrease in cell proliferation. However, TSA was more effective in monotherapy than DAC. These results are in agreement with our previously results in Acute Lymphoblastic Leukemia (ALL) cell lines (MOLT-3 and MOLT-4 cells) [7,23] showing that incubation with TSA is more effective in reducing cell proliferation and viability. Furthermore, the studies of Hollenbach *et. al* (2010) in Acute

Myeloid Leukemia (AML) cell lines (THP-1, HL-60, KG-1a and OCI-AML3 cells) show that DAC induce dose-dependent responses on cell viability, cell cycle, protein synthesis, DNA-methyltransferase 1 (DNMT1) depletion, DNA hypomethylation, induction of apoptosis, DNA damage, and in gene expression [24]. In our study, DAC, in the same range of concentration, did not reduce cell proliferation below 50% at any concentration used, and this fact can be related to the half-live of DAC in cell culture (8–12 hours) [24]. We hypotheses that daily treatments with DAC could enhance the reduction of cell viability, since that will ensure continued exposure to DAC.

However, we observed a synergistic effect in the therapeutic combination of TSA and DAC, in agreement with other studies in ALL [7,23] and AML [25] cell lines, respectively in MOLT-3, MOLT-4, HL-60 and KG1a cells.

Epidrugs in general are known to target aberrantly heterochromatic regions, leading to reactivation of tumor suppressor genes and/or other genes that are crucial for the normal functioning of cells [19]. But, what is confirmed with this work is that the association of TSA with DAC could have an enhanced effect than either drug alone and even in lower doses. This is in agreement with the fact that demethylating agents only have a transient effect on treated cells and abnormal methylation patterns return with the removal of the drug [6,19]. Possibly, HDACis prevent the chromatin compaction, opening the DNA access either for transcription factors or for DNMTis. Both tested drugs are able to reduce DNA methylation, being the simultaneous combination the best approach. Further studies must be done to confirm this theory. Studies indicate that the use of hypomethylating agents could have therapeutic benefit in acute myeloid leukemia [26] and lymphoid malignancies [13], namely in acute lymphoblastic leukemia [23].

Besides the antiproliferative effect, our results show that TSA and DAC induce cell death mainly by apoptosis, as confirmed by flow cytometry analysis and optical

microscopy. Shin *et al* (2012) have observed the same effect in U937 and HL-60 cells, where DAC-induced apoptosis was correlated with downregulation of anti-apoptotic BCL-2, XIAP, cIAP-1 and cIAP-2 protein expression levels, caspases activation and reduction of mitochondrial membrane potential (MMP). Besides that, they also indicate that DAC induce production of reactive oxygen species (ROS) in these human leukemia cells, which are key mediators of MMP collapse, which induces apoptosis followed by caspase activation [27].

Besides these mechanisms, DAC is a hypomethylant agent that could re-express tumour suppressor genes as cell cycle regulators, apoptotic modulators and DNA repair enzymes, as *p15, p16, DAPK, p53* and *MGMT* genes, respectively, that could explain the results observed. Although TSA is more effective in reducing cell proliferation and viability, DAC showed a higher efficacy in reducing the methylation status of *p15*, only surpassed by the simultaneous association of DAC and TSA. These results are in agreement to our previously results with ALL cell line [7], where a better reduction of the methylation status was obtained with the association of both drugs. Although TSA is mainly a histone deacetylases inhibitor, it induced a slight decrease in the methylation pattern of p15. However, we didn´t evaluate the deacethylant effect, which is its main mechanism of action. Stamatopoulos *et al.* (2010) found that other HDACi, the suberoylanilide hydroxamic acid, induces apoptosis and down-regulates the CXCR4 chemokine receptor, leading to decreased CLL-B cells migration, being then a promising therapeutic approach through inhibition of CLL-B cell survival and potentially in overcoming drug resistance [28]. Our results show the same methylation pattern in *p15* and in *p16.* These results are in concern with other diseases such as myelodysplastic syndromes [29], however in contradiction with what was observed in ALL cell lines [7].

MGMT is a DNA repair enzyme that leads to the removal of alkylation adducts from the O6-position of guanine in DNA. So, when *MGMT* gene is silenced, it has two main consequences. First, it uncovers a new mutator pathway that causes the accumulation of G-to-A transition mutations possibly leading to a genomic instability. Second, having the *MGMT* promoter gene hypermethylated, it increases tumor sensitivity to alkylating drugs. Many tumors express a specific *MGMT* hypermethylation profile gene in several human cancers, such as gliomas, lymphomas, colon, head and neck and non-small cell lung carcinomas [30]. In EHEB cells, the *in vitro* cell model use in this study, the *MGMT* promoter gene is unmethylated. Future studies could confirm if the hypomethylation of this gene in CLL-B patients could be useful as a biomarker of the response to alkylating drugs and DNMTis.

DAPK encodes an actin-filament-associated, calcium calmodulin-dependent, serine-threonine kinase that promotes apoptosis. Loss or reduced expression of this protein underlies cases of heritable predisposition to CLL-B and the majority of sporadic CLL-B. Epigenetic silencing of *DAPK-1* by gene promoter hypermethylation occurs in almost all sporadic CLL-B cases [31]. *DAPK-1* gene is also methylated in other hematological neoplasias, such as multiple myeloma [17]. However, in the EHEB cells this gene was unmethylated. On the other hand, in CLL-B, *DAPK-1* overexpression results in upregulation of *p53*, suggesting a signalling feedback loop in which *DAPK-1* and *p53* regulate the expression of each other. Besides that, *DAPK-1* suppresses cMYC- and E2F-induced cell transformation by activating p19ARF/p53-dependent apoptosis and also by blocking tumor metastasis *in vivo* [31]. This feedback may be presented here and may explain why p53 is partially hypomethylated in this CLL-B model. P53 is a tumor suppressor protein that is important in different cellular tasks as cell cycle and apoptosis regulation and in DNA repair mechanisms. The *P53* gene that codes for this

protein is frequently mutated or silenced by methylation in several cancer types, but fewer studies are done regarding this epigenetic phenomenon in CLL-B.

The integration of new prognostic markers could lead to refine risk stratification for individual patients in a wise and timely fashion that incrementally improve the ability to identify patients that may benefit from these type of drug treatment.

Our study suggests that epigenetic modulation might constitute a new therapeutic approach to the treatment of Chronic Lymphocytic Leukemia. However, drugs administration approach may interfere with their therapeutic efficacy. So, the choice of the optimal schedule of drugs administration may be crucial to the success of the therapy.

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