FACULDADE DE MEDICINA DA UNIVERSIDADE DE COIMBRA

TRABALHO FINAL DO 6º ANO MÉDICO COM VISTA À ATRIBUIÇÃO DO GRAU DE MESTRE NO ÂMBITO DO CICLO DE ESTUDOS DE MESTRADO INTEGRADO EM MEDICINA

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EPIGENETIC MODULATION – A NEW THERAPEUTIC APPROACH IN DIFFUSE LARGE B CELL LYMPHOMA

ARTIGO CIENTÍFICO ORIGINAL

ÁREA CIENTÍFICA DE HEMATOLOGIA

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OUTUBRO/2013

Title: Epigenetic modulation – a new therapeutic approach in Diffuse Large B Cell Lymphoma

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Abstract:

Background/Aims: Diffuse Large B Cell Lymphoma (DLBCL) is the most frequent of non-Hodgkin lymphoma and a prevailing neoplasm worldwide. It affects mainly the elderly, but not exclusively. Despite the majority of the patients achieve satisfactory results with R-CHOP standard treatment, some of them fail to respond, progress during chemotherapy or relapse. In that way, there has been some concern in finding alternative therapies, where recent development and comprehension in epigenetics resemble a promise. This work aims to study the potential therapeutic role of hypomethylating agents (HM) and histone deacetylase inhibitors (HDACi's) in monotherapy and in association of both drugs and with vincristine (VCR) in a DLBCL cell line.

Material and methods: Farage cells, a DLBCL cell line, were incubated with an HM agent – decitabine (DAC), and a HDACi – trichostatin A (TSA), at different concentrations during 24 to 72 hours; proliferation studies were performed using rezasurin test; cell death was analyzed by flow cytometry using annexin V and propidium iodide double staining and optical microscopy (May-Grünwald Giemsa staining).

Results and discussion: Our results show a decrease in cell proliferation in a time dependent manner. After 72 hours of incubation, cell proliferation was of 68,5%±7,6% for TSA 50 nM, and 83,2%±9,7% for DAC 2,5 µM. The association of these two epigenetic modulators in low-dose showed a synergistic effect (cell proliferation of 42,7%±11,2% for TSA 10 nM plus DAC 1 µM), as well as in combination with VCR (cell proliferation of 32,3%±8,2% for TSA 10 nM plus VCR 0,1 nM and of 36,6%±16,5% for DAC 1 µM plus VCR 0,1 nM). Moreover, the combination of the three drugs was the best therapeutic regimen tested (cell proliferation of 13,3%±15,3% for TSA 10 nM plus DAC 1 µM plus VCR 0,1 nM). Cell death in tested drug conditions occurred preferably by apoptosis, regarding the increase in activated caspases expression and morphological studies.

Conclusion: This study suggests that epigenetic modulation might be a new approach to the treatment of lymphomas, namely DLBCL, as in monotherapy as in combination with conventional chemotherapy.

Key words: diffuse large B cell lymphoma, epigenetics, decitabine, trichostatin A, methylation profile

List of Abbreviations and Acronyms:

ABC: Activated B Cell

ANOVA: Analysis Of Variance

ATCC: American Type Culture Collection

AV: Annexin V

CNS: Central Nervous System

DAC: Decitabine

DLBCL: Diffuse Large B Cell Lymphoma

DNA: DeoxyribonucleicAcid

DNMTi: DNA Methyltransferase Inhibitor

EBV: Epstein-Barr Virus

EPOCH: Etoposide, Prednisone, Vincristine, Cyclophosphamide, Doxorubicin

FITC: Fluorescein Isothiocyanate

GCB:Germinal Center B-Cell

GEP: Gene Expression Profile

HDACi: Histone Deacetylase Inhibitor

HIV: Human Immunodefficiency Virus

HM: Hypomethylating Agents

IPI: International Prognostic Index

LDH: Lactate Dehydrogenase

NHL: Non-Hodgkin Lymphoma

NOS: Not Otherwise Specified

PBS: Phosphate Buffer Saline

PI: Propidium Iodide

PMLBCL: Primary Mediastinal Large-Cell Lymphoma

R-CHOP: Rituximab, Cyclophosphamide, Doxorubicin, Vincristine, Prednisone

R-DHAP: Rituximab, Dexamethasone, Cytarabine (Ara-C), Cisplatin

R-ESHAP: Rituximab, Etoposide, Solumedrol, Ara-C, Platinum

RICE: Rituximab, Ifosfamide, Carboplatin, Etoposide

SD: Standard Deviation

TSA: Trichostatin A

VCR: Vincristine

WHO: World Health Organization

Background:

Diffuse Large B Cell Lymphoma (DLBCL) is the prototype of the classic "aggressive" or "high grade" lymphoma (1–3) because of its high proliferation rate, rapid evolution, and diffuse growth pattern that usually involves nodal sites; less frequently it involves extra-nodal sites, namely infiltrating the bone marrow, gastrointestinal tract, brain, spinal cord, orbits or kidneys, in addition to other organs (1,2,4–6). DLBCL is the most common form of non-Hodgkin lymphoma (NHL), representing between 25-40% of adult non-Hodgkin lymphomas in western countries, and a similar percentage of all lymphomas (2–12). Its estimated incidence is higher an increasing in developing countries (5).

Accordingly to World Health Organization's (WHO) (2008), DLBCL is a neoplasm of large B lymphoid cells with nuclear size equal or exceeding normal macrophage nuclei or more than twice the size of a normal lymphocyte (5). The designation of DLBCL is above all an umbrella for a diverse set of entities, as seen on morphological, immunophenotypical, genetical and clinical studies (4,5,13,14). Among the various subtypes considered in WHO classification, gene expression profiling (GEP) allowed the identification and highlighting of three of them: germinal center B-cell (GCB), activated B-cell (ABC) and primary mediastinal large-cell lymphoma (PMLBCL) (5,12,14–16). However, it is not always possible to define clearly the causative pathogenic subtype, thus it is often referred as not otherwise specified (NOS) (4,5). Histological patterns are diversified, and include centroblastic, immunoblastic and anaplastic variants (1,5). DLBCL biomarkers include surface antigens CD5, CD10, CD19, CD20, CD22, CD43, CD79a, BCL-6, FOXP1, GCET1, and MUM1; some of them are used to distinguish ABC from GCB subtypes (5,6,14).

This lymphoma is more prevalent among the elderly; median patient age is the sixth to seventh decade of life (4,5,8,14,17). Nevertheless, it may also present in children or younger adults (5,6). DLBCL shows in general a slight masculine preponderance (55%) (2,6), but certain subtypes such as PMLBCL affect primarily females in her thirties (14). This neoplasm most frequently arises *de novo* from B cells, but it can also emerge from transformation of a preexisting low-grade B cell lymphoma (e.g. follicular lymphoma) (6,7). Infectious agents involved in etiopathogenesis include Human Immunodeficiency virus (HIV) and Epstein-Barr virus (EBV) (in the particular case of primary Central Nervous System [CNS] DLBCL) (2,14). Some genetic anomalies associated with this spectra of diseases include: 1) over-expression of *BCL-2* gene (located on chromosome 18) leading to apoptosis suppression due to t(14;18), which occurs in approximately 30% of patients; 2) t(3;-)(q27;-) in relation with *BCL-6* gene, a key transcriptional regulator of the germinal center

reaction (12); and 3) t(17;-)(p13;-), related to tumor suppressor oncogene *p53* (2).

International Prognostic Index (IPI) is widely used prior to therapy in order to stratify patients' risk and categorize them with differential prognosis (1,12,14,16). It is based on five adverse risk factors: age greater than 60 years old, poor performance status, elevated lactate dehydrogenase (LDH), advanced Ann Arbor stage and presence of more than one extra-nodal site (1,16).

Although rituximab's introduction (monoclonal antibody directed to surface B cell antigen CD20) in the standard CHOP chemotherapy regimen (composed by cyclophosphamide, doxorubicin, vincristine, and prednisone) had meant an improvement in outcomes and overall survival rates, treatment failure due to refractory or relapsing disease persists in approximately 30-40% of cases, who have poorer prognosis (4,8–12,17,18). For them, rescue therapy encompasses salvage high-dose chemotherapy regimens like R-ESHAP (rituximab, etoposide, methylprednisolone [solumedrol], cytarabine [ara-C], platinium), RICE (rituximab, ifosfamide, carboplatin, etoposide), R-DHAP (rituximab, dexamethasone, ara-C, cisplatin), dose-adjusted EPOCH (etoposide, prednisone, vincristine, cyclophosphamide, doxorubicin), just to name few, that may be followed by haematopoietic stem cell transplantation on those who are chemotherapy-sensitive (12,15–17,19). However, 40% of patients remain incurable (18) and response rates range from 30% to 60%, with frequent relapses (17).

Consequently, the need of a new rationale led to targeted therapy, expected to hold the solution (9,12,18). Intensive investigation made in the past decades brought new potential agents such as epigenetic modulators, some of them on ongoing clinical trials (8,11,15,17,19–22).

Epigenetics, defined as regulation in gene expression that are independent of changes in the DNA sequence and persist over cell divisions (23) represents a new concept in the treatment of cancers (15), because of its key role in carcinogenesis and tumor progression (24), especially in haematological neoplasms. Epigenetic factors interfere with gene expression, whether by gene silencing, up-regulation or modulation and therefore with protein production (22). Epigenetic alter the degree of DNA compaction and the accessibility of the transcription machinery to the DNA strand (i.e. through DNA methylation and histone deacetylation), thus altering gene expression, phenotype and disease susceptibility (25). These facts are critical for lymphoma cells survival which depend on disruption of the apoptosis pathway (4). Moreover, epigenetics may also play, by itself, a role in treatment's failure and resistance (23).

This work aimed to evaluate the potential therapeutic effect of two different classes of epigenetic modulators, an hypomethylating agent/DNA methyltransferase inhibitor (DNMTi),

decitabine (DAC), and an histone deacetylase inhibitor (HDACi), trichostatin A (TSA), in a DLBCL cell line (Farage cells), as single agents, and in associative therapy with each other and with the anti-neoplastic drug vincristine (brand name Oncovin®).

Materials and methods

Cell culture: The Farage cells (ATCC® CRL-2630™) originally obtained from a metastatic site (a lymph node) biopsy of an adult white female patient with diffuse large cell non-Hodgkin's lymphoma (DLCL) (26) were used as DLBCL cell line. It was provided by American Type Culture Collection (ATCC, Rockville, Maryland, USA). Cells were maintained in culture in RPMI-1640 Medium (Gibco) supplemented with 20% fetal calf serum (renovated each 48 hours or 72 hours), in an atmosphere of f 5% CO_2 at 37ºC. Cells were culture at initial density of $0,5x10^6$ cells/mL. Cell density and cell viability was accessed by cell counting in a Neubauer chamber using trypan blue exclusion test (27). Farage cells were incubated during 72 hours in the absence and presence of epigenetic modulators (epidrugs) and vincristine (VCR). The epidrugs tested were the hypomethylant agent 5'-Aza-2'-deoxycytidine or Decitabine (DAC), in ranging concentrations from 1 µM to 25 µM, the HDACi Trichostatin A (TSA), with a range of concentrations from 10 nM to 500 nM, administered whether alone or in combination with each other. The chemotherapeutic agent vincristine was associated with epigenetic modulators in a concentration of 0,1 nM.

Cell proliferation analysis: Cell proliferation was accessed every 24 hours during 72 hours, using the rezasurin metabolic test. Rezasurin was prepared as a stock solution of 100 µg/mL in PBS. It was then filtered with a sterile 0.20 µm-pore filter and stored in the freezer at -20ºC, in the absence of light. Every 24 hours of incubation, 10 µg/mL of rezasurin were added to each well of the cells' plate, being incubated afterwards at 37ºC in an atmosphere of 5% CO_2 (28). Once controls wells changed color from blue to pink, the absorbance was measured at 560 and 600 nm using a Synergy™ HT Multi-Mode Microplate Reader (BioTek Instruments). The IC_{50} (drug concentration that reduce cell viability in 50%) was evaluated through a dose-response curve.

Cell death analysis by flow cytometry: Cell death was analyzed using flow cytometry, staining cells with annexin V-FITC (AV) and propidium iodide (PI) (29,30), and by caspase expression

levels, using monoclonal antibodies labeling with fluorescent probes. After 48h or 72h, depending on the experimental condition, cells cultured in the presence and absence of the drugs were collected, washed, centrifuged at 1.000 xg for 5 minutes and incubated for 15 minutes in 100 µL AV binding buffer and 5 µL FITC-labeled AV and 5 µL PI. After incubation time, cells were diluted in 400 µM of binding buffer and analyzed by flow cytometry. Results were expressed in percentage of viable (AV^-/PI^-), early apoptotic (AV^+/PI^-), late apoptotic/necrotic (AV^+/PI^+) and necrotic cells (AV^-/PI^+). To quantify the activated caspases levels we used the ApoStat Apoptosis Detection Kit FITC (31), by flow cytometry. Briefly, cells cultured in the same conditions were directly stained during the last 30 minutes of the apoptosis induction period, at 37˚C, with 10 µL of ApoStat per 1 mL culture volume. After the staining period, the cells were harvest, centrifuged at 500 xg for 5 min and wash once with PBS to remove unbound reagent. Then, cells are analyzed by flow cytometry for the presence of bound reagent and an increased fluorescence indicates caspases activity within cells. Flow cytometry analyses were performed using a six-parameter, four-color FACSCalibur™ flow cytometer (Becton Dickinson). For each assay, 1×10^6 cells were used and at least 10,000 events were collected by acquisition using Cell-Quest software (Becton Dickinson) and analyzed using Paint-a-Gate software (Becton Dickinson).

Morphologic studies: Morphologic studies were performed in order to confirm flow cytometry results. After a period of incubation of 48 or 72 hours, depending on the experimental condition, cells were centrifuged and supernatant was excluded. Pellet was resuspended in 10 µL of fetal bovine serum and spread on slides. Cells were then stained for 3 minutes with May-Grünwald solution diluted in a 1:1 ratio with distilled water, followed by staining with Giemsa solution diluted 1:8 in distilled water for 15 minutes. After rinsing 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.

Statistical analysis: Results represent at least three independent experiments and are expressed as mean ± standard deviation (SD). We used GraphPad Prism software version 6.0c for Mac OS X (GraphPad Software, San Diego, CA, USA). Differences between data sets were evaluated by analysis of variance (ANOVA) test, considering a 0.05 significance level (p value). To results obtained on cell death assays we applied also the Bonferroni test.

Results

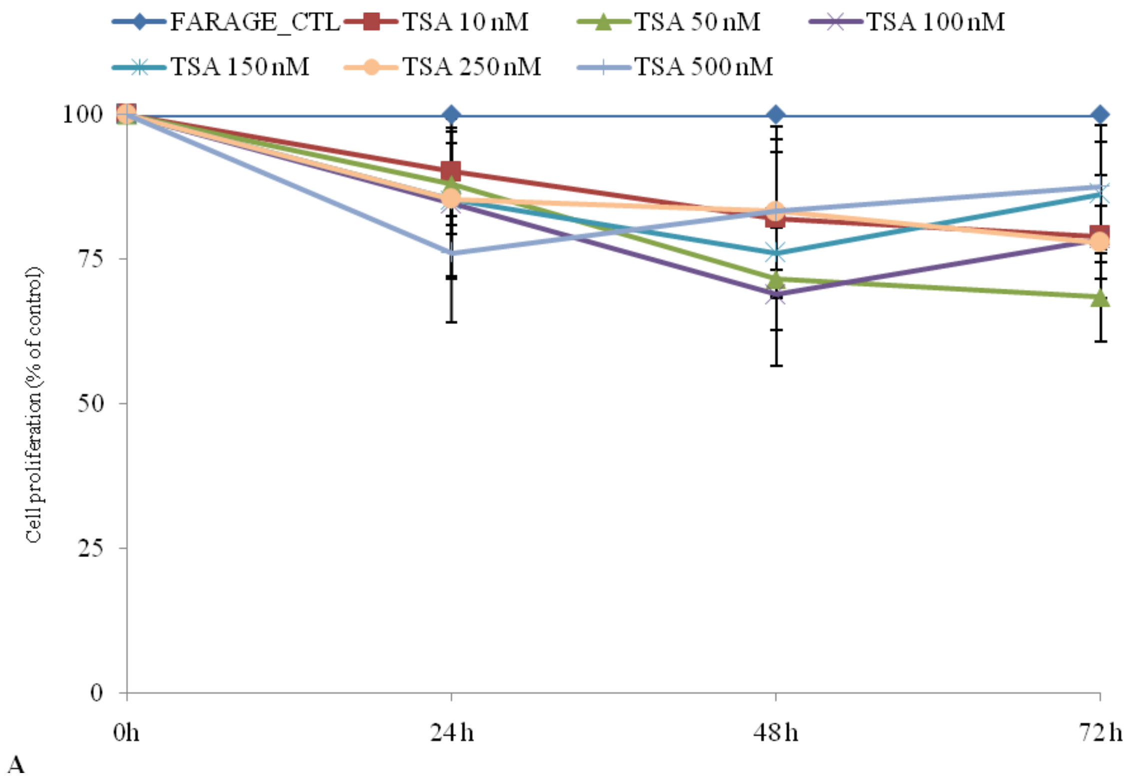
Cell proliferation analysis:

To evaluate the therapeutic potential of epigenetic modulators in DLBCL, Farage cells were cultured in absence and in presence of DAC and TSA during 72 hours, alone and in therapeutic combination with each other and/or VCR.

We observed that TSA and DAC, in a monotherapy regimen, leaded to a decrease in cell proliferation, being TSA slightly more effective than DAC, reducing cell proliferation in approximately 30% at 50 nM (cell proliferation of 68,5%±7,6%) after 72 hours of incubation (Figure 1-A). In all tested DAC concentrations, cell proliferation decrease approximately 20% after 72 hours of incubation (Figure 1-B). Furthermore, none of the drugs administered alone and in single dose show an effective reduction in cell proliferation. In fact, when cells were treated with the drugs in monotherapy and administered in single dose we have never achieved the IC_{50} for none of the tested drugs concentration.

Moreover, we observed that after 24 hours of incubation with TSA the effect was essentially dose-dependent (Figure 1-A), achieving the highest reduction of cell proliferation for the highest drug concentration (500 nM). However, in cells treated with this TSA concentration we observed reversibility of drug effect after 24 hours of incubation from which there was an increase in cell proliferation, an effect that was sustained till 72 hours. We also observed a similar effect in relation to other concentrations (100 nM and 150 nM) between 48 hours and 72 hours, with a recovery of the cellular proliferation. Thus, after 72 hours with TSA treatment, the concentration that appears to be the most effective in reducing cells' proliferation is 50 nM.

As we can see in Figure 1-B, after 24 hours of incubation with DAC there was a 25% reduction in cellular proliferation (cell proliferation: 74,5%±12,9%) regarding the concentration of 10 μM. After this period of time, a reversibility of drug effect for all drugs concentrations, except 1 μM, was observed.



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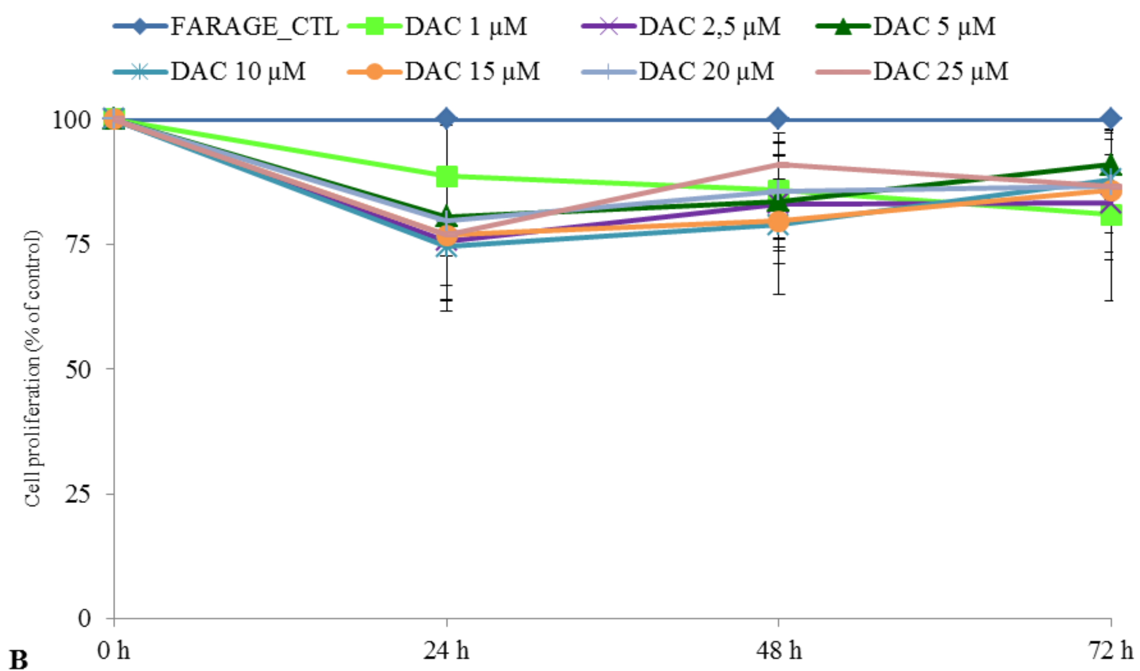


B

Figure 1: Dose-response curves for trichostatin A (TSA) and decitabine (DAC) in Farage cells. Cell proliferation was analyzed every 24 hours during a period of incubation of 72 hours with the drug concentrations indicated in the figure. In (**A**) is represented the effect of TSA alone and in (**B**) the effect of DAC. The results are expressed as percentage (%) compared to control (100%) and represent the average of six independent experiments±SD.

Since we observed reversibility in TSA and DAC anti-proliferative effects, we evaluated if daily administration regimes were more efficient in reducing cell proliferation than single dose administration. In this context, we compare the cytostatic effects of the daily administration of 10 nM of TSA with50 nM of TSA in single administration, during 72 hours. In daily addition, TSA 10 nM had a remarkable effect when compared to the single dose of 50 nM (Figure 2-A). Particularly at 72 hours, daily TSA 10 nM was more powerful than the single administration of TSA 50 nM (cell proliferation was respectively of 38,5%±12,5% *vs* 68,5%±7,6%). When Farage cells were treated daily with 1 µM of DAC we observed also a higher reduction in cellular proliferation compared to cells treated with 5 µM of DAC at single administration (Figure 2-B). At 72 hours, DAC 1 µM added daily had extensively reduced cell population (cell proliferation of 42,9%±20,3%), while DAC 5 µM administered in single dose didn't show any effect (cell proliferation about 90,9%±7,0%).

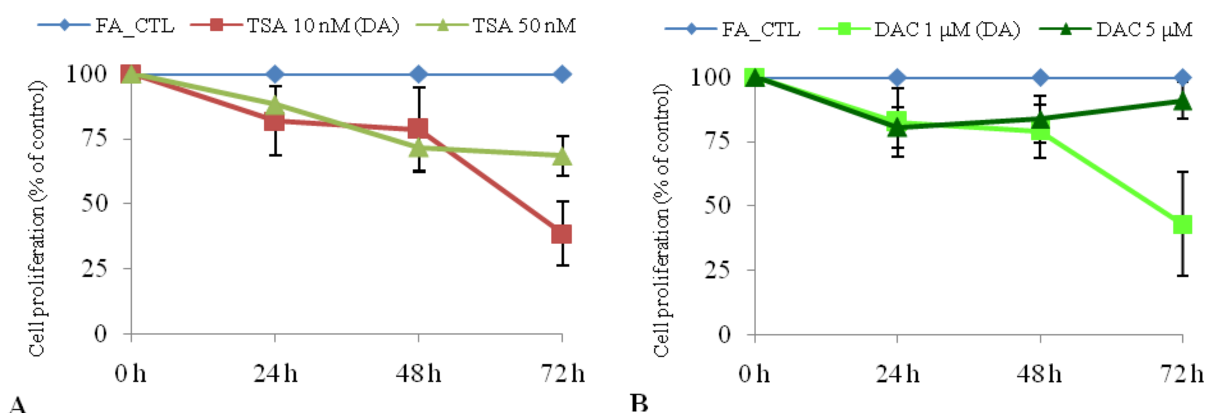


Figure 2: Dose-response curves of trichostatin A (TSA) (A) and decitabine (DAC) (B) in a daily dose administration regime in Farage cells. In both graphics, a daily drug administration in low-dose is compared with the highest equivalent dose at single take. Cell proliferation was analyzed each 24 hours during a period of incubation of 72 hours. (DA) – Daily administration.The results are expressed as percentage (%) compared to control (100%) and represent the average of five independent experiments±SD.

In order to investigate the possibility of a synergistic antiproliferative effect of DAC and TSA, we tested the drugs in association regimes, with each other and with VCR. As we can observed in Figure 3-A, the combination of TSA 10 nM and VCR 0,1 nM induced a highest antiproliferative effect than any effect obtain the drugs alone. There wasn't any noteworthy

difference between simultaneous and out of phase drug administration. This therapeutic strategy conduced to a sustained decrease of cell proliferation whose average values were 80,7%±13,6% at 24 hours, 69,3%±16,5% at 48 hours and 32,3%±8,2% at 72 hours. Similar effects were observed with the association of VCR 0,1 nM and DAC 1 μM in monotherapy (Figure 3-B). Likewise, as previously described for TSA 10 nM and VCR 0,1 nM double therapy, combined therapy of DAC 1 μM and VCR 0,1 nM also shown to be more efficient in reducing cell proliferation that DAC or VCR alone. Once again, the administration schedule of the drugs didn't seem to play a major role in the cytotoxic effect (similar antiproliferative effects).

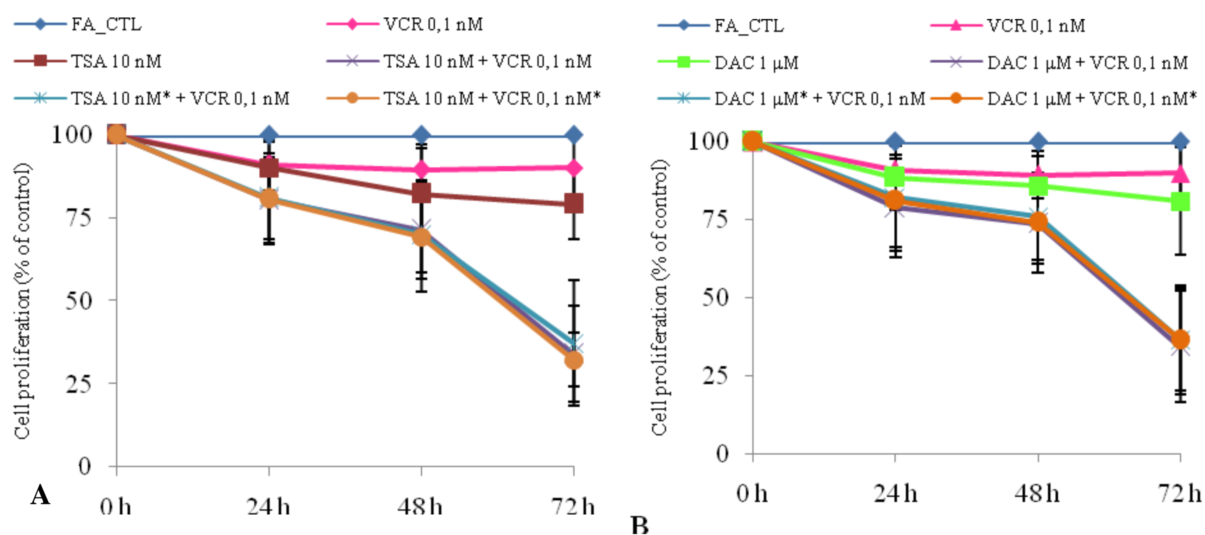


Figure 3: Therapeutic effect of the combination of trichostatin A (TSA) and decitabine (DAC) with vincristine (VCR) in Farage cells. In (**A**) is represented the effect of trichostatin A (TSA) plus vincristine (VCR) and in (**B**) decitabine (DAC) plus VCR. Each one was applied at single low-dose regimen, simultaneously or with a 3h gap, and cell proliferation was analyzed every 24 hours during 72 hours. The star (*) marks the drug administered in first place; the second drug was administered 3 hours later.The results are expressed as percentage (%) compared to control (100%) and represent the average of seven independent experiments±SD.

Next, we analyzed the potential synergistic effect of TSA and DAC in combination using lower doses than dose used to obtain an antiproliferative effect in monotherapy. As shown in Figure 4, all tested conditions had an analogous behavior until 24 hours, time from which the delayed addition of DAC 1 μM to TSA 10 nM seems to be the most effective therapeutic schedule in reducing cell proliferation. Besides, the drugs in monotherapy showed a similar rate of cell death induction, they didn´t reach a decrease in cell proliferation to 25%. Simultaneous treatment of TSA

and DAC (69,3%±28,9%) induced less cytostatic effect than addition out of phase (TSA plus DAC 3h later: 42,7%±11,2%; DAC plus TSA 3h later: 49,7%±17,1%).

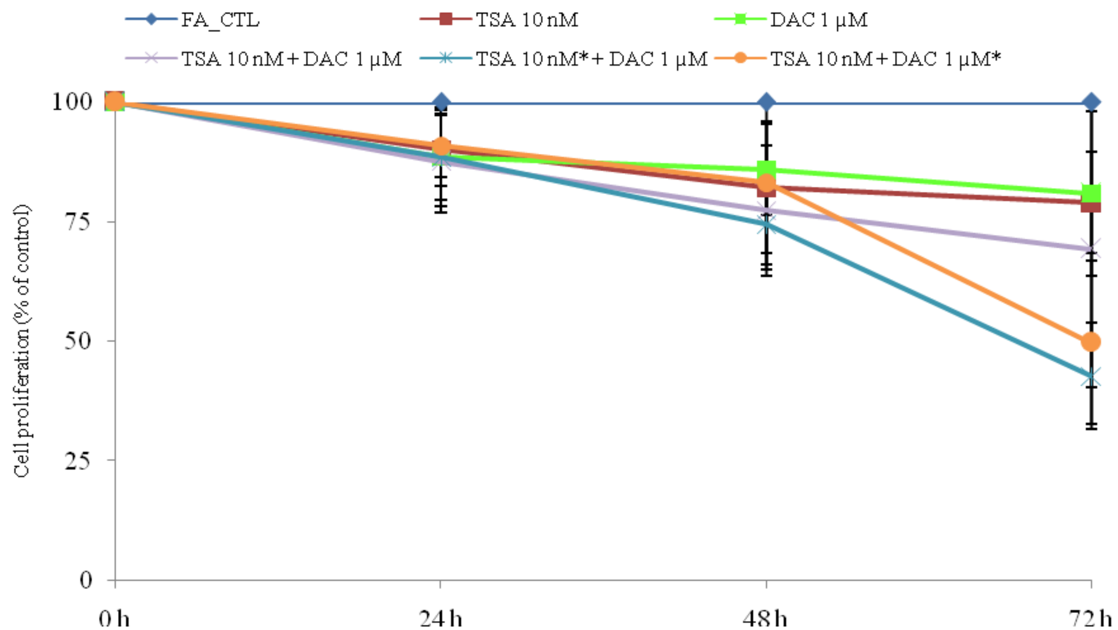


Figure 4: Dose-response curves of trichostatin A (TSA) plus decitabine (DAC) in combination therapy. Cell proliferation was accessed every 24 hours during a period of incubation of 72 hours. The star (*) marks the drug administered in first place; the second drug was administered 3 hours later. The results are expressed as percentage (%) compared to control (100%) and represent the average of six independent experiments±SD.

Finally, we analyzed the therapeutic potential of triple drug associations using TSA 10 nM to VCR 0,1 nM and DAC 1 µM. Our results shown that triple association regimes induced more antiproliferative effects that the drugs in monotherapy, all over the 72 hours (Figure 5). Simultaneous administration of TSA 10 nM, DAC 1 µM and VCR 0,1 nM didn't differ considerably from delayed combinations strategies. The results were indeed compelling inducing a decrease in cell proliferation around 15%. Furthermore, the IC_{50} of the drugs added in triple therapy is attaint earlier (before 48 hours).

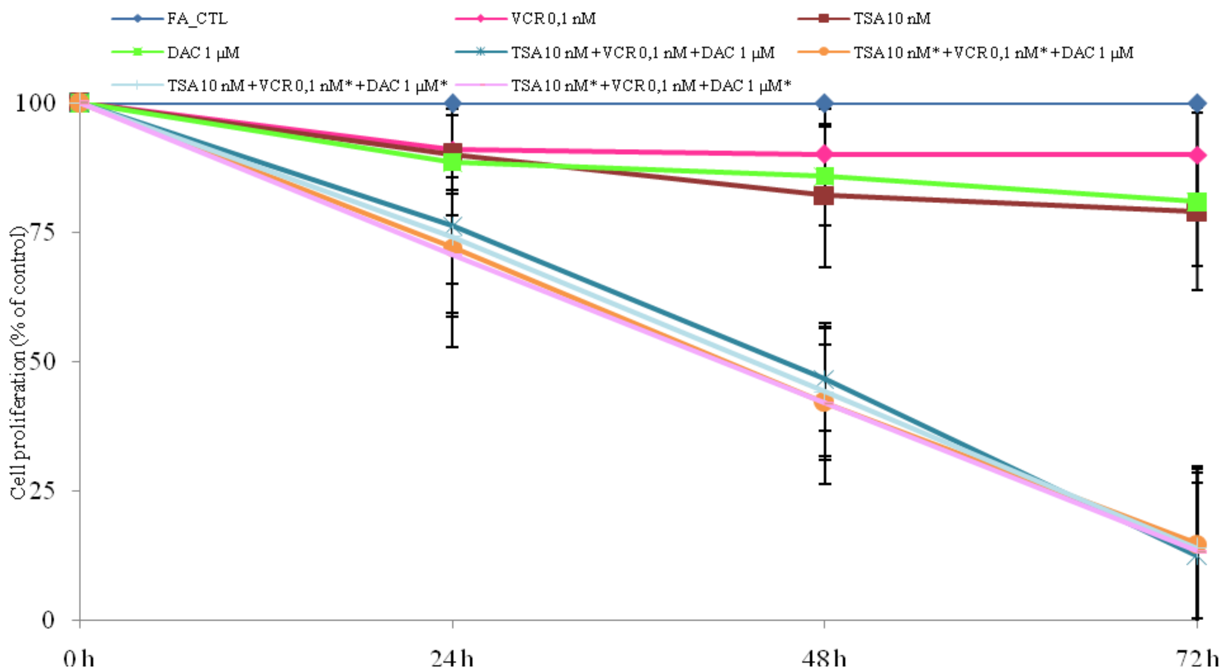


Figure 5: Dose-response curves for triple association of trichostatin A (TSA), decitabine (DAC) and vincristine (VCR) in Farage cells. Cell proliferation was accessed every 24 hours during a period of incubation of 72 hours; drug concentrations were those depicted in the figure. It was used a single low-dose regimen. The star (*) marks the drugs administered in first place; the other was administered 3 hours later. The results are expressed as percentage (%) compared to control (100%) and represent the average of seven independent experiments±SD.

Cell death analysis: Cytotoxic effect was evaluated by flow cytometry using annexin V and propidium iodide (PI) (Figure 6). When testing and comparing monotherapy *vs* combination regimens (Figure 6-A), we found out that in all therapeutic regimes, the predominant pattern of cell death was early apoptosis and late apoptosis/necrosis (Figure 6-A). Besides that, TSA 750 nM was the one who induced the largest ratio of necrotic cells (9,0%±3,5%), while TSA 10 nM induced the lowest (2,7%±1,2%). In general, the necrosis rate was low for all concentrations tested, even in comparison with control cells.

When Farage cells were treated with DAC 1 µM and TSA 10 nM, no significant decreases in cell viability was observed (79,3%±8,1% and 77,3%±7,2%, respectively). Furthermore, we observed cumulative cytotoxic effect for double and triple low-dose regimens in comparison with low-dose monotherapy. For example, cell treated with TSA 10 nM, DAC 1 µM and VCR 0,1 nM in association present 38%±6% viable cells, while it was of 81%±4% for VCR 0,1 nM, 79%±8% for DAC 1 µM and 77%±7% for TSA 10 nM in monotherapy. In summary, the therapeutic regimes tested induced cell death mainly by apoptosis.

As observed in Figure 6-B, Farage cells treated with daily DAC 1µM induced a larger decrease in cell viability (of approximately 34,3%±9,1%), inducing cellular death by early apoptosis (20,7%±3,7%), but also by late apoptosis and necrosis (12%±7%). Daily administration of 10nM of TSA induced essentially death by early apoptosis (17,5%±4%), and a smaller percentage of late apoptotic and necrotic cells (10,0%±6,2%).

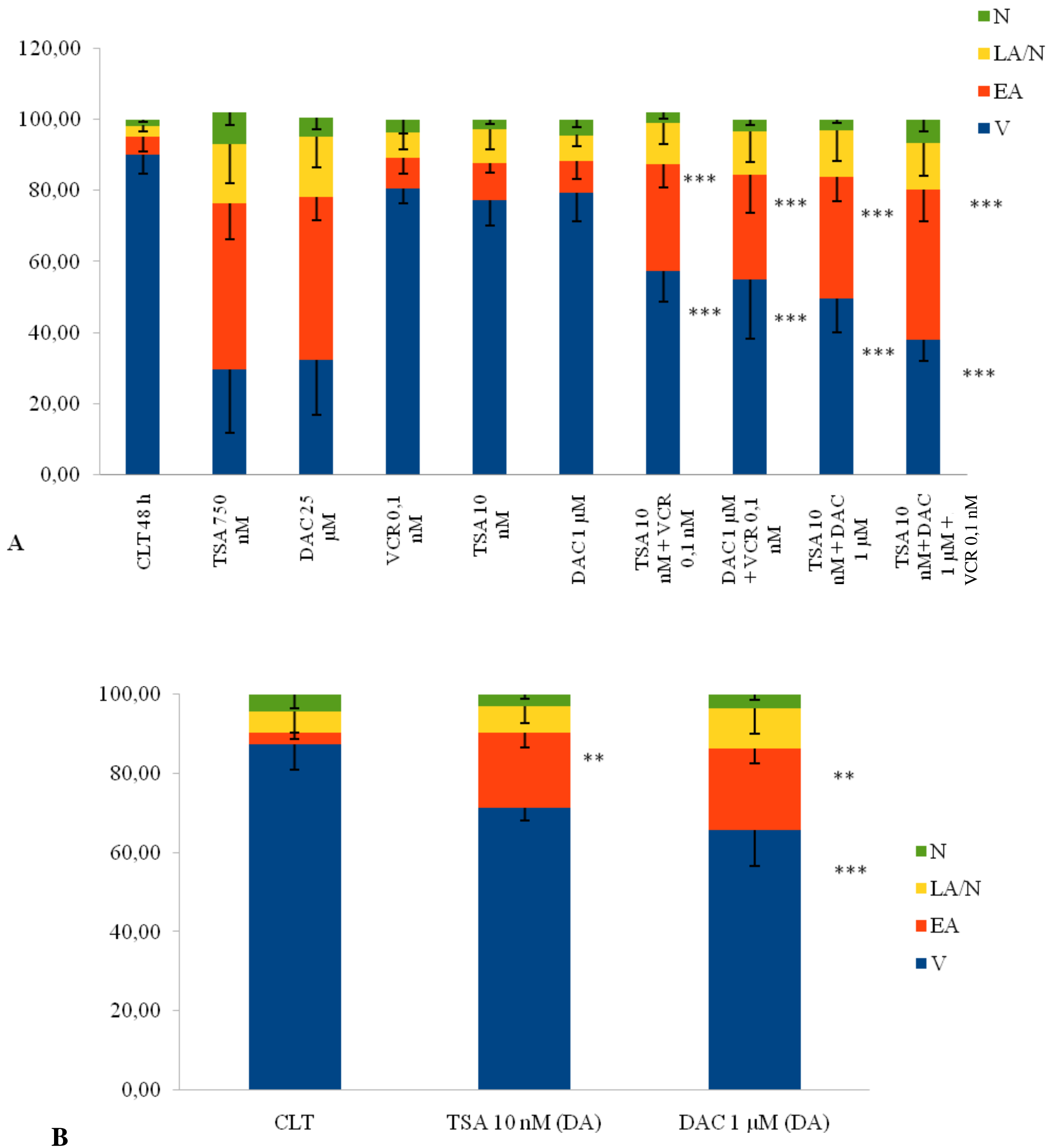


Figure 6: Cell death analysis by flow cytometry. Farage cells were cultured in the absence (control) and in the presence of TSA and DAC in monotherapy at single dose administration and in combination regimens during 48 hours (**A**); and in a daily posology regimen, after 72 hours of

incubation (B). Cell death was accessed using annexin V and propidium iodide staining and analyzed by flow cytometry. N – Necrosis; LA/N – Late apoptosis/ Necrosis; IA – Early apoptosis; V – Viable cells; (DA) – Daily drug dose; CLT – Control; TSA – Trichostatin A; DAC – Decitabine; VCR – Vincristine. ** - p value < 0.01 (in relation to control); *** - p value < 0.001 (in relation to control). The results are expressed as percentage (%)and represent the average of three independent experiments±SD.

In order to confirm the pattern of cells' death induced by epigenetic modulators we analyzed the morphological aspects of untreated (control) and treated Farage cells. Figure 7 show some representative cell smears of tested conditions. As observed, cells treated with DAC and TSA, alone or in combination with each other or with VCR, display typical morphological characteristics of apoptosis, such as cell contraction, nuclear fragmentation, blebbing and apoptotic bodies. These results confirmed what our previous flow cytometry studies.

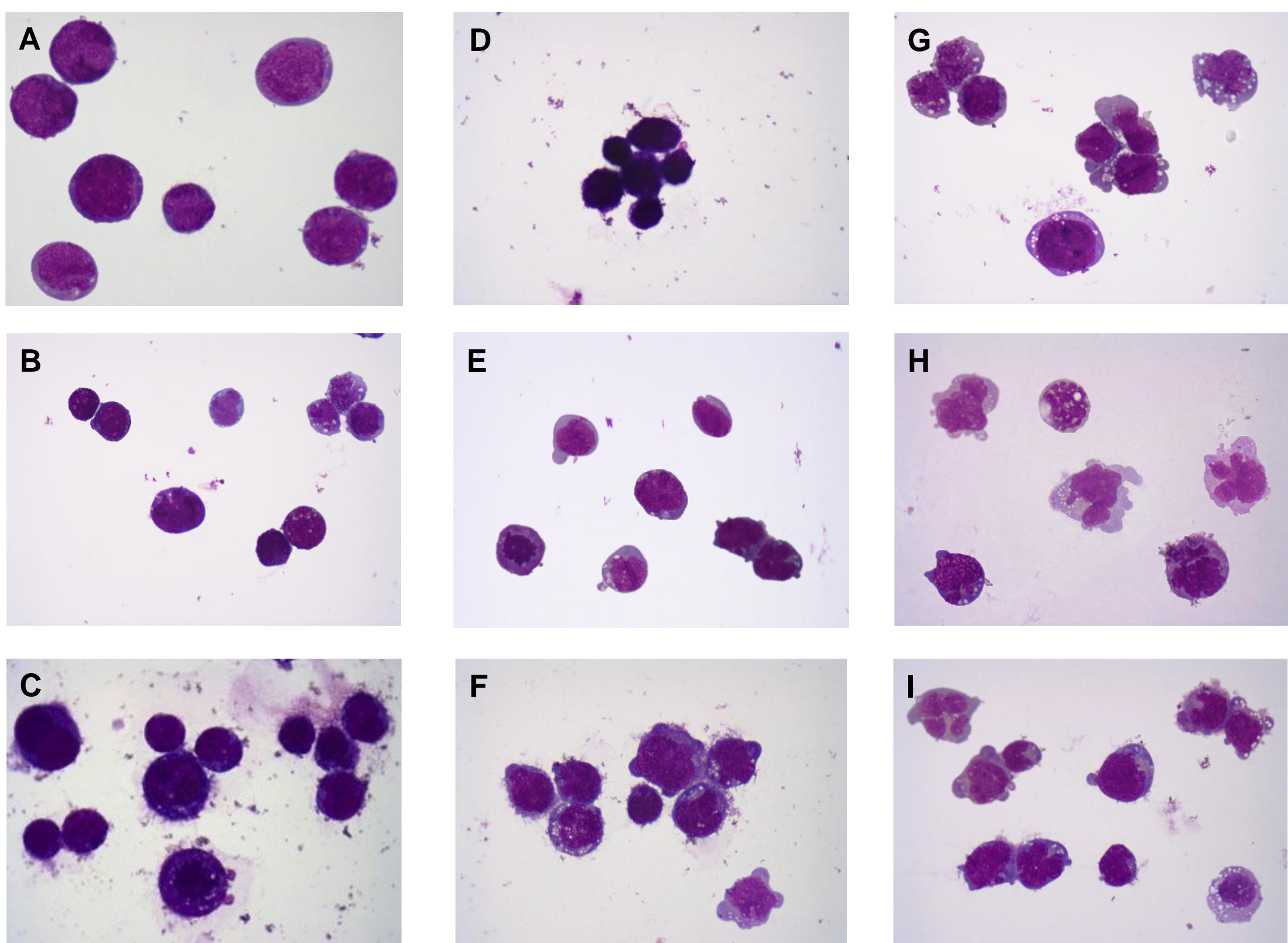
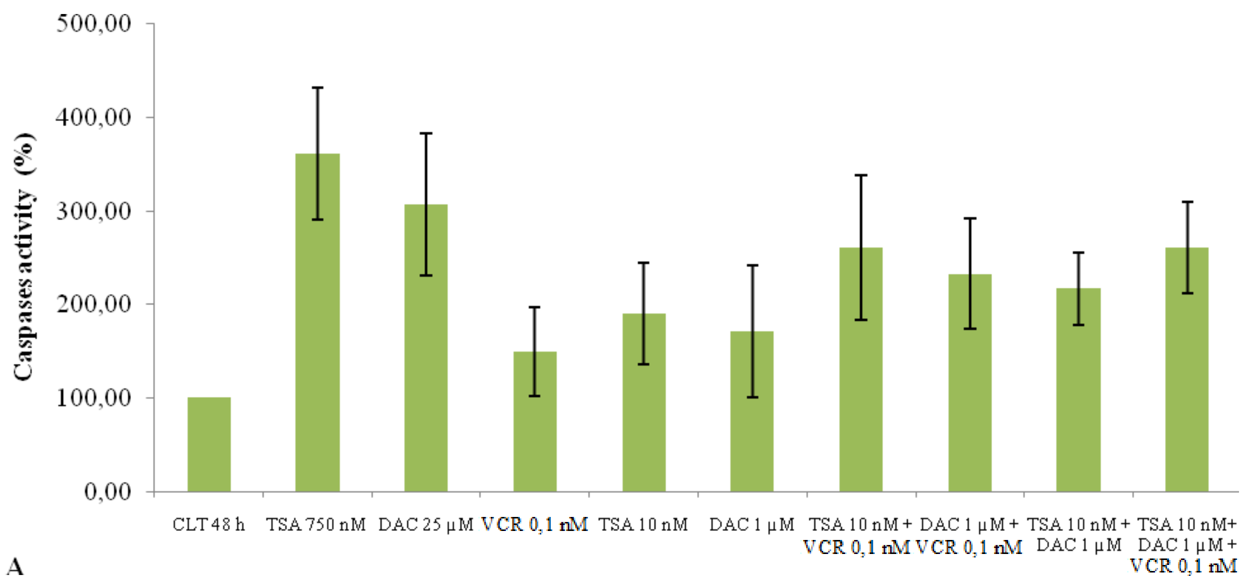


Figure 7: Morphological aspects of Farage cells cultured in the absence and presence of epigenetic modulators in monotherapy and in therapeutic combination with vincristine. A,

control cells; **B**, daily administration of TSA 10 nM (72 h); **C**, daily administration of DAC 1 μM (72h); **D**, single administration of TSA 750 nM (48h); **E**, single administration of DAC 25 μM (48h); **F**, TSA 10 nM plus VCR 0,1 nM (48h); **G**, DAC 1 μM plus VCR 0,1 nM (48h); **H**, TSA 10 nM plus DAC 1 μM (48h); **I**, TSA 10 nM, DAC 1 μM and VCR 0,1 nM (48h). Amplification 1000x.

After cell death analysis, we evaluated if cell death induced by epigenetic modulators is mediated by caspases activation. As observed in Figure 8, DAC and TSA in monotherapy and in therapeutic combination with each other and/or VCR induced an overall increase in caspases activity, compared to control, in a dose dependent manner. These results are in agreement with the previous obtained on flow cytometry using annexin V/PI staining, thus suggesting that these compounds induce cell death in Farage cells mainly by apoptosis.



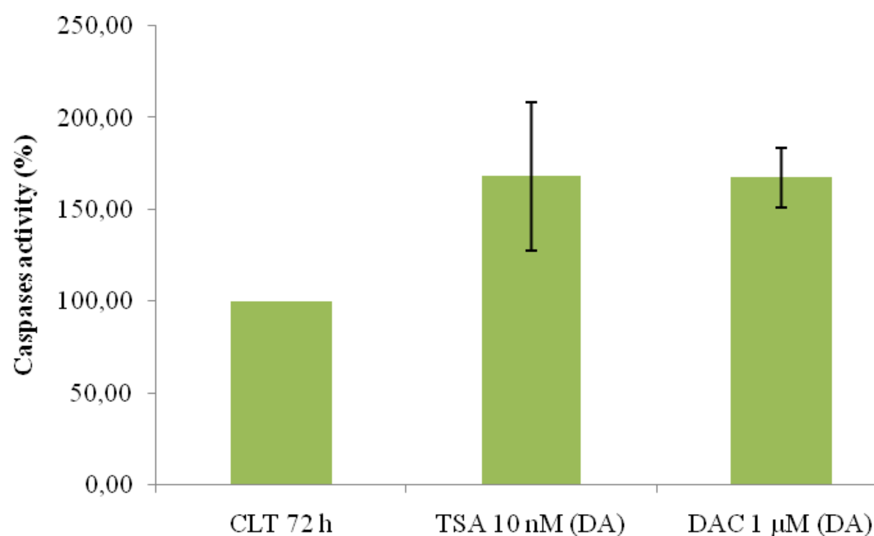


Figure 8: Evaluation of Caspases activity in Farage cells treated with trichostatin A (TSA), decitabine (DAC) and vincristine (VCR) by Flow cytometry. Farage cells were cultured in the absence (control) and in the presence of TSA, DAC and VCR in monotherapy at single dose administration and in combination regimens during 48 hours (**A**); and in the presence of TSA and DAC in a daily posology regimen during 72 hours of incubation (B). Results are expressed in mean intensity of fluorescence percentage (MIF) and represent the mean±SD of three independent experiments. (DA) – Daily drug dose; CLT – Control; TSA – Trichostatin A; DAC – Decitabine; VCR – Vincristine.

Discussion and conclusion

Two of the most extensively studied epigenetic mechanisms are DNA methylation/demethylation and histone tail modifications (24,32,33). DNA methylation occurs at cytosines (5-methyl-C; 5mC) of CpG islands (DNA sequences positioned in gene promoter regions rich in cytosine and guanine), in a reaction catalyzed by a group of enzymes denominated DNA methyltransferases (DNMTs: DNMT1, 3A, 3B and DNMT3L) (23,25). In general, hypermethylation of CpG islands in gene promoter regions (frequently found in tumor cells) is associated with gene silencing (21,22,24,34). The tumor suppressor gene *pl6* is already known to be hypermethylated in a 5' CpG island in many cancer types, with consequent lack of transcription and complete loss of gene expression (35). On the other hand, *p15* as *p16*, is an inhibitor of cyclin-dependent kinase which is aberrantly methylated in many leukemic cell lines and primary leukemias (35). Thus, *p15* and *p16* gene hypermethylation leads to cell cycle deregulation (36). Hypermethylation of other tumor suppressor genes, such as *p53* leading to silenced gene transcription is also observed in certain cancers. In addition, hypomethylation of growth regulatory genes leads to transcriptional activation, and thus uncontrolled cellular proliferation (22). However, it is important to note that these phenomena also take part in normal cell cycle: long-term silencing of genes is seen on X-linked inactivation, imprinted genes and germ-cell specific genes (23).

Moreover, transcriptional activation can be achieved through histones methylation in some lysine and arginine residues, as histone 3 methylation (H3) at lysine 4 (K4) (37). Histones are a highly conserved group of alkaline proteins found in eukaryotic cells' nuclei that constitute the main protein component of chromatin (21,37). They are categorized into five classes (H1/H5, H2A, H2B, H3, and H4), and in two main groups: core histones (H2A, H2B, H3, H4) and linker histones (H1 and H5) (21,37). Histones form units with DNA designated as nucleosomes, each consisting of an octamer of four pairs of core histones and approximately 146 base pairs of DNA wrapped around (37), whereas histone H1, acting as their linker, binding nucleosomes together and so maintaining the structure organized (21). N-terminal domains of core histones contain many basic amino acids positively charged able to interact with DNA structure (37). Dynamic structural histones undergo several posttranslational (epigenetic) modifications, such as methylation/demethylation, acetylation/deacetylation, phosphorylation, sumoylation, citrullination, and ubiquitination, underlying 'histone code' hypothesis (21,24,37). Acetylation can either increase or decrease the function or stability of the proteins or protein–protein interaction (37). Usually, acetylation of NH_2-terminal lysine residues of core histones (especially H3 and H4) by histone acetyl transferases (HATs), neutralizes the positive charged proteins, weakening their interactions with negative-

charged DNA structure; as a consequence, chromatin unfolds, allowing the binding of transcription factors and gene transcription (24,25,37,38). The opposite process, catalyzed by the group of enzymes histone deacetylases (HDACs) (32) is generally linked with chromatin compaction and genes inactivation (22,24,39). Hematological neoplasms show frequently increased activity of HDACs with repression of essential genes for hematopoietic differentiation, cell cycle and apoptosis regulation (37). Loss of lysine acetylation, rather than the increase in histone methylation, has been identified as the first step in gene silencing (24).

In contrast to gene mutations, mostly permanent, epigenetic aberrations are potentially reversible, allowing the malignant cell population to revert to a more normal state (23), and as a result became an appetizing therapeutic target (15,24,37,40), like HDACs inhibitors (HDACis) and methyltransferase inhibitors. HDACis result in restoration of relevant gene expression since these drugs interact with the catalytic domain of HDACs, in order to block their substrate recognition ability and increasing DNA-histone distance, which favors the binding of transcription factors complexes to DNA (37), like cyclin-dependent kinase inhibitor p21 Waf1/Cip1, but also may result in repression of others (38).

Comparatively to the total genome, HDACis modify only approximately 2-10% of transformed cells' expressed genes (20). Besides that, HDACis also deacetylate other non-histone proteins, like transcription factors (22). HDACis cause the induction of differentiation of cultured cells, as well as growth arrest and/or apoptosis of both cultured cells and tumor animal models, and also down regulate angiogenic genes (19,21,37,41). There are four classes of HDACis, according to their chemical properties: short-chain fatty acids, hydroxamic acids, cyclic peptides, and benzamides (21,37). Trichostatin A (TSA), derived from *Streptomyces* sp., was initially employed as an antifungal antibiotic (24). It became the first natural compound found to be a potent and specific inhibitor of HDAC (21,37,38), classified today as hydroxamate (20,37). TSA is a noncompetitive inhibitor of HDAC activity in cultured mammalian cells and in fractionated cell nuclear extracts at low nanomolar concentrations (38), inhibiting mainly two of the HDACs classes (class I and II) that are dependent on zinc on its catalytic site (42). Its pharmacological profile includes cells arrest in G_1 and G_2 phases of the cell cycle, induction of differentiation, and reversion of transformed morphology of cultured cells (38). More precisely, TSA depletes kinethocore proteins levels and decreases phosphorylation of histone H3 in pericentrometric chromatin during G_2 phase (37). The flip side is that TSA is of limited therapeutic use when administered as single agent, due to side effects, poor bioavailability *in vivo* and poor stability (40). Moreover, in lymphoid cell lines TSA affected merely 2% of 340 expressed genes (20). Additionally, rather than dispensing HDACis in short expositions and at lower doses, some authors said that is preferable to give drugs in consistent

doses using extended administrations (37).

Decitabine (DAC) or 5-aza-2'-deoxycytydine is a nucleoside analogue of 2'-deoxycytidine (43). It acts after phosphorylation and direct incorporation into DNA and inhibition of DNA methyltransferase, resulting in DNA hypomethylation, intra-S-phase arrest of DNA replication, and therefore cellular differentiation or apoptosis (43,44). It is already being used in the treatment of Myelodysplastic Syndromes (34), after being found out it also permits re-expression of silenced genes, induction of cell differentiation, and thus restraining tumor cell growth (23). However the therapeutic effect in DLBCL is not well clarified.

In this study, we observed that the hypomethylating agent/DNMTi DAC and the HDACi TSA were both effective in reducing Farage cell density and proliferation, a model *in vitro* culture of DLBCL. We also observed that these agents were more powerful in daily low-doses than higher single doses, which could be informative to translate into clinical practice. In fact, with daily administration of 10 nM TSA or DAC 1 μM, and using a lower total dose, we observed a higher therapeutic effect than with drugs administered in single administration using higher drug concentration (TSA 50 nM, DAC 5 μM) (Figure 2). These observations applied to medical practice could be relevant as with lower dose concentration we could reduce side effects and secondary toxicity. So, everyday posology regimens seem to be preferable when drugs are used in monotherapy. Yet, it is important to recall that epigenetic changes are reversible, so do are changes induced by these drugs, an effect especially noticeable in single posology, with exception of TSA 10 nM, TSA 50 nM and DAC 1μM concentrations (Figure 1).

One of the major issues in epigenetic therapy is whether hypomethylating treatment should be lifetime, or whether it can be interrupted once response has been achieved. At present, tendency is towards continuous maintenance treatment (45). Still, the problem of lack of specificity sometimes found in some DNMTis and HDACis (and the consequent adverse side effects) remains, since occur other cellular mechanisms than epigenetics modulation in tumor suppression arrest and differentiation (40,41). Moreover, the daily administration regimes may reduce the adverse side effects obverse with these therapies, as refereed.

In agreement with our previous studies, DAC in monotherapy and in single dose does not reduce cell proliferation below 50% in none of the tested conditions, which might be due, as we proposed, to its short half-life in culture (8 to 12 hours) (46). Furthermore, when administered in low concentrations, the capacity to induce cell death is slightly. So, remaining cells are expected to regenerate the population over time, and cell proliferation increases. These results are in agreement with our previous work in ALL (47) where, even in higher concentrations such as 25 μM, DAC doesn't induce a significant decrease in cell proliferation.

Our dose-response curves are in harmony with those obtained in other research groups elsewhere, in the same hematopoietic neoplasms (8, 18, 20), and other human cell lines and tumors – for example, breast cancer (38). We noticed once more that TSA in monotherapy was more effective than DAC (46,47).

As it was described by Nair *et al*. (2001) in Hela cells, TSA shows unexpected paradoxical and contradictory effects related to inhibition of nuclear transcription factor Y subunit alpha (NF-Y)-associated HAT activity, a protein involved in a trimeric complex of highly conserved transcription factor, and phosphorylation of the HAT hGCN5. The ability of inducing apoptosis in a significant number of cells is in general higher for TSA, although differences in relation to DAC here were minimal. In fact, for daily drug administration, we verified that DAC had a more prominent role, which might mean that demethylation of some genes in Farage cells is at least as important as chromatin remodeling that occurs with this particular type of HDACi, so, both mechanisms cooperate in the process of restoration of gene functioning.

It is reported that HDACis like TSA potentiate DAC activity; hence these drugs have been associated in basic investigation using cell lines. Following other studies, including previous work in our lab, which noticed a synergistic effect of TSA plus DAC (47), we evaluated the therapeutic potential of the combination of TSA with DAC on Farage cells, not only simultaneously, but also out of phase. Our results matched what was already reported in the literature for other cell lines and/or other epidrugs associations: there is indeed a synergistic effect between the two drugs (potentiation). In fact, when joined together TSA 10 nM plus DAC 1 μM, we observed cytostatic and cytotoxic cumulative effects. Since any of the drugs alone never reached IC_{50}, this suggests the need of a combination therapy in order to obtain a therapeutic effect. Besides that, in this combination therapy regime, we observed that DAC is less effective if administered before TSA. This is in agreement with our previous studies in Acute Lymphoblastic Leukemia (ALL) (47), and Chronic Lymphocytic Leukemia (CLL-B) cell lines (46). This finding was also reported by Shaker *et al*. (2003), in HL-60 and KG1a cells (Acute Myeloid Leukemia cell lines) (48). We hypothesized back then this could be due to inability of hypomethylating agents to reach methylated DNA sequences that were trapped in coiled segments of chromatin (47).

The beneficial effect of synergism was noticed in other types of neoplasms as well. In cultured colorectal carcinoma cells, four hypermethylated genes were solidly transcriptionally reactivated after a combination of TSA plus a low dose of DAC, but not in the presence of TSA alone (37). Furthermore, Tambaro *et al.* (2010) reported that in endometrial cancer cells, TSA seemed also to decrease the stability of DNMT3b mRNA, resulting in decrease of *de novo* methylation (37). If, on one hand, the HDACis like TSA allow the chromatin to relax and so open,

exposing previously unattainable sequences, on the other hand, DNMTis such as DAC may restore part of gene-expression of genome guardians, by removing repressive methyl groups (47). Therefore, they complete each other, bringing the advantages of improved response, reduced toxicity and overcoming drug resistance therapy (24,37).

In addition, it was also shown that HDACis appear not only to synergize with other epigenetic drug classes, but also with other antineoplastic therapies, like radiation, chemotherapeutic drugs such as docetaxel (antitubulin agent) doxorubicin, etoposide, ellipticine (topoisomerase II inhibitors); cisplatin (DNA cross-linking reagent), and even new targeted agents (37). Nonetheless, there may be antagonistic effects too while making these associations ineffective or even prejudicial (40). This was the reason that motivated us to include VCR on our study, and to associate this drug individually with TSA or DAC, and with both of them simultaneously. Vincristine, a vinca alkaloid, is part of R-CHOP regimen, as above mentioned. In summary, this antimitotic and antineoplastic substance binds irreversibly to microtubules and spindle proteins in S phase of the cell cycle, interfering with the formation of the mitotic spindle, thereby arresting tumor cells in metaphase. This agent also depolymerizes microtubules and may also interfere with amino acid, cyclic AMP and glutathione metabolism, calmodulin-dependent Ca^{2+}-activated ATPase activity, cellular respiration, and nucleic acid and lipid biosynthesis (49).

Neither TSA 10 nM or VCR 0,1 nM administered in monotherapy and single administration were very efficient in the reduction of Farage cells proliferation. Since the rate of death of TSA 10 nM plus VCR 0,1 nM was superior to the sum of each one alone, we verified a synergistic effect in this therapeutic combination. The association of DAC 1 µM plus VCR 0,1 nM induced a major rate of cell death too, superior than the sum of each drug in monotherapy, therefore suggesting also a synergistic effect. Since there was a highly synergistic effect when standard-chemoterapy drug VCR was added to TSA and DAC, with an especially dramatic effect particularly noticed when all the three drugs were all added and combined, this may indicate the possibility of adding in the future TSA and/or DAC to first line therapy, as an adjuvant, or as a rescue therapy in non-responsive patients, if further studies confirm our results. Again, association therapy may be beneficial because directing therapy to different cellular targets (two directly involved with DNA structure and functionality, and one interfering with microtubules assembling and thus inhibiting cell division) may warrant its success, with the advantages above numerated.

Besides the antiproliferative effect observed, the epidrugs used also induced cell death mainly by apoptosis which is caspases dependent. A statistically significant decrease in cell proliferation was observed in cells treat with daily DAC 1 µM ($p<0.001$) in relation to control cells,

as well as when the drug is added to TSA 10 nM. These results mean that daily addition of DAC 1 μM has also cytotoxic effects, having the ability to induce apoptosis in a significant number of cells ($p<0.001$), comparatively to control cells. In the same way, we observed a reduction statistically significant of cell proliferation and induction of cell death by apoptosis when cells were treated with TSA 750 nM, DAC 25 μM, TSA 10 nM plus VCR 0,1 nM, DAC 1 μM plus VCR 0,1 nM, TSA 10 nM plus DAC 1 μM, TSA 10 nM plus DAC 1 μM plus VCR 0,1 nM ($p<0.001$). In these conditions, we observed an increase tendency of activated caspase levels, in relation to control, however there wasn't a statistically significant result.

In summary, and accordingly to what we found out, daily low-doses combination schedules using TSA and DAC would be preferable to single drug administrations. Even though it was already demonstrated that TSA (38) is reliably safe in low-doses, and DAC in low-doses has an acceptable pattern of toxicity (45,50), their use in controlled conditions would overwhelm problems such as renal toxicity TSA-induced as refereed by *Dong et al.* (2008) (51) or myelotoxicity DAC-induced, especially neutropenia as mentioned by others (45,50). *Dong et al.* (2008) shown that at micromolar concentrations, TSA induced approximately 60% apoptosis in a renal proximal tubular cell line, perhaps due to its pan-HDACi effects (51). Furthermore, TSA toxicity may be related to protein acetylation and decrease of antiapoptotic proteins including BCL-2 and BCL-XL (51). Although our results are encouraging, further studies are needed, in order to clarify the real role-played by these epidrugs on cell machinery. Future experiments may clarify through molecular analysis and methylation studies whether DAC induces a cytotoxic effect on Farage cells, by directly incorporating into DNA CpG sites and binding DNMT, sequestering its activity or contributing to up-regulation of DNA repair genes and crucial tumor suppressor genes in DLBCL such as BCL-6, p53, HSP, and nuclear factor-B, that would consequently lead to the production of proteins or metabolites toxic to cells (8,52). However, more detailed studies related to molecular mechanisms involved in the apoptotic pathway induced by these drugs would also be of interest.

This study suggests that epigenetic modulation might be a new approach to the treatment of lymphomas, namely DLBCL, as in monotherapy as in combination with conventional chemotherapy.

Acknowledgments

This project was supported by Center of Investigation in Environment, Genetics and Oncobiology (CIMAGO).

Lídia Costa thanks frankly all the team support, her family and Marina for her friendship.

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