

## CD26/DPPIV expression and 8-azaguanine response in T-acute lymphoblastic leukaemia cell lines in culture

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### Abstract

Dipeptidyl peptidase IV, a cell membrane surface protease also known as CD26 (CD26/DPPIV), is known to play multiple functions in human organism, where it is largely expressed, for instance, in the development of human cancer and metastasis as well as in chemotherapy response.

The objective of this work was to study the CD26 membrane expression and DPPIV activity in T-acute leukaemia cell lines (CEM and MOLT3) in culture, in order to observe the modification of its expression under the 8-azaguanine treatment.

Cell line samples were incubated, some without different azaguanine concentration and others with, ranging from 10 to 100  $\mu$ M. Cell surface CD26 expression has been identified by flow cytometry and DPPIV activity, in cultured medium, was fluorimetrically measured.

Results we have observed showed that 8-azaguanine induced a decrease in cell viability in a dose, time and cell type dependent manner with MOLT3 cells being the most sensitive to 8-azaguanine cytotoxic effects (24 h IC50:  $\pm 10 \mu$ M) when compared with CEM cells (24 h IC50:  $\pm 100 \mu$ M).

In the same experimental conditions, MOLT3 cell treated with 8-azaguanine shows an increase in CD26 expression (MIF) compared with that of CEM cell submitted to the same conditions ( $65.4 \pm 1.3$  versus  $18.7 \pm 1.7$ ). DPPIV activity in culture medium supernatant of CEM versus MOLT3 controls cells ( $1.91 \pm 0.43$  versus  $2.06 \pm 0.50$ ) and of CEM versus MOLT3 treated cells ( $2.10 \pm 0.16$  versus  $1.89 \pm 0.04$ ) did not show a significant difference.

These preliminary results suggest that 8-azaguanine stimulates CD26 expression which may be related to cellular sensitivity to 8-azaguanine. © 2006 Elsevier Ireland Ltd. All rights reserved.

**Keywords:** CD26/DPPIV; ATLL; Chemotherapy response

### 1. Introduction

Cell surface-associated peptidases play important roles in human organism where they are largely distributed. They are

involved in many physiological processes ranging from morphogenesis and tissue differentiation to the general regulation of haemostasis. Some of these enzymes also appear to be functionally involved in cell activation and proliferation as well as in cancer cell growth and metastasis [1,2].

Among the membrane-associated peptidases is the serine ectopeptidase dipeptidyl peptidase IV (DPPIV, EC 3.4.14.5) also known as the surface antigen CD26, a T lymphocyte activation marker [3].

Since its first description, by Hopsu-Havu and Glenner in 1966, it has been considered as a unique peptidase that

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cleaves dipeptides from the NH<sub>2</sub>-terminus of proteins having proline, hydroxyproline or alanine residues at the penultimate position, with the highest efficiency observed with proline residues [4,5].

CD26 is constitutively expressed in a variety of different tissues, e.g. intestine epithelial cells and kidney proximal tubules [6,7,8]. DPPiV is involved in the mechanisms of membrane protein turnover, glycosylation events, membrane polarization, in cell adhesion events and in immune response [9,10].

Depending on its cellular localization and cell type expressing CD26/DPPiV, it is a multifunctional molecule that interferes with many immune functions, both in vitro and in vivo. In addition to its membrane-associated form, CD26/DPPiV is also present as a soluble exopeptidase in various body fluids, such as plasma, serum and urine [4,11–15].

Independently of its peptidase activity, CD26/DPPiV is associated to other molecules on the cell surface such as adenosine deaminase (ADA) and CD45. It has been shown to be physically associated with ADA in the T-lymphocytes surface, thus protecting the cell from adenosine-mediated inhibition of proliferation. It is also associated with CD45, a cell surface phosphotyrosine phosphatase, involved in signal transduction. In addition, it has also been proposed that CD26 is involved in the pathophysiology of the acquired immune deficiency syndrome (AIDS) [11–13,16–22].

Both CD26/DPPiV features, as a membrane antigen and as a peptidase, contribute to the co-stimulator function of CD26 in T-cell activation and apoptosis events. However, the CD26/DPPiV role in these events is still unclear [13,23,24].

CD26/DPPiV is involved in the control of cell growth and differentiation by controlling the access of mitogenic peptides to their receptors on the cell membrane. Abnormalities in its expression pattern could result in altered peptide activity, which may contribute to neoplastic transformation, cancer progression and therapeutic response [25,26].

The potential contribution of CD26/DPPiV in pathophysiological processes of cancer development, namely haematological cancer, has been studied. Some studies demonstrated that it is an enzyme marker of diagnostic value in the lymphoproliferative diseases of T-cell origin [27–29]. There is a lack of information regarding the relationship between the CD26/DPPiV expression and complete phenotypic features of T-cells acute leukaemia. Although some studies reported that CD26 was mostly expressed on CD30 anaplastic large cell lymphoma (ALCL), and mainly in T-cell acute lymphoblastic leukaemia (T-cell ALL), non-Hodgkin's B cell lymphomas did not express CD26.

The exact picture of all biological functions of CD26/DPPiV is difficult to know. For example, in therapeutic strategies, one knows relatively little about the relationship of CD26/DPPiV expression and chemotherapy response.

The objective of conventional cancer chemotherapy is the destruction of cancer cells by disrupting its normal

structure. With this purpose, the nucleoside analogues have been used in therapeutic protocols of ALL. 8-Azaguanine is a purine analogue that shows antineoplastic activity. It functions as an antimetabolite and easily incorporates into ribonucleic acids, interfering with normal biosynthetic pathways, thus inhibiting cellular growth. However, the development of chemotherapy resistance is an obstacle to successful leukaemia chemotherapy; therefore, identification of new potential targets for cancer therapy is essential.

### 1.1. Objectives

CD26/DPPiV has established relevance as a modulator of cell activity. Little is known about its role in chemotherapy response. The aim of this work was to determine both the expression of CD26 on cell surface of acute leukaemia cell lines in culture (CEM and MOLT3), and the DPPiV activity (in culture medium supernatant, collected from cellular cultures) after 8-azaguanine treatment.

## 2. Materials and methods

### 2.1. Cell culture

The CEM-CCRF and MOLT3 cells (acute lymphoblastic leukaemia T cells) were supplied by American type collection (ATCC), Rockville, USA. They were maintained in culture in RPMI-1640 medium (L-glutamine 2 mM, HEPES-Na 20 mM, NaHCO<sub>3</sub> 2 g/L, penicillin 100 U/mL and streptomycin 100 µg/mL, pH 7.4) (Sigma®), supplemented with 10% foetal calf serum (FCS, GIBCO®) in 5% CO<sub>2</sub> atmosphere, at 37 °C temperature. Cells were incubated in the absence and in the presence of increasing 8-azaguanine (Sigma®) concentrations, ranging from 25 to 200 µM. Culture medium supernatant and cell samples were collected to perform the assays/analysis.

### 2.2. Proliferation, viability and cell morphology

After 24, 48 and 72 h of incubation time, cells were harvested and assessed for proliferation, viability and cell morphology. The mentioned analysis was performed in triplicate. A cell preparation without 8-azaguanine treatment was used as control.

Cell proliferation was accessed by counting in a Neubauer chamber; cell viability was estimated by trypan blue exclusion; and, cell morphology was evaluated by light microscopic examination of May-Grünwald-Giemsa stained cells, using a microscope Leitz Dialux 20 fitted with a photographic chamber.

### 2.3. Flow cytometry analysis

Monoclonal antibodies directly conjugated were used with one of the following fluorochromes: fluoresceine

isothiocyanate (FITC), phycoerythrin (PE). Flow cytometry was performed using a FACSCalibur™ from Becton Dickinson®, collecting 10,000 events by acquisition.

Detection of apoptosis and necrosis was analysed by Annexin V (AV-FITC) and propidium iodide (PI) staining. After incubation in the absence or in the presence of 8-azaguanine, cells were washed (centrifuged at  $300 \times g$  during 5 min) and incubated for 10 min at  $4^\circ\text{C}$  in  $440 \mu\text{L}$  Annexin buffer, from Immunotech®, containing  $5 \mu\text{L}$  AV-FITC and  $5 \mu\text{L}$  PI. Then cells were washed and re-suspended in phosphate buffered saline (PBS) until analysis.

For CD26 analysis,  $1 \times 10^6$  cells (incubated in the previously referred conditions) were centrifuged and incubated for 10 min at room temperature, with  $1 \mu\text{g}$  anti-CD26 (PE) from Immune Source®. After that, cells were washed with PBS, by centrifugation at  $300 \times g$  during 5 min and analysed on FacsCalibur™ from Becton Dickinson, collecting 10,000 events by acquisition, in order to assess the percentages of the total number cells expressing CD26 and the number of molecules of CD26 expressed by each cell mean fluorescence intensity (MFI). This value represents the medium fluorescence intensity detected in the cells, which is proportional to the number of molecules labelled by the monoclonal antibody. Negative controls were established with isotype immunoglobulin G (Ig G), IgG1 and IgG2b, submitted to the same procedures.

#### 2.4. Dipeptidyl peptidase IV (DPPIV) activity

DPPIV activity in culture medium supernatant was measured by a fluorimetric assay as previously described by Sharpé et al. [30].

DPPIV catalyses the cleavage of the fluorogenic substrate Gly-Pro-4-Me-2-NA, Sigma–Aldrich Co. (St. Louis, 63178-MO, USA), releasing a highly fluorescent molecule: 4-Me-2-NA. For the substrate solution, 20 mmol/L, 7.37 mg of substrate was dissolved in 1 mL of DMSO. This solution was stored at  $4^\circ\text{C}$ .

Standard solution, 4-Me-2-NA was acquired from Bachem Feinchemikalien AG (Budendorf-Switzerland). The stock solution was 50 mmol/L 4-Me-2-NA in DMSO. Before use, it was dissolved as required with stopping solution.

Incubation buffer is a 50 mmol/L Tris–HCL solution, pH 8.3 adjusted at room temperature, stored at  $4^\circ\text{C}$ . Stopping solution is a 100 mmol/L citrate solution, pH 4.0, adjusted at room temperature and stored in the refrigerator at  $4^\circ\text{C}$ .

The assays' fluorescence intensity was measured with a JASCO FP-777 spectrofluorimeter, with a quartz cell, at 340 nm of excitation (Ex) and at 425 nm of the emission (Em) wavelengths.

DPPIV activity has been expressed in units/litre (U/L). One unit (U) of DPPIV activity was defined as the enzyme activity that produces  $1 \mu\text{mol}$  of 4-Me-2-NA in 1 min under the reaction conditions.

#### 2.5. Statistical analysis

Student's *t*-test was used to determine the statistical significance, considering a *p*-value of  $\leq 0.05$  significance.

### 3. Results

In order to analyse the effects of the chemotherapeutic agent 8-azaguanine in cell viability, different specimens of CEM and MOLT3 cells were incubated in the absence and in the presence of various 8-azaguanine concentration (ranging from 10 to  $100 \mu\text{M}$ ). Data represent the mean  $\pm$  S.D. of three to six separate experiments. The results show that cell viability decrease after incubation with 8-azaguanine, in a dose and time dependent manner, as represented in Fig. 1A, for CEM cell line, and Fig. 1B, for MOLT3 cell line. However, MOLT3 cells seem to be more sensitive to 8-azaguanine than CEM cells, since in the former, we observe the same antiproliferative effect with lower 8-azaguanine concentrations. Actually after 24 h of treatment with 8-azaguanine the IC50 for CEM cell line is about  $100 \mu\text{M}$  while for MOLT3 cell line the IC50 is about  $10 \mu\text{M}$ , as it can be observed in Fig. 1A and B.

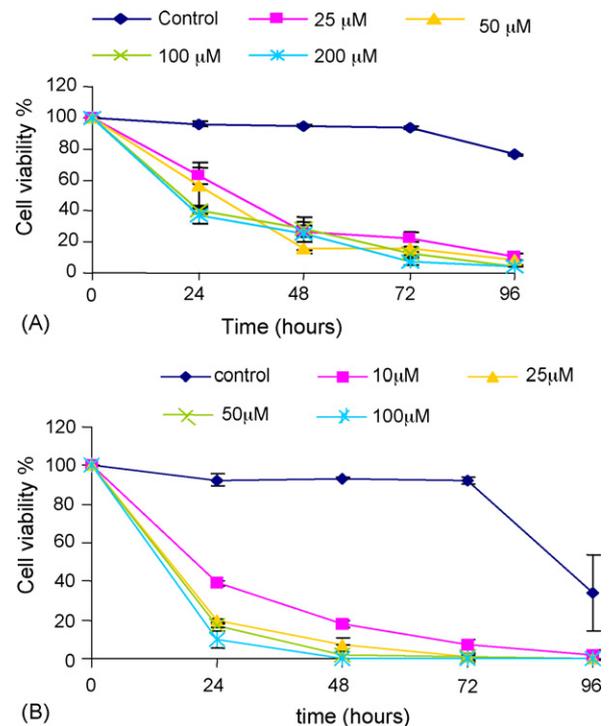


Fig. 1. Dose response curves: CEM (A) and MOLT3 (B) cell viability curves. The cell lines were cultured at  $37^\circ\text{C}$  in RPMI 1640 medium, and incubated in an initial concentration of  $0.3 \times 10^6$  cells/mL for CEM cells, and of  $0.6 \times 10^6$  cells/mL for MOLT3 cells. Cells were incubated in the absence and in the presence of different 8-azaguanine concentrations, as indicated in the figure: 25, 50, 100 and  $200 \mu\text{M}$  for CEM and 10, 25, 50 and  $100 \mu\text{M}$  for MOLT3, during 24, 48, 72 and 96 h. Cell viability was determined by trypan blue exclusion each 24 h. Both cell lines demonstrate a significant decrease of cell viability, after azaguanine-incubation. Data represent the means  $\pm$  S.D. of three to six separate experiments.

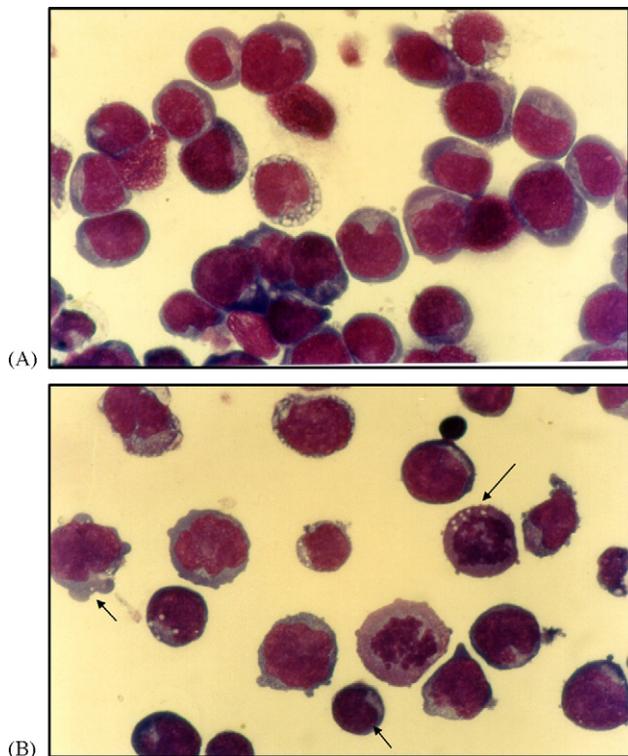


Fig. 2. Cellular morphology analysis of May-Grünwald-Giemsa stained cells (500 $\times$ ), before (A) and after (B) 8-azaguanine treatment. The figure represents the CEM cell line as an example of what it was done in the study. (A) shows the normal morphology of human leukaemia T-cell line; (B) shows morphological changes after 8-azaguanine treatment. Morphological changes are related to apoptotic cell death, namely cellular shrinking, membrane blebs, DNA fragmentation, indicated by the arrows.

In Fig. 1A, it is possible to observe that CEM cell viability, along the time of incubation with different 8-azaguanine concentrations, decreases to a value inferior to 20%. Fig. 1B represents the results of MOLT3 cells treated with 8-azaguanine, in different concentrations and different time of incubation. A decrease in cell viability (about 20%) was also observed. In both cell lines, the observed effects are time and dose dependent.

The observed decrease of cell viability is related to an increase in cell death. Fig. 2A shows the morphological characteristics of control cells. Fig. 2B shows the morphological characteristics of incubated cells with 8-azaguanine demonstrating apoptotic cell death, namely cellular shrinking, membrane blebs and DNA fragmentation (indicated by the arrows). The morphological changes observed in microscopic slides are in agreement with those observed by flow cytometry analysis using AV/PI staining as they are represented in Fig. 3. Fig. 3A, which represents the control cells, shows that almost all cells are alive. In Fig. 3B, which represents cells after 8-azaguanine treatment, an increase in the percentage of cells in early stages of apoptosis and in later apoptotic/necrosis cell death was observed. These results are time, dose and cell type dependent.

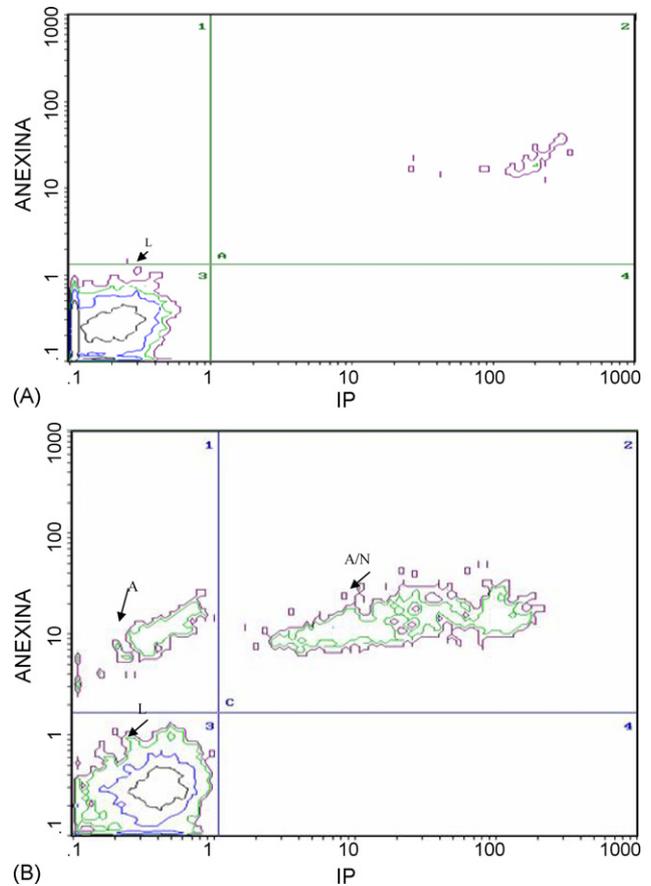


Fig. 3. Evaluation of cellular death by flow cytometry. Cell lines were incubated in the absence (control, A) and in the presence (B) of 8-azaguanine. Cell death was detected by Annexin V/propidium iodide (AV/IP) staining and analysed by flow cytometry. Live cells (L), are Annexin V/propidium iodide (AV/IP) negative, as indicated by the arrow, in control conditions. Early stage of apoptosis are Annexin V (AV) positive (A), and cells in late stage of apoptosis or in necrosis are AV/IP positive (A/N).

CEM and MOLT3 cells surface expression of CD26, Fig. 4, increases after treatment with 8-azaguanine in a concentrations dependent manner. However as we can observe, MOLT3 cells have a higher basal surface CD26 expression (MFI) compared with that observed in CEM cells ( $18.7 \pm 1.7$  versus  $15.1 \pm 2.6$ ), submitted to the same experimental conditions, although without significant difference. After MOLT3 8-azaguanine treatment the expression of CD26 shows a significant augmentation ( $18.7 \pm 1.7$  versus  $65.4 \pm 1.4$ ).

In order to confirm how the mentioned results are due or not to the 8-azaguanine effect, we have incubated cells with another agent, the oxidative stress inducer hydrogen peroxide in various concentrations (ranging from 10 to 200  $\mu\text{M}$ ) (data not shown), during 24 and 48 h and, after that, the expression of CD26 was analysed in cells submitted to 25  $\mu\text{M}$ . No significant change in CD26 cellular expression was observed under these new experimental conditions (Table 1).

The DPPiV activity measured in cell culture medium collected from the wells, where CEM and MOLT3 cells were incubated in the different conditions of the study, did not

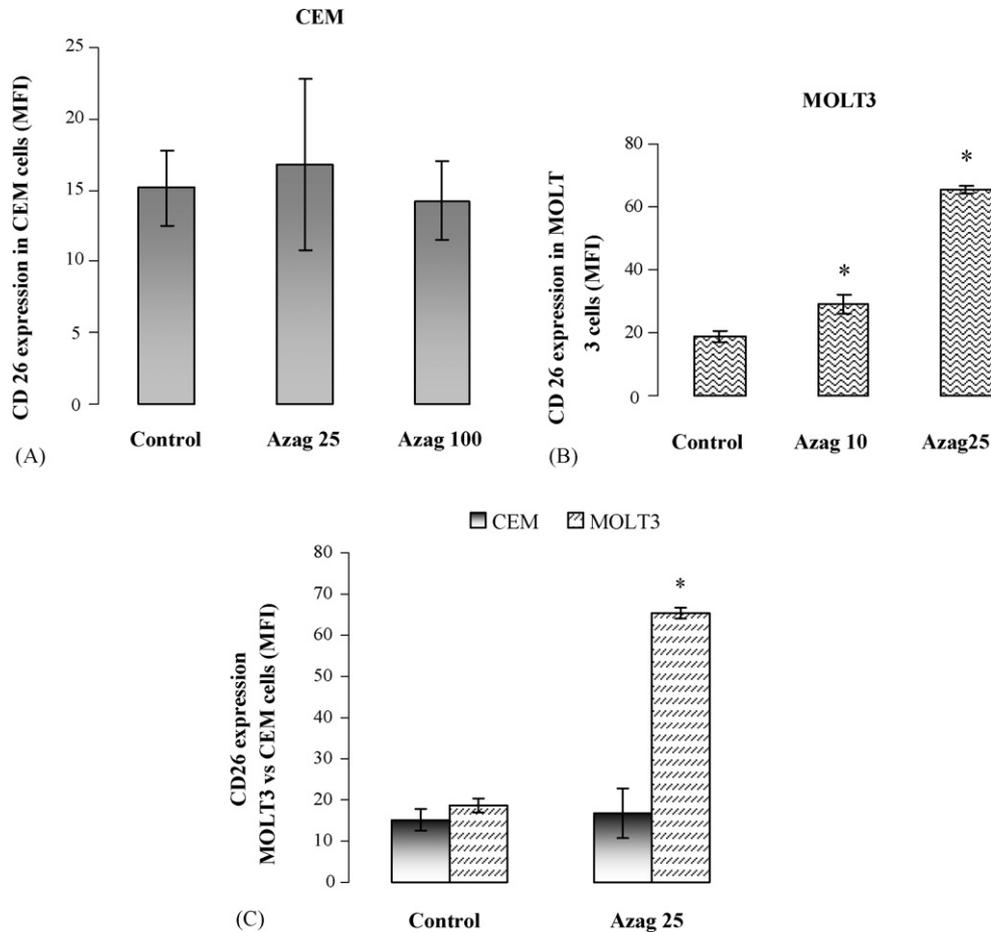


Fig. 4. Cell surface CD26 expression in human leukaemia cell lines CEM and MOLT3, before and after 48 h of 8-azaguanine treatment. Results are expressed in mean fluorescence intensities (MFI). This value represents the medium fluorescence intensity detected in each cell, which is proportional to the number of molecules labelled by the monoclonal antibody. As one can see, the expression of CD26 increased after treatment in a dose and cell type dependent manner. For the same dose (25  $\mu$ M) and incubation time (48 h) MOLT3 cells show a significant increase in CD26 expression, compared with CEM cells, which may be related with a higher cellular sensitivity of MOLT3 cell to 8-azaguanine. \* $p < 0.05$ . (A) represents cell surface CD26 expression in CEM cell line. (B) represents cell surface CD26 expression in MOLT3 cell line. (C) represents cell surface CD26 expression in CEM cells compared with MOLT3 cell line.

Table 1  
CEM and MOLT3 cells CD26 expression in control condition and after 8-azaguanine (25  $\mu$ M) and hydrogen peroxide (25  $\mu$ M) treatment

	Control	Azag25	H <sub>2</sub> O <sub>2</sub> 25
CEM	15.1 $\pm$ 2.6	16.8 $\pm$ 6.0	14.1 $\pm$ 1.6
MOLT3	18.7 $\pm$ 1.7	65.4 $\pm$ 13	17.7 $\pm$ 1.3

The results are expressed in mean fluorescence intensity (MFI) and represent the means  $\pm$  S.D. of three separate experiments.

show any significant difference in our experimental conditions, as we can observe in Fig. 5.

#### 4. Discussion

CD26/DPPIV is a broadly distributed molecule with several functions in human cell physiology. It is constitutively expressed in a variety of different human cell types and tissues, T-lymphocytes, epithelial cells of the intestine, prostate

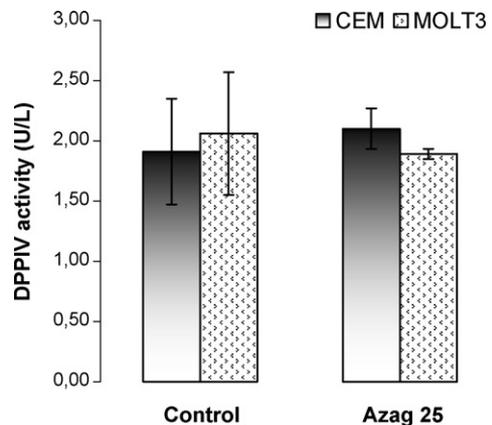


Fig. 5. DPPIV activity (U/L) in culture medium supernatant of CEM and MOLT3 cells cultured under control conditions and after 8-azaguanine treated cells. No significant differences are seen.

gland and kidney proximal tubules. Its enzymatic activity is detectable in serum, urine, seminal plasma, synovial fluid and amniotic fluid. However, the origin of soluble DPPIV is not completely understood [1,3,31,32].

Due to the presence of the soluble form of CD26/DPPIV in serum, any peptide circulating in the blood carrying a proline residue in the penultimate N-terminal position is a candidate substrate to its biological enzymatic activity. For instance, degradation by DPPIV represents a rate-limiting step for the intestinal and renal transport of proline-containing peptides [13,18].

It has been demonstrated that CD26/DPPIV plays an essential role in immune regulation, particularly through its involvement in T lymphocyte activation, being described as a co-stimulatory molecule present in T lymphocyte surface, its expression being markedly enhanced following T lymphocyte activation [28]. It has also been identified as the binding protein for ADA, an enzyme active in T lymphocyte that regulates the levels of adenosine (Ado) and d-Ado by catalyzing their irreversible hydrolytic deamination, which further adds to the importance of this membrane protein in T lymphocyte protection and activation [16,28,33,34].

CD26/DPPIV also appears to play a role in the development or biological behaviour of selected human cancer [2,35,36]. Its potential contribution to the development of human haematological malignancies has been investigated [3,27,28,37–39], but its exact role in cancer biology remains to be elucidated.

Preliminary results suggest that CD26 may contribute to the biology of selected T-cell malignancies influencing the growth of tumour cell lines through its DPPIV activity. CD26 expression has been described to be restricted to those pathological entities characterized by an aggressive clinical evolution such as T-cell acute lymphoblastic leukaemia (T-ALL) and T-cell CD30 positive anaplastic large-cell lymphoma suggesting a potential role for CD26 in the pathogenesis of these malignancies [28,37,39].

T-cell lymphoid malignancies represent a heterogeneous group of diseases that are generally aggressive and often resistant to current treatment modalities [28]. In fact, chemotherapy resistance is an impediment to the cancer therapies' success.

In view of the multi-functional roles of CD26/DPPIV in human body physiology, it is our hypothesis that CD26/DPPIV may be an appropriate target for novel treatment modalities for T lymphocyte leukaemia. Sato and Dang [28] investigated the role of CD26/DPPIV expression in cellular sensitivity to doxorubicin of human T leukaemia cell line Jurkat and concluded that the CD26, especially its DPPIV enzymatic activity, results in enhanced cellular sensitivity to DNA damage by doxorubicin. Authors concluded that it implies a potential role in the clinical setting for CD26/DPPIV in the treatment of haematological malignancies [28,38].

Our objective with this work was to contribute to the clarification of whether the expression of DPPIV/CD26

in leukaemia cell line in culture change, or not, after 8-azaguanine treatment and what changes could we observed.

Present results showed that 8-azaguanine treatment reduce cell viability in a time and dose dependent manner (Fig. 1). Morphological cellular changes consistent with apoptosis was also observed. This cellular programmed cell death was confirmed by flow cytometry studies with AV/IP (Fig. 3).

Indeed the observed CEM cells morphological changes (Fig. 2) are related to apoptosis, as well as in MOLT3 cells (data not shown), namely cellular shrinking, cytoplasm vacuolation and blebbing. Flow cytometry analysis of cell death demonstrated that the number of cells in early and in late stages of apoptosis or in necrosis increase when incubated with 8-azaguanine in a time and dose dependent manner. CEM and MOLT3 cells are acute leukaemia cell lines, nevertheless MOLT3 reveals much more sensitivity to 8-azaguanine effect than CEM. This result suggests that cytotoxicity induced by azaguanine is time, dose and cell type dependent.

The analysis of CD26 expression demonstrated that 8-azaguanine treated cells express more CD26 than the control ones (not treated cells) do. After 8-azaguanine treatment, there was an increase in the absolute number of cells expressing CD26 as well as in the number of expressed CD26 molecules/cell.

In presence of the observation that 8-azaguanine stimulated CD26 expression and reduced cell viability it was our objective to investigate whether the cellular response can be modified by other agents or not. For these we incubated cells with hydrogen peroxide, an oxidative stress inducer and analysed the expression of CD26. CD26 expression did not show significant difference in cells treated with hydrogen peroxide, as it can be seen in Table 1. It seems reasonable to conclude that the modifications we observed are due to 8-azaguanine effects, in the experimental conditions of the study.

This CD26 over expression may contribute to the sensitivity of leukaemia cell lines to 8-azaguanine. That may lead to future treatment strategies targeting CD26 for selected human cancers.

Nevertheless, DPPIV enzymatic activity in culture medium did not show significant variation, neither with incubation time, nor with different azaguanine concentration (Fig. 5). This implied that there could be discordance between these two parameters and that cell surface expression of CD26 can be disassociated from soluble levels of enzymatic DPPIV activity, as others have mentioned before [7,29].

Our present study demonstrates that the membrane expression of CD26 and DPPIV enzymatic activity, soluble form, seem to have no relation. This is, in accordance with others studies on Lymphoblastic HL-60 cell line, which suggested that a decrease in cell CD26 expression is not associated with increased cleavage of DPPIV into the cultured medium [40]. It may be related to the fact that in these conditions the CD26 molecules expressed in the surface of the cells may be necessary to play a key role in cancer cells and, therefore, it could not be released from cellular surface.

Our present results showed augmented CD26 surface expression, after 8-azaguanine cell treatment. We suggest that this may be related to a biological active role for this molecule, in T-ALL cell lines, since it follows the incubation time and seems related to 8-azaguanine cell pre treatment, as it improved after it. The modification of CD26 expression is more evident in MOLT3 cell. This might reflect a contribution to improvement in the cellular sensitivity of this cell line to 8-azaguanine. Thus, it could be a reflex of how different cells with different molecular characteristics have different treatment response to therapeutic agents, and in this way, it renders a greater or lesser capacity to control the disease.

According to some authors, CD26/DPPIV may coexist with other molecules in human lymphocytes where it may play a direct or indirect role in pathophysiology of some disease and therapeutic response [7]. Thus, modulation of its activity and expression might affect essential molecules for cancer progression or intracellular signal transduction. Because T-cell lymphoid malignancies are generally aggressive and resistant to current treatments, the development of new treatment approaches, based on a detailed understanding of the molecular processes involved in the development of cancer and chemotherapy response, would have potential benefit.

In this work, our preliminary results demonstrated that CD26 cellular expression varies with chemotherapy treatment, in a significant way, and is accompanied by a decrease in cell viability. This may be related to cellular chemotherapy response.

Although further work is required to elucidate the molecular mechanisms of CD26/DPPIV in cancer cell biology, the present study suggests that CD26/DPPIV may become an important marker of chemotherapy response.

We conclude that a better understanding of the molecular mechanisms underlying the action of DPPIV/CD26 would help determine how it influences normal cell function as well as pathological processes of cancer and chemotherapy response and how it would be an appropriate target for novel therapeutic intervention with conventional chemotherapy in human leukaemia.

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