BIOCHEMICAL PHARMACOLOGY 75 (2008) 857–865

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Modulation of the human equilibrative nucleoside transporter1 (hENT1) activity by IL-4 and PMA in B cells from chronic lymphocytic leukemia

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ARTICLE INFO

Article history:
Received 25 August 2007
Accepted 15 October 2007

Keywords:
hENT1
IL-4
Chronic lymphocytic leukaemia
PKC
Fludarabine
Nucleoside analogs

ABSTRACT

Nucleoside transporters (NTs) are essential for the uptake of therapeutic nucleoside analogs, broadly used in cancer treatment. The mechanisms responsible for NT regulation are largely unknown. IL-4 is a pro-survival signal for chronic lymphocytic leukemia (CLL) cells and has been shown to confer resistance to nucleoside analogs. The aim of this study was to investigate whether IL-4 is able to modulate the expression and function of the human equilibrative NT1 (hENT1) in primary cultures of CLL cells and, consequently, to affect cytotoxicity induced by therapeutic nucleosides analogs. We found that treatment with IL-4 (20 ng/ml for 24 h) increased mRNA hENT1 expression in CLL cells without affecting that of normal B cells. Given that the enhanced mRNA levels of hENT1 in CLL cells did not result in increased transport activity, we examined the possibility that hENT1 induced by IL-4 may require post-translational modifications to become active. We found that the acute stimulation of PKC in IL-4-treated CLL cells by short-term incubation with PMA significantly increased hENT1 transport activity and favoured fludarabine-induced apoptosis. By contrast, and in line with previous reports, IL-4 plus PMA protected CLL cells from a variety of cytotoxic agents. Our findings indicate that the combined treatment with IL-4 and PMA enhances hENT1 activity and specifically sensitizes CLL cells to undergo apoptosis induced by fludarabine.

1. Introduction

The uptake of nucleosides required for DNA and RNA synthesis in lymphoid cells is mediated by specific nucleoside transporters (NTs). There are two major classes of NTs: the solute carrier family 28 and the solute carrier family 29 (SLC28 and SLC29, respectively) [1,2]. The SLC28 genes encode the concentrative NTs (CNT) proteins and comprise three members: CNT1, CNT2 and CNT3, that mediate high-affinity Na-dependent translocation of nucleosides inside the cells [1,2]. The SLC29 genes encode the equilibrative NTs (ENT), which are found in virtually all cell types. ENT mediate nucleosides

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0006-2952/$ – see front matter © 2007 Published by Elsevier Inc.
doi:10.1016/j.bcp.2007.10.017

transport in both directions depending on the nucleoside concentration gradient across the plasma membrane [1,2]. The ENT protein family comprises four members: ENT1 and ENT2 which are broad-selectivity nucleoside carrier proteins, and the recently characterized ENT3 and ENT4 which do not appear to mediate the translocation of nucleosides across the plasma membrane [1–3]. ENT are the most widely expressed NT in leukocytes and can be further defined by their differential sensitivity (ENT1 and ENT3) or insensitivity (ENT2) to nanomolar concentrations of the inhibitor, nitrobenzyl thioinosine (NBTI) [4].

While there have been important advances in the knowledge of NT structure and distribution, the mechanisms that regulate their expression and activity remain largely unknown. A number of studies showed that exposition to different hormones such as triiodo-L-thyronine [5], glucagon [6] or insulin [7] up-regulate nucleoside transport in different cell types. Also cytokines are able to modulate the expression and function of NTs. Studies conducted in macrophages have shown that macrophage-colony stimulating factor (M-CSF) specifically up-regulated the ENT-mediated transport while interferon γ only induced the CNT-mediated transport and interfered with M-CSF-induced expression of ENT [8]. Finally, it was found that B cell activators such as phorbol 12-myristate 13-acetate (PMA) and lipopolysaccharide (LPS) differentially modulate the expression of CNT and ENT in human B cell lines [9]. Given that NT not only mediate the uptake of physiologic nucleosides, but they also play a key role in the uptake of most nucleoside analogs used in cancer therapy [2,3,10–12], a better understanding of their regulation *in vivo* might be helpful to improve drug efficacy.

B cell chronic lymphocytic leukemia (CLL) is the most common form of adult leukemia in the Occident and is characterized by the progressive accumulation of slowly proliferating $CD5^+$ B-lymphocytes [13–15]. Increasing evidence indicates that CLL cell accumulation *in vivo* depends on essential survival factors that are able to delay spontaneous and drug-induced apoptosis [16–18]. Although at present there is no curative treatment for CLL patients, nucleoside analogs, particularly fludarabine, have substantially improved the clinical outcome of the disease in the last 20 years [19–21]. Fludarabine uptake in CLL cells is mostly, if not exclusively, mediated by ENT-type transporters whose biological activity was clearly correlated with its cytotoxicity [22]. Whether hENT transporters in CLL cells can be modulated by physiological factors present in the CLL microenvironment thus affecting nucleoside analogs accumulation and cytotoxicity is currently unknown. Given that interleukin-4 (IL-4) is one of the most relevant survival factors for CLL [23–25], the present study was undertaken to evaluate the effect of IL-4 in hENT1 expression and function in primary cultures of CLL cells.

2. Methods and materials

2.1. Reagents

Hystopaque, acridine orange, ethidium bromide and propidium iodide, natural uridine, S-(4-nitrobenzyl)-6-thioinosine (NBTI), phorbol 12-myristate 13-acetate (PMA), dexamethasone and fludarabine (9-β-D-arabinosyl-2-fluoroadenine-monophosphate) were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal calf serum, RPMI 1640 culture medium, streptomycin and penicillin were obtained from Life Technologies (Grand Island, NY). Etoposide and Idarubicin were from Bristol–Myers Squibb Argentina and Bortezomib was from Janssen-Cilag Argentina. (5,6-^{3}H)uridine was purchased form Perkin-Elmer Life Sciences (Boston, MA). IL-4 was from Peprotech (Mexico, DF).

Monoclonal antibodies (MoAbs) anti-human CD3 (clone UCHT1, mouse IgG1), anti-human CD14 (clone RM052, mouse IgG2a), anti-human CD16 (clone 3G8, mouse IgG1) and anti-human CD56 (clone C218, mouse IgG1), employed in B-CLL purification, were obtained from Beckman Coulter. Anti-cleaved PARP (clone F21-852) was from BD Pharmingen. Anti-mouse IgG-FITC was obtained from Immunotech (Marseille, France). Cells were fixed and permeabilized by using Fix & Perm from Caltag Laboratories (Burlingan, CA).

2.2. Patients

Peripheral blood samples were obtained from CLL patients. At the time of the analysis, all patients were free from clinically relevant infectious complications and were untreated or had received no treatment over the prior 6 months. Approval for these studies was obtained from the institutional review board (Ethics Committee, Academia Nacional de Medicina, Buenos Aires, Argentina). Informed consent was provided by all patients according to the Declaration of Helsinki.

2.3. Cell isolation and culture

Mononuclear cells (PBMC) were isolated from peripheral blood by centrifugation over a Ficoll-Hypaque layer (Hystopaque), washed twice with saline and resuspended in complete medium (RPMI 1640, 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin). B cell purification from PBMC were performed as previously described [16]. Briefly, PBMC were incubated with MoAbs specific for CD3, CD14, CD16 and CD56 during 45 min at 4 °C, washed twice and treated with magnetic beads coated with anti-mouse IgG antibodies (Dynabeads M450, Dynal, Oslo, Norway), according to the manufacturer's instructions. The purity of CLL population was checked by flow cytometry analysis using anti-CD19 MoAb and was found to be always >98%. Tonsils were obtained from patients undergoing routine tonsillectomies for obstructed breathing disorders. Patients were informed about the objectives of the study and gave their consent. Tonsils were cut and pushed through mesh using the flat end of a 60 ml plastic syringe plunger. Mononuclear cells from tonsils samples were isolated by centrifugation over a Ficoll-Hypaque layer and B cell purification were performed as described above.

2.4. Cells treatment

Aliquots of cell suspensions (5×10^6 ml^{-1}) were placed in 12-well flat-bottom plates and cultured at 37 °C in an humidified atmosphere containing 5% CO_2, with or without IL-4 (20 ng/ml) for 24 h. Cells were then washed twice and incubated with PMA 100 nM (or vehicle 0.03% dimethyl sulfoxide) for 10 min

(short-term stimulation of PKC) and thoroughly washed with PBS. For transport measurements, cells were assessed immediately or after incubation at 37 °C for different periods as indicated under Section 3. In those experiments aimed to evaluate apoptotic rates, cells were exposed to chemotherapeutic drugs for 1 h, washed three times with PBS and cultured for an additional 48 h in complete medium.

2.5. Quantitative PCR

The levels of mRNA expression of the hENT1 nucleoside transporters were assessed by quantitative real-time PCR in purified CLL cells and tonsillar B-lymphocytes incubated in the presence of IL-4 or saline for 24 h. Cellular RNA extraction and cDNA synthesis were performed as previously described [26]. Quantitative real-time PCR was carried out in a Lightcycler detection system (Roche) as previously described [26]. Briefly, cDNA (5 μl) was mixed with primers (300 nM each), LightCycler-FastStart DNA Master SYBR Green I (Roche) (hENT1) or LightCycler-FastStart DNA master hybridization probes (Roche) (18S) and probes (130 nM; if necessary) in a total volume of 20 μl for 40 cycles. Primer and probe sequences are published elsewhere [26]. The data was expressed as C_t, which is the PCR cycle number at which the accumulated fluorescent signal in each reaction crosses a threshold above background. Mean C_t values were then normalized to the expression level in reference to an 18S ribosomal RNA: C_t = sample mean C_t − control mean C_t. The results were then expressed as $2^{-\Delta C_t}$. For each sample, a ratio between the studied gene $2^{-\Delta C_t}$ values and 18S ribosomal $2^{-\Delta C_t}$ values were calculated and considered as final amount of mRNA. All samples were analyzed in three separate experiments

2.6. Quantitation of cellular apoptosis and viability by fluorescence microscopy

Quantitation was performed as previously described [27] using the fluorescent DNA-binding dyes acridine orange (100 μg/ml) to determine the percentage of cells that had undergone apoptosis, and ethidium bromide (100 μg/ml) to differentiate between viable and non-viable cells. With this method, non-apoptotic cell nuclei show variations in fluorescent intensity that reflects the distribution of euchromatin and heterochromatin. By contrast, apoptotic nuclei exhibit highly condensed chromatin that is uniformly stained by acridine orange. To assess the percentage of cells showing morphologic features of apoptosis, at least 100 cells were scored in each experiment.

2.7. Quantitation of cellular apoptosis by PARP fragmentation and flow cytometry

For intracellular detection of cleaved PARP, 5×10^5 cells were fixed with formaldehyde 1% and permeabilized with methanol before the addition of a specific monoclonal antibody (mouse anti-cleaved PARP, 10 μg/ml) or isotype-matched monoclonal antibody (10 μg/ml). Cells were cultured for 30 min at room temperature, washed twice and incubated with anti-mouse IgG-FITC for an additional 30 min. Samples were then washed and stored at 4 °C until acquisition on a FACSCalibur™ (BD Biosciences). Data was analyzed with CELLQuest™ software.

2.8. Transport assay

Transport assays were performed as previously described [28]. Briefly, cells were pelleted and resuspended in a medium containing 10 mM Hepes, 25 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$ and either 137 mM NaCl or 137 mM choline chloride, at pH 7.4. The cells were used immediately in the transport assay or after a 10 min pre-incubation with 0.5 μM NBTI, a nucleoside transport inhibitor. This drug inhibits hENT1 (but not hENT2) activity in the range of 0.1–1 μM [3,4]. Uptake assays were started by mixing 0.15 ml of cell suspension with an equal volume of buffer, supplemented with 1 μM (5,6 ^{3}H)uridine at a specific activity of 5000 dpm/pmol. The mean cell concentration used during the incubation time was 5×10^6 cells/ml. After 4 min (5,6 ^{3}H)uridine uptake was terminated by addition 4 ml of ice-cold buffer. The mixture was immediately poured onto Whatman GF/B filters under vacuum. The filters were washing twice with 4 ml aliquots of ice-cold buffer and the radioactivity on the filters was counted in a liquid scintillation counter. Non-specific uptake of (5,6 ^{3}H)uridine into cells was assessed by adding an excess of uridine (200 μM). Uptake rates at the indicated times are expressed as pmol of uridine per 10^6 cells.

2.9. Statistical analysis

A Mann–Whitney *U*-test was used to analyze the statistical significance of the experimental results. $p < 0.05$ was considered statistically significant.

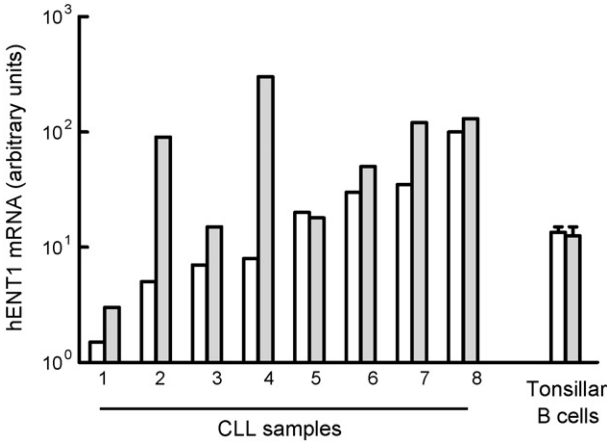


Fig. 1 – Effect of treatment with IL-4 on hENT1 expression in CLL cells and tonsillar B-lymphocytes. Cell suspensions (5×10^6 ml^{-1}) were cultured in the presence (grey bars) or absence (white bars) of IL-4 (20 ng/ml) for 24 h. Total RNA was isolated, retrotranscribed and real-time quantitative RT-PCR of hENT1 was performed as described under Section 2. C_t values from cDNA samples have been normalized to an endogenous reference gene (18 S). The mRNA expression levels are given in arbitrary units. Results are shown as mRNA levels for each CLL sample analyzed and mean ± S.E.M. of six tonsils. Data from untreated *vs.* IL-4-treated CLL samples were significantly different ($p < 0.05$, Mann–Whitney *U*-test for paired samples). There was no statistically significant difference between untreated and IL-4-treated tonsillar B cells.

3. Results

3.1. IL-4 increases hENT1 expression in CLL cells

The effect of IL-4 on hENT1 expression was evaluated in leukemic lymphocytes from CLL patients and in normal B cells purified from tonsils by quantitative RT-PCR. As shown in Fig. 1, incubation of CLL cells for 24 h in the presence of IL-4 (20 ng/ml) significantly increased mRNA levels of hENT1 in seven out of eight samples analysed without affecting normal B cells expression. In agreement with a previous report [22], we found a significant heterogeneity in hENT1 mRNA levels among patients, being the response to IL-4 heterogeneous as well.

Next, we analyzed whether the increased expression of hENT1 induced by IL-4 resulted in enhanced hENT1-dependent uridine uptake. To this aim, we first established the basal measurement conditions for Na^+-independent uridine transport into CLL cells. Data from Fig. 2A shows that uridine uptake in choline chloride buffer rapidly increased during the first 5 min in a saturable way. Taking this into account, a 4 min time point was used for subsequent analysis. As depicted in Fig. 2B, there were no significant differences between transport activity evaluated in NaCl or choline chloride buffer, which confirmed that the major component of uridine uptake in CLL cells was equilibrative. We also confirmed that almost all uridine transport was hENT1-mediated as the addition of 0.5 μM NBTI almost inhibited uridine uptake (Fig. 2B).

Once established the basal conditions to assess hENT1 activity, we analysed if it was affected by IL-4 incubation. To this aim, purified CLL cells were incubated with IL-4 or medium alone for 24 h, cells were washed in a choline buffer and transport assay was performed. As shown in Fig. 2C, we found no significant differences in uridine uptake between IL-4-treated or untreated CLL cells, indicating that increased mRNA levels of hENT1 induced by IL-4 did not result in enhancement of transport activity.

3.2. Acute PKC stimulation increases hENT1 activity in IL-4-treated CLL cells

Given that hENT1 has a number of consensus kinases sites [29] and that it was shown that acute stimulation of PKC causes a rapid increase in hENT1 nucleoside uptake in human carcinoma cell lines [30], we examined the possibility that hENT1 induced by IL-4 may require post-translational modifications to become active. To this aim, cells were exposed to IL-4 for 24 h, washed and incubated with the PKC agonist, phorbol 12-myristate 13-acetate (PMA, 100 nM) for 10 min. Then, cells were washed and hENT1 activity was assessed at different times by measuring uridine uptake. We observed no changes in uridine uptake when transport assay was done

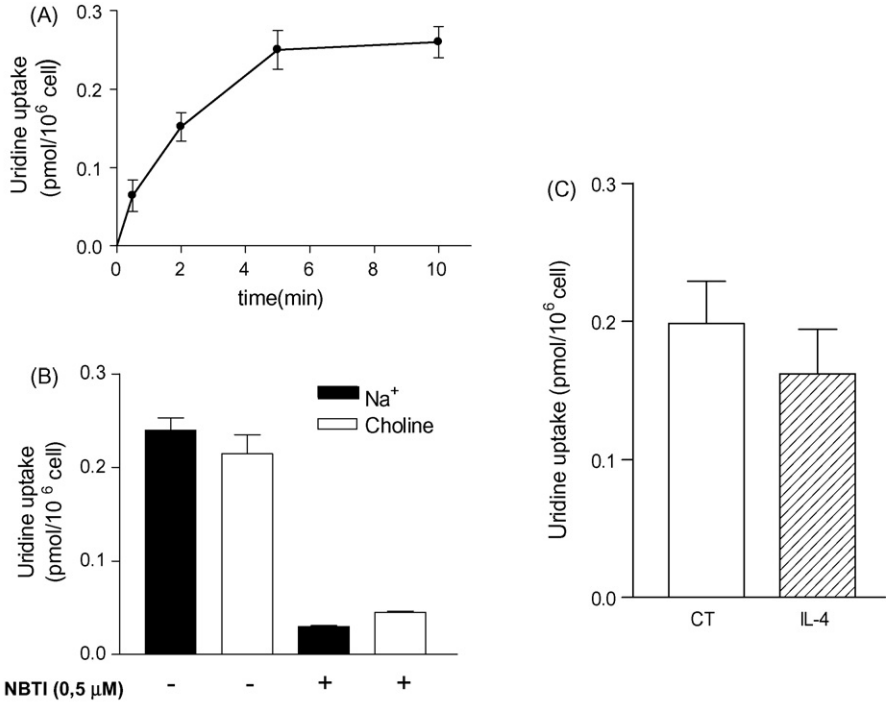


Fig. 2 – Effect of treatment with IL-4 on hENT1 transport activity in CLL cells. (A) Time course of Na^+-independent uridine transport into CLL cells. Purified CLL cells (1.5×10^6) were incubated with (5,6 ^{3}H)uridine in choline chloride buffer for the indicated times. Uridine uptake was assessed as described under Section 2. Results are the mean ± S.E.M. of four samples. (B) Inhibition of uridine uptake into CLL cells by NBTI (0.5 μM). Cells, suspended in NaCl or choline chloride buffer, were pre-incubated for 10 min with NBTI before transport assays. Results are the mean ± S.E.M. of three samples. (C) Effect of IL-4 on uridine transport into CLL cells. Purified CLL cells (5×10^6 ml^{-1}) were incubated with IL-4 (20 ng/ml) for 24 h, washed and resuspendend in choline chloride buffer. Uridine uptake was immediately assessed. Results are the mean ± S.E.M. of 11 samples.

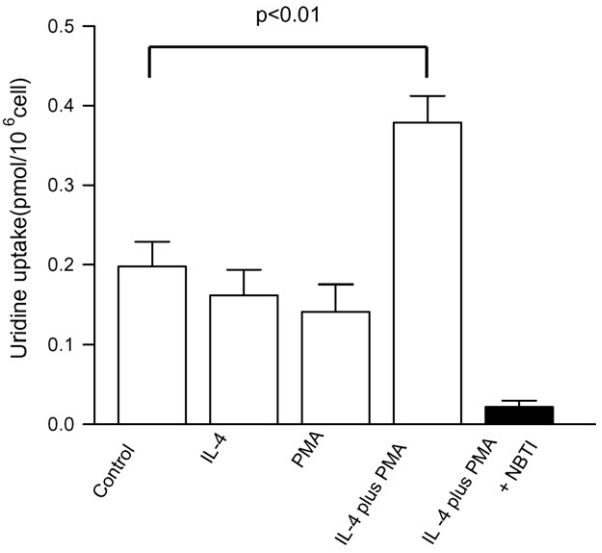


Fig. 3 – Effect of treatment with IL-4 and PMA on hENT1 transport activity in CLL cells. Purified CLL cells (5×10^6 ml^{-1}) were incubated in the presence or absence of IL-4 (20 ng/ml) for 24 h, washed and exposed to PMA (100 nM) or vehicle (0.03% DMSO) for 10 min. Then cells were thoroughly washed, resuspended in choline chloride buffer and incubated for an additional 2 h. When NBTI (0.5 μM) was employed, it was added to cells 10 min before transport assays. Uridine uptake was assessed as described under Section 2. Results are the mean ± S.E.M. of eight samples.

immediately after PKC activation (data not shown). By contrast, when assays were performed 2 h after PKC acute stimulation, there was a significant increase in uridine uptake in IL-4-treated CLL cells ($n = 12$) (Fig. 3), and this increase was sustained for at least 18 h. Fig. 3 also shows that NBTI (0.5 μM) completely abrogated the enhancement in uridine uptake induced by PKC stimulation. Since NBTI used at this concentration inhibits hENT1 transport activity without affecting that of hENT2, we concluded that increased uridine uptake depended on hENT1. Of note short-term incubation with PMA did not increase hENT1 activity in CLL cells in the absence of IL-4 pre-treatment, which differs from what was reported in human carcinoma cell lines [30].

3.3. *Acute PKC stimulation increases fludarabine-induced apoptosis in IL-4-treated CLL cells*

Previous data have shown that fludarabine cytotoxicity in CLL cells correlates with hENT activity [22]. To determine whether the increased hENT1 activity induced by combined IL-4/PMA treatment resulted in enhanced levels of fludarabine-induced apoptosis, we induced acute stimulation of PKC on CLL cells incubated with IL-4 or saline for 24 h. Then, cells were exposed to fludarabine (50 μM) for an hour, washed twice and cultured in drug-free medium for 24 or 48 h. The induction of apoptosis was evaluated in 26 CLL samples by fluorescence microscopy (Fig. 4A). As can be seen in Fig. 4B, IL-4 inhibited both spontaneous and fludarabine-induced apoptosis. On the other hand, short-term incubation with PMA reduced spontaneous apoptosis and slightly decreased fludarabine cytotoxicity. A dramatic inhibition of spontaneous apoptosis was observed in IL-4-treated cells exposed to short-term incubation with PMA, indicating a synergistic protective effect of IL-4 plus PMA. Notably, IL-4-treated cells exposed to PMA were highly sensitive to fludarabine-induced apoptosis. Thus, whereas IL-4 protected cells from fludarabine-induced apoptosis ($47 \pm 4\%$ *vs.* $20 \pm 1\%$, $p < 0.01$), short-term incubation with PMA on IL-4-treated cells almost quadruplicated apoptotic levels ($73 \pm 6\%$ *vs.* $20 \pm 1\%$, $p < 0.01$). To confirm that the increased apoptosis was dependent on a higher uptake of fludarabine mediated by hENT1 we used NBTI (0.5 μM). Treatment of cells with this inhibitor abrogated the enhanced cytotoxic effects of fludarabine in IL-4 plus PMA CLL cells ($19 \pm 9\%$; $p > 0.01$), obtaining apoptotic levels similar to those observed in the presence of IL-4 alone. These results were corroborated in seven independent samples by evaluation of PARP cleavage by flow cytometry as a second apoptosis detection method. Representative histograms are depicted in Fig. 4C.

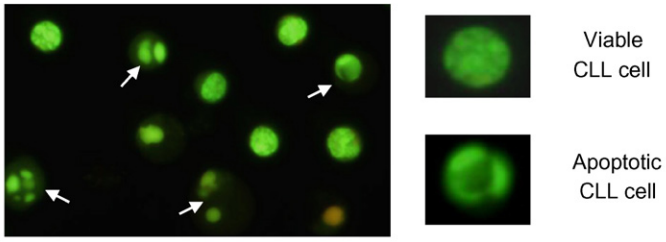
Finally, we ruled out the possibility that IL-4 plus PMA sensitized CLL cells to undergo drug-induced apoptosis by testing four different anti-neoplastic agents, none of which uses NT to be incorporated to cells. To this aim, we chose the proteasome inhibitor, bortezomib (10 nM), the anthracycline antibiotic, idarubicin (5 μM), the topoisomerase II inhibitor, etoposide (10 μM) and the glucocorticoid steroid, dexamethasone (1 μM). As shown in Table 1, we found that short-term incubation with PMA in IL-4-treated CLL cells did not enhance but rather significantly inhibited apoptotic rates induced by

Table 1 – Apoptosis induced by anti-neoplastic agents in CLL cells treated with IL-4 plus PMA

	Percentage of apoptotic cells					
	Saline ($n = 26$)	Fludarabine ($n = 26$)	Bortezomib ($n = 8$)	Etoposide ($n = 8$)	Idarubicin ($n = 8$)	Dexamethasone ($n = 8$)
No treatment	15.3 ± 4.3	47.0 ± 4.6	53.0 ± 10.0	44.2 ± 2.8	54.2 ± 1.5	54.9 ± 5.6
IL-4 plus PMA	1.0 ± 0.1**	73.4 ± 5.7*	38.2 ± 9.8*	15.0 ± 2.0**	16.0 ± 0.2**	10.4 ± 2.5*

CLL cells were incubated in the presence or absence of IL-4 (20 ng/ml for 24 h) plus PMA (100 nM for 10 min) as described in Fig. 4. Then cells were exposed to fludarabine (50 μM), bortezomib (10 nM), etoposide (10 μM), idarubicin (5 μM) or dexamethasone (1 μM) and apoptosis was assessed at 24–48 h by fluorescence microscopy.
Results are the mean ± S.E.M. of *n* samples.
* $p < 0.05$.
** $p < 0.01$: IL-4 plus PMA *vs.* no treatment.

(A)



(B)

	% of apoptotic cells				
	control	IL-4 24h	PMA 10 min	IL-4 24 h plus PMA 10 min	IL-4 24 h plus PMA 10 min + NBTI
without fludarabine (n=26)	15.3 ± 4.3	10.1 ± 1.5	4.0 ± 1.3	1.0 ± 0.1	3.1 ±0.2
with fludarabine (n=26)	47.0 ± 4.6	20.4 ± 1.8*	42.3 ± 3.4	73.4 ± 5.7**	19.0 ±9.2

(C)

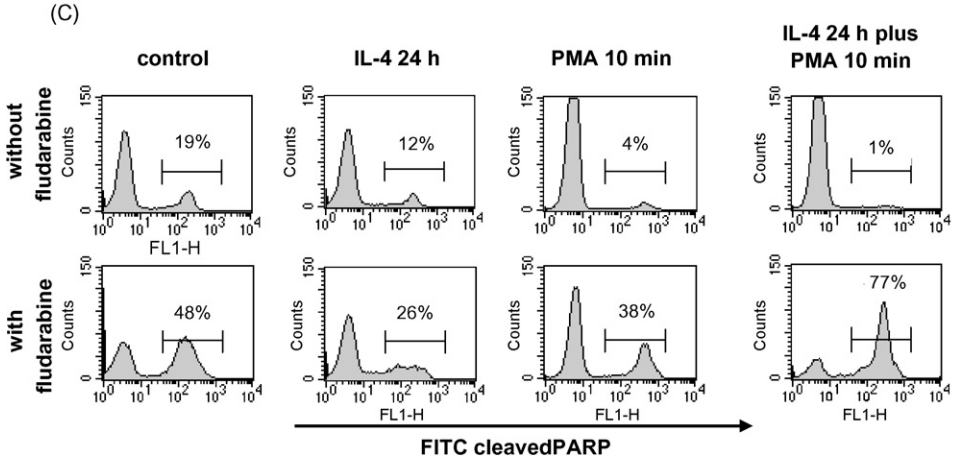


Fig. 4 – Effect of treatment with IL-4 and PMA on fludarabine-induced apoptosis of CLL cells. Purified CLL cells (5×10^6 ml^{-1}) were incubated in the presence or absence of IL-4 (20 ng/ml) for 24 h, washed and exposed to PMA (100 nM) or vehicle (0.03% DMSO) for 10 min. Then cells were thoroughly washed, resuspended in culture medium and treated with fludarabine (50 μM) for an hour. After washing, cells were incubated in drug-free medium for 24–48 h. (A) Fluorescence microscopy of CLL cells as assessed by dual staining with acridine orange and ethidium bromide. Cells undergoing apoptosis (indicated by arrows) display characteristic nuclei with chromatin condensation (magnification, 600×); (B) percentage of apoptosis as evaluated by fluorescence microscopy. *$p < 0.05$: IL-4 *vs.* control in fludarabine-treated cells; **$p < 0.01$: IL-4 plus PMA *vs.* IL-4 in fludarabine-treated cells; (C) percentage of apoptosis as determined by expression of cleaved PARP in permeabilized CLL cells. Representative histograms from one CLL sample ($n = 7$).

any of these cytotoxic drugs. Taken together, these results suggest that the acute stimulation of PKC in CLL cells exposed to IL-4 specifically sensitized cells to undergo fludarabine-induced apoptosis, most probably by favouring its uptake.

4. Discussion

The present study was undertaken to determine whether IL-4 was able to modulate the expression and function of hENT1 in CLL cells, and consequently to affect cytotoxicity induced by therapeutic nucleoside analogues. We found that exposure of CLL cells to IL-4 enhanced the expression of hENT1 at mRNA level but it did not result in increased transport activity. Interestingly, under our experimental conditions, IL-4 did not affect the expression of hENT1 in normal B cells. Although there is no clear explanation for this difference, CLL cells appear to be more responsive to IL-4 than normal B-lymphocytes, probably because of the expression of higher levels of IL-4 receptors [31,32]. Moreover, it was shown that

IL-4 increases the activity of the transcription factor NFκB in CLL cells but not in normal B-lymphocytes [33] suggesting that differences in the signaling pathways triggered by IL-4 also exist. A better understanding of the molecular mechanisms responsible for hENT1 expression will be helpful to explain the present results. Given that the enhanced expression of hENT1 did not result in increased transport activity, we evaluated the possibility that neo hENT1 protein might require post-translational modifications to become active. We demonstrated that acute stimulation of PKC with PMA enhances hENT1 activity (as showed by an increased uridine uptake) only in CLL cells that have been exposed to IL-4. Moreover, we found that the combined treatment with IL-4 and PMA specifically sensitized CLL cells to undergo apoptosis induced by fludarabine. Of note, inhibition of hENT1 transport activity with NBTI (0.5 μM) reversed the sensitization effect induced by the IL-4/PMA treatment. To our knowledge, this is the first study indicating that IL-4 may affect sensitivity of CLL cells to fludarabine by modifying hENT1 transport activity.

Our results showing that incubation of CLL cells with IL-4 induced an increase of mRNA levels of hENT1 were unexpected findings. In fact, the ''*in vitro*'' cytotoxic assays showed that pre-incubation of CLL cells with IL-4 protected from fludarabine-induced apoptosis, confirming previous results found by other authors [34–36]. Thus, our original hypothesis was that a low-hENT1 expression would partially lead to the observed IL-4-induced fludarabine-resistance. To explore how hENT1 was involved in conferring fludarabine-resistance after IL-4 treatment, we assessed hENT1 activity in CLL cells. Although these CLL cells exhibited high levels of hENT1 mRNA, we found no evidence of increased uridine uptake. Similar results concerning the hCNT3 transporters were reported by Mackey et al. [37]. Although the work described by Mackey and colleagues did not take into account the IL-4 context, these authors found that fludarabine-resistant patients presented a higher expression of hCNT3 mRNA and protein levels without showing transport activity.

Acute regulation of hENT by PKC has been previously studied in different cell types. Coe et al. [30] demonstrated that short-term incubation with PMA (10 min, 100 nM PMA) causes a rapid increase in NBTI-sensitive uridine uptake in two carcinoma cell lines. More recently, this group described that acute stimulation of PKC (20 min, 500 nM PMA) increases adenosine uptake in a mouse cardiomyocyte cell line and primary cardiomyocyte cultures [38]. Their data suggest a post-translational modification of the hENT1 transporter by direct phosphorylation or through PKC activation of other enzymes. In contrast, and although the mechanisms responsible are still unclear, evidence indicates that long-term activation of PKC inhibits hENT transport activity. It was reported that differentiation of HL-60 leukemia cells with PMA decreased NBTI-sensitive nucleoside transport probably by inducing conformational changes of the carriers through a PKC-mediated mechanism [39]. Comparable results have been found in chromaffin cells exposed to PMA during 24 h [40] and in B cell lines incubated with low concentration of PMA for at least 3 h [9]. In this latter case, the decrease in NBTI-sensitive nucleoside transport was accompanied by a decrease in hENT1 mRNA amounts.

Our data indicate that pharmacologically acute PKC stimulation prior to fludarabine treatment should be an interesting strategy to reverse fludarabine-resistance induced by IL-4. In this sense, pre-clinical studies suggested that bryostatin-1, a potent PKC activator might be used to increase nucleoside analogues-induced apoptosis. For example Beck et al. have previously shown that bryostatin-1 pre-treatment increased the cellular uptake and the cytotoxic effects of cladribine [41]. Mohammad also found that bryostatin-1 followed by cladribine led to enhanced leukemic cell growth inhibition *in vitro* [42] and in a murine CLL xenograft model [43]. Similar results were observed for the combination of bryostatin-1 followed by the pyrimidine analogue ara-C but not for the reverse sequence [44]. However, Vrana et al. demonstrated that exposure of U937 cell line to bryostatin-1 followed by fludarabine-induced less apoptosis and a lower reduction in colony formation than the reverse combination (fludarabine followed by bryostatin-1) [45]. In either case, subsequent treatment with bryostatin-1 failed to increase F-ara-ATP retention. In these experiments, U937 cells were chronically exposed to bryostatin-1 (at least 24 h) and this could affect fludarabine intracellular uptake. Thus, PKC activation effects would depend in time of exposure to PKC activator (acute *vs.* chronic), as well as the analyzed cell context (cell lines *vs.* ''*ex vivo*'' CLL cells). For example, the pharmacodynamics of bryostatin-1 with regard to PKC is quite complex as bryostatin leads to the activation of PKC isoforms and later to a downmodulation [46,47] that may lead to fludarabine-resistance [48].

Nevertheless, additional effects of IL-4 and/or PMA should also be considered. For example, it has been reported that activation of PKC by bryostatin-1 increases dCK/cN-II ratio in CLL cells and, in this way, potentiates the anti-tumor activity of cladribine [41]. Other authors also evocated that bryostatin-1-induced PKC activation favours the appearance of modifications in the levels of anti-apoptotic proteins as well as membrane lipid alterations or favours the acquisition of a more differentiated state that may increase fludarabine cytotoxicity [45,49,50].

In summary, our data indicate that PKC activation in CLL cells exposed to IL-4 may play a relevant role in plasma membrane permeation to nucleoside analogs, i.e. fludarabine, by enhancing hENT-1 activity. Increased availability of research tools for the study of membrane nucleoside transporters will help to understand the regulatory pathways involved and to develop new strategies to overcome drug resistance due to deficiency in the nucleoside transport activity.

Acknowledgments

The authors thank Ms. Selma Tolosa and Ms. Evelia López for their excellent technical assistance. They are also grateful to Servicio de Otorrinolaringología (Hospital de Clínicas José de San Martín, UBA) for providing tonsil samples.

This work was supported by grants from CONICET, Agencia Nacional de Promoción Científica y Tecnológica (FONCyT) and Fundación Alberto J. Roemmers.

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