Expression of human equilibrative nucleoside transporter 1 (hENT1) and its correlation with gemcitabine uptake and cytotoxicity in mantle cell lymphoma

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Background and Objectives. Nucleoside transporters might play a relevant role in the intracellular targeting of many nucleoside analogs used in anticancer therapy. Two gene families (*SLC28* and *SLC29*) encode the two types of human nucleoside transporters, concentrative nucleoside transporter (CNT) and equilibrative nucleoside transporter (ENT) proteins. Chronic lymphocytic leukemia (CLL) cells express both *SLC28*- and *SLC29*-related mRNA, although transport function seems to be mostly related to ENT-type transporters. Here we have analyzed the role of nucleoside transporters in nucleoside-derived drug bioavailability and action in mantle cell lymphoma (MCL) cells.

Design and Methods. The relative amounts of *hENT1* and *hENT2*-related mRNA and protein were analyzed in five MCL cell lines and 20 primary MCL tumors by real-time quantitative reverse transcriptase polymerase chain reaction and western blots. Cell viability, measured by annexin V-FITC staining, and nucleoside-derived drug transport were also studied.

Results. MCL cells express higher levels of hENT1 protein than do CLL cells, and a good correlation was found between protein and mRNA levels of hENT1, thus indirectly suggesting that hENT1 might be transcriptionally regulated in MCL cells. More importantly, a significant correlation between these two parameters, drug uptake and sensitivity to gemcitabine, was also observed.

Interpretation and Conclusions. These results further support the concept that nucleoside transporters are implicated in the therapeutic response to nucleoside analogs, and suggest a particular and novel role for hENT1 in the genotoxic response to selected nucleoside analogs, such as gemcitabine, in MCL cells.

Key words: nucleoside transporters, equilibrative nucleoside transporters, gemcitabine, cytotoxicity, mantle cell lymphoma

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Mantle cell lymphoma (MCL) is a lymphoid malignancy derived from a subset of mature B cells with co-expression of CD5.[1,2] MCL is characterized by a chromosomal translocation t(11;14)(q13;q32) that results in cyclin D1 overexpression[3] with the consequent deregulation of cell cycle control at the G_1-S checkpoint. The overall prognosis is poor, with a median survival of only 3 years. Although patients may respond to a variety of chemotherapy regimens, response durations are short and no curative therapy has been identified.[1]

Fludarabine is one of the most widely used purine analogs in lymphoid malignancies.[4] Although fludarabine alone possesses moderate efficacy in patients with MCL, fludarabine-containing combinations are efficient in the first-line treatment of MCL. However no improvement in the remission rates has been reported.[5] Recently, our group has described a lack of *in vitro* response to fludarabine in MCL cells, with high doses of fludarabine required to obtain a cytotoxic effect.[6] The metabolism of nucleoside analogs has been broadly studied and appears to be a necessary step in the development of cytotoxicity; however, the major routes of uptake of nucleoside derivatives in MCL cells have not been extensively investigated. Nucleoside transport across the plasma membrane is mediated by transporter proteins belonging to the solute carrier families 28 and 29 (*SLC28* and *SLC29*). *SLC28* genes encode the concentrative nucleoside transporter (CNT) proteins and comprise three members CNT1, CNT2 and CNT3. CNT proteins mediate high-affinity Na-dependent translocation of nucleosides. *SLC29* genes encode the equilibrative nucleoside transporter (ENT) proteins and comprise four members. ENT1 and ENT2 are broad-selectivity equilibrative carrier proteins and ENT1 shows high sensitivity to pharmacological inhibition by the adenosine analog nitrobenzylthioinosine (NBTI). The other two members, ENT3 and ENT4 have recently been characterized.[7,8] CNT and ENT transporters recognize most nucleoside-derived drugs used in anti-cancer treatment and therefore they are pharmacological targets that may influence response to treatment.[9]

Leukemia cells express both *CNT*- and *ENT*-type transporter mRNA.[10] Recently we have detected a significant correlation between fludarabine uptake via ENT carriers, hENT2 protein expression and *ex vivo* sensitivity of chron-

ic lymphocytic leukemia (CLL) cells to fludarabine,[11,12] suggesting a role of the equilibrative nucleoside transporter hENT2 in fludarabine responsiveness in CLL. In contrast to CLL cells, which show defects in the apoptotic machinery, MCL cells are characterized by cell cycle deregulation and additional alterations in cell cycle regulators, particularly in blastoid variants characterized by higher proliferation rates and more aggressive clinical behavior.[13] Since it has previously been suggested that nucleoside transporter proteins play differential roles in cell cycle regulation,[14-17] we wondered whether nucleoside transporter-dependent cytotoxicity might depend upon different transporter isoforms in particular lymphoproliferative malignancies.

Furthermore, the lack of response of MCL cells to fludarabine *in vitro*[6] prompted us to analyze the expression of these nucleoside transporters in MCL cells. For this purpose we analyzed the equilibrative transporters in human MCL cell lines (Granta 519, NCEB-1, Rec-1, JVM-2 and Jeko-1) as well as in primary MCL cells. Furthermore the cytotoxic effect of gemcitabine (2,2'-difluorodeoxycytidine), a deoxycytidine analog, and its correlation with drug uptake and with expression of ENT transporters were also analyzed in MCL cells.

Design and Methods

Cell lines

Five cell lines carrying the t(11;14)(q13;q32) translocation were used: Granta 519, Rec-1, NCEB-1, Jeko-1 and JVM-2. All these cell lines have been described and characterized previously.[18]

Patients

Twenty MCL tumors were studied, four of them corresponding to blastoid variant samples (cases #2, #3, #4 and #8). The diagnosis was established according to the World Health Organization classification.[19] The immunophenotype of the tumor was analyzed by immunohistochemistry on tissue sections and/or by flow cytometry on cell suspensions. For cytotoxic studies, cryopreserved cells from eight of these MCL patients were also used. In all these cases, cells were obtained either at diagnosis or relapse, but patients had not been previously treated with nucleoside analogs. In these cases the status of p53 and ATM has been previously analyzed.[6] Informed consent was obtained from each patient in accordance with the Ethical Committee of the Hospital Clinic (Barcelona, Spain).

Isolation of MCL cells

Mononuclear cells from peripheral blood samples were isolated on a Ficoll/Hypaque (Seromed, Berlin, Germany) gradient. Tumor cells were obtained after squirting spleen biopsies with RPMI 1640 culture medium using a fine needle. Cells were used either immediately or after thawing cryopreserved samples. Manipulation due to freezing/thawing did not influence cell response.

Cell culture

JVM-2, Rec-1 and NCEB-1 cell lines (0.5×10^6 cells/mL) and tumor cells from patients with MCL (1×10^6 cells/mL) were cultured in RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 50 μg/mL penicillin/streptomycin (Gibco, BRL, Paisley, Scotland) and 100 μg/mL normocin (Amaxa, Khöl, Germany), at 37°C in a humidified atmosphere containing 5% CO_2. The Jeko-1 cell line was incubated in the same conditions but supplemented with 20% FCS. The Granta 519 cell line was cultured at a concentration of 0.5×10^6 cells/mL in DMEM culture medium. Absence of mycoplasma infection was regularly assessed by polymerase chain reaction (PCR) and experiments were performed in mycoplasma-free cells.

Cell viability assays

Cell viability were determined simultaneously by double staining with fluorescein isothiocyanate (FITC)-conjugated annexin V (Bender MedSystem, Vienna, Austria) and propidium iodide (PI), as described previously.[20] Cytotoxicity was measured as the percentage of annexin V and PI-positive cells. The LD_{50} was defined as the concentration of drug required to reduce cell viability by 50%.

Real-time quantitative reverse transcriptase (RT)-PCR

Total RNA was isolated from each tumor sample and from MCL cell lines using the guanidine thiocyanate method (Ultraspec, Biotek laboratories, Houston, TX, USA). RNA was treated with DNAse (Ambion, Austin, TX, USA) to eliminate contaminating DNA. In total, 1 μg of RNA was retrotranscribed to cDNA and the analysis of *hENT1*, *hENT2* and β-glucoronidase (*GUS*) mRNA was performed by RT-PCR as previously described.[11] The expression of Ki-67 was analyzed using a pre-designed Assay-on-demand (Applied Biosystems, Foster City, CA, USA). The amounts of mRNA were given as arbitrary units using the ΔCT method (User Bulletin #2, Applied Biosystems) with *GUS* as an internal control.

Western blot analysis

Protein extracts for *hENT1* and *hENT2* analysis were obtained with 10 mM Tris-HCl (pH 7.4), 0.5% Triton X-100, as described elsewhere.[21] Proteins were separated on 10% sodium dodecylsulfate polyacrylamide gels, transferred to Immobilon-P (Millipore, Bedford, MA. USA) membranes and incubated with monospecific polyclonal antibodies against *hENT1* and *hENT2*, which have been recently characterized.[22] *hENT1* and *hENT2* were detected using secondary antibodies conjugated to horseradish peroxidase and an enhanced chemiluminiscence (ECL) detection kit (Amersham, Buckinghamshire, UK). Protein loading was confirmed with α-tubulin (Oncogene Research, Boston, MA, USA). Densitometric analyses were performed with Image Gauge Reader Software (Las3000, Fujifilm, Tokyo, Japan). Ratios of hENT to α-tubulin are given as arbitrary units.

Nucleoside transport

Nucleoside transport into MCL cell lines was measured using a rapid filtration method, as previously described.[11] [5,6-^{3}H]-uridine (Amersham Biosciences, Buckinghamshire, England), [8-^{3}H]-fludarabine and [5-^{3}H]-gemcitabine (Moravek Biochemicals, Brea, CA, USA) were used as substrates at a concentration of 1 μM. The incubation time

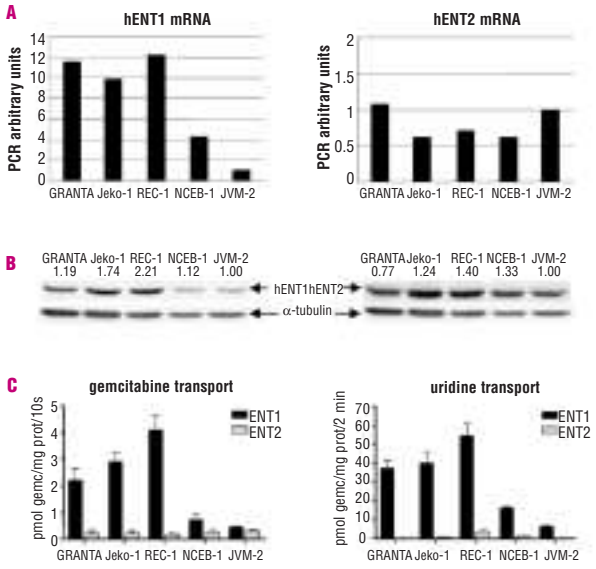

Figure 1. Equilibrative nucleoside transporters (hENT1 and hENT2) in MCL cell lines. Normalized *hENT1* and *hENT2*-related mRNA expression levels in MCL cell lines. CT values for each nucleoside transporter were normalized to an endogenous reference gene (*GUS*). mRNA expression levels are given in arbitrary units, using JVM-2 as the reference control (A). The experiments were done in duplicate. Western blots of hENT1 and hENT2 and α-tubulin in MCL cell lines. Normalized protein expression levels in these MCL cell lines. Protein amounts were calculated as the densitometric ratio of hENT to α-tubulin, and are shown as arbitrary units (B). Equilibrative nucleoside transport in MCL cell lines. Gemcitabine and uridine transport was measured at 10 seconds and 2 minutes, respectively. NBTI (1 μM) was used to discriminate between NBTI-sensitive (hENT1) and NBTI-insensitive (hENT2) transport rates. Results are given as the mean±SE of three to six independent experiments measured in triplicate (C).

was 2 minutes for uridine and 10 seconds for fludarabine and gemcitabine. To discriminate between NBTI-sensitive (hENT1) and NBTI-insensitive (hENT2) transport rates, 1 μM NBTI (Sigma-Aldrich, St. Louis, MO, USA) was used.

Statistical analysis

Correlations between nucleoside transporter expression, nucleoside transport and gemcitabine-induced cytotoxicity were analyzed using the SPSS 11.0 software package (SPSS, Chicago, IL, USA). The significance of correlations was assessed by the Pearson and Mann-Whitney tests.

Results

hENT1 and hENT2 expression in MCL cell lines

The expression of *hENT*-related mRNA was analyzed by quantitative RT-PCR in human MCL cell lines. Figure 1A shows the amounts of *hENT1* and *hENT2*-related mRNA in five MCL cell lines carrying the t(11;14)(q13;q32). The human JVM-2 cell line was used as a relative calibrator, therefore the expression levels of *hENT1* and *hENT2* in this cell line were assigned the value of 1 as an arbitrary unit. *hENT* transporters were expressed in all these cell lines, although a high variability in the levels of expression of *hENT1* was observed. Thus, whereas *hENT1*-related mRNA levels showed a range of variability of nearly 12-fold, *hENT2* variability was less than 1-fold. Three cell lines (Granta 519, Jeko-1 and Rec-1) have higher levels of *hENT1* compared to the other two cell lines analyzed: NCEB-1 and JVM-2. Amounts of hENT protein were analyzed by western blot in these MCL cell lines using specific antibodies against hENT1 and hENT2 proteins. As recently reported,[22] these antibodies specifically recognize single bands of 50-55 kDa. Figure 1B shows western blots of the five cell lines in which hENT1, hENT2 and α-tubulin were analyzed. Semiquantitative analysis of hENT1 and hENT2 expression was achieved by calculating the densitometry ratios of the hENT to α-tubulin, in a range of protein concentrations in which the densitometric signal had been previously shown to be linear. Figure 1C shows the values of these densitometric analyses of the five MCL cell lines. The human JVM-2 cell line was again used as the relative calibrator and its protein amounts assigned the value of 1. The amount of hENT2 protein showed less variability than that of hENT1 protein in MCL cell lines. These results are relatively concordant with the pattern of *hENT*-related mRNA expression observed using quantitative RT-PCR.

Nucleoside uptake into MCL cell lines

Equilibrative nucleoside transport uptake was analyzed in the five human MCL cell lines. Transport of the natural nucleoside uridine was measured at 2 minutes, as its transport was linear up to 10 minutes (*data not shown*). Gemcitabine and fludarabine transport rates were measured at 10 seconds, because transport processes were extremely rapid, and thus linear velocity conditions were lost before the first minute of incubation (*data not shown*). In order to discriminate between hENT1 and hENT2-mediated transport (NBTI-sensitive and -insensitive components, respectively), nucleoside transport was monitored either in the presence or in the absence of 1μM NBTI. Although both hENT1 and hENT2 protein and mRNA were detected in all cell lines, uridine and gemcitabine transport was almost exclusively mediated by the hENT1 transporter (Figure 1C). As was observed in the expression analysis of hENT1, uridine and gemcitabine uptake showed a high range of variability, the Rec-1, Jeko-1 and Granta 519 cell lines having the highest rates for both uridine and gemcitabine uptake. The uptake of gemcitabine mediated by the hENT1 transporter is shown in Table 1. The addition of 1 μM NBTI blocked the gemcitabine uptake, indicating that gemcitabine's transport is hENT1-mediated (*data not shown*). Uridine transport significantly correlated with *hENT1*-related mRNA expression (Figure 2A), determined by quantitative RT-PCR and with hENT1 protein levels (Figure 2B) (p=0.009 and 0.042, respectively). Similar results were obtained for gemcitabine transport (Figure 2C and D) (p=0.036 and p=0.013, respectively), but no correlation was found for fludarabine uptake (*data not shown*). Furthermore, the amounts of hENT2 protein and *hENT2*-related mRNA did not correlate with hENT2-mediated uridine and gemcitabine transport rates (*data not shown*).

Sensitivity of MCL cell lines to gemcitabine

Since in MCL cell lines, the expression levels of *hENT1*-related mRNA are higher than those of *hENT2*, we ana-

Table 1. Cytotoxicity, uptake of gemcitabine and the status of DNA response damage genes in MCL cell lines.

	LD_{50} Gemcitabine (μg/mL)	Gemcitabine uptake (pmoL/mg protein/10 sec)	DNA response damage genes	
			p53[a]	ATM[b]
GRANTA	2.75±0.37	2.20±0.43	wt	del
JEKO-1	0.28±0.013	2.91±0.31	mut	wt
REC-1	0.02±0.016	4.10±0.52	wt	wt
NCEB-1	NR[c]	0.72±0.19	mut	del
JVM-2	NR[c]	0.44±0.05	wt	wt

[a]*p53 mutational status assessed by single strand conformational polymorphism analysis (SSCP) and sequencing;* [b]*ATM status assessed by fluorescent in situ hybridization (FISH);* [c]*NR: no response.*

lyzed the sensitivity of these cells to gemcitabine, a nucleoside analog whose uptake is known to be mediated preferentially by ENT1 transporters. Jeko-1, Rec-1, Granta 519, JVM-2 and NCEB-1 cell lines were incubated for 24 hours with different concentrations of gemcitabine (0.003-50 μg/mL) (Lilly, Hampshire, UK), and the cytotoxic effect was measured by annexin V-FITC/PI staining. The LD_{50} for gemcitabine is shown in Table 1. A cytotoxic effect was observed at low doses of gemcitabine (< 3 μg/mL) in the Jeko-1, Granta 519 and Rec-1 cell lines, whereas JVM-2 and NCEB-1 cells were resistant to gemcitabine-induced cytotoxicity. The highest sensitivity was detected in Rec-1 cells (20 ng/mL) and this cell line also had the highest rates of gemcitabine uptake. In accordance with this, the two resistant cell lines (JVM-2 and NCEB-1) had the lowest levels of gemcitabine uptake. These results suggest that lack of response to gemcitabine correlates with low drug uptake rates. In contrast, no direct relationship was observed between gemcitabine cytotoxicity and *p53* and *ATM* status. Thus, no alterations in the DNA damage response genes (*p53* and *ATM*) were detected in Rec-1 cells, although the other two sensitive MCL cell lines carried *p53* (Jeko-1) or *ATM* (Granta 519) alterations. Furthermore, JVM-2 (wild type *p53* and *ATM*) and NCEB-1 (alterations in *p53* and *ATM*) showed no response to gemcitabine.

Correlation of hENT1 protein and mRNA amounts, drug uptake and sensitivity to gemcitabine in MCL cell lines

We did a correlation analysis to assess possible relationships between hENT expression and sensitivity to gemcitabine and the uptake of these drugs into the cells. Sensitivity to gemcitabine (5 μg/mL) was directly correlated with both *hENT1*-related mRNA expression (Figure 3A) (r=0.9; p=0.04) and hENT1 protein levels (Figure 3B) (r=0.90; p=0.04). Similar results were obtained when cells were incubated with other doses of gemcitabine (*data not shown*). No correlation between sensitivity to gemcitabine and *hENT2* mRNA or protein expression was detected (*data not shown*). Furthermore, gemcitabine transport also correlated with sensitivity to gemcitabine (r=0.97; p=0.005) in these MCL cell lines (Figure 3C).

Equilibrative nucleoside transporter expression pattern in MCL cells

Expression of *ENT* was analyzed by quantitative RT-PCR in tumor cells from 20 primary MCL tumors. Figure 4A shows the expression of *hENT1* and *hENT2*-related mRNA in the whole cohort of MCL tumors analyzed in this study, using JVM-2 cells as the relative calibrator. The mean values of *hENT1* and *hENT2*-related mRNA in primary chronic lymphocytic leukemia (CLL) cells, described previously,[12] are also included in Figure 4A. The variability in *hENT1* and *hENT2*-related mRNA expression was very

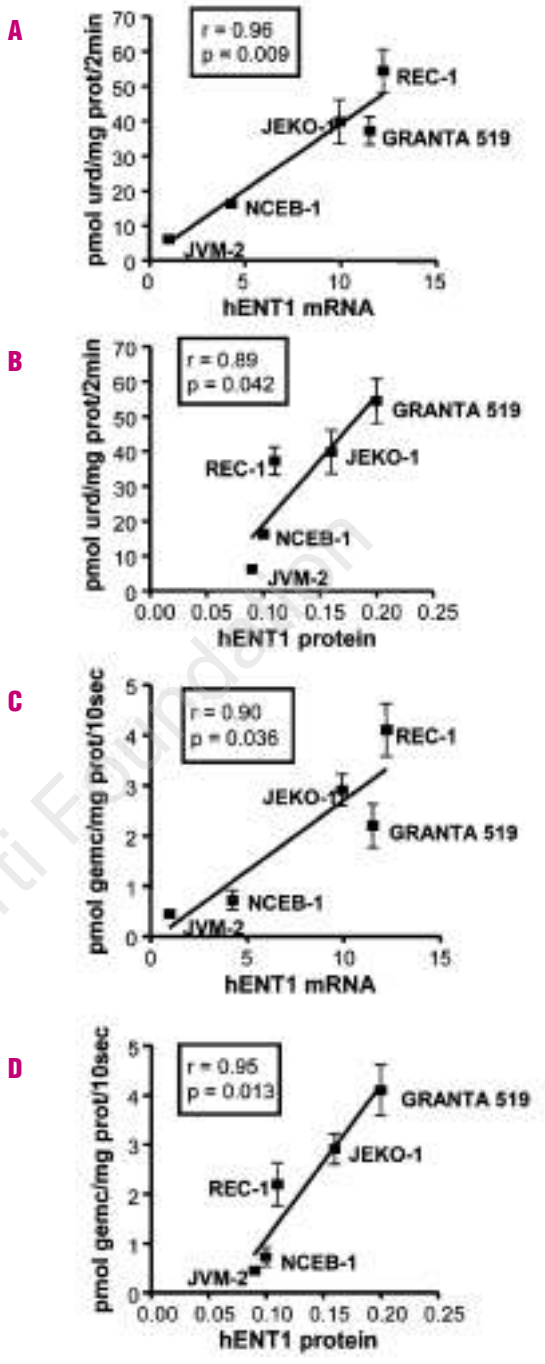

Figure 2. Correlations of nucleoside transport with protein and *SLC29* amounts, in MCL cell lines. hENT1 mRNA and protein expression levels were plotted against uridine (A and B) and gemcitabine (C and D) uptake rates in MCL cell lines. Results are given as the mean ± SE of three to six independent experiments measured in triplicate. Correlation coefficients and *p*-values are shown.

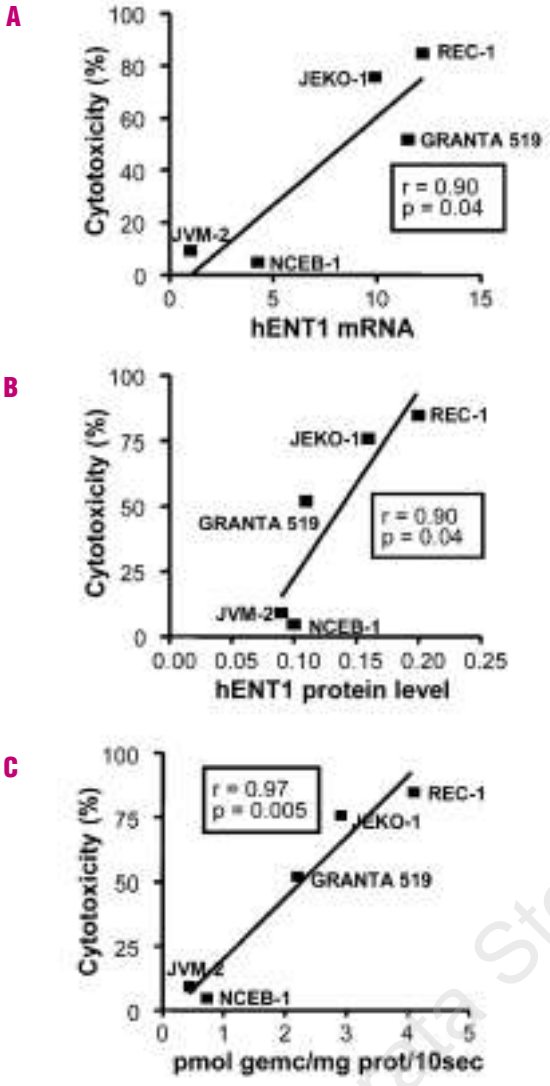

Figure 3. Correlation between gemcitabine cytotoxicity and expression of hENT1 and gemcitabine uptake in MCL cell lines. Sensitivity to gemcitabine (5 μg/mL), expressed as the percentage of apoptotic cells after 24 hours of treatment, was plotted against *hENT1* mRNA levels (A), hENT1 protein expression (B) and gemcitabine transport into the cells (C). Correlation coefficients and *p*-values are shown.

high. The expression patterns of these transporters in primary MCL cells differed from those observed previously in CLL cells. Thus, the average expression of *hENT1*-related mRNA in MCL cells was significantly higher (0.76±0.48) than that reported previously for primary CLL cells[12] (n=22; 0.13±0.10) (p<0.001) and the amount of *hENT2*-related mRNA in MCL cells was significantly lower (0.89±0.52) compared to that observed in CLL cells (n=22; 4.04±1.83) (p<0.001). The expression of hENT1 and hENT2 transporters did not correlate with either the morphological MCL variant or the proliferation index of the tumor cells. In these primary MCL tumors, protein extracts were obtained and ENT were analyzed by western blotting. Figure 4B shows a representative western blot of five independent cases of primary MCL, in which the amounts of hENT1, hENT2 and α-tubulin were analyzed. The semiquantitative analysis of hENT1 and hENT2 expression is shown in Figure 4C. Again, the amounts of hENT1 protein were significantly higher in primary MCL cells (0.15±0.14) compared to those reported previously for CLL cells (0.08±0.02) (p<0.001), and hENT2 protein expression was lower in primary MCL cells (0.26±0.20) than in CLL cells (0.46±0.010) (p< 0.001).

Ex vivo sensitivity to gemcitabine in primary cells from MCL patients

Primary cells from eight MCL patients, which had been included in the expression analysis of ENT transporters, were incubated with different concentrations of gemcitabine (0.5-50 μg/mL) for 48 hours. The biological characteristics of these patients and LD_{50} for gemcitabine are reported in Table 2. In all primary MCL cases analyzed, the doses of gemcitabine necessary to induce a cytotoxic effect were higher than those necessary in sensitive MCL cell lines. In cells from six MCL patients (#13, #14, #15, #17, #18 and #19), the LD_{50} for gemcitabine were less than 50 μg/mL. In the other two cases (#16 and #20) higher doses of gemcitabine were necessary to achieve a cytotoxic effect. Gemcitabine cytotoxicity did not correlate with *p53* and *ATM* status nor with the proliferation index analyzed by Ki-67 quantification in these primary MCL cells.

Correlation of hENT expression with mRNA expression, and ex vivo gemcitabine cytotoxicity in MCL primary cells

The amounts of *hENT1*-related mRNA correlated with the hENT1 protein levels (r=0.62; p=0.013) (Figure 5A). As we observed in MCL cell lines, there was a significant correlation between *hENT1*-related mRNA levels and the cytotoxic effect of gemcitabine at 5 μg/mL (r=0.72; p=0.04) (Figure 5B). Similar results were obtained when primary cells were incubated with other doses of gemcitabine (*data not shown*). Amounts of *hENT2*-related mRNA did not correlate with either hENT2 protein level or with cell viability after exposure to different doses of gemcitabine. Furthermore, neither *hENT1*-related mRNA nor protein expression correlated with the cytotoxic effect induced by other non-nucleoside analog chemotherapeutic agents such as mitoxantrone, a topoisomerase inhibitor (*data not shown*).

Discussion

CNT and ENT proteins are responsible for the uptake of many nucleoside derivatives used in anticancer therapies.[23] Tumor cells show highly variable patterns of CNT and ENT expression, which might affect drug bioavailability and action.[9,10,24] Our previous studies demonstrated that fludarabine accumulation in CLL cells is mostly mediated by ENT-type transporters[11] and we have recently proposed that hENT2, in particular, may play a role in fludarabine responsiveness in CLL patients.[12]

MCL-derived cell lines and primary MCL cells express both ENT-type transporters proteins. We have detected higher levels of hENT1 protein and *hENT1*-related mRNA in MCL cell lines compared to in primary MCL cells and

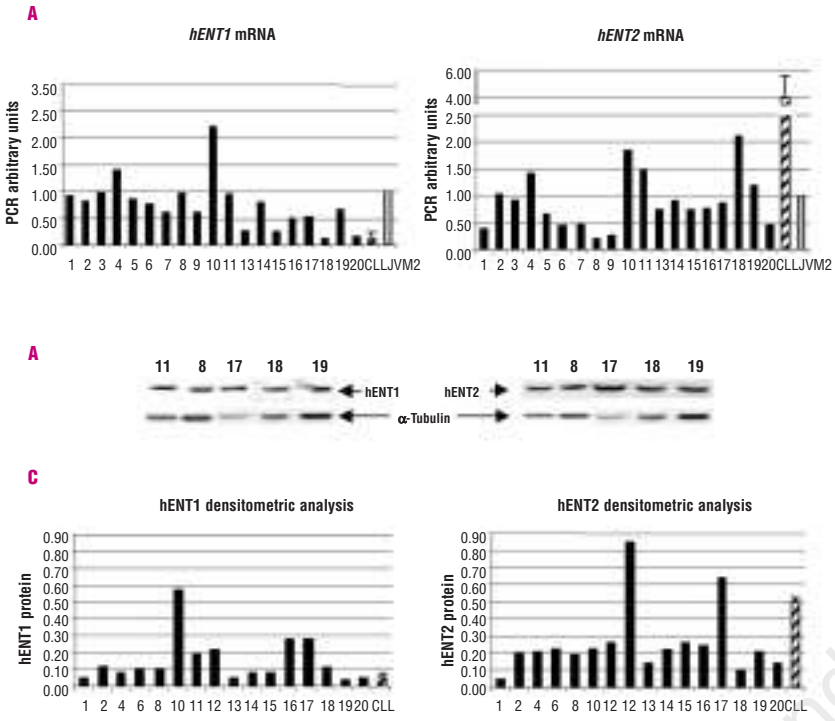

Figure 4. *hENT1* and *hENT2*-related mRNA and protein expression in primary MCL cells. Normalized *hENT1* and *hENT2*-related mRNA expression levels in the cohort of MCL-patients were analyzed (A). The JVM-2 cell line was used as the relative calibrator and assigned the value of 1 as an arbitrary unit. The mean of *hENT*-related mRNA expression in CLL cells is also represented. Western blot of total protein extracts from five representative independent MCL cases (B). Relative amounts of hENT1 and hENT2 in primary MCL cases. Protein amounts were calculated as the densitometric ratio of hENT to α-tubulin, and are given in arbitrary units (C). The mean of hENT1 and hENT2 protein expression in CLL samples obtained previously[12] is also plotted.

CLL cells. In contrast, CLL cells, characterized by the accumulation of B cells in the G_0 phase of the cell cycle,[25] showed higher expression of the hENT2 isoform than did MCL cells. These results are in agreement with previous reports suggesting a significant role for hENT1 in cell proliferation[14,15] and with higher levels of hENT1 expression in immortalized cell lines than in their primary counterparts.[26] Furthermore, high levels of *hENT1* related-mRNA had been previously reported in an isolated case of MCL.[27] Heterogeneity of the amounts of hENT1 in human tissues has been described,[22,28] particularly in non-Hodgkin's lymphomas, where it has been proposed that expression of hENT1 is linked to B-cell differentiation.[29] Moreover, in contrast to the lack of relationship between hENT proteins and mRNA observed in CLL cells,[12] a significant correlation was found between the amount of hENT1 protein and corresponding mRNA levels in MCL cells. This correlation supports the view that hENT1 expression might be transcriptionally regulated in this malignancy.

In MCL cell lines, hENT1 expression was significantly correlated with nucleoside transport when uridine and gemcitabine were used as substrates, but not when fludarabine was used. We demonstrated that gemcitabine transport was almost exclusively mediated by the hENT1 transporter. These results might further reflect a different role for hENT1 and hENT2 proteins in gemcitabine and fludarabine-induced cytotoxicities.

Evidence demonstrating that gemcitabine acts as a genotoxic agent in proliferating tumors[30,31] along with previous *in vitro* and *in vivo* reports showing a lack of responsiveness of MCL cells to fludarabine[6,32,33] prompted us to analyze the effect of gemcitabine in MCL cells. Gemcitabine is a deoxycytidine analog that has more effective cellular kinetics, including intracellular incorporation, phosphory-

Table 2. Characteristics of MCL patients and LD_{50} for gemcitabine.

Patient	*Cell source*[a]	*Morphological variant*[b]	*% Tumoral cells*	*LD_{50} Gemcitabine (μg/mL)*	*p53*[c]	*ATM*[d]	*KI67*[e]
13	PB	C	95	26.93±0.01	wt	del	26
14	Spleen	C	95	13.36±0.76	wt	del	46
15	PB	C	84	30.70±0.02	wt	del	6
16	PB	C	95	58.14±0.01	mut	wt	40
17	Spleen	C	80	5.05±0.01	wt	wt	42
18	PB	C	86	8.42±0.01	wt	wt	23
19	PB	C	90	7.72±0.01	mut	wt	25
20	PB	C	70	62.56±0.01	wt	wt	13

[a]Source of the cells used for the in vitro analysis. PB: peripheral blood; [b]C: classical variant; [c]p53 status was assessed by FISH, SSCP and sequencing; [d]ATM status was assessed by FISH; [e]Ki-67 expression was assessed by RT-PCR. CLL mRNA was used as the relative calibrator and assigned the value of 1 as an arbitrary unit.

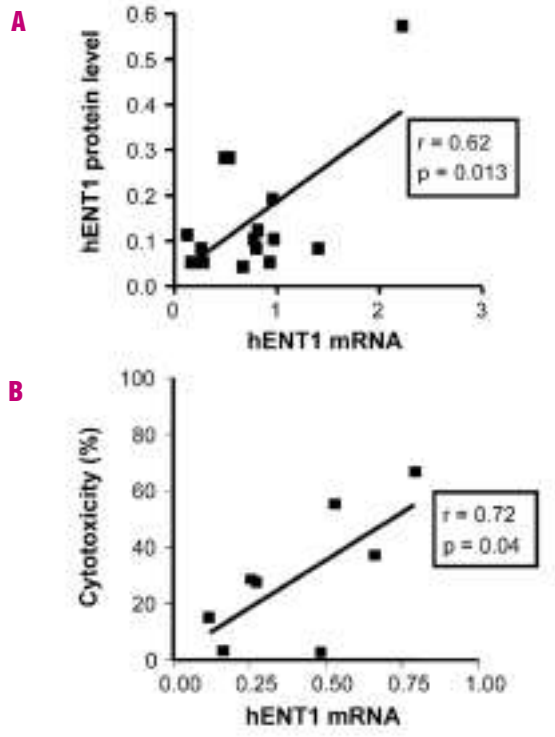

Figure 5. Correlations between hENT1 protein and mRNA expression and *ex vivo* gemcitabine cytotoxicity in primary MCL cells. Correlation between *hENT1*-related mRNA levels and protein expression in primary MCL cells (A). Correlation of *ex vivo* sensitivity to gemcitabine with *hENT1*-related mRNA in gemcitabine-sensitive cases (B). These results are expressed as the percentage of non-viable cells observed after 48 hours treatment with 5 μg/mL gemcitabine. Correlation coefficients and *p*-values are shown.

lation and retention.[34] Furthermore, gemcitabine is phosphorylated more rapidly and eliminated more slowly than other nucleoside-derived drugs.[9] Gemcitabine is commonly used to treat patients with solid tumors.[23,35-38] The major effect of gemcitabine is directed against DNA synthesis[39] and it is preferentially transported by hENT1 proteins.[40] In this study we demonstrated that incubation with gemcitabine at pharmacological doses induced a cytotoxic effect in most cells from primary and MCL cell lines. Moreover, we detected a significant correlation between *ex vivo* sensitivity to gemcitabine, hENT1-mediated gemcitabine transport and the amounts of hENT1 in the MCL cell lines analyzed. Previous studies have shown that the abundance of hENT1 protein is a major determinant of the efficiency of cellular accumulation of several nucleoside analogs. Thus, *hENT1*-related mRNA expression inversely correlated with *in vitro* resistance to ara-C in acute lymphoblastic leukemia[41] and to cytarabine in chilhood acute myeloid leukemia (AML).[42] Furthermore, it has also been reported that AML patients who did not express hENT1 had a shorter disease-free survival after treatment with cytarabine.[43] Recently, a correlation between hENT1 expression and response to gemcitabine as a single agent was detected in patients with pancreatic adenocarcinoma.[28] Pharmacological inhibition of hENT1 resulted in either increased retention and cytotoxicity of cladribine in cells from CLL patients[44] and in cultured human leukemic lymphoblasts,[45] or in inhibition of acadesine-induced cell death due to the inhibition of acadesine entry into CLL cells.[46] Nevertheless, positive correlations of these transporters with a number of anti-metabolite drug-analogs have been reported for the NCI-60 panel of cell lines.[47]

Although gemcitabine induced a cytotoxic effect in most primary MCL cells, some insensitive cases were also found. Resistance to gemcitabine might involve mechanisms other than transport processes, related to intracellular accumulation, metabolism and targeting.[48-50] There is a report of a MCL patient who exhibited resistance to fludarabine with a deficiency in both nucleoside uptake and accumulation, with no major changes in mRNA levels of the genes involved in nucleoside analog uptake and metabolism[27] indicating that resistance to the nucleoside analogs was downstream of gene transcription or involved other genes. Moreover, MCL cells often show alterations in genes implicated in the DNA damage pathway such as *p53* and *ATM*, which might also explain resistance to nucleoside analogs.[6] In spite of these putative mechanisms of resistance, here it is shown that hENT1 plays an important role in drug cytotoxicity in those primary MCL cells that respond to pharmacological doses of gemcitabine.

In summary, this study demonstrates that MCL cells express high levels of hENT1 compared to CLL cells and that these cells might be more sensitive to nucleoside analogs whose uptake is mediated preferentially by the hENT1 transporter. The results presented in this paper further support the hypothesis that nucleoside transporters are implicated in the therapeutic response to nucleoside analogs, suggesting that levels of hENT1 expression might be useful to predict response to nucleoside analogs known to be taken up via ENT1 carriers, such as gemcitabine in MCL patients. A better understanding of nucleoside analog transport may extend therapeutic strategies and improve the prognosis of MCL patients.

SM and MM-A performed all the experiments reported in this paper. They contributed to the interpretation of data and drafting the manuscript. They created the tables and figures of the manuscript. FJ-C participated in the design of the experimental work focused on the nucleoside transporter analysis. NV and EC participated in the design of the experimental work and contributed to the interpretation of data, and the revision of the manuscript. MP-A and DC developed the project, designed the experimental procedures and coordinated them. They contributed to the interpretation of data and drafting the article.

All the authors approved the final version of the manuscript to be published and declare that they have no potential conflict of interest.

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