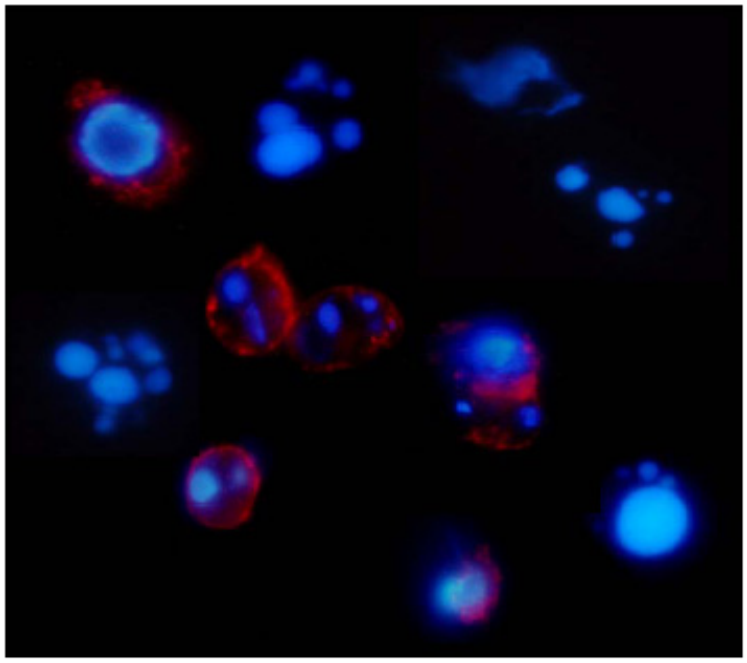
Bases moleculares de la apoptosis inducida por drogas en neoplasias linfoides



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ARTÍCULOS

ARTÍCULO 1

ACTIVACIÓN DE LA APOPTOSIS MITOCONDRIAL EN EL LCM: ELEVADA SENSIBILIDAD A MITOXANTRONA EN CASOS CON GENES DE RESPUESTA A DAÑO EN EL DNA FUNCIONALES

Objetivo

Estudiar los mecanismos de muerte celular inducida por tres fármacos genotóxicos (fludarabina, mitoxantrona y ciclofosfamida) en células de líneas celulares que presentan la t(11;14)(q13;q32) y en células primarias de linfoma de células del manto (LCM), y analizar las vías implicadas.

Resumen

En este estudio se han determinado los mecanismos de inducción de muerte celular por fármacos en el LCM. Para ello se ha trabajado con tres fármacos utilizados en el tratamiento de neoplasias linfoides, como son la fludarabina (1-10 μg/mL), la mitoxantrona (0.05-0.5 μg/mL) y la ciclofosfamida (1-10 μg/mL). La mitoxantrona, un inhibidor de la topoisomerasa II, indujo el mayor efecto citotóxico en tres de las cuatro líneas celulares estudiadas (JVM-2, REC-1 y GRANTA 519) y en células primarias de pacientes diagnosticados de LCM. Estas dosis son inferiores a las utilizadas en la LLC-B. Se obtuvo una LD_{50} de 0.18 μg/mL en las líneas celulares y de 0.37 μg/mL en las células primarias de LCM. La mafosfamida, forma activa de la ciclofosfamida, indujo efecto citotóxico en células primarias de LCM a dosis superiores a las farmacológicas (LD_{50}= 5.70 μg/ml) mientras que solamente indujo efecto en la línea celular REC-1 a una LD_{50} de 5.48 μg/mL. Por otro lado, la fludarabina no ejerció ningún efecto citotóxico en ninguna de las líneas celulares y sólo en uno de los diez casos de células primarias de LCM se observó un efecto citotóxico a altas concentraciones (5-10 μg/mL) y tiempos largos de incubación (48h). Ninguno de los tres fármacos analizados ejerció efecto alguno en la línea celular NCEB-1.

La combinación de los fármacos no demostró efecto aditivo ni sinérgico en las líneas celulares, en cambio sólo se detectó un efecto aditivo de mitoxantrona y mafosfamida en 3 de los 10 casos de LCM analizados.

El efecto citotóxico observado era debido a la inducción de apoptosis, ya que se acompañaba de cambios en los patrones tamaño (FCS) y complejidad (SSC) de la célula, pérdida de potencial de membrana mitocondrial, cambios de conformación de Bax y Bak, proteolisis de las caspasas -9, -8 y activación de la caspasa-3, con la consecuente proteolisis de PARP, disminución de XIAP y disminución de las proteínas de la familia de Bcl-2, Mcl-1 y Bcl-X_L.

El empleo de un inhibidor general de caspasas, el Z-VAD.fmk, restauró la viabilidad celular e impidió la proteolisis de caspasa-3 y PARP indicando que el efecto citotóxico inducido por estos fármacos era debido a la activación de las caspasas.

El inhibidor de la caspasa-9, Z-LEHD.fmk, demostró que al inhibir la activación de la caspasa-9 también se inhibía la proteolisis de la caspasa-8, confirmando que la vía apoptótica que se estaba activando en estos casos era la vía intrínseca o mitocondrial, y que la activación de la caspasa-8 era debida al *loop* de amplificación de la señal de la caspasa-9.

Los mecanismos de respuesta a daño al DNA juegan un papel importante en la patogénesis y el comportamiento clínico de los LCM. El gen ATM codifica para una proteína implicada en la fosforilación de la proteína p53 en respuesta a daño al DNA, promoviendo parada de ciclo y apoptosis. Las alteraciones en p53 y ATM estan asociadas a resistencia a fármacos. Alteraciones en ATM son frecuentes en pacientes de LCM y mutaciones de p53 se han asociado a variantes blásticas (Hernandez L et al, 1996). Los tres fármacos analizados en nuestro estudio son fármacos genotóxicos, que inducen daño al DNA. En este sentido, la línea celular NCEB-1 fue la única en la que no se observó ningún efecto citotóxico inducido por ninguno de los tres fármacos estudiados, a pesar de que la incubación con estaurosporina demostró que la maquinaria apoptótica mitocondrial de esta línea celular era funcional. Esta línea celular se caracteriza por presentar deleción tanto de *p53* como de *ATM*, sugiriendo que es necesaria la funcionalidad de sólo uno de los dos genes implicados en la reparación del DNA para ver un efecto citotóxico inducido por fármacos genotóxicos.

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Activation of mitochondrial apoptotic pathway in mantle cell lymphoma: high sensitivity to mitoxantrone in cases with functional DNA-damage response genes

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Mantle cell lymphoma (MCL) is a mature B-cell proliferation characterized by the presence of translocation t(11;14)(q13;q32), an aggressive clinical course, and poor response to chemotherapy. The majority of drugs currently used in the treatment of lymphoproliferative disorders induce cell death by triggering apoptosis, but few data concerning drug-induced apoptosis in MCL have been reported. We have analysed the mechanisms of drug-induced cell death in four cell lines with the t(11;14) and in primary cells from 10 patients with MCL. Mitoxantrone, a topoisomerase II inhibitor, induced a strong cytotoxic effect in three cell lines (JVM-2, REC-1, and Granta 519), and in primary MCL cells. This cytotoxic effect due to apoptosis induction was observed despite the presence of either p53 or ATM abnormalities. However, no cytotoxic effect was detected after incubation with DNA-damaging agents in the NCEB-1 cell line, carrying p53 and ATM alterations, despite the presence of functional mitochondrial machinery. These results support that mitoxantrone can be effective in the treatment of MCL but that this activity requires the integrity of functional DNA-damage response genes.
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Introduction

Mantle cell lymphoma (MCL) is a lymphoproliferative disorder derived from a subset of naive pregerminal center cells with a mature B-cell phenotype and coexpression of CD5 (Swerdlow *et al*., 2001; Campo, 2003). MCL is characterized by the chromosomal translocation t(11;14)(q13;q32) that results in cyclin D1 overexpression (Bosch *et al*., 1994; Campo *et al*., 1999; Bertoni *et al*., 2004) with the consequent deregulation of cell cycle control at the G_1–S checkpoint. In addition to classical MCL, a blastoid variant of the disease has been described, which is characterized by high mitotic rate and that is associated with *INK4a/ARF* deletions, *p53* mutations, and complex karyotypes (Hernandez *et al*., 1996; Pinyol *et al*., 1997; Schlegelberger *et al*., 1999).

MCL is characterized by an aggressive clinical course and poor response to conventional therapy due to either rapid relapse after an initial response or primary resistance to drugs (Argatoff *et al*., 1997; Bosch *et al*., 1998). The DNA damage response pathway plays a crucial role in the pathogenesis and clinical behavior of tumoral cells. The ataxia-telangiectasia-mutated (*ATM*) gene codifies for a protein involved in p53 phosphorylation in response to DNA damage, thus promoting cell cycle arrest and apoptosis (Banin *et al*., 1998). *ATM* and *p53* alterations are associated with drug resistance in lymphoproliferative disorders. *ATM* inactivation is commonly observed in MCL patients (Camacho *et al*., 2002) and *p53* alterations are associated with blastoid variants (Hernandez *et al*., 1996; Fang *et al*., 2003). The mechanisms of resistance to chemotherapy in MCL are poorly understood. Experimental evidences demonstrate that most of the chemotherapeutic agents currently used exert their cytotoxic effects by inducing apoptosis. Moreover, recently it has been proposed that alterations in the apoptotic pathways may contribute to the development of MCL (Hofmann *et al*., 2001; Martinez *et al*., 2003).

The aim of this study was to analyse the mechanisms of drug-induced apoptosis in MCL. Towards this end, four cell lines carrying the t(11;14)(q13;q32), as well as primary MCL cells, were incubated *in vitro* with several drugs currently used in the treatment of lymphoproliferative disorders and drug-induced cell death was characterized.

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Results

Cytotoxic effect of fludarabine, mafosfamide, and mitoxantrone on cell lines

JVM-2, REC-1, Granta 519, and NCEB-1 cell lines were incubated for 24 and 48 h with different concentrations of fludarabine (1–10 μg/ml), mafosfamide (1–10 μg/ml), and mitoxantrone (0.05–0.5 μg/ml), and the cytotoxic effect of these drugs was measured by annexin V-FITC/PI staining. The LD_{50} for mitoxantrone, fludarabine, and mafosfamide is shown in Table 1. Mitoxantrone induced the strongest cytotoxic effect in JVM-2, REC-1, and Granta 519 cell lines at concentrations lower than those achieved with the dose currently used in clinical practice (0.5 μg/ml). In contrast, pharmacological doses of fludarabine (1 μg/ml) and mafosfamide (1 μg/ml) were not able to induce a significant cytotoxicity on any of these cell lines. REC-1 was the unique cell line for which the LD_{50} for mafosfamide was less than 10 μg/ml whereas the LD_{50} for fludarabine was lower than 10 μg/ml only for Granta 519. No cytotoxic effect was observed after incubation of NCEB-1 cells with mitoxantrone, fludarabine, or mafosfamide, even when higher doses of these drugs were used (data not shown).

To study the possible additive or synergistic effect of these drugs, cells from the four cell lines were incubated during 24 h with 0.25 μg/ml of mitoxantrone, in the presence or absence of 1 μg/ml of fludarabine and/or 1 μg/ml of mafosfamide. Although concentrations of fludarabine and mafosfamide corresponded to those used in the FCM regimen (fludarabine + cyclophosphamide + mitoxantrone) (Bellosillo *et al*., 1999), mitoxantrone concentration was reduced from 0.5 to 0.25 μg/ml due to the massive cytotoxic effect observed at 0.5 μg/ml. As shown in Granta 519 cell line (Figure 1), none of these combinations resulted in a significant increase of cytotoxicity to that observed with mitoxantrone alone. The same results were detected in JVM-2 and REC-1 cell lines (data not shown).

Drug-induced apoptosis on MCL cell lines

In order to determine whether the cytotoxic effect was due to induction of apoptosis, the role of caspases and the proteolytic cleavage of PARP were analysed. Cells from the four cell lines were incubated with 10 μg/ml fludarabine and 0.25 μg/ml mitoxantrone. The decrease on cell viability correlated with loss of $\Delta\Psi_m$, and with the detection of the active form of caspase-3 in Granta 519 (Figure 2a), JVM-2, and REC-1 cell lines. These results demonstrated that drug cytotoxicity was due to apoptosis induction. No changes in $\Delta\Psi_m$ or activation of caspase-3 were observed in NCEB-1 cells (Figure 2b).

To study the mechanisms leading to the activation of caspase-3, the proteolysis and subsequent activation of caspase-9 and -8 were analysed. Mitoxantrone and fludarabine induced processing of both proteases, as shown by the decrease of the procaspase-9 and -8 and the appearance of the intermediate cleavage product (43/41 kDa) of caspase-8. Activation of caspase-3 was also accompanied by proteolysis of PARP (Figure 2c). No activation of caspase-9, -8 or -3 or cleavage of PARP were detected in NCEB-1 cells (Figure 2d).

Apoptosis-related proteins in drug-induced cell death

Western blot analysis revealed that MCL cell lines expressed Bcl-2, Bax, Bak, Bcl-X_L, and Mcl-1 proteins. After incubation with 10 μg/ml fludarabine and 0.25 μg/ml mitoxantrone, no changes in the overall protein levels of Bcl-2, Bax, and Bak were detected, whereas downregulation of Mcl-1 was observed in the sensitive cell lines. Furthermore, a minor decrease on Bcl-X_L expression was also detected. We also determined the behavior of some IAP (*Inhibitor of Apoptosis Proteins*) family members. A downregulation of XIAP protein was observed after drug incubation of sensitive cell lines, whereas no changes in the survivin levels were detected. No downregulation of any Bcl-2 family member or IAP protein was observed in NCEB-1 cell line (Figure 3a).

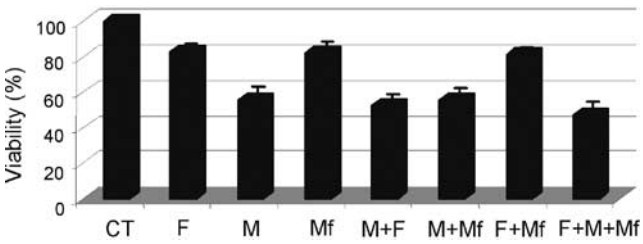

Figure 1 Cytotoxic effect of the combination of mitoxantrone with fludarabine and/or mafosfamide. Cells from Granta 519 cell line were incubated for 24 h in the absence of any factor (CT), with mitoxantrone alone (M; 0.25 μg/ml), and in combination with fludarabine (F; 1 μg/ml), and/or mafosfamide (Mf; 1 μg/ml). Cell viability was determined by annexin V binding as described in Materials and methods. Data are shown as the mean value ± s.d. of triplicate cultures

Table 1 LD_{50} for mitoxantrone (M), fludarabine (F), and mafosfamide (Mf) in MCL cell lines

	Drug (μg/ml)[a]			
	M	*F*	*Mf*	*p53/ATM status*[b]
JVM-2	0.13 ± 0.044	28.31 ± 18.7	13.57 ± 1.44	wt/wt
REC-1	0.17 ± 0.025	12.41 ± 4.93	5.48 ± 0.64	wt/wt
GRANTA 519	0.23 ± 0.004	8.55 ± 4.46	12.42 ± 3.31	wt/del
NCEB-1	NR[c]	NR[c]	NR[c]	del/del

[a]Data are shown as the LD_{50} for each drug (mean ± s.d.) [b]*p53* and *ATM* status assessed by FISH [c]NR: not reached

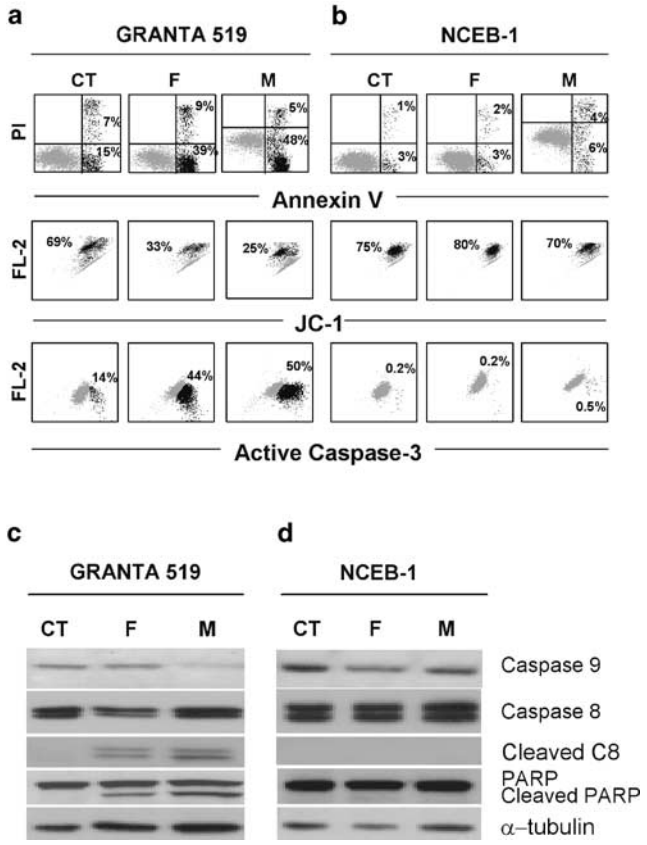

Figure 2 Activation of mitochondrial apoptotic pathway during drug-induced cell death. Cells from Granta 519 (**a**) and NCEB-1 (**b**) cell lines were incubated in the absence (CT) or presence of fludarabine (F; 10 μg/ml) or mitoxantrone (M; 0.25 μg/ml) for 24 hours. Cell viability was determined by annexin V binding. Changes in $\Delta\Psi_m$ were assessed by staining with JC-1. Active form of caspase-3 was quantified by anti-active caspase-3 immunostaining. In mitoxantrone-treated cells, a shift in the signal of fluorescence 3 (630 nm) was observed owing to the incorporation of this drug. The percentage of positive cells is indicated in each panel and represents cells undergoing apoptosis as assessed by the different methods of analysis. Whole-cell lysates were obtained from 2×10^6 cells from Granta 519 (**c**) and NCEB-1 (**d**) in the same conditions, and analysed by Western blot. Blots were also probed with α-tubulin antibody as an internal control

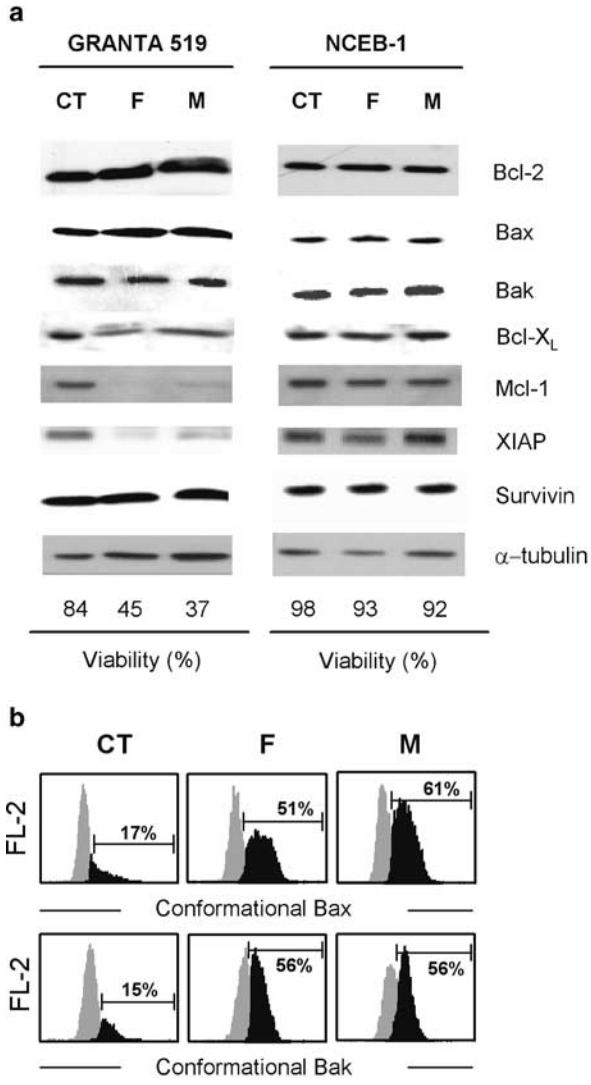

Figure 3 Apoptosis-related proteins in drug-induced apoptosis. **(a)** Whole-cell lysates were obtained from 2×10^6 cells from Granta 519 and NCEB-1 cell lines incubated for 24 h in the absence (CT) or presence of fludarabine (F; 10 μg/ml) or mitoxantrone (M; 0.25 μg/ml), and analysed by Western blot. Blots were also probed with α-tubulin antibody as an internal control. Cell viability is indicated as the percentage of annexin V negative cells analysed by flow cytometry. (**b**) Cells from Granta 519 cell line were incubated for 24 h in the absence (CT) or presence of fludarabine (F; 10 μg/ml) or mitoxantrone (M; 0.25 μg/ml). Bax and Bak conformational changes were determined by staining with anti-Bax and anti-Bak antibodies and flow cytometry analysis, as described in Material and methods. The percentage of positive cells is indicated in each panel

Involvement of Bax and Bak proteins in drug-induced apoptosis was also analysed using anti-Bax and -Bak antibodies directed against the NH_2-terminal region of both proteins. This region is occluded in unstressed intact cells and it is only available for binding to these antibodies following conformational changes of these proteins during the apoptotic process. An increase in the number of Bax and Bak-positive cells was detected in Granta 519 (Figure 3b), JVM-2, and REC-1 cells after incubation with 10 μg/ml fludarabine and 0.25 μg/ml mitoxantrone. No changes in Bax and Bak conformation were observed in NCEB-1 cell line after incubation with these drugs.

Preincubation of Granta 519 cells with 200 μM Z-VAD.fmk, a broad inhibitor of caspases, reversed drug-induced phosphatidilserine exposure, loss of $\Delta\Psi_m$, and caspase-3 activation, thus confirming the role of caspases in drug-induced cell death. In contrast, Bax and Bak conformational changes were observed despite inhibition of the caspase cascade, suggesting that these conformational changes occur upstream of the caspase activation or in a caspase-independent manner (data not shown).

Cell cycle analysis after drug exposure

The effect of mitoxantrone and fludarabine on cell cycle distribution and DNA content was analysed.

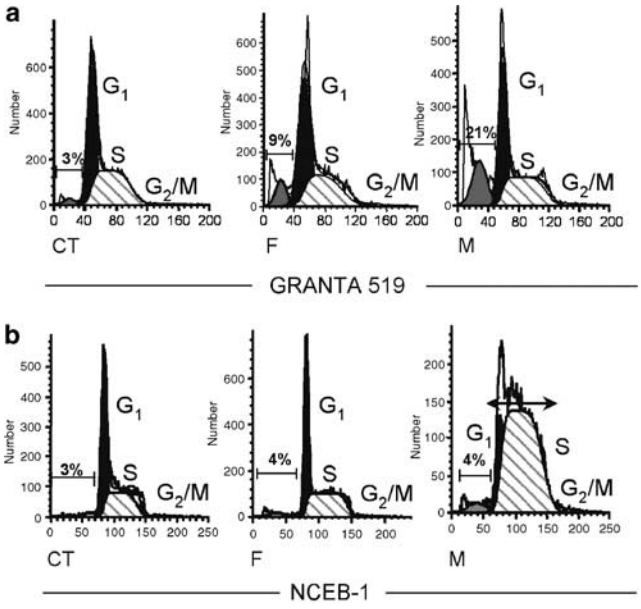

Figure 4 DNA content analysis after drug exposure. Cells from Granta 519 **(a)** and NCEB-1 **(b)** were incubated for 24 h with medium alone and in the presence of fludarabine (F; 10 μg/ml) or mitoxantrone (M; 0.25 μg/ml). DNA content was quantified by PI staining and flow cytometry analysis as described in Material and methods. NCEB-1 cell line displays a 4n DNA content, according to the nearly tetraploid karyotype observed by cytogenetic analysis

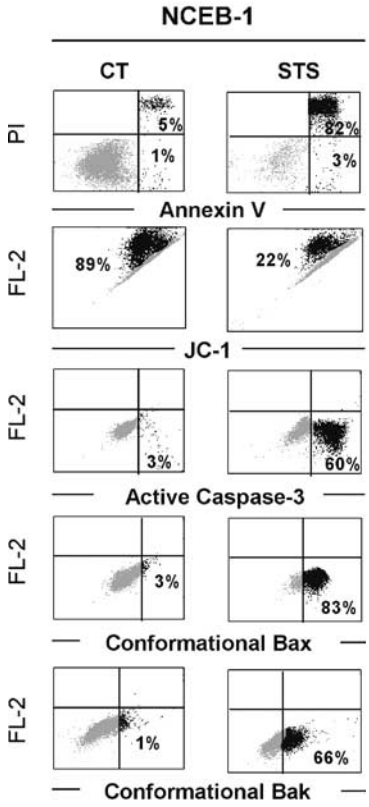

Figure 5 Staurosporine-induced apoptosis in NCEB-1 cells. Flow cytometry analysis of cell viability by annexin V binding, loss of $\Delta\Psi_m$ by JC-1 staining, active form of caspase-3, and conformational changes of Bax and Bak of a representative experiment. Cells from NCEB-1 cell line were incubated with medium alone (CT) or in the presence of staurosporine (STS; 5 μM) for 24 h. The percentage of positive cells is indicated in each panel. All experiments were performed in triplicate

A sub-G_0/G_1 subpopulation corresponding to apoptotic cells was detected in Granta 519 (Figure 4a), JVM-2, and REC-1 cell lines, and progressively increased after 48–72 h incubation. NCEB-1 cells showed a 4n DNA content in agreement with the nearly tetraploid karyotype observed by cytogenetic analysis. After incubation with mitoxantrone, NCEB-1 exhibited a marked arrest in the transition from S phase to G_2–M without apoptotic peak in the sub G_0/G_1 region, even when cell cycle analysis was performed at longer periods of time (48 and 72 h). No changes in the cell cycle pattern were detected after incubation of NCEB-1 cells with fludarabine (Figure 4b).

Staurosporine-induced apoptosis in NCEB-1 cells

To ascertain if the apoptotic machinery was functional in NCEB-1 cell line, cells were incubated with staurosporine, an inducer of mitochondrial apoptotic pathway (Xue *et al*., 2003). After 24 h of drug exposure, a cytotoxic effect in NCEB-1 cells was observed. This effect was accompanied by loss of $\Delta\Psi_m$, activation of caspase-3, and conformational changes of Bax and Bak, demonstrating an efficient mitochondrial apoptotic machinery in this cell line (Figure 5).

Drug-induced apoptosis in cells from MCL patients

Tumoral cells from 10 MCL patients were incubated with mitoxantrone, mafosfamide, and fludarabine at the previously mentioned concentrations. The characteristics of these patients are summarized in Table 2. The 17p deletion by FISH and consequently *p53* mutations were detected in three cases. The deletion of *ATM* was detected in three additional patients. No cases lacking both *p53* and *ATM* were found. Pharmacological concentrations of mitoxantrone induced the strongest cytotoxic effect, being the median LD_{50} for all the patients 0.37 μg/ml (range 0.10–0.76 μg/ml). A significant decrease in cell viability was also observed after incubation with mafosfamide 1 μg/ml ($P = 0.03$), although this effect was due to the cytotoxicity observed in only three out of the 10 MCL patients. The median LD_{50} was 5.70 μg/ml (range 2.6–11.5 μg/ml). No cytotoxic effect was observed after incubation with fludarabine for 24 h, and only in one patient a significant cytotoxicity was detected after 48 h of incubation with high doses of this drug (5–10 μg/ml) (data not shown).

The combination of pharmacological doses of mitoxantrone (0.5 μg/ml) and mafosfamide (1 μg/ml) had a significant additive effect ($P = 0.04$) (Figure 6). However, using the method of Chou and Talalay (1984) this additive effect was only observed in cells from three MCL patients. No additive or synergistic effect was detected after the addition of fludarabine to this combination. The cytotoxic effect was accompanied by loss of $\Delta\Psi_m$, activation of caspase-9, -8, and -3, PARP

Table 2 Characteristics of MCL patients

Patient no.	*Disease status*	*Cell source*[a]	*Morphologic variant*[b]	*% Tumoral cells*	*p53 status*[c]	*ATM status*[d]	*Mitoxantrone LD_{50} (μg/ml)*
1	Diagnosis	PB	C	95	wt	Deleted	0.61
2	Diagnosis	PB	C	85	wt	wt	0.76
3	Diagnosis	Spleen	C	95	wt	Deleted	0.33
4	Diagnosis	PB	C	84	wt	Deleted	0.37
5	Relapse	PB	C	95	CGT→CAT Codon 273	wt	0.38
6	Diagnosis	Spleen	C	80	wt	wt	0.29
7	Diagnosis	PB	C	86	wt	wt	0.49
8	Relapse	PB	C	90	CGC→CCC Codon 72	wt	0.37
9	Relapse	PB	B	70	wt	wt	0.33
10	Relapse	Lymph nod	B	99	Mutated[e]	wt	0.10

[a]Source of the cells used for the *in vitro* analysis. PB: peripheral blood. [b]C: classical, B: blastoid. [c]*p53* status assessed by FISH and mutational status analysed by SSCP and sequencing. [d]*ATM* status assessed by FISH. [e]Positive *p53* staining by inmunohistochemistry

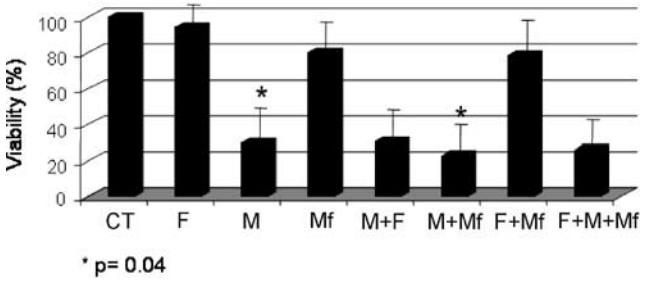

Figure 6 Cytotoxic effect of fludarabine, mitoxantrone, and mafosfamide alone or in combination in primary MCL cells. Lymphocytes from 10 MCL patients were incubated at 37°C for 24 h in the absence of any factor (CT), with pharmacological doses of fludarabine (F; 1 μg/ml), mitoxantrone (M; 0.5 μg/ml), and mafosfamide (Mf: 1 μg/ml), or with the combination of these drugs. Cell viability was determined by annexin V binding as described in Material and methods and is expressed as the percentage with respect to the viability of cells in medium alone at the beginning of the culture. Data are shown as the median value ± range

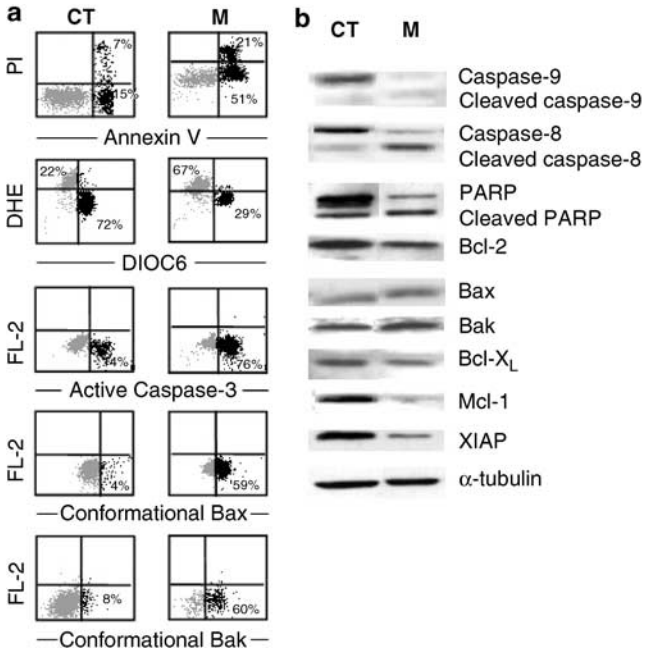

Figure 7 Drug-induced apoptosis in MCL primary cells. **(a)** Cells from a representative MCL patient were incubated with medium alone (CT) or in the presence of mitoxantrone (M; 0.5 μg/ml) for 24 h. Flow cytometric dot plots show cell viability as determined by annexin V binding, loss of $\Delta\Psi_m$ by DiOC6 staining, quantification of active form of caspase-3, and Bax and Bak conformational changes, as described in Material and methods. In mitoxantrone-treated cells, a shift in the signal of fluorescence 3 (630 nm) was observed owing to the incorporation of this drug. The percentage of positive cells is indicated in each panel. **(b)** Whole-cell lysates were obtained from 2×10^6 cells from the same patient incubated in the presence or absence (CT) of mitoxantrone (M; 0.5 μg/ml) for 24 h and analysed by Western blot. Blots were also probed with α-tubulin as an internal control

proteolysis, and conformational changes of Bax and Bak. Furthermore, downregulation of Mcl-1, Bcl-X_L, and XIAP was detected, whereas no changes in the overall protein levels of Bcl-2, Bax, and Bak were observed (Figure 7).

Discussion

MCL is an aggressive lymphoproliferative disorder highly resistant to currently available therapy. In fact, only few patients achieve a complete response and in most cases the prognosis is very poor (Argatoff *et al*., 1997; Bosch *et al*., 1998). Although the current therapy is based on the combination of several drugs, no data regarding the effect of conventional cytotoxic drugs alone or in combination on MCL primary cells have previously been reported. In this study, the cytotoxic effect of different drugs used in the treatment of lymphoproliferative disorders has been analysed in cells from MCL patients, as well as in cell lines carrying the t(11;14)(q13;q32).

Our results demonstrate that mitoxantrone, an inhibitor of topoisomerase II, exerts the highest cytotoxic effect in three MCL cell lines (JVM-2, REC-1, and Granta 519) as well as in primary MCL cells from all patients tested. The LD_{50} in these cases was lower than that previously observed in primary CLL cells (Bellosillo *et al*., 1998).

Topoisomerase II inhibitors may act by interfering with the religation activity of the enzyme to DNA double-strand breaks. Several cell lines display natural resistance to mitoxantrone which is independent of drug transport and drug-target alterations (Bailly *et al*., 1997). Furthermore, low expression of topoisomerase II is one of the mechanisms of resistance to topoisomerase II inhibitors (De Isabella *et al*., 1991; Rasheed and Rubin, 2003). Thus, mitoxantrone rapidly induced apoptosis in sensitive cells, whereas it produced a G_2–M block in resistant ones. In agreement with these results, cell cycle analysis of NCEB-1 cell line revealed a G_2–M arrest in mitoxantrone- but not in fludarabine- or mafosfamide-treated cells. Recently, it has been described that topoisomerase II is one of the proliferation-related genes in MCL cells (Rosenwald *et al*., 2003) and that topoisomerase-IIα expression is a prognostic factor for clinical outcome in MCL (Schrader *et al*., 2004). In this regard, our results demonstrate a high cytotoxic effect in MCL cell lines and primary cells from blastic MCLs, accordingly to their proliferative index.

Fludarabine, alone or in combination, has demonstrated efficacy in the treatment of CLL and follicular lymphoma (Solal-Celigny *et al*., 1996; Flinn *et al*., 2000; Bosch *et al*., 2002). However, in our experience high doses of fludarabine were necessary to induce a minor cytotoxic effect on MCL cells *in vitro*. Recently, a case of a patient with MCL resistant to fludarabine has been reported, due to a deficient fludarabine uptake (Reiman *et al*., 2002). In this regard, a relationship between *in vitro* sensitivity to fludarabine and drug uptake via equilibrative transport system has also been described in primary CLL cells (Molina-Arcas *et al*., 2003).

In primary cells from three MCL patients, the combination of pharmacological doses of mitoxantrone and mafosfamide produced an additive cytotoxic effect. We had previously demonstrated that, in CLL cells, the combination of fludarabine with mafosfamide produced a significant synergistic effect, whereas the addition of mitoxantrone to this combination induced a significant increase in cytotoxicity only in previously treated CLL patients (Bellosillo *et al*., 1999). The mechanism of the additive effect between mafosfamide and mitoxantrone in B-cell lymphoproliferative disorders could be related to the primary DNA damage produced by mafosfamide and the inability of the cellular machinery to repair it due to the mitoxantrone-mediated inhibition of topoisomerase II.

The present study demonstrates that cytotoxic drugs exert their effect by activating the mitochondrial apoptotic pathway in MCL cells. Thus, these drugs induced loss of mitochondrial transmembrane potential and conformational changes of Bax and Bak that triggered caspase-9 activation and apoptosis. Activation of caspase-8 was also observed and it could be explained by the occurrence of a loop involving caspase-9 and -3 or due to the involvement of other caspases upstream of mitochondria (Slee *et al*., 1999). Inhibition of caspase activity using the pan-caspase inhibitor Z-VAD.fmk abolished the cleavage of caspase-3 and restored cell viability indicating that genotoxic-induced cell death in MCL cells depends on caspase activity. As previously described (Bellosillo *et al*., 2002), conformational changes of Bax and Bak were completely independent on caspase activation.

The Bcl-2 family of proteins plays a central role in controlling the apoptosis mitochondrial pathway (Cory and Adams, 2002; Cory *et al*., 2003). In primary MCL cells as well as in MCL cell lines, conformational changes of Bax and Bak were detected after drug treatment and it was also accompanied by downregulation of Mcl-1 and Bcl-X_L. In this regard, downregulation of Mcl-1 has been described in CLL cells after several drug treatments (Bellosillo *et al*., 1999) and, recently, a downregulation of Bcl-X_L after treatment of MCL cells with NF-κB inhibitors has also been reported (Pham *et al*., 2003). Furthermore, high expression of the antiapoptotic proteins Bcl-2, Mcl-1, and Bcl-X_L in NHL has been associated with chemotherapy resistance (Khoury *et al*., 2003; Zhao *et al*., 2004). We have also detected a downregulation of XIAP, a member of the IAPs that inhibits the activity of caspases.

NCEB-1 was the unique cell line in which no cytotoxic effect was observed after incubation with genotoxic drugs, even when higher drug concentrations, longer incubation periods, and combination therapy were employed. In contrast, incubation of NCEB-1 cells with staurosporine induced the typical features of apoptosis, demonstrating that the mitochondrial apoptotic machinery is functional in NCEB-1 cells. The lack of response to genotoxic conventional drugs might be explained by the alteration of upstream regulators of Bcl-2 family proteins and/or in DNA damage response genes. In this regard, NCEB-1 cells showed a complex karyotype that included alterations in *p53* and *ATM*. These anomalies have also been found in patients with CLL and MCL and are associated with therapy failure and shorter survival (Wattel *et al*., 1994; Dohner *et al*., 1995; Hernandez *et al*., 1996; Camacho *et al*., 2002).

Apoptosis induced by genotoxic drugs is accompanied by the stabilization of p53 after its phosphorylation by ATM (Banin *et al*., 1998; Stankovic *et al*., 2004). Since p53-mediated apoptosis is thought to underlie the cytotoxicity of most genotoxic drugs, and *ATM* mutations represent another potential mechanism of drug resistance, the simultaneous dysfunction of both *p53* and *ATM* might explain the failure in the induction of apoptosis in the NCEB-1 cell line. In accordance with this, no apoptosis was observed by topoisomerase I and II agents in *p53*-null mouse embryonic fibroblast with no *ATM* function (Fedier *et al*., 2003). Recently, and in agreement with our results, an MCL cell line with alterations in both *p53* and *ATM* genes was completely resistant to apoptotic cell death although was highly sensitive to *in vitro* radiation. In contrast, a MCL cell line with only *p53* mutation underwent apoptosis (M'kacher *et al*., 2003).

Our results demonstrate that MCL cells have a functional mitochondrial apoptotic machinery and suggested that cells lacking both ATM and p53, but not cells lacking one of these proteins, are resistant to genotoxic apoptotic stimuli. Finally, these *in vitro*

studies provide experimental support for including mitoxantrone in clinical trials.

Materials and methods

Cell lines

Cell lines carrying the t(11;14)(q13;q32) translocation were studied: Granta 519, NCEB-1, and REC-1 cell lines, all derived from MCL patients, and the JVM-2 cell line, derived from a B-prolymphocytic leukemia. Granta 519 and JVM-2 cell lines were purchased from the *DSMZ-German Collection of Microorganisms and Cell Cultures* (Braunschweig, Germany). NCEB-1 and REC-1 cell lines were kindly provided by Dr Niels S Andersen (Department of Hematology, Rigshospitalet, Copenhagen, Denmark) and Dr Christian Bastard (Department of Hematology, Centre Henri Becquerel, Rouen, France), respectively. *p53* mutations were detected in NCEB-1 cell line (Bogner *et al*., 2003) and 11q alterations involving *ATM* in Granta 519 (Vorechovsky *et al*., 1997) and NCEB-1. No ATM protein expression was detected by Western blot in these two cell lines (data not shown).

Patients

Ten patients diagnosed with MCL according to the World Health Organization classification (Swerdlow *et al*., 2001) who had not received treatment for the previous 3 months were studied. Tumoral cells were obtained from peripheral blood in seven patients, splenic tissue in two, and lymph node in one. The percentage of malignant cells CD19+, CD5+, CD23−, and showing light chain restriction were analysed by flow cytometry. Cyclin D1 overexpression was demonstrated in all cases by immunohistochemistry. An 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 by Ficoll/Hypaque sedimentation (Seromed, Berlin, Germany). Tumoral cells were obtained after squirting lymph node or spleen biopsies with RPMI 1640 culture medium using a fine needle. Cells were either used directly or cryopreserved in liquid nitrogen in the presence of 10% dimethyl sulfoxide and 20% heat-inactivated fetal calf serum (Gibco BRL, Paisley, Scotland). Manipulation due to freezing/thawing did not influence cell response.

Cell culture

JVM-2, REC-1, Granta 519, and NCEB-1 cell lines (0.5×10^6/ml). and mononuclear cells from patients with MCL (1 to 5×10^6/ml) were cultured in RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine and 50 μg/ml penicillin–streptomycin, at 37°C in a humidified atmosphere containing 5% carbon dioxide. Cells were incubated for 24–48 h with fludarabine monophosphate (Schering; Berlin, Germany), mitoxantrone (Lederle Laboratories; Gosport, Hampshire, UK), mafosfamide, the active form of cyclophosphamide (ASTAMedica; Frankfurt, Germany), and staurosporine (Sigma Chemicals; St Louis, MO). When Z-VAD.fmk (*N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone) (Bachem; Bubendorf, Switzerland) was employed, cells were preincubated for 1 h prior to the addition of drugs.

Antibodies

Monoclonal and polyclonal antibodies against active caspase-3, and Bax (BD-Pharmingen, San Diego, CA, USA); caspase-8, Bak (Ab-1), p53 (Ab-2), ATM (Ab-3) and α-tubulin (Oncogene Research, Boston, MA, USA); caspase-9 (New England Biolabs Inc., Beverly, MA, USA); poly-adenosine diphosphate ribose polymerase (PARP) (Roche Diagnostics, Mannheim, Germany); Bcl-2 antibody (DAKO, Glostrup, Denmark); Mcl-1 (S-19) (Santa Cruz Biotechnology, CA); XIAP (Transduction Laboratories, Lexington, UK); survivin (Novus Biologicals, Littelton, CO, USA) and Bax (clone YTH-6A7) (Trevigen, Gaithersburg, MD, USA) were employed.

Cell viability by annexin V binding

Exposure of phosphatidylserine residues was quantified by surface annexin V staining as previously described (Bellosillo *et al*., 2002). Experiments were performed in triplicate. LD_{50} was defined for each drug as the concentration of drug required to reduce a 50% the cell viability.

Mitochondrial transmembrane potential ($\Delta\Psi_m$) and reactive oxygen species

Changes in $\Delta\Psi_m$ were evaluated by staining with 1 nM 3,3′-diexyloxacarbocyanine iodide ($DiOC_6[3]$) (Molecular Probes, Eugene, OR, USA) or with 1.5 mM 5,5′,6,6′-tetrachloro-1,1′3,3′-tetraethilbenzimidazolylcarbocyanine iodide (JC-1) (Molecular Probes). Reactive oxygen species (ROS) production was determined by staining with 2 μM dihydroethidine (DHE) (Molecular Probes). Cells were incubated with dyes for 30 min at 37°C, washed, resuspended in PBS, and analysed by flow cytometry (Bellosillo *et al*., 2001). A total of 10 000 cells per sample were acquired in a FACScan flow cytometer. Experiments were performed in triplicate.

Detection of intracellular proteins

Cells were fixed and permeabilized as previously described (Bellosillo *et al*., 2002). Cells were stained with antibodies against the active form of caspase-3, Bax ($0.25\,\mu g/ml \times 10^6$ cells), and Bak ($2.5\,\mu g/ml \times 10^6$ cells) for 20 min at room temperature, followed by goat anti-rabbit-FITC (SuperTechs, Bethesda, MD, USA) or goat anti-mouse-FITC (DAKO), and analysed in a FACScan.

Western blot analysis was performed as previously described (Bellosillo *et al*., 2002). Antibody binding was detected using a chemiluminiscence (ECL) detection system (Amersham, Buckinghamshire, UK). Equal protein loading was confirmed with α-tubulin blots.

Cell cycle analysis

Cells were fixed in 80% ethanol for 5 min at 5°C, centrifuged, and washed twice in PBS. Cells were incubated for 15 min at room temperature in a citrate–phosphate buffer (1:24), centrifuged, resuspended in 0.25 ml of PBS containing PI (5 μg/ml) and Ribonuclease A (100 μg/ml) (Sigma Chemicals, St Louis, MO, USA), and incubated for 10 min in the dark (Nicoletti *et al*., 1991). The percentage of cells in G_0/G_1, S, G_2–M, and the presence of a sub-G_0/G_1 peak were evaluated with ModFit LT (Verity Software House, Inc., Topsham, MA, USA).

Fluorescence in situ *hybridization*

Fluorescence *in situ* hybridization (FISH) was performed on fixed cells from the NCEB-1 cell line and from 10 patients with MCL. The LSI *p53* (17p13) SpectrumOrange-labeled and LSI

ATM (11q22.3) SpectrumGreen-labeled probes (Vysis, Downers Grove, IL) were tested. Two different observers scored 200 nuclei. A true deletion was considered when more than 5% of nuclei showed one hybridization signal. This threshold was established using fixed material from 10 normal subjects.

p53 molecular studies

p53 mutational analysis was performed in nine MCL patients. Exons 4–8 were amplified by PCR. Single-stranded conformational polymorphism (SSCP) analysis and direct sequencing were performed as previously described (Pinyol *et al*., 2000).

Statistical analysis

Comparisons were performed by using the Mann–Whitney test as appropriate. A P-value <0.05 was considered to be statistically significant.

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ARTÍCULO 2

LA EXPRESIÓN DEL TRANSPORTADOR EQUILIBRATIVO DE NUCLEÓSIDOS-2 (hENT2) CORRELACIONA CON LA SENSIBILIDAD *EX VIVO* A LA FLUDARABINA EN CÉLULAS DE LEUCEMIA LINFÁTICA CRÓNICA B (LLC-B)

Objetivo

Analizar el papel de las proteínas hENT1 y hENT2 en el transporte y la citotoxicidad de fludarabina en células de LLC-B.

Resumen

La fludarabina es uno de los fármacos más utilizados en el tratamiento de la leucemia linfática crónica de tipo B (LLC-B). Sin embargo, algunos casos presentan resistencia a este fármaco. La fludarabina es un análogo de las purinas que debe entrar en el interior de las células para ejercer su efecto. En este trabajo hemos analizado el papel de los transportadores de nucleósidos hENT1 y hENT2 de la membrana plasmática de las células en el efecto citotóxico de la fludarabina. La efectividad de la terapia basada en la utilización de análogos de nucleósidos depende tanto del transporte del análogo al interior de la célula como de la metabolización del fármaco. Se han descrito dos familias de genes implicados en el transporte de estos análogos, SLC28 y SLC29 (*solute carrier families 28 and 29*), que codifican para transportadores concentrativos (CNT) y equilibrativos (ENT), respectivamente (Baldwin SA et al, 2004; Gray JH et al, 2004).

Células primarias de LLC-B expresan los mRNA de hCNT2, hCNT3, hENT1 y hENT2, sin embargo, estudios de transporte de nucleósidos naturales y de fludarabina al interior de las células de LLC-B, demostraron que este transporte era básicamente de tipo equilibrativo (hENT) (Molina-Arcas M et al, 2003). Los niveles de expresión de mRNA de hENT1 y hENT2 no correlacionaban con la sensibilidad *ex vivo* a la fludarabina. Estos mRNA pueden estar sometidos a regulaciones transcripcionales y postranscripcionales que influyan en la falta de correlación. La utilización de anticuerpos monoespecíficos policlonales nos ha permitido detectar una correlación entre la expresión de la proteína hENT2, por Western blot, y el transporte de fludarabina al interior de las células y la sensibilidad *ex vivo* de las células de LLC-B. Estos resultados nos sugieren que la proteína hENT2 desempeña un importante papel en la respuesta de la LLC-B a la fludarabina.

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Equilibrative nucleoside transporter-2 (hENT2) protein expression correlates with *ex vivo* sensitivity to fludarabine in chronic lymphocytic leukemia (CLL) cells

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Fludarabine is considered the treatment of choice for most patients with chronic lymphocytic leukemia (CLL). We have analyzed the role of plasma membrane transporters in nucleoside-derived drug bioavailability and action in CLL cells. Among the known plasma membrane transporters, we have previously observed a significant correlation between fludarabine uptake via ENT carriers and *ex vivo* sensitivity of CLL cells to fludarabine, although mRNA amounts of the equilibrative nucleoside transporters hENT1 and hENT2 do not show any predictive response to treatment. In this study, using polyclonal monospecific antibodies we have observed a significant correlation between the expression of hENT2 by Western blot and fludarabine uptake via hENT carriers and also with *ex vivo* sensitivity of CLL cells to fludarabine. These results suggest that the equilibrative nucleoside transporter hENT2 plays a role in fludarabine responsiveness in CLL patients.

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Introduction

B-cell chronic lymphocytic leukemia (CLL) is characterized by the accumulation of long-lived, functionally inactive, mature appearing, neoplastic B lymphocytes.[1] The clonal excess of B cells is mainly caused by a decrease in cell death rather than by increased cell proliferation.[2] CLL is currently an incurable disease but several drug therapies, including the purine analog fludarabine, are now known to cause clinical improvement in CLL patients.[3] Several *in vitro* studies indicate that in CLL cells fludarabine induces apoptosis, suggesting that programmed cell death is the mechanism of their therapeutic action.[4–9] As CLL cells are predominantly quiescent cells, the proapoptotic activity of fludarabine could be due to inhibition of RNA synthesis or alteration of DNA repair. Both p53-dependent and -independent mechanisms have been described.[10,11]

Anticancer therapy using nucleoside-derived analogs is dependent on drug uptake and metabolic activation. Although some enzymes are suitable biomarkers of drug metabolism and may determine response to therapy,[12–14] the role of transporters in determining nucleoside-derived drug bioavailability is less well known. The uptake of these drugs into cells is mediated by one or more of the nucleoside transporters (NT), which have recently been cloned. These proteins belong to two nonrelated gene families, SLC28 and SLC29, encoding CNT (concentrative nucleoside transporters) and ENT (equilibrative nucleoside transporters) proteins, respectively.[15,16]

Leukemia cells appear to express both CNT- and ENT-type transporter mRNAs.[17] Primary CLL cells express hENT1, hENT2, hCNT2 and hCNT3. hCNT3 and hCNT2 are high-affinity Na^+-dependent NT showing broad selectivity and purine preference, respectively. hENT1 and hENT2 are broad-selectivity equilibrative carrier proteins, the former showing high sensitivity to pharmacological inhibition by the adenosine analog NBTI. Despite the apparent co-expression of at least these four transporter genes in CLL cells, most of the measurable natural nucleoside transport and all detectable fludarabine uptake into CLL cells rely on hENT-type carriers.[18]

Clinical correlations using the pattern of NT isoform expression have recently been obtained when monitoring NT occurrence in nearly 300 gynecological tumors using a tissue array approach. hENT1 and hENT2 expression was significantly retained in a high percentage of tumors, whereas hCNT1 expression was frequently reduced or lost,[19] particularly in some histotypes characterized by poor prognosis. Similarly, in a previous study using a small cohort of breast-cancer patients, only a few tumors were found to be negative for hENT1,[20] thus suggesting that, although hENT1 expression might show variability among tumors, a complete lack of hENT1 protein may be rare.

In hematological malignances, a significant correlation between mRNA levels and ara-C-induced cytotoxicity has been reported in acute myeloid leukemia.[21] Nevertheless, in CLL patients, although they show high mRNA levels for hENT1 and hENT2, mRNA amounts do not correlate with *ex vivo* sensitivity to fludarabine, whereas fludarabine influx does.[18] However, uptake measurements may not be a suitable tool to predict response to treatment in CLL because this technique is complex: it requires the use of radioactive-labeled drug, as well as a large number of cells. Since the closest measurable parameter to influx rates is the determination of transporter protein amounts, we have recently used characterized polyclonal monospecific antibodies to establish, firstly, whether protein levels associate with substrate influx and cytotoxicity, and secondly, which particular isoform can predict response to treatment.

Materials and methods

Patients

Cells from 21 patients with CLL who had not been previously treated with fludarabine were included in the study. The

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diagnosis was established according to the World Health Organization classification.[22] In all patients, informed consent was obtained at the time of diagnosis from Hospital Clinic.

Isolation and culture of cells

Mononuclear cells were isolated from blood using gradient centrifugation with Ficoll/Hypaque (Seromed, Berlin, Germany). These cells were more than 95% positive for CD19 and CD5, as assessed by flow cytometry. Cells were used either immediately or after thawing cryopreserved samples.

Cell viability assay

CLL lymphocytes (5×10^5 cells/well) were incubated in 96-well plates in the absence or presence of fludarabine (3, 7.5 and 15 μM) (Schering; Berlin, Germany) in a final volume of 100 μl. Cell viability was determined at 48 h by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as previously described.[4,18]

Western blot analysis

For the analysis of hENT1 expression, protein extracts were obtained as previously described.[23] Briefly, cells were lysed in 20 mM Tris (tris-hydroxymethyl-aminomethane)-HCl, pH 7.5; 0.3 M saccharose; 2 mM EGTA (ethylene glycol-bis-(β-amioethylether) *N,N,N′,N′*-tetraacetic acid); 2 mM EDTA (ethylenediaminetetraacetic acid); 10 mM 2-mercapto-ethanol; 1 mM PMSF; 0.01 mg/ml leupeptin; 0.5% aprotinin; 1% Nonidet P-40. After sonication three times for 10 s, cells were centrifuged at 14 000 rpm for 30 min at 4°C. The remaining pellets were lysed with the same buffer plus 0.15% Nonidet P-40 and 3 mM EGTA, incubated on ice for 30 min, sonicated again and centrifuged. Pellets were lysed in 80 mM Tris-HCl, pH 6.8; 2% sodium dodecyl sulfate (SDS); 10% glycerol; 0.1 M DTT (dithiothreitol). Protein extracts for hENT2 analysis were obtained using 10 mM Tris-HCl (pH 7.4), 0.5% Triton X-100, as described.[24] Proteins were separated on 10% SDS polyacrylamide gels, and transferred onto Immobilon-P (Millipore, Bedford, MA, USA) membranes.

Monospecific polyclonal antibodies against hENT1 and hENT2 have recently been characterized.[19] They were raised against isoform-specific domains at the intracellular loop between transmembrane domains 6 and 7. Following incubations with these primary antibodies against hENT1 and hENT2, proteins were detected with the use of 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).

Real-time quantitative RT-PCR

Total RNA was isolated using the guanidinium thiocyanate method (Ultraspec, Bioteck 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 using the Taqman reverse transcription reagents as previously described.[18] Real time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of hENT1, hENT2 and human β-glucoronidase (GUS) mRNA was performed as previously described.[18] Relative quantification of gene expression was performed as described in the TaqMan user's manual using GUS as an internal control.

Nucleoside transport

Nucleoside transport into CLL cells was measured using a rapid filtration method, as previously described.[18] [8-^{3}H]guanosine and [8-^{3}H]fludarabine (Moravek Biochemicals) were used as substrates at a concentration of 1 μM. Incubation time was 10 s.

Statistical analysis

Correlations between nucleoside transporter expression, nucleoside transport and fludarabine-induced cytotoxicity were analyzed using SPSS 10.0 software package (SPSS, Chicago, IL, USA). Significance of the correlation was assessed by Pearson test.

Results

hENT1 and hENT2 mRNA expression in patients with CLL

Figure 1 shows the amount of hENT1 (a) and hENT2 (b) mRNAs in cells from 21 CLL patients. The expression of each nucleoside transporter in one representative CLL patient was used as a relative calibrator and the expression levels of these cells were assigned the value of 1 as an arbitrary unit. Although a high variability in each transporter was detected, hENT1 mRNA expression showed highest variation than hENT2. Whereas

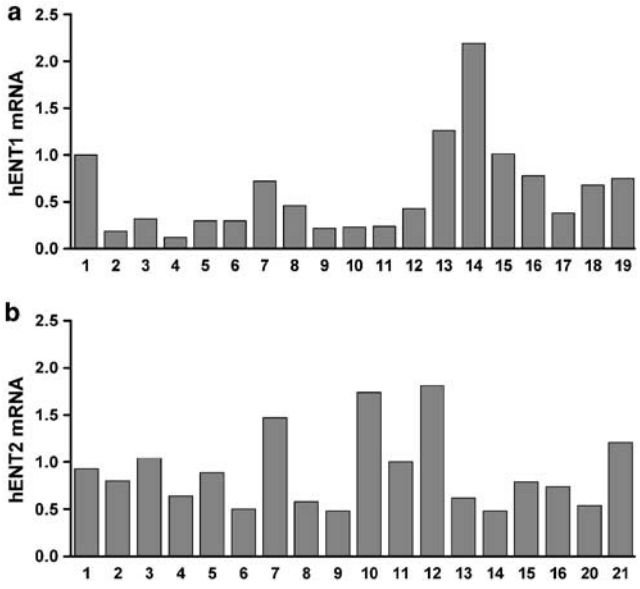

Figure 1 mRNA expression levels of nucleoside transporters in CLL cells. Normalized hENT1 (a) and hENT2 (b) mRNA expression levels in the cohort of CLL-patients analyzed. C_T values for each nucleoside transporter from CLL cDNA samples have been normalized to an endogenous reference gene (GUS). mRNA expression levels are given in arbitrary units, using one CLL cDNA sample as reference control.

hENT2 mRNA levels showed a range of variability of nearly four-fold, and hENT1 variability was 19-fold.

Equilibrative nucleoside transporter expression pattern in CLL cells

The expression of NT hENTs was analyzed by Western blot in CLL samples. As recently reported[19] these antibodies specifically recognized single bands of 50–55 kDa in CLL protein extracts. Figure 2 shows representative Western blots of five independent CLL samples in which hENT1 (a), hENT2 (b) and α-tubulin were analyzed. Antibodies against hENT1 and hENT2 consistently identified a single band of 50–55 kDa in all samples. Western blot using different quantities of protein showed that hENT1 and hENT2 densitometric signals were lineal up to 70 μg of loaded protein (data not shown).

Densitometric analysis of blot was carried out using Phoretix 1D Software. Semiquantitative analysis of hENT1 and hENT2 expression was achieved by calculating the densitometry ratios *vs* α-tubulin, in a range of protein concentrations in which densitometric signal has been previously shown to be linear. Similar results were obtained when actin was used as a constitutive control (not shown). The expression of hENT1 and hENT2 in one CLL case was used as a relative calibrator and the expression level of these cells were assigned the value of 1 as an arbitrary unit. Figure 3 shows the values of hENT1 and hENT2 for the whole cohort of CLL patients analyzed in this study. Contrary to the mRNA expression of hENT1 and hENT2, Western blots analysis show that inter-patient variation of hENT2 expression is 11-fold, whereas for hENT1 expression is three-fold.

Correlation of hENT expression with mRNA expression, transporter function and ex vivo *fludarabine cytotoxicity*

The amounts of hENT1 protein did not correlate significantly with hENT1 mRNA expression, determined by real time RT-PCR, or transporter function, or *ex vivo* fludarabine cytotoxicity (not shown). In contrast, although hENT2 protein did not show any significant relationship with hENT2 mRNA amounts in CLL patients (Figure 4a), it correlated with transport activity when either a natural nucleoside (guanosine) (Figure 4b) or fludarabine (Figure 4c) was the substrate. This close relationship between protein and function extended to cytotoxicity itself, as shown in Figure 4d. *Ex vivo* sensitivity to fludarabine, at a pharmacological concentration (7.5 μM), directly correlated with the amount of hENT2 protein. Similar correlations were observed when the drug was tested at 3 and 15 μM (not shown).

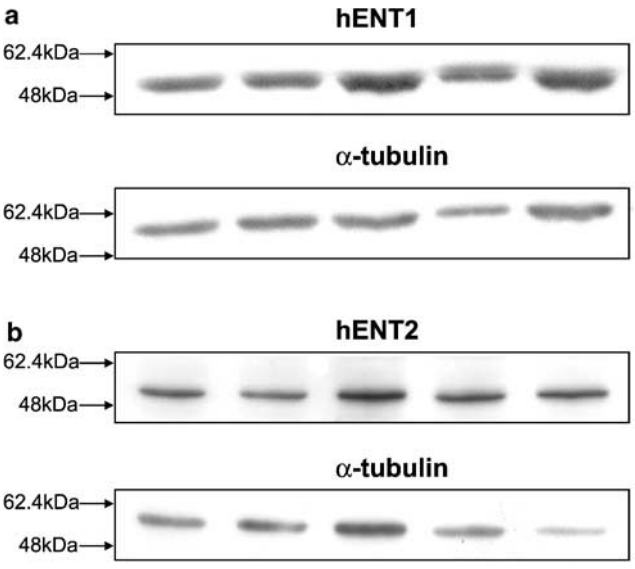

Figure 2 hENT1 and hENT2 western blots. Representative Western blots of hENT1 (a) and hENT2 (b) and their corresponding α-tubulin controls of five randomly chosen independent CLL samples.

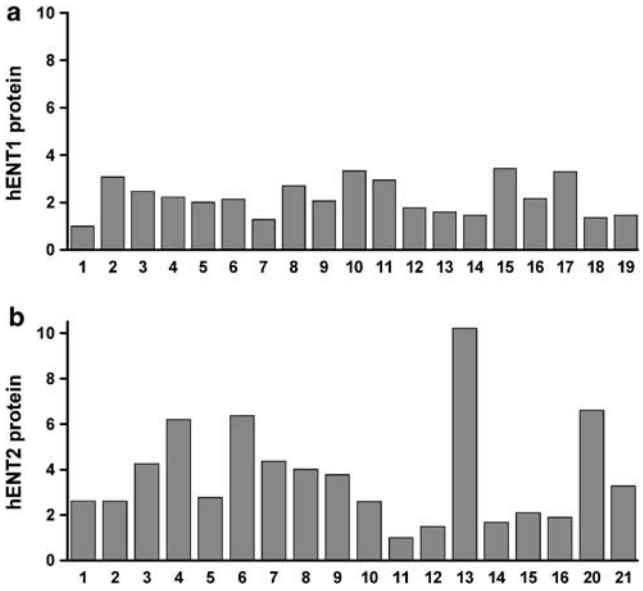

Figure 3 hENT1 and hENT2 protein expression in CLL cells. Normalized hENT1 (a) and hENT2 (b) protein expression levels in the cohort of CLL-patients analyzed. Protein amounts were calculated as the densitometric ratio of hENT *vs* α-tubulin, and were given as arbitrary units using one CLL sample as reference control.

Discussion

The role of nucleoside-derived transporters in modulating drug bioavailability in lymphoid cells has been addressed by analyzing how the pharmacological inhibition of the hENT1 transporter by NBTI modifies drug sensitivity, depending on whether the inhibitor is added either before or after *in vitro* nucleoside treatment. Although ENT proteins may indeed facilitate transport in both directions depending on the relative transmembrane substrate gradient, it has been reported that blocking of hENT1 results either in increased retention and cytotoxicity of cladribine in cells from CLL patients[25] and in cultured human leukemic lymphoblasts,[26] or, alternatively, inhibition of acadesine-induced apoptosis due to the blockade of acadesine entry into CLL cells.[27] Similarly, high-affinity binding of NBTI has been used to monitor hENT1-type binding sites at the membrane surface and has been shown to correlate with sensitivity to citarabine.[28] Nevertheless, the lack of appropriate molecular tools (ie cDNAs, antibodies) has been a bottleneck in the analysis of cytotoxicity associated with specific nucleoside transporter isoforms. This is particularly relevant for hENT2, for which no specific ligands are known and, to date, no

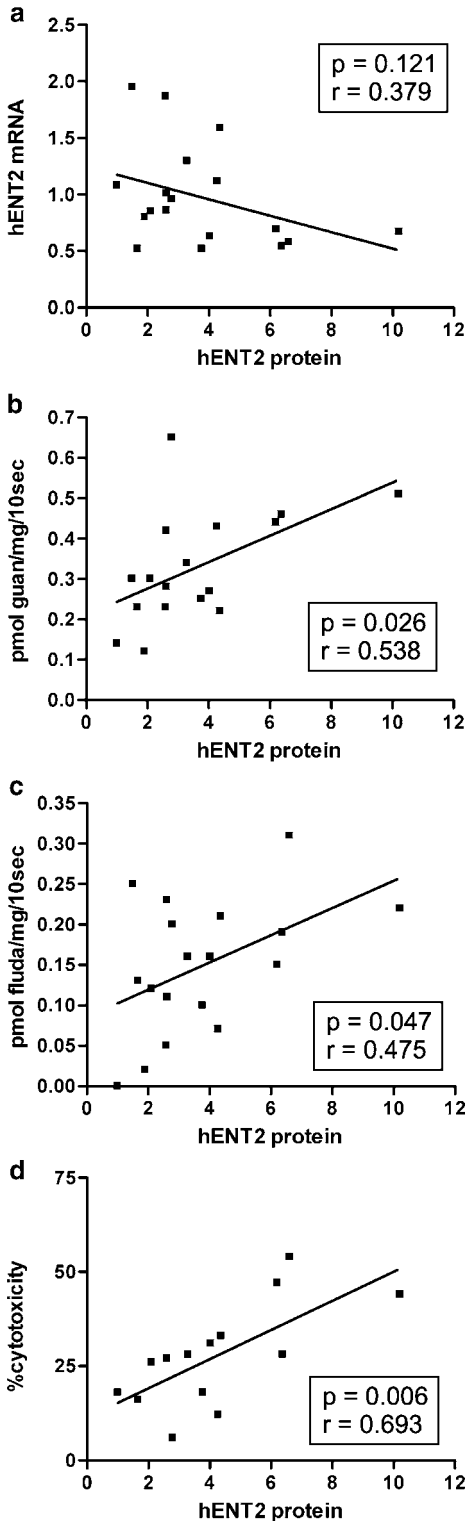

Figure 4 Correlations between hENT2 protein and mRNA, nucleoside transport activities and *ex vivo* fludarabine cytotoxicity. Human ENT2 protein expression levels were plotted against (a) hENT2 mRNA amounts, (b) guanosine uptake rates (10 s, 1 μM), (c) fludarabine uptake rates (10 s, 1 μM), and (d) *ex vivo* sensitivity to fludarabine, expressed as percentage of non viable cells after 48 h treatment with 7.5 μM fludarabine, assessed by the MTT assay. Correlation coefficients and *P*-values are shown in inboxes.

proof that hENT2 has a role in nucleoside-derived cytotoxicity has yet been reported.

In this study we show a lack of correlation between the amount of hENT proteins and their corresponding mRNA levels, as determined by real time RT-PCR. This agrees with previous observations,[18] showing that hENT-mediated accumulation of fludarabine into CLL cells does not correlate with mRNA amounts. However, the evidence that transporter function reflects *ex vivo* fludarabine cytotoxicity prompted us to attempt to identify the particular transporter isoform(s) responsible. This is a relevant issue because it incorporates a new biomarker of nucleotide metabolism as a predictive agent of response to treatment.

Interestingly, the total amount of hENT1 protein does not correlate with either fludarabine uptake or *ex vivo* cytotoxicity. Total hENT1 protein may not reflect function, since it has been reported that hENT1 can be located intracellularly[29,30] and hENT1 related transport activity is also under post-translational control via Protein kinase C.[31] However, this observation might also be explained by the fact that fludarabine uptake measurements, at least in our hands, do not discriminate between hENT1- and hENT2-mediated uptake processes. Nevertheless, fludarabine is also an hENT2 substrate[13] and, according to our data, hENT2 alone seems to predict *ex vivo* sensitivity to the drug. This is based upon the fact that, irrespective of the contribution of hENT1 in transport function, fludarabine uptake rates significantly correlate with hENT2 protein amounts and transporter protein also shows a significant correlation with the *ex vivo* sensitivity to the drug, when used at a variety of pharmacologically relevant concentrations. The possibility that hENT1-related transport activity, rather than hENT2 function, is under complex post-translational control, involving either translocation into the membrane or phosphorylation-dependent processes, will require further research.

In summary, this study strongly indicates that fludarabine action on CLL cells is determined by the equilibrative nucleoside transporter hENT2. This is the first time hENT2 has been implicated in the therapeutic response to a nucleoside analog and these findings might be used to predict fluradabine response in CLL patients. Whether this applies to other lymphoproliferative diseases will require further research.

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ARTÍCULO 3

LA EXPRESIÓN DEL TRANSPORTADOR EQUILIBRATIVO hENT1 CORRELACIONA CON EL TRANSPORTE DE GEMBITABINA Y LA CITOTOXICIDAD *IN VITRO* DEL FÁRMACO EN CÉLULAS DE LINFOMA DE CÉLULAS DEL MANTO

Objetivo

Determinar la implicación de los transportadores equilibrativos de nucleósidos en la respuesta a gemcitabina y la resistencia a fludarabina que presentan las células de linfoma de células del manto (LCM).

Resumen

Las células de leucemia expresan mRNA tanto de transportadores concentrativos (CNT) como equilibrativos (ENT) (Pastor-Anglada M et al, 2004). Como hemos comentado en el trabajo anterior, existe una correlación entre los niveles de proteína hENT2 y la sensibilidad *ex vivo* a la fludarabina en células de LLC-B.

Las células de linfoma del manto (LCM) presentan bajas tasas de respuesta a la fludarabina, requiriéndose dosis muy elevadas para obtener un efecto citotóxico. Este hecho nos llevó a estudiar el estado de los transportadores equilibrativos en células de LCM. Para ello, analizamos cinco líneas celulares derivadas de LCM y células primarias procedentes de 22 pacientes diagnosticados de LCM y determinamos la expresión de los mRNA's y las proteínas de hENT1 y hENT2 mediante RT-PCR y Western blot, observando una correlación significativa entre la expresión del mRNA de hENT1 y la proteína.

Algunas de estas células fueron incubadas con gemcitabina, un análogo de la deoxicitidina con efecto contra muchos tumores sólidos, que se ha descrito que entra en la célula preferentemente vía ENT1, y fludarabina, que es transportada principalmente por ENT2. Tras la incubación con estos fármacos determinamos el efecto citotóxico que inducían sobre las células de LCM y correlacionamos este efecto con la expresión, tanto de mRNA como de proteína. Mientras que requeríamos concentraciones muy altas de fludarabina para ejercer un efecto sobre estas células, las dosis de gemcitabina necesarias para ejercer este efecto citotóxico no superaban las farmacológicas. Al mismo tiempo, se observó una correlación entre la sensibilidad a la gemcitabina y la expresión de mRNA y proteína hENT1, atribuyendo un importante papel de este transportador en el LCM y sugiriendo que la posible resistencia a la fludarabina que presentaban estas células era debida a la expresión de niveles más bajos de hENT2 que de hENT1.

Expression of Human Equilibrative Nucleoside Transporter 1 (hENT1) Correlates with Gemcitabine Uptake and Cytotoxicity in Mantle Cell Lymphoma

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Running title: hENT1 correlates with the response to gemcitabine

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

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Full Text: 3513

Number of figures: 5

Abstract

Background and Objectives: Nucleoside transporters (NTs) might play a relevant role in the intracellular targeting of many nucleoside analogues used in anticancer therapy. Two gene families (SLC28 and SLC29) encode the two types of human NTs, Concentrative Nucleoside Transporter (CNT) and Equilibrative Nucleoside Transporter (ENT) proteins. Chronic lymphocytic leukemia (CLL) cells express both SLC28- and SLC29-related mRNAs, although transport function seems to be mostly related to ENT-type transporters. Here we have analyzed the role of NTs 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 5 MCL cell lines and 20 primary MCL tumors by real-time quantitative RT-PCR and Western blots. Cell viability measured by annexin V-FITC staining and nucleoside-derived drug transport were also studied.

Results: MCL cells express high levels of hENT1 protein compared to 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 that NTs are implicated in the therapeutic response to nucleoside analogues, and suggest a particular and novel role for hENT1 in the genotoxic response to selected nucleoside analogues, such as gemcitabine, in MCL cells.

INTRODUCTION

Mantle cell lymphoma (MCL) is a lymphoid malignancy derived from a subset of mature-B cells with coexpression 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 analogues 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 have 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 analogues have been broadly studied and appears to be a necessary step in the development of cytotoxicity, however, the major routes of nucleoside derivatives uptake in MCL cells have not extensively been 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. CNTs proteins mediate high-affinity Na-dependent translocation of nucleosides. *SLC29* genes encode the equilibrative nucleoside transporter (ENT) proteins and comprise 4 members. ENT1 and ENT2 are broad-selectivity equilibrative carrier proteins and ENT1 shows high sensitivity to pharmacological inhibition by the adenosine analogue 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 mRNAs.[10] Recently we have detected a significant correlation between fludarabine uptake via ENT carriers, hENT2 protein expression and *ex vivo* sensitivity of chronic lymphocytic leukemia (CLL) cells to fludarabine,[11,12] suggesting a role of the equilibrative nucleoside transporter hENT2 in fludarabine responsiveness in CLL. Conversely 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 depending on the particular lymphoproliferative malignancy. 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 have analyzed these 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 analogue, and its correlation with drug uptake and with expression of ENT transporters was also analyzed in MCL cells.

DESIGN and METHODS

Cell lines

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 were described and characterized previously.[18]

Patients

Twenty MCL tumors were studied, four of them corresponding to blastoid variants 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 were not previously treated with nucleoside analogues. In these cases the status of p53 and ATM has been previously analyzed.[6] An 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. Tumoral 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 ($0.5x10^6$ cells/mL) and tumoral cells from patients with MCL ($1x10^6$ cells/mL) were cultured in RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 50 σg/mL penicillin/streptomycin (Gibco, BRL, Paisley, Scotland) and 100 σg/mL normocin (Amaxa, Khöl, Germany), at 37ºC in a humidified atmosphere containing 5% carbon dioxide. Jeko-1 cell line was incubated at the same conditions but supplemented with 20% FCS. Granta 519 was cultured at a concentration of $0.5x10^6$ cells/mL in DMEM culture medium. Absence of mycoplasm infection was regularly assessed by PCR and experiments were performed in mycoplasm-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. LD_{50} was defined as the concentration of drug required to reduce cell viability by 50%.

Real-time quantitative 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). RNA was treated with DNAse (Ambion, Austin, TX) to eliminate contaminating DNA. In total, 1 σg of RNA was retrotranscribed to cDNA and the analysis of *hENT1*, *hENT2* and η-glucoronidase (*GUS*) mRNA was performed by real time quantitative reverse transcriptase-polymerase chain reaction as previously described.[11] Expression of Ki-67 was done using a pre-designed Assay-on-demand (Applied Biosystems, Foster City,

CA). . The amounts of mRNA were given as arbitrary units using the ΔΔCT method (User Bulletin #2, Applied Biosystems) using *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.[21] Proteins were separated on 10% SDS polyacrylamide gels, transferred to Immobilon-P (Millipore, Bedford, MA) membranes and incubated with monospecific polyclonal antibodies against hENT1 and hENT2, that 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 ζ-tubulin (Oncogene Research, Boston, MA, USA). Densitometric analysis were performed with Image Gauge Reader Software (Las3000, Fujifilm, Tokyo, Japan). Ratios of hENT vs ζ-tubulin were 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) were used as substrates at a concentration of 1μM. Incubation time was 2 minutes for uridine and 10 seconds for fludarabine and gemcitabine. To discriminate between NBTI-sensitive (hENT1) and NBTI-insensitive (hENT2) transport rates, 1μM NBTI (Sigma-Aldrich, Saint Louis, MO) was used.

Statistical analysis

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

RESULTS

hENT1 and hENT2 expression in MCL cell lines

The expression of equilibrative nucleoside transporters *hENTs*-related mRNAs were analyzed by quantitative RT-PCR in human MCL cell lines. Figure 1A shows the amounts of *hENT1* and *hENT2*-related mRNAs in 5 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 expression levels of *hENT1* were 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 high levels of *hENT1* compared to the other two cell lines analyzed: NCEB-1 and JVM-2.

hENT protein amounts 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 recognized single bands of 50-55 kDa. Figure 1B shows Western blots of the five cell lines in which hENT1, hENT2 and ζ-tubulin were analyzed. Semiquantitative analysis of hENT1 and hENT2 expression was achieved by calculating the densitometry ratios versus ζ-tubulin, in a range of protein concentrations in which densitometric signal has been previously shown to be lineal. Figure 1C shows the values of these densitometric analysis of the five MCL cell lines. The human JVM-2 cell line was also used as a relative calibrator and its protein amounts adopted the value of 1. hENT2 protein showed less variability than hENT1 protein in MCL cell lines. These results are relatively concordant with the pattern observed in *hENT-related* mRNA expression by quantitative RT-PCR.

Figure 1

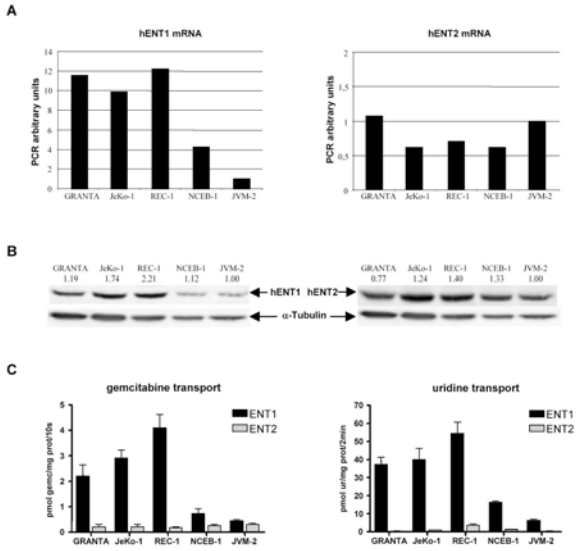

Figure 1. Equilibrative Nucleoside Transporters (hENT1 and hENT2) in MCL cell lines. Normalized *hENT1* and *hENT2*-related mRNA expression levels in MCL cell lines. C_T values for each nucleoside transporter were normalized to an endogenous reference gene (GUS). mRNA expression levels are given in arbitrary units, using JVM-2 as reference control (A). The experiments were done in duplicate. Western blots of hENT1 and hENT2 and ζ-tubulin in MCL cell lines. Normalized protein expression levels in these MCL cell lines. Protein amounts were calculated as the densitometric ratio of hENT versus ζ-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. 1 σM NBTI was used to discriminate between NBTI-sensitive (hENT1) and NBTI-insensitive (hENT2) transport rates. Results were given as the mean ∂ SE of three to six independent experiments measured in triplicate (C).

Nucleoside uptake into MCL cell lines

Equilibrative nucleoside transport uptake was analyzed in the 5 human MCL cell lines. Transport of the natural nucleoside uridine was measured at two minutes, as it was lineal up to 10 minutes (data not shown). Nevertheless, 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 it has been observed in the expression analysis of hENT1, uridine and gemcitabine uptake showed a high range of variability, being Rec-1, Jeko-1 and Granta 519 the cell lines with the highest rates for both uridine and gemcitabine uptake. The uptake of gemcitabine mediated by the hENT1 transporter is showed in Table 1. The addition of 1 σM NBTI blocked the gemcitabine uptake, indicating that the 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).

Figure 2

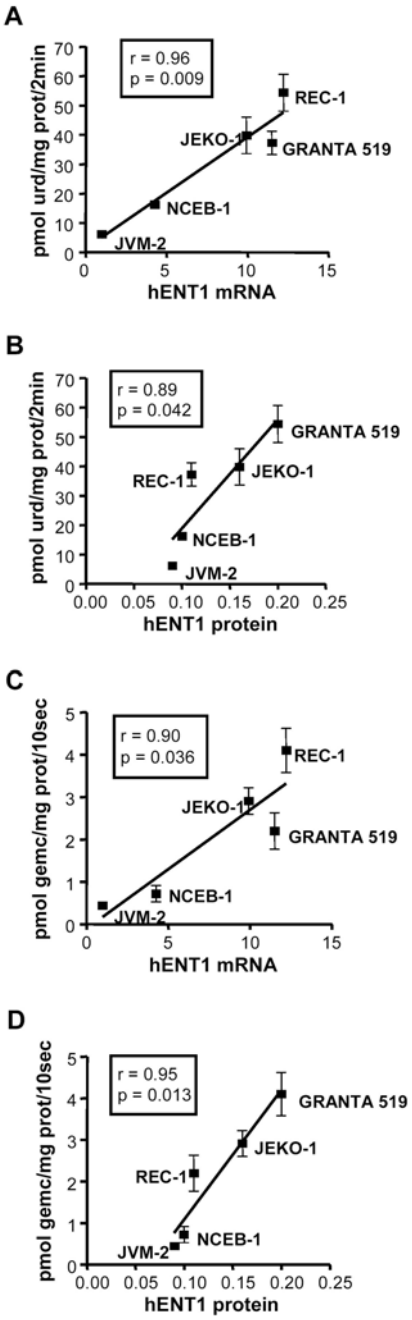

Figure 2. Correlations of nucleoside transport with protein and SLC29 amounts, in MCL cell lines. hENT1 mRNA and protein expression levels were plotted against uridine (A and B) and gemcitabine (C and D) uptake rates in MCL cell lines. Results were given as the mean ∂ SE of three to six independent experiments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

Sensitivity to gemcitabine in MCL cell lines

Since in MCL cell lines, the expression levels of hENT1-related mRNA are higher than those of hENT2, we analyzed the sensitivity of these cells to gemcitabine, a nucleoside analogue that 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 σg/ml)(Lilly, Hampshire, UK), and the cytotoxic effect was measured by annexin V-FITC/PI staining. The LD_{50} (lethal dose 50) for gemcitabine is shown in Table 1. A cytotoxic effect was observed at low doses of gemcitabine (< 3 σg/ml) in 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 (20 ng/mL) and this cell line had also the highest rates of gemcitabine uptake. According to this, resistant cell lines (JVM-2 and NCEB-1) presented the lowest levels of gemcitabine uptake. These results suggested that lack of response to gemcitabine correlated 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 have done a correlation analysis to assess a possible relationship between hENT expression and sensitivity to gemcitabine and the uptake of these drugs into the cells. Sensitivity to gemcitabine (5 σg/ml), directly correlated with *hENT1*-related mRNA

expression (Figure 3A) (r= 0.9; p=0.04) and the 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).

Figure 3

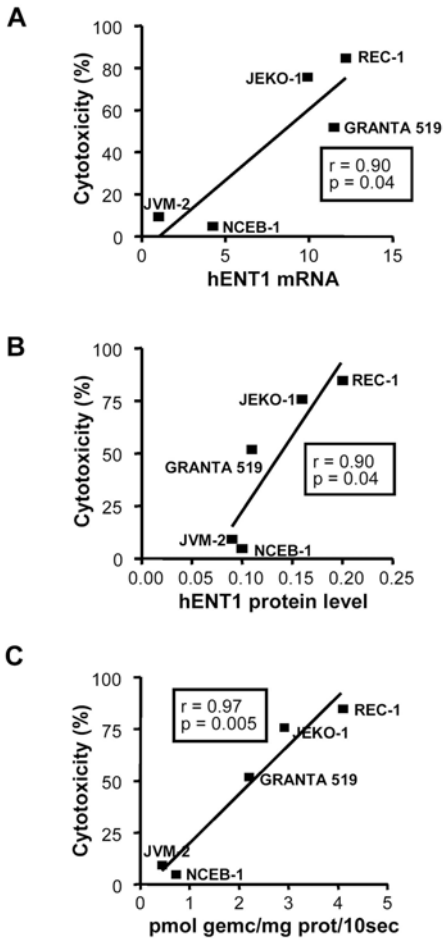

Figure 3. Correlation between gemcitabine cytotoxicity and expression of hENT1 and gemcitabine uptake in MCL cell lines. Sensitivity to gemcitabine (5σg/mL), expressed as the percentage of apoptotic cells after 24 hours of treatment, was plotted against hENT1 mRNA levels (A), hENT1 protein expression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

Equilibrative nucleoside transporter expression pattern in MCL cells

Expression of equilibrative nucleoside transporters was analyzed by quantitative RT-PCR in tumoral cells from 20 primary MCL tumors. Figure 4A shows the expression of *hENT1* and *hENT2*-related mRNAs in the whole cohort of MCL tumors analyzed in this study, using JVM-2 cells as a relative calibrator. The mean values of *hENT1* and *hENT2*-related mRNAs in primary CLL cells described previously[12] were also included in Figure 4A. The variability in *hENT1* and *hENT2*-related mRNA expression was very high. The expression pattern of these transporters in primary MCL cells differed that those observed previously in CLL cells. Thus, the average of the expression of *hENT1*-related mRNA in MCL cells is significantly higher (0.76 ∂ 0.48) than those reported previously in primary CLL cells[12] (n = 22; 0.13 ∂ 0.10) (p< 0.001) and the amount of *hENT2*-related mRNA in MCL cells is significantly lower (0.89 ∂ 0.52) compared to the amounts of *hENT2*-related mRNA observed in CLL cells (n = 22; 4.04 ∂ 1.83) (p< 0.001). The expression of hENT1 and hENT2 transporters did not correlate with the morphological MCL variant nor the proliferation index of tumoral cells.

In these primary MCL tumors, protein extracts were obtained and ENTs were analyzed by Western blot. Figure 4B shows a representative Western Blot of five independent cases of primary MCL, in which the amounts of hENT1, hENT2 and ζ-tubulin were analyzed. The semiquantitative analysis of hENT1 and hENT2 expression is shown in Figure 4C. Again, the amounts of hENT1 protein are significantly higher in primary MCL cells (0.15 ∂ 0.14) compared to those reported previously in CLL cells (0.08 ∂ 0.02) (p < 0.001), and hENT2 protein expression is lower in primary MCL cells (0.26 ∂ 0.20) than CLL cells (0.46 ∂ 0.010) (p < 0.001).

Figure 4

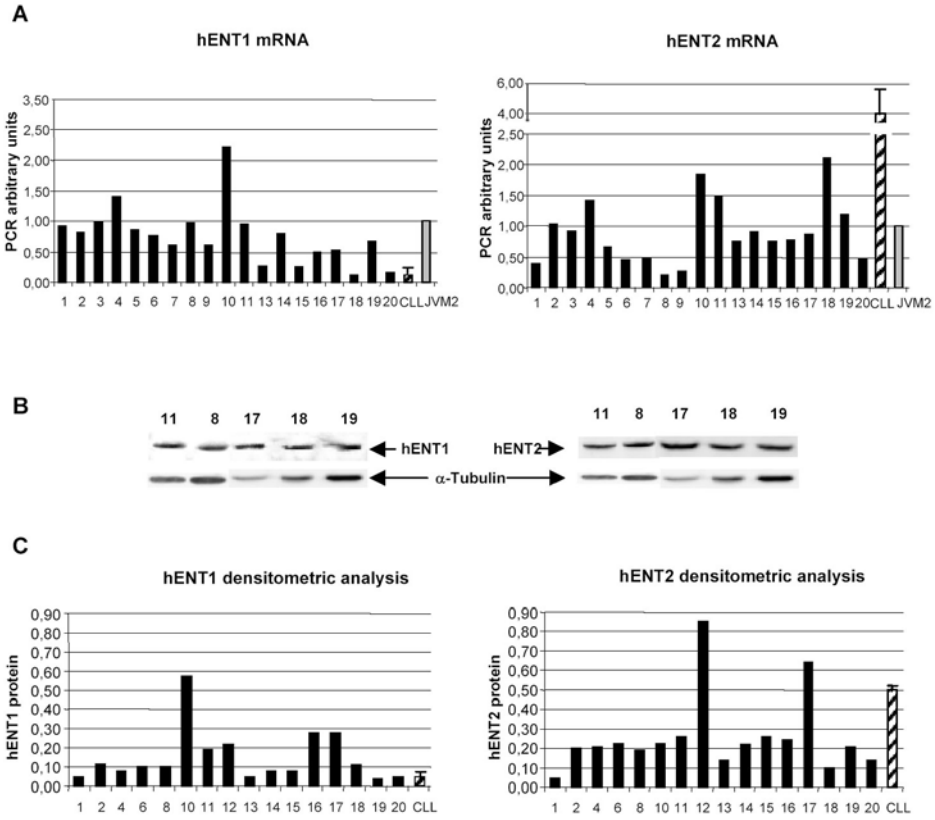

Figure 4. *hENT1* and *hENT2*-related mRNA and protein expression in primary MCL cells. Normalized *hENT1* and *hENT2*-related mRNA expression levels in the cohort of MCL-patients analyzed (A). JVM-2 cell line was used as a relative calibrator and adopted the value of 1 as an arbitrary unit. The mean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

***Ex vivo* sensitivity to gemcitabine in primary cells from MCL patients**

Primary cells from eight MCL patients that have been included in the expression analysis of ENT transporters, were incubated with different concentrations of gemcitabine (0.5-50 σg/ml) for 48 hours. The biological characteristics of these patients and LD_{50} for gemcitabine are described in Table 2. In all primary MCL cases analyzed, the doses of

gemcitabine necessary to induce a cytotoxic effect are higher than those used 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 σg/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 correlate with the hENT1 protein levels (r = 0.62; p=0.013) (Figure 5A). As we have observed in MCL cell lines, a significant correlation between *hENT1*-related mRNA levels and cytotoxic effect of gemcitabine at 5 σg/ml (r = 0.72;p=0.04) was observed (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 neither with hENT2 protein nor with cell viability after exposure to different doses of gemcitabine. Furthermore, neither *hENT1*-related mRNA nor protein expression correlate with the cytotoxic effect induced by other non nucleoside analogues chemotherapeutic agents such as mitoxantrone, a topoisomerase inhibitor (data not shown).

Figure 5

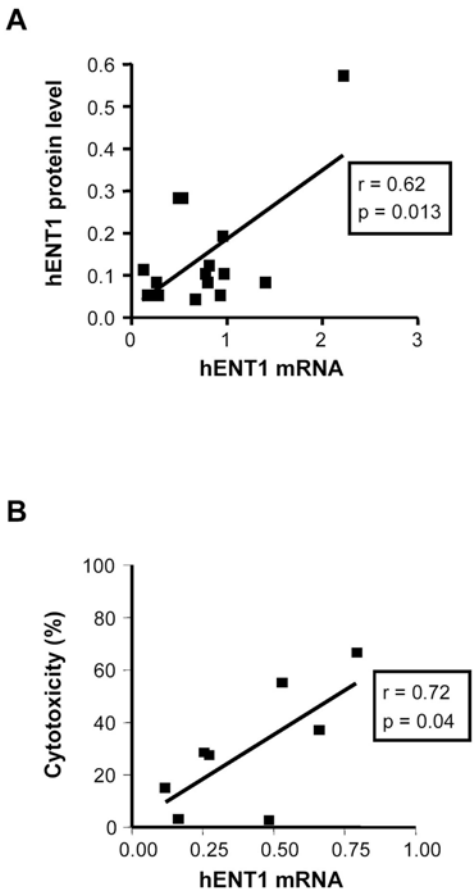

Figure 5. Correlations between hENT1 protein and mRNA expression and *ex vivo* gemcitabine cytotoxicity in primary MCL cells. Correlation between *hENT1*-related mRNA levels and protein expression in primary MCL cells (A). Correlation of *ex vivo* sensitivity to gemcitabine with *hENT1*-related mRNA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

DISCUSSION

CNT and ENT proteins are responsible for the uptake of many nucleoside derivatives used in anticancer therapies.[23] Tumoral cells show highly variable patterns of CNT and ENT expression, which might affect drug bioavailability and action.[9,10,24] Our previous studies have 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 primary MCL cell and to CLL cells. In contrast, CLL cells, characterized by the accumulation of B cells at G_0 of cell cycle,[25] showed higher expression of the hENT2 isoform than MCL cells. These results would be in agreement with previous reports suggesting a significant role for hENT1 in cell proliferation[14,15] and with high hENT1 expression levels in immortalized cell lines compared 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 has been described in human tissues[22,28] and particularly, in non-Hodgkin's lymphomas, where it has been proposed that expression of hENT1 is linked to B cell differentiation.[29]

Moreover, conversely to the lack of relationship between hENTs proteins and mRNA observed in CLL cells,[12] the significant correlation between the amount of hENT1 protein and corresponding mRNA levels in MCL cells would favor the view that hENT1 expression might be transcriptionally regulated in this malignancy.

In MCL cell lines, hENT1 expression 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 analogue that has more effective cellular kinetics, including intracellular incorporation, phosphorilation and retention.[34] Furthermore, gemcitabine is phosphorylated more rapidly and eliminated more slowly than other nucleoside-derived drugs.[9] Gemcitabine is commonly used in treating patients with solid tumors.[23,35-38] The major effect of gemcitabine is directed against DNA synthesis[39] and is preferentially transported by hENT1 proteins.[40] In this study we have demonstrated that incubation with gemcitabine at pharmacological doses induced a cytotoxic effect in most cells from primary and MCL cell lines.

Moreover, we have detected a significant correlation between *ex vivo* sensitivity to gemcitabine, hENT1-mediated gemcitabine transport and the amounts of hENT1 in 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 analogues. 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, correlation of hENT1 expression with response to gemcitabine as a single agent has been detected in patients affected of pancreatic adenocarcinoma.[28] Similarly, pharmacological inhibition of hENT1 results either in

increased retention and cytotoxicity of cladribine in cells from CLL patients[44] and in cultured human leukemic lymphoblasts,[45] or, alternatively, 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-analogues has been reported in 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] It has been reported a case of a MCL patient who exhibited resistance to fludarabine with a deficiency in both nucleoside uptake and accumulation, with no major changes at mRNA levels of genes involved in nucleoside analogue uptake and metabolism[27] indicating that resistance to nucleoside analogues 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, that might also explain resistance to nucleoside analogues.[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 analogues whose uptake is mediated preferentially by hENT1 transporter. The results presented in this paper further support the hypothesis that nucleoside transporters are implicated in the therapeutic response to nucleoside analogues, suggesting that hENT1 expression might be useful to predict response to nucleoside analogues known to be taken up via ENT1 carriers, such as gemcitabine in MCL patients. A better understanding of the nucleoside analogues transport may extend the therapeutic strategies and improve the prognosis of MCL patients.

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

	LD_{50} Gemcitabine (σg/mL)	Gemcitabine uptake (pmol/mg prot/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 SSCP and sequencing
[b] ATM status assessed by FISH
[c] NR: non response

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

Patient	Cell source[a]	Morphological variant[b]	% Tumoral cells	LD_{50} Gemcitabine (σg/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 analysis was assessed by FISH, SSCP and sequencing

[d] ATM status assessed by FISH

[e] Ki-67 expression assessed by RT-PCR. CLL mRNA was used as a relative calibrator and adopted the value of 1 as an arbitrary unit.

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ARTÍCULO 4

LA PÉRDIDA DE EXPRESIÓN DE MTAP EN EL LINFOMA DE CÉLULAS DEL MANTO ESTÁ ASOCIADA A UNA CORTA SUPERVIVENCIA. IMPLICACIÓN PARA UNA POSIBLE TERAPIA DIRIGIDA

Objetivo

Analizar las alteraciones del gen metilthioadenosina fosforilasa (*MTAP*) y la expresión de la proteína en células de linfoma de células del manto (LCM) y determinar si los casos con deleción del gen *MTAP* podrían beneficiarse de una terapia dirigida contra la vía de síntesis *de novo* de AMP, con L-alanosina (SDX-102).

Resumen

Aproximadamente un 30% de los casos de linfoma de células del manto (LCM) presentan deleción del locus *INK4a-ARF*, que localiza en 9p21 y codifica para el inhibidor de ciclina dependiente de quinasas p16, y el regulador de la vía MDM-2/p53, p14. La deleción de estos genes se ha correlacionado con formas agresivas con elevados índices de proliferación y corta supervivencia en paciente con LCM. Por su proximidad a *p16* (100 kb telomérico), muchos casos presentan, también, una codeleción del gen *MTAP* que codifica para una enzima, la metilthioadenosina fosforilasa, implicada en la síntesis de adenina, metionina y ATP, la fuente de energía de la célula. Sin embargo, las células que presentan la deleción de este gen pueden seguir proliferando y viviendo por la presencia de una vía de síntesis *de novo* de AMP y un suministramiento exógeno de metionina.

La pérdida selectiva de *MTAP* en células malignas puede utilizarse para buscar tratamientos dirigidos utilizando inhibidores de la vía *de novo* de AMP. En este sentido, la L-alanosina (SDX-102) es un análogo de aminoácidos que actúa bloqueando la síntesis *de novo* de AMP, inhibiendo la enzima *adenosil succinato sintasa*, de modo que esas células que presenten deleción de *MTAP* estarán condicionadas a entrar en un proceso de muerte celular cuando sean incubadas en presencia de L-alanosina. Las células MTAP positivas, también presentan una ligera pérdida de viabilidad celular al ser tratadas con L-alanosina pero este efecto es revertido cuando se preincuban con un análogo de la proteína MTA, la *9-β-D-eritrofuranosiladenina* (EFA), aumentando así el sustrato de la enzima MTAP. En las células MTAP negativas, el aumento de sustrato mediante el análogo de MTA no induce ningún efecto. Así, esta minoria de células de LCM con deleción de *MTAP* podría beneficiarse de una terapia dirigida con L-alanosina.

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Senior Editor

Lack of MTAP Expression in Mantle Cell Lymphoma is Associated with Shorter Survival. Implications for a Potential Targeted Therapy

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DC and EC should be considered co-senior authors

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Abstract

Purpose: To determine the methylthioadenosine phosphorylase (*MTAP*) gene alterations in mantle cell lymphoma (MCL) and to investigate whether the targeted inactivation of the alternative *de novo* AMP synthesis pathway may be an useful therapeutic strategy in tumors with inactivation of this enzyme.

Experimental design: *MTAP* gene deletion and protein expression were studied in 64 and 52 primary MCL, respectively, and the results were correlated with clinical behavior. Five MCL cell lines were analyzed for MTAP expression and for the *in vitro* sensitivity to L-alanosine, an inhibitor of adenylosuccinate synthetase (ASS) and hence *de novo* AMP synthesis.

Results: No protein expression was detected in 8/52 (15 %) tumors and one cell line (Granta 519). Six of these MTAP negative tumors and Granta 519 cell line had a codeletion of *MTAP* and *p16* genes, one case showed a deletion of *MTAP* but not *p16*, and one tumor had no deletions in neither of these genes. Patients with *MTAP* deletions had a significant shorter overall survival (mean: 16.1 months) than patients with wild type *MTAP* (mean: 63.6 months) ($p<0.0001$). L-alanosine induced cytotoxicity and activation of the intrinsic mitochondrial-dependent apoptotic pathway in MCL cells. 9-β-D-Erythrofuranosyl adenine (EFA), an analogue of MTA, selectively rescued MTAP positive cells from L-alanosine toxicity.

Conclusions: *MTAP* gene deletion and lack of protein expression are associated with poor prognosis in MCL and might identify patients who might benefit from treatment with *de novo* AMP synthesis pathway targeted therapies.

Introduction

Mantle cell lymphoma (MCL) is a mature B-cell neoplasm genetically characterized by the chromosomal translocation t(11;14)(q13;q32) that leads to the overexpression of cyclin D1 gene with the consequent deregulation of cell cycle at G1-S checkpoint (1, 2). This lymphoma is very aggressive from the clinical standpoint, the median survival being 3-5 years. Most patients have a poor response to current treatments (3, 4). Several studies have indicated that the aggressive biological behavior and the poor prognosis in MCL are closely related to the proliferative index of the tumor (5), and to the presence of frequent genetic alterations in cell cycle regulatory elements that contribute to increase cell proliferation (6- 9). One of the most common genetic alterations reported in aggressive MCL is the homozygous deletion of 9p21 where the *INK4a-ARF* locus is mapped. This locus encodes for the cyclin-dependent kinase inhibitor p16/INK4a and the MDM2/p53 upstream regulator p14/ARF. Homozygous deletions of this locus are associated with a high proliferation index and short survival of patients with MCL (10). These deletions include other genes that may be important in the biological behavior of MCL. The methylthioadenosine phosphorylase (*MTAP*) gene is 100Kb telomeric to the *INK4a-ARF* locus and both are frequently co-deleted in aggressive tumors (11- 13).

MTAP is an enzyme that is essential for normal activity of the salvage pathway for both adenine and methionine synthesis. MTAP catalyzes the cleavage of 5' methylthioadenosine (MTA) into adenine and 5-methylthio-D-ribose-1-phosphate (MTR-1-P). Adenine is then used to generate adenosine monophosphate (AMP), while MTR-1-P is converted into methionine (14). MTAP is expressed in all normal cells and tissues, although frequently lost in different human tumors usually due to gene deletions associated with the coincident loss of the *INK4a-ARF* locus (12, 13, 15). Malignant cells lacking *MTAP,* and consequently having an impaired AMP and methionine salvage pathway*,* are completely dependent on *de novo* AMP synthesis

and exogenous methionine supply and thus are expected to be more sensitive to chemotherapy with antimetabolites blocking this pathway, such as L-alanosine, an amino acid analogue obtained from *Streptomyces alanosinicus* that blocks *de novo* AMP synthesis from inosine monophosphate via the inhibition of the adenylosuccinate synthetase (ASS) activity (16, 17). Since MTAP-deficient cells cannot salvage adenosine, and L-alanosine interferes with the *de novo* AMP synthesis, this compound is an ideal candidate therapy for *MTAP* deleted tumors (18, 19).

The aims of this study were to investigate whether the frequent losses of the *INK4a-ARF* locus in MCL also implicates *MTAP* gene deletions and a corresponding lack of protein expression and if tumors with these alterations could be candidates for therapeutic strategies based on the inhibition of the *de novo* AMP synthesis pathway.

Material and Methods

Patients

Frozen tumor samples from 64 MCL patients diagnosed between 1989 and 2002, in the Department of Pathology from Hospital Clinic, Barcelona and the Institute of Pathology in Wurzburg, Germany were studied. The samples corresponded to 48 typical MCL tumors and 16 blastoid variant samples. Fresh tissue was obtained at the moment of biopsy, embedded in OCT ™ Tissue Tek (Sakura, Finetek Europe, Zoeterwoude, NL) frozen at –40ºC in 2-methyl-butane and stored at –80ºC. An informed consent was obtained from each patient of the both institutions according to their Ethical Committees.

Cell lines

Cell lines carrying the t(11;14)(q13;q32) translocation were studied: Granta 519, REC-1, NCEB-1, JeKo-1 and JVM-2. The genetic and molecular characteristics of these cell lines have been described previously (20- 22).

JVM-2, REC-1, and NCEB-1 cell lines ($0.5x10^6$ 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 biosystems Inc., Köln, Germany), at 37ºC in a humidified atmosphere containing 5% carbon dioxide. JeKo-1 cell line was incubated at the same conditions but supplemented with 20% FCS and Granta 519 was cultured at $0.5x10^6$ cells/mL in DMEM culture medium. Cell cultures were periodically tested for mycoplasm and all experiments were conducted in mycoplasm free cells. Cells were incubated in the absence (CT) or presence of L-alanosine (L-Ala; 20-100 µM) and the MTA analog, 9-β-D-erythrofuranosyladenine (EFA; 100 µM) (Salmedix Inc., San Diego, CA).

p16 and MTAP deletion analysis

Genomic DNA was obtained using proteinase K/ Rnase treatment and phenol/ chloroform extraction. To study deletions of the *p16* and *MTAP* locus, real-time quantitative PCR (qPCR) was performed under universal real time standard conditions with the ABI Prism 7900 (Applied Biosystems, Foster City, CA) in a total reaction volume of 25 µL using 25 ng of genomic DNA. Primers and probes used for *MTAP* and *p16* analysis have been published previously (23, 24). At least, three replicates were done for each sample. Albumin and β-actin were used as control genes. The calculated copy number of *MTAP* and *p16* were normalized by the copy number of each control gene, and the relative values were obtained using the △△CT method (User Bulletin #2, Applied Biosystems). Three control DNA samples obtained from peripheral blood lymphocytes of healthy donors were used to establish the cutoff ratio for *p16* and *MTAP* deletions. The normalized ratio of *MTAP* and *p16* genes to control gene is expected to be close to 1 if no deletions were present and close to 0 for homozygous deletions. Considering the potential for contamination of MCL tumor samples with normal cells, values less than 0.4 were judged to be deleted in *MTAP* or *p16* genes.

Immunohistochemistry

Formalin fixed paraffin-embedded tissue was available in 52 of the MCL cases. In the rest of our cases other fixation methods were used (B-5, Bouin) that were not suitable for a proper immunostaining. MTAP immunostaining was performed using a mouse monoclonal antibody (Clone 6.9.5) and a staining technique developed by Salmedix Inc. (San Diego, CA). Unstained sections were deparaffinized by routine techniques. Antigen retrieval was performed by incubating the tissue sections in H.I.E.R. (Pressure Cooker, BORG) at 120°C for 3 minutes followed by trypsin incubation for one minute at room temperature. Slides were washed three times in PBS (DAKO, Carpinteria, CA) and endogenous peroxidase activity was blocked with 5-minute incubation in a hydrogen peroxide solution. The slides were then incubated with

20 µg/mL of the primary antibody or the appropriate negative reagent control for 30 minutes at room temperature. The slides were washed three times in PBS and incubated with Labeled Polymer from the EnVision Plus detection Kit™ (DAKO) for 30 minutes at room temperature. Following three PBS washes, the peroxidase reaction was visualized by incubating with 3,3',-diaminobenzidine tetrahydrochloride (DAB) solution (DAKO) for five minutes. Tissue sections were thoroughly washed with tap water and counterstained with Harris hematoxylin solution.

The proliferative activity of tumors was determined in 58 MCL cases by the immunohistochemical detection of Ki67 using the MIB monoclonal antibody (Immunotech, Marseille, France) at a 1:400 dilution. Antigen retrieval was done with a 10 % EDTA solution (pH 8) in a pressure cooker. Detection was performed with the EnVision Plus detection Kit™ using DAB as chromogen (DAKO) (25).

Analysis of MTAP Promoter-Associated CpG Island Methylation Status

We established the *MTAP* gene CpG island methylation status by PCR analysis of bisulfite modified genomic DNA, using two procedures. First, methylation status was analyzed by bisulfite genomic sequencing of multiple clones as previously described (26). The second analysis used methylation-specific PCR (27) using primers specific for either the methylated or modified unmethylated DNA. Primer sequences of *MTAP* for the unmethylated reaction were 5´-GAAGGATAAATTTTGTTTTTGTTGT-3´ (sense) and 5´-AACATTCCAAAAATTCTCACAAA-3´ (antisense) and for the methylated reaction, 5´-ATAAATTTTGTTTTCGTCGC-3´ (sense) and 5´-GACATTCCAAAAATTCTCGC-3´ (antisense). The annealing temperature for both unmethylated and methylated reactions was 58°C. DNA from normal lymphocytes was used as a positive control for unmethylated alleles and DNA from normal lymphocytes treated *in vitro* with SssI methyltransferase was used as a positive control for methylated alleles. PCR products were loaded onto nondenaturing 2% agarose gels, stained with ethidium bromide and visualized under ultraviolet light.

Protein extraction and Western blot analysis

Total protein extracts of the five MCL cell lines were obtained as previously described (28). 100 µg of protein were separated on SDS-polyacrylamide gels, and transferred to Immobilon membranes (Millipore, Bedford, MA). Western blot was done using the monoclonal MTAP antibody (clone 6.9.5) at a 1:1000 dilution. Antibody binding was detected using a chemiluminiscence (ECL) detection system (Amersham, Buckinghamshire, UK) and the Image Gauge Reader Software (Las3000, Fujifilm Tokyo, Japan). Equal amounts of protein were confirmed using α-tubulin as a control protein.

Quantitation of intracellular ATP levels

ATP levels were measured with the Cell Titer-Glo^{TM} Luminescent Cell Viability Assay (Promega Corporation, Madison, WI), which indicates the presence of metabolically active cells. Cells were incubated in a final volume of 100 µL of culture medium. After 24h, the same volume of Cell Titer-Glo Reagent was added to each test well. The mixture was incubated for 10 min and the luminescence was analyzed using a luminometer (Berthold Technologies, Bad Wildbad, Germany) with an integration time of 0.5 seconds. Experiments were performed in triplicate.

Detection of apoptotic cells.

Membrane translocation of phosphatidylserine residues was quantified by surface annexin V binding as previously described (28). Cytotoxicity was measured as the percentage of annexin V and propidium iodide (PI)-positive cells. Changes in mitochondrial transmembrane potential ($\Delta\Psi_m$) were evaluated by staining with 1 nM 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6[3]$) (Molecular Probes, Eugene, OR) and

reactive oxygen species (ROS) production was determined by staining with 2 μM dihydroethidine (DHE) (Molecular Probes) as previously described (28). Briefly, cells were incubated with dyes for 30 min at 37ºC, washed, resuspended in PBS and analyzed by flow cytometry. A total of 10000 cells per sample were acquired in a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Experiments were performed in triplicate.

For the detection of intracellular proteins by flow cytometry, cells were fixed with paraformaldehyde 4% (Sigma Chemicals, St Louis, MO) over 20 min at 4ºC and permeabilized with saponin 0.1% (Sigma Chemicals, St Louis, MO) for 5 minutes at room temperature. Cells were stained with antibodies against the active form of caspase-3 (BD Pharmingen, San Diego, CA), Bak (Oncogene Research, Boston, MA), and Bax (Trevigen, Gaithersburg, MD) for 20 min at room temperature, followed by goat anti-rabbit-FITC (SuperTechs, Bethesda, MD) or goat anti-mouse-FITC (DAKO, Glostrup, Denmark), and analyzed in a FACScan. Western blot analysis for PARP (Roche Diagnostics, Mannheim, Germany), caspase-9 (New England Biolabs, Beverly, MA), caspase–8 (Oncogene Research) and caspase–3 (BD Pharmingen) was performed as previously described (21).

Cell cycle analysis

Cells were fixed in 80% ethanol for 5 min at 5ºC, centrifuged and washed twice in PBS. Cells were incubated for 15 min at room temperature in a citrate-phosphate buffer (1:24), centrifuged, resuspended in 0.25 mL PI (5μg/mL) and Ribonuclease A (100 μg/mL) (Sigma Chemicals, St Louis, MO), and incubated for 10 min in the dark. The percentage of cells in G_0/G_1, S, G_2/M and the presence of a sub-G_0/G_1 peak were evaluated with ModFit LT software (Verity Software House, Inc., Topsham, MA) as previously described (21).

Statistical analysis

Statistical analysis was done using the SPSS software package version 10 (SPSS, Chicago, IL). The association between MTAP gene status and MTAP protein expression were compared using Fisher's exact test. The statistical analysis of overall survival, defined as the time to death, as influenced by MTAP deletions and proliferative index, was done according to the method described by Kaplan and Meier, and the curves were compared by the log-rank test. $p<0.05$ was considered to reflect statistical significance. Multivariate analysis of *MTAP* gene deletion, MTAP protein expression and proliferative index were performed using COX regression index.

Results

MTAP and p16 gene deletions

Exon 8 of the *MTAP* gene and exon 2 of the *p16* gene were amplified by qPCR to detect gene deletions using genomic DNA from five cell lines carrying the translocation t(11;14)(q13;q32). Homozygous deletion of the *MTAP* gene was detected only in Granta 519 cells. In the other MCL cell lines no changes were seen compared to normal lymphocytes. Granta 519 and REC-1 cell lines showed a homozygous *p16* deletion and Jeko-1 displayed a heterozygous *p16* deletion (Table 1).

To determine whether *MTAP* was also deleted in primary MCL we analyzed by qPCR the DNA from 64 tumors. *MTAP* homozygous deletions were detected in 9 (14%) tumors, 5 (11%) of them typical and 4 (25%) of blastoid variant MCL. *p16* homozygous deletions were observed in 8 (13%) of these cases, 5 of them typical and 3 blastoid variants. These results are summarized in Table 2.

MTAP protein expression

MCL cell lines were next screened for MTAP protein expression by Western blot. Concordant with results from the genetic study a band of approximately 30 KDa was detected in denaturing conditions, corresponding to a subunit of the trimeric 90 KDa MTAP holoenzyme in REC-1, JVM-2, NCEB-1, and Jeko-1 cell lines, while no MTAP expression was observed in Granta 519 cell line (Fig. 1A). These results were confirmed by immunohistochemistry. Cytoplasmic staining was detected in wild type MTAP MCL cell lines, while lack of MTAP labeling was observed in Granta 519 cells (Table 1). Fig. 1B showed a representative immunostaining pattern in wild type MTAP MCL cells (JVM-2) and MTAP-deleted cells (Granta 519).

MTAP protein expression was examined immunohistochemically in 52 primary MCL tumors. Biopsies were considered MTAP-positive when a cytoplasmic staining was observed in tumor cells, and negative when tumor cells showed no immunoreactivity while normal cells (histiocytes and endothelial cells) showed

immunostaining, hence serving as an internal positive control. Nuclear positivity of tumor cells was detected in some of the cases (Fig. 1C). Cytoplasmic MTAP staining was detected in 44 (85%) cases, 34 (89%) being typical and 10 (71%) blastoid variant MCL. Complete loss of MTAP immunollabeling was seen in 8 (15%) tumors, 4 (11%) of them typical and 4 (29%) blastoid variants (Table 2). Six of these 8 tumors lacking MTAP protein expression had a codeletion of the *MTAP* and *p16* genes, one case showed a deletion of *MTAP* but not *p16*, and one tumor had no deletions of either genes (Table 2). We confirmed that the case with non deleted *p16* showed two *p16* signals by FISH. Furthermore, p16 mRNA expression levels analyzed by quantitative PCR were similar to other cases with wild type *p16* and *MTAP* genes (data not shown). The methylation analysis of *MTAP* promoter of the case with no expression of the protein and an apparent non deleted gene revealed a wild type configuration with no evidence of hypermethylation (data not shown).

Correlation between MTAP gene alterations, proliferation, and survival

Survival information was available in 41 cases (32 typical and 9 blastoid variant MCL). The median survival in this series was 41 months (range 1-136 months), being 20 months in blastoid and 61 months in typical MCL variants. *MTAP* gene deletions were detected in 5 of these patients (2 blastoid and 3 typical variants). Patients with *MTAP* gene deleted tumors had a significant shorter overall survival (mean 16.1 months) than patients with wild type *MTAP* tumors (mean 63.6 months) (Fig. 2A). Similarly, lack of MTAP protein expression had a significant predictive value for shorter survival, with a mean of 20 months for patients with loss of MTAP expression and 63.2 months for cases with normal MTAP expression (Fig. 2B).

Since proliferation index is the main prognostic factor in MCL, we compared the proliferation index of these tumors with *MTAP* gene deletion and protein expression. The proliferative index that best predicts survival of the patients in this series was a Ki67 > 35%. We found a statistically significant correlation between the proliferation

index and *MTAP* gene deletions. Eight out of nine cases (89%) with *MTAP* gene deletion were included in the group of high proliferation index (*p< 0.005*). Similarly, all cases with lack of MTAP protein expression by immunohistochemistry were included in the high Ki67 proliferation index group *(p< 0.005).* Four out of the five cases with codeletion of *MTAP* and *p16* genes and typical histology were included in the high proliferation index group, with overall survival ranging from 7.7 to 28 months and a mean of 18.4 (*p< 0.005*). In a multivariate analysis, including the proliferative index and *MTAP* or *p16* deletions, only proliferative index kept independent significative value as survival predictor.

L-Alanosine induced cytotoxicity in MCL cells

As MTAP is necessary for de novo AMP synthesis, we analyzed whether cells from MCL with lack of MTAP expression may be sensitive to the inhibition of this pathway. For this purpose, we incubated MTAP wild type (REC-1 and JVM-2) and MTAP-deleted (Granta 519) MCL cell lines with L-alanosine, a selective inhibitor of this pathway.

After incubation with several doses of L-alanosine (20-100 μM), a decrease in intracellular ATP levels was detected. This effect was dose- and time-dependent. Fig. 3A and B showed the decrease of ATP levels after L-alanosine incubation for 24 hours in two representative MCL cell lines, one with a *MTAP* deletion (Granta 519) and one with a wild type *MTAP* (JVM-2). To verify the specificity of the observed intracellular ATP depletion we used a “rescued strategy” by pre-incubating the selected cell lines with EFA, a MTAP substrate, before the exposure of L-alanosine. It has recently been described that EFA potentiates the alternative pathway for the synthesis of purines in cases with wild type MTAP (29). No cytotoxicity was detected when MCL cells were incubated with different concentrations of EFA alone (data not shown). Preincubation of MCL cells with EFA at the same concentrations that L-alanosine, rescued MTAP-positive cell lines (JVM-2 and REC-1) from L-alanosine-induced depletion of

intracellular ATP levels (Fig. 3B). In contrast, EFA did show any rescuing activity in *MTAP*-deleted Granta 519 cell line exposed to L-alanosine (Fig. 3A).

We also analyzed the effect of L-alanosine on cell cycle distribution in MTAP-positive and MTAP-negative cells. A sub-G_0/G_1 peak corresponding to apoptotic cells was detected in Granta 519 and JVM-2 cell lines after 48 hours of treatment with 100 μM L-alanosine. Furthermore, L-alanosine, induced an arrest in the transition from S phase to G_2/M with a reduction of cells in G_2/M phase of 9% in Granta 519 and 12% in JVM-2. This arrest was reversed when cells with wild type MTAP (JVM-2) were preincubated with EFA 100 μM. In contrast, EFA did not rescue *MTAP*-deleted cells (Granta 519) from L-alanosine induced cell cycle arrest (Fig. 3C).

L-alanosine induces activation of the mitochondrial apoptotic pathway

After incubation with 100 μM L-alanosine for 48 hours, the decrease of the ATP levels was associated with membrane translocation of phosphatidylserine residues and activation of mitochondrial apoptotic pathway, characterized by a loss of mitochondrial transmembrane potential ($\Delta\Psi_m$), ROS production, conformational changes of Bax and Bak and activation of caspase-3 (Fig. 4A). Furthermore, a decrease of pro-caspase 9, -8 and -3, and proteolysis of PARP were also observed by Western Blot (Fig. 4B). These typical characteristics of activation of the mitochondrial apoptotic pathway were more pronounced in Granta 519 (MTAP-deleted) than in JVM-2 (MTAP-expressing) cells. All these changes were reversed by preincubation of MTAP positive cell lines with the MTA analog EFA 100 μM but not in the case of Granta 519 cell line (Fig. 4A and B).

Discussion

MCL is an aggressive B-cell neoplasm with a very poor prognosis that frequenly develops resistance to current chemotherapy regimens (3, 4). For this reason, novel therapeutic strategies taking advantage of the biological alterations of the tumor might be useful to improve the outcome of the patients. In this sense, the recent introduction of proteasome inhibitors as antineoplastic agents have provided evidence of their potent *in vitro* effect against MCL, offering a promising approach in the treatment of patients with refractory MCL (22, 30, 31). Rapid disease progression and poor response to therapy particularly occurs in patients with blastoid variants of MCL, high number of chromosomal alterations or a high proliferative index (2). One of the most common genetic alterations in this subset of aggressive tumors is the deletion of 9p21 targeting the *INK4a-ARF* locus encoding for the CDK4 inhibitor $p16^{INK4a}$ and the MDM2 regulator $p14^{ARF}$ (10). Interestingly, 100Kb telomeric to these genes is the locus encoding for *MTAP,* which is co-deleted with the previous genes in many solid tumors (32- 34), acute lymphoblastic leukemia (35) and high-grade malignant lymphomas (12). However, the status of this gene in MCL has not been previously examined. MTAP is an ubiquitous enzyme that plays a role in the alternative pathway for the synthesis of purines and it is essential for the salvage of adenine and methionine synthesis. Cells lacking MTAP rely exclusively on the *de novo* pathway for the purine synthesis of these elements and might therefore be candidates to treatments based on the inhibition of the purines salvage pathway (36, 37).

In this study we have demonstrated that *MTAP* gene deletion is a relatively frequent phenomenon in MCL occurring in 14% of the cases, 11% of cases with typical morphology and in 25% of the blastoid variants. Our study also shows a high correlation between deletion of *p16* and *MTAP* genes, with just one case harboring a deletion of *MTAP* and wild type *p16*. This case showed a lack of MTAP protein expression by immunohistochemistry confirming the deletion of the *MTAP* gene. Although most deletions in this chromosomal region target *INK4a-ARF* locus (38- 40),

these results indicate that 9p21 deletions may also involve other genes in this region (41).

The immunohistochemical analysis of MTAP protein expression showed a good concordance with the status of the gene. Only one case had a discordant negative expression for the protein whereas the genomic analysis showed no deletion of the gene. This suggests that other mechanisms may be involved in silencing *MTAP* gene expression. In this regard, although hypermethylation of the *MTAP* promoter region has recently been described (39, 42, 43), we could not detect MTAP hypermethylation in our MCL cases. The close correlation between *MTAP* gene deletion and lack of protein expression indicates that this is the main mechanism for inactivation of MTAP in these lymphomas. This observation is concordant with previous findings indicating that the *INK4a-ARF* locus is commonly inactivated by homozygous deletions in MCL whereas hypermethylation, although present in other lymphomas, is uncommon in MCL (44, 45).

MTAP deletions and loss of protein expression in this series of MCL were significantly associated with higher proliferation indices for these tumors and shorter survival of the patients. This phenomenon is most probably due to the close correlation between the deletion of *MTAP* gene and *INK4a-ARF* locus in these tumors, and suggests that the immunohistochemical detection of MTAP may be a good surrogate marker of the inactivation of the whole locus. Interestingly, some recent studies have indicated that *MTAP* by itself may also act as a tumor suppressor gene and its inactivation may contribute to the progression of the tumors. Thus, reintroduction of *MTAP* in a breast cancer cell line in which the gene was deleted, abolished the anchorage independent cell growth and inhibited the tumorigenesis of the cell lines (46). Similarly, a forced expression of MTAP induced a strong reduction in the invasive potential in melanoma cell lines (32). In addition, inactivation of MTAP has also been involved in an indirect inhibition of the STAT1 pathway (47).

The inactivation of *MTAP* gene in MCL cells with high proliferative index and clinical aggressive behavior provides a tumor-specific biochemical feature that could be targeted using inhibitors of the *de novo* AMP synthesis pathway, such as L-alanosine. Several clinical trials have been conducted in the past with L-alanosine, but in tumors where the deletion of MTAP was not documented (48, 49). There is evidence that MTAP deficient tumors, unable to salvage adenine from MTA, are more dependent upon *de novo* synthesis of AMP.

In this study, we reported that in MCL cell lines L-alanosine is cytotoxic against MTAP-negative and MTAP-positive MCL cell lines, as it has been described in other models (18, 50). We also demonstrated that L-alanosine induces the typical features of activation of the mitochondrial apoptotic pathway. Furthermore, we described that EFA, a new MTAP substrate analog, rescued wild type *MTAP* cells from L-alanosine toxicity. EFA has been described as a salvage agent for MTAP positive cells to enhance the therapeutic effect of L-alanosine, since the MTAP substrate provides a source of adenine for normal cells (29).

In summary, MCL cases displaying *MTAP* gene deletions and lack of protein expression are associated with poor prognosis. Moreover MTAP analysis may help to identify patients who might benefit from therapeutic inhibition of *de novo* AMP synthesis pathway. Our results give background to the use of a combination of L-alanosine and EFA as treatment of MTAP-deficient MCL cells.

Figures and Tables

Fig. 1. MTAP expression in MCL cells. A. Western Blot analysis of MTAP protein in total extracts from MCL cell lines. α-tubulin was used as a loading control. B. Immunostaining for MTAP in cells from Granta 519 and JVM-2 MCL cell lines. C. Immunostaining for MTAP: i and ii. Reactive tonsil. MTAP shows an ubiquitous staining for MTAP in a hyperplasic tonsil (positive control). The immunostaining is stronger in mantle cells and shows a cytoplasmic positivity, although some nuclear staining is also seen; iii. Immunostaining for MTAP in a representative MCL tumor with wild type *MTAP*; iv. The same case than in iii incubated with the negative reagent to assess the specificity of the antibody; v. Immunostaining for MTAP in a representative MCL tumor with codeletion of *MTAP* and *p16* genes. Histiocytes and endothelial cells are MTAP positive, serving as an internal positive control, while tumor cells are MTAP negative.

Fig. 2. Overall survival of MCL patients as predicted by *MTAP* gene deletion status (A) and MTAP protein expression by immunohistochemistry (B).

Fig. 3. Cytotoxicity of L-alanosine in MCL. Intracellular ATP levels were determined in Granta 519 (A) and JVM-2 (B) after 24 hours of incubation without (CT) or with different doses of L-alanosine (L-Ala) (20-100 μM). Preincubation of these cells with EFA, at the same doses that L-Ala, preserved ATP levels in the MTAP-positive cell line (JVM-2). C. Analysis of DNA content. Cells from Granta 519 and JVM-2 were incubated for 48 h with medium alone (CT), L-Ala (100 μM) or L-Ala + EFA (100 μM). DNA content was quantified as described in Material and methods.

Fig. 4. Activation of mitochondrial apoptotic pathway after L-alanosine treatment. A. Exposure to L-alanosine (L-Ala, 100 μM) for 48 h induces exposure of cell membrane phosphatidylserine residues, loss of $\Delta\Psi_m$, conformational changes of Bax and Bak and caspase-3 activation. All experiments were performed in duplicate. B. Western blot of activation of caspases and proteolysis of PARP after L-alanosine (100 μM) incubation. EFA (100 μM) rescues JVM-2 from L-alanosine toxicity.

Table 1. *MTAP* and *p16* gene deletions and MTAP protein expression (IHC) in MCL cell lines

	PCR *MTAP*	PCR *p16*	MTAP IHC
JVM-2	wt / wt	wt / wt	+
REC-1	wt / wt	del / del	+
JeKo-1	wt / wt	wt / del	+
Granta 519	del / del	del / del	-
NCEB-1	wt / wt	wt / wt	+

Abbreviations: wt, wild type; del, deleted; IHC: immunohistochemistry

Table 2: MTAP protein expression (IHC) and *MTAP* and *p16* gene deletions in MCL tumors

	MTAP protein expression (n=52)		***MTAP* gene (n=64)**		***p16* gene (n=64)**	
MCL variant	**+**	**-**	**WT**	**DEL**	**WT**	**DEL**
TYPICAL	34/38(89%)	4/38(11%)	43/48(89%)	5/48(11%)	43/48(89%)	5/48(11%)
BLASTOID	10/14(71%)	4/14(29%)	12/16(75%)	4/16(25%)	13/16(81%)	3/16(19%)
TOTAL	**44/52**	**8/52**	**55/64**	**9/64**	**56/64**	**8/64**

Abbreviations: WT, wild type; DEL, deleted

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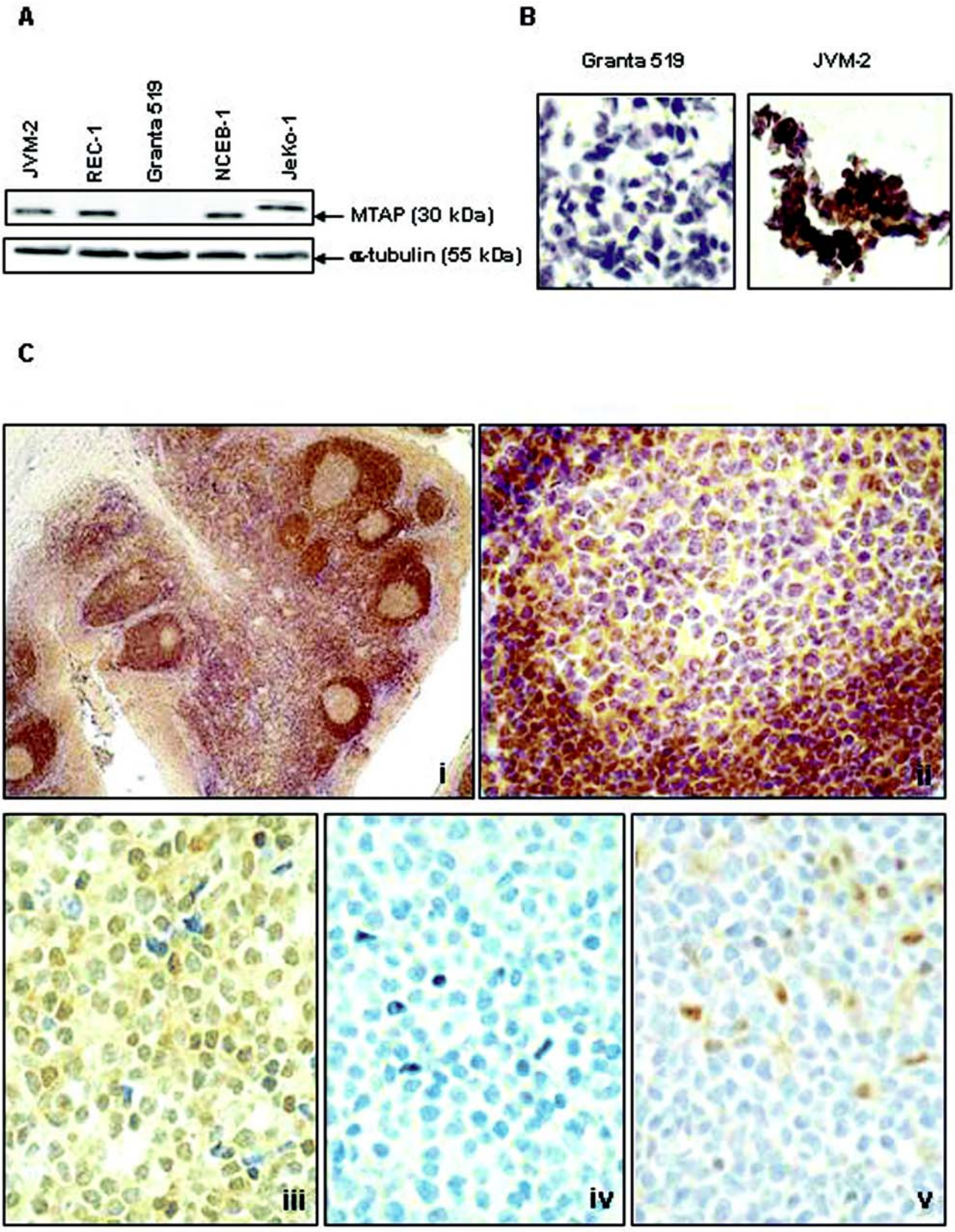
Figure 1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i
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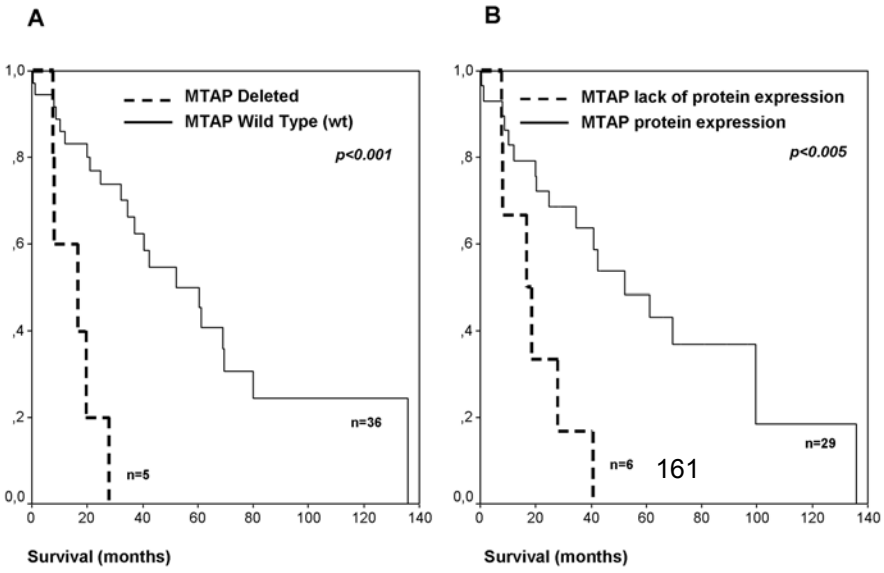
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20
40
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80
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Survival (months)
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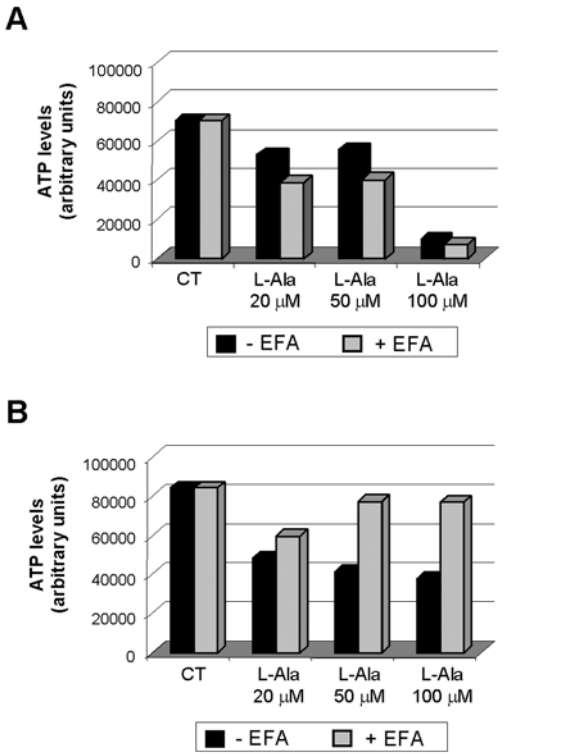
120
140
Survival (months)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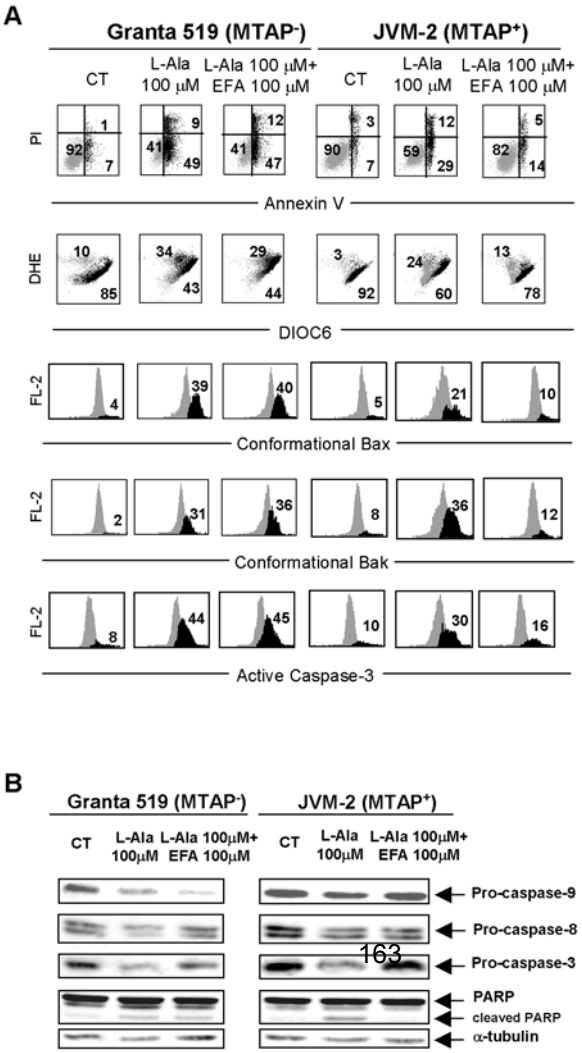
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