Enhancement of fludarabine sensitivity by all-trans-retinoic acid in chronic lymphocytic leukemia cells

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ABSTRACT

Background
A subset of patients with fludarabine-resistant chronic lymphocytic leukemia has previously been shown to express elevated intracellular levels of the concentrative high-affinity fludarabine transporter hCNT3, without any detectable related activity. We have recently shown that all-*trans*-retinoic acid is capable of inducing hCNT3 trafficking to plasma membrane in the MEC1 cell line. We, therefore, evaluated the effect of all-trans-retinoic acid on hCNT3 in primary chronic lymphocytic leukemia cells as a suitable mechanism to improve fludarabine-based therapy of chronic lymphocytic leukemia.

Design and Methods
Cells from 23 chronic lymphocytic leukemia patients wild-type for P53 were analyzed for *ex vivo* sensitivity to fludarabine. hCNT3 activity in chronic lymphocytic leukemia cell samples was evaluated by measuring the uptake of [8-^{3}H]-fludarabine. The amounts of transforming growth factor-β1 and hCNT3 messenger RNA were analyzed by real-time polymerase chain reaction. The effect of all-*trans*-retinoic acid on hCNT3 subcellular localization was analyzed by confocal microscopy and its effect on fludarabine-induced apoptosis was evaluated by flow cytometry analysis using annexin V staining.

Results
Chronic lymphocytic leukemia cases showing higher *ex vivo* basal sensitivity to fludarabine also had a greater basal hCNT3-associated fludarabine uptake capacity compared to the subset of patients showing *ex vivo* resistance to the drug. hCNT3 transporter activity in chronic lymphocytic leukemia cells from the latter patients was either negligible or absent. Treatment of the fludarabine-resistant subset of chronic lymphocytic leukemia cells with all-*trans*-retinoic acid induced increased fludarabine transport via hCNT3 which was associated with a significant increase in fludarabine sensitivity.

Conclusions
Improvement of *ex vivo* fludarabine sensitivity in chronic lymphocytic leukemia cells is associated with increased hCNT3 activity after all-*trans*-retinoic acid treatment.

Key words: chronic lymphocytic leukemia, hCNT3, fludarabine, treatment, nucleoside.

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Introduction

Chronic lymphocytic leukemia (CLL) is a common type of leukemia in adults and is characterized by the persistent accumulation of CD5$^+$ B lymphocytes,[1] primarily due to defects in apoptosis. The disease has a highly variable clinical course and treatment is usually restricted to patients with advanced and symptomatic disease. Fludarabine, a purine nucleoside analog, has long been a major choice for CLL chemotherapy. This drug is cytotoxic both against dividing and resting cells.[2,3] In dividing cells, fludarabine inhibits ribonucleotide reductase and DNA synthesis,[4,5] whereas in quiescent cells the main mechanism of cytotoxicity appears to be inhibition of cellular DNA repair processes leading to the induction of apoptosis.[6,7] Fludarabine monotherapy is associated with higher rates of long-lasting complete remission and improved overall response when compared with treatment with alkylating agents.[8-11] However, since the late 1990s, combination chemotherapies have become the recognized gold standards of care. Purine analogs and alkylating agents have different mechanisms of action and, therefore, have partially non-overlapping toxicity profiles. Moreover, synergistic effects are evident when therapy with two such compounds is prescribed.[12-15] More recent reports suggest that the administration of monoclonal antibodies, such as rituximab, can significantly improve the course of CLL. Indeed, the latest results show that chemoimmunotherapy, such as fludarabine, cyclophosphamide, and rituximab, is the optimal first-line treatment; however, some patients do not respond to this regimen.[16-18] Resistance to fludarabine is a major problem in CLL treatment. As a DNA-damaging agent, fludarabine increases P53 levels by promoting post-translational stabilization of the protein, thereby inducing P53-dependent cell death. Although mutations in the *P53* gene have been correlated with resistance to fludarabine and reduced survival of patients,[19-21] the nucleoside analog can also induce apoptosis of CLL cells *in vitro* in a P53-independent manner.[22] Furthermore, the cellular microenvironment likely influences chemoresistance. Bone marrow stromal cells protect CLL cells from fludarabine, dexamethasone, and cyclophosphamide by a mechanism requiring cell-cell contact.[23] Indeed, several mechanisms may contribute to fludarabine resistance in patients wild-type for P53.

Before entering cells, fludarabine is rapidly dephosphorylated by membrane ectonucleotidases (CD73) and then transported inside the cell via nucleoside-selective plasma membrane transporters. Once inside the cell, the drug is phosphorylated by deoxycytidine kinase before its cytotoxic activity can be exerted.[5,24] Nucleoside uptake into cells is mediated by specific nucleoside transporter (NT) proteins belonging to two unrelated gene families, SLC28 and SLC29, encoding CNT (concentrative nucleoside transporter) and ENT (equilibrative nucleoside transporter) proteins, respectively.[25,26] Previous work by our group showed that primary CLL cells co-express hENT1, hENT2, hCNT2, and hCNT3, but accumulation of fludarabine in CLL cells is mediated mostly, if not exclusively, by ENT-type transporters.[27] hCNT3 but not hCNT2 can also transport fludarabine.[28-30] Indeed, hCNT3 is even more efficient than is hENT2 in this regard; the former transporter has a higher affinity for the drug and, more importantly, it is a CNT, thus concentrating nucleoside analogs inside cells.

Fludarabine-resistant CLL cells express high but variable levels of hCNT3 mRNA and cytosolic hCNT3 protein,[31] thus suggesting that hCNT3 is localized mainly in intracellular compartments of such CLL cells. We have recently reported that all-*trans*-retinoic acid (ATRA), a natural vitamin A derivative currently used in the treatment of acute promyelocytic leukemia,[32] can increase hCNT3 activity in MEC1 cells via a mechanism mediated by transforming growth factor β1 (TGFβ1).[33]

We hypothesized that P53 wild-type cases showing resistance to fludarabine, might have low plasma membrane hCNT3 activity, thus compromising efficient fludarabine uptake into CLL cells, probably by retaining hCNT3 intracellularly. Moreover, we sought to determine whether the effect of ATRA on hCNT3, described to date only in a cell line,[33] was evident in primary CLL cells. This would provide a basis for modulating fludarabine-based therapies.

Design and Methods

Chemicals and reagents

ATRA and TGFβ1 were purchased from Sigma-Aldrich. D-[1-^{14}C]-mannitol was purchased from Amersham Biosciences. [8-^{3}H]-fludarabine was from Moravek Biochemicals. Fludarabine (9-b-D-arabinosyl-2-fluoroadeninemonophosphate) (Schering) and dexamethasone (Merck) were kindly provided by the Pharmacy Department of our hospital. Bortezomib was kindly provided by Millenium Pharmaceuticals. The monoclonal antibody against hCNT3 was raised against a synthetic hCNT3 peptide (conjugated to keyhole limpet hemocyanin) which corresponded to amino acid residues 45-69 in the amino terminus of the protein.

Isolation and culture of primary cells

Twenty-three untreated patients diagnosed with CLL according to the International Working Group on CLL (IWCLL) criteria[34] were included in the present study. Informed consent was obtained from each patient in accordance with guidelines of the Institutional Ethics Committee of our hospital (approval register number 2009/4706) and the Declaration of Helsinki. The biological characteristics of the patients are listed in Table 1. The percentages of tumor cells (CD19$^+$, CD5$^+$, and with light-chain restriction) and CD38 expression were analyzed by flow cytometry, and ZAP70 expression level was quantified as previously described.[35] Cytogenetic alterations were assessed by fluorescence *in-situ* hybridization (FISH) using a multiprobe commercial kit from Vysis; the kit contains locus-specific probes to determine the deletions of 17p (*TP53*), 11q (*ATM*), and 13q, and a centromeric probe to detect trisomy 12. The cut-off point for detection of such alterations in CLL cells was 20%. *P53* mutational analysis was performed according to the recommendations of the IARC TP53 consortium (*http://p53.iarc.fr*). IGHV gene mutational status was determined according to European Research Initiative on CLL (ERIC) guidelines.[36]

Peripheral blood mononuclear cells were isolated by Ficoll/Hypaque sedimentation (Seromed, Berlin, Germany). Cells were cryopreserved in liquid nitrogen with 10% (v/v) dimethyl sulfoxide and 60% (v/v) heat-inactivated fetal bovine serum from Gibco. Previous studies have shown that freezing has no effect on cell response compared to that of freshly isolated CLL cells.[37] After thawing, mononuclear cells from CLL patients were cultured in RPMI 1640 (Invitrogen), supplemented with 10% fetal bovine serum, 2 mM glutamine (Invitrogen), and 50 μg/mL penicillin-

Table 1. Biological characteristics of the CLL patients studied.

Patient n.	Age at diagnosis (years)	IGHV mutational status	Gender	Stage	CD19/CD5 (%)*	ZAP70 (%)*	CD38 (%)*	P53 status¬	Cytogenetic alterations (FISH)±	Apoptosis induction over control with Flu (%) +
CLL R1	46	n.d.	F	B/C	90	> 20	n.a.	w.t.	13q del	<10
CLL R2	52	M	F	B/C	97	1	0,1	w.t.	13q del	<10
CLL R3	54	n.d.	M	B/C	90	> 20	n.a.	w.t.	normal	<10
CLL R4	72	M	M	A	92	0,4	4	w.t.	13q del	<10
CLL R5	49	n.d.	F	B/C	90	n.a.	n.a.	w.t.	normal	<10
CLL R6	63	M	F	A	95	50	6	w.t.	13q del	<10
CLL R7	73	UM	M	B	90	70	9	w.t.	13q del	<10
CLL R8	83	M	M	B	97	1	98	w.t.	normal	<10
CLL R9	58	UM	F	C	96	44	19	w.t.	13q del	<10
CLL R10	59	M	M	B	95	0	1	w.t.	13q del	<10
CLL R11	74	M	M	A	92	4	38	w.t.	normal	<10
CLL R12	49	M	F	A	87	3	8	w.t.	13q del	<10
CLL S1	61	M	F	n.a.	92	1	3	w.t.	13q del	11,0
CLL S2	91	UM	M	A	98	n.a.	46	w.t.	13q del	13,5
CLL S3	58	M	M	A	95	8	3	w.t.	normal	54,5
CLL S4	47	n.d.	M	A	97	15	2	w.t.	13q del	29,0
CLL S5	88	UM	F	C	85	2	0,5	w.t.	n.a.	29,0
CLL S6	62	M	M	B/C	85	3	1	w.t.	normal	25,0
CLL S7	44	UM	M	n.a.	93	12	6	w.t.	13q del	26,0
CLL S8	64	UM	F	A	94	27	94	w.t.	normal	42,0
CLL S9	62	UM	M	B	90	53	n.a.	w.t.	13q del	23,3
CLL S10	75	UM	M	B	91	28	99	w.t.	11q del	11,0
CLL S11	50	M	F	B/C	82	>20	99	w.t.	n.a.	12,2

The principal characteristics of the 23 patients diagnosed with chronic lymphocytic leukemia. Gender: M: male; F: female; IGHV *mutational status: M: mutated; UM: unmutated; n.d: not detected; Stage according to Binet's classification; n.a: not available; *Quantified by flow cytometry; ZAP70: +, > 20% expression; -, < 20%; CD38: +,>30 % expression, -,< 30%; n.a: not available; n.d: not done; P53 status determined by FISH (17p deletion) and direct sequencing; wt: wild type and ± assessed by FISH. del: deletion; n.a: not available; + cells were incubated with fludarabine 10 μg/mL for 90 min, washed and cultured in drug-free complete medium. Cytotoxicity was measured after 48 h by annexin V staining. The difference between basal (not treated) apoptosis and fludarabine-induced apoptosis is shown.*

streptomycin (Invitrogen) at a density of 2×10^6 cells/mL, in a humidified atmosphere at 37°C containing 5% carbon dioxide.

Cell treatment and apoptosis detection by flow cytometry

Cells were incubated for 90 min with fludarabine, bortezomib, or dexamethasone, washed twice with phosphate-buffered saline (PBS), and next cultured in complete medium for 48 h. When indicated, cells were pre-incubated with either ATRA for 5 h or TGFβ1 for 40 min before exposure to the previously mentioned drugs. Cell viability was quantified 48 h later by double-staining with annexin V conjugated to fluorescein isothiocyanate and propidium iodide (BenderMedsystems). Ten thousand cells per sample were acquired in a FACScan flow cytometer and the proportions of labeled cells were analyzed using Paint-A-Gate software (Becton Dickinson).

Fludarabine transport assay

For transport measurements, CLL cells were assessed immediately after thawing or after incubation at 37°C for different periods with or without ATRA. Uptake of [8-^{3}H]-fludarabine (1 μM) was measured using a rapid filtration method adapted from a previously described technique.[38] Cells were resuspended in either a sodium chloride buffer or a choline chloride buffer. Each uptake assay started by mixing a cell suspension with an equal volume of buffer, supplemented with 1 μM [8-^{3}H]-fludarabine at a specific activity of 17,500 dpm/pmol. After incubation, aliquots were placed in ice-cold 0.4-mL needle Eppendorf tubes, containing an upper buffer phase, an intermediate oil layer (dibutylphthalate/bis-3,5-trimethylhexyl) phthalate [3:2, v/v]), and a 10% (v/v) $HClO_4$/25% (v/v) glycerol solution at the bottom. The tube was immediately centrifuged (at 15,000 g for 60 sec), separating cells from the radioactive incubation medium; the cells became pelleted below the $HClO_4$ layer. D-[^{14}C]-mannitol (specific activity 4,000 dpm/pmol) was included in the incubation medium to assess the amount of extracellular medium that became trapped in the bottom acidic layer. To ensure that all cells were recovered prior to counting of radioactivity, each tube was blade-cut at the level of the oil layer level, releasing the bottom part into vials for scintillation counting. Double counting quantified the levels of transported substrate (tritiated fludarabine) and the extracellular marker (D-[1-^{14}C]-mannitol). The protein level in the aliquots was then determined using the Bradford technique (Bio-Rad).

Both ENT- and CNT-type proteins are functional in the presence of sodium, although only CNT are sodium-dependent. This means that, in the absence of the cation, only ENT will be active.

Accordingly, sodium-dependent nucleoside uptake (CNT-associated) was calculated by subtracting the uptake rate measured in choline chloride-containing buffer (almost exclusively associated with ENT1 and ENT2 activities) from that obtained using sodium-containing buffer (in which both ENT and CNT are active).

RNA isolation and quantitative reverse transcriptase polymerase chain reaction

Total RNA was isolated using the TRIzol reagent (Invitrogen). One microgram of total DNase-treated RNA was used to generate cDNA, using M-MLV Reverse Transcriptase and random hexamers for the reverse transcription (Life Technologies). RNA retrotranscription and reverse transcriptase polymerase chain reaction (RT-PCR) were performed as previously described.[33] Quantitative real-time PCR amplification of mRNA encoding ULBP3, hCNT3 and TGFβ1 was performed using primers and probes from Applied Biosystems. PCR amplification of cDNA was monitored in real-time by using the TaqMan Universal Master Mix (Applied Biosystems), 700 nmol/L probe and 150 nmol/L of each primer in the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Relative gene expression was quantified using the $2^{-\Delta\Delta Ct}$ method and the method was validated as described in the TaqMan user's manual with β-glucuronidase (*GUS*) as the internal control and the basal conditions as calibrator.

Immunofluorescence staining and confocal microscopy analysis

Primary CLL cells from fludarabine-resistant CLL patients were cultured in the presence or not of ATRA (10 μM) for 5.5 h. After stimulation, cells were rinsed twice with PBS and fixed on poly-L-lysine-coated glass coverslips with 4% paraformaldehyde for 15 min at 4°C. Coverslips were rinsed in PBS and kept overnight at 4°C. Cells were subjected to WGA-TRITC (wheat germ agglutinin, MolecularProbes) for plasma membrane staining for 10 min at room temperature and rinsed three times with PBS. The cells were then permeabilized with a solution containing 0.1% saponin and 10% fetal bovine serum followed by incubation for 30 min with the monoclonal antibody anti-hCNT3. After three washes in PBS, preparations were incubated with Alexa Fluor 488-labeled goat anti-mouse antibody (Molecular Probes) for an additional 30 min at room temperature in the dark. After three washes in PBS, cells were mounted in Vectashield (Vector Laboratories) and subjected to DAPI (4,6 diamidino-2-phenylindole; SIGMA) for nuclear staining. Images were obtained using a Leica TCS SL laser scanning confocal spectral microscope (Leica Microsystems) with argon and HeNe lasers attached to Leica DMIRE2 inverted microscope lasers.

Statistical analysis

The statistical significance between groups was analyzed using Student's t -test. In the figure legends, $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, and $^{*}P \leq 0.05$ indicate statistically significant differences between treatments.

Results

Sensitivity of primary chronic lymphocytic leukemia cells to fludarabine

Fludarabine sensitivity was evaluated ex vivo in cells from 23 CLL patients who were wild-type for TP53. The cohort comprised 10 women and 13 men with a median age at the time of CLL diagnosis of 62.3 years (range, 44-88 years). The general clinical and biological features of these patients are shown in Table 1.

CLL cells were cultured with fludarabine (10 μg/mL) for 90 min, washed twice in PBS, and next cultured in drug-free complete medium for an additional 48 h, at which time cytotoxicity was assessed by annexin V/propidium iodide staining. A heterogeneous profile of sensitivities to the nucleoside analog was observed among patients, with relative apoptotic rates ranging from 0 to 44% above basal levels. This allowed us to split the cohort into two subsets, one resistant and the other sensitive to fludarabine, as determined by the choice of an arbitrary threshold [a 10% increase in fludarabine-induced apoptosis compared to that of control non-treated CLL cells (Table 1)]. Twelve fludarabine-resistant patients (CLL-R) and 11 fludarabine-sensitive patients (CLL-S) were identified in this way. We focused most of this study on the resistant patients aiming to establish whether ATRA treatment could improve fludarabine sensitivity via a mechanism consistent with up-regulation of hCNT3-related transport activity.

The effect of all-trans-retinoic acid on TGFβ1, hCNT3 and ULBP3 expression in primary chronic lymphocytic leukemia cells

It has been described that the level of expression of UL16-binding protein 3 (ULBP3) in primary CLL cells changes following ATRA treatment.[39] We, therefore, monitored changes in the level of mRNA encoding ULBP3 in cells exposed to ATRA by quantitative real time-PCR as a way to check that CLL cells from fludarabine-resistant patients might be sensitive to ATRA. We found that ULBP3 expression increased in all CLL samples tested for the action of ATRA (Figure 1A). As ATRA has recently been shown to induce the expression and release of TGFβ1 in the MEC1 cell line,[33] we next analyzed the effect of ATRA on TGFβ1 expression in primary cells from fludarabine-resistant CLL cases. ATRA promoted an increase in TGFβ1 mRNA level (Figure 1B) in all instances. Furthermore we analyzed the action of ATRA on hCNT3 observing no changes in the transporter expression when the CLL cells were cultured in the presence of ATRA (Figure 1C).

The effect of all-trans-retinoic acid on fludarabine transport into chronic lymphocytic leukemia cells

To determine the effect of ATRA on hCNT3 activity, [^{3}H]-fludarabine uptake by primary CLL cells from six fludarabine-resistant CLL patients, cultured in the presence or the absence of 10 μM ATRA, was monitored over time, as described in the Design and Methods section. [^{3}H]-fludarabine uptake into untreated CLL cells was attributable principally to hENT-mediated sodium-independent transporters (*data not shown*) whereas sodium-coupled fludarabine transport was very low or even negligible in the cells of some CLL cases (Figure 2A). Interestingly, ATRA treatment of CLL cells caused a time-dependent increase in sodium-coupled fludarabine uptake (Figure 2A); the peak of activity occurred 5-6 h after ATRA treatment. As mentioned above, CLL cells express only the hCNT2 and hCNT3 concentrative transporters, and, of these, only hCNT3 transports fludarabine, thus suggesting that hCNT3 activity is up-regulated in ATRA-treated cells from fludarabine-resistant CLL patients.

We also analyzed the effect of ATRA on primary cells from three CLL patients who were shown to be sensitive to fludarabine *ex vivo*. In contrast to the results described

above, drug-sensitive CLL cells showed significant basal hCNT3 activity which was minimally affected by ATRA treatment (Figure 2B). In addition to the three samples in which the time course studies were performed, basal fludarabine hCNT3-related uptake was assayed in four additional fludarabine-sensitive CLL cases showing that all CLL-S cells had a significant basal fludarabine uptake mediated by hCNT3 (Figure 2C) when compared with the CLL-R samples, in which fludarabine-hCNT3-mediated basal uptake was almost null (Figure 2A).

The effect of all-trans-retinoic acid on hCNT3 subcellular localization in chronic lymphocytic leukemia cells

To determine whether induced hCNT3-related activity correlates with a change in the subcellular localization of the hCNT3 protein in CLL cells, confocal microscopy immunofluorescence was performed in three independent samples derived from fludarabine-resistant CLL patients previously shown to have negligible fludarabine hCNT3-mediated uptake. In basal conditions, hCNT3 staining was easily observed in the cytosol of most cells (Figure 3). After ATRA treatment for 5.5 h, the intracellular signal was markedly depleted, consistent with a change in the subcellular localization of the transporter protein. Nevertheless this was not paralleled by an equivalent increase in plasma membrane labeling, although some co-localization of hCNT3 with plasma membrane markers was observed. An explanation for this low immunoreactivity may lie in the choice of the peptide to which the antibody was raised. This peptide is within the N-terminus tail of the protein, which is intracellular and may interact with other protein partners depending upon their subcellular localization. In any case, these observations favor the view that the increase in fludarabine uptake is the consequence of a change in the subcellular localization of hCNT3 in this subset of CLL patients.

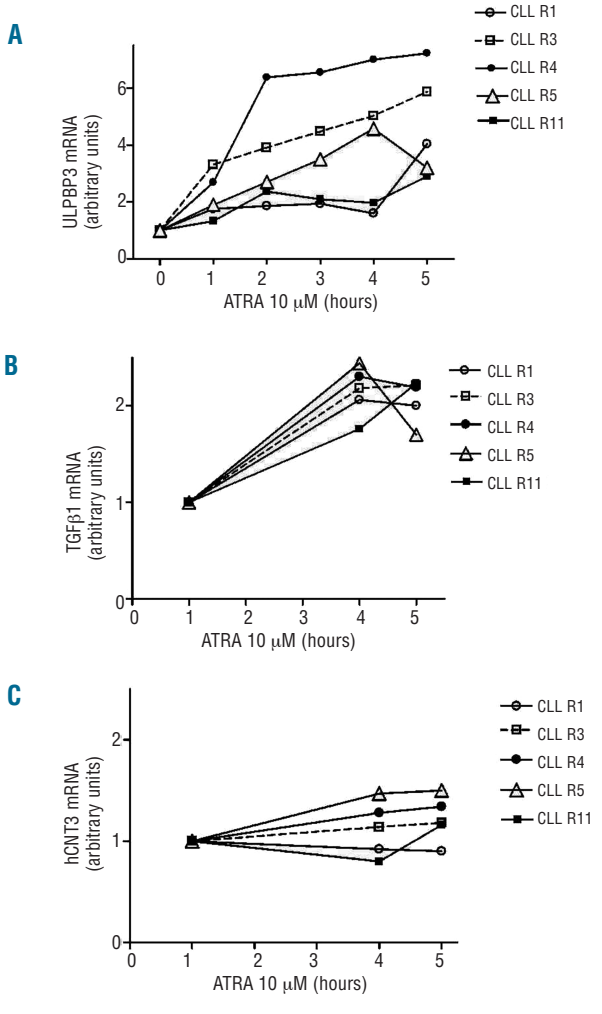

Figure 1. Effect of ATRA on the expression of ULBP3, TGFβ1 and hCNT3. (A) ULBP3, (B) TGFβ1 and (C) hCNT3 mRNA levels in CLL cells were determined by quantitative RT-PCR and are expressed relative to the level of an endogenous reference gene (β-glucuronidase), as described in the *Design and Methods* section. Relative mRNA levels, in arbitrary units, are shown.

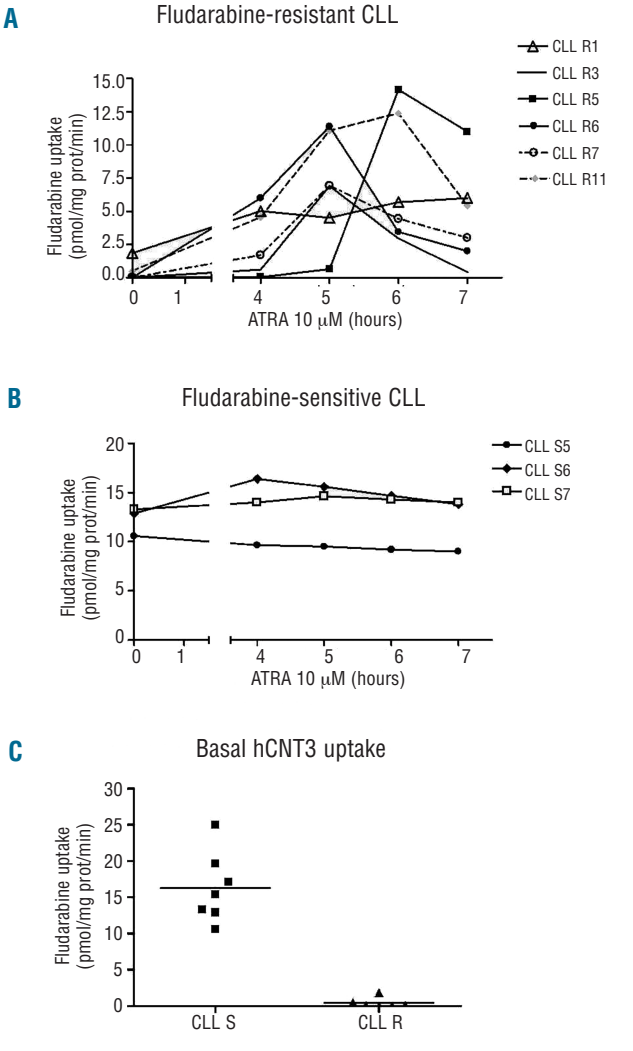

Figure 2. Effect of ATRA on hCNT3-related fludarabine transport activity. Time courses of sodium-dependent [8-^{3}H]-fludarabine uptake by CLL cells from (A) six fludarabine-resistant patients and, (B) three fludarabine-sensitive patients cultured with ATRA (10 μM) for the indicated times. (C) Basal [8-^{3}H]-fludarabine uptake by CLL cells from the previous six fludarabine-resistant and seven fludarabine-sensitive cases is shown. Data are expressed as means of triplicate measurements.

The effect of all-trans-retinoic acid on fludarabine-induced cytotoxicity in primary chronic lymphocytic leukemia cells

As ATRA seems to induce trafficking of hCNT3 to the plasma membrane and a concomitant increase in fludarabine uptake, we next evaluated the effect of ATRA on fludarabine cytotoxicity in CLL cells from the whole cohort of patients (Table 1). Cells were pretreated with ATRA (1 or 10 μM) for 5 h before exposure to fludarabine for 90 min. For a more precise determination of the role transport processes might play in fludarabine-induced apoptosis, the 90 min exposure time was chosen to bracket the peak of hCNT3 activity induced by ATRA. This short time exposure to the drug also explains why higher than usual fludarabine concentrations were used in this study. After incubation with the drug, cells were cultured for an additional 48 h in drug-free complete medium and assayed for apoptosis. The apoptotic response differed among patients. The subset of fludarabine-sensitive cells (11 cases) showed no changes in fludarabine-induced apoptosis after incubation with ATRA (Figure 4A), whereas fludarabine-resistant cells (12 cases) showed significantly elevated apoptotic levels after pre-treatment with ATRA when fludarabine was used at 10 μg/mL, a dose to which the cells were completely insensitive when the drug was employed alone for 90 min (Figure 4B).

We previously showed that TGFβ1 is involved in the effect triggered by ATRA in the CLL cell line MEC1.[33] In the present study, we demonstrated that ATRA induced TGFβ1 expression in primary CLL cells. To evaluate the role played by TGFβ1 in fludarabine-triggered cytotoxicity, CLL cells were cultured in the presence of TGFβ1 for 40 min before addition of fludarabine (10 μg/mL for 90 min). Apoptosis levels were measured 48 h later by annexin V/propidium iodide staining. As shown in Figure 5A, a significant increase in fludarabine-induced apoptosis was evident in cells from drug-resistant patients (n=12) when the cells were pretreated with TGFβ1. On the other hand, TGFβ1 had no effect on apoptosis triggered by fludarabine in drug-sensitive cells (n=11) (Figure 5B).

The effect of all-trans-retinoic acid on the cytotoxicity of non-nucleoside-derived drugs in chronic lymphocytic leukemia cells

The possibility that ATRA could sensitize CLL cells to apoptosis triggered by any cytotoxic anticancer drug, which would indicate that induction of fludarabine-triggered cytotoxicity was non-specific, was addressed by

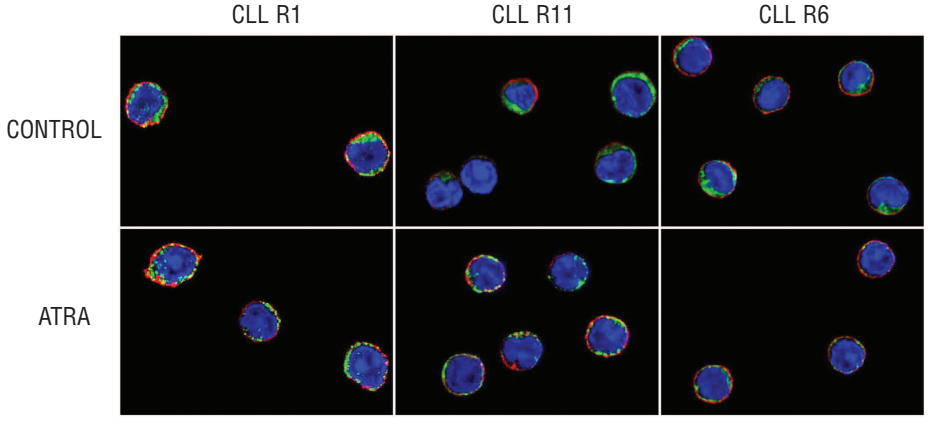

Figure 3. Effect of ATRA treatment on hCNT3 subcellular localization in CLL cells by immunofluorescence microscopy. CLL cells were cultured with ATRA for 5.5 h, fixed, and stained with WGA, a plasma membrane marker (red), a hCNT3 monoclonal antibody (green) and DAPI for nuclear staining (blue). Coverslips were analyzed by confocal microscopy. A representative group of cells from three independent CLL patients is shown.

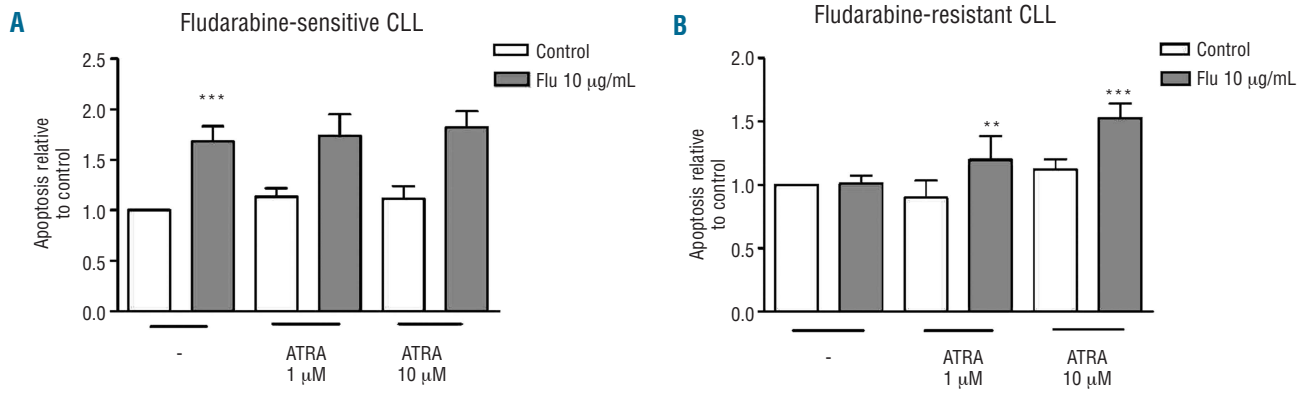

Figure 4. Effect of ATRA on fludarabine-induced cytotoxicity. Primary CLL cells were incubated in the presence or absence of ATRA (1 or 10 μM) for 5 h and further exposed to fludarabine (10 μg/mL) for 90 min. After washing, cells were incubated in drug-free medium for 48 h. (A) Eleven fludarabine-sensitive and (B) 12 fludarabine-resistant CLL samples were analyzed in terms of viability by annexin V binding. Data were normalized to the control (1) for each CLL case and means ± SEM are shown. Statistical significance (**P*<0.05; ***P*<0.01; ****P*<0.001) indicates (in A) a significant difference with respect to the control and (in B) a significant difference relative to fludarabine-treated cells in the absence of ATRA.

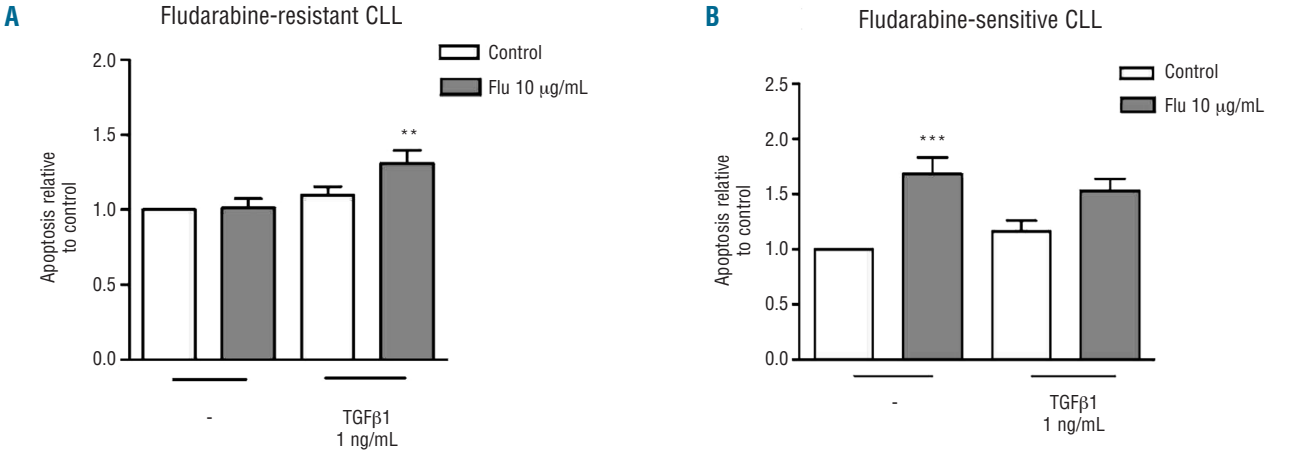

Figure 5. Effect of TGFβ1 on fludarabine-induced cytotoxicity. Primary CLL samples were incubated in the presence or absence of TGFβ1 (1 ng/mL) for 40 min and further exposed to fludarabine (10 μg/mL) for 90 min. After washing, cells were incubated in drug-free medium for 48 h. (A) Twelve fludarabine-resistant and (B) 11 fludarabine-sensitive samples were analyzed in terms of viability by annexin V binding followed by flow cytometry. Data were normalized to the control (1) for each CLL case and means ± SEM are shown. Statistical significance (*P<0.05; **P<0.01; ***, p<0.001) indicates (in A) a significant difference with respect to fludarabine-treated cells in the absence of ATRA and (in B) a significant difference relative to control cells.

testing the effects triggered by two anti-neoplastic agents, the proteasome inhibitor bortezomib (5 nM) and the glucocorticoid steroid dexamethasone (1.5 μM), neither of which is taken up via a nucleoside transporter. CLL cells were pretreated with ATRA for 5 h before addition of either bortezomib or dexamethasone for 90 min. Cells were next washed and cultured for a further 48 h in drug-free complete medium. Neither ATRA nor TGFβ1 increased the extent of apoptosis induced by either cytotoxic drug in any cell sample tested (Figure 6).

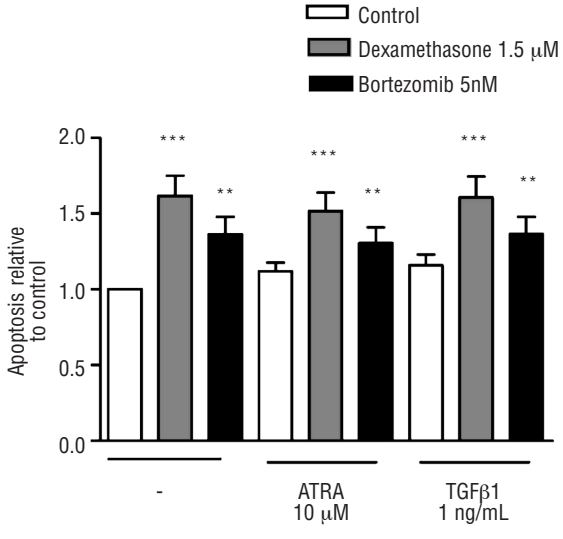

Figure 6. Effect of ATRA on bortezomib- and dexamethasone-induced cytotoxicity. Primary CLL samples were incubated in the presence or absence of either ATRA (10 μM) for 5 h or TGFβ1 (1 ng/mL) for 40 min and further exposed to bortezomib (5 nM) or dexamethasone (1.5 μM) for 90 min. After washing, cells were incubated in drug-free medium for 48 h. Eight CLL samples were analyzed in terms of viability by annexin V binding followed by flow cytometry. Data were normalized to the control (1) for each CLL case and means ± SEM are shown. Statistical significance (P<0.01; ***P<0.001) indicates significant differences relative to control values.**

Discussion

The nucleoside analog fludarabine is one of the most effective drugs in the treatment of CLL. However, some patients do not respond adequately to this therapy which is why the treatment of fludarabine-refractory disease is so important nowadays.[40,41] Previous studies revealed a correlation between intracellular expression of the broad-selectivity nucleoside drug transporter hCNT3 and reduced time-to-progression in patients.[42] Furthermore, on the basis of a study from our laboratory in a CLL-derived cell line that showed regulated trafficking of hCNT3 into the plasma membrane,[33] we hypothesized that early steps (i.e., transport across the plasma membrane) in the pharmacological action of fludarabine may be a limiting factor for the induction of cytotoxicity in fludarabine-resistant patients wild-type for P53, thereby becoming suitable targets for therapy improvement. The present study provides "proof of concept" evidence, demonstrating that modulation of transport function in primary CLL cells improves chemosensitivity in this subset of CLL patients. A model of hCNT3-regulated trafficking in MEC-1 cells recently developed by our group suggests that ATRA can promote movement of hCNT3-enriched vesicles into the plasma membrane.[33] Furthermore, we have previously shown that primary CLL cells only express hCNT2 and hCNT3 as sodium-coupled nucleoside transporters.[27] Thus, since hCNT2 is not a fludarabine transporter, fludarabine uptake can only be mediated by hCNT3.[27] In fact, here it was shown that *ex vivo* treatment of fludarabine-resistant primary CLL cells with ATRA resulted in an increase in fludarabine influx and enhanced cytotoxicity.

ATRA seems to have a variety of effects on CLL cells, apart from the novel post-translational regulation of a drug transporter described in the present study. ATRA induces apoptosis, in a dose-dependent manner, in CLL cells treat-

ed with the drug for 48 h,[43] and this effect is mediated by caspase-3 activation. In our experiments, ATRA alone did not significantly induce apoptosis in cell samples tested, probably because ATRA treatment lasted only 5 to 6 h in our model. In any case, any ATRA-triggered pro-apoptotic effect on CLL cells would benefit patients further.

As ATRA increases TGFβ1 mRNA levels, and considering that this cytokine itself potentiates fludarabine action, it is very likely that the enhanced fludarabine sensitivity of primary CLL cells is mediated by a similar mechanism, if not identical, to that recently described in the MEC-1 CLL cell line. Moreover, neither ATRA nor TGF-β1 induced any change in either the amounts of hCNT3 mRNA or protein (*data not shown*). Thus, the increase in hCNT3-related function after the addition of ATRA cannot be the result of changes in hCNT3 expression levels, in accordance with the observations in MEC1 cells.[33] This might be consistent with the fact that ATRA promotes expression and release of TGFβ1, which would then enhance the trafficking of hCNT3-enriched vesicles into the plasma membrane.[33]

In summary, the data presented here support the view that the fludarabine resistance of primary CLL cells from patients wild-type for P53 can be overcome, to a significant extent, by ATRA treatment, leading to TGFβ1 synthesis and, probably, to a change in the subcelullar localization of hCNT3. In fact, fludarabine-resistant untreated cells were shown to contain high intracellular levels of hCNT3 protein, in line with previous observations.[31]

On the other hand, CLL cells from the subset of patients showing *ex vivo* sensitivity to fludarabine had significant basal hCNT3-related activity, indicating that such cells could take up the drug more efficiently under basal conditions. Such uptake, even if moderately low, might be adequate to sustain the hCNT3 concentrative capacity required to potentiate the pharmacological action of fludarabine. It may be argued that hENT-related activity of both drug-resistant and -sensitive cell populations could mediate fludarabine-related effects, but hENT show lower affinity for fludarabine than hCNT3 and, more importantly, hENT mediate bidirectional transport across the plasma membrane, whereas hCNT3 is a highly concentrative drug influx transporter, as two sodium ions are translocated along with every drug molecule.[29,44] This makes hCNT3 a suitable target for chemotherapeutic intervention in tumor cells in which hCNT3 intracellular stores may be present and eventually recruited to the plasma membrane, thereby enhancing the drug uptake capacity of such cells.

Authorship and Disclosures

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