

REVIEW

Nucleoside transporters in chronic lymphocytic leukaemia

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Nucleoside derivatives have important therapeutic activity in chronic lymphocytic leukaemia (CLL). Experimental evidence indicates that in CLL cells most of these drugs induce apoptosis *ex vivo*, suggesting that programmed cell death is the mechanism of their therapeutic action, relying upon previous uptake and metabolic activation. Although defective apoptosis and poor metabolism often cause resistance to treatment, differential uptake and/or export of nucleosides and nucleotides may significantly modulate intracellular drug bioavailability and, consequently, responsiveness to therapy. Two gene families, SLC28 and SLC29, encode transporter proteins responsible for concentrative and equilibrative nucleoside uptake (CNT and ENT, respectively). Furthermore, selected members of the expanding ATP-binding cassette (ABC) protein family have recently been identified as putative efflux pumps for the phosphorylated forms of these nucleoside-derived drugs, ABC11 (MRP8) being a good candidate to modulate cell sensitivity to fluoropyrimidines. Sensitivity of CLL cells to fludarabine has also been recently correlated with ENT-type transport function, suggesting that, besides the integrity of apoptotic pathways and appropriate intracellular metabolism, transport across the plasma membrane is also a relevant event during CLL treatment. As long as nucleoside transporter expression in leukaemia cells is not constitutive, the possibility of regulating nucleoside transporter function by pharmacological means may also contribute to improve therapy.

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Nucleoside-derived drugs in chronic lymphocytic leukaemia (CLL) therapy

B-cell CLL is characterized by the accumulation of long-lived, functionally inactive, mature appearing, neoplastic B-lymphocytes arrested in the G0 phase of the cell cycle.^{1,2} The clonal excess of B cells is mainly caused by a decrease in cell death rather than by increased cell proliferation.³ CLL is currently incurable but several drugs, including pyrimidine and purine analogues, cause clinical improvement in CLL patients.⁴ The two primary purine analogues in CLL therapy are fludarabine⁵ and 2-chlorodeoxyadenosine (2-CdA).⁶ These drugs have been used mostly alone, but also in combination, for the treatment of low-grade B-cell malignancies, including CLL, follicular lymphoma and hairy-cell leukaemia.^{7,8}

The nucleoside derivatives that induce cytotoxicity in CLL cells include: deoxycoformycin (pentostatin),^{9,10} 2-CdA (cladri-

bine) and its derivatives,^{11–13} fludarabine,¹⁴ cytarabine,¹⁵ gemcitabine¹⁶ and loxoribine.¹⁷ Very recently, we found that the precursor of nucleotide biosynthesis acadesine (5-aminomidazole-4-carboxamide (AICA) riboside) also induces apoptosis in CLL cells.¹⁸

Experimental evidence indicates that in CLL cells most of these drugs induce apoptosis *ex vivo*, suggesting that programmed cell death is the mechanism of their therapeutic action.^{11,14,19–22} Thus, in response to apoptotic signals, cytochrome *c* is released from mitochondria and binds to the adaptor protein Apaf-1. This results in the activation of caspase-9, which triggers downstream caspases, such as caspase-3. Moreover, nucleoside analogues may directly affect the mitochondria²³ or activate Apaf-1.²⁴ As CLL cells are quiescent and do not replicate DNA, the proapoptotic activity of nucleoside analogues could be due to the inhibition of RNA synthesis and/or alteration of DNA repair. Both p53-dependent and -independent mechanisms have been discussed.^{12,25}

The cytotoxic effect of nucleoside analogues in combination with other chemotherapeutic drugs has also been reported. A number of studies have analysed the effect of the combination of fludarabine with other drugs *ex vivo* on CLL cells. Mitoxantrone may synergize with fludarabine and pentostatin,²⁶ whereas chlorambucil synergizes with fludarabine and 2-CdA.²⁷ We have also observed that cyclophosphamide synergizes with fludarabine in inducing cytotoxicity and apoptosis in CLL cells and that the addition of mitoxantrone to this combination may increase the cytotoxic effect in previously treated patients.¹⁹ Although crossresistance in these combined treatments has not been studied in detail, 2-CdA and fludarabine do not induce crossresistance *ex vivo* in a significant percentage of CLL samples.^{19,26} Interestingly, these *ex vivo* assays of drug-induced cytotoxicity may reflect to some extent *in vivo* therapeutic indexes for some of these drugs. For instance, *ex vivo* sensitivity to a combination of fludarabine with several drugs correctly predicted the clinical outcome of some patients.^{28,29}

Defective apoptosis and poor metabolism resulting in low accumulation of the triphosphate analogues often cause resistance to treatment.^{30–33} However, for nucleoside-derived drugs to exert their pharmacological action, mediated transport across the plasma membrane is needed.³⁴ This process may contribute to drug bioavailability, and hence, to cytotoxicity. This review will focus on this particular aspect of drug action.

How nucleoside-derived drugs are transported into cells?

The molecular cloning of the cDNAs encoding the membrane proteins responsible for nucleoside-derived drug uptake into cells is relatively recent^{35–37} and the putative role of nucleoside carriers in determining drug bioavailability and cytotoxicity has

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Table 1 Kinetic and molecular properties of concentrative and equilibrative nucleoside transporters

Transporter	Gene	Protein length	Stoichiometry	Substrates (K_m)	References
<i>Concentrative systems</i>					
hCNT1	SLC28A1	649	1Na/1nucl	Uridine (40–60 μ M) Thymidine (6 μ M) Cytidine (34 μ M)	41,100
hCNT2	SLC28A2	658	1Na/1nucl	Adenosine (8 μ M) Guanosine (21 μ M) Uridine (80 μ M) Inosine (4.5 μ M)	101–103
hCNT3	SLC28A3	698	2Na/1nucl	Uridine (21.6 μ M) Cytidine (15.4 μ M) Thymidine (21.2 μ M) Adenosine (15.1 μ M) Guanosine (43.0 μ M) Inosine (52.5 μ M)	45
Transporter	Gene	Protein length	Inhibitors (IC_{50})	Substrates (K_m)	References
<i>Equilibrative systems</i>					
hENT1	SLC29A1	456	NBTI (0.4 nM) Dipyr. (5 nM)	Adenosine (40 μ M) Guanosine (140 μ M) Inosine (170 μ M) Uridine (260 μ M) Thymidine (300 μ M) Cytidine (580 μ M)	44
hENT2	SLC29A2	456	NBTI (2.8 μ M) Dipyr. (356 nM)	Adenosine (100 μ M) Inosine (50 μ M) Uridine (250 μ M) Thymidine (710 m μ M) Cytidine (5610 μ M)	44

not been appropriately addressed due to a lack of suitable molecular tools.

Two gene families are involved in the uptake of natural nucleosides and analogues: SLC28 and SLC29 (Solute Carrier Families 28 and 29). The former encoding concentrative nucleoside transporter (CNTs) proteins and the latter equilibrative nucleoside transporters (ENTs). Three subtypes of sodium-dependent CNT transporters hCNT1, hCNT2 and hCNT3 have been cloned, whereas up to four members of the equilibrative ENT transporters have been identified so far. hENT1 and hENT2 are well-characterized nucleoside plasma membrane transporters, hENT2 bearing additional selectivity for certain nucleobases. hENT1 is selectively inhibited by nanomolar concentrations of the nucleoside analogue nitrobenzylthioinosine (NBTI), whereas no pharmacological inhibitors are known for CNTs. ENT3 and ENT4 are less well-characterized members of the SLC29 gene family that may be located inside the cell. The basic biochemical and molecular properties of these two gene families have recently been reviewed^{38,39} and are briefly summarized in Table 1. Basically, high-affinity translocation of natural nucleosides is mediated by CNTs *via* the transmembrane sodium gradient, whereas facilitative nucleoside influx and efflux is mediated by ENTs. The latter show significantly lower affinities for substrates than CNTs and may be reversible depending on how the substrate concentration gradients change across the plasma membrane. Substrate selectivity and their pharmacological profiles,^{35–37,40–43} still incomplete, are summarized in Table 2.

Although hENT1 and hENT2 show broad substrate selectivity for natural nucleosides, apparent K_m has not been measured for many nucleoside-derived drugs, although when available (as for

Table 2 Pharmacological properties of concentrative and equilibrative nucleoside transporters

	Drugs as substrates (apparent K_m)	References
<i>Concentrative systems</i>		
hCNT1	Gemcitabine + (17 μ M) 5'-DFUR (209 μ M) Cytarabine + Fludarabine – Cladribine –	41–43, 104 46, 104–106 45
hCNT2	Cladribine – Fludarabine – Gemcitabine –	
hCNT3	Cladribine + Fludarabine + Gemcitabine +	
<i>Equilibrative systems</i>		
hENT1	Gemcitabine + (160 μ M) Cytarabine + Fludarabine + Cladribine +	42, 104, 107–109
hENT2	Gemcitabine + (740 μ M) Fludarabine + Cladribine + Cytarabine ^a +	42, 44, 104

(+) Transported; (–) not transported.

^adrug tested as inhibitor.

5'-DFUR: 5'-deoxy-5'-fluorouridine.

gemcitabine), values are much higher than those reported for CNTs. Significant differences between the two isoforms in terms of drug recognition should be expected, since some natural nucleosides are taken up by hENT2 with a much lower affinity than hENT1.⁴⁴ Several nucleoside analogues used in the treatment of lymphoid malignancies also appear to be better substrates for hENT1 than for hENT2 (Table 2). Substrate selectivity in the CNT gene family is narrower than for ENTs. For instance, gemcitabine is a high-affinity substrate for hCNT1 but it is not recognized by hCNT2⁴² and appears to be less effectively taken up by the hCNT3 isoform.⁴⁵ In contrast, hCNT3 takes up fludarabine with high affinity, while hCNT1 does not recognize it and hCNT2, although inhibited by it, does not take it up. A similar specificity profile seems to apply to cladribine.^{41,46,47}

Which nucleoside transporters do CLL cells express?

In general, leukaemia cells appear to express both CNT- and ENT-type transporters.^{48–52} We have recently examined the expression profiles in leukaemia cell lines and primary CLL cells using quantitative RT-PCR for the five cloned transporters, hCNT1, hCNT2, hCNT3, hENT1 and hENT2. All cell lines analysed showed high hENT1 and hENT2 mRNA expression. hCNT1 was absent in all cell lines, although all of them expressed hCNT2 with great variability, and a few cell lines, in particular, NCEB, JVM-2 and NB4, also showed significant hCNT3 mRNA levels.⁵³ These observations are consistent with the classical kinetic data supporting the coexpression of CNT- and ENT-type transporters in leukaemia cells. Indeed, hCNT1, although present in macrophages,^{54,55} would be mostly expressed in epithelia. This is relevant because this transporter is responsible for the high-affinity uptake of various fluoropyrimidines, including the capecitabine intermediate 5'-deoxy-5-fluorouridine (5-DFUR), a direct precursor of the anticancer drug 5-fluorouracil (5-FU).^{41,43} Leukaemia cell lines such as NB4, Raji and BLS1 also show a Na-dependent, guanosine preferring, nucleoside transport activity that appears to be highly sensitive to inhibition by the analogue NBTI,^{47,56,57} a unique property among putative CNT transporters. Nevertheless, no cDNA related to this transport activity has been isolated so far and there is no evidence that additional SLC28 members remain to be discovered.

The expression of these nucleoside transporters was also explored in cells from 22 CLL patients.⁵³ Interestingly, hCNT1 was not detected in any of the samples, in agreement with the data discussed above showing that this particular isoform was not found in a broad panel of human lymphoid cell lines. The other four genes did not cluster into patient subgroups. The isoform showing the highest individual variability was hCNT3, and that showing least heterogeneity was hENT2. No correlation was found between hCNT2, hCNT3, hENT1 and hENT2. Although some Na-dependent guanosine uptake was detected in 12 patients, fludarabine accumulation was mostly if not exclusively mediated by hENT1 and hENT2, thus suggesting that the only Na-dependent nucleoside transporter with functional activity in patients was hCNT2, which does not recognize fludarabine as substrate, as mentioned above (Table 2).

Is nucleoside transporter expression constitutive in leukaemia cells?

Regulation of nucleoside transport activity in an isoform-specific manner would be a suitable approach to modulate drug

bioavailability. Thus, whether nucleoside transporter expression is constitutive or not may be a clinically relevant question. There is little information on mechanisms of nucleoside transporter regulation, particularly in lymphoid cells. Nevertheless, evidence has been provided for 'short'- and 'long'-term-mediated processes differentially affecting CNT and ENT functions in epithelial and immune system cell models by our laboratories and others. Results obtained in lymphoid cells will be briefly summarized below.

By 'long'-term we mean changes in transporter expression and activity that are likely to be mediated by *de novo* protein synthesis. Human leukaemia B-cell lines expressing both CNT- and ENT-type transporters respond to PMA, LPS and TNF-alpha treatments by upregulating concentrative transport activity and downregulating equilibrative nucleoside uptake, associated with a decrease in hENT1 mRNA amounts. Inhibition of PKC activity by bisindolylmaleimide blocks the upregulation of CNT-related activity triggered by these three agents, whereas the decrease in hENT1-related transport induced by TNF-alpha is insensitive to PKC inhibition, suggesting that different pathways are involved in the opposite response of concentrative and equilibrative transporters to cytokine treatment.⁴⁷ The differentiation by PMA of the promyelocytic cell line HL-60 leads to the downregulation of ENT-type transport systems and the emergence of a Na-dependent, CNT-type, transport activity.⁵⁸ Indeed, the recently cloned hCNT3 transporter is highly upregulated in HL-60 cells when differentiation is induced.⁴⁵ Putative transcription factors involved in this type of regulatory responses are unknown. Nevertheless, fludarabine specifically depleted STAT1 in normal resting B cells and in a CLL patient,⁵⁹ whereas CLL cells show constitutive Ser-727 STAT1 phosphorylation.⁶⁰ In contrast, normal B cells may phosphorylate STAT1 on this serine residue after treatment with PMA.⁶⁰ Indeed, phorbol esters downregulate ENT1 activity in B-cell lines,⁴⁷ which is interesting because in murine bone marrow macrophages the downregulation of ENT1 activity triggered by IFN- γ is STAT1 dependent.⁶¹

Nucleoside transporter expression may also be modulated by nucleosides and nucleoside-derived drugs by other means, probably involving interference with endogenous nucleotide metabolism. Fludarabine and 2-CdA seem to upregulate hENT1 expression in CLL cells, as determined by NBTI binding, probably as a result of an increase in the percentage of cells in S phase.⁶² Moreover, as for many other transporter proteins, the primary sequence of both CNTs and ENTs reveal many putative phosphorylation sites that could be recognized by a variety of kinases and be responsible for short-term-mediated changes in transport activity. Whereas some indirect evidence in favour of this type of regulation has recently been provided in nonlymphoid cell types, nothing is known about the 'short'-term post-translational regulation of these proteins in leukaemia cells.

Besides the physiological regulation of nucleoside transporters, modulation of transport function by pharmacological means is limited due to a lack of drugs to either inhibit or activate these proteins. hENT1 is the only nucleoside transporter that can be pharmacologically inhibited using NBTI at concentrations in the nanomolar range (IC₅₀ values are 0.4 nM vs 2.8 μ M for hENT1 and hENT2, respectively)⁴⁴ (Table 1). Sensitivity of hENT1 and hENT2 to dipyrindamole is also quantitatively different, but IC₅₀ values are closer to each other (5 vs 350 μ M, respectively) than for NBTI (Table 1). Unfortunately, no specific inhibitors against CNTs have been described so far, nor have activators of any of the known transporters. Moreover, the molecular basis for hENT1 inhibition is poorly understood as is the transporter structure itself.^{63–65}

The question then is how nucleoside transporter function determines drug bioavailability, and there are few studies on this. Recently, it has been shown that Na-independent cladribine uptake in the fludarabine-resistant CLL cell line WSU-CLL can be downregulated by bryostatin-1, a potent PKC inhibitor, whereas Na-dependent concentrative drug uptake is upregulated under similar conditions.⁶⁶ Indeed, bryostatin-1 promotes IFN- γ synthesis and leads to differentiation of CLL cells in a STAT1-dependent manner.⁶⁷ Apparently, bryostatin-1 may also modulate nucleotide metabolism beyond transport processes in WSU-CLL cells, since it also increases the deoxycytidine kinase/5'-nucleotidase activity ratio, thus potentiating the antitumour action of 2-CdA.^{68,69} This type of responses may not be common to all cell types, since this drug does not increase the retention of phosphorylated fludarabine in the human monocyte leukemic cell line U937.⁷⁰ However, it sensitizes two breast cancer cell lines to gemcitabine without any significant effect in a nontransformed cell line counterpart.⁷¹ In any case, preclinical studies have prompted researchers to incorporate bryostatin-1 in a variety of clinical trials, including a phase II CLL study⁷² and other leukaemias.⁷³

Modulation of nucleoside transporter function by physiological effectors, such as granulocyte-colony-stimulating factor (G-CSF), influences nucleoside accumulation in leukaemia cells, basically as a consequence of an increase in concentrative transport activity (CNT-type).⁷⁴ However, in this particular study, G-CSF promoted an increase in the accumulation of F-ara-ATP inside cells but a depletion in the ara-CTP intracellular pool. Whether this relates to differential responses to treatment of CNT isoforms remains to be elucidated.

Recently, it has been described that p38MAPK may regulate cytarabine-dependent differentiation of erythroleukaemic K562 cells.⁷⁵ Conventional p38MAPK inhibitors blocked hENT1-mediated nucleoside transport activity. Interestingly, the list of compounds that interact with hENT1 is increasing and now includes inhibitors of tyrosine kinases, PKC, cyclin-dependent kinases and the inhibitor of the TOR pathway rapamycin.⁷⁵ These findings suggest that nucleoside derivatives and protein kinase inhibitors may antagonize each other in some combined therapies.

What role do nucleoside transporters play in nucleoside-derived drug cytotoxicity?

So far, we know that leukaemia cells express CNT- and ENT-type carrier proteins and their expression is not constitutive. Transporter modulation seems to be possible and may contribute to drug bioavailability. Now, do nucleoside transporters play a role in drug cytotoxicity.

ENT carrier proteins have a dual role in maintaining the nucleoside balance across the plasma membrane. Although in principle, active nucleoside accumulation into cells could be accounted for by an ENT-type transport activity efficiently coupled to substrate phosphorylation, evidence suggests that ENTs mediate reversible translocation of substrates, thus responding to concentration imbalances across the plasma membrane. The inhibition of hENT1 by NBTI increases retention and cytotoxicity of 2-CdA in cells from CLL patients⁷⁶ and in cultured human leukemic lymphoblasts.⁷⁷ This occurs when drugs reach cells before the inhibition of transporter function. In contrast, treatment of cells after exposure to NBTI results in resistance.⁷⁸ Thus, NBTI blocks acadesine-induced apoptosis, demonstrating that uptake of acadesine through NBTI-sensitive transporters is necessary for its apoptotic effect.¹⁸ In principle,

either potentiation of drug accumulation or induction of drug resistance by manipulating hENT1 function would occur as long as no concentrative, high-affinity drug transporters are coexpressed. Heterologous expression of hCNT1 in nonlymphoid cells increases sensitivity to 5-DFUR, a fluoropyrimidine derivative recognized by the transporter.⁴³ Similarly, the acquisition of hCNT2 function by gene transfer into a drug-resistant T-lymphoblast cell line devoid of nucleoside transporter activity increased sensitivity to halogenated uridine analogues.⁷⁹ The role of CNT isoforms in nucleoside-derived drug anticancer therapy remains unclear, but it is likely that the two nucleoside influx pathways contribute to drug cytotoxicity.

Variability in nucleoside influx into human leukaemic cells has been recognized for years. Our laboratory has recently addressed the issue of whether nucleoside transporters determine drug sensitivity in CLL cells.⁵³ hENT-mediated fludarabine transport showed high variability among patients (20-fold range), although it did not bear any relationship with hENT1 or hENT2 mRNA levels, but showed a significant correlation with *ex vivo* sensitivity to fludarabine. Whether the lack of correlation between hENT1 mRNA and hENT1 transport activity in CLL is related to the altered STAT1 phosphorylation status is not known, but, in any case, it would be consistent with the finding that ENT1 activity but not mRNA levels depending on STAT1 function in murine bone marrow macrophages.⁶¹ A lack of correlation between hENT1 mRNA levels and drug transport activity has also been described for the hENT1 transporter in MCL cells.⁸⁰

For some reason, little CNT-type transport activity is found in CLL and other B-cell lymphomas, although mRNA for hCNT2 and hCNT3 have been detected. Expression of hCNT2 seems better preserved in cells from CLL patients, but this is not a suitable transporter for fludarabine, thus making hENT1 and hENT2 key players in the uptake of this drug in lymphoproliferative diseases. Consistent with this view, the putative role of nucleoside-metabolizing enzymes and hENT1 in ara-C sensitivity has recently been analysed in cells from childhood ALL patients.⁸¹ A significant correlation between hENT1 mRNA levels and ara-C-induced cytotoxicity has been reported, further demonstrating the key role of nucleoside transporters in nucleoside-derived drug cytotoxicity. This is also consistent with previous observations demonstrating that sensitivity of acute leukaemia cells to cytarabine is associated with hENT1 carrier protein amounts.⁸²

Pathways involved in resistance of CLL cells to purine nucleoside analogues differ from those implicated in the therapeutic action of anthracyclines, vinca alkaloids and alkylating agents. Bax expression correlates with sensitivity to doxorubicin, cyclophosphamide and chlorambucil, but not to fludarabine or cladribine.⁸³ The former are not substrates of nucleoside transporters. Whether pathways for uptake determine particular intracellular events beyond the metabolic activation steps, which may also differ among drugs, requires further research.

How do nucleotides exit cells?

Although hENT1 and hENT2 are putative candidates to mediate nucleoside efflux from cells, under tight metabolic coupling with kinases, nucleoside-derived drugs should be easily retained in their phosphorylated form inside cells.

The best candidates for pumping monophosphorylated nucleosides (nucleotides) outside mammalian cells are members of the expanding ABC protein family. Its members are plasma

Table 3 Kinetic and molecular properties of MRP4, MRP5 and MRP8 transporters

Gene	Protein length	Tissue distribution	Substrates (K_m) ^{a,99}	Putative substrates ^{a,b}
MRP4 ABCC4	1325	Broad	cGMP (9.7 μ M) ⁹⁵ cAMP (44.5 μ M) ⁹⁵ Thio-IMP ⁹⁶ Thio-GMP ⁹⁶ AZT ⁹⁰ PMEA ⁹⁸	6-Mercaptopurine ⁹⁵ 6-Thioguanine ⁹⁵ Ganciclovir ¹¹⁰ PMEG ⁹⁰ 3TC ⁹⁰⁰ Ddl ⁹⁰ d4T ^{c90} Abacavir ⁹⁸ Cladribine ⁹⁸
MRP5 ABCC5	1437	Ubiquitous	cGMP (379 μ M) ⁹³ cAMP (2.1 μ M) ⁹³ Thio-IMP ⁹⁶ Thio-XMP ⁹⁶ Thio-GMP ⁹⁶ PMEA ⁹⁴ d4TMP ⁹⁸ Alaninyl-d4TMP ⁹⁸	6-Mercaptopurine ⁹⁴ 6-Thioguanine ⁹⁴ Abacavir ⁹⁸ Cladribine ⁹⁸ Gemcitabine ⁹⁷ Cytarabine ⁹⁷ Cladribine ⁹⁷
MRP8 ABCC11	1382	Breast, testis, liver, brain, placenta, kidney ^{111,112}	PMEA 5-FdUMP	PMEA ddC 5-FU 5-FUdR 5-DFUR

^aOnly those putative substrates that are nucleoside derivatives are depicted in this table.

^bFor which heterologous expression induces resistance.

^cReported to confer resistance,⁹⁰ but no evidence of being transported.⁹⁸

AZT, 3'-azido-3'-deoxythymidine; PMEA, 9-(2-phosphonylmethoxyethyl)adenine; PMEG, 9-(2-phosphonylmethoxyethyl)guanine; 3TC, (-)2',3'-dideoxy-3'-thiacytidine; ddl, 2',3'-dideoxyinosine; d4T, 2',3'-didehydro-3'-deoxythymidine; d4TMP, 2',3'-didehydro-3'-deoxythymidine monophosphate; 5-FdUMP, 5-fluoro-2'-deoxyuridine monophosphate; 5-FU, 5-fluorouracil; 5-FUdR, 5-fluoro-2'-deoxyuridine; 5-DFUR, 5'-deoxy-5-fluorouridine; ddC, 2',3'-dideoxycytidine.

membrane proteins that contain ATP-binding cassettes and transmembrane domains distributed in modules with a variable number of domains depending on the subfamily of proteins they belong to. These are primary active export pumps that rely on ATP hydrolysis to mediate the efflux of structurally different compounds.⁸⁴

The three genes that encode ABC proteins that have been proven to be putative export pumps for nucleotides and, possibly, for the phosphorylated forms of some nucleoside-derived drugs, are MRP4, MRP5 and MRP8. Basic properties of these transporters are shown in Table 3.

Evidence for variable expression in CLL cells of members of the ABC protein family had been provided for phosphoglycoprotein MDR1 and MRP1.^{85,86} In contrast, no data are available for MRP4, MRP5 or MRP8, which may be relevant in nucleoside-derived drug cytotoxicity. Moreover, their pharmacology is not well known.

MRP5 appears to be relatively ubiquitous, but MRP4 may be more restricted.⁸⁷ Although commercial antibodies are available,⁸⁸ there is no published information on MRP4 or MRP5 tissue distribution patterns at the protein level. It has been postulated that endogenous protein levels may be too low and only transfectants heterologously overexpressing these two isoforms may bear protein levels high enough to be detected with the available antibodies.⁸⁴ Very recently new antibodies against MRP5 have been raised, characterized and used on a multitumour microarray, thus demonstrating a significant variability in MRP5 expression in a set of sarcomas and carcinomas.⁸⁹

MRP4 was shown to be responsible for the efflux of nucleoside monophosphate analogues.⁹⁰ Interestingly, MRP4 overexpression induced resistance to short-term exposure to methotrexate, but not to other non-nucleoside-derived compounds such as anthracyclines, etoposide, vinca alkaloids, and paclitaxel (Taxol).⁹¹ Methotrexate is indeed a substrate for MRP4.⁹² MRP4 has recently been identified as the apical efflux pump for cyclic nucleotides in renal proximal tubules.⁹² MRP5 has been implicated in the efflux of cyclic nucleotides, such as cGMP and cAMP, which points to these membrane proteins as putative pharmacological targets beyond their putative role in mediating drug efflux from cells.^{93,94} Both MRP4 and MRP5 overexpression induce resistance against thiopurine anticancer drugs, such as 6-MP and 6-thioguanine.^{94,95} Monophosphorylated nucleotides such as thio-IMP and thio-GMP are recognized by both MRP4 and MRP5, but thioxanthosine monophosphate is only transported by MRP5, which suggests that these two transporters have different pharmacological profiles.⁹⁶ MRP5 seemed to confer resistance to cytarabine (five- to seven-fold), cladribine (two- to seven-fold) and gemcitabine (two- to three-fold), but not to fludarabine.⁹⁷ Assuming that cytotoxicity is somehow dependent on the MRP5 pharmacological profile, this suggests that phosphorylated fludarabine derivatives are not MRP5 substrates and favours the view that these membrane proteins are selective. Recently, it has been shown that heterologous expression of MRP4 and MRP5 in the same cell line Hek293 does not induce a significant resistance for gemcitabine, cytarabine, fludarabine or 5-fluorouracil.⁹⁸ One explanation for this discrepancy could be

the differences in the expression profiles of nucleoside metabolizing enzymes in different stocks of Hek293 cells.

MRP8 is the last member cloned and functionally characterized and a good candidate to mediate nucleoside-derived drug resistance.⁹⁹ MRP8 is not only involved in the efflux of cyclic nucleotides but also its overexpression clearly confers resistance to fluoropyrimidines.⁹⁹

A definite conclusion on substrate profiles of MRP transporters will require direct estimation of drug fluxes. This is a mandatory step to establish the candidate protein pumps for fludarabine-phosphate efflux and to analyse their putative roles in the therapeutic response of CLL to fludarabine.

Future perspectives

The molecular identification of the proteins involved in nucleoside translocation across the plasma membrane is relatively recent and still incomplete. This is an important bottleneck in the analysis of the putative relationship between drug availability and transport processes. It is indeed probable that the intracellular concentration of phosphorylated nucleoside-derived drugs is dependent not only on metabolism but also on the balance between nucleoside influx, although CNT and ENT transporters and nucleotide efflux, mostly in their monophosphate form through MRP proteins. Although this general view may apply to any kind of cancer, the relative importance of these different transport proteins probably depends on the drug and the type of cancer being treated. CLL is an example of a disease for which fludarabine cytotoxicity is generally dependent on ENT-mediated drug uptake. Particular deviations from this behaviour may reflect a variety of other complementary mechanisms of resistance, which may involve, among others, plasma membrane processes such as those responsible for nucleotide efflux. Combined analysis of influx, efflux and metabolic pathways should contribute to a better understanding of particular responses of CLL and other leukaemias to nucleoside derivatives.

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