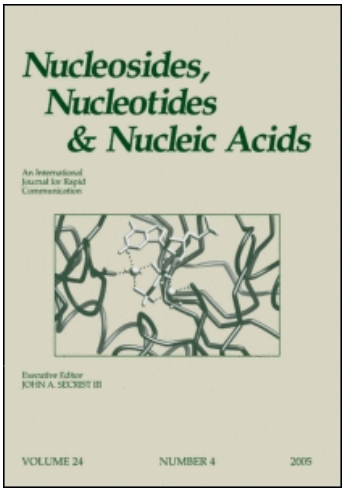

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PHYSIOLOGICAL AND PHARMACOLOGICAL ROLES OF NUCLEOSIDE TRANSPORTER PROTEINS

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□ *Nucleoside transporter proteins, CNT and ENT, encoded by gene families SLC28 and SLC29, respectively, mediate the uptake of natural nucleosides (among them adenosine) and are major routes of entry for a variety of nucleoside analogs used in anticancer and antiviral therapies. Expression of NT proteins is apparently redundant in most cell types, and the elucidation of their particular physiological roles still remains elusive. Moreover, transporter-mediated uptake of nucleoside-derived anticancer drugs is crucial for the pharmacogenomic response triggered by these molecules in tumor cells. This review focuses on recent data demonstrating that nucleoside transporters, particularly CNTs, can play physiological roles other than salvage, whereas particular NT isoforms can significantly contribute to the transcriptomic response triggered by nucleoside analogs in cancer cells.*

Keywords Nucleoside transporter; nucleoside analog; ENT; CNT; adenosine

NUCLEOSIDE TRANSPORTERS IN NATURE

De novo synthesis of purine and pyrimidine nucleotides is energetically costly. Several ATP molecules and sources of carbon and nitrogen atoms in the form of aspartate, glutamate, glycine, and formyl groups are required to build up one inosine monophosphate (IMP) molecule from D-ribose-5-phosphate. Thus, it is not surprising that cells often rely upon extracellular

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nucleosides and nucleobases to build up nucleotides to support cell growth. Moreover, since these molecules are hydrophilic, their efficient transport across the plasma membrane requires a mediated process which is facilitated by transporter proteins that belong to gene families SLC28 and SLC29, encoding CNT (concentrative nucleoside transporter) and ENT (equilibrative nucleoside transporter) proteins, respectively.

Although the major biochemical and molecular properties of these proteins have been reviewed elsewhere (see references[1–5] for recent reviews on general aspects of nucleoside transporter (NT) proteins), it is interesting to remind the reader that CNT and ENT proteins, with some exceptions, differ, among many other features, in the driving force implicated in the translocation process of nucleosides across the membrane. Whereas CNTs are Na^+-coupled, ENTs can mediate either the uptake or even the efflux of nucleosides (and nucleobases, in the case of ENT2) depending on the *cis/trans* relative substrate concentrations across the membrane. Exceptions to these rules are, firstly, ENT4, a suitable adenosine transporter, which is known to be H^+-coupled and, secondly, CNT3, which shows tolerance to Na^+ substitution per H^+, thus being actually a Na^+/H^+-coupled transporter. This feature, although unequivocally shown in cells heterologously expressing CNT3 and ENT4, should be put in a physiological context. H^+-coupled nucleoside transport may be relevant in some tissues, such as the small intestine. H^+-coupled transport processes are well known to occur for both amino acids and small peptides in the jejunum. These uptake mechanisms are mediated by apically located proton-coupled amino acid transporters (PAT) and peptide transporters (PEPT), encoded by gene families SLC36 and SLC15, respectively (see Thwaites et al.[6] and Daniel et al.[7] for recent reviews on PATs and PEPTs, respectively).

The apical localization of CNTs in epithelia would be also consistent with H^+-coupling playing a physiological role in nucleoside absorption (see Pastor-Anglada et al.[8] for a recent review). Similarly, the H^+-dependence of ENT4 has recently been suggested to be physiologically relevant in cardiomiocytes in which hypoxia and subsequent metabolic acidosis can facilitate an H^+-coupled mechanism for adenosine uptake.[9] Actually some ENT orthologs, such as those expressed in protozoa show H^+-dependence, which means that the acronym ENT (for "equilibrative") is not probably too accurate, because ENT-related transport processes in nature are not merely equilibrative, but sometimes H^+-coupled.[1–5] Although ENT orthologs are not found in bacteria, CNTs are. Bacterial CNT orthologs (among them NupC being the best characterized so far) are smaller proteins (they lack the putative first three N-terminus transmembrane domains), show broad substrate selectivity and are H^+-coupled.[10] This observation would anticipate that CNTs are phylogenetically older than ENT proteins, but also supports the view that the need for nucleoside salvage has been preserved during evolution and still is broadly present in nature.

PHYSIOLOGICAL ROLES OF NT PROTEINS

NT-related mRNAs are widely expressed in mammalian tissues. They show substantial variability in their amounts, thus anticipating complex tissue/cell-specific NT expression patterns.[11] Knowledge on the distribution of NT proteins is comparatively limited. Actually, a lack of suitable isoform-specific antibodies against these transporters has been for years a major bottleneck in the field. However, available evidence anticipates that, in most cases, there might be an apparent lack of relationship between mRNA and protein expression profiles.[11,12] In any case, co-expression of several NTs in most cell types, often with overlapping selectivity, argues against a simple role for these plasma membrane transporters as mediators of nucleoside salvage.

In epithelia, heterogeneous distribution of NT proteins at the apical and basolateral poles of cells determines vectorial flux of nucleosides and nucleoside-derived drugs.[8,13–15] A basic mechanism to sustain intestinal absorption and renal reabsorption of these molecules would consist of CNT type transporters being localized at the brush border whereas ENT type proteins would be predominantly found in the basolateral compartment. CNT protein abundance at the brush border is nutritionally regulated in enterocytes.[12] This means that either fasting or nucleotide depletion, when feeding semi-artificial diets, results in increased amounts of CNT proteins in enterocytes. Moreover, treatment of intestinal epithelial cells with agents known to promote differentiation in vivo (i.e., glucocorticoids), results in a selective up-regulation of CNT type proteins, whereas growth factors (EGF and TGF-α) and cell injury promote a positive modulation of ENT transporters, being ENT1 a major target.[16] This is consistent with nucleosides coming from the blood side of epithelia as main contributors to support cell proliferation, for instance, after injury (i.e., gastrointestinal surgery). Actually, it is known that supplementation of parenteral nutrition formulae with nucleosides or nucleotides, significantly ameliorates intestinal mucosa atrophy often caused by parenteral nutrition.[17] Recent immunohistochemical and in situ hybridization data on human duodenum also supports this view, since ENTs are highly expressed in crypt cells whereas CNT expression is associated with differentiated enterocytes.[18]

The concept that CNT and ENT heterologous distribution in epithelia is crucial for nucleoside vectorial flux has probably encouraged the search for CNT type proteins in highly specialized tissues in which nucleoside provision can be essential. Thus, Na+-coupled adenosine transport in primary cultures of rat brain endothelial and rat choroid plexus epithelial cells is polarized and apparently at the surfaces facing the interstitial and cerebrospinal fluids, respectively.[19] Similarly, CNT-type function has recently been identified in the blood-testis barrier. The whole panel of plasma membrane NTs is present in primary cultures of Sertoli cells (ENT1, ENT2,

CNT1, CNT2, and CNT3), whereas a Na+-coupled activity has been identified in these cultures, thus providing a first evidence for a combined role of ENTs and CNTs in supplying nucleosides for spermatogenesis.[20]

Although CNT and ENT proteins in epithelial and, maybe, in some endothelial barriers determine vectorial flux of nucleosides, this phenomenon should not be understood as a novel physiological role for NT proteins, but rather as a more complex evidence of nucleoside salvage in specialized tissues. Actually, nucleoside re-absorption from the renal tubular lumen is extremely efficient, which makes, as far as we know, "nucleosiduria," a very rare phenomenon, and highlights to what extent it is important to prevent nucleoside disposal into urine. Some metabolically inherited diseases, such as mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), are characterized by increased tissue concentrations of selected nucleosides (mostly thymidine and deoxyuridine in MNGIE patients).[21] MNGIE patients lack a functional thymidine phosphorylase (TP) enzyme and efficient reabsorption of luminal nucleosides might be contributing to the pathological phenotype of these individuals, even making NT proteins as putative targets for therapy.

Nevertheless, a body of evidence has been recently built up by us and others supporting the view that NT proteins play roles other than salvage. Primary cultures of murine bone marrow macrophages have been used to dissect physiological responses associated with either cell proliferation or cell activation. Proliferation triggered by Macrophage Colony Stimulating Factor (MCSF) is associated with the up-regulation of ENT1, thus promoting the incorporation into DNA of extracellular thymidine.[22] Pharmacological blockade of ENT1 function results in the inhibition of thymidine incorporation into DNA and cell proliferation as well, whereas the ability of macrophages to take up and incorporate extracellular uridine into RNA remains unaltered under these conditions.[22] This observation is relevant because it highlights, firstly, the role ENT1 plays in proliferation, and, secondly, the possibility of NTs mediating a channeled uptake of nucleosides into particular cellular targets. Interestingly, treatment of murine bone marrow macrophages with either IFN-γ or LPS, selectively upregulates CNT type transporters, even promoting growth arrest and decreased ENT1 related activity and expression.[23] Since LPS treatment eventually leads to apoptosis, the possibility that CNT type proteins contribute to these phenomenon should be taken into consideration. Actually, CNT2 is a high-affinity adenosine transporter and a candidate to modulate extracellular adenosine concentration, thus regulating purinergic responses via P1 receptors. CNT2 is a target of the pro-apoptotic agent TGF-β1 in hepatocytes, by a mechanism that depends on the transcriptional activation of the CNT2 encoding gene via JNK activation.[24] CNT1 is insensitive to TGF-β1 but is a target of multifunctional cytokines implicated in hepatocyte priming prior to proliferation, such as TNF-α and Interleukin 6.[25] Differential regulation of

CNT1 and CNT2 is also consistent with different roles for CNT proteins in cell physiology. Interestingly, CNT1 is not an adenosine transporter[26] and this is a major difference between both CNTs, with probable physiological implications. Consistent with this view, CNT2 is under purinergic regulation in hepatocytes, via A1 receptors, by a mechanism that is dependent on the opening of Katp channels.[27] Interestingly, this observation links CNT2 function with the energy status of the cell, because Katp channels are sensors of the intracellular ADP/ATP ratio. Actually, extracellular adenosine is able to modulate the AMP-dependent kinase, AMPK, via its transport through CNT2 in IEC-6 cells.[28] AMPK is directly implicated in the control of energy metabolism both at the cellular and whole-organ level (see Hardie[29] for a recent review). AMPK activates energy-producing pathways and downregulates energy-consuming metabolic processes and thus, nucleoside transporters (predominantly CNT2, but maybe other adenosine transporter proteins, even with higher concentrative capacity than CNT2, like CNT3) might be novel players in the regulation of AMPK and energy metabolism.

In summary, these, and other evidences not reviewed here, strongly support the view that co-expression of several NT proteins in a single cell type, and the broader distribution of CNT proteins than initially expected as well, might reflect the need for a fine tuning of extracellular (and probably intracellular) adenosine concentrations, whereas selective transporters might also channel particular nucleosides into intracellular targets. The acquisition of this basic knowledge is crucial for a better understanding of the NT-dependent pharmacological actions of most nucleoside-derived drugs used in anticancer therapies. Functional genomics will be extremely helpful in pursuing this goal.

PHARMACOLOGICAL ROLES OF NT PROTEINS

Most nucleoside-derived drugs used in anticancer therapy are NT substrates (see King et al.,[4] Pastor-Anglada et al.[5,30,35] for recent reviews). Nevertheless, not all nucleoside-derived drugs are recognized by every single NT, whereas, when they are, substantial heterogeneity in substrate specificity is also found. This is supported by highly variable apparent Km values for a single transporter protein and a particular set of putative substrates. In practice, in some cases, substrate-transporter interaction might be poor enough as to rule out a particular transporter protein as a suitable target in vivo. Some of the pharmacological profiles of NT proteins are shown in Table 1.

Several clinical and ex vivo studies have linked NT function with sensitivity to nucleoside-derived drugs (see Table 2 for a summary of recent studies). In most cases, the human ortholog of ENT1 (hENT1) seems to play a major role in promoting the cytotoxic action of nucleoside-derived drugs. For instance, hENT1 expression is associated with longer survival in patients

TABLE 1 Pharmacological properties of equilibrative and concentrative nucleoside transporters

	hENT1	hENT2	hCNT1	hCNT2	hCNT3
Citarabine	S	inh	S	NT	
Gemcitabine	S (160 μM)	S (740 μM)	S (17 μM)	NT	S (60 μM)
5′-DFUR	S	S	S (209 μM)	inh	S
Fludarabine	S	S	NT	NT[a]	S
Cladribine	S	S	NT	NT[a]	S
Troxacitabine	Passive difusion				
Clofarabine	S	S	NT	S	

S: substrate. When it is known, apparent Km value is given; inh: drug tested as inhibitor; NT: no transported; NT[a]: no transported but inhibit

suffering from pancreatic adenocarcinomas treated with gemcitabine as single therapy,[32] whereas hENT1 also correlates with gemcitabine sensitivity *ex vivo* in Mantle Cell Lymphoma (MCL) patients.[33] As a way to better understand how nucleoside-derived drugs exert their cytotoxic action and how particular NT proteins contribute to it, our laboratory has recently undertaken several pharmacogenomic approaches to address these issues.

In MCF7 cells, a suitable model of breast cancer, treatment with 5′-deoxy-5-fluorouridine (5′-DFUR) exerts a transcriptomic response that affects, mostly, p53-dependent genes.[34] Among these genes, some were unexpected, such as aquaporin 3 (AQP3). The rationale for the induction of AQP3 expression triggered by genotoxic nucleoside-derived drugs, such as 5′-DFUR, is being addressed at our laboratory and may help to identify putative new targets for therapy (work in preparation by authors).

5′-DFUR is a direct precursor of the active drug 5-fluorouracil (5-FU) and a metabolite of the orally administered prodrug capecitabine

TABLE 2 Clinical and ex vivo studies linking NT function to drug sensitivity

Transporter	Disease	Drug	Correlation	References
hENT1	AML, ALL	ara-C	+	(38–41)
	AML, ALL	ara-C, 2CdA, Fara-A	+	(42)
	AML	ara-C, 2CdA, dFdC	+	(43)
	ALL	2CdA	ns	(41)
	MCL	dFdC	+	(33)
	pancreas tumor	dFdC		(32, 44)
hENT2	CLL	Fara-A	+	(45)
hCNT1	Breast cancer	CMF	−	(36)
hCNT3	pancreas tumor	dFdC	ns	(32)
	LLC	Fara-A	−	(46)

AML: acute myeloid leukemia; ALL: acute lymphoblastic leukemia; MCL: mantle cell lymphoma; CLL; chronic lymphocytic leukemia; ara-C: citarabine; 2-CdA: cladribine; Fara-A: fludarabine; dFdC: gemcitabine; CMF: cyclophosphamide-methotrexate-5-fluorouracil.
+: positive correlation; −: negative correlation; ns: no significative correlation.

(5′-deoxy-5-fluoro-N-[(pentoxy)carbonyl]-cytidine). Capecitabine is currently used in the treatment of a variety of solid tumors, including breast cancer. 5′DFUR is a suitable substrate for hENT1 and hENT2, both expressed in MCF7 cells.[34] Selective pharmacological inhibition of hENT1, using the nucleoside analog NBTI, induces some resistance to 5′DFUR, but not to 5-FU action in this cell line, which is consistent with 5-FU not being an ENT substrate. Interestingly, under these conditions, even when hENT2 is functional, the transcriptomic response induced by this nucleoside-derived drug is blunted, which highlights the important role hENT1 plays in the development of the full transcriptional response to 5′-DFUR treatment.[34] Nevertheless, hCNT1 is a high-affinity transporter of 5′DFUR and its heterologous expression confers sensitivity to this drug.[35] Moreover, hCNT1 is known to be expressed in breast cancer cells.[36] Thus, we have recently engineered MCF7 cells to express hCNT1 and, under conditions of hENT1 inhibition, hCNT1 function is sufficient to maintain the transcriptomic and cytotoxic response associated with 5′DFUR treatment.[37] Thus, NT patterns rather than single NT proteins might modulate the therapeutic performance of most nucleoside-derived drugs. This highlights the need of clinical approaches in which several biomarkers of nucleotide metabolism (not only transporters but also enzymes) will have to be analyzed as putative predictors of drug sensitivity, thus allowing individualized therapies.

CONCLUDING REMARKS

The field of nucleoside transporters faces several challenges. Although significant information about substrate requirements for transportability is already available (not reviewed here), a detailed knowledge of NT structures is essential for a better understanding of drug-transporter interaction and improved drug design. This will also help to understand how particular polymorphisms in NT structure result in altered function, eventually modifying drug pharmacodynamics. Moreover, it is also important to unveil the roles particular NTs play in cell physiology. This should be addressed by using functional genomics, both at the cell and whole animal levels. Actually, the discovery of unexpected novel physiological roles for these membrane proteins will probably reveal NTs as pharmacological targets in the treatment of diseases other than cancer.

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