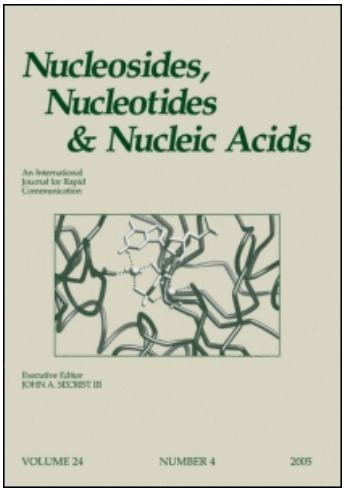

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Role of Nucleoside Transporters in Nucleoside-Derived Drug Sensitivity

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ROLE OF NUCLEOSIDE TRANSPORTERS IN NUCLEOSIDE-DERIVED DRUG SENSITIVITY

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□ *Nucleoside-derived drugs are currently used clinically as anticancer drugs. To exert their pharmacological action first they need to enter into the cell across plasma membrane transporters and be metabolized. Thus, efficacy of treatment and acquisition of resistance can rely on a variety of events. In this article, we will focus in the role of nucleoside transporters in the sensitivity to nucleoside-derived drugs used in chemotherapy. Evidence of different transporter protein expression patterns in tumors compared to normal tissues, besides inter-individual variability in the levels of nucleoside transporters in tumors, suggest a major role of nucleoside transporters in the cytotoxicity of nucleoside analogs. In fact, different studies have linked nucleoside transporter function to drug sensitivity and clinical outcome in cancer patients. However, prospective clinical studies analysing nucleoside transporters and metabolic enzymes, as biomarkers of drug metabolism and action are required to better establish the role these proteins might play in cancer chemotherapy.*

Keywords Nucleoside transporters; SLC28; SLC29; nucleoside analogs; chemotherapy; tumor

MECHANISM OF ACTION OF NUCLEOSIDE ANALOGS

Nucleoside-derived drugs are currently used clinically as anticancer drugs. The purine analogs fludarabine (F-ara-A, 9-β-D-arabinosyl-2-fluoroadenine) and cladribine (2-CdA, 2-chlorodeoxyadenosine) are widely used in the treatment of lymphoproliferative malignancies. The cytidine derivative cytarabine (ara-C, 1-β-D-arabinosilfuranosilcytosine) has a major role in the therapy of acute leukaemia. Whereas, the pyrimidine analogs

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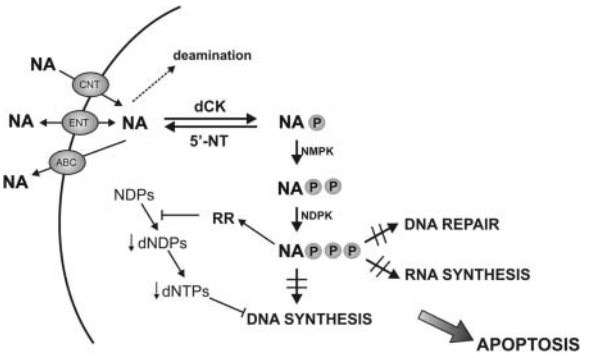

FIGURE 1 Mechanism of action of nucleoside analogs: Nucleoside analogs (NA) enter into the cell across the nucleoside transporters (ENT and CNT). Once inside the cell NA are phosphorylated by nucleoside kinases (dCK, deoxycytidine kinase; NMPK, nucleoside monophosphate kinases; and NDPK, nucleoside diphosphate kinases). Phosphorylated NA can incorporate into DNA or RNA, inhibit DNA repair or metabolic enzymes, such as ribonucleotide reductase (RR), producing at the end cell apoptosis. Drug efficiency can be reduced by 5′-nucleotidases (5′-NT) or by membrane efflux pumps (ABC transporters).

gemcitabine (dFdC, 2′,2′-difluorodeoxycytidine) and capecitabine, a prodrug that yields 5-fluorouracil inside tumor cells, being 5′-DFUR (5′-deoxy-5-fluorouridine) its immediate precursor, are frequently used in the treatment of solid tumors. Moreover, the group is expanding with the synthesis of new molecules as clofarabine (2-chloro-2′-fluoro-deoxy-9-β-arabinosyladenine) and troxacitabine ((-)-2′-deoxy-3′-oxacitabine).

Nucleoside analogs present slight structural modifications with respect to natural nucleosides, thus, they retain most of the metabolic properties of natural nucleosides. In fact, they need to enter into the cell across plasma membrane transporters and be metabolized to exert their pharmacological action. The mechanism of action of nucleoside analogs is summarized in Figure 1. First, nucleoside transporters mediate the uptake of nucleoside-derived drugs. Nucleoside transporters belong to the Solute Carriers families 28 and 29 (SLC28 and SLC29), which encode concentrative nucleoside transporters (CNT) and equilibrative nucleoside transporters (ENT) proteins, respectively.[1,2] Biochemical and molecular characteristics of nucleoside transporters will be detailed below. Once inside the cell, nucleoside analogs are phosphorylated by nucleoside kinases. Deoxycytidine kinase (dCK) is the enzyme responsible of the phosphorylation and activation of most nucleoside analogs.[3] However, this step can also be performed by deoxyguanosine kinase (dGK), thymidine kinase 1 (TK1), and thymidine kinase 2 (TK2).[4] These enzymes differ in their substrate selectivity and their localization. Monophosphate forms can be dephosphorylated by 5′-nucleotidases, decreasing drug efficiency.[5] Moreover, nucleoside analogs can also be degraded through action of some deaminases, although some nucleoside derivatives such as fludarabine are resistant to deamination.[6] Drug

activity can also be reduced by the activity of membrane efflux pumps. Three genes that encode ABC transporter proteins (MRP4, MRP5, and MRP8) have been suggested to be export pumps for nucleotides.[7–9] After the first phosphorylation, which usually is the rate-limiting step, monophosphorylated nucleoside analogs are phosphorylated to di- and triphosphorylated forms by nucleoside monophosphate kinases (NMPK) and nucleoside diphosphate kinases (NDPK).[4] The di- and triphosphorylated nucleoside analogs are the active forms of these drugs and exert their cytotoxic effect by different mechanisms. Phosphorylated nucleoside analogs can incorporate into enlongating DNA, into RNA, produce DNA strand breakage or perturbation of intracellular nucleotide pools by inhibiting some metabolic enzymes such as ribonucleotide reductase (RR). All these actions will induce the activation of apoptotic signals producing cell death.[4] Although nucleoside analogs share some features they also posses specific properties in terms of metabolism and drug-target interactions, which may explain their differences in activity in various diseases.

The use of nucleoside analogues in the cancer treatment is limited by primary and acquired resistance. The mechanisms of resistance are multiple and can be divided into three groups. The first mechanism is produced by low levels of active forms that can be produced by a reduced activity of nucleoside transporters or by an increased activity of membrane efflux pumps. Moreover, the ratio between the enzymes responsible for nucleoside analog phosphorylation, such as dCK, and the enzymes that dephosphorylate or deaminate nucleoside analogs will determine the levels of active forms. The second mechanism is produced when active forms of nucleoside analogs are not able to produce enough damage into the DNA or perturbations in nucleotides pools. For example, high levels of ribonucleotide reductase will require higher amounts of diphosphate nucleoside analog to produce an effective inhibition. Finally, the third mechanism is the alteration in the apoptotic machinery, responsible to produce cell death. Mutations in proteins necessary for the apoptotic response can potentially produce a decrease in cell death, which is the final objective of nucleoside analog treatment.

The resistance to cytotoxic nucleoside analogs is an active area of study. All the mechanisms of resistance proposed have been extensively studied and different correlations have been found.[10,11] In this article, we will focus in the role of nucleoside transporters in the sensitivity to nucleoside-derived drugs used in chemotherapy.

TRANSPORT OF NUCLEOSIDE ANALOGS

Nucleoside analog uptake is mediated by nucleoside transporters. Equilibrative nucleoside transporters (ENT, SLC29) facilitate a passive diffusion

transport, thus, they are potentially bidirectional carriers which mediate the influx and efflux of substrates. Four members of ENTs have been identified so far.[2] The two well characterized transporters hENT1 and hENT2 differ in their sensitivity to the adenosine derivative nitrobenzylthioinosine (NBTI), being hENT1 selectively inhibited by nanomolar concentrations of NBTI. hENT1 and hENT2 present low affinity and wide selectivity for their substrates (Table 1). Both transport purines and pyrimidines with Km values in the high micromolar range. Moreover, hENT2 is selective for certain nucleobases. hENT1 and hENT2 transport most of nucleoside analogs used in cancer treatment (Table 1), but similarly to natural nucleosides apparent Km values are lower than those reported for hCNTs.

hENT3 and hENT4 have been cloned more recently and are less well-characterized members of the SLC29 family.[12,13] Interestingly hENT3 has recently been associated with an inherited disease in humans, the H syndrome,[14] which is to some extent consistent with a mitochondrial disease. In fact, hENT3, although initially reported to be a lysosomal protein, has recently been identified in mitochondria.[15] Moreover, it seems to show even broader selectivity than hENT1 and hENT2, thus anticipating a key role for hENT3 in mediating mitochondrial toxicity triggered by nucleoside derivatives.

Concentrative nucleoside transporters (CNT, SLC28) mediate the unidirectional uptake of nucleosides in an active, energy costly process coupled to the trans-membrane sodium gradient. hCNT family consists of three members which differ in their substrate selectivity. hCNT1 is a pymidine-nucleoside preferring transporter, whereas, hCNT2 transports purine-nucleosides and uridine. Finally, hCNT3 shows much broader selectivity, translocating both purine and pyrimidine nucleosides.[2] Moreover, CNT transporters show higher affinities for their substrates than ENTs, with Km values in the low micromolar range (Table 1). Similarly to natural nucleosides, concentrative transporters are more selective for nucleoside analogs than equilibrative transporters, but they show higher affinities. hCNT1 transport most of the pyrimidine-derived drugs.[16] hCNT3 seems to be the best drug carrier protein because it can transport most of the pyrimidine and purine nucleoside analogs and with higher affinities than hENT1 and hENT2.[17] Finally, although hCNT2 is a purine nucleoside transporter it does not take up some purine derivatives such as fludarabine and cladribine.[18]

EXPRESSION OF NUCLEOSIDE TRANSPORTERS

Equilibrative nucleoside transporters may be considered ubiquitous transporters, although with significant variability in tissue abundance.[19] Concentrative nucleoside transporters were initially thought to be expressed in few epithelia tissues, but now they are known to have a much broader tissue

TABLE 1 Substrate selectivity and pharmacological properties of nucleoside transporters

Concentrative nucleoside transporter			Equilibrative nucleoside transporter		
	Substrate (K_m)			Substrate (K_m)	
Transporter	Nucleoside	Nucleoside analog	Transporter	Nucleoside	Nucleoside analog
hCNT1	Urd (40–60 μM)	Cytarabine	**hENT1**	Ado(40 μM)	Cytarabine
	Thd (6 μM)	Gemcitabine (17 μM)		Guo(140 μM)	Gemcitabine (160 μM)
	Cyd (34 μM)	5′-DFUR (209 μM)		Ino (170 μM)	5′-DFUR
hCNT2	Guo(21 μM)			Urd (260 μM)	Fludarabine (107 μM)
	Ino(4.5 μM)	Clofarabine (81 μM)		Thd (300 μM)	Cladribine (23 μM)
	Urd (21.6 μM)			Cyd (580 μM)	Clofarabine (108 μM)
hCNT3	Cyd (15.4 μM)	Cytarabine	**hENT2**	Ado(100 μM)	Cytarabine
	Thd (21.2 μM)	Gemcitabine (60 μM)		Ino (50 μM)	Gemcitabine (740 μM)
	Ado (15.1 μM)	5′-DFUR		Urd (250 μM)	5′-DFUR
	Guo (43.0 μM)	Fludarabine		Thd (710 μM)	Fludarabine
	Ino (52.5 μM)	Cladribine		Cyd(5610 μM)	Cladribine
		Clofarabine (52 μM)		Guo	Clofarabine (328 μM)
				nucleobases	

distribution.[20,21] Therefore, nucleoside transporters (NTs) are coexpressed in a variety of cells and tissues, and they show different substrate selectivity and specificity. To analyze the role of NTs in nucleoside analogs sensitivity it is important to understand better the expression of these transporters. We should study, first, if there is evidence of different transporter protein expression patterns in tumors compared to normal tissues and, secondly, if there is interindividual variability in the levels of NTs in tumors.

Evidence of selective loss of NTs in tumors was first provided in rat models of hepatocarcinogenesis using anti-rCNT1 and anti-rCNT2 polyclonal antibodies.[22] In human cells, the use of a commercial tumor RNA array demonstrated variability in NT expression profiles from different individuals and, in general, decreased NT expression in tumor tissue compared with normal.[23] Immunohistochemical analysis of hENT1, hENT2, and hCNT1 in 300 gynaecologic tumors demonstrated variability in NT expression. Moreover, hENT1 and hENT2 protein expression was highly retained, whereas a significant number of tumors were hCNT1 negative.[24] hENT1-negative tumors were detected when analyzing a cohort of 33 breast cancer patients.[25] Moreover, regulation studies have demonstrated that ENT1 expression is mostly linked to cell proliferation, whereas CNT-type related activity is basically dependent upon differentiated functions of particular cell types.[26–28] Thus, the fact that ENT1 is the major provider of nucleosides for proliferation would explain why it is highly expressed in tumors, whereas CNT transporter expression is most frequently loss in tumors.

Recently, the availability of suitable molecular tools such as isoform specific anti-NT antibodies, have allowed the study of NTs in tumors.[24,29,30] In the last years, a large number of studies evidence variability and different transporter protein expression patterns in tumors, which would suggest variability in drug uptake capabilities.

DRUG TRANSPORT AND RESPONSIVENESS TO TREATMENT

To exert their pharmacological action, nucleoside analogs must enter the cell via nucleoside transporter proteins. This, together with the heterogeneity in NT expression observed in tumors, suggest a major role of NTs in the cytotoxicity of nucleoside derived-drugs.

Evidence obtained from in vitro cultured cell models clearly suggest that NTs contribute to nucleoside-derived drug cytotoxicity. Cells without NT-related activity were highly resistant to gemcitabine treatment.[31] Similarly, nucleoside-transport deficient CCRF-CEM cells were resistant to gemcitabine and cytarabine, but not to troxacitabine, which is taken up by passive diffusion. In contrast, a deoxycytidine kinase (dCK) deficient variant was resistant to all three drugs.[32] CCRF-CEM cells resistant to cytarabine, express ENT1

with a single missense mutation that results in reduced hENT1-related activity.[33] Other mutations in the hENT1 and dCK encoding genes have been detected in cell lines resistant to cytarabine.[34] Inhibition of hENT1 activity using NBTI reduces 5′-DFUR sensitivity in the breast cancer cell line MCF7, although this cell line also presents hENT2 activity. Moreover, microarray experiments demonstrated that most of the transcriptional changes produced by 5′-DFUR treatment where blocked when hENT1 activity was inhibited.[35] These results highlight the relevant role of a particular transporter isoform in the nucleoside-derived triggered transcriptomic response.

The role of concentrative transporters in in vitro models has usually been addressed by analyzing the effect of their heterologous expression. Expression of hCNT1 in Chinese hamster ovary cells increases cytotoxic action of 5′-DFUR. Moreover, inhibition of endogenous ENT transporters using dypiridamole does not affect drug sensitivity, suggesting that the high-affinity hCNT1 transporter itself is enough to achieve the maximum cytotoxic effect.[36] Similar results where obtained with pancreatic adenocarcinoma and breast cancer cell lines.[37,38]

Studies linking NT function to drug sensitivity and clinical outcome in cancer patients were initially focused on lymphoproliferative malignancies, because it is easier to obtain samples and cytotoxicity assays can be performed ex vivo. Several studies have reported a correlation between hENT1 expression and cytarabine sensitivity in acute myeloid and lymphoid leukaemia (AML and ALL).[39–41] In mantle cell lymphoma hENT1 expression correlated with gemcitabine sensitivity.[42] In chronic lymphocytic leukaemia (CLL) a correlation between fludarabine uptake rates, measured at 5 seconds, and ex vivo sensitivity to this analog, measured at 48 hours, was detected.[43] Moreover, hENT2 protein amounts correlated with fludarabine sensitivity, demonstrating that in this disease is hENT2 the transporter that plays an important role in drug response. Consistent with this, transcriptional changes produced by fludarabine were not blocked when hENT1 activity was inhibited.[44] CLL is characterized by a decrease in cell death rather by an increase in cell proliferation.[45] Fludarabine has been demonstrated to be effective in non resting cells mainly through inhibition of DNA repair processes.[46] Therefore, these characteristics would explain why in highly proliferative leukemias hENT1 seems to be important for drug cytotoxic effect, whereas in CLL hENT2 is the transporter that mediates this response.

More recently, some studies have also demonstrated a link between nucleoside transporter expression and clinical response in solid tumors. Patients with pancreatic adenocarcinoma with detectable hENT1 protein expression have a significantly longer survival after gemcitabine chemotherapy than tumors without detectable hENT1.[47] Analysis of 102 pancreas cancer patients treated with gemcitabine demonstrated that hENT1-related mRNA

levels correlated with longer overall survival.[48] Similarly, in a prospective study hENT1 protein expression was associated with increased overall and disease-free survival in pancreatic cancer patients who received gemcitabine, but not in those who received 5-fluorouracil, which is not hENT1 substrate.[49]

In contrast to the studies showed above, it has also been described that CLL subjects with elevated hCNT3 expression had lower complete response rate to fludarabine therapy.[30] Similar results were obtained using immunohistochemical analysis.[50] However, no hCNT3-related nucleoside transport was detected; indeed, all hCNT3 protein was located intracellularly. Similarly, in breast cancer patients intracellular expression of hCNT1 is indicative of poor prognosis.[29] These results could suggest a different role of nucleoside transporters besides plasma membrane transport. In this sense, a splice variant that lacks 69 aminoacids of the N-terminus has been described. This variant is located in the endoplasmic reticulum where it is active, probably contributing to intracellular nucleoside recycling.[51]

FUTURE PERSPECTIVES

A large number of studies on clinical samples have demonstrated the importance of levels of expression of a particular NT isoform on the bioavailability and cytotoxic action of nucleoside-derived drugs in cancer treatment. However, the transporter or transporters important in nucleoside analog response may depend on the tumor analyzed and on the drug used for the treatment. Therefore, more prospective studies are required to better understand this relationship.

Genetic polymorphisms could also explain some of the differences observed between cancer patients in response to nucleoside analogue based treatment. Compared with other transporter gene families, nucleoside transporters are not very polymorphic, however some of the polymorphic variants identified have functional implications which could affect pharmacokinetics and drug response. In fact, some haplotypes identified at the hENT1-encoding gene promoter decrease its transcriptional activity and alter cytarabine chemosensitivity.[52] However, the role of genetic polymorphisms of NTs in nucleoside-derived drug sensitivity in cancer patients has not been studied so far.

Chemotherapeutic response depends on the coordination of transporters, enzymes and intracellular targets and that can vary depending on the drug used and the tumor treated. New techniques and new approaches should be used to analyze all these parameters together in order to detect more subtle correlations. In this sense, acquired resistance to gemcitabine correlated with the ratio of hENT1 x dCK / RRM1 x RRM2 gene expression in pancreatic cell lines.[53] This would lead to the identification of those genes important for drug response for a specific nucleoside analog

in a specific tumor. Once these genes had been identified, not only new biomarkers of response would be available, but also new therapeutic strategies could be envisaged. In this regard, in selected tumors, such as pancreatic adenocarcinomas, in which the role of a particular transporter protein in nucleoside-derived drug (i.e., gemcitabine) response has been unveiled, strategies to overcome transporter function are being developed. Strategies to make nucleoside-derivatives more lypophylic to bypass membrane transport processes are currently being assayed.[54] Alternatively, other strategies could involve the pharmacological modulation of the drug activation pathways, by enhancing either transport or subsequent metabolism, or both.

In summary, efforts should be put in order to find a coordinate view of how transporters, enzymes and intracellular targets interact to define chemotherapeutic responses. Measurement of proteins responsible of drug resistance at the time of diagnosis and application of strategies designed to overcome this resistance, could contribute to a targeted therapy in the future.

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