Expression and Functionality of Anti-Human Immunodeficiency Virus and Anticancer Drug Uptake Transporters in Immune Cells

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ABSTRACT

Almost all drugs used in anti-human immunodeficiency virus (HIV)-1 and anticancer therapies require membrane proteins to get into the cell to develop their proper activity. Nevertheless, little is known regarding the expression and activity of specific carriers involved in the uptake of these drugs in immune cells. Here, we assessed the mRNA levels, protein expression profile, and activity of the gene families SLC28 (coding for concentrative nucleoside transporters, hCNT1–3), SLC29 (equilibrative nucleoside transporters, hENT1–2), and SLC22 (organic cation transporters, hOCT1–3 and hOCTN1–2). Both hENTs and hCNT2 were abundant in primary lymphocytes, with a preferential activity of hENT1. A significant up-regulation in hENTs expression (100-fold) and activity (30-fold) was seen under stimulation of primary T lymphocytes. In contrast, monocytess, monocyte-derived macrophages (MDMs), and immature monocyte-derived dendritic cells predominantly expressed hCNT3, a functional transporter in MDMs. Finally, in immune cells, hOCTs showed a more heterogeneous expression profile and a lower activity than human nucleoside transporters (hNTs), although up-regulation of hOCTs also occurred upon lymphocyte activation. Overall, the expression and activity of most of the studied transporters emphasize their relevance in relation to anti-HIV and anticancer therapies. The identification of the transporter involved in each specific drug uptake in immune cells could help to optimize pharmacological therapeutic responses.

Most of the drugs currently used in the treatment of the human immunodeficiency virus type-1 (HIV-1) infection and in anticancer therapies need to cross cell membranes to develop their pharmacological activity. Thus, host factors, including drug transporters, drug-metabolizing, and detoxifying enzymes, might affect intracellular drug concentrations, limiting the ability of drug regimens to inhibit HIV-1 replication or proliferation of cancerous cells (Evans and Relling, 1999; Fridland et al., 2000). However, little is known regarding drug uptake transporters expression and activity in immune cells.

During the past few years, many membrane proteins have been identified as putative transport systems that regulate cell entry of many antiviral and anticancer drugs. The best

ABBREVIATIONS: HIV, human immunodeficiency virus; hCNT, human concentrative nucleoside transporter; hENT, human equilibrative nucleoside transporter; hOAT, human organic anion transporter; hOCT, human organic cation transporter; hOCTN, human organic cation/zwitterion transporter; PHA, phytohemagglutinin; ddC, zalcitabine; ddl, didanosine; IL, interleukin; MDM, monocyte-derived macrophages; D-22, 1,1'-diethyl-2,2'-cyanine iodide; MDCCs, monocyte-derived dendritic cells; NBTI, nitrobenzylthioinosine; DIP, dipiridamole; MPP+, N-methyl-4-phenylpyridinium; ET, ergothioneine; GM-CSF, granulocyte/macrophage colony-stimulating factor; HRP, horseradish peroxidase; AZT, azidothymidine; TBS, Tris-buffered saline; LPS, lipopolysaccharide; PCR, polymerase chain reaction; C57, threshold cycle; mMDDC, mature MDDC; 5'-DFUR, 5'-deoxy-5-fluorouradine; Rani, ranitidine; hNT, human nucleoside transporters; DAB, 3,3'-diaminobenzidine.
known uptake transporters belong to the SLC29 and SLC28 gene families, which correspond to human equilibrative nucleoside transporters (hENTs) and human concentrative nucleoside transporters (hCNTs), respectively. The best characterized isoforms of hENTs, hENT1 and hENT2, are low-affinity (K_m 50–700 μM), bidirectional, and equilibrative nucleoside transporters that are widely distributed in human tissues (Baldwin et al., 2004). hENT1 has been described as transporter of the anti-HIV nucleoside analogs zalcitabine (ddlC) and didanosine (ddI), whereas hENT2 is considered a low-affinity azidothymidine (AZT) transporter (Baldwin et al., 2004). Both hENTs mediate the entry of most of the drugs used in anticancer treatment: fludarabine, cladribine, cytarabine, and gemcitabine (Zhang et al., 2007). In relation to hCNTs, the sodium-dependent nucleoside family with a high substrate affinity (K_m 1–50 μM), three isoforms have been described with differential substrate selectivity, whereas hCNT1 can transport pyrimidines, hCNT2 is a sodium-preferring transporter that can also transport uridine and hCNT3 is a broad selective nucleoside transporter (Gray et al., 2004). Although hCNT1 has been described as the uptake transporter for ddI and stavudine (d4T), we and others (Cano-Soldado et al., 2004; Chang et al., 2004) have demonstrated that the lack of the 3′-OH group in the ribose ring of the nucleoside analogs structure causes a substantial reduction in hCNT1/2 and hENT1 affinity for these drugs. Nevertheless, hCNT3 seems to be a good transporter for AZT, ddC, ddI, and some important anticancer drugs, such as fludarabine, gemcitabine, and 5′-DFUR (Pastor-Angliada et al., 2005; Hu et al., 2006; Errasti-Murugarren et al., 2007).

On the other hand, some members of SLC22 gene family that comprises human organic anion transporters (hOATs) and organic cation/wtzerion transporters (hOCTs and hOCTNs, respectively) have also been studied as uptake transporters of different antiretroviral and anticancer drug families in secretion and reabsorption tissues (Aiba et al., 1995; Zhang et al., 2000; Lee and Kim, 2004). Four hOATs members (hOAT1–4) have been cloned and identified so far, and their substrates include cyclic nucleotides, prostaglandins, uric acid, and different types of drugs (including antivirus and anticancer nucleosides) (Koepsell and Endou, 2004). Nevertheless, we have recently demonstrated that these transporters are not expressed in T-lymphocytes or macrophages (Purcut et al., 2006). With regard to hOCTs, they mediate the first step in hepatic or renal excretion of many cationic and amphiphilic drugs and affect interstitial concentrations of endogenous compounds, drugs, and xenobiotics in a variety of tissues (Koepsell et al., 2007). hOCT1 and hOCT2 mediate the uptake of oxaliplatin, a platinum-derived drug used in cancer (Zhang et al., 2006). hOCT1 also transports imatinib, and its expression is important in the clinical response of this drug broadly used in chronic myeloid leukemia (White et al., 2006; Wang et al., 2007). Moreover, some hOCTs could be involved in the uptake of anti-HIV drugs as it has been described for AZT and lamivudine (3TC) uptake by rat OCTs and a nondetermined type of organic cation-proton exchanger (Aiba et al., 1995; Hong et al., 2001; Leung and Bendayan, 2001). Finally, hOCTN1 and hOCTN2, belonging to organic cation/wtzerion transporters, are linked to H^+ and Na^+ cotransport, respectively. hOCTN1 mainly transports cationic molecules, whereas hOCTN2 is a high-affinity transporter for l-carnitine (Koepsell and Endou, 2004).

It is interesting to note that, whereas in secretory tissues only carrier-dependent mechanisms are described in the uptake of anti-HIV drugs, the few studies published in leukocytes suggest nonfacilitated diffusion mechanisms (Mahony et al., 2004). Nevertheless, we have recently shown that approximately 60% AZT entry in the T-cell line Molt-4 involves a facilitated mechanism (Purcut et al., 2006).

In the present study, we have assessed the mRNA levels, protein expression profile, and physiological activity of membrane transporters codified by SLC29, SLC28, and SLC22 genes in target cells for HIV-1 infection and some leukemias, including peripheral blood mononuclear cells (PBMCs), T-lymphocytes (lymphoblastoid T-cells and purified CD4^+ T-cells), monocytes, macrophages, and dendritic cells.

### Materials and Methods

#### Tissue Specimens from Human Donors

PBMCs were obtained from healthy human volunteers, and a lysate of normal kidney tissue was obtained from renal biopsy. The institutional ethics review board approved the study in adherence to the Helsinki Declaration.

**Reagents.** Uridine ([5,6-^3^H]), 37 Ci/mmol, l-carnitine hydrochloride (H_L, C_3^2^-H), 83 Ci/mmol, and mannitol (≥1-[1^-1^4^C]), 59 mCi/mmol were purchased from Amersham Biosciences (Buckinghamshire, UK). Cytidine ([5^-2^-H(IN)], 22 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA), and N-methyl-4-phenylpyridinium (MPP^+^) ([H_L, C, H^2^-3^H]), 85 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO).

The inhibitors of nucleoside transporters nitrobenzylthioninosine (NBFI), dipryridamole (DIP), and substrates and inhibitors of organic cation transport ranitidine (Rani), ergothioneine (ET), and D-22 were obtained from Sigma-Aldrich (St. Louis, MO).

#### Cell Culture

Six different lymphoblastoid cell lines (MT2, MT4, Molt-4/CRC5, Hut78, A3F7, and CEM13) were used to quantify nucleoside and organic cation transporter expression by real-time PCR. The T-cell leukemia cell lines MT2 and MT4 were obtained from Dr. D. Richman; T4-lymphoblastoid human cell line Molt-4/CRC5 was from Dr. M. Baba; and Hut78 human cutaneous T-cell lymphoma cell line was from Dr. A. F. Gazdar, all of these through the National Institutes of Health AIDS Research and Reference Reagent Program. The T4-cell line A3.01/CRC5 F7 (abbreviated here as A3F7) was obtained from Dr. Q. Sattentau through the Centre for AIDS Reagents. Clone 13 (CEM13), which was derived from the T4-cell line CEM, was kindly donated by Dr. L. Montagnier from Institute Pasteur (Paris, France). Cells were routinely cultured in RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mML-glutamine, and 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen, Paisley, UK), with the exception of the A3F7 cells, which were also supplemented with 1 mg/ml G-418 (Invitrogen) and maintained at 37°C in a humidified atmosphere containing 5% CO_2_.

PBMCs were isolated from HIV-1-seronegative donors by Ficoll-Hypaque density gradient centrifugation of heparin-treated venous blood. CD4^+ T-cells were isolated from PBMC by negative selection with the CD4^+ Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany), and all of the isolated populations had a high purity (>95%). Cells were stimulated for 3 days by adding 3 μg/ml phytohemagglutinin (PHA; Invitrogen) and 10 IU/ml interleukin-2 (IL-2; Roche, Basel, Switzerland). Monocytes were isolated with a high purity (>97%) from PBMC with CD14^-positive selection magnetic beads (Miltenyi Biotec). We differentiated macrophages by culturing monocyte populations (0.8 × 10^6 cells/ml) with 100 ng/ml macrophage-colony-stimulating factor (Peptech, London, UK); we maintained them in culture for 5 to 6 days replacing medium at day 3.

To obtain immature dendritic cells populations, we cultured monocytes (0.5 × 10^6 cells/ml) during 7 days with 1000 IU/ml granulocyte/macrophage colony-stimulating factor (GM-CSF) and 1000 IU/ml IL-4 (R&D Systems, Minneapolis, MN). Medium was replaced every 2 to 3 days adding fresh GM-CSF and IL-4. To obtain mature den-
dritic cells population, 100 ng/ml lipopolysaccharide (LPS; Sigma-Aldrich) was added at day 5, and the culture was maintained until day 7.

All primary cells were maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine, 20% fetal bovine serum (10% for macrophages), and 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen).

**Quantitative Real-Time Reverse Transcriptase-PCR Analysis.** Total RNA was isolated from different lymphoblastoid cell lines, CD4+ lymphocytes, PBMCs, macrophages, and kidney lysates using RNaseasy Mini Kit or RNaseasy Micro Kit (QIAGEN, Barcelona, Spain). RNA was treated with DNase I from RNase-free DNase set (QIAGEN) to eliminate contamination of DNA. In total, 1 μg of RNA was retrotranscribed to cDNA using the TaqMan reverse transcription reagents (including Multiscribe reverse transcriptase and random hexamers) as described by the manufacturer (Applied Biosystems, Foster City, CA). Real-time quantitative reverse transcriptase-PCR analysis of hENT1, hENT2, hCNT1, hCNT2, hCNT3, and human β-glucuronidase mRNA was performed as described previously (Molina-Arcas et al., 2003). The mRNA expression of hOCT1, hOCT2, hOCT3, hOCTN1, and hOCTN2 was assessed using the commercial Gene Expression Assays (Applied Biosystems). Absolute quantification of gene expression was performed by using DNA plasmids containing each of the analyzed transporters to construct standard curves based on serial dilutions of the plasmids. Standard curves were optimized in terms of correlation, slope, and efficiency and were run in duplicate simultaneously with the samples in the ABI Prism 7000 (Applied Biosystems). The threshold cycle (CT) is defined as the cycle number at which the fluorescence corresponding to the amplified PCr product is detected. The standard curves allowed us to correlate CT values of the samples with the mRNA copy number of each gene per microgram of total RNA.

**Immunocytochemistry.** Cytocentrifuge preparations (cytopsin) of PBMCs or monocyte-derived macrophages (MDMs) cultured on poly-l-lysine-treated coverslips (Nunc, Roskilde, Denmark) were air-dried overnight and fixed in 100% acetone for 10 min. Slides were incubated with polyclonal antibodies diluted in Tris-buffered saline (TBS) containing 2% normal human serum or fetal bovine serum to block unspecific binding. Primary rabbit polyclonal antibodies anti-hCNT2, -hCNT3, -hENT1, and -hENT2 were produced in our laboratories (Molina-Arcas et al., 2001; Farre et al., 2004). The newly generated anti-hCNT2 and anti-hCNT3 antibodies were characterized by competing them with an excess of the purified peptide used to immunize the rabbit, as well as by analyzing hCNT2 and hCNT3 expression in transfected cells expressing these particular transporter proteins (data not shown). For cytopsins of PBMCs or MDMs cultured on coverslips, all primary antibodies were detected using a polyclonal goat anti-rabbit-conjugated antibody (Cultek, Barcelona, Spain) followed by development with liquid DAB' substrate (DakoCytoMation, Madrid, Spain) as described by the manufacturer. After washing with TBS and fixing with 4% paraformaldehyde, samples were incubated in Nuclear Fast Red (DakoCytoMation) for 45 min at room temperature and then revealed with ECL-Plus solution (Amersham Biosciences). Images were collected using Kodak Gel Logic 440 Imaging System and Kodak Molecular Imaging Software (Kodak, Barcelona, Spain).

**Uptake Measurements.** The uptake of uridine, the organic cation model (MPP+), and L-carnitine into PBMCs and PHA-stimulated PBMCs was measured using a rapid filtration method as described previously (Molina-Arcas et al., 2003). Having previously determined in time-course experiments with radiolabeled substrates that the uptake linearity of the substrates was maintained at least until 5 min for uridine and MPP+ and until 10 min for L-carnitine in those cells, PBMCs were incubated for 2 (uridine and MPP+) or 5 min (L-carnitine) in an uptake buffer containing 137 mM NaCl or 137 mM choline chloride plus 5.4 mM KCl, 1.8 mM CaCl2, 1.2 mM MgSO4, and 10 mM HEPES, pH 7.4, supplemented with radiolabeled uridine or MPP+, or L-carnitine and d-[1-14C]mannitol as an extracellular marker and inhibitor of both nucleoside or organic cation transport when necessary. Substrates were routinely used at a concentration of 1 μM as done previously in the case of uridine (Purcut et al., 2006) and based on kinetic parameters (Km and Vmax) for MPP+ and L-carnitine as reported previously (Koeppel and Endou, 2004; Koeppel et al., 2007).

Transport studies in MDMs were carried out on 24-well plates as described previously (Soler et al., 2003) by incubating semiconfluent cell monolayers for 10 min in an uptake buffer of choline chloride or sodium chloride supplemented with radiolabeled uridine or cytidine or the organic cation model (MPP+) at a final concentration of 1 μM in all cases. The uptake was measured either in the presence or absence of the equilibrative nucleoside inhibitors NBTI (1 μM) or dipyridamole (10 μM) in nucleoside uptake experiments and the organic cation inhibitor D-22 (20 μM) or the high-affinity competitive substrates Rani and ET at 1 mM in organic cation uptake measurements. Incubations were stopped by rapid aspiration of the uptake buffer, followed by immediate washing in ice-cold stop solution (137 mM NaCl, 10 mM HEPES, pH 7.4).

**Statistical Analysis.** The unpaired and paired Student's t tests were used for the statistical comparison of experimental data. These analyses were carried out using GraphPad Prism version 4.0 software (GraphPad Software Inc., San Diego, CA).

**Results**

mRNA Expression of Nucleoside and Organic Cation Transporters. Because of the importance of the SLC28 and SLC29 families in nucleoside-derived drug transport, especially those used in cancer and HIV treatment, we first focused our attention on the expression of hNTs in lymphoblastoid T-cell lines by examining the mRNA levels of both concentrative (hCNT1–3) and equilibrative nucleoside transporters (hENT1–2) (Fig. 1A). hCNT2 was the most expressed concentrative isoform in all of the cell lines tested, being more expressed in MT4, whereas hCNT1 and hCNT3 showed either low or negligible expression. Both hENTs showed a...
and in purified CD4⁺ T-cells either in basal conditions or after PHA stimulation of these cells. As shown in Fig. 1B, similar to T-lymphoblastoid cells, hCNT2 was the only concentrative nucleoside transporter substantially expressed in PBMCs and CD4⁺ T-cells. Under PHA stimulation, all hCNTs appeared to up-regulate their expression, although only the increase in hCNT2 transcripts in PBMCs was statistically significant (p = 0.005). hENT-related mRNA levels were similar to those of hCNT2 in nonstimulated PBMCs (approximately 10⁵ copies/µg of RNA). In that case, the PHA stimulation caused a statistically significant up-regulation in both PBMCs and CD4⁺ T-cells, with hENT1 being the most up-regulated transporter (p < 0.01 and 0.005, respectively). Moreover, the greatest difference between PBMCs and CD4⁺ T-cells, when looking at basal transporter expression, was associated with hENTs, both showing higher mRNA-related levels in PBMCs (p = 0.005 for hENT1 and p = 0.035 for hENT2) than in CD4⁺ T-cells.

To further study hNT expression patterns in immune cells, we determined the mRNA levels for SLC28 and SLC29 gene family transporters in monocytes, MDMs and MDDCs. In contrast to PBMCs and CD4⁺ T-cells, monocytes showed a remarkably higher expression of hCNT3. Moreover, monocyte differentiation to MDMs and MDDCs produced a significant increase of hCNT3 expression (p = 0.03 and 0.005, respectively). As for T-cell lines and primary lymphocytes, hCNT2 and both hENTs isofoms showed a notable expression, with hENT1 being the most expressed transporter. The high expression of hENT1 and hENT2 in monocytes (approximately 10⁵ and 10⁴ copies mRNA/µg RNA, respectively) could explain the higher mRNA amounts of those transporters in PBMCs compared to CD4⁺ T lymphocytes.

Focusing on SLC22 gene family, hOATs were ruled out from this study because they were expressed in Molt-4, but not in primary T-cells or in macrophages (Purcet et al., 2006). Even though we aimed at studying the expression of hOCTs and hOCTNs, which also belong to the SLC22 gene family and are known to be involved in antiviral and anticancer drug uptake (Koepsell et al., 2007). Organic cation transporters showed lower expression and a more heterogeneous expression profile than nucleoside transporters in T-cell lines (Fig. 1B). It is noteworthy that none of the cell lines tested had detectable hOCT2 mRNA levels.

In nonstimulated primary PBMCs and CD4⁺ T-cell cultures (Fig. 2B), hOCT1 and hOCTN2 had nearly the same mRNA levels in both populations, whereas CD4⁺ T-cells showed a significantly lower hOCT3 and hOCTN1 mRNA expression than PBMCs (p = 0.002 for both transporters). As seen in T-cell lines, hOCT2 mRNA was not found in any of primary lymphocytes tested. Under PHA stimulation, all hOCTs and hOCTNs previously expressed underwent a significant up-regulation. The greatest up-regulation in PBMCs and CD4⁺ T-cells was seen for hOCT3 and hOCTN2 (p < 0.01 for hOCT3 and p < 0.001 for hOCTN1 in both cell types).

Regarding monocytes/macrophage lineage primary cultures (Fig. 2C), hOCTs and hOCTNs showed the most heterogeneous and cell type depending on mRNA expression pattern. In regard to monocytes, they showed the highest mRNA expression of hOCT3 and hOCTN1. The differentiation of monocytes to macrophages with macrophage-colony-stimulating factor or to dendritic cells with GM-CSF/IL-4 seems to have an antagonistic effect on hOCT and hOCTN expression.

very high expression (between 10⁵ and 10⁷ mRNA copy number/microgram of RNA).

We then studied the mRNA expression of hNTs in PBMCs
Whereas for hOCT1 and hOCTN2 transcripts, there is a statistically significant up-regulation (p < 0.05 and 0.001, respectively), hOCT3 and hOCTN1 mRNA copy numbers decrease in a significant manner. When iMDDCs were matured with LPS, the expression of hOCTN1 increased respect to MDMs.

**Protein Expression of hNTs and hOCT/Ns.** Having studied mRNA expression of hNTs and hOCT/Ns and knowing that there might not be systematic correlation between mRNA and protein expression (Molina-Arcas et al., 2005), we wanted to assess the protein expression profile in PBMCs and MDMs and the effect that PHA would cause in the transporter expression in PBMCs. As shown in Fig. 3, we analyzed both hCNTs and hENTs expression in PBMCs and PHA-stimulated PBMCs by immunocytochemistry developed by horseradish peroxidase (HRP). In those cells, we found that mRNA and protein had the same expression profile, nonstimulated PBMCs expressing hCNT2 and hENT1 and 2 proteins, whereas hCNT1 and hCNT3 were not detected (data not shown). Moreover, the expression of hCNT2 and both hENTs in PHA-stimulated PBMCs was increased, with hENT2 up-regulation being the highest. Immunocytochemistry performed in MDMs cultured on coverslips showed a high expression of hCNT2, hCNT3, and both hENT isoforms (Fig. 4) and a negligible expression of hCNT1 (data not shown).

We analyzed the protein expression of hOCT1, hOCT2, hOCT3, and hOCTN1 in nonstimulated and PHA-stimulated PBMCs, purified CD4 T-cells and monocytes MDMs and

**Fig. 2.** hOCT and hOCTN mRNA expression in T-cell lines, primarily lymphocytes, monocytes, MDMs, and MDDCs cultures. A, mRNA expression of hOCTs and hOCTNs in six different T-lymphoblastoid cell lines (MT2, MT4, Molt-4, CEM13, A3F7, and Hut78). B, mRNA expression of hOCTs and hOCTNs in nonstimulated PBMCs (PBMCs) or purified CD4 T-lymphocytes (CD4+) and 3 days of PHA-stimulated PBMCs (Stim PBMCs) or CD4 T-lymphocytes (Stim CD4+). Statistical significance between PBMCs/CD4+ and Stim PBMCs/CD4+ (*, p < 0.05; **, p < 0.01; ***, p < 0.001) and between PBMCs and CD4+ (#, p < 0.01) was assessed by paired Student’s t test. C, mRNA expression of monocytes, MDM, and iMDDC and mMDDC. Statistical significance between monocytes and MDMs/iMDDCs/mMDDCs (*, p < 0.05; **, p < 0.01; ***, p < 0.001) was assessed by paired Student’s t test. The data represent the mean ± S.E.M. of at least six independent experiments performed in duplicate. DL represents the theoretical detection limit of the technique.

**Fig. 3.** Immunocytochemistry of hCNT2 and hENTs in nonstimulated and PHA-stimulated PBMC cytospins. Cytocentrifuge preparations of PBMCs (nonstim PBMCs) or PHA-stimulated PBMCs (PHA-stim PBMCs) were fixed, and primary rabbit-specific polyclonal anti-hCNT2, -hENT1, and -hENT2 antibodies were used to detect protein expression. A secondary conjugate and the development of activity with DAB substrate (brown) were used for the detection of primary antibody binding. Nuclei were counterstained with Nuclear Fast Red (light pink). For negative controls, slides were stained with isotype-matched (IgG) human antibodies. Intense brown circular stained cells are residual erythrocytes (with a high peroxidase content in their membranes) present in the cytospin preparations.

**Fig. 4.** Immunocytochemistry of hCNT3 and hENTs in MDMs cultured on coverslips. Monocyte-derived macrophages were cultured in poly-L-lysine-treated coverslips and fixed. Primary rabbit specific polyclonal anti-hCNT3, -hENT1, and -hENT2 antibodies were used to detect protein expression. A secondary conjugate and the development of activity with DAB substrate (brown) were used for the detection of primary antibody binding. Nuclei were counterstained with hematoxylin (blue-violet). For negative controls, slides were stained with isotype-matched (IgG) human antibodies.
MDDCs by Western blot (Fig. 5). In agreement with mRNA expression levels, we observed an increase in hOCT1, hOCT3, and hOCTN1 protein amounts following PHA stimulation of PBMC and CD4+ T-cells. hOCT2 protein was not detected in any of the blood cells studied (data not shown). hOCT1 protein expression could not be detected in monocytes, macrophages, and MDDCs, even though mRNA levels were high. In relation to hOCT3, showing less associated mRNA levels in MDMs and MDDCs, a higher protein expression in monocytes and macrophages than in MDDCs was observed. hOCTN1 showed a notable expression in MDMs.

Functional Studies of Nucleoside and Organic Cation Transport in PBMCs. We aimed at determining the physiological function of both nucleoside transporters and organic cation transporters in primary lymphocytes (in both nonstimulated and PHA-stimulated PBMCs). To accomplish this objective, we used uridine and MPP+ model substrates of hNTs and hOCTs, respectively, and we carried out inhibition experiments. As shown in Fig. 6A, equilibrative uridine transport in nonstimulated PBMCs was very low (0.3–1.0 pmol/mg protein) and could be completely inhibited (p < 0.05) both by DIP and NBPT, thus confirming that all equilibrative nucleoside transport was mainly due to hENT1 activity. Under PHA stimulation, a significant up-regulation of uridine transport was seen (p = 0.004; 5.5–9.0 pmol/mg protein), and this could also be almost completely inhibited by NBPT (p = 0.001). This reflects the minimal contribution of hENT2 activity to nucleoside transport in primary lymphocytes. In addition, the Na+ component of nucleoside transport (that would be attributed to hCNT2) seemed to be residual, nearly negligible. However, it is important to remark that the PBMCs of some of the donors showed partial Na+ dependence of uridine uptake, suggesting detectable hCNT2 function.

When addressing OCT-related functional activity, we observed a relatively high percentage of transport in nonstimulated PBMCs (0.5–1.3 pmol/mg protein) (Fig. 6B) that could be related to the high lipophilicity of MPP+. Actually, MPP+ transport could not be inhibited by either Rani, D-22, or ET. When PBMCs were stimulated with PHA, MPP+ transport was increased 3-fold above basal (nontreated) cells (2.3–3.0 pmol/mg protein). A notable inhibition of MPP+ uptake could be observed with the addition of ranitidine (32.7% inhibition; p < 0.001) and D-22 (34.4%; p = 0.001). Moreover, the high-affinity hOCNT1 substrate ET could inhibit MPP+ transport significantly (24.2%, p = 0.018) but to a lesser extent than that exerted by hOCT substrates or inhibitors.

Functional Studies of L-Carnitine Transport in PBMCs. To assess the functionality of hOCNT2, a Na+-dependent high-affinity transporter for L-carnitine (Tamai et al., 2000; Duran et al., 2005), we performed uptake of L-[3H]carnitine in nonstimulated and PHA-stimulated PBMCs in the absence (choline medium) and presence of Na+ (NaCl). In the presence of Na+, we found a notable L-carnitine transport in both nonstimulated and PHA-stimulated PBMCs (Fig. 7), with the increase of transport in stimulated cells (447 ± 25 vs. 765 ± 66 fmol/10⁶ cells; p = 0.01) being statistically significant. It is surprising that we also found a high transport rate of L-carnitine in the absence of Na+ both in nonstimulated PBMCs (365 ± 6 fmol/10⁶ cells; 82% transport in NaCl) and in PHA-stimulated PBMCs (516 ± 87 fmol/10⁶ cells; 67% transport in NaCl). Finally, the difference between the transport in the absence and presence of Na+ in nonstimulated PBMCs was statistically significant (365 ± 6 versus 447 ± 25 fmols/10⁶ cells; p = 0.03).

Functional Studies of Nucleoside and Organic Cation Transport in MDMs. To further study the function of drug uptake transporters in immune cells, we investigated the importance of hNTs and hOCTs in MDMs. This was done in macrophages due to their importance in inflammatory processes during cancer and viral infections and their role as important latent reservoirs for HIV-1 (Aquaro et al., 2002).
The expression of drug transporters is relatively well studied in secretory and reabsorptive tissues in relation to toxicity, drug-drug interactions, and drug resistance in antineoplastic and antiviral therapies (Lee and Kim, 2004; McRae et al., 2006; Koepsell et al., 2007). Moreover, in relation to peripheral blood cells exists a broad knowledge about the expression and activity of efflux transporters (Kock et al., 2004). However, little is known regarding the expression profile and activity of uptake transporters in human leukocytes within the context of anticancer and antiretroviral therapies. In the present study, we have assessed the mRNA levels, protein expression profile, and activity of drug uptake transporters involved in anti-HIV and antineoplastic drug uptake in cells of the immune system. Taking into account that HIV retrotranscriptase inhibitors and anticancer drugs are mostly nucleoside derivatives, our first focus was on the nucleoside transporters from the SLC28 family.

Discussion

As we can see in Fig. 8A, normalizing data to 100% transport with uridine transport in Na+ medium (with both hCNTs and hENTs being operative), the hCNT-associated transport of uridine (57.5%) is much higher than the one found in PBMCs (3.4%; nearly negligible). Uridine transport associated with hENT-type activities could be significantly inhibited by 1 mM guanosine, thus revealing hCNT3-related activity. Cross-inhibition of pyrimidine nucleoside transport by purines was also highly variable in donors (from 40 to 95%).

Finally, we studied MPP+ transport processes in MDMs (Fig. 8C). As for PHA-stimulated PBMCs, MPP+ basal transport (12.7 ± 4.2 pmol/mg protein) could be inhibited by the hOCT substrate ranitidine (34.4% inhibition; p < 0.01), the hOCT1 substrate ergothioneine (27.9%; p < 0.01), and the hOCT inhibitor D-22 (57.3%; p < 0.05). This reflects the importance of hOCT and hOCTN function in macrophages and corroborates our results of mRNA and protein expression.

As we can see in Fig. 8A, normalizing data to 100% transport with uridine transport in Na+ medium (with both hCNTs and hENTs being operative), the hCNT-associated transport of uridine (57.5%) is much higher than the one found in PBMCs (3.4%; nearly negligible). Uridine transport associated with hENT-type activities could be significantly inhibited by 1 mM guanosine, thus revealing hCNT3-related activity. Cross-inhibition of pyrimidine nucleoside transport by purines was also highly variable in donors (from 40 to 95%).

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Discussion

The expression of drug transporters is relatively well studied in secretory and reabsorptive tissues in relation to toxicity, drug-drug interactions, and drug resistance in antineoplastic and antiviral therapies (Lee and Kim, 2004; McRae et al., 2006; Koepsell et al., 2007). Moreover, in relation to peripheral blood cells exists a broad knowledge about the expression and activity of efflux transporters (Kock et al., 2004). However, little is known regarding the expression profile and activity of uptake transporters in human leukocytes within the context of anticancer and antiretroviral therapies. In the present study, we have assessed the mRNA levels, protein expression profile, and activity of drug uptake transporters involved in anti-HIV and antineoplastic drug uptake in cells of the immune system. Taking into account that HIV retrotranscriptase inhibitors and anticancer drugs are mostly nucleoside derivatives, our first focus was on the nucleoside transporters from the SLC28 family.

Likewise, members of the SLC28 family (OATs and OCTs) have also been described as drug transporters in other tissues. We had demonstrated that OATs were not expressed in immune system cells (Purcet et al., 2006); thus, we have only analyzed OCT transporters, which have not been previously characterized in immune system cells.

First, we analyzed the mRNA and protein expression of hCNTs and hENTs. In both T-cell lines and primary lymphocytes (PBMCs and CD4+ T-cells), hENTs were the most expressed transporters, whereas hCNT2 was the only sodium-dependent nucleoside transporter remarkably expressed. These results are in agreement with the previously described expression profile of nucleoside transporters in primary lymphocytes (Smith et al., 1989; Wiley et al., 1989; Molina-Arcas et al., 2003; Molina-Arcas et al., 2005). Second, PHA stimulation significantly up-regulated hENTs and hCNT2 in PBMCs and CD4+ T-cells. A similar up-regulation had been described for CNT1 and CNT2 upon stimulation of murine bone marrow macrophages with interferon-γ/α/β/γ, tumor necrosis factor-α, or LPS (Soler et al., 2001, 2003). In contrast, all of these stimuli slightly down-regulated the expression of ENT1 in murine macrophages (Soler et al., 2001), suggesting that the up-regulatory effect of PHA on hENTs might act through a different pathway. Moreover, the PHA-
induced up-regulation of hENT1 is in agreement with the 30-fold increase in the number of hENT1 molecules previously found in peripheral blood lymphocytes cultured within 48 h with the T-cell mitogen (Smith et al., 1989). It is also important to remark that PHA stimulation of PBMCs and CD4+ T-cells enhances hENT expression to a level similar to that reported in T-acute lymphoblastic leukemia. In fact, an increased proliferative rate of acute myeloid leukemia or lymphoma is associated with higher numbers of nucleoside transporters in the cell membrane (Wiley et al., 1989). As in the case of T-lymphocytes and T-cell lines, significant mRNA levels were also seen for hCNT2, hENT1, and hENT2 in monocyte/macrophage lineage cells. However, monocyte/macrophage lineage cells expressed important quantities of hCNT3, whereas T-lymphocytes did not. Thus, iMDDC and MDM showed the highest number of hCNT3 mRNA copies even though monocytes and mMDDCs also showed greater expression than PBMCs or CD4+ T-cells. The abundance of hCNT3 protein in macrophages was also confirmed by immunocytochemistry. Of note, the differential expression profile of this transporter in monocyte/macrophage lineage cells is a novel observation in this field.

With regard to hNT function, uridine transport in PBMCs revealed a preferential role of hENT1. In contrast, there was a minimal contribution of sodium-dependent transporters (only hCNT2 was expressed) and a nearly negligible role of hENT2 in spite of its high and ubiquitous expression, as suggested by the complete inhibition of uridine transport in the presence of NBTI and DIP. It is important to point out that two mRNA splice variants resulting in nonfunctional transporters have been described previously (Mangravite et al., 2003). Therefore, it is possible that the protein and mRNA found for hENT2 correspond to one of these variants. Moreover, the PHA stimulation also only resulted in greater hENT1 activity accordingly with the up-regulatory effect seen on its mRNA and protein expression, whereas hENT2 did not show an increase in activity. Finally, hCNT2 activity seemed to be so low in PHA-stimulated PBMCs, although the PBMCs of some of the specific donors showed Na+/H+ dependence of uridine uptake. This is in agreement with previous findings.
results shown by our group in B-cells from chronic lymphocytic leukemia in which, although CNT2 expression was detected in 22 patients, Na\(^+\)-dependent guanosine transport activity was only detected in 12 patients (Molina-Arcas et al., 2003).

Functional analysis of hNT in MDMs mainly paralleled that in PBMCs, thus confirming that hENT2 plays a negligible role in nucleoside transport in leukocytes despite its high level of mRNA expression. The main difference found between PBMCs and MDMs was the relevant sodium component of uridine uptake in MDMs, presumably due to the presence of hCNT3. As hypothesized, the contribution of hCNT3 function was confirmed by cytidine transport inhibition by guanosine. Because cytidine is a pyrimidine nucleoside and could only be transported by hCNT1 (not expressed in MDMs) and hCNT3, the inhibition of sodium-dependent cytidine transport by guanosine demonstrates the occurrence of hCNT3 function. This finding is noteworthy by itself because it is well documented that hCNT3 is a broadly selective, potent, and high-affinity transporter for its natural substrates and, more importantly, a suitable transporter for nucleoside analogs used in anti-HIV (such as AZT, dC, or ddi) and in anticancer therapies (such as cladribine, gemcitabine, 5-fluorouridine, and fludarabine) (Hu et al., 2006; Errasti-Murugarren et al., 2007).

With regard to hOCTs and hOCTNs, we did the same mRNA and protein expression analysis as done for hNTs. Overall, the expression profile of hOCTs and hOCTNs was more heterogeneous with a quantitative general lower expression than hNTs. PHA stimulation of PBMCs and CD4\(^+\) T-cells increased the mRNA levels and protein of hOCTs and hOCTNs, with the exception of hOCT2, which was not expressed in any of T-cell lines or primary leukocytes studied. Similar mRNA up-regulatory effects for hOCTN1 have been shown upon cell incubation with tumor necrosis factor-α (Tokuhiro et al., 2003). This expression profile of hOCTs is identical to the one that had recently been described in haemophils (Schneider et al., 2005).

The physiological role that hOCTs might play in immune cells remains to be well understood, but we believe that these transporters might be important for the regulation of inflammatory processes and, as known for hOCT3, immune response related to pro-Th2 cytokines and histamine (Schneider et al., 2005). In fact, histamine for itself has a wide variety of functions in immune regulation, including vascular endothelial growth factor production via H\(_2\) receptor stimulation, mast cell chemotaxis via H\(_4\) receptor stimulation, T-cell proliferation, and dendritic cell maturation (Jutel et al., 2002). It is noteworthy that the treatment of patients with drugs with high affinity for hOCT3 might somehow impair immune response. Moreover, hOCTs might also be involved in the regulation of membrane potential, which could be important in some drug therapies using positively charged molecules, such as corticosterone, which is taken up by hOCTs (Koepsell et al., 2007).

With regard to hOCTNs, their occurrence in the immune system has only been reported so far for the hOCTN1 isoform, which is present in human cord blood cells (CD71\(^+\) cells) and leukocytes (Grundemann et al., 2005). In monocyte/macrophage lineage cells, the expression of hOCTs and hOCTNs was notably high, with monocytes being the cells with a higher expression of hOCT3 and hOCTN1. This result agrees with the maximal expression of hOCTN1 previously found in a subset of CD14\(^+\) lymphocytes (Tokuhiro et al., 2003). Moreover, monocyte differentiation to MDMs or iMDDCs caused antagonistic effects in hOCTNs mRNA expression; whereas hOCT1 and hOCTN2 up-regulation was observed, hOCT3 and hOCTN1 showed significant down-regulation. Furthermore, LPS maturation of iMDDCs also had an antagonistic effect on different iMDDCs hOCT/N expression. The physiological basis for this differential effect remains unexplained. Protein expression in monocyte/macrophages lineage cells could not be found for hOCT1. Nevertheless, hOCT3 showed a high protein expression in monocytes and macrophages and a lower expression in MDDCs (as seen for mRNA levels). Finally, hOCTN1 protein was highly expressed in MDMs.

The activity of hOCTs, analyzed by MPP\(^+\) transport, was lower in PBMCs with respect to the hNTs, probably because of the higher lipophilicity of organic cations and therefore higher rates of simple diffusion, even though in PHA-stimulated PBMCs, the MPP\(^+\) transport could be partially inhibited by the competing substrates ranitidine and ET, as well as by the hOCTs inhibitor D-22. In addition, because hOCTN1 is a low-affinity transporter for MPP\(^+\) (Koepsell et al., 2007) and ET is only transported by hOCTN1 with a high affinity (Grundemann et al., 2005), the inhibition seen with ET, together with the mRNA expression profile, is direct evidence that hOCTN1 is an active transporter in stimulated PBMCs. Finally, the Na\(^+\)-coupled L-carnitine transport found in nonstimulated and PHA-stimulated PBMCs confirms that hOCTN2 is active in those cells. The L-carnitine uptake in the absence of Na\(^+\), which was also found, would be attributed to the recently described transporter OCTN3, which seems to be a Na\(^+\)-independent L-carnitine transporter highly expressed in enterocyte basolateral membrane (Duran et al., 2005). For MDMs, we found a similar activity pattern than that for PHA-stimulated PBMCs; the substrates ranitidine and ET significantly inhibited MPP\(^+\) transport, whereas D-22 did it to a lesser extent. As far as we know, these results in PBMCs and MDMs are the first evidence of hOCT and hOCTN activity studied in primary leukocytes.

In conclusion, all of these data strengthen the importance of drug transporters associated to cell entry of hydrophilic drugs used in anticancer and antiviral therapies in immune cells. Of note, we have described the abundant presence of both hNTs and hOCTs in primary lymphocytes, monocytes, macrophages, and dendritic cells and the high activity of both transporter types in PHA-stimulated PBMC and CD4\(^+\) T-cells and MDMs. Moreover, further studies on the specific implication of each isoform in relation to antineoplastic or anti-HIV drug transport across cell membrane would help us to understand drug-drug interactions, drug resistance, and therapy failure and would provide us new insights in relation to drug pharmacokinetics and drug metabolism.

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References


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