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Departament de Biologia Cel·lular, de Fisiologia i d'Immunologia
Facultat de Medicina

Universitat Autònoma de Barcelona

Uptake Transporters of Nucleoside-Derived Anti-HIV Drugs: Expression and Functional Analysis in Immune Cells

Gerard Minuesa Dinarés

Fundació IrsiCaixa
Hospital Universitari Germans Trias i Pujol

Memòria de la tesi presentada per obtenir el grau de Doctor en Immunologia per la Universitat Autònoma de Barcelona

Bellaterra, 18 de maig del 2009

Director: Dr. Javier Martínez-Picado
Tutora: Dr. Paz Martínez Ramírez

Amb el suport del Departament d'Educació i Universitats i l'Agència de Gestió d'Ajuts Universitaris i de Recerca de la Generalitat de Catalunya

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La Dra. Paz Martínez Ramírez, Coordinadora de Tercer Cicle de Biologia Cel·lular, Fisiologia i Immunologia de la Universitat Autònoma de Barcelona,

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I per tal que quedi constància, signa aquest document a Badalona, el 18 de Maig de 2009.

Dra. Paz Martínez Ramírez

A la meva família,
pel temps que us dec, pels ànims, per la paciència

Als meus Amics,
per haver-me salvat tantes vegades de *tanta* ciència

"¿De dónde venimos?, ¿a dónde vamos?,
¿hay posibilidad de tarifa de grupo?"
Woody Allen

No entiendes realmente algo a menos que seas
capaz de explicárselo a tu abuela
Albert Einstein

Abbreviations Commonly Used

3TC: Lamivudine

AIDS: Acquired Immunodeficiency Virus
ABC: Abacavir / ATP-Binding Cassette
Atrop: Atropine
AZT: Azidothyimidine

CHO: Chinese Hamster Ovary
CNT: Concentrative Nucleoside Transporter
C_t: Threshold Cycle

D-22: 1,1'-diethyl-2,2'-cyanine iodide
d4T: stavudine
DCs: Dendritic Cells
ddC: zalcitabine
ddI: didanosine
DIP: Dipyridamole
dNTP: Deoxynucleotide Triphosphate
DNA: DeoxyriboNucleic Acid

ELISA: Enzyme-Linked Immunosorbent Assay
ENT: Equilibrative Nucleoside Transporter
ET: Ergothioneine

FACS: Fluorescence-Activated Cell Sorting
FBS: Foetal Bovine Serum
FTC: Emtricitabine

GFP: Green Fluorescence Protein
GM-CSF: Granulocyte/Macrophage Colony Stimulating Factor

HAART: Highly Active Antiretroviral Therapy
HIV-1: Human Immunodeficiency Virus Type 1
HRP: Horseradish Peroxidase
HTLV-1: Human T-Lymphotropic Virus Type 1

IFN: Interferon
IL: Interleukin
iMDDC: immature Monocyte-Derived Dendritic Cell

LPS: Lipopolysaccharide

M-CSF: Macrophage Colony Stimulating Factor
MDM: Monocyte-Derived Macrophage
Metformin: 1,1-[dimethyl]-biguanide hydrochloride

mMDDC: mature Monocyte-Derived Dendritic Cell
MPP+: *N*-Methyl-4-PhenylPyridinium
MRP: Multidrug Resistant Protein

NBTI: Nitrobenzylthioinosine
NRTI: nucleoside reverse transcriptase inhibitor
NNRTI: non-nucleoside reverse transcriptase inhibitor
NT: Nucleoside Transporter

OAT: Organic Anion Transporter
OATP: Organic Anion Transporting-Polypeptide Transporter
OCT: Organic Cation Transporter
OCTN: Organic Cation/Carnitine Transporter

PBS: Phosphate Buffer Saline
PCR: Polymerase Chain Reaction
PHA: Phytohemagglutinin
PI: Protease Inhibitor

Rani: Ranitidine
RT: Reverse Transcriptase

SLC: SoLute Carrier

TDF: Tenofovir Disoproxil Fumarate
TBS: Tris-Buffered Saline
TBuA: TetraButylAmmonium
TEA: TetraEthylAmmonium

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Introduction

1. Human Immunodeficiency Virus

1.1. The discovery of Human Immunodeficiency Virus (HIV)

In 1981 a number of strange case reports from medical practice appeared in the United States of America: a succession of homosexual men presented clinical profiles with unusual opportunistic infections (especially pneumonia caused by *Pneumocystis carinii* and cytomegalovirus infections), Kaposi's sarcoma and/or lymphoadenopathies (Centers for Disease Control (CDC), 1981; Gottlieb et al., 1981). All of them shared an acquired shortfall of cellular immunity, basically a depletion of CD4+ T cells, that often caused them to die (Masur et al., 1981). That same year, the disease was described as Acquired Immunodeficiency Syndrome (AIDS). In 1983 the laboratory of Professor Luc Montagnier at the Pasteur Institute in Paris isolated a new retrovirus from the biopsy of an AIDS patient (Barre-Sinoussi et al., 1983). The virus, called *Lymphadenopathy-Associated Virus* (LAV), and the *Human T cell Lymphotropic Virus* (HTLV) discovered at the same time, were T lymphotropic viruses from a retrovirus family that was horizontally transmitted and caused different pathologic malignancies, including AIDS (Barre-Sinoussi et al., 1983). The following year, Robert Gallo and his co-workers provided more scientific evidence that the infection by the identified and named HTLV-III virus – which was actually the LAV virus, isolated the year before by Professor Montagnier – was the cause of AIDS (Gallo et al., 1984). During 1984, the virus was independently isolated by the group of Jay Levy at the University of California in San Francisco. He demonstrated the presence of LAV (or HLTV-III) in AIDS patients and in asymptomatic individuals from risk groups (Levy et al., 1984). In 1986 the International Committee on Taxonomy of Viruses "baptized" the previously named LAV and/or HTLV-III virus as Human Immunodeficiency Virus (HIV). Remarkably, from the first AIDS cases reported in 1981 until the identification of the virus in 1983, only two years had elapsed, the same time needed to develop a viable test to detect HIV in blood. These two findings can be considered as two of the most successful discoveries in the history of biomedicine.

1.2. AIDS epidemic

Twenty-five years ago, HIV was identified as the infectious agent causing AIDS. Since then, the scientific community has engaged in ceaseless effort to control and eradicate the infection. Firstly, the appearance of azidothymidine (AZT) in 1985, represented a step forward: it was the first drug that inhibited HIV replication and controlled the infection. Almost ten years later, the introduction of the *Highly Active*

Antiretroviral Therapy (HAART) – a combination of three or more drugs that act against HIV – reduced the mortality and morbidity of the disease, leading AIDS to come to be considered a chronic illness in developed countries.

Unfortunately, the incidence, prevalence and the presence of the disease around the world are broader than could have been initially foreseen, even today. In 2007, 2.7 million people were newly infected with HIV. Moreover, there were 2 million of deaths related with the virus. Today, around **33 million people** are estimated to be **infected with HIV**, with 22 million infected in Sub-Saharan Africa alone, or nearly 70% of the total infected population in the world (UNAIDS/WHO, 2007) (*Figure 1*). Even though the rate of infection is decreasing in some countries globally, this favourable trend is concealing increases in the number of new infections in other countries. In China, the most populous country in the world, transmissions have increased by about 8% from 2006 to the present (Lu et al., 2008). AIDS has become the main cause of death in Sub-Saharan African countries and the fourth in the World, behind heart attacks, cerebral ischemia and acute respiratory diseases. The most worrying factor is that more than 95% of those infected live in developing countries, with limited access to antiretroviral therapy and particularly to new anti-HIV drugs.

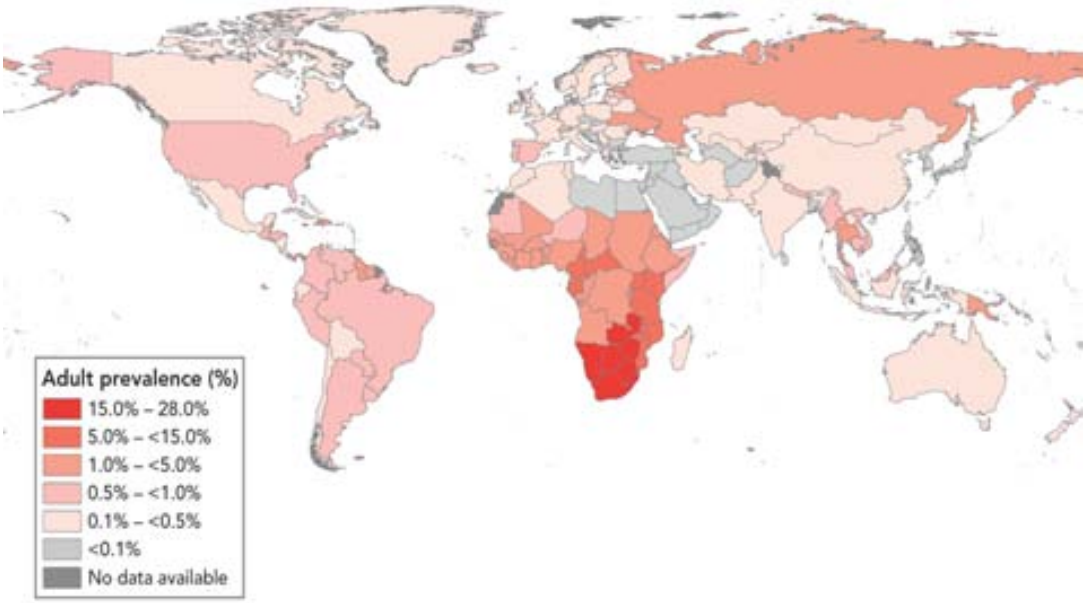


Figure 1. A global view of HIV infection in 2007. Percentage of HIV adult prevalence is shown in a range of grey and red colours (*see legend* on the left bottom). Taken from the Joint United Program on HIV/AIDS (UNAIDS) and the World Health Organization (WHO) 2007. *AIDS epidemic update: December 2007* (www.unaids.org).

1.3. HIV characteristics

HIV belongs to the *Retroviridae* family, which groups together RNA viruses that require the action of the *Reverse Transcriptase (RT)* enzyme to transform their RNA genome to DNA and thereby integrate it into the host cell genome and replicate. Within the retroviruses, HIV belongs to the lentiviruses, which generally take a long time to produce adverse effects in infected patients. Genetically, we can differentiate between two HIV strains: HIV-1 and HIV-2. These strains have a 40 to 60% protein homology, are transmitted in the same way and both produce AIDS indistinctly (Reeves and Doms, 2002). The majority of HIV infections are caused by HIV-1 because HIV-2 is a less common strain, and even though it is frequent in West Africa, it is up to eight times less transmissible and pathogenic than HIV-1 (Rowland-Jones and Whittle, 2007; de Silva et al., 2008). HIV-1 is then classified in three main groups: M (from *major*), O (from *outlier*) and N (from *new*). The M group is also divided into the subgroups A, B, C, D, F, G, H, J, K, CRFs.

HIV has three main routes of transmission: (1) **sexual contact** with an infected person (direct contact with secretions: blood, semen etc.); (2) **parenteral transmission** by sharing needles and/or syringes – primarily for drug injection – or, less commonly (and now very rarely in countries where blood is screened for HIV antibodies) through transfusions of infected blood or blood clotting factors; and (3) **mother-to-child/vertical transmission** during pregnancy, birth or through breast-feeding.

One essential requirement of both HIV-1 and HIV-2 is that cells that are going to be infected must express the CD4+ receptor (Dalgleish et al., 1984) and either CXCR4 or CCR5 (chemokine receptors present mainly in immune cells) (Berger et al., 1999). These chemokine receptors serve as co-receptors that guide the viral envelope glycoproteins into a conformation permitting membrane fusion and entry into the cell. Due to these requirements, HIV preferentially infects immune system cells. The main HIV targets are CD4+ T-lymphocytes, but other cells expressing CD4+ in their surface such as monocytes, macrophages, dendritic cells or CD8+ T lymphocytes, are susceptible to infection. In fact, it has been described that some of these cell types are infected *in vivo* and could be acting as HIV reservoirs, increasing the latency period and the development towards AIDS (Schrager and D'Souza, 1998; Stevenson, 2003; Montaner et al., 2006).

Focusing on the co-receptor use, HIV strains are divided according to their **tropism**, hence a preference for the use of one or other co-receptor. Those strains that use CCR5 are commonly known as “R5” strains, while those that use CXCR4 are called “R4”

strains. Those strains that can use CCR5 or CXCR4 indistinctly are called *dual-tropic* or "X4R5". There are numerous studies in which the differences in infectivity between these different strains are analyzed. It is known that R5 strains are *macrophage*-tropic (or M-tropic) so that they mainly infect monocytes, macrophages and dendritic cells (Deng et al., 1996; Deng et al., 1997). R5 strains are preferentially transmitted from one person to another and are normally detected during the early stages of the disease. In contrast, X4 strains are called T cell-tropic (or T-tropic) because they mainly infect CD4+ T lymphocytes and lymphoblastic T cells *in vitro*, and have a low capacity for infecting macrophages (Doranz et al., 1996; Feng et al., 1996). Usually, X4 or X4R5 strains appear during the late stages of the disease (Connor et al., 1997).

1.4. HIV structure

High-resolution electronic microscope techniques help study how mature HIV virions are organized structurally. HIV particles are roughly spherical and 100–150 nm in size. The following can be distinguished in the particles (*Figure 2*):

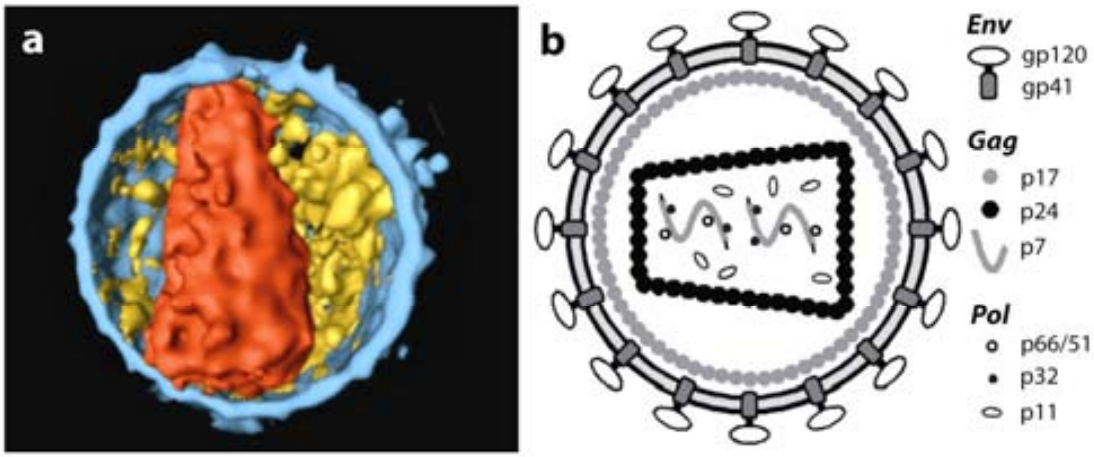


Figure 2. Schematic representation of the structure of HIV. *(a)* Reconstruction of a mature HIV particle after analysis of viral cryosections. Adapted from (Briggs et al., 2006). *(b)* Schematic picture showing viral proteins (gp120, gp41, p17, p24, p7, p66/51, p32 and p11) encoded by the corresponding genes (*Env, Gag and Pol*). Adapted from the *University of Zambia Medical Library website* (www.medguide.org.zm).

- **Envelope:** derived from the host cell membrane. Formed of a lipid bilayer containing host cell proteins and spikes on its outer surface corresponding to viral envelope glycoproteins *gp120* and the transmembrane *gp41*, responsible for the initial virus–cell interaction, receptor binding and membrane fusion required for virus entry.

- **Matrix:** essentially formed of the protein p17, crucial for virion integrity.
- **Nucleocapsid:** a core shaped like a truncated cone shape encapsulates the genetic material protected by the nucleocapsid protein (p7). This core, along with viral enzymes (reverse transcriptase –RT-, protease –PR- and integrase –IN-) is contained in the capsid shell formed by the capsid protein (p24), the most abundant in the virus. Additionally, several accessory proteins, *Nef, Vif* and *Vpr* are also present in the mature virus. Moreover, other accessory proteins (*Tat*, *Vpu* and *Rev*) are synthesized in the host cell.

1.5. HIV lifecycle

The HIV replication cycle is complex. To simplify and reduce complexity, it is usually divided into different stages (*Figure 3*):

- **Virus entry into host cell:** this stage involves *adhesion*, *co-receptor interaction*, *fusion* and *virus internalization*. HIV adhesion with the host cell surface occurs due to a high affinity interaction between the envelope protein gp120 and the host cell CD4 receptor (Dalgleish et al., 1984). The gp120–CD4 complex interacts with the CCR5 or CXCR4 co-receptors, and the glycoprotein gp41 allows membrane fusion and the internalization of the virus into the host cell. After fusion, the nucleocapsid loses its structure and its content is released into the cytoplasm.
- **Reverse transcription and transport into the nucleus:** single-stranded RNA is transformed into double-stranded DNA by *Reverse Transcriptase* (RT). The enzyme has a high frequency for misincorporation of nucleotides, contrary to other mammal polymerases, because it lacks *proofreading* mechanisms. The synthesized DNA is then translocated into the nucleus.
- **Viral DNA integration into genomic DNA:** viral DNA, once in the nucleus, is processed and transferred into the host genome by *integrase* (IN) enzyme activity. When the viral DNA is integrated, the infected cell is "permanently infected". The provirus can be inactive (*latent*) in the genome for a long time (months or even years) or it can undergo active viral production, depending on the activation state of the cellular polymerases.

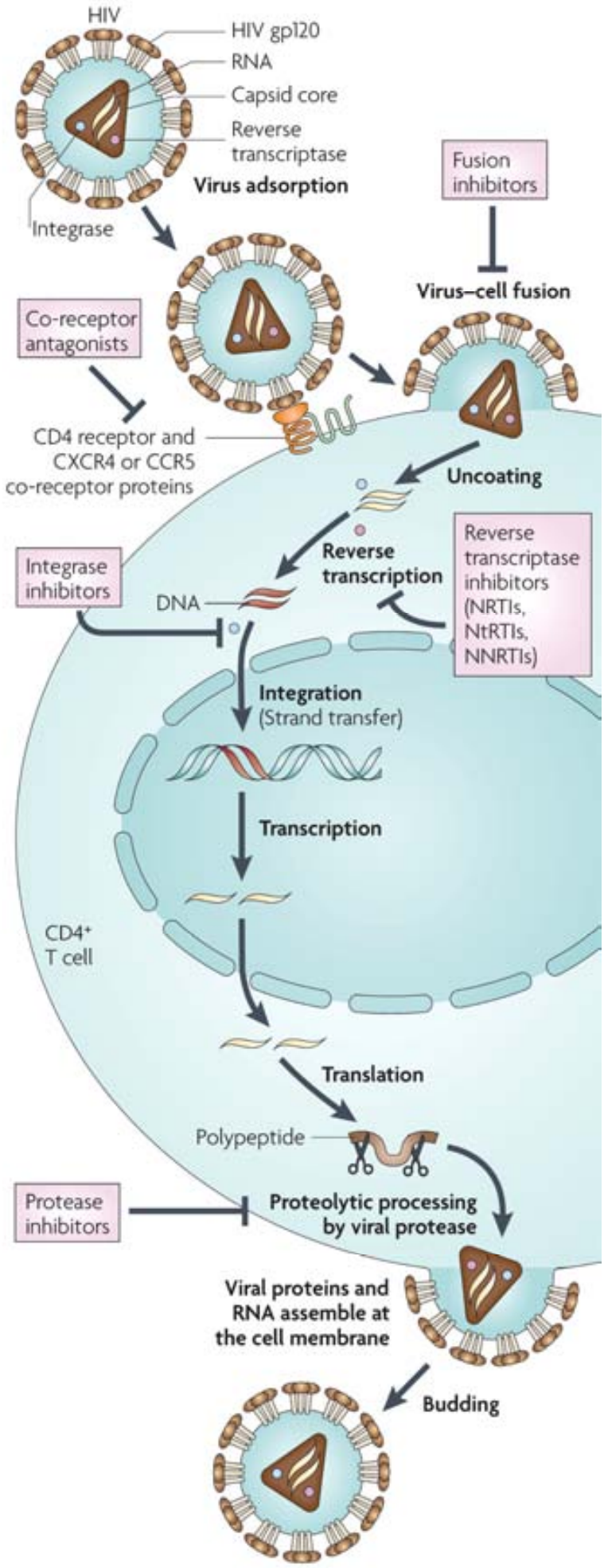


Figure 3. HIV lifecycle and established and potential anti-retroviral drug targets. Viral entry into the cells requires interaction with the $CD4^+$ receptor and co-receptor (CCR5 or CXCR4). Co-receptor inhibitors inhibit this step and fusion inhibitors inhibit virus–cell fusion. Once the virus has fused and internalized the capsid, Reverse Transcriptase (RT) plays one of the main roles in the replicative cycle of HIV, as it is responsible for the transcription of the viral single-stranded (+)RNA genome to proviral double-stranded DNA. Two classes of anti-HIV act ın this RT step: NRTIs and NNRTIs, both inhibiting at the same level but by different mechanisms. Then the viral dsDNA enter the nucleus and the virally encoded integrase enzyme causes its integration into the host cell genome. The latest drug to be developed and approved for use against HIV acts by inhibiting the integrase step. If the cycle is not inhibited, the transcription and translation machinery from the host cells translate the messenger (t)RNAs into a unique polypeptide. This polypeptide, through the action of the protease, is cut into mature proteins to be assembled into new virions. This step of proteolytic procession is inhibited by PIs. The maturation continues after the virus particles have been released by budding from the cells. Taken from (De Clercq, 2007).

- **Transcription and translation:** the activation of the provirus occurs through the replicative cellular machinery (such as polymerases or selective and constitutive transcriptional factors) and as a result favours the production of mRNAs from the virus, which are later translated into the regulatory proteins *Tat*, *Rev*, *Vpu* and *Nef*. After this, their expression as polyprotein precursors of structural gens, such as *gag, gag-pol* and *env,* occurs.
- **New virion production and budding:** the assembly of the regulatory proteins, enzymes and viral RNA near the host cell membrane is required to form a new viral core. Budding then occurs by pinching off a part of the cell membrane.
- **Virion maturation:** the action of protease (PR) allows the processing of the polyprotein precursors *gag* and *gag-pol* into structurally and functionally mature proteins.

1.6. Natural history of HIV-1 infection

The natural history of HIV-1 infection is characterized by chronic development with high variability among patients. A schematic view of the course of a typical HIV-1 infection is depicted in *Figure 4*. Clinically, it is characterized by an initial **acute infection** (1) – also known as *primary infection* – that lasts between two and four weeks. It is a period of rapid virus replication during which a pronounced increase in viral load (copies of viral RNA/ml plasma) results in *seroconvertion*. At the same time, a very drastic decrease in the number of CD4+ T cells occurs, which is inversely proportional to the increase in viral load. Two to three weeks after infection, the immune system is heavily depressed as a consequence of the high viral load in the blood (>10^4 copies viral RNA/ml plasma). This immune depression makes patients manifest a similar symptomathology to patients suffering infectious mononucleosis (fever, sweating, lymphoadenopathies, pharyngitis, rash, myalgia and arthralgia etc.). However, symptoms disappear in one to two weeks due to a strong specific cytotoxic response and a high HIV-specific antibody production. Following this, an equilibrium is achieved between viral replication and cellular and humoral immune response over a period of years know as the **chronic phase** (2). During this phase the patient remains in an state of clinical asymptomatic latency. As time goes by (Hupfeld, 1931), the immune system is progressively deteriorated and due to the rapid reduction in the number of CD4+ T cells, the capacity for controlling viral replication decreases dramatically. At that point, the patient arrives at the **AIDS phase** (3). The AIDS

phase is characterized by an uncontrolled replication of the virus and a decrease in the number of CD4+ T lymphocytes to below 200 cells/ml plasma. The immune system becomes inefficient in controlling the virus and clinically the patient shows opportunistic infections, tumours such as the Kaposi's sarcoma, cervical cancer or neurophysiological disorders. The neuropsychological disorders associated with HIV infection can result in anorexia and asthenia and finally in weight loss. Weight loss can also result from malabsorption, diarrhoea or altered metabolism. This group of syndromes associated with the AIDS phase is known as the *wasting syndrome*.

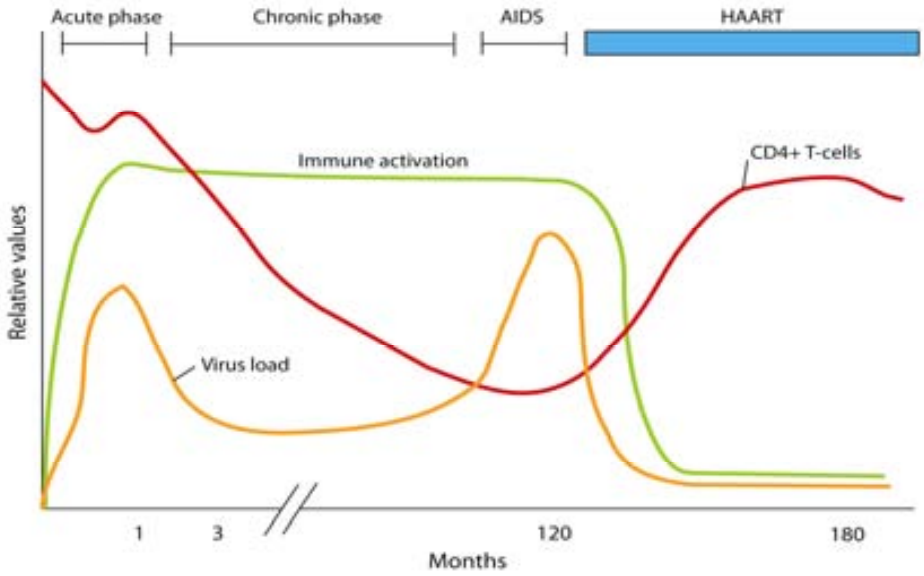


Figure 4. Natural history of HIV-1 infection. Modified from (Gougeon, 2003).

2. Antiretroviral Drugs and their targets

2.1. History of Antiretroviral Therapy (ART)

From the detection of the first patients with AIDS until the discovery of HIV by Luc Montagnier and his colleagues, only two years of scientific research were required. After that achievement, knowledge of the structure and natural infection of the virus grew, thanks to scientific research from all over the world. In that context, the possibility of developing drugs that would constitute an efficient therapy against HIV became increasingly likely every day and, taking into account the "extent of the tragedy", it was an urgent necessity.

The history of antiretroviral therapy (ART) begins with a curious event, despite it being a common feature of the history of Medicine and scientific innovation. **Azydothyimidine (AZT)**, a drug initially designed to combat cancer, was the **first anti-HIV drug** approved by the *Food and Drug Administration* (FDA) in 1985 when its antiviral activity was discovered *in vitro* (Mitsuya et al., 1985). Since then, AZT, the first *Nucleoside Reverse Transcriptase Inhibitor* (NRTIs), has been in use in antiretroviral therapy and is still frequently used developing countries today, twenty-one years after its approval and with up to twenty-five other drugs available in the fight against HIV infection. In fact, for years it was the only antiretroviral drug to combat infection and to increase life expectancy for AIDS patients. However, later it was demonstrated that monotherapy with AZT was inefficient in acting against some of the pathological effects of AIDS, and, more importantly, in some cases caused high toxicity and abundant side effects (Hamilton et al., 1992; Volberding et al., 1995).

Between 1991 and 1994, other NRTIs – such as didanosine (ddI), zalcitabine (ddC) and stavudine (d4T) – were added to ART as alternative drugs to AZT (*Figure 5*). In 1995 lamivudine (3TC), today one of the most commonly used NRTIs, was approved. With the approval of these drugs the success of anti-HIV therapy was expected to increase but, simultaneously, the questions of which drug and what dose would be most effective for each patient remained to be answered. At that time, after ten to fifteen years of peaking HIV infections in USA, mortality had grown considerably. Concern about the spread of HIV at the social, clinical and scientific levels, and the need for a more effective antiretroviral therapy increased. With the drugs available at that time, in 1996 the efficacy of treatment with two analogues was proven to be higher than with monotherapy (Hammer et al., 1996; Hammer et al., 1997).

Meanwhile, and thanks to better knowledge of the molecular structure of HIV, the *protease inhibitors* (PIs) appeared on the ART scene. PIs interfere in the catalytic action of HIV protease, inhibiting the formation of new virions. The **first approved PI** was **saquinavir (SQV)** followed by ritonavir (RTV) and indinavir (INV). Between 1995 and 1998, the addition of a PI to treatment with NRTIs was demonstrated to improve the efficacy of ART, increase life expectancy and prolong the survival of AIDS patients (Palella et al., 1998). This finding represented a radical change in the ARV drug therapy guidelines and a revolution in HIV treatment. This novel pharmacological guideline, based on the combined treatment with NRTIs and PIs, was called *Highly Active Antiretroviral Therapy* (HAART).

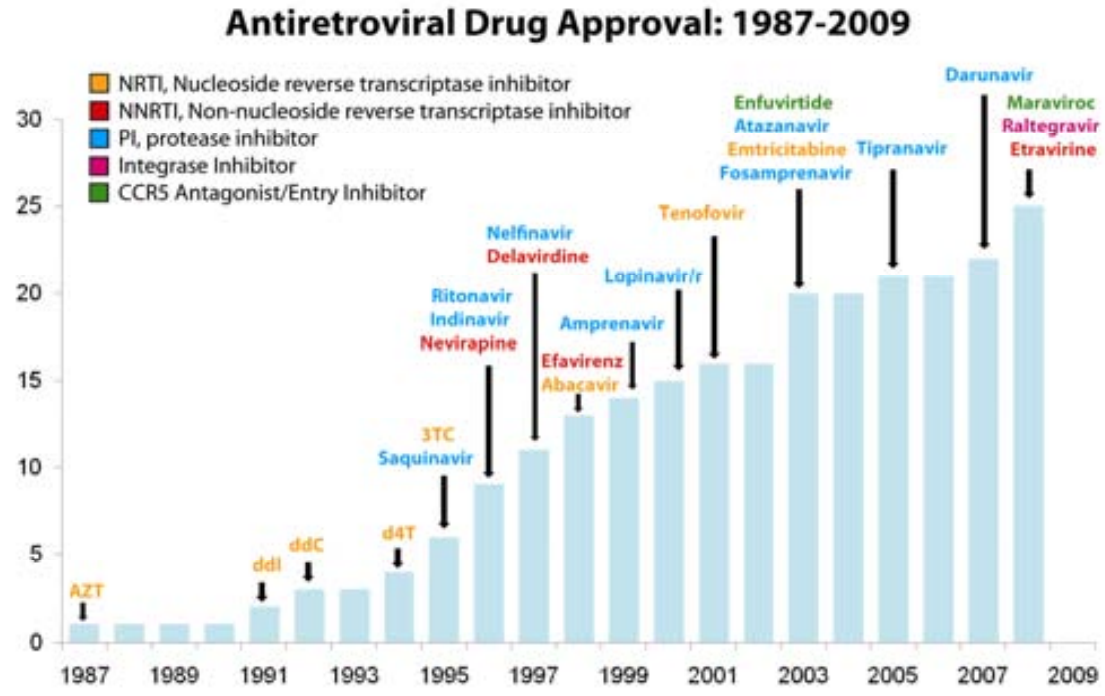


Figure 5. Antiretroviral drug approval from 1987 to 2009. Blue bars indicate the number of anti-HIV drugs available each year. The different anti-HIV drug families are indicated in different colours: yellow for NRTIs, red for NNRTIs, blue for PIs, pink for Integrase Inhibitors and green for CCR5 and Entry Inhibitors. Of note, today, twenty-five years after the discovery of AIDS, there are twenty-five drugs available to combat HIV. Adapted from (Back, 2009).

The intense pharmacological research, simultaneous to the growing knowledge of the molecular mechanisms involved in HIV infection, led to the appearance of new chemically distinct antiretroviral drugs. The first two classes of compounds that could be

categorised as *Non-Nucleoside Reverse Transcriptase Inhibitors* (NNRTIs) were the HEPT and TIBO derivatives (Baba et al., 1989; Pauwels et al., 1990). It was not until 1996 that **nevirapine (NVP)**, the **first NNRTI targeting HIV-1,** was approved (De Clercq, 1996). These drugs acted against RT (like NRTIs) but differed from NRTIs in their chemical structure and mechanism of action. Today, nevirapine (NVP) and efavirenz (EFZ) are the NNRTIs most frequently used in HAART.

Some years later, in 2003 the first fusion inhibitor, enfurtivide (also known as T-20) was approved in the USA and Europe. This drug spelt a revolution in HIV treatment because it was the first compound to act outside the cells, impeding HIV entry to cells by blocking the membrane's fusion process. Later on, in April 2007 the CCR5 antagonist maraviroc, from a new anti-HIV drug class of co-receptor inhibitors, was approved by the FDA for treatment of drug-experienced patients. Finally, two new types of anti-HIV drugs have appeared in ARV therapy: integrase and maturation inhibitors. The FDA has not approved any drug from the latter family and it may be some time before this happens.

The **use of ARV therapy** to control HIV replication has **changed the face of AIDS disease in developed countries**. Regarding disease progression, mortality and morbidity, relatively good results have been achieved. ARV therapy has simultaneously brought new discoveries in pathogenesis and in the cellular and viral dynamics of HIV infection. However, there remain **many obstacles to overcome**. The treatment is not suppressing viral replication in all patients and the emergence of resistant viruses represents a worrying problem, forcing a change in drug combinations in HAART. Moreover, chronic therapy usually results in a certain toxicity for various organs. Furthermore, the persistence of integrated virus in the genome of quiescent cells and in tissues as isolated compartments called "reservoirs", is also a new challenge (Montaner et al., 2006). The existence of these new challenges and threats force the field of HIV-related pharmacology to work hard, all the more so since a preventive vaccine is still so far from becoming a reality. The main aim of the present thesis is to study the drug uptake mechanisms of NRTIs in immune cells, in order to try to relate this mechanism with cellular resistance, drug–drug interactions and toxicity mechanisms.

2. Antiretroviral Drug Families

2.2.1. Reverse Transcriptase (RT) inhibitors

2.2.2.1. Nucleoside Reverse Transcriptase Inhibitors (NRTIs)

Being the first antiretroviral drugs class discovered and approved for HIV treatment, NRTIs are dideoxynucleosides (generally, 2',3'-dideoxynucleosides) that act like alternative substrates for the RT enzyme, competing directly with physiological nucleosides (2'-deoxynucleosides) for the active polymerase site. To act properly they **need to efficiently enter the cells and be phosphorylized** in the cytoplasm by cellular kinases. The kinases involved will vary depending on the nucleoside of which they are analogues. Therefore, as an example, 2',3'-dideoxycytidine (ddC) is phosphorylized in the first step by the deoxycytidine kinase (dCyd kinase), then by the deoxycytidine-monophosphate kinase (dCMP kinase) and finally, in a common step for all NRTIs, 5'-nucleoside diphosphate kinase (NDP kinase) acts, giving the triphosphate form (*Figure 6*). The resulting triphosphate dideoxy-nucleosides (ddNTPs) inhibit proviral DNA replication when incorporated by RT into the newly synthesized viral DNA strand: as they lack the hydroxyl group (-OH) at 3' position of the ribose ring, the phosphodiester bond necessary for DNA polymerization cannot be formed.

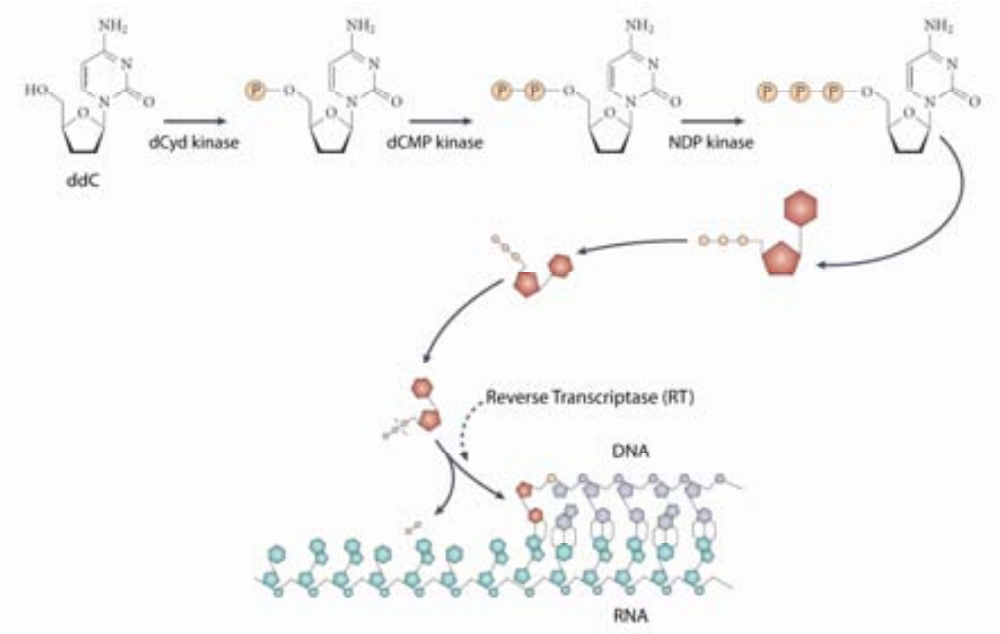


Figure 6. Mechanism of action of Nucleoside Reverse Transcriptase Inhibitors (NRTIs). The scheme shows the intracellular phosphorylation steps of zalcitabine (2',3'-dideoxycytidine; ddC) and its subsequent incorporation by RT into a newly synthesized DNA strand to stop the polymerisation due to the lack of the 3'-hydroxyl in the ribose ring. Modified from (De Clercq, 2007).

This family of drugs are the *protagonists* in the this thesis' *story*, which will explore the nature of the drug transporters involved in the uptake of some of the most important NRTIs currently used in clinical practice.

2.2.2.1.1. NRTIs classification and characteristics

NRTIs are usually classified depending on the nucleoside of which they are analogues (*Figure 7*). Thus, we distinguish:

Thymidine (T) analogues

Azidothymidine (3'-azido-2',3'-dideoxythimidine; **AZT**) and **stavudine** (2',3'-didehydro-3'-deoxythymidine; **d4T**) are thymidine analogues. Since they compete for the same nucleobases and share thymidine kinase 1/2 (TK1, TK2) and thymidilate kinase intracellular phosphorylation steps, their co-administration is not recommended..

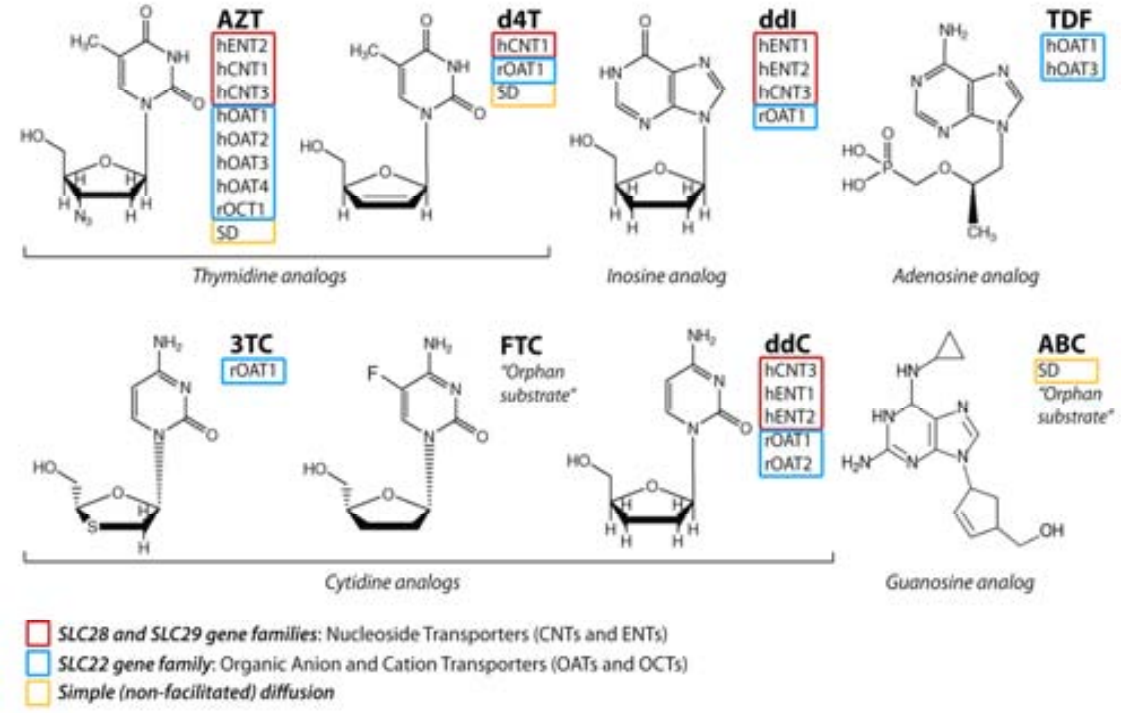


Figure 7. Chemical structures of NRTIs used in HIV therapy and the *putative* transporters from *Solute Carrier (SLC)* families involved in their uptake. NRTIs are classified by the nucleoside or the nucleobase from which they are derived. Membrane transporters that have been described in the literature as being involved in NRTI uptake are indicated and grouped in families (in red, Nucleoside Transporters from the *SLC28* and *SLC29* gene families; in light blue, Organic Cation and Anion Transporters from the *SLC22* gene family). Note that simple diffusion (in yellow, *SD*) mechanisms have also been described for AZT, d4T and ABC. Adapted from (Pastor-Anglada et al., 2005).

For AZT, the first phosphorylation step is mediated principally by TK1, though TK2 may be important in those tissues or cell lines where there is no active cell division (Arner et al., 1992). We have already mentioned that AZT was the first drug used in HIV therapy and that since its approval it has been widely used to combat AIDS. d4T is not used in HAART frequently, as the drug has many side effects and risk factors associated with it, such as lactic acidemia, pancreatitis, lipodystrophy and peripheral neuropathy (Thomas and Segal, 1998; Price et al., 2006). Interestingly, AZT is much more lipophylic than the rest of the NRTIs due to the presence of an azide group in 3' of the ribose ring (see *Table 1*, page 26). This chemically relevant feature will be more extensively detailed in the *Anti-HIV Drug Uptake Mechanism* section.

Cytidine (C) analogues

Lamivudine ((-)β-L-2',3'-Dideoxy-3'-thiacytidine; **3TC**), **emtricitabine** ((-)β-L-2',3'-Dideoxy-3'-thia-5'-fluorocytidine; **FTC**) and **zalcitabine** (2',3'-dideoxycytidine; **ddC**) are cytidine analogues and are not usually co-administered. Among these cytidine analogues, ddC is not usually present in HAART regimens today due to its high toxicity and its low effectiveness, even though it has a potent anti-HIV activity (Anderson et al., 2004). 3TC and FTC are the only NRTIs used in anti-HIV therapy that are β-L-nucleosides, conformations not present in endogenous nucleosides. Both drugs, with identical structures except for a fluorine at the nitrogen base, have very similar mechanisms, but FTC has been shown to be between four to ten times more active than 3TC in some cell types (Hazen and Lanier, 2003). Both drugs are currently being widely used in HAART regimens in the following co-formulations: Kivexa® from *GlaxoSmithKline* (co-formulation of **3TC** and ABC), Truvada® from *Gilead Sciences* (co-formulation of **FTC** and TDF), Combivir® and Trizivir® from *GlaxoSmithKline* (AZT/**3TC** and AZT/ABC/**3TC** co-formulations, respectively) and Atripla® from *Bristol-Myers Squibb* and *Gilead Sciences* (consisting of **FTC**/TDF/EFZ). The lack of antagonism between ABC, 3TC and TDF against wild-type and drug-resistant HIV-1 has been demonstrated in peripheral blood mononuclear cells and MT-4 cells *in vitro* (Lanier et al., 2005).

Guanosine (G) analogue

Another drug still widely used is the guanosine analogue **abacavir** (**ABC**; [[(1S, 4R)-4-[2-amino-6-(cyclopropylamino)purin-9-yl]-cyclopent-2-enyl]methanolsulfate), especially when combined with 3TC (Kivexa®) or AZT and 3TC (Trizivir®). It presents a

different phosphorylation pattern than the rest of the NRTIs. The first step towards its final conversion into its active metabolite, carbovir triphosphate, is phosphorylation by adenosine phophotransferase. Then a cytosolic enzyme, adenosine monophosphate deaminase, acts to convert the ABC monophosphate to carbovir monophosphate, which will be double phosphorylized to the final carbovir triphosphate firstly by guanylate kinase and secondly by the common nucleoside diphosphate kinase. Contrary to the rest of the NRTIs, this sequential enzymatic phosphorylation pathway does not present a limiting step.

Inosine (I) analogue

Didanosine (2'3'-dideoxyinosine; **ddI**) is the only structural inosine analogue in the NRTI family. Originally, one of the main concerns was its poor solubility at low pH values. As stated for d4T and ddC, it is not currently being used due to its high toxicity (associated mainly to lactic acidemia, steatosis, pancreatitis and peripheral neuropathy) (Carr and Cooper, 2000; Anderson et al., 2004).

Adenosine (A) analogue

Tenofovir disoproxil fumarate (**TDF**) is the only nucleotide analogue (NtRTI) approved by the FDA which acts against HIV RT, although it is commonly classified in the NRTI group. TDF is a prodrug of **tenofovir** that acts as an analogue of the nucleotide adenosine 5'-monophosphate and similar to NRTIs, behaves as a "false substrate" for RT. As it already has phosphorylation in its structure, it only requires two phosphorylation steps in the cell cytoplasm. It is a highly active drug and presents a high **bioavailability**, hence it is administered once a day. It is a highly tolerable drug and its resistance profile is favourable. It is considered a very "attractive" drug in clinics and it is, in fact, one of the most commonly used today, especially in co-formulations with FTC (Truvada®) and FTC/EFZ (Atripla®). Nevertheless, nephrotoxicity mechanisms and tubular abnormalities have been associated with its use (Izzedine et al., 2006; Labarga et al., 2009) although researchers from *Gilead Sciences* laboratories insist in demonstrating a low probability of nephrotoxicity due to uptake and extrusion mechanisms at the renal epithelial level (Cihlar et al., 2001; Ray et al., 2006; Ray and Cihlar, 2007). Importantly, the combination of protease inhibitors (PIs) with TDF seems not to affect the NtRTI clearance (Cihlar et al., 2007). New nucleotide and nucleoside analogues are under investigation and some are at advanced phases in clinical trials. Some examples are amdoxovir (AMDX) in full Phase II

efficacy studies and apricitabine (AVX754), elvucitabine (ACH126), fosalvudine, racivir and dioxolane thyimidine (DOT) currently under Phase I or I/II (Martinez-Picado, personal communication).

2.2.1.2. Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)

The compounds from this family of RT inhibitors are agents with a non-nucleoside derived structure that act through a non-competitive mechanism: they couple allosterically to a hydrophobic and asymmetrical cavity 10 Å, far from the RT polymerase catalytic centre. The junction involves a conformational change to the cavity formed by the residues Asp110, Asp185 and Asp186, inhibiting the polymerisation process. There are four NNRTIs approved by the FDA: efavirenz (EFZ), nevirapine (NVP), delavirdine (DLV) and etravirine (ETV) (*Figure 8*).

As the NNRTI binding site is near the substrate (dNTP) binding site, NNRTIs are assumed to interfere with the active (catalytic) site, thus disturbing the normal functioning of the RT. The amino acids with which the NNRTIs interact within the NNRTI binding pocket may be prone to mutate, and this has proven to be the case for the amino acid residues lysine at position 103 (K103N) and tyrosine at position 181 (Y181C), among others. Moreover, it has been described that only one of the mentioned mutations in the RT gene can confer resistance to all or almost all NNRTIs (Domaoal and Demeter, 2004; De Clercq, 2009).

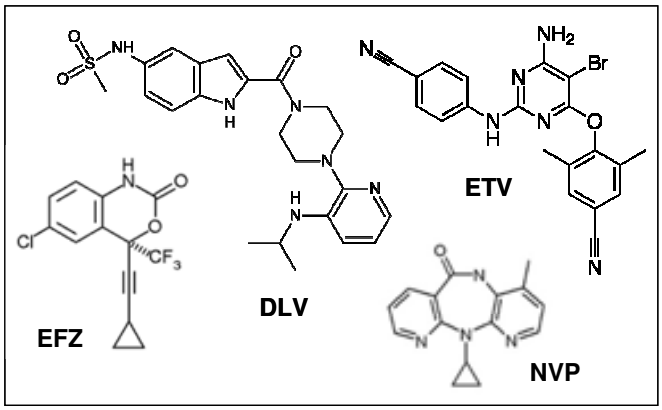


Figure 8. Chemical structures of NNRTIs. Efavirenz (EFZ), nevirapine (NVP), delavirdinde (DLV) and etravirine (ETV) are depicted.

Importantly, around 2004 a second generation of NNRTIs was developed to solve the problems related with resistant mutations in commercial NNRTIs. The approach consisted of maximizing the interactions with the carbonate bone and with the most conserved residues from the RT (such as W229) (Pelemans et al., 2000). Two drugs

designed following this principle are capravirine and ETV (also known as TMC125). Unfortunately, early Phase II studies failed to demonstrate any statistically significant advantages associated with the use of capravirine in people who had tried and failed other NNRTIs in the past, and the development of the drug was halted by its manufacturer in June 2005. On the other hand, ETV, having had its efficacy and safety in treatment-experienced HIV-1-infected patients demonstrated (Lazzarin et al., 2007; Madruga et al., 2007), was the first drug to be approved by the FDA in 2008.

2.2.4. Protease Inhibitors (PIs)

HIV genome has three principal genes: *gag*, *pol* and *env*. The product resulting from *gag* is a polyprotein of 55 kDa that contains the structural proteins of the matrix, capsid and nucleocapsid along with other peptides that are involved in the virion's assembly and the morphogenesis. The *pol* gene contains the sequences for the three viral enzymes (RT, protease -PR- and integrase -IN-) that are translated as a polyprotein complex of 160 kDa. The protease cuts these 55 and 160 kDa polyproteins to produce the corresponding functional proteins, a crucial process for the development of new virions. The protease is a non-covalent homodimer of the aspartil proteases family. Its active site is located in the dimeric interphase and contains a catalytic Asp residue from each monomer (D25 and D125) (Wlodawer and Gustchina, 2000). So far, ten protease inhibitors (PIs) have been licensed for clinical use in the treatment of HIV infections. With the exception of tipranavir, all these PIs are based on the 'peptidomimetic' principle, in that they contain a hydroxyethylene scaffold which mimics the normal peptide linkage but which cannot be cleaved itself. They thus prevent the HIV protease from carrying out its normal function, the proteolytic processing of precursor viral proteins into mature viral proteins. The ten PIs presently available for the treatment of HIV infections are saquinavir (SQV), ritonavir (RTV), indinavir (INV), nelfinavir (NFV), amprenavir (AMV), lopinavir (LPV), atazanavir (ATV), fosamprenavir, tipranavir (TPV) and darunavir (DRV).

Although all these compounds are highly active *in vitro* and present high activity in clinical trials, they have some disadvantages regarding the appearance of resistant viruses and their cross-resistance. Moreover, due to their peptidic character, some protease inhibitors present a low bioavailability, high toxicity and side effects such as lipodystrophy and the increase of cholesterol and triglycerides in the blood stream.

2.2.5. Entry Inhibitors: Fusion and Co-receptor Inhibitors

HIV-1 entry is a complex process divided into three main steps: adhesion, interaction with the co-receptor and fusion. Therefore, this mechanism is an essential stage that offers several potential new targets for antiviral agents to block these different steps of the HIV-1 entry process (Kuritzkes, 2009). So far, two entry inhibitors have been approved for HIV treatment, while some others are in clinical trials. Enfuvirtide (T-20), a fusion inhibitor, was the first entry inhibitor approved by the FDA, and in April 2007, maraviroc, the first co-receptor inhibitor (a CCR5 antagonist) was approved for the treatment of drug-experienced patients. Another, vicriviroc, is forthcoming: it might be approved for clinical use in 2009 (De Clercq, 2009; Kuritzkes, 2009). Other CCR5 antagonists have been or are currently being tested in clinical trials. The other type of co-receptor inhibitors are CXCR4 inhibitors: bicyclams were the first class of CXCR4 agents described that block HIV replication (Donzella et al., 1998), but different compounds have been developed since then. Some were stopped at some phase of the clinical trials because of toxicity issues, others are under Phase III clinical trials, waiting to be approved soon (De Clercq, 2009; Kuritzkes, 2009). Importantly, both types of drugs act extracellularly by inhibiting the entry of HIV particles into host cells, so even though they are important in HIV treatment in developed countries and their use is very promising, they are not relevant to this thesis.

2.2.6. The Newest Anti-HIV Inhibitors: Integrase and Maturation Inhibitors

Although viral integrase has been pursued for many years as a potential target for the development of new anti-HIV compounds, the first integrase inhibitor (INI) licensed for clinical use, raltegravir (RAL), has only recently been approved by the FDA (October 2007). HIV integrase has essentially two important catalytic functions: 3'-processing and strand transfer (Hicks and Gulick, 2009). Raltegravir is targeted at the strand transfer reaction, as is elvitegravir, which is at present still in clinical (Phase III) development. Elvitegravir is intended for once-daily oral dosing, whilst raltegravir has to be administered twice daily. The latter has proven highly effective in reducing viral loads in HIV infected patients (Grinsztejn et al., 2007).

Mature inhibitors act through the specific blocking of the final rate-limiting step in *Gag* processing, thus preventing the release of mature capsid protein from its precursor, resulting in the production of immature, non-infectious virus particles. Bevirimat, the

most promising maturation inhibitor, along with other drugs, is still undergoing clinical trials, although none have been approved so far (Adamson et al., 2006; Smith et al., 2007).

2.3. Viral Resistance and Toxicity of Anti-HIV Drugs

Morbidity and mortality due to infection with HIV have been considerably reduced since the introduction of HAART. However, this is often limited by the emergence of drug-resistant HIV-1 strains. HIV infection is characterized by a high replication rate (Coffin, 1995) and along with the extremely error-prone process of reverse transcription, numerous mutations occur during this step. Such mutations generate a **genetically diverse population** within an infected person that can lead to the selection of viruses that replicate better in a drug-containing environment. For this reason, when treatment is not able to totally inhibit viral replication, a **resistant viral population** can emerge. Recently, resistant viral mutations and their consequences on viral fitness have been extensively discussed (Martinez-Picado and Martinez, 2008). The drug-resistance emergence, along with the latency of HIV and the **presence of viral reservoirs** (where the drugs cannot achieve their optimal concentrations) (Stevenson, 2003; Levy, 2009), renders current treatments unable to eradicate the virus from infected individuals.

No drug is without toxicity. When antiretroviral drugs were first introduced, risks and toxicities were tolerated in the face of an imminently life-threatening disease. With prolonged chronic therapy and the disappearance of the common symptoms of underlying HIV disease, the adverse complications of antiretroviral drugs are being identified and characterized. Today it is well known that all the anti-HIV compounds **present long-term toxicity** and **adverse effects** (Carr, 2003), which in some cases lower the adherence to treatment, leading to a suboptimal concentration of the compounds and a subsequent therapy failure and the development of viral resistance.

Although NRTIs and NtRTIs are capable of diminishing viral RNA levels efficiently, systemic and mitochondrial toxicity, along with the emergence of resistant HIV strains, are inconveniences usually associated with the antiretroviral therapy. In addition, prolonged use of NRTIs has been associated with adverse effects like lactic acidosis, polyneuropathy, pancreatitis and lipoatrophy, which usually result in changes to the drug regimen (Anderson et al., 2004). On the one hand, due to their widespread use in HAART – especially of the combinations 3TC plus ABC (Kivexa®) and FTC plus TDF (Truvada®) – the basic study of the mechanisms associated with NRTI mitochondrial toxicity, drug–drug interactions and resistance are of great interest. On the other hand, the challenge of

finding new nucleoside or nucleotide analogues with low toxicity and without cross-resistance is still open (Balzarini et al., 2003; Barral et al., 2003; Cihlar et al., 2008).

2.4. Anti-HIV Drug Uptake Mechanisms and Cellular Resistance

Drug efficacy represents an interplay between multiple processes that regulate drug availability (pharmacokinetics) and response (pharmacodynamics) (Ross, 2001; Wilkinson, 2001). For orally administered drugs, their pharmacologic action is dependent on adequate intestinal absorption and distribution to sites of action, before their elimination by metabolic and excretory pathways. From the beginning of HIV therapy, most studies have focused on viral resistance mechanisms (Martinez-Picado and Martinez, 2008) while others have focused on the contribution of drug-metabolizing enzymes to the drug disposition process (Rotger et al., 2007; Arab-Alameddine et al., 2009). However, over the past decade, it has become increasingly apparent that **transporters** – carrier-mediated processes – also **play critical roles in** the overall **disposition** of numerous **drugs in antiviral and anticancer** treatments (Pastor-Anglada et al., 2004; Izzedine et al., 2005; Pastor-Anglada et al., 2005; Koepsell et al., 2007; Zhang et al., 2007).

Regarding the general transport processes across biological membranes, the two main transport types must be distinguished: **passive** and **active transport** (*Figure 9*). Passive transport requires no energy from the cell and move solutes or ions across a membrane according to their transmembrane concentration gradient. In this group we find **(1) simple/non-facilitated diffusion**, which represents free movement across a cell membrane or movement across a channel and **(2) carrier-mediated/facilitated diffusion**, which involves a protein carrier (*Figure 9a*). Importantly, the simple diffusion is a non-regulated and non-inhibitable process – the more concentration of solute there is at one side of the membrane, the more solute diffuses and crosses the cell membrane. On the one hand, some gases (O_2, CO_2, N_2), small uncharged polar molecules (water, ethanol, urea etc.) and small and highly lipophilic molecules can freely diffuse through the cell membrane without the need for a carrier. On the other hand, sugars, amino acids and other charged molecules (anions and cations) need channels or carriers to cross the lipid bilayer. This carrier-mediated process – or facilitated diffusion – is a saturable process, meaning that it reaches a maximum rate of transport at a determined concentration of substrate (*Figure 9b*). Of the transporters that we are going to investigate in the course of this thesis, *Equilibrative Nucleoside Transporters*, *Organic Cation Transporters* and some *Organic Anion Transporters* from the *SLC29* and *SLC22* gene families are the

carriers that mediate the facilitated diffusion of nucleosides, nucleoside-derived drugs and cationic and anionic compounds, and thus correspond to the third depicted type of proteins (*Figure 8a*).

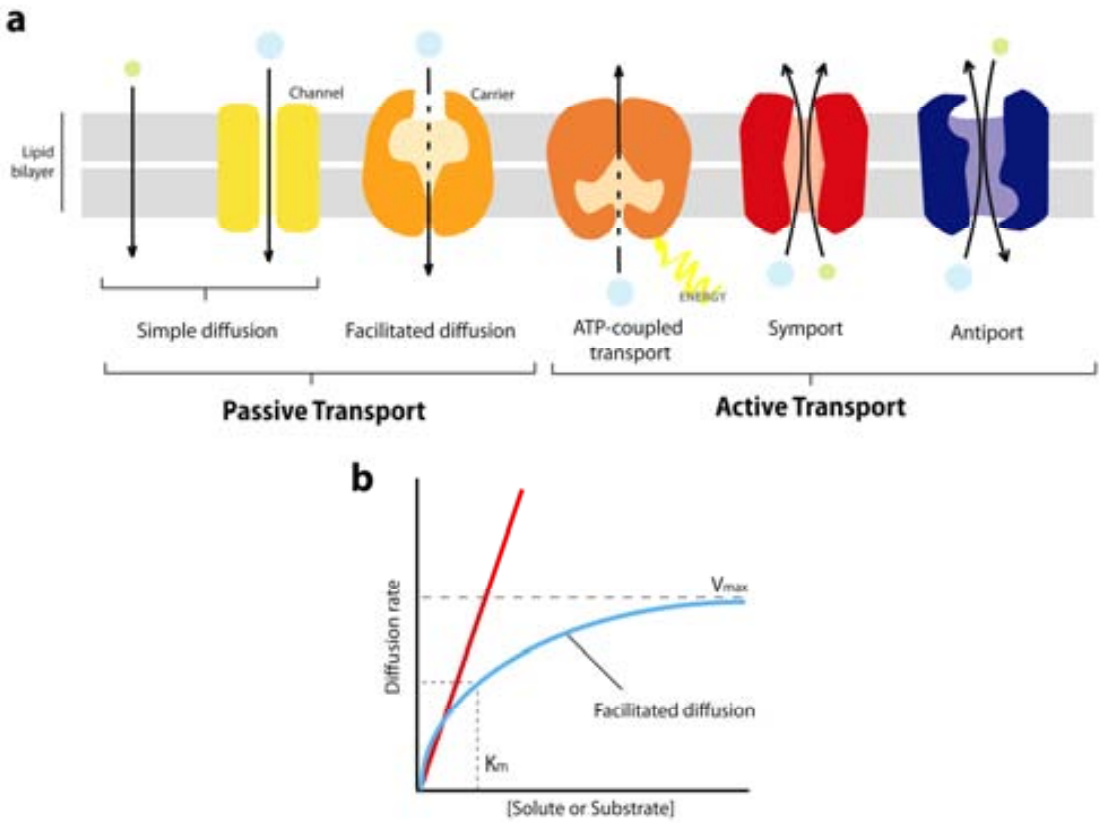


Figure 9. Transport processes across cell membranes. *(a)* Transport process types across biplasma membranes are divided between passive and active transport groups. In the former we distinguish between simple (non-facilitated) and carrier-mediated (facilitated) diffusion, and in the latter between ATP-coupled transport and symporter/antiporters. *(b)* Chart of the rate of diffusion *versus* the concentration of the solute – or named substrate, for a carrier – in simple (non-facilitated) and carrier-mediated (facilitated) diffusion processes. Adapted from Harper's Illustrated Biochemistry. 26*th Edition,* 2003.

In contrast to passive transport, active transport requires the expenditure of energy in the form of ATP (commonly called *primary active transport*) or by coupling the anti/co-transport (*secondary active transport*) of ions or other molecules and moving solutes across the membrane against their concentration gradient. Therefore, in that group we distinguish the **(3) ATP-coupled carrier mechanisms** (e.g. P-glycoprotein or Multidrug Resistance Proteins) and the **(4) symport or antiport mechanisms** (e.g. a Na^+/Ca^{2+} exchanger). All these transport types are simplified and depicted in *Figure 9b*.

Regarding the transporter families of interest, both *Concentrative Nucleoside Transporters* (co-transporting Na^+) and some members of *Organic Anion Transporters* (co-transporting α-ketoglutarate) form part of this latter group of secondary active transporters.

A reductionist view of the crucial cellular steps that could result in cellular resistance to NRTIs – and intracellularly active anti-HIV drugs – is shown in *Figure 10*. Those steps are: (1) **drug (NRTI) cellular uptake**; (2) **drug intracellular metabolism** (activation by phosphorylation) and (3) **nucleic acid incorporation** into DNA strands newly synthesized by the RT. With relation to the first step, the main focus of this thesis, it is known that the reduced entry or increased efflux of anti-HIV drugs could compromise intracellular drug levels, thus favouring the emergence of resistant viruses (Fridland et al., 2000; Turriziani and Antonelli, 2004).

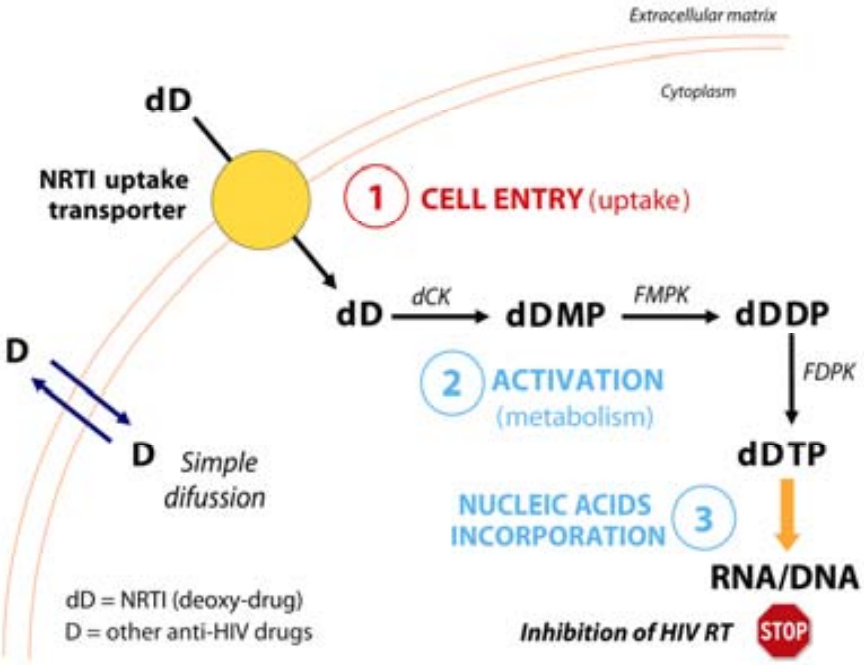


Figure 10. Possible mechanisms involved in cellular resistance to NRTIs in the activation pathway developing their pharmacological action. The first step of the activation pathway is the *cell entry (1)* of the NRTI (deoxy-drug; *dD*) into the cell cytoplasm through a membrane transporter. After this, NRTIs must be *activated (2)* by three enzymatic steps of phosphorylation with some limiting steps. Finally, deoxy-triphophated drugs (dDTP) must be *incorporated into the DNA (3)* strand undergoing synthesis by HIV RT. A reduced entry, a slow metabolism or an inappropriate incorporation of dDTP into the DNA would result in cellular resistance to the NRTI, and could indirectly generate viral resistance by permitting virus to replicate more inside the cell. Scheme adapted from (Galmarini et al., 2001).

Almost all NRTIs, such as natural nucleosides, are hydrophilic drugs that need to cross lipid bilayers through a carrier-mediated transport; otherwise their entry would be tremendously slow and its subsequent pharmacological action would often be inefficient. Therefore, knowledge of the entry mechanisms that NRTIs use to cross cell membranes is crucial to answering questions of cellular resistance, drug–drug interactions, drug toxicity and pharmacokinetics. Although drug uptake mechanisms have been studied for some NRTIs, much remains unknown, as the controversy about which transporters or which mechanisms are involved in NRTIs cell entry continues in some cases (Pastor-Anglada et al., 2008; Young et al., 2008). In that respect, although nucleoside analogues share a hydrophilic character and a low capacity for non-facilitated diffusion, some authors have described non-mediated entry mechanisms for NRTIs such as ABC, d4T and AZT or the antiviral acyclovir (Zimmerman et al., 1987; Mahony et al., 1988; Mahony et al., 2004).

An important physicochemical characteristic to pay attention to when exploring NRTI entry mechanisms is the **partition coefficient (P_{app})**. This is a measure of the differential solubility of a compound in two immiscible solvents. The most commonly used solvent system is composed of the organic solvent 1-octanol (or *n*-octanol) and water (or an aqueous solution). Thus, the partition coefficient is the **descriptor of lipophilicity** for neutral compounds or, where the compound exists in a single form (for instance, a fixed physiological; e.g. pH = 7.4). To simplify, we can state that compounds with $P_{app} \leq 1$ would have a low lipophilicity, where $P_{app} = 1$ would be low to moderately lipophilic and $P_{app} >> 2$, highly lipophilic. In *Table 1* lipophilicity values, expressed as the *n-octanol/water partition coefficient,* are shown. Values were obtained directly by transforming Log*P* values found throughout the literature. Log*P* values represent the logarithm at base 10 of "1-octanol to water" partition coefficient ($\text{Log}P = \log_{10} P_{app}$) and are predicted values obtained using bioinformatic tools. Regarding NRTIs, one of the important features that affects the lipophilicity value is the functional group that replaces the 3'-hydroxyl of the ribose. Therefore, for instance, AZT, with an azido group at the 3' position is one of the most lipophilic of all NRTIs together with ABC ($P_{app} >> 5$), whereas 3TC, ddI or ddC, with a sulphur atom or a hydrogen at the same position, are much more hydrophilic ($P_{app} < 0.5$).

Many protein members from the *Solute Carrier* (SLC) superfamily of transporters mentioned above have been described as possible transporters involved in the uptake of NRTIs in different tissues (see *Figure 6*), but it is not clear which are expressed and active in immune cells and which are crucial *in vivo* (Pastor-Anglada et al., 2005). Moreover, their specific repercussions on antiretroviral drug pharmacokinetics and

pharmacodynamics have not been extensively clarified. Fortunately, the amazing increase in pharmacogenetic knowledge is helping us understand inter-individual differences in drug response with relation to not only to drug transporters and drug-metabolizing enzymes (Lubomirov et al., 2007; Telenti and Zanger, 2008) but also to hypersensitivity reactions and metabolic complications (Phillips and Mallal, 2007; Tarr and Telenti, 2007).

Nucleoside-derived drug	P_{app} (n-octanol/water)	Reference(s)
Thymidine analogs		
AZT	0.98 - 1.26[a]	[1], [2], [3]
d4T	0.18[a]	[1]
Cytidine analogs		
3TC	0.11[a]	[4]
FTC	0.37[b]	[5]
ddC	0.05[a,b]	[6], [7]
Guanosine analog		
ABC	6.6[a] - 15.8[b]	[8], [9]
Inosine analog		
ddI	0.07[a]	[10]
Adenosine analog		
TDF (prodrug of PMPA)	17.8[b]	[5]
Tenofovir (PMPA)	0.0134[a]	[11]
Other antiviral nucleoside-derived drugs		
Acyclovir (ACV)	0.018-0.022[b]	[12], [13], [14]
Ganciclovir (GCV)	0.027[b]	[15], [16]
Carbovir (CBV)	0.30[a]	[17]

Notes. References: [1] (August et al., 1991); [2] (Zimmerman et al., 1987); [3] (Collins and Unadkat, 1989); [4] (Gibbs et al., 2003); [5] *Gilead Sciences*, product information sheet; [6] (Shojaei et al., 1999); [7] (Gibbs and Thomas, 2002); [8] (Thomas et al., 2001); [9] (Mahony et al., 2004); [10] (Ahluwalia et al., 1987); [11] (Anthonypillai et al., 2006); [12] (De Miranda et al., 1981); [13] (De Miranda et al., 1982); [14] (Dias et al., 2002a); [15] (Tirucherai et al., 2002); [16] (Dias et al., 2002b) ; [17] (Anderson and Taphouse, 1981).

Table 1. Partition coefficient values for NRTIs and other nucleoside-derived antiviral drugs. [a] Values taken directly from the references mentioned; [b] P_{app} values are mathematically converted values from Log*P* values found in the literature. As stated in the text and to summarize: compounds with $P_{app} \leq 1$ are low lipophilic (hydrophilic), with $P_{app} = 1$, low-moderately lipophilic and $P_{app} >> 2$, highly lipophilic.

Some authors have also focused their studies on the differences in pharmacological features between active and resting immune cells. Thus, for instance, it has been described that cells treated with phytohemagglutinin or granulocyte-macrophage colony-stimulating factor generated 2 to >150-fold higher triphosphate concentrations of ddC, 3TC, d4T, AZT and ddI than resting cells *in vitro* (Perno et al., 1992; Gao et al., 1993; Gao et al., 1994; Robbins et al., 1998; Robbins et al., 2003). These differences in concentrations of NRTI

triphophates in active and resting cells suggest a mediated uptake mechanism, as non-facilitated diffusion through membranes cannot be regulated.

Finally, it is important to remark here that only a **few studies relating NRTI uptake in immune cells and/or T lymphocytes** (the main targets for HIV-1) had been published at the commencement of this thesis project. The need for further investigation in that direction and the possibility of initiating some pharmacogenomic approaches stimulated our research. Nevertheless, unexpectedly, what at first seemed to be a simple approach, resulted in a more complex pharmacological issue that led us to a more membrane biology-related study.

3. Antiviral Drug Transporters

Membrane transporters play pivotal roles in sustaining the normal life of cells. The ***Solute Carrier* (*SLC*) superfamily** in humans constitutes a large series of membrane transporters currently consisting of 362 putatively functional protein-coding genes in 55 gene families (Hediger et al., 2004; He et al., 2009). Transporters from the *SLC* superfamily share at least 20–25% amino acid sequence identity and are thought to have evolved from a common ancestor. Whereas it is generally anticipated that transporters from the same gene family possess similar functional properties, several *SLC* families appear to consist of members with great functional diversity (Saier, 2000; Wright et al., 2004). From this vast group of transporter-based gene families with huge functional diversity, only a few gene families have so far been related to antiviral or anticancer drug uptake. Those, extensively reviewed in the literature, are depicted in *Figure 11* (Pastor-Anglada et al., 2004; Izzedine et al., 2005; Pastor-Anglada et al., 2005; Koepsell et al., 2007; Zhang et al., 2007).

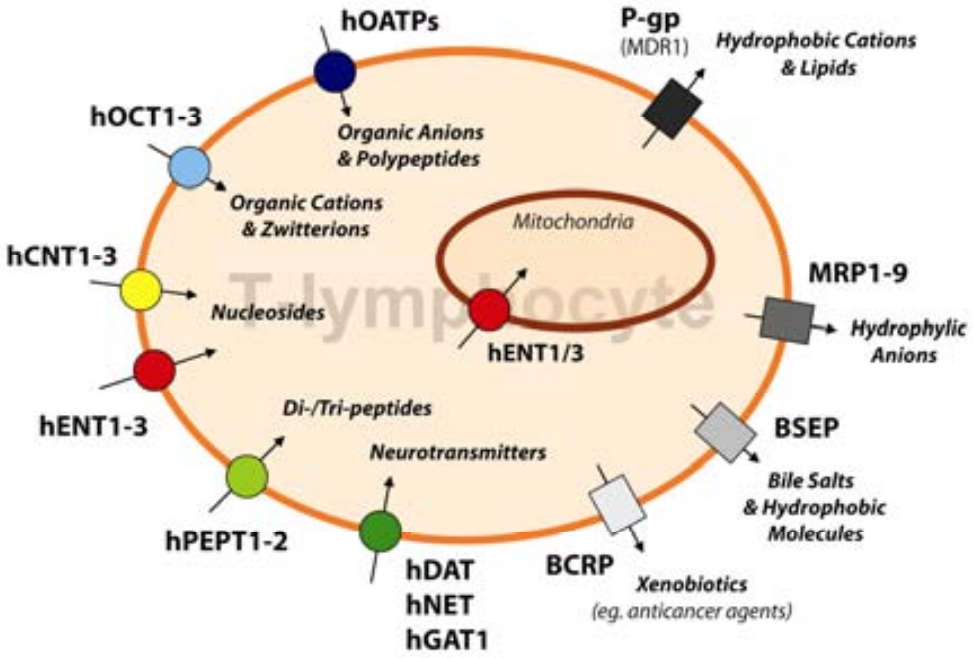


Figure 11. *The usual suspects* in drug uptake and efflux mechanisms from the *SLC* (coloured circles) **and the *ABC*** (grey squares) **superfamilies and their common substrates.** Transporters are grouped by transporter family (e.g. hOCT1–3 belong to the *SLC22* family; hCNT1–3 belong to the *SLC28* family; hENT1–3 belong to the *SLC29* family). Physiological substrates for each family of transporters are listed. The direction of the common transport action is indicated by an arrow pointing into the cell (influx) or out of the cell (efflux). Adapted from (Leabman et al., 2003).

To be concrete, the most important **families related to antiviral drug uptake** in the literature are ***SLC28, SLC29, SLC22*** and some members from the ***SLC15*** and ***SLCO*** gene families. Here, based on these previous studies and on the chemical structures of the NRTIs – the main anti-HIV drugs of interest to us – we review the functional and structural characteristics of the *usual suspects* involved in NRTI uptake, starting with Nucleoside Transporters, our first targets.

3.1. Nucleoside Transporters: the *SLC28* and *SLC29* gene families

Nucleosides play an important role as metabolic precursors in nucleic acid synthesis, being recycled through *salvage* pathways, and are crucial to the control of cell and tissue growth (Pastor-Anglada et al., 2008). Importantly, many nucleoside analogues are currently used in anticancer and antiviral therapies, as previously explained for HIV in the *Antiretroviral drugs and their targets* section, thus highlighting the pharmacological role that these molecules can play in disease. Nucleosides and nucleoside-derived drugs are hydrophilic molecules and diffuse very slowly across cell membranes. Therefore, to facilitate their uptake, specific membrane proteins mediate their translocation from the extracellular milieu into the cytoplasm.

Studies performed decades ago described up to seven systems that could be responsible for nucleoside transport in mammalian cells (Griffith and Jarvis, 1996). Among them, two mechanisms of nucleoside transport have been identified: the equilibrative, bi-directional facilitators and the concentrative, inwardly directed Na^+/nucleoside co-transporters (Griffith and Jarvis, 1996). The proteins responsible for these processes belong to two structurally unrelated protein families: the **Concentrative Nucleoside Transporter** family (**CNT**, encoded by *SLC28* gene family) and the **Equilibrative Nucleoside Transporter** family (**ENT**, encoded by the *SLC29* gene family). While ENTs are present in most, possibly all, cell types, CNTs are found in intestinal and renal epithelial cells – and other specialized cells – where they function in tandem with ENTs during the absorption, distribution and elimination of nucleosides and nucleoside drugs. There are seven known nucleoside transport proteins in human cells (hCNT1/2/3 and hENT1/2/3/4), with corresponding orthologues present in other mammalian species (Pastor-Anglada et al., 2008; Young et al., 2008).

3.1.1. Concentrative Nucleoside Transporters (hCNT): the *SLC28* gene family

3.1.1.1. Human family members and their functional characteristics

Human Concentrative Nucleoside Transporter (hCNT1) was the first transporter isolated from the kidneys by hybridization cloning and was found to be 83% identical to the rat CNT1 orthologue in amino acid sequence (Ritzel et al., 1997). In epithelia, CNT1 is localized to the apical membrane and works in concert with equilibrative nucleoside transporters (ENTs), which are localized predominantly in the basolateral membranes of these tissues to mediate transepithelial nucleoside flux (Mangravite et al., 2001; Lai et al., 2002; Mangravite et al., 2003).

In the late 90s, two human isoforms of *CNT2 (hCNT2)* were isolated at the same time (Wang et al., 1997; Ritzel et al., 1998). The amino acid sequence was identical except for a polymorphism at residue 75 (Arg substituted by Ser). At the protein level, hCNT2 was found to be 83% and 72% identical to rat CNT2 (rCNT2) and hCNT1, respectively. hCNT2 is present in cells from the kidneys, liver, heart, brain, placenta, pancreas, skeleton, muscle, colon, rectum, duodenum, jejunum and ileum (Wang et al., 1997; Pennycooke et al., 2001).

Human CNT3 (hCNT3) was cloned from human mammary gland tissue and differentiated human myeloid HL-60 and it showed 79% identity at the protein level with its mouse orthologue (Ritzel et al., 2001b). However, the homology between hCNT3 and hCNT1/2 was only around 50% and unlike either hCNT1 or hCNT2, hCNT3 was shown to be capable of coupling transport to protons. Moreover, the hCNT3 gene mapped to chromosome 9q22.2 (Ritzel et al., 2001a; Ritzel et al., 2001b). High levels of hCNT3 mRNA transcripts are found in the pancreas, trachea, bone marrow and mammary gland (Ritzel et al., 2001b).

3.1.1.2. Substrate selectivity

CNT substrate selectivity has been determined basically by combining the following techniques: (1) labelled substrate flux measurements (2) cross-inhibition studies and (3) electrophysiology. In order to analyze substrate selectivity and the pharmacological profiles of CNT-mediated substrate translocation, electrophysiology has been very helpful in the absence of putative labelled substrate molecules. This technique takes advantage of the currents induced by the mandatory Na^+-coupled transport found in all CNT isoforms,

which can be measured by a two-electrode voltage clamp. The experiments are usually performed in *Xenopus laevis* oocytes expressing a concrete CNT isoform.

Human CNTs show high affinity for their substrates, with apparent K_m values in the low micromolar range (10–100 μM) and more restricted selectivity than equilibrative transporters (*Table 2*) Despite all hCNT orthologues accepting uridine as a permeant, they differ functionally with respect to their selectivities for other permeants. **hCNT1 is a pyrimidine nucleoside-preferring transporter**, and although adenosine is able to bind with high affinity (K_d = 14 μM), it is not translocated (Larrayoz et al., 2004). **hCNT2**, on the other hand, **is a purine nucleoside-preferring transporter**, though it also transports uridine. Finally, **hCNT3 is a broadly selective transporter** and translocates both purine and pyrimidine nucleosides (Ritzel et al., 1997; Wang and Giacomini, 1997; Ritzel et al., 1998; Ritzel et al., 2001b; Smith et al., 2004). The Na^+/nucleoside stoichiometry of the CNT-mediated translocation process varies between transporters: human CNT1 and CNT2 are thought to show a 1:1 (Na^+: nucleoside) stoichiometry (Ritzel et al., 1998; Smith et al., 2004), whereas hCNT3 employs a 2:1 (Na^+:nucleoside) coupling ratio (Ritzel et al., 2001b), although a similar stoichiometry has also been proposed for hCNT1 (Larrayoz et al., 2004).

	hCNT1	**hCNT2**	**hCNT3**
Substrate	K_m (μM)	K_m (μM)	K_m (μM)
Uridine	22 - 37[a,b]	40 - 116[a,b]	1.1 - 21.6[a,b]
Cytidine	3.1[a], 29[b], 120[a]	-	3.5 - 15.4[a,b]
Thymidine	26[b]	-	3.7 - 21.2[a,b]
Inosine	-	4.5-13[a,b]	4.3[a], 52.5[b]
Guanosine	-	-	5.1 - 43[a,b]
Adenosine	-	8[b]	2.4 - 15[a,b]
References	[6], [11-12], [4-5]	[6-8], [9,10]	[1-3]

[a] Values for K_m in cell line system

[b] Values for K_m in *Xenopus laevis* oocytes system

Notes. References: [1] (Toan et al., 2003); [2] (Errasti-Murugarren et al., 2008); [3] (Ritzel et al., 2001a); [4] (Smith et al., 2004); [5] (Larrayoz et al., 2004); [6] (Lang et al., 2004); [7] (Shin et al., 2003); [8] (Schaner et al., 1999); [9] (Ritzel et al., 1998); [10] (Wang et al., 1997); [11] (Graham et al., 2000); [12] (Cano-Soldado et al., 2004).

Table 2. Substrate specificity of human Concentrative Nucleoside Transporters (hCNTs). K_m values found in cultured cell systems or in Xenopus laevis oocytes systems are shown. Of note, hCNT1 preferentially transports pyrimidine nucleosides, hCNT2 prefers purine nucleosides whereas hCNT3 is a broadly selective transporter that takes up both purinic and pyrimidinic nucleosides.

Moreover, hCNT3 can also act to translocate other ions, such as Li^+ or H^+, showing a 1:1 (H^+:nucleoside) stoichiometry when using H^+ alone (Ritzel et al., 2001b).

3.1.1.3. Physiological implications

Human Concentrative Nucleoside Transporters (hCNTs) are thought to play critical roles in nucleoside homeostasis: at the organism level they maintain systemic blood levels of nucleosides through absorption and elimination mechanisms; at the cellular level they mediate the influx of extracellular nucleosides into cells through the *salvage pathway*. Although most cells are capable of *de novo* synthesis of nucleosides, the ***salvage pathway*** is favoured due to its **lower energy requirements** (Gray et al., 2004). Nucleoside transporters (NTs) expressed in intestinal and renal epithelial cells are differentially distributed at the plasma membrane, often with CNTs at the apical membrane and ENTs at the basolateral domain (Mangravite et al., 2001; Mangravite et al., 2003; Errasti-Murugarren et al., 2007). The perfect coupling between these two membrane compartments allows cells to perform active absorption/re-absorption or even active extrusion of nucleoside and nucleoside-derived drugs (Lai et al., 2002; Errasti-Murugarren et al., 2007). This putative role of NTs in absorption and re-absorption of nucleosides is supported by their localization along both the intestinal tract and the renal tubule (Rodriguez-Mulero et al., 2005). The expression of CNTs and ENTs at the apical and basolateral poles of epithelial cells, respectively, supports vectorial absorption and reabsorption of nucleosides and their derivatives, thus contributing to whole body nucleoside homeostasis, and probably to nucleoside-derived drug pharmacokinetics. Finally, another important role for CNTs is the high affinity Na^+-coupled adenosine transport they mediate in the central nervous system, as adenosine is known to be a neuromodulator implicated in the circadian clock and dark-adaptive processes in the retina (Huang et al., 2005; Ribelayga and Mangel, 2005). Of NTs, CNT2 shows the highest affinity for adenosine (Wang et al., 1997; Ritzel et al., 1998) (*Table 2*). Therefore, particular CNT-type transporters can act as putative "transceptors", as their ability to translocate adenosine triggers intracellular signals relevant to cell physiology.

3.1.1.4. Structure–function relationships

All CNTs share a general topology based on thirteen putative transmembrane domains (TMs), with proteins length of 650, 659 and 691 amino acids for hCNT1, hCNT2 and hCNT3, respectively. The thirteen TMs' topologies would be consistent with the N-terminus tail facing the cytosol, and an extracellular C-terminus domain (*Figure 12)*.

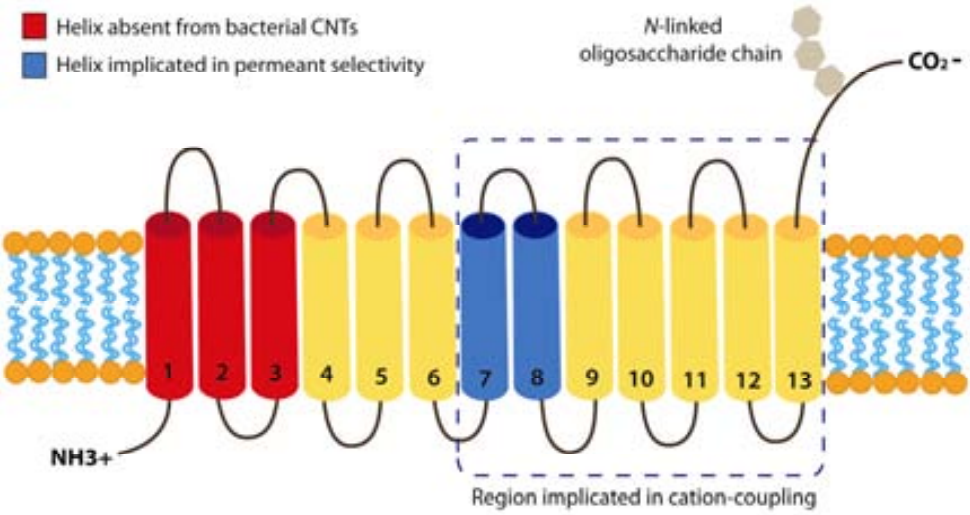


Figure 12. Predicted transmembrane topology of mammalian members of CNT family. The locations of *N*-linked glycosylation sites in CNT1–3 are shown. TM regions implicated in nucleoside inhibitor recognition by site-directed mutagenesis experiments or characterization of chimaeras, are also indicated. The first three putative TM regions of CNT proteins from eukaryotes are absent from their eubacterial counterparts and are not essential for transport activity. Adapted from (King et al., 2006a).

The membrane orientation of the C and N termini was confirmed using antibodies and immunocytochemistry, and by studying the glycosylation sites at the N-site for CNT1 (Hamilton et al., 2001). CNT1 and 2 have two N-glycosylation sites in the extracellular C-terminus. The hCNT3 isoform has also been demonstrated to be N-glycosylated (Toan et al. 2003). Moreover, conformational studies, GFP-fused chimeras and site-directed mutagenesis analysis of rat and human orthologues have provided a great deal of information on which amino acids are crucial for substrate recognition and for the trafficking and functional activity of the transporter proteins, as reviewed in (Pastor-Anglada et al., 2008).

Substrate recognition requirements studies have been another important issue in the study of the structure–function of CNTs. Using structure-inhibitory analysis we have learnt that hCNT1 and hCNT2 binding sites differ in their interaction with uridine and adenosine analogues and that hCNT1 exhibits higher affinity for the uridine ones (Patil et al., 2000; Lang et al., 2001; Lang et al., 2004). Pharmacophore models were generated using computational studies, molecular modelling, structure building and previous data on hCNT1 and hCNT2 inhibitors. These models suggested that steric and electrostatic interactions play an important role in hCNT1 inhibition and that hydrogen bonding is

important in hCNT2 inhibition (Chang et al., 2004). hCNT1 and 2 showed much more sensitivity to modifications in the base and in the sugar moiety than hCNT3 (Zhang et al., 2003; Zhang et al., 2005a). As *Figure 13* shows, when studying the *cis*-inhibition of [^{3}H]uridine uptake in the presence of increasing concentrations of cytidine and two cytidine analogues, we could clearly demonstrate that the 3'-hydroxyl group at the ribose ring was crucial for substrate recognition. When using 2',3'-dideoxycytidine (ddC), the K_i value was decreased by three orders of magnitude (from approximately 10 to 10,000 μM) whereas using 2'-deoxycytidine (without a hydroxyl group at the 2' position of the ribose) the K_i value experienced no change with respect to cytidine. Importantly, we were the first to show that the ribose C(3')-OH appears to be crucial for the recognition of substrates and inhibitors by the CNTs (Cano-Soldado et al., 2004). Computational approaches performed by other authors have supported this finding (Zhang et al., 2003; Cano-Soldado et al., 2004; Chang et al., 2004; Zhang et al., 2005a). All these findings suggest the view that slight changes in substrate structure can provoke a dramatic shift in selectivity.

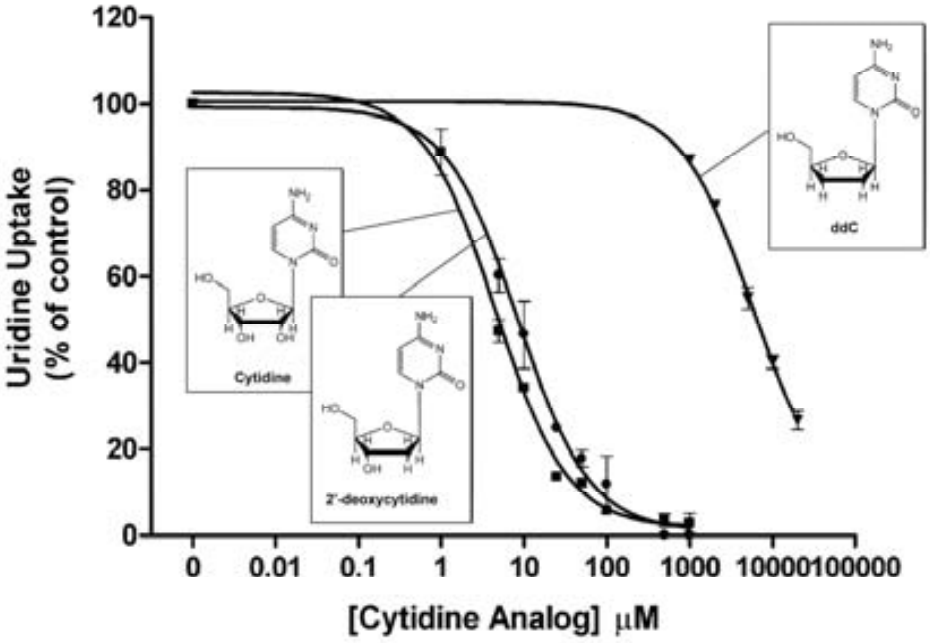


Figure 13. Structural determinants of CNT1 substrates. As an example of how slight structural modifications in a nucleoside determine its transportability by a specific transporter protein, we show the cross-inhibition of CNT1-mediated uridine uptake by three closely related pyrimidine nucleosides. Recombinant CNT1 expressed in CHO-K1 cells can be inhibited by cytidine (■) with a K_i value in the low micromolar range. The loss of the hydroxyl group at the 2' position does not significantly affect the interaction with the transporter protein, as demonstrated by the inhibition of CNT1-mediated uridine transport triggered by 2'-deoxycytidine (●). Nevertheless, when ddC (2',3'-dideoxycytidine) is used as the inhibitor (▼), the interaction with the transporter is dramatically reduced.

3.1.1.6. CNTs pharmacological profiles

Like natural nucleosides, therapeutic nucleoside analogues follow similar uptake and metabolic processes: they are incorporated into newly synthesized DNA and/or RNA, resulting in chain termination (see *Figure 6,* page 14) and, in the case of anticancer drugs, leading to cell apoptosis (Galmarini et al., 2001). Different CNT subtypes possess overlapping and variable substrate selectivity for nucleoside-derived anticancer and antiviral drugs (Pastor-Anglada et al., 2005; King et al., 2006a; Pastor-Anglada et al., 2008) (*Table 3,* next page). With relation to anti-HIV NRTIs, it has been described that hCNT1 transports azidothymidine (AZT), whereas stavudine (d4T) and zalcitabine (ddC) are very low affinity substrates of the transporter. hCNT2 efficiently transports the anti-HCV ribavirin (K_m = 18 μM) and the anti-HIV didanosine (ddI) (Lang et al., 2004). Human CNT3 exhibits a broader range of permeant selectivities for various therapeutic nucleoside analogues: it transports both pyrimidine (AZT; K_m = 310 μM) and purine (ribavirin; K_m = 14 μM) nucleoside derivatives efficiently and even nucleobase derivatives used in cancer, such as 6-mercaptopurine or 6-thioguanine (Fotoohi et al., 2006; Hu et al., 2006). Although poorly transported, ddC and ddI seem to be substrates for hCNT3 (Ritzel et al., 2001b). Importantly, AZT and ribavirin are readily transferred in a Transwell model expressing CNT3 (Errasti-Murugarren et al., 2007). hCNT3 could be of key importance in their pharmacokinetics as neither drug is easily transported by hCNT1 (Cano-Soldado et al., 2004).

Drug	Substrate	hCNT1 K_m (μM)	hCNT2 K_m (μM)	hCNT3 K_m (μM)	References
Anti-HIV drugs					
AZT	**hCNT1/3**	**450**	-	**310**	[1-4]
d4T	**hCNT1/3**	**15600**	-	*n.d.*	[1], [4]
3TC	-	-	-	-	[5]
FTC	Unknown	-	-	-	[5]
ddC	**hCNT1/3**	*n.d.*	-	*n.d.*	[2], [6]
ddI	**hCNT2/3**	-	*n.d.*	*n.d.*	[1], [2], [6]
TDF	Unknown	-	-	-	[5]
Anticancer drugs	*Substrate*	K_m (μM)	K_m (μM)	K_m (μM)	
Cytarabine	**hCNT1/3**	*n.d.*	-	*n.d.*	[2], [7]
Gemcitabine	**hCNT1/3**	**24**	-	**59.7**	[1], [3-4], [8-9]
5'-DFUR*	**hCNT1-3**	**15.2**	*n.d.*	*n.d.*	[3], [10-11]
Fludarabine	**hCNT3**	-	-	*n.d.*	[7], [10-12]
Cladribine	**hCNT1-3**	*n.d.*	**187**	*n.d.*	[2], [12-14]
Clofarabine	**hCNT2/3**	-	**81**	**52**	[12]
Troxacitabine	*Passive non-facilitated diffusion*				[15]

n.d. = K_m not determined; in cases, K_i have been determined but as those values reflect the interaction with the transporter rather than the transporter specificity, they are not detailed here. For further information *see references*.

* 5'-DFUR = commonly used abreviation for 5'-deoxy-5-fluorouridine.

Notes. References: [1] (Hu et al., 2006); [2] (Smith et al., 2004) ; [3] (Errasti-Murugarren et al., 2007) ; [4] (Cano-Soldado et al., 2004) ; [5] (Pastor-Anglada et al., 2005); [6] (Ritzel et al., 2001a); [7] (Errasti-Murugarren et al., 2008); [8] (Mackey et al., 1999); [9] (Clarke et al., 2006); [10] (Lang et al., 2001); [11] (Mata et al., 2001); [12] (King et al., 2006b); [13] (Owen et al., 2006); [14] (Smith et al., 2004); [15] (Gourdeau et al., 2001).

Table 3. Nucleoside-derived drugs used in HIV therapy and in cancer described as substrates of human Concentrative Nucleoside Transporters (hCNTs). K_m values found in cultured cell systems or in *Xenopus laevis* oocytes systems are shown. Of note, those drugs that are substrates but the K_m of which have not been determined yet, the term n.d. (not determined) is indicated whereas those durgs that are not substrates for hCNTs are marked with a dash (-).

3.1.2. *SLC29* genes and human Equilibrative Nucleoside Transporters

3.1.2.1. Human family members and their functional characteristics

Human ENT1 (hENT1) is a 456-residue protein 78% identical in sequence to its 457-residue rat homologue and 79% identical to the 460-residue mouse protein (Griffiths et al., 1997a; Yao et al., 1997; Handa et al., 2001). Several single nucleotide polymorphisms (SNPs) resulting in non-synonymous variant hENT1 transporters have been identified (Osato et al., 2003; Kim et al., 2006). Nevertheless, those SNPs functionally characterized show normal kinetics for nucleoside and nucleoside drug (Osato et al., 2003). ENT1 is almost ubiquitously distributed in human and rodent tissues, a fact confirmed by studies at both the mRNA and protein levels. Despite this, its abundance varies between different tissues and cell types (Griffiths et al., 1997a; Handa et al., 2001). Expressing hENT1 tagged with green or yellow fluorescent protein (GFP or YFP, respectively) in Madin-Darby canine kidney cells, the transporter targeted similarly to the basolateral membrane (Lai et al., 2002; Mangravite et al., 2003). ENT1 in the kidney may operate in tandem with CNT proteins to mediate the transepithelial flux/reabsorption of nucleosides or nucleoside-derived drugs. Nevertheless, recent evidence suggests that hENT1 is not only found in the basolateral membrane but also in the apical membranes, working in association with hCNT3 (Damaraju et al., 2007). ENT1 also potentially contributes to transepithelial movement of nucleosides across other polarized cells such as intestinal, hepatic and placental cells (Barros et al., 1995; Govindarajan et al., 2007).

Human ENT2 (hENT2) is a 456-residue protein 46% identical in amino acid sequence to hENT1 and 88% to its 456-residue mouse and rat homologues (Griffiths et al., 1997b; Yao et al., 1997; Crawford et al., 1998; Kiss et al., 2000). ENT2 mRNA is expressed in a wide variety of different cell types and tissues. With relation to tissue distribution, it is remarkable that it is particularly abundant in skeletal muscle, but it is also expressed in brain, heart, placenta, thymus, pancreas, prostate and kidney cells (Griffiths et al., 1997b; Crawford et al., 1998). ENT2 seems to be mainly targeted at the basolateral membranes in human epithelial cells (Mangravite et al., 2003; Elwi et al., 2006; Damaraju et al., 2007).

Human ENT3 (hENT3) is a 475-residue protein 29% identical in sequence to hENT1 and 74% identical to its 475-residue mouse homologue (Hyde et al., 2001; Baldwin et al., 2005). The structure of hENT3 differs from ENT1 and ENT2 in possessing a very long hydrophilic N-terminal region preceding the first transmembrane domain (TM1). hENT3 is particularly abundant in the placenta, the tissue from which the cDNA was originally cloned. Some years ago, it was demonstrated that hENT3 is predominantly

localized intracellularly, mainly in lysosomes (Baldwin et al., 2005). Nevertheless, very recently, it has been found that hENT3 localization is cell-type dependent and that it is also substantially expressed in mitochondria (Govindarajan et al., 2009).

Human hENT4, the most recently discovered hENT isoform to date, is a 530-residue protein 86% identical in sequence to its mouse homologue (mENT4) (Barnes et al., 2006). It was originally identified by genome database analysis (Acimovic and Coe, 2002) but it was not functionally characterized until 2004 (Baldwin et al., 2004; Engel et al., 2004). By gene ontology, ENT4 is the fourth isoform of the ENT family and that is the reason why it was initially designated ENT4. Multiple tissue RNA array analysis suggest that hENT4 is ubiquitously expressed in human tissues. More recent immunocytochemistry analyses revealed that the transporter is predominantly present in cardiomyocytes and endothelial cells (Barnes et al., 2006).

3.1.2.2. Substrate selectivity

Human ENTs show a low affinity for their permeants (high K_m values), usually in the 0.1–3.0 mM range (*Table 4*) (Young et al., 2008). hENTs substrate selectivity is broader than that for hCNTs. While the three CNTs orthologues differ in their selectivity for nucleosides, hENT1–3 accept all nucleosides as permeants. Concretely, hENT1 transports a wide range of purine and pyrimidine nucleosides, with K_m values ranging from 0.04 mM (adenosine) to 0.60 mM (cytidine), but is unable to transport the pyrimidine base uracil (Griffiths et al., 1997a; Ward et al., 2000). hENT2 transports a broad range of purine and pyrimidine nucleosides, although with lower apparent affinity (from 0.14 mM to 5.60 mM) than hENT1, except in the case of inosine (0.05 *versus* 0.17 mM) (Ward et al., 2000). The most remarkable difference between hENT1 and hENT2 is that the latter has the capacity to transport purine and pyrimidine nucleobases while the former does not. hENT3 has broad selectivity for both nucleosides and nucleobases but, unlike hENT2, does not transport hypoxanthine. In contrast to hENT1 and hENT2, the transport activity of hENT3 is strongly dependent upon pH, with its optimal value at 5.5, probably reflecting the location of the transporter in acidic, intracellular compartments (Baldwin et al., 2005). More recently, hENT3 has been confirmed as a mitochondrial pH-dependent transporter that is also localized in the cell membrane in certain placental cells. It has also been shown to transport both organic cations such as MPP+ or serotonin, with an optimal transport of MPP+ at pH 7.4 (Govindarajan et al., 2009).

Substrate	hENT1 K_m (μM)	hENT2 K_m (μM)	hENT3 K_m (μM)	hENT4 K_m (μM)	References
Nucleosides					
Uridine	44.1[a], 240[b], 260[c]	195[a], 200[b], 250[c]	2000[b]		[1-6]
Cytidine	234[a], 580[c]	5610[c]	-		[1-3], [5-6]
Thymidine	300[c]	710[c]	-		[1-3], [5-6]
Inosine	28.5[a], 170[c]	180[a], 50[c]	-		[1-3], [5-6]
Guanosine	47.5[a], 140[c]	2700[c]	-		[1-3], [5-6]
Adenosine	17.8[a], 40[c]	106[a], 140[c]	1900[b]	780[b]	[1-7]
Nucleobases					
Hypoxanthine	-	700[b]	-	-	[6]
Organic Cations					
MPP+	-	-	*n.d.*	33[c]	[8]
TEA	-	-	-	6600[c]	[9]
Neurotransmitters					
Serotonin	-	-	*n.d.*	114[c]-1900[b]	[7-8]
Norepinephrine	-	-	-	2600[c]	[8]
Dopamine	-	-	-	329[c]	[8]
Histamine	-	-	-	>10000[c]	[9]
Epinephrine	-	-	-	>15000[c]	[8]

Values for K_m found in [a]*Saccharomyces cerevisiae* system, [b]*Xenopus laevis* oocytes system, [c]Cell culture system. *n.d.* = *Not determined*. In principle, those compounds with an *n.d.* are putative substrates described in the literature but the K_m has not been determined yet.

Notes. References: [1] (Visser et al., 2005) ; [2] (Griffiths et al., 1997a) ; [3] (Ward et al., 2003) ; [4] (Baldwin et al., 2004); [5] (Griffiths et al., 1997b); [6] (Yao et al., 2002) ; [7] (Barnes et al., 2006) ; [8] (Engel et al., 2004) ; [9] (Engel and Wang, 2005).

Table 4. Substrate specificity of human Equilibrative Nucleoside Transporters (hENTs). K_m values found in *Saccharomyces cerevisiae*[a], in *Xenopus laevis* oocytes system[b] or cell culture system[c] are shown. Of note, uridine and adenosine are common substrates for all hENTs. hENT1 and hENT2 are preferentially nucleoside transporters with the latter being also a good nucleobase transporter. hENT3 and hENT4 can also transport organic cations (such as MPP+) and neurotransmitters (serotonin, norepinephrine, or dopamine) although in some cases the K_m has not been determined yet (in that cases, an *n.d.= not determined* is shown).

However, ENT4 does not significantly interact with the majority of nucleosides or structurally related compounds. In fact, it was demonstrated that the transporter protein, renamed Plasma Membrane Monoamine Transporter (PMAT), functions as a polyspecific organic cation transporter that efficiently transports classic OCT substrates (TEA^+, MPP^+ or guanidine) and neurotransmitters, thus being closer to OCTs or Neurotransmitter Transporters than to ENTs in substrate selectivity (Engel et al., 2004; Engel and Wang, 2005). It has also been shown that at acidic pH, hENT4 (or PMAT) would act as an adenosine-preferring transporter, whereas at physiological pH (7.4) it would be mainly translocate organic cations (Barnes et al., 2006).

3.1.2.3. ENT inhibitors

Prior to the identification of their genes, the ENTs were classified on the basis of their sensitivity to inhibition by the drug nitrobenzylthioinosine (NBTI), as equilibrative NBTI-sensitive or equilibrative NBTI-insensitive (Baldwin et al., 1999). Thus, with regard to ENTs inhibitors, the main difference between hENT1 and hENT2 is that the former is inhibited by NBTI at very low concentrations (K_i = 0.4 nM) whereas the latter needs much higher concentrations to be inhibited (K_i = 2.8 μM) *(Table 5)*.

		hENT1	**hENT2**	**hENT3**	**hENT4**	*References*
Drug	*Inh. transporter(s)*		K_i (nM)			
Inhibitor						
NBTI*	**hENT1 -** hENT2	0.4	2800	-	-	[1], [2]
Dipyridamole	**hENT1/2 -** hENT3/4	5	360	>5000	>5000	[1], [3-6]
Dilazep	**hENT2 -** hENT3/4	-	>5000	>5000	>5000	[1], [3-6]
Draflazine	**hENT2**	-	*n.d.*	-	-	[1]

n.d. = K_m not determined; in cases, K_i have been determined but as those values reflect the interaction with the transporter rather than the transporter specificity, they are not detailed here. For further information *see references*.

* NBTI = commonly used abreviation for Nitrobenzylthioinosine

Notes. References: [1] (Griffiths et al., 1997a); [2] (Griffiths et al., 1997b); [3] (Visser et al., 2002); [4] (Baldwin et al., 2004); [5] (Engel et al., 2004); [6] (Barnes et al., 2006).

Table 5. Inhibitors of human Equilibrative Nucleoside Transporters. Commonly used inhibitors of hENTs and their K_i values are shown. NBTI inhibits with a really high-affinity the transporter hENT1 (in bold) as well as it does dipyridamole (DIP) for both hENT1 and hENT2 and with less potency hENT3 and 4 (not in bold). Dilazep and draflazine (coronary vasodilators as DIP) mainly inhibit hENT2, with the former also inhibiting hENT3 and hENT4 but with much less potency.

Moreover, it is well known that the coronary vasodilator drugs dipyridamole (DIP), dilazep and draflazine inhibit hENT1–3 with different potency. Dipyridamole, the most commonly used inhibitor *in vitro*, shows a K_i of 5 nM for hENT1 and one of 360 nM for hENT2. In comparison with hENT1, the hENT3 and hENT4 proteins are much less sensitive to inhibition by NBTI and coronary vasodilator drugs, but the latter is inhibited by OCTs inhibitors (such as D-22) (Engel et al., 2004; Baldwin et al., 2005; Barnes et al., 2006). All these structurally diverse inhibitors interact with ENT1/2 through a common mechanism involving high-affinity binding to overlapping sites within or closely adjacent to the outward-facing nucleoside recognition site of the transporter.

3.1.2.4. Physiological implications

In those cells deficient in *de novo* nucleoside biosynthetic pathways, hENTs play an important role in the provision of nucleosides and nucleobases derived from diet or produced by tissues such as liver for *salvage* pathways of nucleotide synthesis. In mammals, the former cells include erythrocytes, leukocytes, bone marrow cells and some cells in the brain (Murray, 1971; Griffith and Jarvis, 1996). As stated previously, only hENT2 transports nucleobases. Thus, the coexistence in many cell types of both hENT1 and hENT2, with similar nucleoside specificities, may reflect the importance of hENT2 to the transport of nucleobases for the *salvage* pathway. Moreover, there is a direct relationship between adenosine responses and ENTs. Processes regulated by adenosine include coronary blood flow, the myocardial O_2 supply–demand balance, inflammation and neurotransmission (Shryock and Belardinelli, 1997).

Finally, it is not well known why so many cells and tissues display multiple nucleoside and nucleobase transporters with overlapping selectivities and how this multiplicity of function influences the pharmacology of nucleoside drugs. All human ENTs transport adenosine, although with different affinities, and all ENTs (except hENT4) transport uridine (*Table 4*). It seems clear that the genetic variation in both ENTs and CNTs is very low compared with other transporter proteins of the *SLC* superfamily (e.g. Organic Cation Transporters). This fact suggests that genes from the *SLC29* and *SLC28* families are under high selective pressure and therefore are individually important (Osato et al., 2003; Kim et al., 2006; Owen et al., 2006).

3.1.2.4. Structure–function relationships

Ever since the discovery of hENT1, an eleven transmembrane (TM) domain architecture was proposed. Some years later, the use of newer and more sophisticated

techniques – such as the use of antibodies in combination with glycosylation scanning mutagenesis – confirmed the topology proposed and contributed to clarifying that hENTs have a cytoplasmatic N-terminus and an extracellular C-terminus (*Figure 14*) (Sundaram et al., 1998; Sundaram et al., 2001). With respect to glycosylation sites, it has been described that hENT1 is N-glycosylated at a single site and that hENT2 has two sites in the large extracellular loop that links TM1 and TM2, although glycosylation does not seem to be essential for activity or plasma membrane targeting (Vickers et al., 1999; Ward et al., 2003). Similarly, hENT3 and mENT3 bear a glycosylation site in the TM1–2 loop, and hENT4 and mENT4 locate it in the C-terminal tail (Barnes et al., 2006).

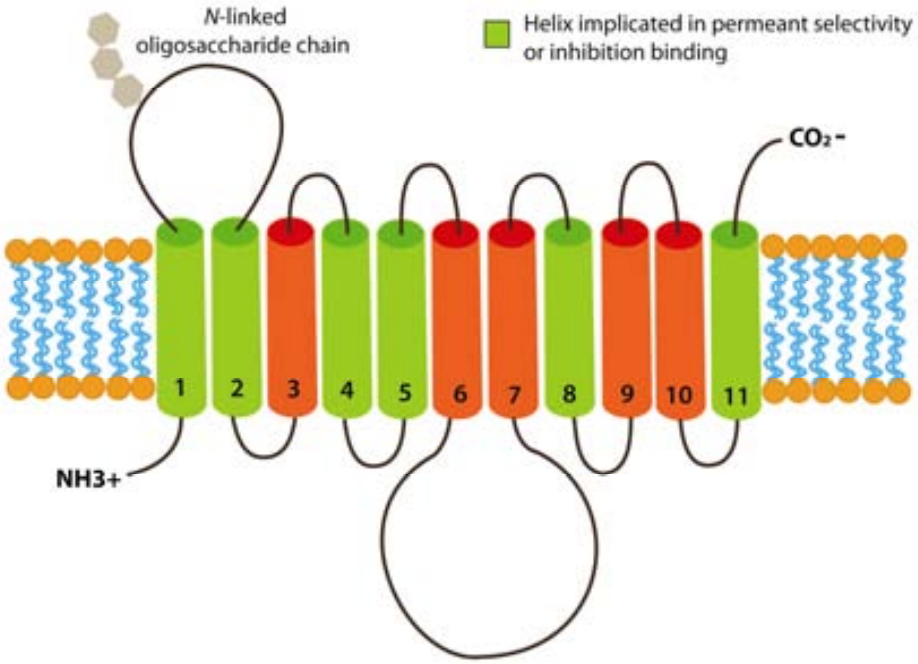


Figure 14. Predicted transmembrane topologies of mammalian members of the ENT family. The location of *N*-linked glycosylation is shown. TM domains implicated in nucleoside selectivity or inhibitor binding in ENT1–4 found by site-directed mutagenesis experiments or characterization of chimaeras are also indicated. Adapted from (King et al., 2006a).

Chimaera constructs produced between different species (normally human and rat) have also thrown considerable light on the transporter regions involved with permeants and the interaction with inhibitors. Such studies have revealed that domain swaps within the amino–terminal halves of hENT1 and rENT1 identified residues 100–231 (incorporating TMs 3–6) of hENT1 as the major sites of coronary vasodilators and NBTI interaction (Sundaram et al., 1998; Sundaram et al., 2001). Later on, TMs in hENT2, possibly involved in nucleobase and nucleoside derived drug recognition, were identified:

TM1–6 appeared to be responsible for the transport of 3'-deoxynucleosides (Yao et al., 2001), while TM5–6 were implicated in nucleobase acceptance and transportability (Yao et al., 2002).

Point mutation studies have always been revealing. Thus, the mutagenesis of specific amino acids have helped us understand which transporter structure residues are crucial for substrate or inhibitor binding and which are important for trafficking and anchoring to the plasma membrane. In reciprocal studies involving hENT1 and hENT2, position 33 in TM1 has been demonstrated to be crucial for the binding of both nucleosides and the coronary vasodilators inhibitors (Visser et al., 2002; Visser et al., 2005b). Further mutagenesis studies have revealed that Leu442 of hENT1 has an important specific role on dipyridamole sensitivity (Visser et al., 2005a). Extensive mutagenesis studies have also identified residues in TM2 (M89 and L92), TM4 (G154, S160) (Endres et al., 2004; SenGupta and Unadkat, 2004; Endres and Unadkat, 2005) and TM5 (G179) (SenGupta et al., 2002) involved in permeant translocation and/or NBTI binding. Furthermore, residue G154 in TM4 seems to be especially important: substituting residue G154 of hENT1 with serine in hENT2 converts hENT1 to a transporter that exhibits partial characteristics of hENT2, losing NBTI sensitivity. Importantly, this conversion in hENT1 implies less sensitive inhibition by anti-HIV azidothymidine (AZT), dideoxyinosine (ddI) and the nucleobase hypoxanthine (SenGupta and Unadkat, 2004). In TM8, hENT1 residues F334 and N338 also influence coronary vasodilator interactions, but are also important determinants of protein folding and catalytic turnover (Visser et al., 2007). To summarize, multiple transmembrane regions seem to contribute to ENT function, with TM5 and TM8 being the regions with the greatest concentration of operationally important residues.

3.1.2.6. ENTs pharmacological profiles

Antiviral nucleoside analogues seem not to be good substrates of ENT1 or CNT1/2, although the implication of nucleoside transporters in anti-HIV drug bioavailability remains unclear (Pastor-Anglada et al., 2005; Varatharajan and Thomas, 2009). hENT1 has been described as poorly transporting the antiviral nucleosides 2',3'-dideoxycytidine (ddC) and ddI, and as not transporting AZT (*Table 6*). The low efficiency of transport of nucleoside anti-HIV drugs, all of them lacking a 3'-hydroxyl group (*see Figure 7, page 14* and *Figure 13, page 33*), indicates the importance of this position for substrate recognition and transportability in relation not only to ENT1 but also to CNT1 and 2 (Yao et al., 2001; Zhang et al., 2003; Cano-Soldado et al., 2004; Chang et al., 2004; Vickers et al., 2004;

Zhang et al., 2005a). Remarkably, anticancer nucleoside analogues, with a 3'-hydroxyl group in their structure are generally well tolerated and robustly transported.

		hENT1	hENT2	hENT3	References
Drug	*Transporter(s)*				
Anti-HIV drugs			K_m (mM)		
AZT	**hENT2/3**	-	*n.d.*	*n.d.*	[1], [10]
d4T	**hENT3**	-	-	*n.d.*	[11]
3TC	**hENT3**	-	-	*n.d.*	[11]
ddC	hENT1 - **hENT2/3**	*n.d.*	>7.5	*n.d.*	[1], [2], [10]
ddI	hENT1 - **hENT2/3**	*n.d.*	3	*n.d.*	[1], [2], [10]
Anticancer drugs			K_m (μM)		
Cytarabine	hENT1	*n.d.*	-	-	[3], [6]
Gemcitabine	**hENT1/2**	160	740	*n.d.*	[4], [10]
5'-DFUR*	**hENT1**	*n.d.*	-	-	[5]
Fludarabine	**hENT1/2/3**	107	*n.d.*	*n.d.*	[6], [10]
Fialuridine	**hENT1/3**	*n.d.*	-	*n.d.*	[11], [12]
Cladribine	**hENT1/2/3**	23	*n.d.*	*n.d.*	[7-8], [10]
Clofarabine	**hENT1/2**	108	328	-	[7], [9]
Other antivirals			K_m (μM)		
Ribavirin	**hENT1/3**	321 (mENT1)	-	*n.d.*	[11], [13]

n.d. = K_m not determined; in cases, K_i have been determined but as those values reflect the interaction with the transporter rather than the transporter specificity, they are not detailed here.

* 5'-DFUR = commonly used abreviation for 5'-deoxy-5-fluorouridine.

Notes. References: [1] (Yao et al., 2001) ; [2] (Vickers et al., 2004); [3] (Clarke et al., 2006); [4] (Mackey et al., 1999) ; [5] (Mackey et al., 2005) ; [6] (Zhang et al., 2007) ; [7] (King et al., 2006a); [8] (King et al., 2006b); [9] (Zhang et al., 2006a) ; [10] (Baldwin et al., 2005); [11] (Govindarajan et al., 2009); [12] (Lai et al., 2004); [13] (Endres et al., 2009).

Table 6. Nucleoside-derived drugs used in HIV therapy and in cancer, described as substrates of human Concentrative Nucleoside Transporters (hENTs). K_m values found in cultured cell systems or in *Xenopus laevis* oocytes systems are shown. Of note, those drugs that are substrates but for which the K_m has not yet been determined are marked *n.d. (= not determined)*, whereas those that are not substrates are marked with a dash (-). The K_m value for ribavirin corresponds to mouse ENT1 orthologue (mENT1).

Firstly, with relation to anti-HIV drugs transport, the main difference between hENT1 and hENT2 is that the latter can transport AZT, although it does it with low affinity. Moreover, it also exhibits a much greater capacity to transport ddC and ddI (Yao et al., 2001). Similar to hENT1, hENT2 transports a broad range of anticancer nucleoside-derived drugs, especially cladribine, fludarabine and clofarabine. Remarkably, hENT2 shows a weak ability to interact with cytosine and cytidine. The presence of the amino group at the 4 position of the base seems to be a structural feature which impedes it from being a good ENT substrate. In agreement with that, hENT2 has a lower apparent affinity

for anticancer cytidine analogues (Mackey et al., 1999). Interestingly, no interaction or transport through ENT2 for the anti-HIV cytidine analogues lamivudine (3TC) and emtricitabine (FTC) has been described to date (Varatharajan and Thomas, 2009).

With relation to hENT3, it has been described as translocating the adenosine analogues used in cancer chemotherapy and the anti-HIV NRTIs ddI, ddC and AZT (Baldwin et al., 2005). Importantly, while this thesis was being written, an significant finding came to light, obtained from relating mitochondrial antiviral and anticancer nucleoside drug import by hENT3 (Govindarajan et al., 2009). The authors demonstrated that the transporter protein is intracellularly expressed, mainly in mitochondria. Expressing an N-terminal deleted hENT3 (Δ36-hENT3), they showed pH-dependent interaction with several classes of nucleosides that produce mitochondrial toxicity (anti-HIV NRTIs 3TC, ddC, ddI, d4T and AZT, the anticancer drugs gemcitabine and fialuridine, and the anti-HCV drug, ribavirin). This expression of hENT3 in mitochondria correlates with hENT3-mediated transport of nucleoside drugs, which may play an important role in mediating mitochondrial toxicity (Govindarajan et al., 2009).

The recently identified hENT4/PMAT, as stated, mainly transports organic cations, and adenosine in acidic environments (pH 5.5). Importantly, it has been demonstrated to transport metformin, an antihyperglucemic drug widely used for the treatment of type II diabetes mellitus, although with a low apparent affinity (K_m = 1.32 mM) (Zhou et al., 2007).

3.2. Organic Anion and Cation Transporters (hOCTs and hOATs): the *SLC22* gene family

The SLC22 family is a broad gene family that comprises three families of transporters: Organic Cation Transporters (OCTs), Organic Cation/Carnitine Transporters (OCTNs) and Organic Anion Transporters (OATs). Most members of the *SLC22* family share a predicted membrane topology with twelve α-helical transmembrane domains and several common motifs. Whereas many transporters from the *SLC* superfamily are highly specialized or "oligospecific", mediating facilitated transport of essential molecules (e.g. nucleosides for the *SLC28* and *SLC29* families or glucose for the *SLC3* and *SLC5* transporters) others are more generalized and, due to their broad substrate specificity, are usually termed as "polyspecific" transporters (Koepsell et al., 2007). Accordingly, members from the *SLC22* family are polyspecific and transport multiple different substrates (in size and molecular structures). Importantly, they not only have different permeants but also

numerous other compounds which can act as inhibitors, these probably being modulators of the physiological function of the transporters (Koepsell and Endou, 2004; Koepsell et al., 2007).

3.2.1. Human Organic Cation and Cation/Carnitine Transporters (hOCT and hOCTNs)

The first subgroup of the *SLC22* family discovered comprises the Organic Cation Transporters (OCTs) subtypes from 1 to 3 (OCT1–3, gene products of *SLC22A1*, *A2* and *A3*, respectively). Transport of organic cations by any of the three OCT subtypes is: (1) electrogenic, (2) Na^+-independent and (3) bidirectional (reversible with respect to direction). The driving force is solely supplied by the electrochemical gradient of the transported organic cation. At the same time, members from the other subgroup, termed the Organic Cation/Carnitine Transporter family (OCTNs) – often included in a general OCT group – were discovered. This subgroup comprises the transporters OCTN1–2 (*SLC22A4–A5*), OCTN3 (without gene identified yet) and the hCT2 (also known as hOCT6 and hFLIPT2, gene product of SLC22A16). These transporters may function as (1) organic cation uniporters or H+/Organic cation antiporter (e.g. OCTN1) or (2) as uniporters for organic cations or Na^+/Carnitine cotransporters (e.g. OCTN2) (Koepsell, 2004; Ciarimboli, 2008). Of note, almost all members from *SLC22* family and all the generally termed *"Polyspecific Organic Cation Transporters"* share a predicted topology of twelve transmembrane domains (TM), a large hydrophilic loop between TM1 and TM2 carrying several glycosylation sites, and a multiple potential phosphorylation site on the large intracellular loop between TM6 and TM7. All of them have both intracellular N and C-termini (*Figure 17*, page 54).

3.2.1.1. Human family members and their functional characteristics

Human OCT1 (hOCT1; *SLC22A1*) was first cloned in 1997, nearly simultaneously by two groups (Gorboulev et al., 1997; Zhang et al., 1997). The gene encoded a 554-amino acid protein with a 78–80% identity with the previously cloned rat OCT1. In humans OCT1 is strongly expressed in epithelial cells and some neurons. Regarding human tissues, it is mainly expressed in the small intestine, kidney and liver concretely localized to the sinusoidal membrane of the hepatocytes (unpublished data from Lips, K.S., Kummer, W., Ciarimboli, G., Schlatter, E. and Koepsell, H.). Nevertheless, in humans and rodents it has been also detected in many other organs and/or in tumour cells and basophilic

granulocytes (Koepsell et al., 2007). The presence of hOCT1 on the luminal membrane of ciliated epithelial cells in bronchial tissue (Lips et al., 2005; Kummer et al., 2006) has also been functionally identified along with hOCT3 in the placenta (Wessler et al., 2001).

Human OCT2 (hOCT2; encoded by *SLC22A2* gene) was first cloned in 1997 by Koepsell *et al.* (Gorboulev et al., 1997). It encodes a 555-amino acid protein with 81% identity with the previously cloned rOCT2 and 68% identity with rOCT1 (Okuda et al., 1996; Gorboulev et al., 1997). Interestingly, hOCT2 has a more restricted expression pattern than OCT1 or OCT3. OCT2 is most strongly expressed in the kidney but RT PCR analyses also reveal transcription of hOCT2 in a variety of other organs, including the small intestine, lung, spleen, placenta, skin, brain and choroid plexus (Koepsell et al., 2007; Ciarimboli, 2008). In the human kidney OCT2 is expressed in all three segments of proximal tubules (Motohashi et al., 2002). By immunofluorescence analysis of human bronchi, OCT2 was mainly detected in the apical membrane of ciliated cells, and less intensely at the plasma membrane of basal cells (Lips et al., 2005).

Human OCT3 (hOCT3, also known as extraneuronal monoamine transporter, EMT; *SLC22A3*) was first cloned from Caki-1 cells, a human kidney carcinoma cell line which consists of 556 amino acids (Grundemann et al., 1998). The prototypical organic cation TEA^+ is not a substrate for hOCT3. The tissue expression pattern of this transporter is very broad. In contrast to OCT1 and 2, OCT3 is not only expressed in epithelial cells and neurons but also in muscle cells and glial cells (Grundemann et al., 1998; Koepsell, 2004; Koepsell et al., 2007). In humans the strongest expression was found in skeletal muscle, the liver, the placenta and the heart; however, OCT3 was also expressed in many other organs including the brain and also in some cancer cells (Koepsell et al., 2007). The OCT3 protein was localized at the basolateral membrane of the trophoblast in the placenta and in epithelial cells of renal proximal tubules (Sata et al., 2005), at the sinusoidal membrane of hepatocytes and at the luminal membranes of bronchial epithelial cells and small intestinal enterocytes (Lips et al., 2005; Muller et al., 2005).

Human Organic Cation/Carnitine Transporter 1 (hOCTN1; encoded by gene *SLC22A4*) was first cloned in 1997 by Tamai et al. (Tamai et al., 1997). It consists of 551 amino acids with eleven putative transmembrane segments, four N-glycosylation sites and five protein kinase C phosphorylation sites. When first cloned, it was found to be mainly expressed in the kidney, skeletal muscle, the placenta, the prostate and the heart. In humans the strongest expression has been observed in the kidney, skeletal muscle, bone marrow and the trachea (Tamai et al., 1997; Tokuhiro et al., 2003). Interestingly, in bone marrow OCTN1 mRNA was detected in $CD86^+$ macrophages, $CD34^+$ T cells and $CD14^+$

mononuclear cells (Tokuhiro et al., 2003; Peltekova et al., 2004). More recently, it has been reported that OCTN1 is intracellularly expressed in mitochondria (Lamhonwah and Tein, 2006).

Human Organic Cation/Carnitine Transporter 2 (hOCTN2; *SLC22A5*) was cloned in 1998 by two groups nearly simultaneously from a human placental trophoblast cell line (Wu et al., 1998) and from a human kidney cDNA library (Tamai et al., 1998). OCTN2 cDNA encoded a polypeptide of 557 amino acids with 75.8% similarity to OCTN1 (Tamai et al., 1998). It is expressed in epithelial cells, muscle cells, glial cells, macrophages, lymphocytes and sperm (Koepsell et al., 2007). In humans the strongest expression of the transporter was observed in kidney, liver, skeletal muscle, heart and placenta cells, although it is also expressed in several other tissues (Koepsell et al., 2007; Ciarimboli, 2008). OCTN2 is a Na^+/Carnitine cotransporter with a high affinity for carnitine, though it can function alternatively as a polyspecific and Na^+-independent organic cation uniporter.

Organic Cation/Carnitine Transporter 3 (OCTN3; *Slc22a9*) has only been cloned from mice and rats and it is mainly expressed in the testis. Nevertheless, the expression of OCTN3 has been also detected in other organs and neurons (Tamai et al., 2000b; Duran et al., 2005; Alnouti et al., 2006). In the small intestine of rat OCTN3 has been assigned to the basolateral membrane of the enterocytes (Duran et al., 2005).

Human Carnitine Transporter 2 (hCT2 or hOCT6; *SLC22A16*) is mainly expressed in the testis (Enomoto et al., 2002; Gong et al., 2002). In addition, it was detected in embryonic liver, in hematopoietic cells, in leukemias and in some cancer cell lines (Gong et al., 2002; Okabe et al., 2005). In the testis, hCT2 is located in the plasma membranes of Sertoli cells and in the luminal membrane of epithelial cells in the epididymus (Enomoto et al., 2002). It is a high-affinity carnitine transporter.

3.2.1.2. Substrate selectivity

Regarding OCT1–3, the basic transport characteristics of the three transporters are similar in various species. First, OCT1–3 translocate a variety of organic cations with widely differing molecular structures and are inhibited by a large number of non-transported compounds (*Table 7, page 49*) (Koepsell and Endou, 2004; Koepsell et al., 2007; Ciarimboli, 2008). The relative molecular mass of most compounds transported by OCT1–3 is below 500 g/mol and the smallest diameter of the molecules is below 4 A° (Schmitt and Koepsell, 2005). Second, OCTs translocate organic cations in an electrogenic manner. Nevertheless, for the human transporters, the electrogenicity of transport has only been shown for hOCT1 and hOCT2 (Gorboulev et al., 1997; Busch et al., 1998; Dresser

et al., 2000). Third, OCTs operate independently of Na^+, and are independent of proton gradients when the effect of proton gradients on the membrane potential is excluded(Gorboulev et al., 1997; Busch et al., 1998; Kekuda et al., 1998; Keller et al., 2005). Fourth, OCTs are able to translocate organic cations across the plasma membrane in either direction. In addition to cation influx, cation efflux has been demonstrated for rOCT1-3 and hOCT2 and 3 (Koepsell et al., 2007).

As stated previously, most substrates of OCTs are organic cations and weak bases that are positively charged at physiological pH. However, as our knowledge of OCT substrate specificity increases, the number of non-charged compounds that could possibly be transported by OCTs also increases. Compounds transported by the OCT family comprise (1) endogenous compounds (e.g. choline, creatinine and neurotransmitters); (2) drugs (e.g. quinine, quinidine, metformin and acyclovir); (3) xenobiotics/model substrates (e.g. TEA, MPP+) (*Table 7, next page*). The most commonly used model substrate cation in the study of drug interactions and substrate specificity of OCTs *in vitro* is the neurotoxin *N*-methyl-4-phenylpyridinium (MPP^+) because it exhibits very high uptake rates and similar K_m values for the three OCTs (*Table 7, next page*). A large variety of cations (e.g. TBuA, TPA, decynium-22), non-charged compounds and even anions inhibit OCT function but are not transported by any of the OCT subtypes. This finding has also been reviewed here for the *SLC28* and *SLC29* families, and it is a common feature in most *SLC* gene families.

Briefly, with relation to hOCTN and hCT2 substrate selectivity and specificity, it is important to mention that the main difference with OCTs is that the former are capable of transporting L-carnitine. The broad spectrum of substrates and inhibitors of hOCTNs are also shown in *Table 7*. Regarding hOCTN1, it has been recently described that it transports the zwitterionic antioxidant ergothioneine with much higher affinity than L-carnitine or TEA^+ (Grundemann et al., 2005; Grigat et al., 2007). For that reason, it has also been named Ergothioneine Transporter (ET) (Grundemann et al., 2005). Apparently, hOCTN1 employs translocation mechanisms for different substrates and is able to operate in both directions. hOCTN2 functional characterization revealed that it is a Na^+-dependent, high affinity transporter of L-carnitine, acetyl-L-carnitine and the zwitterionic β-lactam antibiotic cephaloridine; however, alternatively, it can function as a polyspecific Na^+-independent cation transporter (Tamai et al., 1998; Ohashi et al., 1999; Wu et al., 1999; Wagner et al., 2000; Ohashi et al., 2001). Na^+-dependent transport of L-carnitine by OCTN2 is electrogenic and stereospecific (Ohashi et al., 1999; Wagner et al., 2000) and

Na^+ increases the affinity for L-carnitine (Inano et al., 2004). There is evidence that hOCTN2 is a Na^+-L-carnitine co-transporter rather than a transporter and that it is activated by extracellular Na^+. OCNT3, has only been cloned from mice and at variance to OCTN2, mOCTN3 transports carnitine independently of Na^+ (Tamai et al., 2000b). Finally, hCT2 (also OCT6) is a high affinity carnitine transporter that also translocates various organic cations such as TEA^+ and doxorubicin (Enomoto et al., 2002; Okabe et al., 2005).

	hOCT1	**hOCT2**	**hOCT3**	**hOCTN1**	**hOCTN2**
Drug	K_m or (IC_{50}) [µM][a,b]				
Model Cations					
MPP+	**15, 32**	**19, 78 (24)**	**47 (54)**		(>1000)
TEA	**229 (160)**	**76 (48-270)**	**(1372)[c]**	**195-1280**	**300**
Inhibitors					
Decynium-22 (D-22)	(2.7-4-7)	(0.10-1-10)	(0.1)		
Disprocynium 24			(0.015)		
TPrA*	**(102)**	(20, 128)			
TBuA+	**(30)**	(20, 120)		(535)	
TPeA**	(1.5, 7.5)	(1.5, 11)	4.5[c]	(70)	
Endogenous substrates/inhibitors					
Metabolites					
L-Carnitine	**(12400)[c]**	**(13000)**	(5600)	**(24)**	**4.3, 4.8**
Guanidine	**(5,030)[c]**	**(2300)**	(1300, 6200)		
Neurotransmitters					
Acethylcholine	**(580)**	**117 (149)**	(10490)[c]		
Dopamine	(>20000)[c]	**390-1400**	1200[c]		
Epinephrine	(>30000)[c]	**400**	**240**		
Histamine	(>20000)[c]	**940, 1300**	**180, 220**		
Norepinephrine	(7100)[c]	**1500, 1900**	**510-2600**		
Serotonin	(>20000)[c]	**80, 290 (310)**	(1000)[c]		
Hormones / Miscellaneous					
Corticosterone	(7, 22)	(34)	(0.12, 0.29)		(<100)
Agmantine	**(24000)**	**1400 (3251)**	**2500**		
Ergothioneine[d]				**21**	
Stachydine[d]				**270**	

[a] K_m and IC_{50} values were measured in oocytes of Xenopus laevis or cell lines expressing the transporters; [b] Bold face indicates cations for which transport has been demonstrated; [c] Koepsell et al., unpublished data; [d] Compounds in the blood that are normally taken up with the food.

*TPrA = Tetrapropylammonium; †TBuA = Tetrabutylammonium; **TPeA = Tetrapentylammonium

Table 7. Substrates and inhibitors of polyspecific Organic Cation Transporters. K_m or IC_{50} values found in cultured cell systems or in *Xenopus laevis* oocytes systems are shown. Bold face indicates cations for which transport has been demonstrated. Adapted from (Koepsell et al., 2007).

3.1.2.3. Physiological implications

Although OCTs and OATs have been extensively studied in relation to cation and anion transport, the role some specific subtypes play in the availability and elimination of physiological endogenous substrates is not fully understood. Nevertheless, to understand the functional roles of individual organic cation transporters, the functional interplay of transporters with overlapping specificity in individual cells, the specific function of the respective cells and the physiologic function of the respective organ must be considered.

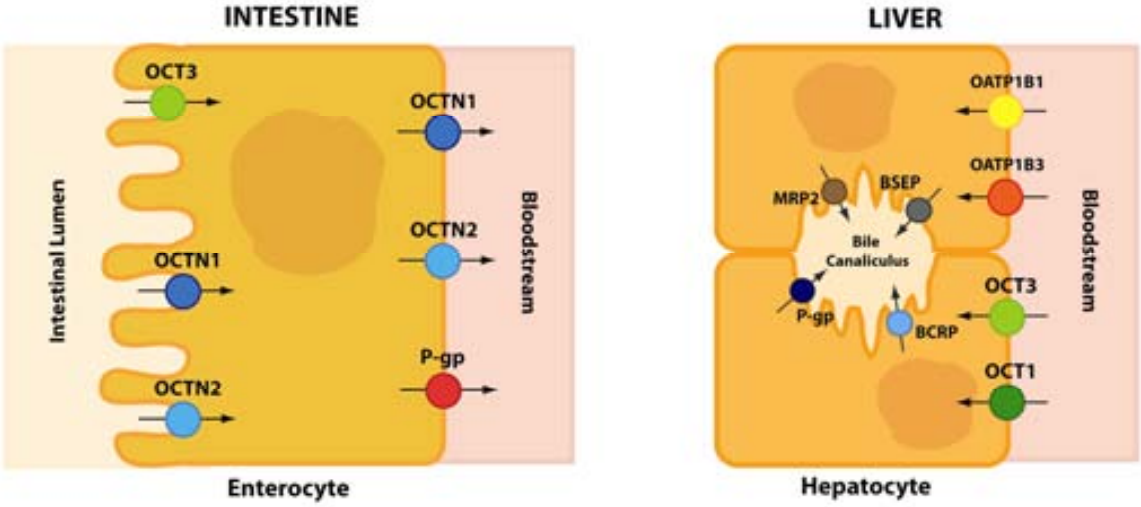


Figure 15. Small intestine and liver-specific expression of OCTs and other important transporters involved in absorption and excretion of nutritional compounds and drugs. OCT, Organic cation transporter; OCTN, Organic Cation/Carnitine Transporter; OAT, organic anion transporter; P-gp, P-glycoprotein (MDR1); BSEP, bile salt export pump; OATP, organic anion transporting polypeptide; MRP, multidrug resistance–associated protein; BCRP, breast cancer resistance protein. Adapted from (Ho and Kim, 2005; Koepsell et al., 2007).

Cationic food components, drugs and xenobiotics are absorbed in the *small intestine* but can also be excreted. In humans the first step in the absorption of organic cations from the intestinal lumen is mediated by hOCTN1 and 2 and/or hOCT3 in the brush border membrane, whereas the efflux is probably mediated by hOCT1 (*Figure 15*) (Muller et al., 2005). The secretion of organic cations is mediated by the combined action of hOCT1 and several transporters (hOCTN1/2 and P-glycoprotein, encoded by the MDR1 gene) in the brush border membrane. P-glycoprotein mainly translocates hydrophobic compounds that are neutral or positively charged (Ho and Kim, 2005; Koepsell et al., 2007).

Most cationic compounds that have been absorbed in the small intestine enter the *liver*. In the hepatocytes, drugs and nutritional compounds may undergo biotransformation. Normally, they are excreted across the biliary membrane into the bile or across the sinusoidal membrane, back into the blood stream. In humans hOCT1 and hOCT3, expressed in sinusoidal membrane of hepatocytes (unpublished data from H. Koepsell's lab) (Meyer-Wentrup et al., 1998) (*Figure 15*), mediate the first step in the biliary excretion of most cationic drugs; however, they can also mediate the release of organic cations into the blood. The sinusoidal membrane of hepatocytes also contains the organic anion transporting polypeptides (OATPs; SLC21 gene family) that translocate some organic cations and contribute to the translocation of organic cations across this membrane (Van Montfoort et al., 2001; Van Montfoort et al., 2003; Ho and Kim, 2005).

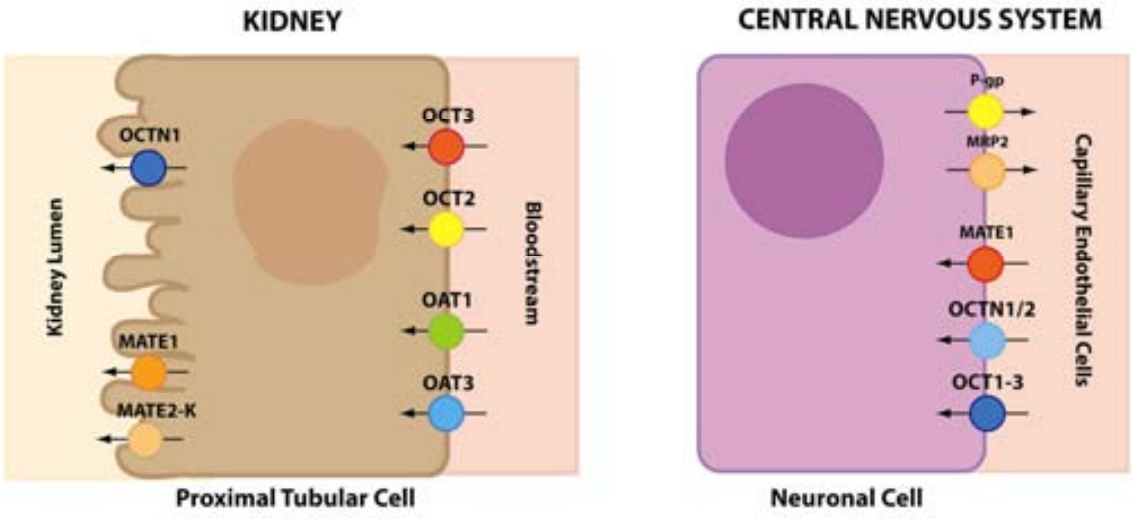


Figure 16. Kidney and brain-specific expression of OCTs and other important transporters involved in the absorption and excretion of nutritional compounds and drugs. OCT, Organic cation transporter; OCTN, Organic Cation/Carnitine Transporter; OAT, organic anion transporter; P-gp, P-glycoprotein (MDR1); MATE, multidrug and toxin extrusion; OATP, organic anion transporting polypeptide; MRP, multidrug resistance-associated protein; BCRP, breast cancer resistance protein. Adapted from (Ho and Kim, 2005; Koepsell et al., 2007).

In the *kidneys*, organic cations may be ultrafiltrated in the glomeruli or secreted in the renal proximal tubules. Hydrophilic organic cations that do not bind to plasma proteins are readily ultrafiltrated and may be reabsorbed in the proximal tubule (*Figure 16*). Nevertheless, many endogenous cations and cationic drugs are bound to plasma proteins and are not filtrated efficiently, and many of these are secreted actively. In a first

step in the secretion of organic cations in the proximal tubule, cations are translocated across the basolateral membrane. In humans OCT2 and OCT3 are thought to be important for basolateral uptake (Izzedine et al., 2005; Wright, 2005; Koepsell et al., 2007). Importantly, hOATs (especially OAT1 and OAT3) are also expressed in the kidneys and their role in the transport of weak bases and perhaps also organic cations could be important (Cha et al., 2001; Izzedine et al., 2005). In the second step of secretion, the transport from the intracellular milieu to the lumen is mediated by OCTN1 and/or the proton/cation exchangers MATE1 and MATE2-K (Tamai et al., 1997; Otsuka et al., 2005; Masuda et al., 2006).

In the Central Nervous System, hOCT1–3, hOCTN1–2 and MATE1 expression in the brain has been reported. OCT2 and OCT3 are expressed in the neurons, and the former along with OCTN2 are also expressed in the glial cells (Grundemann et al., 1998; Koepsell et al., 2007). A large amount of data is available in the literature regarding the distribution of OCT3, and OCT function in neurotransmitter uptake (Grundemann et al., 1998; Grundemann et al., 2002; Koepsell et al., 2007), which strongly suggests a crucial modulatory role for OCTs in brain functions. In fact, Gründemann et al., and other authors, defend the thesis that OCT3 is an extraneuronal monoamine transporter (EMT) present in the peripheral non-neuronal tissues (Grundemann et al., 1999; Martel et al., 2001). The named EMT was first identified as a low-affinity uptake system for catecholamines in isolated rat heart (Iversen, 1965). In addition to OCT3 (EMT), OCT1 and OCT2 have also been shown to mediate the transport of catecholamines and other biogenic amines (Breidert et al., 1998; Grundemann et al., 1998).

More recently, it has been shown that all polyspecific OCTs are expressed in the lungs. In humans concretely, all subtypes have been found in the respiratory epithelium of the trachea and bronchi and all but hOCT2 in the alveolar epithelial cells (Lips et al., 2005; Horvath et al., 2007; Lips et al., 2007). hOCT1 and 2, capable of transporting acethylcholine, are supposed to mediate the release of this compound involved in non-neuronal autocrine and paracrine cholinergic regulation. Furthermore, although the specific physiological and biomedical roles of individual OCTs in the lung are not understood, it is believed that they play a significant role in the distribution of cationic drugs in the lung.

3.2.1.4. Structure-function relationships

Members of the OCT family are generally 550–560 amino acids in length and have common structural features, namely a characteristic membrane topology including twelve

putative transmembrane spanning α-helices, C and N-termini within the cell, an intracellular loop with phosphorylation sites between the 6th and the 7th transmembrane domains and a large extracellular loop between the 1st and 2nd TM containing glycosylation sites (*Figure 17*).

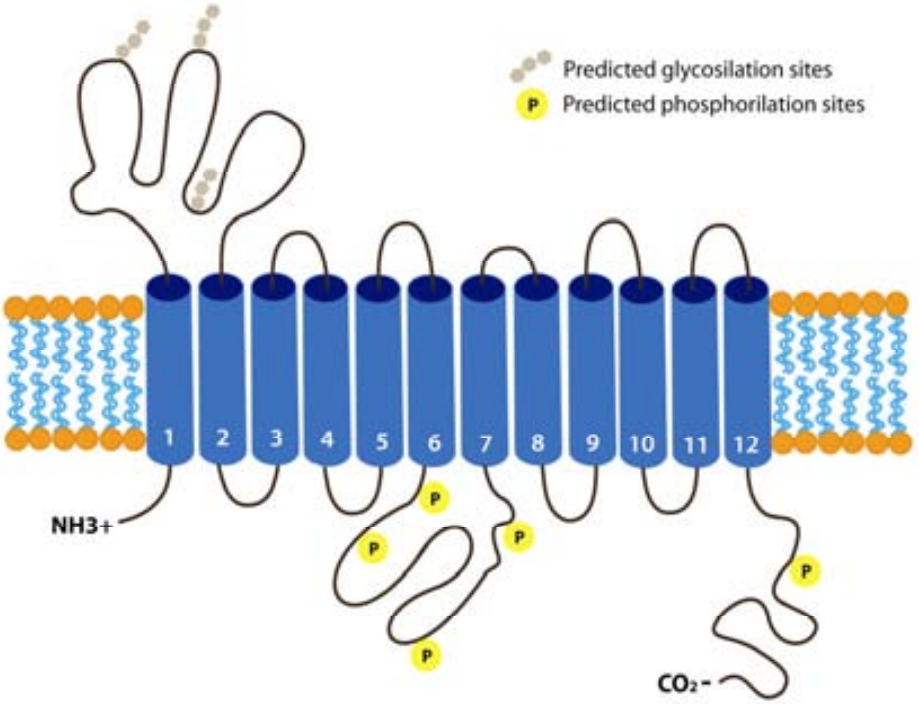


Figure 17. Predicted topology of hOCT1 as a model for transporters of *SLC22* families. Both hOCTs and hOATs from this gene family share twelve transmembrane domains and a long connection loop between TM1 and TM2. Predicted topology of the OCT1 organic cation transporter in humans. Predicted glycosylation sites (grey polygons) on the large extracellular loop and predicted phosphorylation sites (yellow circles, *P*) are indicated. Adapted from (Koepsell and Endou, 2004).

To date, all the structure–function relationship experiments for OCTs have only been performed on rat and rabbit orthologues of OCTs, though these models are indeed very helpful in understanding what might be occurring in human subtypes. Using site-directed mutagenesis with rOCT1, seven amino acids are identified as being involved in cation binding: tryptophan 218, tyrosine 222, threonine 226 (in TM4), alanine 443, leucine 447, glutamine 448 (in TM10) and aspartate 475 (in TM11) (Gorboulev et al., 1999; Popp et al., 2005). Moreover, studies of both rOCT1/2 and rabbit OCT2 have demonstrated that changes in those positions change substrate affinity for TEA^+, choline and/or MPP^+, and have suggested that the substrate binding region of rOCT1 contains partially overlapping binding sites for different substrates (Gorboulev et al., 2005; Zhang et al., 2005b).

As stated before, the crystallization and determination of tertiary structures of two members of the MFS transporters from *Escherichia coli* (prokaryote) was achieved in 2003 (Abramson et al., 2003; Huang et al., 2003). Based on these structures, Dr. Koepsel's group in collaboration with Dr. Müller have modelled the tertiary structure of both rOCT1 and rOCT2 (Gorbunov et al., 2008; Schmitt et al., 2009). Importantly, the seven amino acids assigned to the substrate binding region using site-directed mutagenesis are located at a similar depth within this cleft. A comparison of the substrate binding region in the rOCT1/2 models with the sizes of the substrates suggests that more than one compound could bind at the same time.

3.2.1.5. OCTs pharmacological profiles

The presence of OCTs in excretory organs such as the kidneys, liver and intestine is well documented and reviewed, and the importance of transporters from the *SLC22* family in detoxifying mechanisms involving the secretion of potentially toxic compounds is highlighted elsewhere in the literature (Koepsell and Endou, 2004; Lee and Kim, 2004; Ho and Kim, 2005; Izzedine et al., 2005; Koepsell et al., 2007). Moreover, the role of OCTs (and OATs) in the disposition, absorption and elimination of many clinically important drugs is increasing day by day. As the range of interactions and drug transportability for hOCT is so huge *(Table 8, next page),* only the most important will be summarized here, focusing on those that match the objectives of this thesis.

Biguanides such as metformin and phenformin have been developed for the treatment of hyperglycaemia in diabetic patients and currently, metformin is a widely used drug in clinical practice. Rat OCT1 has been shown to transport metformin and the other two biguanides with different affinities (Wang et al., 2002). Moreover, $OCT1^{(-/-)}$ mice showed a lower distribution of the former drug to the liver and intestine than $OCT1^{(+/+)}$ mice (Wang et al., 2002). These results suggested that OCT1 is responsible for the hepatic and intestinal uptake of metformin. Importantly, in the kidney, hOCT2 – which is abundantly expressed – has been characterized as transporting metformin with a ten-fold greater capacity than hOCT1. Therefore, hOCT2 may play a dominant role in metformin pharmacokinetics (Kimura et al., 2005a; Kimura et al., 2005b). More recently, Shu et al. in two key papers demonstrated that genetic variation in OCT1 affects both metformin action and pharmacokinetics (Shu et al., 2007; Shu et al., 2008). Investigating a large series of non-steroidal anti-inflammatory drugs (NSAIDs), they demonstrated that some of these important and globally widely used drugs not only inhibit some organic anion uptake (related to hOAT1-4) but also organic cation mediated by hOCT1 and 2. However, none of

these two transporters mediated the transport of NSAIDs (Khamdang et al., 2002). Interestingly, cisplatin and oxaliplatin, two effective but highly nephrotoxic antineoplasic drugs, have been demonstrated to be hOCT2 permeants in renal proximal tubules and HEK293 cells (Ciarimboli et al., 2005; Yonezawa et al., 2006). Finally, imatinib, a drug used effectively in the treatment of chronic myeloid leukaemia (CLL), has shown a temperature-dependent and active uptake process likely to be mediated by hOCT1, as the transport could be blocked by competing with hOCT inhibitors (Wang et al., 2008). Human OCT3 also transports antiarrhythmic drugs such as procainamide, quinidine and lidocaine (Hasannejad et al., 2004).

Compound	*Class*	**hOCT1**	**hOCT2**	**hOCT3**	**hOCTN1**	**hOCTN2**
		K_m or (IC_{50}) [μM][a,b]				
Receptor antagonists						
Atropine	AChE receptor (muscarinic)	(1.2)	(29)	**(466)**		
Phenoxybenzamine (PbA)	α-adrenoreceptor	(2.7)	(4.9)	(6.1)		
Cimetidine	Histamine H_2 receptor	**(166)**	**8.6, 73 (70)**	(17)		
Ranitidine	Histamine H_2 receptor	(28)	**265 (40)**	(372)		
Receptor agonists						
Nicotine	AChE receptor (nicotinic)	(186)	(42)	(101)	(>5000)	(>1000)
Etilefrine	α-adrenoreceptor	(447)	(4009)	**2800 (4450)**		
Receptor agonists						
Verapamil	Ca^{2+} channel	(2.9)	(206)[c]	(24)	**(8.4)**	**(<50)**
Procainamide	Na^+ channel	(74)	(50, 58)	(738)	(<500)	(<3000)
Quinidine	Na^+ channel	**(18)**			**(69)**	**(<100)**
Cocaine	Noradrenaline transporter	(85)	(113, 277)	(>1000)		
Citalopram	Serotonin transporter	(2.8)[c]	(21)[c]	(158)[c]		
Drugs and xenobiotics						
Cephaloridine	Antibiotic				(<1000)	**230**
Metformin	Antidiabetic	**1470 (2010)**	**990 (340-1700)**			
Quinine	Antimalarial	**(13, 23)**	**(23, 34)**	(37)	(<500)	(<100)
Aciclovir (ACV)	Antiviral	**151**				
Ganciclovir (GCV)	Antiviral	**516**				
Indinavir (INV)	Anti-HIV protease inhibitor	(62)				
Nelfinavir (NFV)	Anti-HIV protease inhibitor	(22)				
Ritonavir (RTV)	Anti-HIV protease inhibitor	(5.2)				
Cisplatin	Anticancer drug (cytostatic)	(>100)	**(1.5)**			
Oxaliplatin	Anticancer drug (cytostatic)		**>5000**	**>5000**		
Mitoxantrone	Anticancer drug (cytostatic)	(16)[c]	(800)[c]	(440)[c]		

[a] K_m and IC_{50} values were measured in oocytes of Xenopus laevis or cell lines expressing the transporters; [b] Bold face indicates cations for which transport has been demonstrated; [c] Koepsell et al., unpublished data.

Table 8. Drugs and xenobiotics that interact or are substrates for polyspecific Organic Cation Transporters. K_m or IC_{50} values found in cultured cell systems or in *Xenopus laevis* oocytes systems are shown. Bold face indicates cations for which transport has been demonstrated. Adapted from (Koepsell et al., 2007).

3.2.2. Human Organic Anion Transporters (hOATs)

All Organic Anion Transporters (OATs) are encoded by the *SLC22* gene family and share with OCTs a membrane topology including α-helical transmembrane domains and one extracellular loop between TMs 1 and 2. To date, five human isoforms have been cloned and functionally analyzed: human OAT1 (hOAT1), hOAT2, hOAT3, hOAT4 and hOAT7 (Koepsell and Endou, 2004; Srimaroeng et al., 2008). OAT5 has only been partially characterized in mice and rats (Youngblood and Sweet, 2004; Anzai et al., 2006) and OAT6 was cloned in mouse tissue some years ago (Monte et al., 2004). As for hOCTs, these transporters are known to transport a broad range of unrelated chemical structural substrates including numerous endogenous organic anions (cyclic nucleotides, dicarboxylates and neurotransmitter metabolites) as well as exogenous organic anionic environmental chemicals (mycotoxins or sulphate, cysteine, glycine and glucuronide conjugates). Of this huge range of substrates, the best characterized and most used compounds for *in vitro* inhibition experiments are: *p*-aminohippuric acid (PAH) – known as the best prototypic substrate for OATs – probenecid, prostaglandins and/or estrone-3-sulphate (Koepsell and Endou, 2004). Importantly, all OATs are functionally coupled to metabolic energy: their transport activity is driven by an exchange of α-ketoglutarate, so they require an intracellular > extracellular α-ketoglutarate gradient to act properly (Pitchard, 1990). OATs also transport a wide variety of clinically important drugs, such as β-lactam antibiotics, diuretics, non-steroidal anti-inflammatory drugs (NSAIDs), anti-HIV drugs, anticancer drugs and the inhibitors for angiotensin-converting enzyme. Importantly, since OAT can recognize multiple substrates and other compounds can act as inhibitors, drug–drug interaction can take place at its transport site or sites. Given the efficacy of the uptake process in the kidneys, renal organic anion transport plays a critical role in controlling drug and xenobiotic concentration and retention within the body. Moreover, the involvement of OATs in the nephrotoxicity of drugs and environmental substances has been reported (Endou, 1998; Jariyawat et al., 1999), an issue specifically important for the antiviral drugs adefovir and cidofovir (Ho et al., 2000).

3.2.3. The *SLC22* gene family of Transporters and Antiviral Drugs

With regard to antiviral drugs, both human OATs and OCTs have been characterized as mediating the influx of a broad spectrum of these drugs, and P-glycoprotein (*MDR1* gene product) and multidrug resistance proteins (MRPs) 2–5 are thought to act as efflux transporters in many tissues. Along with drug-metabolizing

enzymes, these transporters are important determinants of drug effectiveness and toxicity (Lee and Kim, 2004; Ho and Kim, 2005; Izzedine et al., 2005). It has been consistently stated in this introduction that anti-HIV nucleoside-derived drugs are the *focus* of this thesis. As early as 1982 it was recognized that mouse kidney slices accumulated the nucleoside analogue 2'-deoxytubercidin (dTub) through a saturable and metabolically dependent process with characteristics of an organic cation transport system (Kuttesch et al., 1982). Later, it was demonstrated that kidney proximal tubule cells express a cimetidine-sensitive ability to efflux TEA^+, which was nucleoside-sensitive (Chen et al., 1999). Other classic OCT inhibitors and several nucleosides also inhibited TEA^+ efflux by these cells in a manner reflecting structural specificity for the carrier. Whereas nucleosides are not substrates of rOCT2 (Chen et al., 1999), rOCT1 transports dTub, cytosine arabinoside, 2-chlorodeoxyadenosine, and AZT when expressed in a *Xenopus laevis* oocyte translation system (Chen and Nelson, 2000; Wada et al., 2000). When looking specifically at anti-HIV agents, we find a first approach for the implication of OATs and OCTs in AZT renal clearance in rats, since when in the presence of probenecid (OATs inhibitor) and cimetidine (OCT substrate), AZT renal secretion was significantly reduced (Aiba et al., 1995). Similarly, the excretion ratio of lamivudne (3TC) was found to be reduced in an isolated perfused rat kidney model in the presence of trimethoprim, a drug used to combat *Pneumocystis jirovencii* pneumonia and commonly administered to HIV+ patients (Sweeney et al., 1995). The interaction between 3TC and trimethoprim clearance was later confirmed in a randomized study in humans (Moore et al., 1996) and in a rat model using tritium-labelled 3TC (Takubo et al., 2000b). This OCT-related renal clearance has recently been confirmed again in an isolated perfused rat kidney model for emtricitabine (FTC): both the OCT substrates cimetidine and trimethoprim significantly decreased FTC clearance (Nakatani-Freshwater and Taft, 2008b; Nakatani-Freshwater and Taft, 2008a).

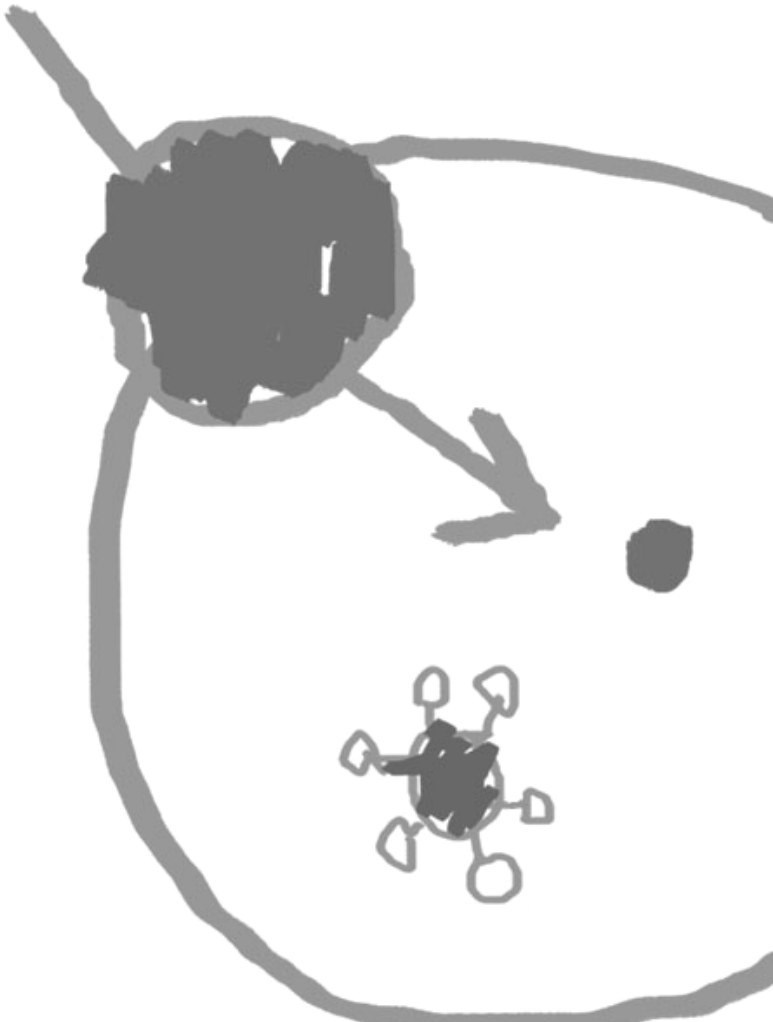
Looking back at hOATs, both hOAT1 and the rat orthologue of OCT1 have been involved in the uptake and cytotoxicity of the nucleoside analogues adefovir (anti-HIV NRTI) and cidofovir (anti-cytomegalovirus drug) (Cihlar et al., 1999; Ho et al., 2000). Moreover, non-steroidal anti-inflammatory drugs inhibited adefovir hOAT1-mediated transport and cytotoxicity in a cell line stably expressing the transporter (Mulato et al., 2000). More importantly, tenofovir disoproxil fumarate (TDF), the only nucleotide analogue drug approved by the FDA and widely used in HIV therapy today, has been related with hOAT1 and hOAT3 uptake and with MRP4 efflux in renal epithelial cells, suggesting a low nephrotoxicity associated with the balance of influx/efflux mechanisms (Cihlar et al., 2001; Cihlar et al., 2004; Ray et al., 2006; Ray and Cihlar, 2007). hOAT1

would be quantitatively the main renal transporter for TDF, adefovir and cidofovir (Uwai et al., 2007). In 2001 two transport mechanisms related to a unidentified organic cation transporter were described for AZT (in microglia) and 3TC (in a renal epithelial cell line) (Hong et al., 2001; Leung and Bendayan, 2001). Later on, AZT uptake was demonstrated to be mediated by hOAT1–4 in renal tissue whereas hOCT1 mediated renal acyclovir and ganciclovir transport (Takeda et al., 2002).

3.3. Other *Putative* Antiviral Drug Uptake Transporters

Even though those have not been studied in this thesis, it worth mentioning a few families from the *SLC* superfamily that have also been related to anti-HIV drug uptake throughout the history of HIV pharmacology. As depicted in *Figure 11* (page 27), some members of the *SLC15* family (Peptide Transporters; PEPTs) and *SLCO* family (Organic-Anion Transporting Polypeptides; OATPs) have been related to antiviral drug uptake. For instance, both PEPT1 and PEPT2 transporters have been shown to transport valacyclovir, an amino acid ester prodrug of acyclovir – a drug widely used to combat *Herpes zoster* infections (Balimane et al., 1998; Ganapathy et al., 1998). Interestingly, it was also demonstrated that 5' amino acid esters prodrugs of acyclovir and AZT where readily transported at rates three to ten times higher than the parental nucleoside derivatives (Han et al., 1998). With relation to *SLCO*, the members *SLCO1A2* and *SLC1B1* – corresponding to OATP-A and OATP-C, respectively – have been shown to transport the protease inhibitors saquinavir, lopinavir and darunavir in a *Xenopus laevis* model (Su et al., 2004; Kwan et al., 2008). Remarkably, OATP-A is mainly expressed in the brain (and less expressed in the liver, kidneys and lungs), whereas OATP-C is predominant in the liver. Moreover, OATP3A1 has been shown to be expressed in T lymphocytes (Tamai et al., 2000a; Janneh et al., 2008) and it may be involved in the cellular uptake and disposition of saquinavir but not lopinavir (a much more lipophilic drug) (Janneh et al., 2008). Rat Oatp2 is found at the cerebral capillary endothelium and the choroid plexus epithelium, suggesting a role for these carriers in the transport of drugs between the blood and central nervous system (CNS) compartments (Gao et al., 1999). An Oatp-2-like transporter has been implicated in the uptake of the NRTIs, ddI and 3TC into the choroid plexus (Gibbs and Thomas, 2002; Gibbs et al., 2003a,b). HIV may enter the CNS via the choroid plexus and therefore transporters present at this site could play an important role in the CNS efficacy of certain drugs.

Hypothesis & Objectives



Nucleoside Reverse Transcriptase Inhibitors (NRTIs) need an efficient uptake into immune cells susceptible to HIV infection to act properly within the cells inhibiting HIV replication. This core process, also important for other hydrophilic antiretroviral drug families, might be involved in cellular resistance to NRTIs and could also play a role in the development of viral resistance, by letting the virus replicate freely inside infected cells. Moreover, knowing NRTIs and antiretroviral uptake mechanisms in detail would provide new insights into drug pharmacokinetics/pharmacodynamics, drug–drug interactions and toxicity.

Due to the lack of clear and concise information regarding NRTI entry mechanisms we pursued the following objectives:

1. To study the uptake mechanisms of azidothymidine (AZT) in T lymphocytes and the contribution of simple diffusion to its entry.

2. To characterize the expression and activity of anti-retroviral drug transporters from *SLC28*, *SLC29* and *SLC22* gene families in T lymphoblastic cell lines and immune cells.

3. To evaluate the effect of HIV infection on Nucleoside Transporters and assess the possible antiviral effect of Dipyridamole, an Equilibrative Nucleoside Transporter inhibitor, in T cells.

4. To explore the interaction and transportability of NRTIs with Organic Cation Transporters 1, 2 and 3 and the possible drug–drug interactions between lamivudine (3TC), abacavir (ABC) and azidothymidine (AZT).

Materials & Methods

1. Cell lines

Lymphoblastoid cell lines

Six different lymphoblastoid cell lines (MT2, MT4, Molt-4/CCR5, Hut78, A3F7 and CEM13) were used to quantify nucleoside and organic cation transporter expression by real-time PCR. The T cell leukaemia cell lines MT2 and MT4 were obtained from Dr. D. Richman; the T4 lymphoblastoid human cell line Molt-4/CCR5 was from Dr. M. Baba; the Hut78 human cutaneous T cell lymphoma cell line was from Dr. A. F. Gazdar, all obtained through the *National Institutes of Health AIDS Research and Reference Reagent Program*. The T4 cell line A3.01/CCR5 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 the Institute Pasteur (Paris, France). Cells were routinely cultured in RPMI 1640 culture medium (Invitrogen, Paisley, UK) supplemented with 10% heat-inactivated foetal bovine serum (FBS), 2 mM L-glutamine, and 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen); 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% CO2.

MCF-7 cell line

The MCF-7 cell line (HTB-22, ATCC-LGC Promochem Partnership, USA), derived from human breast cancer, was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine and antibiotics, in the same conditions as those described above. RPMI 1640, DMEM media, FBS and antibiotics were purchased from Invitrogen.

CHO cell line

The CHO-K1 cell line was derived as a subclone from the parental CHO cell line initiated from a biopsy of an ovary of an adult Chinese hamster by T. T. Puck in 1957 (Puck et al., 1958). Chinese hamster ovary (CHO) cells are the most widely used mammalian cells for transfection, expression and large-scale recombinant protein production. Since CHO cells provide stable and accurate glycosylation, they offer a post-translationally modified product and are thus a more accurate in vitro rendition of the natural protein (Sheeley et al., 1997).

CHO cells stably expressing hOCTs were routinely cultured in F-12 (Ham's) medium (Invitrogen) supplemented with 10% heat-inactivated foetal bovine serum in the presence of 300 μg/ml hygromycin B (see *Stable transfection of hOCTs in CHO cell line*) and maintained at 37°C in a humidified atmosphere containing 5% CO2. The F-12 (Ham's) medium was obtained from Invitrogen.

2. Primary cultures

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's adherence to the Helsinki Declaration. Moreover, the studies were approved by the Hospital Universitari Germans Trias i Pujol review board on biomedical research.

Peripheral blood mononuclear cells (PBMCs) and $CD4^+$ T cells and PHA-stimulation

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 the PBMCs by negative selection using the CD4+ Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany), and all of the isolated populations had a high purity (>95%), as determined by immunophenotype in-flow cytometry analyses. Cells were stimulated for three days by adding 3 μg/ml phytohemagglutinin (PHA; Invitrogen) and 10 IU/ml interleukin-2 (IL-2; Roche, Basel, Switzerland). After this period of stimulation, activation was confirmed by flow cytometry, observing the change in forward-scatter and side-scatter (SSC) light gating. This gating analysis was also used to exclude dead cells and debris from the analysis.

Monocytes and Monocyte-derived Macrophages (MDMs)

Monocytes were isolated at a high purity (>97%) from PBMCs using CD14+ selection magnetic beads (Miltenyi Biotec). We differentiated macrophages by culturing monocyte populations (0.8 x 10^6 cells/ml) with 100 ng/ml macrophage-colony-stimulating factor (M-CSF; Peprotech, London, UK). We maintained them in culture for five to six days replacing medium on day 3. Once the monocyte population had been isolated and the MDMs differentiated, an immunophenotype analysis was performed on each case as explained in the *Immunophenotype* section.

Immature and Mature Monocyte-derived Dendritic Cells (iMDDC and mMDDCs)

To obtain immature dendritic cell populations, we cultured monocytes (0.8 x 10^6 cells/ml) over seven days with 1000 IU/ml granulocyte/macrophage colony-stimulating factor (GM-CSF) and 1000 IU/ml IL-4 (R&D Systems, Minneapolis, MN). The medium was replaced every two to three days by adding fresh GM-CSF and IL-4. To obtain a population of mature dendritic cells, 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% foetal bovine serum (10% for macrophages), and 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen).

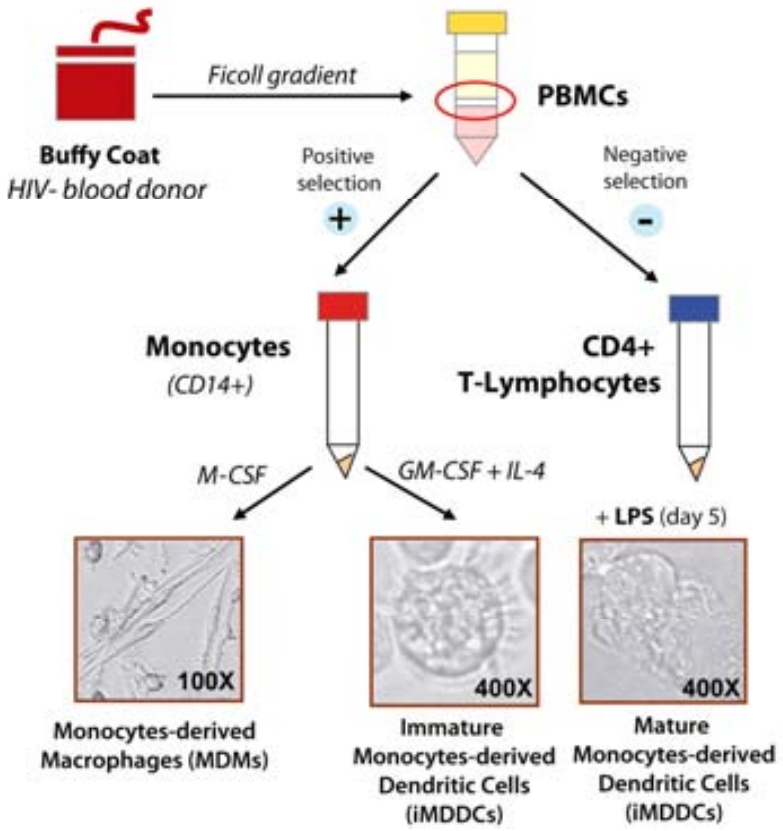


Figure 18. A schematic view of the procedure followed to obtain the primary cells used throughout this thesis. Primary cells obtained include PBMCs, $CD4^+$ T cells, monocytes, MDMs, iMDDCs and mMDDCs.

3. Immunophenotype

$CD4^+$ T lymphocytes and monocytes were immunophenotyped immediately after isolation from blood donor PBMCs, while monocyte-derived macrophages (MDMs) and immature and mature monocyte-derived DCs (iMDDCs and mMDDCs) were stained at day 5 (macrophages) and at day 7 (MDDCs). All the cells bar the $CD4^+$ T lymphocytes were previously blocked with 1 mg/ml of human IgG (Baxter, Hyland Immuno) to prevent binding to Fc receptors through the Fc portion of the antibody. All cells were stained for twenty minutes at 4°C (except for $CD4^+$ T lymphocytes stained at room temperature) to avoid antibody internalization, then washed and resuspended in PBS containing 2% formaldehyde. Samples were analyzed with a FACSCalibur flow cytometer (Beckton Dickinson) using CellQuest software to evaluate the data collected.

Cells	mAb-Fluorochrom	Brand
CD4+ T-lymphocytes	CD3-PerCP	BD
	CD4-FITC	
	CD8-PE	
Monocytes	CD3-PerCP	BD
	CD4-PE	
	CD14-FITC	Pharmigen
Monocytes-Derived Macrophages (MDM)	CD14-FITC	Pharmigen
	HLA-DR-PerCP	BD
	αV-PE	Santa Cruz
Monocyte-Derived Dendritic Cells (MDDC)	CD3-PerCP	BD
	CD4-PE	
	HLA-DR-PerCP	
	CD14-FITC	Pharmigen
	CD83-PE	
	CD86-FITC	
	DC-SIGN-PE	R&D

BD, Beckton Dickinson; R&D, R & D Systems

Table 9. Monoclonal antibodies (mAb) used to immunophenotype $CD4^+$ T lymphocytes, monocytes, MDMs and MDDCs by flow-cytometry.

Forward-scatter and side-scatter light gating were used to analyse the activation of PBMCs and CD4+ T cell cultures in the presence of PHA/IL-2 at days 3 to 4 of stimulation, as the activation tends to enlarge the cells and accumulate those cells in aggregates.

Forward-scatter and side-scatter light gating analysis was also used to exclude dead cells and debris from the analysis. Cells were stained using the previously titered monoclonal antibodies (mAbs), listed in *Table 9*, using their matched isotype controls. The basic immunophenotype of $CD4^+$ T lymphocytes and monocytes (CD3-/CD4+/CD14+) was evaluated before further culture. Adequate differentiation of monocytes to macrophages was based on the loss of CD14 and the acquisition of HLA-DR, and the integrins from the αV family. Moreover, the correct differentiation of macrophages could be also confirmed by microscope observation, as the rounded monocytes were clearly transformed into longer and more linear forms. iDC differentiation was based on the loss of CD14 and the acquisition of DC-SIGN, while maturation with LPS up-regulated the expression of CD83, CD86 and HLA-DR in DCs.

4. Reagents

Radiolabelled nucleosides, organic cations, NRTIs and others

The nucleoside Uridine ([5, 6-^{3}H], 37 Ci/mmol), L-carnitine hydrochloride ([H_3C-^{3}H], 83 Ci/mmol) and mannitol (D-[1-^{14}C], 59 mCi/mmol) were purchased from Amersham Biosciences (Buckinghamshire, UK). Cytidine ([5-^{3}H(N)], 22 Ci/mmol), guanosine ([8-^{3}H], 6.7 Ci/mmol) and the nucleobase hypoxanthine ([2,8-^{3}H], 42.6 Ci/mmol) were from Moravek Biochemicals (Brea, CA, USA). The model organic cation *N*-Methyl-4-phenylpyridinium (MPP+) ([H_3C-^{3}H], 85 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO) or from BioTrend (Köln, Germany). Finally, the NRTIs ABC ([^{3}H], 0.5 Ci/mmol), AZT ([H_3C-^{3}H]; 12.7 Ci/mmol), 3TC ([5-^{3}H(N)]; 9 Ci/mmol) and the anti-diabetic drug metformin (dimethyl-[^{14}C]; 112 mCi/mmol) were purchased from Hartmann Analytic GmbH (Braunschweig, Germany).

Nonradiolabelled reagents

Uridine, guanosine, hypoxanthine, 3'-azido-2',3'-dideoxythymidine (azido-thymidine, AZT), 2',3'-dideoxycytidine (zalcitabine; ddC), 2',3'-dideoxyinosine (didanosine; ddI), 2',3'-didehydro-3'-deoxythymidine (stavudine; d4T), ribavirin, probenecid and p-aminohippuric acid (PAH) were obtained from Sigma-Aldrich (Saint Louis, MO, USA). 5'-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) was purchased from Toronto Research Chemicals Inc. (North York, Canada). The inhibitors of nucleoside transporters nitrobenzylthioinosine (NBTI), dipyridamole (DIP), and substrates and inhibitors of organic cation transport tetraethylammonium (TEA),

tetrabutylammonium (TBuA), MPP+, ranitidine (Rani), ergothioneine (ET), atropine (Atrop), and 1,1'-diethyl-2,2'-cyanine iodide (D-22) were obtained from Sigma-Aldrich (St. Louis, MO). The remaining reagents used for uptake measurements were also purchased from Sigma-Aldrich, unless otherwise indicated in the text. During the first experimental approaches, 3TC was kindly provided by *GlaxoSmithKline*, Inc. (London, UK) and TDF was donated by Gilead Sciences (Foster City, CA, USA). Later, all the NRTIs used (3TC, AZT, d4T, ddC, ABC, FTC, and TDF) were routinely obtained through the *AIDS Research and Reference Reagent Program*, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health (Bethesda, MD).

5. Stable Transfection of hOCT in Flp-In™-CHO Cell Line

Flp-In™ Cell Lines (Invitrogen, Carlsbad, CA) are designed for the rapid generation of stable cell lines that express a protein of interest from a Flp-In™ expression vector. These cells contain a single stably integrated FRT site at a transcriptionally active genomic locus. Targeted integration of a Flp-In™ expression vector ensures high-level expression of your gene of interest. There are six Flp-In™ Cell Lines available for the generation of isogenic stable cell lines The Flp-In™-CHO Cell Line was created by Invitrogen by transfecting CHO cells with pFRT/lacZeo2 and selecting for Zeocin™-resistant clones. Co-transfection of the Flp-In™ Cell Lines with a Flp-In™ expression vector and the Flp recombinase vector, pOG44, results in targeted integration of the expression vector to the same locus in every cell, ensuring homogeneous levels of gene expression.

To construct CHO cell lines stably expressing hOCTs, hOCT1 (GenBank accession number X98322), hOCT2 (GenBank accession number X98333), and hOCT3 cDNAs (GenBank accession number AJ001417, kindly provided by Dr. V. Ganapathy, Augusta, GA) were recloned into the pcDNA5/FRT/TO vector (Invitrogen). The eukaryotic expression vectors and an empty vector (pcDNA5), used as a control in uptake experiments, were then transfected into the Flp-In™ CHO cell line (Invitrogen) using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's recommendations and selected for positive clones with 600 µg/ml hygromycin B (PAA Laboratories GmbH, Linz, Austria). The CHO cells with the highest transport activity were chosen for further study and routinely cultured in F-12 (Ham's) medium (Invitrogen) supplemented with 10% heat-inactivated foetal bovine serum in the presence of 300 µg/ml hygromycin B. They were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

6. Quantitative Real-Time Reverse Transcriptase-PCR Analysis

Total RNA was isolated from the different lymphoblastoid cell lines mentioned above (MT2, MT4, Molt-4, Hut78, A3F7 and CEM13), PBMCs, $CD4^+$ T lymphocytes, monocytes, monocyte-derived macrophages (MDMs), immature and mature monocyte-derived dendritic cells (iMDDCs and mMDDCs) and kidney lysates using RNeasy Mini Kit (limits: 1-10 x 10^7 cells or 0.5-30 mg tissue) and/or RNeasy Micro Kit (limits: <5 x 10^5 cells or <5 mg tissue; QIAgen, Barcelona, Spain) depending on the number of the cells that we disposed of. Therefore, T cell line and PBMC RNA was usually extracted with an RNeasy Mini kit, whereas the RNA from primary monocytes, MDMs and MDDCs (with less cells were available) was purified by using an RNeasy Micro kit. When cell pellets were frozen at -80ºC -to extract RNA after a few days- a reagent was added to the samples to stabilize RNA following the protocol provided by the manufacturer (RNA*later* RNA Stabilization Reagent, QIAgen). During the RNA isolation, RNA was treated with DNase I from RNase-free DNase set (QIAgen) to eliminate DNA contamination. 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 (hGUSB) 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 following commercial Gene Expression Assays (Applied Biosystems): Hs00427554_m1, Hs00161893_m1, Hs02382478_s1, Hs01548721_m1, Hs00161895 m1, respectively. 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. The plasmids were constructed by cloning a PCR amplicon of the gene of interest (e.g. hENT1; GenBank accession number NM_001078174) into a commercial pGEM-T-Easy Vector (Promega). Standard curves were optimized in terms of *precision* (intra-assay variation) and *reproducibility* (inter-assay variation). With regard to *reproducibility*, the curves were optimized in terms of correlation ($r^2 \geq 0.98$), slope (-*a*, from the curve $y = -ax + b$, between -3.0 and -4.0), and efficiency ($E \geq 95\%$ where $E = 10^{-1/\text{slope}} - 1$) and were run in duplicate simultaneously with the samples in the ABI Prism 7000 (Applied Biosystems). The threshold cycle (C_t) is defined as the cycle number at which the fluorescence corresponding to the amplified PCR product is detected.

The standard curves allow us to correlate the C_t values of the samples with the mRNA copy number of each gene per microgram of total RNA. An example of three typical standard curves obtained for the hCNT1–3 genes in our experiments is shown in *Figure 19*.

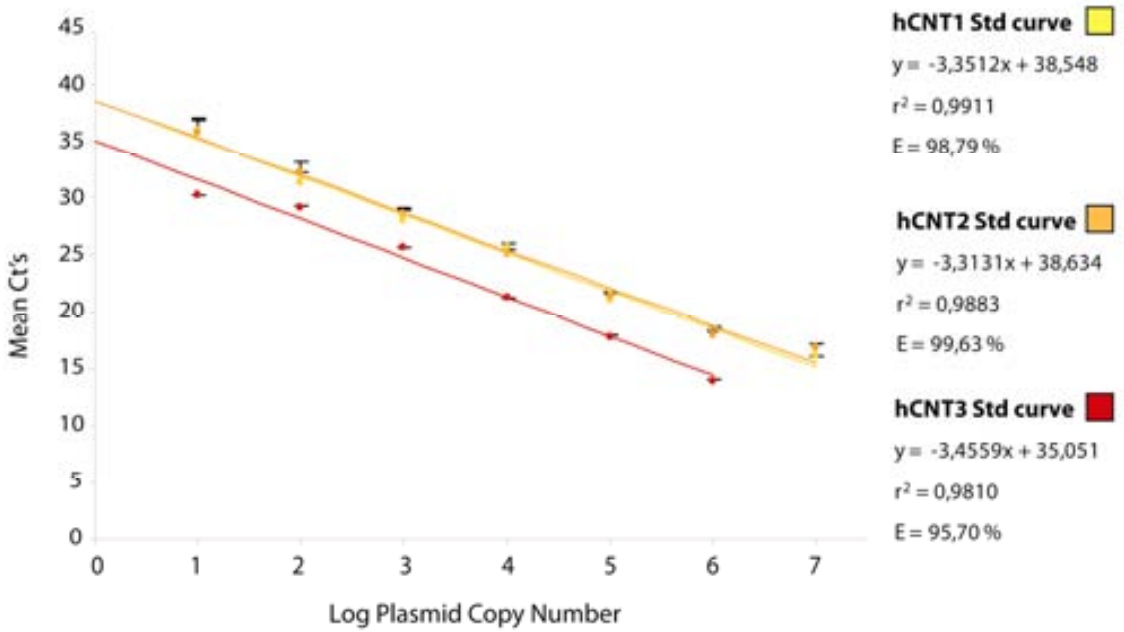


Figure 19. Representative standard curves for hCNT1 (yellow)**, hCNT2 (orange) and hCNT3 genes.** The curve equation ($y = -ax + b$), correlation (r^2) and efficiency (E) are shown for each gene. Graph colours are: yellow for hCNT1, orange for hCNT2 and red for hCNT3.

7. Qualitative RT-PCR analysis

The expression of organic anion transporters 1–4 (hOAT1–4) in the Molt-4 cell line and immune cells (PBMCs, PHA-stimulated PBMCs, $CD4^+$ T cells, PHA $CD4^+$ T cells and macrophages) was assessed by RT-PCR analysis. The oligonucleotides used for hOATs amplification were: hOAT1: 5'-GCC TGC CGA TGC CAA CCT CAG-3' and 5'-GAC TCT CTG CAG GGC CCT CAG-3'; hOAT2: 5'-CAA AGT GGC CGC CGG GGA AC-3' and 5'-CAG CTC GTC ACC ACT GCC C-3'; hOAT3: 5'-GTC GAC AGC ACT CGG GTA CTG-3' and 5'-CAA AGG TGA GAG CCA AGA TGG CC-3'; and hOAT4: 5'-CTT CTG ACC ATC TCC ATC CCG C-3' and 5'-CTG AGC TCC TGA AGT GCT TGG-3'. The reaction mixture was heated to 94°C for five minutes and then to 80°C. At this step, 2.5 units of *Taq* Polymerase (Promega, Madison, WI, USA) were added. The PCR conditions were as follows: one minute, 94°C; one minute, 60°C (for hOAT1), 65°C (for hOAT2), 58°C (for hOAT3) or 56°C (for hOAT4); and three minutes, 72°C for 40 cycles. Finally, the PCR was heated to 72°C for fifteen

minutes and cooled to 4ºC. The amplified fragments were run in a 1% agarose gel. The expected sizes of the PCR products were 0.72, 0.96, 0.55 and 0.66 kb for each OAT 1–4.

8. Immunocytochemistry

Cytocentrifuge preparations (cytospins) of PBMC or monocyte-derived macrophages (MDMs) cultured on poly-Lysine-treated coverslips (Nunc, Roskilde, Denmark) were air-dried overnight and fixed in 100% acetone for ten minutes. Slides were incubated with polyclonal antibodies diluted in Tris-buffered saline (TBS) containing 2% normal human serum or FBS to block unspecific binding. Primary rabbit polyclonal antibodies anti-hCNT2, hCNT3, hENT1 and hENT2 were produced in the Biochemistry and Molecular Biology Laboratory of Dr. Pastor-Anglada at the University of Barcelona (Mata et al., 2001; Farre et al., 2004). The newly generated anti-hCNT2 and anti-hCNT3 antibodies were also characterized at the University of Barcelona laboratory by competing them with an excess of the purified peptide used to immunize the source rabbit, as well as by analyzing hCNT2 and hCNT3 expression in transfected cells expressing these particular transporter proteins (data not shown). For cytospins 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 PBMCs or hematoxylin (DakoCytomation) for MDMs for 5 minutes to counterstain the nuclei, then they were washed in TBS, dehydrated with ethanol and xylene, and mounted with Distyrene-Plasticizer-Xylene medium (Sigma-Aldrich). For negative controls, slides were stained with isotype-matched human or rat antibodies. Images were collected at 400x using an Axioskop Microscope (Carl Zeiss, Barcelona, Spain) and a Leica 330 DFX camera using the Leica Cam software (Leica, Barcelona, Spain).

9. Western Blot Analysis

HEK293T, a human embryonic kidney cell line (15×10^6), and primary cultures of PBMCs, PHA-stimulated PBMCs, $CD4^+$ T cells, PHA-stimulated $CD4^+$ T cells, monocytes, MDMs, immature monocyte-derived dendritic cells (iMDDCs), and mature MDDCs (mMDDCs) (20×10^6 in all cases) were harvested, washed with phosphate-buffered saline, and lysed in chilled hypotonic buffer (20 mM Tris-HCl, 140 mM NaCl, 10% glycerol, 1%

Nonidet P-40, and 2 mM EDTA, pH 8.0), freshly supplemented with a cocktail of protease inhibitors and 100 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich). Cell debris was removed by centrifugation, and quantification of protein concentration was performed by bicinchoninic acid (Pierce, Madrid, Spain). Protein samples were run using 4 to 12% *v*-polyacrylamide gel electrophoresis gels (Invitrogen) and blotted onto nitrocellulose membranes using the iBlot Dry Blotting System as described by the manufacturer (Invitrogen). Membranes were blocked with 0.5% phosphate-buffered saline Tween-20 and 5% non-fat milk (blocking solution) for four hours at room temperature and incubated overnight with rabbit polyclonal antibodies for rat OCT1 and OCT3, or for mouse OCTN1 (diluted 1/100 in blocking solution; Alpha Diagnostic, San Antonio, TX), all cross-reacting with human orthologues or the mouse monoclonal antibody for actin (1/3000 in blocking solution; Chemicon, Barcelona, Spain) at 4°C. After washing, membranes were incubated with goat anti-rabbit horseradish peroxidise (1/2000 in blocking solution; Cultek) or rabbit antimouse (Invitrogen) for 45 minutes at room temperature and then revealed with ECL-Plus solution (Amersham Biosciences). Images were collected using a Kodak Gel Logic 440 Imaging System and Kodak Molecular Imaging Software (Kodak, Barcelona, Spain).

10. HIV infections in MT4 and PHA-stimulated PBMCs

MT4 infection with GFP-HIV and flow cytometry measurements

MT4 culture volume corresponding to 5 x 10^6 cells was separated and spun at 1400 rpm for 5 minutes in a 15 ml tube. Using an HIV-GFP virus previously constructed from an HIV-1 hemiplasmid (p83-10) and characterized and titrated in MT4 cells (Weber et al., 2006) (50% *Tissue Culture Infective Dose* ($TCID_{50}$) = 1.6 x 10^7 x ml) we resuspended 5 x 10^6 cell pellets with a volume corresponding to 5000 $TCID_{50}$ (31.5 µl) to act at a *Multiplicity of Infection* (MOI) of 0.001 [MOI = 5000 $TCID_{50}$/5 x 10^6 cells = 0.001] and topped them up to 1 ml (968.5 µl) with RPMI supplemented with 10% FBS (R10), then mixed. We incubated the HIV-GFP with the MT4 cells for two to three hours at 37ºC and 5% CO_2 (shaking the pellet every 45 minutes to one hour, approximately). Cells were then spun down (five minutes, 1400 rpm) and supernatants were removed. Then, two washing steps with 10 ml 1X PBS were performed. Infected MT4 cells were resuspended in 10 ml R10 to allow the culture to grow at a concentration of 0.5 x 10^6 cells/ml at 37ºC and 5%CO_2. In those cultures in which treatment with NBTI or dipyridamole (DIP) was required, drugs were added after MT4 infection, when the cells were put into culture

separately. For NBTI the final concentration used was 1 μM, whereas DIP was added at 10 μM. The culture was kept until day 4 or 5 and the infection was followed by flow cytometry by removing 100 μl of the culture and counting the percentage of GFP positive cells (% GFP+ cells) by flow cytometry (FACSCalibur). As described for the PHA stimulation of PBMCs and CD4+ T cells, forward-scatter and side-scatter light gating analysis was used to exclude dead cells and debris from the analysis. For MT4 cultures, the number of viable cells was followed using the automatic cell counter *Vi-Cell* Cell Viability Analyzer (Beckman Coulter, London, UK).

PHA-stimulated PBMC infection with HIV-1 and p24 antigen ELISA

After three days of PHA stimulation of PBMCs (as described above, see *PBMCs and CD4⁺ T cells and PHA stimulation*) culture volume corresponding to 1 x 10^7 cells was separated and spun at 1400 rpm for 5 minutes in a 15 ml tube. Using the laboratory strain HIV-1 virus (NL-4.3) and having titrated it before in PBMCs, we followed the same infection procedure as for MT4 cells and HIV-GFP. As for MT4, we incubated the HIV-1 with PBMCs cells for two to three hours at 37ºC and 5% CO_2 (shaking the pellet every 45 minutes to one hour, approximately). The cells were then spun down (5 minutes, 1400 rpm) and supernatants were removed. Then, two washing steps with 10 ml 1X PBS were performed. Infected PBMCs cells were resuspended in 10 ml R20/IL-2 (RPMI supplemented with 20% FBS plus 10 IU/ml IL-2) to let the culture to grow at a concentration of 1 x 10^6 cells/ml at 37ºC and 5% CO_2. In those cultures where treatment with dipyridamole (DIP; 1, 10 and 100 μM) was required, drugs were added after PBMC infection, when the cells were put in culture separately. The culture was kept running until day 9 and the infection was followed using the p24 antigen measure from ELISA, as described by the manufacturer (Alliance HIV-1 p24 antigen ELISA kit, Perkin-Elmer, Waltham, MA, USA). For PBMC cultures, the number of viable cells and cytotoxicity was analyzed by the MTS viability assay (Promega, Madrid, Spain).

11. MTS viability assay

To assess PBMCs and MT4 viability, the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (also *MTS viability assay*; Promega) was used. This assay is a colorimetric method for determining the number of viable cells in proliferation or cytotoxicity assays. It contains a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazo-lium, inner salt; MTS]

(different from the most commonly used MTT; (Berridge and Tan, 1993)) and an electron coupling reagent (phenazine ethosulfate; PES). PES has enhanced chemical stability, which allows it to be combined with MTS to form a stable solution. The assay is based on the bioreduction of the MTS tetrazolium compound by cells (accomplished using NADPH or NADH in active cells) into a coloured formazan product that is soluble in tissue culture medium (*Figure 20*). The quantity of the formazan product as measured by absorbance at 492 nm is directly proportional to the number of living cells in culture (Figure 3). Because the MTS formazan product is soluble in tissue culture medium, the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay requires fewer steps than procedures that use other tetrazolium compounds such as MTT or INT (Berridge et al., 2005).

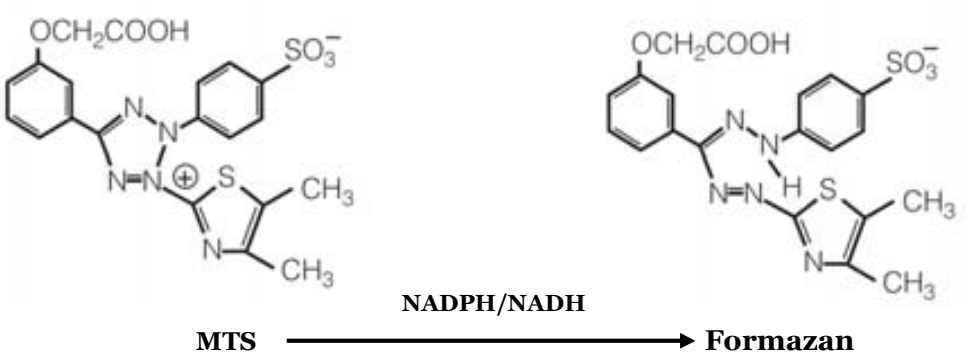


Figure 20. MTS tetrazolium chemical reduction to formazan by NADPH/NADH of living cells in the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay.

The assay was performed following the manufacturer's protocol (Promega). Briefly, after thawing the CellTiter 96® AQ_{ueous} One Solution Reagent for 60 minutes at room temperature (or, when needed urgently, ten minutes in a water bath at 37ºC), cell cultures were well mixed and 100 µl of each culture was taken to perform the assay. Then, 20 µl of the reagent was added to each well of the 96-well assay plate containing the 100 µl samples. We incubated the plate for 2–2.5 hours at 37ºC in a humidified, 5% CO2 atmosphere. After that, the absorbance at 492 nM (using a reference absorbance at 620 nm) was recorded using a Labsystems Multiskan MS (Type 352) 96-well plate reader (Thermo Scientific, Barcelona, Spain).

12. Uptake Measurements

Rapid filtration method

The uptake of nucleosides, hypoxanthine, the organic cation model (MPP+), L-carnitine and AZT into the Molt-4 cell line, PBMCs and PHA-stimulated PBMCs was measured using a rapid filtration method adapted from a technique previously characterized by Pastor-Anglada's group at the University of Barcelona (Mercader et al., 1996). Cells were washed and resuspended in either a NaCl (to measure Na^+-dependent and Na^+-independent transport) or a choline chloride buffer (to measure Na^+-independent transport alone), as previously reported. Uptake assays were started by mixing cell suspensions with an equal volume of buffer, supplemented with a radionucleoside ([^{3}H]uridine, [^{3}H]cytidine or [^{3}H]guanosine), [^{3}H]hypoxanthine, [^{3}H]MPP+, [^{3}H]L-carnitine or [^{3}H]AZT at a specific activity and D-[1-^{14}C]mannitol (at a specific activity ranging from 900 to 3500 dpm/pmol) as an extracellular marker. Moreover, substrate(s) and/or inhibitor(s) of both nucleoside or organic cation transport were added to the buffer when necessary. Nucleosides and the nucleobase hypoxanthine were routinely used at a concentration of 1 μM. The organic cation MPP+ was also used at 1 μM in this rapid filtration method but commonly at 12.5 nM when using *"Short-Time" Uptake Measurements* (see next section of *Uptake Measurements*). The NRTI AZT was used at 2 μM. When the selected incubation times (from 30 seconds to 10 minutes) had elapsed, 125 μl aliquots (containing ~1.25 x 10^6 cells) were taken and added to ice-cold 0.4-mL needle Eppendorf tubes, containing an upper buffer phase (either NaCl or choline chloride one), an intermediate oil layer (dibutylphthalate/bis-3,5-trimethylhexyl)phthalate [3:2, vol/vol]), and a 10% $HClO_4$/25% glycerol solution at the bottom. The tube was immediately centrifuged (15000 g for 60 seconds), thus enabling the cells to be separated from the incubation medium and pelleted into the $HClO_4$ layer (lysing solution). To ensure that pelleted cells were fully recovered for radioactivity counting, the tubes were blade cut at the oil layer level, releasing the bottom part into scintillation counting vials. Double counting permitted discrimination between the transported substrate (a tritiated [^{3}H]nucleoside) and the extracellular marker (D-[1-^{14}C] mannitol). Protein in the transport mixture was measured by the Bradford method (Bio-Rad, Hercules, CA). Linearity was determined in time-course experiments with radiolabelled substrates in those cells. In our experiments with primary lymphocytes, the uptake linearity of the substrates was maintained for at least 5 minutes for uridine and MPP+, and for 10 minutes for L-

carnitine. Therefore, PBMCs were routinely incubated for 2 (uridine and MPP+) or 5 minutes (L-carnitine) in the uptake buffer.

Transport in cell monolayers

Transport studies of the MCF7 cell line and the MDMs were carried out on 24-well plates (35 mm tissue culture dishes) as described previously (Soler et al., 2003) by incubating semiconfluent cell monolayers for 10 minutes in an uptake buffer of choline chloride or sodium chloride supplemented with radiolabelled 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 after 2 minutes (for [^{3}H]uridine, [^{3}H]cytidine, [^{3}H]hypoxanthine and [^{3}H]AZT) and 10 minutes (for [^{3}H]MPP+) 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). Cells were lysed in 0.5 ml of 0.5% Triton X-100. Subsequently, 0.4 ml of cellular extract was used for radioactivity counting and the rest for protein determination following the Bradford technique (Bio-Rad protein reagent; Bio-Rad Laboratories, Madrid, Spain). Concentrative (Na^+-dependent) uptake was calculated as the difference between transport rates in the presence of Na^+ and in the absence of Na^+ (choline medium). The relative contribution of the CNT3 transport system in MDMs (which expressed both CNT2 and CNT3) was assessed by performing [^{3}H]cytidine uptake in the absence or presence of 1 mM guanosine (only transported by the purine-preferring transporter CNT1 – not expressed in MDMs – and CNT3).

"Short-time" uptake measurements

To determine the IC_{50} values of the NRTIs and to assess which of the NRTIs could be substrates of hOCTs, [^{3}H]MPP+, [^{3}H]3TC, [^{3}H]ABC and [^{3}H]AZT uptake in CHO-hOCTs and/or CHO-pcDNA5 (empty vector) cells were measured after 1-second (MPP+, ABC and AZT) and 15-seconds (3TC) incubations using a *"short-time" uptake protocol* previously established at Dr. Koepsell's laboratory (Lips et al., 2005).

As shown in *Figure 21*, after detaching cells with a soft EDTA/HEPES/NaHCO3 buffer (0.02%/10 mM/28 mM) and resuspending them in a transport solution (1X phosphate-buffered saline -PBS- with 0.5 mM $MgCl_2$ and 1 mM $CaCl_2$, pH 7.4) at 10^8 cells/ml, 90 μl (9 x 10^6 cells) was placed at the bottom of four 2-ml tubes (Sarstedt AG &

Co., Nümbrecht, Germany) and shaken in a water bath at 37°C. The uptake measurement was then made tube by tube: 10 µl of radioactive solution (containing the appropriate concentration of the corresponding substrate or inhibitors) was placed on the inner wall of each tube approximately 1 cm above the cells. Uptake measurement was started by vortexing the tube, enabling the radioactive solution and the cells to be mixed, then bringing incubation to an immediate stop with 1 ml of stop buffer (cold 1X PBS plus 100 µM quinine solution).

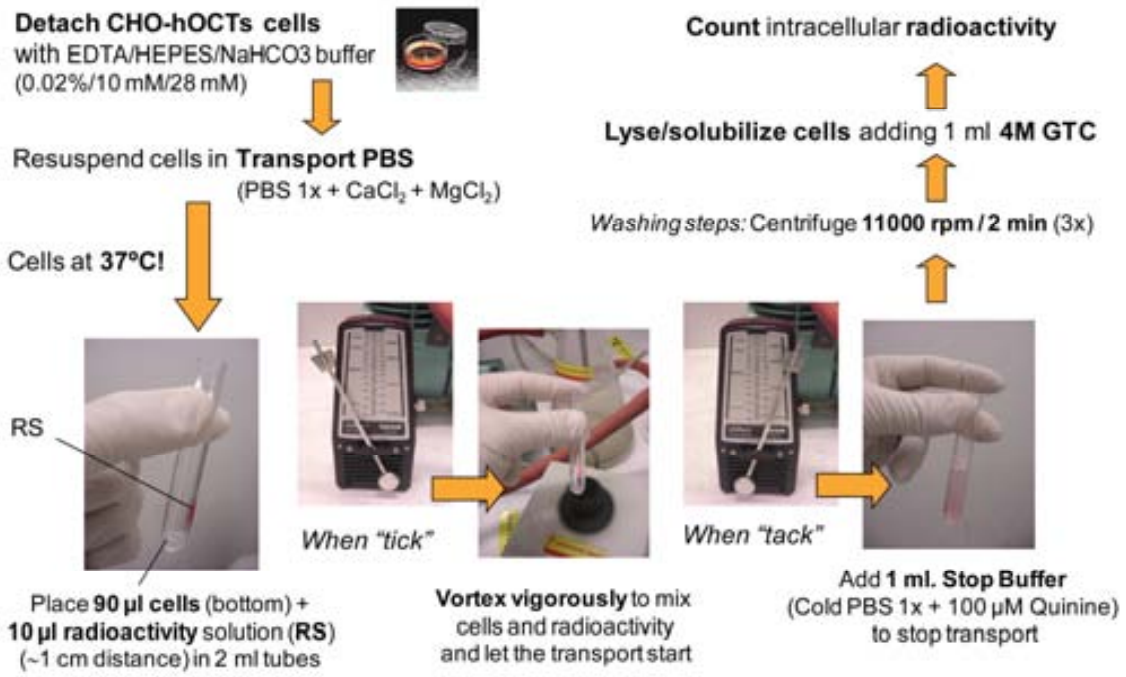


Figure 21. Schematic protocol for *1-second* uptake measurements performed in CHO-hOCT cells with [^{3}H]or [^{14}C] labelled substrates. The protocol for 15-second uptake measurements for [^{3}H]3TC was the same but applied a 15-second incubation with a timer instead of a metronome.

The incubation time was determined using a metronome for 1-second measurements or a timer for 15-seconds measurements. After two centrifugation/washing steps with stop buffer, the cells were lysed and solubilized with 200 µl guanidine thiocyanate (4 M), mixed with 2 ml scintillation liquid, and put into the scintillation counter to determine the levels of intracellular radioactivity. The advantage of performing this short-time uptake measurement lies in the reduction of the passive diffusion of the radiolabelled substrates used (MPP+, 3TC, ABC, or AZT). For time course measurements, we used the same procedure as described above, with incubation times extended to three

minutes. To measure uptake at 0-seconds incubation, ice-cold stop solution was added to the cells first, and radioactive substrates were added thereafter.

Tracer uptake experiments in X. laevis oocytes expressing hOCTs

Oocytes of *Xenopus laevis* were prepared as described previously (Arndt et al., 2001). Briefly, oocytes were defolliculated with collagenase A and stored for several hours in Ori buffer [5 mM 3-(N-morpholino)propanesulfonic acid-NaOH, pH 7.4, 100 mM NaCl, 3 mM KCl, 2 mM CaCl2, and 1 mM MgCl2] containing 50 mg/l gentamycin. Before cRNA injection, the oocytes were incubated for 5–15 minutes in hyperosmolar Ori buffer (130 mM NaCl). Oocytes expression vectors (pOG2 or pRSSP) containing hOCT1 or hOCT2 were linearized and sense cRNAs were transcribed using T7 or SP6 RNA polymerase as described earlier (Veyhl et al., 1993). Then, oocytes were injected with 10 ng hOCT1 or hOCT2 cRNAs in a volume of 50 nl of H2O. Non-injected oocytes were used as controls. After injection with cRNA, oocytes were stored for 3 to 5 days in Ori buffer at 16°C. The uptake of radioactively labelled compounds into *X. laevis* oocytes was measured as described previously (Arndt et al., 2001). rOCT2-expressing and non-injected control oocytes from the same batch were incubated at 19°C in Ori buffer containing 12.5 nM [^{3}H]MPP+, 2 µM [^{3}H]ABC, 0.10 µM [^{3}H]AZT or 156.25 nM [^{3}H]3TC with and without 1 mM of the non-labelled OCT-substrate (MPP+) or 2 mM of the non-labelled NRTI (ABC, AZT or 3TC). Uptake was stopped after 30 minutes and the oocytes were washed with ice-cold Ori buffer. The amount of substrate taken up into the oocytes was determined by liquid scintillation counting.

Kinetic and statistical analysis

For the IC_{50} and K_i analyses, data were fitted to the Hill equation:

$$(1)\ V = \frac{V_o}{\left[1 + \left(\frac{I}{IC_{50}}\right)^n\right]},$$

where V is the uptake of [^{3}H]MPP+ (1 second) or [^{3}H]3TC (15 second) in the presence of the inhibitor (a specific NRTI), V0 is the uptake of [^{3}H]MPP+ (1 second) or [^{3}H]3TC (15 seconds) in the absence of the inhibitor, I is the inhibitor concentration (nanomolar), and

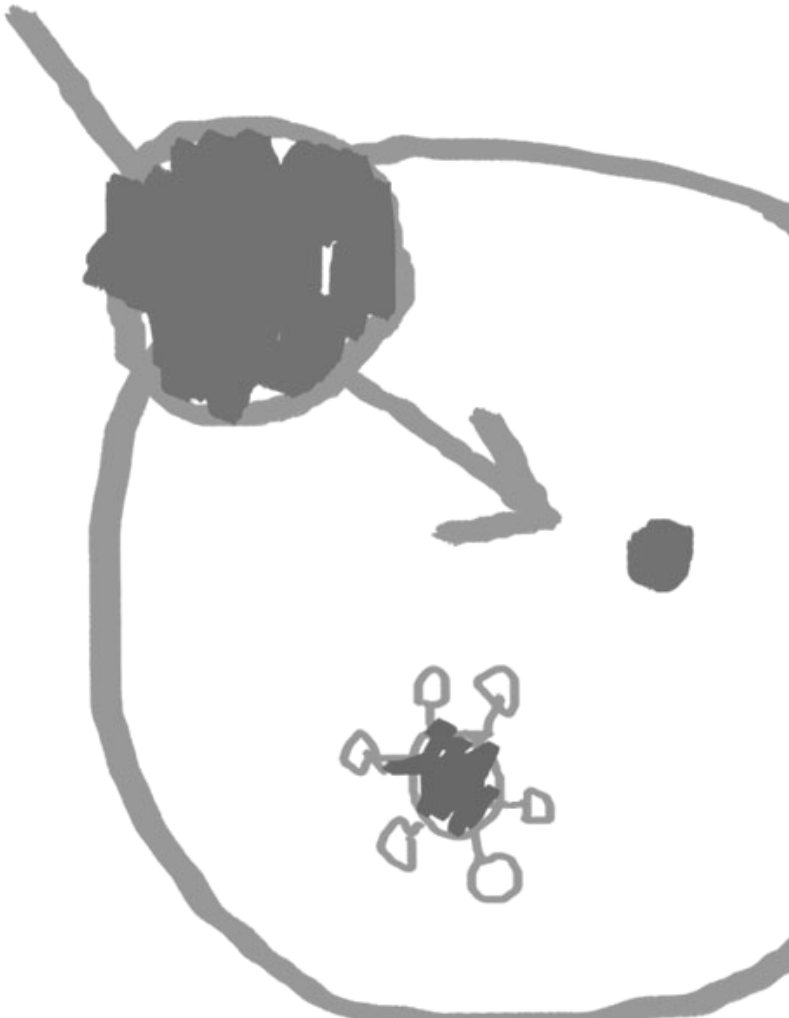
n is the Hill coefficient. The kinetics graphs (uptake velocity of 3TC *versus* substrate concentration) were fitted using the classic Michaelis-Menten equation:

$$(2)\ V_o = \frac{V_{\max} \cdot [S]}{K_m + [S]},$$

where V_0 and V_{max} represent initial and maximal transport velocities, respectively (in picomoles per milligram of protein per minute), [S] is the initial substrate concentration (micromolar), and Km is the substrate concentration at half-maximal transport velocity (micromolar). We also used the equation to calculate the quotient V_{max}/K_m, which represents the transport efficiency.

Paired and unpaired Student's *t* tests and the analysis of variance test (ANOVA) were used for the statistical comparison of experimental data. We also compared which of the fits for the 3TC inhibition curves (two-binding-site competition *versus* one-binding-site competition) was best. The analysis of variance test (ANOVA) was performed with Statgraphics 4.0 software (Statpoint, Inc., Herndon, VA, USA) and the rest of the analyses were carried out using GraphPad Prism 4.0 and 5.0 software (GraphPad Software Inc., San Diego, CA, USA).

Results



Chapter I. 3'-Azido-2',3'-Dideoxythymidine (AZT) uptake mechanisms in T lymphocytes

ABSTRACT

The nucleoside reverse transcriptase inhibitors (NRTIs) make up a family of antiretroviral drugs widely used in the treatment of human immunodeficiency virus type 1 (HIV-1) infection. Since nucleoside transporters (hCNTs and hENTs), encoded by the SLC28 and SLC29 gene families respectively, are known to be involved in the uptake of a variety of nucleoside analogues used in anticancer treatment, we examined whether SLC28 and SLC29-encoded proteins contribute to the entry of these NRTIs into the human leukemic T cell line Molt-4. *Cis*-inhibition experiments demonstrated that nucleoside transporters play a negligible role in antiviral drug uptake. Moreover, the previously identified 3'-azido-2',3'-dideoxythymidine (AZT) carriers, known as organic anion transporters (hOATs, members of the SLC22 gene family), have not been detected in T cells, neither functionally nor at the mRNA level, thus ruling out a role for hOATs in antiviral drug uptake in these cells. Nevertheless, the data provided here argue against the hypothesis of simple diffusion across the plasma membrane as the unique mechanism of AZT uptake. Actually, this pyrimidine derivative seems to have a temperature sensitive route of entry, a finding which – along with the evidence that AZT inhibits its own uptake, and that its transport into phytohemagglutinin-stimulated peripheral blood mononuclear cells is up-regulated – strongly supports the idea that AZT uptake into T cells is associated with a mediated and regulated transport mechanism.

Characterization of nucleoside transporter expression and activity in the Lymphoblastic T cell line, Molt-4

First, in order to determine the expression pattern of nucleoside transporters in the Molt-4 cell line, a real-time quantitative PCR was performed with the hGUSB gene as an internal control (*Figure 22a*). The expression of hENT1, hENT2, hCNT2 and, to a lesser extent, hCNT1 was detected. hCNT3 transcripts were not detected in Molt-4 cells. In addition, to characterize the main mechanisms implicated in nucleoside uptake in Molt-4 cells, the time-course accumulation of uridine (a substrate for all known nucleoside plasma membrane transporters, hENT1, hENT2, hCNT1, hCNT2 and hCNT3) and guanosine (a substrate for all but hCNT1 proteins) was monitored, either in the presence (i.e. with operative CNT-type carriers) or in the absence of sodium (CNT-type transporters inoperative) in the medium (*Figures 22b and 22c*).

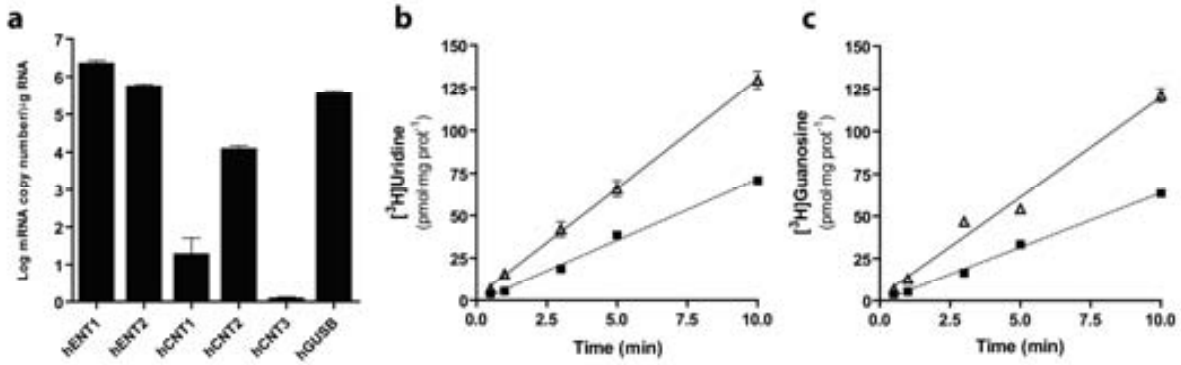


Figure 22. Characterization of Nucleoside Transporters in Molt-4 cells. *(a)* mRNA expression of Equilibrative (hENT1 and hENT2) and Concentrative Nucleoside Transporters (hCNT1, hCNT2 and hCNT3) determined by Quantitative real-time PCR. Results are expressed as log mRNA copy number per microgram of RNA, as the mean ± SEM of at least six experiments performed in duplicate. Human β-glucoronidase (hGUSB) was used as a control gene. Time course of *(b)* [3H]Uridine (substrate for all hNTs) and *(c)* [3H]Guanosine (substrate for all hNTs but hCNT1) uptake in Molt-4 cell line. Cells were incubated with 1 μM nucleoside, either in a choline chloride medium (■) or in a NaCl medium (Δ). Incubation was stopped at 30 s and 1, 3, 5 and 10 min.

Uptake was linear during the chosen experimental times and significant Na^+-dependent uridine and guanosine transport was detected. This component was associated exclusively with the hCNT2 expression. This association was based upon the lack of hCNT3 expression and the experimental evidence that Na^+-dependent uridine uptake could not be inhibited by cytidine (not shown), thus discounting a role for hCNT1.

Inhibition of hENT1 and hCNT2-mediated uridine uptake by several antiviral drugs in Molt-4 cells

To establish whether antiviral nucleoside-derived drugs interact with hENT1, *cis*-inhibition of NBTI-sensitive uridine transport by AZT and other nucleoside analogues currently used in HAART was monitored (*Figure 23*). Of the molecules tested, only AZT at 1 mM significantly inhibited hENT1 function. One mM TDF also appeared to induce a slight but non-significant inhibition, which is unlikely to be associated with TDF transport by hENT1, because TDF is a monophosphorylated derivative and nucleoside transporters, in principle, cannot take up nucleotides (Kong et al., 2004). The putative interaction of these antiviral drugs with the hCNT2-related transport activity was also studied, by determining their ability to inhibit Na^+-dependent guanosine transport into cells (*Figure 23b*). AZT significantly inhibited guanosine uptake via hCNT2, although this inhibition was slight (20%), whereas the purine-nucleoside derivative ddI significantly inhibited Na^+-dependent guanosine transport.

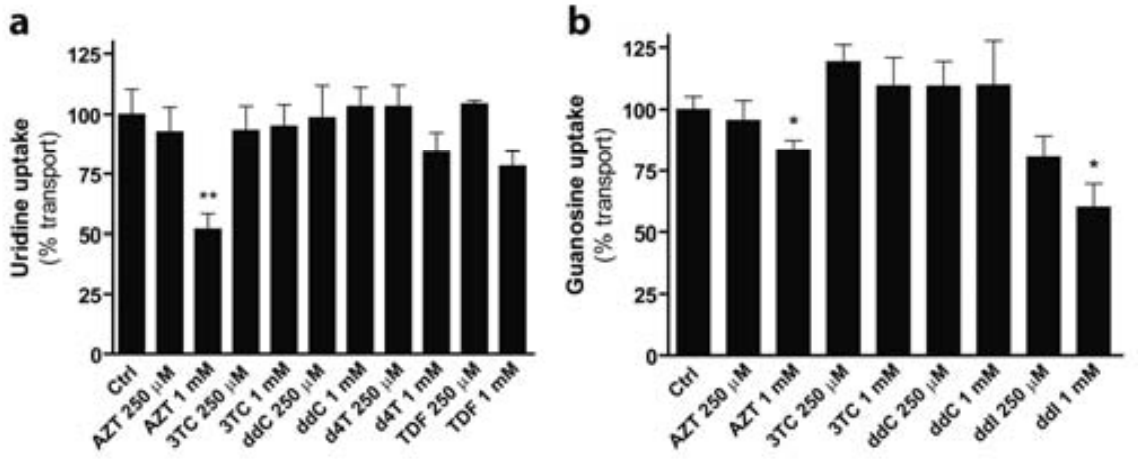


Figure 23. Inhibition of hENT1 and hCNT2-mediated uridine uptake by several antiviral drugs in Molt-4 cells. *(a)* The hENT1 dependent uptake of 1 µM [^{3}H]uridine was calculated by subtracting 1 µM NBTI-insensitive uridine uptake from the uridine transport in a choline chloride medium. *(b)* The hCNT2-dependent transport of 1 µM [^{3}H]guanosine was measured in a NaCl medium plus 10 µM dipyridamole. Transport was measured over a 2 minute incubation period, either in the absence (Ctrl) or the presence of two concentrations (250 µM and 1 mM) of competing non-radioactive antiviral drugs. Data are expressed as a percentage of uptake versus the control value (absence of inhibitor). Results are expressed as the mean ± SEM of four independent experiments performed in triplicate. Statistical significance was assessed using a Student's *t*-test: ctrl versus antiviral drug (*$p < 0.05$, **$p < 0.01$).

Characterization of AZT Transport in Molt-4

Under the same experimental conditions as in *Figure 22*, radiolabelled AZT time-course accumulation in Molt-4 cells was performed (*Figure 24a*). The pyrimidine derivative accumulation was also linear, but one order of magnitude lower than that found for natural nucleosides. Although a minimal accumulation appeared to also be dependent on sodium in the uptake medium, this was not statistically relevant. A complementary approach was followed to identify the putative transporter implicated in AZT uptake into T cells. Therefore, AZT uptake in the absence of sodium (the main route for AZT entry into cells, as shown in *Figure 24a*) was directly measured in the presence of a series of molecules, some of them known to be transported by hENT proteins (nucleosides such as uridine and guanosine) – well characterized as pharmacological blockers of hENTs (dipyridamole) – other antiretroviral nucleoside-derived drugs (3TC and ddI), the nucleobase hypoxanthine, a nucleoside analogue AMPK activator (AICAR) (Campas et al., 2003), a nucleoside-derived antiviral used in hepatitis C treatment (ribavirin) (Maag et al., 2001), but also non-nucleoside compounds such as PAH and probenecid – common substrates with different specificities of the four OAT carriers described so far (Koepsell and Endou, 2004).

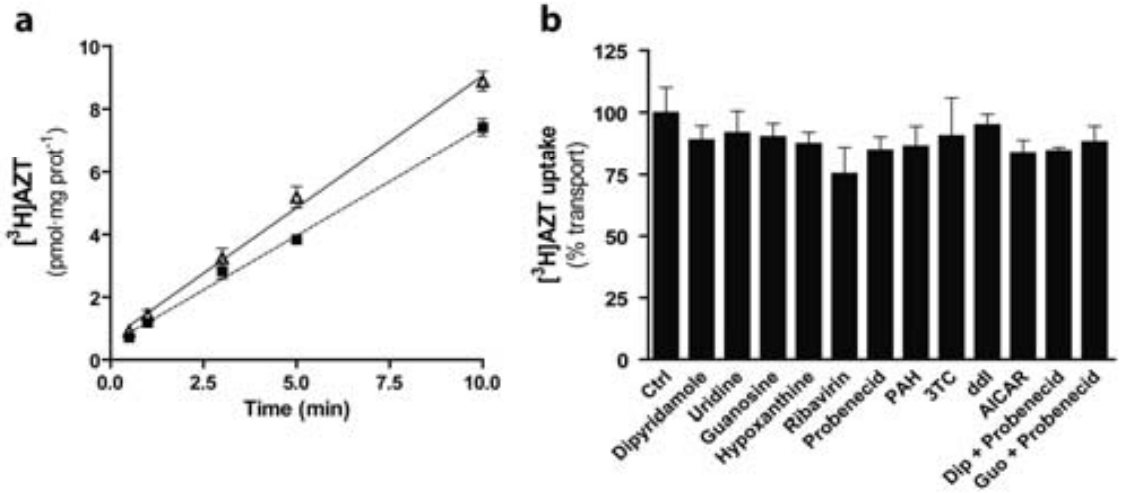


Figure 24. Characterization of AZT transport in Molt-4 cells. *(a)* Time course of [^{3}H]AZT uptake by Molt-4 cell line. Cells were incubated with 1 μM AZT, either in a choline chloride medium (■) or in a NaCl medium (Δ). Incubation was stopped at 30 seconds, and 1, 3, 5 and 10 minutes *(b)* The equilibrative uptake of 2 μM [^{3}H]AZT was monitored after 2 minutes incubation in a choline chloride medium either in the absence (Ctrl) or the presence of competing substrates. Dipyridamole was used at 10 μM, uridine and guanosine were used at 100 μM, whereas hypoxanthine, ribavirin, probenecid, PAH, 3TC, ddI and AICAR were all used at 1 mM. Data are expressed as a percentage of uptake versus the control value (absence of inhibitor). Results are expressed as the mean ± SEM of three to five independent experiments performed in triplicate. Statistical significance was assessed using Student's t-test.

In addition, combined inhibitory experiments including nucleoside transporters and organic anion transporter inhibitors were performed (*Figure 24b*). None of them significantly inhibited AZT uptake at the concentrations used.

hENT2-mediated AZT and hypoxanthine uptake in the MCF7 cell line and Expression of hOATs in Molt-4, T cells and macrophages

The possibility that AZT could be transported by hENT2, as shown previously when the recombinant hENT2 was expressed in *Xenopus laevis* oocytes (Yao et al., 2001), was not consistent with the lack of inhibition of AZT accumulation by hypoxanthine (*Figure 3b*), a nucleobase known to be a hENT2 substrate (Yao et al., 2002). However, for a better analysis of this possibility, we monitored AZT transport in the non-lymphocyte cell line MCF7, which expresses significant hENT2-related activity. Equilibrative hypoxanthine uptake was measured in parallel. As shown in *Figure 25*, hypoxanthine transport was not inhibited by NBTI (a hENT1 specific blocker at the concentration used), but was dramatically inhibited by dipyridamole (a pharmacological blocker of both hENT1 and hENT2), thus suggesting that carrier-mediated hypoxanthine transport was almost exclusively due to hENT2. Under the same experimental conditions, AZT transport was unaffected by these blockers, thus ruling out a role for hENT2 in AZT uptake (*Figure 25*).

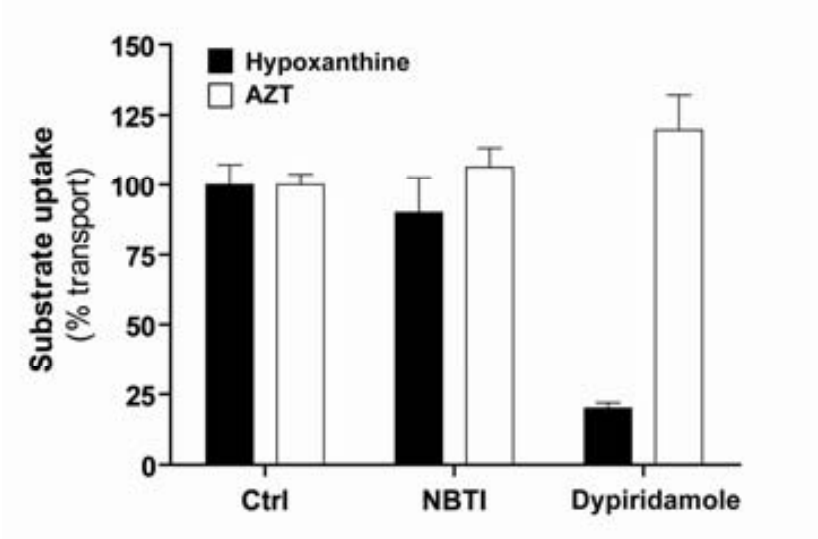


Figure 25. hENT2-mediated AZT and hypoxanthine uptake in MCF7 cell line. The equilibrative uptake of 2 μM [^{3}H]AZT (white bars) and 1 μM [^{3}H]hypoxanthine (black bars) was measured after 2 minutes incubation in a choline chloride medium either in the absence (Ctrl) or the presence of equilibrative nucleoside transporters inhibitors NBTI (1 μM) and dipyridamole (10 μM). A single representative experiment (performed in quadruplicate) is shown.

The lack of inhibition of AZT transport by known hOAT substrates, such as probenecid and PAH (*Figure 24b*), was consistent with a lack of expression of any of the hOAT proteins in T cells. hOATs are known to be suitable candidates for mediating AZT uptake and all isoforms can transport this nucleoside-derived drug with relatively high apparent affinity, at least when measured in cell systems in which the recombinant proteins were heterologously expressed (Takeda et al., 2002). *Figure 26* shows an RT-PCR experiment to identify whether any of the cloned hOATs are expressed in Molt-4 cells, blood derived macrophages and both PHA-stimulated or unstimulated primary PBMC or purified $CD4^+$ T lymphocytes. A human kidney sample was used as a positive control, since the four hOATs are expressed in renal tubular epithelia (Sekine et al., 2000). mRNAs for the four transporters – hOAT1, hOAT2, hOAT3 and hOAT4 – were amplified from the kidney sample (*Figure 26*). Under the same conditions, none of these mRNA were detected in any of the T cells or macrophages analyzed (*Figure 26*), strongly suggesting that hOAT proteins are not expressed in T cells, which accords with the kinetic data reported above.

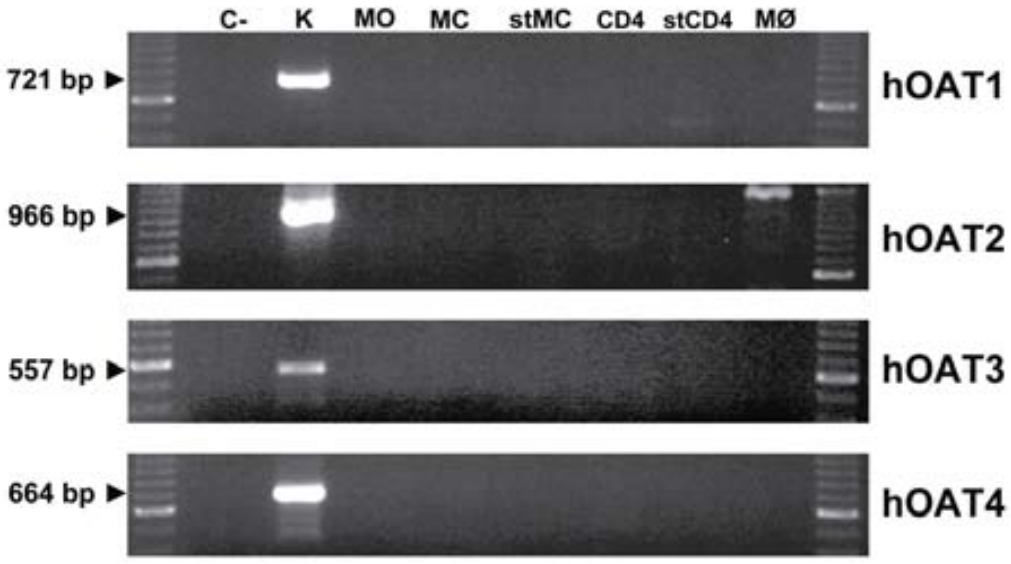


Figure 26. Expression of human organic anion transporters (hOATs) 1–4 in Molt-4, T cells and macrophages. An RT-PCR was used to amplify hOAT1, hOAT2, hOAT3 and hOAT4 mRNA from Molt-4 (MO), peripheral blood mononuclear cells (MC), PHA-stimulated peripheral blood mononuclear cells (stMC), CD4+ lymphocytes (CD4), PHA-stimulated CD4+ lymphocytes (stCD4) and macrophages (MØ). Kidney (K), was used as a positive control for RT-PCR amplification. Representative agarose gels showing the amplification of cDNA fragments of the anticipated molecular weights are shown.

Temperature Dependence of AZT and Uridine Uptake in Molt-4 Cell Line

To further determine whether AZT accumulation into T cells is protein-mediated or actually the result of simple diffusion across the plasma membrane, the temperature dependence of AZT uptake into Molt-4 cells was determined (*Figure 27*). Nearly 80% of uridine uptake in a Na-free medium (hENT-mediated) was eliminated when cells were incubated at 4°C instead of room temperature (20-22°C). Inhibition of hENT1 using NBTI showed nearly 20% of the basal uridine uptake, thus reflecting the low occurrence of hENT2-related transport activity in Molt-4 cells. This residual transport rate was still temperature sensitive and was completely eliminated when dipyridamole was used as a blocker (inhibition of both hENT1 and hENT2) (*Figure 27*).

When AZT was used as substrate, an equally dramatic sensitivity to temperature as that found for uridine uptake was observed (*Figure 27*). Moreover, in agreement with the inhibition studies described above, neither NBTI nor dipyridamole significantly modified AZT uptake at either temperature.

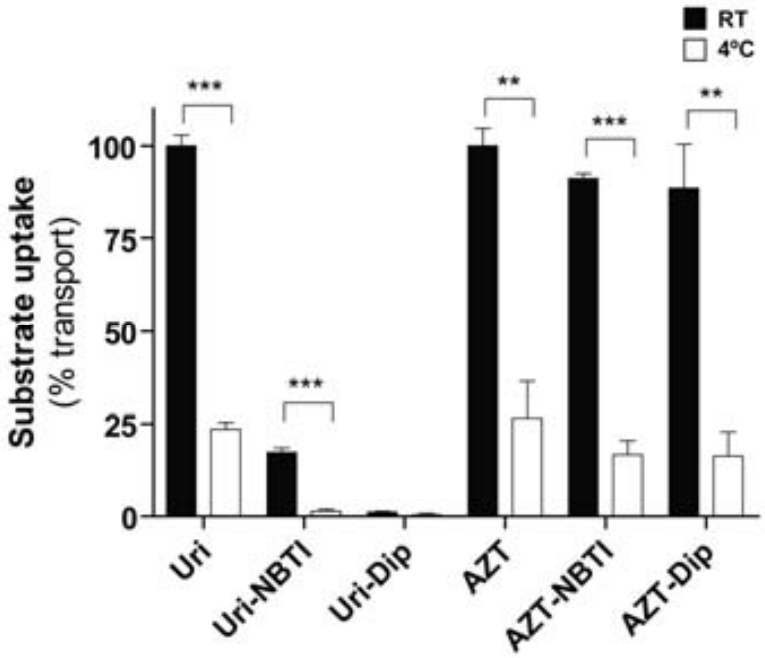


Figure 27. Temperature dependence of AZT and uridine uptake in the Molt-4 cell line. The equilibrative uptake of 2 μM [^{3}H]AZT or 1μM [^{3}H]uridine was measured in a choline chloride medium at room temperature (RT) (black bars) and 4°C (white bars), in the absence or the presence of 1 μM NBTI or 10 μM dipyridamole. Data are expressed as a percentage of the uptake versus the control value (uridine or AZT at room temperature in the absence of inhibitor). Results are expressed as the mean ± SEM of three independent experiments performed in triplicate. Statistical significance was assessed using a Student's *t*-test: RT vs. 4°C (**$p < 0.01$, ***$p < 0.001$).

Inhibition of [^{3}H]AZT Uptake by Increasing Concentrations of Non-Labelled AZT

To obtain further evidence of the occurrence of a mediated process in AZT uptake into T cells, we monitored the *cis*-inhibition of tritiated AZT uptake by nonradiolabelled AZT. This experiment is based upon the fact that simple diffusion cannot be inhibited, whereas a mediated process can. As shown in *Figure 28*, AZT inhibited tritiated AZT transport into Molt-4 cells. At the highest AZT concentrations used (2–5 mM) inhibition appeared to be maximal, although some residual transport was still present, thus suggesting the presence of a AZT-insensitive component of uptake, probably associated with the simple diffusion of the drug across the plasma membrane.

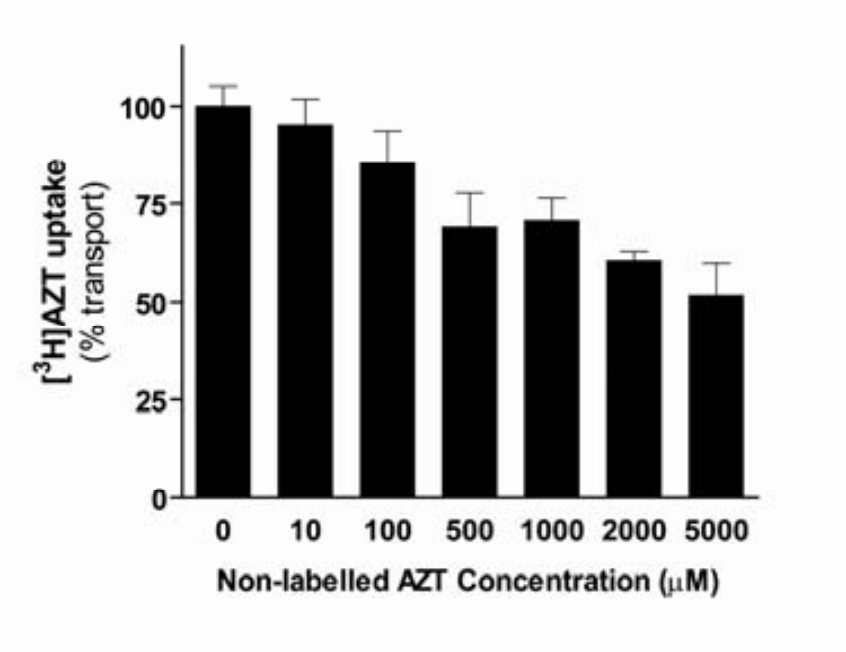


Figure 28. Inhibition of AZT uptake in a choline chloride medium by increasing concentrations of non-labelled AZT. The equilibrative uptake of 2 μM [^{3}H]AZT was measured in a choline chloride medium either in the absence (Ctrl) or the presence of increasing non-labelled AZT concentrations (10 to 5000 μM). Data are expressed as a percentage of the uptake *versus* the control value (no additional AZT). Results are expressed as the mean ± SEM of five independent experiments performed in triplicate. Statistical significance was assessed using an analysis of variance test: F-ratio 7.47, p-value 0.0001.

Azidothymidine (AZT) and Uridine Uptake in PBMC from Healthy Donors

Finally, we decided to characterize AZT transport in PBMC from four healthy donors. The drug uptake was measured either in the presence or the absence of sodium (*Figure 29*). Transport in the absence of this cation was also checked for its sensitivity to the known hENT-type carrier inhibitors NBTI, and dipyridamole. This experiment was

performed both under basal conditions and in PHA-stimulated PBMC. As shown in *Figure 29*, AZT uptake was Na^+-independent and mostly insensitive to hENT inhibitors, thus ruling out, in accordance with the findings for Molt-4 cells, the involvement of this family of nucleoside transporters in AZT accumulation. Interestingly, stimulation resulted in a significant increase in AZT uptake rates, a fact that can also be observed in the uridine control (*Figure 29*). This finding further supports the notion that the entry of this nucleoside analogue into T cells is actually a mediated, and probably a regulated, process.

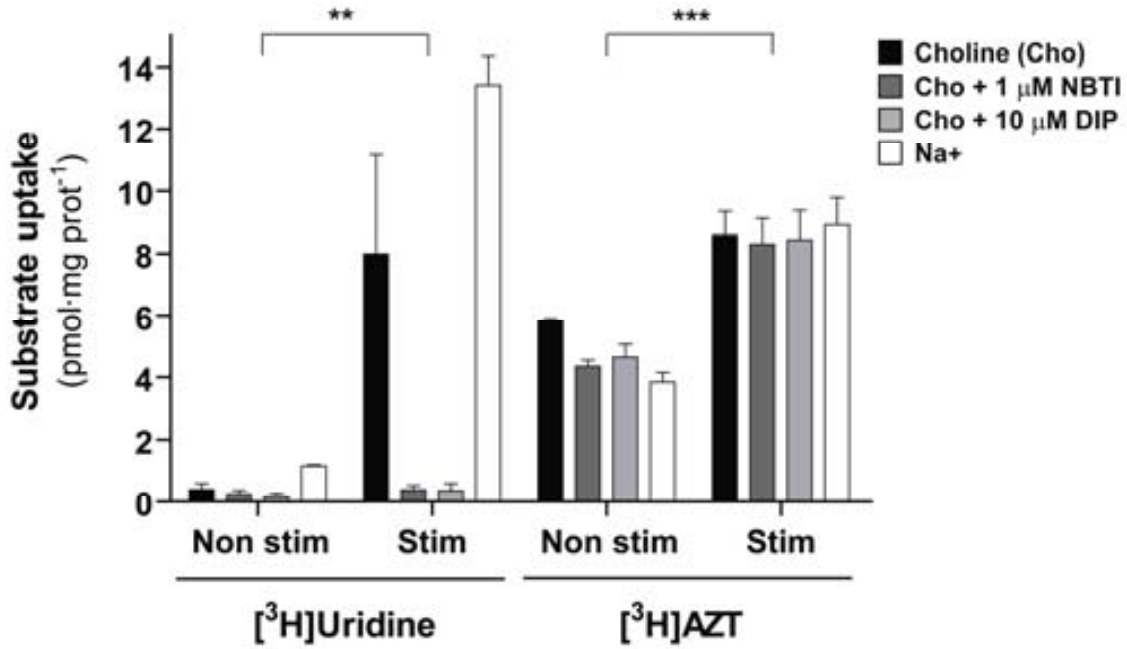


Figure 29. Uridine and AZT uptake in peripheral blood mononuclear cells (PBMCs). The equilibrative (black bars), NBTI inhibited (dark gray bars), dipyridamole inhibited (light gray bars) and concentrative (white bars) uptake of 1 μM [3H]uridine and 2 μM [3H]AZT was measured either in a choline chloride or NaCl medium in PBMC cells from four independent healthy donors (performed in triplicate). The experiment was carried out with both resting and stimulated PBMCs. Results are expressed as the mean ± SEM of the four independent donors. Statistical significance between non stimulated and stimulated groups was assessed using an analysis of variance test: F-ratio 30.09, p-value 0.0027 for uridine uptake; F-ratio 36.76, p-value < 0.0001 for AZT uptake.

Chapter II. Expression and functionality of anti-HIV and anticancer drug uptake transporters in immune cells

ABSTRACT

Almost all drugs used in anti-HIV-1 and anticancer therapies require membrane proteins in order to enter cells and develop their proper activity. Nevertheless, little is known regarding the expression and activity of the 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 Nucleotide Transporters, hENT1–2), and SLC22 (Organic Cation Transporters, hOCT1–3 and hOCTN1–2). Both hENTs and hCNT2 were abundant in primary lymphocytes, with preferential activity for hENT1. A significant up-regulation in hENTs expression (100-fold) and activity (30-fold) was observed under stimulation of primary T lymphocytes. Conversely, monocytes, 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 hNTs, although up-regulation of hOCTs also occurred upon lymphocyte activation. Overall, the expression and activity of most of the studied transporters emphasizes their relevance with relation to anti-HIV and anticancer therapies. The identification of the transporter involved in each specific drug uptake in immune cells could help optimize pharmacological therapeutic responses.

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 human Nucleoside Transporters (hNTs) in lymphoblastoid T cell lines by examining the mRNA levels of both Concentrative (hCNT1–3) and Equilibrative Nucleoside Transporters (hENT1–2) (*Figure 30a*). hCNT2 was the most expressed concentrative isoform in all cell lines tested, being more frequently expressed in MT4, while hCNT1 and hCNT3 showed either low or negligible expression. Both hENTs showed a very high expression (between 10^5 and 10^7 mRNA copy number/μg RNA).

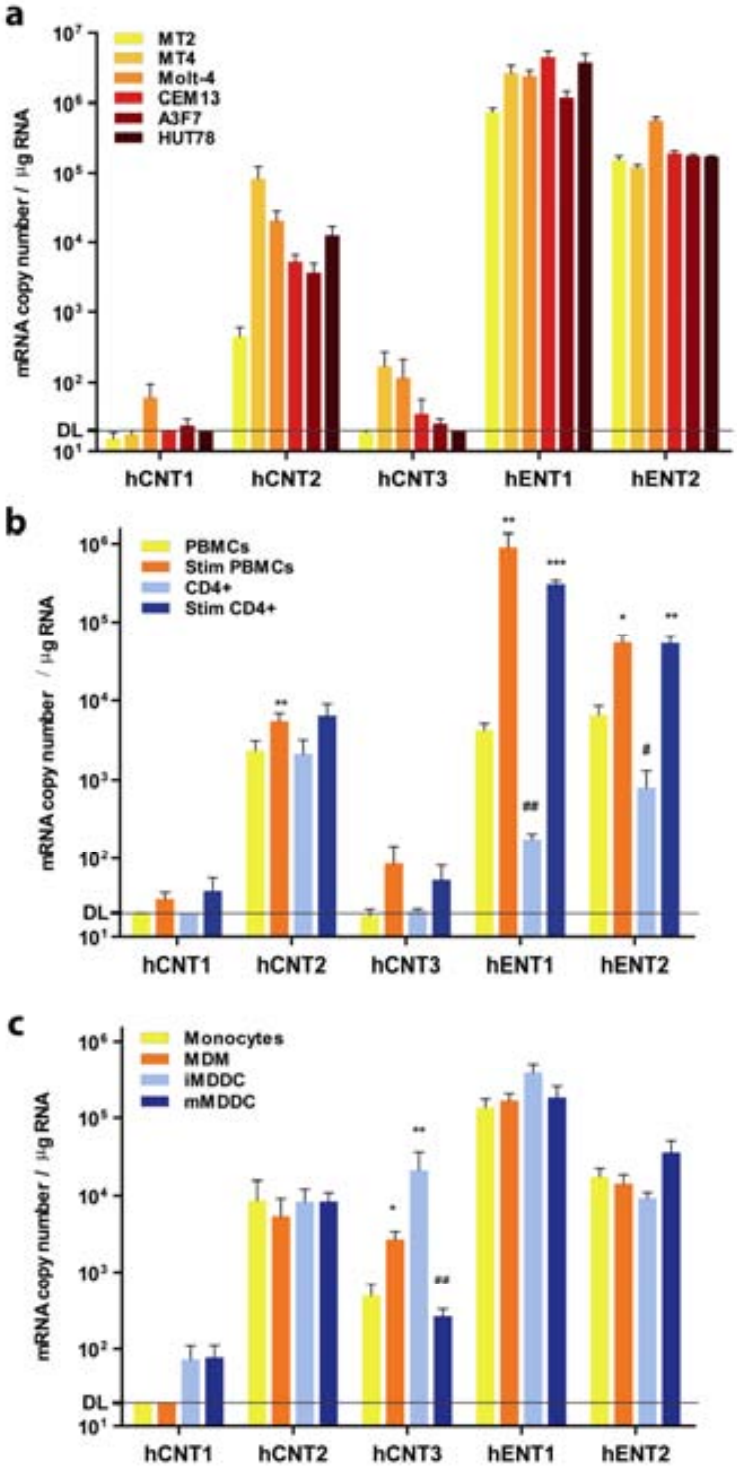


Figure 30. hCNT and hENT mRNA expression in T cell lines, primary lymphocytes, monocytes, MDMs and MDDC cultures. *(a)* mRNA expression of hNTs in six different T lymphoblastoid cell lines (MT2, MT4, Molt-4, CEM13, A3F7 and Hut78). *(b)* mRNA expression of hNTs in non-stimulated PBMCs (PBMCs) or purified $CD4^+$ T lymphocytes (CD4+) and three 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 $CD4^+$ (#, $p < 0.005$ and ##, $p < 0.01$) was assessed by paired Student's t test. *(c)* mRNA expression of monocytes, MDM and monocyte-derived dendritic cells, both iMDDC and mMDDC. Statistical significance between monocytes and MDMs/iMDDCs (*, $p < 0.05$; **, $p < 0.01$) and between iMDDCs and mMDDCs (##, $p < 0.01$) was assessed by paired Student's t test. The data represent the mean ± SEM of at least four independent experiments performed in duplicate. DL represents the theoretical detection limit of the technique.

We then studied the mRNA expression of hNTs in PBMCs and in purified $CD4^+$ T cells, either under basal conditions or after PHA stimulation of these cells. As shown in *Figure 30b*, similarly 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 non-stimulated PBMCs (about 10^3 copies/µg 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 $p < 0.005$, respectively). Moreover, the greatest difference between PBMCs and $CD4^+$ T cells with respect to 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 the *SLC28* and *SLC29* gene transporter families in monocytes, monocyte derived macrophages (MDMs) and dendritic cells (MDDCs) (*Figure 30c*). In contrast to PBMCs and $CD4^+$ T cells, monocytes showed a remarkably higher expression of hCNT3. Moreover, monocyte differentiation into MDMs and immature MDDCs (iMDDCs) produced a significant increase of hCNT3 expression ($p = 0.03$ and $p < 0.005$, respectively). As for T cell lines and primary lymphocytes, hCNT2 and both the hENTs isoforms showed a notable expression, with hENT1 being the most expressed transporter. The high expression rate of hENT1 and hENT2 in monocytes (about 10^5 and 10^4 copies mRNA/µg RNA, respectively) could explain the higher mRNA amounts of those transporters in PBMCs compared to $CD4^+$ T lymphocytes.

Focusing on the *SLC22* gene family, human Organic Anion Transporters (hOATs) were ruled out from this second study because, *as shown in Chapter I (Figure 26, page 92)*, they are not expressed in Molt-4, neither in primary T cells nor in macrophages. Nonetheless, we aimed to study the expression of human Organic Cation/Zwitterion Transporters (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 profiles than Nucleoside Transporters in T cell lines (*Figure 31a*). Importantly, none of the cell lines tested had detectable hOCT2 mRNA levels.

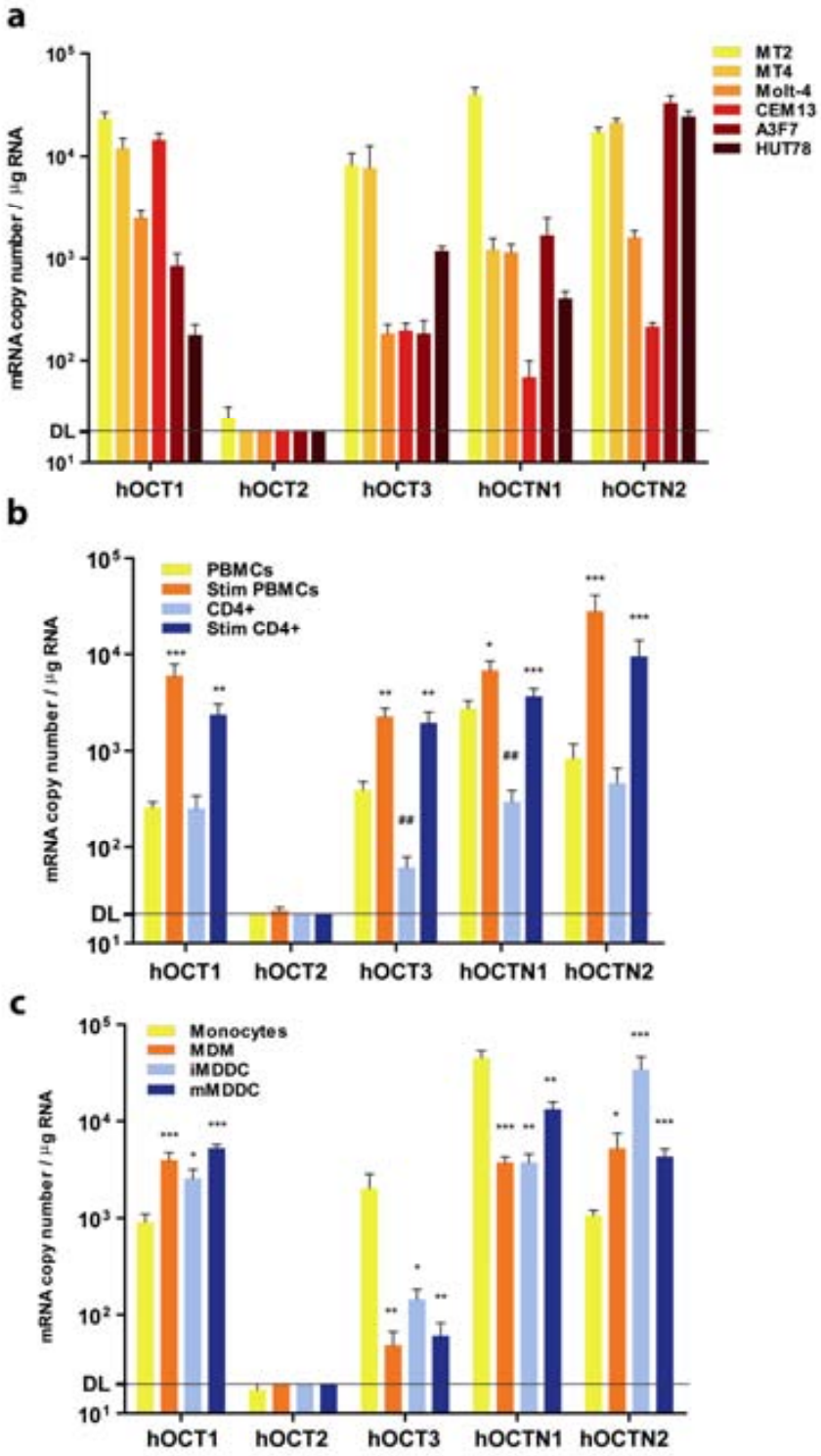


Figure 31. hOCT and hOCTN mRNA expression in T cell lines, primary lymphocytes, monocytes, MDMs and MDDC cultures. *(a)* mRNA expression of hOCTs and hOCTNs in six different T lymphoblastoid cell lines (MT2, MT4, Molt-4, CEM13, A3F7 and Hut78) *(b)* mRNA expression of hOCTs and hOCTNs in non-stimulated PBMCs (PBMCs) or purified $CD4^+$ T lymphocytes (CD4+) and three 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 monocyte-derived dendritic cells, both iMDDC and mMDDC. Statistical significance between monocytes and MDMs/iMDDCs (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) and between iMDDCs and mMDDCs (##, $p < 0.01$) was assessed by paired Student's *t* test. The data represent the mean ± SEM of at least six independent experiments performed in duplicate. DL represents the theoretical detection limit of the technique.

In non-stimulated primary PBMCs and $CD4^+$ T cell cultures (*Figure 31b*), 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 the primary lymphocytes tested. Under PHA stimulation, all hOCTs and hOCTNs previously expressed underwent 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 (*Figure 31c*), hOCTs and hOCTNs showed the most heterogeneous and cell-type dependent mRNA expression pattern. With regard to monocytes, they showed the highest mRNA expression of hOCT3 and hOCTN1. Monocyte differentiation into macrophages with M-CSF or to dendritic cells with GM-CSF/IL-4 seems to have an antagonistic effect on hOCT and hOCTN expression. While there is a statistically significant up-regulation for hOCT1 and hOCTN2 transcripts ($p < 0.05$ and $p < 0.001$, respectively), hOCT3 and hOCTN1 mRNA copy numbers decrease significantly. When iMDDCs were matured with LPS, the expression of hOCTN1 increased with respect to MDMs.

Protein Expression of hNTs and hOCT/Ns

Having studied the mRNA expression of hNTs and hOCT/Ns, and knowing that there might not be a 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 have on the transporter expression in PBMCs. As show in *Figure 32*, we analyzed both hCNTs and hENTs expression in cytospun PBMCs by immunocytochemistry developed by horseradish peroxidase (HRP). In those cells we found that the mRNA and the protein had the same expression profiles, with non-stimulated 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, the hENT2 up-regulation being the highest. Immunocytochemistry performed on MDMs cultured on coverslips showed a high expression of hCNT2, hCNT3 and both hENT isoforms (*Figure 33*), and a negligible expression of hCNT1 (data not shown).

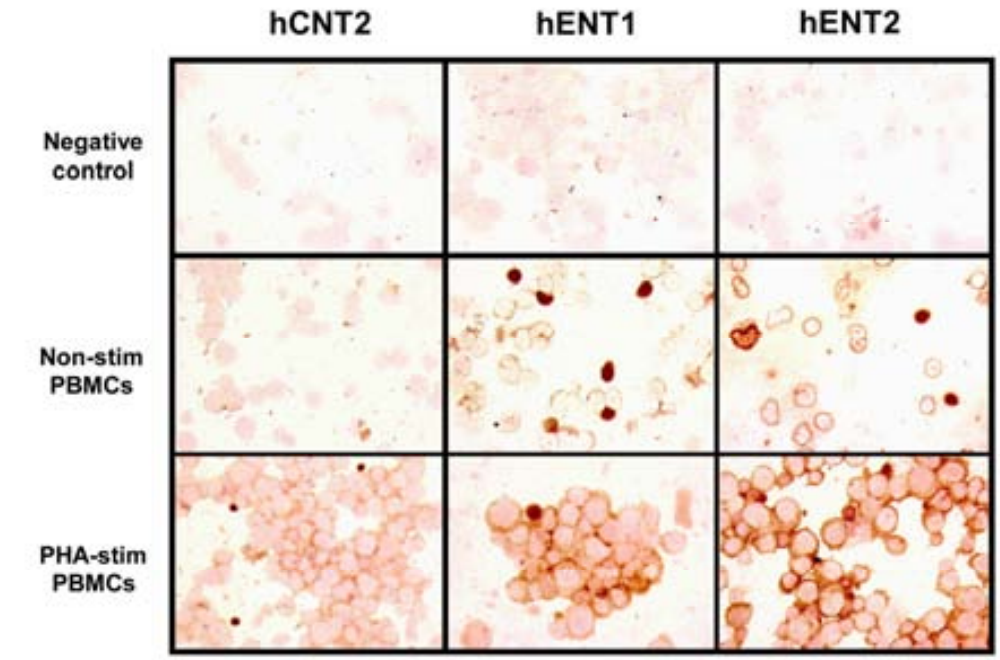


Figure 32. Immunocytochemistry of hCNT2 and hENTs in nonstimulated and PHA-stimulated PBMC cytospins. Cytocentrifuge preparations of PBMCs (nonstim PBMCs) or PHA-stimulated PBMCs (PHA-stim PBMCs) were fixed, and primary rabbit-specific polyclonal anti-hCNT2, hENT1 and hENT2 antibodies were used to detect protein expression. A secondary conjugate and a development of activity with a 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 cytospun preparations.

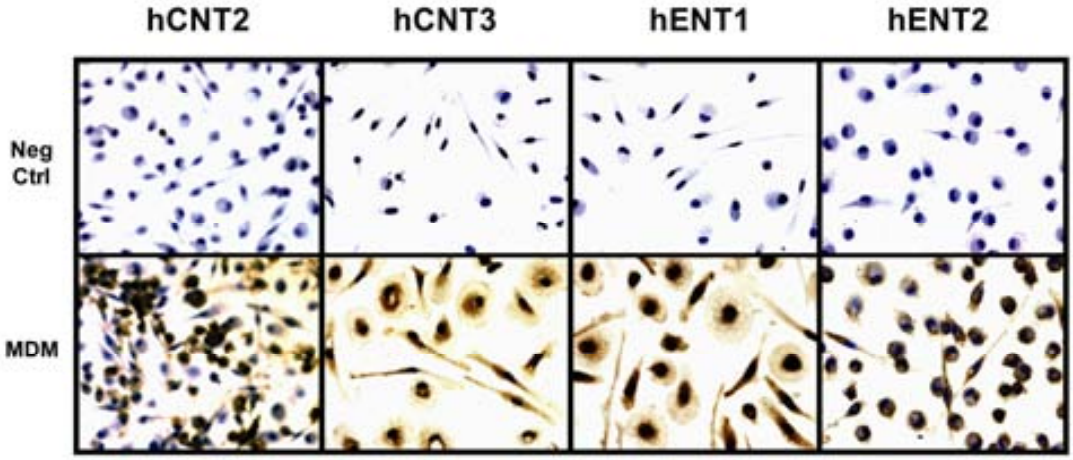


Figure 33. Immunocytochemistry of hCNT3 and hENTs in MDMs cultured on coverslips. Monocyte-derived macrophages were cultured in poly-Lysine-treated coverslips and fixed. Primary rabbit-specific polyclonal anti-hCNT3, hENT1 and hENT2 antibodies were used to detect protein expression. A secondary conjugate and a development of activity with a 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.

We analyzed the protein expression of hOCT1, hOCT2, hOCT3 and hOCTN1 in non-stimulated and PHA-stimulated PBMCs, purified $CD4^+$ T cells and in monocytes, MDMs and MDDCs by western blot (*Figure 34*). In agreement with mRNA expression levels, we observed an increase in hOCT1, hOCT3 and hOCTN1 protein amounts following the 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. With relation to hOCT3, lower associated mRNA levels in MDMs and MDDCs, and a higher protein expression in monocytes and macrophages than in MDDCs was observed. hOCTN1 showed a notable expression in MDMs.

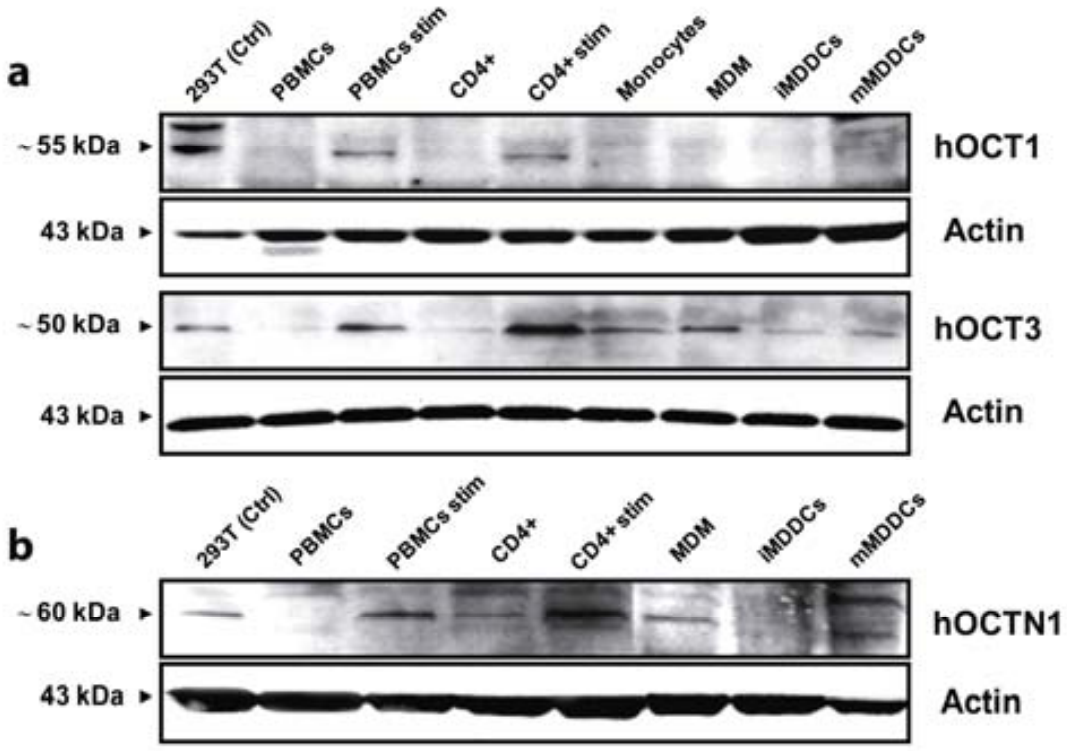


Figure 34. Western blot analysis of hOCT1, hOCT3 *(a)* and hOCTN1 *(b)* in immune cells. Lines correspond to HEK293T (293T-Ctrl-), PBMCs, PHA-stimulated PBMCs (PBMCs stim), $CD4^+$ T cells, PHA-stimulated $CD4^+$ T cells ($CD4^+$ stim) and monocytes MDMs, iMDDCs and mMDDCs. Actin was used as a control for protein loading.

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 non-stimulated and PHA-stimulated PBMCs). To accomplish this objective, we used uridine and *N*-methyl-4-phenylpyridinium (MPP+) – model substrates of hNTs and hOCTs, respectively – and we carried out *cis*-inhibition experiments.

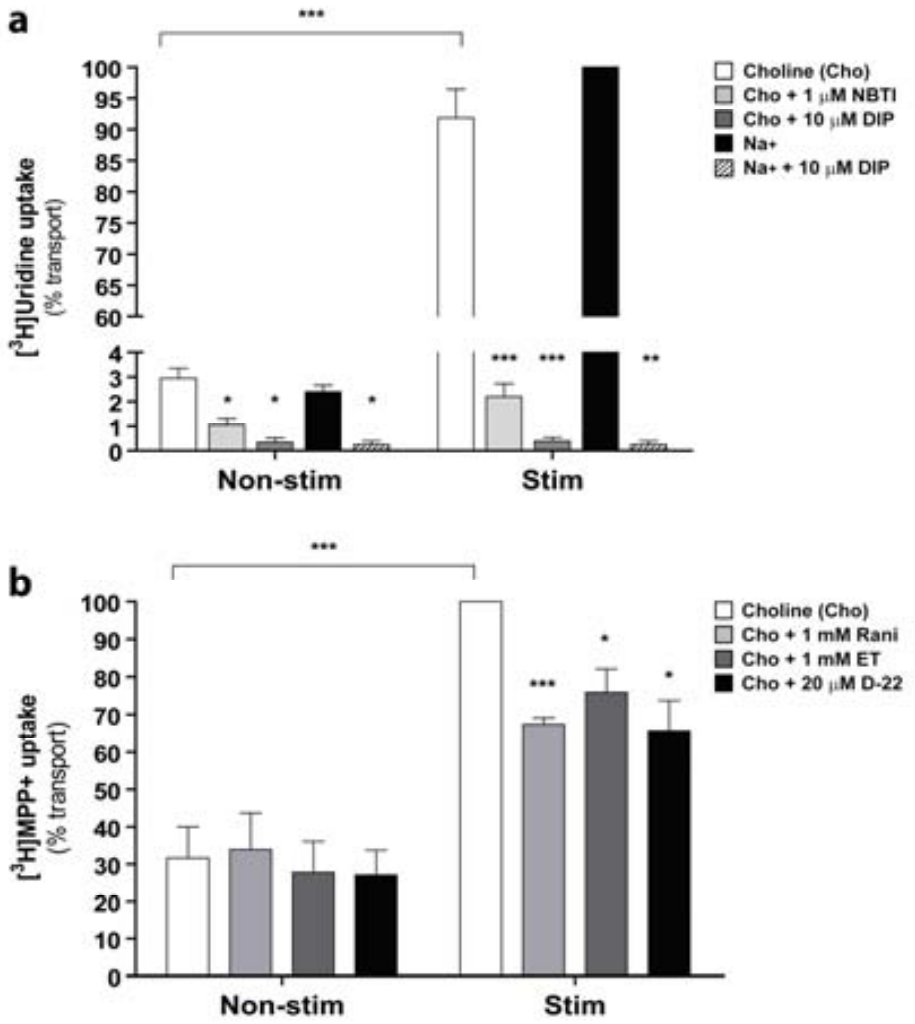


Figure 35. Uridine and MPP+ transport in non-stimulated and PHA-stimulated PBMCs. A, the equilibrative (Cho; white bars), NBTI-inhibited (light gray bars) and DIP-inhibited uptake (dark gray bars); the amount of concentrative and equilibrative (Na+; black bars); and the amount of concentrative and equilibrative DIP-inhibited uptake (white-grated bars) of 1 μM [3H]uridine was measured for 2 minutes either in a choline chloride or NaCl medium in PBMC cells from at least four independent healthy donors (performed in triplicate). **B**, the Na^+-independent uptake of 1 μM [3H]MPP+ was measured for 2 minutes in a choline chloride medium in the absence (Ctrl; white bars) or in the presence of the hOCT/N substrates Rani (1 mM; light gray bars) and ET (1 mM; dark gray bars), and hOCT inhibitor D-22 (20 μM; black bars) in PBMCs from at least four independent healthy donors (performed in triplicate). The experiments were carried out in both non-stimulated (Nonstim) and 3 day PHA-stimulated PBMCs (PHA-stim). Results are expressed as the mean ± SEM. The statistical significance of Cho/control versus different transport conditions and of nonstim Cho/control versus stimulated Cho/control was assessed by paired Student's *t*-test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

As shown in *Figure 35a*, equilibrative uridine transport in non-stimulated PBMCs was very low (0.3–1.0 pmols/mg protein) and could be completely inhibited ($p < 0.05$) both by dipydiramole (DIP) and nitrobenzylthioinosine (NBTI), thus confirming that all equilibrative nucleoside transport was mainly due to hENT1 activity. Under PHA stimulation, a significant up-regulation of uridine transport was observed ($p = 0.004$; 5.5–9.0 pmols/mg protein) and this could also be almost completely inhibited by NBTI ($p = 0.001$). This reflects the minimal contribution of hENT2 activity to nucleoside transport in primary lymphocytes. In addition, the Na^+ component of nucleoside transport (which would be attributed to hCNT2) seemed to be residual, nearly negligible. However, it is important to remark that some donors' PBMCs showed partial Na^+-dependence for uridine uptake, suggesting detectable hCNT2 function.

When addressing OCT-related functional activity we observed a relatively high percentage of transport in non-stimulated PBMCs (0.5–1.3 pmols/mg protein) (*Figure 35b*) which could be related to the high lipophilicity of MPP+. Actually, MPP+ transport could not be inhibited by either ranitidine (Rani), ergothioneine (ET) or D-22. When PBMCs were stimulated with PHA, MPP+ transport was increased three-fold above basal (non-treated) cells (2.3–3.0 pmols/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 caused by hOCT substrates or inhibitors.

Functional studies of L-Carnitine transport in PBMCs

To assess the functionality of hOCTN2, a Na^+-dependent high affinity transporter for L-carnitine (Tamai et al., 2000a; Duran et al., 2005), we performed uptake of [^{3}H]-L-carnitine in non-stimulated 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 non-stimulated and PHA-stimulated PBMCs (*Figure 36*), with the increase of transport in stimulated cells being statistically significant (447 ± 25 *versus* 765 ± 66 fmols/10^6 cells; $p = 0.01$). Surprisingly, we also found a high transport rate of L-carnitine in the absence of Na^+, both in non-stimulated PBMCs (365 ± 6 fmols/10^6 cells; 82% of transport in NaCl) and in PHA-stimulated PBMCs (516 ± 87 fmols/10^6 cells; 67% of transport in NaCl). Finally, the difference between the transport in the absence and presence of Na^+ in non-stimulated PBMCs was statistically significant (365 ± 6 vs. 447 ± 25 fmols/10^6 cells; $p = 0.03$).

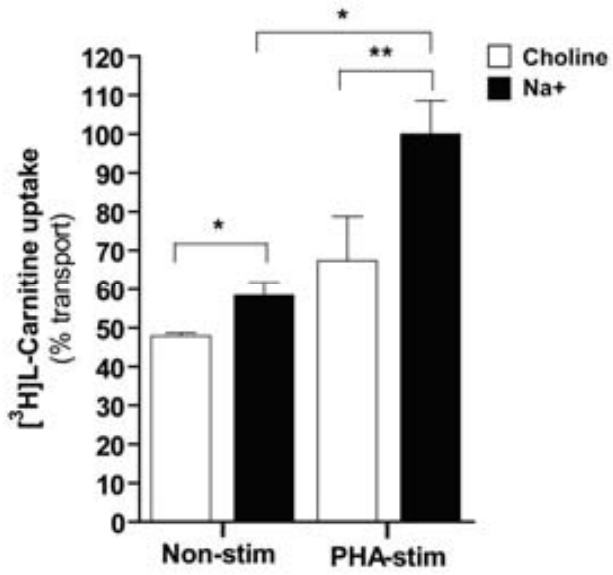


Figure 36. L-Carnitine transport in non-stimulated and PHA-stimulated PBMCs in the presence and absence of sodium. The Na^+-independent (Choline; white bars) and the Na^+-dependent uptake (Na+; black bars) of 1 μM [^{3}H]L-carnitine was measured for 5 minutes in non-stimulated (Non-stim) and PHA-stimulated PBMCs (PHA-stim) from three independent healthy donors (performed in triplicate). Results are expressed as the mean ± SEM. Statistical significance of choline (Cho) *versus* Na+ in non-stimulated PBMCs and PHA-stimulated PBMCs was assessed by paired Student's *t*-test (*, $p < 0.05$; **, $p < 0.01$).

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 monocyte-derived macrophages (MDMs). This was performed on macrophages due to their importance in inflammatory processes during cancer and viral infections, and due to their role as latent reservoirs for HIV-1 (Schrager and D'Souza, 1998; Aquaro et al., 2002). As shown in *Figure 37a* (page 101), normalizing data to 100% transport with uridine transport in Na^+ medium (with both hCNTs and hENTs operative), the hCNT-associated transport rate of uridine (57.5%) is much higher than that found for PBMCs (3.4%; nearly negligible). Uridine transport associated with hENT type activities could be significantly inhibited by both: the specific hENT1-inhibitor NBTI ($p < 0.05$) and the generic hENT inhibitor DIP ($p < 0.05$). This agrees with data obtained for PBMCs, where hENT2 function was absent in spite of the high mRNA levels. Moreover, the difference between uridine transport rates measured in a choline chloride medium (where only hENTs are functional) and a sodium chloride medium (with all hNTs actively transporting) was statistically significant (9.6 ± 1.6 *versus* 27.6 ± 10.4 pmols/mg protein; $p = 0.003$), a feature that highlights the relevance of hCNTs in macrophages.

To elucidate whether this Na^+-coupled transport activity was related to hCNT3 expression in MDMs, the uptake of cytidine was monitored in the presence of 1 mM guanosine. hCNT2 is a purine-preferring transporter (can transport guanosine but not cytidine) and hCNT3 is known to transport both purines and pyrimidines (can transport guanosine and cytidine). As found at mRNA level, hCNT1 is not expressed in MDMs so the guanosine inhibition of cytidine uptake would be attributable to hCNT3 function. *Figure 37b* (overleaf) shows a representative cytidine transport experiment in MDMs for only one blood donor, because in fact, a high variability of cytidine transport rates among donors was observed (1.7–7.0 pmols/mg protein in choline chloride and 5.7–14.0 pmols/mg protein in NaCl medium). Results showed that concentrative cytidine uptake could be 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 (*Figure 37c,* next page). As for PHA-stimulated PBMCs, MPP+ basal transport (12.7 ± 4.2 pmols/mg protein) could be inhibited by the hOCTs substrate ranitidine (34.4% inhibition; $p < 0.01$), the hOCTN1 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 corroborate our results for mRNA and protein expression.

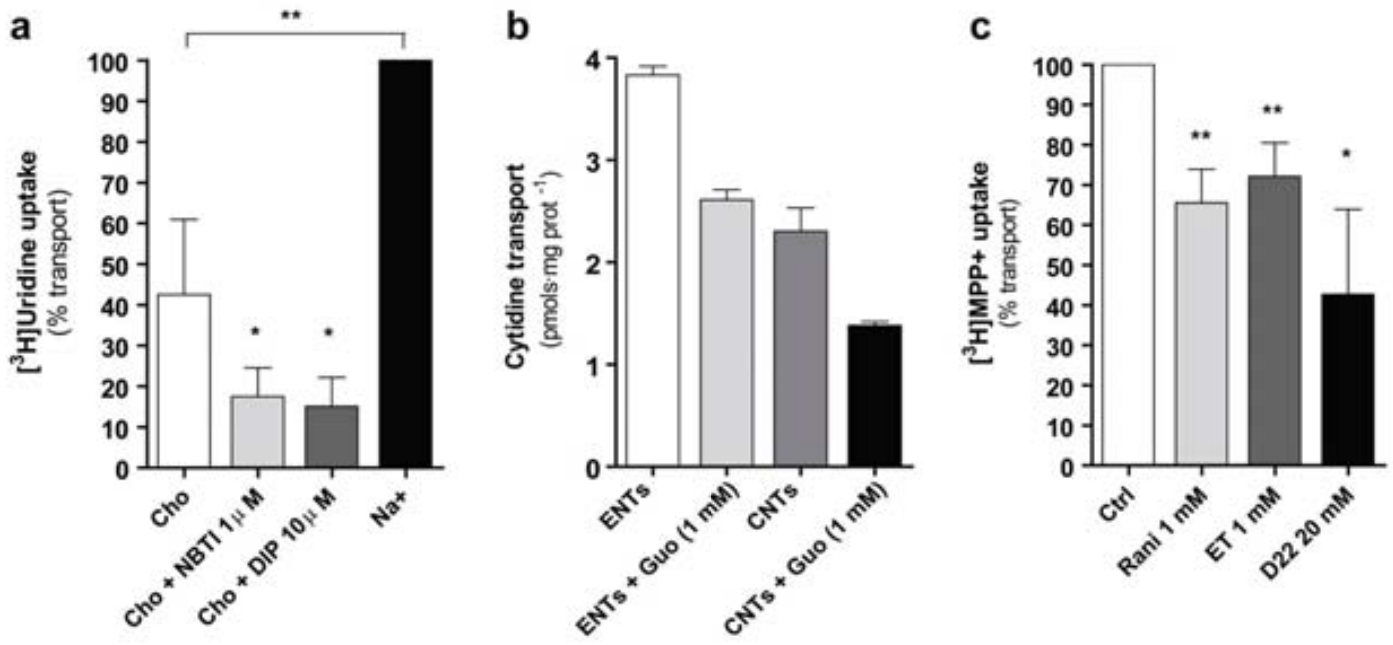


Figure 37. Uridine, cytidine and MPP+ transport in MDMs. *(a)*, the equilibrative (Cho; white bars), NBTI-inhibited (light gray bars) and DIP-inhibited uptake (dark gray bars), and the amount of concentrative and equilibrative uptake (Na+, black bars) of 1 μM [^{3}H]uridine were measured over 2 minutes either in a choline chloride or NaCl medium in MDMs from four independent healthy donors (performed in triplicate). *(b)*, the uptake of 1 μM [^{3}H]cytidine over 2 minutes in choline chloride medium in the absence (ENTs; white bar) or presence of 1 mM guanosine (ENTs + Guo 1 mM; light gray bar) and the Na^+-dependent cytidine transport in the absence (CNTs; dark gray bar) or presence of 1 mM guanosine (CNTs + Guo 1 mM; black bar) is shown. A representative experiment (performed in triplicate) from one healthy donor is shown. *(c)*, the Na^+-independent uptake of 1 μM [^{3}H]MPP+ was measured for 10 minutes in a choline chloride medium the absence (Ctrl; white bar) and the presence of hOCT/N substrates Rani (1 mM; light gray bar) and ET (1 mM; dark gray bar) and the inhibitor of hOCT D-22 (20 μM; black bar) in MDMs from four independent healthy donors (performed in triplicate). Results are expressed as the mean ± SEM of the four independent donors or the mean ± SEM from one donor in *(b)*. In *(a)* and *(c)*, the statistical significance of Cho/Ctrl versus different conditions of transport was assessed by paired Student's *t*-test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Chapter III. HIV-1 Infection *in vitro* does not affect Nucleoside Transporters' mRNA expression but it is inhibited by Dipyridamole in MT4 cells and PBMCs

ABSTRACT

It is almost completely unknown how HIV-1 affects the membrane-bound proteins of immune cells and specifically whether HIV-1 modifies drug uptake transporter expression and/or activity. Only a few reports have addressed this question in protein members from the ABC superfamily of transporters, finding interesting expression and activity changes in ABCA1 and multidrug resistant proteins (Jorajuria et al., 2004; Mujawar et al., 2006; Ronaldson et al., 2008). Here, we first studied whether the HIV-1 infection *in vitro* in MT4 and PBMCs affects the expression of human Concentrative Nucleoside Transporters (hCNTs) and/or human Equilibrative Nucleoside Transporters (hENTs) at mRNA level. Moreover, by using the hENT inhibitors nitrobenzylthioinosine (NBTI) and dipyridamole (DIP), we aimed to assess whether the inhibition of the nucleoside salvage route through these transporters decreases or impairs HIV-1 infection by limiting the main types of nucleosides in T cells. First, we found that *in vitro* HIV-1 infection, with 25% of cells infected at day 2 post-infection, only affected hCNT2 mRNA expression, down-regulating it >nine-fold (2306 *versus* 253 copies mRNA/µg RNA_{total}) in MT4 cells. When exploring the changes in mRNA of hCNT2 in PBMCs and $CD4^+$ T cells, they could not be found in either cell subset. DIP strikingly decreased the percentage of infected cells at days 2 and 3 post-infection in MT4 (from 40 to 13.35% and from 98 to 59.5%, respectively) whereas NBTI did not. DIP inhibition of HIV replication was confirmed in PBMCs from different donors. In summary, these results suggest that HIV-1 infection might not alter hNTs expression *in vivo*, at least not at the transcriptional level. The hENTs inhibitor DIP shows a good antiviral effect but its mechanism of action needs further research to be understood.

hCNT2 down-regulation during HIV-1 infection in vitro in MT4 cells

It has been previously reported that PBMCs from HIV patients and macrophages have increased and/or decreased expression of efflux transporters from the ABC superfamily at both mRNA and/or protein levels (Jorajuria et al., 2004; Mujawar et al., 2006; Ronaldson et al., 2008). No information on the effect of HIV-1 infection on the protein members of the *SLC28*, *SLC29* or *SLC22* gene families is available to date. Accordingly, we initially aimed to study the direct effect of HIV-1 infection *in vitro* on mRNA expression of human Nucleoside Transporters. To do so, we used a previously constructed and characterized HIV-GFP virus (Weber et al., 2006). It represents a useful HIV-1 in *in vitro* experiments (constructed from an original p83-10 strain of HIV-1) that emits green fluorescence when a productive infection is occurring, so that the percentage of infected cells can be followed by flow cytometry. The mRNA expression of the concentrative subtypes hCNT1, hCNT2, hCNT3 and the equilibrative hENT1 and hENT2, was measured as described in *Materials & Methods*. As previously mentioned, human β-glucoronidase was used as a control (housekeeping gene). As *Figure 38a* shows, the infection spread rapidly in MT4 cells (MOI=0.001): at day 2 post-infection 25% of cells were infected while at day 3, nearly all the cells were green (≥95% were infected).
were green (≥95% were infected).

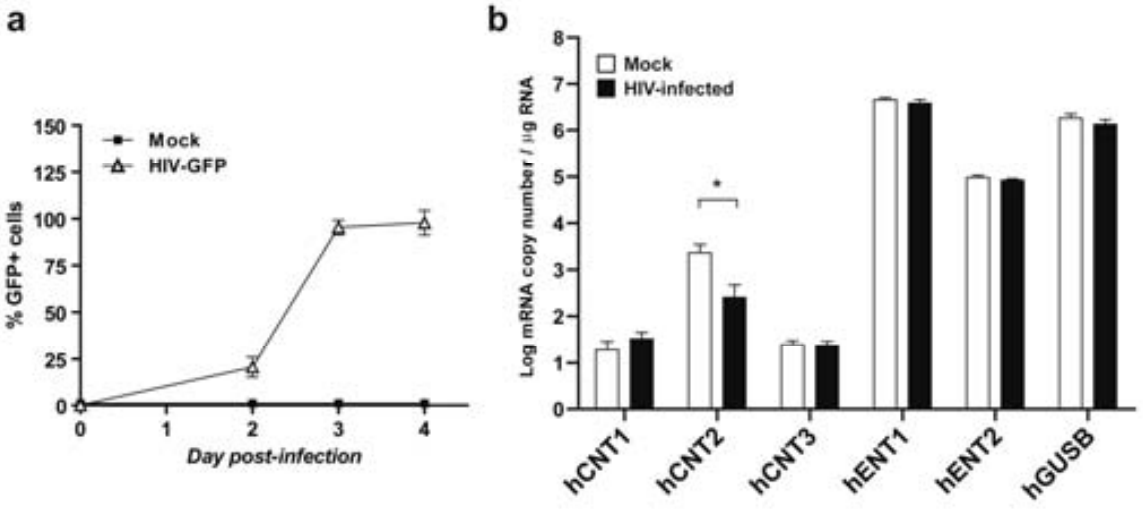


Figure 38. Effect of HIV-infection on mRNA expression of hCNTs and hENTs in MT4 cells. MT4 cells were cultured in the absence (Mock) or presence of HIV-GFP (MOI = 0.001) over 4–5 days. *(a)* The percentage of GFP positive cells (%GFP+ cells) were followed over 4 days by flow cytometry. *(b)* mRNA expression of hCNT1, hCNT2, hCNT3, hENT, hENT2 and the housekeeping gene hGUSB was measured at day 2 post-infection as detailed in *Materials & Methods* by Quantitative Real-time PCR. Results are expressed in log mRNA copy number per microgram of total RNA as the mean ± SEM of three independent measurements performed in triplicate.

We decided to determine the hCNTs and hENTs mRNA levels at days 2 and 3, when cell viability was still up to 90–95%. Focusing on mRNA expression of hNTs in HIV-infected MT4 cells, we can see in *Figure 38b* that only hCNT2 transcripts seemed to be affected at day 2 post-infection, experiencing a great down-regulation of about ten-fold in the case of HIV infection (3.36 ± 0.36 *versus* 2.40 ± 0.54 in log mRNA/µg total RNA). Of note, at day 3 post-infection no changes were found (data not shown). The underlying mechanism by which hCNT2 is down-regulated is not clear, and the consequences that down-regulation could bring were not studied more profoundly at this time. Nevertheless, a putative mechanism linked to apoptosis of HIV-infected cells is depicted in *Figure 39*.

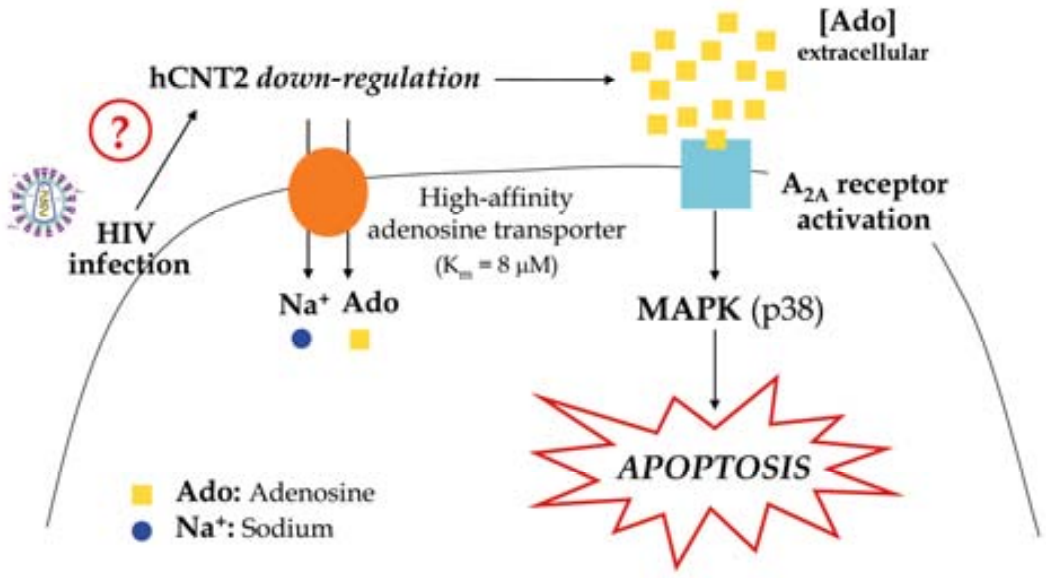


Figure 39. Scheme of probable HIV-infection mechanism linked to hCNT2 down-regulation and apoptosis. HIV-infection would provoke a dramatic down-regulation in hCNT2 expression. hCNT2 is a high-affinity adenosine transporter (K_m=8 µM) while hENT2, even though it is a major adenosine transporter and is highly-expressed in T lymphocytes, it is not very active functionally. Therefore, hCNT2 down-regulation would mean higher concentrations of adenosine in the extracellular milieu. This adenosine could interact with the A_{2A} adenosine receptors that, through p38-MAPK pathway would lead to cell apoptosis.

hCNT2 is adenosine's highest-affinity transporter (Wang et al., 1997; Ritzel et al., 1998) while hENT2 is the major adenosine low-affinity transporter (Baldwin et al., 2004). Even though we had determined that hENT2 is strongly expressed (and up-regulated when PBMCs or $CD4^+$ T cells were activated with PHA) we found near-negligible hENT2 activity, with hENT1 and, to a lesser extent hCNT2, being the most functionally active transporters in T cells. According to this background, down-regulation of hCNT2 as a consequence of

HIV-infection would mean higher concentrations of extracellular adenosine that would interact with high-affinity with A_{2A} adenosine receptors and, via a p38-mitogen-activated protein kinase (MAPK) pathway, would lead to cell apoptosis (Schulte and Fredholm, 2003). Though this remains to be proven, this theoretical mechanism could be regulating the apoptosis of HIV-infected cells through extracellular adenosine, a nucleoside best known for its role in modulating cell responses in many tissues and in the central nervous system (Fredholm et al., 2005).

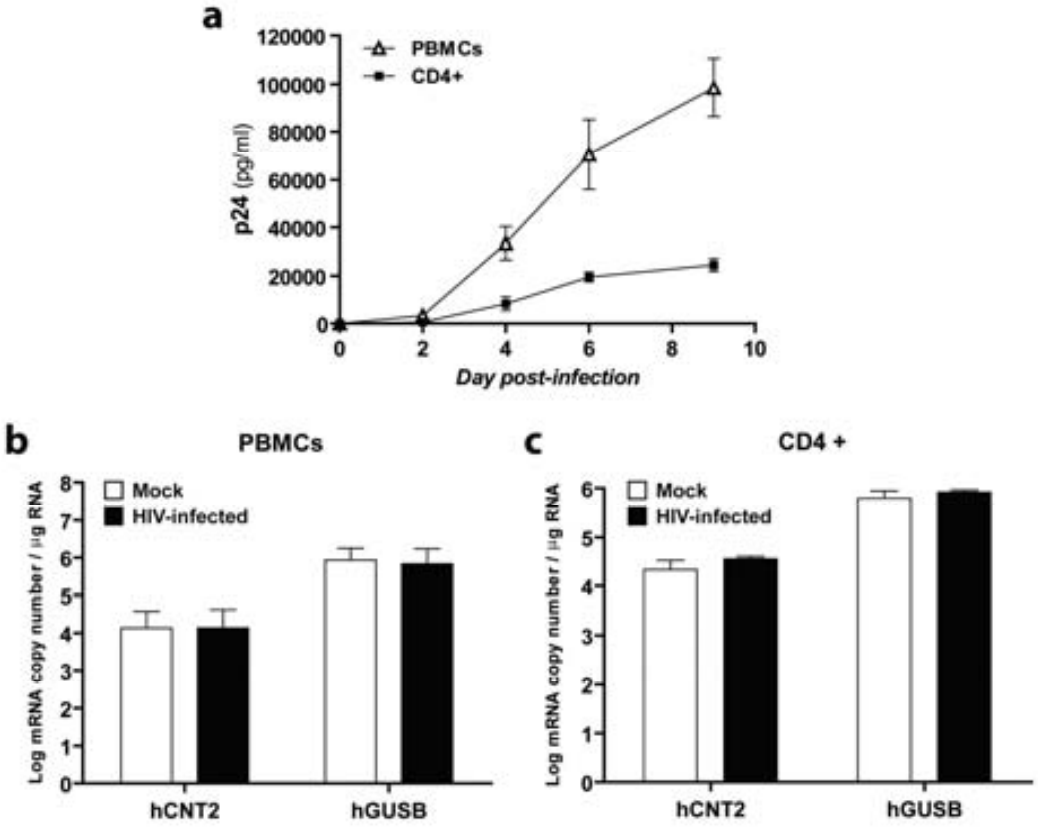


Figure 40. Effect of HIV-infection on mRNA expression of hCNT2 in PBMCs and CD4⁺ T cells. PBMCs and isolated CD4⁺ T cells were cultured in the absence (Mock) or presence of HIV p83-2R (MOI = 0.01) over 9 days. *(a)* The HIV antigen p24 was measured at 2, 4, 6 and 9 days post-infection using a commercial ELISA kit (see *Materials & Methods*). Results are expressed as the mean ± SEM in pg/ml of three experiments performed in duplicate. *(b)* PBMC and *(c)* CD4⁺ T cell mRNA expression of hCNT2 and the housekeeping gene hGUSB were measured at day 4 post-infection by Quantitative real-time PCR. Results are expressed in log mRNA copy number per microgram of total RNA as the mean ± SEM of three independent measurements performed in triplicate.

To further assess the putative hCNT2 down-regulation linked to HIV-infection in T cells, we performed the same type of experiments in primary cultures of PBMCs and isolated $CD4^+$ T cells (*Figure 40*). The infection was followed by measuring the HIV antigen p24 by ELISA. As shown in *Figure 40a*, PBMC infection was much higher and showed faster kinetics than $CD4^+$ T cells. Nevertheless, at day 4 post-infection both PBMCs and $CD4^+$ T cells gave good measurable p24 levels. Our hypothesis for higher replication levels in PBMCs would be that factors secreted by monocytes, B cells and other immune cells in the PBMC pool would facilitate the infection. Surprisingly, when observing mRNA expression of hCNT2 in both cell types, it can clearly be stated that this expression was not affected, at variance with what we had observed for hCNT2 transcripts in HIV-infected MT4 cells. It should be noted, however, that at that time, the infection was performed with a wild-type HIV virus (NL4-3) followed by p24 antigen and not with the GFP-labelled one. It is not clear why the down-regulation for hCNT2 could not be observed in HIV-infected primary cultures, but one plausible explanation could be that, since HIV-infection in primary T cells (as $CD4^+$ T cells) or whole PBMCs requires previous activation with PHA, and this phenomenon is known to dramatically increase the expression of hCNT2 (and hENTs), the possible down-regulation of hCNT2 would somehow be masked or simply avoided by PHA-stimulation.

Slow down of HIV kinetics by dipyridamole (DIP) treatment in MT4

Our second main hypothesis for this fourth chapter was that, since the virus needs a nucleoside *salvage* route for the construction of new virions during its replication, by inhibiting hENTs – with hENT1 being the main entry of nucleosides salvage in T cells – HIV replication would be reduced or slowed down. We therefore performed a number of experiments concerning the putative effect that the hENT1 inhibitor nitrobenzylthioinosine (NBTI) and the hENTs-mediated transport inhibitor dipirydamole (DIP) have on HIV infection. Using the HIV-GFP virus, we initially performed the experiments on the T cell line MT4. As depicted in *Figure 41,* MT4 cells were infected with HIV-GFP (MOI = 0.001, see *Materials and Methods*) and treated with 1 μM NBTI (inhibiting only hENT1 function) or 10 μM DIP (inhibiting both hENTs) for 4 days. The infection was followed by flow cytometry counting the percentage of GFP positive cells, with the number of viable cells being followed by an automatic cell counter. According to our hypothesis, the presence of DIP in the media at days 2 and 3 post-infection reduced the percentage of GFP+ cells from 40 to 15% and from 98 to 60%, respectively; although at day 4 post-infection, ≥95% of the cells where infected in nearly all cases. Surprisingly, NBTI did not have any effect on the

percentage of GFP+ cells, which in principle would mean that the inhibition of the contribution of nucleoside through the salvage routes is not the cause of the slowdown observed in HIV-infection kinetics. In *panel c* of *Figure 41* we can see three microscope photographs of the percentage of green cells in MT4 cultures. Of note, the greatest difference in GFP positive cells occurred at day 3 post-infection in the presence of 10 µM DIP.

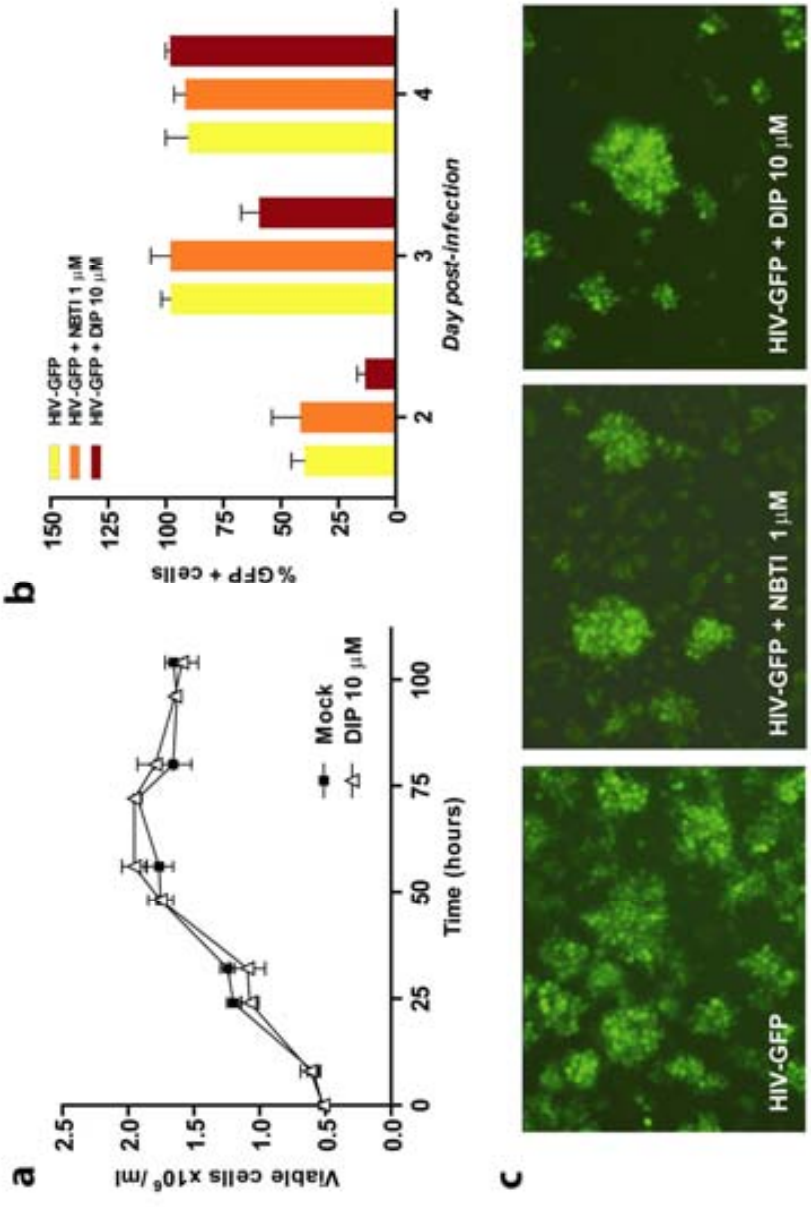


Figure 41. Effect of nitrobenzylthioinosine (NBTI) and dipirydamole (DIP) on HIV infection in MT4 cells. MT4 cells were infected with HIV-GFP at an MOI = 0.001 and treated with 1 µM NBTI or 10 µM DIP over 4 days. *(a)* The toxicity was followed in the case of DIP treatment by an automatic cell counter counting viable cells every 8 and/or 16 hours until day 4. Results are expressed as the mean ± SEM of viable cells x10^6 per millilitre of three counts *(b)* The percentage of GFP-positive cells (GFP+ cells) was followed by flow cytometry (as an equivalent of the percentage of infected cells) at days 2, 3 and 4 post-infection. *(c)* Three representative microscope images (200X) of MT4 cultures infected with HIV-GFP in the presence of NBTI and DIP at day 3 post-infection are shown. The decrease in GFP+ cells (green cells, infected cells) can be clearly distinguished in the treatment with DIP (last image on the right corner).

Inhibition of HIV replication by dipyridamole (DIP) treatment in PBMCs

Following these results, we aimed to confirm the slowdown in HIV infection kinetics observed during dipyridamole (DIP) treatment of primary cultures of PBMCs from blood donors infected *in vitro* with HIV. As shown in *Figure 42a*, the toxicity of three different concentrations of DIP (1, 10 and 100 μM) was assessed by MTS viability assay. It is clear that the lower concentration of DIP used (1 μM) was not toxic for PBMCs. However, 10 μM DIP showed a slight toxicity after day 5, decreasing cell viability from nearly 90% (day 5) to 65% (day 9), approximately. The highest concentration tested, 100 μM DIP, was clearly toxic for the cells, showing a viability of 40% at day 3 and decreasing to 25% at day 9. We therefore used 1 and 10 μM DIP for the assays. When testing the effect of DIP in HIV infected PBMCs from three different donors (*Figure 42b, c and d*), we observed the same effect as seen in MT4 cells: 1 and/or 10 μM DIP clearly inhibited HIV infection. The phenomenon can be regarded as a slowdown in HIV infection kinetics, showing a reproducible decrease in p24 concentration in all PBMC cultures from three different donors. At day 3, the decrease in p24 concentration was present, though it cannot be distinguished clearly in the graphs and, as the virus was still in an early replication phase, the inhibition was not as high as at day 5. Of note, PBMCs from different donors showed different infection rates (from 7.200 to 115.000 pg/ml p24 at day 5 post-infection, for instance) which is the reason they are shown separately. Despite these different infection rates, the presence of 1 or 10 μM DIP provoked a significant decrease in p24 both at day 5 and day 9 post-infection in all cases.

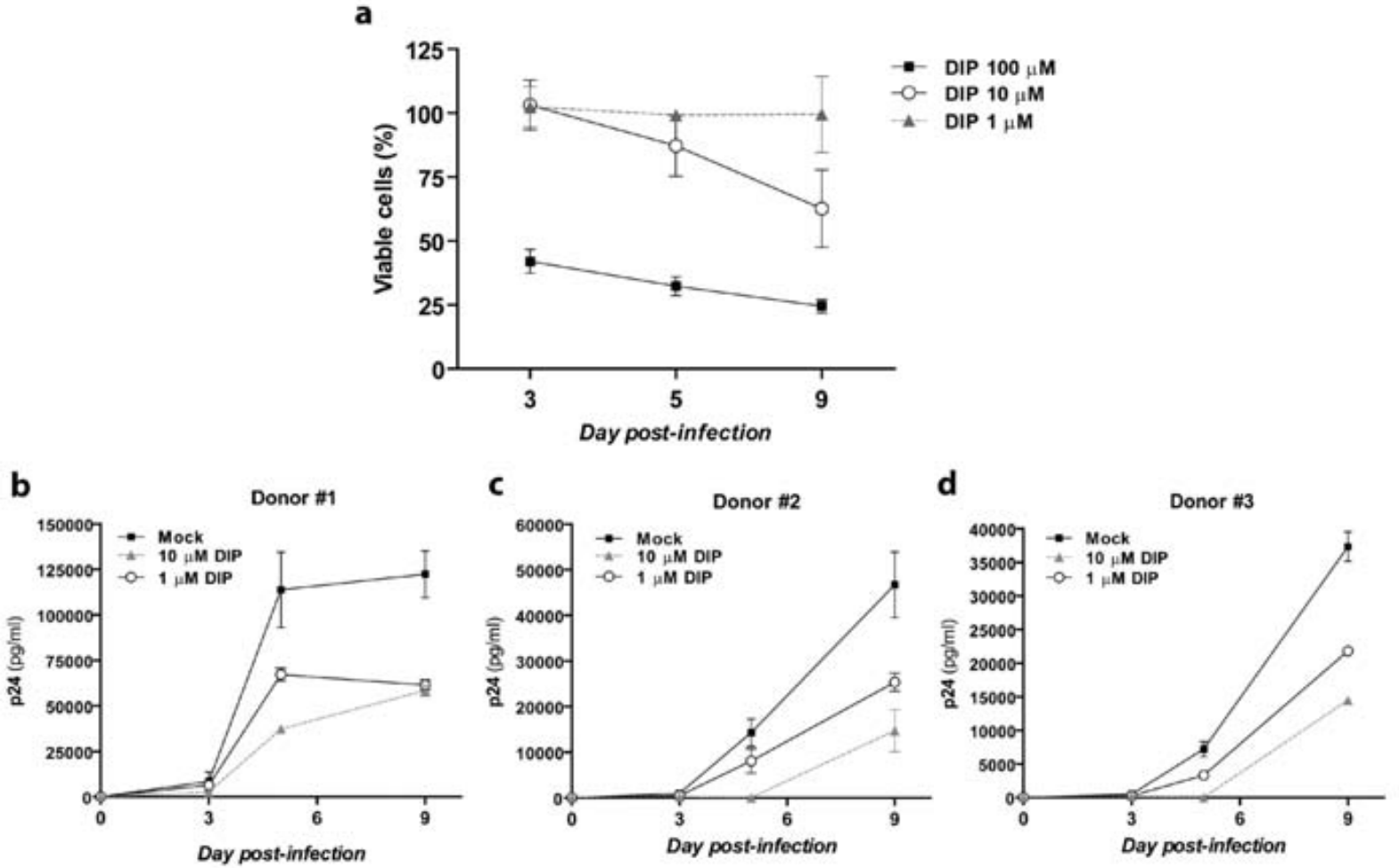


Figure 42. Effect of dipirydamole (DIP) on HIV infection in PBMCs from different donors. PBMCs were infected with HIV (NL4-3) at an MOI = 0.01 and treated with 1 µM, 10 µM or 100 µM DIP over 9 days. *(a)* The cytotoxicity of the three different DIP concentrations was followed by MTS viability assay (see *Materials & Methods*) at days 3, 5 and 9 post-infection. Results are expressed as the mean percentage of viable cells ± SEM of three measurements performed in duplicate. The HIV p24 antigen concentration was measured in the PBMC culture media of donor #1 *(b),* donor#2 *(c)* and donor#3 *(d)* using a commercial ELISA kit. Results are expressed as picogrammes of p24 per ml of media. The deviation bars are the SD of three measurements of p24 antigens from each time point and culture (Mock, 10 µM DIP or 1 µM DIP).

Chapter IV. Transport of Lamivudine (3TC) and High-Affinity Interaction of Nucleoside Reverse Transcriptase Inhibitors (NRTIs) with Human Organic Cation Transporters 1, 2 and 3

ABSTRACT

Nucleoside reverse transcriptase inhibitors (NRTIs) need to enter cells to act against HIV-1. Human organic cation transporters (hOCT1–3) are expressed and active in $CD4^+$ T cells, the main targets of HIV-1, and have been associated with antiviral uptake in different tissues. In this study, we examined whether NRTIs interact and are substrates of hOCT in cells stably expressing these transporters. Using [^{3}H]N-methyl-4-phenylpyridinium, we found a high-affinity interaction among abacavir [[(1S, 4R)-4-[2-amino-6-(cyclopropylamino) purin-9-yl]-cyclopent-2-enyl]methanol sulphate] (ABC)] (<0.08 nM), azidothymidine [3'-azido-3'-deoxythymidine (AZT)] (<0.4 nM), tenofovir disoproxil fumarate (<1.0 nM), and emtricitabine (<2.5 nM) and hOCTs. Using a wide range of concentrations of lamivudine [(-)-β-L-2',3'-dideoxy-3'-thiacyitidine (3TC)], we determined two different binding sites for hOCTs: a high-affinity site (K_{d1} = 12.3–15.4 pM) and a low-affinity site (K_{d2} = 1.9 –3.4 mM). Measuring the direct uptake of [^{3}H]3TC and inhibition with hOCT substrates, we identified 3TC as a novel substrate for hOCT1, 2, and 3, with hOCT1 as the most efficient transporter (K_m = 1.25 ± 0.1 mM; V_{max} = 10.40 ± 0.32 nmol/mg protein/min; V_{max}/K_m = 8.32 ± 0.40 μl/mg protein/min). In drug-drug interaction experiments, we analyzed cis-inhibition of [^{3}H]3TC uptake by ABC and AZT and found that 40 to 50% was inhibited at low concentrations of the drugs (K_i = 22–500 pM). These data reveal that NRTIs experience a high-affinity interaction with hOCTs, suggesting a putative role for these drugs as modulators of hOCT activity. Finally, 3TC is a novel substrate for hOCTs and the inhibition of its uptake at low concentrations of ABC and AZT could have implications on the pharmacokinetics of 3TC.

High-Affinity Interaction of NRTIs with hOCTs

Our first objective was to study the interaction of the currently most commonly used NRTIs with the three subtypes of hOCTs we had cloned previously and stably expressed in CHO cells. To do so, we performed one-second measurements of [^{3}H]MPP+ (12.5 nM) uptake (within the linearity range; see *Figure 52, page 129*) in the presence of different low-range concentrations (from 10^3–10^4 nM) of ABC, AZT, FTC and TDF. All NRTIs tested showed a high-affinity interaction with the three subtypes of hOCTs (*Figure 43*). FTC showed the highest affinity for hOCT1 (IC_{50} = 0.020 nM), followed by ABC, AZT and TDF (*Table 10*). The mean percentage of MPP+ transport inhibition ranged from 45 to 60%, with a higher value for ABC (70% inhibition). For hOCT2, all NRTIs also showed a high-affinity interaction; ABC had the highest interaction value (IC_{50} = 0.041 nM), followed by AZT, TDF and FTC (*Table 10*). For hOCT2, the percentage of MPP+ uptake inhibition was no higher than 60% for all drugs. Finally, for hOCT3, all NRTIs again showed high values and a notable percentage of MPP+ uptake inhibition. The drug that interacted with the highest affinity was TDF (IC_{50} = 0.005 nM) (IC_{50} = 0.005 nM), followed by ABC, AZT and FTC (*Table 10*).

	IC_{50} (nM)		
NRTIs	**hOCT1**	**hOCT2**	**hOCT3**
ABC	0.072 ± 0.033	0.041 ± 0.020	0.050 ± 0.029
AZT	0.155 ± 0.111	0.270 ± 0.105	0.396 ± 0.270
FTC	0.020 ± 0.006	2.400 ± 1.162	0.530 ± 0.260
TDF	0.854 ± 0.012	0.566 ± 0.549	0.005 ± 0.003

Table 10. IC_{50} values for inhibition of [^{3}H]MPP+ uptake by NRTIs in CHOhOCT1, hOCT2, and hOCT3 cell lines. Data are shown as IC50 values ± SEM from at least three independent experiments, with each point performed in quadruplicate.

Interaction between 3TC and hOCT1, 2 and 3: Identification of High and Low-Affinity Binding Sites

The existence of two different binding sites in hOCTs for the substrates TEA, choline and MPP+, and three binding sites for the non-transported inhibitor TBuA have been described elsewhere (Gorbunov et al., 2008). Because of the widespread prescription of 3TC and previous evidence that it could be a substrate for hOCTs (Takubo et al., 2000a; Takubo et al., 2002), we focused on the interaction between 3TC and hOCTs by studying the inhibitory effect of a wide range of concentrations of 3TC on radiolabelled MPP+ uptake.

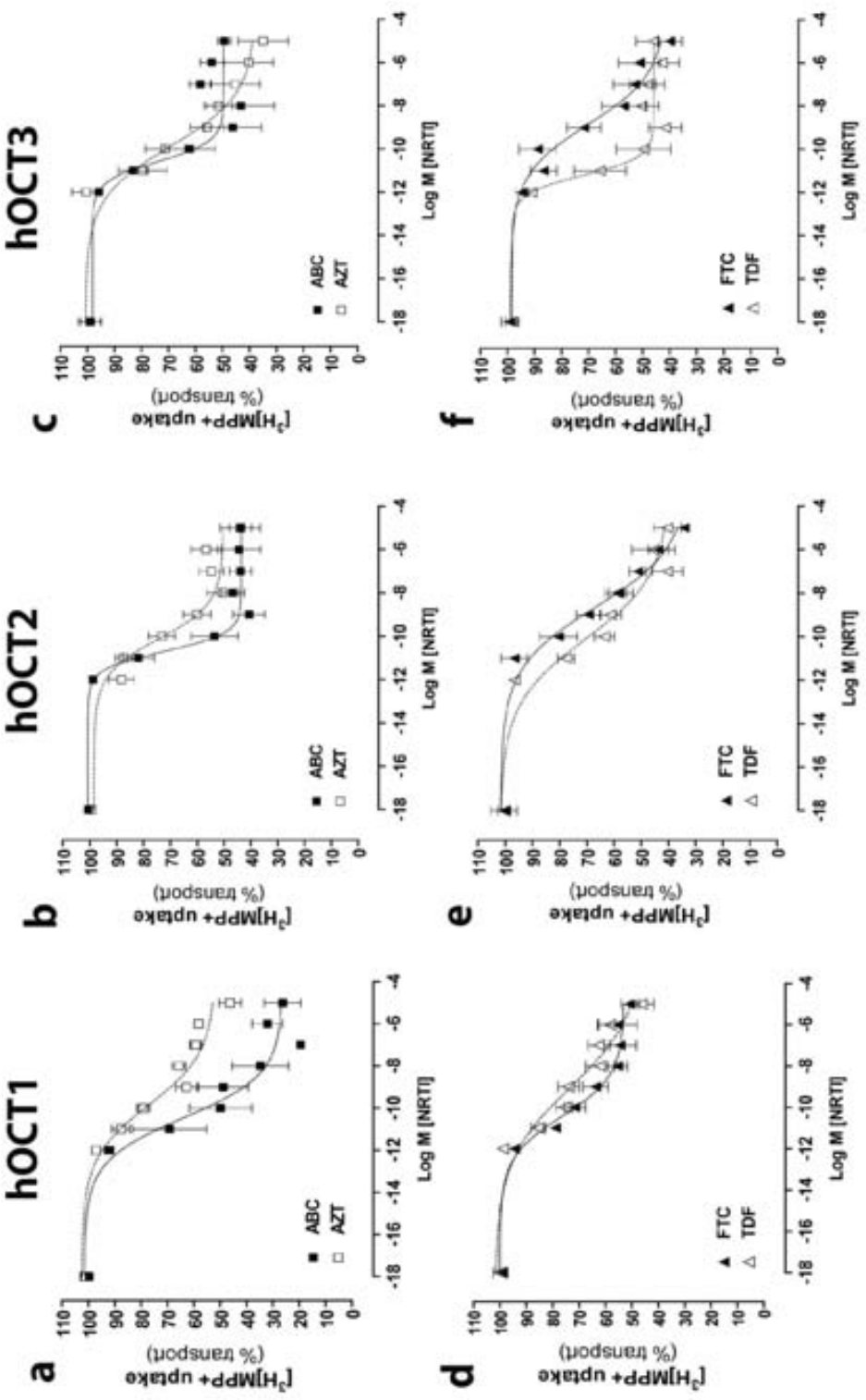


Figure 43. Use of NRTIs for *cis*-inhibition of [³H]MPP+ uptake in CHO-hOCT1, hOCT2 and hOCT3 cell lines. ABC (■) and AZT (□) at different low-range concentrations (10^{-3}, 10^{-2}, 0.1, 1.0, 10, 10^{2}, 10^{3} and 10^{4} nM) were used to perform *cis*-inhibition of 1-second [³H]MPP+ uptake (12.5 nM) by CHO-hOCT1 *(a)*, hOCT2 *(b)* and hOCT3 *(c)* transfected cells at 37°C. Likewise, FTC (▲) and TDF (Δ) at the same low-range concentrations (10^{-3}-10^{4} nM) were used to perform *cis*-inhibition of 1-second [3H]MPP+ uptake (12.5 nM) by CHO-hOCT1 *(d)*, hOCT2 *(e)* and hOCT3 *(f)* transfected cells at 37°C. Table 10 depicts IC_{50} values and the SEM from at least three independent experiments, each point performed in quadruplicate.

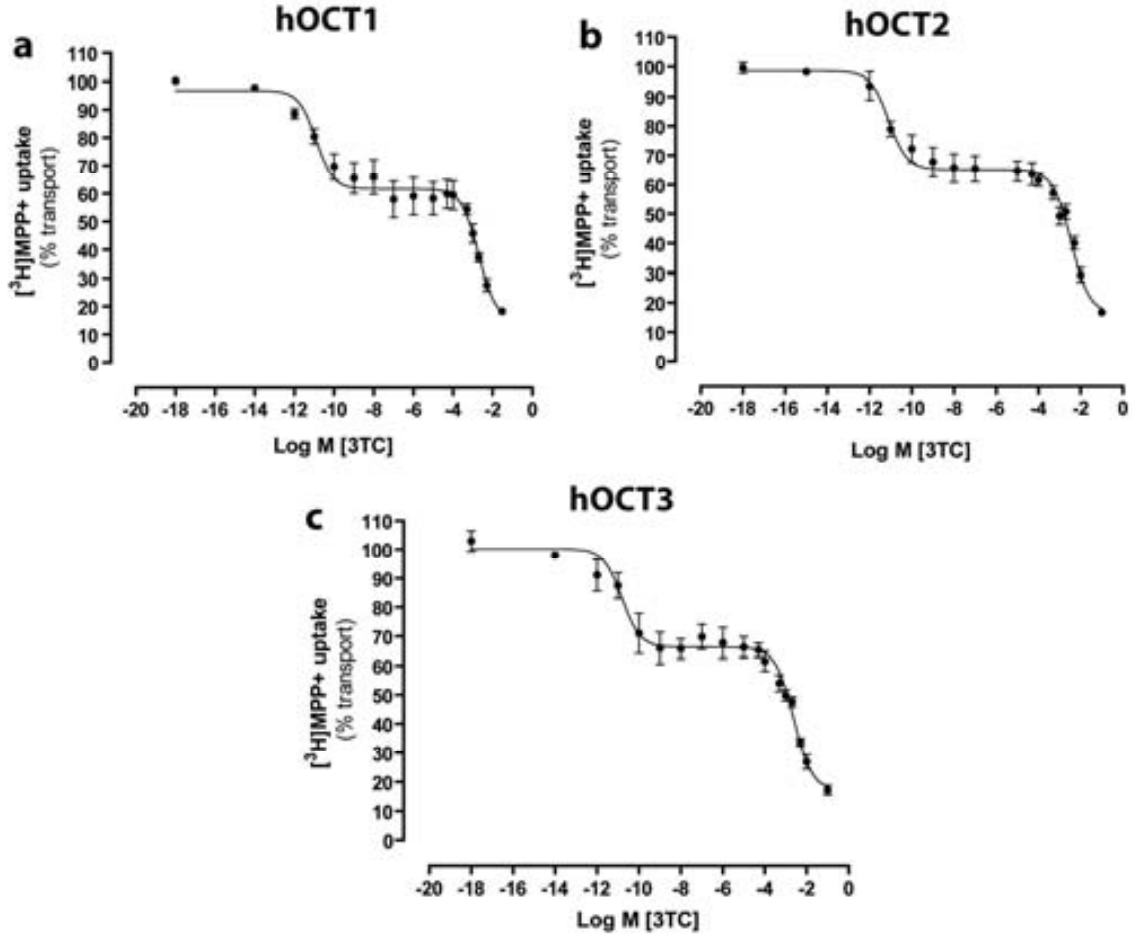


Figure 44. Two-site interaction of 3TC with hOCT1, 2 and 3. *cis*-inhibition of [3H]MPP+ (12.5 nM) uptake in CHO-hOCT1 *(a)*, hOCT2 *(b)* and hOCT3 *(c)* cells was performed in the presence of a wide-ranging concentration of 3TC (low and high concentrations ranging from 10^{-3}–10^{4} pM to 10^{1}–10^{5} μM, respectively). *Table 2* presents low-affinity (picomolar) and high-affinity (millimolar) K_d values obtained by fitting the data to the two-site competition model and the p values for the statistical comparison between the two and one-site models.

The inhibition of MPP+ uptake by 3TC clearly followed a biphasic curve (*Figure 44*). At low concentrations of non-labelled 3TC, the inhibition was clear for the three hOCT subtypes but did not reach more than 35 to 40% (*Figure 44*). It is interesting that the cells were incubated with concentrations of 3TC greater than 10 μM, with inhibition increased to 80 to 85%. In all cases, the K_d values for the high-affinity binding site were in the picomolar range (*Table 11*), whereas the K_d values for the low-affinity binding site were in the millimolar range (*Table 11*). A comparison between fitting the data by one-binding site competition versus two-binding site competition indicated that the latter was preferred in all cases, with statistical significance ($p < 0.0001$).

	High-affinity binding site	*Low-affinity binding site*	*Two-binding vs. One-binding-site*
	K_{d1} (pM)	K_{d2} (mM)	
hOCT1	12.3 ± 0.25	1.90 ± 0.14	*P<0.0001*
hOCT2	8.13 ± 0.17	3.45 ± 0.11	*P<0.0001*
hOCT3	15.4 ± 0.23	2.40 ± 0.13	*P<0.0001*

Table 11. K_d for high-affinity and low-affinity binding sites and *p* values for statistical comparison of two-binding versus one-binding site fitting of the inhibition curve shown in *Figure 2*. Data are shown as Kd values ± SEM from at least three independent experiments with each point performed in quadruplicate.

To determine whether high- and low-affinity inhibition by 3TC is due to an interaction with previously described high and low-affinity MPP+ binding sites in OCT1 (Gorbunov et al., 2008), we tested the interaction of 3TC using three different MPP+ concentrations (12.5 nM, 125 nM, and 5 µM) (*Figure 45*). The results showed that IC_{50} values for the high-affinity binding site increased at higher concentrations of substrate (MPP+), whereas IC_{50} values for the low-affinity binding site remained unchanged (*Table 12*).

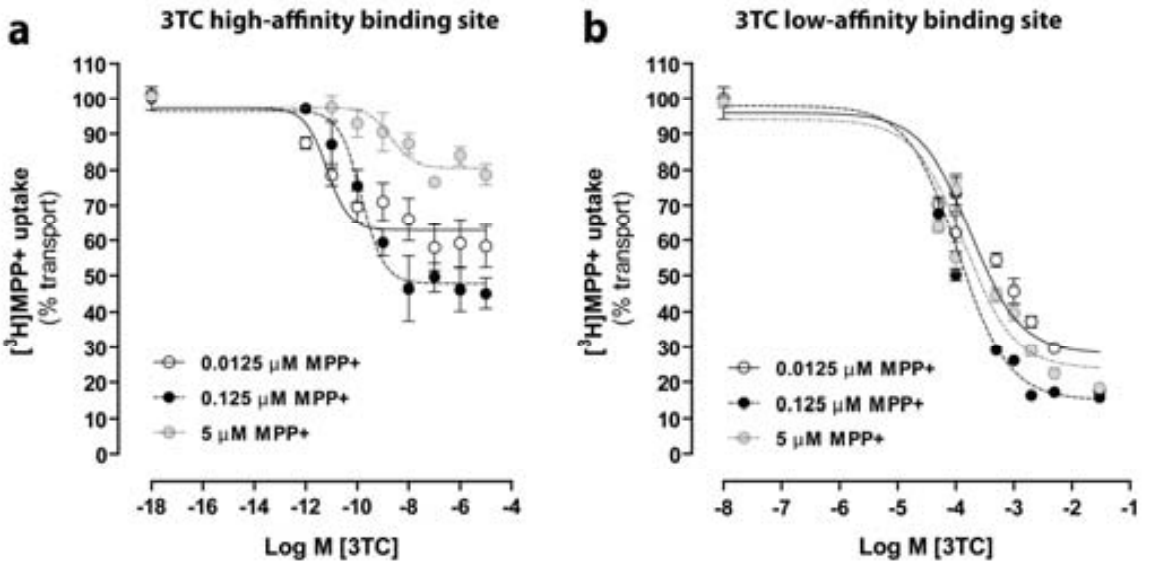


Figure 45. Competition experiments of MPP+ uptake with high-affinity (picomolar) and low-affinity (millimolar) binding sites of 3TC in CHO-hOCT1. *cis*-Inhibition of [3H]MPP+ uptake at three different concentrations 12.5 nM (○), 125 nM (●), and 5 µM (◌) in CHO-hOCT1 was performed in the presence of low 3TC concentrations (10^{-3}-10^{4} pM) *(a)* and high 3TC concentrations (10^{1}-10^{5} µM) *(b)*. Data are expressed as the mean percentage of inhibition ± one half the range of the two mean uptake values of two independent experiments, with each point performed in quadruplicate. Values are shown in *Table 3*.

	CHO-hOCT1	
	IC_{50} values of 3TC two-binding sites	
[MPP+] (μM)	*High-affinity binding site* (pM)	*Low-affinity binding site* (mM)
0.0125	12.3 ± 0.25	1.90 ± 0.14
0.125	145.5 ± 2.15*	1.12 ± 0.07
5.0	2127.5 ± 38.5*	1.42 ± 0.08

*$P<0.05$, IC_{50} different from the reference one (0.0125 μM MPP+)

Table 12. IC_{50} values for high-affinity binding site and low-affinity binding site for inhibition of [^{3}H]MPP+ uptake (0.0125, 0.125, or 5.0 μM) by 3TC in CHO-hOCT1. The statistical comparison between reference IC_{50} value (MPP+ at 0.0125 μM) and the other two MPP+ concentrations was done (*, $p<0.05$).

hOCTs Show a Saturable Time Course and Facilitate Transport of 3TC

We then tested the uptake of radiolabelled 3TC in CHO cell lines stably expressing hOCTs compared to the uptake in CHO cells stably expressing the empty vector (pcDNA5). We found a substantial difference between uptake rates; although CHO-pcDNA5 cells transported very low quantities of 3TC, showing a non-saturable and linear uptake, CHO-hOCT1, hOCT2 and hOCT3 cell lines showed saturable uptake (up to 60–90 seconds) and high transport of 3TC (*Figures 46*). [^{3}H]3TC uptake was shown to be linear for hOCT1 during the first 15 seconds (inset, *Figure 46a*).

3TC Uptake Can Be Inhibited Either by hOCT Substrates and Their Inhibitors or by Nonradiolabelled 3TC

Having demonstrated that 3TC was taken up by the three hOCTs, we assessed whether this uptake could be inhibited by substrates and inhibitors of hOCTs, and by the nonradiolabelled drug. Therefore, we performed radiolabelled 3TC uptake in CHO-hOCT cell lines inhibited by the substrate MPP+ (2 mM), by the low-affinity inhibitors TBuA (2 mM), Rani (2 mM) and Atrop (2 mM), by the high-affinity inhibitor D-22 (200 μM) and by nonradiolabelled 3TC (2 mM). The inhibition achieved for hOCT1 and hOCT2 uptake was greater than for hOCT3 uptake (*Figure 47*), although they were all statistically significant ($p<0.005$). In the case of hOCT1, the highest inhibition was found in the presence of Rani, Atrop, and D-22. For hOCT2, the highest inhibition was found with TBuA followed by Rani, although the remaining drugs also inhibited at similar levels. hOCT3 showed the lowest inhibition, 60% with D-22 and Rani, and nearly 50% with the remaining

compounds. Nevertheless, inhibition was statistically significant in all cases and showed that 3TC was a substrate for hOCTs.

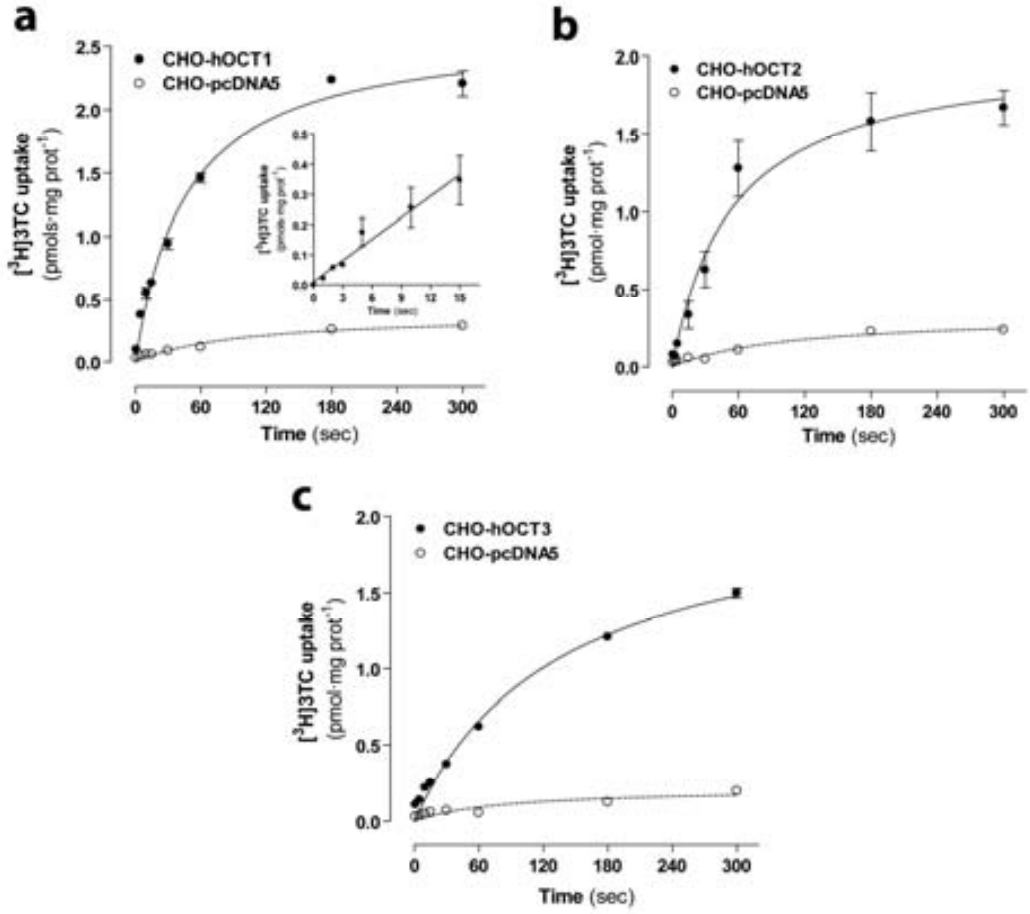


Figure 46. Time courses of [³H]3TC uptake in CHO-hOCT1, -hOCT2, -hOCT3, and -pcDNA5 (empty vector) cells. [³H]3TC uptake (156.25 nM) at 37°C was performed at different times (1, 5, 15, 30, 60, 120, 180, and 300 seconds) in CHO-hOCT1 *(a)*, -hOCT2 *(b)*, and -hOCT3 *(c)* (●) to assess the linearity and behaviour of 3TC uptake and the involvement of each transporter subtype (compared with control cells/CHO-pcDNA5, ○). Inset graph in *(a)*: CHO-hOCT1 [³H]3TC uptake values for the first 15 seconds of transport. Data are expressed as the mean uptake (picomoles per milligram of protein) ± one half the range of the two mean uptake values of two independent experiments with each point performed in quadruplicate. Error bars are not shown if the deviation was smaller than the size of the symbol.

ABC and AZT Are Not hOCTs substrates and Have a High-Percentage of Simple Diffusion

After studying [³H]3TC uptake and inhibition in detail, we performed the uptake of radiolabelled AZT and ABC in CHO-hOCTs to compare it with the uptake in CHO-pcDNA5 (empty vector). First, the time course experiments with [³H]ABC showed some significant differences between CHO-hOCT1 and hOCT3 versus CHO-pcDNA5 for ABC. For AZT

uptake, only CHO-hOCT1 showed significant differences versus CHO-pcDNA5, although all time course experiments showed a linear uptake up to 300 seconds. These linear uptake rates indicate that most probably, a high-percentage of non-mediated diffusion is occurring. To confirm the putative transport of ABC and the high diffusion and hence low involvement of hOCTs in AZT transport, we performed *cis*-inhibition experiments with OCT substrates and inhibitors, as done for 3TC (*Figure 48 and 49*).

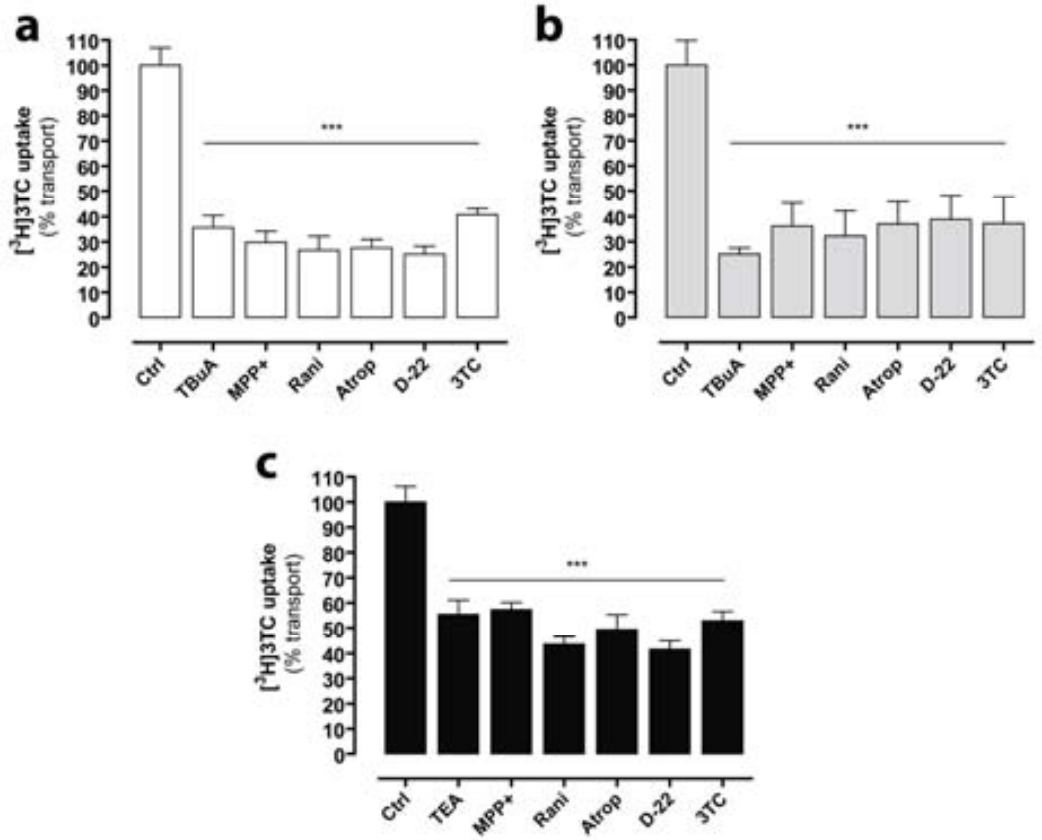


Figure 47. Inhibition of [3H]3TC uptake by hOCT substrates and inhibitors in CHO-hOCT1 *(a)*, hOCT2 *(b)* and hOCT3 *(c)* cells. [3H]3TC uptake (156.25 nM) at 15 seconds (linear range) was performed in the absence (Ctrl) or presence of 2 mM TBuA (TEA, in the case of hOCT3), MPP+, ranitidine, atropine, nonradiolabelled 3TC and 200 µM D-22 at 37°C. Results are expressed as the transport percentage (normalized by the uptake in control cells) and are represented as the mean ± SEM of three independent experiments, with each point performed in quadruplicate. Statistical significance was assessed using a paired Student's *t*-test (***, $p < 0.001$).

Surprisingly, with ABC we found no significant inhibition for either CHO-hOCT1 or CHO-hOCT3. In addition, no inhibition of [3H]ABC uptake was found for 2 mM of cold ABC. Only ranitidine and TBuA exerted a slight inhibition (~20%) of ABC uptake.

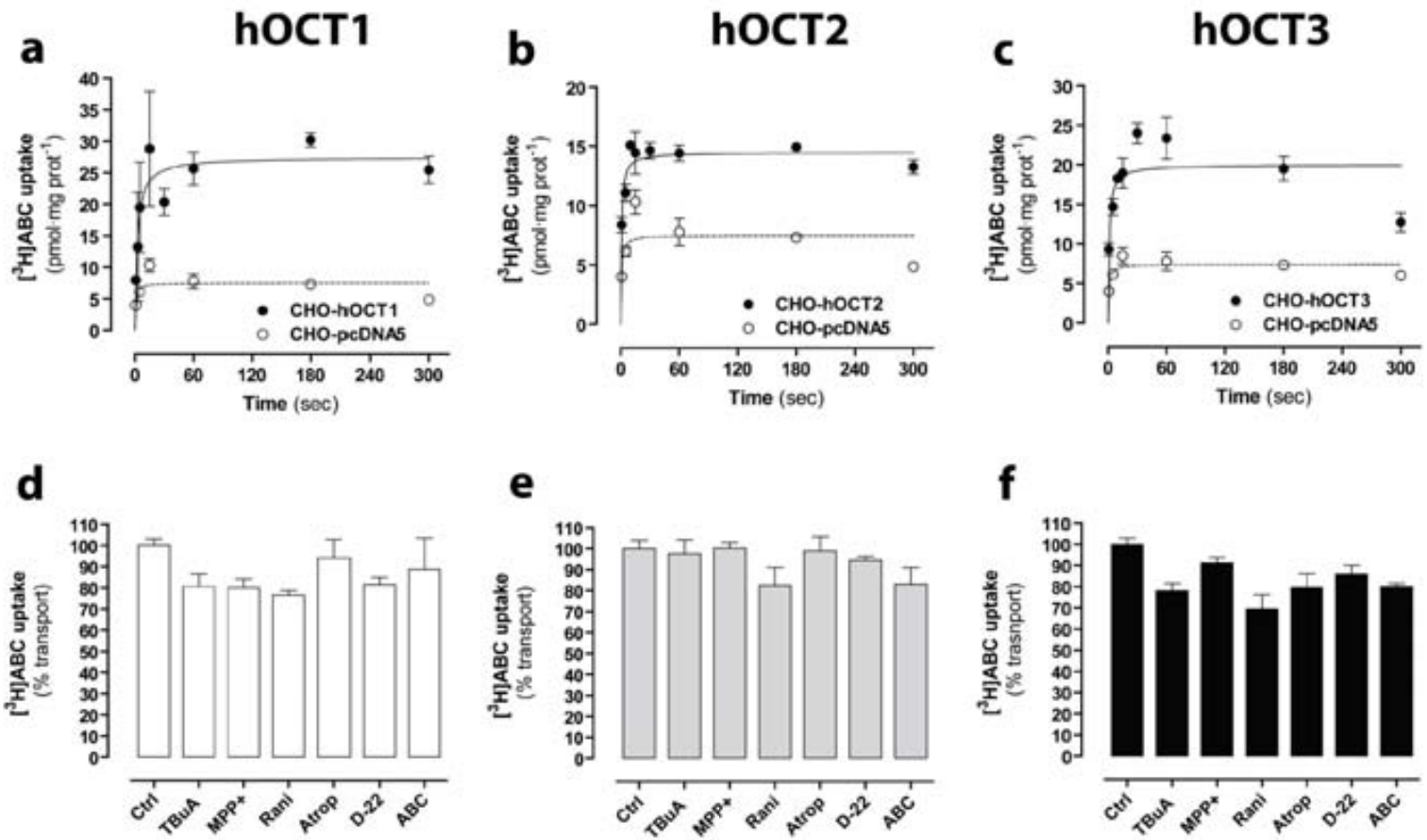


Figure 48. Time-courses of [3H]ABC uptake and inhibition by hOCT substrates and inhibitors in CHO-hOCT1, hOCT2 and hOCT3 cells. *(a, b, c)* [3H]ABC uptake (2 µM) at 37°C was performed at different time points (1, 5, 15, 30, 60, 120, 180 and 300 seconds) to assess the linearity and behavior of ABC uptake and the involvement of each transporter subtype (compared with control cells). Data are expressed as the mean uptake (pmol/mg prot) ± SEM of two independent experiments performed in quadruplicate. *(d, e, f)* [3H]ABC uptake (2 µM) at *15 seconds* (linear range) was performed in the absence (Ctrl) or presence of 2 mM TBuA (TEA, in the case of hOCT3), MPP+, ranitidine, atropine, non-radiolabelled ABC and 200 µM D-22 at 37°C. The results are expressed as a percentage of uptake inhibition (compared with the uptake of control cells) and are represented as the mean ± half the range of the two mean uptake values of two independent experiments, with each point performed in quadruplicate. Statistical significance was assessed using a paired *t*-test.

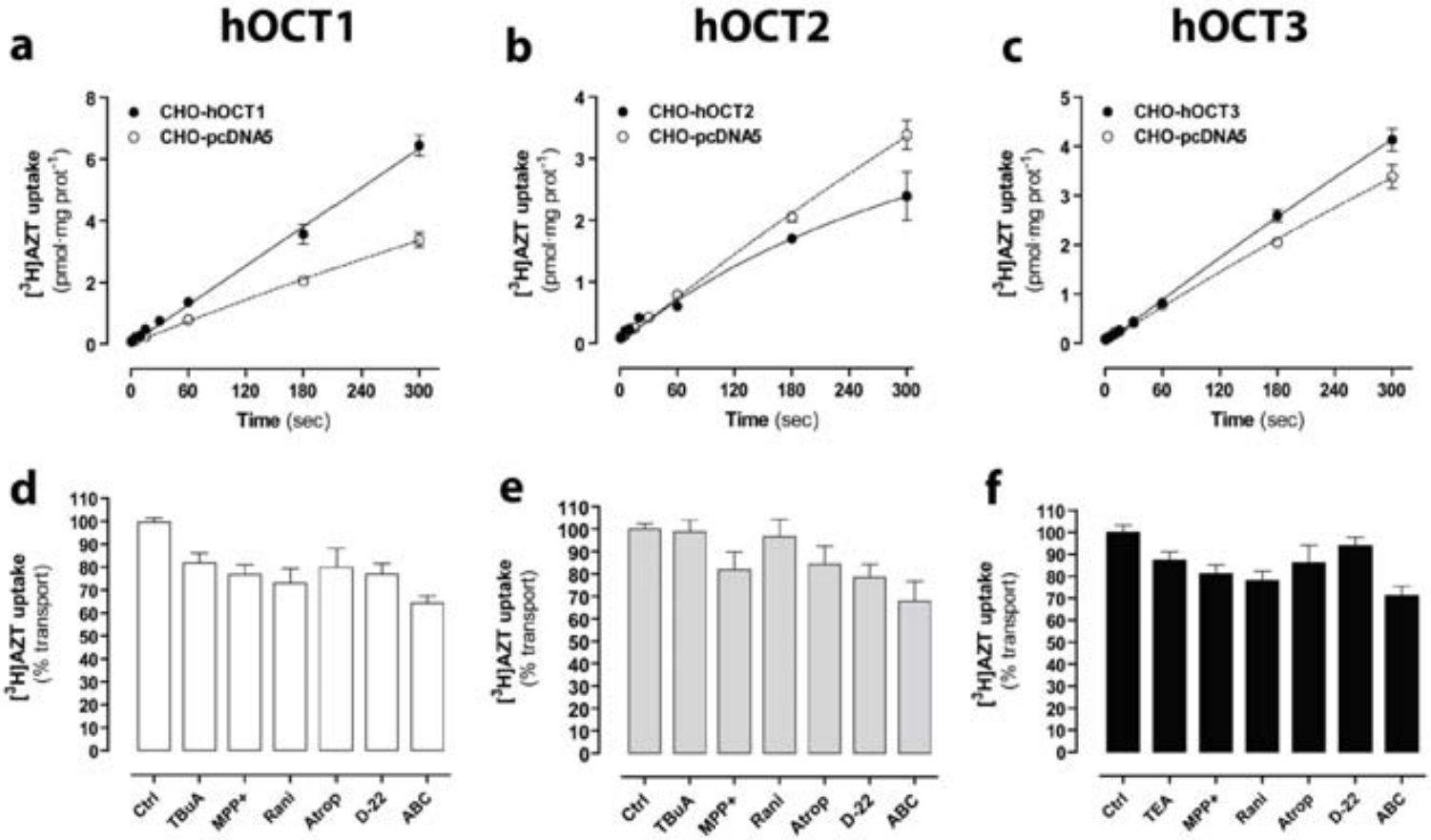


Figure 49. Time-courses of [^{3}H]AZT uptake and inhibition by hOCT substrates and inhibitors in CHO-hOCT1, hOCT2 and hOCT3 cells. *(a, b, c)* [^{3}H]AZT uptake (0.10 µM) at 37°C was performed at different time points (1, 5, 15, 30, 60, 120, 180 and 300 seconds) to assess the linearity and behaviour of AZT uptake and the involvement of each transporter subtype (compared with control cells). Data are expressed as the mean uptake (pmol/mg prot) ± SEM of two independent experiments performed in quadruplicate. *(d, e, f)* [^{3}H]AZT uptake (0.10 µM) at *15 seconds* (linear range) was performed in the absence (Ctrl) or presence of 2 mM TBuA (TEA, in the case of hOCT3), MPP+, ranitidine, atropine, non-radiolabelled AZT and 200 µM D-22 at 37°C. The results are expressed as a percentage of uptake inhibition (compared with the uptake of control cells) and are represented as the mean ± half the range of the two mean uptake values of two independent experiments, with each point performed in quadruplicate. Statistical significance was assessed using a paired *t*-test.

This would indicate, as anticipated for AZT, that a non-mediated diffusion mechanism is mainly involved in ABC entry into CHO cells. Nevertheless, part of a mediated mechanism related to endogenous transporters up-regulated in CHO-hOCTs cells (since CHO-pcDNA5 transported less [3H]ABC) could also play a role. As expected for AZT, only very weak inhibition (~20–25%) was found in the presence of hOCT substrates in CHO-hOCTs cells, although no statistical significance was found. Cold AZT at 2 mM inhibited [3H]AZT up to 25%, suggesting a 75% non-mediated diffusion mechanism for AZT in CHO cells.

hOCTs selectivity for 3TC, ABC and AZT in Xenopus laevis uptake experiments

To additionally confirm the conclusions of hOCT selectivity for NRTIs, we carried out uptake experiments on *Xenopus laevis* oocytes injected with cRNA of hOCT1 and hOCT2 (see *Materials & Methods*). Interestingly, confirming our previous results for the CHO cell system, the uptake of [3H]3TC and [3H]MPP+ was inhibited by 2 mM 3TC and 1 mM MPP+, respectively, whereas the uptake of [3H]ABC and [3H]AZT was not affected by 2 mM ABC and AZT, respectively. This would mean, as stated previously, that 3TC is a substrate of, at least hOCT1 and hOCT2, whereas ABC and AZT are not, with non-facilitated diffusion as the main mechanism involved in their entry (*Figure 50*).

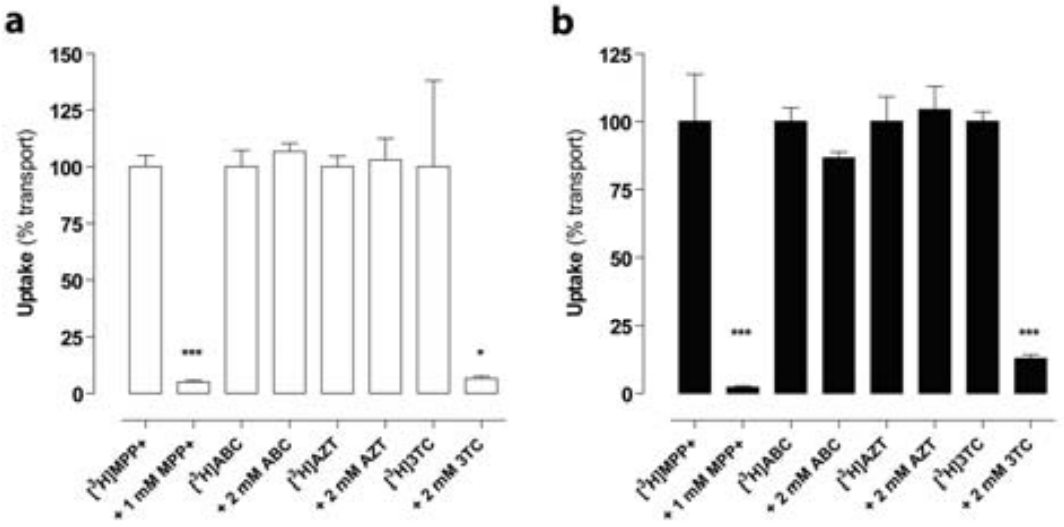


Figure 50. Inhibition of [3H]MPP+ and [3H]NRTIs uptake by cold MPP+ and NRTIs in *Xenopus laevis* oocytes expressing injected cRNA of hOCT1 and hOCT2. Oocyte preparation and uptake measurements were performed as described previously (Gorboulev et al., 2005). [3H]MPP+ (12.5 nM), [3H]ABC (2 μM), [3H]AZT (0.10 μM) and [3H]3TC (156.25 nM) uptake was measured for 30 minutes in the absence (Ctrl) or presence of 1 mM MPP+ or 2 mM ABC, AZT and 3TC at room temperature. Results are expressed as the transport percentage (normalized by the uptake in control cells). The figure shows a representative experiment with each point showing the mean value ± SEM of 6–9 oocytes. Statistical

significance was assessed using a paired *t*-test (* $p < 0.05$; *** $p < 0.001$ compared to the respective uninhibited controls).

Transport Kinetics Revealed hOCT1 to Be the Most Efficient 3TC Transporter of hOCTs

To kinetically characterize the uptake of 3TC by the three hOCT subtypes and compare the efficacy of transport and affinity for the substrate, we performed 3TC uptake at increasing concentrations of the drug (*Figure 51*). All three hOCTs showed saturable transport kinetics and followed a Michaelis-Menten curve for 3TC uptake, with K_m being in the same order of magnitude (millimolar range) and only slight differences in V_{max} values (*Table 13*). To be specific, hOCT1 showed the highest affinity for the 3TC substrate, with a saturation curve with an estimated K_m of 1.25 ± 0.1 mM and a V_{max} of 10.40 ± 0.32 nmol/mg protein/min, followed by hOCT2 and hOCT3.

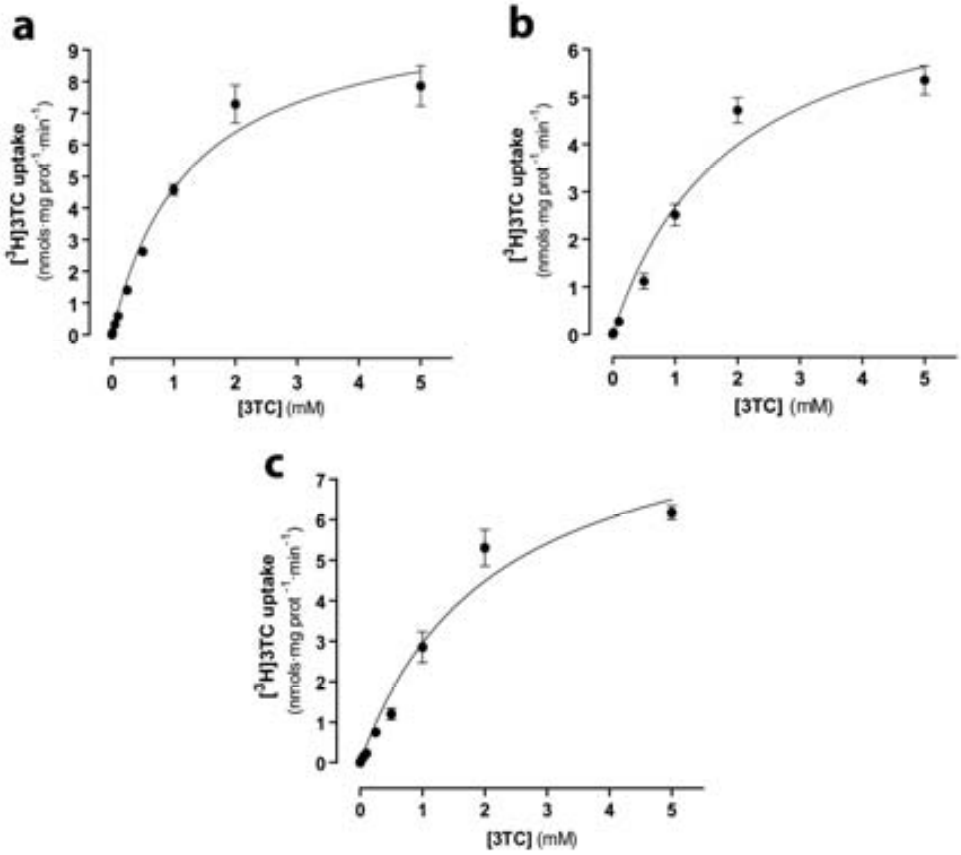


Figure 51. Uptake kinetics of 3TC by hOCT1, hOCT2 and hOCT3. CHO-hOCT1 *(a)*, hOCT2 *(b)* and hOCT3 *(c)* were incubated with [3H]3TC (156.25 nM) for 15 seconds (linear range) in the presence of increasing concentrations of nonradiolabelled 3TC (10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 0.25, 0.5, 1.0, 2.0 and 5.0 mM) at 37°C. Kinetic parameters (K_m and V_{max}) were estimated by fitting hOCT-specific uptake rates to a Michaelis-Menten nonlinear equation (*Table 13*). Data represent the mean (in nanomoles per milligram of protein per minute) ± S.E.M. from three independent experiments, with each point performed in quadruplicate. Error bars are not shown if the S.E.M. values were smaller than the size of the symbol.

Moreover, hOCT1 also showed the highest transport efficiency, with a V_{max}/K_m quotient of 8.33 ± 0.40 µl/mg protein/min, that is, twice as high as hOCT2 and hOCT3, which showed the same transport efficiency (4.10 ± 0.30 versus 4.30 ± 0.30 µl/mg protein/min, respectively).

	K_m (mM)	V_{max} (nmol·mg prot^{-1}·min^{-1})	V_{max}/K_m (µl·mg prot^{-1}·min^{-1})
hOCT1	1.25 ± 0.10	10.04 ± 0.32	8.03 ± 0.40
hOCT2	1.90 ± 0.25	7.80 ± 0.45	4.10 ± 0.30
hOCT3	2.14 ± 0.24	9.27 ± 0.50	4.30 ± 0.31

Table 13. Kinetic parameters of 3TC uptake in CHO-hOCT1, -hOCT2, and -hOCT3. The K_m and V_{max} values were estimated by fitting the data from *Figure 34* to a Michaelis-Menten nonlinear equation. Values are mean ± SEM of at least three experiments.

Comparison of Transport Efficiency (TE) Values for 3TC with the Model Substrate MPP+ and the Antidiabetic Drug Metformin

It is well accepted that in order to discriminate between "good" and "bad" substrates, the transport efficiency (TE) must be determined, which is defined – analogously to catalytic efficiency for enzymes – by K_{cat}/K_m. To extract the TE as defined, one must determine the number of active carriers (E_{total}) in the plasma membrane of the system used (cell line or oocytes). As measuring E_{total} is a very difficult measurement, TE is usually determined as V_{max}/K_m, which is a measure directly proportional to K_{cat}/K_m (since $V_{max} = K_{cat} \cdot E_{total}$). It is clear that the transport efficiencies are markedly different among the carriers (Schomig et al., 2006).

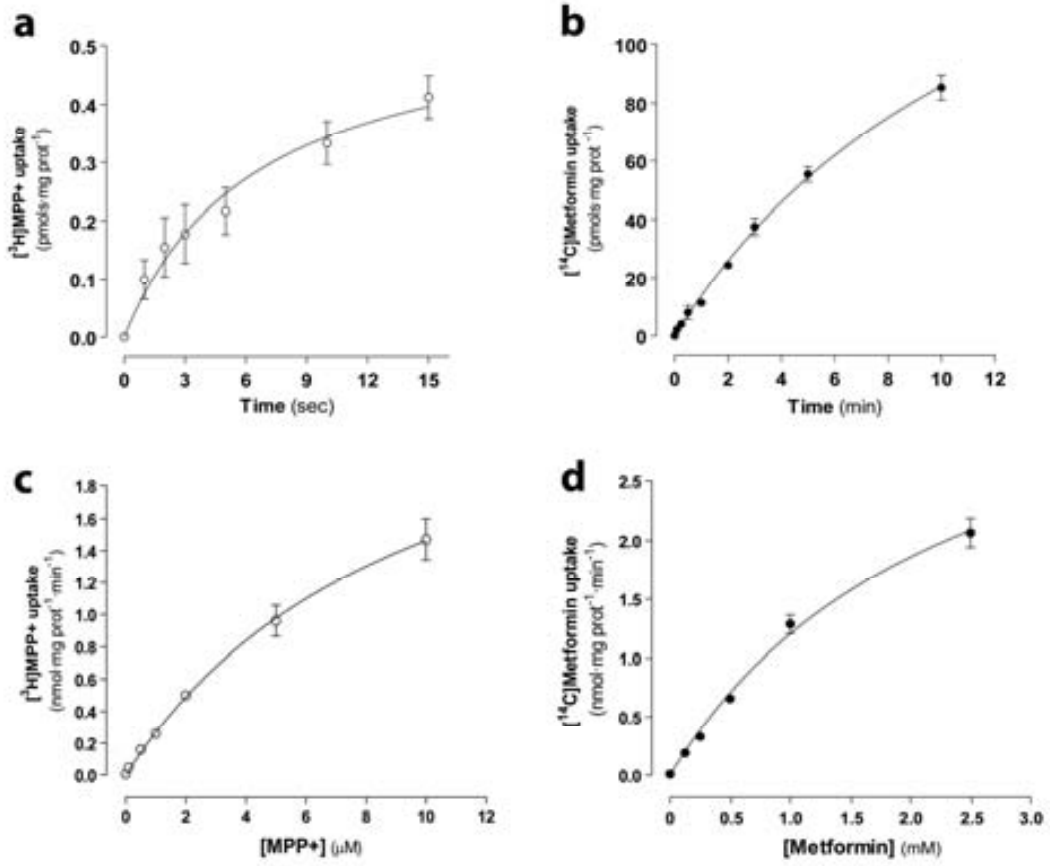


Figure 52. Time-courses of [^{3}H]MPP+ and [^{14}C] metformin uptake and concentration-dependence studies by hOCT substrates and inhibitors in CHO-hOCT1. *(a)* [^{3}H]MPP+ uptake (12.5 nM) at 37°C was performed at different time points (0, 1, 2 , 3, 5, 10 and 15 seconds) in CHO-hOCT1 to assess the linearity and behaviour of MPP+ uptake at the transport rates of the first few seconds. *(b)* Similarly, [^{14}C] metformin uptake (5 μM) at 37°C was performed at different time points (0, 5, 15, 30 seconds, and 1, 2, 3, 5 and 10 minutes). Time course data are expressed as the mean uptake (pmol·mg prot^{-1}) ± half the range of the two mean uptake values, with each point performed in quadruplicate. *(c)* CHO-hOCT1 were incubated with [^{3}H]MPP+ (12.5 nM) for 1 second (linear range) in the presence of increasing concentrations of nonradiolabelled MPP+ (0.1, 0.5, 1.0, 2.0, 5.0 and 10.0 μM) at 37°C, and *(d)* were incubated with [^{14}C] metformin (5 μM) for 1 minute (linear range) in the presence of increasing concentrations of nonradiolabelled metformin (0.125, 0.25, 0.5, 1.0 and 2.5 mM). Kinetic parameters were estimated by fitting hOCT-specific uptake rates to a Michaelis-Menten nonlinear equation. Results are expressed as the mean (in nmol·mg prot^{-1}min^{-1}) ± half the range of the two mean uptake values of two independent experiments.

	K_m (μM)	V_{max} (nmol·mg prot^{-1}·min^{-1})	V_{max}/K_m (μl·mg prot^{-1}·min^{-1})
MPP+	9.95 ± 0.32	2.92 ± 0.45	293.50 ± 4.00
Metformin	2390 ± 220	4.08 ± 0.225	1.70 ± 0.01
3TC	1250 ± 103	10.04 ± 0.32	8.03 ± 0.40

Table 14. Kinetic parameters of 3TC uptake in CHO-hOCT1, hOCT2 and hOCT3. The K_m and V_{max} values were estimated by fitting the data from *Figure 34* to a Michaelis-Menten nonlinear equation. Values are the mean ± SEM of at least three experiments.

Therefore, we aimed to compare the TE of 3TC with MPP+ and metformin for hOCT1 in our cell system. MPP+ is a good model substrate of hOCTs. Metformin, an antidiabetic drug recently discovered to be an hOCT1 and hOCT2 substrate with a K_m also in the millimolar range, was thus the best example to establish a comparison in terms of TE. To do so, we first performed a time course for metformin (transport being quite linear until 10 minutes) and then kinetic curves with both MPP+ and metformin (*Figure 52*). Fitting the curves with the Michaelis-Menten equation, we found a very high V_{max}/K_m for MPP+ (293.5 μl/mg protein/min), as expected, but a five-fold lower transport efficiency for metformin (1.7 μl/mg protein/min) than for 3TC (8.03 μl/mg protein/min; *Table 14*).

ABC and AZT Inhibit 3TC Uptake at Very Low Concentrations

Finally, because we had observed that all the NRTIs interacted with a high affinity with hOCTs (*Figure 43*) and that 3TC was a substrate for these transporters, we wanted to explore whether other NRTIs usually taken in combination with 3TC during highly active antiretroviral therapy (HAART) could inhibit 3TC uptake in our stably transfected cell system (*Figure 53*). Using low concentrations of ABC and AZT (up to 10 μM), we found inhibition of hOCT-mediated transport of 3TC, with K_i ranging from 6.2 ± 4.1 pM (ABC inhibition for hOCT2-mediated transport) to 330 ± 280 pM (ABC inhibition for hOCT1-mediated transport) (*Table 15, page 126*). It is significant that both NRTIs showed considerably different affinities for the transporter subtypes; for example, ABC had a more than fifty-fold greater affinity for hOCT2 and a five-fold greater affinity for hOCT3 than for hOCT1. Nevertheless, no inhibition was more than 45 to 55% for either drug at the highest concentration tested.

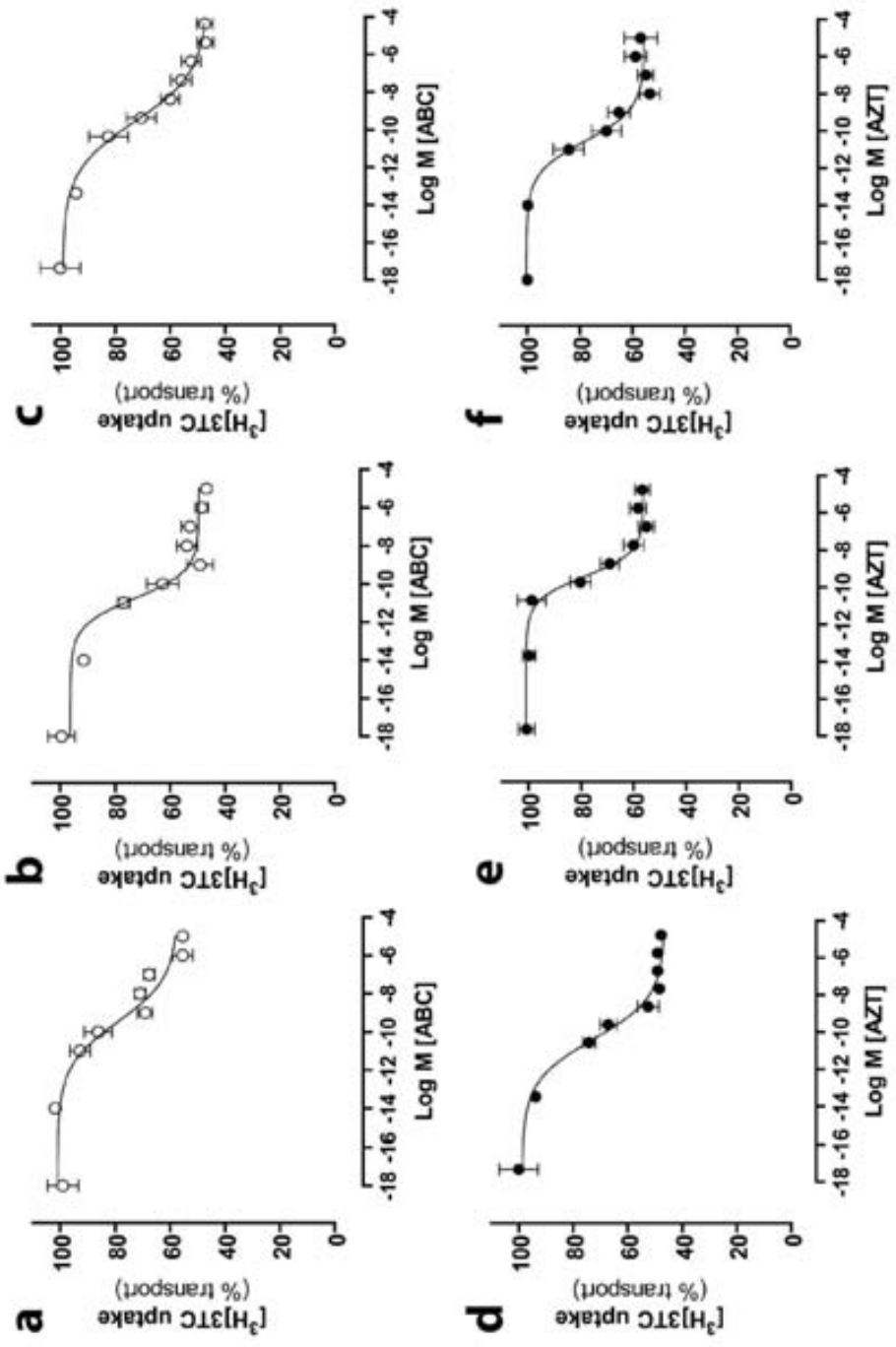


Figure 53. Use of ABC and AZT for *cis*-inhibition of [^{3}H]3TC uptake in CHO-hOCT1, hOCT2 and hOCT3 cell lines. Different low-range concentrations (10^{-3}, 10^{-2}, 10^{-1}, 1.0, 10, 10^2, 10^3 and 10^4 nM) of ABC were used to perform *cis*-inhibition for 15 second [^{3}H]3TC uptake (156.25 nM) by CHO-hOCT1 *(a)*, hOCT2 *(b)* and hOCT3 *(c)* transfected cells at 37°C. The same low-range concentrations (10^{-3}–10^4 nM) of AZT were used to perform *cis*-inhibition of [^{3}H]3TC uptake (156.25 nM) by CHO-hOCT1 *(d)*, hOCT2 *(e)* and hOCT3 *(f)* transfected cells at 37°C. The results are expressed as the percentage of inhibition (mean ± SEM) from at least three independent experiments, with each point performed in quadruplicate. The inhibition constant (K_i) values were obtained by fitting the data to the Hill Inhibition equation (*Table 15*).

	K_i (nM)		
NRTI	**hOCT1**	**hOCT2**	**hOCT3**
ABC	0.330 ± 0.280	0.006 ± 0.004	0.070 ± 0.045
AZT	0.021 ± 0.016	0.205 ± 0.098	0.030 ± 0.021

Table 15. K_i values of ABC and AZT inhibition of [^{3}H]3TC uptake in CHO-hOCT1, -hOCT2, and -hOCT3. Data are shown as K_i values ± S.E.M. from at least three independent experiments, with each point performed in quadruplicate.

Discussion

General Overview

This thesis has addressed the question of which **molecular mechanisms take part in the entry of the nucleoside-derived drugs used in HIV therapy**, focusing on the putative transporters previously indicated in the literature as candidate transporters for antiviral drugs. To do so, the **entry mechanisms of azidothymidine (AZT)** were analysed first, taking into account that this has probably been the most widely used drug since the beginning of HIV therapy, and perhaps the one with the most complex entry mechanism to solve, due to its more highly lipophylic character. Following that, we attempted to address the lack of information regarding the **expression and activity of drug uptake transporters in immune cells**, including T cells and cells from the monocyte/macrophage families, the main targets of HIV infection. As a preliminary approach to the direct effect of HIV on the expression of transporters, we also explored the changes in mRNA levels in infected PBMCs and MT4. Moreover, **the implication of nucleoside salvage and the possible antiviral effects of dipyridamole (DIP)** was also analysed in these cells. Finally, focusing on a family of highly expressed and active transporters in immune cells, the Organic Cation Transporter (OCT) family, we studied the **interactions and transportability of all NRTIs currently used in Highly Active Antiretroviral Therapy (HAART) with the three main OCT types**. Throughout the course of the investigation, we discovered that NRTIs do not seem to have a common entry pathway, contrary to what it was initially thought, neither in the immune cells nor in the other cells tested (such as CHO or HeLa cells). We have corroborated the finding that slight changes in the nucleoside-derived anti-HIV drugs can result in considerable differences in the selectivity of the compounds for one or another transporter family. Importantly, we demonstrated that some protein members of the *SLC28, SLC29* and *SLC22* gene families do participate in NRTI uptake in immune cells, which could also be significant to absorption and re-absorption tissues. In the following, each part of the results chapters will be discussed in detail in order to arrive at the conclusions for this work.

Chapter I. 3'-Azido-2',3'-Dideoxythymidine (AZT) Uptake Mechanisms in T-Lymphocytes

Cross-Inhibition Studies with NRTIs Do Reveal False Clues of Substrate Selectivity for hNTs

AZT was the first drug used against the HIV virus and for that reason it has been one of the most commonly used drugs in HIV therapy (Mitsuya et al., 1985; De Clercq, 2009). The first part of this thesis addresses the question of **whether AZT uptake into T cells is a carrier-mediated process or**, alternatively**, the result of non-facilitated diffusion** of the drug across the plasma membrane, as suggested for this particular nucleoside-derived molecule in other cell systems (Zimmerman et al., 1987; Lopez-Anaya et al., 1990). Although simple diffusion of AZT into T cells might indeed contribute to drug accumulation, clear evidence was obtained to suggest that mediated processes can also occur.

Firstly, by performing radiolabelled uridine uptake. Upon competition with two different concentrations of each NRTI tested (AZT, 3TC, ddC, d4T and TDF) as an initial screening of NRTIs interaction with NTs, we only found significant inhibition for 1 mM AZT. Performing the same type of experiment with radiolabelled guanosine, the same AZT concentration inhibited uptake, although to a lesser extent than ddI 1 mM. These inhibition experiments led us to consider AZT as a good candidate for hNTs transport, as well as ddI specifically for hCNTs. However, this is a common error in membrane transporter studies: the pharmacological profile of NTs (and other transporters) has often been analysed using cross-inhibition studies when, unfortunately, transportability cannot necessarily be deduced from this type of experimental approach (Schomig et al., 2006; Pastor-Anglada et al., 2008). By **cross-inhibition studies one can only deduce the interaction between a drug and a transporter (or a family of transporters)** and, depending on the substrate used and the equation used to fit the data, an IC_{50} or a K_i value can be obtained. In this sense many drugs and various compounds could be interacting and/or inhibiting one or another hCNT or hENT isoform without being translocated (Baldwin et al., 2004; Pastor-Anglada et al., 2008). This phenomenon has also been described for many drugs with relation to other transporter families and it is especially well known for the *SLC22* gene family (human Organic Cation and Anion Transporters) (Koepsell, 2004; Koepsell et al., 2007). Therefore, to confirm that a drug is a substrate of a specific transporter, direct entry with the radiolabelled drug must be tested. Using a cell line stably expressing or over-expressing a concrete isoform of the transporter of interest

would be very useful. Nevertheless, experiments are also commonly performed by inhibiting drug uptake with specific substrates or inhibitors of the transporter isoform. Therefore, to test whether AZT was a good NT substrate, we performed [^{3}H]AZT uptake experiments with Molt-4.

Is AZT a Real hCNT or hENT Substrate in Immune Cells?

AZT is a nucleoside-derived drug. Simply based on its chemical structure, it could be initially hypothesized that NTs are the transporter proteins involved in its cell entry. Surprisingly, **a major contribution of the nucleoside transporter proteins** hCNTs (encoded by the *SLC28* gene family) and hENTs (encoded by the *SLC29* gene family**) in AZT transport**, at least **into T cells**, **can be ruled out** on the basis of the data found in the first study. The Na^+-dependence of AZT entry into Molt-4 cells was found to be negligible, hence the involvement of hCNTs – the Na^+-dependent nucleoside transporters – was also supposed to be very low or negligible. Even though we know that hCNT3 is a good antiviral transporter (Errasti-Murugarren et al., 2007), we showed in the second part of this thesis that it was not found to be expressed in Molt-4 cells, as concluded from the limited amounts of mRNA and protein levels found in the majority of lymphoblastic T cell lines (including Molt-4), primary cultures of PBMCs and CD4+ T lymphocytes. More importantly, we had previously found for hCNT1 that the **3'hydroxyl group of the sugar**, which is modified in AZT, **is a key structural determinant for substrate recognition** (Cano-Soldado et al., 2004). The fact that slight differences in the nucleoside structures can determine its NT transportability were confirmed later not only for hCNT1 but also for hCNT2 and hENT1 (Zhang et al., 2003; Chang et al., 2004; Zhang et al., 2005a).

The role of NTs in AZT uptake in the Na^+-dependent hCNTs and the hENT passive facilitators, was clearly discounted when AZT uptake could not be inhibited by high concentrations (1 mM) of either the specific ENT1 inhibitor nitrobenzylthioinosine (NBTI), the ENTs inhibitor dipyridamole (DIP), the natural nucleosides uridine and guanosine, or the nucleobase hypoanthine. Previous studies using recombinant hENT2 protein expressed in *Xenopus* oocytes anticipated a role for this transporter in AZT uptake (Yao et al., 2001). Our data on MCF7, a cell line that endogenously expresses this carrier, would rule out this possibility, since little or no DIP inhibition of the AZT uptake was found. Nevertheless, based upon the putative **slight interaction of AZT with hENT1** described here, a **residual role for the equilibrative nucleoside transporters** in the uptake of this antiviral drug in other cell types or tissues **cannot be fully excluded**.

Looking for Other Means of Egress: human Organic Anion Transporters and Non-Mediated Diffusion

The other candidate high-affinity AZT uptake mediators were human Organic Anion Transporters, proteins encoded by the *SLC22* gene family which show lower apparent K_m values (between 20 and 150 μM) than those determined for nucleoside transporters (Yao et al., 2001; Cano-Soldado et al., 2004; Errasti-Murugarren et al., 2007). The lack of inhibition of AZT transport by known hOAT substrates such as probenecid or PAH, is consistent with the mRNA profiles, which strongly suggest **that immune system cells in general do not express hOAT proteins**, even after stimulation with PHA. According to the available data on hOAT tissue distribution (Koepsell, 2004), we believe that these membrane proteins are probably restricted to absorptive and re-absorptive epithelia and might play a significant role in determining drug pharmacokinetics, but do not contribute to drug accumulation in T lymphocytes and macrophages.

With regard to passive diffusion, lipophilicity is an important physicochemical characteristic related to drug chemical structure and size that must be considered. The more lipophilic a drug is, the larger the proportion of non-mediated diffusion it may have. Therefore, it is notable that **AZT is probably the most lipophilic of the NRTIs** available to clinics. Lipophilicity is normally expressed as the *n*-octanol/water coefficient, a value generally called the *partition coefficient* (P_{app}). Along with ABC, AZT has a partition coefficient number above 1, whereas the other NRTIs do not reach 0.5. Thus, the azido group ($-N_3$) at the 3' position of the ribose in AZT, and the cyclopropylamino moiety at the 6 position of the purine ring in ABC, confer a more lipophilic character to these drugs ($P_{app} > 1$; *see* Table 1 in *Introduction*), **which could result in a high percentage of non-mediated diffusion through cell membranes**. In fact, the suggestion that AZT entered erythrocytes and primary lymphocytes by means of a non-facilitated diffusion mechanism was made a long time ago (Zimmerman et al., 1987). Intestinal transport studies have shown that AZT is transported by non-facilitated membrane diffusion via systemic circulation (Park and Mitra, 1992). Similarly, ABC has more recently been described as entering erythrocytes and the CEM cell line via non-mediated diffusion compared to the more hydrophilic ABC analogue, carbovir ($P_{app} = 0.30$), which showed a carrier-mediated mechanism (Mahony et al., 2004). Importantly, these membrane permeation characteristics common to AZT and ABC are consistent with their superior oral bioavailability and their impressive ability to penetrate the central nervous system (Letendre et al., 2008; Varatharajan and Thomas, 2009). The possibility that a significant fraction of transport is associated with simple diffusion is not restricted to the nucleoside-

derived drugs used in HIV-1 therapy. It has recently been shown that the pyrimidine nucleoside analogue troxacitabine, an anticancer drug candidate, is taken up almost exclusively by simple non-facilitated diffusion (Gourdeau et al., 2001). More astonishing is the finding that d4T is taken into the lymphoblastoid H9 cell line via non-facilitated diffusion (August et al., 1991) as the low partition coefficient of the drug (P_{app} = 0.18) – indicating a low lipophilicity – suggests a carrier-mediated mechanism rather than a free diffusion mechanism. However, in intestinal transport studies this thymidine analogue was shown to permeate the intestine via a carrier-mediated transport, accounting for approximately 30–40% of the total apparent permeability (Waclawski and Sinko, 1996).

AZT has, in part, a Carrier-Mediated Diffusion Mechanism

The data provided in our first study suggest that a route for **AZT** carrier-mediated **transport** also contributes to drug intracellular accumulation, and this is based upon various lines of evidence. The process **is temperature sensitive**, as also shown for uridine uptake mechanisms (mostly hENT1 in Molt-4 cells). It is widely known that protein-mediated cell processes suffer a dramatic decrease in activity at low temperatures. In contrast, a non-facilitated diffusion process would be less affected by temperature changes, hence our data indicate a mediated component in AZT uptake. The **capacity of AZT to inhibit its own transport by up to 45–50%** is additional evidence arguing against the simple diffusion hypothesis, although this 50-55% non-inhibitable component could be non-facilitated diffusion of the drug through the lipid bilayer.

Most interestingly, the **up-regulation of AZT uptake when PBMC is PHA-stimulated** is a further evidence for a mediated and probably regulated carrier, since simple diffusion is a passive non-regulated process. Nevertheless, the whole range of inhibitors used has not yet permitted us to approach the molecular identity behind this process. In that respect, in experiments with Molt-4, we used a range of nucleoside transporters substrates such as uridine (a common hNTs substrate), guanosine (taken up mainly by hCNT3 and hENTs), hypoxanthine (only substrate for hENT2), ribavirin (an anti-HCV drug known to be substrate of hENTs) and dipyridamole (high-affinity inhibitor of hENTs). None of these substrates or inhibitors induced a significant inhibition of AZT uptake. Previous reports had described that the efflux rate of AZT from the brain is significantly inhibited by probenecid and PAH but not by thymidine (Takasawa et al., 1997a; Takasawa et al., 1997b). Moreover, it is well known that OATs from the *SLC22* gene family, especially the rat orthologue of OAT1 and the four human OATs (hOAT1–4), are capable of transporting AZT with high-affinity (e.g. K_m = 43–70 μM for rOAT1 and K_m =

27–152 μM for hOATs) (Chen and Nelson, 2000; Wada et al., 2000; Takeda et al., 2002). To test hOATs' implications on AZT cell entry, we tried inhibition with the hOATs substrates probenecid and PAH alone and in combination with nucleoside transporter substrates but we could not find significant inhibition rates in either case. This lack of inhibition by hOATs could correspond with a lack or negligible expression of hOATs in Molt-4 cells (and presumably in general in T cells), as stated previously. Finally, since other antiviral nucleoside derivatives (3TC and ddI) could not inhibit AZT, this suggests that **AZT uptake might not share the same routes as those used by the other antiviral drugs**. At that point, we also asked ourselves if a common route of entry exists for all NRTIs or, alternatively, whether each drug – with its chemical and structural particularities – uses a transporter subtype different from any *SLC* family expressed and active in immune cells. This question will be discussed further.

In summary, in the first study we provide evidence for mediated routes for AZT transport across the plasma membrane of T cells, which may contribute to drug accumulation, in addition to the generally accepted non-facilitated diffusion component of AZT uptake. Moreover, considering that the mediated component of AZT uptake appears to be regulated, we provide the first basis for a putative modulation of the entry routes of antiviral drugs as a future means to improve drug accumulation and action. This will only become feasible once the AZT carrier is identified, a goal that is still being pursued at our laboratories.

Chapter II. Expression and Functionality of Anti-HIV and Anticancer Drug Uptake Transporters in Immune Cells

The expression of drug transporters is relatively well studied in secretory and re-absorptive tissues with relation to toxicity, drug–drug interactions and drug resistance in antineoplasic and antiviral therapies (Lee and Kim, 2004; McRae et al., 2006; Koepsell et al., 2007). Moreover, there is broad knowledge of the expression and activity of efflux transporters in peripheral blood cells (Kock et al., 2007). However, **little is known of the expression profile and activity of uptake transporters in human leukocytes within the context of anticancer and antiretroviral therapies**. Therefore, once the AZT mechanisms of entry had been studied, we sought to assess the mRNA levels, the protein expression profile and activity of the putative drug uptake transporters involved in anti-HIV and antineoplasic drug uptake in immune system cells. Though AZT has been shown to not be taken up by nucleoside transporters, we zeroed in

on the members of the *SLC28* and *SLC29* families, given that HIV reverse transcriptase inhibitors and anticancer drugs are mostly nucleoside derivatives. Similarly, members of the *SLC22* family (OATs and OCTs) have also been described as antiviral drug transporters in other tissues. Since we had demonstrated that OATs are not expressed in immune system cells, we turned to analyse OCT transporters, which have not been previously characterized in immune system cells.

High hNTs' mRNA and Protein Expression in T cells and the Up-Regulatory Effect of PHA

We commenced the second part of this thesis by analysing the mRNA and protein expression of hCNTs and hENTs. In all the **T cell lines** tested (MT2, MT4, Molt-4, CEM13, A3F7 and Hut78) and in **primary lymphocytes** (PBMCs and $CD4^+$ T cells), **hENTs were the most expressed transporters, whereas hCNT2 was the only Na^+-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). As HIV infection is accompanied by the state of activation of these cells, we wanted to assess the effect of the strong mitogen phytohemmagglutinin (PHA) on transporter expression. PHA causes the transformation of resting lymphocytes into rapidly dividing lymphoblasts, with the synthesis of DNA beginning at 24 hours and peaking around 48 to 72 hours, preceded by an increase in the rate of synthesis of RNA and protein (Hausen and Stein, 1968). In our experiments, **PHA stimulation significantly up-regulated hENTs and hCNT2 in PBMCs and $CD4^+$ T cells**. As early as 1971, studies by Peters and Hausen demonstrated that PHA results in an increase in uridine uptake on lymphocyte membrane transport, a change that is intimately related to a change in the number of functional carrier sites of the membrane transport system (Peters and Hausen, 1971b). Similarly, they observed that PHA stimulates the transport of 3-*O*-methyl-glucose in bovine lymphocyte membranes (Peters and Hausen, 1971a). Moreover, the PHA-induced up-regulation of hENT1 is in agreement with the thirty-fold increase in the number of hENT1 molecules previously found in peripheral blood lymphocytes cultured within 48 hours of the introduction of the T cell mitogen (Smith et al., 1989). Studying CNT activity in bone marrow macrophages, a highly specialized cell type that coexpresses CNTs and ENTs, Soler et al. found that the activation of macrophages by either lipopolysaccharide (LPS) or interferon-γ (IFN-γ) resulted in the selective induction of CNT2 (Soler et al., 2001a; Soler et al., 2001b; Soler et al., 2003). In

contrast, LPS or IFN-γ slightly down-regulated the expression of ENT1 in murine macrophages (Soler et al., 2001), suggesting that the up-regulatory effects of PHA and other stimuli on hENTs might act through different pathways. Moreover, the addition of endocrine factors known to induce differentiation of foetal hepatocytes (e.g. dexamethasone and T_3) results in selective up-regulation of CNT2 expression (del Santo et al., 2001). 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 cell acute lymphoblastic leukaemia. In fact, an increased proliferative rate in acute myeloid leukaemia or lymphoma is associated with higher numbers of nucleoside transporters in the cell membrane (Wiley et al., 1989).

hENT1 is the Most Important Nucleoside Transporter in T lymphocytes and hCNT2 makes a Low Contribution to Transport Despite High mRNA and Protein Levels

Regarding hNT function, **uridine transport in PBMCs revealed a preferential role for hENT1**. Conversely, sodium-dependent transporters made a minimal contribution (only hCNT2 was expressed) and the **role of hENT2 was nearly negligible despite 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 non-functional transporters have previously been described (Mangravite et al., 2003). It is therefore 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, in accord with the up-regulatory effect observed for the mRNA and protein expression, whereas hENT2 did not show an increase in activity. hNT genes and the regulation of their expression and activity are poorly understood. Even though some of the studies explaining regulatory mechanisms started in the mid 80s, these were largely limited to ENT1. In 1971 the studies by Peters and Hausen mentioned above showed the same up-regulatory effect of PHA observed here but, at that time, they could not distinguish between hENTs subtypes. Studying the effect of DIP they found similar inhibition curves for non-treated and PHA-treated bovine lymphocytes in agreement with our inhibition rates (Peters and Hausen, 1971b). In contrast, much more recently, it has been shown that the differentiation of the human promyelocytic cell line HL-60 with phorbol myristate acetate (PMA) produced a decrease in ENT1 activity, whereas that of hCNT3 was concomitantly increased (Ritzel et al., 2001a). In addition to changes in transporter levels, there is a rapid activation of cell surface

hENT1 in cultured cells after phorbol ester treatment, perhaps involving PKC δ and/or ε, possibly by activation of transporters at the cell membrane by post-translational modification (Coe et al., 2002). Extracellular adenosine has been implicated as the central signalling molecule during hypoxia. Remarkably, an examination of the ENT1 promoter identified a hypoxia-inducible factor 1-dependent repression of ENT1 during hypoxia (Eltzschig et al., 2005). An extensive study of the ENT1 promoter has recently appeared, finding other transcription factors possibly involved in regulating ENT1 expression and activity (Abdulla and Coe, 2007). Therefore, in summary, the regulatory effects of different stimuli seen on hENT expression would be generally linked to hENTs activity and, depending on the stimulus and its associated regulatory pathway, the transporters would be either up or down-regulated at the expression and/or functional level. Finally, **hCNT2 activity seems to be very low in PHA-stimulated PBMCs, even though some specific donors' showed Na^+-dependence of uridine uptake**, which accords with previous results shown for B cells from Chronic Lymphocytic Leukaemia, in which although CNT2 expression was detected in twenty-two patients, Na^+-dependent guanosine transport activity was only detected in twelve patients (Molina-Arcas et al., 2003). Importantly, thus far the cellular activation state for a given cell type has been determined as being the most influential factor for increased generation of intracellular NRTI phosphate concentrations, although this was elucidated *in vitro*. Cells treated with PHA or granulocyte macrophage colony stimulating factor generated 2 to 1150-fold higher triphosphate concentrations of ddC, 3TC, d4T, AZT and ddI than resting cells *in vitro* (Perno et al., 1992; Gao et al., 1993; Gao et al., 1994; Robbins et al., 2003). Elevated cell activation results in high nucleic acid synthesis and the up-regulation of kinases that phosphorylate NRTIs (Gao et al., 1993; Soler et al., 2001). It must be noted that AZT, 3TC and ddI are more virologically active in resting cells than in activated cells, which has been attributed to a more favourable NRTI triphosphate ratio to endogenous nucleoside triphosphate in resting cells (Gao et al., 1993; Gao et al., 1994).

Relevant hCNT3 Expression and Activity in Monocyte/Macrophage Lineage Cells and its Putative Role in Antiviral Drug Entry

As in the case of T lymphocytes and T cell lines, significant mRNA levels were also observed for hCNT2, hENT1 and hENT2 in monocyte/macrophage lineage cells. However, **monocyte/macrophage lineage cells expressed significant quantities of hCNT3, whereas T lymphocytes did not**. Therefore, iMDDC and MDM showed the highest number of hCNT3 mRNA copies even though monocytes and mMDDCs also

showed higher expression than PBMCs or $CD4^+$ T cells. The abundance of hCNT3 protein in macrophages was confirmed by immunocytochemistry. Of note, the differential expression profile of this transporter in monocyte/macrophage lineage cells is a novel observation in this field. 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 guanosine-induced cytidine transport inhibition. As cytidine is a pyrimidine nucleoside and could only be transported by hCNT1 (not expressed in MDMs) and hCNT3, the inhibition of Na^+-dependent cytidine transport by guanosine demonstrates the occurrence of hCNT3 function. This finding is noteworthy in 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 the nucleoside analogues used in anti-HIV** (such as AZT, ddC or ddI) and in anticancer therapies (such as cladrabine, gemcitabine, 5-fluorouridine or fludarabine) (Hu et al., 2006; Errasti-Murugarren et al., 2007).

Heterogeneous and Lower but Notable Expression of hOCTs and hOCTNs in T Lymphocytes and Up-Regulatory Effect of PHA

Regarding **hOCTs and hOCTNs** overall, their **mRNA and protein expression profiles were more heterogeneous, with a quantitatively generally lower expression than hNTs**. PHA stimulation of PBMCs and CD4+ T cells increased the mRNA and protein levels of hOCTs and hOCTNs, with the exception of hOCT2, which was not expressed in any of the T cell lines or primary leukocytes studied. Similar mRNA up-regulatory effects for hOCTN1 have been shown upon cell incubation with TNF-α (Tokuhiro et al., 2003). More importantly, this expression profile of hOCTs is identical to that which was recently described for basophiles (Schneider et al., 2005). The physiological role that hOCTs could play in immune cells remains to be properly understood but we believe that **these transporters could be important in 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 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).

Importantly, 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 respect to hOCTNs, their occurrence in the immune system has been reported so far only 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 the highest hOCT3 and hOCTN1 expression. This result agrees with the maximal expression of hOCTN1 previously found in a subset of CD14+ lymphocytes (Tokuhiro et al., 2003), as monocytes are CD14+ cells. Moreover, monocyte differentiation into MDMs or iMDDCs caused antagonistic effects in hOCT/Ns mRNA expression: while hOCT1 and hOCTN2 upregulation was observed, hOCT3 and hOCTN1 showed significant down-regulation. Furthermore, LPS maturation of iMDDCs also had an antagonistic effect on various iMDDC 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 high protein expression for monocytes and macrophages and a lower expression for MDDCs (as seen for mRNA levels), with hOCTN1 protein being highly expressed in MDMs.

Importance of hOCTs Regulation in Excretion and (re)Absorption Tissues

It is notable that **OCTs are widely distributed and active in the liver, kidneys and intestine**, all of which have to deal with rapidly changing amounts of substances as a consequence of variable fluid and meal intakes, as well as metabolic activities. These organs are the targets of many hormones which can activate a series of regulatory pathways, meaning that the **rapid regulation of their activity is both conceivable and desirable** (Ciarimboli and Schlatter, 2005). On the other hand, there are situations where the body undergoes substantial changes, for example during **development or in disease states**, which can also modify the expression of OCTs, giving rise to **long-term regulatory periods**. With regard to rapid and short-term regulation of OCTs performed in cell culture models, it has been described that both PKA and Ca^{2+}/calmodulin pathways can affect OCT function, the first by inhibiting the uptake of the fluorescent substrate 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide in hOCT1 and 2, and the second by stimulating cation uptake (Martel et al., 2001; Cetinkaya

et al., 2003). In isolated segments of rabbit kidney, proximal tubules PKC also stimulated the transport of the hOCT substrate TEA^+ (Hohage et al., 1994). While hOCT1 was not regulated by cGMP (Ciarimboli et al., 2004), the renal hOCT2 was found to be significantly inhibited by cGMP (Schlatter et al., 2002). With relation to long-term regulation, briefly, expression of OCT1–3 is gender dependent (with higher expression of rOCT2 in male rats), regulated by steroid hormones such as testosterone, estradiol, dexamethasone and hydrocortisone (Urakami et al., 1999; Urakami et al., 2000; Shu et al., 2001) and dependent on developmental status (Pavlova et al., 2000). Interestingly, it has been recently described that hepatocyte nuclear factor 4α interacts with two DNA response elements of the hOCT1 promoter and activates the transcription of the membrane protein (Saborowski et al., 2006).

Lower Absolute hOCT/Ns Expression and Activity with respect to hNTs and Higher Lipophilicity of hOCT substrates

The **activity of hOCTs**, analysed by MPP+ transport**, was lower in PBMCs with respect to hNTs**, probably due to **the higher lipophilicity of organic cations** and therefore to **higher rates of simple diffusion**. However, in PHA-stimulated PBMCs, MPP+ transport could be partially inhibited by the competing substrates ranitidine and ergothioneine (ET), as well as by the hOCTs inhibitor D-22. In addition, as hOCTN1 is a low affinity transporter for MPP+ (Koepsell, 2004) and ET is only transported by hOCTN1 with a high affinity (Grundemann et al., 2005), the inhibition observed with ET, along with the mRNA expression profile, are direct evidence that hOCTN1 is an active transporter in stimulated PBMCs. Finally, the Na^+-coupled L-carnitine transport found in non-stimulated 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, could be attributed to the recently described transporter OCTN3, which seems to be a Na^+-independent L-carnitine transporter highly expressed in enterocytes basolateral membrane (Duran et al., 2005). For MDMs, we found a similar activity pattern as for PHA-stimulated PBMCs: the substrates ranitidine and ET significantly inhibited MPP+ transport, while D-22 did so 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.

To conclude, the data for this second part strengthen the importance of drug transporters associated with the 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 study of the specific implications of each isoform with relation to antineoplasic or anti-HIV drug transport across cell membrane would help us understand drug–drug interactions, drug resistance and therapy failure, and would provide us with new insights with relation to drug pharmacokinetics and drug metabolism.

Chapter III. HIV-1 Infection *in vitro* does not affect Nucleoside Transporter mRNA Expression but it is Inhibited by Dipyridamole in MT4 cells and PBMCs

Having previously determined the expression of human Nucleoside Transporters and their activity in immune cells, we aimed to assess the straight effect of HIV infection specifically on the mRNA expression of these transporters. This objective was pursued because we found throughout the literature that both hCNT and hENTs expression can be altered during cancer and other malignancies (Farre et al., 2004) so it was possible that the virus could be somehow modulating hNTs expression and/or activity. Moreover, as dipyridamole (DIP) had been described to potentiate the inhibitory effects of AZT and ddC against HIV replication (Szebeni et al., 1989), we aimed to assess the effect of both hENTs inhibitors, NBTI and DIP, in HIV infection in MT4 cells and in PBMCs.

A Down-Regulation of hCNT2 after HIV Infection in MT4 cells was Not Confirmed in PBMCs: Negligible Role in vivo*?*

First, by using an HIV-GFP virus which permitted us to easily follow the infection kinetics by flow cytometry, we found 25% of cells infected at day 2 and a nearly 100% of cells infected at day 3 post-infection. After determining the hCNT and hENT mRNA levels at these two time points, we found that only **at day 2 post-infection**, were **hCNT2 transcripts significantly down-regulated**. The mechanism by which the virus could be specifically down-regulating hCNT2 transcripts at day 2 remains to be elucidated. Nevertheless, in trying to understand why this phenomenon could be occurring, we hypothesized a theoretical mechanism (depicted and briefly described in the *Results* section) that still needs to be proved. As hCNT2 is the highest affinity adenosine transporter (Wang et al., 1997; Ritzel et al., 1998), its down-regulation provoked by HIV infection would lead to higher concentrations of adenosine in the extracellular milieu. The greater number of interactions between adenosine and A_{2A} adenosine receptors would then

result in a greater response associated with this specific receptor which, through a p38-mitogen-activated protein kinase (MAPK) pathway, could result in turn in cell apoptosis (Schulte and Fredholm, 2003; Melani et al., 2006). Unfortunately, **though we attempted to corroborate the down-regulation of hCNT2 mRNA levels in PBMCs** and isolated $CD4^+$ T cells in the presence of a wild-type HIV, **we were unable to find it**. One possible explanation is that GFP-HIV and the wild-type HIV act differently, thus provoking different effects on the membrane and transcriptional machinery of T lymphocytes. However, the most plausible explanation is that by activating PBMCs and $CD4^+$ T cells to infect them with HIV, we were highly up-regulating hNT expression, as seen in the previous section, and this phenomenon could have somehow masked the possible effect of the virus on hCNT2. Contrastingly, MT4 cells – an immortalized T cell line – show high endogenous expression of hNTs that could be modulated by the virus. As we did not explore the transporter protein content in our studies, further experiments are required to explain the differences in hCNT2 expression between MT4 and PBMCs infected cells, whether they are due to the use of GFP-HIV virus instead of HIV and, specifically, to discover whether the protein content and activity of the transporters is truly affected by HIV itself. Nevertheless, as far as we know, **this is the first approach**, though preliminary, **to the direct effect of HIV infection on the expression of nucleoside transporters in immune cells**. To date, only one study relates HIV-1 infection and anti-HIV treatment with hNTs expression, but it does so in human adipose tissue. HIV-1 infection, prior to HAART and lipodystrophy development in HIV+ naïve-treated patients, was associated with the upregulation of the mRNA of hCNT1, hCNT3 and hENT2 (Guallar et al., 2007). These differences could be due to the increase in the action of interferon-α (IFN-α), a cytokine with enhanced expression in adipose tissue following HIV-1 infection, as it was found in adipocytes *in vitro* (Guallar et al., 2007). In contrast to our *in vitro* studies with MT4, hCNT2 mRNA levels were not altered by HIV itself in adipose tissue. The development of HIV-1 treatment-associated lipodystrophy syndrome was associated with an increase in CNT2 and ENT2 encoding mRNA levels (Guallar et al., 2007). To conclude, as in primary leukocytes, since we could not find any differences in any of the hNTs explored during *in vitro* HIV-1 infection, the virus itself most probably does not affect nucleoside transporters *in vivo*.

With regard to this, of the studies *in vitro* relating HIV-1 infection to the expression of transporters, the most clear effect of HIV-1 infection on the ATP binding cassette (ABC) transporter expression is a study by Mujawar et al. in which they demonstrate a significant

down-regulation of ABCA1 protein and a decrease of its activity in macrophages by the viral protein *Nef* (Mujawar et al., 2006). After HIV-1 gp120 treatment, immunoblotting analysis showed a significant decrease in P-glycoprotein expression in astrocytes (Ronaldson and Bendayan, 2006). In contrast, other studies support the idea of up-regulation of transporters provoked by HIV-1 infection: MRP1 mRNA and protein, and the mRNA of MRP1, MRP4 and MRP5 were increased in gp120-treated astrocytes and in human MDMs, respectively (Jorajuria et al., 2004; Ronaldson et al., 2008). With relation to the *SLC* protein members, it has been recently demonstrated that the *Tat* HIV-1 protein can decrease dopamine transporters function (Ferris et al., 2009; Zhu et al., 2009). However, the concrete mechanism by which the virus (or some specific proteins from its structure) modifies the expression of transporters and/or membrane-bound molecules remains unexplored.

In addition, **not only could the expression of hNTs or other transporters be influenced by the direct effect of HIV-1 infection but also by the indirect effects of cytokines** (such as IFN-α) or other factors. For instance, in astrocyte cultures, P-glycoprotein expression and activity has been shown to increase in the presence of TNF-α and IL-1β but to decrease profoundly when IL-6 is added (Ronaldson and Bendayan, 2006; Bauer et al., 2007). In a very recent study, the effect of TNF-α and IL-6 on hepatic uptake transporters was assessed: the treatment down-regulated the mRNA of some protein members from the OATP family, OAT2, OCT1 and the sodium-taurocholate co-transporting polypeptide (NTCP), and concomitantly reduced NTCP and OATPB1 protein expression and NTCP, OATP and OCT1 transport activities (Vee et al., 2009).

The effect of a specific drug on the expression or activity of transporters has also been addressed in some studies. With relation to efflux pumps – which pump out specific antiviral drugs – some authors have described that some anti-HIV protease inhibitors (PIs) can affect not only the expression levels of transporters (mainly P-glycoprotein) but also their activity in Caco-2 cell monolayers and in PBMCs (Profit et al., 1999; Lucia et al., 2001; Janneh et al., 2005). Moreover, a correlation between CXCR4 – the HIV-1 coreceptor – with P-glycoprotein have been described in healthy volunteers and HIV+ patients, finding in the former group a colocalization of both proteins in the cell membranes of PBMCs (Owen et al., 2004; Chandler et al., 2007).

Antiviral Effects of Dipyridamole (DIP) in T cells: Some Questions Remain to Be Answered

Exploring **dipyridamole (DIP) as a possible inhibitor of HIV replication**, we initially performed inhibition experiments in MT4 cells with the GFP-HIV virus. At that time point, we also hypothesized that HIV requires new nucleosides to be taken by the host cells from the extracellular milieu in order to construct new virions during its replication. Therefore, by inhibiting hENTs, with hENT1 being the most important transporter for nucleoside salvage in T cells, HIV replication would be reduced or slowed. In that respect we performed experiments on the putative effect that DIP and the hENT1 inhibitor nitrobenzylthioinosine (NBTI) could produce on HIV infection. In MT4 cells infected over 4–5 days, the treatment with **DIP** did not affect cell viability and on days 2 and 3 **produced a significant decrease in the percentage of infected cells**. Nevertheless, in all cases on day 4 more than the 95% of MT4 cells were infected. Surprisingly, NBTI did not affect HIV infection in any case (neither on day 2 nor on days 3 or 4). This first approach in MT4 cells revealed that the **inhibitory effect of DIP is most probably not linked to the nucleoside salvage route**: the lack of HIV inhibition by NBTI is an argument against a significant need for nucleosides in HIV replication. Nevertheless, taking into account that MT4 is an immortalized cell line that most probably has a high *de novo* synthesis of ribonucleotides, it is possible that despite inhibiting the main salvage route in T cells with NBTI (associated with hENT1), cells can produce new viral DNA strands by using nucleosides from nucleotide intracellular pools. Of note, it has been shown that T lymphocytes from AIDS patients are unable to synthesize ribonucleotides *de novo* in response to mitogenic stimulation by PHA (Bofill et al., 1995). Importantly, if we had analysed the effect of NBTI on HIV replication in PBMCs, we would have been able to conclude on whether the inhibition of the salvage route through hENT1 in primary leukocytes is sufficient to inhibit HIV replication. Unfortunately, we did not check the effect of NBTI in PBMCs.

When performing the experiments with wild-type HIV **in PBMCs** and following HIV replication by p24 antigen measurement **we clearly found the same inhibitory effect of DIP as we found with MT4** (at non-toxic 1 and 10 μM concentrations). This potent inhibition effect on HIV replication by DIP had been studied alone and in combination with AZT and ddC in primary monocyte/macrophages (Szebeni et al., 1989). The time-course of HIV infection in elutriated monocyte/macrophages from blood donors treated with DIP alone showed similar inhibition rates to ours with PBMCs but, contrastingly, in their case the increase in concentration (from 2 to 20 μM) did not show a

greater effect on the decrease in HIV p24 levels. In further experiments published on uninfected monocyte/macrophages, DIP significantly inhibited cellular salvage of deoxycytidine, whereas it did not affect the salvage of ddC, suggesting that the inhibition of the salvage of competing physiological nucleosides could explain or contribute to the potentiating effect of DIP on NRTIs (especially AZT and ddC) (Patel et al., 1991). Therefore, in AZT, if DIP is present in media, for example, the cellular entry of thymidine would be highly repressed resulting in an advantageous reduction in the thymidine triphosphate:AZT triphosphate ratio, thus the capacity of AZT to inhibit RT would be favoured and more quickly accomplished. This would result in a stronger AZT effect at the same concentrations of the drug: in other words, a decrease in IC_{50} of AZT, as demonstrated by Szebeni et al. (Szebeni et al., 1989). Disappointingly, the only *in vivo* study performed at that time did not find differences in AZT pharmacokinetics when administered alone or in combination with an adjusted dose of DIP (Hendrix et al., 1994).

In our experiments with MT4 and PBMCs, without NRTIs present in the media, the antiviral effect of DIP must be explained using other arguments. Interestingly, apart from the inhibition of hNT function, several different mechanisms by which DIP could be contributing to this antiviral activity have been proposed throughout the literature: *(1)* DIP could alter the surface properties of cells as, for instance, it decreases erythrocyte membrane deformability (Sowemimo-Coker et al., 1983), and through such action it might influence virus binding or release; *(2)* DIP has been reported as inducing IFNs in a variety of cell types and, perhaps on that basis, as inhibiting a wide range of viruses (other than HIV) (Galabov and Mastikova, 1982; Tonew et al., 1982); *(3)* DIP can affect the metabolism of adenosine (FitzGerald, 1987) and thereby influence the expression of the CD4 molecule (Sipka et al., 1988). In our case, as we moved on to other aspects of antiviral drug transporters and were performing other types of experiments at that time, we only analysed the influence of DIP on the expression of CD4 molecules. In MT4 cultures treated with 10 μM DIP until day 4, CD4 expression was not altered (data not shown in *Results* section), thus, in principle, discounting the direct effect of DIP on the expression of the receptor for HIV-1. Among the other possible mechanisms probably contributing to the antiviral effects of DIP, the induction of IFN-α release in T cells, with known potent antiviral effects (Gendelman et al., 1990; Francis et al., 1992), would be the most plausible explanation, even though neither we nor other authors have demonstrated this effect yet in this cell type and for this specific IFN.

Finally, it is important to mention that DIP is a very well tolerated medication commonly used in clinical practice in conjunction with aspirin in the secondary prevention of strokes and transient ischaemic attacks. In addition, it is very inexpensive. In that respect, a clinical trial with DIP in HIV+ patients under HAART regimens would be plausible not only for assessing the differences in pharmacokinetics as performed previously (Hendrix et al., 1994), but also to attempt an effective inhibition of HIV replication and the consequent recovery of the patients' $CD4^+$ T cells.

Chapter IV. Transport of Lamivudine (3TC) and the High-Affinity Interaction of Nucleoside Reverse Transcriptase Inhibitors (NRTIs) with Human Organic Cation Transporters 1, 2 and 3

The fourth part of this thesis assesses the **interaction of NRTIs with hOCTs and the cell uptake transport of three of the most widely prescribed NRTIs**: 3TC, ABC and AZT. Even though some drug uptake transporters have been described in renal epithelial cells and hepatocytes, it is still unknown which ones are implicated in NRTI transport across the biplasma membranes of immune cells. The *SLC22* gene family, which encodes for hOATs and hOCTs proteins, has been associated with the uptake of antiviral drugs used in HIV and other viral infections (Chen and Nelson, 2000; Takeda et al., 2002). We determined in the second chapter that hOCTs are highly expressed, active and up-regulated in immune cells (including CD4+ T cells, the main targets of HIV).

All NRTIs Interact with High-Affinity with hOCTs and Could Have a Role in Modulating their Physiological Activity

With this background, and with the construction of CHO cell lines that stably express hOCTs, we first studied the interaction of hOCTs with NRTIs, finding a high affinity (IC_{50} in the pM range) interaction in all cases. This means that **all NRTIs** (with C_{max} in plasma ranging from 2–10 μM) **could physiologically inhibit the *in vivo* function of the three hOCT subtypes tested** and potentially inhibit the transport of xenobiotics or endogenous substrates such as hormones, neurotransmitters, metabolites or the newly identified substrates cyclo(his-pro) and salsosinol (Koepsell et al., 2007; Taubert et al., 2007). The expression and activity of hOCTs is increasingly important in the clinical response to drugs such as metformin (used to treat type 2 diabetes), imatinib (used against chronic myeloid leukaemia), or cisplatin and oxaliplatin (used in chemotherapy against some solid tumours) (Yonezawa et al., 2006; Zhang et al., 2006b; Shu et al., 2007; Wang et

al., 2008). Consequently, this **high-affinity interaction of NRTIs with hOCTs could play a role in drug–drug interactions**.

To the best of our knowledge, this high-affinity interaction of NRTIs with hOCTs is a new finding. Other authors have described the interaction of protease inhibitors (PIs) and the anti-infective drugs pentamidine and trimethoprim with hOCTs, but the IC_{50} were much higher (Zhang et al., 2000; Jung et al., 2008). The inhibition effect of both NRTIs and PIs at different concentrations could have implications on the clinical outcomes of combinations of these drug families during highly active antiretroviral therapy (HAART). Nevertheless, further studies are necessary before we can draw conclusions on the possible antagonistic effects of specific drug combinations.

3TC Shows a High-Affinity and a Low-Affinity Binding Site: Possible type of Competition and Mechanistic Role for each Site

3TC is frequently included in HAART regimens and is one of the first choices in the treatment of therapy-naïve patients. Previous studies had suggested that it could be a substrate for OCTs (Leung and Bendayan, 2001; Takubo et al., 2002). Therefore, we tested the interaction of the three hOCT subtypes with a wide range of concentrations of **3TC**. Interestingly, we found a **two-interaction site inhibition curve with a first, high-affinity binding site in the pM range, and a second, low-affinity binding site in the mM range**. The presence of two-binding-site interactions has recently been shown for choline, TEA, and MPP+ for rat OCT1 (rOCT1) in epifluorescence measurements performed with *Xenopus* oocytes (Gorbunov et al., 2008). Of note, the existence of a high-affinity MPP+ binding site for hOCT1, with a K_d also in the pM range, has also been demonstrated with the solubilized and immobilized transporter using liquid chromatography (Moaddel et al., 2005). Previously, we showed that high affinity binding of the non-transported inhibitor TBuA inhibited MPP+ uptake by an rOCT1 mutant but not by the wild-type transporter (Gorbunov et al., 2008). This study provides evidence that binding of transported or nontransported compounds to high-affinity sites may lead to the inhibition of human wild-type OCTs as all NRTIs investigated partially inhibited uptake of 3TC and/or MPP+ by the hOCTs.

Functional, molecular and structural characterization of rOCTs has provided evidence that OCTs contain a large binding cleft that switches from an outward-facing to an inward-facing conformation during the transport cycle (Volk et al., 2003; Gorboulev et al., 2005). The high-affinity binding sites for organic cations are probably located outside the innermost cavity of the outward-facing binding cleft (Gorbunov et al., 2008). The

functional role of the high-affinity binding sites remains unclear. Assuming a rate constant for association (K_{on}) lower than 1 x 10^9 $M^{-1} \cdot S^{-1}$, the K_d values (pM) found for NRTIs, suggest half-time for dissociation as something in the order of hours (Corzo, 2006). Therefore, these sites would be occupied over long periods in the presence of low concentrations of individual ligands and could modulate the substrate selectivity for transport and/or the transport velocity (V_{max}). In addition, in the presence of other ligands (e.g. NRTIs), these release the previously bound molecules by either direct competition at the same binding site or by allosteric effects on other high-affinity binding sites nearby. Of note, in competition experiments, when we increased [^{3}H]MPP+ concentrations, IC_{50} values for 3TC at the high-affinity site showed a significant shift, evidence of direct competition between MPP+ and 3TC at these sites. The type of competition between MPP+ and 3TC (and/or other NRTIs) remains to be understood.

With relation to the low-affinity site, the **similarity between the IC_{50} value for 3TC inhibition and the K_m value for 3TC uptake suggests that the low-affinity inhibition site of 3TC is identical with the transport site for 3TC**. Despite this, this site might not overlap significantly with the low-affinity MPP+ binding site because no competition between MPP+ and 3TC was found at the low-affinity 3TC binding site. Accordingly, recent data from our laboratories suggests that different hOCT-transported compounds may have partially different binding regions in the low-affinity binding site (H. Koepsell, unpublished data).

3TC is a Novel hOCTs Substrate with hOCT1 being the Best Transporter whereas AZT and ABC are Transported mainly by Non-Mediated Transport in CHO cells: Important Consequences in vivo

Some studies have investigated the role of organic anion and cation transporter proteins in the uptake of antiretroviral drugs (Takeda et al., 2002; Cihlar et al., 2004; Cihlar et al., 2007; Uwai et al., 2007). Moreover, since many of these transporters are highly expressed in the intestine, liver and kidneys, the *SLC22* family is thought to play a pivotal role in drug absorption and excretion (Koepsell et al., 2007). Therefore, *SLC22* family members have been largely studied in those tissues with relation to the handling of therapeutic agents and xenobiotics, and to drug pharmacokinetics and bioavailability. In fact, there is broad knowledge in this field, and many good reviews have appeared over the last few years in the literature with relation to human OAT and OCT families (Jonker and Schinkel, 2004; Koepsell, 2004; Lee and Kim, 2004; Wright, 2005; Koepsell et al., 2007). All these reviews emphasize the importance of the transport of both organic anion and

cations in order to recycle some endogenous compounds (e.g. 5-hydroxytryptamine, noradrenaline or histamine) and also in the need to detoxify the body from exogenous drugs (e.g. cimetidine, quinine or procainamide). Importantly, in our study, **the direct uptake of [^{3}H]NRTIs indicated that 3TC is a substrate for hOCTs, whereas ABC and AZT are not**. Although AZT influx involves a 40–50% protein associated mechanism in T lymphocytes, as seen in the first chapter, hOCTs do not seem to be relevant to uptake. For 3TC, the proportional inhibition effect of substrates of hOCT, the kinetic parameters (K_m, V_{max}), and the transport efficiency (V_{max}/K_m) all allow us to conclude that **hOCT1 is the best transporter, although hOCT2 and hOCT3 can also participate in its uptake**. A recent study has described 3TC and zalcitabine as substrates for hOCT1 and hOCT2, but shows only one interaction site with an IC_{50} in the low μM range (Jung et al., 2008). With relation to kinetic parameters, both studies showed the same transport efficiency values and agreed on the fact that hOCT1 was the most efficient transporter. As hOCT1, and especially hOCT2 and hOCT3, are highly expressed in the kidneys (Koepsell et al., 2000; Koepsell et al., 2007),), the role of hOCTs in the renal clearance of 3TC could be noteworthy. Moreover, the known hOCT1 expression and activity in the intestine could be relevant to the absorption and bioavailability of the drug. Although in the brain of rodents and/or humans the expression of OCT1, OCT2, OCT3 and OCTN1 and OCTN2 have been reported (Koepsell, 2004; Koepsell and Endou, 2004), the role of hOCTs in CNS is not yet clearly understood. We hypothesize that the presence of hOCTs at the blood–brain barrier (Varatharajan and Thomas, 2009) could be relevant for the entry/efflux of 3TC from the central nervous system, a known sanctuary site for HIV-1. Of all NRTIs, 3TC, along with FTC and d4T, is classified as “intermediate” in a Central Nervous System (CNS) penetration effectiveness rank established by Letendre et al., whereas AZT and ABC are classified as “high” (Letendre et al., 2008). The high penetration rates of the latter drugs might be related to their higher lipophilicity and their higher capacity to freely cross the blood–brain barrier. Recent progress strongly suggests that certain transporters in the blood–brain barrier impede the access of anti-HIV drugs to the CNS and that pharmacological modulation of these transporters could be used to our advantage. Overall, the *in vivo* importance of hOCTs in NRTI uptake in different tissues must be more extensively studied and clearly elucidated. To understand the physiological and biomedical significance of polyspecific OCTs, their regulation over short term and long term adaptations must also be understood. This would help optimize drug action and pharmacokinetics for currently available drugs, and also to aid in the development of drugs that modify the excretion of xenobiotics.

Even though our K_m values for 3TC are in the mM range (1.25-2.14 mM) and the transport efficiency of OCTs for MPP+ is much higher than that for 3TC, **the transport of 3TC by OCTs could be relevant *in vivo***. This assertion is supported by the fact that the genetic polymorphisms of hOCT1, which transports metformin with a K_m of 2.42 mM, play a role in modulating the clinical response to the drug by influencing plasma disposition and pharmacokinetics (Shu et al., 2007; Shu et al., 2008). Moreover, in our system, hOCT1 showed a transport efficiency for metformin five times lower than the efficiency of hOCT1 for 3TC.

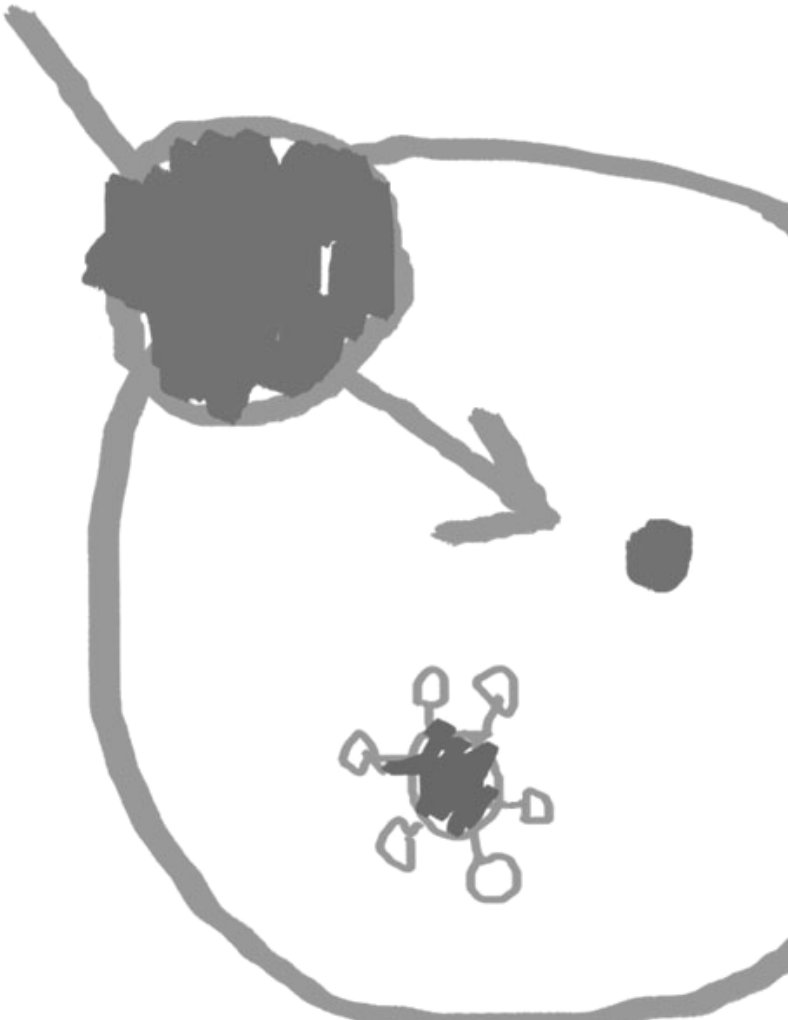
Inhibition of 3TC uptake by Low Concentrations of AZT and ABC could be Relevant in 3TC Pharmacokinetics in vivo

Finally, we wanted to assess the issue of drug–drug interactions, since some NRTIs are frequently coadministered. Two co-formulations are frequently prescribed as first-line antiretroviral regimens for HIV-1 infection: Kivexa® (ABC and 3TC), and Truvada®, (FTC and TDF), both in combination with either a PI or non-NRTI. In our study we focused on ABC and AZT taken separately at low concentrations. As expected, both NRTIs inhibited 3TC uptake by up to 50%. The implications to clinical practice are significant, as both hOCT1 and hOCT2 are highly expressed in the kidney and 3TC is mainly eliminated via the kidneys (Epivir® drug information sheet, *GlaxoSmithKline*). **ABC and/or AZT could inhibit hOCTs function as a modulator of 3TC renal clearance and pharmacokinetics.** This inhibitory phenomenon of 3TC renal clearance and the maintenance of higher levels of 3TC in plasma have been previously described for trimethoprim, a drug widely used against *Pneumocystis jiroveci* pneumonia in HIV+ patients (Sweeney et al., 1995). Moreover, subsequent clinical trials have confirmed that this interaction might be clinically relevant (Moore et al., 1996). The effect of trimethoprim on the clearance of 3TC, emtricitabine and apricitabine – the latter two having similar chemical structures to 3TC – have been confirmed in rat kidney (Nakatani-Freshwater et al., 2006; Nakatani-Freshwater and Taft, 2008b). Similarly, the effect of organic anion, cation and nucleoside transport inhibitors on FTC excretion have been recently studied and cimetidine especially decreased the clearance of the drug (Nakatani-Freshwater and Taft, 2008a). Extended studies to clarify the role of hOCTs in the uptake of other NRTIs with similar structures to 3TC are under way in our laboratories. The possibility of regulating the activity of renally expressed drug transporters has substantial pharmacological and pathophysiological outcomes: a negative regulation of tubular secretion may, for example, increase the exposure of the body to potentially dangerous

synthetic and natural xenobiotics, whereas on the other hand, the activation of tubular transport processes might be useful for prevention or treatment of occupational diseases by the elimination of environmental toxins (Berkhin and Humphreys, 2001; Ho and Kim, 2005; Izzedine et al., 2005).

To conclude, this study provides evidence of hOCTs as important determinants of 3TC intracellular and plasma concentrations, as all three hOCT subtypes transport 3TC and are expressed in both immune cells and excretion tissues. The finding that 3TC is a substrate of hOCTs and that NRTIs are high-affinity inhibitors of hOCT function provides new insights into drug–drug interactions. Due to the co-administration of ABC and AZT with 3TC in HAART, these observations could have important implications for clinical practice, especially with regard to 3TC clearance and pharmacokinetics.

Conclusions



Objective 1

To study the uptake mechanisms of azidothymidine (AZT) in T lymphocytes and the contribution of simple diffusion to its entry.

1.1. AZT has a putative carrier-mediated and regulated mechanism in T cells that is not related with either human Nucleoside Transporters (hNTs) or human Organic Anion Transporters (hOATs).

1.2. Non-mediated diffusion can make a 40–50% contribution to AZT entry in T lymphocytes.

Objective 2

To characterize the expression and activity of antiretroviral drug transporters from the *SLC28*, *SLC29* and *SLC22* gene families in T-lymphoblastic cell lines and primary immune cells.

2.1. Human Nucleoside Transporters (hNTs, the *SLC29* gene family) are highly expressed and up-regulated by PHA in immune cells. From a functional perspective, hENT1 is the most relevant transporter in T cells.

2.2. Only hCNT2 (the *SLC28* gene family) is expressed at mRNA and protein levels in immune cells, although its transporter activity seems to be negligible under the conditions studied.

2.3. In contrast to T lymphocytes, hCNT3 is highly expressed and functional in monocyte/macrophage lineage cells. It could be an important antiviral transporter in these cell types.

2.4. Human Organic Cation and Cation/Carnitine Transporters (hOCT/Ns, *SLC22* gene family) are less highly expressed and more heterogeneous than hNTs in immune cells.

2.5. All hOCT/Ns, except hOCT2, are active and highly up-regulated by PHA in $CD4^+$ T cells – the main target of HIV-1 – and could greatly contribute to the uptake of antiretroviral drugs in these cells.

Objective 3

To evaluate the effect of HIV-1 infection on human Nucleoside Transporters and assess the possible antiviral effect of Dipyridamole, an Equilibrative Nucleoside Transporter inhibitor, in T cells.

3.1. HIV-1 down-regulated hCNT2 mRNA expression in MT4 cells but none of the transcripts of hNTs were affected in PBMCs.

3.2. Dipyridamole (DIP) showed an antiviral effect in both MT4 cells and PBMCs and this effect seems not to be linked to *nucleoside salvage*.

Objective 4

To explore the interaction and transportability of NRTIs with Organic Cation Transporter 1, 2 and 3 and the possible drug–drug interactions between lamivudine (3TC), abacavir (ABC) and azidothymidine (AZT).

4.1. 3TC is a novel substrate for hOCTs, with hOCT1 being the most efficient transporter.

4.2. ABC, AZT, TDF and FTC interact with hOCTs with a high affinity and transporter activity is inhibited by 50–70%. These drugs could be modulators of physiological function of hOCTs *in vivo*.

4.3. 3TC showed two binding sites with hOCTs: a high-affinity one (in the pM range) and a low-affinity one (in the mM range). The low-affinity site seems to correspond to the drug translocation site.

4.4. ABC and AZT (NRTIs frequently coadministered with 3TC) inhibit the transport of 3TC through hOCTs at low concentrations by up to 50%, which could lead to consequences for 3TC pharmacokinetics.

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Un cop acabat el bloc d'Irsi, que no és curt però així *ha de ser*, he d'agrair als nostres col·laboradors habituals i amics de la **UB** el haver-me format (i tant!) en el bioquímic món del transport de membrana:

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Chinpón.