Impact of membrane transporters polymorphisms on nucleoside-derived drug bioavailability and action

Impacte dels polimorfismes en transportadors de membrana en la biodisponibilitat i acció de fàrmacs anàlegs de nucleòsids

Cristina Arimany Nardi

Tesi doctoral

IMPACT OF MEMBRANE TRANSPORTERS POLYMORPHISMS ON NUCLEOSIDE-DERIVED DRUG BIOAVAILABILITY AND ACTION

"Impacte dels polimorfismes en transportadors de membrana en la biodisponibilitat i acció de fàrmacs anàlegs de nucleòsids"

Cristina Arimany Nardi

UNIVERSITAT DE BARCELONA

Departament de Bioquímica i Biologia Molecular

Facultat de Biologia

2013

MEMÒRIA PER A OPTAR AL GRAU DE DOCTORA PER A LA UNIVERSITAT DE BARCELONA

PROGRAMA DE DOCTORAT EN BIOMEDICINA

Memòria presentada per

Cristina Arimany Nardi

La interessada,

Cristina Arimany Nardi

Vistiplau del director,

Dr. Marçal Pastor Anglada

Catedràtic de Bioquímica i Biologia Molecular

DEPARTAMENT DE BIOQUÍMICA I BIOLOGIA MOLECULAR

FACULTAT DE BIOLOGIA

UNIVERSITAT DE BARCELONA

Setembre 2013

“No t’assenyalis fites: fes camí”

Miquel Martí i Pol (1929-2003)

“El nostre major triomf no està en no fallar,
si no en aixecar-nos cada vegada que ens equivoquem”

Ralph Waldo Emerson (1803-1882)

LIST OF ABBREVIATIONS

3TC: lamivudine

ABC: abacavir

ALL: Acute Lymphoblastic Leukaemia

AUC: area under the concentration-time curve

AZT: zidovudine

BBB: Blood-Brain Barrier

CLL: Chronic Lymphocytic Leukaemia

Cmax: maximal plasma concentration

CNT: Concentrative Nucleoside Transporter

CHB: Chronic Hepatitis B

d4T: stavudine

ddC: zalcitabine

ddI: didanosine

DNMT: DNA methyltransferase

EFV: efavirenz

ENT: Equilibrative Nucleoside Transporter

FDA: Food and Drug Administration

FTC: emtricitabine

HBV: Hepatits B Virus

HIV: Human Immunodeficiency Virus

ICGC: International Cancer Genome Consortium

IL-2: interleukin-2

ITC: International Transporter Consortium

LBL: Lymphoblastic Lymphoma

MDMs: Monocyte-Derived Macrophages

MDS: Myelodisplastic Syndrome

MPP+: 1-methyl-4-phenylpyridinium

NBTI: nitrobenzylthioinosine

NNRTIs: Non-Nucleoside Reverse Transcriptase Inhibitors

NRTIs: Nucleoside-derived Reverse Transcriptase Inhibitors

NT: Nucleosdie Transporter

OAT: Organic Anion Transporter

OCT: Organic Cation Transporter

PAH: p-amminohippurate

PBMCs: Peripheral blood mononuclear cells

PHA: phytohaemagglutinin

PIs: Protease Inhbitors

PMAT: Plasma Membrane Monoamine Transporter

RAL: raltegravir

SLC: Solute Carrier

TMD: Transmembrane Domain

TABLE OF CONTENTS

INTRODUCTION 1

1- Nucleoside-derived drugs 3

1.1 Nucleoside-derived drugs 3

1.2 Mechanisms of action 5

1.2.1 Termination of chain elongation 5

1.2.2 Accumulation of mutations in the viruses 5

1.2.3 Induction of apoptosis 6

1.3 Antiviral Nucleoside Analogues in HIV infection therapy 6

1.3.1 Nucleoside Reverse Transcriptase Inhibitors (NRTIs) 6

1.3.1.1 Cytidine (C) analogues 7

1.3.1.2 Thymidine (T) analogues 8

1.3.1.3 Guanosine (G) analogues 9

1.3.1.4 Inosine (I) analogues 9

1.3.1.5 Adenosine (A) analogues 9

1.3.2 Other non-nucleoside analogues used in retroviral therapy 9

1.3.2.1 Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs) 9

1.3.2.2 Protease Inhibitors (PIs) 10

1.3.2.3 Integrase Inhibitors 10

1.3.2.4 Entry Inhibitors 10

1.4 Antiviral Nucleoside Analogues in other viral infections 11

1.4.1 Herpes Simplex virus 11

1.4.2 Hepatitis 11

1.5 Anticancer Nucleoside Analogues 12

1.5.1 Haematological malignancies 12

1.5.1.1 DNA methyltransferase inhibitors 13

1.5.1.2 Bendamustine 16

1.5.2 Solid tumours 17

1.6 Other indication 18

1.6.1 Immunosuppression 18

1.6.2 Neuroprotectors and cardioprotectors 18
1.6.3 Hyperuricaemia 19
2- Nucleoside and nucleoside-derived drug transporters 20
2.1 Nucleoside transporters 21
2.1.1 Concentrative nucleoside transporters (CNTs) 21
2.1.1.1 Human family members 21
2.1.1.2 hCNT structure 22
2.1.2 Equilibrative nucleoside transporters (ENTs) 24
2.1.2.1 Human family members 24
2.1.2.2 hENT structure 25
2.1.3 Tissue distribution of nucleoside transporters 26
2.1.3.1 NT expression changes in cancer cells 28
2.1.4 Pharmacological profiles of nucleoside transporters 28
2.1.5 Clinical implications of nucleoside transporters 30
2.2 Other nucleoside-derived drug transporters 31
2.2.1 *SLC22* gene family 31
2.2.1.1 Human Organic Cation Transporters (hOCTs) 32
2.2.1.1.1 Organic Cation Transporters structure 33
2.2.1.2 Human Organic Anion Transporters (hOATs) 34
2.2.1.2.1 Human Organic Anion Transporters structure 35
2.2.1.3 hOCTs and hOATs tissular distribution 36
2.2.1.4 OCTs and OATs role in nucleoside-analogues internalisation 38
2.2.2 *SLC15* gene family 39
3- Pharmacogenomics of drug transporters 40
3.1 Introduction to pharmacogenomics 40
3.2 Membrane transporters pharmacogenomics 40
3.2.1 *SLC28* family polymorphisms 41
3.2.2 *SLC29* family polymorphisms 42
3.2.3 *SLC22* family polymorphisms 43

OBJECTIVES 47

RESULTS 51

1- Role of Organic Cation Transporter 1 (hOCT1) polymorphisms on lamivudine (3TC) bioavailability and drug-drug interaction 53

1.1 Effect of hOCT1 polymorphisms in lamivudine, metformin and MPP^+ uptake 53

1.2 Oligomerization of hOCT1 and two of its polymorphic variants located at the large extracellular loop 54

1.3 Kinetic parameters of MPP+ and 3TC uptake mediated by hOCT1 SNPs 56

1.4 hOCT1 expression in HIV-1 infected PBMCs from healthy donors 58

1.5 Drug-drug interactions in the hOCT1 polymorphic variants 59

2- Identification of hOCT1 as a bendamustine transporter and determination of the role its polymorphic variants play in its cytotoxicity 63

2.1 Screening of bendamustine interaction with *SLC28* and *SLC29* transporters 63

2.2 Role of human Organic Cation Transporter 1 (hOCT1) in the uptake of bendamustine 64

2.3 Effect of hOCT1 polymorphic variants on bendamustine internalization 66

3- Determination of transporters mediating DNMT inhibitors translocation through the plasma membrane 71

3.1 Interaction of DNMT inhibitors with nucleoside transporters 71

3.2 Role of nucleoside transporters in the uptake of DNMT inhibitors 72

3.3 Role of nucleoside transporters in determining the vectorial flux of DNMT inhibitors across epithelial barriers 76

3.4 Role of organic cation transporters uptake of DNMT inhibitors 79

3.5 Effect of hOCT1 polymorphic variants on zebularine efflux 81

4- hCNT3 modelling 85

4.1 Model of hCNTs nucleoside binding site 86

4.2 Identification of hCNT3 primary sodium binding site 88

4.3 Identification of hCNT3 secondary sodium binding site 90

GENERAL DISCUSSION 93

CONCLUSIONS 101

EXPERIMENTAL PROCEDURES 105

1- Cell culture 107

1.1 Introduction 107

1.2 Working area 107

1.2.1 Required equipment 108

1.3 Maintaining cells in culture 108

1.3.1 Passaging and plating 108

1.3.1.1 Materials 108

1.3.1.2 Procedures 109

1.3.2 Freezing and thawing 110

1.3.2.1 Freezing 110

1.3.2.2 Thawing 110

1.4 Used cell lines 110

1.4.1 HeLa cells 110

1.4.2 MDCK cells 111

1.4.2.1 Culture in polycarbonate filters 111

1.4.3 HEK293 cells 112

2- Heterologous expression methods 113

2.1 Introduction 113

2.2.1 Calcium phosphate 113

2.2.1.1 Buffers 114

2.2.1.2 Transfection 114

2.2.2 Lipofectamine 115

2.2 Stable cell lines generation 115

3- Cytotoxicity assays **117**
3.1 Introduction 117
3.2 Procedure 117
4- Transport measurements **118**
4.1 Uptake in cells grown in monolayers 118
4.1.1 Transport media and buffers 118
4.1.2 Multi-well plates 120
4.1.3 Transwell-chambers 121
4.1.4 Protein measurement using the bicinchoninic acid assay (BCA) 121
4.1.5 Calculations 122
4.2 Uptake in detached cells 122
4.2.1 Short-time measurements 122
4.2.2 Protein measurement using the bradford assay 123
4.2.3 Calculations 124
4.3 Oocytes 124
5- Protein expression analysis **125**
5.1 Cell lysate obtention 125
5.2 SDS-PAGE electrophoresis 125
5.2.1 Buffers and gel 126
5.2.2 Sample preparation 127
5.2.3 Gel running 127
5.3 Western blotting 128
5.3.1 Buffers 128
5.3.2 Transferring 128
5.3.3 Ponceau staining 129
5.3.4 Immunodetection 129
5.3.4.1 Buffers 129
5.3.4.2 Blocking 130
5.3.4.3 Antibody incubation 130
5.3.4.4 Gel development 130

6- Polymerase chain reaction (PCR) and related techniques **131**
6.1 Introduction 131
6.2 Restriction sites introduction 131
6.2.1 Procedure 132
6.3 Site directed mutagenesis 133
6.4 PCR sequencing 136
7- Molecular biology **138**
7.1 Competent bacteria 138
7.1.1 Media 138
7.1.2 Bacteria growth 139
7.2 Transformation 140
7.3 Plasmid DNA isolation 141
7.4 Agarose gel 141
7.4.1 Buffers 142
7.4.2 Gel running procedure 142
7.5 Plasmid preparation 143
7.5.1 Digestion with endonuclease enzymes 143
7.5.2 DNA ligation 144
8- cDNA expression analysis **145**
8.1 RNA isolation 145
8.2 cDNA synthesis from RNA 145
8.3 Real-time PCR 146
8.3.1 Data analysis 147
9- Statistics and data representation **149**

REFERENCES **151**

RESUM DE LA TESI DOCTORAL **175**

INTRODUCTION

1

NUCLEOSIDE-DERIVED DRUGS

1.1 Nucleoside-derived drugs

Natural nucleosides include purine nucleosides such as adenosine, guanosine and inosine; and pyrimidine nucleosides such as uridine, cytidine and thymidine (Figure 1). Nucleosides play essential roles in cellular processes such as DNA and RNA synthesis, cell signalling, enzyme regulation and metabolism. They are precursor molecules for ATP, nucleic acids, coenzymes (such as NADP and FAD) and intracellular signalling molecules (such as cAMP and cGMP). Adenosine, moreover, is an agonist for cell surface adenosine receptor and can facilitate a variety of physiological responses including coronary vasodilatation, neuromodulation, and platelet aggregation (Leung and Tse, 2007).

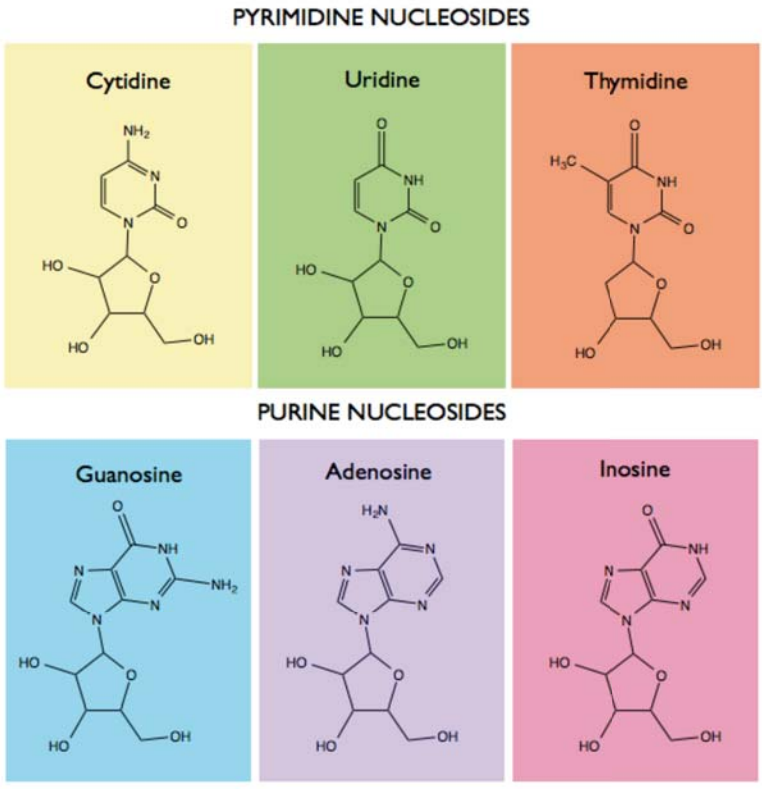


Figure 1. Structures of natural nucleosides. Pyrimidine and purine natural nucleosides structure.

Chemical modifications in natural nucleosides can lead to pharmacologically active derivatives which mimic their physiological equivalents and share similar uptake and metabolic processes (Figure 2).

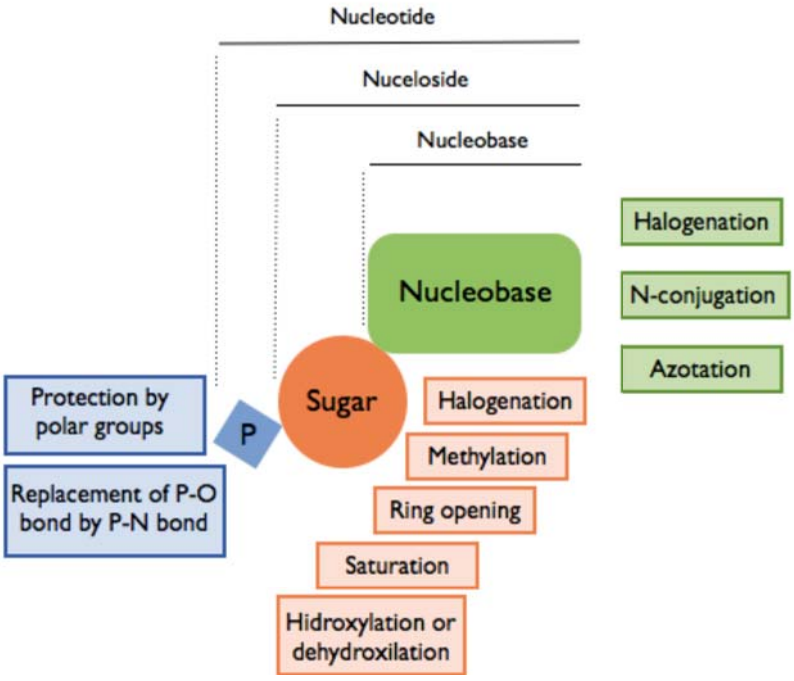


Figure 2. General structural and chemical modifications of nucleoside-derived drugs. Adapted from Jordheim et al., 2013. The nucleobase modifications are shown in green, the sugar modifications in orange and the phosphate modifications in blue.

Nucleoside analogues share common characteristics with natural nucleosides. Most of them require, due to its hidrophilicity, specific membrane transporters to mediate its translocation through the plasma membrane in order to reach the cytoplasm. Once internalised, they have to be activated by intracellular metabolic steps that retain the resulting nucleotide in the cell and form the active phosphate derivatives.

The active phosphate compounds can be incorporated into DNA and/or RNA to inhibit cellular division and viral replication. In addition to their incorporation into nucleic acids, nucleoside and nucleotide analogues can interact with and inhibit essential enzymes such as human and viral polymerases, kinases, ribonucleotide reductase, DNA methyltransferases, purine and pyrimidine nucleoside phosphorylases and thymidylate synthase.

Nucleobase analogues, nucleoside derivatives and phosphorylated nucleoside-derived drugs are currently used as first-line treatments of many human diseases, including cancer (solid tumours and haematological malignancies), viral infection (HIV and others) and autoimmune and inflammatory diseases, such as ulcerative colitis and Crohn's disease (Galmarini et al., 2002; Jordheim et al., 2013).

1.2 Mechanisms of action

Currently used therapeutic nucleoside and nucleotide analogues use the same metabolic pathways as endogenous nucleosides. They enter cells through specific transporters. Once inside the cells, the drug is phosphorylated by a nucleoside kinase and a nucleoside monophosphate kinase. Then a nucleoside diphosphate kinase, creatine kinase or a 3-phosphoglycerate kinase leads to the triphosphorylated drug which can be accumulated in cancer or virus-infected cells. Mono-, di- and tri-phosphorylated nucleosides are the active forms of the drug and they act by inhibiting intracellular enzymes, such as viral or human polymerases or ribonucleotide reductases, as well as being incorporated into newly synthesised DNA or RNA causing the termination of the chain elongation, the accumulation of mutations in the virus or the induction of apoptosis (Jordheim et al., 2013; Pastor-Anglada et al., 1998).

1.2.1 Termination of chain elongation

The absence of 3' hydroxyl group on the sugar ring of the analogue prevent the formation of 3'-5' phosphodiester bonds between the analogue and the upcoming nucleotide resulting in an early termination of the growing DNA or RNA chain. Viral polymerases have often weaker specificity for nucleotides than the mammalian ones and are more prone to incorporate analogues.

1.2.2 Accumulation of mutations in the viruses

Some of these analogues including 5-fluorouracil, 5-azacytidine and other deoxyguanosine derivatives have a 3'-hydroxyl group which enables chain elongation after incorporation into DNA or RNA leading to a mismatching which can cause mutagenesis, resulting in reduced infectivity. Ribavirin has also been described to induce lethal mutations in the viral genomes (Chevaliez et al., 2007; Leyssen et al., 2008).

1.2.3 Induction of apoptosis

The exact mechanisms by which nucleoside derivatives induce apoptosis are still not clearly understood. They generally cause a block on the S phase of the cell cycle. Nucleoside analogues are commonly combined with agents that are toxic to DNA such as alkylating or platinum compounds, as they both inhibit the repair of DNA and can also cause lesions (Ewald et al., 2008).

1.3 Antiviral Nucleoside Analogues in HIV infection therapy

Some classical antiviral drugs used in HIV infection therapy are nucleoside-derived reverse transcriptase inhibitors (NRTIs). This include the pyrimidine analogues zidovuidine (AZT; azydothymidine), zalcitabine (ddC; 2',3'-dideoxycytidine), stavudine (d4T; 2'-3'-didehydro-2'-3'-dideoxythymidine), lamivudine (3TC; 2',3'-dideoxy-3'-thiacytidine) and emtricitabine (FTC); and the purine derivatives didanosine (ddI; 2',3'-dideoxyinosine), abacavir (ABC) and entecavir. In this group we can also find the nucleotide analogues tenofovir (TDF). Some of these drugs can be used as well for the treatment of other viral infections together with ribavirin and entecavir (Figure 3) (Errasti-Murugarren and Pastor-Anglada, 2010).

1.3.1 Nucleoside Reverse Transcriptase Inhibitors (NRTIs)

NRTIs were the first class of antiretroviral drugs discovered and approved for HIV treatment, NRTIs are dideoxynucleosides (generally, 2',3'-dideoxynucleosides) that act like alternative substrates for the retrotranscriptase enzyme, competing directly with physiological nucleosides (2'-deoxynucleosides) for the active polymerase site (Figure 4).

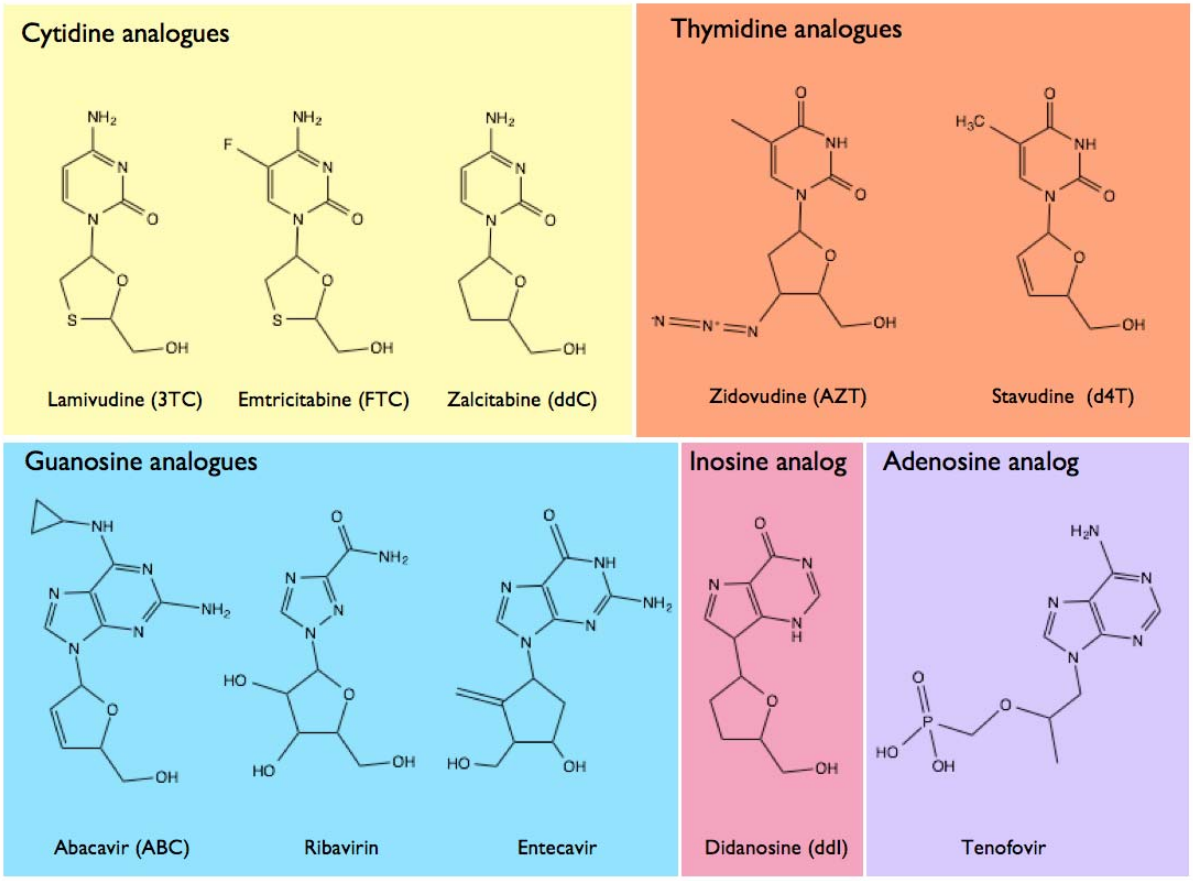


Figure 3. Antiviral nucleoside analogues structures. Currently used nucleoside analogues in antiviral therapy classified depending on their natural nucleoside analogues.

NRTIs are usually classified depending on the nucleoside of which they are analogues to:

1.3.1.1 Cytidine (C) analogues

Lamivudine ((-)b-L-2',3'-Dideoxy-3'-thiacytidine; 3TC), **emtricitabine** ((-)b-L-2', 3'-Dideoxy-3'-thia-5'-fluorocytidine; FTC) and **zalcitabine** (2',3'-dideoxycytidine; ddC) are cytidine analogues. Despite its potent anti-HIV activity, ddC is not currently used in the treatment due to its high toxicity and its low effectiveness (Anderson et al., 2004). 3TC and FTC have identical structures except for a fluorine at the 5' position of the cytosine ring. They are the only NRTIs used in anti-HIV therapy that are b-L-nucleosides, conformations not present in natural nucleosides. Both compounds share a common pathway, they both require 2'-deoxycytidine kinase to yield the triphosphate active form (Hazen and Lanier, 2003).

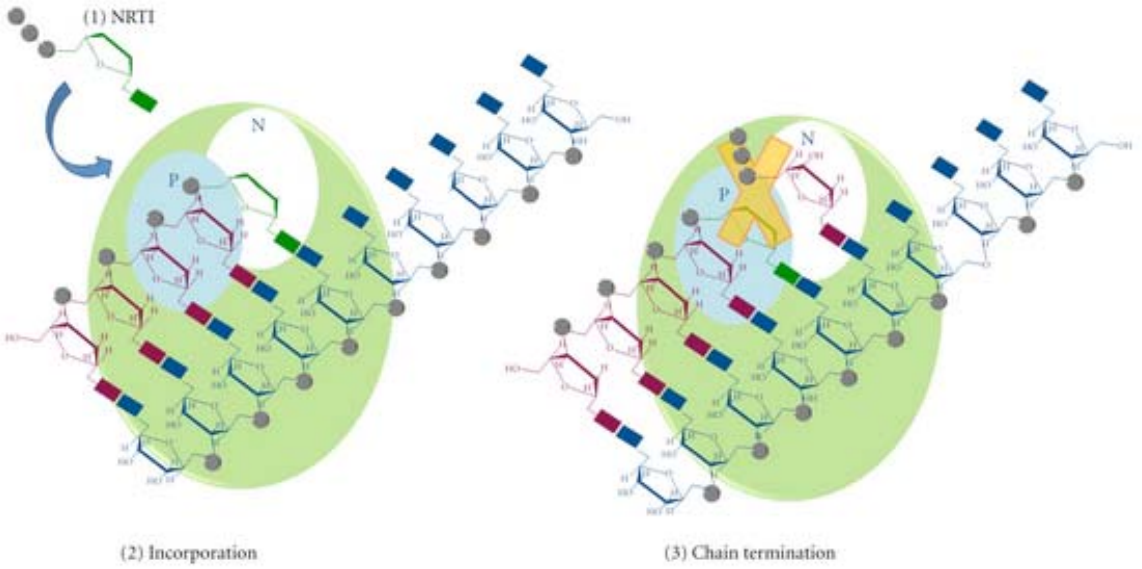


Figure 4. Mechanism of action of RT inhibitors acting as chain terminators. The RT is represented as a green circle with the priming binding site in blue (P) and the nucleotide binding site in white (N). The RNA template is showed in blue and the (−)strand DNA in purple. The NRTI triphosphate (dark green) (1) competes for the binding with the natural dNTPs, it is incorporated into the growing DNA (2) and it blocks the further DNA elongation because it lacks the 3′-hydroxyl group (3) (Esposito et al., 2012).

Both drugs are currently widely being used in therapy in combination with other antiretroviral drugs regimens in the following co-formulations: Kivexa® (co-formulation of 3TC and ABC), Truvada® (co-formulation of FTC and TDF), Combivir® and Trizivir® (AZT/3TC and AZT/ABC/3TC co-formulations, respectively) and Atripla® (consisting of FTC/TDF/EFZ).

They can also be used in the treatment of Hepatitis B.

1.3.1.2 Thymidine (T) analogues

Azidothymidine (3'-azido-2',3'-dideoxythimidine; AZT) and **stavudine** (2',3'-didehydro-3'-deoxythymidine; d4T) are thymidine analogues. They both share the same intracellular enzymes thymidine kinase 1/2 (TK1, TK2) and thymidilate kinase to be activated and, thus, their co-administration is not recomended. The azide group in AZT increases its lipophilicity, facilitating its passive diffusion through the plasma mebrane. d4T is not used in the clinic as it has many side effects such as lactic acidemia, pancreatitis, lipodystrophy and peripheral neuropathy (Price et al., 2006).

1.3.1.3 Guanosine (G) analogues

Abacavir ([[(1S,4R)-4-[2-amino-6-(cyclopropylamino)purin-9-yl]-cyclopent-2-enyl] methanolsulfate); ABC) is a widely used guanosine analogue. It differs from the other NRTIs in its phosphorylation pattern. To be activated it is first phosphorylated by adenosine phosphotransferase into ABC monophosphate and then adenosine monophosphate deaminase converts it to carbovir monophosphate which will be double phosphorylated by guanylate kinase and diphosphate kinase to the active metabolite carbovir monophosphate.

Abacavir is currently used in the treatment of the infection with HIV in combination with 3TC (Kivexa®) or AZT and 3TC (Trizivir®).

1.3.1.4 Inosine (I) analogues

Didanosine (2'3'-dideoxyinosine; ddI), an inosine analogue, is not currently used in the clinics due to its high toxicity as for ddC and d4t (Anderson et al., 2004; Carr and Cooper, 2000).

1.3.1.5 Adenosine (A) analogues

Tenofovir disoproxil fumarate (TDF) is the only nucleotide analogue (NtRTI) approved by the US Food and Drug Administration (FDA) which inhibits the retrotranscriptase, thus being classified in the NRTI group. It is a prodrug of tenofovir, which mimics adenosine 5'-monophosphate and behaves as a false substrate for retrotranscription. As it is a monophosphate it only requires two additional phosphorylations in the cytoplasm.

TDF is one of the most commonly used drugs in the clinics nowadays, specially in co-formulations with FTC (Truvada®) and FTC/EFZ (Atripla®).

1.3.2 Other non-nucleoside analogues used in retroviral therapy

As many NRTIs are used in combination with other antiretroviral drugs, some non-nucleoside analogues from other antiretroviral drug families were also studied in this thesis.

1.3.2.1 Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)

The non-nucleoside reverse transcriptase inhibitors (NNRTIs) act through a non-competitive mechanism, coupling allosterically to a hydrophobic cavity of 10Å, far from

the catalytic centre of the enzyme. As the NNRTIs bind very close to the substrate they are thought to impair the substrate binding, thus avoiding the proper work of the retrotranscriptase.

There are four NNRTIs approved by the FDA: **efavirenz** (EFZ), **nevirapine** (NVP), **delavirdine** (DLV) and **etravirine** (ETV)**.** In this thesis we have only worked with efavirenz.

1.3.2.2 Protease Inhibitors (PIs)

The viral protease proteolyses precursor viral proteins into mature viral proteins, an essential stage for the development of new virions of the HIV virus. Almost all developed PIs contain a hydroxyethylene scaffold which mimics the normal peptide linkage but cannot be cleaved, preventing the protease from carrying out its normal function.

There are ten PIs currently available for the treatment of HIV infections. They are **saquinavir** (SQV), **ritonavir** (RTV), **indinavir** (INV), **nelfinavir** (NFV), **amprenavir** (AMV), **lopinavir** (LPV), **atazanavir** (ATV), **fosamprenavir**, **tipranavir** (TPV) and **darunavir** (DRV).

None of them were assessed in this work because, although they seem to show a high activity in vitro and in clinical trials, they have some disadvantages related to the appearance of resistant viruses and their cross-resistance. Moreover, due to their peptidic character, some protease inhibitors present low bioavailability, high toxicity and side effects such as lipodystrophy and the increase of cholesterol and triglycerides in the blood stream.

1.3.2.3 Integrase Inhibitors

HIV-1 integrase has two essential catalytic functions: 3'-processing and strand transfer, allowing the virus genetic material to be integrated into the DNA of the infected cells. **Raltegravir** (RAL), the first integrase inhibitor approved by the FDA and as **elvitegravir**, just approved by the FDA in 2012, target the strand transfer reaction.

1.3.2.4 Entry Inhibitors

HIV-1 entry is an essential stage for the infection and a complex process divided into three main steps: adhesion, interaction with the co-receptor and fusion. Two entry inhibitors have been approved so far: **enfuvirtidine,** a fusion inhibitor, and **maraviroc**, a

co-receptor inhibitor. Both types of drugs act extracellularly by inhibiting the entry of HIV particles into host cells, so they are not relevant to this thesis.

1.4 Antiviral Nucleoside Analogues in other viral infections

1.4.1 Herpes Simplex virus

Nucleoside analogues are the major drugs used in the treatment of Herpes Simplex viral infection.

Acyclovir, **famciclovir** and **ganciclovir** are guanosine analogues. The acyclovir active form, acyclovir-triphosphate, is synthesised by viral thymidine kinase phosphorylation followed by cellular kinases. Acyclovire-triphosphate acts as a substrate for DNA polymerase and its incorporation into the viral DNA chain results in termination of viral replication. Famciclovir is a prodrug of **penciclovir** which follows the same phosphorylation pathway as acyclovir. Ganciclovir also undergoes a tri-phosphorylation to become active being first phosphorylated by UL97-encoded kinase and subsequently by cellular kinases. Acyclovir is also approved for the treatment of *Varicella Zoster* virus.

Brivudin is a 5'halogenated thymidine-derived drug. Its mode of action is similar to that reported for acyclovir, being its active form brivudin-triphosphate then incorporated into DNA and terminating viral DNA synthesis (Razonable, 2011).

1.4.2 Hepatitis

Hepatitis infections are also treated with nucleoside-derivatives, some times shared with the treatment of AIDS as co-infection with HIV might be common.

Ribavirin is a synthetic analogue of guanosine. Its mechanism of action is not completely understood. It may act as a competitive inhibitor of cellular enzymes. It inhibits a broad range of viruses in vitro, however, it is only approved for the use in combination with Interferone-α for the treatment of chronic hepatitis C and it is not effective when given in mono-therapy (Razonable, 2011).

The treatment of chronic hepatitis B (CHB), as mentioned before, is sometimes shared with the treatment of HIV infection being emtricitabine, lamivudine and tenofovir used in both cases. Adefovir is an acyclic nucleotide analogue of adenosine monophosphate. Its active form adefovir-diphosphate competitively inhibits Hepatitis B

Virus (HBV) DNA polymerase. It is the least potent anti-HBV drug currently available so it is not the first choice for treatment (Razonable, 2011). Entecavir, on the other hand, is one of the most potent agents in the treatment of CHB. It is a guanosine analogue with a unique mechanism of action, inhibiting three specific functions of the Hepatitis B virus: viral DNA-polymerase priming, reverse transcription of the negative strand and synthesis of the positive strand. The last drug found in this group is Telbivudine, a thymidine analogue which inhibits the virus by competitive inhibition of the DNA polymerase.

1.5 Anticancer Nucleoside Analogues

The anticancer nucleoside drugs include several analogues of natural pyrimidine and purine nucleosides as well as nucleobase-derivatives.

1.5.1 Haematological malignancies

Some purine derivatives such as **fludarabine** (FA) and **cladribine** (2-chlorodeoxyadenosine; 2-CdA) have established clinical activity and are extensively used in haematological malignancies (Molina-Arcas and Pastor-Anglada, 2013). **Clofarabine** has also been approved for the treatment of refractory patients with acute lymphoblastic leukaemia (ALL) and lymphoblastic lymphoma (LBL). **Acadesine** is a water soluble nucleoside with a different mechanism of action compared to current approved nucleoside analogues. It has been described to activate in some cases AMP protein kinase but also to cause a pyrimidine starvation in an AMPK independent manner (Bardeleben et al., 2013). Clinical trials have been performed for the use of acadesine for Chronic Lymphocytic Leukaemia (CLL) treatment (Van Den Neste et al.). However, it has not yet been approved by the FDA for the treatment of any haematological malignancy.

The pyrimidine nucleoside analogues currently used for the treatment of haematological malignancies include **azacitidine**, **decitabine**, **zebularine** and **cytarabine** (AraC) (Robak, 2011).

These nucleoside analogues are shown in figure 5.

The anticancer analogues studied in this thesis are DNA methyltransferase inhibitors and bendamustine.

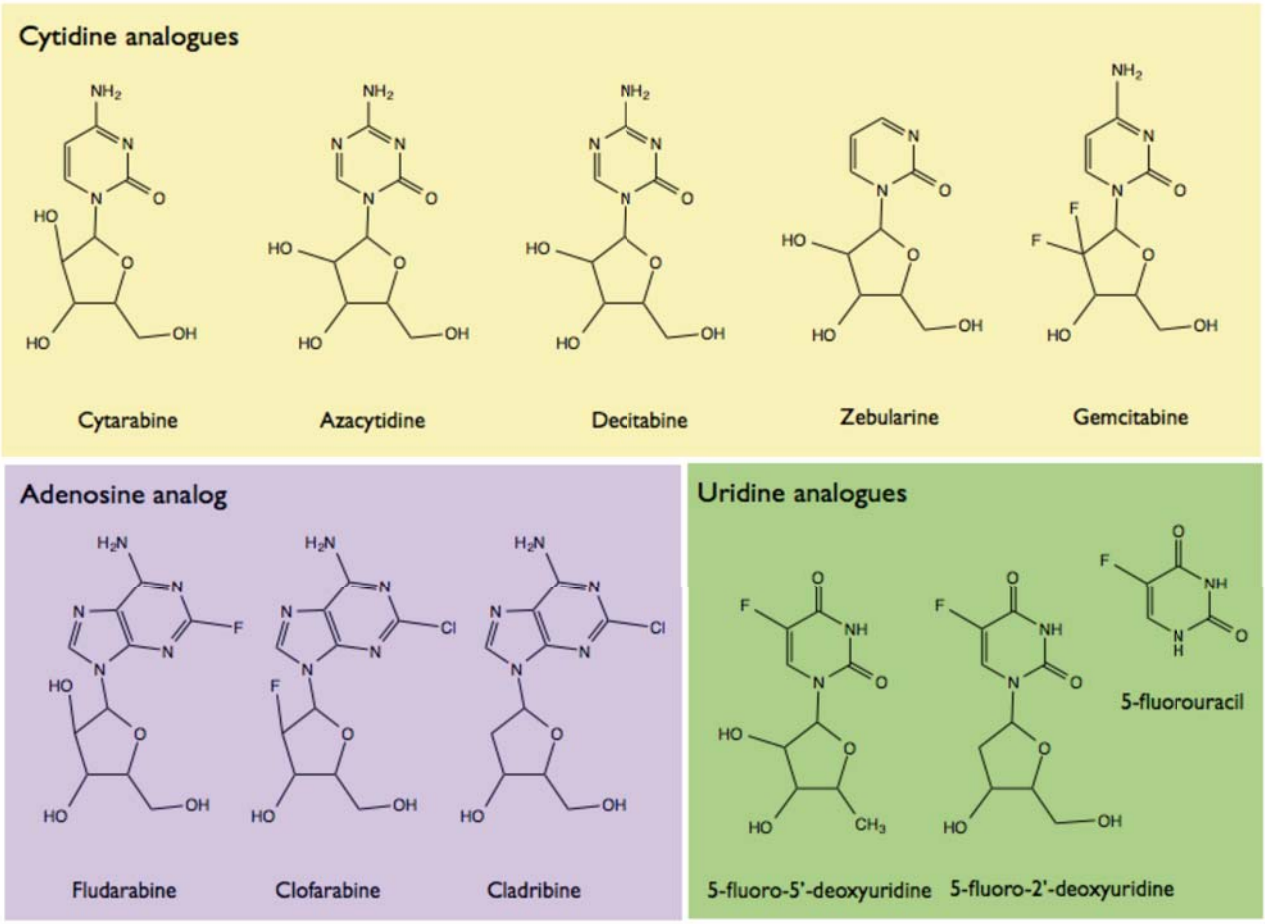


Figure 5. Structures of some nucleoside analogues used in cancer treatment. Currently used nucleoside analogues in cancer treatment classified upon their natural nucleoside analogues.

1.5.1.1 DNA methyltransferase inhibitors

DNA methyltransferases are a family of enzymes that catalyse the addition of a methyl group to the 5 carbon of a cytosine that is immediately 5' linked to a guanine (CG dinucleotide). Surprisingly, many tumours are characterised by a global hypomethylation with localised regions of hypermethylation on CpG islands, which leads to transcriptional inactivation when it occurs within a promoter region (Ewald et al., 2008).

Both hyper- and hipomethylation can cause tumour growth. On one hand, the down regulation by methylation of certain tumour suppressor genes containing CpG-rich promoter regions links promoter methylation and tumour initiation. On the other hand, hypermethylation-mediated silencing of p15 and p16 represents some of the most

common and earliest epigenetically mediated losses of tumour suppressor gene function in haematological, breast, colon and lung cancers (Ewald et al., 2008).

In general, it has been estimated that around 10% of CpG islands in DNA are abnormally methylated in tumours (Ahluwalia et al., 2001; Yan et al., 2001). The fact that these modifications can be reversible, offers a novel target for cancer therapy (Ewald et al., 2008).

Among all the nucleoside inhibitors described, azacitidine and decitabine have been approved by the FDA in 2004 and 2006, respectively, for the treatment of Myelodysplastic Syndrome (MDS) and Acute Myeloid Leukaemia (AML), with one additional indication for azacitidine: Chronic Myelomonocytic Leukaemia (CMML) (Gros et al., 2012). Although they have only been approved for hematopoietic malignancies, they might prove useful for other types of cancer, including solid tumours (Garcia-Manero, 2012; Ghai et al., 2013; Quintas-Cardama et al., 2010; Rius and Lyko, 2012).

The drugs are phosphorylated by uridine–cytidine kinase and deoxycytidine kinase, respectively (Stresemann and Lyko, 2008) and accumulate in cells as their active triphosphates. Azacitidine, being a ribonucleoside analogue is preferentially incorporated into RNA and thereby interferes with protein synthesis. However, a minor portion (10%) is incorporated into DNA (Li et al., 1970). Decitabine, being the deoxyribose analogue of azacitidine is generally assumed to be more specific since it is more directly incorporated into DNA (Brueckner et al., 2007). Once incorporated, both aza-analogues covalently trap the DNA methyltransferases and mediate their degradation, leading to a passive loss in DNA methylation in the cell (Stresemann and Lyko, 2008).

The efficacy of azacitidine or decitabine as antineoplastic agents results from two distinct mechanisms: cytotoxicity when administered at high doses and inhibition of DNA methyltransferases when given at low doses. At higher doses, decitabine induces a classical DNA damage response. At lower doses, hypomethylation associated reactivation of genes appears to mediate its antileukaemic action.

Although effective against certain haematopoietic disorders, azacitidine and decitabine have some toxicity both in vitro and in vivo and are unstable in neutral solutions. Moreover, deamination of these analogues by cytidine deaminases results in

their inactivation. Hence zebularine, a non-toxic, highly stable, effective DNMT inhibitor was developed (Billam et al., 2010).

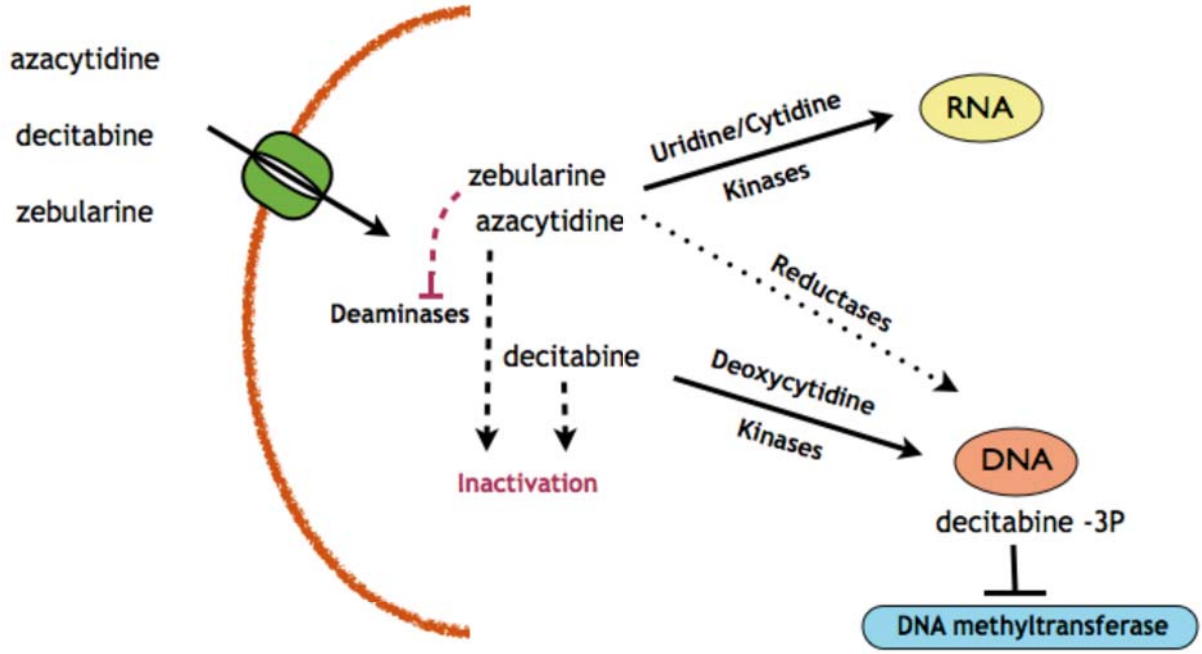


Figure 6. DNMT inhibitors mode of action

Zebularine is, like 5-azacytidine, phosphorylated by uridine-cytidine kinase and incorporated into RNA, the deoxy form of zebularine is not a good substrate for deoxycytidine kinase, so zebularine can only be incorporated into DNA via the ribonucleotide reductase pathway. Unlike azacitidine and decitabine, which are subject to deamination by cytidine deaminase being thereby inactivated, zebularine also acts as an inhibitor of the enzyme cytidine deaminase and is therefore not subject to deactivation (Yoo et al., 2004).

Zebularine, is stable at acidic and neutral pHs and in aqueous solution, and has been shown to be less toxic than the 5-aza-nucleosides in cultured cells, making oral administration possible (Cheng et al., 2004). However, high doses of zebularine are required and studies in rodents and primates (Hollenbach et al., 2010; Yoo et al., 2004) have shown limited bioavailability thus not allowing the drug to progress into clinical trials.

1.5.1.2 Bendamustine

Bendamustine is not a classical nucleoside or nucleotide analogue. It has the benzimidazole ring structure common to many purine analogues. However, instead of being linked to a ribose it has a nitrogen-mustard group at position 5 of the benzimidazole ring (Figure 7). Bendamustine was originally synthesised in the early 1960s in the former East German Democratic Republic, and until 1990 was only commercialised in East German Democratic Republic (Leoni, 2011). Not until 2008 was it approved by the FDA to be used in the treatment of CLL and indolent B-cell non-Hodgkin's lymphoma.

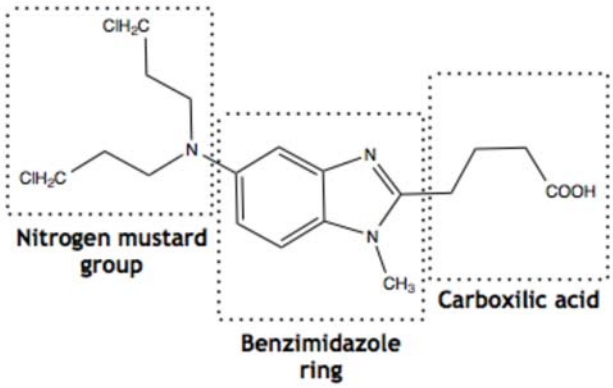


Figure 7. Bendamustine struture

Although the precise mechanism of action of bendamustine is as yet unknown, the basis of its anti-neoplastic effects is the cross-linking of DNA single and double strands by alkylation. Inter-strand DNA cross-links are created when the electrophilic alkyl groups of bendamustine form covalent bonds with electron-rich nucleophilic moieties, with the covalent linkage impairing DNA matrix functions and DNA synthesis and repair, leading to cell death via several pathways including the inhibition of mitotic checkpoints and the base excision repair DNA damage response pathway, with subsequent mitotic catastrophe, and apoptosis (Hoy, 2012).

The benzimidazole ring is thought to increase bendamustine alkylating activity by facilitating the nuclear transport and elevating concentrations within the cell nucleus, or via the inhibition of DNA repair, or directly confer antimetabolite (i.e. purine analogue) activity, thereby permitting the incorporation of the agent (or its metabolites) into the newly synthesised DNA or the inhibition of enzymes involved in the generation of deoxynucleoside triphosphates. However, with no direct evidence to date, further studies are required to determine the role the benzimidazole ring plays in bendamustine anti-tumour activity (Leoni, 2011).

1.5.2 Solid tumours

Pyrimidine analogues are mainly used for the treatment of solid tumours. **Gemcitabine** is a cytarabine analogue which presents at the 2‘ position of the ribose two fluorine atoms that make gemcitabine more suitable for the treatment of solid tumours. The presence of the fluorine atoms confer gemcitabine more lipophylicity and it is more efficiently phosphorylated by dCK. It is the most common nucleoside analogue used for the treatment of solid tumours including pancreatic, bladder, breast, ovarian and non-small-cell lung cancer (Errasti-Murugarren and Pastor-Anglada, 2010). It has also activity in some haematological malignant diseases such as leukaemia and non-Hodgkin’s lymphoma.

The fluoropyrimidines, **5-fluorouracil (5-FU)**, **5-fluoro-5’-deoxyuridine** and **5-fluoro-2’-deoxyuridine** have shown activity in colorectal, gastric and breast cancers (Errasti-Murugarren and Pastor-Anglada, 2010; Galmarini et al., 2002). In order to overcome the low bioavailability of 5-FU, **capecitabine** was synthesised (Lamont and Schilsky, 1999). Capecitabine is a pro-drug of 5-FU which is orally administrated and metabolised in the liver. To be activated, it has to be firstly transformed into 5’-deoxy-5-fluorouridine (5’-DFUR) by a carboxylesterase and cytidine deaminase at the liver. 5’-DFUR has then to be converted into 5-FU by thymidine phosphorylase at the tumour cells (Walko and Lindley, 2005). In fact, the levels of this enzyme as well as the amount of the inactivating enzyme, dihydropyrimidine dehydrogenase determine the levels of active compound at the target cells. Capecitabine has been approved by the FDA for the treatment of metastatic breast and colorectal cancers.

The structures of all these analogues are shown in figure 5.

1.6 Other indication

Besides their classical use in cancer and virology, some nucleoside and nucleotide analogues have been used in various other indications (Figure 8).

1.6.1 Immunosuppression

In addition to antiviral and antitumoural effects, some nucleoside analogues exhibit immunosuppressive effect.

The thiopurine drugs, **azathioprine**, **6-mercaptopurine**, and 6-**thioguanine**, are widely used to treat malignancies, rheumatic diseases, dermatologic conditions, inflammatory bowel disease, and solid organ transplant rejection as immunosuppressive drugs (Sahasranaman et al., 2008).

Cladribine has recently been tested in patients with multiple sclerosis and other autoimmune disorders including autoimmune haemolytic anaemia, rheumatoid arthritis, systemic lupus erythematosus, psoriasis and in patients with refractory factor VIII inhibitors due to its specific activity on lymphocytes (Robak et al., 2006).

Mizoribine is an inhibitor of purine synthesis and has been used as an orally available immunosuppressive agent in human renal transplantation (Mori et al., 2008).

1.6.2 Neuroprotectors and cardioprotectors

The cellular uptake of adenosine as well as the intracellular activity of adenosine kinase have been considered potential targets for the protection of neurones and cardiac cells (Jordheim et al., 2013). Adenosine accumulates extracellularly to achieve high enough concentrations to activate its specific cell surface receptors and to initiate a response within the cells and the vasculature, leading to neuroprotection and cardioprotection. This can be pharmacologically achieved by either mimicking adenosine activation with adenosine analogues that activate adenosine receptors or by increasing the extracellular adenosine by inhibiting the nucleoside transporters responsible for adenosine internalisation (Sharma et al., 2013).

1.6.3 Hyperuricaemia

A structural isomer of hypoxanthine, allopurinol, acts as an inhibitor of xanthine oxidase and has been used since 1966 for the treatment of chronic hyperuricaemia.

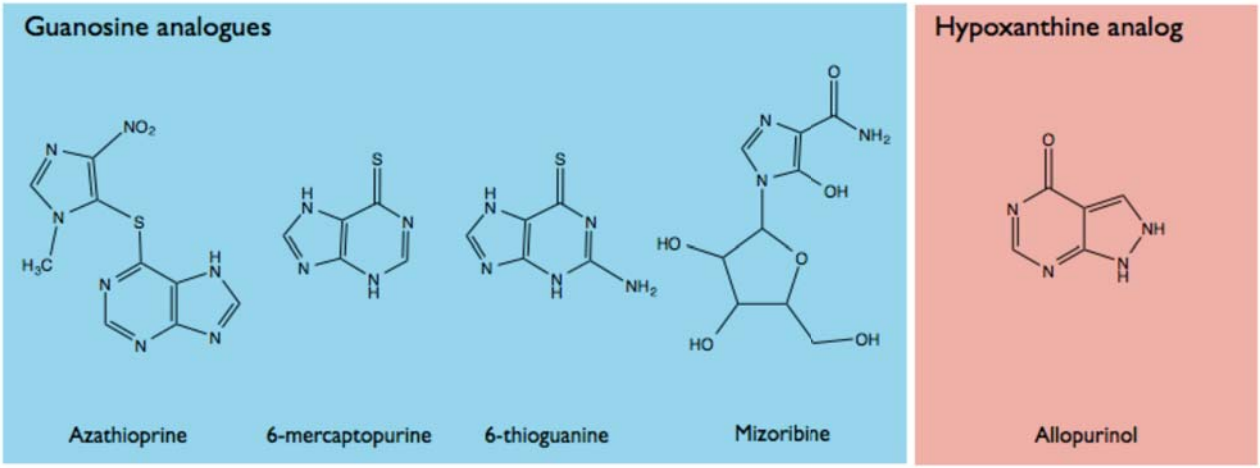


Figure 8. Structures of nucleoside analogues used for other indications. Currently used nucleoside analogues in other treatments classified upon their natural nucleoside analogues.

2

NUCLEOSIDE AND NUCLEOSIDE-DERIVED DRUG TRANSPORTERS

The first step in the pharmacological action of all nucleoside-derived drugs is its uptake into the cytoplasm. Nucleosidic drugs are in general hydrophilic molecules, for this reason, they require specific membrane transporters to translocate them through the plasma membrane.

About one third of all proteins are embedded in biological membranes and about one third of these proteins function to facilitate the transport of molecules across the membrane. Membrane transporters play crucial roles in basal cellular functioning and normal physiology processes but some can also interact with drugs and their metabolites being essential for drug bioavailability. They are composed by multiple transmembrane domains that form a pore through which the substrate passes (Yan, 2005). Transporters are of increasing interest across numerous therapeutic areas, due to the role they play both in regulating pharmacokinetic properties of drugs (absorption, distribution and elimination) and in the ocurrence of cellular drug resistance by either decreasing the uptake or increasing the efflux of the drug. In drug development, particular attention has been paid to transporters expressed in epithelia of intestine, liver, kidney and the endothelium of the blood-brain barrier. For this, the International Transporter Consortium (ITC) comprising industrial, regulatory and academic scientists with expertise in drug metabolism, transport and pharmacokinetics was created. The aim of this consortium was to set up a working team provide consensus on the role transporters must play during drug development. The ITC provided some guidelines that industry and regulatory agencies can apply to assist in the development of safe and effective medications. Consideration must be given to the role of transporters in the absorption (for example, intestinal P-gp and BCRP); distribution (for example, P-gp at the blood-brain barrier, and OATP1B1, OATP1B3 and OATP2B1 for hepatocyte uptake); and excretion (for example, OATs and OCTs for renal elimination) of new molecules in development (Giacomini et al., 2010).

Membrane transporters belong to two major superfamilies, the solute carrier (SLC) superfamily and the ATP-binding cassette (ABC) superfamily. The Solute Carrier (SLC) superfamily in humans encodes for a large number of membrane transporters classified in 52 gene families (He et al., 2009). Transporters from this family have at least 20-25% homology and are thought to have evolved from a common ancestor. There are numerous transporter proteins with different but overlapping selectivities, thus, one drug might be translocated not only by a single transporter but by more than one transporter from different families. Only a few genes from this group have been related so far to the uptake of nucleoside-derived drugs.

Concentrative nucleoside transporters (CNTs) encoded by the gene family *SLC28* and equilibrative nucleoside transporters (ENTs) encoded by *SLC29* are the transporters of natural nucleosides. However, chemical modification of nucleosides may alter their ability to interact with those transporters. Thus, additional carrier proteins that do not transport natural nucleosides become major players in drug uptake and bioavailability (Cano-Soldado and Pastor-Anglada, 2012; Errasti-Murugarren and Pastor-Anglada, 2010). In particular, proteins encoded by selected *SLC22* and *SLC15* family genes have also been implicated in the cellular uptake of some nucleoside derivatives used in chemotherapy.

2.1 Nucleoside transporters

2.1.1 Concentrative nucleoside transporters (CNTs)

2.1.1.1 Human family members

Concentrative nucleoside transporters are encoded by the gene family *SLC28*. They are high-affinity transporters, showing apparent *Km* values in the low micro molar range (10–100μM) and more restricted selectivity than equilibrative transporters (Table 1). They can translocate natural nucleosides and some nucleoside-analogues in a sodium dependent manner. This family is composed of three members which differ in their substrate selectivity and Na^+:nucleoside stoichometry.

hCNT1 (*SLC28A1*) is a pyrimidine-preferring transporter able to translocate pyrimidine nucleosides but also pyrimidine nucleoside derivatives currently used in the clinics, such as gemcitabine and cytarabine, with a 1:1 sodium:nucleoside stoichometry. Adenosine can bind to the transporter with high affinity but can not be translocated

(Errasti-Murugarren and Pastor-Anglada, 2010; Larrayoz et al., 2004; Pastor-Anglada et al., 2008). Interestingly, hCNT1 has recently been described to act as a transceptor, being able to generate intracellular signals relevant to cell physiology in a translocation-independent manner (Perez-Torras et al., 2013).

hCNT2 (*SLC28A2*) mediates the uptake of natural purine nucleosides as well as antiviral purine analogues such as ribavirin or purine derivatives used for the treatment of cancer such as fludarabine. Despite being a purine-preferring transporter it can also internalize uridine. The sodium:nucleoside coupling ratio is the same as for hCNT1.

hCNT3 (*SLC28A3*) is able to mediate the uptake both of purine and pyrimidine nucleosides. hCNT3 is the only member of the family with a sodium:nucleoside stoichometry of 2:1 and it has been described that it can also translocate in a proton specific manner with and stoichometry 1:1 proton:nucleoside (Ritzel et al., 2001).

Transporter	Gene	Substrate (*Km*)	Stoichometry (Na^+:nucleoside)
hCNT1	*SLC28A1*	Uridine (22-37 μM) Thymidine (26 μM) Cytidine (29 μM)	1:1
hCNT2	*SLC28A2*	Inosine (4 μM) Adenosine (8 μM) Uridine (40 μM) Guanosine (21 μM)	1:1
hCNT3	*SLC28A3*	Uridine (22 μM) Thymidine (21 μM) Cytidine (15 μM) Inosine (52 μM) Adenosine (15 μM) Guanosine (43 μM)	2:1

Table 1. Concentrative nucleoside transporter kinetics and properties.

2.1.1.2 hCNT structure

Human CNT1, CNT2, and CNT3 are 649, 658, 691 residue proteins with a still controversial topology. Until recently a 13 transmembrane domain (TMD) topology with an intracellular N-terminus and an extracellular C-terminus domain was broadly

accepted. However, the recent crystallisation of the concentrative nucleoside transporter of *Vibrio cholerae* has changed the predicted topology from 13 TMD to 11 TMD. The bacterial transporter is homo-trimeric, instead of 11 TMD, as expected, it has 8 TMD, 3 domains parallel to the plane of the membrane in the interfacial region and two re-entrant hairpins (HP) with opposite orientations in the membrane (Figure 8). Transmembrane domains TM4 and TM7 are discontinuous with and unwound region in the middle (Johnson et al., 2012). The homologous human proteins have extended C-terminal regions containing multiple glycosylation sites, plus three transmembrane helices and big extracellular domains at their N-termini characteristic of the eukaryotic transporters, not essential for transport activity but thought to be important in intracellular protein-binding, signalling and trafficking (Errasti-Murugarren et al., 2010a; Pinilla-Macua et al., 2012; Young et al., 2013).

Residues implied in uridine binding are located at HP1 and HP2 and in the unwound regions of TM4 and TM7, the sodium binding site has been identified between the tip of HP1 and the unwound portion of TM4. The location of the additional sodium binding site present in hCNT3 has not yet been described (Young et al., 2013).

A number of sites in human CNT3 that exhibit cation-induced changes in protein conformation have been previously identified from experiments on cysteine mutants using the membrane-impermeant inhibitor para-chloromercuribenzene sulfonate (Young et al., 2013). However, all models have to be re-evaluated after the first crystal structure of this family has been elucidated.

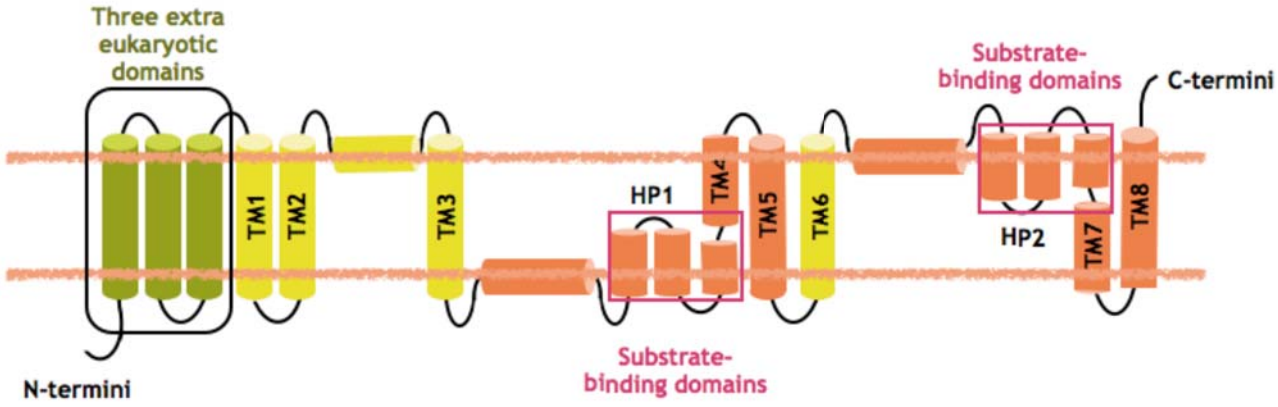


Figure 8. Concentrative nucleoside transporter structure model. This model is based on the *Vibrio cholerae* nucleoside transporter crystal **(Johnson et al., 2012)**.

2.1.2 Equilibrative nucleoside transporters (ENTs)

Equilibrative nucleoside transporters mediate facilitated diffusion of nucleosides across the plasma membrane bidirectionally and depending on the substrate concentration gradient.

2.1.2.1 Human family members

Two members of this family (hENT1 and hENT2) are expressed at the plasma membrane and have been broadly studied and characterised for the transport of natural nucleosides as well as for nucleoside-derived drugs. Both hENT1 and hENT2 can translocate purine and pyrimidine nucleosides with a lower affinity than concentrative nucleoside transporters. hENT2 is also able to translocate nucleobases. They differ in their interaction affinity to nitrobenzylthioinosine (NBTI), hENT1 binds NBTI in a noncovalent interaction at a high affinity binding site with a *Ki* of 1-10nM, whereas hENT2 is not affected by nanomolar conentrations of NBTI but becomes inhibited at concentrations higher than 10μM (Kong et al., 2004). Both transporters can be inhibited by cardiovascular drugs such as dipyridamole, dilazep and draflazine.

hENT3 is a pH-dependent intracellular transporter. It was initially localised in lysosomes, however, it was later described to be located in mitochondria were it could play a role in the mitochondrial toxicity of nucleoside-derived drugs (Govindarajan et al., 2009).

The fourth member of the *SLC29* family, hENT4, also known as Plasma Membrane Monoamine Transporter (PMAT), exhibits low sequence identity to the other family members. It is a pH-dependent transporter which functions as a polyspecific organic cation transporter that can also transport neurotransmitters, thus being closer to OCTs or Neurotransmitter Transporters than to ENTs in terms of substrate selectivity (Engel and Wang, 2005; Engel et al., 2004). Although at first it was reported not to interact with nucleosides, later it was reported that at acidic pH, hENT4 would transport adenosine efficiently, but not at physiological pH 7.4 (Barnes et al., 2006).

Transporter	Gene	Substrate (*Km*)	Inhibitor (*Ki*)
hENT1	*SLC29A1*	Uridine (260 μM) Thymidine (300 μM) Cytidine (580 μM) Adenosine (40 μM) Guanosine (140 μM) Inosine (170 μM)	NBTI (1-10 nM) Dypiridamole (5nM)
hENT2	*SLC29A3*	Uridine (250 μM) Thymidine (710 μM) Cytidine (5610 μM) Adenosine (100 μM) Guanosine (2700 μM) Inosine (50 μM) Nucleobases	NBTI (>10 μM) Dypiridamole (356nM)

Table 2. Equilibrative nucleoside transporters kinetic and molecular characteristics

2.1.2.2 hENT structure

hENT1 and hENT2 contain 456 amino acids with a 46% sequence identity. Their predicted topology is 11 TMDs with an intracellular amino terminus and an extracellular carboxyl terminus. Two large loops containing N-glycosylation sites have been predicted, an intracellular loop linking TM6 and TM7 and an extracellular loop linking TM1 and TM2 (Figure 9). hENT1 is N-glycosylated at a single site and hENT2 at two sites, in the large extracellular loop, but glycosylation is not required for activity or plasma membrane targeting of the proteins (Baldwin et al., 2004). Chimeric studies with human and rat ENT proteins have determined that the regions between TM3 and TM6 contain residues responsible for sensitivity to NBTI and other coronary vasodilator inhibitors (Baldwin et al., 2004). TM1-6 have been identified to play a key role in the transport of 3'-deoxynucleosides by human and rat ENT2, whereas TM5-6 have been implied in nucleobase transport (Baldwin et al., 2004).

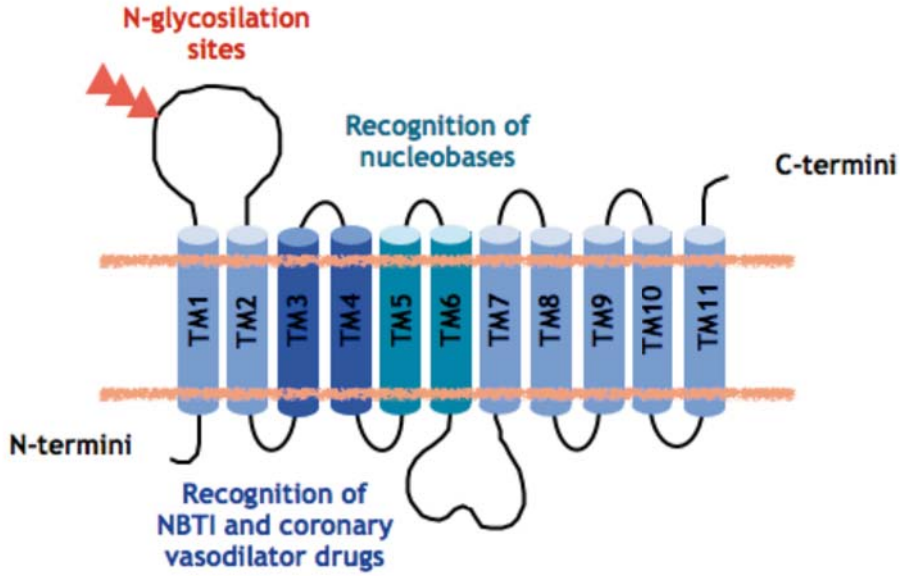


Figure 9. Equilibrative nucleoside transporters structure model. This model is based on the results published by Baldwin et al 2004.

2.1.3 Tissue distribution of nucleoside transporters

Human ENT1 and hENT2 are considered ubiquitous transporters, but their abundance varies among tissues and cell types. They are expressed mainly, but not exclusively, at the basolateral side of polarised epithelia, contributing to the vectorial flux of nucleoside transporters across these barriers.

Concentrative nucleoside transporters were initially thought to be only expressed in a few epithelial tissues, but now they are known to have broader tissue distribution. In polarised enterocytes and renal tubule epithelial cells, concentrative carriers are located at the apical domain.

This asymmetric distribution of hCNTs and hENTs at the apical and basolateral poles of mature enterocytes and renal tubule epithelial cells might permit vectorial flux of nucleosides and their derivatives, thus contributing to whole body nucleoside homeostasis and to nucleoside-derivative (re)absorption (Errasti-Murugarren et al., 2007; Lai et al., 2002) (Figure 10).

The NT polarised distribution, however, may differ among epithelial barriers. Hepatocytes show a less asymmetrical distribution of hCNT and hENT proteins. In human hepatocytes, hCNT1, hCNT2 and hENT1 appear to be located both in the basal

(sinusoidal) and apical (canalicular) membrane domains (Govindarajan et al., 2008) (Figure 10).

In case of the blood-brian barrier (BBB), hCNT2 seems to be the major CNT-type protein (Cansev, 2006; Li et al., 2001) whereas the only CNT-type protein expressed in syncytiotrophoblasts is hCNT1 and it is found together with ENTs in both membranes (Errasti-Murugarren et al., 2011).

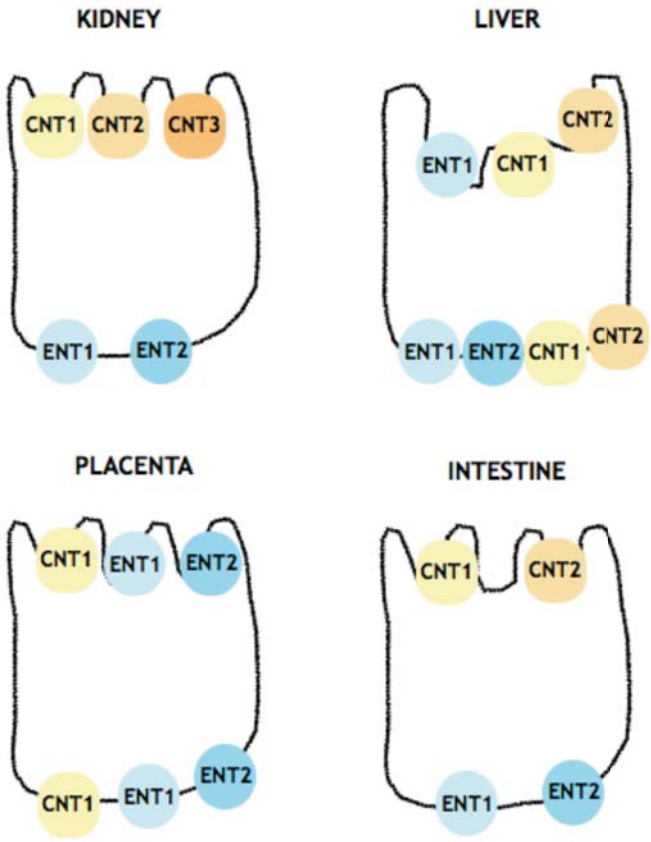


Figure 10. Nucleoside transporters distribution in polarised epithelia. Equilibrative nucleoside transporters (blue) and concentrative nucleoside transporters (yellow-orange) distribution in kidney, liver, placenta and intestine polarised cells.

CNT proteins are also expressed in other non-epithelial cell types including murine bone marrow macrophages (Soler et al., 2001a; Soler et al., 2001b) and B cell-derived lines (Soler et al., 1998). The expression of hENT1, hENT2, hCNT2 and hCNT3 proteins has been detected in primary CLL cells. However, hCNT3 function is very low or

negligible in these cells (Molina-Arcas et al., 2003). Interestingly, it has been recently shown that hCNT3 activity can be up-regulated by treatment with all-transretinoic acid (ATRA) in the CLL-derived cell line MEC1 as well as in cells from CLL patients *ex vivo* (Fernandez-Calotti and Pastor-Anglada, 2010; Fernandez-Calotti et al., 2012).

Both hENTs and hCNT2 are also present in primary T lymphocytes, while monocyte-derived macrophages (MDMs) and immature monocyte-derived dendritic cells express mainly hCNT3 (Minuesa et al., 2008). Up to date, no detailed information on NT expression in hematopoietic progenitor cells is available.

2.1.3.1 NT expression changes in cancer cells

Evidence of selective loss of NTs in tumours was first determined in rat models of hepatocarcinogenensis (Dragan et al., 2000). In humans, several studies have assessed NT expression levels in different tumours, showing variability in NT expression profiles from different individuals, but in general, a decrease in NT expression in tumour tissue compared to normal tissue (Pennycooke et al., 2001). The immunohistochemical analysis of hENT1, hENT2 and hCNT1 proteins in 300 gynecologic tumours demonstrated again a great variability in NT expression. However, a great number of tumours were hCNT1 negative whereas all tumours retained equilibrative transporter expression (Farre et al., 2004)

hENT1 expression has been linked to proliferation, whereas hCNT-type related activities are more dependent on differentiated functions of particular cell types (Aymerich et al., 2004; Soler et al., 2001a; Soler et al., 2001b). This could explain why hENT-expression is mainly retained in tumours whereas hCNT expression is more frequently lost.

2.1.4 Pharmacological profiles of nucleoside transporters

Nucleoside transporters pharmacological profiles have been assessed using either substrate flux measurements, *cis*-inhibition studies, electrophysiology or a combination of all these techniques. Drug selectivity is similar to that observed for natural nucleosides, being the affinities for nucleoside-derived drugs, in general lower, than those determined for natural nucleosides (Table 3). hENT1 and hENT2 exhibit broad substrate selectivity and they are able to transport both purine and pyrimidine analogues. Both hENT1 and hENT2 are able to transport most drugs with variable efficacy. hCNT3 is also a broad selectivity transporter and it is able to transport also

both purine and pyrimidine analogues with higher affinities than those of equilibrative transporters. hENT2 and hCNT3 can also internalise some nucleobase derivatives such as 6-mercaptopurine and 6-thioguanine (Fotoohi et al., 2006; Molina-Arcas and Pastor-Anglada, 2013)

Small changes in the derivative structure can effect the drug-transporter interaction. For instance, hCNT2 can translocate the adenosine analogue clofarabine whereas it does not take up fludarabine, even though the structures are similar.

Anticancer nucleoside derivatives					
Drug	**hCNT1**	**hCNT2**	**hCNT3**	**hENT1**	**hENT2**
Cytarabine	S	NT	S	S	S
Gemcitabine	S (24μM)	NT	S (60μM)	S (160μM)	S (740μM)
5-azacytidine	S (63μM)			S	
Zebularine			S		
5-fluoro-5'-deoxyuridine	S (209μM)	S	S	S	S
5-fluoro-2'-deoxyuridine	S	S	S		
Fludarabine	NT	INH	S	S (107μM)	S
Cladribine	NT	S	S	S (23μM)	S
Clofarabine	NT	S (81μM)	S (52μM)	S (108μM)	S (328μM)
AraG				S	S
6-mercaptopurine			S		S
6-thioguanine			S		S

S: substrate (when known *Km* values are given); NT: not substrate; INH: not substrate but inhibitor.

Table 3. Pharmacological profiles of nucleoside transporters: Anticancer drugs.

Antiviral NRTIs are in generally less efficiently translocated by nucleoside transporters. The main difference between antiviral and anti-tumour nucleoside derivatives is the lack of 3'-hydroxyl group of the antiviral drug sugar. This confirms the fact that slight modifications in the structure of the drug can lead to huge changes in

transportability (Table 4). Interestingly, the antiviral drug AZT is translocated by hCNT1, hCNT3, and hENT2 but not by hENT1 (Cano-Soldado et al., 2004; Errasti-Murugarren et al., 2007; Govindarajan et al., 2009; Yao et al., 2001), although it can inhibit hENT1-mediated transport. The same happens with ddC, which can inhibit hCNT1-mediated transport but is not translocated by hCNT1. Thus, similarly to what happens with the natural nucleoside adenosine, which can bind to hCNT1 with high affinity but is not translocated (Larrayoz et al., 2004), some drugs can also bind to nucleoside transporters and block the transport of other nucleosides.

Antiviral nucleoside derivatives					
Drug	**hCNT1**	**hCNT2**	**hCNT3**	**hENT1**	**hENT2**
Zidovudine (AZT)	S (450μM)	NT	S (310μM)	INH	S
Zalcitabine (ddC)	NT	NT	S	S	S (>7.5mM)
Didanosine (ddI)	NT	S	S	S	S (3mM)
Stavudine (d4T)	S (15.6mM)	NT	S	NT	
Lamivudine (3TC)	NT	NT		NT	
Ribavirin	NT	S (81μM)	S (52μM)	S (160μM)	S (328μM)

S: substrate (when known *Km* values are given); NT: not substrate; INH: not substrate but inhibitor.

Table 4. Pharmacological profiles of nucleoside transporters: antiviral drugs.

2.1.5 Clinical implications of nucleoside transporters

As nucleoside transporters belonging to *SLC28* and *SLC29* families have been implicated in the uptake of most nucleoside-derived drugs, the expression levels of these transporters are important not only in the bioavailability of the drug but also in its action.

In vitro studies showed that cells lacking nucleoside transporter-activity were resistant to the action of nucleoside derivatives (Aran and Plagemann, 1992). On the other hand, over-expression of a hCNT increased the sensitivity of the cells to nucleoside analogues (Mata et al., 2001).

As commented above, NT expression levels in human tumours are very heterogeneous. The first work trying to correlate NT expression and drug sensitivity and clinical outcomes of cancer patients was initially performed in patients with lymphoproliferative diseases. Different studies have shown that the expression of hENT1 correlates with the responsiveness to cytarabine treatment (Gati et al., 1997; Hubeek et al., 2005; Wright et al., 2002) and in mantle cell lymphoma patients it correlates with the therapeutic response to gemcitabine (Marce et al., 2006).

NT expression in solid tumours had not been studied until more recently. It was shown that the response to gemcitabine in pancreatic adenocarcinoma patients was better when tumours expressed hENT1 than when hENT1 levels were very low or undetectable (Giovannetti et al., 2006; Marechal et al., 2009; Spratlin et al., 2004).

From all this data, it would seem reasonable to conclude that higher expression of transporters responsible for drug uptake would lead to a better response to treatment. However, other studies have pointed on a different direction. The expression levels of hCNT1 in breast cancer patients inversely correlated with response to the treatment and disease-free survival (Gloeckner-Hofmann et al., 2006). Just recently, the role of hCNT1 as a transceptor has been elucidated (Perez-Torras et al., 2013), thus, the correlation between transporter expression and clinical outcome might have not only to do with its ability to translocate the drug used in treatment but also with the role the transporter plays in cell signalling.

2.2 Other nucleoside-derived drug transporters

Some clinically relevant nucleoside derivatives and prodrugs can not be efficiently translocated by nucleoside transporters of any type. Transporters encoded by *SLC22* and *SLC15* gene families have been implicated in the uptake of some of these analogues.

2.2.1 *SLC22* gene family

The *SLC22* gene family encodes for organic cation transporters (OCTs) and for organic anion transporters (OATs). Although, transporters from the SLC superfamily are very specialised, OCTs and OATs, have a broad substrate selectivity, being able to transport multiple compounds different in size and molecular structures. OCTs and OATs do not appear to be good transporters of natural nucleoside (Ciarimboli, 2008;

Srimaroeng et al., 2008) but have been implicated in the uptake of nucleoside and nucleobase derivatives that can not be translocated by nucleoside transporters and are currently used in the antiviral and anticancer therapies.

2.2.1.1 Human Organic Cation Transporters (hOCTs)

Organic cation transporters (OCT1-3) can translocate a broad variety of molecules with widely different molecular structures but can also be inhibited by a large number of compounds which are able to interact with the transporters but are not translocated. The molecular mass of most OCT substrates is below 500 and the smallest diameter is below 4Å (Koepsell et al., 2007). Transport of organic cations by any of the three OCT subtypes is (1) equilibrative and/or electrogenic, (2) Na^+-independent and (3) bidirectional. The driving force is supplied by the electrochemical gradient of the transported organic cation. They are bidirectional transporters able to translocate substrates across the plasma membrane in either direction with different affinities depending upon the site of drug-transporter interaction (Volk et al., 2009).

OCT substrates include endogenous compounds, drugs, xenobiotics and model compounds (Table 5). 1-Methyl-4-phenylpyridinium (MPP^+) is a model cation substrate which can be transported by all OCTs and shows high uptake rates and similar *Km* values (Koepsell et al., 2007). A variety of cations such as tetrapentylammonium or decynium-22, non-charged compounds such as corticosterone and anions like probenecid can inhibit OCT activity but they are not transported by these proteins. Most substrates and inhibitors of organic cation transporters broadly overlap among the different members of the family.

Transporter (gene)	Model substrate (*Km* µM)	Endogenous substrate (*Km* µM)	Inhibitors (*Ki* µM)
hOCT1 (*SLC22A1)*	MPP+ (15-25) ASP+ (9) TEA (69-566) N-methylquinine (20) TBuMA (53)	Agmatine (1900) Prostaglandine E2 (0.66) Prostaglandine F2α (0.48)	Amantadine (236) Cimetidine (149) Cocaine (85) Decynium-22 (2.73) Quinidine (17.5) Ranitidine (28-33) Quinine (13-23)
hOCT2 (*SLC22A2)*	MPP+ (8-25) ASP+ (24) TEA (48-500)	Acetylcholine (117) Agmatine (1800) Choline (102-210) Dopamine (330-1400) Epinephrine (420) Norepinephrine (1500) Histamine (940-1300) 5-Hydroxytriptamine (80-290) Prostaglandine E2 (0.03) Prostaglandine F2α (0.33) Ranitidine (65) Serotonin (80-290)	Amantadine (23-28) Cimetidine (110-373) Cocaine (113) Decynium-22 (0.1) Procainamide (50) Quinine (2.9-3.4) Trimethoprim (49.7) Verapamil (13-85)
hOCT3 (*SLC22A3)*	MPP+ (47-114)	Dopamine (1200) Epinephrine (240) Histamine (140-220) Norepinephrine (510-2630) Serotonin (2500)	Amantadine (>1000) Cocaine (>1000) Decynium-22 (0.1) Quinine (37)

Table 5. Substrates and inhibitors of human organic cation transporters. The table shows selected model substrates, endogenous substrates and inhibitors of human organic cation transporters (Stocker et al., 2013).

2.2.1.1.1 Organic Cation Transporters structure

Members of the OCT family are 550-560 amino acids in length and have common structural features. The predicted membrane topology includes twelve putative TMDs with intracellular C- and N-termini. It presents two large loops, an intracellular loop with phosphorylation sites between the sixth and the seventh transmembrane domains

and a large extracellular loop containing glycosylation sites between TM1 and TM2 (Figure 11) (Ciarimboli, 2008; Koepsell et al., 2007). The cysteines in the extracellular loop have recently been described to be essential for the oligomerization of organic cation transporters and the subsequently proper insertion in the plasma membrane (Brast et al., 2011; Keller et al., 2011).

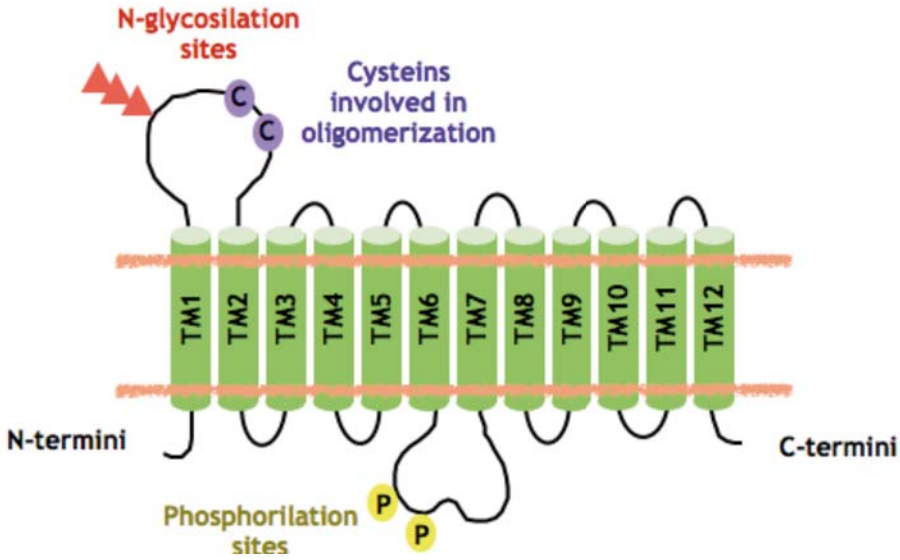


Figure 11. Organic cation transporter. This model is based on the model published by Koespell et al, 2007.

Moreover, a two binding-sites model has been proposed for organic cation transporters. A high affinity cation binding sites may bind low concentrations of xenobiotics and drugs. However, translocation of the substrate may only be induced when a low-affinity binding site is loaded (Ciarimboli, 2008; Gorbunov et al., 2008; Minuesa et al., 2009). As there is no crystal for OCT proteins, homology modelling has been used to predict the putative three-dimensional structure of OCT1.

2.2.1.2 Human Organic Anion Transporters (hOATs)

The best characterised organic anion transporters OAT1 and OAT3 have been shown to transport organic anions against a negative membrane potential in exchange for α-ketoglutarate. The α-ketoglutarate gradient is maintained by the secondary active sodium-dicarboxylate co-transporter. They have the capacity to concentrate anions against their chemical gradient and they are able to force anions into an already negative charged environment in the cells (VanWert et al., 2010).

OAT1 is known for its high-affinity transport of p-aminohippurate (PAH) from renal tubule cells with apparent affinity (*Km*) in the low micromolar range (Roth et al., 2011). OAT3 can also transport PAH but with lower affinity than OAT1. Similarly to OCTs, OATs substrates are chemically diverse. They include pharmacological agents, steroid hormones and metabolites and xenobiotics and toxins among others. OAT-function is a main determinant of mammalian capacity for mitigation substrate toxicity.

Transporter (gene)	substrate (*Km* μM)	substrate/inhibitors
hOAT1 (*SLC22A6)*	PAH (11-85.1) Prostaglandine E2 (0.97) Prostaglandine F2α (0.58)	antiviral agents uraemic toxins fluorescein indoxyl sulfate penicillin G diclofenac furosemide probenecid
hOAT2 (*SLC22A7)*	Prostaglandine E2 (0.72) Prostaglandine F2α (0.43)	glutarate PAH cAMP methotrexate
hOAT3 (*SLC22A8)*	PAH (87.2) Prostaglandine E2 (0.35) Prostaglandine F2α (1.1)	cortisol cAMP urate salicylate

Table 6. Substrats and inhibitors of human organic anion transporters. The table shows selected substrates with determined *Km* values and other substrate/inhibitors. Data was obtained from Srimaroeng et al, 2008.

2.2.1.2.1 Human Organic Anion Transporters structure

The size of OATs ranges from 542 to 563 amino acids. Like OCTs, they are predicted to have 12 TMDs with both ends facing the cytosol. They also have a large extracellular loop between TM1 and TM2 and an intracellular loop between TM6 and TM7. As for OCTs, the large extracellular loop contains potential N-glycosylation sites and the intracellular loop, phosphorylation sites. The extracellular loop, as for hOCTs

has been related with the oligomerization of the transporter (Keller et al., 2011). There is so far no crystal structure available for any OAT, therefore, homology modelling has been used to predict their putative tridimensional structure (Roth et al., 2011).

2.2.1.3 hOCTs and hOATs tissular distribution

Organic cation transporters and organic anion transporters are expressed in different tissues throughout the body.

Human Organic Cation Transporter 1 (hOCT1) is usually considered a liver-specific transporter. However, weak mRNA expression of hOCT1 has also been detected in other tissues such as heart, skeletal muscle, kidney, brain and placenta (Roth et al., 2011). hOCT1 is mainly localised to the sinusoidal membrane of the hepatocytes (Nies et al., 2008). hOCT1 protein was also localised to the luminal membrane of lung epithelial cells (Lips et al., 2005). rOCT1 has been identified at the basolateral membrane of enterocytes and proximal tubule epithelial cells. However, hOCT1 has not been detected in human kidney (Gorboulev et al., 1997).

Human Organic Cation Transporter 2 (hOCT2) is mainly a kidney transporter, although low mRNA levels have been detected in spleen, placenta, small intestine and brain. In human kidney OCT2 is expressed in all three segments of proximal tubules and it is localised to the basolateral membrane (Koepsell et al., 2007; Roth et al., 2011). It has also been detected in pyramidal cells of cerebral cortex as well as the luminal membrane of lung epithelia.

Human Organic Cation Transporter 3 (hOCT3) is the OCT with a widest tissue distribution. Strong mRNA expression has been observed in liver, placenta, kidney and skeletal muscle, and weaker in lung, heart and brain (Roth et al., 2011; Wu et al., 2000). The protein expression has been confirmed at the basolateral membrane of hepatocytes, the basal membrane of trophoblasts, the apical membrane of enterocytes and the luminal membrane of lung epithelial cells.

Human Organic Anion Transporter 1 (hOAT1) presents the highest mRNA expression levels in the kidney followed by skeletal muscle, brain and placenta. At the protein level, hOAT1 has been detected at the basolateral membrane of proximal tubules (Hosoyamada et al., 1999; Roth et al., 2011) and in the plasma membrane of skeletal muscle.

Human Organic Anion Transporter 2 (hOAT2) shows the highest mRNA levels in liver with lower levels also detected in kidney. Based on finding in rodents, it is also assumed to be expressed at the basolateral membrane of hepatocytes.

Human Organic Anion Transporter 3 (hOAT3) also presents high mRNA expression in kidney and lower levels in brain and adrenal tissue. hOAT3 protein has been localised at the basolateral membrane of proximal tubules (Cha et al., 2001; Roth et al., 2011).

Regarding immune cells, hOATs are not expressed in primary T-cells, B-cells or macrophages (Purcet et al., 2006). hOCTs are present in T-cells, primary Peripheral blood mononuclear cells (PBMCs), monocytes and macrophages with a lower expression level and a higher heterogeneity than nucleoside transporters. Interestingly, they only express hOCT1 and hOCT3 but not hOCT2. hOCT up-regulation can be observed upon lymphocyte stimulation with phytohaemagglutinin (PHA) (Minuesa et al., 2008).

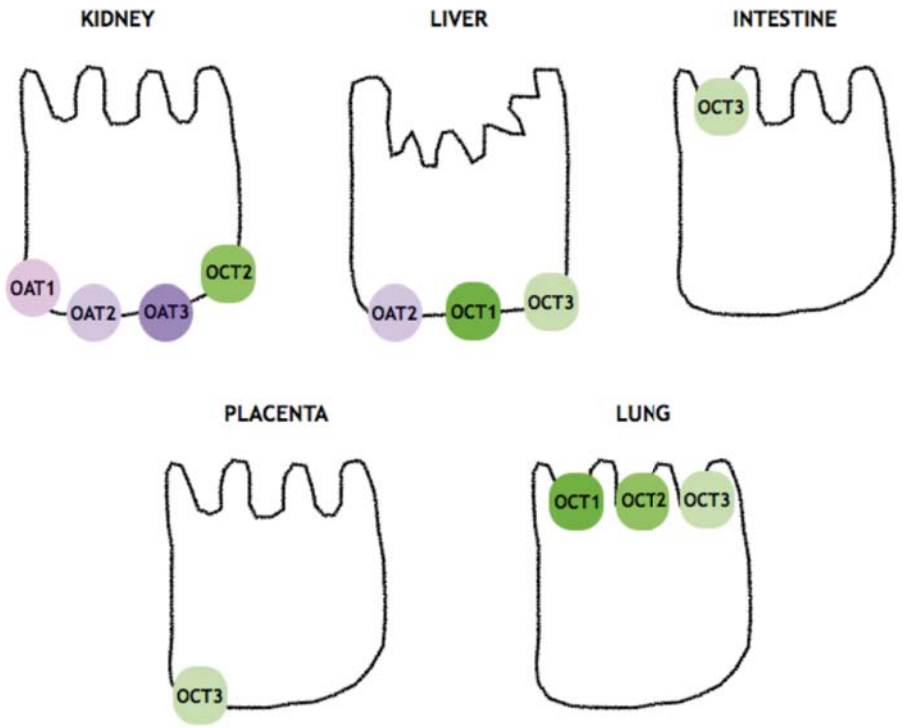


Figure 12. Organic cation transporters and organic anion transporters distribution in polarised epithelia. Organic cation transporters (green) and organic anion transporters (purple) distribution in kidney, liver, placenta, lung and intestine polarised cells.

In lymphoma cell lines as well as in leukaemia cell samples from patients with untreated CLL, hOCT1 was over-expressed compared to control lymphocytes. No

expression of hOCT2 or hOCT3 was detected in either the lymphoma cell lines or the primary cell samples (Gupta et al., 2012).

2.2.1.4 OCTs and OATs role in nucleoside-analogues internalisation

hOCTs and hOATs are novel pharmacological targets for the chemotherapeutical response to some nucleoside-derivatives. These derivatives include some antiviral analogues lacking the 3'-OH group as well as some nucleobase derivatives.

The first data suggesting the implication of these proteins in the translocation of nucleoside analogues was obtained when studying AZT renal clearance in rats. Data showed that in the presence of probenecid (OAT inhibitor) or cimetidine (OCT inhibitor), AZT renal excretion was significantly reduced (Aiba et al., 1995). Since that, other studies have pointed to the same direction. For instance, in an isolated perfused rat kidney model, two OCT substrates (cimetidine and trimethoprim) significantly reduced emtricitabine (FTC) clearance (Nakatani-Freshwater and Taft, 2008a; Nakatani-Freshwater and Taft, 2008b).

hOAT1 and the rat orthologue of hOCT1 have also been involved in the uptake and cytotoxicity of adefovir and cidofovir (Cihlar et al., 1999; Ho et al., 2000).

More recently, all hOCTs have been shown to mediate the uptake of the nucleoside-derived antiviral drug lamivudine, widely used in anti-HIV therapy (Minuesa et al., 2009). Moreover, zidovudine uptake was shown to be mediated by hOAT1-3 in renal tissue, whereas hOCT1 appears to mediated renal acyclovir and ganciclovir transport (Takeda et al., 2002).

The localisation of these proteins to the basolateral domain of polarised renal epithelia suggests they might be major players in the renal excretion of antiviral nucleosides.

Table 7 summarises the transportability profiles of these transporters and nucleoside derived antiviral drugs.

Antiviral nucleoside derivatives						
Drug	**hOCT1**	**hOCT2**	**hOCT3**	**hOAT1**	**hOAT2**	**hOAT3**
Zidovudine (AZT)	INH	INH	INH	S	S	S
Zalcitabine (ddC)	S	S		S	CYT	CYT
Didanosine (ddI)				CYT		CYT
Stavudine (d4T)				CYT		CYT
Lamivudine (3TC)	S	S	S	CYT		CYT

S: substrate; NT: not substrate; INH: not substrate but inhibitor; CYT: suggested substrate by cytotoxicity studies

Table 7. Pharmacological profile of organic cation transporters and organic anion transporters in the uptake of nucleoside-derived antiviral drugs.

2.2.2 *SLC15* gene family

Some *SLC15* gene family members encode either peptide transporters 1 and 2 (PepT1 and PepT2). PepTs mediate the translocation of di- or tri-peptides in a proton-coupled manner. A part from natural peptides they can also transport pharmacologically active molecules against a substrate concentration gradient. This proteins are predicted to have 12 TMDs with a large extracellular loop between TM9 and TM10 and cytoplasmatic N- and C-termini (Rubio-Aliaga and Daniel, 2008). Although no work in the present thesis was performed with these transporters, they are relevant because they are expressed in many epithelial barriers and can translocate some nucleoside prodrugs. Valacyclovir, a derivative amino acid ester of acyclovir, is a PepT1 substrate whereas acyclovir is not (Ganapathy et al., 1998). More recently, an amino acid ester pro-drug of the anticancer agent 5-fluoro-2'-deoxyuridine has also been reported to be transported by PepT1 (Landowski et al., 2005).

3

PHARMACOGENOMICS OF DRUG TRANSPORTERS

3.1 Introduction to pharmacogenomics

Genetic variants include single nucleotide polymorphisms (SNPs) which are typically present in more than 1% of the population. Variants present in less percentage of the population are considered mutations. SNPs present in the protein coding regions can be classified into two groups, synonymous and non-synonymous, depending on whether the amino acid sequence is altered or conserved in the variant allele. It is also possible that the SNPs lead to frameshift of amino acid sequence or premature truncation of the protein, due to amino acid insertions or deletions.

Pharmacogenomics study the genetic basis of individual variations in response to drug therapy. The goal of pharmacogenomics is to achieve optimal therapy for individual patients, using genetic and genomic principles to facilitate drug discovery and development and to improve drug therapy. When the term pharmacogenomics was first introduced back in 1959, the field was primarily concentrated in genetic variants of drug-metabolising enzymes. However, nowadays, the term is used to represent the entire spectrum of genes that determine drug pharmacokinetics and pharmacodynamics.

3.2 Membrane transporters pharmacogenomics

Polymorphic variants in a membrane transporter can modulate an individual's overall exposure to a drug. Depending on the expression and localisation of the transporter, the presence of a polymorphism can cause an increased or decreased systemic exposure to the drug. Sometimes, when the transporter is present in multiple tissues and it has overlapping functions with other transporters of similar expression patterns, it might be difficult to determine the *in vivo* contribution of a particular genetic variant of a membrane transporter.

To date, multiple transporter polymorphisms have been identified. Some variants have been shown to cause altered trafficking of the transporter to the plasma membrane, resulting from incorrect folding of the transporter or inability to interact with partner proteins. Other variants can affect substrate recognition and binding, sometimes in a substrate-specific manner, making it difficult to predict the effect of a

polymorphism on the transport of a specific compound without testing that compound directly (Errasti-Murugarren and Pastor-Anglada, 2010; Molina-Arcas and Pastor-Anglada, 2013; Urban et al., 2006). Although a high amount of polymorphisms have been identified in membrane transporters, not all of them affect transporter function. In fact, in a study in which polymorphic variants in SLC22, SLC28 and SLC29 genes were identified and functionally characterised, only 14% out of 88 polymorphic variants identified had a decreased or altered transporter function in vitro (Urban et al., 2006).

3.2.1 *SLC28* family polymorphisms

SLC28 gene family does not appear to be highly polymorphic in humans. However, due to its tissue distribution any variants affecting transport function are expected to have a marked impact on the (re)absorption of nucleoside derivatives, thus also affecting drug disposition.

Within *SLC28A1* gene, encoding for hCNT1, 58 SNPs have been identified so far, 15 of which alter the amino acid sequence. All but two of the variants, hCNT1-S546P and hCNT1-1153del, retain functional activity (Gray et al., 2004). The first variant affects a highly conserved serine residue in transmembrane domain 12, while the second one results in a truncated protein. Some other variants, despite being functional, exhibit changes in thymidine uptake capacity. For instance, hCNT1-L635V has a reduced thymidine uptake ability, whereas hCNT1-V189I and -D521N, which have allele frequencies higher than 20% in the studied population, are gain of function variants that increase hCNT1-mediated thymidine uptake (Gray et al., 2004). In addition, V189I is associated to higher mizoribine bioavailability in kidney transplant recipients (Naito et al., 2010) whereas D521N, has been shown to be associated with haematological toxicity in non-small cell lung cancer (NSCLC) patients treated with gemcitabine (Soo et al., 2009).

Within *SLC28A2* gene, encoding for hCNT2, a total of 5 non-synonymous variants in the coding region have been identified, all of which retain function although some are associated with either increased (hCNT2-S75R) or reduced (hCNT2-F355S, hCNT2-E385K and hCNT2-M612T) uptake of natural nucleosides or derivatives (Li et al., 2007; Owen et al., 2005). Interestingly, the hCNT2-P22L mutant, which seems to retain normal transporter activity, has been associated with a more favourable prognosis in lung carcinoma patients treated with gemcitabine, a cytidine analogue which is not substrate

for hCNT2 (Soo et al., 2009). Six non-coding variants with substitutions in a non-coding putative promoter region of *SLC28A2* gene have been described to alter basal transcriptional activity of the transporter (Li et al., 2009; Yee et al., 2009). To date, the clinical relevance and transport activity of these variants has not yet been fully assessed.

With regard to the *SLC28A3* gene, encoding for hCNT3, 16 coding region variants, 10 of which are non-synonymous, have been identified (Badagnani et al., 2005). Only the polymorphic variant hCNT3-G267R showed to have altered transport activity, showing a 80-85% reduction in the uptake rates of both purine and pyrimidine nucleosides. The other variants encode for proteins that have similar kinetic and ion-interaction properties to the reference hCNT3 protein. A rare non-synonymous mutation has recently been identified in Spanish population with an allele frequency of 1%. This hCNT3-C602R contains a charged residue in the last transmembrane domain. It is associated with low-level of transport activity which seems to be related to an impaired binding of one of the Na^+ ions known to be necessary for efficient hCNT3-mediated translocation of nucleosides. Moreover, the hCNT3-C602R protein shows missorting to non lipid-raft fractions, where the transporter appears to be less active than when it reaches the appropriate location. This variant affects the selectivity of nucleoside-derived drugs (Errasti-Murugarren et al., 2008; Errasti-Murugarren et al., 2010b). Just recently, the variant haplotype rs10868138G/rs56350726T (allelic frequency 0.074) was associated with protection against haemolytic anaemia in patients with chronic hepatitis C under ribavirin treatment (Doehring et al., 2011).

3.2.2 *SLC29* family polymorphisms

Genetic variants in *SLC29* gene family may be relevant both in drug pharmacodynamics and action as the proteins they encode for are widely distributed both in normal and tumour cells.

For *SLC29A1* gene, en*coding* for hENT1, the most abundantly and widely distributed transporter of the family, several studies on polymorphic variants have been carried out. Two common promotor region SNPs (-1050G>A and -1345C>G) have been shown to increase the transcriptional activity of the gene in a luciferase reporter assay (Myers et al., 2006). However, *in vivo,* hENT1 mRNA levels in haplotypes with these

variants are not significantly different. To date, there is no evidence of encoding region variants resulting in altered ENT1-mediated uptake.

Within *SLC29A2* gene, encoding for hENT2, four non-synonymous variants have been identified until now. All but one of the variants yield active proteins. The non-functional variant, hENT2-845-846del, results in a truncated protein. Two other functional variants, hENT2-D5Y and -551-556del, resulted in an uptake reduction of both natural nucleosides and nucleoside drugs, respectively, whereas hENT2-N68K exhibited an increased guanosine uptake activity (Owen et al., 2006). To date, no clinical correlations between hENT2 variants and clinical outcome have been reported.

SLC29A3, encoding for hENT3, is the only gene in the family in which described mutations are associated with a human disease. These clinical conditions include H syndrome, an autosomal recessive genodermatosis with systemic manifestations (Huber-Ruano et al.), and pigmented hypertrichosis with insulin-dependent diabetes mellitus syndrome (Cliffe et al., 2009). All mutations of the gene detected to date are associated with rare diseases but polymorphic variants remain yet to be studied.

3.2.3 *SLC22* family polymorphisms

Polymorphic variants in *SLC22* gene family are major contributors to variation in drug disposition, therapeutic and adverse drug responses as well as risk for human disease.

SLC22A1 gene, encoding for hOCT1, is a highly polymorphic gene. Over 200 SNPs have been identified in hOCT1, some of them ethnic-specific. Out of these, 24 are non-synonymous with four (F160l, S189L, M408V and M420del) being present in most ethnic groups. In Caucasians, 14 non-synonymous SNPs have been identified four of which (G38A, C88R, S189L and G465R) are specific to this ethnic group. Many of the non-synonymous polymorphisms in hOCT1 are situated in conserved regions of the gene suggesting a fundamental role of the residues for transport activity. Seven polymorphic variants in hOCT1 from multiple ethnic populations (R61C, C88R, G220V, P283L, R287G, G401S and G465R), have been shown to mediate a reduced or negligible uptake of MPP^+. Four out of these (R61C, G220V, G401S and G465R) also showed reduced uptake of metformin. Additionally, six more variants which showed no altered MPP^+ uptake, have also been demonstrated to show reduced uptake of metformin. Accordingly, the

potential pharmacokinetic consequences of hOCT1 polymorphic variants may vary depending on the administrated drug (Stocker et al., 2013).

hOCT1 genetic variants have been associated to changes in the pharmacokinetics and pharmacodynamics of metformin (Shu et al., 2008; Shu et al., 2007). A study in healthy volunteers showed an increase in the area under the concentration-time curve (AUC) and higher maximal plasma concentration (*Cmax*) in those individuals carrying reduced function alleles (R61C, G401S, M420del and G465R) comparing to those carrying the wild type transporter (Shu et al., 2008). Moreover, recent studies showed that hOCT1 proteins with reduced function polymorphisms, such as R61C and M420del, are more susceptible to inhibition by drugs compared to the reference hOCT1. Interestingly, metformin uptake could be inhibited with lower concentrations of verapamil when checking the M420del variants when compared to the reference hOCT1 transporter (Ahlin et al., 2011). hOCT1, has in fact, been included in the white paper from the ITC as a key drug transporter which should be evaluated for cross-interactions at the early stages of drug development (Giacomini et al., 2010).

The antiemetic drug topisetron, has also been shown to be a hOCT1 substrate. This drug is primarily metabolised by CYP2D6, an hepatic enzyme. So in order to be metabolised, it has to be internalised in the liver by hOCT1; thus, polymorphic variants in the transporter are postulated to alter drug response. A recent study by Tzvetkov et al showed an increase in plasma concentrations and drug response in patients carrying reduced functioning hOCT1 variants (R61C, C88R, G401S, M420del and G465R) independently of polymorphic variants in the metabolising enzyme (Tzvetkov et al., 2012).

Imatinib, is a tyrosine kinase inhibitor currently used in the treatment of chronic myeloid leukaemia (CML). Although some *in vitro* studies indicated that imatinib is a weak hOCT1 substrate (Minematsu and Giacomini, 2011), some studies have found that the expression of hOCT1 gene and its activity may be the most important determinants for imatinib treatment outcome in CML patients (Crossman et al., 2005; Wang et al., 2008; White et al., 2006). Moreover, a recent clinical study in newly diagnosed CML patients showed that the polymorphic variant M420del could modulate the outcome during imatinib treatment (Giannoudis et al., 2013).

Sorafenib is a novel tyrosine kinase inhibitor which has been reported to be beneficial in the treatment of hepatocellular carcinoma (HCC) and cholangiocarcinoma (CGC). A recent study identified hOCT1 as a sorafenib transporter. Two novel variants, hOCT1-R61S and hOCT1-C88A, which were identified either in HCC, CGC or both but not in the adjacent healthy tissue, showed impaired sorafenib transportability which may affect the ability of sorafenib to reach active intracellular concentrations in the tumours expressing them (Herraez et al., 2013).

SLC22A2, encoding for hOCT2, is less polymorphic than *SLC22A1*. However, some clinically relevant variants have also been identified in this transporter. For instance, hOCT2-T199I, hOCT2-T201M and hOCT2-A270S have been shown to impact the renal clearance of metformin (Song et al., 2008a; Song et al., 2008b). The basolateral localisation of hOCT2 in proximal renal tubule cells is responsible for more than 80% of total metformin clearance. Thus, reduced hOCT2 function results in a reduced clearance and therefore in an increase in plasma concentrations of metformin. The more common non-synonymous variants (M165I, K432Q and R400C) also exhibit functional differences from the reference hOCT2. hOCT2-M165I and hOCT2-R400C variants result in a decreased MPP^+ uptake while hOCT2-K432Q exhibits an increase in the apparent affinity for MPP^+ (Leabman et al., 2002). In general, the more frequent non-synonymous variants result in a more subtle functional reference when compared to the reference hOCT2. Only the hOCT2-A270S polymorphism has a high allele frequency and is present in different populations. Interestingly, this variant shows reduced metformin uptake in oocytes (Kang et al., 2007; Song et al., 2008b), although the uptake of metformin in HEK293 cells expressing this variant is increased (Chen et al., 2009). The reason for this discrepancy remains unclear. hOCT2-A270S variant has also been associated with hypertension and the correlation is more pronounced in patients with diabetes mellitus (Lazar et al., 2006).

Cisplatin, a widely used anticancer agent, is primarily eliminated by the kidney and has been shown to be a hOCT2 substrate (Ciarimboli et al., 2005; Zhang et al., 2006). Cellular studies have shown the importance of hOCT2 in mediating cisplatin and its analogues oxaliplatin and carboplatin cytotoxicity (Zhang et al., 2006). Although Caucasian patients receiving cisplatin and carrying the hOCT2-A270S variant presented no alteration in the pharmacokinetic parameters including urinary excretion (Filipski et

al., 2009), patients with A270S variant had a reduced risk of developing cisplatin induced nephrotoxicity (Filipski et al., 2009).

Information on SLC22A3 gene, encoding for hOCT3, polymorphic variants is very limited. Only one variant, hOCT3-T44M, has been reported to alter MPP^+ uptake. However, three variants demonstrated altered metformin transport, two polymorphisms (T400I and V423F) showed reduced uptake while one polymorphisms (T44M) showed increased uptake (Chen et al., 2010). Interestingly, one non-synonymous SNP (A411A) and a non-coding 3'UTR (rs3088442) polymorphism in hOCT3 have been associated with decreased hOCT3 mRNA and protein expression in Caucasian liver samples (Nies et al., 2009). The presence of genetic polymorphisms in hOCT3 coding and proximal promoter regions has been identified and associated with an increase risk of coronary artery disease (Tregouet et al., 2009), obsessive-compulsive disorder (Lazar et al., 2003; Lazar et al., 2008) and prostate cancer (Eeles et al., 2008).

In *SLC22A6* gene, encoding for hOAT1, only two non-synonymous variants with functional implications have been described until now. hOAT1-R50H shows increased affinity for some of its substrates including the nucleoside derivatives adefovir, cidofovir and tenofovir whereas hOAT1-R454Q is a non-functional variant (Bleasby et al., 2005; Fujita et al., 2005). A clinical study of hOAT1-R454Q association with altered *in vivo* renal clearance of adefovir, showed no association, suggesting that renal hOAT1 is not the only transporter responsible for adefovir renal clearance.

SLC22A8, encoding for hOAT3, variants show a high degree of functional heterogeneity. Three of the described polymorphisms (R149S, Q239Stop and I260R) encode non-functional transporters. By contrast, the variant hOAT3-I305F shows altered substrate specificity, it exhibits impaired estrone-3-sulphate uptake but it maintains its ability to transport cimetidine (Erdman et al., 2006).

Membrane transporters may have other roles a part from substrate translocation. For instance, hCNT1 protein expression in pancreatic adenocarcinoma can induce a variety of intracellular signalling cascades, promote cell cycle arrest and inhibit cell migration in a translocation-independent manner (Perez-Torras et al. 2013). The impact of genetic polymorphic variants on the additional biological functions will have to be addressed in the near future (Pastor-Anglada, 2013).

OBJECTIVES

Membrane transporters play an essential role in drug pharmacokinetics and pharmacodynamics. Moreover, the presence of polymorphic variants in transporter proteins can alter not only the drug transportability profiles but also drug-drug interactions.

Nucleoside-analogues require specific membrane transporters to be internalised in cells and be pharmacologically effective. A clear understanding of drug uptake mechanisms and drug-drug interaction as well as a better knowledge of the effect genetic variants may have on them, is essential for a clear understanding of the pharmacokinetics/pharmacodynamics and adverse effects of drugs.

Furthermore, good transporter structural models would help to predict the implications of particular SNPs on transporter function but would also help to anticipate how drug-transporter and drug-drug interactions occur.

Based upon this background, the objectives of this thesis were:

1- To elucidate the role hOCT1 polymorphic variants play on lamivudine (3TC) transportability and drug-drug interactions.

2- To identify the transporter responsible for bendamustine uptake and to analyse the effect its polymorphic variants may have in its cellular handling.

3- To identify and functionally characterise the transporter proteins responsible for the uptake and cellular handling of DNA methyltransferase inhibitors used in the epigenetic treatment of cancer.

4- To generate a hCNT3 structural model suitable for the prediction of drug-transporter interactions.

RESULTS

1

ROLE OF ORGANIC CATION TRANSPORTER 1 POLYMORPHISMS IN LAMIVUDINE (3TC) BIOAVAILABILITY AND DRUG-DRUG INTERACTION

1.1 Effect of hOCT1 polymorphisms in lamivudine, metformin and MPP^+ uptake

Some nucleoside analogues, specially dose antivirals which lack the 3'-hydroxyl group, are not good substrates for nucleoside transporters. However, some of them such as lamivudine (3TC) and zalcitabine (ddC) can interact with and be internalised through organic cations transporters (Jung et al., 2008; Minuesa et al., 2009). *SLC22A1* encoding for hOCT1 is a highly polymorphic gene with some of its variants presenting a high allelic frequency in Caucasian population. Some of the described SNPs have shown to alter pharmacokinetics and bioavailability of some drugs currently used in clinics such as metformin, morphine or imatinib (Giannoudis et al., 2013; Shu et al., 2008; Shu et al., 2007; Tzvetkov et al., 2013).

The role that these polymorphic variants play in the uptake of 3TC was assessed in this thesis by generating the most common polymorphic variants by site-directed mutagenesis and transiently transfecting their cDNAs in HeLa cells. The uptake of 10μM 3TC and 1μM MPP^+ was measured for 1 minute exposure and compared to the uptake rates of metformin published by Shu et al (Shu et al., 2007). The percentages of activity versus those of the wild type transporter are shown in table 1.

Interestingly, polymorphic variants did not affect equally the uptake of all tested drugs. In order to further characterise the role of the polymorphic variants in 3TC uptake, those variants with high allelic frequency which showed significant changes in substrate transportability (R61C, C88R, S189L, M420del and G465R (highlighted in table 1)) were chosen for further studies. They were stably transfected in HEK293 cells using the commercially available FlpIn method together with the wild type hOCT1 and a control vector.

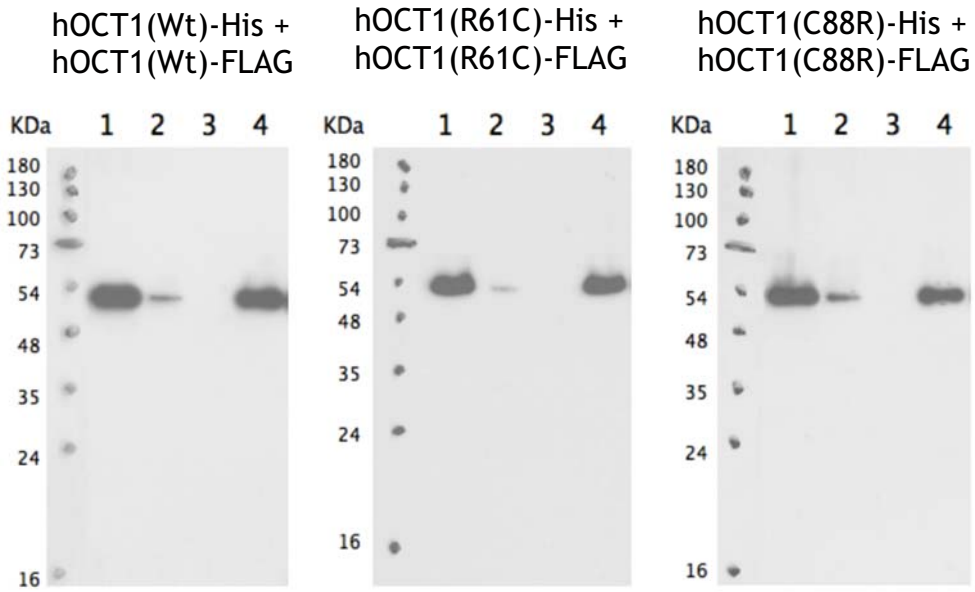
dbSNP	AA change	Allele Freq Caucasians (%) [1]	MPP^+ uptake 1min	3TC uptake 1 min	Metformin uptake 5 min [3]
rs12208357	R61C	7.2	75	40	5
rs55918055	C88R	0.62 [2]	10	25	n.d
rs683369	F160L	0.65	100	100	100
rs34104736	S189L	0.5	80	50	25
rs2282143	P341L	0	100	85	100
rs34130495	G401S	1.1	10	25	10
rs628031	M408V	59.8	100	100	100
rs35167514	M420del	18.5	80	50	30
rs34059508	G465R	4	10	20	5

[1] (Shu et al., 2003) [2] (Kerb et al., 2002) [3] (Shu et al., 2007)

Table 1. hOCT1 SNPs used in this study defined by rs number and amino acid (AA) change. The uptake of the different compounds here tested is expressed as percentage of hOCT1 wild type uptake. MPP^+ and 3TC uptake measurements were performed in transiently transfected HeLa cells. Those polymorphic variants highlighted in grey were chosen for being further characterised and stable HEK293 cells expressing them were generated.

1.2 Oligomerization of hOCT1 and two of its polymorphic variants located at the large extracellular loop

Cysteines located at the first extracellular loop of the organic cation transporters have recently been implicated in the oligomerization of the transporter and its correct insertion into the plasma membrane (Brast et al., 2011; Keller et al., 2011). Two of the chosen polymorphic variants are located at the large extracellular loop and implicate cysteines, either by adding a new cysteine (R61C) or by substituting an already existing one (C88R).



Construct		Coprecipitation (percentage of total)
His-tagged	FLAG-tagged	
rOCT1	rOCT1	93.3 ± 2.1 #
rOCT1	rOAT1	12.3 ± 2.5 #
hOCT1	hOCT1	89.7 ± 4.2
hOCT1(R61C)	hOCT1(R61C)	90 ± 4.4
hOCT1(C88R)	hOCT1(C88R)	87.7 ± 4.5

(Keller et al., 2011)

Figure 2. Oligomerization of polymorphic variants located at the extracellular loop. The samples were diluted with 200µL of Tris buffer containing 1% CHAPs and 10mM imidazole (lanes 1). After 1h incubation at 4ºC, Ni^{2+}-NTA-agarose beads were added, the suspension was incubated for 1h and centrifuged. Supernatants were collected (lanes 2). The beads were washed five times with 1mL of buffer and pelleted (supernatants lanes 3). His-tagged proteins were eluted by incubating the beads with 400µL of buffer containing 1% CHAPS and 100mM imidazole (supernatant lanes 4). Proteins were separated by SDS-PAGE, transferred to a blotting membrane, and stained with an antibody against the FLAG tag. The experiment was performed three independent times. The mean ± S.D. are shown in the table.

In collaboration with the group of Dr. Koepsell, oligomerization studies were carried out, to determine if those polymorphic variants affecting cysteines at the extracellular loop could form oligomers or not. For this, His-tagged and FLAG-tagged hOCT1 WT or the polymorphic variants of interest were incubated with Ni^{2+}-NTA-agarose beads, known to bind the His-tagged proteins. FLAG-tagged proteins were shown to bind all His-tagged proteins assayed except OAT1 which was used in this experiment as a negative control.

These oligomerization studies showed that both R61C and C88R polymorphic variants, although one of them substituted a cysteine located at the extracellular loop for an arginine and the other one added a new cysteine to the extracellular loop, did not affect the ability of the transporter to oligomerize. Moreover, this suggests that the cysteine at the 88 position is not essential for the correct oligomerization of the transporter.

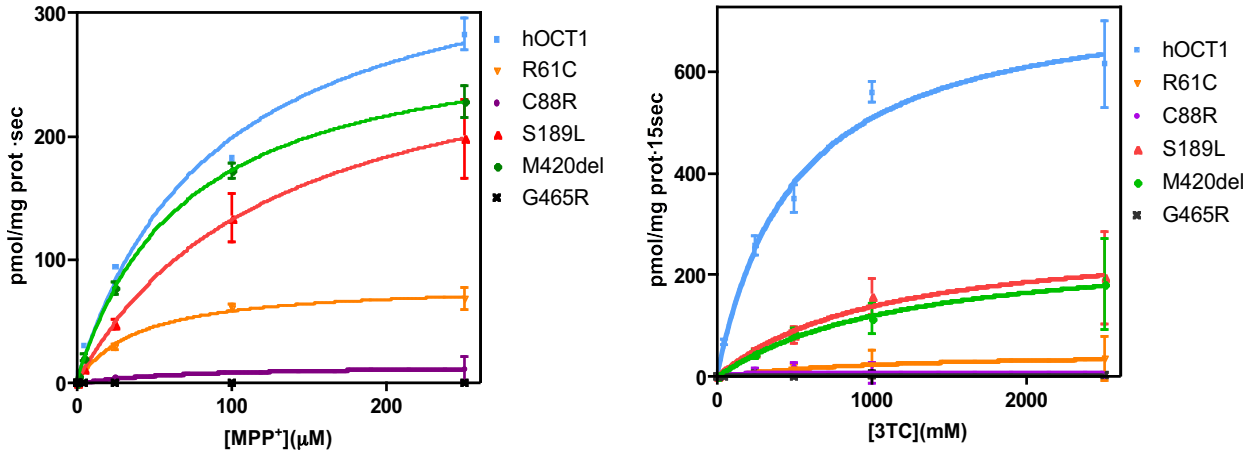
1.3 Kinetic parameters of MPP^+ and 3TC uptake mediated by hOCT1 SNPs

The polymorphic variants R61C, C88R, S189L, M420del and G465R were chosen for further analysis because they showed different transport rates for the different substrates. HEK293 stably expressing them where generated and the cells were subsequently used to carry out kinetic studies for MPP^+ and 3TC.

The kinetic measurements where performed at one second for MPP^+ and 15 seconds for 3TC to ensure we were only measuring the uptake mediated by the stably expressed transporter and no passive diffusion or unspecific transport altered the results. Results are shown in figure 3.

Km and *Vmax* values were calculated by fitting the data experimentally obtained to a *Michaelis Menten* curve. In the case of C88R and G465R variants, data could not be fitted to a curve because uptake rates mediated by these variants were too low. *Km* values for MPP^+ were similar for all tested variants to the wild type transporter. However, for 3TC, the *Km* value for S189L was significantly higher ($p<0.01$) than for the wild type transporter. Although *Vmax* values were significantly lower in all polymorphic variants for all drugs, in case of 3TC values were even much lower compared to those

for MPP+. Transport efficiency was calculated by dividing *Vmax* by *Km* values. If this ratio is higher than 1 the transporter is considered to be a good transporter for the assayed substrate, whereas if it is lower than 1, it is not considered and efficient transporter. For MPP+, even though the transport efficiency was significantly reduced in the R61C variant ($p<0.01$), it could still be considered a good transporter for MPP+ because this ratio was higher than 1. For 3TC, all polymorphic variants here tested showed impaired transport efficiency, with ratios being much lower than 1 ($p<0.01$).



		hOCT1	R61C	S189L	M420del
MPP+	*Km* (µM)	103 ± 21	85 ± 23	112 ± 23	101 ± 19
	Vmax (pmol/ mg prot ·sec)	361 ± 11	73 ± 13***	206 ± 48**	191 ± 33**
	Vmax/*Km* (µL/ mg prot·sec)	3.94 ± 0.62	1.17 ± 0.36**	2.25 ± 0.73	2.18 ± 0.71
3TC	*Km* (µM)	786 ± 84	1008 ± 104	1161 ± 63**	806 ± 9
	Vmax (pmol/ mg prot·15 sec)	851 ± 103	104 ± 49***	267 ± 11***	206 ± 46***
	Vmax/*Km* (µL/ mg prot·15sec)	1.09 ± 0.13	0.10 ± 0.05**	0.23 ± 0.02**	0.26 ± 0.06**

Figure 3. Kinetic studies for 3TC and MPP+. Kinetc studies were performed in HEK293 stably expressing hOCT1 wild type or one of the polymorphic variants. For MPP+ the uptake was measured at 1s and for 3TC at 15s to enhance the sensibility. Kinetic parameters shown in the table were calculated fitting data to a *Michaelis Menten* curve. Unpaired t-Student test was performed. (**, $p<0.01$; ***, $p<0.001$)

1.4 hOCT1 expression in HIV-1 infected PBMCs from healthy donors

hOCT1 is expressed in immune cells and can be up-regulated upon lymphocyte activation (Minuesa et al., 2008). HIV-1 infection has been shown to up-regulated the mRNA levels of genes encoding hCNT1, hCNT3 and hENT2 in adipose tissue (Guallar et al., 2007). Therefore, we wanted to determine whether *ex vivo* infection of PBMCs from healthy donors could modulate hOCT1 expression. For this, in collaboration with the laboratory of Dr Xavier Martinez-Picado (Retrovirology Laboratort, IRSI-Caixa), PBMCs were stimulated using two different methods: Interleukin-2 (IL2) or the combination of IL2 and phytohaemagglutinin (PHA) and subsequently infected with two strains of HIV-1 virus at two different concentrations. RNA was extracted from the cells and quantitative RT-PCR was performed. GAPDH expression was measured as endogenous control (Figure 4).

The results showed an up-regulation of hOCT1 mRNA expression levels when PBMCs where infected with the strain NL4-3 in a dose dependent manner following both types of stimulation. The other tested strain, NFN-SX, only up-regulated the expression of hOCT1 mRNA when the cells had been stimulated with the combination of IL2-PHA.

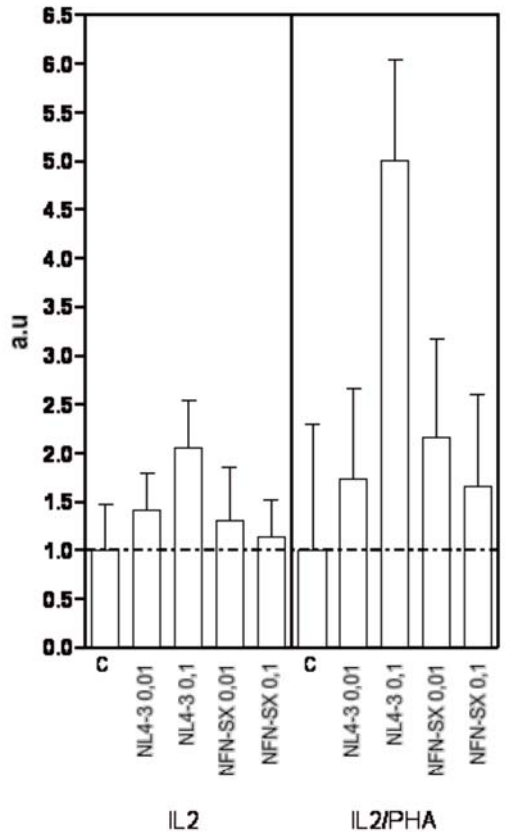


Figure 4. hOCT1 levels in PBMCs *ex vivo* infecetd with HIV-1. PBMCs from 4 healthy donors were in vitro stimulated (PHA/IL2 or IL-2) and subsequently infected for 4h with two concentrations of two tropisms of HIV-1 virus (NL4 or NFN-SX). Cells were pelleted and RNA was extracted. The graph shows the expression of hOCT1 in arbitrary units of each infection in reference to control cells, stimulated the same way but not infected.

1.5 Drug-drug interactions in the hOCT1 polymorphic variants

The management of HIV-1 infection usually includes the combination of multiple antiretroviral drugs. The fact that other NRTI antiviral drugs such as abacavir (ABC) have been shown to interact with hOCT1, despite not being translocated by the transporter (Minuesa et al., 2009) and that in the case of metformin, the polymorphic variant M420del has been determined to be more sensitive to drug-drug interactions than the wild type transporter (Ahlin et al., 2011) has raised the question of what would be the pharmacological relevance of these variants when facing possible drug-drug interactions phenomena.

To determine which antiretroviral drugs could be implicated in drug-drug interactions with 3TC, a panel of antiretroviral drugs belonging to different classes were studied. The tested drugs were abacavir (ABC), zidovudine (AZT), emtricitabine (FTC) and tenofovir diproxil fumarate (TDF) from the NRTIs group; efavirenz (EFV) from the NNRTI group and raltegravir (RAL) form the integrase-1 inhibitors group. A fixed concentration (500μM) of all these drugs and 10μM, 2μCi of 3TC were used to perform

these *cis-inhibition* studies using HEK293 cells stably expressing the hOCT protein (Figure 5).

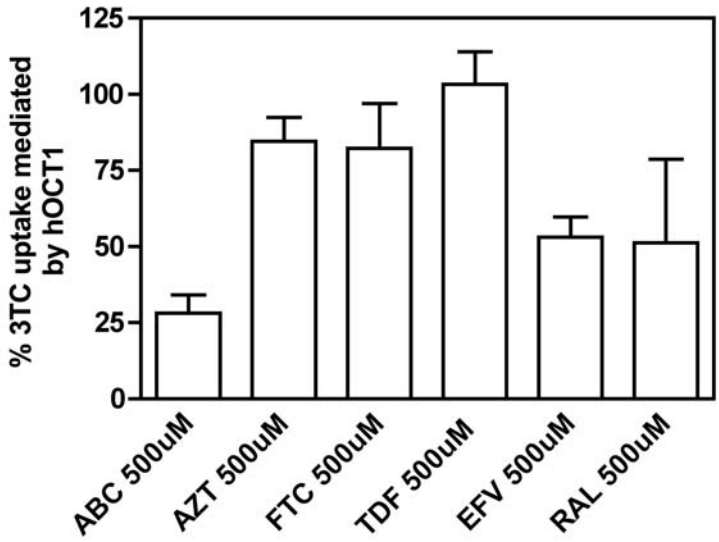


Figure 5. 3TC uptake inhibited by other antiretroviral drugs. Percentage of inhibition of 500µM antiretroviral drugs to the uptake of 10µM, 2µCi of 3TC mediated by hOCT1. The uptake was measured at 15s. The graph shows the mean ± S.E.M of three independent experiments.

Results depicted in figure 5, showed that ABC, EFV and RAL are the best inhibitors of 3TC uptake. Thus, we decided to pursue the study with these drugs. Moreover, we also decided to include AZT, because, although it could not inhibit the WT hOCT1 it was still possible that it could inhibit the uptake mediated by some of the polymorphic variants with impaired transport capacity. Only those variants for which *Km* and *Vmax* values could be calculated were used for the drug-drug interaction measurements. In order to make the study clinically relevant, the maximum drug concentrations used were derived from the peak drug levels found in blood (*Cmax*) shown in table 2.

Drug	Class	*Cmax* (µM)	Used concentration (µM)
3TC	**NRTI**	15,29	10
ABC	**NRTI**	13,48	13
AZT	**NRTI**	4,45	4
EFV	**NNRTI**	12,97	13
RAL	**InST1**	4,50	4

Table 2. *Cmax* (Jilek et al., 2012) **and concentration used of the antiretroviral drugs for the cis-inhibition studies.**

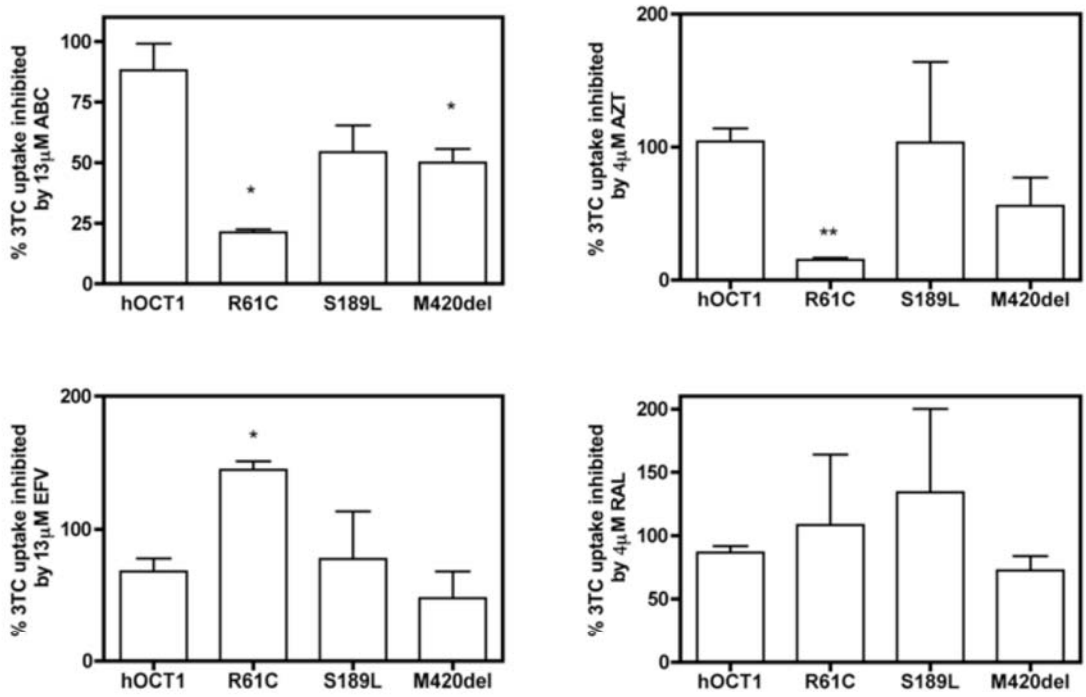


Figure 6. Cis-inhibition studies of 3TC with other antiretroviral drugs in hOCT1 polymorphic variants. 3TC (10μM, 2μCi) uptake was inhibited with a dose close to the *Cmax* value of other antiretroviral drugs in HEK93 stably expressing the WT transporter or one of its polymorphic variants. Graphs show the mean ± S.E.M of two-three single experiments.

C*is-inhibition* studies were performed using the HEK293 cells stably expressing either the wild type transporter or selected polymorphic variants. The results showed a clear difference among drug-drug interaction found for ABC, AZT and EFV. In the case of ABC and AZT, R61C and M420del variants were more sensitive to drug-drug interaction than the WT transporters. However, additional experiments appear to be necessary to determine whether difference found for AZT when studying the M420del variant are in fact significant. For EFV, the WT protein, S189L and M420del showed similar inhibition levels. However, the R61C variant, showed an increased uptake of 3TC in the presence of 13μM EFV. For RAL, the experiment needs to be repeated, because the standard error is too big to rule out the possibility that there is no difference in the inhibition profiles among the polymorphic variants (Figure 6).

3TC is used in therapy mainly in combination with ABC and AZT, the fact that R61C and M420del variants sowed a higher drug-drug interaction than the WT, highlights the

importance that these polymorphisms can have in drug bioavailability and also in the pharmacokinetics and pharmacodynamics of the drug not only when used in mono-therapy but also when it is administrated in drug combinations.

2

IDENTIFICATION OF HOCT1 AS A BENDAMUSTINE TRANSPORTER AND DETERMINATION OF THE ROLE ITS POLYMORPHIC VARIANTS PLAY IN ITS CYTOTOXICITY

2.1 Screening of bendamustine interaction with *SLC28* and *SLC29* transporters

Bendamustine is a purine analogue/alkylator hybrid currently used in the treatment of CLL (Leoni et al., 2008). Although it is widely used in clinics, the pathways facilitating the uptake of the drug into target cells remain poorly understood. Like all nucleoside-derived drugs, due to its hydrophilicity, it requires specific membrane transporters to be internalised. Due to its nucleobasic nature, nucleoside transporters were the first transporters to be tested as they were though to be good candidates for the internalization of the drug. Proteins from *SLC28* and *SLC29* families (encoding for hCNT and hENTS transporters) were screened for interaction with bendamustine. For this purpose, concentrative nucleoside transporter (hCNT) cDNAs were transiently transfected in HEK293 cells and interaction between the drug and the transporter was measured by *cis*-inhibition studies. In accordance with the transporter nucleoside selectivity, [^{3}H]cytidine (1μM, 1μCi) was used as a natural substrate for hCNT1 and hCNT3 transporters while [^{3}H]guanosine (1μM, 1μCi) was used for hCNT2. The natural nucleoside uptake was measured either in the presence or in the absence of 100μM bendamustine. For interaction studies with equilibrative nucleoside transporters (hENTS) endogenous hENTs in HEK293 cells were used and the same approach as for hCNTs was performed. In case that bendamustine could interact with any of the transporters, it would compete with the natural substrate, thus inhibiting its uptake and showing a clear reduction on the transport activity of the transporter.

In our study, no inhibition was observed in any case discarding nucleoside transporters as responsible for the uptake of bendamustine in the cells (Figure 1).

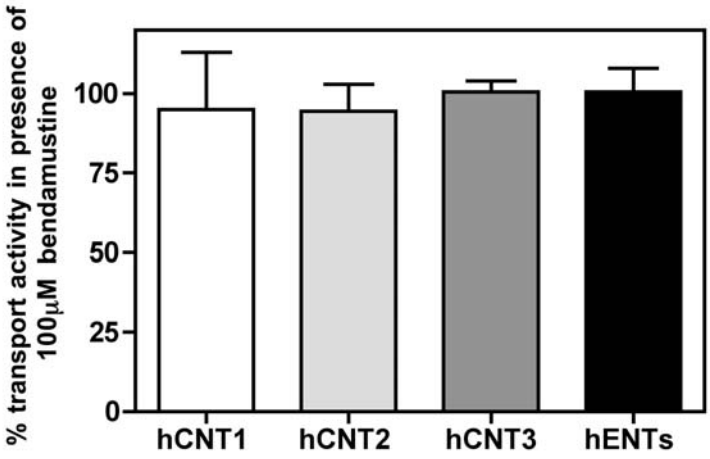


Figure 1. Bendamustine interaction with nucleoside transporters. Percentage of total transport activity mediated by hCNT1, hCNT2, hCNT3 and hENTs in presence of 100µM bendamustine, measured in HEK293 cells transiently transfected, expressing the concentrative transporters and measuring endogenous activity for hENTs (n=3±S.E.M.)

2.2 Role of human Organic Cation Transporter 1 (hOCT1) in the uptake of bendamustine

As mentioned before, hOCTs have also been described to mediate the uptake of selected nucleoside analogues (Errasti-Murugarren and Pastor-Anglada, 2010; Minuesa et al., 2009) and to be expressed in immune system and lymphoma cells (Gupta et al., 2012; Minuesa et al., 2008). Therefore, hOCT1 was also considered as a possible candidate to mediate bendamustine transport.

To study the ability of hOCT1 to interact with bendamustine, HEK293 cells stably expressing hOCT1 were used to perform *cis*-inhibition studies, using in this case MPP^+ as a model substrate. When 100µM bendamustine was added to the transport media, the uptake of [^{3}H]MPP^+ mediated by hOCT1 was significantly reduced ($p<0.05$). However, the same amount of bendamustine could not significantly inhibit the uptake mediated by hOCT1-M420del, one of the most frequent polymorphic variants (18.5% in Caucasian population (Shu 2003)) which has been shown to cause differences in substrate transportability (Figure 2).

For the hOCT1 wild type transporter and for the M420del polymorphic variant, drug-transporter interaction studies were performed to calculate the inhibition constants. *Ki* values for hOCT1 (138.1 µM±10.9) and the polymorphic variant M420del (233.6µM±.24.5) showed statistically significant changes ($p<0.05$) (Figure 2).

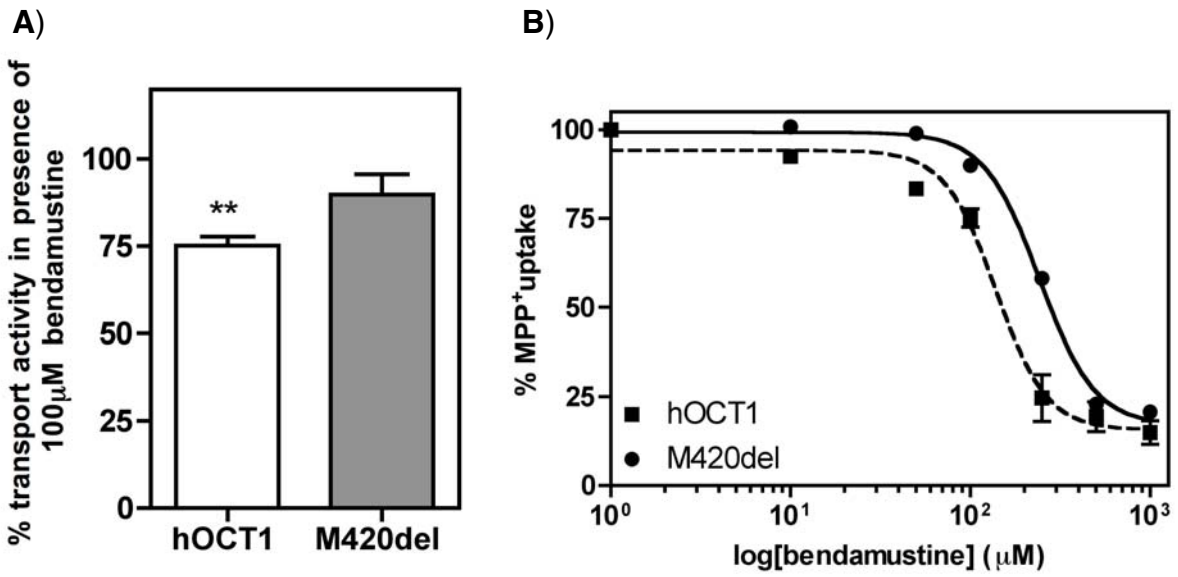


Figure 2. Bendamustine interaction with organic cation transporter 1. (A) Percentage of total transport mediated by hOCT1 and its polymorphic variant M420del in the presence of 100μM bendamustine measured in HEK293 stably expressing the transporters. **(B)** Interaction between bendamustine with either hOCT1 or the polymorphic variant M420del. Experimental data was fitted to a non-lineal curve (variable slope) and the *Ki* values were calculated. (n=4-5 ± S.E.M)

All those data showed that bendamustine could interact with the transporter but did not demonstrate that the drug could be internalised by hOCT1. Bendamustine is not available in any radiolabelled form, so direct uptake measurements, which could prove that bendamustine was an actual substrate of the transporter, or kinetic studies to calculate the *Km* and *Vmax* could not be performed. Therefore, to elucidate the relevance of the transporter in determining drug bioavailability, cytotoxicity experiments were carried out. HEK293 cells stably expressing either hOCT1 or an empty vector (pcDNA5) used as a control were incubated for 24h with increasing concentrations of bendamustine and 48h after removing the drug, cell viability was measured with MTT. The results showed a significant sensitisation of HEK293 cells to bendamustine when expressing hOCT1 (Figure 3). This result was consistent with the previous observation that bendamustine could inhibit the uptake of the model substrate probably by competition for the translocation site and confirmed that hOCT1 could mediate the uptake of bendamustine and also highlighted the role that this transporter may have in increasing sensitivity to bendamustine treatment *in vivo*.

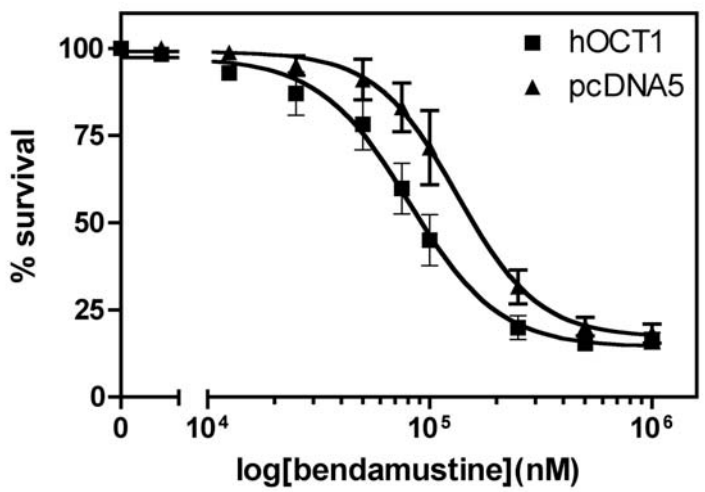


Figure 3. Bendamustine cytotoxicity in hOCT1-expressing and control HEK293 cells. Cell viability measured 48h after a 24h treatment with increasing concentrations of bendamustine. Data was fitted to a non-lineal curve (variable slope) and the EC_{50} values were calculated and showed to be significantly different (77 ± 11 for hOCT1-expressing HEK293 *versus* 155 ± 28 for control HEK293; $p<0.05$) in a T-student test (n=5 ± S.E.M)

2.3 Effect of hOCT1 polymorphic variants on bendamustine internalization

As commented before, *SLC22A1*, the gene encoding for hOCT1 is highly polymorphic and many of the described variants have a high allelic frequency in Caucasian population. We had access to the data generated for CLL within the framework of the International Cancer Genome Consortium (ICGC) and could calculate the allelic frequency of hOCT1 polymorphic variants in 233 sequenced Spanish CLL patients (Table 1). The values for the frequency of already described polymorphic variants turned out to be similar to those already published for Caucasians (Kerb et al., 2002; Shu et al., 2003). Some novel variants were identified, but their allelic frequency was so low that they could be rather the result of spontaneous mutations instead of polymorphic variants, so they were not considered for further characterisation.

Interestingly, all variants detected were mainly located at the ends of the transporter (exons 1-3 or 6-9).

Those polymorphic variants which have shown significant differences in drug uptake in previous studied drugs (Shu et al., 2007) (this thesis) were chosen to perform further cytotoxicity assays and determine the role they could have in sensitising HEK293 cells to bendamustine. R61C, S189L, M420del and G465R were all stably expressed in HEK293 cells, and the same cell viability measurements as previously described for the WT transporter and the control cells transfected with the empty vector were performed.

Exon	dbSNP	Nucleoside change	Amino acid change	Allele frequency CLL population (%)	Allele frequency European American population (%)
1	rs35888596	113G>A	G38D	1.1	n.d.
1	rs12208357	181C>T	R61C	5.6	7.2 [1]
1	rs55918055	262T>C	C88R	0.2	0.62 [2]
1		295G>A	A99T	0.2	n.d.
2	rs683369	480G>C	L160F	79.2	99.35 [1]
3		520G>A	G174S	0.4	n.d.
3	rs34104736	566C>T	S189L	0.4	0.5 [1]
6	rs2282143	1022C>T	P341L	1.7	0 [1]
7	rs34130495	1201G>A	G401S	2.6	1.1 [1]
7	rs628031	1222A>G	M408V	60.1	59.8 [1]
7	rs35167514	1257delATG	M420del	11.6	18.5 [1]
8	rs35956182	1320G>A	M440I	1.1	0 [1]
9	rs34059508	1393G>A	G465R	1.7	4 [1]

(1) (Shu et al., 2003) (2) (Kerb et al., 2002)

Table 1. hOCT1SNPs found in 233 CLL patients defined by rs number, nucleoside change and amino acid change.

The results showed two clear sensitisation profiles. Two of the studied polymorphic variants (R61C and M420del) showed an increase in the *EC50* values which was not significantly different from the wild type transporter (Figure 4A). Thus, they were classified as semi-functional. The other two studied variants (S189L and G465R) had *EC50* values significantly different from hOCT1 WT ($p>0.05$) but similar to control cells expressing the empty vector (Figure 4b). They were classified as non-functional variants. *EC50* values for all tested variants, the wild type transporter and control cells are shown in Table 2.

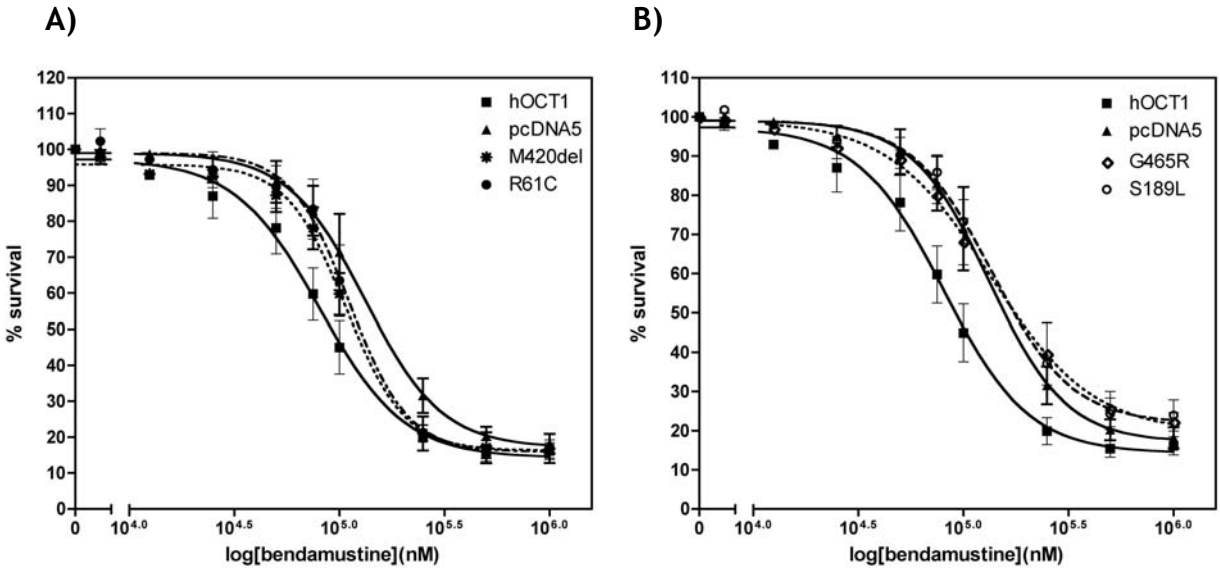


Figure 4. Bendamustine cytotoxic effect in hOCT1 polymorphic variants. (A) HEK293 stably expressing hOCT1 semi-functional polymorphic variants viability after bendamustine treatment compared to either hOCT1 or pcDNA5. **(B)** HEK293 stably expressing hOCT1 non-functional polymorphic variants viability after bendamustine treatment compared to hOCT1 or pcDNA5. (n=3-5 ± S.E.M)

	hOCT1	R61C	S189L	M420del	G465R	pcDNA5
EC50 (μM)	77 ± 11	104 ± 14	129 ± 10 *	90 ± 8	127 ± 14 *	155 ± 28 *

Table 2. Bendamustine EC_{50} values for HEK293 cells expressing either hOCT1, its polymorphic variants or the control vector. EC_{50} values calculated by fitting the results to a non-lineal curve (variable slope). Unpaired t-student test were performed to compare hOCT1 with the variants or the empty vector. (*, $p<0.05$) (n=3-5 ± S.E.M)

Cytotoxicity was also measured in collaboration with the group of Dr. Dolors Colomer in genotyped CLL patient cells *ex vivo*. CLL cells were incubated for 24h with 25μM bendamustine and after that the cytotoxic effect of the drug was measured. The expression of hOCT1 mRNA was correlated with the cytotoxic effect of bendamustine in the CLL cells but no lineal-regression was detected between the amount of hOCT1 mRNA expressed and the cytotoxicity of the drug. To further discard any relation between the expression and the effect, patients in which bendamustine could kill more than 35% of the cells were considered sensitive while those with less than 35% effect

were considered resistant. There was neither any relation between the expression and the resistance to the treatment. (Figure 5)

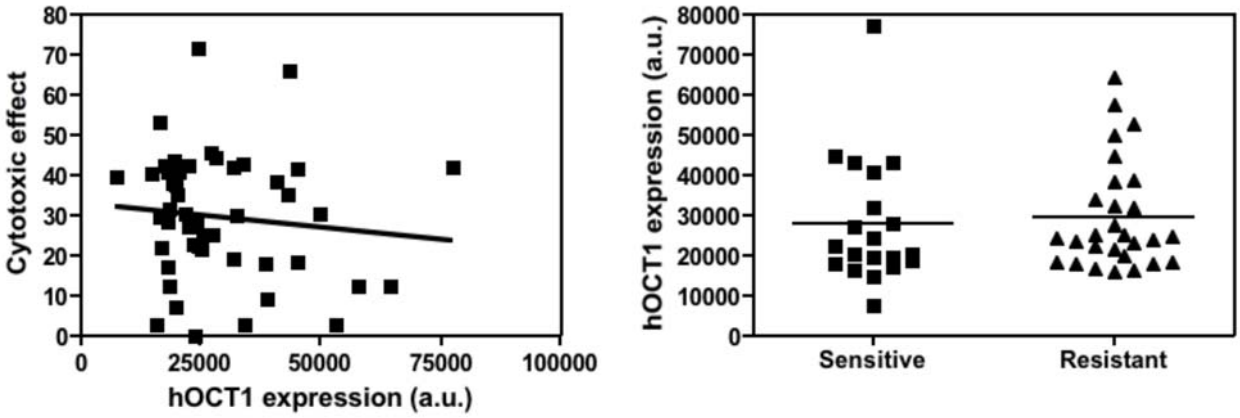


Figure 5. Relation between hOCT1 mRNA expression and bendamustine effect in CLL cells. Lineal-regression between bendamustine cytotoxic effect and hOCT1 mRNA expression (left) and hOCT1 mRNA expression of sensitive and resistant patients. The cut-off value to determine sensitive and resistance patients was situated at 35%.

No relation could be found between the mRNA expression of hOCT1 and the effect triggered by bendamustine. However, the role that the presence of polymorphic variants in the CLL patients cells played in determining the cytotoxic effect had yet to be evaluated. For this, patients were classified into three groups depending on their genotype. hOCT1-L160F and hOCT1-M408V polymorphic variants, had been studied before for metformin by the group of Dr. Giacomini (Shu et al., 2007) and for 3TC by our own group (this thesis) and they have always shown similar transport efficiency to the WT ransporter, and so, these variants were not included in the drug-transporter interaction studies. All patients carrying them, were considered to express functional transporters. Heterozygous patients for M420del or R61C polymorphic variants were included in the semi-functional group and heterozygous patients for S189L or G465R, in the non-functional group. Patients with other polymorphic variants not characterised in this study were not included.

When we classified the patients in each group in either resistant or sensitive to bendamustine, only half of the patients in the functional group were resistant whereas a higher number of resistant patients were found in the semi-functional and non-functional group (51.6% versus 72.2% and 66.7%)

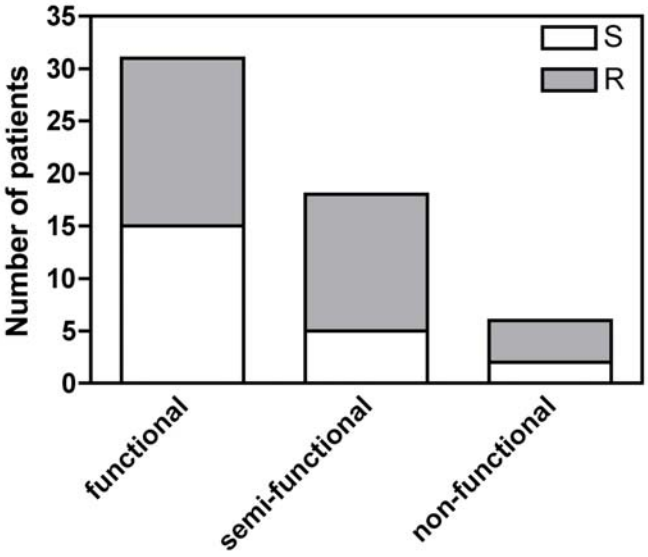


Figure 5. Polymorphic variants function and bendamustine resistance or sensitiveness. Number of resistant (R) and sensitive (S) patients classified by the hOCT1 variant they express. Cytotoxicity higher than 35% was considered sensitive.

In general, hOCT1 expression seems to correlate with the cytotoxic effect of bendamustine in accordance with the drug interaction found with the hOCT1 wild type transporter. Thus, we can consider hOCT1 as a suitable bendamustine transporter. The fact that it does not correlate with the mRNA levels *in vitro* does not discard the possibility that it could correlate with the protein levels not measured in this thesis.

In summary, hOCT1 is a highly polymorphic protein and some of the polymorphisms present in caucasian CLL population can modulate the cytotoxic efficiency of the drug and cause more resistance to the treatment *ex vivo* highlights the importance that the presence of polymorphic variants in CLL patients can have in bendamustine bioavailability and action.

3

DETERMINATION OF TRANSPORTERS MEDIATING DNMT INHIBITORS TRANSLOCATION THROUGH THE PLASMA MEMBRANE

3.1 Interaction of DNMT inhibitors with nucleoside transporters

DNMT inhibitors are cytidine analogues used in the treatment of myelodysplastic syndrome. To be internalised and act as inhibitors of the intracellular DNA methyltransferase they require specific membrane transporters. They only bear minor changes compared to the natural nucleoside cytidine in their molecular structures (Figure 1), so the candidate transporters to mediate their internalization are those which are able to transport the natural nucleoside cytidine, ruling out, hCNT2 because of its well known purine-selectivity (Errasti-Murugarren and Pastor-Anglada, 2010; Pastor-Anglada et al., 2008).

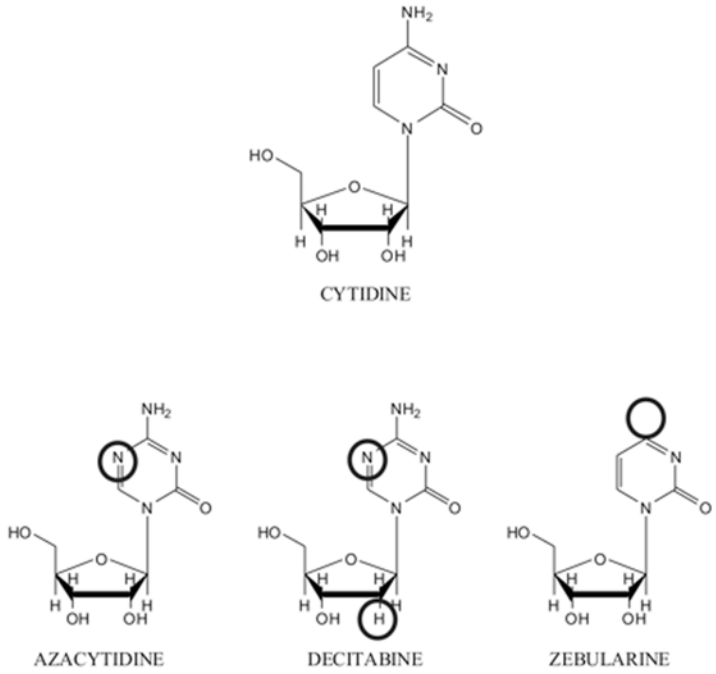


Figure 1. Structure of cytidine and cytidine-analogues inhibitors of DNA methyltransferases. The differences between the cytidine analogues and the natural nucleoside are highlighted.

To study the ability of nucleoside-derived DNMT inhibitors to interact with nucleoside transporters, we assayed the effect of increasing concentrations of these drugs on the uptake of 1μM [^{3}H]uridine in either hCNT1 or hCNT3-transfected HeLa cells. The same approach was used for the endogenously expressed equilibrative nucleoside transporters, hENT1 and hENT2. hENT1- and hENT2-related activities were discriminated by using specific inhibitors (1μM NBTI for hENT1 and 10 μM dypiridamole for both, hENT1 and hENT2). The K_i values calculated from the drug concentration-dependent inhibition of uridine uptake are listed in Table 1. *Ki* values for the interaction of DNMT inhibitors with hCNTs and hENT2 were in the low-medium micromolar range, whereas interaction with hENT1 was comparatively poor, being the *Ki* values in the mM range, particularly for decitabine and zebularine.

Ki (μM)	hENT1	hENT2	hCNT1	hCNT3
Azacitidine	379.5 ± 3.5	25.0 ± 0.9	11.4 ± 1.6	3.5 ± 1.2
Decitabine	1000-5000	5.6 ± 0.5	21.6 ± 3.0	14.4 ± 4.6
Zebularine	≈ 1000	22.6 ± 6.1	31.3 ± 5.0	4.6 ± 0.5

Table 1. Inhibitor constants of DNMT inhibitors for nucleoside transporters. *Ki* values for inhibition of [^{3}H]uridine uptake by DNA methyltransferase inhibitors in HeLa cells transiently transfected with either hCNT1 or hCNT3 encoding cDNAs. Drug interactions with hENT1 and hENT2 were determined based upon the endogenous activities present in HeLa cells. *Ki* values were obtained by fitting data by non-linear regression in Graph Pad prism 4.0 software. Data are shown as *Ki* values ± S.E.M. from 2-3 independent experiments.

3.2 Role of nucleoside transporters in the uptake of DNMT inhibitors

To check whether the nucleoside-derived DNMT inhibitors were substrates of the hCNT-type transporters, direct uptake measurements using radiolabeled drugs were performed. Because azacitidine is not commercially available in any radiolabeled form the study was restricted to decitabine and zebularine. Nevertheless, azacitidine is the only azanucleoside for which transport properties by hCNT1 and hCNT3 had been

determined earlier using a radiolabeled form that had been synthesized by the authors (Rius et al., 2010; Rius et al., 2009). [^{3}H]Decitabine and [^{3}H]zebularine uptake was measured in transiently transfected HeLa cells. The results, shown in Figure 2, confirmed that zebularine was a suitable substrate for both hCNTs, which is consistent with its low *Ki* value obtained from the *cis*-inhibition experiments (Table 1). Zebularine uptake rates via hCNT1 and hCNT3 were significantly higher than those of the natural hCNT substrate uridine, when used at the same concentrations (Figure 2). A sodium-independent component of transport was also observed for zebularine which corresponds to the sum of the hENT1- and hENT2-related activities (Figure 2). Interestingly, decitabine was not internalized by either hCNT1 or hCNT3 although it showed high affinity interaction with hCNT1, hCNT3 and hENT2 (Figure 2 and Table 1). The three molecules retain the 3' hydroxyl group which is known to be essential for high affinity interaction with nucleoside transporters (Cano-Soldado et al., 2011; Cano-Soldado and Pastor-Anglada, 2012; Chang et al., 2004), but not necessarily for substrate translocation. However, the only difference between azacitidine and decitabine is the 2'-hydroxyl group. The fact that decitabine did interact with all these nucleoside transporters but could not efficiently be transporter suggests that the 2' hydroxyl group plays a key role in determining substrate transportability in these cytidine analogues.

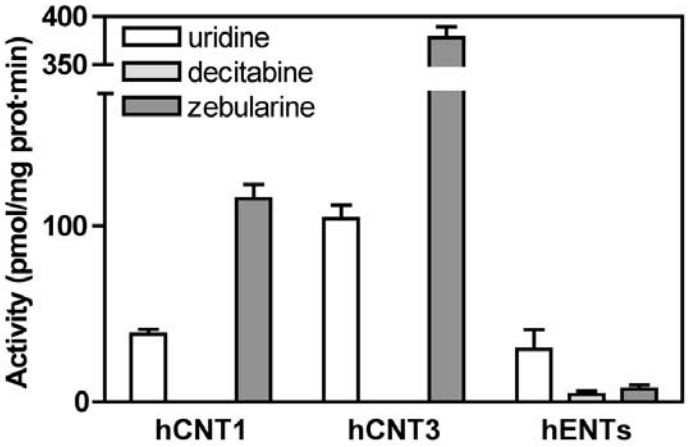


Figure 2. DNMT inhibitors and uridine uptake mediated by nucleoside transporters. Sodium-dependent uptake of [^{3}H]-labeled nucleoside-derived drugs and uridine (1μM, 1min) by either hCNT1 or hCNT3 in HeLa transiently transfected cells. For equilibrative transport, the endogenous activity was measured in HeLa cells using sodium-depleted medium. (n=3 ± S.E.M)

To determine the relevance of hCNT1 and hCNT3 in assessing intracellular drug bioavailability and action, cell proliferation studies in the presence of these drugs were performed. The experiments were initially done by incubating the cells in the presence of a particular drug for 24h, 48h and 72h. The 48h time point was chosen because 24h treatments did not result in maximum cytotoxicity, whereas the 72h time point yielded similar results to those obtained after incubating the cells for 48h. Results are shown in Figure 3 and the EC_{50} values derived from these curves listed in Table 2. Independently of expression of hCNT1 and hCNT3 decitabine showed very low toxicity. The EC_{50} values could not be properly calculated but were in the high micromolar/ low milimolar range. Azacitidine and zebularine which are internalized by hCNT1 and hCNT3 proteins induced cytotoxicity at lower concentrations in those cells expressing the transporters compared to pcDNA3.1-transfected cells (Figures 3). The evidence that the highest drug concentrations used did not apparently reach maximal toxicity (100%) can be explained by the fact that these DNMT inhibitors are likely to induce cell cycle arrest rather than apoptosis. Transporter mediated sensitisation to both analogues resulted in significant decreases in the EC_{50} values (Table 2). For zebularine these effects were more pronounced. Expression of hCNT3 seemed to induce greater sensitivity to both azacitidine and zebularine compared to hCNT1. Overall, the effect of transporter expression on cell sensitivity of the DNMT inhibitors was consistent with the interaction and transport-kinetic values reported in the previous experiments.

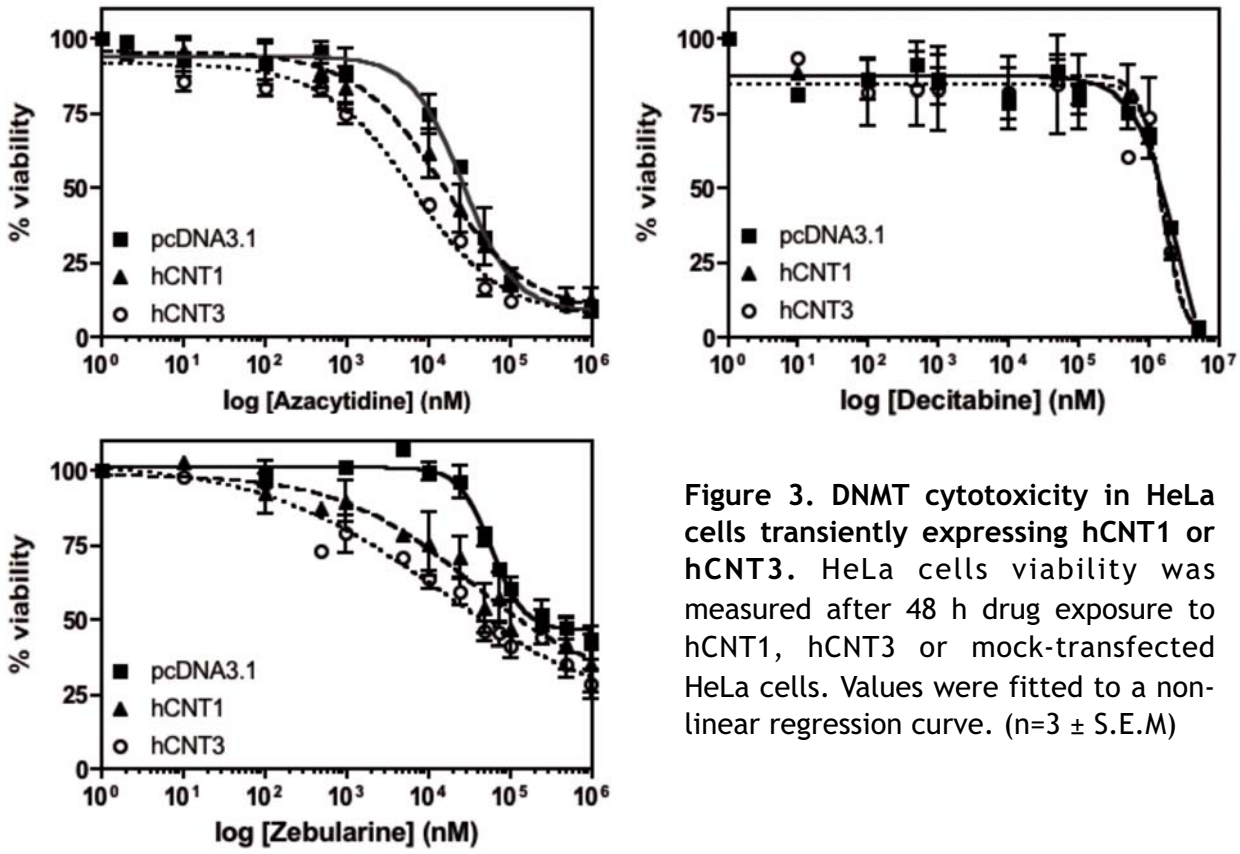


Figure 3. DNMT cytotoxicity in HeLa cells transiently expressing hCNT1 or hCNT3. HeLa cells viability was measured after 48 h drug exposure to hCNT1, hCNT3 or mock-transfected HeLa cells. Values were fitted to a non-linear regression curve. (n=3 ± S.E.M)

EC_{50} (μM) 48h	pcDNA3.1	hCNT1	hCNT3
Azacitidine	29.5 ± 2	13.6 ± 0.7 **	6.8 ± 0.7 ***
Decitabine	>1000	>1000	>1000
Zebularine	62.0 ± 5.9	18.5 ± 6.6 **	8.9 ± 2.0 ***

Table 2. *EC_{50}* values for DNA methyltransferase inhibitors. EC_{50} values were calculated by fitting data by non-linear regression in Graph Pad prism 4.0 software. Data are expressed as the mean ± S.E.M of at least three independent experiments performed on different days using different passages of cells. Unpaired t-student test was performed comparing the EC_{50} value for mock-transfected cells and hCNT1, hCNT3. (**, <0.01; ***, <0.001)

Heterologous expression of hCNT1 and hCNT3 in HeLa cells resulted in a significant sensitisation of cells to azacitidine and zebularine, whereas no matter these

transporters were present or not, cells remained resistant to decitabine, due to its lack of permeability via NT-type transporter proteins. The highest sensitisation of cells to azacitidine and zebularine associated with hCNT3 expression is consistent with the observed high-affinity interaction of both drugs with this transporter protein and with the fact that hCNT3 is the only member within the *SLC28* gene family able to translocate 2 Na^+ per nucleoside (Cano-Soldado and Pastor-Anglada, 2012; Errasti-Murugarren and Pastor-Anglada, 2010; Minuesa et al., 2011). This would explain a probable higher hCNT3-driven capacity to concentrate these two cytidine analogues within the cells, than that associated with hCNT1 expression. Moreover, the finding that decitabine cannot be translocated via these transporter proteins, but can inhibit them with high affinity, suggests that, if combined therapies implicating hCNT-related substrates would have to be used, undesired drug-drug interactions may occur. This possibility might be also relevant to the pharmacokinetics of these DNMT inhibitors

3.3 Role of nucleoside transporters in determining the vectorial flux of DNMT inhibitors across epithelial barriers

The impact of the apical insertion of hCNT type proteins on the trans-epithelial fluxes of DNMT inhibitors was assessed on polarised MDCK cells grown on transwell inserts, as previously reported (Errasti-Murugarren et al., 2007; Pastor-Anglada et al., 2008). Apical-to-basolateral and basolateral-to-apical fluxes of the DNMT inhibitors were measured. For decitabine, no sodium-dependent flux was observed in either direction (Figure 4A). This is consistent with its lack of transport by hCNT1 and hCNT3 (Figure 2A). Additionally, decitabine sodium-independent fluxes in both directions, mediated by endogenous equilibrative nucleoside transporters, were smaller than those observed for cytidine (data not shown). Cytidine was used as a control of hCNT-type protein proper expression, hCNT correct apical insertion had previously been verified by immunohistochemistry (Errasti-Murugarren et al., 2007). For zebularine, a clear apical-to-basal sodium-dependent flux was detected after expression of either hCNT1 or hCNT3 (Figure 4B). Nevertheless, the magnitude of the apical-to-basolateral zebularine flux in

the presence of hCNT3 was nearly 4-fold greater than that observed when hCNT1 was expressed. Basal-to-apical flux of zebularine after transfection of either hCNT1 or hCNT3 in presence of sodium was much lower compared to apical-to-basal flux (Figure 4B), thereby demonstrating that apical insertion of these transporters do contribute to drug vectorial flux across this epithelial barrier. As previously reported for other nucleoside analogues (Errasti-Murugarren et al., 2007), the basal to apical flux of zebularine, alhough much lower than that found in the apical to basal direction, was even lower in the presence of sodium than when fluxes were measured in the choline chloride medium. This can be explained by the fact that hCNT3 proteins at the apical side of the barrier are highly efficient in removing the drug from the apical compartment, thereby reducing the net basal to apical flux. In fact, this had been previously demonstrated by blocking the apical hCNT3 function with phloridzin (Errasti-Murugarren et al., 2007).

Drug cellular accumulations were measured 20 minutes after addition of the DMNT inhibitors under the same conditions used for the analysis of vectorial fluxes (Figures 4C and D). As expected, accumulation of decitabine was negligible (around 1pmol/mg protein/20min) and totally independent of the presence of sodium in either compartment (Figure 4C). Zebularine accumulated within hCNT1- and hCNT3-MDCK expressing cells more than decitabine (Figure 4D). The highest intracellular accumulation of zebularine was observed, as expected, in the presence of sodium, when the drug was added to the apical compartment, where hCNT-type proteins are expressed.

It is known that hCNT and hENT expression in (re)absorptive epithelia is asymmetric, thereby facilitating vectorial flux of substrates across these barriers (Govindarajan et al., 2007; Govindarajan et al., 2008; Mangravite et al., 2003a). As for other nucleoside analogues used in anticancer and antiviral therapies (Errasti-Murugarren et al., 2007), the apical expression of hCNT-type transporters in polarised epithelia (in this case polarised MDCK cells grown on transwell inserts) determined the apical-to-basal vectorial flux of zebularine.

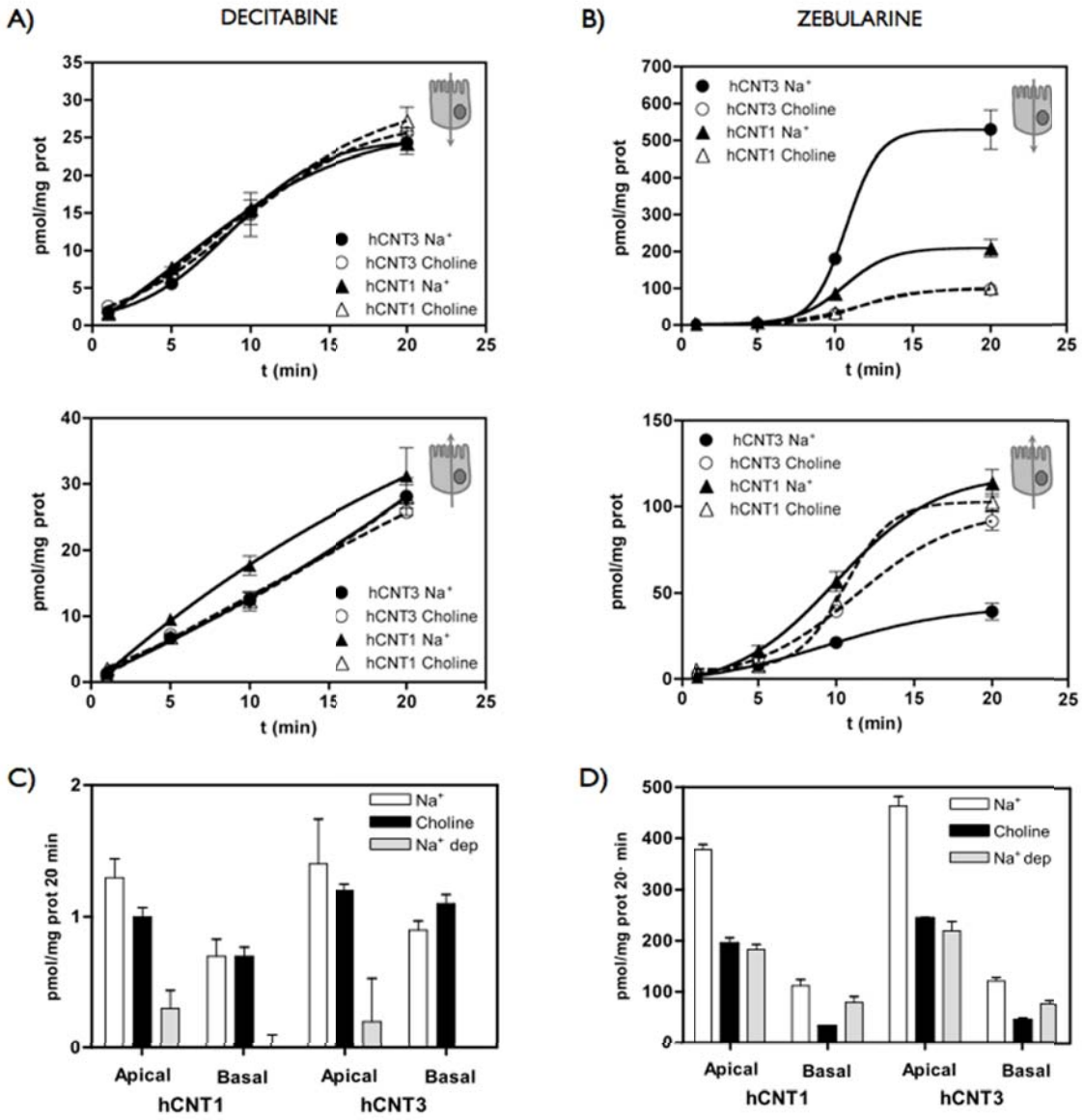


Figure 4. Transepithelial flux and cellular accumulation of DNMT inhibitors in hCNT1 or hCNT3 transfected MDCK cells grown as monolayers. Trans-epithelial fluxes of [^{3}H]decitabine (**A**) and [^{3}H]zebularine (**B**) (1 μM, 2 μCi) in sodium and choline chloride medium from apical to basolateral compartments (upper panel) and from basolateral to apical compartments (lower panel) are shown. The MDCK cells transfected with hCNT1 or hCNT3 were grown on filters. Accumulation of decitabine (**C**) and zebularine (**D**) after 20 min incubation with radioactive DNMT inhibitors added to the apical or basal compartments. The measurements were performed in sodium and choline media and the differences between sodium-containing and sodium-free corresponding to the hCNT activities, are indicated as sodium dependent (Na^+-dep) accumulation. Data are expressed as the mean ± S.E.M of uptake values obtained in 3 wells. (n=3)

3.4 Role of organic cation transporters uptake of DNMT inhibitors

As for bendamustine, we investigated whether human organic cation transporters hOCT1 and hOCT2 transported decitabine and/or zebularine. HeLa cells transiently transfected with either hOCT1 or hOCT2 showed a 40-fold higher accumulation of the hOCT model substrate [^{3}H]MPP$^+$ than control cells (transfected with the empty vector) (47.3±1.1 and 45.5±5 versus 7.5±1.1). This resulted in similar net hOCT1 and hOCT2 MPP + mediated uptake rates (Figure 4A), which is consistent with both transporter proteins being similarly expressed and properly inserted at the plasma membrane of HeLa cells. hOCT-mediated [^{3}H]decitabine was low (hOCT2) or even negligible (hOCT1). Surprisingly, the heterologous expression of hOCT1 and hOCT2 resulted in reduced accumulation of [^{3}H]zebularine compared to control cells (Figure 5). This observation could be interpreted on the basis that the transporter rather than mediating the uptake of zebularine facilitates its release from cells. When these experiments were done in the presence of 100μM uridine, which can inhibit nucleoside transporters, no differences were observed between hOCT-expressing and non-expressing cells and no zebularine accumulated at all. (Figure 6)

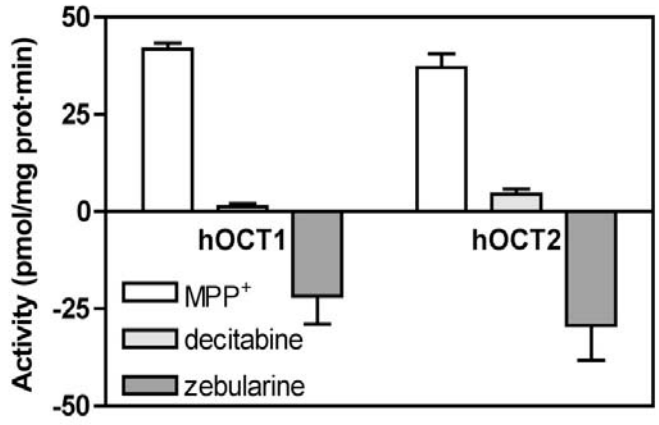


Figure 5. hOCT1-mediated accumulation in HeLa cells. Difference between the accumulation in hOCT expressing cells and the accumulation in mock-transfected cells after 1 min uptake measurement. (n=3±S.E.M)

In fact, HeLa cells express significant basal hENT-related transport activities (i.e. 43±2.5 and 11±2.5pmol uridine/min·mg protein via hENT1 and hENT2, respectively, n=4, mean+/-S.E.M.), thereby suggesting that zebularine is preferentially taken into hOCT-expressing cells via hENT type transporters, although once inside cells it is rapidly released via hOCTs (Figure 6)

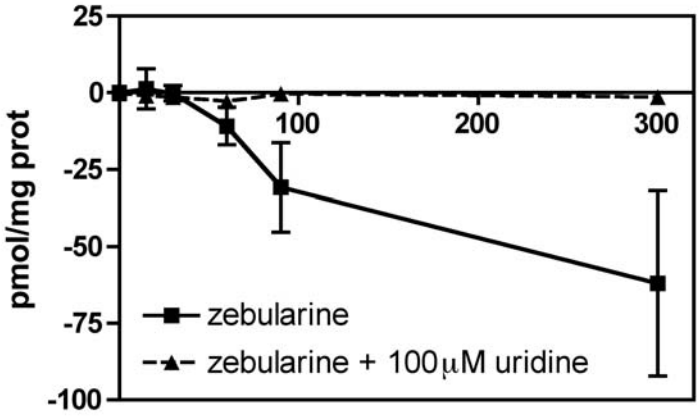


Figure 6. Zebularine time dependent accumulation in hibiting nucleoside transporters activity. Time course of hOCT1-mediated efflux of zebularine in the presence or absence of 100μM uridine in transiently transfected HeLa cells. The graph is a representative graph of two single experiments performed in quadruplicates

Since the study of the substrate efflux properties of transporters in mammalian cells is challenging, we decided to corroborate the role of hOCTs in zebularine efflux using *Xenopus laevis* oocytes, a much cleaner system than HeLa cells. While hOCT1 and hOCT2, as expected, promoted a significant uptake of radiolabeled MPP^+ into oocytes, zebularine accumulation into hOCT1 and hOCT2 expressing cells was significantly reduced when compared to non-injected oocytes (Figure 7). This mimics the results using a human cell background (HeLa cells) (Figure 5).

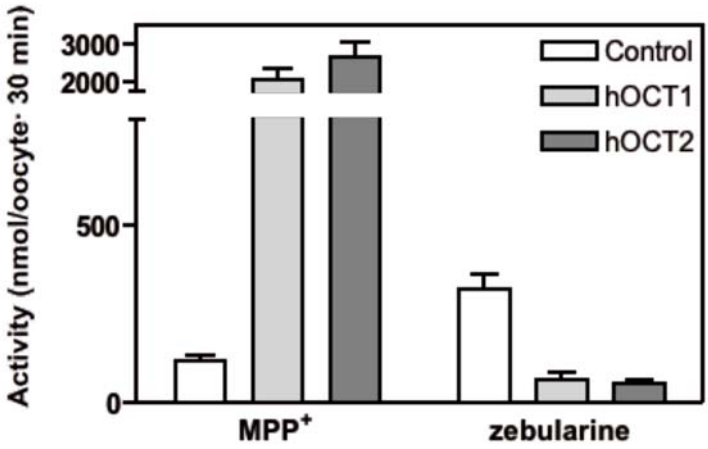


Figure 7. Zebularine accumulation in oocytes. [^{3}H] MPP^+ (10 nM, 1 μCi) and [^{3}H] zebularine (100 nM, 1 μCi) accumulation in oocytes after 30 min drug exposure. Data shown as mean ± S.E.M of 20 single oocytes from two different batches.

To definitely validate that both hOCT1 and hOCT2 were responsible for zebularine efflux, the radiolabeled drug was injected into oocytes expressing either hOCT1 or hOCT2 and control oocytes as well, and the release of [^{3}H]zebularine was measured at different time points in Ori buffer either containing or not 100μM quinine, a well-known hOCT inhibitor (Koepsell et al., 2007). A large efflux of zebularine was observed only in hOCT1/2-injected oocytes, whereas efflux from control-oocytes was minimal. Of note, hOCT1 and hOCT2 mediated zebularine efflux was completely blocked by quinine (Figure 8).

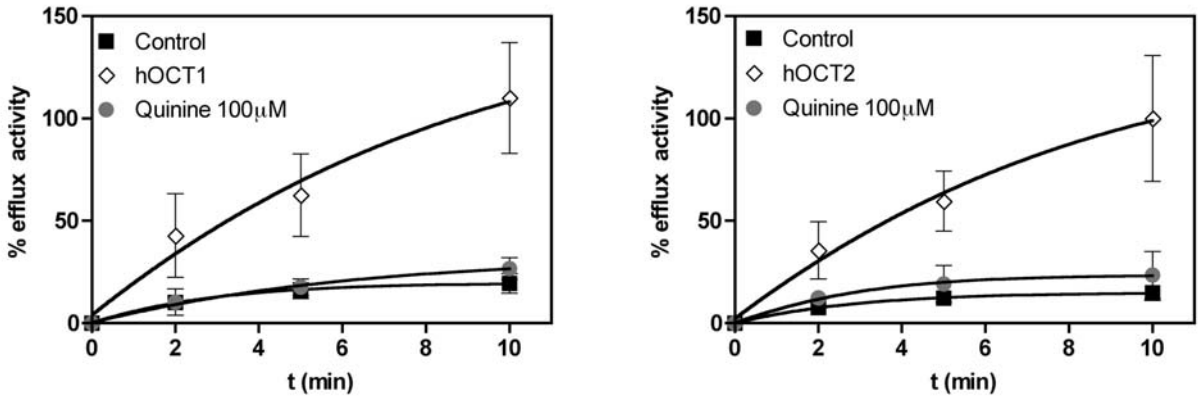


Figure 8. Zebularine efflux in oocytes. Efflux of [^{3}H]zebularine from previously injected (0.013 μCi, 26.8 nM) control oocytes in ORI buffer or oocytes expressing hOCT1 (**left**) or hOCT2 (**right**) in ORI buffer or in ORI buffer containing quinine 100 μM. All values have been calculated considering the maximum efflux 100%. Data are representative of four experiments carried out on different days using different batches of oocytes.

3.5 Effect of hOCT1 polymorphic variants on zebularine efflux

As mentioned above, the gene encoding the hOCT1 protein, *SLC22A1,* is highly polymorphic and several of its genetic variants show relatively high allelic frequency in humans (Errasti-Murugarren and Pastor-Anglada, 2010; Shu et al., 2003). The functional consequences on zebularine efflux of some of the most frequent polymorphic variants found in Caucasian population were assessed. Site-directed mutagenesis of the wild-type

transporter allowed the generation of the following variants: hOCT1-C88R, hOCT1-M408V, hOCT1-M420del and hOCT1-G465R. These cDNAs were transcribed *in vitro* and cRNAs injected into oocytes to allow transporter protein synthesis and efflux measurements. Efflux was determined by quantifying the accumulation of the radiolabeled substrate in the Ori buffer 10 minutes after injection of the hOCT substrates inside the oocytes. The efflux profile for MPP^+ was very similar to that of its uptake (Figure 9 and (Shu et al., 2003)). In the case of zebularine, all tested polymorphic variants promoted a significantly lower efflux than the wild-type transporter (Figure 9).

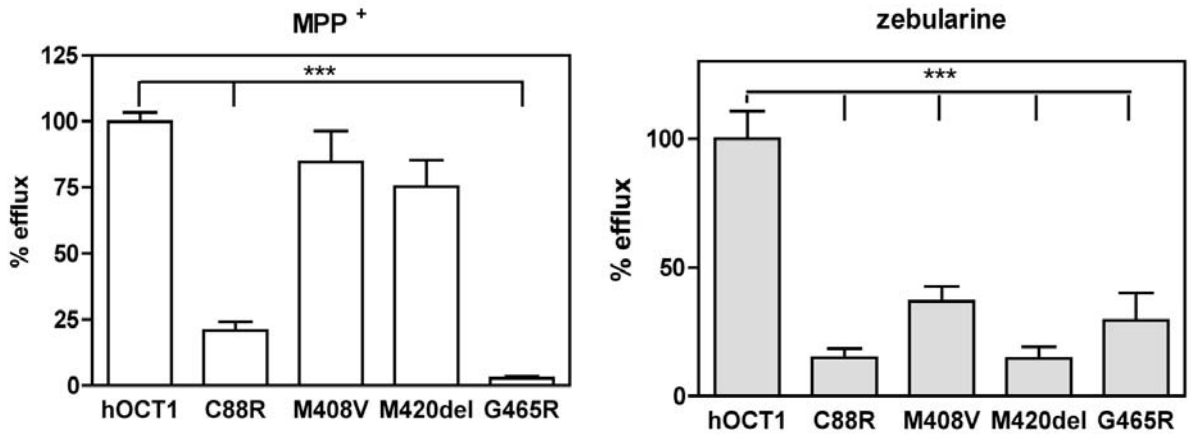


Figure 9. MPP^+ and zebularine efflux mediated by hOCT1 polymorphic variants. [^{3}H] MPP^+ (0.025 µCi, 6 nM) and [^{3}H]zebularine (0.013 µCi, 26.8 nM) efflux measured in 15 single oocytes from three different experiments performed at different days with different batches of oocytes, injected with the cRNA of hOCT1, hOCT1C88R, hOCT1M408V, hOCT1M420del, hOCT1G465R. hOCT1 all values were expressed as percentage of maximal efflux, which was set at a value of 100%.

To determine whether these efflux differences could play a role in the bioavailability and action of zebularine, cytotoxicity assays were performed. HEK293 stably-transfected cell lines expressing either the wild type transporter or the hOCT1M420del or hOCT1G465R variants were treated for 24h with zebularine. Then, they were washed and kept for 48 additional hours before zebularine induced toxicity was quantified. Data were fitted to non-linear regression analysis using GraphPad Prism 4.0 software and the EC_{50} values were calculated. These values were 256µM ±42; 116µM

±31 and 133µM±27 for hOCT1WT, hOCT1-M420Del and hOCT1-G465R, respectively (Figure 10). A unpaired t-student test showed a significant difference ($p<0.05$) between hOCT1WT EC_{50} value and those obtained when expressing both polymorphic variants.

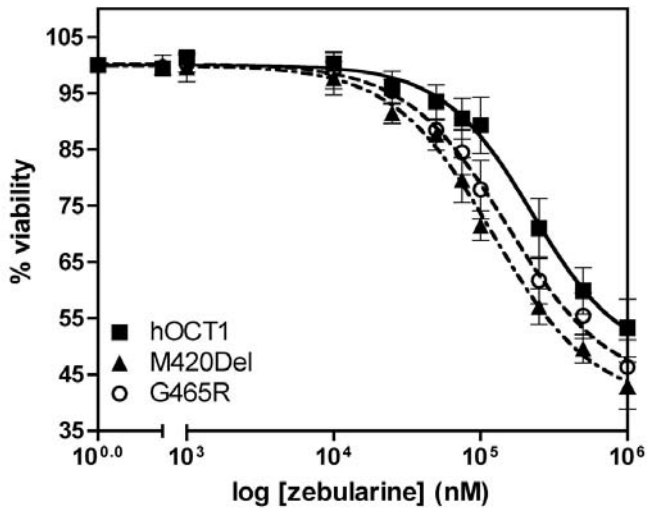


Figure 10. hOCT1 variants effect on zebularine cytotoxicity. Cytotoxicity assay in stable HEK293 cell line expressing WT hOCT1 and its polymorphic variants measured after 24h of drug treatment followed by 48h. Data shown is the average of three single experiments performed on different days with different batches of cells.

In summary, this study outlines the scenario for the transport phenomena determining the intracellular levels of DNMT inhibitors in cancer cells. Immune system cells do express the combination of transporter proteins (hENT1, hENT2, hCNT3 and hOCT1) here reported to interact with these inhibitors in a differential manner (see Figure 10). Unexpectedly, interaction with hOCT1 was shown to be variable depending on the substrate used but, more importantly, asymmetry in the interaction could confer this transporter protein a novel role in target cells as contributor to chemoresistance, thereby changing the classical view of how selected polymorphic variants of hOCT1 can determine drug bioavailability and action. DNMT inhibitors are used with variable results in highly heterogeneous myelodysplastic syndromes, for which patterns of drug transporter expression are not yet known. This study strongly supports the need for the analysis of drug transporter profiles in target cells, and eventually genotyping for hOCT1 polymorphisms, when dealing with DNMT inhibitors based therapies.

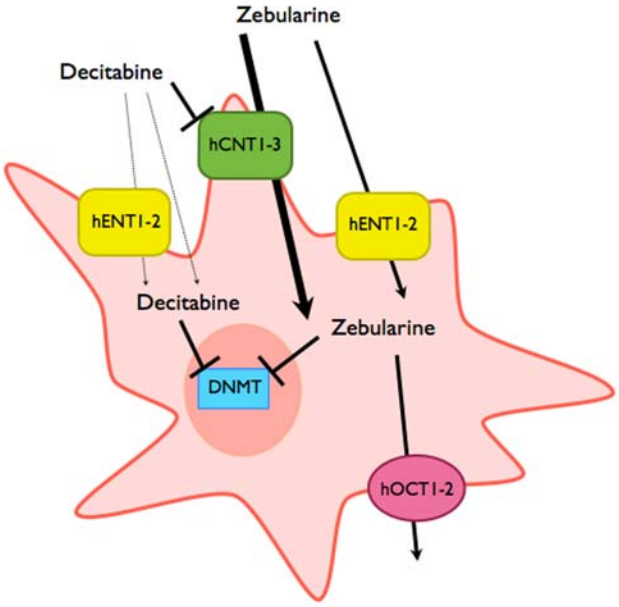


Figure 11. Theoretical model showing the role of nucleoside transporters and organic cation transporters in DNA methyltransferase internalisation and efflux.

4

hCNT3 MODELLING

Protein modelling is essential for an easy and clear understanding of how point mutations or polymorphic variants affect protein function. In case of membrane transporters, few crystals have been obtained so far, so generating good topology models has been challenging. The elucidation of CNT structures is a crucial step for a better nucleoside-derived drug design as well as for the understanding of the role polymorphic variants might have in drug interaction and transportability. Since recently, no nucleoside transporters or related proteins had been crystallised, so obtaining a good model for any of the transporters in this family was almost impossible because homology with better known transporters is very low. The crystallisation of the concentrative nucleoside transporter of *Vibrio cholerae* (Johnson et al., 2012) opened the opportunity to generate the first human concentrative nucleoside transporter protein model. This has been done preliminarily in collaboration with the bioinformatics group of Dr. Xavi Barril and Dr Axel Bidon-Chanal. The fact that the *Vibrio cholerae* nucleoside transporter is 39% homologue to hCNT3 and that almost all sodium binding amino acids and substrate binding amino acids determined for the *V. cholerae* transporter are conserved not only in hCNT3 but also in the other hCNT family members makes it a good template to model human CNT-proteins (Figure 1).

```
hCNT1           AFLGLVLWLSLDTSQR-PEQLVSFAGICVFVALLFACSKHHCAVSWRAVSWGLGLQFVLG 218
hCNT2           SLVGLILWLALDTAQR-PEQLIPFAGICMFILILFACSKHHSAVSWRTVFSGLGLQFVFG 213
hCNT3           LVLAVIFWLAFDTAKLGQQQLVSFGGLIMYIVLLFLFSKYPTRVYWRPVLWGIGLQFLLG 240
vcCNT           -----------------MSLFMSLIGMAVLLGIALLLSSNRKAINLRTVGGAFAIQFSLG 43
                                  . ::.: *: : : : :  *.    :  *.*  .:.:** :*

hCNT1           LLVIRTEPGFIAFEWLGEQIRIFLSYTKAGSSFVFG--------EALVKD--VFAFQVLP 268
hCNT2           ILVIRTDLGYTVFQWLGEQVQIFLNYTVAGSSFVFG--------DTLVKD--VFAFQALP 263
hCNT3           LLILRTDPGFIAFDWLGRQVQTFLEYTDAGASFVFG--------EKYKDH--FFAFKVLP 290
vcCNT           AFILYVPWGQELLRGFSDAVSNVINYGNDGTSFLFGGLVSGKMFEVFGGGGFIFAFRVLP 103
                 ::: .  *   :  :.  :  .:.*   *:**:**        :       .***:.**

hCNT1           IIVFFSCVISVLYHVGLMQWVILKIAWLMQVTMGTTATETLSVAGNIFVSQTEAPLLIRP 328
hCNT2           IIIFFGCVVSILYYLGLVQWVVQKVAWFLQITMGTTATETLAVAGNIFVGMTEAPLLIRP 323
hCNT3           IVVFFSTVMSMLYYLGLMQWIIRKVGWIMLVTTGSSPIESVVASGNIFVGQTESPLLVRP 350
vcCNT           TLIFFSALISVLYYLGVMQWVIRILGGGLQKALGTSRAESMSAAANIFVGQTEAPLVVRP 163
                 ::**. ::*:**::*::**::  :.  :  : *::  *:: .:.****. **:**::**
```

Figure 1. Sequence alignment of hCNT1, hCNT2, hCNT3 and vcCNT. Residues implicated in uridine binding have been highlighted in green. In blue, residues implicated in sodium binding are highlighted.

4.1 Model of hCNTs nucleoside binding site

A model was generated for all concentrative nucleoside transporters binding sites, having among them only small differences in some of the residues that conform the cavity in concordance with its different substrate selectivity. Some residues were found to be responsible for the different selectivity. The pair Ser318/Gln319 in hCNT1 that is Gly/Met hCNT2 and Gly/Gln in hCNT3. And also Ala350 in hCNT1, that is a serine in hCNT2 and alanine in hCNT3.

```
hCNT1           VLYHVGLMQWVILKIAWLMQVTMGTTATETLSVAGNIFVSQTEAPLLIRPYLADMTLSEV 338
hCNT2           ILYYLGLVQWVVQKVAWFLQITMGTTATETLAVAGNIFVGMTEAPLLIRPYLGDMTLSEI 333
hCNT3           MLYYLGLMQWIIRKVGWIMLVTTGSSPIESVVASGNIFVGQTESPLLVRPYLPYITKSEL 360
                :**::**:**:: *:.*:: :* *::. *:: .:*****. **:***:****  :* **:

hCNT1           HVVMTGGYATIAGSLLGAYISFGIDATSLIAASVMAAPCALALSKLVYPEVEESKFRREE 398
hCNT2           HAVMTGGFATISGTVLGAFIAFGVDASSLISASVMAAPCALASSKLAYPEVEESKFKSEE 393
hCNT3           HAIMTAGFSTIAGSVLGAYISFGVPSSHLLTASVMSAPASLAAAKLFWPETEKPKITLKN 420
                *.:**.*::**:*::***:*:**: :: *::*****:**.:** :** :**.*:.*:  ::
```

Figure 2. Sequence alignment of hCNT proteins. In orange, residues thought to be responsible for substrate selectivity have been highlighted.

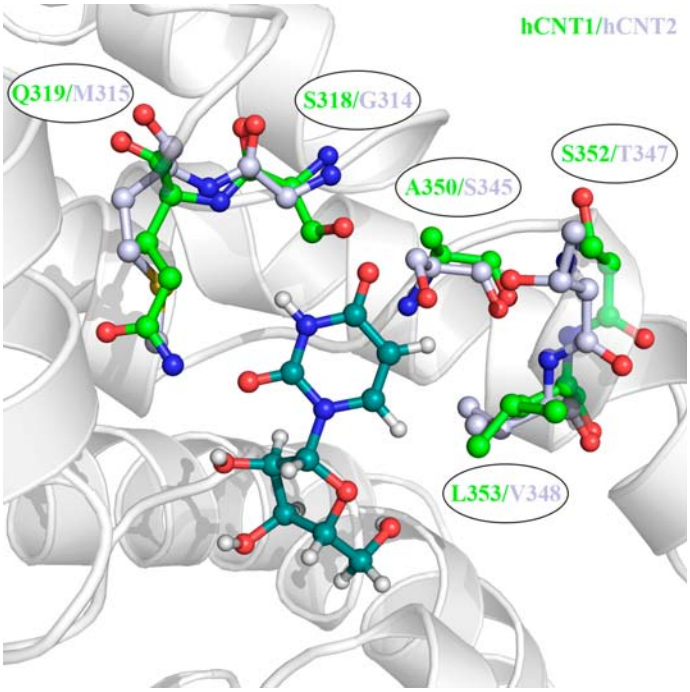


Figure 3. hCNT1/hCNT2 nucleoside binding site. Model of hCNT1 and hCNT2 uridine binding site. hCNT1 (green) and hCNT2 (grey) different residues have been highlighted.

The experimental prove of the binding site residues was not done because the proposed residues had already been studied by Loewen et al. (Loewen et al., 1999). Interestingly, they described that the mutation of serine 318 in hCNT1 to glycine and the mutation of glutamine 319 to methionine, present at the same locations in hCNT2, caused a change in the transporter selectivity from pyrimidine selectivity to a broad selectivity transporter. The additional mutation of the alanine 350 to serine, characteristic of hCNT2, maintained the broad substrate selectivity of the transporter. However, the single mutation of the residue at this position did not alter the transporter

selectivity. These results, obtained by others before the modelling of the transporter binding pocket, proved that these residues are essential for substrate selectivity in hCNT1 and hCNT2. Moreover, they also support the consistency of our model.

4.2 Identification of hCNT3 primary sodium binding site

The primary sodium binding site was localised at the hCNT3 model protein by homology with the sodium binding site in *V. cholerae* protein. Three residues, T370, I371 and N336, were determined to be important for the first sodium coupling. The threonine at position 370 and the asparagine at the 336 position were mutated to alanine. And the isoleucine at 371 was mutated into glutamate. The former two mutations were aimed so the residues loss their binding capacities whereas the latter was aimed to disrupt the whole sodium binding cavity by adding a negative charged residue.

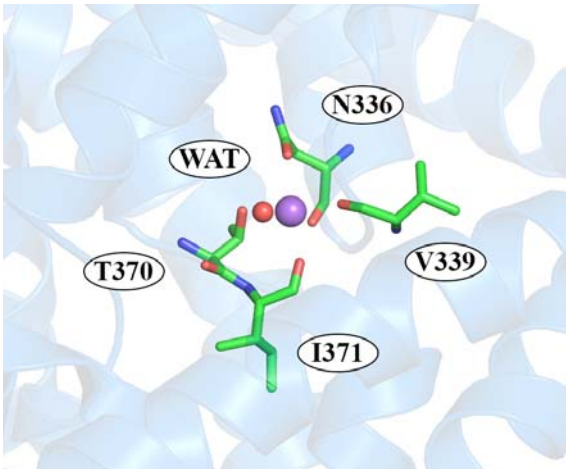


Figure 3. First sodium binding site.

The generation of mutations in this primary sodium binding site were thought to cause a total loss of activity. However, experimental results in HEK293 cells transiently transfected with the mutated transporters showed similar uptake rates for T370A and N336A whereas the mutant I371E presented no transport activity. To ensure all proteins were properly expressed by the transiently transfected HEK293 cells, cell extracts were obtained and, hCNT3 proteins were detected by Western Blot using an antibody against the N-terminal tail. Interestingly. mutants T370A and N336A seemed to be expressed in higher amounts than the wild type transporter. Expression levels were semi-quantified by densitometry and uptake rates corrected by the protein expression (Figure 4).

The fact that two of the three mutants had some activity allowed the possibility of measuring the Na^+:nucleoside stoichometry. The hill coefficient which is normally two

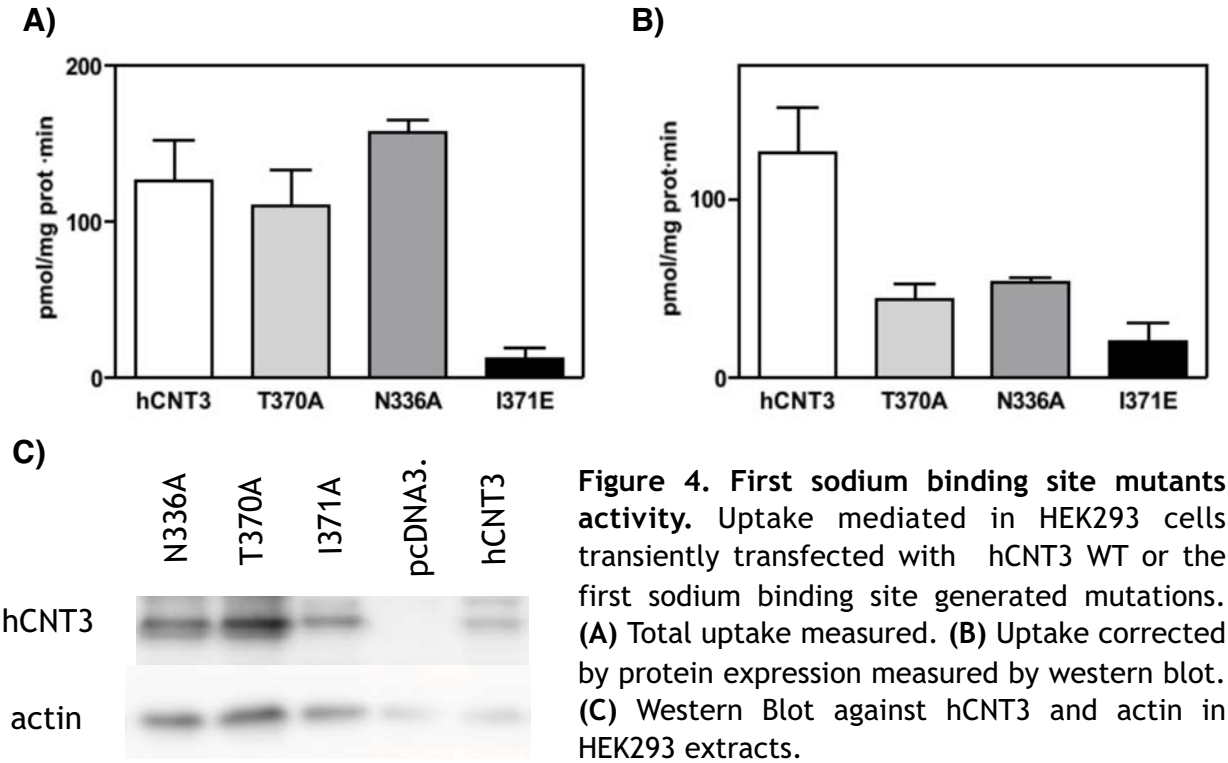


Figure 4. First sodium binding site mutants activity. Uptake mediated in HEK293 cells transiently transfected with hCNT3 WT or the first sodium binding site generated mutations. **(A)** Total uptake measured. **(B)** Uptake corrected by protein expression measured by western blot. **(C)** Western Blot against hCNT3 and actin in HEK293 extracts.

for hCNT3 was expected to shift to one when any residue implicated in sodium binding was changed. hCNT3 wild type was used as a control. The results showed hill values of 2 ± 0.1 for hCNT3 WT and 0.9 ± 0.1 for I370A as expected. However, the value for N336A was 2 ± 0.03. This result does not discard that asparagine 336 is important for the first sodium binding. The fact that we do not see a shift of the Hill coefficient value could be explained by a water molecule substituting the asparagine chain in coordination with the water molecule. (Figure 5)

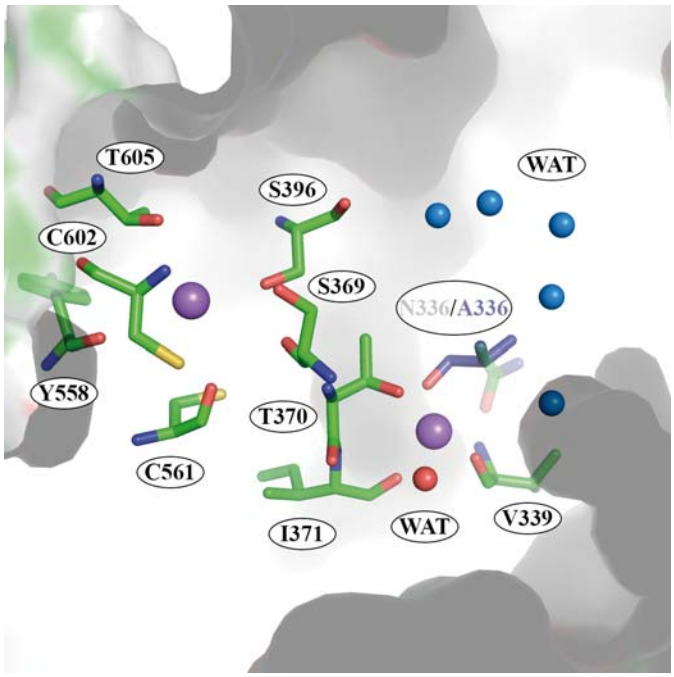


Figure 5. Sodium binding sites showing N336A mutation. Asparagine chain is substituted by a water molecule.

4.3 Identification of hCNT3 secondary sodium binding site

The cysteine at 602 position, which had previously been described as being implicated in sodium binding as it presents a hill coefficient of 1 (Errasti-Murugarren et al., 2008), was not located at the first sodium binding site. However, the hCNT3 model showed that it could be one of the key residues in the secondary sodium binding site. The possible second sodium binding site was identified close to the primary one. To validate the model, two of the putative residues of the second sodium binding sites: the threonine at the 605 position and the serine at the 396 position were mutated into alanines. Hill coefficients were calculated for these variants and the results were 1±0.1 for S396A and 1.1±0.05 for T605A.

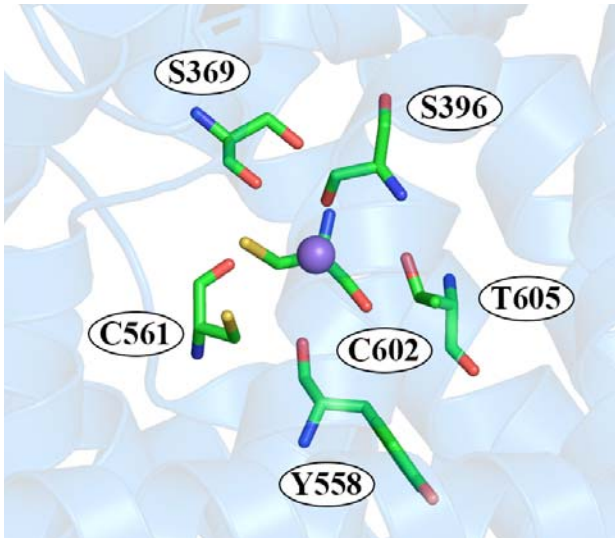


Figure 6. Secondary sodium binding site.

The second sodium binding site is also known for being able to bind H^+ instead of Na^+ with and stoichometry H^+:nucleoside (1:1) (Errasti-Murugarren and Pastor-Anglada, 2010; Pastor-Anglada et al., 2008). pH dependence experiments will be performed in order to determine wether the mutations of the residues involved in the second sodium binding could alter somehow the pH dependency of hCNT3.

So far, the model we are working with seems to be a good hCNT3 model. It will help to understand why hCNT3 polymorphic variants such as hCNT3-C602R affect differentially the transportability of different substrates (Errasti-Murugarren et al., 2010b) and why some substrates such as decitabine can interact with the transporter without being translocated (this thesis).

The model, however, has some limitations. Prokaryotic transporters lack the 3 N-termini transmembrane domains which seem to be implicated in interactions with partner proteins as well as in transceptor functions (Huber-Ruano et al.). These domains are highly disordered, thus, enabling multiple protein-protein interactions which might modulate in an allosteric manner transport functions. Moreover, although it is a good binding and docking model, the substrate translocation cycle is complex and implicate

sometimes big structural changes. To understand the translocation cycle multiple crystals of the transporter in all possible intermediate states is required. For the *Vibrio cholerae* nucleoside transporter only one crystal has been obtained so far, so molecular dynamic studies which are essential to understand how a transporter protein works, are still far from being elucidated for hCNT.

GENERAL DISCUSSION

Comprehensive analysis of the functional implications of transporter polymorphic variants on either drug-transporter or drug-drug interactions may help to optimise drug treatments. The aim of this work was to determine how genetic variations in nucleoside-derived drug transporters could modulate its bioavailability.

The first drug studied was lamivudine (3TC), an antiretroviral drug which has been described to be internalised through hOCT1 (Minuesa et al., 2009). Similarly to what happens for other hOCT1 substrates such as metformin, morphine or imatinib (Giannoudis et al., 2013; Shu et al., 2007; Tzvetkov et al., 2013), some hOCT1 polymorphic variants (R61C, S189L and M420del) affect 3TC uptake mediated by the transporter variants, resulting in transport efficiency values lower than 1. This makes this variants not suitable for mediating lamivudine uptake into cells. For the hOCT1 model substrate MPP^+, although transport was also impaired in some cases, efficiency values were always higher than 1. This shows that the effect that the polymorphic variants have on substrate transportability is somehow substrate dependent and highlights the need of carefully analysing each particular polymorphic variants for every substrate of interest.

Cysteines located at the large extracellular loop of hOCT1 proteins have been implicated in their oligomerization and its correct insertion into the plasma membrane as well (Brast et al., 2011; Keller et al., 2011). Two of the reported polymorphic variants here analysed are located within this loop. One of them, R61C, results in a change of an arginine by a cysteine and the other one, C88R, substitutes a cysteine residue within this loop by an arginine. Although both variants involved a cysteine residue, none seemed to alter the oligomerization capacity of hOCT1. This finding rules out the possibility of cysteine 88 being essential for the correct oligomerization of the transporter.

hOCTs have been detected in immune cells. Stimulation of these cells results in an increase in hOCT1 and hOCT3 transporters mRNA levels (Minuesa et al., 2008). In this thesis it is shown that the infection of PBMCs with HIV-1 virus results in an increase of hOCT1 mRNA levels similar to what has been described for hCNT1, hCNT3 and hENT2 in adipose tissue of HIV-1 infected patients (Guallar et al., 2007). Interestingly, the fact that cells infected with target cell being more sensitive to those drugs internalised via this transporter protein. In our case this relates to 3TC availability.

In the treatment of AIDS, 3TC is normally co-administered with other antiretroviral drugs such as AZT or ABC. Some other antivirals have been shown to inhibit hOCT1 despite not being internalised by the transporter (Minuesa et al., 2009). Polymorphic variants R61C and M420del seemed to modify drug-drug interactions being *Cmax* concentrations of ABC and AZT able to inhibit 3TC uptake to a higher extent than when using the WT transporter. This is similar to what has been reported for metformin. Metformin drug-drug interactions were measured in the variant M420del and it also showed to be more sensitive to drug-drug interactions than the WT transporter (Ahlin et al., 2011).

In summary, responsiveness to lamivudine therapy appears to be somehow dependent upon the occurrence of selected hOCT1 polymorphic variants. This might be even more relevant when the drug is co-administered with other antiretroviral drugs able to interact with hOCT1. Genetic studies are warranted when facing cases in which the therapy using these drugs do not result in the desired effects.

hOCT1 has also been identified in this thesis as a putative bendamustine transporter. Although direct transport of bendamustine through hOCT1 could not be determined due to the lack of a radiolabeled form of this drug, the capacity of bendamustine to inhibit MPP^+ uptake and the sensitisation of HEK293 cells to bendamustine when expressing hOCT1, strongly supported this possibility.

The fact that bendamustine does not interact with hCNT proteins is consistent with their selectivity for nucleosides (Errasti-Murugarren and Pastor-Anglada, 2010; Huber-Ruano and Pastor-Anglada, 2009). hENT2 is the only nucleoside transporter known to mediate the uptake of natural nucleobases, being a good hypoxanthine transporter. hENT1 has recently been suggested to translocate some nucleobase-derived drugs, such as 5-Fluorouracil (5-FU) (Yao et al., 2011). However, the lack of inhibition of hENT-mediated transport by bendamustine suggests that hENTs do not play a significant role in its uptake and cytotoxicity.

hOCT1 is significantly expressed in immune cells, including peripheral blood mononuclear cells (PBMCs), $CD4^+$ T cells, monocytes, monocyte-derived macrophages and dendritic cells (Minuesa et al., 2008). Its expression also appears to be somehow modulated because it is up-regulated after stimulation of PBMCs and $CD4^+$ cells with phytohemagglutinin (PHA) (Minuesa et al., 2008). Significant expression of the SLC22A1

mRNA, encoding hOCT1, was also reported to occur in a limited set of primary cells from CLL patients where it appears to contribute to irinotecan- and paclitaxel-induced cytotoxicities (Gupta et al., 2012). In fact, this has also been corroborated here when analysing the transcriptomic profiles of the whole cohort of CLL patients used in this study. The mRNA levels for hOCT1 in these patients, although variable, did not significantly correlate with the *ex vivo* sensitivity to bendamustine, although this might not necessarily reflect the hOCT1 protein amounts present in CLL cells.

hOCT1 is also widely expressed at the sinusoidal membrane of the hepatocytes. Thus, not only bendamustine action could be modulated but also bendamustine handling and detoxification by the liver are likely to be mediated by this particular transporter protein.

As comprehensively discussed above, hOCT1 is a highly polymorphic transporter. The impact of genetic heterogeneity within the SLC22A1 gene on the pharmacokinetics and pharmacodynamics of some drugs and on the clinical outcome of patients treated with hOCT1 substrates has been recently addressed by different laboratories (Giannoudis et al., 2013; Shu et al., 2007; Tzvetkov et al., 2013; Tzvetkov et al., 2012). We calculated the allelic frequencies of the polymorphic variants in a cohort of 233 CLL patients genotyped within the framework of the International Genome Consortium (ICGC) and compared them with the ones reported by Kerb et al. and Shu et al. (Kerb et al., 2002; Shu et al., 2003). As summarised above, frequencies are in general within the range of what has been previously published for Caucasians. Both semi-functional variants, R61C and M420del, showed to be less frequent, being the former (5.6% *vs* 7.2%) and the latter (11.6% *vs* 18.5%) in the cohort of CLL patients than in the random Caucasian population genotyped by others (Shu et al., 2003). Our findings determined that hOCT1 polymorphisms are likely to modulate the sensitivity of CLL cells to bendamustine and, simultaneously, determine bendamustine bioavailability and pharmacodynamics.

Following the comprehensive analysis of nucleoside analogues used in chemotherapy, this study also addressed the determination of the transporter proteins implicated in the cellular handling of DNA methyltransferase (DNMT) inhibitors. These drugs, despite presenting among them minor changes in their molecular structures have shown significant differences in their transportability profiles. Although all these three

molecules retain the 3'-hydroxyl group which is known to be important for drug interaction with most nucleoside transporters (Cano-Soldado and Pastor-Anglada, 2012), it has been here found that decitabine, despite being a good nucleoside transporter inhibitor, cannot be translocated by any of the studied transporters. This is similar to what has been reported for adenosine and its interaction with the high affinity nucleoside transporter hCNT1 (Larrayoz et al., 2004). The only structural difference between azacitidine and decitabine is in the 2'-hydroxyl group. This suggests that the hydroxyl group at 2' plays a key role in determining substrate-transporter interaction and transportability. This could also be confirmed by cytotoxic studies showing that the presence of either hCNT1 or hCNT3 could sensitise cells to either azacitidine or zebularine but not to decitabine.

hCNT and hENT asymmetric expression in (re)absorptive epithelia facilitates the vectorial flux of substrates across these barriers (Govindarajan et al., 2007; Govindarajan et al., 2008; Mangravite et al., 2003b). Similarly to what has been described for other nucleoside analogues (Errasti-Murugarren et al., 2007), the apical expression of both hCNT1 and hCNT3 in polarised MDCK cells grown on transwell filters, determined the apical-to-basal vectorial flux of zebularine.

hCNT expression has been found not only in epithelia but also in cells from patients with lymphoproliferative disease. Although in all cell types analysed hENT1 and hENT2 appear to be highly expressed, hCNT3, which is a suitable transporter for azacitidine and zebularine, is similarly highly expressed in monocytes, MDMs and MDDCs. However, hCNT3 expression in PBMCs and $CD4^+$ T cells is negligible although significantly induced in (PHA)-stimulated cells (Minuesa et al., 2008). CLL cells express hCNT2 and hCNT3 (Molina-Arcas et al., 2003). It has also been shown that hCNT3 activity can be up-regulated by treatment with all-transretinoic acid (ATRA) in the CLL-derived cell line MEC1 as well as in cells from CLL patients *ex vivo* (Fernandez-Calotti and Pastor-Anglada, 2010; Fernandez-Calotti et al., 2012). Overall this anticipates that all immune cell types but especially myeloid populations and CLL cells can be targeted by selected DNMT inhibitors, based upon their transporter expression patterns.

As mentioned above, hOCT expression has also been found in immune cells and has been identified as a suitable transporter for some nucleoside analogues. Thus, it was considered as a suitable candidate to mediate the uptake of DNMT inhibitors.

Unexpectedly, hOCT1 expression resulted in a reduced zebularine accumulation in HeLa cells, thereby suggesting that hOCT1 could have a role in zebularine efflux rather than in its uptake into cells. This was further confirmed by measurements using *Xenopus laevis* oocytes as the cell background in which to express hOCT proteins. Asymmetry in the translocation properties of hOCTs could be anticipated as this has been already reported for the interaction of corticosterone with hOCT1, being its binding properties different depending upon whether the molecule binds to the inward or to the outward pocket of the protein (Volk et al., 2009).

The study of the effect of hOCT1 polymorphic variants on zebularine efflux, interestingly, showed that the efflux capacity of the transporter was impaired when any of the studied polymorphic variants affecting hOCT1 performance were present. If hOCT1 plays a role in zebularine efflux instead of facilitating its uptake into target cells, hOCT1 expression would not result in increased sensitivity to the drug but, instead, in chemoresistance. More interestingly, selected genetic hOCT1 variants known to determine poor response to therapy (Giannoudis et al., 2013; Shu et al., 2007; Tzvetkov et al., 2009) would confer more sensitivity to zebularine than the wild type transporter, as it was here demonstrated with the cytotoxicity assays. In this particular case these variants would contribute to retain zebularine inside tumour cells once previously internalized via either hCNT or hENT-type proteins.

Drug-transporter and drug-drug interactions appear to be relatively complex and this may be even more complicated when facing genetic variants known to differentially affect drug-transporter interactions. To better understand these phenomena, knowledge of the structure of these drug transporters is becoming essential. Structures have been a bottleneck in this field. However, the recent crystallisation of a *Vibrio cholerae* nucleoside transporter (vCNT) has opened the possibility to obtain good docking models for hCNT type proteins, particularly considering that the *V. cholerae* ortholog shows a 39% identity with the human CNT3. The different selectivity of hCNT1 and hCNT2 to pyrimidinic or purinic nucleosides could be explained by the generated model as different amino acids were present at the nucleoside binding pocket of both transporters. Interestingly, the residues proposed to be important had already been studied by Loewen et al (Loewen et al., 1999). The mutation of serine 318 and glutamine 319 present in hCNT1 to glycine and methionine which are present at the

same position in hCNT2 resulted caused a shift of hCNT1 selectivity from being pyrimidine-selective to become a broad selectivity transporter. Site-directed mutagenesis studies could identify the two sodium binding sites in hCNT3. The first sodium binding site, is located close to the nucleoside binding site and it was described by homology with the sodium binding site in vCNT (Johnson et al., 2012). Cysteine 602 in hCNT3 had previously been shown to be important for sodium binding as the polymorphic variant showing and arginine at this position instead of a cysteine showed a hill coefficient of 1 (Errasti-Murugarren et al., 2008). Interestingly, this cysteine appears to be located in the second sodium binding site which is found close to the first one.

In summary, similar drugs may have different transportability profiles and genetic polymorphic variants may affect differently related substrates. As we do not yet have good transporter models, a good experimental characterisation of drug transportability should be performed to further understand pharmacokinetics, pharmacodynamics and toxicity of the drugs.

CONCLUSIONS

- Human Organic Cation Transporter 1 (hOCT1) appears to be a suitable transporter protein for bendamustine and zebularine, although it mediates the uptake of the former and the efflux of the latter, thereby anticipating different roles for this protein in drug action depending upon the substrate it translocates.

- Polymorphic variants of hOCT1, with significant allelic frequency in humans, do modify differentially the interaction of known substrates of hOCT1, such as lamivudine, and the interaction of novel hOCT1 substrates here identified, such as bendamustine and zebularine.

- Polymorphic variants implicating cysteine residues in the extracellular loop between TMDs 1 and 2 of hOCT1 do not impair hOCT1 oligomerization.

- hOCT1 genetic variants also appear to differentially affect antiviral drug-drug interactions with the transporter protein. Infection of PBMCs with Human Immunodeficiency Virus (HIV) results in the up-regulation of the expression of hOCT1 mRNA.

- Polymorphic variants of hOCT1 might be associated with either chemoresistance (i.e. for bendamustine) or chemosensitivity (i.e. for zebularine), depending upon an apparent asymmetry of the transporter protein regarding its substrate selectivity.

- Genotyping of hOCT1 polymorphic variants in a cohort of Spanish Chronic Lymphocytic Leukaemia (CLL) patients revealed allelic frequencies similar to those previously reported for Caucasians. Those variants associated with reduced or null hOCT1 activity were more abundant in those subgroups showing *ex vivo* resistance to bendamustine.

- Transporter proteins belonging to gene families *SLC22* (hOCT1, hOCT2), *SLC28* (hCNT1, hCNT3) and *SLC29* (hENT1, hENT2) might determine the cellular handling of DNA methyltransferase (DNMT) inhibitors in a differential manner, being nucleoside transporters responsible for azacitidine and zebularine uptake, and hOCT1 and hOCT2 efflux transporters for zebularine.

- Decitabine, despite showing a minor structural variation versus azacitidine (lack of the 2'OH group) is not translocated by any of the studied transporter proteins, although it can interact with hENT and hCNT type transporters.

- A model for hCNT substrate docking is put forward and preliminarily validated. Residues T370, I371, N336, S396 and T605 appear to be implicated in the first and second sodium binding sites of hCNT3, thereby explaining mechanistically the hCNT3 polymorphic variant (hCNT3-C602R) previously identified in Spanish population.

EXPERIMENTAL PROCEDURES

1

CELL CULTURE

1.1 Introduction

Cell culture is the process by which cells are grown *ex vivo* under controlled conditions in order to maintain their original physiological, biochemical and genetic properties utmost. It is one of the major tools used in cellular and molecular biology, providing excellent model systems for studying the normal physiology and biochemistry of cells and the effects of drugs and toxic compounds on them. It is also used in drug screening and development. The major advantage of using cell culture for any of these applications is the consistency and reproducibility of results that can be obtained from using a batch of clonal cells.

Primary cultures are those obtained directly from an animal tissue and they have a limited life span. When a finite cell line undergoes transformation, which can occur spontaneously or can be chemically or virally induced, and acquires the ability to divide indefinitely, it becomes a continuous cell line. Cell lines allow longer term experiments than primary cultures. However, as they do not have proliferation control they may lose cellular characteristics which lead them to a less differentiated state than the original tissue. Most cells coming from organs are grown in monolayers attached to a substrate; on the other hand, cells from the immune system are grown in suspension.

1.2 Working area

Successful cell culture depends on keeping the cells free from contamination by microorganisms. For this reason, cell cultures must be always performed under sterile conditions. To that effect, it is required to work in a laminar flow hood previously sterilised with UV light and all material used have to be sterile as well as all media and reagents.

1.2.1 Required equipment

- Cell culture laminar flow hood with UV sterilisation system and aspiration pump.
- Cell incubator with controlled atmosphere (37ºC, 5% CO_2, 95% humidity)
- Inverted microscope.
- Water bath.
- Centrifuge.
- Refrigerator and freezer (4ºC, -20ºC and -80ºC).
- Liquid nitrogen (N_2) freezer and cryo-storage container.
- Cell counter.
- Other material: flasks, multiwell plates, pipettes, pipettors, etc

1.3 Maintaining cells in culture

1.3.1 Passaging and plating

In order to obtain large number of cells and to prolong their life, cells need to be subcultured. Passaging involves re-suspending and splitting the cells and transferring a small number into a new flask. For cells grown attached to a substrate, disaggregation, using substances such as trypsin or EDTA, is required. Trypsin is a serine protease which cleaves proteins bonding the cultured cells to the dish, so that the cells can be suspended whereas EDTA chelates the calcium involved in cell-cell adhesion.

1.3.2.1 Materials

- PBS:

137mM	NaCl
2.7mM	KCl
1.4mM	KH_2PO_4
4.3mM	Na_2HPO_4
7.3	pH

- Cell culture medium supplemented with fetal bovine serum (FBS).

- 0.05% Trypsin-EDTA from Life Technologies.

1.3.2.2 Procedure

Procedures are to be done in the Biological Safety Cabinet using aseptic techniques and all reagents are to be warmed up to 37ºC.

Cells are grown in an incubator with controlled atmosphere in 75 cm^2 flasks. When they get to the desired confluence the medium is aspirated and 10ml PBS is added to eliminate any residual serum, which can interfere in trypsin action. Then the PBS is removed and 1-2ml trypsin (depending on the cells) is added and incubated for 1-10min at 37ºC depending on the attaching capacity of the cells. Once the cells are completely off the flask surface, 10ml fresh medium is added to inhibit any further enzymatic reaction and the cells are mechanically disaggregated with a sterile pipette in order to obtain an homogeneous suspension. At this point cells are ready for passaging, seeding or freezing.

For passaging, a small number of cells are transferred to a new flask and new medium is added up to the desired volume. To use the cells in further experiments, they need to be plated in plates, for that, cells are counted using the cell counter (Invitrogen) and plated in the required density depending on the experiment considering the number of cells per cm^2 (Table 1).

		Area (cm^2)	Number of cells at confluence ($\cdot 10^6$)	Cell culture medium volume(mL)
Round dishes	35mm	9.62	1.2	2
	65mm	28.27	3.2	3
	100mm	78.54	8.8	10
	150mm	176.71	20	20
Multi-well plates	6 wells	9.62	1.2	3-5
	12 wells	4.01	0.4	1-2
	24 wells	2.00	0.2	0.5-1
Flasks	T-25	25.00	2.8	3-5
	T-75	75.00	8.4	8-15
	T-160	160.00	18.4	15-30

Table 1. Useful information for cell plating. Cell number in confluence depends on the cell type. HeLa cells have been used as reference in the table.

1.3.2 Freezing and thawing

After some time in culture, cells can accumulate mutations which can lead to phenotypic changes altering the experimental results. Cell freezing pretends to keep the original cells stored in liquid nitrogen for further use. Cryoprotectors such as DMSO (dimetilsulfoxide) are required to ensure cell viability after freezing. Cell lines used in this thesis have been frozen in FBS supplemented with 10% DMSO.

1.3.2.1 Freezing

Trypsinized cells are centrifuged in 15mL tubes for 4 minutes at 1200rpm. The supernatant is removed and the cells are resuspended in freezing medium (FBS + 10% DMSO) and aliquoted in 2mL cryovials. The vials are subsequently stored in the cell freezing container which provides the 1°C/min cooling rate required for successful cryopreservation of cells. The container is placed in a -80ºC freezer for from 4 days to 1 month and afterwards the cryovials are stored in a liquid nitrogen tank at -196ºC.

1.3.2.2 Thawing

To obtain fresh cells, a selected cryotube from the liquid nitrogen tank is rapidly thawed in a 37ºC bath and transferred in a 15mL tub containing fresh medium. The tube centrifuged for 4 minutes at 1200rpm and the supernatant is discarded to get rid of the remaining DMSO. Cells are resuspended in new medium and plated in a new flask.

1.4 Used cell lines

1.4.1 HeLa cells

HeLa cells are epithelial cells which were derived from cervical cancer cells from Henrietta Lacks, a patient who eventually died of her cancer on 1951. It is the oldest and most commonly used human cell line due to its ability to express heterologous expressed proteins.

The cell growth medium used for these cells is:

500mL	DMEM *High Glucose*
10%	FBS
2mM	glutamine
100units/mL	penicillin
100µg/mL	streptomycin
0.025µg/mL	amphotericin B

These cells have a high proliferation rate so they need to be passaged at least twice a week. The number of passages can affect the transient transfection efficiency so a maximum of 30-35 passages is recommended.

1.4.2 MDCK cells

Madin-Darby Canine Kidney Epithelial Cells (MDCK) was derived from the kidney tissue of an adult female cocker spaniel. The MDCK line is commonly used as a general model for epithelial cells and under the adequate growth conditions it is able to polarise and develop many typical properties from the renal epithelia. For this reason, it is used as a model to study a great variety of proteins.

The cell growth medium used for these cells is:

500mL	DMEM *High Glucose*
10%	FBS
2mM	glutamine
100units/mL	penicillin
100µg/mL	streptomycin
0.025µg/mL	amphotericin B

As well as HeLa cells, MDCK have a high proliferation rate and need to be subcultured twice a week.

1.4.2.1 Culture in polycarbonate filters

In order to measure epithelial flux in polarised MDCK cells, they have to be grown in polycarbonate filters. The polycarbonate filters need to be treated with 200µL serum-free growth medium supplemented with type I rat tail collagen (0.003%) for 3h. After

removing the medium, 190,000 cells are seeded per polycarbonate filter resuspended in 200µL cell growth medium. After one hour, 1mL growing medium is added to the apical and basolateral compartments of the transwell filter, always in this order. The next morning the cells are ready to be transfected.

1.4.3 HEK293 cells

Human Embryonic Kidney 293 cells (HEK293) are a cell line obtained from a single apparently healthy foetus legally aborted under Dutch law in the early 70s. HEK293 cells are very easy to grow and transfect very readily.

The cell growth medium used for HEK293 cells is:

500mL	DMEM *High Glucose*
10%	heat-inactivated FBS
2mM	glutamine
100units/mL	penicillin
100µg/mL	streptomycin

In case of the HEK293 FlpIn cells the medium is supplemented with 200µg/mL zeocin and for the HEK293 stably expressing hOCT1 or its polymorphic variants 100µg/mL of hygromicin B is added to the regular medium.

HEK293 also have a high proliferation rate and need to be passaged before they reach confluence.

HEK293 cells easily de-attach when transport measurements are performed. To avoid this, before platting them 24 well plates are treated with 0.01mg/mL Poly-D-lysine (sigma) diluted in PBS for 3h. The PBS is than removed by aspiration and the cells are plated.

2

HETEROLOGOUS EXPRESSION METHODS

2.1 Introduction

Transfection techniques allow the introduction of nucleic acids into eukaryotic cells. In this thesis it has been used to over-express the proteins of interest in order to characterise its function.

For the most used DNA constructs, stable cell lines were generated whether for those only used in seldom studies transient transfection was used.

2.2 Transfection techniques

Transfection techniques which are commonly used today can be classified into three groups: (1) methods that make use of genetically engineered viruses (2) chemical methods that rely on carrier molecules and (3) physical methods that deliver nucleic acids directly to the cytoplasm. The methods used in this thesis are all chemical-based. Calcium-phosphate technic is used to transfect HeLa or HEK293 cell lines, whether Lipofectamine® (Life Technologies) is used to transfect those cell lines which can not be transfected using the calcium-phosphate method.

2.2.1 Calcium phosphate

This method is based on the formation of calcium-DNA precipitates in a phosphate saline solution. Small changes in the phosphate solution pH can vary significantly the transfection efficiency. For this, the phosphate pH has to be tested for an optimal transfection in every cell line. In our case pH 6.4 showed to be the best for both HeLa and HEK293 cells.

2.2.1.1 Buffers

- Calcium buffer:

500mM	$CaCl_2$
100mM	N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES)
6.95	pH

- Phosphate buffer:

280mM	NaCl
0.75mM	Na_2HPO_4
0.75mM	NaH_2PO_4
6.4	pH

Both solutions as well as Milli-Q water have to be filtered through a 0,22µm filter.

2.2.1.2 Transfection

Cells are seeded so they are 60-80% confluent at the time of transfection (25,000cells/cm^2). The transfection mix is prepared by adding in the following order DNA, sterile water, calcium buffer and phosphate buffer. The mixture is homogenised, bubbling with a pipette, and left for 15 minutes before adding the required volume to the seeded cells. The transfection solution is left overnight and replaced the next morning for new medium. The expression of the protein will be optimal at 40hours post-transfection.

The required amounts of reagents used are shown in Table 2.

	96 well plate	24 well plate	60mm plate	100mm plate
DNA	0.1µg	0.7µg	7µg	20µg
Water	2.5µL	12.5µL	125µL	250µL
Calcium buffer	2.5µL	12.5µL	125µL	250µL
Phosphate buffer	5µL	25µL	250µL	500µL
Volume	10µL	50µL	500µL	1000µL

Table 2. Transfection volumes used for HeLa and HEK293 cells.

2.2.2 Lipofectamine

Lipofectamine is a cationic liposome formulation which can bind negatively charged DNA and facilitate its uptake into the cytosol. In this thesis it was used to transfect MDCK cells seeded on transwell filters as they could not be transfected using calcium phosphate.

Before transfecting cells with lipofectamine the cell medium needs to be changed to non-supplemented medium. 190,000 cells/transwell insert are seeded so 24h later they are 95% confluent. Two solutions are prepared, one containing 0.7µg DNA in 50µL of non-supplemented medium and another one containing 1µL lipofectamine in 50µL of non-supplemented medium. The two solutions are mixed and left for 25minutes before adding 50µL to each compartment. After 5h the medium is changed to supplemented medium. The cells can be tested 48h later when they are confluent and the polarised monolayer is formed.

2.2 Stable cell lines generation

The FlpIn™ System allows integration and expression of the gene of interest in mammalian cells at a specific genomic location. It involves the introduction of a Flp Recombination Target (FRT) site into the genome of the mammalian cell line of choice. An expression vector containing the gene of interest is then integrated into the genome via Flp recombinase-mediated DNA recombination at the FRT site. Targeted integration of a FlpIn expression vector ensures high-level expression of the gene of interest and the fact that all gene are integrated in the same gene region allows the generation of isogenic stable cell lines.

The first step in stable cell line generation consists in the introduction of a FRT site in the genome of the selected cell line to generate a FlpIn host cell line. For that, pFRT/lacZeo target site vector is used. The vector contains a lacZ-Zeocin™ fusion gene whose expression is controlled by the SV40 early promoter. A FRT site has been inserted just downstream of the ATG initiation codon of the lacZ-Zeocin™ fusion gene. The FRT site serves as the binding and cleavage site for the Flp recombinase. The pFRT/lacZeo plasmid is transfected into the mammalian cell line of interest and cells are selected for zeocin resistance. Zeocin-resistant clones are screened to identify those containing a

single integrated FRT site. The resulting FlpIn host cell line contains an integrated FRT in which the gene of interest will be inserted.

hOCT1 and its polymorphic variants are cloned into the pcDNA5/FRT/TO vector (Invitrogen). This vector also contains the hygromycin B resistance gene with a FRT site embedded in the 5' coding region. The pcDNA5/FRT vector containing hOCT1, its polymorphic variants or the empty vector used as a control and pOG44 plasmid, which constitutively expresses the Flp recombinase, are co-transfected into the FlpIn™ host cell line in a ratio 1:9 by using the calcium phosphate method. Upon co-transfection, the Flp recombinase expressed from pOG44 mediates an homologous recombination event between the FRT sites (integrated into the genome and on pcDNA5/FRT).

Positive clones are selected with 100µg/mL hygromycin B and tested for activity. The HEK293 cells with the highest transport activity were chosen for further study and routinely cultured as described before.

3

CYTOTOXICITY ASSAYS

3.1 Introduction

Cell viability was measured in this thesis using the MTT method, a quantitative and colorimetric assay. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is reduced from yellow tetrazole, in living cells, to purple formazan crystals which can be solubilized in DMSO. The optic density of this dissolved compound correlates with the number of metabolic active cells as only those cells with NAD(P)H-dependent cellular oxidoreductase activity will be able to promote the reaction.

3.2 Procedure

HeLa or HEK293 cells are seeded (40,000 cells/cm^2) in 96-well plates. In the case that the cells need to be transfected, transfection is performed 24h post-seeding with the calcium phosphate method. The cells are treated 24h post-transfection or post-seeding, in case of HEK293 which are already expressing the transporter of interest, with increasing concentrations of the drug. The drug is left for 24h-48h depending on the drug being tested. The viability is measured by adding 100µL/well of 7.5mg/mL MTT dissolved in PBS and supplemented-culture medium (1:9). This solution is photosensitive so it has to be kept protected from the light. Cells are incubated with the solution for 30-60minutes at 37ºC. After this time a purple precipitate can be observed. The medium is removed and 100µL/well DMSO is added in order to dissolve the formazan crystals. The absorbance is measured at 550nm.

4

TRANSPORT MEASUREMENTS

4.1 Uptake in cells grown in monolayers

In order to measure the uptake activity of nucleoside transporters, cells are incubated at room temperature in either sodium medium or choline medium. As concentrative nucleoside transporters are sodium dependent, the difference between the uptake in sodium-containing medium and the uptake in sodium-free medium measures the transport mediated by concentrative nucleoside transporters. On the other hand, the sodium independent uptake is that mediated by equilibrative nucleoside transporters and passive diffusion. To distinguish between hENT1 and hENT2 uptake activity we used specific inhibitors. 1μM NBTI is used to inhibit hENT1 and 10μM dipyridamole to inhibit both hENT1 and hENT2.

To determine hOCT-mediated transport the uptake is measured in NMDG medium in cells expressing the hOCT of interest and in parallel in cells expressing the empty vector. Being in this case the difference between these two measurements the hOCT-mediated transport.

4.1.1 Transport media and buffers

- Sodium medium:

137mM	NaCl
5.4mM	KCl
1.8mM	$CaCl_2$
1.2mM	$MgSO_4$
10mM	HEPES
7.4	pH

- Choline medium:

137mM	Choline chloride
5.4mM	KCl
1.8mM	$CaCl_2$
1.2mM	$MgSO_4$
10mM	HEPES
7.4	pH

- NMDG medium:

137mM	N-metil-D-glucamina (NMDG)
5.4mM	KCl
1.8mM	$CaCl_2$
1.2mM	$MgSO_4$
10mM	HEPES
7.4	pH

- STOP medium:

137mM	NaCl
10mM	HEPES
7.4	pH

- Lysis buffer (multi-well plate):

0.5%	Triton X-100
100mM	NaOH

- Lysis buffer (transwell):

0.1%	SDS
100mM	NaOH

- Detaching buffer (in PBS):

0.02%	EDTA
10mM	HEPES
28mm	$NaHCO_3$

- ORI buffer:

5mM	3-(N-morpholino)propanesulfonic acid-NaOH (MOPS)
100mM	NaCl
3mM	KCl
2mM	$CaCl_2$
1mM	$MgCl_2$
7.4	pH

4.1.2 Multi-well plate

Transport studies of HeLa and HEK293 cells are carried out on 24-well plates. Transport media are supplemented with un-labeled substrates at a concentration of 1µM and 1µCi of the corresponding tritium-radiolabeled substrate. In the case of drug-transporter interaction assays or kinetic studies, increasing concentrations of the drug is added to the transport media.

Semi-confluent cell monolayers are washed twice in sodium, choline or NMDG medium and subsequently incubated for 1 minute in 220µl of the corresponding transport medium. Uptake is stopped after 1 minute by two rapid washings with ice-cold stop solution. Cells are then, lysed in 100µL of lyses solution for 1 hour shaking to ease the liberation of the intracellular accumulated radioactivity. Once homogenised, 10µL are collected for further protein quantifying, the remaining 90µL are used for radioactivity counting. 5µL of the transport media used are also collected in duplicate for radioactivity measurement to be used as standard. All samples are measured in a beta-counter which measures disintegration per minute (dpm).

For the measurements of Na^+:nucleoside stoichiometry, media with 0-100mM Na^+ and the necessary amount of choline to maintain the osmolarity are used. The transport measurements and radioactivity counting are performed as described for 24 well plate measurements.

4.1.3 Transwell-chambers

Vectorial flux measurements have been performed at 37ºC so the cells have to be kept in an incubator and all media used was warmed in a water bath. Transport media are prepared the same way as those used for multi-well plate measurements, being the only difference the volume used per well, which is 500µL for transwell assays.

Growing media is removed by aspiration always following the order basolateral-apical and the cells are washed twice, before 500µL of radioactive medium is added to the desired compartment (apical or basolateral) and the same volume of the corresponding medium is added to the opposite compartment. For initial-velocity measurements, after 1minute the transport is stopped by rapid aspiration of the uptake buffer, followed by immediate washing in ice-cold stop solution. To quantify the vectorial flux, 50µL of the opposite compartment to the one containing the radiolabelled substrate are collected at 1, 5 10 and 20 minutes to quantify the radioactivity released. Once the transport is finished, the filters are washed in ice-cold buffer. Afterwards, each filter is separated from the well, introduced into an eppendorf containing 200µl of lyses buffer and incubated for 2 hours at 37ºC. From the remaining lysate, 10µL are collected per duplicate to quantify the protein amount. Moreover, 100µL are used to determine the accumulated radioactivity which will determine the substrate accumulation.

4.1.4 Protein measurement using the bicinchoninic acid assay (BCA)

Although cells are counted before seeding, the amount of cells at the transport moment is never exactly the same. Consequently, in order to have comparable results, the uptake values need to be corrected by protein concentration.

To quantify the protein amount, the bicinchoninic acid (BCA) assay (Pierce) is used. The BCA Protein Assay combines the well-known reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{1+}) by bicinchoninic acid. The chelation of two molecules of BCA with one cuprous ion produces an intense purple-colored product which is water-soluble and exhibits a strong linear absorbance at 562nm with increasing protein concentrations.

10µL from each transport well are deposited in a 96well plate and 200µL of the mixture, from the commercially available kit, in a proportion 1:50, are added. Alongside a calibration curve with 0-2000µg/mL of bovine serum albumin (BSA) is measured to determine the exact amount of protein per well.

4.1.5 Calculations

The radioactivity counts obtained in disintegration per minute (dpm) have to be corrected by the amount of protein and by the amount of radioactivity used in order to have pmol/mg prot·min. First of all, it has to be considered that the dpm value provided by the beta-counter is not the total dpms of the sample as part of it has been kept for protein quantifying. Once this has been corrected the calculation to be done is the following:

$$Activity(pmol/mgprot) = \frac{dpmsample \times 10^3}{samplevolume} \times AE * \times \left[prot(\mu g/\mu L \right]$$

*AE corresponds to standard activity (dpm/pmol) obtained by dividing the dpms of the standard by the volume counted.

4.2 Uptake in detached cells

To determine the uptake of hOCTs or its polymorphic variants in HEK293 stably expressing the transporters or HEK293-pcDNA5 uptake is measured after 1 second (MPP^+) and 15 seconds (3TC) incubations in detached cells using a "short-time" uptake protocol previously established at Dr. Koepsell's laboratory (Lips et al., 2005). The advantage of performing this short-time uptake measurements lies in the reduction of the passive diffusion or unspecific uptake of the radiolabelled substrate.

4.2.1 Short-time measurement

After detaching cells with a detaching buffer, washing them with NMDG medium and resuspending them in NMDG medium, 90µL of cells (1,000,000 cells aprox.) are placed at the bottom of 2mL eppendorf and left in a water bath at 37°C for 5 minutes.

The uptake measurement is then made tube by tube by placing 10µL of radioactive solution (containing the appropriate concentration of the corresponding substrate or inhibitors) on the inner wall of each eppendorf. Uptake measurement is started by vortexing the tube and enabling the radioactive solution and the cells to mix. Then, the measurement is ended by bringing incubation to an immediate stop with 1mL of stop buffer.

The incubation time is determined using a metronome, for 1 second measurements, or a timer for 15 seconds measurements. Once the incubation is stopped two centrifugation/washing steps with stop buffer are performed and then the cells are lysed and solubilized with 300µL of 0,5% Triton X-100, 100mM NaOH, mixed with 2mL of scintillation liquid, and put into the scintillation counter to determine the levels of intracellular radioactivity. 5µL of the radioactive medium are also counted per duplicate to be used as standards.

4.2.2 Protein measurement using the bradford assay

For short time measurements, cells are detached and split into eppendorf tubes before measuring their activity so the amount in protein in each eppendorf should be exactly the same. Therefore, it is not necessary to measure each experiment point protein individually.

When splitting the total amount of cells into eppendorfs two extra ones are filled to quantify the protein amount. These will be centrifuged to remove the NMDG medium and lysed with 300µL of 0,5% Triton X-100, 100mM NaOH. Then 5µL of the protein lysate will be quantified using the Bradford assay. The Bradford assay is based on an absorbance shift of the dye Coomassie Brilliant Blue from 465 to 595nm upon binding a protein.

Commercially available Biorad solution “Bio-Rad protein assay” (BioRad) is diluted 1:4 in distillate water and 1mL is added to each plastic cuvette containing the sample. In parallel a BSA calibration curve is prepared so the exact amount of protein per sample can be calculated. The absorbance is measured at 595nm.

4.2.3 Calculations

Calculations for detached cells uptake measurements are the same that those for cells grown in monolayers. The only difference is that in this case the dpms given by the beta-counter are the total dpms of the transport point as the protein is measured apart.

4.3 Oocytes

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. Before cRNA injection, the oocytes were incubated for 5–15 minutes in hyperosmolar Ori buffer (130mM NaCl). Oocytes expression vectors (pOG2 or pRSSP) containing hOCT1, hOCT2 or a polymorphic variant of hOCT1 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 10ng cRNAs in a volume of 50nL of H_2O. 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). Oocytes expressing hOCT1 or hOCT2 were incubated for 30 min at room temperature in ORI buffer containing [^{3}H]MPP$^+$ (10nM, 1µCi) or [^{3}H]zebularine (100nM, 1µCi). Non-injected oocytes from the same batch were used as control. After incubation in uptake buffer, oocytes were washed three times with ice-cold Ori buffer and solubilized in 5% SDS solution, and the intracellular radioactivity was analysed by liquid scintillation counting.

For efflux experiments, control oocytes and oocytes expressing hOCT1, hOCT2 or a polymorphic variant of hOCT1 were injected [^{3}H]MPP$^+$ (0.025 µCi, 6 nM) or [^{3}H] zebularine (0.013 µCi, 26.8 nM) and washed with ice-cold ORI buffer. The efflux was initiated by adding one oocyte to 100µL of ORI buffer with or without quinine 100 µM and the efflux rate was measured at different time points, taking samples and quantifying the radioactivity released in the buffer.

5

PROTEIN EXPRESSION ANALYSIS

5.1 Cell lysate obtention

Cellular lysates are obtained from confluent 60mm or 100mm plates. The growing medium of each plate is removed and the plates are washed twice with PBS making sure no medium traces are left. The plates can at this point be stored at -80ºC or the procedure can be continued by adding 100-150µL lysis buffer (50mM Tric-HCl pH 7,5; 150mM NaCl; 1% NP40; 5mM sodium pyrophosphate; 50mM NaF; 1mM Na_3VO_4 and proteases inhibitor) per plate. From this point all the process has to be done on ice or at 4ºC to avoid protein degradation. Using a scrapper, the cells are detached and the resulting suspension is collected in 1.5mL eppendorfs which are left for some minutes on ice. With a 20G needle the cell suspension is homogenised and left again on ice for 10 minutes, then the samples are centrifuged at 10,000rpms at 4ºC for 20 minutes. The resulting supernatants can be quantified following the Bradford protocol (4.2.2) and stored at -20ºC.

5.2 SDS-PAGE electrophoresis

Electrophoresis is a technique used for protein separation. When separated on a polyacrylamide gel, the procedure is called SDS-PAGE (Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis). SDS is an anionic detergent applied to protein sample to impart a negative charge to linearized proteins. The binding of SDS to the polypeptide chain imparts an even distribution of charge per unit mass, thereby resulting in a fractionation by approximate size during electrophoresis.

Polyacrylamide gels are formed from the polymerization of two compounds, acrylamide and N,N-methylenebisacrylamide (Bis). Bis is a cross-linking agent for the gels. The polymerization is initiated by the addition of ammonium persulfate along with TEMED. The separation of molecules within a gel is determined by the relative size of the pores formed within the gel. The gel is composed of two parts, the stacking gel,

which has a low acrilamide percentage (5%) and allows the alignment of the samples before they get in the resolving gel which will be in charge of the separation. The acrilamide concentration can be from 5% to 15% depending on the size of the protein of interest being gels of higher percentage more suitable for smaller proteins.

5.2.1 Buffers and gel

The following buffers have to be prepared:

1- Electrophoresis buffer 10X (before using it has to be dissolved 10 times with distilled water):

250mM	Tris-base
1.91M	glycine
1%	SDS

2- Loading buffer 5X :

62.5mM	Tris-HCl pH 6.8
2%	SDS
20%	glycerol
0.01%	bromophenol blue

Before using 100µL of ß-mercaptoethanol per 1mL of loading buffer is added to reduce the disulphide bonds.

To prepare the gel the resolving part is prepared first and the stacking part is added once the resolving is polymerised.

1- Resolving gel:

For 1 gel (10mL):

Depending on the desired percentage:

10%	3.3mL	30% acrilamide/bis-acrilamide
12%	4mL	
	2.5mL	Tris 1,5M pH 8.8
	0.1mL	10% SDS
	0.1mL	10% amonium persulfate
	4µL	TEMED
	up to 10mL	Water

2- Stacking gel:

For 1 gel (4mL):

0.67mL	30% acrilamide/bis-acrilamide
0.5mL	Tris 1M pH 6.8
40µL	10% amonium persulfate
40µL	10% SDS
4µL	TEMED
2.7mL	Water

5.2.2 Sample preparation

The amount of sample loaded depends on the experimental purpose, being 30µg the optimal amount for good hOCT1 or hCNT3 detection. Considering the total amount loaded (20-25µL), protein sample, loading buffer (5X) and water are mixed. Before loading them into the gel, the samples are incubated for 30 minutes at 37ºC to denature the proteins.

5.2.3 Gel running

To prepare the gel, the glasses have to be cleaned with ethanol and assembled in the plastic cassette. Once they are ready the resolving gel is prepared and added. To ensure a uniform gel, water is added over the gel until it is polymerised. After

polymerisation, the water is removed and the stacking gel is prepared an added together with the comb.

When the gel is ready, the combs are removed and the gel is introduced in the electrophoresis tank. The tank is filled with electrophoresis buffer and the samples, which have been previously prepared adding to the required amount of protein, loading buffer 5X and water up to 20-25µL, as well as a molecular weight marker (dual colour-Biorad) are loaded in the gel.

The gel is run at 40mA per gel until the front reaches the bottom of the gel (aprox. 90min).

5.3 Western blotting

Western blotting consists of the electrotransference of proteins previously separated by SDS-PAGE to a nitrocellulose membrane and its posterior detections by specific antibodies.

5.3.1 Buffers

1- Transfer buffer 10X (before using 100mL have to be dissolved with 700mL water and 200mL methanol):

250mM Tris-base

1.91M glycine

5.3.2 Transferring

Before starting preparing the transferring sandwich, membranes and whatman paper have to be cut 6cm x 9cm big. To activate the membrane, it has to be incubated for 15 seconds with methanol, followed with a 2 minute wash in distilled water and equilibration in transfer buffer.

The transferring is prepared submerged in transfer buffer following the order shown in figure M1. It is very important to make sure no bubbles are formed as they would effect the correct transferring.

The transferring is run at 100V for 90 minutes. An ice block is added to the tank and it is agitated during the transfer to prevent excessive heating.

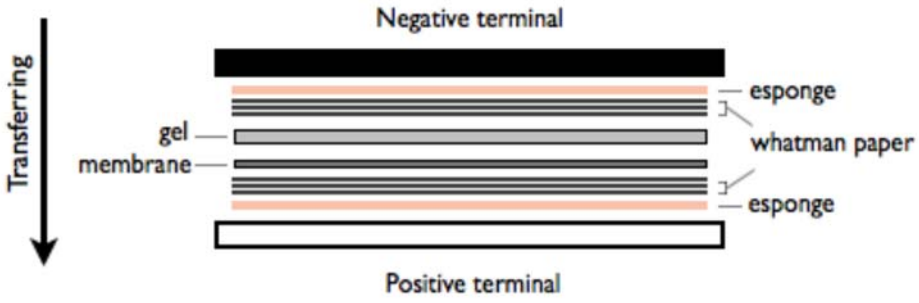


Figure 1 Transferring assembling for western blotting.

5.3.3 Ponceau staining

Ponceau staining is used for rapid reversible detection of protein bands in nitrocellulose. Once the transferring is over, the membrane is incubated for a few seconds with Ponceau stain. If the transferring was successful red protein bands will appear on the membrane. The stain can be easily removed by continued washing.

5.3.4 Immunodetection

The proteins are detected by using sequentially two antibodies. The first antibody is directed specifically to the target protein whether the second antibody binds to the first one amplifying the signal and allowing the detection due to a horseradish peroxidase binding to the heavy chain of the antibody.

5.3.4.1 Buffers

1- TBS-tween:

20mM	Tris-base
150mM	NaCl
0.1%	Tween
7.6	pH

2- Blocking solution:

50mL	TBS-Tween
2.5mg	powder milk

5.3.4.2 Blocking

After the stain is washed, the membrane is incubated with 10mL of blocking solution in plastic bags to block the non-specific binding sites. The blocking is performed for at least 1h at room temperature, being also possible to incubate the membrane overnight at 4ºC.

5.3.4.3 Antibody incubations

For the primary antibody incubation, once the blocking time is over, the membrane is transferred to a new plastic bag containing the solution with the primary antibody. In case of hOCT1 the antibody is prepared 1:5000 in blocking solution. For hCNT3 and actin, which is used as a loading control, it is prepared 1:2000 in blocking solution. The incubation can be left for 1h at room temperature or overnight at 4ºC in agitation.

Next, the membrane is washed 3x10 minutes with TBS-tween before incubating it with the secondary antibody. In our case, the secondary antibody used for hOCT1 and hCNT3 detection is anti-mouse and it is prepared in blocking solution 1:2000. For actin, an anti-rabbit antibody is used and it is also prepared 1:2000 in blocking solution. The incubation lasts 1h in agitation at room temperature. Before developing the gel it has to be washed with TBS-tween 3x10 minutes.

5.3.4.4 Gel development

The horseradish peroxidase linked to the secondary antibody can react and cause chemiluminescence which can be captured. To do so, ECL (Biological Industries) is used, following the protocol, solution A and solution B are mixed 1:1 preparing a total volume of 500µL per membrane. The membrane is covered with the mix and incubated for 1-3minutes at room temperature. The membrane is then developed using a LAS3000 camera.

6

POLYMERASE CHAIN REACTION (PCR) AND RELATED TECHNIQUES

6.1 Introduction

Polymerase chain reaction (PCR) is an in vitro technique (Mullis et al, 1986) that allows the enzymatic amplification of DNA. The method relies on thermal cycling, consisting of repeated heating and cooling of the reaction for DNA denaturalisation and enzymatic replication of the DNA. The reaction consists of a DNA template, a thermostable enzyme with DNA polymerase activity, dNTPs, a pair of primers and the reaction buffer. The mixture is placed into a thermal cycler where the reaction takes place. In each cycle, the DNA copies are duplicated so 20 PCR cycles represent approximately a million copies of the original template.

In this thesis, PCR techniques have been used to insert new restriction sites and subsequently re-clone insert into new plasmids, for site-directed mutagenesis and also in sequencing to check the correct sequences of our constructs.

6.2 Restriction sites introduction

To generate the stable cell lines expressing hOCT1 and its polymorphic variants, hOCT1 and the polymorphic variants inserts had to be re-cloned into pcDNA5 vector. As there were no compatible restriction sites, new sites where inserted by PCR. The primers used (Table 3) were designed by adding at the 5' segment of the oligonucleotide the desired restriction site followed by the number of nucleotides necessary for a correct function of the restriction enzyme (checked at www.neb.com). The restriction sites have to be chosen discarding the possibility that the insert contains the selected restriction site in the middle, and ensuring both endonuclease enzymes are compatible (also checked at www.neb.com).

The primers used need to have:

- Restriction site plus the needed extra bases at 5'.

- At least 15 bases from the original sequence at 5' (forward) or 3' (reverse) of the sequence of interest.

hOCT1fw KpnI	5'-CGGGGTACCATCATGCCCACCGTGGATGACA-3'
hOCT1rv BamHI	5'- CGCGGATCCCTCTCAGGTGCCCGAGGGTTC-3'

Table 3. Primers used for the introduction of restriction sites. Introduced restriction sites have been underlined in the oligonucelotide.

6.2.1 Procedure

When performing PCR it is very important to use globes, sterile solutions and sterile material in order to avoid any possible contaminations. This technique is very sensitive and it could easily amplify a wrong template and give a false positive. Thus, a tube control containing sterile water instead of the DNA template is always prepared to be used as a negative control.

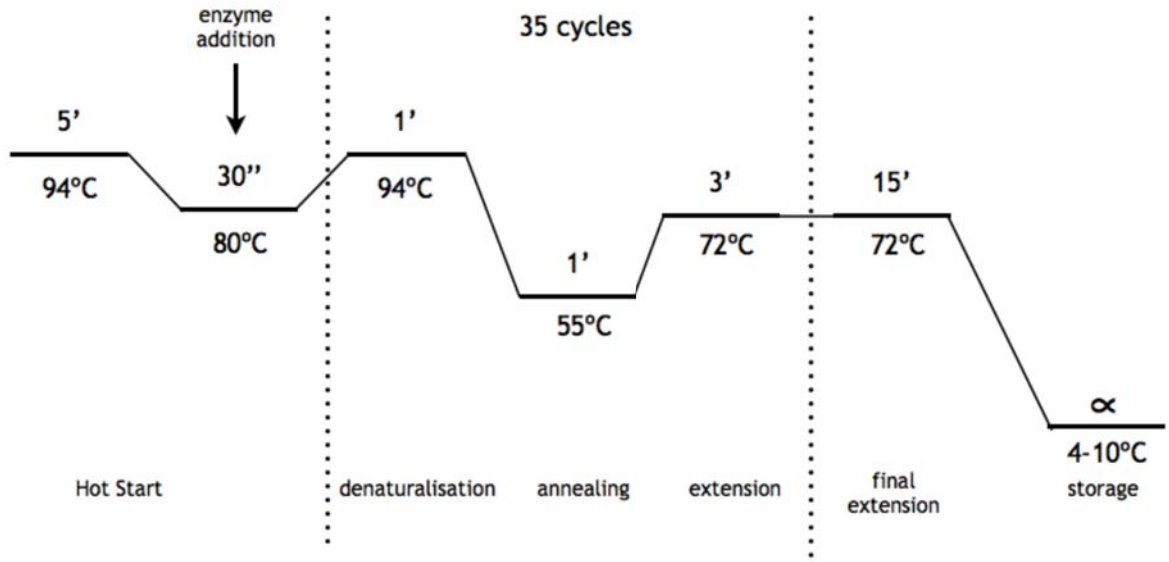
The reaction mixed contains:

5µL	Buffer 10X with $MgCl_2$
1µL	primer 20µM (forward and reverse)
1µL	dNTPs mix 10mM (2.5mM of dATP, dCTP, dGTP and dTTP)
1-5µg	DNA template
0.75µL	*Faststart High Fidelity Taq polymerase*
up to 50µL	milliQ water

The polymerase used for this reaction is the *Faststart High Fidelity Taq polymerase* (Roche) since being a high fidelity polymerase reduced the incidence of mutations appearance.

Before adding the Taq polymerase enzyme the sample is heated for 5min at 94ºC, known as hot start, to ensure the primers bind specifically. This is specially important when working with genomic DNA.

The PCR conditions used are:



The PCR product is purified using an agarose gel (see 7.4).

6.3 Site directed mutagenesis

hOCT1 polymorphic variants as well as hCNT3 mutations have been generated by site-directed mutagenesis to study structure-function relation. For this the *Pfu turbo* enzyme (Agilent Technologies) is used.

The primers used for site directed mutagenesis have to have the following characteristics:

- Both primers (forward and reverse) bind to the same sequence of the template.

- The primers have to be 25-45b long with a melting temperature (T_m)≥78ºC. If longer primers have to be used, the probability of forming secondary structures is higher.

- The desired mutations has to be at the middle of the primers with 10-15b of the correct sequence at each site.

- Ideally, primers should contain minimum 40% of CG and should have C or G at the terminal zones.

Primers used in this thesis to generate mutants are shown in table 4 and 5.

Mutation		Sequence (5'-3')
R61C	Fw	CTGGGGTGGCTGAGCTGAGCCAGTGCTGTGGCTGGAGCCCTGCGGAGG
	Rv	CCTCCGCAGGGCTCCAGCCACAGCACTGGCTCAGCTCAGCCACCCCAG
C88R	Fw	GGGCGAGGCCTTCCTTGGCCAGCGCAGGCGCTATGAAGTGGACTGG
	Rv	CCAGTCCACTTCATAGCGCCTGCGCTGGCCAAGGAAGGCCTCGCCC
F160L	Fw	GTCCTGTTTGAATGCGGGCTTCCTCTTTGGCTCTCTCGGTGTTGGC
	Rv	GCCAACACCGAGAGAGCCAAAGAGGAAGCCCGCATTCAAACAGGAC
S189L	Fw	GAACTGTGCTGGTCAACGCGGTGTTGGGCGTGCTCATGGCCTTCTCGCC
	Rv	GGCGAGAAGGCCATGAGCACGCCCAACACCGCGTTGACCAGCACAGTTC
P341L	Fw	CATTTGCAGACCTGTTCCGCACGCTGCGCCTGAGGAAGCGCACCTTC
	Rv	GAAGGTGCGCTTCCTCAGGCGCAGCGTGCGGAACAGGTCTGCAAATG
G401S	Fw	CCCTCATCACCATTGACCGCGTGAGCCGCATCTACCCCATGGCCATGTC
	Rv	GACATGGCCATGGGGTAGATGCGGCTCACGCGGTCAATGGTGATGAGGG
M408V	Fw	GGGCCGCATCTACCCCATGGCCGTGTCAAATTTGTTGGCGGGGGCAG
	Rv	CTGCCCCCGCCAACAAATTTGACACGGCCATGGGGTAGATGCGGCCC
M420Del	Fw	GGCGGGGGCAGCCTGCCTCGTCATTTTTATCTCACCTGACCTGC
	Rv	GCAGGTCAGGTGAGATAAAAATGACGAGGCAGGCTGCCCCCGCC
G465R	Fw	CCCCACATTCGTCAGGAACCTCCGAGTGATGGTGTGTTCCTCCCTG
	Rv	CAGGGAGGAACACACCATCACTCGGAGGTTCCTGACGAATGTGGGG

Table 4. Primers used for hOCT1 site-directed mutagenesis. Substituted nucleotides have been underlined.

Mutation		Sequence (5'-3')
N336A	Fw	CTGTAGTTGCTTCTGGCGCTATATTTGTTGGACAAAC
	Rv	GTTTGTCCAACAAATATAGCGCCAGAAGCAACTACAG
T370A	Fw	GACCGCCGGGTTCTCTGCCATTGCTGGAAGCGTGC
	Rv	GCACGCTTCCAGCAATGGCAGAGAACCCGGCGGTC
I371E	Fw	GCCGGGTTCTCTACCGAGGCTGGAAGCGTGCTAG
	Rv	CTAGCACGCTTCCAGCCTCGGTAGAGAACCCGGC
T605A	Fw	CGTGGCCTGCTTCATGGCAGCCTGCATCGCAGGC
	Rv	GCCTGCGATGCAGGCTGCCATGAAGCAGGCCACG

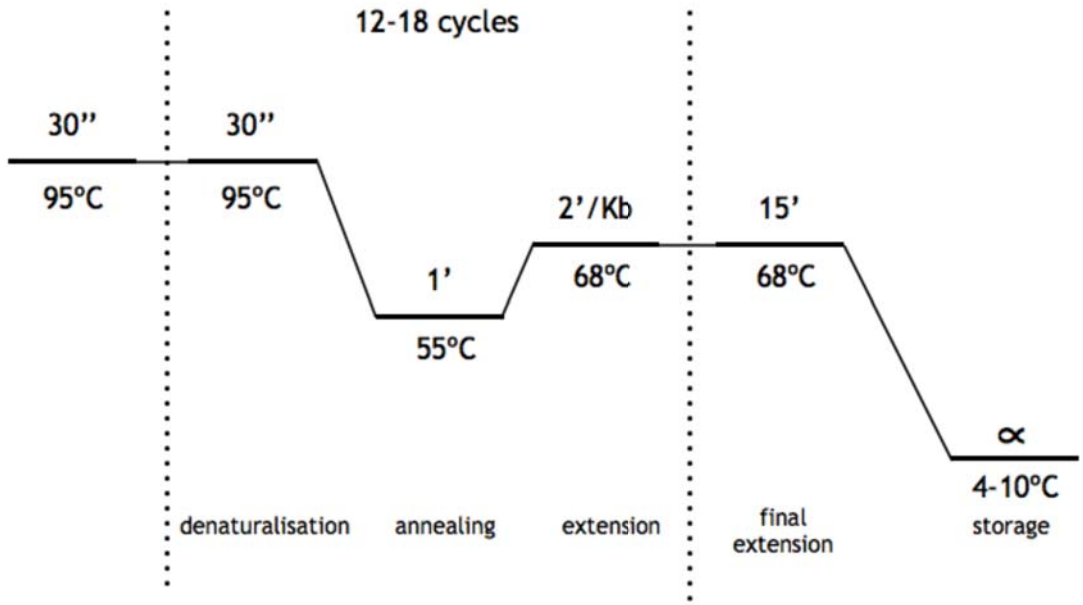
Table 5. Primers used for hCNT3 site-directed mutagenesis. Substituted nucleotides have been underlined.

The PCR reaction mix contains:

5µL	Buffer 10X
1µL	primer 20µM (forward and reverse)
1µL	dNTPs mix 10mM (2.5mM of dATP, dCTP, dGTP and dTTP)
100ng	DNA template
1µL	*Pfu turbo* enzyme
up to 50µL	milliQ water

Pfu turbo enzyme allows the generation of mutations with a high efficiency. To reduce the possible apparition of random mutations a low quantity of template is used and only a few PCR cycles are performed. The number of cycles can vary from 12 if the aim is only to generate a punctual mutation to 18 when multiple insertions or delations are being generated.

The PCR conditions are:



The PCR product is digested with *DpnI* endonuclease enzyme for 1h at 37ºC. *DpnI* digests methylated DNA so it eliminates the template DNA. The product is then transformed in competent bacteria (see 7.2).

6.4 PCR sequencing

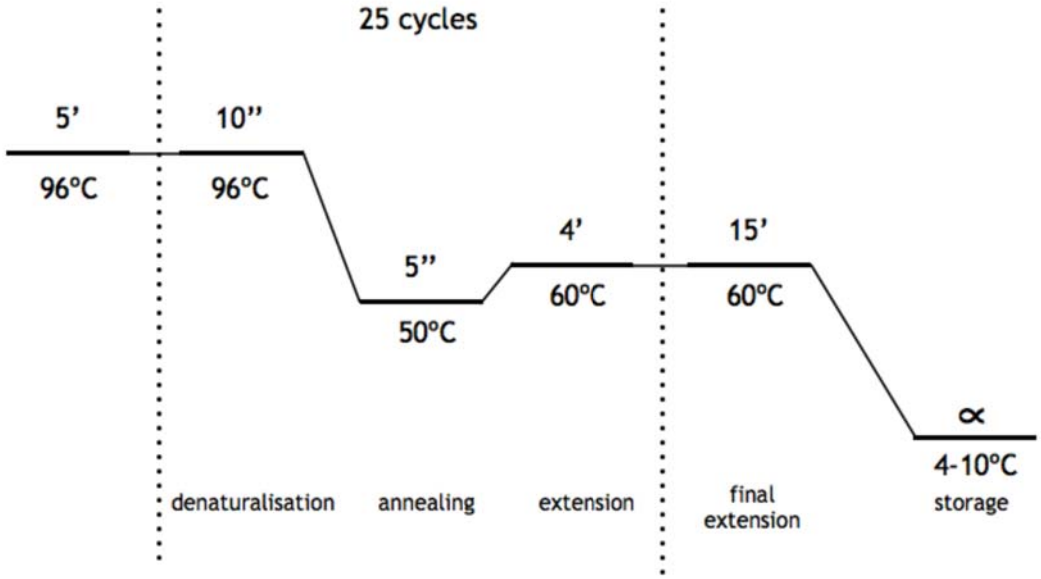
This technique is based on the use of fluorescent labelled dideoxynucleotides lacking the 3'-hydroxyl group, which terminate the elongation of the chain during the PCR reaction. This way, different size fragments of DNA are generated depending on the moment in which the labelled dideoxynucleotide is incorporated. The obtained mix of different size fragments are separated by capillary electrophoresis and excited at the base of the capillary with an excitation laser, causing the fluorescence emission. The fact that the different dideoxynucleotides (A, G, C and T) are labelled with different colours, generates an electrofluorogram which elucidates the DNA sequence.

The *Big Dye Terminator Cycle Sequencing Ready Reaction kit v.3.1* (Applied Biosystems) is used to perform the sequencing. An specific oligonucleotide either in 5'-3' or 3'-5' direction is used.

The reaction mix contains:

2µL	*Ready reaction mix*
3µL	*Big dye sequencing buffer*
1µL	Primer (10µM)
100ng/Kb	DNA template
up to 20µL	milliQ water

The PCR program used is:



The PCR product is sent to the Sequencing Platform to get the DNA sequence. The results are then compared to the original sequence using http://www.ebi.ac.uk/Tools/msa/clustalw2/ web page to align the sequences.

7

MOLECULAR BIOLOGY

This section contains all technics used to generate and amplify the DNA plasmids used in the thesis.

7.1 Competent bacteria

Competent bacteria are bacteria adapted for easier receiving of heterogeneous DNA. Such bacteria enable fast and highly efficient transformation of recombinant plasmids in molecular cloning. Bacteria used in this thesis are *Escherichia coli* XL1-blue generated in the laboratory and stored at -80ºC.

All the process has to be performed next to a Bunsen flame and using sterile material.

7.1.1 Media

- Luria-Broth medium (LB):

1% (W/v)	Tryptone
0.5%(w/v)	Yeast extract
1%(w/v)	NaCl

- SOB medium:

2%(w/v)	Tryptone
0.5%(w/v)	Yeast extract
10mM	NaCl
2,5mM	KCl

This medium is autoclaved and supplemented with previously filtrated:

2.5mL	$MgCl_2 \cdot 6H_2O$ 2M
2.5mL	$MgSO_4 \cdot 7H_2O$ 2M

- RF1 medium (has to be sterilised by filtration):

100mM	RbCl
50mM	$MnCl_2 \cdot 4H_2O$
30mM	KAc pH7,5
10mM	$CaCl_2$
15% (w/v)	glycerol

- RF2 medium (has to be sterilised by filtration):

10mM	MOPS pH 6.8
10mM	RbCl
75mM	$CaCl_2$
15% (w/v)	glycerol

7.1.2 Bacteria growth

E. coli X-Blue bacteria are grown overnight in 3mL LB buffer at 37ºC in a shaker. To ensure sterility, in parallel, a tube with no-bacteria is also incubated overnight under the same condition. The next morning, 1mL bacteria growth is inoculated in 50mL SOB in a sterile erlenmeyer and is left in the shaker at 37ºC. Every 15-30 minutes, aliquots of the medium are taken, always keeping its sterility, and the absorbance is determined at 550nm. When the optic density reaches 0.375-0.4, the culture is in exponential growth and optimal to obtain competent bacteria. At this point, the growth is split into two tubes and left on ice for 15 minutes. Then, the tubes are centrifuged at 3000rpm for 15minutes at 4ºC. The supernatant is discarded and the cells are resuspended with a third of the original volume with RF1. The tubes are subsequently incubated for 20minutes on ice and centrifuged again under the same conditions. This time, the pellet is resuspended with 1/12.5 of the original volume with RF2. Finally bacteria are incubated on ice for 15 minutes more and they are ready to be used for transformation or aliquoted and frozen at -80ºC.

7.2 Transformation

Transformation is a process by which exogenous genetic material can be introduced into a bacterial cell. *E. coli* X-Blue are submitted to a heat-shock which allows the introduction of foreign DNA into the bacteria.

Agar-LB plates are prepared:

1%(W/v)	Tryptone
0.5%(w/v)	Yeast extract
1%(w/v)	NaCl
15%	agar

The medium is supplemented with the selection antibiotic (ampicillin 30µg/mL or kanamycin 100µg/mL), autoclaved and split into plates and left to solidify.

Bacteria are thawed on ice. The volumes of bacteria and DNA depend on the origin of the experiment, volumes used are shown in Table 6. DNA is added to bacteria, mixed gently and left for 30 minutes on ice. The thermic shock is performed at 42ºC during 1 minute 45 seconds followed by 2 minutes on ice. Immediately after, 1mL LB supplemented with sterile 20mM $MgSO_4$ and incubated for 1h in the shaker at 37ºC.

	DNA	Bacteria	Seeding Volume
mini-midi prep transformation	1000µg (1-5µL)	50µL	150µL
ligation	total ligation volume (10µL)	100µL	Total Volume
mutagenesis	10µL	100µL	Total Volume

Table 6. Transformation volumes

Once the incubation is over, bacteria have grown and are ready to be plated. The seeding volume is plated on agar-LB plates with selection antibiotic to avoid the further growth of any bacteria which has not incorporated the plasmid. In case of ligation or mutagenesis, in which the total volume needs to be seeded, it is centrifuged at 5000rpm

for 5 minutes and the pellet is resuspended in 150µL and plated. The plates are incubated over night at 37ºC. The day after, individual colonies can be picked and grown again, this time in 3mL LB supplemented with the selection antibiotic in bacterial tubes overnight, shaking, at 37ºC.

For midi-prep obtention, a third growth is required this time 40mL LB supplemented with the selection antibiotic is inoculated with 60µL growth in a sterile erlenmeyer and left over night, shaking, at 37ºC.

7.3 Plasmid DNA isolation

The DNA isolation protocols can be classified depending on the amount and purity of DNA obtained. When the protocol is fast but a small amount of impure plasmid is obtained, it is called mini-prep. When a greater amount of purer plasmid is obtained it is called midi or maxi-prep.

To obtain a mini-prep, 2mL from a 3mL overnight growth are needed. The commercially available kit mini-prep (Promega) is used following the manufacturer's protocol. For midi-preps 40mL of an overnight growth are used. In this case, the NucleoBond midi-maxi-prep (Cultek) is used following the manufacturer's protocol. In brief, both protocols consist in re-suspending the bacterial pellets, realise and alkaline lysis and a neutralisation to discard cellular debris. Then, plasmid DNA binds to an anionic-exchange resin under the optimal conditions and it is washed several times to eliminate RNA, protein or any other impurities before being eluted. In case of midi-prep the DNA is precipitated with isopropanol and resuspended with sterile water.

7.4 Agarose gel

Agarose gel electrophoresis is an easy, fast and efficient way to separate and identify DNA fragments depending on its size but also to purify DNA fragments.

7.4.1 Buffers

- 5X TBE buffer (for 1L):

54g	Tris-base
27.5g	Boric acid
20mL	EDTA 0.5M pH8
uo to 1L	water

- 10X TAE buffer (for 1L):

48.4g	Tris-base
11.4mL	Glacial acetic acid
20mL	EDTA 0.5M pH8
uo to 1L	water

- Loading dye (10mL)

4g	sacarose
25mg	xylene cyanol
25mg	bromophenol blue
up to 10mL	water

7.4.2 Gel running procedure

The agarose gel can be prepared with TBE or TAE buffer depending on its purpose. TAE buffer is used when the DNA fragment has to be purified whereas TBE is used for other applications.

1% gels are prepared by dissolving 0.6g of agarose in 60mL TBE (0.5X) or TAE (1X) for the small gel or 1.2g in 120mL for the big gel in the microwave. In case the band of interest is smaller than 300pb higher percentages can be used. Once the solution cool down, 3µL or 6µL of *Sybr Safe DNA gel stain* (Invitrogen) is added. The mixture is poured into a cast and a comb is placed in the cast to create wells for loading the samples and left to polymerase.

The samples are mixed with loading dye 1:9 (loading dye:sample) and will be loaded concurrently with a molecular weigh marker. The gel is run at 100V until the bands are separated as desired. The gel can be developed under the UV light.

7.4.3 DNA band purification

DNA samples can be purified from an agarose gel by cutting the band with the help of a blade. The band is purified with a silica column (*QIAquick Gel Extraction Kit* (Qiagen)) following the manufacturers protocol.

7.5 Plasmid preparation

In order to generate HEK293 cells stably expressing hOCT1 or its polymorphic variants, the pcDNA5 vector containing all the constructs of interest had to be generated. For this, restriction sites are introduced at the 5' and 3' sites of the sequence of interest by PCR (see 6.2). The PCR product is purified with an agarose gel (see 7.4). The obtained insert with the terminal restriction sites and the pcDNA5 vector have to be digested with the same oligonucleotides and subsequently ligated.

7.5.1 Digestion with endonuclease enzymes

DNA enzymatic digestion allows the separation of the DNA fragments necessary to generate recombinant constructions leaving the specific terminal sequences which will facilitate its subsequently ligation.

In a tube the reaction components have to be mixed:

3µg	DNA
0.5µL	enzyme 1
0.5µL	enzyme 2 (0.2µL in case of *KpnI*
1µL	buffer 10X
up to 10µL	water

The buffer used depends on the enzymes required. The total amount of enzymes should never exceed 10% of total reaction volume otherwise the high amount of glycerol in which enzymes are dissolved would alter the reaction. In case the two enzymes required are not compatible sequential digestions will have to be performed. For most restriction endonucleases the optimal temperature is 37ºC. The incubation time is from 1-3h. Longer times are not recommended because of the enzymes star activity which digests non-specifically the DNA.

The digestion product can be purified using an agarose gel (see 7.4).

7.5.2 DNA ligation

DNA fragments ligation is performed with *T4 DNA ligase* enzyme (Promega) which catalyses the formation of a di-ester bond between a 5'-phosphate group and a 3'-hydroxyl group allowing the union of two DNA fragments, either with cohesive or blunt ends, obtained by previous enzymatic digestion (see 7.5.1). It is highly recommended to try different ratios vector:insert. To calculate the needed DNA insert for the reaction the following calculations need to be done:

$$ng_{vector} \frac{Kb_{insert}}{Kb_{vector}} = ng_{insert}$$

This will give us the value for a 1:1 ratio. For 1:3 ratio the value will have to be multiplied by three.

The reaction mix will contain:

1µL	10X ligation buffer
1µL	*T4 DNA ligase* enzyme
100ng	vector
x ng	insert
up to 10µL	water

The ratios used for the ligation will be 1:0 as a negative control, 1:1 and 1:3. The incubation will be left overnight at 16ºC. The ligation product will be transformed and amplified in competent bacteria (see 7.2, 7.3).

8

cDNA EXPRESSION ANALYSIS

8.1 RNA isolation

To purify RNA the "SV Total RNA Isolation System" (Promega) is used following the manufacturer's protocol. It is based on the combined effects of guanidine thiocyanate, beta-mercaptoethanol and SDS which allow the nucleic acids to be isolated essentially free of proteins such as RNases.

Confluent 60-100cm plates are used. After two washings with PBS the lysis buffer is added. In brief, after the centrifugation of the cell lysate, the RNA is selectively precipitated and purified with a silica column in which it is attached. After some washings the bound RNA is eluted with nuclease free water. Once eluted the RNA can be immediately used or stored at -80ºC to avoid its degradation.

To determine the quantity and quality of the obtained RNA, the absorbance of the RNA diluted in DEPC water is measured at 260nm and 280nm. Nucleic acids present maximal absorbance at 260nm so the value obtained at this wave length let us calculate the concentration of the purified RNA. At 280nm proteins present maximal absorbance. The ratio DO_{260}/DO_{280} should be between 1.8 and 2.0 to ensure the purity of the samples. This same procedure is used to quantify DNA and the ratio has to be between 1.8 and 2.0 too.

8.2 cDNA synthesis from RNA

To obtain cDNA the RNA obtained as explained above is used. The reactions synthesis requires a retro-transcriptase, in our case M-MLVRT, which comes from Moloney murine leukaemia virus, as well as primers. In our case, random hexanucleotides, which bind to various RNA sites generating short cDNAs, were used.

In total, 1µg of RNA is retro-transcribed to cDNA. The reaction is started by denaturalising de RNA at 65ºC for 5minutes, after that, the RNA is left on ice and the reaction mix is added. The reaction mix contains per sample:

1µM	DTT (Life Technologies)
625µM	dNTPs (Promega)
250ng	random primers (Life Technologies)
1X	Buffer first strand *(Life technologies)*
6U	M-MLVRT (Invitrogen)
0,8U	RNAsin (Promega)

The samples are then incubated for 2h at 37ºC followed by 10 minute incubation at 65ºC. The samples are now ready to be further used or stored at -20ºC.

8.3 Real-time PCR

The Real-time PCR technique (RT-PCR) is used to amplify and simultaneously quantify a targeted cDNA molecule. The quantity can be either an absolute number of copies or a relative amount when normalised to normalising genes. There are two common methods for the detection of products in quantitive PCR: (1) non specific fluorescent dies that intercalate with any double-stranded DNA such as SYBER Green and (2) sequence-specific DNA probes, consisting of specific oligonucleotides labelled with a fluorescent reporter. From these two options the latter is the most specific, giving more reliable and reproducible results and its the one used in this thesis.

The oligonucleotides used present two types of molecules linked to their structure: a fluorescent marker (reporter) at 5' and a quencher at 3'. When the probe is intact the proximity between the reporter and the quencher reduces the fluorescence of the reporter due to FRET (Förster resonance energy transfer). When the probe binds downstream one of the primers at the target sequence, it is degraded due to the 5'-nuclease activity of the Taq DNA polymerase separating the reporter form the quencher and subsequently increasing the fluorescence signal of the reporter. In each PCR cycle more reporter molecules are liberated, increasing the fluorescence in a quantity-related manner. One advantage of this technique is that the probe has to bind specifically to the target to generate a fluorescent signal. Thus, not generating any signalling if the probe binds incorrectly to the wrong sequence.

The primers used for the RT-PCR were purchased at Life technologies. The hOCT1 probe (Hs00427554) was used to detect hOCT1 cDNA expression and as endogenous control the gliceraldehyde-3-dehydrogenase (GAPDH) gene probe (4310884E) was used.

In each 96 multi-well plate well is loaded:

2µL cDNA

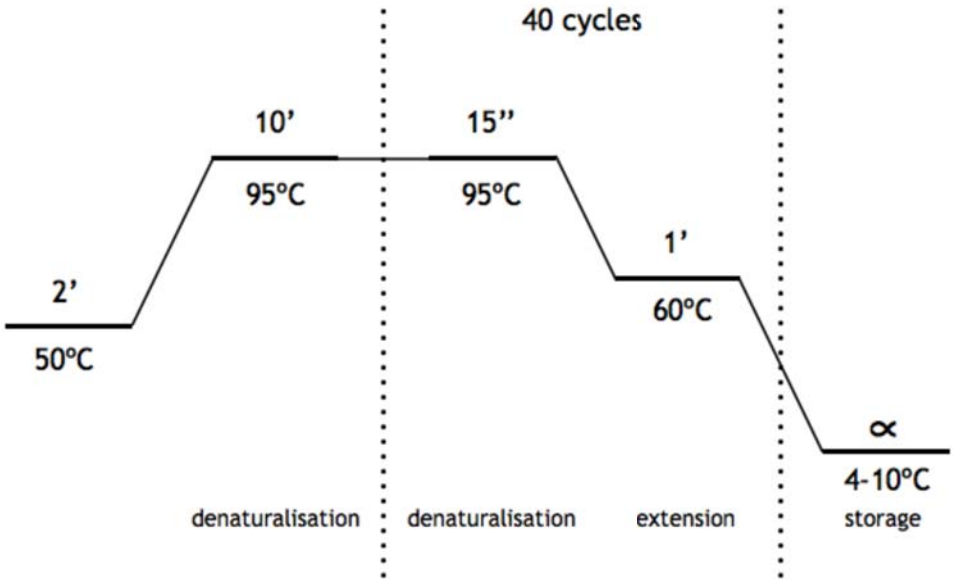
7µL water

10µL master mix 10X (*Life technologies*)

1µL probes 20X

A well containing water instead of cDNA is used as negative control to discard any contamination.

The RT-PCR conditions used are:



8.3.1 Data analysis

The RT-PCR results give us the Ct value. The Ct value is the number of cycles required for the fluorescent signal to exceed the background level. Ct levels are inversely proportional to the amount of target nucleic acid in the sample.

In this thesis the RT-PCR method has been used to compare the hOCT1 levels in HIV-1 infected PBMCs to those of PBMCs stimulated under the same conditions. The

results were first normalised with the endogenous control (GAPDH) and then compare it to the control sample in our case the non-infected stimulated PBMCs.

The calculations carried out are:

1- Calculate average Ct of each sample and the endogenous control.

2- Calculate ΔCt as Ct_{sample}-$Ct_{endogenous\ control}$

3- The ΔCt standard error (SE) can be calculated as $\sqrt{}(SE_{sample})$-($SE_{endogenous\ control}$)

4- Calculate ΔΔCt as ΔCt_{sample}-$\Delta Ct_{contol\ sample}$

5- To determine the expression of the sample related to the control sample in arbitrary units it is needed to calculate $2^{-\Delta\Delta Ct}$.

9

STATISTICS AND DATA REPRESENTATION

Data processing has been done with the softwares Microsoft Excel 2004 and Numbers'09. The graphic representation and the statistic tests have been performed with the GraphPad Prism 4.0 program.

Results shown in this thesis are the mean ± standard error of n experiments. Routinely, in each experiment three to four replicates per conditions have been measured. Statistic differences have been measured by t-Student tests, calculating the *p* value. Two groups are considered statistically different when $p<0.05$. [$p<0.05$ (*); $p<0.01$(**); $p<0.001$(***)].

REFERENCES

A

Ahlin G, Chen L, Lazorova L, Chen Y, Ianculescu AG, Davis RL, Giacomini KM and Artursson P (2011) Genotype-dependent effects of inhibitors of the organic cation transporter, OCT1: predictions of metformin interactions. *Pharmacogenomics J* **11**(6): 400-411.

Ahluwalia A, Yan P, Hurteau JA, Bigsby RM, Jung SH, Huang TH and Nephew KP (2001) DNA methylation and ovarian cancer. I. Analysis of CpG island hypermethylation in human ovarian cancer using differential methylation hybridization. *Gynecol Oncol* **82** (2): 261-268.

Aiba T, Sakurai Y, Tsukada S and Koizumi T (1995) Effects of probenecid and cimetidine on the renal excretion of 3'-azido-3'-deoxythymidine in rats. *J Pharmacol Exp Ther* **272** (1): 94-99.

Anderson PL, Kakuda TN and Lichtenstein KA (2004) The cellular pharmacology of nucleoside- and nucleotide-analogue reverse-transcriptase inhibitors and its relationship to clinical toxicities. *Clin Infect Dis* **38**(5): 743-753.

Aran JM and Plagemann PG (1992) Nucleoside transport-deficient mutants of PK-15 pig kidney cell line. *Biochim Biophys Acta* **1110**(1): 51-58.

Arndt P, Volk C, Gorboulev V, Budiman T, Popp C, Ulzheimer-Teuber I, Akhoundova A, Koppatz S, Bamberg E, Nagel G and Koepsell H (2001) Interaction of cations, anions, and weak base quinine with rat renal cation transporter rOCT2 compared with rOCT1. *Am J Physiol Renal Physiol* **281**(3): F454-468.

Aymerich I, Pastor-Anglada M and Casado FJ (2004) Long term endocrine regulation of nucleoside transporters in rat intestinal epithelial cells. *J Gen Physiol* **124**(5): 505-512.

B

Badagnani I, Chan W, Castro RA, Brett CM, Huang CC, Stryke D, Kawamoto M, Johns SJ, Ferrin TE, Carlson EJ, Burchard EG and Giacomini KM (2005) Functional analysis of genetic variants in the human concentrative nucleoside transporter 3 (CNT3; SLC28A3). *Pharmacogenomics J* **5**(3): 157-165.

Baldwin SA, Beal PR, Yao SY, King AE, Cass CE and Young JD (2004) The equilibrative nucleoside transporter family, SLC29. *Pflugers Arch* **447**(5): 735-743.

Bardeleben C, Sharma S, Reeve JR, Bassilian S, Frost P, Hoang B, Shi Y and Lichtenstein A (2013) Metabolomics identifies pyrimidine starvation as the mechanism of 5-aminoimidazole-4-carboxamide-1-beta-riboside-induced apoptosis in multiple myeloma cells. *Mol Cancer Ther* **12**(7): 1310-1321.

Barnes K, Dobrzynski H, Foppolo S, Beal PR, Ismat F, Scullion ER, Sun L, Tellez J, Ritzel MW, Claycomb WC, Cass CE, Young JD, Billeter-Clark R, Boyett MR and Baldwin SA (2006) Distribution and functional characterization of equilibrative nucleoside transporter-4, a novel cardiac adenosine transporter activated at acidic pH. *Circ Res* **99**(5): 510-519.

Billam M, Sobolewski MD and Davidson NE (2010) Effects of a novel DNA methyltransferase inhibitor zebularine on human breast cancer cells. *Breast Cancer Res Treat* **120**(3): 581-592.

Bleasby K, Hall LA, Perry JL, Mohrenweiser HW and Pritchard JB (2005) Functional consequences of single nucleotide polymorphisms in the human organic anion transporter hOAT1 (SLC22A6). *J Pharmacol Exp Ther* **314**(2): 923-931.

Brast S, Grabner A, Sucic S, Sitte HH, Hermann E, Pavenstadt H, Schlatter E and Ciarimboli G (2011) The cysteines of the extracellular loop are crucial for trafficking of human organic cation transporter 2 to the plasma membrane and are involved in oligomerization. *FASEB J* **26**(3): 976-986.

Brueckner B, Kuck D and Lyko F (2007) DNA methyltransferase inhibitors for cancer therapy. *Cancer J* **13**(1): 17-22.

C

Cano-Soldado P, Gorraitz E, Errasti-Murugarren E, Casado FJ, Lostao MP and Pastor-Anglada M (2011) Functional analysis of the human concentrative nucleoside transporter-1 variant hCNT1S546P provides insight into the sodium-binding pocket. *Am J Physiol Cell Physiol* **302**(1): C257-266.

Cano-Soldado P, Lorrayoz IM, Molina-Arcas M, Casado FJ, Martinez-Picado J, Lostao MP and Pastor-Anglada M (2004) Interaction of nucleoside inhibitors of HIV-1 reverse transcriptase with the concentrative nucleoside transporter-1 (SLC28A1). *Antivir Ther* **9**(6): 993-1002.

Cano-Soldado P and Pastor-Anglada M (2012) Transporters that translocate nucleosides and structural similar drugs: structural requirements for substrate recognition. *Med Res Rev* **32**(2): 428-457.

Cansev M (2006) Uridine and cytidine in the brain: their transport and utilization. *Brain Res Rev* **52**(2): 389-397.

Carr A and Cooper DA (2000) Adverse effects of antiretroviral therapy. *Lancet* **356**(9239): 1423-1430.

Ciarimboli G (2008) Organic cation transporters. *Xenobiotica* **38**(7-8): 936-971.

Ciarimboli G, Ludwig T, Lang D, Pavenstadt H, Koepsell H, Piechota HJ, Haier J, Jaehde U, Zisowsky J and Schlatter E (2005) Cisplatin nephrotoxicity is critically mediated via the human organic cation transporter 2. *Am J Pathol* **167**(6): 1477-1484.

Cihlar T, Lin DC, Pritchard JB, Fuller MD, Mendel DB and Sweet DH (1999) The antiviral nucleotide analogs cidofovir and adefovir are novel substrates for human and rat renal organic anion transporter 1. *Mol Pharmacol* **56**(3): 570-580.

Cliffe ST, Kramer JM, Hussain K, Robben JH, de Jong EK, de Brouwer AP, Nibbeling E, Kamsteeg EJ, Wong M, Prendiville J, James C, Padidela R, Becknell C, van Bokhoven H, Deen PM, Hennekam RC, Lindeman R, Schenck A, Roscioli T and Buckley MF (2009) SLC29A3 gene is mutated in pigmented hypertrichosis with insulin-dependent diabetes mellitus syndrome and interacts with the insulin signaling pathway. *Hum Mol Genet* **18** (12): 2257-2265.

Crossman LC, Druker BJ, Deininger MW, Pirmohamed M, Wang L and Clark RE (2005) hOCT 1 and resistance to imatinib. *Blood* **106**(3): 1133-1134; author reply 1134.

Cha SH, Sekine T, Fukushima JI, Kanai Y, Kobayashi Y, Goya T and Endou H (2001) Identification and characterization of human organic anion transporter 3 expressing predominantly in the kidney. *Mol Pharmacol* **59**(5): 1277-1286.

Chang C, Swaan PW, Ngo LY, Lum PY, Patil SD and Unadkat JD (2004) Molecular requirements of the human nucleoside transporters hCNT1, hCNT2, and hENT1. *Mol Pharmacol* **65**(3): 558-570.

Chen L, Pawlikowski B, Schlessinger A, More SS, Stryke D, Johns SJ, Portman MA, Chen E, Ferrin TE, Sali A and Giacomini KM (2010) Role of organic cation transporter 3 (SLC22A3) and its missense variants in the pharmacologic action of metformin. *Pharmacogenet Genomics* **20**(11): 687-699.

Chen Y, Li S, Brown C, Cheatham S, Castro RA, Leabman MK, Urban TJ, Chen L, Yee SW, Choi JH, Huang Y, Brett CM, Burchard EG and Giacomini KM (2009) Effect of genetic variation in the organic cation transporter 2 on the renal elimination of metformin. *Pharmacogenet Genomics* **19**(7): 497-504.

Cheng JC, Yoo CB, Weisenberger DJ, Chuang J, Wozniak C, Liang G, Marquez VE, Greer S, Orntoft TF, Thykjaer T and Jones PA (2004) Preferential response of cancer cells to zebularine. *Cancer Cell* **6**(2): 151-158.

Chevaliez S, Brillet R, Lazaro E, Hezode C and Pawlotsky JM (2007) Analysis of ribavirin mutagenicity in human hepatitis C virus infection. *J Virol* **81**(14): 7732-7741.

D

Doehring A, Hofmann WP, Schlecker C, Zeuzem S, Sarrazin C, Berg T, Muller T, Herrmann E, Geisslinger G and Lotsch J (2011) Role of nucleoside transporters SLC28A2/3 and SLC29A1/2 genetics in ribavirin therapy: protection against anemia in patients with chronic hepatitis C. *Pharmacogenet Genomics* **21**(5): 289-296.

Dragan Y, Valdes R, Gomez-Angelats M, Felipe A, Javier Casado F, Pitot H and Pastor-Anglada M (2000) Selective loss of nucleoside carrier expression in rat hepatocarcinomas. *Hepatology* **32**(2): 239-246.

E

Eeles RA, Kote-Jarai Z, Giles GG, Olama AA, Guy M, Jugurnauth SK, Mulholland S, Leongamornlert DA, Edwards SM, Morrison J, Field HI, Southey MC, Severi G, Donovan JL, Hamdy FC, Dearnaley DP, Muir KR, Smith C, Bagnato M, Ardern-Jones AT, Hall AL, O'Brien LT, Gehr-Swain BN, Wilkinson RA, Cox A, Lewis S, Brown PM, Jhavar SG, Tymrakiewicz M, Lophatananon A, Bryant SL, Horwich A, Huddart RA, Khoo VS, Parker CC, Woodhouse CJ, Thompson A, Christmas T, Ogden C, Fisher C, Jamieson C, Cooper CS, English DR, Hopper JL, Neal DE and Easton DF (2008) Multiple newly identified loci associated with prostate cancer susceptibility. *Nat Genet* **40**(3): 316-321.

Engel K and Wang J (2005) Interaction of organic cations with a newly identified plasma membrane monoamine transporter. *Mol Pharmacol* **68**(5): 1397-1407.

Engel K, Zhou M and Wang J (2004) Identification and characterization of a novel monoamine transporter in the human brain. *J Biol Chem* **279**(48): 50042-50049.

Erdman AR, Mangravite LM, Urban TJ, Lagpacan LL, Castro RA, de la Cruz M, Chan W, Huang CC, Johns SJ, Kawamoto M, Stryke D, Taylor TR, Carlson EJ, Ferrin TE, Brett CM, Burchard EG and Giacomini KM (2006) The human organic anion transporter 3 (OAT3; SLC22A8): genetic variation and functional genomics. *Am J Physiol Renal Physiol* **290**(4): F905-912.

Errasti-Murugarren E, Cano-Soldado P, Pastor-Anglada M and Casado FJ (2008) Functional characterization of a nucleoside-derived drug transporter variant (hCNT3C602R) showing altered sodium-binding capacity. *Mol Pharmacol* **73**(2): 379-386.

Errasti-Murugarren E, Casado FJ and Pastor-Anglada M (2010a) Different N-terminal motifs determine plasma membrane targeting of the human concentrative nucleoside transporter 3 in polarized and nonpolarized cells. *Mol Pharmacol* **78**(5): 795-803.

Errasti-Murugarren E, Diaz P, Godoy V, Riquelme G and Pastor-Anglada M (2011) Expression and distribution of nucleoside transporter proteins in the human syncytiotrophoblast. *Mol Pharmacol* **80**(5): 809-817.

Errasti-Murugarren E, Molina-Arcas M, Casado FJ and Pastor-Anglada M (2010b) The human concentrative nucleoside transporter-3 C602R variant shows impaired sorting to lipid rafts and altered specificity for nucleoside-derived drugs. *Mol Pharmacol* **78**(2): 157-165.

Errasti-Murugarren E and Pastor-Anglada M (2010) Drug transporter pharmacogenetics in nucleoside-based therapies. *Pharmacogenomics* **11**(6): 809-841.

Errasti-Murugarren E, Pastor-Anglada M and Casado FJ (2007) Role of CNT3 in the transepithelial flux of nucleosides and nucleoside-derived drugs. *J Physiol* **582**(Pt 3): 1249-1260.

Esposito F, Corona A and Tramontano E (2012) HIV-1 Reverse Transcriptase Still Remains a New Drug Target: Structure, Function, Classical Inhibitors, and New Inhibitors with Innovative Mechanisms of Actions. *Mol Biol Int* **2012**: 586401.

Ewald B, Sampath D and Plunkett W (2008) Nucleoside analogs: molecular mechanisms signaling cell death. *Oncogene* **27**(50): 6522-6537.

F

Farre X, Guillen-Gomez E, Sanchez L, Hardisson D, Plaza Y, Lloberas J, Casado FJ, Palacios J and Pastor-Anglada M (2004) Expression of the nucleoside-derived drug transporters hCNT1, hENT1 and hENT2 in gynecologic tumors. *Int J Cancer* **112**(6): 959-966.

Fernandez-Calotti P and Pastor-Anglada M (2010) All-trans-retinoic acid promotes trafficking of human concentrative nucleoside transporter-3 (hCNT3) to the plasma membrane by a TGF-beta1-mediated mechanism. *J Biol Chem* **285**(18): 13589-13598.

Fernandez-Calotti PX, Lopez-Guerra M, Colomer D and Pastor-Anglada M (2012) Enhancement of fludarabine sensitivity by all-trans-retinoic acid in chronic lymphocytic leukemia cells. *Haematologica* **97**(6): 943-951.

Filipski KK, Mathijssen RH, Mikkelsen TS, Schinkel AH and Sparreboom A (2009) Contribution of organic cation transporter 2 (OCT2) to cisplatin-induced nephrotoxicity. *Clin Pharmacol Ther* **86**(4): 396-402.

Fotoohi AK, Lindqvist M, Peterson C and Albertioni F (2006) Impaired transport as a mechanism of resistance to thiopurines in human T-lymphoblastic leukemia cells. *Nucleosides Nucleotides Nucleic Acids* **25**(9-11): 1039-1044.

Fujita T, Brown C, Carlson EJ, Taylor T, de la Cruz M, Johns SJ, Stryke D, Kawamoto M, Fujita K, Castro R, Chen CW, Lin ET, Brett CM, Burchard EG, Ferrin TE, Huang CC, Leabman MK and Giacomini KM (2005) Functional analysis of polymorphisms in the organic anion transporter, SLC22A6 (OAT1). *Pharmacogenet Genomics* **15**(4): 201-209.

G

Galmarini CM, Mackey JR and Dumontet C (2002) Nucleoside analogues and nucleobases in cancer treatment. *Lancet Oncol* **3**(7): 415-424.

Ganapathy ME, Huang W, Wang H, Ganapathy V and Leibach FH (1998) Valacyclovir: a substrate for the intestinal and renal peptide transporters PEPT1 and PEPT2. *Biochem Biophys Res Commun* **246**(2): 470-475.

Garcia-Manero G (2012) Myelodysplastic syndromes: 2012 update on diagnosis, risk-stratification, and management. *Am J Hematol* **87**(7): 692-701.

Gati WP, Paterson AR, Larratt LM, Turner AR and Belch AR (1997) Sensitivity of acute leukemia cells to cytarabine is a correlate of cellular es nucleoside transporter site content measured by flow cytometry with SAENTA-fluorescein. *Blood* **90**(1): 346-353.

Ghai V, Sharma K, Abbi KK, Shimko S and Epner EM (2013) Current approaches to epigenetic therapy for the treatment of mantle cell lymphoma. *Adv Exp Med Biol* **779**: 257-266.

Giacomini KM, Huang SM, Tweedie DJ, Benet LZ, Brouwer KL, Chu X, Dahlin A, Evers R, Fischer V, Hillgren KM, Hoffmaster KA, Ishikawa T, Keppler D, Kim RB, Lee CA, Niemi M, Polli JW, Sugiyama Y, Swaan PW, Ware JA, Wright SH, Yee SW, Zamek-Gliszczynski MJ and Zhang L (2010) Membrane transporters in drug development. *Nat Rev Drug Discov* **9** (3): 215-236.

Giannoudis A, Wang L, Jorgensen AL, Xinarianos G, Davies A, Pushpakom S, Liloglou T, Zhang JE, Austin G, Holyoake TL, Foroni L, Kottaridis PD, Muller MC, Pirmohamed M and

Clark RE (2013) The hOCT1 SNPs M420del and M408V alter imatinib uptake and M420del modifies clinical outcome in imatinib-treated chronic myeloid leukemia. *Blood* **121**(4): 628-637.

Giovannetti E, Del Tacca M, Mey V, Funel N, Nannizzi S, Ricci S, Orlandini C, Boggi U, Campani D, Del Chiaro M, Iannopollo M, Bevilacqua G, Mosca F and Danesi R (2006) Transcription analysis of human equilibrative nucleoside transporter-1 predicts survival in pancreas cancer patients treated with gemcitabine. *Cancer Res* **66**(7): 3928-3935.

Gloeckner-Hofmann K, Guillen-Gomez E, Schmidtgen C, Porstmann R, Ziegler R, Stoss O, Casado FJ, Ruschoff J and Pastor-Anglada M (2006) Expression of the high-affinity fluoropyrimidine-preferring nucleoside transporter hCNT1 correlates with decreased disease-free survival in breast cancer. *Oncology* **70**(3): 238-244.

Gorboulev V, Ulzheimer JC, Akhoundova A, Ulzheimer-Teuber I, Karbach U, Quester S, Baumann C, Lang F, Busch AE and Koepsell H (1997) Cloning and characterization of two human polyspecific organic cation transporters. *DNA Cell Biol* **16**(7): 871-881.

Gorbunov D, Gorboulev V, Shatskaya N, Mueller T, Bamberg E, Friedrich T and Koepsell H (2008) High-affinity cation binding to organic cation transporter 1 induces movement of helix 11 and blocks transport after mutations in a modeled interaction domain between two helices. *Mol Pharmacol* **73**(1): 50-61.

Govindarajan R, Bakken AH, Hudkins KL, Lai Y, Casado FJ, Pastor-Anglada M, Tse CM, Hayashi J and Unadkat JD (2007) In situ hybridization and immunolocalization of concentrative and equilibrative nucleoside transporters in the human intestine, liver, kidneys, and placenta. *Am J Physiol Regul Integr Comp Physiol* **293**(5): R1809-1822.

Govindarajan R, Endres CJ, Whittington D, LeCluyse E, Pastor-Anglada M, Tse CM and Unadkat JD (2008) Expression and hepatobiliary transport characteristics of the concentrative and equilibrative nucleoside transporters in sandwich-cultured human hepatocytes. *Am J Physiol Gastrointest Liver Physiol* **295**(3): G570-580.

Govindarajan R, Leung GP, Zhou M, Tse CM, Wang J and Unadkat JD (2009) Facilitated mitochondrial import of antiviral and anticancer nucleoside drugs by human equilibrative nucleoside transporter-3. *Am J Physiol Gastrointest Liver Physiol* **296**(4): G910-922.

Gray JH, Mangravite LM, Owen RP, Urban TJ, Chan W, Carlson EJ, Huang CC, Kawamoto M, Johns SJ, Stryke D, Ferrin TE and Giacomini KM (2004) Functional and genetic diversity in the concentrative nucleoside transporter, CNT1, in human populations. *Mol Pharmacol* **65**(3): 512-519.

Gros C, Fahy J, Halby L, Dufau I, Erdmann A, Gregoire JM, Ausseil F, Vispe S and Arimondo PB (2012) DNA methylation inhibitors in cancer: recent and future approaches. *Biochimie* **94**(11): 2280-2296.

Guallar JP, Cano-Soldado P, Aymerich I, Domingo JC, Alegre M, Domingo P, Villarroya F, Javier Casado F, Giralt M and Pastor-Anglada M (2007) Altered expression of nucleoside transporter genes (SLC28 and SLC29) in adipose tissue from HIV-1-infected patients. *Antivir Ther* **12**(6): 853-863.

Gupta S, Wulf G, Henjakovic M, Koepsell H, Burckhardt G and Hagos Y (2012) Human organic cation transporter 1 is expressed in lymphoma cells and increases susceptibility to irinotecan and paclitaxel. *J Pharmacol Exp Ther* **341**(1): 16-23.

H

Hazen R and Lanier ER (2003) Relative anti-HIV-1 efficacy of lamivudine and emtricitabine in vitro is dependent on cell type. *J Acquir Immune Defic Syndr* **32**(3): 255-258.

He L, Vasiliou K and Nebert DW (2009) Analysis and update of the human solute carrier (SLC) gene superfamily. *Hum Genomics* **3**(2): 195-206.

Herraez E, Lozano E, Macias RI, Vaquero J, Bujanda L, Banales JM, Marin JJ and Briz O (2013) Expression of SLC22A1 variants may affect the response of hepatocellular carcinoma and cholangiocarcinoma to sorafenib. *Hepatology* **58**(3): 1065-1073.

Ho ES, Lin DC, Mendel DB and Cihlar T (2000) Cytotoxicity of antiviral nucleotides adefovir and cidofovir is induced by the expression of human renal organic anion transporter 1. *J Am Soc Nephrol* **11**(3): 383-393.

Hollenbach PW, Nguyen AN, Brady H, Williams M, Ning Y, Richard N, Krushel L, Aukerman SL, Heise C and MacBeth KJ (2010) A comparison of azacitidine and decitabine activities in acute myeloid leukemia cell lines. *PLoS One* **5**(2): e9001.

Hosoyamada M, Sekine T, Kanai Y and Endou H (1999) Molecular cloning and functional expression of a multispecific organic anion transporter from human kidney. *Am J Physiol* **276**(1 Pt 2): F122-128.

Hoy SM (2012) Bendamustine: a review of its use in the management of chronic lymphocytic leukaemia, rituximab-refractory indolent non-Hodgkin's lymphoma and multiple myeloma. *Drugs* **72**(14): 1929-1950.

Hubeek I, Stam RW, Peters GJ, Broekhuizen R, Meijerink JP, van Wering ER, Gibson BE, Creutzig U, Zwaan CM, Cloos J, Kuik DJ, Pieters R and Kaspers GJ (2005) The human

equilibrative nucleoside transporter 1 mediates in vitro cytarabine sensitivity in childhood acute myeloid leukaemia. *Br J Cancer* **93**(12): 1388-1394.

Huber-Ruano I, Errasti-Murugarren E, Godoy V, Vera A, Andreu AL, Garcia-Arumi E, Marti R and Pastor-Anglada M Functional outcome of a novel SLC29A3 mutation identified in a patient with H syndrome. *Biochem Biophys Res Commun* **428**(4): 532-537.

Huber-Ruano I and Pastor-Anglada M (2009) Transport of nucleoside analogs across the plasma membrane: a clue to understanding drug-induced cytotoxicity. *Curr Drug Metab* **10**(4): 347-358.

Huber-Ruano I, Pinilla-Macua I, Torres G, Casado FJ and Pastor-Anglada M Link between high-affinity adenosine concentrative nucleoside transporter-2 (CNT2) and energy metabolism in intestinal and liver parenchymal cells. *J Cell Physiol* **225**(2): 620-630.

J

Jilek BL, Zarr M, Sampah ME, Rabi SA, Bullen CK, Lai J, Shen L and Siliciano RF (2012) A quantitative basis for antiretroviral therapy for HIV-1 infection. *Nat Med* **18**(3): 446-451. Johnson ZL, Cheong CG and Lee SY (2012) Crystal structure of a concentrative nucleoside transporter from Vibrio cholerae at 2.4 A. *Nature* **483**(7390): 489-493.

Jordheim LP, Durantel D, Zoulim F and Dumontet C (2013) Advances in the development of nucleoside and nucleotide analogues for cancer and viral diseases. *Nat Rev Drug Discov* **12**(6): 447-464.

Jung N, Lehmann C, Rubbert A, Knispel M, Hartmann P, van Lunzen J, Stellbrink HJ, Faetkenheuer G and Taubert D (2008) Relevance of the organic cation transporters 1 and 2 for antiretroviral drug therapy in human immunodeficiency virus infection. *Drug Metab Dispos* **36**(8): 1616-1623.

K

Kang HJ, Song IS, Shin HJ, Kim WY, Lee CH, Shim JC, Zhou HH, Lee SS and Shin JG (2007) Identification and functional characterization of genetic variants of human organic cation transporters in a Korean population. *Drug Metab Dispos* **35**(4): 667-675.

Keller T, Egenberger B, Gorboulev V, Bernhard F, Uzelac Z, Gorbunov D, Wirth C, Koppatz S, Dotsch V, Hunte C, Sitte HH and Koepsell H (2011) The large extracellular loop of organic cation transporter 1 influences substrate affinity and is pivotal for oligomerization. *J Biol Chem* **286**(43): 37874-37886.

Kerb R, Brinkmann U, Chatskaia N, Gorbunov D, Gorboulev V, Mornhinweg E, Keil A, Eichelbaum M and Koepsell H (2002) Identification of genetic variations of the human organic cation transporter hOCT1 and their functional consequences. *Pharmacogenetics* **12**(8): 591-595.

Koepsell H, Lips K and Volk C (2007) Polyspecific organic cation transporters: structure, function, physiological roles, and biopharmaceutical implications. *Pharm Res* **24**(7): 1227-1251.

Kong W, Engel K and Wang J (2004) Mammalian nucleoside transporters. *Curr Drug Metab* **5**(1): 63-84.

L

Lai Y, Bakken AH and Unadkat JD (2002) Simultaneous expression of hCNT1-CFP and hENT1-YFP in Madin-Darby canine kidney cells. Localization and vectorial transport studies. *J Biol Chem* **277**(40): 37711-37717.

Lamont EB and Schilsky RL (1999) The oral fluoropyrimidines in cancer chemotherapy. *Clin Cancer Res* **5**(9): 2289-2296.

Landowski CP, Song X, Lorenzi PL, Hilfinger JM and Amidon GL (2005) Floxuridine amino acid ester prodrugs: enhancing Caco-2 permeability and resistance to glycosidic bond metabolism. *Pharm Res* **22**(9): 1510-1518.

Larrayoz IM, Casado FJ, Pastor-Anglada M and Lostao MP (2004) Electrophysiological characterization of the human Na(+)/nucleoside cotransporter 1 (hCNT1) and role of adenosine on hCNT1 function. *J Biol Chem* **279**(10): 8999-9007.

Lazar A, Grundemann D, Berkels R, Taubert D, Zimmermann T and Schomig E (2003) Genetic variability of the extraneuronal monoamine transporter EMT (SLC22A3). *J Hum Genet* **48**(5): 226-230.

Lazar A, Walitza S, Jetter A, Gerlach M, Warnke A, Herpertz-Dahlmann B, Grundemann D, Grimberg G, Schulz E, Remschmidt H, Wewetzer C and Schomig E (2008) Novel mutations of the extraneuronal monoamine transporter gene in children and adolescents with obsessive-compulsive disorder. *Int J Neuropsychopharmacol* **11**(1): 35-48.

Lazar A, Zimmermann T, Koch W, Grundemann D, Schomig A, Kastrati A and Schomig E (2006) Lower prevalence of the OCT2 Ser270 allele in patients with essential hypertension. *Clin Exp Hypertens* **28**(7): 645-653.

Leabman MK, Huang CC, Kawamoto M, Johns SJ, Stryke D, Ferrin TE, DeYoung J, Taylor T, Clark AG, Herskowitz I and Giacomini KM (2002) Polymorphisms in a human kidney

xenobiotic transporter, OCT2, exhibit altered function. *Pharmacogenetics* **12**(5): 395-405.

Leoni LM (2011) Bendamustine: rescue of an effective antineoplastic agent from the mid-twentieth century. *Semin Hematol* **48 Suppl 1**: S4-11.

Leoni LM, Bailey B, Reifert J, Bendall HH, Zeller RW, Corbeil J, Elliott G and Niemeyer CC (2008) Bendamustine (Treanda) displays a distinct pattern of cytotoxicity and unique mechanistic features compared with other alkylating agents. *Clin Cancer Res* **14** (1): 309-317.

Leung GP and Tse CM (2007) The role of mitochondrial and plasma membrane nucleoside transporters in drug toxicity. *Expert Opin Drug Metab Toxicol* **3**(5): 705-718.

Leyssen P, De Clercq E and Neyts J (2008) Molecular strategies to inhibit the replication of RNA viruses. *Antiviral Res* **78**(1): 9-25.

Li JY, Boado RJ and Pardridge WM (2001) Cloned blood-brain barrier adenosine transporter is identical to the rat concentrative Na+ nucleoside cotransporter CNT2. *J Cereb Blood Flow Metab* **21**(8): 929-936.

Li L, Koo SH, Hong IH and Lee EJ (2009) Identification of functional promoter haplotypes of human concentrative nucleoside transporter 2, hCNT2 (SLC28A2). *Drug Metab Pharmacokinet* **24**(2): 161-166.

Li L, Tan CM, Koo SH, Chong KT and Lee EJ (2007) Identification and functional analysis of variants in the human concentrative nucleoside transporter 2, hCNT2 (SLC28A2) in Chinese, Malays and Indians. *Pharmacogenet Genomics* **17**(9): 783-786.

Li LH, Olin EJ, Buskirk HH and Reineke LM (1970) Cytotoxicity and mode of action of 5-azacytidine on L1210 leukemia. *Cancer Res* **30**(11): 2760-2769.

Lips KS, Volk C, Schmitt BM, Pfeil U, Arndt P, Miska D, Ermert L, Kummer W and Koepsell H (2005) Polyspecific cation transporters mediate luminal release of acetylcholine from bronchial epithelium. *Am J Respir Cell Mol Biol* **33**(1): 79-88.

Loewen SK, Ng AM, Yao SY, Cass CE, Baldwin SA and Young JD (1999) Identification of amino acid residues responsible for the pyrimidine and purine nucleoside specificities of human concentrative Na(+) nucleoside cotransporters hCNT1 and hCNT2. *J Biol Chem* **274**(35): 24475-24484.

M

Mangravite LM, Badagnani I and Giacomini KM (2003a) Nucleoside transporters in the disposition and targeting of nucleoside analogs in the kidney. *Eur J Pharmacol* **479** (1-3): 269-281.

Mangravite LM, Xiao G and Giacomini KM (2003b) Localization of human equilibrative nucleoside transporters, hENT1 and hENT2, in renal epithelial cells. *Am J Physiol Renal Physiol* **284**(5): F902-910.

Marce S, Molina-Arcas M, Villamor N, Casado FJ, Campo E, Pastor-Anglada M and Colomer D (2006) Expression of human equilibrative nucleoside transporter 1 (hENT1) and its correlation with gemcitabine uptake and cytotoxicity in mantle cell lymphoma. *Haematologica* **91**(7): 895-902.

Marechal R, Mackey JR, Lai R, Demetter P, Peeters M, Polus M, Cass CE, Young J, Salmon I, Deviere J and Van Laethem JL (2009) Human equilibrative nucleoside transporter 1 and human concentrative nucleoside transporter 3 predict survival after adjuvant gemcitabine therapy in resected pancreatic adenocarcinoma. *Clin Cancer Res* **15**(8): 2913-2919.

Mata JF, Garcia-Manteiga JM, Lostao MP, Fernandez-Veledo S, Guillen-Gomez E, Larrayoz IM, Lloberas J, Casado FJ and Pastor-Anglada M (2001) Role of the human concentrative nucleoside transporter (hCNT1) in the cytotoxic action of 5[Prime]-deoxy-5-fluorouridine, an active intermediate metabolite of capecitabine, a novel oral anticancer drug. *Mol Pharmacol* **59**(6): 1542-1548.

Minematsu T and Giacomini KM (2011) Interactions of tyrosine kinase inhibitors with organic cation transporters and multidrug and toxic compound extrusion proteins. *Mol Cancer Ther* **10**(3): 531-539.

Minuesa G, Huber-Ruano I, Pastor-Anglada M, Koepsell H, Clotet B and Martinez-Picado J (2011) Drug uptake transporters in antiretroviral therapy. *Pharmacol Ther* **132**(3): 268-279.

Minuesa G, Purcet S, Erkizia I, Molina-Arcas M, Bofill M, Izquierdo-Useros N, Casado FJ, Clotet B, Pastor-Anglada M and Martinez-Picado J (2008) Expression and functionality of anti-human immunodeficiency virus and anticancer drug uptake transporters in immune cells. *J Pharmacol Exp Ther* **324**(2): 558-567.

Minuesa G, Volk C, Molina-Arcas M, Gorboulev V, Erkizia I, Arndt P, Clotet B, Pastor-Anglada M, Koepsell H and Martinez-Picado J (2009) Transport of lamivudine [(-)-beta-L-2',3'-dideoxy-3'-thiacytidine] and high-affinity interaction of nucleoside reverse transcriptase inhibitors with human organic cation transporters 1, 2, and 3. *J Pharmacol Exp Ther* **329**(1): 252-261.

Molina-Arcas M, Bellosillo B, Casado FJ, Montserrat E, Gil J, Colomer D and Pastor-Anglada M (2003) Fludarabine uptake mechanisms in B-cell chronic lymphocytic leukemia. *Blood* **101**(6): 2328-2334.

Molina-Arcas M and Pastor-Anglada M (2013) Nucleoside Transporters (SLC28 and SLC29) family, in *Pharmacogencomics of Human Drug Transporters* (Ishikawa T, Kim R and König J eds), John Wiley & Sons, New Jersey.

Mori N, Yokooji T, Kamio Y and Murakami T (2008) Characterization of intestinal absorption of mizoribine mediated by concentrative nucleoside transporters in rats. *Eur J Pharmacol* **586**(1-3): 52-58.

Myers SN, Goyal RK, Roy JD, Fairfull LD, Wilson JW and Ferrell RE (2006) Functional single nucleotide polymorphism haplotypes in the human equilibrative nucleoside transporter 1. *Pharmacogenet Genomics* **16**(5): 315-320.

N

Naito T, Tokashiki S, Mino Y, Otsuka A, Ozono S, Kagawa Y and Kawakami J (2010) Impact of concentrative nucleoside transporter 1 gene polymorphism on oral bioavailability of mizoribine in stable kidney transplant recipients. *Basic Clin Pharmacol Toxicol* **106**(4): 310-316.

Nakatani-Freshwater T and Taft DR (2008a) Renal excretion of emtricitabine I: effects of organic anion, organic cation, and nucleoside transport inhibitors on emtricitabine excretion. *J Pharm Sci* **97**(12): 5401-5410.

Nakatani-Freshwater T and Taft DR (2008b) Renal excretion of emtricitabine II. effect of trimethoprim on emtricitabine excretion: In vitro and in vivo studies. *J Pharm Sci* **97** (12): 5411-5420.

Nies AT, Herrmann E, Brom M and Keppler D (2008) Vectorial transport of the plant alkaloid berberine by double-transfected cells expressing the human organic cation transporter 1 (OCT1, SLC22A1) and the efflux pump MDR1 P-glycoprotein (ABCB1). *Naunyn Schmiedebergs Arch Pharmacol* **376**(6): 449-461.

Nies AT, Koepsell H, Winter S, Burk O, Klein K, Kerb R, Zanger UM, Keppler D, Schwab M and Schaeffeler E (2009) Expression of organic cation transporters OCT1 (SLC22A1) and OCT3 (SLC22A3) is affected by genetic factors and cholestasis in human liver. *Hepatology* **50**(4): 1227-1240.

O

Owen RP, Gray JH, Taylor TR, Carlson EJ, Huang CC, Kawamoto M, Johns SJ, Stryke D, Ferrin TE and Giacomini KM (2005) Genetic analysis and functional characterization of polymorphisms in the human concentrative nucleoside transporter, CNT2. *Pharmacogenet Genomics* **15**(2): 83-90.

Owen RP, Lagpacan LL, Taylor TR, De La Cruz M, Huang CC, Kawamoto M, Johns SJ, Stryke D, Ferrin TE and Giacomini KM (2006) Functional characterization and haplotype analysis of polymorphisms in the human equilibrative nucleoside transporter, ENT2. *Drug Metab Dispos* **34**(1): 12-15.

P

Pastor-Anglada M (2013) Transporter pharmacogenetics: do we need function? Do we need motion? *Pharmacogenomics* **In press**.

Pastor-Anglada M, Cano-Soldado P, Errasti-Murugarren E and Casado FJ (2008) SLC28 genes and concentrative nucleoside transporter (CNT) proteins. *Xenobiotica* **38**(7-8): 972-994.

Pastor-Anglada M, Felipe A and Casado FJ (1998) Transport and mode of action of nucleoside derivatives used in chemical and antiviral therapies. *Trends Pharmacol Sci* **19**(10): 424-430.

Pennycooke M, Chaudary N, Shuralyova I, Zhang Y and Coe IR (2001) Differential expression of human nucleoside transporters in normal and tumor tissue. *Biochem Biophys Res Commun* **280**(3): 951-959.

Perez-Torras S, Vidal-Pla A, Cano-Soldado P, Huber-Ruano I, Mazo A and Pastor-Anglada M (2013) Concentrative nucleoside transporter 1 (hCNT1) promotes phenotypic changes relevant to tumor biology in a translocation-independent manner. *Cell Death Dis* **4**: e648.

Pinilla-Macua I, Casado FJ and Pastor-Anglada M (2012) Structural determinants for rCNT2 sorting to the plasma membrane of polarized and non-polarized cells. *Biochem J* **442**(3): 517-525.

Price CJ, George JD, Marr MC, Myers CB, Bieler GS, Williams RL and Jahnke GD (2006) Prenatal developmental toxicity evaluation of 2',3'-dideoxyinosine (ddI) and 2',3'-didehydro-3'-deoxythymidine (d4T) co-administered to Swiss Albino (CD-1) mice. *Birth Defects Res B Dev Reprod Toxicol* **77**(3): 207-215.

Purcet S, Minuesa G, Molina-Arcas M, Erkizia I, Casado FJ, Clotet B, Martinez-Picado J and Pastor-Anglada M (2006) 3'-Azido-2',3'-dideoxythymidine (zidovudine) uptake mechanisms in T lymphocytes. *Antivir Ther* **11**(6): 803-811.

Q

Quintas-Cardama A, Santos FP and Garcia-Manero G (2010) Therapy with azanucleosides for myelodysplastic syndromes. *Nat Rev Clin Oncol* **7**(8): 433-444.

R

Razonable RR (2011) Antiviral drugs for viruses other than human immunodeficiency virus. *Mayo Clin Proc* **86**(10): 1009-1026.

Ritzel MW, Ng AM, Yao SY, Graham K, Loewen SK, Smith KM, Ritzel RG, Mowles DA, Carpenter P, Chen XZ, Karpinski E, Hyde RJ, Baldwin SA, Cass CE and Young JD (2001) Molecular identification and characterization of novel human and mouse concentrative Na+-nucleoside cotransporter proteins (hCNT3 and mCNT3) broadly selective for purine and pyrimidine nucleosides (system cib). *J Biol Chem* **276**(4): 2914-2927.

Rius M, Keller D, Brom M, Hummel-Eisenbeiss J, Lyko F and Keppler D (2010) Vectorial transport of nucleoside analogs from the apical to the basolateral membrane in double-transfected cells expressing the human concentrative nucleoside transporter hCNT3 and the export pump ABCC4. *Drug Metab Dispos* **38**(7): 1054-1063.

Rius M and Lyko F (2012) Epigenetic cancer therapy: rationales, targets and drugs. *Oncogene* **31**(39): 4257-4265.

Rius M, Stresemann C, Keller D, Brom M, Schirrmacher E, Keppler D and Lyko F (2009) Human concentrative nucleoside transporter 1-mediated uptake of 5-azacytidine enhances DNA demethylation. *Mol Cancer Ther* **8**(1): 225-231.

Robak T (2011) New nucleoside analogs for patients with hematological malignancies. *Expert Opin Investig Drugs* **20**(3): 343-359.

Robak T, Wierzbowska A and Robak E (2006) Recent clinical trials of cladribine in hematological malignancies and autoimmune disorders. *Rev Recent Clin Trials* **1**(1): 15-34.

Roth M, Obaidat A and Hagenbuch B (2011) OATPs, OATs and OCTs: the organic anion and cation transporters of the SLCO and SLC22A gene superfamilies. *Br J Pharmacol* **165**(5): 1260-1287.

Rubio-Aliaga I and Daniel H (2008) Peptide transporters and their roles in physiological processes and drug disposition. *Xenobiotica* **38**(7-8): 1022-1042.

S

Sahasranaman S, Howard D and Roy S (2008) Clinical pharmacology and pharmacogenetics of thiopurines. *Eur J Clin Pharmacol* **64**(8): 753-767.

Sharma NK, Mahadevan N and Balakumar P (2013) Adenosine transport blockade restores attenuated cardioprotective effects of adenosine preconditioning in the isolated diabetic rat heart: potential crosstalk with opioid receptors. *Cardiovasc Toxicol* **13**(1): 22-32.

Shu Y, Brown C, Castro RA, Shi RJ, Lin ET, Owen RP, Sheardown SA, Yue L, Burchard EG, Brett CM and Giacomini KM (2008) Effect of genetic variation in the organic cation transporter 1, OCT1, on metformin pharmacokinetics. *Clin Pharmacol Ther* **83**(2): 273-280.

Shu Y, Leabman MK, Feng B, Mangravite LM, Huang CC, Stryke D, Kawamoto M, Johns SJ, DeYoung J, Carlson E, Ferrin TE, Herskowitz I and Giacomini KM (2003) Evolutionary conservation predicts function of variants of the human organic cation transporter, OCT1. *Proc Natl Acad Sci U S A* **100**(10): 5902-5907.

Shu Y, Sheardown SA, Brown C, Owen RP, Zhang S, Castro RA, Ianculescu AG, Yue L, Lo JC, Burchard EG, Brett CM and Giacomini KM (2007) Effect of genetic variation in the organic cation transporter 1 (OCT1) on metformin action. *J Clin Invest* **117**(5): 1422-1431.

Soler C, Felipe A, Mata JF, Casado FJ, Celada A and Pastor-Anglada M (1998) Regulation of nucleoside transport by lipopolysaccharide, phorbol esters, and tumor necrosis factor-alpha in human B-lymphocytes. *J Biol Chem* **273**(41): 26939-26945.

Soler C, Garcia-Manteiga J, Valdes R, Xaus J, Comalada M, Casado FJ, Pastor-Anglada M, Celada A and Felipe A (2001a) Macrophages require different nucleoside transport systems for proliferation and activation. *FASEB J* **15**(11): 1979-1988.

Soler C, Valdes R, Garcia-Manteiga J, Xaus J, Comalada M, Casado FJ, Modolell M, Nicholson B, MacLeod C, Felipe A, Celada A and Pastor-Anglada M (2001b) Lipopolysaccharide-induced apoptosis of macrophages determines the up-regulation of concentrative nucleoside transporters Cnt1 and Cnt2 through tumor necrosis factor-alpha-dependent and -independent mechanisms. *J Biol Chem* **276**(32): 30043-30049.

Song IS, Shin HJ, Shim EJ, Jung IS, Kim WY, Shon JH and Shin JG (2008a) Genetic variants of the organic cation transporter 2 influence the disposition of metformin. *Clin Pharmacol Ther* **84**(5): 559-562.

Song IS, Shin HJ and Shin JG (2008b) Genetic variants of organic cation transporter 2 (OCT2) significantly reduce metformin uptake in oocytes. *Xenobiotica* **38**(9): 1252-1262.

Soo RA, Wang LZ, Ng SS, Chong PY, Yong WP, Lee SC, Liu JJ, Choo TB, Tham LS, Lee HS, Goh BC and Soong R (2009) Distribution of gemcitabine pathway genotypes in ethnic Asians and their association with outcome in non-small cell lung cancer patients. *Lung Cancer* **63**(1): 121-127.

Spratlin J, Sangha R, Glubrecht D, Dabbagh L, Young JD, Dumontet C, Cass C, Lai R and Mackey JR (2004) The absence of human equilibrative nucleoside transporter 1 is associated with reduced survival in patients with gemcitabine-treated pancreas adenocarcinoma. *Clin Cancer Res* **10**(20): 6956-6961.

Srimaroeng C, Perry JL and Pritchard JB (2008) Physiology, structure, and regulation of the cloned organic anion transporters. *Xenobiotica* **38**(7-8): 889-935.

Stocker S, Riedmaier A, Schwab M and Giachini K (2013) OCT (SLC22A) and OCTN family, in *Pharmacogenomics of Human Drug Transporters*, John Wiley & Sons, New Jersey.

Stresemann C and Lyko F (2008) Modes of action of the DNA methyltransferase inhibitors azacytidine and decitabine. *Int J Cancer* **123**(1): 8-13.

T

Takeda M, Khamdang S, Narikawa S, Kimura H, Kobayashi Y, Yamamoto T, Cha SH, Sekine T and Endou H (2002) Human organic anion transporters and human organic cation transporters mediate renal antiviral transport. *J Pharmacol Exp Ther* **300**(3): 918-924.

Tregouet DA, Konig IR, Erdmann J, Munteanu A, Braund PS, Hall AS, Grosshennig A, Linsel-Nitschke P, Perret C, DeSuremain M, Meitinger T, Wright BJ, Preuss M, Balmforth AJ, Ball SG, Meisinger C, Germain C, Evans A, Arveiler D, Luc G, Ruidavets JB, Morrison C, van der Harst P, Schreiber S, Neureuther K, Schafer A, Bugert P, El Mokhtari NE, Schrezenmeir J, Stark K, Rubin D, Wichmann HE, Hengstenberg C, Ouwehand W, Ziegler A, Tiret L, Thompson JR, Cambien F, Schunkert H and Samani NJ (2009) Genome-wide haplotype association study identifies the SLC22A3-LPAL2-LPA gene cluster as a risk locus for coronary artery disease. *Nat Genet* **41**(3): 283-285.

Tzvetkov MV, Dos Santos Pereira JN, Meineke I, Saadatmand AR, Stingl JC and Brockmoller J (2013) Morphine is a substrate of the organic cation transporter OCT1 and polymorphisms in OCT1 gene affect morphine pharmacokinetics after codeine administration. *Biochem Pharmacol* **86**(5): 666-678.

Tzvetkov MV, Saadatmand AR, Bokelmann K, Meineke I, Kaiser R and Brockmoller J (2012) Effects of OCT1 polymorphisms on the cellular uptake, plasma concentrations and efficacy of the 5-HT(3) antagonists tropisetron and ondansetron. *Pharmacogenomics J* **12**(1): 22-29.

Tzvetkov MV, Vormfelde SV, Balen D, Meineke I, Schmidt T, Sehrt D, Sabolic I, Koepsell H and Brockmoller J (2009) The effects of genetic polymorphisms in the organic cation transporters OCT1, OCT2, and OCT3 on the renal clearance of metformin. *Clin Pharmacol Ther* **86**(3): 299-306.

U

Urban TJ, Sebro R, Hurowitz EH, Leabman MK, Badagnani I, Lagpacan LL, Risch N and Giacomini KM (2006) Functional genomics of membrane transporters in human populations. *Genome Res* **16**(2): 223-230.

V

Van Den Neste E, Cazin B, Janssens A, Gonzalez-Barca E, Terol MJ, Levy V, Perez de Oteyza J, Zachee P, Saunders A, de Frias M and Campas C Acadesine for patients with relapsed/refractory chronic lymphocytic leukemia (CLL): a multicenter phase I/II study. *Cancer Chemother Pharmacol* **71**(3): 581-591.

VanWert AL, Gionfriddo MR and Sweet DH (2010) Organic anion transporters: discovery, pharmacology, regulation and roles in pathophysiology. *Biopharm Drug Dispos* **31**(1): 1-71.

Veyhl M, Spangenberg J, Puschel B, Poppe R, Dekel C, Fritzsch G, Haase W and Koepsell H (1993) Cloning of a membrane-associated protein which modifies activity and properties of the Na(+)-D-glucose cotransporter. *J Biol Chem* **268**(33): 25041-25053.

Volk C, Gorboulev V, Kotzsch A, Muller TD and Koepsell H (2009) Five amino acids in the innermost cavity of the substrate binding cleft of organic cation transporter 1 interact with extracellular and intracellular corticosterone. *Mol Pharmacol* **76**(2): 275-289.

W

Walko CM and Lindley C (2005) Capecitabine: a review. *Clin Ther* **27**(1): 23-44.

Wang L, Giannoudis A, Lane S, Williamson P, Pirmohamed M and Clark RE (2008) Expression of the uptake drug transporter hOCT1 is an important clinical determinant of the response to imatinib in chronic myeloid leukemia. *Clin Pharmacol Ther* **83**(2): 258-264.

White DL, Saunders VA, Dang P, Engler J, Zannettino AC, Cambareri AC, Quinn SR, Manley PW and Hughes TP (2006) OCT-1-mediated influx is a key determinant of the intracellular uptake of imatinib but not nilotinib (AMN107): reduced OCT-1 activity is the cause of low in vitro sensitivity to imatinib. *Blood* **108**(2): 697-704.

Wright AM, Paterson AR, Sowa B, Akabutu JJ, Grundy PE and Gati WP (2002) Cytotoxicity of 2-chlorodeoxyadenosine and arabinosylcytosine in leukaemic lymphoblasts from paediatric patients: significance of cellular nucleoside transporter content. *Br J Haematol* **116**(3): 528-537.

Wu X, Huang W, Ganapathy ME, Wang H, Kekuda R, Conway SJ, Leibach FH and Ganapathy V (2000) Structure, function, and regional distribution of the organic cation transporter OCT3 in the kidney. *Am J Physiol Renal Physiol* **279**(3): F449-458.

Y

Yan L, Yang X and Davidson NE (2001) Role of DNA methylation and histone acetylation in steroid receptor expression in breast cancer. *J Mammary Gland Biol Neoplasia* **6**(2): 183-192.

Yan Q (2005) Pharmacogenomics and systems biology of membrane transporters. *Mol Biotechnol* **29**(1): 75-88.

Yao SY, Ng AM, Cass CE, Baldwin SA and Young JD (2011) Nucleobase transport by human equilibrative nucleoside transporter 1 (hENT1). *J Biol Chem* **286**(37): 32552-32562.

Yao SY, Ng AM, Sundaram M, Cass CE, Baldwin SA and Young JD (2001) Transport of antiviral 3'-deoxy-nucleoside drugs by recombinant human and rat equilibrative, nitrobenzylthioinosine (NBMPR)-insensitive (ENT2) nucleoside transporter proteins produced in Xenopus oocytes. *Mol Membr Biol* **18**(2): 161-167.

Yee SW, Shima JE, Hesselson S, Nguyen L, De Val S, Lafond RJ, Kawamoto M, Johns SJ, Stryke D, Kwok PY, Ferrin TE, Black BL, Gurwitz D, Ahituv N and Giacomini KM (2009) Identification and characterization of proximal promoter polymorphisms in the human concentrative nucleoside transporter 2 (SLC28A2). *J Pharmacol Exp Ther* **328**(3): 699-707.

Yoo CB, Cheng JC and Jones PA (2004) Zebularine: a new drug for epigenetic therapy. *Biochem Soc Trans* **32**(Pt 6): 910-912.

Young JD, Yao SY, Baldwin JM, Cass CE and Baldwin SA (2013) The human concentrative and equilibrative nucleoside transporter families, SLC28 and SLC29. *Mol Aspects Med* **34**(2-3): 529-547.

Z

Zhang S, Lovejoy KS, Shima JE, Lagpacan LL, Shu Y, Lapuk A, Chen Y, Komori T, Gray JW, Chen X, Lippard SJ and Giacomini KM (2006) Organic cation transporters are determinants of oxaliplatin cytotoxicity. *Cancer Res* **66**(17): 8847-8857.

RESUM DE LA TESI DOCTORAL

1

INTRODUCCIÓ

1.1 Fàrmacs derivats de nucleòsids

Petites modificacions en l'estructura dels nucleòsids naturals pot conferir-los propietats farmacològiques. Els fàrmacs anàlegs de nucleòsids, inclouen: anàlegs de nucleobases, derivats de nucleòsids i derivats fosforilats que comparteixen mecanismes d'entrada i metabolisme amb els nucleòsids naturals. Aquests fàrmacs, un cop internalitzats han de ser fosforilats per adoptar la seva forma activa, que es pot incorporar al ADN o ARN i així impedir la proliferació cel·lular, la replicació viral o inhibir la funció d'enzims essencials com quinases, ADN metiltransferases o fosforilases de nucleòsids entre altres.

Els fàrmacs derivats de nucleòsids són actualment utilitzats com a tractament d'elecció en alguns malalties humanes com el càncer, infeccions virals (VIH entre altres) i malalties autoimmunes i inflamatòries com colitis ulcerosa i malaltia de Crohn.

1.1.1 Anàlegs de nucleòsids antivirals

Molts dels fàrmacs clàssicament utilitzats en la teràpia antiretroviral com a inhibidors de la retrotranscriptasa són anàlegs de nucleòsids (NRTIs). En la majoria dels casos, són 2'-3'-desoxinucleòsids que actuen com a substrat alternatiu per a la retrotranscriptasa bloquejant així la seva acció.

Els anàlegs utilitzats en aquesta tesis són l'anàleg de citidina, lamivudina (3TC), l'anàleg de timidina, azidotimidina (AZT), l'anàleg de guanosina, abacavir (ABC), i l'anàleg de nucleòtid, tenofovir diproxil fumarat (TDF). Moltes vegades aquests NRTIs són administrats en combinació amb fàrmacs d'altres famílies. Aquestes altres famílies inclouen, inhibidors de la retrotranscriptasa no anàlegs de nucleòsids com l'efavirenz, inhibidors de la proteasa, inhibidors de la integrasa com el raltegravir i inhibidors de l'entrada.

Els anàlegs de nucleòsids no només s'utilitzen en el tractament de la infecció per VIH, també són utilitzats en el tractament d'altres infeccions virals com hepatitis i el virus de l'herpes. El tractament de l'hepatits comparteix alguns fàrmacs amb el

tractament de la sida, com són la lamivudina i el tenofovir entre altres. La ribavirina i l'entecavir són també ampliament utilitzat en el tractament de les infeccions per hepatitis.

1.1.2 Anàlegs de nucleòsids antineoplàstics

En el tractament de malalties neoplàstiques, els anàlegs de nucleòsids s'utilitzen tant en el tractament de tumors sòlids com de malalties limfoproliferatives. Dins els fàrmacs utilitzats en el tractament de malalties limforproliferatives trobem els anàlegs de citidina inhibidors de la ADN metiltransferasa així com també bendamustina, un anàleg de nucleobase.

Els **inhibidors de la ADN metiltransferasa** són fàrmacs epigenètics; que actuen inhibint l'enzim responsable de la metilació del ADN modificant així l'estat global de metilació de les cèl·lules diana. Dos d'ells, azacitidina i decitabina estan aprovats per la FDA per al tractament de la síndrome mielodisplàstica. Mentre que zebularina està encara en fase de desenvolupament.

La **bendamustina**, no és un anàleg clàssic ja que presenta un anell benzimidazol unit a un grup mostassa nitrogenada en lloc de a una ribosa. La bendamustina s'utilitza en el tractament de leucèmica limfàtica crònica i limfoma de cèl·lules B non-Hodgkin.

En el tractament de tumors sòlids els fàrmacs derivats de nucleòsids més utilitzats són gemcitabina i capecitabina un pro-fàrmac de 5-fluorouracil.

1.1.3 Anàlegs de nucleòsids en altres aplicacions

Els anàlegs de nucleòsids també s'utilitzen per altres indicacions. Les tiopurines així com també la cladribina i la mizoribina s'utilitzen com a immunosupressors; anàlegs de adenosina o inhibidors de la seva recaptació es poden utilitzar com a neuroprotectors i cardioprotectors i l'isomer d'hipoxantina, alopurinol, s'utilitza en el tractament de la hiperuricèmia.

1.2 Transportadors de nucleòsids i fàrmacs anàlegs de nucleòsids

Degut a la seva naturalesa hidrofílica, tant els nucleòsids com els seus anàlegs requereixen transportadors de membrana per tal de poder ser internalitzats i així realitzar la seva funció. Els principals transportadors implicats són els de les famílies *SLC28* (CNTs) i *SLC29* (ENTs). No obstant, petits canvis en l'estructura dels nucleòsids potden alterar la seva capacitat de interaccionar amb aquests transportadors. Per això,

altres transportadors de membrana que no transporten nucleòsids naturals poden ser essencials en la internalització i biodisponibilitat d'aquests anàlegs. Transportadors de les famílies gèniques *SLC22* i *SLC15* estan implicats en la internalització d'alguns d'aquests derivats de nucleòsids utilitzats en quimioteràpia.

1.2.1 Transportadors de nucleòsids

Els transportadors de nucleòsids es poden dividir en dues grans famílies. Els transportadors concentratius, codificats pels gens *SLC28* i els transportadors equilibratius, codificats pels gens *SLC29*.

Els **transportadors concentratius** (CNTs) es caracteritzen per translocar nucleòsids de manera depenent de sodi amb una estequiometria 1:1 (Na^+: nucleòsid) per a hCNT1 i hCNT2 i 2:1 per a hCNT3, essent aquest últim també capaç de transportar nucleòsids de forma depenent de protó amb una estequiometria 1:1 (H^+: nucleòsid). Són transportadors d'alta afinitat amb una selectivitat més restringida que els transportadors equilibratius. hCNT1 és selectiu cap a nucleòsids pirimidínics, hCNT2 és selectiu cap a nucleòsids purics tot i que també pot transportar uridina i hCNT3 és el menys selectiu podent transportar tant nucleòsids purics com pirimidínics.

L'estructura dels transportadors concentratius fins fa poc es creia que era de 13 dominis trans-membrana. No obstant, la recent cristal·lització del transportador concentratiu de *V. cholerae* ha mostrat que en realitat es tracta de transportadors amb 11 dominis transmembrana, els 8 de l'ortòleg procariota amb tres més al extrem N-terminal característics dels transportadors eucariotes.

Els **transportadors equilibratius** (ENTs) medien un transport facilitat i potencialment bidireccional a favor de gradient de concentració. Els principals transportadors equilibratius situats a la membrana plasmàtica són hENT1 i hENT2. Ambdos poden transportar tan nucleòsids purics com pirimidínics amb una menor afinitat que els transportadors equilibratius. hENT2 també és capaç de transportar nucleobases. Es poden diferenciar els dos transportadors segons els seus inhibidors, NBTI inhibeix selectivament hENT1 mentre que dipiridamol inhibeix tant hENT1 com hENT2.

Els transportadors de nucleòsids es troben distribuïts de forma asimètrica en epitelis polaritzats, podent facilitar així un flux dirigit de nucleòsids o dels seus anàlegs. Els CNTs es troben localitzats a la membrana apical d'enteròcits i cèl·lules renals i en ambdues membranes en fetge i placenta. Els transportadors equilibratius, no tenen una localització tan específica trobant-se majoritàriament en la membrana basolateral. La

seva expressió també s'ha detectat a nivell de cèl·lules de sistema immune. L'expressió de CNTs s'ha vist reduïda en alguns tumors mentre que l'expressió d'ENTs és més retinguda i en alguns casos inclús es troba augmentada.

Aquests transportadors poden transportar una gran quantitat d'anàlegs antineoplàstics. Els anàlegs antivirals com que no tenen el grup desoxi en la posició 3' tenen menor afinitat cap aquests transportadors. Diferents estudis *in vitro* han mostrat que l'expressió de transportadors de nucleòsids correlaciona clínicament amb l'efecte de fàrmacs substrat com citarabina o gemcitabina.

1.2.2 Altres transportadors de fàrmacs anàlegs de nucleòsids

Els transportadors codificats per la família genètica ***SLC22*** tot i no ser bons transportadors de nucleòsids naturals, poden transportar alguns anàlegs de nucleòsids.

Els **transportadors de cations orgànics** (OCTs) tenen una àmplia selectivitat de substrat; transporten de manera independent de sodi i a favor de gradient de substrat i de càrrega. Són transportadors bidireccionals que presenten diferent afinitat depenent del lloc d'interacció sigui intra o extracel·lular.

Els **transportadors d'anions orgànics** (OATs) transporten una gran varietat de substrats, intercanviant-los per α-cetoglutarat.

Els transportadors de la família gènica *SLC22*, es troben localitzats bàsicament en epiteli. hOCT1 i hOAT2 es troben majoritàriament a nivell hepàtic mentre que hOCT2 així com també hOAT1 i hOAT3 es troben a nivell renal. hOCTs es troben també a placenta, pulmó i intestí. L'expressió de hOCT1 i hOCT3 s'ha trobat també en cèl·lules del sistema immunitari. No obstant, no s'ha pogut detectar ni hOCT2 ni cap dels hOATs. Un estudi recent determinà que en cèl·lules de CLL l'expressió de hOCT1 podia estar augmentada, facilitant així l'entrada dels fàrmacs substrat.

Tant hOCTs com hOATs han estat implicats en la internalització d'anàlegs de nucleòsids en concret d'antivirals com lamivudina i azidotimidina.

Els transportadors de la família gènica *SLC15*, **transportadors de pèptids** (PepT1 i PepT2) a part de pèptids naturals poden transportar alguns pro-fàrmacs d'anàlegs de nucleòsids com el valaciclovir, un amino éster de l'aciclovir.

1.3 Farmacogenòmica de transportadors de nucleòsids

Les variants polimòrfiques que afecten els transportadors de membrana poden modular la resposta a la teràpia. S'han identificat multiples variants en transportadors de membrana que poden afectar la correcta inserció a membrana plasmàtica, el reconeixement del substrat o la interacció amb altres proteïnes. Les variants que afecten el reconeixement del substrat solen fer-ho de manera específica de substrat, fent difícil predir l'efecte que pot tenir una variant sobre un compost sense provar-ho experimentalment.

Tot i que s'han descrit variants polimòrfiques en tots els transportadors prèviament descrits, el transportador més polimòrfic és hOCT1. Les variants polimòrfiques en aquest transportador han estat àmpliament estudiades i han mostrat canvis en la farmacocinètica i farmacodinàmica de fàrmacs diversos com la metformina.

2

OBJECTIUS

En base a aquests antecedents els objectius d'aquesta tesis doctoral van ser:

1- Identificar el paper que les variants polimòrfiques de hOCT1 juguen en la transportabilitat de lamivudina i les interaccions fàrmac-fàrmac.

2- Identificar el transportador responsable de la internalització de bendamustina i analitzar l'efecte que les seves variants polimòrfiques poden tenir en la disponibilitat cel·lular.

3- Identificar i caracteritzar funcionalment els transportadors responsables de la internalització i disponibilitat cel·lular dels inhibidors de la ADN metiltransferasa utilitzats en el tractament epigenètic del càncer.

4- Generar un model estructural de hCNT3 adequat per a predir interaccions fàrmac-transportador.

3

RESULTATS I DISCUSSIÓ

3.1 Paper dels polimorfismes del transportador de cations orgànics 1 (hOCT1) en la biodisponibilitat de lamivudina i les interaccions fàrmac-fàrmac.

La lamivudina (3TC) és un anàleg de citidina utilitzat actualment en el tractament de la infecció per VIH així com també de l'hepatitis B crònica. Com tots els anàlegs de nucleòsids, la seva naturalesa hidrofílica fa que necessiti transportadors de membrana específics per a poder ser internalitzat i així exercir la seva acció. Els transportadors responsables d'aquesta internalització són hOCT1, hOCT2 i hOCT3 (Minuesa et al., 2009). Els transportadors de cations orgànics (OCTs) són transportadors capaços d'internalitzar una gran varietat de compostos. hOCT1 és altament polimòrfic i els seus polimorfismes poden afectar la biodisponibilitat i l'acció de alguns fàrmacs com metformina o imatinib.

El primer bloc de la tesis es basa en la determinació de com aquestes variants polimòrfiques poden afectar l'eficiència del transportador a l'hora d'internalitzar 3TC i també en com aquestes variants poden alterar les interaccions fàrmac-fàrmac.

Una gran bateria de polimorfismes van ser generats per mutagènesi dirigida i provats per la seva capacitat de transportar tant 3TC com el substrat model MPP^+ . Amb aquells en els que es van veure diferències funcionals més interessants i una freqüència al·lèlica en humans elevada es van generar línies cel·lulars estables utilitzant el mètode FlpIn en HEK293, per tal de poder utilitzar-les en posteriors estudis cinètics. Els estudis cinètics van revelar que tot i que tant pel substrat model com per 3TC hi havia canvis significatius en *Km* o *Vmax,* quan comparàvem els polimorfismes estudiats (R61C, S189L i M420del), només en el cas de 3TC les variants polimòrfiques deixaven de ser eficients en transportar el fàrmac amb valors de *Vmax/Km* per sota de 1.

Com que dues de les variants polimòrfiques es troben al *loop* extracel·lular i aquest està implicat en la dimerització del transportador i la seva correcta inserció a membrana plasmàtica (Keller et al., 2011), en col·laboració amb el laboratori del

Professor Hermann Koepsell es van dur a terme estudis de oligomerització mostrant que tant la variant R61C com la C88R seguien tenint la capacitat de formar oligomers.

La infecció *in vitro* amb dues soques diferents de VIH-1 de de PBMCs de pacients sans, estimulats prèviament amb IL-2 o amb la combinació de IL-2 i PHA, mostrà un augment de l'expressió del ARNm de hOCT1. Aquest augment podria potenciar l'entrada d'aquells fàrmacs que utilitzen hOCT1 per entrar a l'interior de les cèl·lules.

La teràpia de la infecció per VIH sol estar sempre composta per més d'un fàrmac. Per això vam voler veure també com altres fàrmacs utilitzats en la teràpia de la infecció per VIH com els anàlegs de nuclèosids inhibidors de la retrotranscriptasa (NRTIs), abacavir (ABC) i zidovudine (AZT); l'inhibidor de la retrotranscriptasa no anàleg de nucleòsid (NNRTI), efavirenz; l'anàleg de nucleòtid inhibidor de la retrotranscriptasa (NtRTI), tenofovir i l'inhibidor de la integrasa, raltegravir, podien afectar la internalització de 3TC a través de hOCT1. Tres dels 5 fàrmacs semblaven inhibir l'entrada de 3TC a concentracions de 500μM. Els experiments realitzats amb aquests fàrmacs a concentracions pròximes a la seva *Cmax* mostraren interessants diferències entre la interacció fàrmac-fàrmac. La interacció entre 3TC i ABC o AZT, es veia clarament afectada en les variants polimòrfiques ja que la mateixa quantitat de fàrmac inhibia en major grau la internalització de 3TC en les variants que en el transportador *WT*.

El fet que hOCT1 estigui sobreexpressat en cèl·lules infectades faria les cèl·lules diana més accessibles al fàrmac, fet que seria beneficiós per la teràpia. No obstant, els nostres resultats mostren que s'ha de parar especial atenció a la presència de variants polimòrfiques ja que aquestes poden afectar no només la biodisponibilitat del fàrmac si no també la capacitat d'altres fàrmacs d'inhibir la internalització de 3TC.

3.2. Identificació de hOCT1 com al transportador responsable de la internalització de bendamustina. Implicació dels polimorfismes en l'acció citotòxica del fàrmac.

La bendamustina és un fàrmac utilitzat en el tractament de la leucèmia limfàtica crònica (CLL). No és un anàleg de nucleòsids clàssic ja que té un anell de purina unit a

una mostassa nitrogenada en lloc de a una ribosa. Tot i que el fàrmac s'utilitza en clínica amb bons resultats no hi ha res descrit de la seva via d'internalització.

En ser un anàleg de purina els primers transportadors provats foren els clàssics transportadors de nucleòsids concentratius (CNTs) i equilibratius (ENTs) però cap d'ells interaccionava amb el fàrmac. La bendamustina però sí que podia interaccionar amb el transportador de cations orgànics 1 (hOCT1), inhibint l'entrada del substrat model MPP^+ amb una *Ki* de 138.1 μM±10.9. Com que no disposàvem del fàrmac marcat, no vam poder confirmar mitjançant estudis de transport directe que el fàrmac fos internalitzat. No obstant, estudis de citotoxicitat en HEK293 que establement expressen hOCT1 o alguna de les seves variants polimòrfiques, mostraren que la presència del transportador sensibilitzava significativament les cèl·lules al tractament amb bendamustina, confirmant així la seva implicació en la internalització.

Com s'ha comentat anteriorment, el transportador hOCT1, és un transportador altament polimòrfic. Les dades del Consorci Internacional del Genoma del Càncer (ICGC) ens han permès calcular la freqüència al·lèlica de les variants polimòrfiques en 233 pacients de CLL. De les 13 variants trobades, 11 estaven ja havien estat descrites i les seves freqüències en pacients de CLL eren de l'ordre de les ja descrites i publicades anteriorment per a població Caucàsica. Les altres dues variants trobades, tenien la freqüència al·lèlica massa baixa per descartar que no es tractessin de mutacions espontànies i per això van ser descartades per a posteriors estudis. Les variants amb freqüències al·lèliques elevades per a les que s'han descrit diferències significatives d'activitat depenent del substrat van ser elegides per a realitzar estudis de citotoxicitat. S'observà que dues d'elles S189L i G465R no eren capaces de sensibilitzar les cèl·lules a bendamustine com ho feia el transportador *WT*, mostrant unes *EC50* de l'ordre de les observades per les HEK293 control. Les altres dues variants, R61C i M420del, mostraven una sensibilització menor que el transportador *WT*. Així, classificàrem les variants en semi-funcionals (R61C i M420del) i no-funcionals (S189L i G465R).

Es realitzaren paral·lelament estudis de citotoxicitat *ex vivo* en cèl·lules de pacients dels quals teníem la informació genètica. Els pacients es classificaren en resistents o sensibles depenent de si la citotoxicitat era menor o major a un 35%. La sensibilitat al fàrmac no es va poder correlacionar amb la expressió de ARNm de hOCT1.

No obstant, quan es mirà la relació entre polimorfismes en el transportador i l'efecte del fàrmac, s'observà que en el grup dels pacients amb el transportador semi-funcional o no-funcional la freqüència de pacients resistents era major que la del grup amb transportador funcional.

Els resultats mostraren que de manera similar al que passa amb altres fàrmacs com imatinib, la presència de variants polimòrfiques en hOCT1 poden modular la sensibilitat de cèl·lules de CLL al tractament i així determinar la seva biodisponibilitat i acció.

3.3 Determinació dels transportadors implicats en la translocació dels fàrmacs inhibidors de la DNA metiltransferasa (DNMT) a través de la membrana plasmàtica.

Els fàrmacs inhibidors de la DNMT, azacitidina, decitabina i zebularina, són anàlegs de la citidina. Els dos primers estan aprovats per la FDA per al tractament del síndrome mielodisplasic, mentre que la zebularina està encara en fase de desenvolupament clínic.

Estudis de interacció fàrmac-transportador mostraren que tots ells interaccionaven amb valors de *Ki* en el rang micromolar baix amb els transportadors concentratius i el transportador equilibratiu hENT2 mentre que pel transportadors equlibratiu hENT1 els valors de *Ki* eren més alts. Estudis de transport directe, no obstant, mostraren que la decitabina, tot i poder interaccionar amb els transportadors de nucleòsids amb una bona afinitat no podia ser translocada a través d'ells. Els estudis amb azacitidina no es van poder portar a terme ja que no es disposava comercialment del fàrmac marcat radioactivament, tanmateix per aquest fàrmac ja està descrita la capacitat de hCNT1 i hCNT3 de internalitzar-lo. El fet que l'azacitidina pugui ser translocada però la decitabina no, i que la única diferència entre els dos sigui el grup hidroxil en 2', destaca la importància que aquest grup pot tenir en la translocació de fàrmacs a través de transportadors de nucleòsids. La zebularina podia ser transportada tant pels transportadors concentratius com pels transportadors equilibratius. Els estudis de citotoxicitat concorden amb els resultats de transport ja que la presència de hCNT1 o

hCNT3 sensibilitza les cèl·lules al tractament amb azacitidina o zebularina mentre que no es veu cap sensibilització al tractament amb decitabina.

Els transportadors de nucleòsids es troben distribuïts asimètricament en epitelis polaritzats trobant-se els transportadors concentratius al domini apical i els equilibratius majoritàriament al basolateral. La presència tant de hCNT1 com de hCNT3 al domini apical de MDCK transfectades transitòriament causava un flux depenent de sodi apical-basolateral important pera la zebularina, mentre que per a la decitabina era quasi nul.

La decitabina és un fàrmac utilitzat i efectiu en la clínica. Com que no pot ser internalitzat per NTs els següents candidats que es varen provar foren hOCTs. Es realitzaren estudis de transport per a hOCT1 i hOCT2 i no s'observà entrada de decitabina. No obstant, en les cèl·lules que no expressaven el transportador hi havia una acumulació de zebularina més gran que en aquelles que l'expressaven suggerint un possible paper de hOCT1 i hOCT2 en l'extrusió del fàrmac. La inhibició de l'entrada depenent de hENTs, revertia aquesta diferència d'acumulació. Així podriem pensar que els hENTs endògens de les cèl·lules internalitzen el fàrmac i hOCT1 i hOCT2 estan implicats en la seva sortida. En cèl·lules, era molt complicat mesurar directament la sortida del fàrmac. Per això es va decidir treballar amb un model més senzill i a l'hora més net com són els oòcits de *Xenopus laevis*. La injecció de fàrmac marcat en oòcits mostra una sortida de zebularina en aquells que expressaven el transportadors inhibible per quinina, conegut inhibidor de hOCTs (Koepsell et al., 2007), mentre que en els oòcits control no s'observava.

L'estudi de l'efecte d'algunes variants polimòrfiques en aquesta capacitat, mostrà un patró idèntic al d'entrada per al substrat natural, tanmateix per a la zebularina cap de les variants estudiades era capaç d'extreure-la al mateix nivell que el transportador *WT*. Estudis de citotoxicitat en HEK293, que expressen de forma estable hOCT1 o les seves variants polimòrfiques, mostraren que en aquest cas la presència del transportador *WT* conferia una resistència a les cèl·lules al tractament en comparació amb dues de les variants no capaces de portar a terme l'extrusió.

En resum, petits canvis en l'estructura dels fàrmacs poden afectar la seva transportabilitat. La decitabina tot i només tenir la diferència del grup hidroxil en 2' respecte la azacitidina pot interaccionar amb tots els transportadors de nucleòsids estudiats però no pot ser translocada per cap. La zebularina, en canvi, és transportada

per tots els transportadors estudiats (hCNT1, hCNT3, hENTs i hOCTs). En un model en que es co-expressessin els transportadors, zebularina seria internalitzada a través de CNTs i ENTs i un cop a l'interior cel·lular seria excretada a l'exterior a través dels OCTs. En aquest model la presència de variants polimòrfiques en hOCT1 en lloc de conferir resistència a la teràpia, serien beneficials.

3.4 Modelatge de hCNT3

L'obtenció de models bioinformàtics dels transportadors permetrien determinar empíricament la importància de variants polimòrfiques o mutacions en la seva activitat. Ara bé, per poder obtenir un bon model cal tenir cristal·litzada una proteïna amb alta homologia. Fins fa molt poc, cap transportador de la família *SLC28* o *SLC29* havia estat cristal·litzats. Així, obtenir un bon model d'estructura era molt difícil ja que cap dels transportadors cristal·litzats fins al moment presentaven un grau d'homologia suficient amb els tansportadors CNT com per a ser bons motlle. La cristal·lització del transportador concentratiu de nucleòsids de *Vibrio cholerae* ens ha permès en col·laboració amb el grup de Xavier Barril i Axel Bidon-Chanal, generar un model per a hCNT3.

El cristall de *V. cholerare* mostrà que el transportador només tenia 8 dominis transmembrana (Johnson et al., 2012). La diferència principal entre els transportadors procariotes i els eucariotes és que aquests últims tenen 3 dominis més a l'extrem N-terminal, els quals són els que estan relacionats amb la unió a proteïnes i senyalització intracel·lular. Això faria pensar que els transportadors humans tenen 11 dominis transmembrana, en lloc de 13 com s'havia pensat fins al moment.

El model dissenyat per homologia, no permet modelar aquest tres dominis N-terminal, però si que permet obtenir un model bastant acurat del lloc de unió a substrat així com també dels llocs d'unió a sodi del transportador hCNT3.

Els residus implicats en la selectivitat de substrat dels diferents CNTs van ser identificats en el model. Els residus identificats, havien estat estudiats anteriorment per Loewen et al. i s'havia determinat que eren essencials per a la selectivitat de hCNT1 i hCNT2, comprovant així la validesa del nostre model.

També es van identificar els dos llocs d'unió de sodi de hCNT3. Per tal de provar el model, es van generar una sèrie de mutacions. Dues d'elles S396A i T605A afectaven

el segon lloc d'unió a sodi i les estequiometries donaven una relació Na^+:nucleòsid 1:1. Les mutacions que afectaven el primer lloc d'unió a sodi en principi havien de ser no funcionals perquè aquest lloc d'unió és molt proper al d'unió de substrat. No obstant, tot i que una d'elles, I371E, sí que havia perdut la funció, les altres dues eren funcionals, una d'elles amb una estequiometria 1:1 (T370A) i l'altre amb una estequiometria de 2:1 (N336A). El fet que aquesta segona mutació no donés el canvi esperat d'estequiometria es pot justificar en el model, per la substitució de la cadena lateral de l'asparagina per una molècula d'aigua que coordinaria amb l'alanina i amb el sodi.

El segon lloc d'unió de sodi pot unir també protons. Per això, es portaran a terme estudis de dependència de pH per determinar si mutacions en els residus situats en aquest lloc d'unió poden alterar la dependència de pH.

El model amb el que estem treballant, sembla ser un bon model de hCNT3. Aquest model ens ajudarà a entendre com variants polimòrfiques poden alterar la transportabilitat de diferents substrats. No obstant, el model té algunes limitacions. La primera, el fet que el transportador eucariota té tres dominis més a l'extrem N-terminal fa que aquests no siguin modelables, i sembla que són importants en la interacció amb altres proteïnes. A més a més, estem treballant amb un model estàtic, per a tal de poder entendre com funciona el cicle de translocació d'un transportador fa falta tenir-lo cristal·litzats en totes les possibles conformacions intermèdies i en el cas del nostre transportador fins ara només es disposa d'un cristall. Per això, de moment no es poden portar a terme estudis de dinàmica molecular essencials per entendre el funcionament del transportador.

4

CONCLUSIONS

- El transportador de cations orgànics 1 (hOCT1) sembla ser un bon candidat com a proteïna transportadora de bendamustina i zebularina, mediant la internalització de la primera i la extrusió de la segona, anticipant així el diferent paper que pot tenir aquesta proteïna en l'acció dels fàrmacs depenent del substrat que transloqui.

- Les variants polimòrfiques de hOCT1 amb freqüències al·lèliques significatives en humans modifiquen diferencialment la interacció de substrats coneguts de hOCT1 com la lamivudina, i la interacció de nous substrats identificats en aquesta tesis, com la bendamustina i la zebularina.

- Les variants polimòrfiques que impliquen cisteïnes del *loop* extracel·lular situat entre els TMDs 1 i 2 de hOCT1 no afecten la oligomerització de hOCT1.

- Les variants genetiques de hOCT1 també semblen afectar diferencialment les interaccions fàrmac-fàrmac amb la proteïna transportadora. La infecció de PBMCs amb el Virus de la Immunodeficiència Humana (VIH) augmenta l'expressió de ARNm de hOCT1.

- Les variants polimòrfiques de hOCT1 poden estar associades tant en quimoresistència (en el cas de bendamustina) com en quimiosensibilitat (en el cas de zebularina), depenent de l'aparent asimetria de la proteïna transportadora pel que fa a la selectivitat de substrat.

- El genotipat de les variants polimòrfiques de hOCT1 en una cohort de pacients espanyols de Leucèmia Limfàtica Crònica mostrà freqüències al·lèliques similars a les prèviament descrites en Caucàsics. Les variants associades amb una activitat de hOCT1 reduïda o nul·la eren més abundants en els subgrups que mostraven major resistència a bendamustina *ex vivo*.

- Les proteïnes transportadores que pertanyen a les famílies gèniques *SLC22* (hOCT1, hOCT2), *SLC28* (hCNT1, hCNT3) and *SLC29* (hENT1, hENT2) poden determinar la disponibilitat cel·lular dels inhibidors de la ADN metiltransferasa (DNMT) de manera diferencial, essent els transportadors de nucleòsids responsables de la internalització de azacitidina i zebularina, i hOCT1 i hOCT2 responsables de la extrusió de zebularina.

- La decitabina, tot i només presentar petits canvis estructurals respecte a l'azacitidina (la falta del grup 2'OH) no és translocada per cap de les proteïnes transportadores estudiades, tot i que pot interaccionar amb els hENTs i hCNTs.

- S'ha generat i validat preliminarment un model d'unió de substrat per a hCNT. Els residus T370, I371, N336, S396 i T605 semblen estar implicats en la unió del primer i el segon sodi en hCNT3., explicant així mecanísticament la variant polimòrfica (hCNT3C602R) prèviament identificada en la població espanyola.