Structural and Functional Studies of Enzymes in Nucleotide Metabolism

A Detailed Investigation of Two Enzymes and Interaction Profiling of FDA-Approved Nucleoside Analog Drugs with 23 Enzymes

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Cover: An artistic view of the screening results obtained in Paper III

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Structural and Functional Studies of Enzymes in Nucleotide Metabolism – A Detailed Investigation of Two Enzymes and Interaction Profiling of FDA-Approved Nucleoside Analog Drugs with 23 Enzymes

Abstract

Enzymes in nucleotide metabolism serve as the producers of the building blocks for DNA and RNA. From a medical perspective, nucleotide metabolism, and in particular salvage pathway enzymes, have attracted special interest, as nucleoside prodrugs given in the treatment of cancer and HIV are converted into their active metabolite forms by these enzymes.

In this thesis, two enzymes; uridine monophosphate kinase (UMPK) from *Ureaplasma parvum* (*Up*) and human phosphoribosyltransferase domain containing protein 1 (PRTFDC1), have been investigated. Furthermore, a nucleoside analog library (NAL) consisting of 45 FDA-approved nucleoside analogs has been developed.

The structure of *Up*-UMPK revealed that it was a hexamer. Kinetic constants were determined for UMP and ATP. UTP was a competitive inhibitor of UMP, and a non-competitive inhibitor of ATP. In contrast to other bacterial UMPKs, *Up*-UMPK was not activated by GTP.

PRTFDC1 is a homolog of hypoxanthine-guanine phosphoribosyltransferase (HPRT). Mutations in HPRT are associated with Lesch-Nyhan syndrome. The three-dimensional structures of PRTFDC1 and HRPT are very similar. Even though PRTFDC1 recognizes guanine and hypoxanthine as substrates, the functional turnover rates are less than 1% of the activity of HPRT.

NAL was screened using the high-throughput method, differential static light scattering (DSLS). An interaction profile of 23 enzymes involved in nucleotide metabolism and NAL was revealed. Interactions were detected for uridine phosphorylase 1 (UPP1) and guanine deaminase (GDA) with eight and six nucleoside prodrugs, respectively. The knowledge gained from this study can be important in the future search for drug lead candidates for UPP1 and GDA.

Keywords: Nucleotide metabolism, nucleoside analogs, *Ureaplasma parvum*, uridine monophosphate kinase, phosphoribosyltransferase domain containing protein 1, nucleoside analog library, differential static light scattering, enzyme kinetics, crystal structure.

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To my father

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I **Egeblad-Welin, L**., Welin, M., Wang, L., Eriksson, S. (2007). Structural and functional investigations of *Ureaplasma parvum* UMP kinase – a potential antibacterial drug target. *FEBS J* 274, 6403-6414.

II *Welin, M., ***Egeblad, L**., Johansson, A., Stenmark, P., Wang, L., Flodin, S., Nyman, T., Trésaugues, L., Kotenyova, T., Johansson, I., Eriksson, S., Eklund, H., Nordlund, P. (2010). Structural and functional studies of the human phosphoribosyltransferase domain containing protein 1. *FEBS J* 277, 4920-4930.

* Shared first authorship

III **Egeblad, L**., Welin, M., Johansson, A., Flodin, S., Gräslund, S., Wang, L., Eriksson, S., Nordlund, P. Interaction profiling of FDA-approved nucleoside analog drugs with 23 enzymes of human nucleotide metabolism. *Manuscript*.

Paper not included in the thesis:

IV ⋆**Egeblad-Welin, L.**, ⋆Sonntag, Y., Eklund, H., Munch-Petersen, B. (2007). Functional studies of active-site mutants from *Drosophila melanogaster* deoxyribonucleoside kinase – Investigations of the putative catalytic glutamate-arginine pair and of residues responsible for substrate specificity. *FEBS J* 274, 1542-1551.

⋆ Shared first authorship

Abbreviations

Nucleobases

A	Adenine
C	Cytosine
G	Guanine
Hx	Hypoxanthine
T	Thymine
U	Uracil
X	Xanthine

Nucleosides

Ado	Adenosine
dAdo	Deoxyadenosine
Cyd	Cytidine
dCyd	Deoxycytidine
Guo	Guanosine
dGuo	Deoxyguanosine
Ino	Inosine
dIno	Deoxyinosine
Urd	Uridine
dUrd	Deoxyuridine
Thd	Thymidine

Nucleotides

dNMP	Deoxynucleoside monophosphate
dNDP	Deoxynucleoside diphosphate
dNTP	Deoxynucleoside triphosphate
NMP	Nucleoside monophosphate
NDP	Nucleoside diphosphate

NTP	Nucleoside triphosphate

Other abbreviations

AIDS	Acquired immune deficiency syndrome
AlF_x	Aluminofluoride
BeF_x	Beryllofluoride
CMV	Cytomegalovirus
CNT	Concentrative nucleoside transporter
DSLS	Differential static light scattering
ENT	Equilibrative nucleoside transporter
FDA	U.S. Food and Drug Administration
HIV	Human immunodeficiency virus
HSV1	Herpes simplex virus type 1
HSV2	Herpes simples virus type 2
KEGG	Kyoto Encyclopedia of Genes and Genomes
MDS	Mitochondrial DNA depletion syndrome
MNGIE	Mitochondrial neurogastrointestinal encephalomyopathy
MRP	Multidrug resistance proteins
mtDNA	Mitochondrial DNA
MTHF	5,10-methylenetetrahydrofolate
NA	Nucleoside analog
NAL	Nucleoside analog library
NAMP	Nucleoside analog monophosphate
NADP	Nucleoside analog diphosphate
NATP	Nucleoside analog triphosphate
NRTI	Nucleoside reverse transcriptase inhibitor
NtRTI	Nucleotide reverse transcriptase inhibitor
OAT	Organic anion transporter
PEPT	Peptide transporter
PDB	Protein data bank
PP_i	Pyrophosphate
PRPP	Phosphoribosyl pyrophosphate
SCID	Severe combined immunodeficiency
SGC	Structural genomics consortium
T_{agg}	Aggregation temperature
UMPK	Uridine monophoshate kinase
Up	*Ureaplasma parvum*
VZV	Varicella zoster virus

Gene name	Other name	Protein name
***De novo* synthesis of purines**		
ADSL		Adenylosuccinate lyase
ADSS, ADSSL1, ADSS2		Adenylosuccinate synthetase
ATIC		Phosphoribosylaminoimidazole-carboxamide formyltranseferase/ IMP cyclohydrolase
GART		Phosphoribosylglycinamide formyltransferase, Phosphoribosylamine--glycine ligase, Phosphoribosylformylglycinamidine cyclo-ligase
GMPS		GMP synthetase
IMPDH1, IMPDH2	IMP dehydrogenase	Inosine 5'-monophosphate dehydrogenase
PAICS		Phosphoribosylaminoimidazole carboxylase, Phosphoribosylaminoimidazole -succinocarboxamide synthetase
PFAS		Phosphoribosylformylglycinamidine synthetase
PPAT		Amidophosphoribosyltransferase
***De novo* synthesis of pyrimidines**		
CAD	Carbamoyl-phosphate synthetase 2	Glutamine-dependent carbamoyl-phosphate synthetase, Aspartate carbamoyltransferase, Dihydroorotase
CTPS, CTPS2		CTP synthetase
DCTD	dCMP deaminase	Deoxycytidylate deaminase
DHODH		Dihydroorotate dehydrogenase, mitochondrial
TYMS	TS, ThyA	Thymidylate synthase
UMPS	UMP synthase	Uridine 5'-monophosphate synthase
Ribonucleotide reductase		
RRM1, RRM2, RRM2B	RR	Ribonucleotide reductase
RRM1	R1	Ribonucleoside-diphosphate reductase large subunit
RRM2	R2	Ribonucleoside-diphosphate reductase small subunit
RRM2B	P53R2	p53 inducible ribonucleotide reductase small subunit 2-like protein
Salvage pathway		

ADK		Adenosine kinase
APRT		Adenine phosphoribosyltransferase
DCK	dCK	Deoxycytidine kinase
DGUOK	dGK	Deoxyguanosine kinase, mitochondrial
HPRT1	HPRT	Hypoxanthine-guanine phosphoribosyltransferase
PRTFDC1		Phosphoribosyltransferase domain containing protein 1
TK1	TK1	Thymidine kinase 1, cytosolic
TK2	TK2	Thymidine kinase 2, mitochondrial
UCK1, UCK2		Uridine-cytidine kinase
Mono- and diphosphate kinases		
AK1, AK2, AK3, AK4, AK5, AK7	AMPK	Adenylate kinases
DTYMK	TMPK	Thymidylate kinase
GUK1	GMPK	Guanylate kinase
NME1, NME2, NME3, NME4, NME5, NME6, NME7, NME1-2	NDPK	Nucleoside diphosphate kinase
CMPK1, CMPK2	UMP-CMPK	UMP-CMP kinase
Catabolism of purines and pyrimidines		
ADA		Adenosine deaminase
AMPD1, AMPD2, AMPD3		AMP deaminase
CDA		Cytidine deaminase
DPYD		Dihydropyrimidine dehydrogenase [$NADP^+$]
DPYS		Dihydropyrimidinase
DUT	dUTPase	Deoxyuridine 5'-triphosphate nucleotidohydrolase
GDA		Guanine deaminase
GMPR, GMPR2		GMP reductase
ITPA	ITPase	Inosine triphosphate pyrophosphatase
NUDT16	(deoxy)inosine diphosphatase	U8 snoRNA-decapping enzyme
PNP		Purine nucleoside phoshorylase
TYMP		Thymidine phosphorylase
UPB1		Beta-ureidopropionase
UPP1, UPP2		Uridine phosphorylase
XDH	Xanthine oxidase	Xanthine dehydrogenase/oxidase
NT5C2, NT5C, NT5E, NT5C3, NT5M, NT5C1A, NT5C1B	5'-nucleotidase	Purine

1 Introduction

1.1 Enzymes in nucleotide metabolism

Enzymes in nucleotide metabolism serve as the producers of the building blocks/precursors for DNA and RNA. An elaborate collection of enzymes are involved in producing a balanced deoxynucleotide pool, and are often regulated by sophisticated mechanisms. From a medical perspective, nucleotide metabolism, and in particular salvage pathway enzymes, have gained special interest, as prodrugs given in the treatment of cancer and HIV are converted into their active metabolite forms by these enzymes.

1.1.1 Synthesis of precursors for DNA and RNA

Nucleotides are the building blocks of DNA and RNA and therefore essential for the ability of the cell to replicate, synthesize proteins and repair DNA. The nucleotides are divided into purine and pyrimidine nucleotides. ”Nucleotide” is a general term for a structure comprised of a base, a pentose sugar and one to three phosphate groups. The purine nucleobases are adenine (A) and guanine (G), and the pyrimidine nucleobases are cytosine (C), thymine (T) and uracil (U). Thymine is only found in DNA and uracil is only found in RNA. The pentose sugar is either a ribose, which is found in RNA or 2’-deoxyribose, which is found in DNA (Figure 1).

A nucleoside is comprised of a base with an added pentose sugar but without any phosphate groups.

Enzymes involved in nucleotide synthesis can make precursors via either *de novo* or salvage pathways. In the *de novo* pathway, nucleotides are built from amino acids (aspartic acid, glycine and glutamine), CO_2,

phosphoribosyl pyrophosphate (PRPP) and 5,10-methylenetetrahydrofolate (MTHF). Enzymes involved in the salvage pathway can recycle nucleobases, nucleosides and deoxynucleosides obtained from either degradation of RNA and DNA or from the diet.

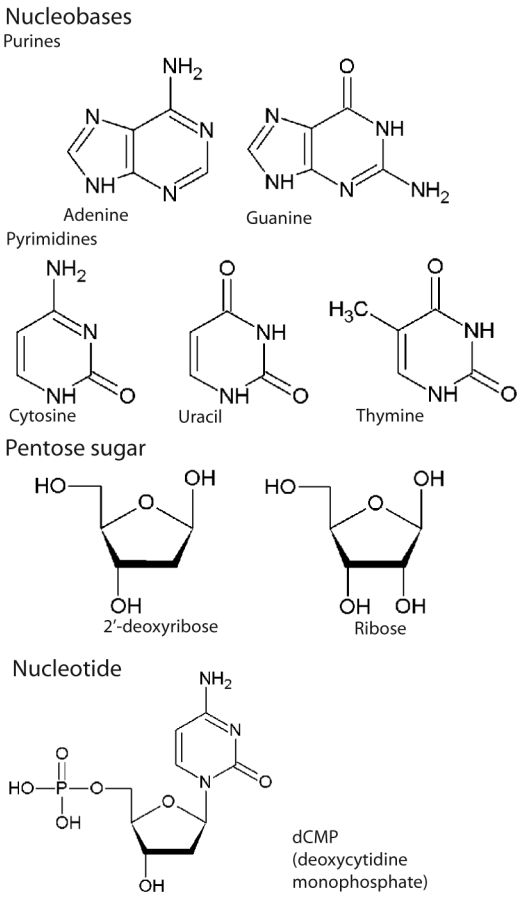

Figure 1. Nucleobases, pentose sugars and one nucleotide. Nucleobases are grouped into purines (adenine and guanine) and pyrimidines (cytosine, uracil and thymine). The pentose sugars are ribose and 2'-deoxyribose. An example of a nucleotide, deoxycytidine monophosphate (dCMP), is also shown.

Nucleotides are also important in other cellular functions, such as an energy currency (ATP and, to a lesser extent, GTP), as carriers of activated intermediates (UDP-glucose and CDP-diacylglycerol), as second messengers in signal transduction pathways (cAMP and cGMP) and as components of cofactors (Coenzyme A, FAD, NAD^+ and $NADP^+$). Furthermore, some

nucleosides and nucleotides can act as extracellular signalling molecules (Burnstock, 2008).

In the following sections, the reaction pathways leading to the synthesis of nucleotides are described. For simplicity, two figures are shown; Figure 2 is an overview of purine metabolism and Figure 3 illustrates pyrimidine metabolism. Each reaction is labeled with a number corresponding to the enzyme(s) performing the reaction. The gene name in humans can be found in the figure text, in which the enzymes will be annotated by their gene names if not otherwise stated.

1.1.2 *De novo* synthesis of purines

In the *de novo* pathway, AMP and GMP are synthesized via the intermediate, IMP. An activated pentose sugar, PRPP, serves as a starting point, and in a ten step reaction, IMP is generated from glutamine, glycine, aspartate, CO_2 and MTHF. These reactions are carried out by six enzymes in humans; three monofunctional enzymes (PPAT, PFAS, ADSL), two bifunctional enzymes (PAICS and ATIC) and one trifunctional enzyme (GART). Once IMP is synthesized, it can be converted into either AMP or GMP in two-step reactions by adenylosuccinate synthetase and ADSL for AMP, and IMP dehydrogenase and GMPS for GMP. A highly regulated step in *de novo* synthesis of purines is the first step leading to the generation of IMP by the enzyme, amidophosphoribosyltransferase (PPAT). This enzyme is inhibited by AMP, GMP and IMP and activated by PRPP (Welin & Nordlund, 2010; Zhang *et al.*, 2008; Smith, 1995).

1.1.3 *De novo* synthesis of pyrimidines

In humans, the biosynthesis of UMP involves three enzymes; a trifunctional enzyme (CAD), dihydroorotate dehydrogenase (DHODH) and a bifunctional enzyme (UMP synthase (UMPS)). The three domains of CAD (Carbamoylphosphate synthetase 2, Aspartate carbamoyltransferase and Dihydroorotase) assemble a six membered ring from glutamine, aspartic acid and CO_2. PRPP is added through the orotate phosphoribosyltransferase domain of UMPS. In order to produce CTP, UMP is phosphorylated by monophosphate and diphosphate kinases to UTP. UTP, in turn, is aminated by CTP synthetase (CTPS and CTPS2) to CTP. Regulation of *de novo* pyrimidine synthesis is exerted on the carbamoylphosphate synthetase domain of CAD by UTP as a feedback inhibitor and PRPP as an activator. CTP synthetase is inhibited by the end product, CTP, and activated by GTP. Together, these regulatory mechanisms contribute to maintaining a

balanced production of purine and pyrimidine nucleotides (Welin & Nordlund, 2010; Evans & Guy, 2004).

Production of dTTP is comprised of several steps; dCMP is converted into dUMP by dCMP deaminase (DCTD), followed by conversion of dUMP into dTMP by thymidylate synthase (TYMS), often referred to as TS or ThyA (Costi, 1998).

1.1.4 Ribonucleotide reductase

Ribonucleotide reductase (RR) is an important *de novo* enzyme, involved in the allosteric regulation of dNTP levels. RR catalyzes the conversion of the 2'-OH of a ribonucleoside diphosphate to a hydrogen atom, generating a 2'-deoxynucleoside diphosphate, through free radical chemistry. RR can be categorized into three different classes; I, II and III. In eukaryotes, class Ia is found. This form of RR is composed of R1 and one of the two R2 subunits (R2 or its homolog p53R2). The R1 subunit contains the catalytic site, the activity site and the specificity site. ATP is an activator and binds to the activity site, whereas dATP is an inhibitor. ATP, dATP, dTTP and dGTP function as effector molecules altering the substrate specificity depending on which molecule binds to the specificity site. The substrate molecules are CDP, UDP, ADP and GDP (Nordlund & Reichard, 2006; Reichard, 2002). The R2 subunit undergoes cell cycle-dependent degradation. p53R2, important in DNA repair, is expressed at a low level throughout the cell cycle, and is over expressed after DNA damage (Nordlund & Reichard, 2006).

1.1.5 The salvage pathway

Nucleobases, nucleosides and deoxynucleosides obtained from either degraded DNA or RNA or from the diet, can be recycled via reactions catalyzed by different families of enzymes. One family is the phosphoribosyltransferases, which includes adenine and hypoxanthine-guanine phosphoribosyltransferase (APRT and HPRT1, in this work called HPRT) (Sinha & Smith, 2001). HPRT is the most well characterized member, as mutations in this enzyme are linked to Lesch-Nyhan syndrome. APRT recycles adenine, producing AMP, and HPRT can use both guanine and hypoxanthine as substrates in the synthesis of GMP and IMP. Both enzymes are expressed ubiquitously (Keough *et al.*, 1999; Thomas *et al.*, 1973). A homolog of HPRT, phosphoribosyltransferase domain containing protein 1 (PRTFDC1) (Nicklas, 2006), is discussed in more detail in **Chapter 3** and **Paper II**.

The deoxynucleoside kinases include thymidine kinase 1 or TK1 (TK1), deoxycytidine kinase or dCK (DCK), thymidine kinase 2 or TK2 (TK2) and deoxyguanosine kinase or dGK (DGUOK). These are the salvage enzymes used for recycling thymidine (Thd), deoxycytidine (dCyd), deoxyuridine (dUrd), deoxyadenosine (dAdo) and deoxyguanosine (dGuo). The deoxynucleosides are phosphorylated by nucleoside triphosphates (NTPs) at the 5´position to become deoxynucleoside monophosphate (dNMP). This is believed to the rate-limiting step for the salvage of dNTPs (Arner & Eriksson, 1995). TK1 and dCK are both cytosolic proteins. TK1 has narrow substrate specificity and phosphorylates only Thd and dUrd, while both pyrimidine and purines i.e. dCyd, dAdo and dGuo can act as substrates for dCK. Deoxycytidine kinase is discussed in more detail in **4.4**. Both TK2 and dGK are mitochondrial enzymes. TK2 can phosphorylate Thd, dCyd and dUrd, while the substrates for dGK are dAdo, dGuo and deoxyinosine. Reactions carried out by deoxynucleoside kinases are feed-back inhibited by their distal end products (dTTP, dCTP, dATP and dGTP) (Eriksson *et al.*, 2002).

Two kinds of nucleoside kinases are identified in humans. Van Rompay *et al.* (2001) characterized two recombinant uridine-cytidine kinases (UCK1 and UCK2), and it was shown that both enzymes could phosphorylate Urd and Cyd in the presence of ATP (Van Rompay *et al.*, 2001). The other nucleoside kinase is an adenosine kinase (ADK), phosphorylating Ado into AMP (Spychala *et al.*, 1996).

1.1.6 Monophosphate kinases and nucleoside diphosphate kinase

Once the nucleoside or deoxynucleoside monophosphates are made, they are phosphorylated further by their corresponding monophosphate kinase and nucleoside diphosphate kinase into final products, either NTPs or dNTPs.

There are four categories of monophosphate kinases: thymidylate kinases or TMPK (DTYMK), UMP-CMP kinases or UMP-CMPK (CMPK1, CMPK2), adenylate kinases or AMPKs (AK1, AK2, AK3, AK4, AK5, AK7) and guanylate kinases or GMPK (GUK1). TMPK phosphorylates both dTMP and dUMP (Huang *et al.*, 1994).

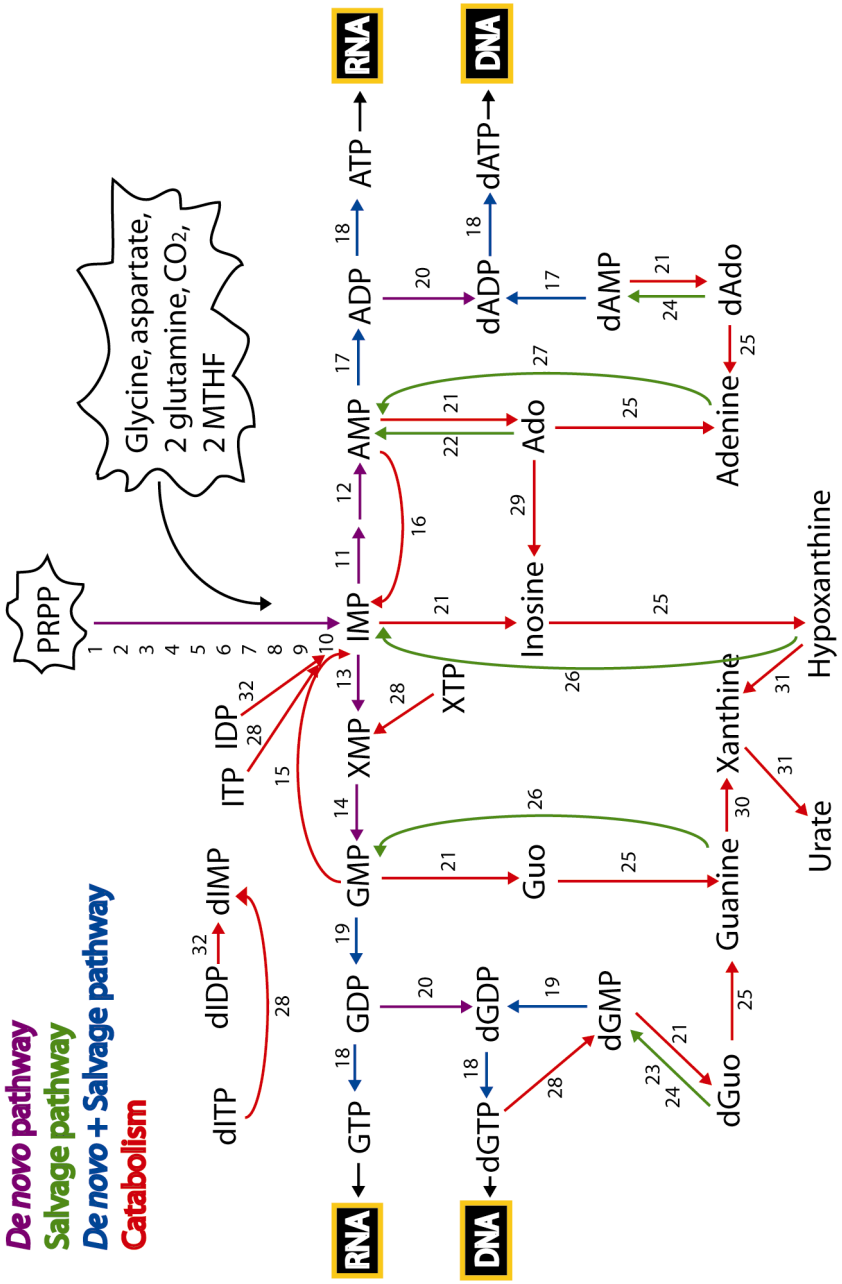

Figure 2. A simplified overview of purine metabolism including the *de novo* pathway (purple), the salvage pathway (green), the *de novo* and salvage pathway (blue) and the catabolic (red) pathway. The map is based on purine metabolism in humans obtained from Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa *et al.*, 2010; Kanehisa *et al.*, 2006; Kanehisa & Goto, 2000). All enzymes annotated in *italics* have been investigated in **Paper**

III. For simplicity, reactions are shown progressing only in the most favored direction. Each reaction is given a number that correlates to the following gene names. If a protein is encoded by several genes, a common name for the protein is given: **1** PPAT, **2** *GART*, **3** *GART*, **4** PFAS, **5** *GART*, **6** *PAICS*, **7** *PAICS*, **8** *ADSL*, **9** ATIC, **10** ATIC, **11** *Adenylosuccinate synthetase* (ADSSL1, ADSS, *ADSS2*) **12** *ADSL*, **13** IMP dehydrogenase (IMPDH1 or IMPDH2), **14** *GMPS*, **15** *GMP reductase* (GMPR and *GMPR2*), **16** AMP deaminase (AMPD1, AMPD2, AMPD3), **17** Adenylate kinase (AK1, AK2, AK4, AK5, AK7), **18** Nucleoside diphosphate kinase (NME1, NME2, NME3, NME4, NME5, NME6, NME7, NME1-2), **19** GUK1, **20** *Ribonucleotide reductase* (*RRM1*, RRM2, RRM2B), **21** *5'-Nucleotidase* (*NT5C2*, NT5C, NT5E, *NT5C3*, NT5M, NT5C1A, NT5C1B), **22** ADK , **23** DGUOK, **24** *DCK*, **25** PNP, **26** HPRT1, **27** APRT, **28** *ITPA*, **29** ADA, **30** *GDA*, **31** XDH, **32** *NUDT16*.

UMP-CMPK phosphorylates CMP and UMP with ATP or dATP as preferred phosphate donors (Liou *et al.*, 2002; Van Rompay *et al.*, 1999). A mitochondrial UMP-CMPK2 was reported in 2008 by Xu *et al.* This enzyme phosphorylates dUMP, dCMP, UMP and CMP, with a preference for deoxynucleotides (Xu *et al.*, 2008). AMPKs preferentially phosphorylate AMP, but some AMPKs can also phosphorylate dAMP (Van Rompay *et al.*, 2000). GMPK phosphorylates GMP and dGMP into their diphosphate forms (Brady *et al.*, 1996).

A new class of UMP kinases was identified in *E. coli* (Serina *et al.*, 1995). The bacterial/archaeal UMP kinase is a member of the amino acid kinase family and is subject to sophisticated regulation. This will be further discussed in **Chapter 2** and **Paper I**.

The nucleoside diphosphate kinases (NDPKs) are ubiquitous enzymes and eight different kinds have been identified in humans (Lacombe *et al.*, 2000). NDPKs catalyze the transfer of a γ-phosphoryl group to a nucleoside diphosphate. The enzymes are promiscuous with regard to phosphate donors and acceptors (Janin & Deville-Bonne, 2002). Besides NDPKs, other enzymes, such as pyruvate kinase, have been shown to have NDPK-like properties (Deville-Bonne *et al.*, 2010).

Although not shown in Figures 2 and 3, the actions of the monophosphate and diphosphate kinases are reversible (Van Rompay *et al.*, 2000).

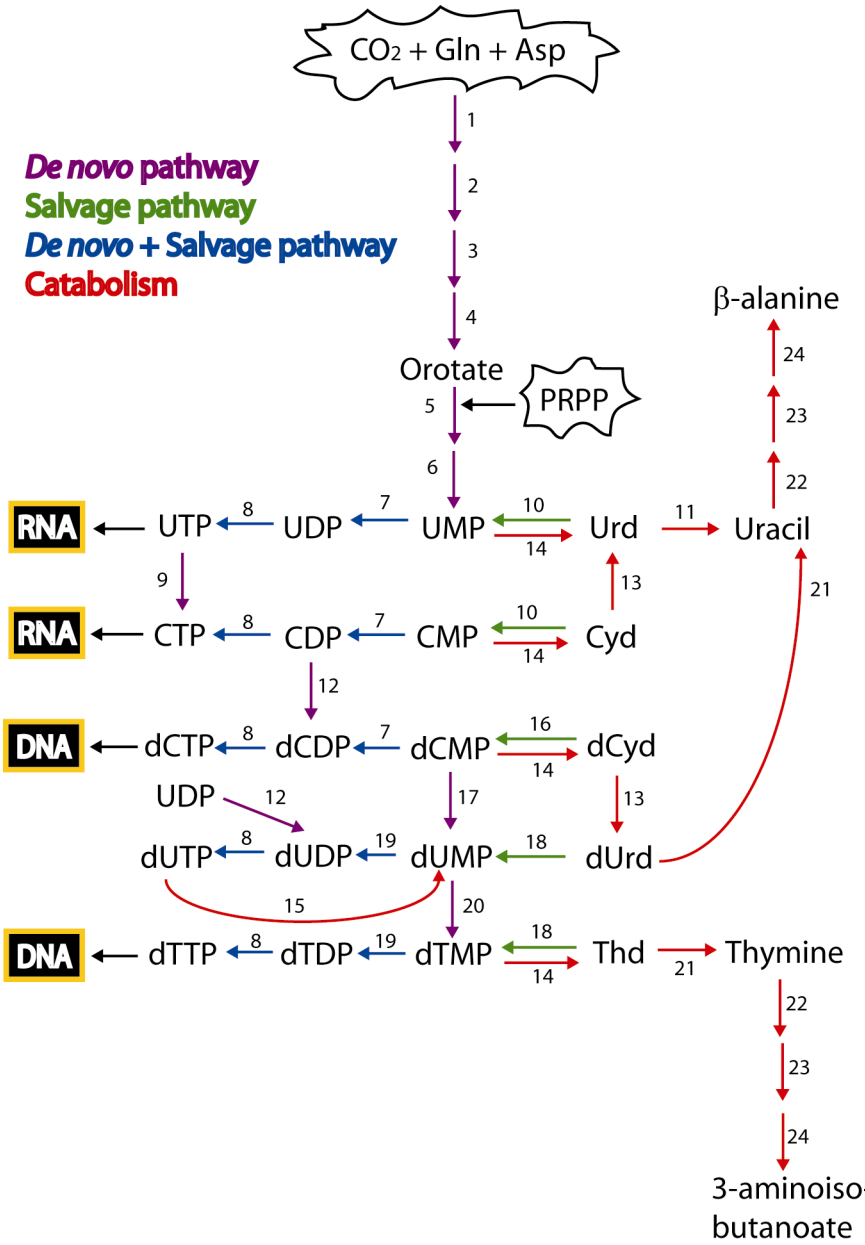

Figure 3. A simplified overview of pyrimidine metabolism including the *de novo* pathway (purple), the salvage pathway (green), the *de novo* and salvage pathway (blue) and the catabolic (red) pathway. The map is based on pyrimidine metabolism in humans obtained from KEGG (Kanehisa *et al.*, 2010; Kanehisa *et al.*, 2006; Kanehisa & Goto, 2000). All enzymes annotated in *italics* have been investigated in **Paper III**. For simplicity, reactions are shown progressing only in the most favored direction. Each reaction is given a number that correlates to gene names listed in this text. If a protein is encoded by several genes, a

common name for the protein is given: **1** CAD, **2** CAD, **3** CAD, **4** DHODH, **5** *UMPS*, **6** *UMPS*, **7** CMPK1 and *CMPK2*, **8** Nucleoside diphosphate kinase (NME1, NME2, NME3, NME4, NME5, NME6, NME7, NME1-2), **9** *CTP synthetase* (CTPS and *CTPS2*), **10** *Uridine-cytidine kinase* (*UCK1*, UCK2), **11** *UPP1* and *UPP2*, **12** *Ribonucleotide reductase* (*RRM1*, RRM2, RRM2B), **13** CDA, **14** *5'-Nucleotidase* (*NT5C2*, NT5C, NT5E, *NT5C3*, NT5M, NT5C1A, NT5C1B), **15** DUT, **16** *DCK*, **17** *DCTD*, **18** TK1 and TK2, **19** DTYMK, **20** TYMS, **21** TYMP, **22** DPYD, **23** *DPYS*, **24** *UPB1*.

1.1.7 Catabolism of purines and pyrimidines

Excessive purines and pyrimidines are degraded and secreted via the urine. Purines are degraded to xanthine (X) and then to urate by xanthine oxidase (XDH). Preceding reactions involve 5'-nuclotidases, purine nucleoside phosphorylase (PNP), guanine deaminase (GDA) and adenosine deaminase (ADA). Pyrimidines are degraded via uracil and thymine, which are themselves degraded to β-alanine and 3-aminoisobutanoate, respectively, by DPYD, DPYS and UPB1. Before uracil and thymine are formed, the nucleotides/nucleosides are degraded by 5'-nuclotidases, cytidine deaminase (CDA), uridine phosphorylases (UPP1 and UPP2) and thymidine phosphorylase (TYMP).

The presence of 5'-nucleotidases are also important for the maintenance of a balanced nucleotide pool. 5'-nucleotidases dephosphorylate (deoxy)nucleoside monophosphates into (deoxy)nucleosides and free phosphate ions. There are seven kinds of 5'-nucleotidases with overlapping substrate specificities and different tissue expression. Cytosolic NT5C2 prefers IMP, dIMP, GMP, dGMP and XMP as substrates, whereas cytosolic NT5C3 only hydrolyzes pyrimidine monophosphates (Hunsucker *et al.*, 2005). Other enzymes important for the regulation of nucleotide pools are GMP reductases and AMP deaminases, which are responsible for the conversion of GMP and AMP back to IMP.

A group of enzymes called house-cleaning NTPases are responsible for the elimination of potentially toxic by-products of nucleotide metabolism, such as dUTP and dITP. ITPase (ITPA) hydrolyses dITP, ITP and XTP into their monophosphate forms, and dUTPase (DUT) can hydrolyse dUTP to dUMP (Galperin *et al.*, 2006). A human (deoxy)inosine diphosphatase (NUDT16) was recently identified by Iyama *et al.* (2010). This enzyme catalyzes the hydrolysis of dIDP and IDP as preferred substrates (Iyama *et al.*, 2010).

1.2 dNTP pools within the cell

1.2.1 Highly regulated dNTP pools within the cell

High fidelity in the maintenance of dNTP pools is of utmost importance. This is regulated at the allosteric level as well as the transcriptional and posttranslational levels, as asymmetries in the natural dNTP pools are known to give rise to increased mutagenesis (Mathews, 2006; Reichard, 1988).

Allosteric regulation is carried out by many enzymes in nucleotide metabolism, such as ribonucleotide reductase, CTP synthetase and DCTD (Evans & Guy, 2004; Reichard, 1988). At a transcriptional and posttranslational level, dNTP pools are regulated in proliferating cells via controlled cell cycle-dependent protein synthesis and protein degradation. Examples of enzymes controlled in this manner include TK1 and R2-subunit from ribonucleotide reductase (Mathews, 2006).

Mitochondrial DNA (mtDNA) replication takes place both during the cell cycle and in non-dividing cells. The mitochondrial dNTP pools are separated from the nuclear precursor dNTP pools by the mitochondrial inner membrane. Precursors for mitochondrial DNA replication or repair can either be imported from the cytosol in dividing cells, or synthesized by mitochondrial salvage enzymes in non-dividing cells (Mathews & Song, 2007; Saada, 2004).

1.2.2 Diseases associated with mutations in enzymes in nucleotide metabolism

Several disorders are directly linked to congenital errors in purine and pyrimidine metabolism, and are characterized by the presence of distorted dNTP pools. The abnormalities are often a result of point mutations in the corresponding genes.

A number of disorders associated with purine metabolism have been found. The most well known disease is Lesch-Nyhan syndrome, caused by mutations in HPRT. Depending on the mutation, either partial HPRT deficiency or Lesch-Nyhan syndrome will develop (see **3.2.2**). APRT deficiency leads to renal stone disease in children. Adenosine deaminase (ADA) deficiency has been successfully treated with gene therapy. If untreated, the deficiency will give rise to severe combined immuno deficiency (SCID), and affected individuals normally die before the age of two. Purine nucleoside phosphorylase (PNP) deficiency also gives rise to

SCID. Xanthine oxidase (XDH) deficiency is characterized by hypouricemia and is associated with renal complications. Mutations in ATIC and ADSL, both of which are involved in the *de novo* biosynthesis of purine, give rise to developmental delay and mental retardation, respectively. Mutations in AMP deaminase (AMPDH1) can cause pains and cramps in patients during exercise (Nyhan, 2005).

Disorders associated with pyrimidine metabolism include the *de novo* enzyme, UMPS, causing orotic aciduria. The disease can be treated by daily doses of uridine (Nyhan, 2005). A rare deficiency, called Miller syndrome, is caused by mutations in the *de novo* enzyme, DHODH, and gives rise to craniofacial defects (Ng *et al.*, 2010). Mutations in the pyrimidine catabolizing enzymes, DPYD, DPYS and UPB1, preventing correct degradation of thymine and uracil, cause clinical manifestations as seizures and mental retardation (Nyhan, 2005).

Increased 5'-nucleotidase activity toward both purines and pyrimidines has been associated with developmental delay and seizures (Nyhan, 2005).

The integrity of the mitochondrial dNTP pools is important for cell survival, as mitochondria are producers of cellular ATP. Point mutations in the two mitochondrial salvage enzymes, dGK and TK2, give rise to mitochondrial DNA depletion syndrome (MDS). Affected individuals usually die in early childhood (Saada, 2004). Mutations in cytosolic thymidine phosphorylase (TYMP) have been shown to be involved in mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), characterized by aberrations in mtDNA, including multiple deletions and depletion. Elevated levels of Thd and dUrd leads to increased levels of dTTP in mitochondria, and may thus distort the mitochondrial dNTP pools (Saada, 2004).

1.3 Nucleoside analogs (NAs)

A brief overview of each nucleoside analog (NA) will be presented here, including an account of the mechanisms underlying the activation of a prodrug into an active metabolite (import into the cell, activation of NAs and resistance mechanisms).

In **Chapter 4** and **Paper III**, development of a nucleoside analog library (NAL) screen, consisting of 45 FDA-approved (U.S. Food and Drug Administration) compounds, has been described. The structures of all 45

NAs are shown in Figures 4 to 7, according to drug application; anti-cancer agents (Figure 4), anti-viral agents (Figures 5 and 6) and agents used in the treatment of other medical conditions (Figure 7).

1.3.1 NAs used as anti-cancer agents

In 1957, fluorouracil was discovered to have anti-tumor activity toward transplanted tumors in rats and mice (Heidelberger *et al.*, 1957). Fluorouracil can be grouped together with floxuridine and capecitabine, the latter being metabolized in the cell to floxuridine and fluorouracil. Fluoropyrimidines are used in the treatment of gastrointestinal, pancreatic, head and neck, renal, skin, prostate, breast and colorectal cancers. Thiopurines, such as thioguanine and mercaptopurine, are used in the treatment of acute leukaemias. Cladribine and fludarabine are both deoxyadenosine derivates used in the treatment of different malignant disorders of the blood. Fludarabine is administered in 5'-monophosphate form and is dephosphorylated in the body before cellular uptake (Galmarini *et al.*, 2002). Clofarabine is a next generation dAdo analog, approved by the FDA in 2004 for the treatment of pediatric leukemic patients (Zhenchuk *et al.*, 2009). Cytarabine, a dCyd analog, is also used against hematological disorders of the blood, while gemcitabine, a dCyd analog, is active against solid tumours, such as pancreatic, breast, lung and bladder cancer (Galmarini *et al.*, 2002). An additional four NAs approved by FDA are used in the treatment of cancer. These are nelarabine, azacitidine, decitabine and pemetrexed.

1.3.2 NAs used in treatment of viral diseases

Nucleoside analogs are used in the treatment or prophylaxis of several diseases caused by viruses such as HIV, herpes simplex virus type 1 and 2 (HSV1, HSV2), Varicella zoster virus (VZV), cytomegalovirus (CMV), and hepatitis B and C viruses. Herpes viruses do not constitute a risk in immunocompetent individuals, but in immunocompromised patients (AIDS, cancer and transplant patients), the different herpes viruses can cause serious illness (Snoeck, 2000).

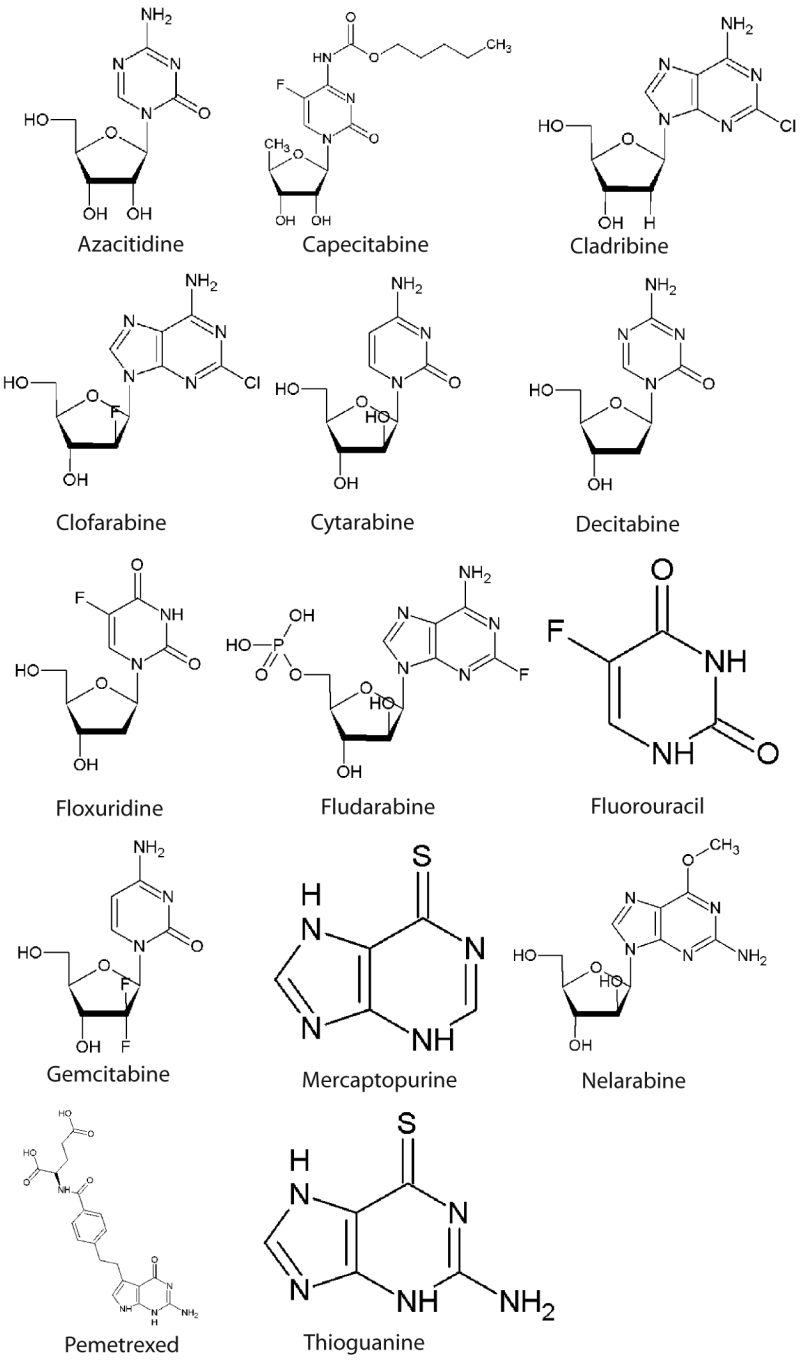

Figure 4. NAs used as anti-cancer agents.

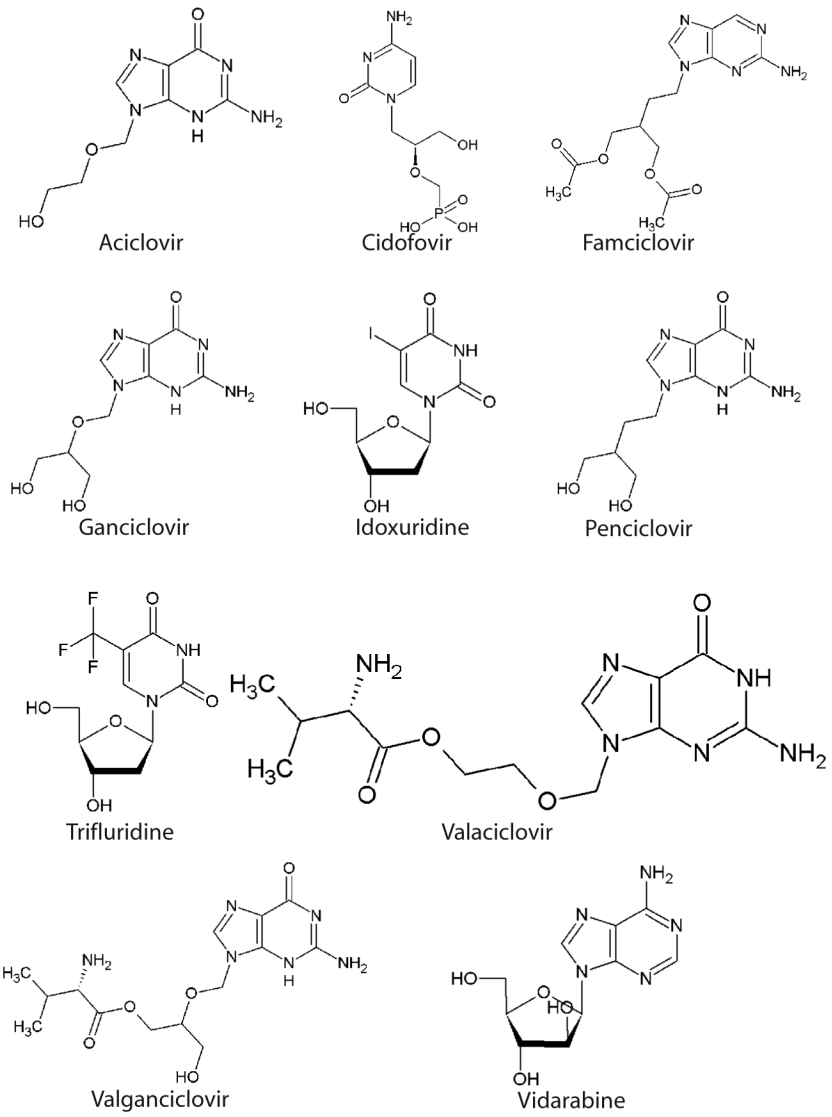

Figure 5. NAs used in the treatment of HSV1, HSV2, VZV or CMV.

Idoxuridine, trifluridine and vidarabine belong to the first generation NAs used in treatment of herpes viruses. These analogs are not specific for viral replication enzymes, and are too toxic for systemic use (De Clercq & Field, 2006; Kleymann, 2003). The second generation compounds used in the treatment of HSV1, HSV2, VZV and CMV, were introduced with the discovery of the acyclic NA, aciclovir in 1974, which entered the market in 1981. The acyclic NAs are recognised by viral enzymes, and are therefore

specific for viral replication. Aciclovir has a limited oral availability and only some 20% is absorbed from the gastrointestinal tract. Great efforts have been made to develop the acyclic concept, and have lead to the development of valaciclovir, a prodrug for aciclovir, with greater oral availability. Other acyclic analogs are penciclovir, ganciclovir, famciclovir and valganciclovir (Pescovitz, 2010; De Clercq & Field, 2006; Pescovitz, 2006; Freeman & Gardiner, 1996).

Cidofovir is a nucleoside phosphonate used in treatment of CMV in AIDS patients (De Clercq & Field, 2006).

Since HIV was identified as the etiological agent of AIDS in 1983, increasing knowledge of the virus and disease, has led to the discovery of 25 approved anti-HIV compounds for the treatment of HIV and AIDS. Seven compounds (didanosine, zalcitabine, stavudine, lamivudine, abacavir, emtricitabine and zidovudine) are nucleoside reverse transcriptase inhibitors (NRTIs). Tenofovir is the only nucleotide reverse transcriptase inhibitor (NtRTI) that has been approved for clinical use against HIV and AIDS (De Clercq, 2009).

Since 1998, five antiviral NAs (tenofovir, adefovir, lamivudine, telbivudine and entecavir) have been approved for the treatment of chronic hepatitis B (Yuen & Lai, 2011). Ribavirin is used in the treatment of chronic hepatitis C (Munir *et al.*, 2010).

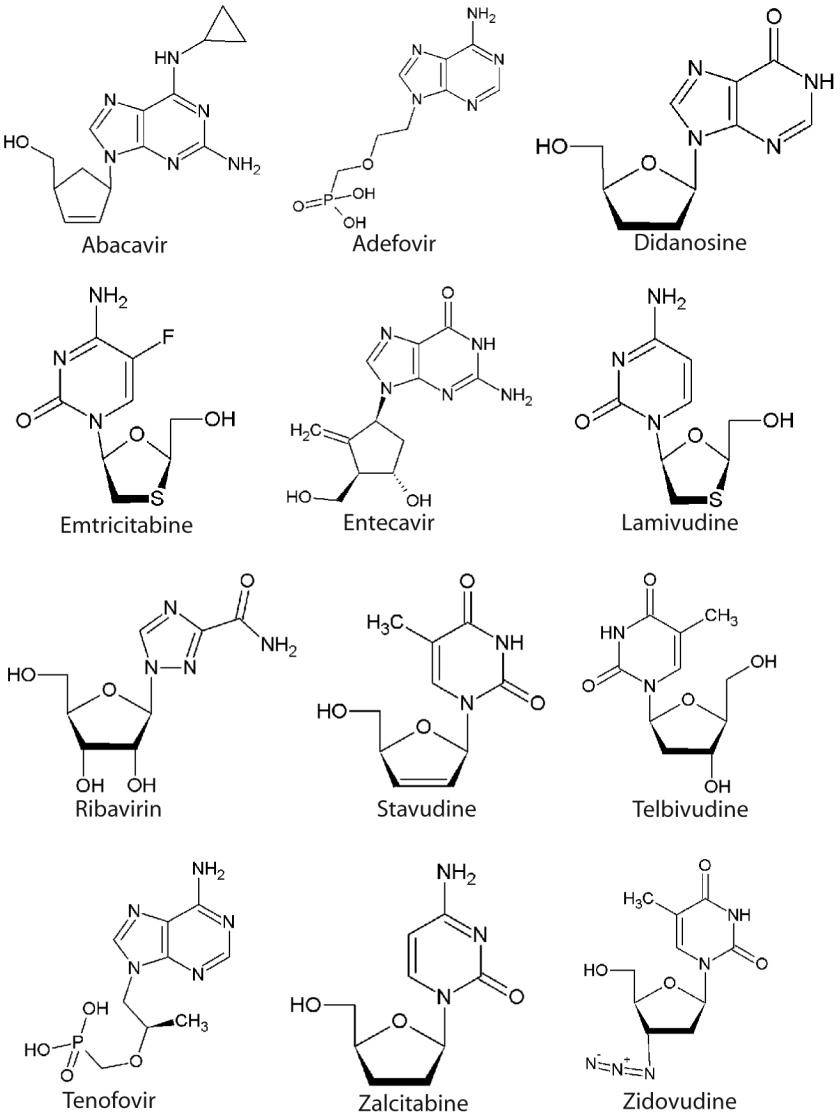

Figure 6. NAs used in the treatment of HIV, AIDS, hepatitis B and hepatitis C. Some NAs, such as tenofovir and lamivudine are used in the treatment of both HIV and hepatitis B.

1.3.3 NAs in the treatment of other medical conditions

Allopurinol is used in the treatment of gout and hyperuricemia (Ferreira, 1965), whereas flucytosine is one of the "old" antifungal agents (Chen & Sorrell, 2007). From the early 1960s until the early 1980s, azathioprine was used as a first choice immune suppressor in combination with steroids after renal transplantation (Briggs, 1991).

Adenosine is used in the treatment of paroxysmal supraventricular tachycardia (Muller & Jacobson, 2011). The xanthine derivates (caffeine, dyphylline, theophylline, theobromine, pentoxyfylline and enprofylline) all have bronchodilator properties. Theophylline has been used in the treatment of asthma (Daly, 2007; Skorodin, 1993).

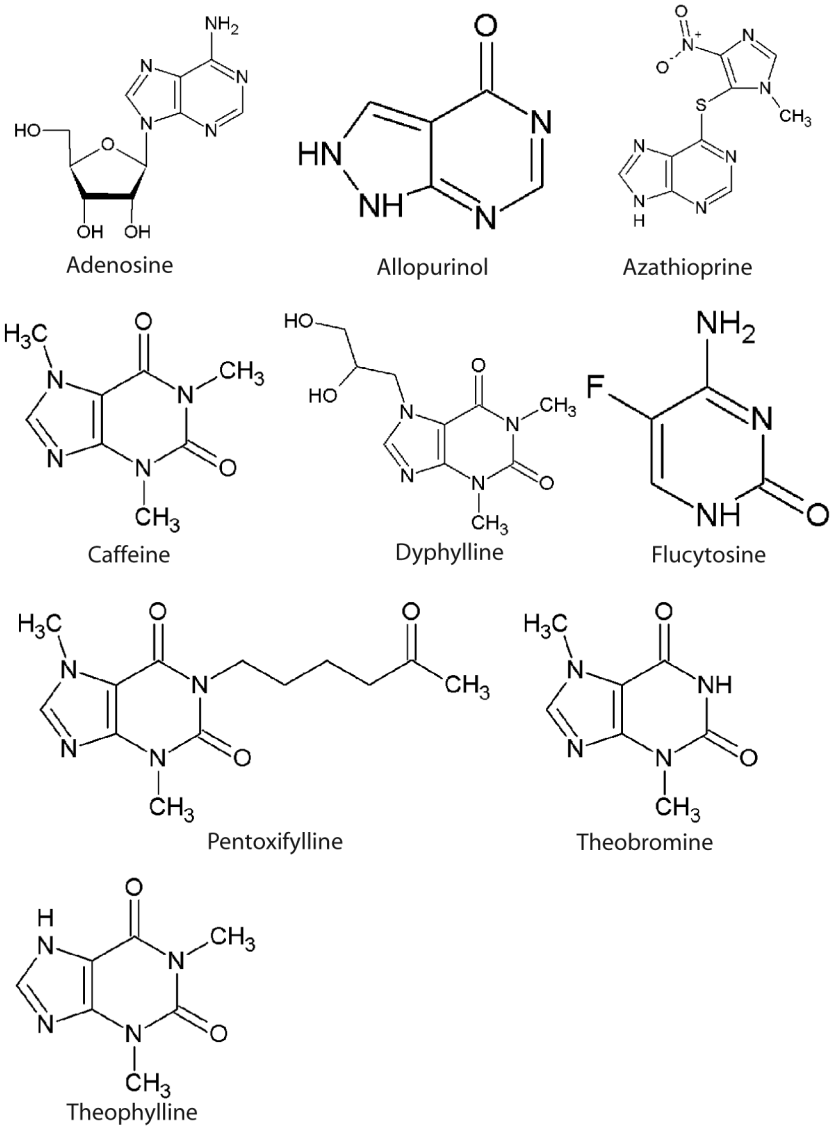

Figure 7. NAs used in the treatment of other medical conditions.

1.3.4 Mechanisms responsible for activation of NAs

Most of the NAs included in our NAL, with exception of the xanthine derivates, adenosine, allopurinol and pemetrexed, are prodrugs. In order to see an effect of an analog, it must be activated to an active substance within the cell. In this chapter, the mechanisms underlying nucleotide metabolism, especially via the salvage pathway, are described, with a focus on the NAs used as anti-cancer agents or in the treatment of viral diseases.

The first step in nucleoside analog action is import into the cell. Transporter systems include equilibrative nucleoside transporters (ENT), concentrative nucleoside transporters (CNT), organic anion transporters (OAT) and peptide transporters (PEPT). A group of multidrug resistance proteins (MRP), pumps controlling the reverse action of transporters, are also involved. The mechanisms the uptake of all NAs are not well known (Pastor-Anglada *et al.*, 2005).

Once inside the cell, the salvage nucleoside and deoxynucleoside kinases, dCK, TK1, TK2, dGK, ADK, UCK1 and UCK2, are responsible for the first phosphorylation of the NAs. The nucleoside analog monophosphates (NAMPs) are phosphorylated further by monophosphate kinases into nucleoside analog diphosphate (NADP) forms, and thereafter by nucleoside diphosphate kinases into the corresponding nucleoside analog triphosphate (NATP) forms. The NATPs are incorporated into cellular DNA/RNA and viral DNA by either DNA polymerase (cellular or viral), RNA polymerase or viral reverse transcriptase (Figure 8) (Van Rompay *et al.*, 2003; Van Rompay *et al.*, 2000). The intracellular pool of phosphorylated NAs, either in mono, di or tri phosphate form, depends on the turnover of each intermediate. The rate-limiting step in the conversion of an NA can thus be either a monophosphate or diphosphate kinase (Deville-Bonne *et al.*, 2010).

The herpes viruses, HSV1, HSV2 and VZV, all possess a viral thymidine kinase responsible for activation of the NA. This explains the low cytotoxicity of the acyclic NAs (De Clercq & Field, 2006). The acyclic nucleoside phosphonate analogs (tenofovir, cidofovir and adefovir) are activated by the monophosphate kinases as a first step (Van Rompay *et al.*, 2000). Activation mechanisms of nucleobase analogs follow different routes. Flucytosine, for example, is converted into fluorouracil by a unique cytosine deaminase found only in fungi and bacteria (Aghi *et al.*, 2000). One activation mechanism for fluorouracil has been suggested to proceed via UPP1 (Cao *et al.*, 2002). The thiopurines can be activated into their monophosphate forms by HPRT (Coulthard & Hogarth, 2005).

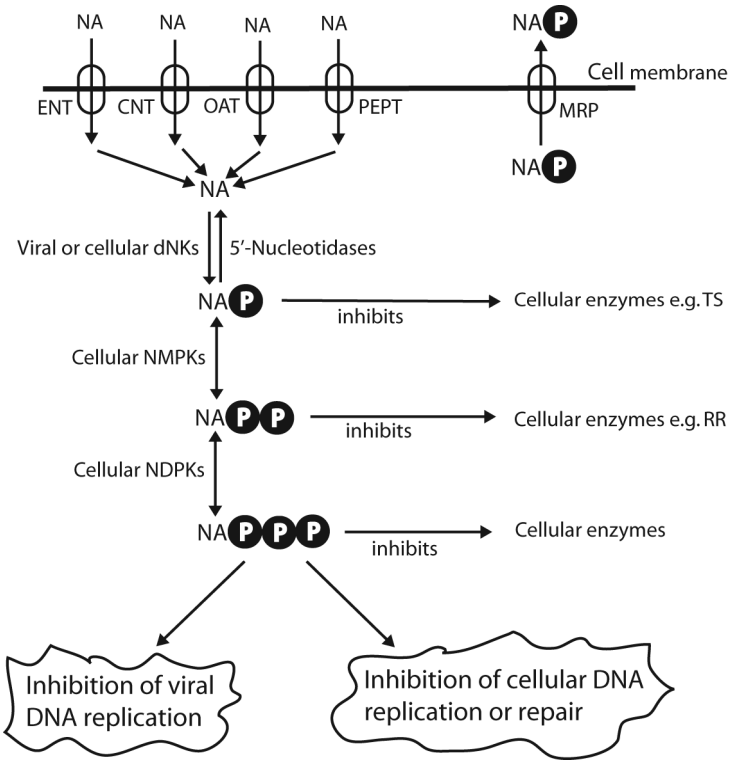

Figure 8. Activation of NAs through the salvage pathway. The NAs are imported via the action of different transporters (ENT, CNT, OAT, PEPT) and exported via MPR. The NAs are activated via viral or cellular (d)NKs, cellular NMPKs and cellular NDPKs. The phosphorylated NAs, in turn, can inhibit or interact with other enzymes involved in nucleotide metabolism. The triphosphate form can inhibit viral DNA replication or cellular DNA replication and DNA repair (Modified from Van Rompay *et al.*, 2000).

The activated NAs can exert their growth inhibiting effect at several levels. For example, (1) NAMPs can inhibit cellular enzymes, such as TS, (2) NADPs and NATPs can inhibit cellular enzymes, such as RR, and (3) NATPs can inhibit either viral DNA replication or cellular DNA replication and repair or inhibit cellular enzymes (Figure 8) (Van Rompay *et al.*, 2000).

A few examples of inhibitory effects may be given to illustrate the complexity in the functions of individual NAs.

Fluorouracil, for example, is activated to FdUMP within the cell, and this intermediate can then react with TS, forming a covalent bond, inactivating the enzyme and leading to depletion of the dTTP pools (Friedman & Sadee, 1978).

Gemcitabine diphosphate inhibits RR, thus blocking the *de novo* synthesis of dNTPs and altering the ratio between gemcitabine triphosphate and dNTPs, favouring incorporation of the analog into DNA (Galmarini *et al.*, 2001).

Incorporation of an NATP into the growing DNA strand can cause chain termination. Dideoxynucleosides, such as zalcitabine, operate in this manner (De Clercq, 2009). In non-dividing cells, the incorporation of cladribine triphosphate into a DNA strand inhibits the DNA repair machinery and accumulation of single stranded DNA breaks can potentially initiate apoptosis (Galmarini *et al.*, 2001).

1.3.5 Resistance towards NAs

An emerging problem in patients treated with NAs is resistance to these compounds. Resistance occurs at several levels, and is probably a combination of multiple factors. Several potential resistance mechanisms are discussed below.

Uptake of NAs via membrane transporters can be affected, thereby lowering the intracellular concentration of the NAs (Galmarini *et al.*, 2001). Increased activity of 5'-nucleotidases has been correlated with lower overall survival for patients with haematological malignancies (Galmarini *et al.*, 2001). A raised level of CDA has been suggested to increase the metabolism of NAs within the cells, thereby lowering the concentration of active metabolites (Galmarini *et al.*, 2001). Finally, up-regulated levels of the dNTP pools have been observed in many tumor cells, which restrict the incorporation of NATPs into the growing DNA strand (Galmarini *et al.*, 2001; Traut, 1994).

1.4 Mollicutes as a model organism for the salvage pathway?

A uridine monophosphate kinase from *Ureaplasma parvum (Up)* is described in **Chapter 2** and **Paper I**. *Up* belongs to the class of bacteria known as Mollicutes.

1.4.1 Mollicutes

Mollicutes belong to the kingdom of bacteria, and are often referred to as mycoplasma. A unique property of this class is that they have no cell wall. This also explains the name of the class, which literally means soft skin (mollis = soft and cutis = skin). Another characteristic trait is the minute size of the genomes; the smallest genome reported is only 580 kb and belongs to *Mycoplasma genitalium* and the larger genomes are up to 2200 kb long (Razin *et al.*, 1998). The genome of *Up* serovar 3 is 751.7 kb, and the G+C content is 25.5% (Glass *et al.*, 2000). This makes the Mollicutes ideal for studying the minimal set-up of genes required for maintenance of life processes (Razin *et al.*, 1998).

Sequencing of complete genomes from *M. genitalium*, *M. pneumoniae* and *Up* in 1995, 1996 and 2000, respectively, revealed that these bacteria have no genes for the *de novo* synthesis of purines and pyrimidines (Glass *et al.*, 2000; Himmelreich *et al.*, 1996; Mushegian & Koonin, 1996; Fraser *et al.*, 1995). In order to survive, precursors for RNA and DNA synthesis must be imported from the surroundings and anabolized via salvage pathways and monophosphate kinases into their final products.

Among the mollicute species, some are known to be disease causing agents, while others are viewed as commensal i.e. the bacteria benefit from living in symbiosis with humans without harming the individual. In recent decades, several mollicute species have been isolated from immunocompromised individuals. Mollicutes are thus listed as opportunistic pathogens (Waites *et al.*, 2005). This highlights the importance of finding new antibiotics toward the different mollicute species. **Chapter 2** and **Paper I** describe attempts to characterize a potential drug target from *Up*.

1.4.2 Ureaplasma parvum

Up is a human opportunistic pathogen found in the urogenital tract. It is referred to as part of the commensal system in the female genital tract, as it is found in 40% of sexually inactive and 67% of sexually active women. Infection with *Up* has been associated with male urethritis and prostatitis (Volgmann *et al.*, 2005). *Up* can cause several obstetrical complications such

as spontaneous abortions, low birth weight and infertility. The bacteria can be transferred vertically from mother to child, either through intrauterine infection or passage through the birth canal. In full-term neonates, *Up* does not constitute a great risk. However, for premature neonates, especially those of birth weights less than 1500 g, infection of *Up* has been directly linked to bacteraemia, congenital and neonatal pneumoniae and development of bronchopulmonary dysplasia (Sung, 2010; Volgmann *et al.*, 2005).

Up has no cell wall and is thus not affected by beta-lactam antibiotics. Instead, antibiotics that interfere with protein synthesis, such as macrolides and tetracyclines, are used to treat *Up* infections (Waites *et al.*, 2005). Only macrolides are considered for the treatment of neonatal *Up*-infections, due to the side effects of tetracyclines (Sung, 2010; Volgmann *et al.*, 2005).

2 Uridine monophosphate kinase from *Ureaplasma parvum* (Paper I)

2.1 Aim of the study

The uridine monophosphate kinase (UMPK) from *Up* has been investigated because it was found to be a potential drug target in the development of new antibiotics against *Up*-infections. *Up*-UMPK fulfils two important criteria for choosing this enzyme as a drug target; first of all, *Up* relies on the salvage pathway and monophosphate enzymes in order to survive, and secondly, *Up*-UMPK belongs to the amino acid kinase family, while its human counterpart, UMP-CMPK, belongs to the nucleoside monophosphate kinase family.

2.2 Bacterial and archaeal UMPKs

UMPK catalyzes the reversible phosphorylation of UMP, using ATP as a phosphate donor (Serina *et al.*, 1995). The protein is encoded by the *PyrH* gene which has been identified in many bacteria and archaea (Labesse *et al.*, 2010; Lee *et al.*, 2010; Tu *et al.*, 2009; Meier *et al.*, 2008; Egeblad-Welin *et al.*, 2007; Evrin *et al.*, 2007; Jensen *et al.*, 2007; Marco-Marin *et al.*, 2005; Fassy *et al.*, 2004; Gagyi *et al.*, 2003; Serina *et al.*, 1995). The *PyrH* gene has been shown to be essential for the survival of *E. coli*, *H. influenzae* and *St. pneumoniae* (Fassy *et al.*, 2004; Akerley *et al.*, 2002; Yamanaka *et al.*, 1992). In general, the bacterial UMPKs are hexamers, and they are subject to allosteric regulation via activation by GTP and inhibition by the distal end-product, UTP (Lee *et al.*, 2010; Evrin *et al.*, 2007; Gagyi *et al.*, 2003; Serina *et al.*, 1995). A group of UMPKs from gram-positive bacteria exhibited positive cooperativity with ATP as a substrate (Evrin *et al.*, 2007; Fassy *et*

al., 2004). An interesting feature is that UMPKs from *Up* and the archaea, *Sulfolobus solfataricus*, are not activated by GTP (Egeblad-Welin *et al.*, 2007; Jensen *et al.*, 2007). Phosphoryl transfer for UMPK in *S. solfataricus* has been suggested to follow a sequential random bi-bi reaction mechanism (Jensen *et al.*, 2007). UMP kinase within the cell is located close to the membrane in *B. subtilis* and *E. coli* (Gagyi *et al.*, 2004; Landais *et al.*, 1999). Furthermore, UMPK from *E. coli* has been suggested to act as a sensor in regulation of the internal pyrimidine nucleotide pools. A high level of nucleotides can thus serve as a signal for repression of the enzyme responsible for the first step in *de novo* pyrimidine synthesis (Kholti *et al.*, 1998).

The first structure of a bacterial UMPK was demonstrated using *Neisseria meningitidis* (Protein data bank (PDB) id: 1YBD). The structure was deposited in the protein data bank in February 2005. This was followed by structures of UMPKs from *E. coli* and *Pyrococcus furiosus* published in 2005, revealing an enzyme belonging to the amino acid kinase family, and with similarities to carbamate kinase and N-acetylglutamate kinase (Briozzo *et al.*, 2005; Marco-Marin *et al.*, 2005). Today, the structures of bacterial UMPKs from 11 different organisms, in complex with substrates, allosteric regulators and/or apo-structures, have been solved (Labesse *et al.*, 2010; Tu *et al.*, 2009; Meier *et al.*, 2008; Meyer *et al.*, 2008; Egeblad-Welin *et al.*, 2007; Jensen *et al.*, 2007; Briozzo *et al.*, 2005; Marco-Marin *et al.*, 2005). Four of the UMPK structures have been deposited in the protein data bank but not yet published (PDB-id's: 2IJ9, 2AIF, 1Z9D and 1YBD). The structures have revealed three binding sites: one site for UMP and one for ATP in the active site, and an allosteric site for GTP.

2.3 Kinetics

The kinetic constants were determined for the interactions between *Up*-UMPK and its two substrates, UMP and ATP. The primary plot of the two-substrate kinetic experiments indicates that the reaction mechanism is sequential. K_m(UMP) was 214 ± 4 μM, $K_{0.5}$(ATP) was 316 ± 54 μM and V_{max} was 262 ± 24 $\mu mol \cdot min^{-1} \cdot mg^{-1}$.

The nature of the inhibition patterns of UTP toward UMP and ATP was investigated, and showed that UTP was a competitive inhibitor of UMP, which is in accordance with structures from *E. coli* in complex with both UMP and UTP. In these structures, it is clear that UTP binds within the active site, with the uracil base in the same position as for UMP (Briozzo *et al.*, 2005). UTP acts as a non-competitive inhibitor of ATP, leading to a decrease in V_{max}, while K_m(ATP) remained at the same level.

The K_i values for UTP as an inhibitor of UMP and ATP are 0.7 mM and 1.2 mM, respectively.

When GTP was tested as an allosteric activator, no effect was observed, and this was the first bacterial UMPK showing this behaviour.

Altogether, the rate of *Up*-UMPK is most likely determined by the intracellular levels of UMP, as the enzyme is less sensitive to UTP feedback inhibition, and, in contrast to other bacterial UMPKs, is not activated by GTP.

2.4 Structure of *Up*-UMPK

Before it became clear that *Up*-UMPK was not activated by GTP, the enzyme was crystallized in the presence of 5 mM GTP, and resulted in crystals diffracting to 2.5 Å. The structure was solved by molecular replacement using the monomer of UMPK from *H. influenzae* (PDB id: 2A1F). A phosphate ion was observed in the active site of each monomer, corresponding to the position of the β-phosphate of UDP and UTP when *Up*-UMPK was superimposed on *E. coli* UMPK in complex with these nucleotides (PDB id's: 2BND and 2BNF).

The enzyme is a hexamer of three dimers related by a three-fold symmetry (Figure 9A). Each monomer has an α/β-fold with a nine-stranded β-sheet surrounded by eight α-helices and one 3_{10} helix (Figure 9B). The primary contact between subunits A and B is predominantly via hydrophobic interactions between the two antiparallel α3 helices. One interaction between subunits A and C is via a hydrophobic interaction formed between amino acid residues T131 and F133 situated in a flexible loop (Figure 9C).

2.5 Mutational study

The structure of *E. coli* revealed a cross-talk region, where amino acid residues, T138 and N140 form two hydrogen bonds with the equivalent amino acid residues in the neighbouring subunit. In *Up*-UMPK this interaction corresponds to the hydrophobic interactions between T131 and F133 between the A and C subunits (Figure 9C). Replacement of either residue with an alanine in *E. coli* UMPK, resulted in enzymes that were not activated by GTP (Briozzo *et al.*, 2005). The cross-talk region in *E. coli* UMPK forms hydrogen bonds, while hydrophobic interactions are observed in *Up*-UMPK. We therefore suggested that the presence of an asparagine in position 140 in *E. coli* is involved in the GTP activation

mechanism. F133 was substituted with an alanine or an asparagine residue; however, neither *Up*-UMPK-F133N nor *Up*-UMPK-F133A were activated by GTP. Similar mutations were created in *B. subtilis* UMPK with substitution of the threonine and asparagine residues with alanine, however these mutated enzymes were still activated by GTP (Evrin *et al.*, 2007). Altogether, these results show that the cross-talk region is not part of a "GTP activation" motif.

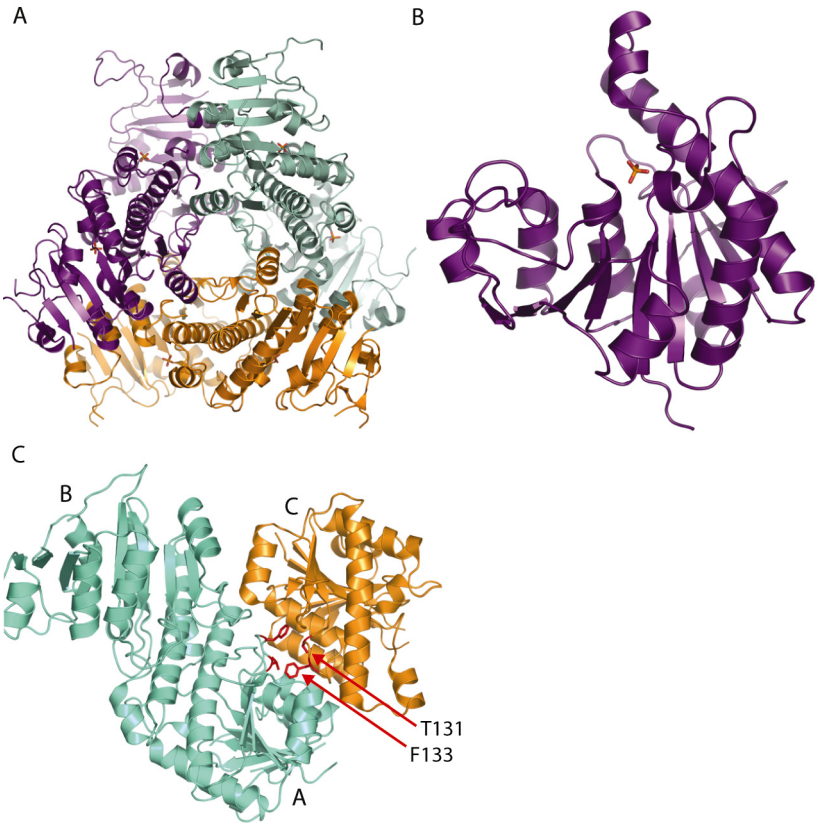

Figure 9. Structure of *Up*-UMPK, A) Hexamer, B) Monomer, C) Subunit A, B and C. Two amino acid residues, T131 and F133, involved in hydrophobic interactions, are marked in red on subunits A and C. The protein structure figures were made using PyMOL (PyMOL © 2005, DeLano Scientific LLC, South San Francisco, California, U.S.A).

2.6 Binding site for GTP

The allosteric site in UMPK responsible for binding GTP was recently identified in four different UMPKs from *E. coli*, *Bacillus anthracis*,

Xanthomonas campestris and *Mycobacterium tuberculosis* (Labesse *et al.*, 2010; Tu *et al.*, 2009; Meier *et al.*, 2008; Meyer *et al.*, 2008). In order to illustrate the two very distinct binding sites, the binding of UMP and GTP is shown for *E. coli* (Figures 10A and 10B). The binding mode of GTP in *E. coli* UMPK differs from those of *X. campestris* UMPK and *M. tuberculosis* UMPK. In *B. anthracis*, an ATP molecule binds in the GTP allosteric site. The allosteric site is situated between two subunits from two dimers. In *E. coli* UMPK, the GTP molecule is more deeply buried, while it is located closer to the central cavity in the other three reported UMPKs. The allosteric site is found in a position that would correspond to the space between subunits B and C of *Up*-UMPK (Figure 9C).

As suggested by Jensen *et al.* (2007), amino acid residues between the end of α-helix 3 and β-strand 5 (i.e. *E. coli* UMPK numbering) are responsible for binding GTP. However, the particular amino acid residues involved in binding GTP depend on the structure under analysis (*E. coli* vs *X. campestris*) (Figure 10E). Attempts to uncover the corresponding residues in *Up*-UMPK have been based on both possible binding modes of GTP.

When GTP is bound to *E. coli* UMPK (Figure 10C), several amino acid residues from position 92 to 130 were shown to be involved in GTP-binding (Meyer *et al.*, 2008). Several arginines (R92, R103, R127 and R130) were found in this region, and were shown to interact with GTP. None of these arginines were found in *Up*-UMPK. Marco-Marín & Rubio (2009) performed site-directed mutagenesis of many of these residues, and showed that substitution of R103 and R130 with alanine completely abolished the ability of *E. coli* UMPK to be activated by GTP. The role of W119 is to stack against the guanine base of GTP; when this residue is substituted with alanine, GTP activation is abolished (Marco-Marin & Rubio, 2009; Meyer *et al.*, 2008). In *Up*-UMPK, the corresponding residue for W119 is A112, and it is therefore unable to stack to the base moiety of GTP.

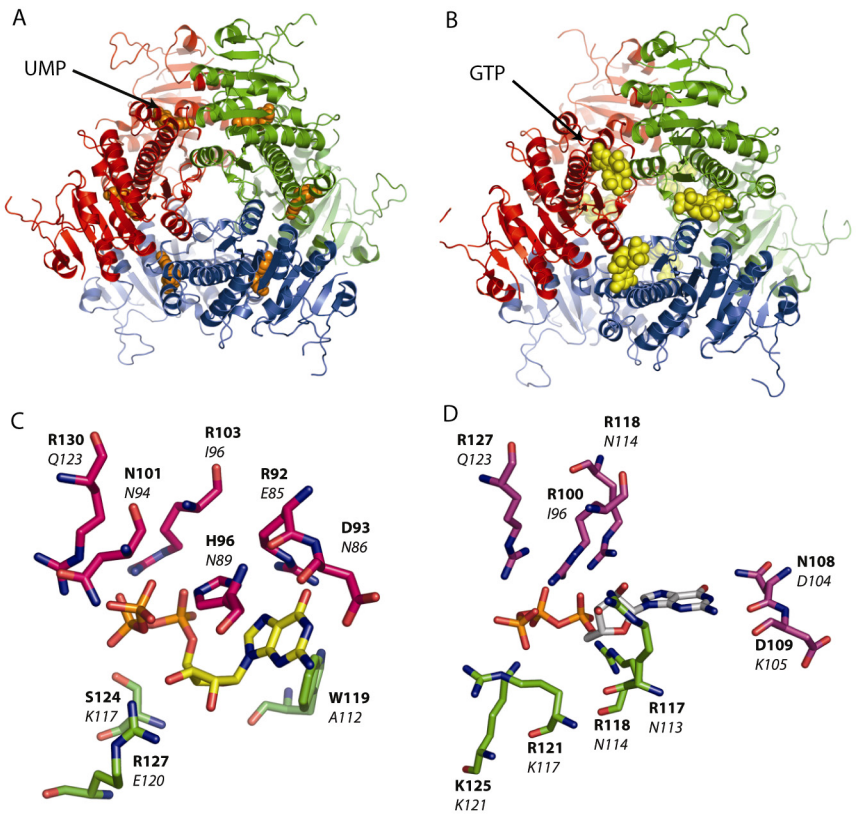

Figure 10. A) *E. Coli* UMPK with UMP (orange) bound (PDB id: 2BNE) (Briozzo *et al.*, 2005). B) *E. coli* UMPK with GTP (yellow) bound (PDB id: 2V4Y) (Meyer *et al.*, 2008). C) GTP binding site in *E. coli* UMPK (Meyer *et al.*, 2008), amino acid residues written in **bold** belong to *E. coli*, while amino acid residues in *italic* belong to the corresponding residues in *Up*-UMPK. D) GTP binding site in *X. campestris* UMPK (Tu *et al.*, 2009), amino acid residues written in **bold** belong to *X. campestris*, while amino acid residues in *italic* belong to the corresponding residues in *Up*-UMPK. E) Local alignment of residues between α-helix 3 and β-strand 5 (of *E. coli*). Amino acid residues binding to GTP have been underlined in the alignment. The accession numbers for the sequences are; *E. coli*, P0A7E9; *H. influenzae*, P43890; *X. campestris*, P59009; *B. subtilis*, O31749; *B. anthracis*, Q81S73; *U. parvum*, Q9PPX6; *P. furiosus*, Q8U122 and *S. solfataricus*, Q97ZE2. The alignment was made using Clustal W2 and ESPript 2.2 (Gouet *et al.*, 1999).

Few interactions between the guanine base and UMPK are involved in GTP binding in *X. campestris* UMPK (Figure 10D). A number of arginines (R100, R117, R118, R121, R127) are found to interact with the ribose moiety and the phosphate groups but none of these arginine residues are found in *Up*-UMPK.

The residues necessary for an allosteric site in *Up*-UMPK appear to have been lost, regardless of whether GTP binds in a manner similar to *E. coli* or *X. campestris* UMPK. The presence of the arginines appears to be essential for binding of GTP as an allosteric activator, yet none of these arginine residues are found in *Up*-UMPK. Three residues are conserved in GTP binding among UMPKs from *E. coli* and *X. campestris* and these are referred to with *E. coli* numbering: R103, S124 and R130. Among all the residues reported to interact with GTP, R103 and R130 seem to play pivotal roles. In *Up*-UMPK, the corresponding amino acid residues are I96 and Q123.

3 Human phosphoribosyltransferase domain containing protein 1, a homolog of HPRT (Paper II)

3.1 Aim of the study

This study was done in collaboration with the Structural Genomics Consortium (SGC) at the Karolinska Institute, Stockholm (see also **4.1**). In the present study, the structure and function of human PRTFDC1 was elucidated. PRTFDC1 is a homolog of HPRT with 65% sequence identity. HPRT has been extensively characterized; mutations in this gene are responsible for partial or full HPRT deficiency, the latter associated with the complex neurological disease, Lesch-Nyhan syndrome (Torres & Puig, 2007).

3.2 Background

3.2.1 HPRT

HPRT catalyzes the reversible reaction in which guanine/hypoxanthine (Hx) and PRPP are converted to GMP/IMP and PP_i (Craig & Eakin, 2000). The forward reaction is suggested to be ordered and sequential, with PRPP being the first substrate to bind, followed by the nucleobase. The pyrophosphate is released prior to the nucleotide (Craig & Eakin, 2000). The enzyme is constitutively expressed in most tissues, although it is more abundant in the brain (Jiralerspong & Patel, 1996). The structure of human HPRT was solved by Eads *et al.* in 1994. The monomer consists of two domains: a core domain and a hood domain (Keough *et al.*, 2005; Eads *et*

al., 1994). A flexible loop, known as loop II, has been shown to cover the active site when a transition state analog is bound to the enzyme (Shi *et al.*, 1999).

Eleven amino acid residues around the active site are conserved among all genes encoding HPRTs. The residues in human HPRT are L67, G69, S103, Y104, E133, D134, D137, K165, G189, D193 and R199 (Craig & Eakin, 2000). Aspartate 137 has been suggested to act as a general base due to its proximity to N7 of the purine ring (Xu & Grubmeyer, 1998). However, a site-directed mutagenesis study on residue D137 of *Trypanosoma cruzi* HPRT suggested that its function is to act as a transition state stabilizer, via a hydrogen bond to N7 of the purine ring, thereby promoting catalysis (Canyuk *et al.*, 2001).

The specific activity (V_{max} values) for recombinant HPRT have been reported to be 46 $\mu mol \cdot mg^{-1} \cdot min^{-1}$ and 27 $\mu mol \cdot mg^{-1} \cdot min^{-1}$ with G and Hx as substrates, respectively. The K_m(app)-values at 1 mM PRPP were reported to be 1.9 $\pm$ 0.3 and 3.1 $\pm$ 0.9 with G and Hx as substrates, respectively (Keough *et al.*, 1999).

Measurement of HPRT activity

The enzymatic activity of HPRT in forward reactions has been determined by several methods; a radiochemical assay using [^{14}C]-labeled substrates and two variants of spectrophotometric assays (Keough *et al.*, 1987; Giacomello & Salerno, 1977). Lately, the method of choice has been the spectrophotometric assay, where the amount of product is measured using a light source of a wavelength between 240 and 260 nm (Keough *et al.*, 1987).

3.2.2 HPRT deficiencies

Mutations in *HPRT* have been associated with partial and full HPRT deficiencies. More than 300 mutations in *HPRT* have been identified in patients, giving rise to primarily point mutations or truncated versions of the protein (Jinnah *et al.*, 2004; Jinnah *et al.*, 2000). Partial HPRT deficiency has been associated with hyperuricemia, gout and potentially dystonia. Full HPRT deficiency is linked to Lesch-Nyhan syndrome. Dystonia, mental retardation and self-injurious behaviour are some of the traits characterizing this disease (Torres & Puig, 2007).

3.2.3 PRTFDC1 - a homolog of HPRT

PRTFDC1 was identified as a homolog of HPRT, when a search for pseudogenes of *HPRT1* was performed. *PRTFDC1* was found to be conserved in vertebrates, and it was therefore suggested that although it was likely to be a functional gene, that function was unknown (Nicklas, 2006). Keebaugh *et al.* (2007) suggested that proteins encoded by *PRTFDC1* had lost their ancestral HPRT enzymatic activity. This hypothesis was based on the presence of 11 conserved residues in *HPRT1*, of which not all were conserved in *PRTFDC1* (Keebaugh *et al.*, 2007; Craig & Eakin, 2000). It was suggested that *PRTFDC1* remained in the genome and possibly evolved a new function (Keebaugh *et al.*, 2007).

Two recent studies have identified *PRTFDC1* as a putative tumor suppressor gene potentially involved in the inhibition of the development of ovarian cancer and oral squamous-cell carcinomas (Cai *et al.*, 2007; Suzuki *et al.*, 2007).

Furthermore, PRTFDC1 has been shown to interact with HPRT (Rual *et al.*, 2005).

3.3 Structure of PRTFDC1

The structure of recombinant PRTFDC1 was solved with GMP bound within the active site at 1.7 Å resolution. The monomer is comprised of the core and hood domains. The core domain contains a six-stranded, twisted parallel β-sheet surrounded by three α-helices. The hood domain consists primarily of residues from the C-terminus, and is made up by a two-stranded anti-parallel β-sheet, and one α-helix. The flexible loop, loop II, is stabilized by part of β4 and β5 (Figure 11).

The structure of PRTFDC1 was superimposed on structures of human HPRT (PDB-ids: 1Z7G (ligand free), 1BZY (transition state analog) and 1HMP (GMP)) (Keough *et al.*, 2005; Shi *et al.*, 1999; Eads *et al.*, 1994). The rmsd values of the superimpositions varied between 1.0 and 1.7 for approximately 200 Cα atoms, thus showing that the structures of HPRT and PRTFDC1 are very similar.

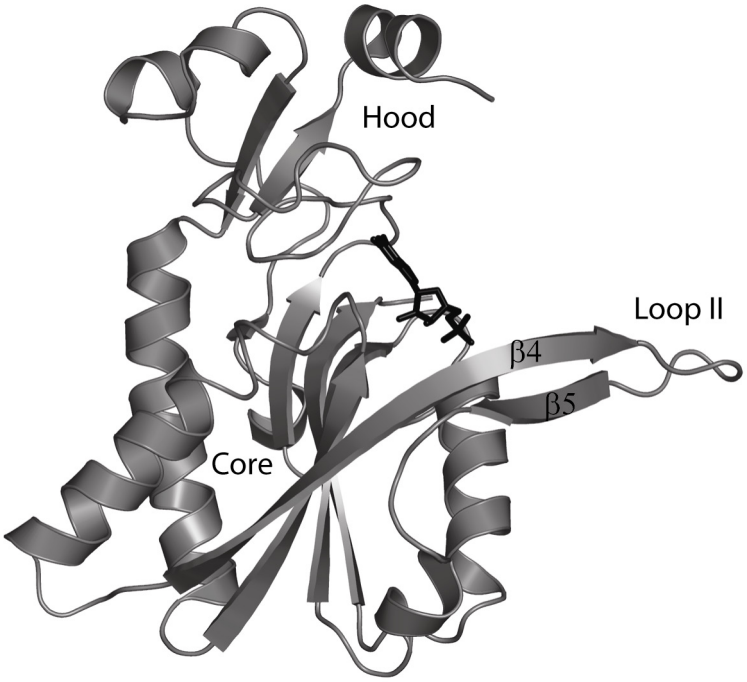

Figure 11. Monomer of PRTFDC1 with GMP.

3.4 Functional studies

3.4.1 Metabolome library

A differential static light scattering (DSLS) thermal-melt assay was used to investigate the function of PRTFDC1 (see **4.3**). A nucleotide metabolome library containing 81 compounds (substrates, products, inhibitors, activators of nucleotide metabolism) was screened with PRTFDC1. Increased thermal shifts for PRTFDC1 in the presence of PRPP, IMP and GMP, indicated a function for PRTFDC1 similar to HPRT. However, thermal shifts for the nucleobases (Hx and G) alone were not observed. When PRPP was added, both Hx and G exhibited higher thermal shifts. This result supports the concept of an ordered sequential bi-bi mechanism, similar to that observed for HPRT (Craig & Eakin, 2000). This suggests that PRPP is the first

substrate to bind to PRTFDC1, followed by the nucleobase. In summary, the ligands found in this study, suggested that PRTFDC1 and HPRT fulfil similar functions.

3.4.2 Kinetics

A radiochemical assay with tritium-labeled nucleobases and PRPP provided a more detailed analysis of the nucleobases as substrates. The kinetic parameters, K_m and V_{max}, were determined for PRTFDC1 with Hx and G in the forward reaction. HPRT was also characterized using this method, as this enzyme, to our knowledge, has never been characterized using this type of assay.

The catalytic efficiency (k_{cat}/K_m) of PRTFDC1 was 0.26% of HPRT when Hx was a substrate. With G as a substrate, the catalytic efficiency of PRTFDC1 was 0.09% of HPRT. For both substrates, the major contribution to the lower catalytic efficiencies was a marked decrease in V_{max} (~ 70- to 310-fold). The K_m values were only slightly increased (4- to 6-fold).

One interesting feature was observed when determining the kinetic constants for HPRT. With Hx as a substrate, the values of K_m and V_{max} were consistent with earlier results. However, with G as a substrate, K_m was found to be 5-fold higher, and V_{max} ~ 20-fold higher when determined with tritium-labeled substrates as compared to the spectrophotometric assay (Keough *et al.*, 1999).

3.5 The role for residue G145

Of the eleven conserved residues in HPRT, one residue, D137, was altered in PRTFDC1. As described earlier (see **3.2.1**), D137 was suggested to be involved in catalysis. In PRTFDC1, this amino acid corresponds to G145. A water molecule was observed in PRTFDC1 at the position corresponding to the aspartic acid side chain in HPRT. We therefore propose that this water molecule may act as weak base. This can also explain the greatly reduced V_{max}-values, and relatively unchanged K_m-values determined for PRTFDC1 with Hx and G.

4 Interaction profiling of FDA-approved nucleoside analog drugs with 23 enzymes of human nucleotide metabolism (Paper III)

4.1 Aim of the study

This study was performed at SGC at the Karolinska Institute in Stockholm. At the SGC facility, one goal has been to elucidate the structures of enzymes involved in nucleotide metabolism (Welin & Nordlund, 2010). The high throughput pipeline used for cloning, purification and structure determination gives a good foundation for protein production suitable in high throughput screening. In this study, the DSLS method has been applied.

A NAL has been set up consisting of 45 FDA-approved NAs already used as anti-cancer agents or in treatment of viral diseases. Results obtained from DSLS can reveal new interactions between NAs and selected enzymes.

Table 1: *Enzymes investigated with NAL using DSLS. Information regarding enzyme number, gene name, full name, genbank ID, accession number, EC number, full-length of protein, construct size, involved in which part of pathway and T_{agg} for the enzyme with no added ligand.*

Number	Gene name	Full name	Genbank ID	Accession number	EC number	Full-length	Construct	Pathway	Tagg-mean/ °C
1	ADSS2	Adenylosuccinate synthetase isozyme 2	gi|15214463	P30520	6.3.4.4	456	1-456	*De novo* purines	52.5
2	ADSL	Adenylosuccinate lyase	gi|12652985	P30566	4.3.2.2	484	1-481	*De novo* purines	61.2
3	BPNT1	3'(2'),5'-bisphosphate nucleotidase 1	gi|116812595	O95861	3.1.3.7	308	6-308	Sulfur metabolism	55.5
4	CMPK2	UMP-CMP kinase 2, mitochondrial	gi|46409274	Q5EBM0	2.7.4.14	449	27-449	*De novo* + salvage	51.6
5	CTPS2	CTP synthetase 2	gi|23271202	Q9NRF8	6.3.4.2	586	1-275	*De novo* pyrimidines	33.9
6	DCTD	Deoxycytidylate deaminase	gi|66840174	P32321	3.5.4.12	178	5-174	Catabolism pyrimidines	57.7
7	DPYS	Dihydropyrimidinase	gi|21707927	Q14117	3.5.2.2	519	4-519	Catabolism pyrimidines	56.8
8	GART	Phosphoribosylglycinamide formyltransferase	gi|4503915	P22102	2.1.2.2	1010	1-1003	*De novo* purines	54.2
		Phosphoribosylamine--glycine ligase			6.3.4.13	1010	1-1003	*De novo* purines	
		Phosphoribosylformylglycinamidine cyclo-ligase			6.3.3.1	1010	1-1003	*De novo* purines	
9	GDA	Guanine deaminase	gi|31566380	Q9Y2T3	3.5.4.3	454	1-454	Catabolism purines	60.1
10	GMPR2	GMP reductase 2	gi|50541956	Q9P2T1	1.7.1.7	348	10-341	Catabolism purines	47.2
11	GMPS	GMP synthetase	gi|4504035	P49915	6.3.4.1/6.3.5.2	693	1-693	*De novo* purine	50.7
12	ITPA	Inosine triphosphate pyrophosphatase	gi|15626999	Q9BY32	3.6.1.19	194	1-194	Catabolism	63.2
13	NT5C2	Cytosolic purine 5'-nucleotidase	gi|6912598	P49902	3.1.3.5	561	1-561	Catabolism	56.6
14	NT5C3	Cytosolic 5'-nucleotidase 3	gi|7706031	Q9H0P0	3.1.3.5	336	52-336	Catabolism	51.1
15	NUDT16	U8 snoRNA-decapping enzyme	gi|24308370	Q96DE0	3.6.1.30	195	1-195	Catabolism	60.3
16	PAICS	Phosphoribosylamino-imidazolesuccinocarboxamide synthetase	gi|16307450	P22234	6.3.2.6	425	1-424	*De novo* purines	64.6
		Phosphoribosylaminoimidazole carboxylase			4.1.1.21	425	1-424	*De novo* purines	
17	PRTFDC1	Phosphoribosyltransferase domain containing protein 1	gi|14250450	Q9NRG1	2.4.2.8	225	1-225	Salvage purines?	55.0
18	RRM1	Ribonucleoside-diphosphate reductase large subunit	gi|4506749	P23921	1.17.4.1	792	75-742	*De novo* pur + pyr	48.8
19	UCK1	Uridine-cytidine kinase 1	gi|60551657	Q9HA47	2.7.1.48	277	22-243	Salvage pyrimidines	56.4
20	UMPS (1)	Orotate phosphoribosyltransferase	gi|4507835	P11172	2.4.2.10	480	7-203	*De novo* pyrimidines	42.6
21	UMPS (2)	Orotidine-5'-phosphate decarboxylase	gi|4507835	P11172	4.1.1.23	480	224-479	*De novo* pyrimidines	56.4
22	UPB1	Beta-ureidopropionase	gi|17373540	Q9UBR1	3.5.1.6	384	1-384	Catabolism pyrimidines	52.0
23	UPP1	Uridine phosphorylase 1	gi|13938418	Q16831	2.4.2.3	310	1-310	Catabolism pyrimidines	57.0
24	UPP2	Uridine phosphorylase 2	gi|27597096	O95045	2.4.2.3	317	35-317	Catabolism pyrimidines	47.2

4.2 Background

4.2.1 Selection of nucleoside analogs for the library

In order to select the NAs that should be included in the nucleoside analog library, two criteria were set up:

- The NAs should be approved by the FDA as pharmaceutical drugs.
- The structure of the compound should include a natural or modified nucleobase, nucleoside or nucleotide.

Drugbank was used as a search engine. Drugbank contains more than 6800 drug entries, where 1431 of these are FDA-approved small molecule drugs (Knox *et al.*, 2011; Wishart *et al.*, 2008; Wishart *et al.*, 2006). Forty-seven NAs were identified as candidates for NAL.

4.2.2 Nucleoside analogs

The 45 NAs included in NAL are described in **1.3**, and the chemical structures are shown in Figures 4, 5, 6 and 7. Activation mechanisms are illustrated in Figure 8. Adefovir is included in NAL in its prodrug form, as adefovir dipivoxil. Each enzyme is screened against NAL at concentrations of 100 and 500 μM.

4.2.3 Enzymes investigated

The majority of the 23 enzymes (Table 1) screened against NAL are involved in nucleotide metabolism, described in **1.1**. One exception is BPNT1, a 3´(2´),5´-bisphosphate nucleotidase, annotated as being involved in sulphur metabolism by KEGG. However, BPNT1 catalyzes the hydrolysis of 3´(2´)-phosphoadenosine 5´-phosphate to AMP and inorganic phosphate, and is therefore included (Yenush *et al.*, 2000). UMPS is mentioned twice, UMPS(1) and UMPS(2), as the domains are screened separately as two independent enzymes. Furthermore, dCK was used as a control protein, as it is well established that this salvage enzyme plays a key role in the activation of several prodrugs (Eriksson *et al.*, 2002). Human TK1 was intended to be used as a control protein, because of its strict substrate specificity. However, TK1 was discarded as a control protein due to the biphasic appearance of the aggregation temperature (T_{agg}) curve when analyzed by DSLS.

4.3 Differential Static Light Scattering

DSLS is a high-throughput method where the T_{agg} of a protein is determined during heat-induced denaturation. The method was originally developed as a screening method for identification of optimal buffer conditions and ligands, being able to promote enzyme stability, and ultimately structure determination (Vedadi *et al.*, 2010; Senisterra & Finerty, 2009; Senisterra *et al.*, 2006; Vedadi *et al.*, 2006).

The theory behind protein aggregation assumes that the protein follows a two-state irreversible model, which is a simplified expression of the three-state reversible-irreversible model:

$$N \xrightarrow{k_{app}} A$$

$$nN \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} nD \xrightarrow{k_2} A$$

where N is the native state, and A is the aggregated state, k_{app} is the apparent rate constant, D is the denatured state, and k_1, k_{-1} and k_2 are rate constants (Senisterra *et al.*, 2006).

The aggregation measurements were performed on a Stargazer instrument (Harbinger Biotechnology and Engineering Corporation, Toronto, Canada). The protein samples in 384-well plates were placed on top of a heating block constructed with light-emitting diodes. The plates were heated from 25 to 80°C at a rate of 1°C per min. Light scattering due to the amount of aggregated protein, was detected by a charge coupled device camera. Images were taken at every 0.5°C increase in temperature. The increase is measured using a thermistor in one well of the plate (Senisterra *et al.*, 2006).

In order to calculate T_{agg}, the intensities (derived from each picture) can be plotted as a function of the temperature. T_{agg} is defined as the temperature where at which 50% of the protein is aggregated. Addition of a ligand will potentially increase the stability of the enzyme, and thus raise the T_{agg}. Opinions vary on the value of ΔT_{agg} that marks a significant increase in thermal stability for identifying ligands. A ΔT_{agg} of 1.0 or 1.5°C has been suggested (Senisterra *et al.*, 2006). In the work presented in this thesis, the cut-off for identifying a ligand is set to $\Delta T_{agg} > 1.0$°C. However, compounds exhibiting ΔT_{agg} in the range 0.5 - 1°C may be tested in a

concentration dependent manner, in order to avoid the exclusion of potential ligands.

Once a compound has been identified as a potential ligand, further experiments such as concentration dependent stabilization in the presence of the potential ligand is performed. If a correlation is found, the ligand can be further explored using techniques such as isothermal titration calorimetry (ITC), enzyme assays and co-crystallisation for the further determination of structure (Vedadi *et al.*, 2010).

Ligands can bind to the native and denatured protein, giving rise to altered T_{agg} (increased or decreased thermal shifts). Even though some compounds exhibit identical thermal shifts, care should be exercised in comparing results, as the affinity for a ligand is an expression of thermodynamic processes. When comparing thermal shifts for ligands, these should have similar physicochemical properties (Vedadi *et al.*, 2010; Senisterra *et al.*, 2006).

4.4 Validation of method using dCK

4.4.1 Background information on human dCK

Human dCK catalyzes phosphorylation of the natural substrates, dCyd, dAdo and dGuo, with dCyd as the preferred substrate. In its active form, dCK is a dimer composed of two subunits (Bohman & Eriksson, 1988). Each subunit is comprised of 260 amino acids with a predicted mass of 30.5 kDa (Chottiner *et al.*, 1991). The end product, dCTP, acts as a strong feedback inhibitor (Bohman & Eriksson, 1988). Although all nucleotides (ATP, UTP, GTP, CTP and dTTP) can act as phosphate donors, UTP has been suggested to be the true intracellular phosphate donor (Hughes *et al.*, 1997; Krawiec *et al.*, 1995). Depending on which donor is used, either ATP or UTP, the reaction mechanism can follow different pathways. With ATP as the phosphate donor, a rapid equilibrium, random bi-bi reaction has been suggested. With UTP as a phosphate donor, binding of the substrates will be ordered, with UTP being the first substrate to bind before dCyd, followed by a random release of the products (Hughes *et al.*, 1997; Datta *et al.*, 1989).

dCK is responsible for the activation of a number of FDA-approved prodrugs e.g. lamivudine, cytarabine, cladribine, gemcitabine, fludarabine, zalcitabine, vidarabine, nelarabine, decitabine, clofarabine, emtricitabine,

didanosine and azacitidine (Peters, 2006; Beausejour *et al.*, 2002; Eriksson *et al.*, 2002). The kinetic parameters such as K_m and k_{cat}, are given for some NAs if available (Table 2), with both ATP and UTP as phosphate donors. As shown in Table 2, the catalytic efficiency (k_{cat}/K_m) is higher for all substrates/NAs where UTP is the phosphate donor.

Table 2. *Kinetic properties of dCK. K_m and k_{cat} for the natural substrates and some NAs in the presence of the phosphate donors, ATP and UTP. The concentration of ATP and UTP is 1 mM, except in the case of clofarabine, which is determined in the presence of 100 μM ATP.*

	ATP			UTP		
Substrates	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
dC [a]	< 1	0.033 ± 0.001	> 33 × 10^3	< 1	0.044 ± 0.001	> 44 × 10^3
dA [a]	100 ± 20	1.7 ± 0.1	17 × 10^3	13 ± 3	0.33 ± 0.02	25 × 10^3
dG [a]	231 ± 20	2.6 ± 0.1	11 × 10^3	21 ± 4	0.33 ± 0.02	16 × 10^3
Zalcitabine [b]	406.8	0.18	0.4 × 10^3	N.D.	N.D.	-
Cytarabine [a]	6.9 ± 2	0.30 ± 0.02	43 × 10^3	2.7 ± 1	0.17 ± 0.02	63 × 10^3
Gemcitabine [b]	22.0	0.37	16.8 × 10^3	N.D.	N.D.	-
Emtricitabine [c]	4.4 ± 0.6	0.036 ± 0.001	8.2 × 10^3	6.2 ± 0.5	0.100 ± 0.002	16.2 × 10^3
Lamivudine [d]	3.4 ± 1.0	0.030 ± 0.001	8.8 × 10^3	8.0 ± 1.0	0.102 ± 0.001	12.8 × 10^3
Cladribine [e]	78 ± 16	0.6 ± 0.04	7.7 × 10^3	5.1 ± 0.9	0.37 ± 0.01	72.6 × 10^3
Clofarabine [f]	3.3 ± 0.6	N.D.	-	N.D.	N.D.	-

a (Godsey *et al.*, 2006), b (Sabini *et al.*, 2003), c (Sabini *et al.*, 2007b), d (Sabini *et al.*, 2007a), e (Sabini *et al.*, 2008a), f (Lotfi *et al.*, 1999). N.D. – Not determined.

Sabini *et al.* determined the structure of human dCK in complex with dCyd as well as two NAs; gemcitabine and cytarabine (Sabini *et al.*, 2003). Since then, a wealth of information about binding of natural substrates and NAs in the presence or absence of phosphate donors has been gathered (Hazra *et al.*, 2010; Sabini *et al.*, 2008a; Sabini *et al.*, 2008b; Sabini *et al.*, 2007a; Sabini *et al.*, 2007b; Godsey *et al.*, 2006; Zhang *et al.*, 2006).

4.4.2 Detailed view of dCK in complex with substrates

The complexes used for the analysis of the binding of substrates and NAs in the active site of dCK were dCyd-ADP (Sabini *et al.*, 2003), clofarabine-ADP (Zhang *et al.*, 2006) and lamivudine-ADP (Sabini *et al.*, 2007a), as

representatives of a natural pyrimidine substrate, a purine NA and an L-nucleoside analog, respectively (Figure 12).

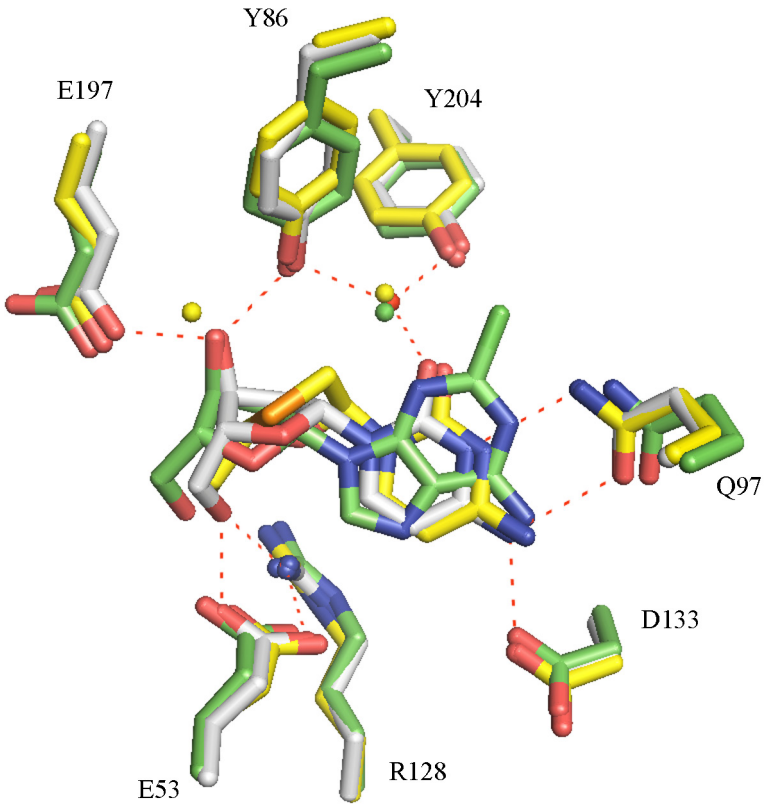

Figure 12. Active site of dCK with dCyd (grey), clofarabine (green) and lamivudine (yellow). Hydrogen bonds are shown for dCK with dCyd.

The main interactions for dCyd are with Q97, D133, Y86, E197 and indirectly to Y204 through a water molecule. E53 and R128 have been suggested to act as a catalytic couple, where R128 anchors the position of E53, so that this glutamate can act as a base (Sabini *et al.*, 2003; Eriksson *et al.*, 2002). With clofarabine bound in the active site, Q97 has moved to accommodate room for the bigger purine base. The 3'-OH of clofarabine is kept in the same position, while the position of 5'-OH together with E53 has moved. The fluorine group at the 2'-position of clofarabine forms a hydrogen bond with R128 (Zhang *et al.*, 2006). The cytosine base of lamivudine binds in the same way as dCyd, however the base is tilted by

10°. A structural water molecule is found in the lamivudine structure, creating hydrogen bonds to E197 and Y86. In order to position the 5'-OH of lamivudine correctly for a nucleophilic attack from E53, the five-membered ring is rotated ~ 30° around the glycosidic bond (Sabini *et al.*, 2007a). This illustrates the plasticity of the active site of dCK, and explains the promiscuous nature of dCK.

4.4.3 DSLS screening of dCK

Metabolome library screen

In order to evaluate dCK as a model protein, the metabolome library screen described in **3.4.1** was used, and the results are shown in Table 3 in **Paper III**. The fingerprint of the dCK metabolome thermal shifts correlated well with what is known about dCK, thus supporting the use of DSLS for identification of biologically relevant interactions. The distal feedback inhibitor, dCTP, induced a positive thermal shift of 16°C for dCK. The natural products are represented by dCMP (ΔT_{agg} = 11.9°C), dGMP (ΔT_{agg} = 3.3°C) and dAMP (ΔT_{agg} = 1.5°C). Of the phosphate donors, ATP was shown to give a decreased T_{agg} for dCK, while UTP stabilized the protein with a ΔT_{agg} of 1.4°C. An interesting observation was that both dADP and dGDP stabilized the protein more effectively than their corresponding triphosphate forms.

NAL

The control protein, dCK, was screened against NAL at different concentrations (100 and 500 μM) and at 500 μM NAL in the presence of 0.1 and 1 mM ATP. The results are shown in Table 2 in **Paper III**. Emtricitabine, lamivudine, decitabine, clofarabine, cladribine, gemcitabine, cytarabine and fludarabine were identified as ligands, both in the absence and presence of ATP, but with higher thermal shifts in the presence of 1 mM ATP (~ 5 to 10°C). It is important to note that cladribine was not identified as a ligand in the absence of ATP, and that fludarabine was only identified as a ligand in the absence of ATP.

All identified ligands, with exception of fludarabine, exhibited positive concentration-dependent stabilization of dCK, while fludarabine induced negative concentration-dependent stabilization going from a ΔT_{agg} around 2°C at 100 μM to just below 0°C at 1000 μM fludarabine. Furthermore, dCK has been co-crystallized with almost all identified ligands in this group, with the two exceptions of decitabine and fludarabine.

A second group of compounds were revealed when 1 mM ATP was added to dCK when screening. This group appeared directly after the top thermal shifts, with ΔT_{agg} values in the range of 0.5 to 1.0°C, but with relative thermal shifts above 1°C after addition of ATP. This group consisted of zalcitabine, entecavir, azacitidine, vidarabine and nelarabine. As it is established from the literature that several of these NAs (zalcitabine, vidarabine, azacitidine and nelarabine) are ligands of dCK, it is possible that the detection limit could be set at 0.5°C instead of 1.0°C for dCK after repeated tests.

As a general rule, interactions between dCK and emtricitabine, lamivudine, decitabine, clofarabine, cladribine, gemcicitabine, cytarabine and fludarabine are revealed even in the absence of ATP. Interactions between dCK and zalcitabine, entecavir, azacitidine, vidarabine and nelarabine are revealed only in the presence of 1 mM ATP, but below the set detection limit at 1.0. This group of NAs would not have been identified without the addition of ATP.

In total, eight ligands were identified as ligands for dCK, while a subgroup appeared with a ΔT_{agg} in the range 0.5 to 1.0°C. However, it is important to include this subgroup as ligands. All reported interactions between dCK and NAs in NAL, with the exception of didanosine, were thus detected. In addition, an interaction between dCK and entecavir was detected.

It is possible that the thermal shifts were greater when ATP was added due to the formation of the products or because the two substrates together led to the formation of a more stable enzyme. However, had dCK and NAL not been screened in the presence of ATP, only eight ligands would have been detected.

4.5 Results

The results for all 23 enzymes screened against 500 µM NAL are shown in Figure 13. T_{agg} for each enzyme is listed in Table 1. Focused follow-up studies were performed on three enzymes; UPP1, GDA and RRM1.

4.5.1 Uridine phosphorylase 1

UPP1 catalyzes the reversible reaction of the conversion of uridine and inorganic phosphate into uracil and ribose-1-phosphate (Renck *et al.*,

2010). Vidarabine, trifluridine, idoxuridine, zidovudine, telbivudine, fluorouracil, floxuridine and thioguanine were all identified as ligands for UPP1. Results are shown in Table 4 and Figures 2A and 3 in **Paper III**.

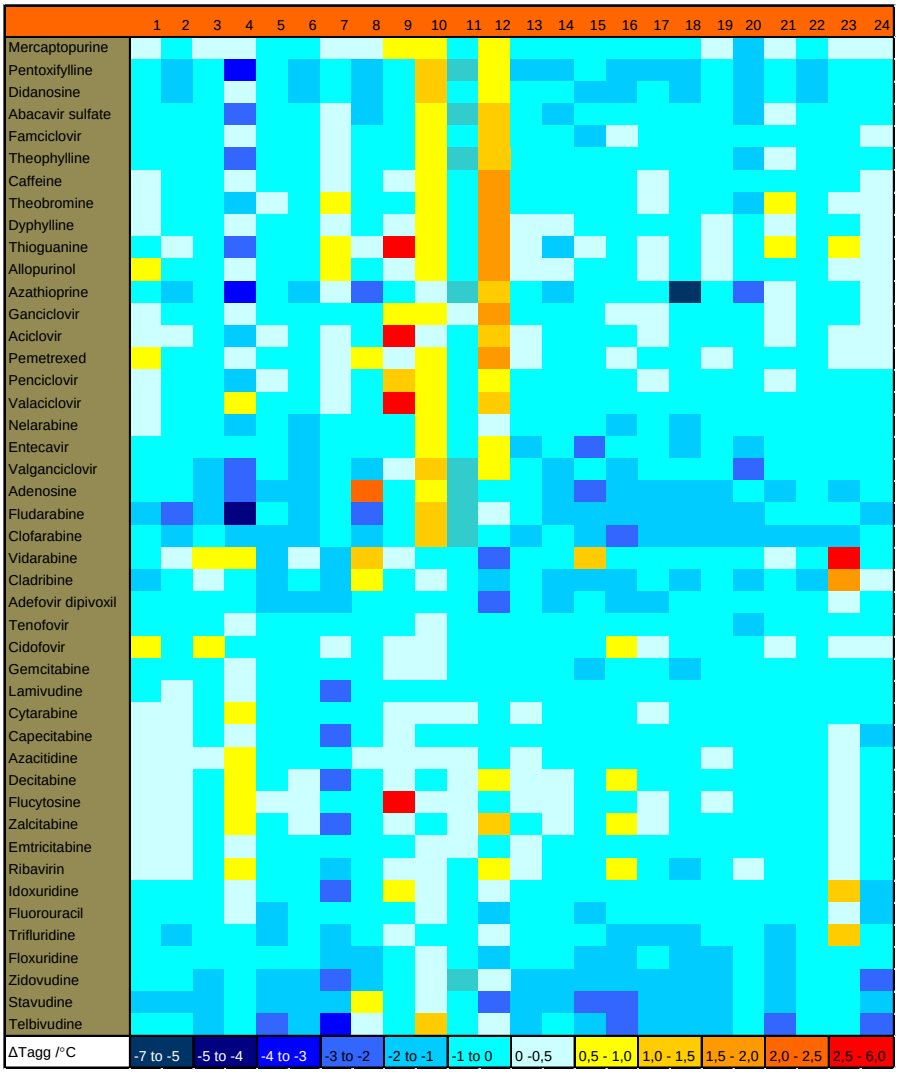

Figure 13. Results for the 23 enzymes screened against NAL. The enzymes are numbered: **1** ADSS2, **2** ADSL, **3** BPNT1, **4** CMPK2, **5** CTPS2, **6** DCTD, **7** DPYS, **8** GART, **9** GDA, **10** GMPR2, **11** GMPS, **12** ITPA, **13** NT5C2, **14** NT5C3, **15** NUDT16, **16** PAICS, **17** PRTFDC1, **18** RRM1, **19** UCK1, **20** UMPS(1), **21** UMPS(2), **22** UPB1, **23** UPP1, **24** UPP2. The results are given as color codes based on ΔT_{agg} calculated from two values within the same screen.

The interaction between UPP1 and vidarabine was highly unexpected, with an elevated thermal shift of ~ 8°C. Trifluridine and idoxuridine exhibited thermal shifts in the range of 4 to 5°C, which is slightly elevated compared to uridine, the natural substrate. Zidovudine, telbivudine, fluorouracil, floxuridine and thioguanine all exhibited positive thermal shifts in the range of 1.7 to 2.4°C. All reported values are obtained in the presence of 1 mM compound, and data is obtained from concentration-dependent stabilization curves in the presence of 1 mM compound.

Two NAs, fluorouracil and floxuridine, have been reported to be substrates for UPP1 (Cao *et al.*, 2002). To our knowledge, interactions between UPP1 and vidarabine, idoxuridine, trifluridine, telbivudine, zidovudine and thioguanine have never been reported before. The fact that zidovudine was identified as a ligand was interesting. In a study by Calabresi *et al.* in 1990, reduced zidovudine-derived bone marrow toxicity was observed when mice were given benzylacyclouridine, an inhibitor of UPP1. This effect was explained by elevated levels of uridine (Calabresi *et al.*, 1990). It is tempting to speculate whether zidovudine is a substrate of UPP1, and if the observed bone marrow toxicity could be caused by zidovudine metabolites.

4.5.2 Guanine deaminase

GDA catalyzes the irreversible reaction in which guanine is deaminated to become xanthine (Yuan *et al.*, 1999). Six ligands were identified and these could be separated into two groups. The first group, containing the mercaptopurine derivatives consisted of mercaptopurine and thioguanine. The second group, containing acyclic guanine derivatives consisted of valaciclovir, aciclovir, penciclovir and ganciclovir. The results are shown in Figures 2B and 4 in **Paper III**.

Valaciclovir stabilized GDA with a positive thermal shift ~ 7.2°C, a shift similar to that for the natural substrate, guanine. Valaciclovir can be regarded as a ligand of GDA, as the structure of GDA was solved with valaciclovir bound in the active site. This is further discussed in **Paper III**.

The effect of different functional groups can be compared within the two groups. The only difference between thioguanine and mercaptopurine is the amino group at the 2-position in thioguanine. The thermal shift for thioguanine is ~ 4.3°C at 1 mM, which is 3.3°C higher than for mercaptopurine, indicating the importance of this amino group. Within the second group for acyclic guanine derivatives, it is evident that the longer

chains found in a compound such as valaciclovir establish a higher thermal shift compared to aciclovir (~ 4.2°C). Branched chains at the 9-position of the purine ring e.g. in penciclovir and ganciclovir, stabilized GDA with thermal shifts between 1.1 and 2.3°C.

Thioguanine is the only ligand which has been described as a weak substrate for GDA isolated from rabbit liver and pig brain (Bergstrom & Bieber, 1979; Rossi *et al.*, 1978). We have determined that valaciclovir is a ligand of GDA by structure determination. For the other ligands, aciclovir, penciclovir, ganciclovir and mercaptopurine, as well as valaciclovir, further experiments will be required to determine the nature of the ligand. However, these results can serve as a platform for a focused study for the identification of new improved ligands of GDA.

4.5.3 Ribonucleotide reductase with azathioprine

RRM1, which is the large subunit (R1) of ribonucleotide reductase, was destabilized in the presence of azathioprine in a dose dependent manner. The result is shown in Figure 2C in **Paper III**. Azathioprine is a prodrug of mercaptopurine. It contains a substituted imidazole ring attached the 6-thio group of a purine ring (Figure 7). Mercaptopurine by itself only destabilizes RRM1 with a slightly negative thermal shift.

One possible explanation for the negative thermal shifts for RRM1 in the presence of azathioprine is that this compound binds to either the catalytic site or the specificity site of RRM1. With the large imidazole ring at the 6-position, azathioprine is able to destabilize RRM1. Further work is needed to substantiate this hypothesis.

4.6 AlF_x – attempts to generate a phosphorylated NAL mimic

In the search for interactions between NAs and cellular enzymes, the NAL should ideally consist of NAMPs, NADPs and NATPs. This may provide an alternative way to identify new interactions.

Aluminofluoride (AlF_x) and beryllofluoride (BeF_x) have been used as phosphate analogs. AlF_x can exist in several complexed states, where x can be between 1 and 6, and AlF_4^- represents the tetrahedral analog (Strunecka *et al.*, 2002; Chabre, 1990). In order to generate a mimic of a phosphorylated NAL we attempted to use AlF_x alone and in different combinations with pyrophosphate (PP_i) and/or free phosphate ions. Once again, dCK was used as a control protein. AlF_x alone, however, did not

stabilize the enzyme in the presence of known ligands (dCyd, emtricitabine and lamivudine). Interestingly, PP_i alone was able to stabilize dCK in the presence of the known ligands. AlF_x alone was tested on the two 5'-nucleotidases, NT5C2 and NT5C3, that had previously been crystallized in the presence of AlF_x and/or BeF_3 (Wallden *et al.*, 2007; Bitto *et al.*, 2006). The result for NT5C2 did show an elevated thermal shift of 8.8°C in the presence of AlF_x (1 mM $AlCl_3$ + 10 mM NaF), although this did not reveal any extra NA interactions for NT5C2. For NT5C3, an increased thermal shift of 1.7°C was observed in the presence of AlF_x, but no additional NAs were identified. Altogether, these results indicate that the addition of AlF_x was not a reliable way to generate a mimic of a NAL for the kinases.

5 Conclusions and future perspectives

In this thesis, some of the enzymes involved in nucleotide metabolism have been studied. Focused studies have been performed on two enzymes in particular; UMPK from *Ureaplasma parvum* and PRTFDC1, a human homolog of HPRT. Furthermore, a nucleoside analog library was created and screened with 23 enzymes within this pathway.

5.1 *Ureaplasma parvum* UMPK

The structure of *Up*-UMPK was determined, and showed a hexameric enzyme belonging to the amino acid kinase family. Interactions between *Up*-UMPK and its natural substrates were characterized, revealing a high K_m value for UMP. The idea was to investigate *Up*-UMPK as a potential target enzyme for antibiotic development. It is unlikely that chemical modifications of UMP could generate substrates for *Up*-UMPK with lower K_m values. We propose, therefore, that if *Up*-UMPK is still viewed as a potential antibiotic target, non-nucleosides/non-nucleotides should be considered as potential inhibitors. An interesting finding about *Up*-UMPK was that this enzyme, in contrast to other bacterial UMPKs, is not activated by GTP. Amino acid residues in a region between α-helix 3 and β-strand 5 have been shown in other UMPKs to be responsible for binding GTP. In many cases, arginines are responsible for interaction with GTP, and in *Up*-UMPK there are no arginines in this region, which most likely explains the lack of GTP-activation. Archaeal UMPKs have also been shown to lack the ability to be activated by GTP. The physiological role for this lack of allosteric regulation in archaeal and mollicute UMPKs may be related to an altered role of GTP in these organisms compared to other major phyla.

5.2 PRTFDC1

PRTFDC1 was reported to be a homolog of HPRT, but the function of the protein was listed as unknown. The structure of PRTFDC1 was determined with GMP in the active site, which showed a fold similar to HPRT. Using a nucleotide metabolome screen, ligands were identified. In accordance with substrates and products for HPRT, Hx, G PRPP, IMP and GMP were identified as ligands. In order to investigate if PRTFDC1 had any enzymatic activity toward these potential substrates, a radiochemical assay revealed that both Hx and G could be converted into their products. However the activity of PRTFDC1 when compared to HPRT was only 0.26% for Hx and 0.09% for G. The presence of G145 in the position of D137 of HPRT, could explain this low catalytic efficiency. A mutational study involving the conversion of residue G145 into D145 in PRTFDC1 would clarify this hypothesis.
Mutations in all regions of *HPRT1*, however, have been shown to alter the kinetic properties significantly, and as sequence identity is 65%, alterations in other regions could also account for the differences.

An interesting observation in our study was that the V_{max} value obtained for HPRT with G as a substrate was 20-fold higher than earlier published results. This discrepancy possibly depends on the different assays used in this and previous studies.

Many questions remain regarding the function of PRTFDC1. Does PRTFDC1 play a role in patients with partial or full HPRT deficiencies, by changing the phenotype of the specific HPRT genotype? Or, is PRTFDC1 a potential tumor suppressor gene, as reported in two studies? Finally, does PRTFDC1 interact with HPRT, and in this way affect HPRT activity? Further studies are needed to answer these questions, but molecular genetic screening of patients with Lesch-Nyhan phenotype may reveal if compensatory mutations in PRTFDC1 play a role in the disease.

5.3 Nucleoside analog library

A NAL has been set up consisting of 45 FDA-approved NAs. This has been screened using DSLS with 23 enzymes involved in nucleotide metabolism. Only a few cross-reactivities were observed between these enzymes and NAs, which demonstrates that the NAs are specific for their putative drug target.

In order to validate the method, the well-characterized salvage enzyme, dCK, was used. Eight interactions between dCK and NAs were identified with thermal shifts above the set detection limit; another five NAs appeared to be potential ligands when 1 mM ATP was added to the screen. Altogether, almost all ligands of dCK were identified using DSLS.

When screening UPP1 and GDA, several NAs gave rise to elevated thermal shifts. Vidarabine, trifluridine, idoxuridine, zidovudine, telbivudine, fluorouracil, floxuridine and thioguanine were all identified as ligands of UPP1. The most unexpected interaction seen for UPP1 was with vidarabine, an adenine analog. Several ligands were identified for GDA; these were valaciclovir, aciclovir, penciclovir, ganciclovir, thioguanine and mercaptopurine. Furthermore, the structure of GDA co-crystallized with valaciclovir was solved, confirming that this was a true ligand. A negative concentration-dependent stabilization was observed for RRM1 with azathioprine. This suggests that azathioprine binds to sites within RRM1, and thus destabilizes the enzyme.

This study serves as an initial screen for the identification of potential ligands. The positive concentration-dependent stabilization curves indicate that these compounds are indeed ligands. However, before these ligands can be classified either as substrates, inhibitors or activators, further experiments will have to be performed.

UPP1 and GDA bind nucleosides/nucleobases and nucleobases, respectively. It is thus not surprising that it was possible to identify new or well known ligands for these enzymes, as the NAs are primarily found as either nucleosides or nucleobases in NAL. Some interactions between selected enzymes and some phosphorylated forms of the NAs have almost certainly been missed.

This sheds light on the importance of having all forms of every NA (i.e. NA, NAMP, NADP and NATP) in the library, in order to reveal the complete map of interactions between the NAL and a target enzyme.

References

Aghi, M., Hochberg, F. & Breakefield, X.O. (2000). Prodrug activation enzymes in cancer gene therapy. *J Gene Med* 2(3), 148-64.

Akerley, B.J., Rubin, E.J., Novick, V.L., Amaya, K., Judson, N. & Mekalanos, J.J. (2002). A genome-scale analysis for identification of genes required for growth or survival of Haemophilus influenzae. *Proc Natl Acad Sci U S A* 99(2), 966-71.

Arner, E.S. & Eriksson, S. (1995). Mammalian deoxyribonucleoside kinases. *Pharmacol Ther* 67(2), 155-86.

Beausejour, C.M., Gagnon, J., Primeau, M. & Momparler, R.L. (2002). Cytotoxic activity of 2',2'-difluorodeoxycytidine, 5-aza-2'-deoxycytidine and cytosine arabinoside in cells transduced with deoxycytidine kinase gene. *Biochem Biophys Res Commun* 293(5), 1478-84.

Bergstrom, J.D. & Bieber, A.L. (1979). Characterization of purified guanine aminohydrolase. *Arch Biochem Biophys* 194(1), 107-16.

Bitto, E., Bingman, C.A., Wesenberg, G.E., McCoy, J.G. & Phillips, G.N., Jr. (2006). Structure of pyrimidine 5'-nucleotidase type 1. Insight into mechanism of action and inhibition during lead poisoning. *J Biol Chem* 281(29), 20521-9.

Bohman, C. & Eriksson, S. (1988). Deoxycytidine kinase from human leukemic spleen: preparation and characteristics of homogeneous enzyme. *Biochemistry* 27(12), 4258-65.

Brady, W.A., Kokoris, M.S., Fitzgibbon, M. & Black, M.E. (1996). Cloning, characterization, and modeling of mouse and human guanylate kinases. *J Biol Chem* 271(28), 16734-40.

Briggs, J.D. (1991). A critical review of immunosuppressive therapy. *Immunology Letters* 29(1-2), 89-94.

Briozzo, P., Evrin, C., Meyer, P., Assairi, L., Joly, N., Barzu, O. & Gilles, A.M. (2005). Structure of Escherichia coli UMP kinase differs from that of other nucleoside monophosphate kinases and sheds new light on enzyme regulation. *J Biol Chem* 280(27), 25533-40.

Burnstock, G. (2008). Purinergic signalling and disorders of the central nervous system. *Nat Rev Drug Discov* 7(7), 575-90.

Cai, L.Y., Abe, M., Izumi, S., Imura, M., Yasugi, T. & Ushijima, T. (2007). Identification of PRTFDC1 silencing and aberrant promoter methylation of GPR150, ITGA8 and HOXD11 in ovarian cancers. *Life Sci* 80(16), 1458-65.

Calabresi, P., Falcone, A., St Clair, M.H., Wiemann, M.C., Chu, S.H. & Darnowski, J.W. (1990). Benzylacyclouridine reverses azidothymidine-induced marrow suppression without impairment of anti-human immunodeficiency virus activity. *Blood* 76(11), 2210-5.

Canyuk, B., Focia, P.J. & Eakin, A.E. (2001). The role for an invariant aspartic acid in hypoxanthine phosphoribosyltransferases is examined using saturation mutagenesis, functional analysis, and X-ray crystallography. *Biochemistry* 40(9), 2754-65.

Cao, D., Russell, R.L., Zhang, D., Leffert, J.J. & Pizzorno, G. (2002). Uridine phosphorylase (-/-) murine embryonic stem cells clarify the key role of this enzyme in the regulation of the pyrimidine salvage pathway and in the activation of fluoropyrimidines. *Cancer Res* 62(8), 2313-7.

Chabre, M. (1990). Aluminofluoride and beryllofluoride complexes: a new phosphate analogs in enzymology. *Trends Biochem Sci* 15(1), 6-10.

Chen, S.C. & Sorrell, T.C. (2007). Antifungal agents. *Med J Aust* 187(7), 404-9.

Chottiner, E.G., Shewach, D.S., Datta, N.S., Ashcraft, E., Gribbin, D., Ginsburg, D., Fox, I.H. & Mitchell, B.S. (1991). Cloning and expression of human deoxycytidine kinase cDNA. *Proc Natl Acad Sci U S A* 88(4), 1531-5.

Costi, M.P. (1998). Thymidylate synthase inhibition: a structure-based rationale for drug design. *Med Res Rev* 18(1), 21-42.

Coulthard, S. & Hogarth, L. (2005). The thiopurines: an update. *Invest New Drugs* 23(6), 523-32.

Craig, S.P., 3rd & Eakin, A.E. (2000). Purine phosphoribosyltransferases. *J Biol Chem* 275(27), 20231-4.

Daly, J.W. (2007). Caffeine analogs: biomedical impact. *Cell Mol Life Sci* 64(16), 2153-69.

Datta, N.S., Shewach, D.S., Mitchell, B.S. & Fox, I.H. (1989). Kinetic properties and inhibition of human T lymphoblast deoxycytidine kinase. *J Biol Chem* 264(16), 9359-64.

De Clercq, E. (2009). Anti-HIV drugs: 25 compounds approved within 25 years after the discovery of HIV. *Int J Antimicrob Agents* 33(4), 307-20.

De Clercq, E. & Field, H.J. (2006). Antiviral prodrugs - the development of successful prodrug strategies for antiviral chemotherapy. *Br J Pharmacol* 147(1), 1-11.

Deville-Bonne, D., El Amri, C., Meyer, P., Chen, Y., Agrofoglio, L.A. & Janin, J. (2010). Human and viral nucleoside/nucleotide kinases involved in antiviral drug activation: structural and catalytic properties. *Antiviral Res* 86(1), 101-20.

Eads, J.C., Scapin, G., Xu, Y., Grubmeyer, C. & Sacchettini, J.C. (1994). The crystal structure of human hypoxanthine-guanine phosphoribosyltransferase with bound GMP. *Cell* 78(2), 325-34.

Egeblad-Welin, L., Welin, M., Wang, L. & Eriksson, S. (2007). Structural and functional investigations of Ureaplasma parvum UMP kinase - a potential antibacterial drug target. *Febs J*.

Eriksson, S., Munch-Petersen, B., Johansson, K. & Eklund, H. (2002). Structure and function of cellular deoxyribonucleoside kinases. *Cell Mol Life Sci* 59(8), 1327-46.

Evans, D.R. & Guy, H.I. (2004). Mammalian pyrimidine biosynthesis: fresh insights into an ancient pathway. *J Biol Chem* 279(32), 33035-8.

Evrin, C., Straut, M., Slavova-Azmanova, N., Bucurenci, N., Onu, A., Assairi, L., Ionescu, M., Palibroda, N., Barzu, O. & Gilles, A.M. (2007). Regulatory mechanisms differ in UMP kinases from gram-negative and gram-positive bacteria. *J Biol Chem* 282(10), 7242-53.

Fassy, F., Krebs, O., Lowinski, M., Ferrari, P., Winter, J., Collard-Dutilleul, V. & Salahbey Hocini, K. (2004). UMP kinase from Streptococcus pneumoniae: evidence for co-operative ATP binding and allosteric regulation. *Biochem J* 384(Pt 3), 619-27.

Ferreira, A. (1965). Allopurinol in Gout. *Br Med J* 1(5438), 810-1.

Fraser, C.M., Gocayne, J.D., White, O., Adams, M.D., Clayton, R.A., Fleischmann, R.D., Bult, C.J., Kerlavage, A.R., Sutton, G., Kelley, J.M., Fritchman, R.D., Weidman, J.F., Small, K.V., Sandusky, M., Fuhrmann, J., Nguyen, D., Utterback, T.R., Saudek, D.M., Phillips, C.A., Merrick, J.M., Tomb, J.F., Dougherty, B.A., Bott, K.F., Hu, P.C., Lucier, T.S., Peterson, S.N., Smith, H.O., Hutchison, C.A., 3rd & Venter, J.C. (1995). The minimal gene complement of Mycoplasma genitalium. *Science* 270(5235), 397-403.

Freeman, S. & Gardiner, J.M. (1996). Acyclic nucleosides as antiviral compounds. *Mol Biotechnol* 5(2), 125-37.

Friedman, M.A. & Sadee, W. (1978). The fluoropyrimidines: biochemical mechanisms and design of clinical trials. *Cancer Chemother Pharmacol* 1(2), 77-82.

Gagyi, C., Bucurenci, N., Sirbu, O., Labesse, G., Ionescu, M., Ofiteru, A., Assairi, L., Landais, S., Danchin, A., Barzu, O. & Gilles, A.M. (2003). UMP kinase from the Gram-positive bacterium Bacillus subtilis is strongly dependent on GTP for optimal activity. *Eur J Biochem* 270(15), 3196-204.

Gagyi, C., Ionescu, M., Gounon, P., Sakamoto, H., Rousselle, J.C. & Laurent-Winter, C. (2004). Identification and immunochemical

location of UMP kinase from Bacillus subtilis. *Curr Microbiol* 48(1), 62-7.
Galmarini, C.M., Mackey, J.R. & Dumontet, C. (2001). Nucleoside analogues: mechanisms of drug resistance and reversal strategies. *Leukemia* 15(6), 875-90.
Galmarini, C.M., Mackey, J.R. & Dumontet, C. (2002). Nucleoside analogues and nucleobases in cancer treatment. *Lancet Oncol* 3(7), 415-24.
Galperin, M.Y., Moroz, O.V., Wilson, K.S. & Murzin, A.G. (2006). House cleaning, a part of good housekeeping. *Mol Microbiol* 59(1), 5-19.
Giacomello, A. & Salerno, C. (1977). A continuous Spectrophotometric assay for hypoxanthine-guanine phosphoribosyltransferase. *Anal Biochem* 79(1-2), 263-7.
Glass, J.I., Lefkowitz, E.J., Glass, J.S., Heiner, C.R., Chen, E.Y. & Cassell, G.H. (2000). The complete sequence of the mucosal pathogen Ureaplasma urealyticum. *Nature* 407(6805), 757-62.
Godsey, M.H., Ort, S., Sabini, E., Konrad, M. & Lavie, A. (2006). Structural basis for the preference of UTP over ATP in human deoxycytidine kinase: illuminating the role of main-chain reorganization. *Biochemistry* 45(2), 452-61.
Gouet, P., Courcelle, E., Stuart, D.I. & Metoz, F. (1999). ESPript: analysis of multiple sequence alignments in PostScript. *Bioinformatics* 15(4), 305-8.
Hazra, S., Konrad, M. & Lavie, A. (2010). The sugar ring of the nucleoside is required for productive substrate positioning in the active site of human deoxycytidine kinase (dCK): implications for the development of dCK-activated acyclic guanine analogues. *J Med Chem* 53(15), 5792-800.
Heidelberger, C., Chaudhuri, N.K., Danneberg, P., Mooren, D., Griesbach, L., Duschinsky, R., Schnitzer, R.J., Pleven, E. & Scheiner, J. (1957). Fluorinated pyrimidines, a new class of tumour-inhibitory compounds. *Nature* 179(4561), 663-6.
Himmelreich, R., Hilbert, H., Plagens, H., Pirkl, E., Li, B.C. & Herrmann, R. (1996). Complete sequence analysis of the genome of the bacterium Mycoplasma pneumoniae. *Nucleic Acids Res* 24(22), 4420-49.
Huang, S.H., Tang, A., Drisco, B., Zhang, S.Q., Seeger, R., Li, C. & Jong, A. (1994). Human dTMP kinase: gene expression and enzymatic activity coinciding with cell cycle progression and cell growth. *DNA Cell Biol* 13(5), 461-71.
Hughes, T.L., Hahn, T.M., Reynolds, K.K. & Shewach, D.S. (1997). Kinetic analysis of human deoxycytidine kinase with the true phosphate donor uridine triphosphate. *Biochemistry* 36(24), 7540-7.

Hunsucker, S.A., Mitchell, B.S. & Spychala, J. (2005). The 5'-nucleotidases as regulators of nucleotide and drug metabolism. *Pharmacol Ther* 107(1), 1-30.

Iyama, T., Abolhassani, N., Tsuchimoto, D., Nonaka, M. & Nakabeppu, Y. (2010). NUDT16 is a (deoxy)inosine diphosphatase, and its deficiency induces accumulation of single-strand breaks in nuclear DNA and growth arrest. *Nucleic Acids Res* 38(14), 4834-43.

Janin, J. & Deville-Bonne, D. (2002). Nucleoside-diphosphate kinase: structural and kinetic analysis of reaction pathway and phosphohistidine intermediate. *Methods Enzymol* 354, 118-34.

Jensen, K.S., Johansson, E. & Jensen, K.F. (2007). Structural and enzymatic investigation of the Sulfolobus solfataricus uridylate kinase shows competitive UTP inhibition and the lack of GTP stimulation. *Biochemistry* 46(10), 2745-57.

Jinnah, H.A., De Gregorio, L., Harris, J.C., Nyhan, W.L. & O'Neill, J.P. (2000). The spectrum of inherited mutations causing HPRT deficiency: 75 new cases and a review of 196 previously reported cases. *Mutat Res* 463(3), 309-26.

Jinnah, H.A., Harris, J.C., Nyhan, W.L. & O'Neill, J.P. (2004). The spectrum of mutations causing HPRT deficiency: an update. *Nucleosides Nucleotides Nucleic Acids* 23(8-9), 1153-60.

Jiralerspong, S. & Patel, P.I. (1996). Regulation of the hypoxanthine phosphoribosyltransferase gene: in vitro and in vivo approaches. *Proc Soc Exp Biol Med* 212(2), 116-27.

Kanehisa, M. & Goto, S. (2000). KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res* 28(1), 27-30.

Kanehisa, M., Goto, S., Furumichi, M., Tanabe, M. & Hirakawa, M. (2010). KEGG for representation and analysis of molecular networks involving diseases and drugs. *Nucleic Acids Res* 38(Database issue), D355-60.

Kanehisa, M., Goto, S., Hattori, M., Aoki-Kinoshita, K.F., Itoh, M., Kawashima, S., Katayama, T., Araki, M. & Hirakawa, M. (2006). From genomics to chemical genomics: new developments in KEGG. *Nucleic Acids Res* 34(Database issue), D354-7.

Keebaugh, A.C., Sullivan, R.T. & Thomas, J.W. (2007). Gene duplication and inactivation in the HPRT gene family. *Genomics* 89(1), 134-42.

Keough, D.T., Brereton, I.M., de Jersey, J. & Guddat, L.W. (2005). The crystal structure of free human hypoxanthine-guanine phosphoribosyltransferase reveals extensive conformational plasticity throughout the catalytic cycle. *J Mol Biol* 351(1), 170-81.

Keough, D.T., McConachie, L.A., Gordon, R.B., de Jersey, J. & Emmerson, B.T. (1987). Human hypoxanthine-guanine phosphoribosyltransferase. Development of a spectrophotometric assay and its use in detection and characterization of mutant forms. *Clin Chim Acta* 163(3), 301-8.

Keough, D.T., Ng, A.L., Winzor, D.J., Emmerson, B.T. & de Jersey, J. (1999). Purification and characterization of Plasmodium falciparum hypoxanthine-guanine-xanthine phosphoribosyltransferase and comparison with the human enzyme. *Mol Biochem Parasitol* 98(1), 29-41.

Kholti, A., Charlier, D., Gigot, D., Huysveld, N., Roovers, M. & Glansdorff, N. (1998). pyrH-encoded UMP-kinase directly participates in pyrimidine-specific modulation of promoter activity in Escherichia coli. *J Mol Biol* 280(4), 571-82.

Kleymann, G. (2003). Novel agents and strategies to treat herpes simplex virus infections. *Expert Opin Investig Drugs* 12(2), 165-83.

Knox, C., Law, V., Jewison, T., Liu, P., Ly, S., Frolkis, A., Pon, A., Banco, K., Mak, C., Neveu, V., Djoumbou, Y., Eisner, R., Guo, A.C. & Wishart, D.S. (2011). DrugBank 3.0: a comprehensive resource for 'omics' research on drugs. *Nucleic Acids Res* 39(Database issue), D1035-41.

Krawiec, K., Kierdaszuk, B., Eriksson, S., Munch-Petersen, B. & Shugar, D. (1995). Nucleoside triphosphate donors for nucleoside kinases: donor properties of UTP with human deoxycytidine kinase. *Biochem Biophys Res Commun* 216(1), 42-8.

Labesse, G., Benkali, K., Salard-Arnaud, I., Gilles, A.M. & Munier-Lehmann, H. (2010). Structural and functional characterization of the Mycobacterium tuberculosis uridine monophosphate kinase: insights into the allosteric regulation. *Nucleic Acids Res*.

Lacombe, M.L., Milon, L., Munier, A., Mehus, J.G. & Lambeth, D.O. (2000). The human Nm23/nucleoside diphosphate kinases. *J Bioenerg Biomembr* 32(3), 247-58.

Landais, S., Gounon, P., Laurent-Winter, C., Mazie, J.C., Danchin, A., Barzu, O. & Sakamoto, H. (1999). Immunochemical analysis of UMP kinase from Escherichia coli. *J Bacteriol* 181(3), 833-40.

Lee, M.J., Chien-Liang, L., Tsai, J.Y., Sue, W.T., Hsia, W.S. & Huang, H. (2010). Identification and biochemical characterization of a unique Mn2+-dependent UMP kinase from Helicobacter pylori. *Arch Microbiol* 192(9), 739-46.

Liou, J.Y., Dutschman, G.E., Lam, W., Jiang, Z. & Cheng, Y.C. (2002). Characterization of human UMP/CMP kinase and its phosphorylation of D- and L-form deoxycytidine analogue monophosphates. *Cancer Res* 62(6), 1624-31.

Lotfi, K., Mansson, E., Spasokoukotskaja, T., Pettersson, B., Liliemark, J., Peterson, C., Eriksson, S. & Albertioni, F. (1999). Biochemical pharmacology and resistance to 2-chloro-2'-arabino-fluoro-2'-deoxyadenosine, a novel analogue of cladribine in human leukemic cells. *Clin Cancer Res* 5(9), 2438-44.

Marco-Marin, C., Gil-Ortiz, F. & Rubio, V. (2005). The crystal structure of Pyrococcus furiosus UMP kinase provides insight into catalysis

and regulation in microbial pyrimidine nucleotide biosynthesis. *J Mol Biol* 352(2), 438-54.
Marco-Marin, C. & Rubio, V. (2009). The site for the allosteric activator GTP of Escherichia coli UMP kinase. *FEBS Lett* 583(1), 185-9.
Mathews, C.K. (2006). DNA precursor metabolism and genomic stability. *Faseb J* 20(9), 1300-14.
Mathews, C.K. & Song, S. (2007). Maintaining precursor pools for mitochondrial DNA replication. *Faseb J* 21(10), 2294-303.
Meier, C., Carter, L.G., Sainsbury, S., Mancini, E.J., Owens, R.J., Stuart, D.I. & Esnouf, R.M. (2008). The crystal structure of UMP kinase from Bacillus anthracis (BA1797) reveals an allosteric nucleotide-binding site. *J Mol Biol* 381(5), 1098-105.
Meyer, P., Evrin, C., Briozzo, P., Joly, N., Barzu, O. & Gilles, A.M. (2008). Structural and functional characterization of Escherichia coli UMP kinase in complex with its allosteric regulator GTP. *J Biol Chem* 283(51), 36011-8.
Muller, C.E. & Jacobson, K.A. (2011). Recent developments in adenosine receptor ligands and their potential as novel drugs. *Biochim Biophys Acta*, doi:10.1016/j.bbamem.2010.12.017.
Munir, S., Saleem, S., Idrees, M., Tariq, A., Butt, S., Rauff, B., Hussain, A., Badar, S., Naudhani, M., Fatima, Z., Ali, M., Ali, L., Akram, M., Aftab, M., Khubaib, B. & Awan, Z. (2010). Hepatitis C treatment: current and future perspectives. *Virol J* 7, 296.
Mushegian, A.R. & Koonin, E.V. (1996). A minimal gene set for cellular life derived by comparison of complete bacterial genomes. *Proc Natl Acad Sci U S A* 93(19), 10268-73.
Ng, S.B., Buckingham, K.J., Lee, C., Bigham, A.W., Tabor, H.K., Dent, K.M., Huff, C.D., Shannon, P.T., Jabs, E.W., Nickerson, D.A., Shendure, J. & Bamshad, M.J. (2010). Exome sequencing identifies the cause of a mendelian disorder. *Nat Genet* 42(1), 30-5.
Nicklas, J.A. (2006). Pseudogenes of the human HPRT1 gene. *Environ Mol Mutagen* 47(3), 212-8.
Nordlund, P. & Reichard, P. (2006). Ribonucleotide reductases. *Annu Rev Biochem* 75, 681-706.
Nyhan, W.L. (2005). Disorders of purine and pyrimidine metabolism. *Mol Genet Metab* 86(1-2), 25-33.
Pastor-Anglada, M., Cano-Soldado, P., Molina-Arcas, M., Lostao, M.P., Larrayoz, I., Martinez-Picado, J. & Casado, F.J. (2005). Cell entry and export of nucleoside analogues. *Virus Res* 107(2), 151-64.
Pescovitz, M.D. (2006). Valganciclovir. *Transplantation Reviews* 20(2), 82-87.
Pescovitz, M.D. (2010). Valganciclovir: Recent Progress. *American Journal of Transplantation* 10(6), 1359-1364.
Peters, G.P. (Ed.) (2006). *Deoxynucleoside Analogs in Cancer Therapy*. Totowa, New Jersey: Humana Press Inc.

Razin, S., Yogev, D. & Naot, Y. (1998). Molecular biology and pathogenicity of mycoplasmas. *Microbiol Mol Biol Rev* 62(4), 1094-156.

Reichard, P. (1988). Interactions between deoxyribonucleotide and DNA synthesis. *Annu Rev Biochem* 57, 349-74.

Reichard, P. (2002). Ribonucleotide reductases: the evolution of allosteric regulation. *Arch Biochem Biophys* 397(2), 149-55.

Renck, D., Ducati, R.G., Palma, M.S., Santos, D.S. & Basso, L.A. (2010). The kinetic mechanism of human uridine phosphorylase 1: Towards the development of enzyme inhibitors for cancer chemotherapy. *Arch Biochem Biophys* 497(1-2), 35-42.

Rossi, C.A., Hakim, G. & Solaini, G. (1978). Purification and properties of pig brain guanine deaminase. *Biochim Biophys Acta* 526(1), 235-46.

Rual, J.F., Venkatesan, K., Hao, T., Hirozane-Kishikawa, T., Dricot, A., Li, N., Berriz, G.F., Gibbons, F.D., Dreze, M., Ayivi-Guedehoussou, N., Klitgord, N., Simon, C., Boxem, M., Milstein, S., Rosenberg, J., Goldberg, D.S., Zhang, L.V., Wong, S.L., Franklin, G., Li, S., Albala, J.S., Lim, J., Fraughton, C., Llamosas, E., Cevik, S., Bex, C., Lamesch, P., Sikorski, R.S., Vandenhaute, J., Zoghbi, H.Y., Smolyar, A., Bosak, S., Sequerra, R., Doucette-Stamm, L., Cusick, M.E., Hill, D.E., Roth, F.P. & Vidal, M. (2005). Towards a proteome-scale map of the human protein-protein interaction network. *Nature* 437(7062), 1173-8.

Saada, A. (2004). Deoxyribonucleotides and disorders of mitochondrial DNA integrity. *DNA Cell Biol* 23(12), 797-806.

Sabini, E., Hazra, S., Konrad, M., Burley, S.K. & Lavie, A. (2007a). Structural basis for activation of the therapeutic L-nucleoside analogs 3TC and troxacitabine by human deoxycytidine kinase. *Nucleic Acids Res* 35(1), 186-92.

Sabini, E., Hazra, S., Konrad, M. & Lavie, A. (2007b). Nonenantioselectivity property of human deoxycytidine kinase explained by structures of the enzyme in complex with L- and D-nucleosides. *J Med Chem* 50(13), 3004-14.

Sabini, E., Hazra, S., Konrad, M. & Lavie, A. (2008a). Elucidation of different binding modes of purine nucleosides to human deoxycytidine kinase. *J Med Chem* 51(14), 4219-25.

Sabini, E., Hazra, S., Ort, S., Konrad, M. & Lavie, A. (2008b). Structural basis for substrate promiscuity of dCK. *J Mol Biol* 378(3), 607-21.

Sabini, E., Ort, S., Monnerjahn, C., Konrad, M. & Lavie, A. (2003). Structure of human dCK suggests strategies to improve anticancer and antiviral therapy. *Nat Struct Biol* 10(7), 513-9.

Senisterra, G.A. & Finerty, P.J., Jr. (2009). High throughput methods of assessing protein stability and aggregation. *Mol Biosyst* 5(3), 217-23.

Senisterra, G.A., Markin, E., Yamazaki, K., Hui, R., Vedadi, M. & Awrey, D.E. (2006). Screening for ligands using a generic and high-

throughput light-scattering-based assay. *J Biomol Screen* 11(8), 940-8.
Serina, L., Blondin, C., Krin, E., Sismeiro, O., Danchin, A., Sakamoto, H., Gilles, A.M. & Barzu, O. (1995). Escherichia coli UMP-kinase, a member of the aspartokinase family, is a hexamer regulated by guanine nucleotides and UTP. *Biochemistry* 34(15), 5066-74.
Shi, W., Li, C.M., Tyler, P.C., Furneaux, R.H., Grubmeyer, C., Schramm, V.L. & Almo, S.C. (1999). The 2.0 A structure of human hypoxanthine-guanine phosphoribosyltransferase in complex with a transition-state analog inhibitor. *Nat Struct Biol* 6(6), 588-93.
Sinha, S.C. & Smith, J.L. (2001). The PRT protein family. *Curr Opin Struct Biol* 11(6), 733-9.
Skorodin, M.S. (1993). Pharmacotherapy for asthma and chronic obstructive pulmonary disease. Current thinking, practices, and controversies. *Arch Intern Med* 153(7), 814-28.
Smith, J.L. (1995). Enzymes of nucleotide synthesis. *Curr Opin Struct Biol* 5(6), 752-7.
Snoeck, R. (2000). Antiviral therapy of herpes simplex. *Int J Antimicrob Agents* 16(2), 157-9.
Spychala, J., Datta, N.S., Takabayashi, K., Datta, M., Fox, I.H., Gribbin, T. & Mitchell, B.S. (1996). Cloning of human adenosine kinase cDNA: sequence similarity to microbial ribokinases and fructokinases. *Proc Natl Acad Sci U S A* 93(3), 1232-7.
Strunecka, A., Strunecky, O. & Patocka, J. (2002). Fluoride plus aluminum: useful tools in laboratory investigations, but messengers of false information. *Physiol Res* 51(6), 557-64.
Sung, T.J. (2010). Ureaplasma infections in pre-term infants: Recent information regarding the role of Ureaplasma species as neonatal pathogens. *Korean J Pediatr* 53(12), 989-93.
Suzuki, E., Imoto, I., Pimkhaokham, A., Nakagawa, T., Kamata, N., Kozaki, K.I., Amagasa, T. & Inazawa, J. (2007). PRTFDC1, a possible tumor-suppressor gene, is frequently silenced in oral squamous-cell carcinomas by aberrant promoter hypermethylation. *Oncogene* 26(57), 7921-32.
Thomas, C.B., Arnold, W.J. & Kelley, W.N. (1973). Human adenine phosphoribosyltransferase. Purification, subunit structure, and substrate specificity. *J Biol Chem* 248(7), 2529-35.
Torres, R.J. & Puig, J.G. (2007). Hypoxanthine-guanine phosophoribosyltransferase (HPRT) deficiency: Lesch-Nyhan syndrome. *Orphanet J Rare Dis* 2, 48.
Traut, T.W. (1994). Physiological concentrations of purines and pyrimidines. *Mol Cell Biochem* 140(1), 1-22.

Tu, J.L., Chin, K.H., Wang, A.H. & Chou, S.H. (2009). Unique GTP-binding pocket and allostery of uridylate kinase from a gram-negative phytopathogenic bacterium. *J Mol Biol* 385(4), 1113-26.

Waites, K.B., Katz, B. & Schelonka, R.L. (2005). Mycoplasmas and ureaplasmas as neonatal pathogens. *Clin Microbiol Rev* 18(4), 757-89.

Wallden, K., Stenmark, P., Nyman, T., Flodin, S., Graslund, S., Loppnau, P., Bianchi, V. & Nordlund, P. (2007). Crystal structure of human cytosolic 5'-nucleotidase II: insights into allosteric regulation and substrate recognition. *J Biol Chem* 282(24), 17828-36.

Van Rompay, A.R., Johansson, M. & Karlsson, A. (1999). Phosphorylation of deoxycytidine analog monophosphates by UMP-CMP kinase: molecular characterization of the human enzyme. *Mol Pharmacol* 56(3), 562-9.

Van Rompay, A.R., Johansson, M. & Karlsson, A. (2000). Phosphorylation of nucleosides and nucleoside analogs by mammalian nucleoside monophosphate kinases. *Pharmacol Ther* 87(2-3), 189-98.

Van Rompay, A.R., Johansson, M. & Karlsson, A. (2003). Substrate specificity and phosphorylation of antiviral and anticancer nucleoside analogues by human deoxyribonucleoside kinases and ribonucleoside kinases. *Pharmacol Ther* 100(2), 119-39.

Van Rompay, A.R., Norda, A., Linden, K., Johansson, M. & Karlsson, A. (2001). Phosphorylation of uridine and cytidine nucleoside analogs by two human uridine-cytidine kinases. *Mol Pharmacol* 59(5), 1181-6.

Vedadi, M., Arrowsmith, C.H., Allali-Hassani, A., Senisterra, G. & Wasney, G.A. (2010). Biophysical characterization of recombinant proteins: a key to higher structural genomics success. *J Struct Biol* 172(1), 107-19.

Vedadi, M., Niesen, F.H., Allali-Hassani, A., Fedorov, O.Y., Finerty, P.J., Jr., Wasney, G.A., Yeung, R., Arrowsmith, C., Ball, L.J., Berglund, H., Hui, R., Marsden, B.D., Nordlund, P., Sundstrom, M., Weigelt, J. & Edwards, A.M. (2006). Chemical screening methods to identify ligands that promote protein stability, protein crystallization, and structure determination. *Proc Natl Acad Sci U S A* 103(43), 15835-40.

Welin, M. & Nordlund, P. (2010). Understanding specificity in metabolic pathways--structural biology of human nucleotide metabolism. *Biochem Biophys Res Commun* 396(1), 157-63.

Wishart, D.S., Knox, C., Guo, A.C., Cheng, D., Shrivastava, S., Tzur, D., Gautam, B. & Hassanali, M. (2008). DrugBank: a knowledgebase for drugs, drug actions and drug targets. *Nucleic Acids Res* 36(Database issue), D901-6.

Wishart, D.S., Knox, C., Guo, A.C., Shrivastava, S., Hassanali, M., Stothard, P., Chang, Z. & Woolsey, J. (2006). DrugBank: a

comprehensive resource for in silico drug discovery and exploration. *Nucleic Acids Res* 34(Database issue), D668-72.
Volgmann, T., Ohlinger, R. & Panzig, B. (2005). Ureaplasma urealyticum-harmless commensal or underestimated enemy of human reproduction? A review. *Arch Gynecol Obstet* 273(3), 133-9.
Xu, Y. & Grubmeyer, C. (1998). Catalysis in human hypoxanthine-guanine phosphoribosyltransferase: Asp 137 acts as a general acid/base. *Biochemistry* 37(12), 4114-24.
Xu, Y., Johansson, M. & Karlsson, A. (2008). Human UMP-CMP kinase 2, a novel nucleoside monophosphate kinase localized in mitochondria. *J Biol Chem* 283(3), 1563-71.
Yamanaka, K., Ogura, T., Niki, H. & Hiraga, S. (1992). Identification and characterization of the smbA gene, a suppressor of the mukB null mutant of Escherichia coli. *J Bacteriol* 174(23), 7517-26.
Yenush, L., Belles, J.M., Lopez-Coronado, J.M., Gil-Mascarell, R., Serrano, R. & Rodriguez, P.L. (2000). A novel target of lithium therapy. *FEBS Lett* 467(2-3), 321-5.
Yuan, G., Bin, J.C., McKay, D.J. & Snyder, F.F. (1999). Cloning and characterization of human guanine deaminase. Purification and partial amino acid sequence of the mouse protein. *J Biol Chem* 274(12), 8175-80.
Yuen, M.F. & Lai, C.L. (2011). Treatment of chronic hepatitis B: Evolution over two decades. *J Gastroenterol Hepatol* 26 Suppl 1, 138-43.
Zhang, Y., Morar, M. & Ealick, S.E. (2008). Structural biology of the purine biosynthetic pathway. *Cell Mol Life Sci* 65(23), 3699-724.
Zhang, Y., Secrist, J.A., 3rd & Ealick, S.E. (2006). The structure of human deoxycytidine kinase in complex with clofarabine reveals key interactions for prodrug activation. *Acta Crystallogr D Biol Crystallogr* 62(Pt 2), 133-9.
Zhenchuk, A., Lotfi, K., Juliusson, G. & Albertioni, F. (2009). Mechanisms of anti-cancer action and pharmacology of clofarabine. *Biochem Pharmacol* 78(11), 1351-9.

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