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The role of 5'-nucleotidases and Deoxynucleoside Kinases in Responses to Nucleoside Analogues

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Some look at things that are, and ask why.
I dream of things that never were and ask why not?

George Bernard Shaw

ABSTRACT

The efficacy of nucleoside analogues (NAs) in treating several hematological malignancies, solid tumors and viral infections is limited primarily by side-effects and the development of drug resistance. The aims of the present thesis were to elucidate mechanism(s) involved in tissue-specific toxicity associated with NA therapy, as well as the mechanisms underlying resistance to these drugs.

The mRNA levels and activities of different cytosolic and mitochondrial deoxynucleoside kinases (dNKs) and 5'-nucleotidases (5'- NTs) exhibit a distinct pattern for each of a variety of mouse tissues. Heart and skeletal muscle, as well as adipose tissue demonstrate low levels of both the anabolic and catabolic enzymes, which may explain at least some of the adverse side-effects of NA treatment.

A novel approach based on high-performance liquid chromatography (HPLC) revealed that each of 14 different mouse and rat tissues exhibits a unique profile of dNK and 5'NT activities, with 2-3-fold species differences for certain of these tissues. These observations have important implications for the choice of an animal model for characterization of NA toxicity.

The cytotoxicity of gemcitabine (a cytidine-containing NA employed clinically to treat patients with cancer) towards human leukemia and melanoma cell lines rendered deficient in either deoxycytidine kinase (dCK) or deoxyguanosine kinase (dGK) with siRNA was compared to the corresponding toxicity towards cells that express these enzymes. Both types of deficient cells were more sensitive to gemcitabine, suggesting that, at least in this system, the toxicity of this drug is not correlated to the levels of activating enzymes.

The activites of various 5'-NTs in peripheral blood cells isolated from CLL patients show considreble inter-individual variation. Degradation of the phosphorylated forms of cladribine and fludarabine, two important anti-leukemic NAs, was characterized in these cells. Significant correlations between the rate of cladribine monophosphate degradation and the activity of cytosolic 5'-nucleotidase 1 (CN1), as well as between the rate of fludarabine monophosphate degradation and the activity of cytosolic 5'-nucleotidase 2 (CN2) were observed.

This investigation provides new insights regarding the patterns of expression of anabolic and catabolic enzymes that hopefully can be used to improve NA therapy in the future.

LIST OF PUBLICATIONS

This thesis is based on the following papers:

I. Svetlena N. Rylova, **Saeedeh Mirzaee**, Freidoun Albertioni, Staffan Eriksson. Expression of deoxynucleoside kinases and 5'-nucleotidases in mouse tissues: Implications for mitochondrial toxicity. Biochemical Pharmacology, 2007, 74, 169-75.

II. **Saeedeh Mirzaee**, Staffan Eriksson, Freidoun Albertioni. Differences in cytosolic and mitochondrial 5'-nucleotidase and deoxynucleoside kinase activities in Sprague- Dawley rat and CD-1 mouse tissues: Implication for the toxicity of nucleoside analogs in animal models. Toxicology, 2010, 267, 159-64.

III. Anna Fyrberg, **Saeedeh Mirzaee**, Freidoun Albertioni, Kourosh Lotfi. RNA interference targeting nucleoside analog activating enzymes in leukemic and solid malignancies enhances the effect of gemcitabine (Submitted).

IV. **Saeedeh Mirzaee**, Xin Wang, Narges Bayat, Anna Fyrberg, Kourosh Lotfi, Karin Karlsson, Gunnar Juliusson, Staffan Eriksson, Freidoun Albertioni. Activity profiles of 5'-nucleotidases in blood cells from untreated patients with B-cell chronic lymphocytic leukemia and in phytohemag-glutinin stimulated cells from healthy subjects: correlation to nucleoside analogues therapy (Manuscript).

CONTENTS

LIST OF ABBREVIATIONS

5’-NTs	5’-nucleotidases
3TC	2’-deoxy-3’-thiacytidine
ADA	Adenosine deaminase
ADP	Adenosine diphosphate
AK	Adenosine Kinase
ALL	Acute lymphocytic leukemia
AML	Acute myeloid leukemia
AMP	Adenosine monophosphate
APAF-1	Apoptosis protein activating factor-1
Ara-CTP	Cytosine arabinoside triphosphate
Ara-G	9 -β-D-Arabinofuranosylguanine, Nelarabine
ATP	Adenosine triphosphate
AZT	3’-Azido-2’-, 3’ –dideoxythymidine
CAFdATP	2-Chloro-2’arabino-fluro-2’deoxyadenosine triphosphate
CdAMP	2-Chloro-2’-deoxyadenosine monophosphate
CdATP	CdA triphosphate
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
CN1	Cytosolic 5’-nucleotidase-1
CN2	Cytosolic 5’-nucleotidase-2
CTP	Cytidine triphosphate
d4T	2’, 3’-Didehydro-3’-deoxythymidine
dCK	Deoxycytidine kinase
dCMP	Deoxycytidine monophosphate
dCTP	Deoxycytidine triphosphate
ddC	2’,3’-Dideoxycytidine
dFdCTP	2’,2’-Difluorodeoxycytidine triphosphate
dGK	Deoxyguanosine kinase
dGMP	Deoxyguanosine monophosphate
dIMP	Deoxyinosine monophosphate
dNKs	Deoxynucleoside kinases
dNT	Deoxynucleotidase
dNTP	Deoxynucleoside triphosphate
dTMP	Deoxythymidine monophosphate
dTTP	Deoxythymidine triphosphate
dUMP	Deoxyuridine monophosphate
Fara-AMP	9-β -D-arabinofuranosyl-2-fluroadenine monophosphate
FLT	3’- Fluorothymidine
GTP	Guanosine triphosphate
hCNT3	Human concentrative nucleoside transporter-3
hENT1	Human equilibrative nucleoside transporter-1
IMP	Inosine monophosphate
mt-DNA	Mitochondrial DNA
NAs	Nucleoside analogues
NRTIs	Nucleoside reverse- transcriptase inhibitors
RR	Ribonucleotide reductase
TK	Thymidine kinase
UTP	Uridine triphosphate

1 BACKGROUND

1.1 GENERAL INTRODUCTION

Nucleosides analogues are small molecules related chemically to physiological nucleosides that are widely employed in the treatment of several hematological malignancies, solid tumors and multiple sclerosis, as well as autoimmune disorders and HIV infection [1, 2]. The importance of nucleoside analogues as chemotherapeutic agents has increased in recent years as a result of the introduction of novel compounds into clinical use, as well as their more extensive application in the field of solid tumors. Many patients are cured with present treatments, but a large group develops resistance to therapy.

Primary and acquired drug resistance are the most serious obstacles to successful chemotherapy today. The mechanisms underlying such resistance are numerous and it is crucial to possess in-depth knowledge in order to propose new strategies for treating resistant cancer cells. Furthermore, the anti-cancer and anti-viral analogues available at present are not particularly selective, leading to unavoidable side-effects. The morbidity associated with these toxic effects often causes diagnostic problems, because it can involve every organ and system of the body and mimic the signs of the underlying disease.

At present, clinical pharmacology is attempting to replace empirical therapeutic approaches with mechanistic and targeted therapy, i.e., treatment designed for each particular individual. Research in molecular pharmacology and pharmacokinetics, together with pharmacogenetic and clinical trials all contribute to this process. No drug response should ever be designated as idiosyncratic. All responses have an underlying mechanism(s), the understanding of which will aid in development of further therapy with that drug or other alternatives [3].

1.2 PRINCIPLES OF CANCER TREATMENT

Over 1.3 million individuals in the United States alone are diagnosed with invasive cancer each year. Currently, 1 in 4 deaths in the United States is due to cancer, which thus ranks second after coronary disease as the leading cause of mortality in that country. Indeed, cancer is the leading cause of death among individuals of both sexes under the age of 65.

The primary goal of cancer treatment is to eliminate the malignant cells. If this cannot be achieved, the focus is switched to palliative therapy, designed to treat the symptoms and maintain quality of life for as long as possible. Current cancer therapies are of four major types: surgery, radiation therapy (including photodynamic therapy), chemotherapy (including hormonal and molecularly targeted therapy), and biological therapy (including immune and gene therapy). These approaches are frequently applied in combination and agents in each class work via different mechanisms. Surgery and radiation therapy are believed to be local treatments, although they can affect tumors at distant sites as well. Chemotherapy and biological therapy are generally considered to be systemic [3].

1.3 CHEMOTHERAPY

The aim of chemotherapeutic regimens is to distribute pharmacological compounds systemically in order to eliminate all tumor cells. The ideal cancer drug would kill tumor cells without damaging healthy tissues, but no such compound yet exists. The efficacy of chemotherapy thus depends on the fact that normal cells have a larger capacity for survival and repair than do many tumor cells.

In general, hematological malignancies are treated with chemotherapy and/or radiation, with surgery being employed mainly for diagnosis and staging. Conversely, with certain exceptions surgery is the primary treatment for non-hematologic malignancies. Chemotherapy is effective against microscopic tumor residues and micro-metastases, and thus adjuvant chemotherapy is frequently employed to improve the response to surgical resection.

Chemotherapeutic drugs are generally classified according to the phase of the cell cycle at which they exert their effects. Drugs whose effects are not pecifically related to the cell cycle are able to kill cells that are not dividing, whereas cell cycle-specific agents are toxic to proliferating cells only. Some drugs can do both.

Anti-metabolites are commonly cell cycle-specific, exerting their major toxicity during the S-phase of the cell cycle and consequently, this group of drugs is effective primarily towards rapidly proliferating tumors. As analogues of nucleic acid precursors, these compounds interfere with the synthesis of nucleic acids by inhibiting key enzymes. Anti-metabolites are used primarily for the treatment of hematological malignancies, but are also effective towards solid tumors [3, 4] (Figure 1).

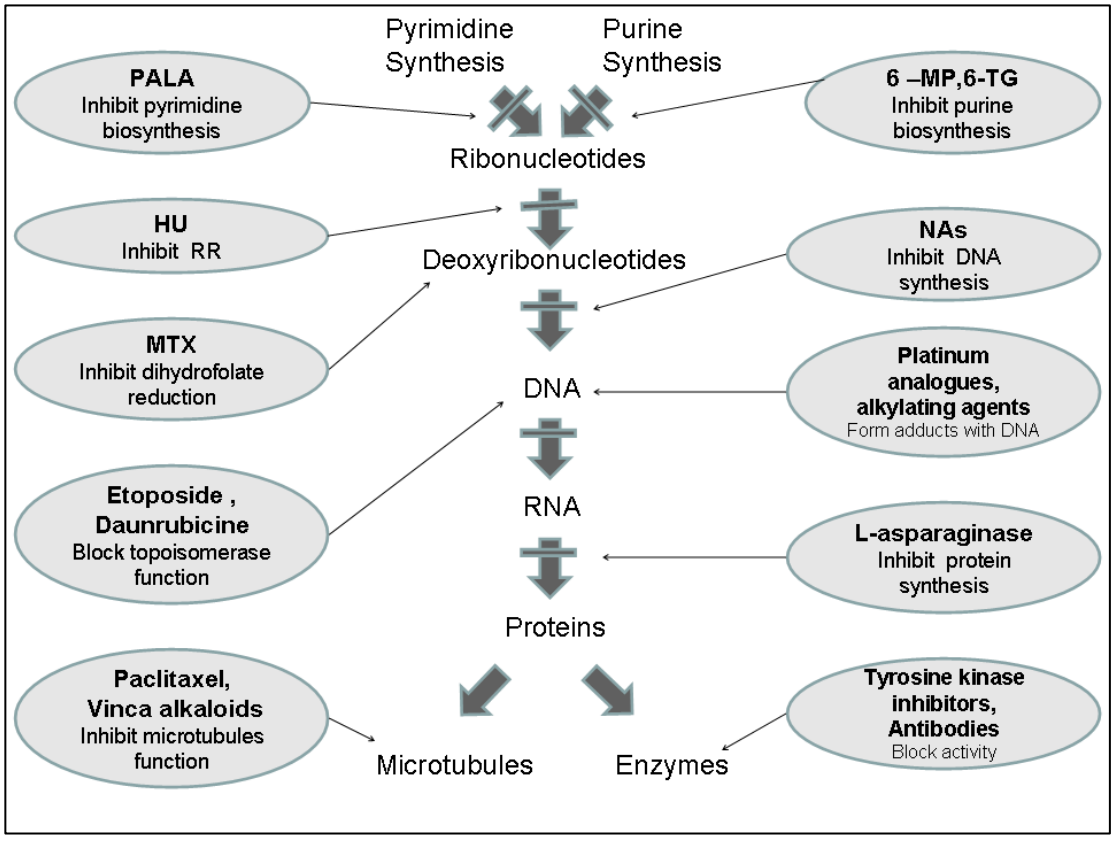


Figure 1 .Mechanisms of action of certain chemotherapeutic compounds used in cancer treatment. PALA: *N-phosphonoacetyl-L-aspartate;* ***6-MP:*** *6-mercaptopurine;* ***6-TG:*** *6-thioguanine;* ***MTX****: methotrexate;* ***HU:*** *hydroxyurea:* ***NAs****: nucleoside analogues.*

1.4 PRODUCTION OF DEOXYRIBONUCLEOTIDES FOR DNA SYNTHESIS

Several biosynthetic and catabolic pathways regulate the metabolism of purine and pyrimidine nucleotides. Deoxyribonucleotides required as building blocks for DNA synthesis and repair are derived either by *de novo* synthesis or from the salvage pathway. Different cell types and organisms rely on exogenous purines and pyrimidines to various extents [5]. In mammals the high rate of *de novo* synthesis in the liver produces excess purine nucleotides for salvage by other tissues [6]. In contrast, lack of such *de novo* synthesis in erythrocytes, interstitial epithelia and the bone marrow renders these tissues dependent on the salvage pathway [7]. The rate of purine synthesis can also depend on the state of the cells: whereas *de novo* purine synthesis is low in resting lymphocytes, its rate increases considerably after activation [8].

De novo synthesis of deoxyribonucleotides occurs by reduction of the corresponding ribonucleoside diphosphates by ribonucleotide reductase (RR) during the S-phase of the cell cycle [9, 10]. In the salvage pathway, deoxyribonucleosides derived from nutrients and DNA degradation are transported into the cell by specific transports [11-14]. Next, these nucleosides are phosphorylated to nucleoside monophosphates, and a step generally considered to be rate-limiting for the salvage pathway. In contrast to *de novo* synthesis, most of the salvage pathway enzymes are active throughout the cell cycle, providing dNTPs continuously for DNA repair and mitochondrial DNA (mt-DNA) replication. This salvage pathway is an interesting area of pharmacological research, since nucleoside analogues used in the treatment of viral infections and cancer are administered as prodrugs that are activated in cells by the salvage enzymes (Figure 2).

The 5’-phosphorylation of deoxyribonucleosides and their analogues in mammalian cells is mediated primarily by 4 deoxyribonucleoside kinases (dNKs): deoxycytidine kinases (dCK; EC2.7.1.74) and thymidine kinase-1 (TK1; EC 2.7.1.21) are localized in the cytosol, whereas deoxyguanosine kinase (dGK; EC 2.7.1.113) and thymidine kinase-2 (TK2; EC 2.7.1.21) are present in mitochondria [15]. In addition, certain other kinases, such as adenosine kinase (AK), can also catalyze the initial phosphorylation step of the salvage pathway [16].

Intracellular phosphorylation of deoxyribonucleoside monophosphates is reversed by a family of enzymes called 5’-nucleotidases (5’-NTs; EC2 3.1.3.5), which are the catabolic members of substrate cycles involved in regulating intra-cellular dNTP pools [17, 18]. Clearly, the relative activities of dNKs and 5′-dNTs are the main determinants of the size of dNTP pools both in the cytosol and mitochondria, and particularly in resting cells that carry out minimal *de novo* synthesis of DNA precursors [19, 20]. The nucleoside monophosphate is converted to the triphosphate in two reversible steps catalysed by members of the nucleoside monophosphate kinase (NMPK) and nucleoside diphosphate kinase (NDPK) families [21].

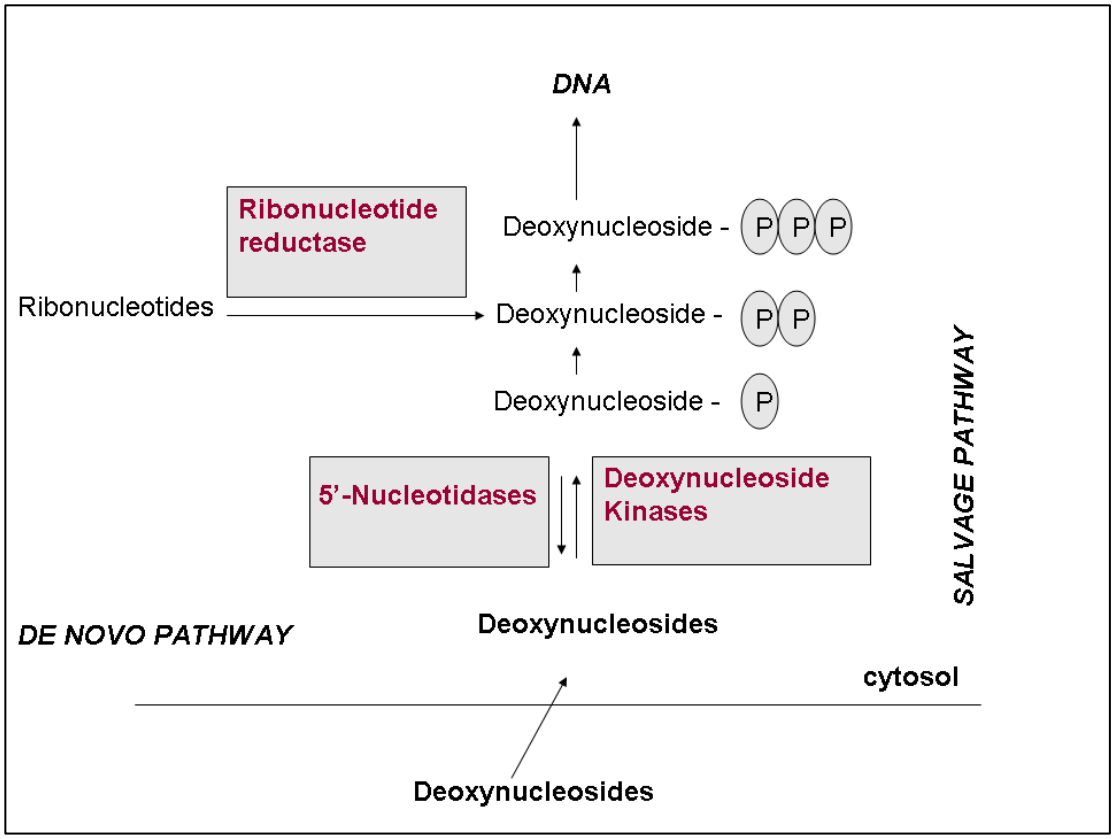


Figure. 2 The de novo and salvage pathways of deoxyribonucleotide synthesis.
In de novo synthesis, ribonucleotides are reduced to the corresponding deoxyribonucleotides by RR. In the salvage pathway, dNKs catalyze the first step of deoxynucleosides phosphorylation. The deoxyribonucleoside triphosphates are then incorporated to DNA. Intracellular phosphorylation of deoxyribonucleoside monophosphates is reversed by 5'-NTs.

1.5 NUCLEOSIDE ANALOGUES

Nucleoside analogues (NAs) were among the first chemotherapeutic agents to be used in cancer treatment. These compounds are anti-metabolites that compete with natural nucleosides and induce cytotoxic effects by interactions with a large number of intracellular targets. NAs are essential drugs in the treatment of hematological malignancies (cladribine, fludarabine, cytarabine and, more recently clofarabine), and solid tumors (gemcitabine), as well as acting as immunosuppressive and anti-viral agents. Their metabolism and mechanisms of action are directly related to those of the physiological purine and pyrimidine nucleosides [22, 23]. Since NAs are hydrophilic, their uptake occurs via nucleoside-specific membrane-based transport systems [24]. As pro-drugs, these analogues need to be activated in order to exert their subsequent cytotoxic effects [25].They inhibit synthesis of DNA, either by direct incorporation into nuclear DNA and inhibition of the synthesis of DNA precursors, which leads to chain termination, or by direct activation of the caspase cascade. Moreover, these compounds potentiate their own action by indirectly inhibiting RR, thus lowering the availability of endogenous nucleosides which compete with the effects of NA triphosphates on DNA synthesis [22, 23]. Although NAs have share common general characteristics, each drug is also involved in specific interactions, which explains the variation in their efficacies toward different kinds of disorders (Figure 3).

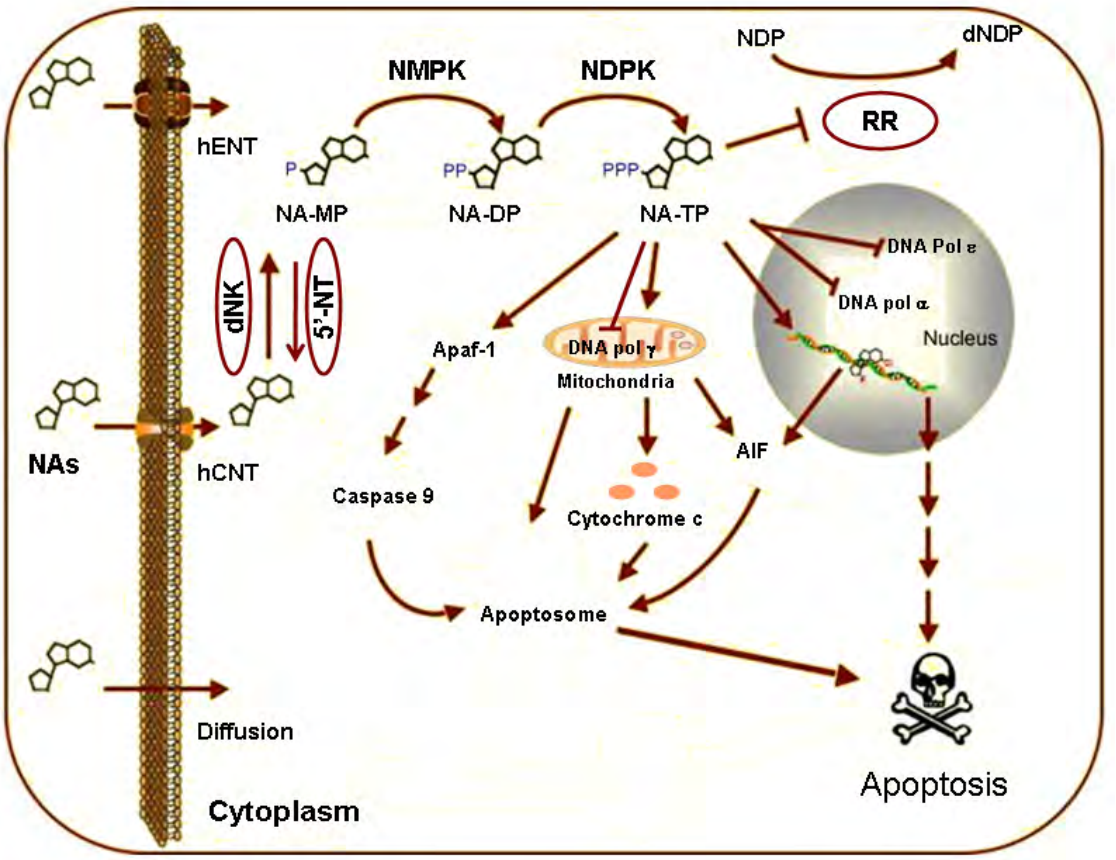


Figure 3. The metabolism and mechanisms of action of NAs.
NAs are transported into the cell either by active transport via specific transporters (including human concentrative and equilibrative nucleoside transports (hCNT and hENT)) or by passive diffusion. Once inside the cells, NAs undergo phosphorylation to mono-di- and activated triphosphate forms by the enzymes dNK, NMPK and NDPK, respectively. NA-triphosphates (NA-TP) can inhibit RR and reduce the size of dNTP pool, as well as inhibit DNA polymerase α, β in the nucleus and γ in mitochondria. Furthermore, NA-TP incorporation into DNA inhibits DNA synthesis and/or repair, which leads to apoptosis. By binding to APAF-1, NA-TPs can directly activate the caspase cascade. In addition, NA-TPs affect the mitochondrial transmembrane potential and cause the release of cytochorome c and apoptosis-inducing factor (AIF).

1.5.1 Purine nucleoside analogues

1.5.1.1 Deoxyadenosine base analogues

1.5.1.1.1 Cladribine (2-chloro-2'-deoxyadenosine, CdA)

CdA was first synthesized in 1972 through substitution of chlorine for the hydrogen atom at the 2' position on the adenine ring [26] (Figure 4). Its resistance to adenosine deaminase (ADA) allows this analogue to be used in the treatment of hairy cell leukemia and low-grade lymphoma, chronic lymphocytic leukemia (CLL), as well as auto-immune disorders [27]. Indeed, 80% of patients with hairy cell leukemia exhibit complete remission following one course of treatment with this compound [28]. CdA is also a secondary agent for treatment of Langerhans cell histiocytosis, cutaneous T-cell lymphomas, Waldensström's macroglobulinemia and myofibromatosis [29-31].

CdA enters the cell via specific transporters, where it undergoes phosphorylation by cytosolic dCK and also mitochondrial dGK [32]. This analogue is toxic to both dividing and quiescent cells [33]. In the case of resting cells, CdA inhibits DNA repair, leading to accumulation of strand breaks, and is eventually responsible for both p53-dependent and -independent apoptosis [34]. Inside dividing cells, CdATP inhibits DNA synthesis by incorporation into DNA, thereby terminating chain elongation by DNA polymerase and inducing S-phase-specific apoptosis. Moreover, this drug inhibits DNA replication by inhibiting indirectly RR through the reduction of dNTP pools. In addition, direct binding of the active drug to pro-apoptotic APAF-1, thereby activating caspase-3 and -9, is also proposed to occur [35-37].
CdA is absorbed moderately well, with a bioavailability of approximately 55% following oral administration [38, 39]. Routinely, this dug is administered intravenously at a continuous infusion rate of 5-10 mg/m^2 during 5-7 days. Its terminal half- life is 6.7 hours and excretion by the kidneys occurs dose-dependently at a rate of 3.5-10.5 mg/m^2/day [39-41]. 30-50% of the dose administered is recovered unchanged in the urine after 1 day and clearance is complete 1-3 days after termination of infusion [40, 42, 43]. CdA crosses the blood-brain barrier, to give a concentration in the cerebrospinal fluid that is 25% of that in the plasma, and it has been shown that children with acute myeloid leukemia (AML) involving the central nervous system benefit from CdA treatment [44]. Myelosuppression, the major dose-limiting toxicity of CdA, is associated with severe opportunistic infections [43].

1.5.1.1.2 Fludarabine (9-B-D-arabinofuranosyl-2-fluroadenine monophosphate, Fara-AMP)

In order to obtain a drug more effective than the antiviral agent vidarabine (adenine arabinoside, araA), which is rapidly deaminated by ADA, the fluorinated analogue, Fara-A was synthesized [45] (Figure 4). Fara-A is poorly soluble in water and is therefore administered clinically as the corresponding 5'- monophosphate. Following rapid dephosphorylation by membrane ecto- 5'-NTs, Fara-A is transported into the cell via a specific transporter, where, like the other NAs, it undergoes phosphorylaion to its active triphosphate form. dCK is responsible for the first step in this activation [46].
Like CdA, Fara-A is cytotoxic towards both dividing and resting cells and these two drugs share very similar mechanisms of action. In addition, Fara-A is incorporated into RNA, thereby inhibiting RNA function and processing and mRNA translation [47]. Moreover, this drug is able to activate the apoptosome pathway more efficiently than other deoxyadenosine analogues [48].
Fara-A is especially effective towards CLL, but is also active towards other indolent lymphoid disorders [49]. In combination with high doses of alkylators, Fara-A has been reported to be a potent immunosuppressive agent used in stem cell transplantation [50]. This drug is administered intravenously, with the recommended dose being 20-30 mg/ m^2 daily for 5 consecutive days. The half-life of this drug is approximately 10 hours and it is eliminated by renal excretion at a clearance rate of 73.53 ml/min/m^2 [51]. The major adverse effects of Fara-A treatment are mylosuppression, opportunistic infections and neurotoxicity [52].

1.5.1.1.3 Clofarabine (2-chloro-2'arabino-fluro-2'deoxyadenosine, CAFdA)

In the early 1990s, CAFdA, a potent second-generation purine nucleoside analogue, was synthesized in attempt to overcome the limitations of both Fara-A and CdA (Figure 4). Following a decade of successful preclinical studies and clinical trials, in 2004 CAFdA (Clolar ™, Genzyme) was approved by the FDA for treatment of pediatric refractory/relapsed acute lymphocytic leukemia (ALL) after at least two other regimens have been tried. In 2006 it was also approved by the European Commission (Clolar™). Placement of a fluorine atom at the 2'- arabino position of CdA increases drug stability at acid pH and decreases susceptibility to cleavage by nucleoside phosphorylases expressed by gastrointestinal *E.coli*, both of which features enhance oral bioavailability [53, 54]. Like the other nucleoside analogues, CAFdA enters cells through specific transporters, in particular hCNT3, and thereafter undergoes phosphorylation to its monophosphate form, catalyzed primarily by cytosolic dCK and also by mitochondrial dGK [55, 56]. CAFdA is more efficiently phosphorylated by purified recombinant dCK than are either Fara-A or CdA. CAFdAMP accumulates in cells at high levels and intracellular retention of CAFdATP is more prolonged than in the case of Fara-A and CdA [54, 57, 58]. CAFdA is believed to act through incorporation into DNA, inhibition of RR (a self-potentiation mechanism), extensive inhibition of DNA polymerases α and ε, and direct induction of apoptosis [58-60].

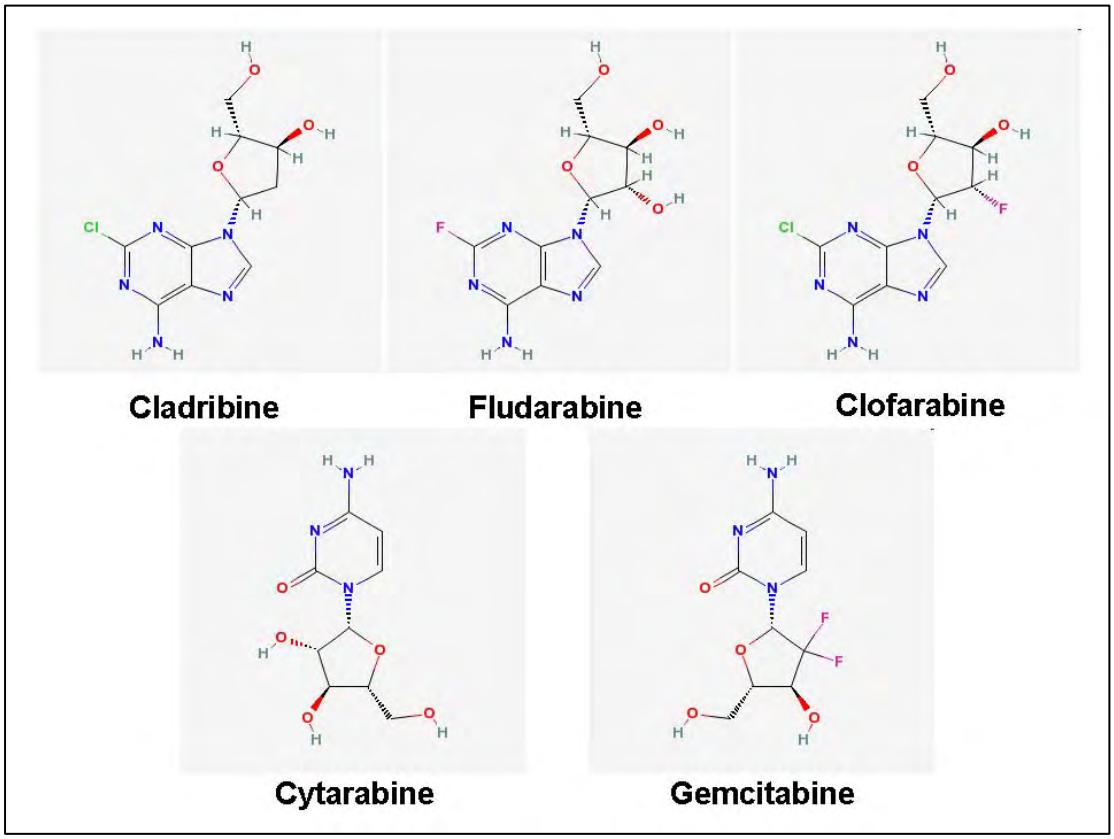


Figure 4. The chemical structures of purine and pyrimidine analogues discussed in this thesis.

1.5.2 Pyrimidine nucleoside analogues

1.5.2.1 Deoxycytidine analogues

1.5.2.1.1 Cytarabine (Cytosine arabinoside, Ara-C)

This cytidine analogue was first synthesized in1950 and introduced into clinical practice in 1963 [61]. It differs from deoxycytidine by the substitution on the hydroxyl group in position 2 of the sugar moiety (Figure 4). Ara-C is the most efficient single agent for treatment of AML. This drug is also active towards ALL and, to a lesser extent, chronic myeloid leukemia (CML), but has no effect on solid tumors.
Intracellular levels of Ara-C depend on its plasma concentration [62]. At a plasma concentration of 0.5-1 μM,which is achieved after conventional doses (100-200 mg/m^2), the level of expression of nucleoside transporters (hENT1) is rate-limiting for uptake of this drug into cells. At concentrations of 50 μM or more, such as those reached with high-dose treatment (2000-3000 mg/m^2), passive diffusion become more important [63, 64].
Inside the cell, Ara-C undergoes phosphorylation to become active, the initial step being catalyzed by dCK. The drug itself is also rapidly deaminated by cytidine deaminase to the inactive metabolite Ara-U, and dephosphorylation of Ara-CMP is regulated by 5'-NTs. Incorporation of Ara-CTP into DNA leads to chain termination and blockage of prolongation and this drug also inhibits DNA polymerase and RR activities [65, 66]. The main side-effect of conventional Ara-C therapy is myelosuppression, while high-dose therapy is also associated with pericarditis and neurotoxicity [67].

1.5.2.1.2 Gemcitabine (2', 2'difluorodeoxycytidine, dFdC)

dFdC (Figure 4), a diflouro analogue of deoxycytidine first synthesized in 1986, is an effective antiviral agent because of its inhibitory activity in both DNA and RNA viruses [68]. In 1996 this drug was approved by the FDA for first-line treatment of high-grade pancreatic adenocarcinoma. dFdC is also an important agent for treatment of cancer of the head and neck, esophagus, ovary, and bladder, as well as non-small cell lung cancer [69].
Although the anabolism and effects of dFdC are generally similar to those of Ara-C, there are some important differences. dFdC is more lipophilic, a better ligand for membrane transporters, and a more efficient substrate for dCK. These characteristics result in more accumulation and longer maintenance of the active drug in cells and, consequently, more cytotoxicity [70].
After entering the cell via specific transporters, dFdC is initially phosphorylated by dCK and then undergoes further phosphrylation to its active triphosphate form. Catabolism of this drug is mediated by cytidine deaminase and 5'-NTs [70, 71]. The active drug is incorporated into DNA, following which one more natural nucleotide is added, a process which hides the dFdCTP from DNA repair enzymes [72]. dFdC is also an effective inhibitor of RR and the *de novo* synthesis pathway. Reduced intracellular levels of dCTP result in increased incorporation of dFdCTP into DNA and, at the same time, attenuated clearance by dCMP deaminase [73, 74]. Routinely, dFdC is infused intravenously for 30 minutes at a dose of 1-1.2 g/m^2/day for three weeks and its main side-effect is myelosuppression.

1.6 METABOLISM OF NUCLEOSIDE ANALOGUES

1.6.1 Anabolic enzymes

1.6.1.1.1 Deoxyribonucleoside kinases

This family of enzymes catalyzes the first step of deoxyribonucleoside phosphorylation, an essential reaction in the biosynthesis of DNA precursors via the salvage pathway (Figure 5). These key enzymes also carry out the initial step in the conversion of the non-toxic nucleoside analogues prodrugs to the corresponding toxic nucleoside triphosphates drugs effective in anti-viral and anti-cancer treatment [75].

1.6.1.1.2 Deoxycytidine kinase (dCK)

The human dCK gene, located on chromosome 4q13.3-q21.1, has been cloned and found to encode a 30.5-kDa protein containing 261 amino acids. dCK (EC 2.7.1.74) phosphorylates purine and pyrimidine deoxyribonucleosides employing ATP or UTP as the phosphate donor [25, 76, 77]. This enzyme is expressed to some extent throughout the cell cycle and the expression of its mRNA is tissue-specific, being very low in differentiated tissues, at intermediate levels in proliferating cells, and particularly high in lymphoid cells [15, 76, 78]. Regulation of this enzyme is primarily post-translational [79]. Although endogenous dCK is a cytoplasmic enzyme, it can be translocated into the nucleus when over-expressed [80, 81]. dCK has a broad substrate specificity, encompassing both natural substrates and NAs, being most efficient towards deoxycytidine (dCyt), followed by deoxyadenosine (dAdo) and deoxyguanosine (dGuo). Moreover, several anti-cancer and anti-viral NAs are phosphorylated efficiently by dCK, including CdA, Fara-A, dFdC, CAFdA, zalcitabine (ddc) and lamivudine (3TC) [15, 82-85].

1.6.1.1.3 Thymidine kinase-1 (TK1)

TK1 (EC 2.7.1.21) is a key enzyme of the pyrimidine salvage pathway in a variety of organisms. The human TK1 gene, found on chromosome 17q25.2-25.3 and encoding a 25.5-kDa protein containing 234 amino acids, was first cloned and sequenced by Flemington and coworkers [86, 87]. In contrast to other kinases, TK1 has a narrow substrate specifity, phosphorylating only deoxythymidine (dThd) and deoxyuridine (dUrd) using (d) ATP as the phosphate donor [88, 89]. Expression of TK1 is regulated strictly during the cell cycle, with little or no activity in resting cells, appearance in late G1phase, increase during the S-phase and disappearance during mitosis [90-92]. TK1 is expressed at high levels in leukemia cell lines, as well as in numerous malignant tumors [93]. Although regulation of TK1 is primarily transcriptional, post-transcriptional mechanisms also play an important role [94, 95]. TK1 also phosphorylates several clinically important anti-viral nucleoside analogues, including zidovudine (AZT), stavudin (d4t) and floxuridine [96, 97].

1.6.1.1.4 Thymidine kinase-2 (TK2)

TK2 (EC 2.7.1.21) is a mitochondrial enzyme involved in mt-DNA precursor synthesis via the pyrimidine deoxynucleoside salvage pathway. The human TK2 gene is located on chromosome 16q22. In contrast to TK1, TK2 is expressed constitutively at low levels in all tissues and all stages of the cell cycle, being the only pyrimidine deoxyribonucleoside kinase in non-proliferating cells [98, 99]. This enzyme

phosphorylates a broad range of natural nucleosides (dCyt, thymidine (Thd) and dUrd), as well as anti-viral and anti-cancer NAs (AZT, 3'-fluorothymidine, Ara-C and dFdC), using ATP and CTP as the phosphate donor [99, 100]. Mutation of the TK2 gene causes a myophatic form of the mitochondrial DNA depletion syndrome [101].

1.6.1.1.5 Deoxyguanosine kinase (dGK)

The mitochondrial enzyme dGK (EC 2.7.1.113) catalyzes phosphorylation of purine nucleosides and their analogues. The human gene is located on chromosome 2q13 and constitutively expressed in all tissues [102, 103]. The dGK protein is a dimer of 30-kDa subunits, each of which contains 277 amino acids [104]. In addition to phosphorylating the natural purines dGuo, dAdo and deoxyinosine (dIno) with different K_m values, dGK also phosphorylates anti-cancer and anti-viral NAs, e.g., Ara-G, CdA, CAFdA, and dFdC, as well as to some extent, Fara-A, ganciclovir and pencyclovir. Ara-G is a very good substrate for dGK, even better than dGuo [32, 56]. Although dGK is a mitochondrial enzyme, it can be relocated from mitochondria to the cytosol during apoptosis [105]. Since dCK has such high activity, it is mainly responsible for the activation of many NAs in the cells in which it is expressed. On the other hand, in tissues with no dCK activity, dGK is the primary activator of purine NAs.

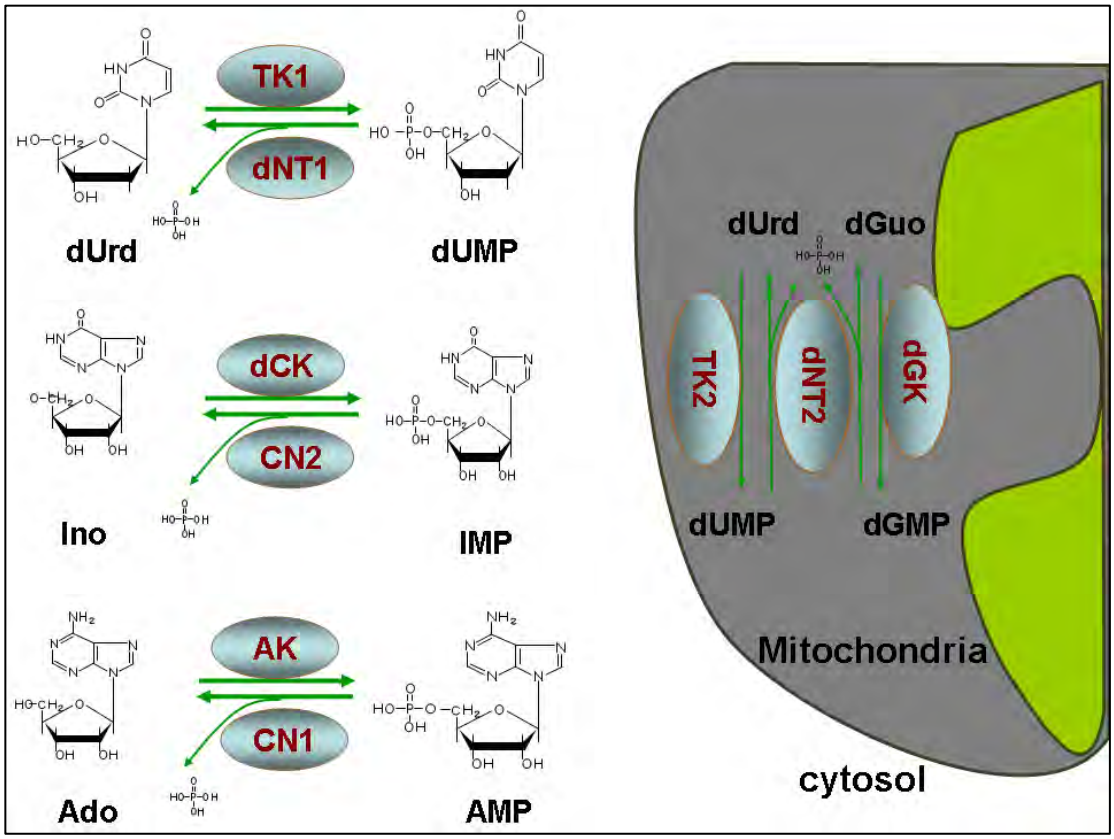


Figure 5. The primary substrates and reactions catalyzed by different dNKs and 5'-NTs.
***dUrd**: deoxyuridine; **Ino**: inosine; **Ado**: adenosine ; **dGuo** : deoxyguanosine ;**dUMP** : deoxyuridine monophosphate ; **IMP** : inosine monophosphate ; **AMP** : adenosine monophoshate ; **dGMP** : deoxyguanosine monophosphate.*

1.6.2 Catabolic enzymes

1.6.2.1 5'-Nucleotidases

While the nucleoside kinases responsible for the initial phosphorylation of salvaged nucleosides have been well characterized, many of the catabolic nucleotidases have only recently been cloned and characterized and further characterization is required.
To date, at least 5 human nucleotidases have been isolated and shown to vary with regards to sub-cellular localization, substrate specificity and tissue distribution. CN1, CN2 and dNT1 are localized in the cytosol, whereas dNT2 is present in the mitochondria and as an ecto-enzyme on the plasma membrane. 5´-NTs catalyze the dephosphorylation of ribo- and deoxyribonucleoside monophosphates by hydrolyzing the bond between the phosphate group and carbon- 5' of ribose or deoxyribose (Figure 5). The different properties of the members of the 5'-NT enzyme family are most likely key factors in adjusting nucleotide pools to the metabolic needs of each particular tissue or type of cell [106]. Earlier investigations have revealed considerable overlap in the substrate specificities of these enzymes and their role in the metabolism of both natural nucleotides and their analogues is still under investigation [107, 108].

1.6.2.1.1 Ecto- 5'-nucleotidase

The gene for this human ecto-enzyme is on chromosome 6q14-q21 [109] and its coding region is 1725-bp long, which translates into a protein containing 574 amino acids [110]. The ecto-protein a dimer of 71-kDa subunits, is attached to the extracellular surface of the plasma membrane by a glycosyl phosphatidylinositol anchor [111]. Ecto-5´-NT is expressed in many tissues and regulated primarily at the level of transcription [112]. Although it has a broad substrate specificity, AMP is considered to be the most efficient physiological substrate and ribonucleotides are better substrates than deoxyribonucleotides. This ecto-activity is inhibited potently by α-β-methylene-ADP, but also by ADP and ATP [113]. In addition to its enzymatic activity, this protein has several other functions, such as acting as a co-receptor in connection with T-cell activation and as a cell adhesion molecule [114, 115]. Since ecto-5´-NT activity is low in undifferentiated hematopoietic cells and increases during cell maturation, this activity is low in connection with ALL and elevated ecto-activity is associated with a poor prognosis [116, 117].

1.6.2.1.2 Cytosolic 5'-nucleotidase-1 (CN1)

The human CN1-A gene, located on chromosome 1p34.3-p33, encodes a tetramer of 41-kDa subunits, which was initially described as AMP-specific and demonstrates a preference for AMP and pyrimidine deoxyribonucleotides. ADP and, to a lesser extent, GTP activate CN1-A [118]. This protein is expressed at high levels in skeletal and heart muscle, where it plays a physiological role in the intracellular formation of adenosine during ischemia and hypoxia [119]. Furthermore, this enzyme may be important in regulating the size of deoxyribonucleotide pools in tissues where it is expressed.
CN1-B is encoded on chromosome 2p24.3 and is expressed ubiquitously, with highest mRNA levels being detected in the testis and lowest in skeletal muscle and the brain. Even though the substrate specificity of this enzyme is not completely known, it appears to be similar to CN1-A. The use of AMP as a substrate in the presence of ADP can not be utilized to distinguish between the activities of these two homologues in tissues or cell lysates [120].

1.6.2.1.3 Cytosolic 5'-nucleotidase-2 (CN2)

Cytosolic 5'- nucleotidase, which prefers IMP/GMP as substrates was first described by Itoh and colleagues in 1967. Its gene is located on chromosome 10q24.32 [121] and encodes a tetramer of 65-kDa subunits, each with 561 amino acids [122]. CN2 is expressed ubiquitously in human tissues, at highest levels in the heart, skeletal muscle and pancreas. At the same time, high activity has been detected in the testis, spleen and lymphoblastiod cells, which all have rapid turnover of nucleic acids or DNA synthesis, and low activity is present in skeletal muscle and erythrocytes [123, 124]. CN2 prefers 6-hydroxypurine nucleoside monophosphates, (d) IMP, as substrates and is most active towards (d) ATP and GTP, being an important regulator of the pools of these latter two nucleotides [125]. Besides acting as a nucleotidase, CN2 can catalyze phosphotransfer from a purine nucleoside monophosphate donor to inosine and also guanosine [126, 127]. It has been proposed that under physiological conditions CN2 acts primarily as a phosphotransferase and several anti-cancer and anti-viral analogues that are not substrates for cellular nucleoside kinases are activated via this reaction [128, 129].

1.6.2.1.4 Cytosolic 5' (3') – deoxyribonucleotidase-1 (dNT1)

The dNT1 gene is located on chromosome 17q25.321-23, encodes a dimer of 23.4 kDa subunits and is ubiquitously expressed in human tissues, with high mRNA levels being detected in the pancreas, heart and skeletal muscle [130]. Although this enzyme is highly active with uracil and thymidine monophosphates, it also dephosphorylates other deoxyribonucleotides, in particular dGMP and dIMP [131, 132]. In combination with nucleoside kinases, dNT1 appears to form substrate cycles designed to regulate deoxynucleotide levels in cells. Over-expression of murine dNT1 in human 293 cells leads to secretion of dCyt, dThd and dUrd from these cells [18].

1.6.2.1.5 Mitochondrial 5' (3') – deoxyribonucleotidase-2 (dNT2)

The dNT2 gene is located on chromosome 17p11.2 and has the same structure as the dNT1 gene. The human dNT2 enzyme is a dimer of 26-kDa subunits which are shortened to 23-kDa by removal of the mitochondrial import sequence. Expression of its mRNA is highest in the brain, heart and skeletal muscles and much lower in the kidney and pancreas.
dNT2 has a narrow substrate specificity for dUMP and dTMP, suggesting that it protects the mitochondria from excessive levels of dTTP, which can be mutagenic during mt-DNA replication [130]. Over-expression of dNT2 in 293 cells did not enhance their secretion of dCyt, dThd and dUrd, indicating that this enzyme does not participate in regulating cytosolic deoxynucleotide pools [133]. In non-cycling cells with minimal *de novo* synthesis, the dNT2/TK2 substrate cycle is important for regulation of dTTP pools [20].

Table 1: Deoxynucleoside kinases and 5'-nucleotidases

Name Abbreviation	Chromosomal location	Subunit size (KDa)	Substrates Natural	Analogues
dCK	4q13.3-q21.1	30.5	dCyt, dAdo, dGuo	CdA, Fara-A, dFdC, CAFdA ddC, 3TC
TK1	17q25.2-25.3	25.5	dThd, dUrd	AZT, D4T
TK2	16q22	29	dCyt, Thd, dUrd	AZT, FLT, Ara-C, dFdC
dGK	2q13	30	dGuo, dAdo, dIno	Ara-G, CdA, dFdC, CAFdA
Ecto	6q14-q21	71	AMP, GMP, IMP, UMP	Fara-A
CN1-A	1p34.3-p33	41	AMP and pyrimidine deoxyribonucleotides	CdAMP, AZTMP
CN2	10q24.32	65	(d) IMP: (6 hydroxy purin)	CdAMP, AraG-MP, AZTMP ddCMP
dNT1	17q25.321-23	23.4	dUMP, dTMP	AZTMP, d4TMP,3TCMP ddCMP dFdCMP, AraG-MP
dNT2	17p11.2	26	dUMP, dTMP	AZTMP, d4TMP

AZT, *Zidovudine;* ***d4t,*** *Stavudine;* ***3TC,*** *Lamivudine;* ***ddc,*** *Zalcitabine;* ***FLT,*** *3'-fluorothymidine.*

1.7 MECHANISMS OF RESISTANCE TO NUCLEOSIDE ANALOGUES

Resistance to nucleoside analogues during the course of treatment is a common clinical problem. Although both clinical and *in vitro* studies suggest that elevated nucleotidase activity can attenuate nucleoside analogue activation and result in drug resistance, the mechanisms underlying such resistance are not yet fully understood. Possible mechanisms include an inadequate concentration of the active form of the drugs, due to inefficient uptake via membrane transporters, a decrease in kinase activity, an increase in catabolism (either by dephosphorylation via 5'-NTs or deaamination by specific deaminases) and/or enhanced efflux via membrane pumps. Other possibilities are a lack of RR inhibition, altered affinity for DNA polymerase, or attenuated induction of apoptosis [22, 134].

1.7.1 Deoxynucleoside kinases

1.7.1.1 dCK and resistance to NAs

dCK carries out the rate-limiting first step of phosphorylation of many NAs to their active monophosphate forms [82, 83, 135]. There is no doubt that as the main enzyme for the activation of NAs, dCK deficiency plays an important role in resistance to these drugs. In several different cell lines reduction in this activity has been correlated to NA resistance [136-140]. In addition, dCK deficiency in murine and human cell lines is associated with resistance to dFdC [141-143]. Our group has reported that dCK deficiency at all levels i.e., mRNA and protein expression and enzyme activity is exhibited by CdA- and CAFdA-resistant CEM cell lines [144]. Other *in vitro* studies have demonstrated that reduced dCK activity is an underlying mechanism for cross-resistance to CdA, dFdC, Fara-A and Ara-C [138, 145]. Furthermore, transfection of the dCK gene into dCK-deficient cancer cell lines restores sensitivity toward CdA and Ara-C [146, 147].

From the clinical point of view, the correlation between dCK activity and treatment outcome is somewhat controversial. Some studies have indicated a relationship between poor outcome and reduced levels of dCK in patients with acute and chronic leukemia [148-152], but others have found no such significant correlation [153, 154]. Moreover, a high level of dCK mRNA was correlated with slower disease progression in a CLL patient receiving Fara-AMP [155].

1.7.2 5'- Nucleotidase

1.7.2.1 Ecto 5'-nucleotidase and resistance to NAs

Since NAs are phosphorylated primarily in the cytosol, the location of this enzyme on the plasma membrane makes its unlikely to play a direct role in resistance to NAs. However, this ecto-enzyme does carry out the initial step in activation of Fara-A, which is administered as Fara-A-monophosphate and must be dephosphorylated prior to cellular uptake [156]. Furthermore, ecto-5´-NT expression has been reported to be correlated to the expression of multidrug resistance protein in resistant cells [157].

1.7.2.2 CN1 and resistance to NAs

CN1-A exhibits a high affinity for deoxyadenosine analogues [118, 158] and CdAMP is a substrate for this enzyme, even though it exhibits no activity with Fara-AMP or Ara-CMP [118]. Over expression of CN1-A in HEK 293 and Jurkat cells leads to resistance to CdA and dFdC and to CdA and zalcitabine, respectively [118]. Another study demonstrated that transfection of CN1 into murine fibroblasts renders these cells resistant to fluorouracil (5-FU) and CdA [159]. The role of CN1-B in resistance to NAs has not yet been examined.

1.7.2.3 CN2 and resistance to NAs

CN2 is the most extensively studied 5'-NTs with respect to resistance to NAs. The purified recombinant human enzyme dephosphorylates several anti-cancer and anti-viral NAs, including CdAMP, Ara-GMP, AZTMP and ddCMP, although all at a much lower rate than IMP [132]. Fara-AMP is also dephosphorylated by CN2 [160].
More recently, of two Ara-C-resistant cell lines, the one with a low level of dCK and high level of CN2 was found to be less sensitive to Fara-A than the other with only a low level of dCK [161]. Increased CN2 activity has also been reported in both an etoposide-resistant CEM cell line, with cross-resistance to Ara-C and CdA, and in a CdA-resistance HL60 cell line [162, 163]. A series of Ara-C-, CdA-, dFdC- and Fara-A-resistant K562 cells demonstrated elevated CN2 activity and also low levels of dCK [138]. Nevertheless, over-expression of CN2 in human 293 cells did not result in resistance to dGuo, dAdo or CdA [124, 164].
Several studies have confirmed that elevated levels of CN2 mRNA in patients with AML are correlated with poor clinical prognosis [165, 166]. Furthermore, among patients with CLL or hairy cell leukemia, non-responders to CdA had higher activity and /or lower dCK activity than responders [150]. In addition, one recent study reported that the outcome of patients with high-risk myelodysplastic syndrome receiving Ara-C was correlated to their level of CN2 mRNA [167]. At the same time, an elevated level of CN2 in patients with non-small cell lung cancer being treated with dFdC was correlated with better prognosis [168].

1.7.2.4 dNT1 and resistance to NAs

With its preference for deoxynucleotide monophosphates, dNT1 is a good candidate for mediatig resistance to NAs [130]. The purified recombinant human enzyme dephosphorylates several NAs, including AZTMP, d4TMP, dFdCMP,3TCMP, ddCMP and Ara-GMP, although all at much lower rates than the natural substrates [132]. Surprisingly, it has been reported that low levels of dNT1 mRNA in the peripheral blood at the time of diagnosis were correlated with shorter overall survival in leukemic patients treated with Ara-C. This observation could be the result of high levels of dCTP, which reduce Ara-CTP incorporation into DNA and also decrease Ara-C phosphorylation by inhibiting dCK [169].

1.7.2.5 dNT2 and resistance to NAs

Mitochondrial doxyribonucleotidase (dNT2) appears unlikely to play a role in resistance to NAs, since most of the analogues are designed to target nuclear DNA. However treatment with inhibitors of nucleoside reverse transcriptase (NRTIs), such as zidovudine and stavudine, is associated with a number of metabolic complications in which depletion of mt-DNA is believed to play a major role [170, 171]. Dephosphorylation of these analogues by mitochondrial dNT2 is supposed to reduce these side-effects without altering drug efficiency toward viral infections, since cytosol activation of the analogues should be undisturbed.

Table 2: Clinical studies on levels of dNK and 5'NT and responses to NAs.

Disease	Treatment	dNKs/5NTs	Results/conclusions	Reference
HCL, CLL	CdA	dCK/CN2	Higher dCK and lower CN2 activites in responders	[150]
CLL	CdA	dCK	No correlation	[153]
AML	AraC	dCK/CN2	Lower dCK and higher CN2 mRNA levels reduce the DFS	[172]
CLL	FaraA	dCK/dNT1	Lower dCK and dNT1 mRNA levels increase the risk for disease progression	[155]
Pancreas cancer	dFdC	dCK	A lower level of dCK protein decreases the OS	[173]
AML	AraC	CN2	Higher CN2 mRNA levels reduce the DFS	[165]
AML	AraC	Cytosolic 5'NT	A higher level of cytosolic 5'NT decreases DFS and OS	[166]
AML	AraC	CN2, CN3	Higher CN2 and lower CN3 mRNA levels decrease the OS	[174]
NSCLC	dFdC	CN2	A higher level of CN2 protein increases the OS	[168]
Myelodysplastic syndrome	AraC	CN2	Higher CN2 mRNA levels decrease the OS	[167]
AML	AraC	dNT1	Higher dNT1 mRNA levels increase the OS	[169]

***HCl**: Hairy cell leukemia; **NSCLC**: Non-small cell lung cancer; **DFS**: Disease-free survival; **OS**: Overall survival.*

1.8 RIBONUCLEOTIDE REDUCTASE

Ribonucleoside reductase (RR) is the key enzyme in the *de novo* pathway of DNA synthesis [175]. Human RR consists of two proteins, R1 and R2, with different activities. The active enzyme most likly contains six R1 and two R2 subunits [176]. The R1 protein contains both the substrate binding site and site for allosteric regulation, whereas the smaller R2 protein contains an iron center and tyrosine residues required for the reducing activity. Expression of RR is regulated in a cell cycle-dependent fashion [177].

Transfection of human pancreas adenocarcinoma cells with R2 results in elevated RR activity and dFdC resistance [178, 179]. Another protein referred to as p53-induced R2 protein (p53R2) , together with R1, is part of the active RR complexin non-proliferating cells [180]. RR is also involved in cellular mechanisms other than the synthesis of deoxyribonucleotides. The R1 protein has been reported to possess tumor suppressor properties both *in vivo* and *in vitro*. On the other hand, together with other oncogene products, R2 plays a role in tumor progression [181-183].

1.8.1 RR and resistance to NAs

RR synthesizes large amounts of dNTPs, which are used in DNA replication, a process that efficiently prevents NA from being incorporated into DNA. Moreover, dNTP pools may also reduce NAs activation by decreasing the activity of dCK [184]. Therefore, drugs which inhibit RR and thereby reduce pools of normal dNTPs might potentiate the cytotoxicity of various NAs.

Several NAs employed clinically interact with RR to alter levels of dNTP [37, 185]. While Fara-A, dFdC and CdA all inhibit RR, a combination of one these drugs with another analogue, usually Ara-C, appears to produce therapeutic synergism. Several *in vitro* and clinical studies have demonstrated the success of this strategy [186-193]. Another inhibitor of RR significantly enhances the cytotoxicity of Ara-C [194, 195].

Because of its important role in determining responses to NAs, elevated RR activity may contribute to resistance. Expression of the R1 and R2 subunits is up-regulated in several cell lines resistant to NAs. In a dFdC-resistant cell line, R1 is over-expressed [196-199] and there are also indications of increased RR activity in Fara-A-resistant cell lines [138, 144].

1.9 INHIBITORS OF NUCLEOSIDE REVERSE TRANSCRIPTASE (NRTIS)

The DNA polymerase of the HIV virus, also known as reverse transcriptase, converts viral RNA into DNA that is subsequently incorporated into the host cell`s DNA. Both NAs and other compounds that inhibit this enzyme are available. The selective toxicity of these drugs relies on their ability to inhibit the reverse transcriptase without inhibiting the host DNA polymerase. Even though the intracellular triphosphate forms of these drugs exhibit low affinity for human DNA polymerase α and β, some do inhibit human mt-DNA polymerase γ, resulting in mitochondrial toxicity, one of the important forms of toxicity common to this category of drugs [200, 201] (Figure 6).

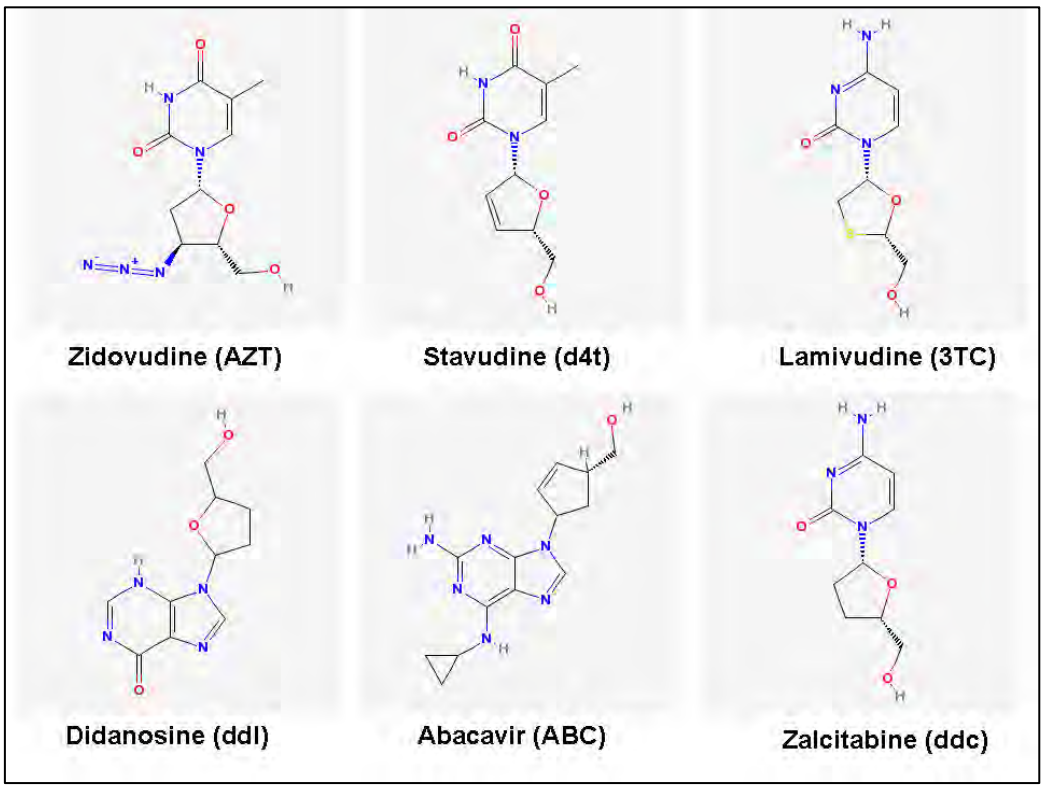


Figure 6. Chemical structures of NRTIs approved for clinical use.

1.10 MITOCHONDRIAL TOXICITY AND THE MITOCHONDRIAL DNA DEPLETION SYNDROME

Treatment with NRTIs is associated with a number of metabolic complications, including myopathy, peripheral neuropathy, cardiomyopathy, hepatotoxicity, lactic acidosis and the lipodystrophy syndrome [170, 202]. It is still not known why the toxicity of these drugs specifically involves skeletal and heart muscles, liver, adipose tissue and peripheral nerves, but this may reflect the pattern of enzyme expression in these tissues and their dependence on mitochondrial function. The mitochondrial toxicity of NRTIs is believed to play a major role in these side-effects, resulting in depletion of mt-DNA and reduced oxidative phosphorylation [171, 203, 204] (Figure 7). Several of these same tissues can be affected by the inherited mt-DNA depletion syndrome caused by mutations in the mitochondrial kinases TK2 and dGK. Mutations in TK2 result in mt-DNA depletion primarily in muscles, while mutations in dGK affect predominantly the liver and brain [101, 205].

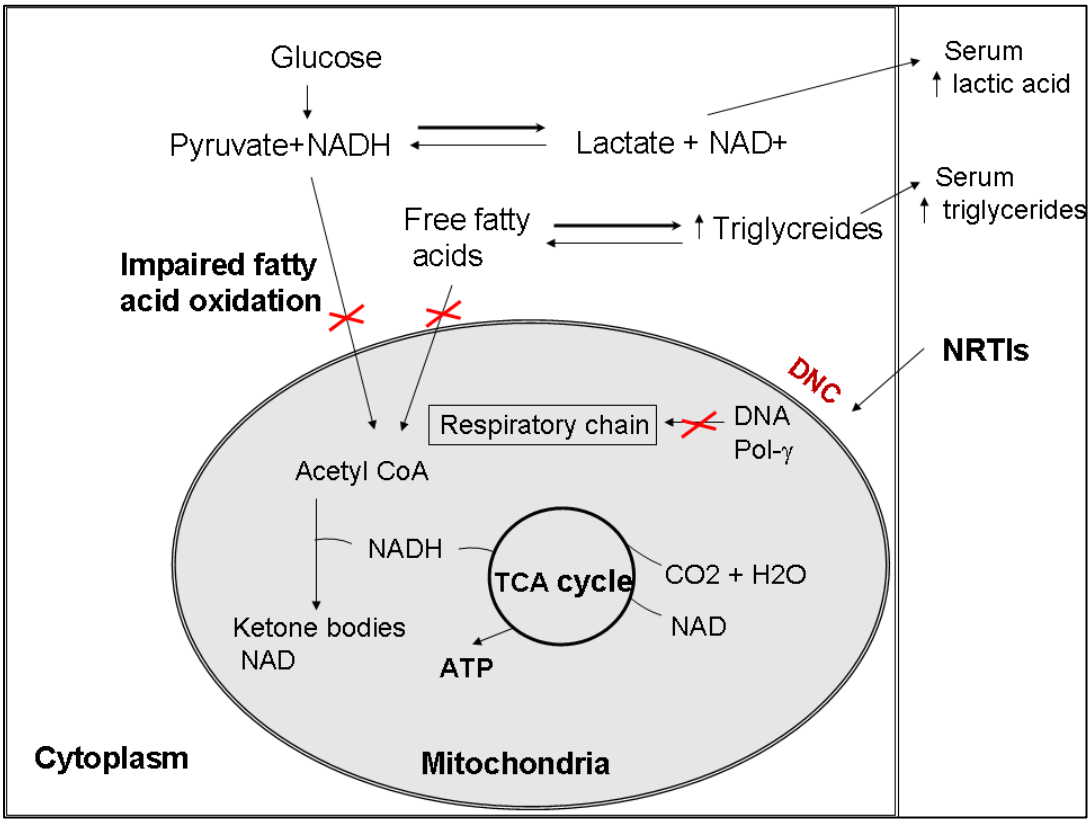


Figure 7. Schematic presentation of the mechanism of NRTI mitochondrial toxicity. *Activated NRTIs are incorporated into mt-DNA and cause chain termination and mt-DNA depletion, which leads to decreased in oxidative phosphorylation, increased production of lactate and, finally, mitochondrial dysfunction.*

***NAD**: nicotinamide adenine dinucleotide; **DNA pol-γ**: DNA plolymerase-γ; **TCA cycle**: tricarboxylic acid cycle; **ATP**: adenosine triphosphate.*

2 AIMS OF THE PRESENT STUDY

The overall goal of the present study was to improve nucleoside analogue therapy by individualizing treatment on the basis of each patient´s pattern of anabolic and catabolic enzymes. Moreover, we have attempted to demonstrate that inhibitors of 5´-nucleotidase may potentiate therapeutic efficacy towards nucleoside-resistant tumors.

Specific aims

- To clarify the mechanisms underlying the tissues-specific toxicity of NRTIs. For this purpose the expression/activity profiles of dNKs (TK1, TK2, dCK, dGK and UK), 5'-NT (dNT) and the DNC transporter in different mouse tissues were characterized.
- To elucidate the dNK (TK and dCK), and 5'-NT (CN1, CN2 and dNT) activities in 14 different tissues of the mouse and rats, two animal models commonly used in studies on cytostatic drugs.
- To characterize the mechanisms underlying resistance towards purine and pyrimidine NAs used clinically. To this end, dCK- and dGK- deficient human leukemia and melanoma cell lines were exposed to NAs.
- To characterize the significance of 5'-NT activities for the outcome of CLL treatment, these activities, as well as CdAMP and Fara-AMP degradation, were measured in samples from patients treated for CLL.

3 MATERIAL AND METHODS

3.1 ANIMAL TISSUES (PAPER I AND II)

For these studies CD-1 male mice (5-6 weeks of age) and male Sprague- Dawley rats (5-6 months of age) were sacrificed and tissues dissected out, frozen in dry ice baths and stored at –80° C until analysis. About 100 mg tissue per ml of extraction buffer (containing 50 mM Tris-Cl, pH 7.6, 100 mM KCl, 2 mM DTT, 20% glycerol, 0.5% NP-40 and complete mini-protease inhibitor cocktail) were extracted at 40° C using a polytron device. The resulting homogenate was then subjected to 3 cycles of freezing and thawing, followed by sonication for 2 × 15 s to release both cytosolic and mitochondrial proteins. This suspension was then centrifuged for 20 minutes at 12,000 × g and 4^o C and the supernatant stored at -80^o C. The protein concentrations of these extracts were determined using the Bradford procedure [206].

3.2 CELL LINES (PAPER III AND IV)

CCRF-CEM and Molt4 (acute T-lymphoblastic leukaemia) and RaH3 and RaH5 (primary melanoma) cells were cultured at 37° C in RPMI-1640 media containing 10% foetal calf serum (FCS), 100 IU penicillin/ml, 100 μg streptomycin/ml and 2 mM L-glutamine under a humidified atmosphere containing 5% CO_2. In addition, sodium bicarbonate (0.1%) was added to the media in which melanoma cells were cultured.

3.3 PATIENT SAMPLES (PAPER IV)

Prior to initiation of treatment, blood samples were obtained from 59 patients with B-CLL presently participating in a Phase III International Randomized Study involving chlorambucil, fludarabine and cladribine as the primary treatment and being performed in Scandinavia and Australia. Peripheral blood mononuclear cells were isolated from these samples employing density gradient centrifugation and thereafter frozen in liquid nitrogen for later analysis.

3.4 PERIPHERAL BLOOD MONONUCLEAR CELLS (PAPER IV)

In this study PBMC were separated from the buffy coats of 10 healthy donors (median age 39 years) and then suspended in RPMI-1640 medium containing 10% heat-inactivated pooled human serum. These cells were stimulated for various periods of time with phytohemagglutinin [(PHA) 10 μg/ml] and then incubated under humidified air containing 5% CO_2 at 37° C. The numbers of cells in the G1, S, and G2+M phases of the cell cycle were determined by flow cytometry.

3.5 THE *IN VITRO* CHEMOSENSITIVITY ASSAY (MTT) (PAPER III)

In this study the cytotoxicity of various drugs was assessed using the methyl thiazol tetrazolium (MTT) assay. In living cells soluble MTT is cleaved by mitochondrial dehydrogenases to form formazan. Cells were incubated with several dilutions of each drug for as long as 72 hours at 37^o C , following which 10 μl at a MTT solution (5 mg/ml) was added and the cells incubated for an additional 4 hours before adding 100 μl of 10% SDS in 10 mM HCl. The absorbance at a 540 nm (with a reference

wavelengh of 650 nm) was then measured in an ELISA plate reader. Cell survival was expressed as the IC_{50} value, i.e., the drug concentration that kills 50% of the cells.

3.6 WESTERN BLOTTING (PAPER III)

In this investigation Western blotting was used to visualize the levels of expression of dCK, dGK, P53R2, and the R1 and R2 subunits. The protein extracts were separated electrophoretically in SDS-polyacrylamide gels and then transferred to an immune-blot PVDF membrane. The primary antibodies employed included: rabbit anti-dCK and anti-dGK antibodies, a monoclonal mouse anti-R1 antibody, polyclonal rat anti-R2 antibody, polyclonal rabbit anti-p53R2 and rabbit anti-β-actin. After incubating the membrane with the appropriate antibody, the secondary peroxidase-labelled antibodies were added and the blots developed using Supersignal® West Pico Chemiluminescent Substrate.

3.7 REAL-TIME QUANTITATIVE PCR (PAPERS I AND III)

Sequence-specific primers and Taqman probes for the different dNKs and 5'-NTs genes were designed using the Primer Express software (PE Applied Biosystem). Equal amounts of cDNA from each sample were amplified in the reaction containing TaqMan Universal PCR master mix (Applied Biosystem) in a total volume of 25 µl, using the ABI PRISM 7700 thermocycler (PE Applied Biosystems) with the following conditions: 1 cycle at 50° C for 2 min and 95° C for 10 min., followed by 40 cycles at 95° C for 15 sec and 60° C for 1 min.

3.8 SMALL INTERFERING RNA (SIRNA) TRANSFECTION (PAPERS III AND IV)

The leukaemia and melanoma cell lines were transfected with the Gene Pulser Xcell Electroporation System, using the square wave procedure at 340 V (pulse length 10 ms in a 0.4-cm electrode cuvette). Prior to transfection the cells were washed, counted and resuspended at a concentration of 4×10^6/ml in RPMI-1640 medium containing 2 mM, L-glutamine and 1.25% dimethyl sulfoxide (for cell membrane stability) and 100-250 nM siRNA was then added. After transfection, the cells were resuspended in RPMI-1640 medium containing 2 mM L-glutamine and 20% FCS, as well as 0.1% sodium bicarbonate in the case of the RaH5 cells. Penicillin and streptomycin were not included during electroporation, but added 24 hours later to avoid contamination.

3.9 FLOW CYTOMETRY (PAPER IV)

Apoptosis was detected by Annexin V / Propidium Iodine staining. For this purpose cells were harvested, centrifuged and resuspended in complete medium at a concentration of 1.5×10^6 per 1.5 ml. The cell pellet was mixed with 100 µl staining mixture containing Annexin V / Propidium Iodine and incubated on ice in the dark for 10 – 15 minutes. Thereafter, 400 µl buffer was added and flow cytometric analysis performed. Fluorescence was measured above 435 nm and 10,000 cells were analyzed. Furthermore, the numbers of cells in the G1, S, and G2+M phases of the cell cycle were determined by propidium iodide staining and fluorescence-activated cell sorting (FACS). Cells were pelleted by centrifugation and resuspended in medium containing

propidium iodine. The fluorescence intensity of the propidium iodine was detected with FL-3 (excitation at 568 nm) by FACS analysis.

3.10 ASSAYS FOR DEOXYNUCLEOSIDE KINASE ACTIVITIES (PAPERS I – III)

Kinase activities in total protein extracts were measured employing radiochemical methods as described previously [207, 208]. Briefly, 30-50 μg of extracted protein in a buffer consisting of 50 mM Tris-Cl (pH 7.6), 5 mM $MgCl_2$, 5 mM ATP, 10 mM NaF, 4 mM DTT and 20 μM [^{3}H]-dCyt was supplemented with 1 mM Thd for relatively selective assay of dCK activity; with [^{3}H]-dGuo plus dCyd and an inhibitor of PNP for selective assay of dGK activity, or with 20 μM [^{3}H]-Thd for assay of total TK activity. In attempt to obtain a more selective assay for TK1 activity, 1 mM dCyd was added, which inhibits TK2 activity by approximately 50% with minimal effect on the TK1 activity [97].

3.11 ASSAYS FOR 5'-NUCLEOTIDASE ACTIVITIES (I – IV)

All of these enzymes were assayed employing HPLC-based radiochemical procedures. CN1 activity with 1 mM [^{3}H]-AMP as substrate was determined in buffer containing 50 mM MOPS, pH 6.9, 100 mM KCl, 3 mM ADP, 6 mM $MgCl_2$, 0.1 mM DTT, 0.05 mM ß-methylene-ADP, 0.02 mM EHNA and 0.2 mg/ml BSA [209]. Following incubation, labeled adenosine and AMP were separated by HPLC. CN2 activity with 0.2 mM [^{3}H]-IMP as substrate was measured in buffer containing 50 mM imidazole, pH 6.5, 3 mM ATP, 1 mM ß-methylene-ADP, 10 mM $MgCl_2$, 5 mM DTT, 0.5 M NaCl and 0.2 mg/ml BSA [209]. Following incubation, labeled inosine and IMP were separated by HPLC. dNT activity with 0.2 mM [^{3}H]-dUMP was assayed in an extraction buffer containing 0.25 mM acetate, pH 5.5, 1 mM ß-methylene-ADP, 20 mM $MgCl_2$, 5 mM DTT, 30 mM KCl and 0.2 mg/ml BSA [209]. After incubation the reaction was stopped by adding PCA and then neutralized with KOH. The samples were centrifuged and supernatants injected into the HPLC system. Nucleosides and nucleotides were separated on a Spherisorb 5 ODS2, Interchrome column. The mobile phase was 0.4 M $NH_4H_2PO_4$, pH 3.7. The enzyme activities are expressed as pmol product formed/mg protein/min.

3.12 STATISTICAL ANALYSES

Statistical analyses were performed using the GraphPad Prism and STATISTICA Software. The data are expressed as means ± standard deviations. A P- value of less than 0.05 was considered to be statistically significant.

4 RESULTS AND DISCUSSION

4.1 PAPER 1

Expression of deoxynucleoside kinases and 5'-nucleotidases in mouse tissues: Implications for mitochondrial toxicity

The aim of this study was to elucidate the mechanism(s) underlying the tissue-specific toxicity of NRTIs. For this purpose we characterized the expression/activity profiles of dNKs (TK1, TK2, dCK, dGK and UK), 5'-NT (dNT) and the DNC transporter in 14 different mouse tissues.

The level of TK1 mRNA was very low in the brain; relatively low in the heart, skeletal muscle and adipose tissue; and very high (6-8- fold higher) in the spleen and liver. dCK expression was low in adipose tissue; relatively low in the liver, heart and skeletal muscle; and 6-8-fold higher in the lung, brain, testis and spleen. The levels of TK2 expression were lower in the heart, skeletal muscle, testis, and adipose tissue. There was less variation in dGK expression among these tissues, except for the almost 2-fold higher levels in the lung, kidney, spleen, brain and skeletal muscle. DNC was expressed at similar low levels in all tissues, with the exception of the kidney and skeletal muscle, where the levels were 2-3-fold higher. The kidney and brain exhibited the highest expression of CN2; whereas in the heart, adipose tissue and testis this expression was relatively low (4-8-fold lower). DNT1 was expressed at more similar levels in these tissues, except for the approximately 2-fold lower levels in the heart and skeletal muscle. CN1-B was absent from the heart, liver and spleen, but expressed at an extremely high level in adipose tissue (73 times higher than in brain); relatively low levels in the brain and testis; and very low levels in the other tissues examined. Thus, our results reveal variations in the levels of these mRNA species and enzyme activities in different mouse tissues. Although previous studies have demonstrated varying degree of correlation between the levels of mRNA and activity for dCK and TK1 [75, 93, 208, 210-212], we could not detect any such variations in mouse tissues.

TK1 activity was very low in most of the tissues examined, expect for the spleen and, to a certain extent the testis. The activity of dCK varied from 0.12 - 55 pmole product formed/mg/min, being lowest in the muscle extracts and highest in the spleen. The activities of the mitochondrial dNKs, TK2 and dGK varied less (10-20 fold) than those of the cytosolic enzymes, but were dissimilar in that in most tissues the TK2 activity was 10-fold lower than the activity of dGK. The highest dGK activities were present in the liver, kidney, lung, spleen, heart and brown fat and the lowest levels in muscle tissues. In all tissues except the spleen, testis and brain, dGK activities were as much as 4-fold higher than the activity of any of the other dNKs. TK2 activity was highest in the brain, kidney, liver and brown fat and lowest in the extracts of biceps muscle. The activities of uridine kinase (UK) were in general approximately 10-fold higher than those of the dNKs and varied about 50-fold between the highest level in spleen extracts and the lowest in vastus muscle (Table 3).

Table 3. Specific activities of dNKs in mouse tissues extracts.

Tissues	TK1	TK2	dCK	dGK	UK
Liver	1.9 ± 0.1	2.5	4.4± 0.3	**37.1± 5.8**	49.3± 1.6
Lung	1.4 ± 0.1	1.9	3.7± 0.4	23.1± 2.3	147± 7.9
Kidney	2.1 ± 0.1	3.1	4.2± 0.6	**36.6± 2.7**	160± 3.2
Brain	4.8 ± 0.3	**8.2**	13± 1.0	15.9± 2.7	160± 2.9
Testis	6.4 ± 0.6	4.6	13± 0.9	8.1± 1.0	42.2± 1.7
Pancreas	1.3 ± 0.1	1.3	3.2± 0.2	7.8± 0.7	157± 3.5
Spleen	**218 ± 89**	n.d.	**56± 9.5**	22.2± 4.0	**504± 27**
Cardiac	0.8 ± 0.1	1.1	1.2± 0.1	20.2± 1.0	70.0± 2.5
Diaphragm	0.3 ± 0.1	0.5	0.6± 0.1	14.0± 1.0	26.6± 1.0
Biceps	0.2 ± 0.1	**0.2**	**0.4± 0.1**	**5.8± 1.5**	**12.8± 0.3**
Vastus	0.1 ± 0.05	0.6	**0.5± 0.4**	**5.1± 0.4**	**10.3± 1.0**
Semitend	0.1 ± 0.05	0.4	**0.4± 0.2**	**5.4± 0.9**	**16.8± 1.6**
Brown fat	1.9 ± 0.2	3.8	5.1± 0.6	23.4± 1.1	145± 2.9
Adipose	2.4 ± 1.0	3.4	5.8± 2.8	20.7± 5.1	79.1± 1.9

Assays were performed with ^{3}H- labeled deoxynucleosides as substrates in total mouse tissue extracts. The results are expressed as pmol product formed/mg protein/min and presented as means ± standard deviation of independent measurements (in duplicate) on three mice.

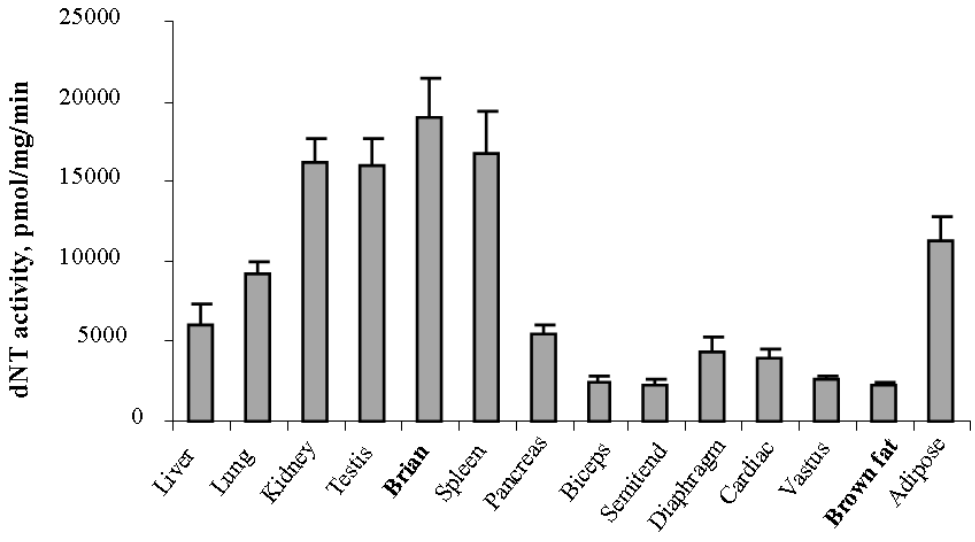


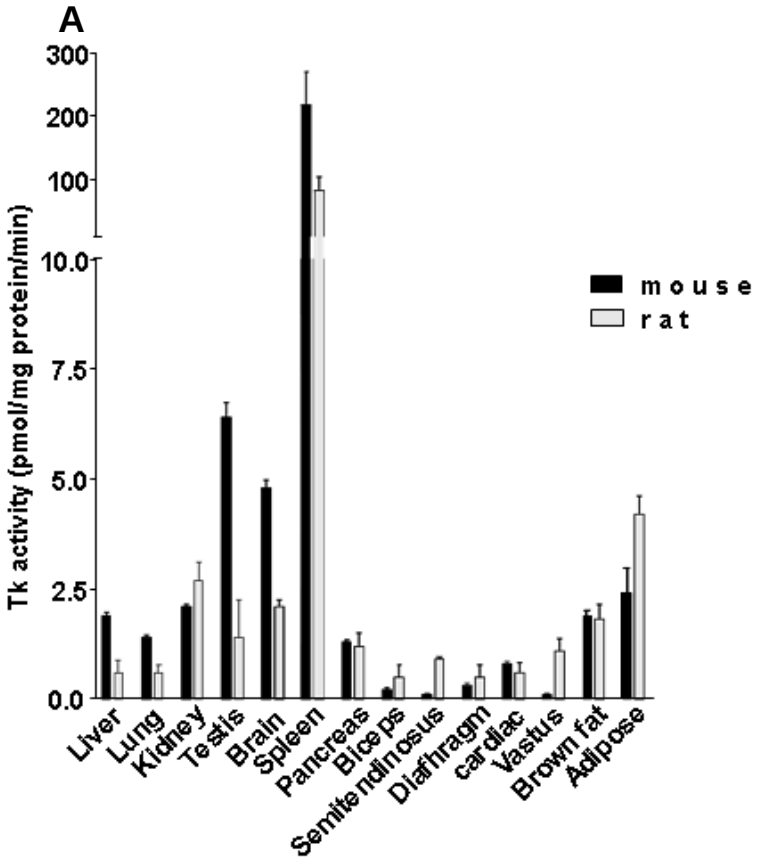
Figure 8. Specific activities of 5'- nucleotidase (dNT) in various murine tissues. *This activity was assayed in total tissue extracts with ^{3}H-dUMP as substrate. The results are expressed as pmol product formed/mg protein/min and presented as means ± standard deviation of independent measurements (in duplicate) on three mice.*

dNT activities in the tissue extracts examined were much higher than those of the nucleoside kinases. The variation in dNT activities was about 8-fold, with brown fat exhibiting the lowest and kidney extracts the highest activity. The activities in the spleen and brain were also high, while those in fat and muscle extracts were several-fold lower (Figure 8).
The levels of dNK and 5'-NT activities demonstrate very pronounced variations, indicating that the capacity to salvage pyrimidine deoxynucleosides is tissue-specific, with the spleen demonstrating the highest and the heart and skeletal muscles the lowest capacities. In summary, these findings indicate that the heart, skeletal muscles and adipose tissue express the lowest levels of both anabolic and catabolic activities, which may explain the side-effects of NRTI therapy associated with these tissues.

4.2 PAPER II

Differences in cytosolic and mitochondrial 5'-nucleotidase and deoxynucleoside kinase activities in Sprague-Dawley rat and CD-1 mouse tissues: Implication for the toxicity of nucleoside analogs in animal models

The aim of this study was to employ selective assay procedures to characterize the distribution of the activities of dNKs (TK and dCK), as well as of 5'-NTs (CN1, CN2, dNT) in 14 different tissues of the mouse and rat, which are commonly used in experimental studies on cytostatic drugs.
In general, our observations indicate that the activities of dCK and TK (most likely TK2) in the liver, lung, testis and brain are approximately 2-5-fold higher in the mouse than in the rat and, furthermore, that the spleen demonstrates the highest dCK activity, being approximately 3-fold higher in the mouse (Figure 9).



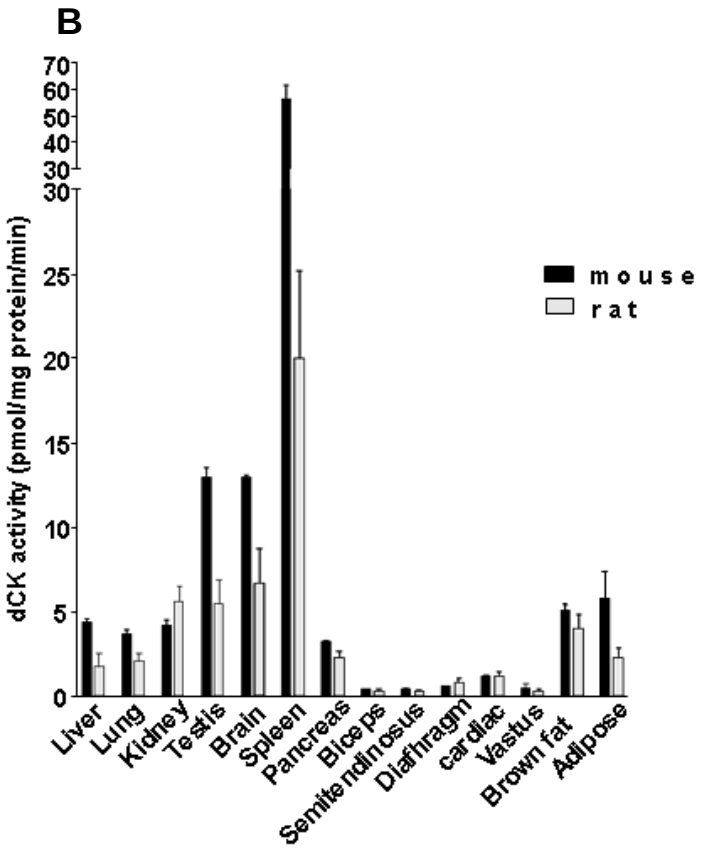
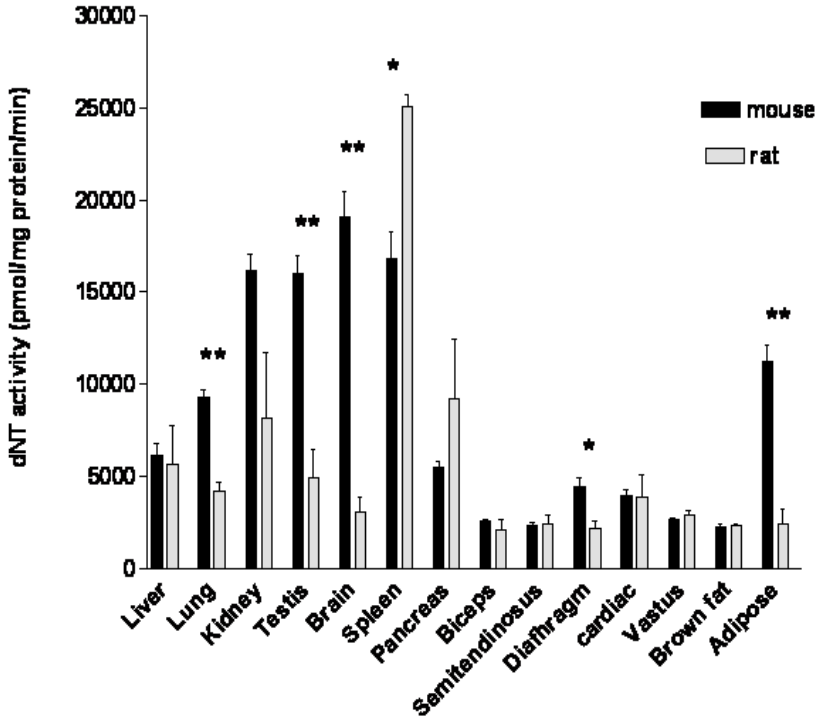


Figure 9. Specific activities of deoxynucleoside kinases in murine and rat tissues. *Phosphorylation of ^{3}H-dCyd and ^{3}H-Thd in total tissue extracts from mice and rats were measured as described in the Materials and the methods. dCK (B) and TK (A) activities are expressed as pmol monophosphates formed/mg protein/minute and presented as means ± standard deviation of independent measurements (in duplicate) on three mice or rats.*

These results are in agreement with the differences in deoxycytidine metabolism between the mouse and rat found by Chan and coworkers [213], as well as with other reports on differences between tissues and species with regards to dNK activities [15, 214]. Chen and colleagues demonstrated that spleen extracts from both of these species exhibit high dCK activity and, in addition, that this same activity in extracts from the liver, spleen and kidney of the mouse is higher than for the rat. These earlier findings correlate well with our present results, with certain exceptions. The study by Chen *et al.* also showed that extracts of mouse organs exhibited deoxycytidine deaminase activity, whereas the rat tissue extracts showed none of this activity [213]. A similar investigation revealed that the distribution of adenosine deaminase in tissues from man, the rat and mouse also differs [215]. Therefore, the pharmacological effects of adenosine and deoxycytidine analogues in the mouse, rat and man might also be expected to differ. The levels of TK activity in mouse tissue extracts documented here are in agreement with those reported previously by Wang and Eriksson [216]. However, the assays were performed with certain differences, which may explain the lower levels of presumptive TK2 activity observed here, except in case of the spleen extracts, which contain mostly TK1 activity. For most tissues the level of TK2 activity was higher in the mouse, with the exception of skeletal muscle, where this level was 6-10-fold higher in the rat extracts. These results indicate that mouse muscle may be

more sensitive to the toxic effects of thymidine and deoxyuridine analogues than is rat muscle.



Figurer 10. Specific activities of 5'-deoxynucleotidases (dNT) in murine and rat tissues. *The total 5'-deoxynucleotidases (dNT) activity in extracts from mouse and rat tissues was measured using ^{3}H-dUMP as described in the Materials and methods and expressed here as nmol per product formed/mg of protein/minute. The results are presented as mean ± standard deviation of independent measurements (in duplicate) on three mice or rats. * = p < 0,05 and ** = p < 0,005*

Here, we also showed that, with the exception of the spleen and pancreas, mouse dNT activities are 2-6-fold higher than those in rat tissues. The highest dNT activity was observed in mouse brain, where this activity was approximately 6-fold higher than in rat brain (Figure 10). The only other study of direct relevance to our own that we are aware of demonstrated considerable variation in nucleotidase activity between these two rodent species [217]. In that investigation, the CN2 activities in mouse testis, spleen, pancreas and diaphragm were 2-3-fold higher than in the corresponding activities in the rat, whereas rat lung CN2 activity was 2-fold higher than in the mouse. The testis exhibited the highest CN2 activity and this activity was approximately 2-fold higher in mouse than in rat. CN2 is also expressed ubiquitously in human tissues, with highest expression in the heart, pancreas and skeletal muscles, but with high activity also being detected in the spleen, testis and lymphoblastoid cells, which have rapid nucleic acid turnover and/or DNA synthesis, and with low activity in skeletal muscle and erythrocytes [123, 124]. This pattern agrees very well with our own findings.
CN1 activity in the liver, kidney and adipose extracts was 2-3-fold higher in the rat than in the mouse, but the extract from mouse testis exhibited 2-fold higher activity than the corresponding extract from rat. The spleen had the highest CN1 activity, which was

approximately 1.5-fold higher in the rat. Expression of CN1-A is largely tissue-specific, being high in skeletal and heart muscle, where this enzyme generates intracellular adenosine under ischemic conditions. On the other hand, human CN1-B is expressed ubiquitously, with highest mRNA levels in the testis and lowest in the brain and skeletal muscle. [120]. Our own findings correlate to a certain extent to the level of CN1-B expression.

In summary, our present findings reveal significant differences between the mouse and rat with respect to these enzyme activities, as well as significant variation between the different tissues of each animal species. Thus, the capacity for metabolism of pyrimidine nucleoside analogues by dNKs and 5'-NTs is both species- and tissue-specific. Such inter- and intraspecies differences in activities and substrate specificities between different animal models and humans with regards to pyrimidine nucleotide dephosphorylation may exert an important impact on strategies designed to introduce inhibitors of these enzymes into the clinic, as well as on drawing conclusions concerning the metabolism and chemotherapeutic use of pyrimidine analogues in humans on the basis of animal studies. Moreover, the results documented here may be helpful in choosing the animal species in which to examine the metabolism of a given compound so as to obtain information of most direct relevance to humans.

4.3 PAPER III

RNA interference targeting nucleoside analog activating enzymes in leukemic and solid malignancies enhances the effect of gemcitabine

The aim of this investigation was to examine mechanisms underlying resistance towards certain purine and pyrimidine nucleoside analogues used in the clinic. Accordingly, we generated dCK- and dGK-deficient leukemia and melanoma cell lines employing the siRNA technique and subsequently tested their sensitivity to NAs.

Untransfected CEM cells were sensitive to all of the nucleoside analogues tested, with IC_{50} values ranging between 0.05 and 5.4 μM. The untreated melanoma cell lines were sensitive to Fara-A, CAFdA, and dFdC with IC_{50} values of 0.04- 5.7 μM. The mean dCK activity in the CEM cells (16 pmol/min/mg) was significantly higher than the corresponding mean activity in three different melanoma cell lines (3.4 pmol/min/mg). At the same time, the melanoma cells exhibited significantly higher dGK activity (mean 3.2 pmol/min/mg) than the CEM cells (1.4 pmol/min/mg). Transfection down-regulated dCK in the CEM cells and dGK in the RaH5 cells. Both expression and activity were reduced for as long as 72 hours following siRNA transfection of CEM cells, with the maximal effect being observed after 24 hours (Figure 11 A, B).

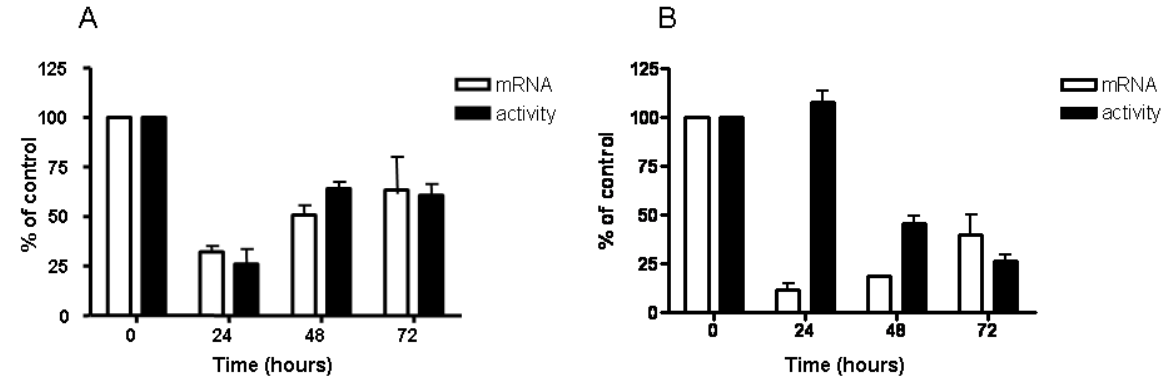


Figure 11. Dow-regulation of dCK in CEM cells and dGK in RaH5 cells using siRNA. *CEM cells (A) or RaH5 cells (B) were transfected employing the siRNA technique, harvested and assayed for dCK or dGK, respectively. Both mRNA levels and enzyme activities are shown.*

After down-regulation of dCK, the sensitivity of the CEM cells to CdA, CAFdA and Ara-C was reduced 3-6-fold, suggesting that the cytotoxicity of these drugs was strongly dependent on activation by dCK. Their sensitivity to Fara-A and Ara-G was unchanged, while these same cells demonstrated significantly enhanced (8-fold, p = 0.002) sensitivity to dFdC (Figure 12 A). In the case of the RaH5 melanoma cells, down-regulation enhanced resistance to Fara-A slightly and, more pontently, to CAFdA, while at the same time enhancing sensitivity to dFdC (3-fold, p = 0.004) (Figure 12 B).

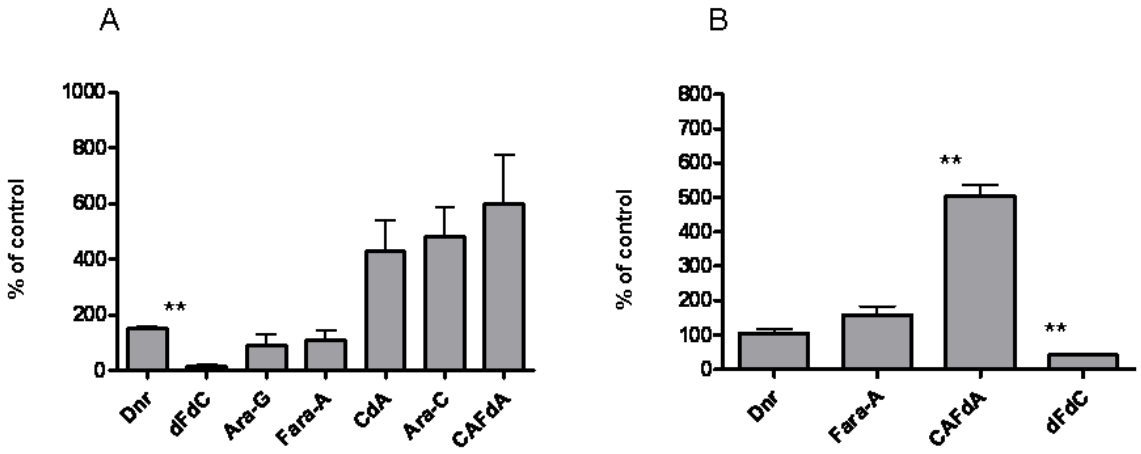


Figure 12. IC_{50} values of siRNA-transfected CEM and RaH5 cells for the different analogues. *siRNA-transfected CEM cells (A) and RaH5 cells (B), exhibiting reduced dCK and dGK expression, respectively, were incubated with the different drugs and the IC_{50} values obtained compared to those of untransfected cells. Daunorubicin (Dnr) was used as a positive control, since its activity is independent of both of these enzymes.*

Here, we down-regulated the dCK and dGK enzymes by approximately 70-80%. dCK is the first and rate-limiting enzyme for the activation of several NAs, but the other kinases also contribute. dGK and TK2 activities increase in a compensatory manner when the activity of dCK is reduced [144, 218]. 24-72 hours after transfection, the activities of dGK and TK2 in the CEM cells were not significantly altered in comparison to the control cells. The activity of dCK in the RaH5 cell line was slightly reduced, i.e., no compensatory mechanism was evident. The expression and activity of TK2 in these siRNA-transfected RaH5 cells were not significantly altered, except for being elevated approximately two-fold 72 hours after transfection. This late elevation may, however, contribute to the enhanced cytotoxicity of dFdC towards the RaH5 cells with lowered dGK activity.

Ara-G is incorporated into mitochondrial and Ara-C into nuclear DNA, suggesting that these two drugs are activated by different enzymes [219]. Our results are in agreement with this conclusion, since the cytotoxicity of Ara-G was almost unaffected in the cells with down-regulated dCK, wherase the cytotoxicity of Ara-C was potentiated as much as 4-fold. Our findings also reveal a pronounced dependence of the cytotoxicity of CdA and CAFdA on phosphorylation by dCK, indicating that this is the major activating enzyme for these drugs.

In the case of Fara-A, although a strong correlation between sensitivity and dCK activity has been found [146], a mechanism other than dCK deficiency has been suggested to underline resistance to this compound [54], allowing dCK-deficient CEM cells to remain sensitive [56], as was also observed here.

dFdC shows cytotoxicity towards both solid tumors [220, 221] and leukemic cells [222]. Although mechanisms of resistance to dFdC have been shown to involve reduced dCK activity [223], the cytotoxicity of this agent is less dependent on the activity of dCK than is the case for other NAs [146, 224, 225]. This difference could reflect changes in the ribo- and deoxyribonucleotide pools, since dFdC exerts a potent effect on intracellular NTP pools [226]. The RaH5 cells with lowered dGK activity were resistant to CAFdA, but not Fara-A or dFdC. All of these NAs are known to be activated by dGK [59, 81, 218, 227], but they can also be phosphorylated efficiently by dCK [144, 228, 229], as well as, to some extent, by TK2 [99]. Our present investigation indicates that dFdC toxicity is not always correlated to the level of activating enzymes, i. e., other enzymes or mechanisms may also be important for the activation of this drug and their levels may vary between different types of cells. Furthermore, certain NAs with a major effect on both leukemic and melanoma cells, such as CAFdA, Fara-A and dFdC, may be a valuable complement to the present treatment of choice for malignant melanoma, involving surgery, targeted therapies and immune-modulating therapies.

4.4 PAPER IV

Activity profiles of 5'-nucleotidases in blood cells from untreated patients with B-cell chronic lymphocytic leukemia and in phytohemag-glutinin stimulated cells from healthy subjects: correlation to nucleoside analogues therapy

This study was designed to improve our understanding of the significance of 5'-NT activities with respect to outcome of treatment for CLL. For this purpose, blood samples from patients with this disease were collected prior to initiation of treatment and their levels of various 5'-NT activities, as well as the rates of CdAMP and Fara-AMP degradation determined employing HPLC-based procedures.
There was pronounced inter-individual variation with respect to the different 5'-NT activities. The median values were 132 (please put units here) for CN1 (95% CI: (119-157), 478 for CN2 (95% CI: 427-616) and 3265 dNT (95% CI: 2690-3573) (Figure 13). To our knowledge, no previous study has examined these three 5'-NTs in patients with leukemia and we conclude that the cytotoxicity of purine nucleoside analogues may be influenced by the levels of 5`-NT expression in the patient´s leukemic cells.

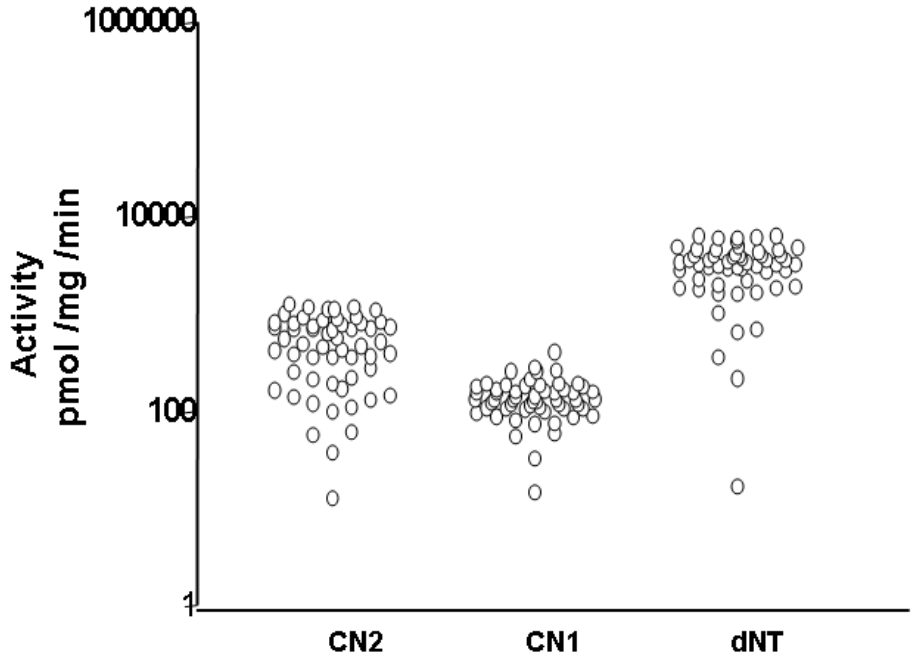


Figure 13. Large inter-individual variations in 5´-nucleotidase activities in PMBCs isolated from 59 patients with B-CLL prior to treatment*. Peripheral leukocytes were isolated from these patients prior to initiation of chemotherapy and CN1, CN2 and dNT activities measured employing radiolabeled AMP, IMP and dUMP, respectively, as described in the Materials and Methods. The median value (95%) for CN1, CN2 and dNT were 132 (119-157), 478 (427-616) and 3265 (2690-3573), pmol/mg/min, respectively.*

Furthermore, with cladribine monophosphate as substrate, we found an excellent correlation between the rate of CdAMP degradation and the activity of the cytosolic 5´-NT, CN1, suggesting this enzyme is primarily responsible for degradation of that particular drug (Figure 14). These findings are in agreement with previous reports demonstrating that CdAMP is a substrate for CN1, although this enzyme exhibits no activity towards Fara-AMP or Ara-CMP. This same earlier study also mentioned that over-expression of CN1 in HEK 293 and Jurkat cells leads to resistance to CdA [118]. More recently, transfection of murine fibroblasts with CN1 was found to render these cells resistant to CdA [159]. Altogether, these observations indicate that the level of CN1 activity may also influence the therapeutic efficacy of certain NAs.
Furthermore, our results demonstrate a clear correlation between the rate of Fara-AMP degradation and the activity of CN2, suggesting that this enzyme may be responsible for such degradation (Figure 14). CN2 dephosphorylates several anti-viral and -cancer analogues, including Fara-AMP, as supported by our present findings [160]. In another recent study one Ara-C-resistant cell line with a high level of CN2 and low level of dCK was less sensitive to Fara-A than another with only a low level of dCK [161]. In addition, a series of K562 cell lines resistant to dFdC, CdA, Ara-C and Fara-A demonstrated elevated CN2 and low dCK activities [138].
From a clinical point of view, some studies have reported that elevated levels of CN2 mRNA are correlated with poor therapeutic prognosis in patients with AML [165, 166]. Another investigation revealed that among patients with CLL or hairy cell leukemia, non-responders demonstrated higher CN2 and /or lower dCK activity [150].
Together, our findings indicate that the outcome of treatment can be significantly influenced by the rate of dephosphorylation by cytosolic 5'NTs enzymes, which reduce the efficacy of the analogues, and that CN2 may become a valuable prognostic indicator in future clinical trials. In addition, the pattern of CN2 activity was found to be altered in response to phytohemagglutinin (PHA)-induced proliferation. This observation may reflect the requirement for dNTPs for DNA synthesis in connection with the cell proliferation, induced by PHA stimulation.

When we also tested cellular sensitivity to the cytotoxicity of nucleoside analogues following down-regulation of 5'-NTs with siRNA, we observed enhanced cytotoxicity of all the drugs tested after such down-regulation of dNT1. In the case of down-regulation of CN1 and CN2, the findings with the different drugs were variable and require further investigation. Nevertheless, our present observations indicate that despite the fact that dNT1 has a preference for deoxynucleoside monophosphates, this enzyme is a good for mediating resistance to NAs. Identification of CN1 and CN2 as two of the nucleotidases involved in the turnover of deoxyribonucleotide pools has consequences for anti-cancer and anti-viral therapy and sensitivity to dephosphorylation by these enzymes may become an important parameter in the development of more effective drugs. Furthermore, our studies indicate that CdAMP and CN1 activities, as well as the activities of Fara-AMP and CN2 are strongly correlated and may be valuable prognostic factors concerning the outcome of CLL therapy, as well as being involved in responses to the purine analogues.

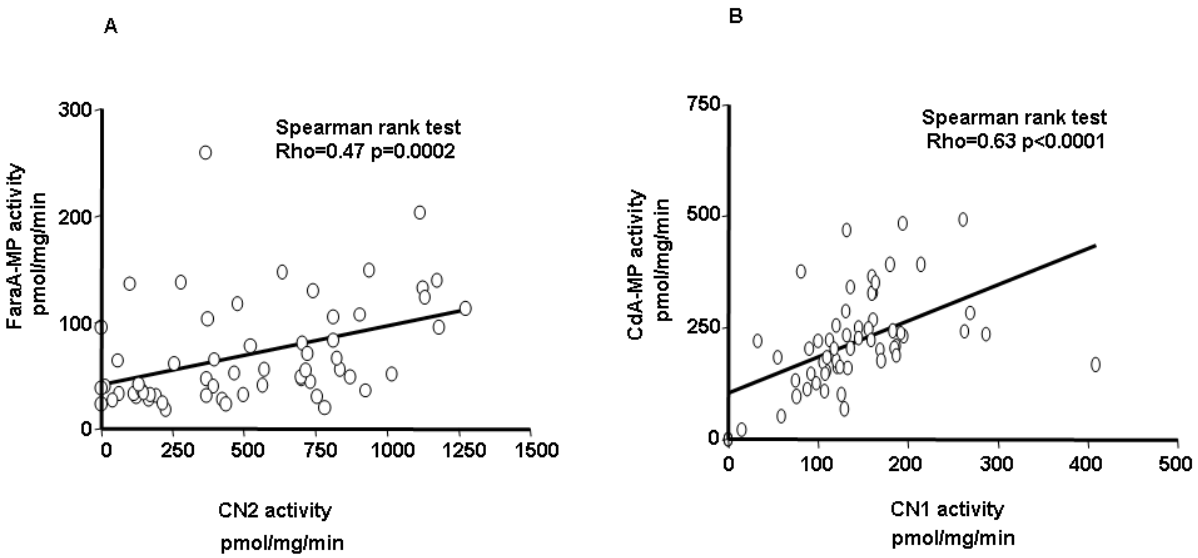


Figure 14.Correlation between CN1 and CN2 activities and the rated of degradation of purine nucleoside analogues in the peripheral leukocytes of patients with CLL. *Peripheral leukocytes were isolated from 55 patients with B-CLL prior to initiation of drug treatment. (A) The correlation between the rate of Fara-AMP degradation and CN2 activity (Spearman rank test: Rho=0.47, p=0.0002); (B) The correlation between the rate of CdAMP degradation and CN1 activity (Spearman rank test: Rho=0.63, p<0.0001).*

5 CONCLUSIONS

The general conclusions to be drawn from this thesis include the following:

- Each tissue in a mouse exhibits its own unique pattern of expression of cytosolic and mitochondrial dNKs and 5'-NTs. Adipose tissue, the heart and skeletal muscles all demonstrate low levels of enzymes involved in both the anabolism and catabolism of NAs, which may explain, at least in part, the adverse side-effects of NRTI therapy on these tissues. These findings may help clarify the tissue-specific toxicity observed with nucleoside analogues used to treat patients infected with HIV, as well as the symptoms of inherited deficiencies in mitochondrial TK2.

- Each tissue of a rat or mouse has a unique pattern of dNK and 5'NT activities, with approximately 2-3- fold differences between the rat and mouse in most cases. There observations may account for the differences in the pharmacological responses of these two animal species to certain nucleosides, which is of key importance in animal studies concerning the mechanisms of toxicity of NAs.

- The cytotoxicity of dFdC does not always correlate with the levels of activating enzyme. Other mechanisms may also be important for activation of this drug and the rates of these processes may vary between different types of cells. NAs such as CAFdA, Fara-A and dFdC, which exert potent effects on both leukemic and melanoma cells, may be a valuable complement to the present treatment of choice for malignant melanoma.

- Rates of CdAMP and Fara-AMP degradation were significantly correlated to the dephosphorylating activity of CN1 and CN2, respectively. Expression of CN2 activity was dependent on stimulation of proliferation by PHA. Thus, differences in outcome following CdA and Fara-A treatment may be explained by different levels of CN1 and CN2 activity, a factor which should be considered in connection with the development of more effective drugs, as well as with regards to the possible addition of inhibitors of 5'-NTs to current therapeutic regimens.

6 FUTURE PERSPECTIVES

The amount of information available concerning the mechanisms of toxicity and resistance to NAs is increasing continuously. One area of research in this field that is growing rapidly is the use of these drugs for treatment of solid tumors, e.g., the use of dFdC for treatment of pancreatic and lung cancer [69]. The extensive use of this latter compound has motivated further studies of the molecular mechanisms underlying its effects. CAFdA is also a potent member of the next generation of purine analogues. Although this drug has only been approved for treatment of pediatric leukemia so far, several studies concerning its oral bioavailability and use for treating elderly patients with AML and combination with other regimens are presently ongoing [230]. Furthermore, our previous studies [54, 144] indicate that the mechanisms of resistance to CAFdA differ, at least in part, from those of other analogues and require further investigation.

Although new analogues presently being developed have similar chemical structures and mechanisms of action, they also have some important differences, especially with regards to their metabolic pathways [231, 232]. Continuing research on cellular responses to NA cytoxicity will improve our understanding of the discrepancies between different drugs in cell model studies, as well as in clinical trials, and will help guide the development of novel drugs that will be more effective and specific.

The processes of membrane transport and initial intracellular activation appear to be reasonably well understood, but the mechanisms involved in cellular deactivation, especially by 5'-NTs, as well as the final steps in the induction of apoptosis have not yet been clarified. Previous studies suggest a role for p53 in the overall activity of NAs, but its role seems to vary in different type of cells [233-235]. Further studies focusing on NA-induced apoptosis should be carried out.

The 5'-NTs play an important role in mediating resistance to NAs and additional studies in which 5'-NT activities are compared to clinical drug resistance in different types of malignancies are required. Elevated expression/activity of nucleotidases could be counteracted either by developing new analogues resistant to 5'-NT-catalyzed dephosphorylation or by thesynthesis of specific inhibitors. On the other hand, from a clinical point of view the accurate determination of different 5'-NT activities in human tissues should provide valuable information concerning whether a particular enzyme might mediate clinical resistance, as well as helping to elucidate the mechanism(s) underlying the tissue-specific toxicity associated with NA therapy.

Most of the present literature deals with 5'-NT activities in cell lines or particular animal tissues and these activities need to be determined in human tissues as well. Human 5'-NTs are localized in the cytosol, mitochondria or outer plasma membrane [106]. Dephosphorylation by cytosolic enzymes reduces efficacy of NAs while dephosphorylation by mitochondrial enzymes may diminish mitochondrial toxicity, both of which effects might influence treatment outcome. This aspect needs to be taken into consideration when developing new drugs.

Finally, chemical alteration of the NAs presently available, e.g., by conjugation or synthesis of pronucleotides, will hopefully allow these drugs to be used effectively for treatment of non-responders or patients who develop resistance.

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