NUCLEOSIDE ANALOG TOXICITY AND NUCLEOSIDE KINASE DEFICIENCY;

effects on mitochondrial DNA

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Stockholm 2008
Till Martin
I may not have gone where I intended to go,  
but I think I have ended up where I needed to be.  
Douglas Adams

When you reach for the stars, you may not quite get one,  
but you won’t come up with a handful of mud either.  
Leo Burnett

Quand on est dans la merde jusqu’au cou,  
il ne reste plus qu’à chanter.  
Samuel Beckett
ABSTRACT

Nucleoside analogs are modified nucleosides used in treatment of cancer and viral infections. They are dependent on intracellular phosphorylation to be pharmacologically active. Deoxyribonucleoside kinases catalyze the rate-limiting step in the phosphorylation of many clinically used nucleoside analogs. Human cells contain four distinct deoxyribonucleoside kinases that have partially overlapping substrate specificities for both naturally occurring deoxyribonucleosides as well as nucleoside analogs.

The deoxycytidine analog 2',3'-dideoxycytidine (ddC) has been used for treatment of human immunodeficiency virus (HIV) infections. ddC causes delayed toxicity, due to mitochondrial DNA (mtDNA) depletion, when cells are exposed to the drug at low concentration for prolonged periods of time. CEM T-lymphoblast cell lines resistant to the delayed toxicity of ddC were generated and studied. Although the cells were resistant to mtDNA depletion they had a retained anti-HIV activity of ddC. The ddC resistant cells were shown to have slightly increased levels of mtDNA and decreased mRNA expression of deoxycytidine kinase and thymidine kinase 2.

Mitochondria are the main providers of energy for cells and these organelles have a separate genome distinct from the nuclear genome. The mitochondrial genome is dependent on the mitochondrial deoxyribonucleoside kinases for normal DNA replication. Inherited deficiency of either of the two mitochondrial deoxyribonucleoside kinases deoxyguanosine kinase (dGK) and thymidine kinase 2 (TK2), causes mtDNA depletion in patients. In order to understand the molecular mechanism of mtDNA depletion due to TK2 deficiency, a Tk2 deficient mouse strain was generated and characterized. The Tk2 deficient mice showed growth retardation, severe hypothermia and a reduced life span. They also exhibited mtDNA depletion in multiple organs. It was concluded that TK2 has a major role in supplying deoxyribonucleotides for mtDNA replication and that other pathways of deoxyribonucleotide synthesis cannot compensate for the loss of this enzyme.

TK2 phosphorylates several nucleoside analogs that are also substrates of other deoxyribonucleoside kinases. To elucidate the role of TK2 mediated phosphorylation in the cytotoxic effects of nucleoside analogs, a human fibroblast cell line with partial TK2 deficiency was studied. A recombinant enzyme with the same mutation found in the TK2-deficient cells was also investigated in order to obtain further insights into the mechanisms of TK2 deficiency. It was concluded that the loss of TK2 activity did not alter the cells’ sensitivity to nucleoside analogs and that TK2 phosphorylation may be less important, compared to other deoxyribonucleoside kinases, for the cytotoxic effects of these compounds.

The deoxyribonucleoside kinase of the fruit fly Drosophila melanogaster (Dm-dNK) is a multisubstrate enzyme that phosphorylates pyrimidine and purine deoxyribonucleosides as well as several anticancer and antiviral nucleoside analogs. The broad substrate specificity together with the high catalytic rate makes it an interesting candidate gene for suicide gene therapy. In order to understand the determinants of the substrate specificity of the enzyme and to find Dm-dNK mutants with improved kinetic properties for application in suicide gene therapy, we performed site directed mutagenesis on the enzyme. It was shown that the mutants showed partial or complete loss of activity compared with the wild type enzyme in terms of thymidine phosphorylation. This decreased phosphorylation efficiency supports their importance for substrate binding.
LIST OF PUBLICATIONS

I. Nicola Solaroli, Mia Bjerke, Marjan Amiri, Magnus Johansson,
   Anna Karlsson
   Active site mutants of *Drosophila melanogaster* multisubstrate
deoxynucleoside kinase

II. Mia Bjerke, Maribel Franco, Magnus Johansson, Jan Balzarini,
    Anna Karlsson
    Increased mitochondrial DNA copy-number in CEM cells resistant to
delayed toxicity of 2',3'-dideoxycytidine

III. Xiaoshan Zhou, Nicola Solaroli, Mia Bjerke, James B Stewart,
     Björn Rozell, Magnus Johansson, Anna Karlsson
     Progressive loss of mitochondrial DNA in thymidine kinase 2 deficient
     mice

IV. Mia Bjerke, Nicola Solaroli, Nicole Lesko, Jan Balzarini,
    Magnus Johansson, Anna Karlsson
    Retained sensitivity to cytotoxic pyrimidine nucleoside analogs in
    thymidine kinase 2 deficient human fibroblasts
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Nucleosides and nucleotides
dAdo, dAMP, dADP, dATP  deoxyadenosine, mono-, di- and triphosphate
dThd, dTMP, dTDP, dTTP  deoxythymidine, mono-, di- and triphosphate
dCyd, dCMP, dCDP, dCTP  deoxycytidine, mono-, di- and triphosphate
dGuo, dGMP, dGDP, dGTP  deoxyguanosine, mono-, di- and triphosphate
dUrd, dUMP, dUDP, dUTP  deoxyuridine, mono-, di- and triphosphate
dN, dNMP, dNDP, dNTP  any deoxyribonucleoside, mono-, di- and triphosphate

Enzymes
TK1  thymidine kinase 1
TK2  thymidine kinase 2
dCK  deoxycytidine kinase
dGK  deoxyguanosine kinase
Dm-dNK  Drosophila melanogaster deoxyribonucleoside kinase
dNK  deoxyribonucleoside kinase
rNK  ribonucleoside kinase
NMPK  nucleoside monophosphate kinase
NDPK  nucleoside diphosphate kinase
RR, R1, R2, p53R2  ribonucleotide reductase and subgroups
5'-NT  5'-nucleotidase
dNT-1, dNT-2  5'-deoxyribonucleotidase 1 and 2
pol γ  polymerase gamma
Tfam  transcription factor A
RT  reverse transcriptase

Nucleoside analogs
ddC  2',3'-dideoxycytidine (zalcitabine)
AZT  3'-azido-2',3'-dideoxythymidine (zidovudine)
ddI  2',3'-dideoxynosine (didanosine)
d4T  2',3'-didehydro-3'-deoxythymidine (stavudine)
3TC  2'-deoxy-3'-thiacytidine (lamivudine)
AraC  1-β-D-arabinofuranosylcytosine (cytarabine)
AraG  9-β-D-arabinofuranosylguanine (nelarabine)
CdA  2'-chloro-2'-deoxyadenosine (cladribine)
dFdC  2',2'-difluorodeoxycytidine (gemcitabine)
BVDU  (E)-5-(2-bromovinyl)-2'-deoxyuridine (brivudine)

Misc
DNA  deoxyribonucleic acid
RNA  ribonucleic acid
mt  mitochondria
ENT, hENT1, 2, 3, 4  equilibrative nucleoside transporter and subgroups
CNT, hCNT1, 2, 3  concentrative nucleoside transporter and subgroups
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>DNC</td>
<td>deoxyribonucleotide carrier</td>
</tr>
<tr>
<td>CEM</td>
<td>T-lymphoblastoid cell line</td>
</tr>
<tr>
<td>MDS</td>
<td>mitochondrial DNA depletion syndrome</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HBV</td>
<td>hepatitis B virus</td>
</tr>
<tr>
<td>HSV</td>
<td>herpes simplex virus</td>
</tr>
<tr>
<td>VZV</td>
<td>varicella zoster virus</td>
</tr>
<tr>
<td>EtBr</td>
<td>ethidium bromide</td>
</tr>
<tr>
<td>SUCLA2</td>
<td>protein associated with encephalomyopathic MDS</td>
</tr>
<tr>
<td>MPV17</td>
<td>protein associated with hepatocerebral MDS</td>
</tr>
<tr>
<td>NRTI</td>
<td>nucleoside analog reverse transcriptase inhibitor</td>
</tr>
<tr>
<td>HAART</td>
<td>highly active anti-retroviral therapy</td>
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</tbody>
</table>
INTRODUCTION

Nucleoside analogs are modified nucleosides and these compounds have been used in chemotherapy since the 1940s (Elion 1989). They are efficient in the treatment of hematological malignancies and solid tumors as well as in the treatment of several viral infections (Arner and Eriksson 1995; Balzarini 1994; Galmarini et al. 2001). Nucleoside analogs are dependent on intracellular phosphorylation to become pharmacologically active. Several different nucleoside and nucleotide kinases from different enzyme families catalyze the phosphorylation of the nucleoside analogs. The kinases involved in the phosphorylation pathways have been studied to elucidate the pharmacological mechanisms of the nucleoside analogs.

The first step in the phosphorylation of many clinically used nucleoside analogs is catalyzed by deoxyribonucleoside kinases. Human cells contain four distinct deoxyribonucleoside kinases that have partially overlapping substrate specificities for both naturally occurring deoxyribonucleosides as well as nucleoside analogs. These enzymes catalyze the rate-limiting step in the phosphorylation of many nucleoside analogs that are dependent on phosphorylation to their triphosphate form. Genetically engineered human nucleoside kinases or nucleoside kinases from other organisms with improved kinetic properties for nucleoside analog phosphorylation have been studied. The aim is to understand the catalytic mechanisms of the deoxyribonucleoside kinases in order to improve nucleoside analog therapy. The deoxyribonucleoside kinases with improved or altered substrate specificities have also been investigated for possible use as suicide genes in gene therapy.

Two of the human deoxyribonucleoside kinases are located in the mitochondria. Mitochondria are intracellular organelles that provide energy for the cell and these organelles have a separate genome distinct from the nuclear genome. The mitochondrial genome is dependent on the mitochondrial deoxyribonucleoside kinases for normal DNA replication. Inherited deficiency of either of the two mitochondrial deoxyribonucleoside kinases causes mtDNA depletion. Several nucleoside analogs cause adverse effects by damaging or depleting mtDNA and interfering with mitochondrial function. The presence of deoxyribonucleoside kinases within the mitochondria suggests that their subcellular location may contribute to the adverse effects of the nucleoside analogs as cells depend on the function of these enzymes to maintain normal mtDNA replication.

This study is focused on investigating molecular mechanisms of nucleoside analogs and their phosphorylation in cells. We studied the multisubstrate nucleoside kinase from Drosophila melanogaster and performed site-directed mutagenesis to alter the nucleoside analog substrate specificity of the enzyme. We have generated cell lines resistant to the nucleoside analog 2',3'-dideoxycytidine and studied the phenotype of the cells and molecular mechanisms associated with the drug resistance.
the mitochondrial deoxyribonucleoside kinase Tk2 and studied the consequences of Tk2 deficiency as well as the role of TK2 for the acute cytotoxicity of nucleoside analogs in a human cell line with partial deficiency of the enzyme.

**DNA SYNTHESIS**

The deoxyribonucleic acid is a linear polymer that stores our genetic information. James Watson and Francis Crick discovered in 1953 that the DNA molecule consisted of two long chains forming a double-stranded helix. Ribonucleic acid (RNA) is a single-stranded polymer, central to the synthesis of proteins. Both the DNA and RNA chains are build up by four types of nucleotide subunits. A nucleotide consists of a nucleoside, i.e. an organic nitrogen ring-structured base with a five-carbon sugar (pentose), and a phosphate-group. The pentose sugars in DNA and RNA are deoxyribose and ribose, respectively. In DNA there are four kinds of organic bases; the purines adenine (A) and guanine (G), and the pyrimidines thymine (T) and cytosine (C). In RNA, thymine is replaced with uracil (U). The phosphate group can be a mono-, di- or triphosphate (Figure 1).

Nucleosides are hydrophilic and have limited passive diffusion across the plasma membrane. The deoxyribonucleosides and ribonucleosides are therefore imported into the cells by nucleoside transport proteins. These proteins either facilitate diffusion or actively transport nucleosides across the membrane (Cass et al. 1999). They also mediate the uptake of both anticancer and antiviral nucleoside analogs (Baldwin et al. 1999). In mammalian cells there are two groups of nucleoside transporters; equilibrative nucleoside transporters (ENT) and concentrative nucleoside transporters (CNT). The ENTs mediate passive transport and are responsible for both the influx and efflux of nucleosides using their equilibrative gradient. They are widely distributed and occur in most cell types and tissues (Yao et al. 2001). The ENT family is subdivided into four different proteins. hENT1 is ubiquitously distributed in several tissues and hENT2 is found in a variety of tissues with the highest level in skeletal muscle. They are both predominantly located in the plasma membrane, but can also be found in the mitochondrial membrane. hENT3 is believed to be a transporter located in intracellular membranes and hENT4 has been suggested to be a transporter in brain and cardiac tissue. However, the role of these two ENT proteins in nucleoside transport processes have not yet been established (Damaraju et al. 2003; Lai et al. 2004; Zhang et al. 2007). The concentrative nucleoside transporters mediate influx of nucleosides, driven by the sodium gradient across the cell membrane. Three subtypes in this transport family have been cloned and characterized; hCNT1, hCNT2 and hCNT3. Both groups of nucleoside carriers also transport several different nucleoside analogs (Damaraju et al. 2003; Yao et al. 2001; Zhang et al. 2007).
The nucleosides have to be phosphorylated to the respective nucleoside triphosphates in the cytosol before they can be incorporated into the growing DNA strand during synthesis. The bases bind to each other as specific sets of complementary pairs in the DNA chain. Adenine pairs only with thymine, and cytosine only with guanine (Figure 2).

The mammalian genome consists of $3 \times 10^9$ base pairs. The nuclear DNA (nDNA) in the cells is organized in chromosomes, i.e. large DNA molecules that contain many genes and are associated with protective proteins such as histones. Eukaryotic cells have in addition to the DNA in their nucleus, a separate DNA genome in their mitochondria (mtDNA).
Spontaneous mutations contribute to genetic variation and are the basis for evolution. In all cells, from bacteria to eukaryotes, error frequencies range from $1$ error per $10^3$ to $10^6$ base replication (Kunkel and Bebenek 2000; Timsit 1999). Error in DNA synthesis is involved in the normal process of ageing but does also play an important role in diseases such as cancer. DNA replication is a process in which each parental strand is the template for the synthesis of a new complementary strand. The accuracy of DNA replication is fundamental to the genetic stability of the cell and is catalyzed by a group of enzymes known as polymerases. The DNA polymerases catalyze the addition of deoxyribonucleoside triphosphates to the $3'$-OH end of the growing DNA strand in the $5' \rightarrow 3'$ direction. Four of the human polymerases, $\alpha$, $\beta$, $\delta$ and $\epsilon$, located in the nucleus, are responsible for the replication and repair of nuclear DNA (Hubscher et al. 2000). The fifth polymerase, pol $\gamma$, is also encoded by the nucleus but is located in the mitochondrial membrane where it is responsible for the replication and repair of the mitochondrial genome (Graziewicz et al. 2006).

The DNA replication is not only based on the fidelity of the polymerases, it also requires a balanced supply of the four deoxyribonucleotides; dTTP, dATP, dCTP and dGTP. An imbalance can have genotoxic effects resulting in point mutations, frame shift mutations, DNA breakdown, cell growth arrest and consequently cell death (Mathews 2006; Reichard 1988; Song et al. 2003). Even when the dNTP pools are balanced, an increased dNTP pool level may result in decreased proofreading efficiency (Mathews 2006).

Nuclear DNA replication occurs only in proliferating cells during the S-phase of the cell cycle. The mitochondrial DNA replication is however active independently of the cell cycle phase (Rampazzo et al. 2000a).
DEOXYRIBONUCLEOTIDE SYNTHESIS

There are no carrier proteins for the nucleotides in the cell membrane and diffusion over the plasma membrane is prevented by the negative charge of the phosphate groups. The nucleotides must therefore be synthesized within the cells before they are incorporated into DNA. The dNTP can diffuse freely through the nuclear pores thus creating equilibrium of the dNTPs between the cytosol and the nuclei (Bertoli et al. 2005). The four pools have different sizes with the dTTP pool normally being the largest and dGTP the smallest, the latter representing only 5-10% of the total dNTP pool (Song et al. 2003). The size of the cytosolic dNTP pools of cultured cells changes with the growth phase of the cell (Rampazzo et al. 2004).

Mitochondria have been shown to have dNTP pools that are separated from the pools in the cytosol. The cytosolic and mitochondrial dNTP pools are however in exchange using mitochondrial transporters (Pontarin et al. 2003). It has been concluded that mitochondrial dNTP pools reflect the size of the cytosolic pools (Rampazzo et al. 2004).

There are two different pathways by which the dNTPs can be synthesized, the \textit{de novo} pathway and the salvage pathway (Figure 3).

![Figure 3. The \textit{de novo} and salvage pathways of the deoxyribonucleoside metabolism in human cells](image-url)
THE \textit{DE NOVO} PATHWAY

In mammalian cells the majority of the deoxyribonucleotides are synthesized by the \textit{de novo} pathway. In proliferating cells this pathway is the main provider of dNTPs needed for DNA replication (Reichard 1988; Wright et al. 1990). Initially ribonucleoside monophosphates are synthesized from low molecular weight precursors like ribose, amino-acids, CO$_2$ and NH$_3$ (Reichard 1988). The monophosphates are then further phosphorylated to their diphosphate counterpart by nucleoside monophosphate kinases (NMPK). The regulatory step in the \textit{de novo} synthesis is the reduction of the 2'-hydroxyl group of the ribonucleoside diphosphate to the corresponding deoxyribonucleoside diphosphate. This reaction is catalyzed by ribonucleotide reductase (RR) (Mathews 2006; Munch-Petersen et al. 1998; Reichard 1988).

The RR enzyme has a dominating role in both the regulation of dNTP pool sizes and their composition by directing the total flow of metabolites into the DNA and dividing the flow into separate channels via an allosteric mechanism, maintaining the balance between the four pools (Gazziola et al. 2001). Mammalian RR is composed of two non-identical subunits with genes located on different chromosomes (Bjorklund et al. 1990). The larger of the subunits, R1, is a homodimer of 86 kDa and contains a substrate binding site and sites for allosteric regulation of the substrate specificity of the enzyme. The smaller R2 subunit, a homodimer of 43 kDa, contains a di-iron centre and two tyrosine residues essential for the reduction activity (Bianchi and Spychala 2003; Jordan and Reichard 1998). The level of R1 is almost constant throughout the cell cycle in proliferating cells (Engstrom et al. 1985). The level of R2 is cell cycle regulated showing strictly S-phase-correlated expression (Bjorklund et al. 1990; Eriksson et al. 1984). RR is an important factor in the regulation of the cell proliferation and in particular the level of the R2 subunit plays an essential role in regulating DNA synthesis (Eriksson et al. 1984; Jordan and Reichard 1998).

A novel RR gene, p53R2, highly similar to RR subunit R2 and forming active RR complexes with R1, has been identified. It contains a p53 binding site and has been reported to be directly involved in p53-regulated DNA repair (Nakano et al. 2000; Tanaka et al. 2000). p53 is a transcription factor involved in the DNA damage response and is frequently mutated in different types of cancers. In normal conditions, p53 exerts a tumor-suppressing function by regulating cell-cycle arrest or by inducing apoptosis. The latter is a process important both for normal events in the development of a complex organism, as well as for abnormal events such as tumorigenesis (Scheffler 2001a). Expression of p53R2 differed from R2 by being constitutively expressed in proliferative and nonproliferative tissues, allowing a low but constant dNTPs synthesis throughout the cell cycle (Hakansson et al. 2006). These findings suggest that there are two pathways in human cells that supply dNTPs \textit{de novo} for DNA synthesis: through the activity of R2, which is involved in normal maintenance of dNTPs for DNA replication during the S-phase in a cell cycle-dependent manner, and through p53R2, which supplies dNTPs for DNA repair in G0/G1 cells in a p53-dependent manner (Yamaguchi et al. 2001). Transcripts encoding R2 are undetectable in tissues with predominantly resting
cells such as heart, brain and muscle, implying that these tissues entirely rely on p53R2 for de novo dNTP synthesis (Bourdon et al. 2007).

The final step in the de novo pathway is the addition of the third phosphate group in a reaction catalyzed by a family of nucleoside diphosphate kinases (NDPK).

THE SALVAGE PATHWAY

In quiescent or terminally differentiated cells the salvage pathway is important to provide deoxyribonucleotides necessary for DNA replication and repair (Arner and Eriksson 1995; Cass et al. 1999). In this pathway the deoxyribonucleosides are imported through nucleoside carrier proteins from the extracellular space. This transport is the first regulatory step (Arner and Eriksson 1995; Griffith and Jarvis 1996; Pastor-Anglada et al. 1998). The following step is the phosphorylation of the nucleosides to the corresponding deoxyribonucleotide monophosphates catalyzed by deoxyribonucleoside kinases (dNK) (Arner and Eriksson 1995; Eriksson et al. 2002). The reaction is irreversible, but the monophosphates can be dephosphorylated by 5′-nucleotidases (5′-NT). The simultaneous activities of two opposing reactions, in this case a deoxyribonucleoside kinase and a 5′-nucleotidase, is called a substrate cycle and it regulates the cytosolic dNTP pools in addition to the allosteric regulation by RR (Arner and Eriksson 1995; Gazziola et al. 1999; Rampazzo et al. 2000a; Reichard 1988).

The continued phosphorylation of the monophosphates to their corresponding diphosphates and triphosphates occurs in two subsequent reversible steps that are catalyzed by nucleoside monophosphate kinases (NMPK) and nucleoside diphosphate kinases (NDPK) respectively (Van Rompay et al. 2000). Because of the constant function of the pathway, salvage enzymes play an essential role in activating several antiviral and cytostatic nucleoside analogs (Arner and Eriksson 1995).
DEOXYRIBONUCLEOSIDE KINASES AND 5'-NUCLEOTIDASES

There are four different deoxyribonucleoside kinases in mammalian cells; thymidine kinase 1 (TK1), deoxycytidine kinase (dCK), thymidine kinase 2 (TK2) and deoxyguanosine kinase (dGK) (Arner and Eriksson 1995; Arner et al. 1992). Three of the kinases, dCK, TK2 and dGK, are evolutionary closely related and their amino acid sequences share 40% identity (Saada et al. 2001). All four kinases have distinct but overlapping specificities (Table 1). Both TK2 and dGK are located in the mitochondria and together they phosphorylate all four natural deoxyribonucleosides (Arner and Eriksson 1995). The kinases are feedback regulated by the end-product triphosphate (Eriksson et al. 2002). dNKs are of medical interest as the phosphorylation of chemotherapeutic nucleoside analogs used in treatment of cancer and viral diseases depends on their phosphorylation activity (Eriksson et al. 2002).

<table>
<thead>
<tr>
<th>Deoxyribonucleotide kinase</th>
<th>Natural substrates</th>
<th>Subcellular location</th>
</tr>
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<tbody>
<tr>
<td>Thymidine kinase 1 (TK1)</td>
<td>dThd, dUrd</td>
<td>cytosol</td>
</tr>
<tr>
<td>Thymidine kinase 2 (TK2)</td>
<td>dThd, dUrd, dCyd</td>
<td>mitochondria</td>
</tr>
<tr>
<td>Deoxycytidine kinase (dCK)</td>
<td>dCyd, dAdo, dGuo</td>
<td>cytosol, nucleus</td>
</tr>
<tr>
<td>Deoxyguanosine kinase (dGK)</td>
<td>dGuo, dAdo, dIno</td>
<td>mitochondria</td>
</tr>
</tbody>
</table>

Table 1. Natural substrates and subcellular locations of the four human deoxyribonucleoside kinases

THYMIDINE KINASE 1

Thymidine kinase 1 is a tetramer of ~100 kDa and the gene is localized on chromosome 17 (Kuo et al. 1996; Petty et al. 1996). The protein is located in the cytosol and widely distributed in all tissues (Arner and Eriksson 1995). TK1 is cell cycle regulated and expressed during the S-phase (Bianchi et al. 1997). TK1 activity is therefore high in proliferating cells but almost absent in quiescent cells (Arner and Eriksson 1995; Eriksson et al. 2002; Sherley and Kelly 1988). TK1 has a restricted substrate specificity and phosphorylates only thymidine and deoxyuridine (Munch-Petersen et al. 1991). However, TK1 can also phosphorylate several clinically important nucleoside analogs such as AZT and d4T, both used in HIV-treatment (Eriksson et al. 2002). It is able to use most phosphate donors but ATP shows the highest efficiency (Ellims and Van der Weyden 1981).

DEOXYCYTIDINE KINASE

Deoxycytidine kinase is a dimer of 60 kDa composed of two 30 kDa subunits (Arner and Eriksson 1995; Innoceta et al. 2002). The gene is located on chromosome 4 and is ubiquitously expressed throughout the cell cycle (Bianchi et al. 1997; Stegmann et al. 1993). The enzyme phosphorylates deoxycytidine, deoxyadenosine and deoxyguanosine. dCK has been intensively studied since this enzyme also phosphorylates a number of clinically important anticancer and antiviral drugs, such as ddC, araC, CdA, dFdC and 3TC (Eriksson et al. 2002;
dCK can use UTP, ATP and other nucleoside triphosphates as phosphate donors (Eriksson et al. 2002; Johansson et al. 1997). The enzyme is tissue specific and has a high expression in lymphatic tissues and T-lymphoblasts such as immature T-cells of the thymus. These cells phosphorylate dCyd more actively than most other cell types (Arner and Eriksson 1995; Bianchi et al. 1997). Although its lymphoid-specific activity, dCK is also expressed in solid tumors (Sasvari-Szekely et al. 1998). Elevated levels of dCK expression have been detected in several malignant tumors and this has been implicated as a mechanism of tissue targeted cytotoxicity of dCK phosphorylated nucleoside analogs in lymphoblasts (Durham and Ives 1969; Ruiz van Haperen et al. 1993; Spasokoukotskaja et al. 1995). dCK has previously been described as a cytosolic enzyme but Johansson et al demonstrated a nuclear localization signal in the N-terminal region of dCK (Johansson et al. 1997). Although the role of the nuclear localization signal remains to be elucidated, the intracellular location appears not to be important for the cytotoxicity of nucleoside analogs.

THYMIDINE KINASE 2

Human thymidine kinase 2 was cloned in the 1990s by two independent research groups, Johansson et al and Wang et al (Johansson and Karlsson 1997; Wang et al. 1999). TK2 is a dimer of 58 kDa with a mitochondrial targeting signal. The TK2 gene is located on chromosome 16 (Johansson and Karlsson 1997). It is expressed in most tissues throughout the cell cycle with the highest expression in liver, pancreas, muscle and brain (Arner and Eriksson 1995; Arner et al. 1992; Johansson and Karlsson 1997). Skeletal muscle has TK2 levels that are 5- to 14-fold lower than in heart, liver and fibroblasts, which may make this tissue specifically sensitive to TK2 malfunctioning (Wang et al. 2005). The TK2 activity in tissue extracts appears to be proportional to the cellular mitochondrial content rather than to the growth state of the cells (Arner et al. 1992). TK2 phosphorylates dThd, dCyd and dUrd as well as the nucleoside analogs BVDU, AZT and ddC (Eriksson et al. 2002; Perez-Perez et al. 2008; Rossi et al. 1999). In proliferating cells TK1 activity is dominating and TK2 represents only a fraction of the total cellular TK levels. When the cells are resting TK1 is almost undetectable and TK2 is the only thymidine phosphorylating enzyme (Johansson and Karlsson 1997). It has been suggested that mitochondrial TK2 activity plays an important role in mitochondrial homeostasis (i.e. maintenance of mitochondrial DNA and proper dNTP pool balances) and mitochondrial toxicity of some nucleoside analogues (Perez-Perez et al. 2008).

DEOXYGUANOSINE KINASE

Deoxyguanosine kinase is a dimer of 58 kDa and the gene is localized on chromosome 2 (Arner and Eriksson 1995; Johansson et al. 1996). The dGK sequence contains a mitochondrial targeting signal and the enzyme is located in the mitochondrial matrix (Gower et al. 1979; Johansson et al. 1996; Jullig and Eriksson 2000; Lewis et al. 2003). dGK phosphorylates dGuo, dAdo and dIno as well as the anticancer analogs araG and CdA (Gower et al. 1979; Wang et al. 1993). In cells
with both dGK and dCK, the latter is most likely the main contributor in activation of many analogs, such as araC, CdA and dFdC. However, in tissues that do not contain dCK, dGK is to a large extent responsible for the activation of purine nucleoside analogs (Eriksson et al. 2002; Zhu et al. 1998). dGK is constitutively expressed throughout the cell cycle with the highest expression in muscle, brain, liver and lymphoid tissues (Johansson and Karlsson 1996; Johansson and Karlsson 1997). The dGK enzyme level is suggested to be proportional to the amount of mitochondria in the tissues (Arner and Eriksson 1995).

**DROSOPHILA MELANOGASTER DEOXYRIBONUCLEOSIDE KINASE**

The fruit fly *Drosophila melanogaster* is one of the most studied organisms in biology and serves as a model system for the investigation of many developmental and cellular processes common to higher eukaryotes, including humans. It contains only one single deoxyribonucleoside kinase (*Dm*-dNK) that was purified and cloned by two independent groups in the late 1990s. The Munch-Petersen-group concluded the protein to be a dimer with a total molecular mass of 60 kDa and the *Dm*-dNK gene to be located on chromosome 3 (Munch-Petersen et al. 1998).

Johansson et al showed that the kinase is closely related to the human deoxyribonucleoside kinase enzyme family, in particular with TK2, but that its substrate specificity was most similar to dCK (Johansson et al. 1999). *Dm*-dNK is targeted to the cell nucleus when expressed in human cell lines (Zheng et al. 2000). The enzyme is multifunctional and is, in contrast to human enzymes, a multisubstrate nucleoside kinase. It phosphorylates all the natural nucleosides as well as several anticancer and antiviral nucleoside analogs, although pyrimidine nucleosides are the preferred substrates (Johansson et al. 1999). The catalytic rate of deoxyribonucleoside phosphorylation of *Dm*-dNK depends on the substrate, but is 10- to 100-fold higher than what has been reported for the mammalian enzymes. These unique properties have lead this enzyme to be evaluated as a suicide gene (Zheng et al. 2000; Zheng et al. 2001a; Zheng et al. 2001b).

The first clinical trials for suicide gene therapy was introduced in 1990 (Verma and Somia 1997). Suicide gene therapy focuses on making cells more sensitive to chemotherapeutics. The principle of this method is the transfer of a new gene into a cell where the inserted gene then encodes an enzyme that can convert an inactive prodrug into a cytotoxic metabolite which then kills selected cells (Lal et al. 2000). The transfer of the gene encoding HSV-1 TK into malignant cells and subsequent treatment with ganciclovir is the most commonly studied strategy of suicide gene therapy (Culver et al. 1992; Moolten and Wells 1990). *Dm*-dNK and *Dm*-dNK mutants with increased specificity towards certain nucleoside analogs are interesting candidates to be used as suicide genes in gene therapy (Eriksson et al. 2002). It has been shown that overexpression of *Dm*-dNK enhances the sensitivity of cancer cells to several cytotoxic nucleoside analogs (Zheng et al. 2000).

The success of suicide gene therapy also depends on the bystander effect, i.e. the killing of adjacent cells (Moolten and Wells 1990). Not all tumor cells in vivo are
transfected with the suicide gene and instead the neighboring cells are killed by the transfer of phosphorylated nucleoside analogs between cells via gap junctions, which results in a larger portion of dead cells (Johansson et al. 1999; Mesnil et al. 1996; Zheng et al. 2000). The mechanism behind this effect is not yet fully understood.

5'-NUCLEOTIDASE
5'-nucleotidases are part of the substrate cycle that regulates dNTP pools, by catalyzing the dephosphorylation of the monophosphates into a nucleoside and a phosphate group. The 5'-NTs consists of a large family of enzymes with different subcellular localization, pH sensitivity, substrate specificity and dependency of ATP (Gazziola et al. 2001; Rampazzo et al. 1999). One nucleotidase (dNT-1) has previously been described and cloned by Rampazzo et al and was reported to be a cytosolic enzyme continuously expressed throughout the cell cycle and responsible for dephosphorylation of both purine and pyrimidine nucleotides (Rampazzo et al. 2000b). Overproduction of dNT-1 have been shown to increase the excretion of pyrimidine nucleosides maintaining the pool balance (Gazziola et al. 2001). Another identified 5'-NT is dNT-2, which is located in the mitochondria (Rampazzo et al. 2000a; Reichard 1988). dNT-2 has in contrast to dNT-1 a more limited substrate specificity and has been suggested to participate in a dThd substrate cycle thereby regulating the intramitochondrial dTTP pool (Rampazzo et al. 2000a).
NUCLEOSIDE ANALOGS

Nucleoside analogs mimic natural nucleosides in terms of uptake and metabolism. They are toxic compounds used as chemotherapeutic agents in both antiviral and anticancer therapy (Table 2).

The toxicity is classified according to the structure and chemical properties of the specific analog. General symptoms of nucleoside analog toxicity include peripheral neuropathy, myopathy, bone marrow suppression and pancreatitis (Johnson et al. 2001; Lewis and Dalakas 1995; Petit et al. 2003). This toxicity can either be acute but sometimes also be delayed and occur after several weeks or months of drug exposure (Lewis et al. 1996). Usually the symptoms are reversible upon withdrawal of drug treatment (Johnson et al. 2001). Effectiveness and toxicity of any given nucleoside analog depend on several factors including uptake, transport, metabolic activation, incorporation and degradation (Lee et al. 2003). Mitochondrial toxicity is a severe side effect of several clinically used nucleoside analogs (Petit et al. 2003).

<table>
<thead>
<tr>
<th>Nucleoside analog</th>
<th>Deoxyribonucleoside kinase</th>
<th>Clinical use</th>
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<td>hairy cell leukemia, CLL</td>
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<tr>
<td>AraC</td>
<td>dCK</td>
<td>acute leukemia</td>
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<tr>
<td>AraG</td>
<td>dGK, dCK</td>
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<td>dFdC</td>
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<td>BVDU</td>
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Table 2. Clinical use of anticancer and antiviral nucleoside analogs

MECHANISM OF ACTION

Permeation through the plasma membrane is the first step of both anticancer and antiviral activity (Yao et al. 2001). Nucleoside transporters mediate the extracellular uptake of anticancer and antiviral nucleoside analogs and have been linked mechanistically with drug resistance and toxicities (Damaraju et al. 2003; Zhang et al. 2007). The nucleoside analogs are then phosphorylated to their triphosphate form and incorporated into cellular or viral DNA, causing termination of DNA chain elongation. The principal mode of action of all analogs is competing with normal dNTPs for the active site of DNA polymerase leading to
the incorporation of the triphosphate form into the replicating DNA strand with a subsequent inhibition of DNA synthesis (Petit et al. 2003).

A side effect of several antiviral analogs is their mitochondrial toxicity. These analogs are phosphorylated to their active triphosphate form, either in the cytosol and shuttled into the mitochondrial matrix by transport proteins, or carried into the mitochondria by transporters and then phosphorylated by mitochondrial nucleoside kinases (Lewis et al. 2003). Inside the mitochondria they inhibit the mitochondrial polymerase \( \gamma \). There is most likely an exchange of deoxyribonucleoside mono-, di-, and triphosphates between the mitochondrial matrix and the cytosol, which could explain the activation of nucleoside analogs through mitochondrial enzymes (Arner and Eriksson 1995). The presence of antiviral nucleoside analogs in the DNA is also believed to damage repair processes leading to chain breaks and eventually cell death. There are different strategies by which the analogs inhibit or impair the DNA synthesis:

1) analogs lacking the 3'-hydroxyl group, such as ddC and AZT, serve as competitive, alternate substrates for DNA pol \( \gamma \), inhibiting the polymerase to extend the mtDNA chain. After incorporation they produce a premature termination of DNA chain elongation (Johnson et al. 2001);
2) analogs with an intact 3'-hydroxyl group do not act as absolute chain terminators. Instead they are incorporated into the DNA, severely impairing chain elongation by their structural modification, creating for example strand breaks. They also compete for the nucleotide binding site on pol \( \gamma \) (Johnson et al. 2001; Lewis and Dalakas 1995);
3) analogs can also interfere with key enzymes of the DNA synthesis involved in the supply of nucleotides, such as DNA polymerase and/or DNA ligase. This leads to changes in the dNTP pool and impaired proper DNA replication.

RESISTANCE

It is common that cells develop resistance towards nucleoside analogs. The resistance mechanisms are complex and together with the aforementioned cytotoxic side effects they comprise major problems in the treatment of cancer and viral infections with nucleoside analogs. Cells resistant to one analog can show cross resistance to other analogs (Agarwal et al. 1999; Han et al. 2004).

Antiviral resistance is mainly acquired through mutations in viral enzymes leading to impaired binding of the nucleoside analogs or inhibited viral replication. Resistance to HIV for example, is usually acquired through mutations in the viral reverse transcriptase leading to reduced efficiency of the nucleoside analog (Agarwal et al. 1999). It has been demonstrated that drug-resistant HIV-strains can even be transmitted to drug-naïve individuals (Balzarini et al. 1998). Multinucleoside resistance in HIV-strains are usually developed starting with a virus with one (or several) mutations reducing the viral replication capacity. The mutant virus eventually acquires optimal replication competence and higher drug
Resistance levels by the introduction of additional compensatory mutations giving rise to cross resistance (Balzarini et al. 1998).

Resistance to cytotoxic nucleoside analogs used in chemotherapy of cancer is caused by cellular mechanisms. Three general mechanisms have been described by which this resistance can be acquired (Galmarini et al. 2001):

1) insufficient intracellular concentrations of nucleoside analog triphosphates which may be due to inefficient cellular uptake, decreased levels of activating enzymes or increased nucleoside analog degradation by increased levels of 5'-NT or deaminases, or expansion of dNTP pools;
2) the inability to achieve sufficient alterations in DNA strands or dNTP pools either by altered interactions with DNA polymerases, lack of inhibition of RR or inadequate p53 exonuclease activity;
3) defective induction of apoptosis (programmed cell death)

*In vitro* studies have demonstrated that cells deficient in nucleoside transporters are highly resistant to nucleoside analogs and that different cell lines exhibit cross resistance to a spectrum of nucleoside analogs due to reduced uptake. It has been shown that the toxicity of several antiviral analogs, such as ddC, AZT and ddI, are highly dependent on the hENT2 level (Lai et al. 2004; Zhang et al. 2007).

Decreased dCK and TK expression in cells have been reported to result in resistance to the nucleoside analogs ddC and AZT respectively. This indicates that the enzyme plays a key role in the metabolism and pharmacological activities of these analogs (Agarwal et al. 1999; Innoceta et al. 2002; Magnani et al. 1995a; Wu et al. 1995).

It has been shown that increased activity of the 5'-nucleotidase dNT-1 can lead to resistance of both antiviral and anticancer analogs including ddC, CdA and dFdC (Hunsucker et al. 2001). Extra- and intracellular deaminases are capable of transforming deoxyribonucleosides and deoxyribonucleotide monophosphates into inactive compounds. Elevated levels of cytidine deaminase have been connected to resistance to araC and dFdC (Neff and Blau 1996).

Overexpression of both the RR subunits R1 and R2 can lead to resistance to nucleoside analogs such as dFdC in cancer cells (Davidson et al. 2004).

In the future, the greatest benefit, in terms of reduced risk of resistance development, may be expected from therapies using multiple drug combinations.
CdA (2-chloro-2’-deoxyadenosine, cladribine) is a deoxyadenosine nucleoside analog, phosphorylated by dCK and dGK. It is effective in the treatment of hairy cell leukemia and of other lymphoproliferative disorders such as chronic lymphocytic leukemia (CLL) (Arner and Eriksson 1995). CdA interferes with DNA causing DNA strand breaks leading to chain termination with subsequent phase specific apoptosis. CdA can also indirectly inhibit DNA replication through inhibition actions on RR, causing a subsequent reduction of the dNTP pools (Arner 1996; Galmarini et al. 2001).

AraC (1β-D-arabinofuranosylcytosin, cytarabine) is a deoxycytidine analog phosphorylated by dCK. It was developed in 1967 as one of the first nucleoside analogs (Damaraju et al. 2003). It is used predominantly in hematological malignancies such as acute myeloid leukemia but also in combination with other anticancer drugs in treatment of leukemias and lymphomas (Galmarini et al. 2001; Van Rompay et al. 2003). Cytotoxicity is caused by a combination of DNA polymerase inhibition and DNA chain termination by incorporation of araCTP.

AraG (9β-D-arabinofuranosylguanine, nelarabine) is a deoxyguanosine analog phosphorylated by dGK but also by dCK, although less efficiently (Curbo et al. 2001). It was approved in 2005 for treatment of T cell acute lymphoblastic leukemia. The limiting factor, when used clinically, is its neurotoxicity. Other adverse effects include myopathy, myelosuppression and loss of peripheral sensitivity (Lewis and Dalakas 1995). The mechanism of action of araG is not yet fully understood, but it has been suggested that the analog induces apoptosis (Curbo et al. 2003).

dFdC (2’,2’-difluorodeoxycytidine, gemcitabine) is a deoxycytidine analog phosphorylated by dCK and TK2. It is approved for treatment of pancreatic, breast and non-small cell lung cancers and other solid tumors (Noble and Goa 1997). dFdC gets incorporated into the growing DNA strand and after an
additional natural nucleotide is added, the incorporated dFdC is masked. The DNA polymerase is unable to proceed, therefore preventing DNA repair by base pair excision. The process is called “masked DNA chain termination” (Huang and Plunkett 1995). dFdC does not terminate DNA replication as effectively as for example araC, but once dFdC-TP gets incorporated, it is much more difficult for the cells to remove the triphosphate from the DNA chain compared to araC-TP (Ruiz van Haperen et al. 1993).

**ANTIVIRAL NUCLEOSIDE ANALOGS**

HIV is a retrovirus with the viral enzyme reverse transcriptase (HIV-RT) necessary for its replication. The most successful anti-HIV therapies include compounds that interfere with the function of this enzyme. HIV-RT lacks a proofreading ability resulting in approximately 1 error per genome. The usage of this error-prone enzyme contributes to viral pathogenicity by creating drug resistant variants or by escaping immune surveillance (Mathews 2006). Different strategies have been developed to delay the emergence of drug resistant HIV-1 during therapy. One strategy involves using combinations of drugs that have interacting drug resistance mutations, such that resistance to one drug results in re-sensitization of the virus to the second drug. Another strategy involves combinations of HIV-RT inhibitors and HIV protease inhibitors suppressing viral replication and significantly delaying the development of resistance to both classes of drugs (Mayers 1997). However, combination treatment using at least three anti-HIV drugs is the most widely used strategy and has become standard when treating HIV-infected patients. The combination comprises one or two nucleoside analogs inhibiting the viral reverse transcriptase (NRTIs) and one non-nucleoside reverse transcriptase inhibitor interacting with the RT at an allosteric, non-substrate binding site, or a protease inhibitor specifically inhibiting the virus-associated protease (De Clercq 2002; Lewis et al. 2003; Perez-Perez et al. 2008). This highly active anti-retroviral therapy (HAART) effectively slows the rate of viral replication sufficiently to reduce the viral load and thereby also slows the rate of evolution of new, resistant forms of the virus. The result is reduced morbidity and prolonged life of HIV-1 infected patients (Groschel et al. 2002; Lee et al. 2003). NRTIs are structurally similar and this would normally suggest that these compounds share similar mechanisms of toxicity. The picture however is much more complex. The most toxic antiviral analogs such as ddC and d4T, all bind more tightly to HIV-RT than their corresponding natural dNTP and this stronger binding is largely responsible for their greater antiviral effect (Lee et al. 2003).

**AZT** (3’-azido-2’,3’-dideoxythymidine, zidovudine) is a deoxythymidine nucleoside analog, phosphorylated by TK1 and TK2 (Eriksson et al. 2002; Van Rompay et al. 2003). It was introduced into clinical practice in 1987, as the first nucleoside compound approved for use in patients with HIV.
infection. It lacks the 3'-hydroxyl group leading to terminated DNA chain elongation (Benbrik et al. 1997). AZT is primarily associated with hematological toxicity and myopathy and decreases mtDNA level in myoblasts and lymphoblasts (Lewis and Dalakas 1995; Lund et al. 2007). AZT resistance increases with the sequential accumulation of multiple mutations during treatment over many months in vivo. The resistant cells are highly cross-resistant to cytotoxic and anti-HIV effects of different thymidine and deoxycytidine analogs such as d4T, 3TC, ddC, dFdC and araC (Groschel et al. 2002; Zhang et al. 1994). The human polymerases α, β and γ can all interact with AZT, although with different efficiencies. The analog is however, most efficiently incorporated by the HIV reverse transcriptase making the drug an important factor in HAART (Copeland et al. 1992).

ddI (2’-3’-dideoxyinosine, didanosine) is a deoxyadenosine analog that followed AZT as being the second antiretroviral drug accepted for clinical use. Together with AZT and ddC it is an anti-HIV drug and can not form 3’-phosphodiester linkages leading to DNA chain termination (Benbrik et al. 1997). ddI decreases mtDNA abundance, causes destruction of mitochondria and increases lactate production in neuronal cell lines (Lewis and Dalakas 1995). ddI causes side effects such as pancreatitis and peripheral neuropathy (Lund et al. 2007).

d4T (2’-3’-didehydro-3’-deoxythymidine, stavudine) is a deoxythymidine analog phosphorylated by TK1. The analog is terminally incorporated into DNA, resulting in the formation of truncated DNA strands (Balzarini 1994). It is associated with lipodystrophy and increased risk of lactic acidosis. It has an anti-HIV activity and is a moderate inhibitor of pol γ (Lund et al. 2007; Medina et al. 1994). One side effect when treating with d4T is a reversible peripheral neuropathy (Lewis and Dalakas 1995).

3TC (2’-deoxy-3’-thiacytidine, lamivudine) is a deoxycytidine analog phosphorylated by dCK. It was approved in 1998 for use against chronic hepatitis B virus (HBV) (Leung 2004). The analog is highly inhibitory to both HIV and HBV DNA polymerase. The triphosphate form is subsequently incorporated into DNA by HIV-RT, resulting in DNA chain termination and ultimately cessation of viral replication (Chang et al. 1992; Medina et al. 1994; Van Rompay et al. 2003). HBV-resistance to 3TC is mainly associated with a single
point mutation, Met550 → Val (Balzarini et al. 1998). Mutated viral variants are harbored in patients and when treatment with the 3TC is initiated, these viral variants rapidly emerge as the predominant circulating virus (Mayers 1997).

BVDU ((E)-5′-(2-bromovinyl)-2′-deoxyuridine, brivudine) is a deoxyuridine analog selectively phosphorylated by TK2 (Franzolin et al. 2006). The triphosphate form of the analog interferes as a competitive inhibitor or alternate substrate with both the viral or cellular DNA polymerases. It is one of the most efficient inhibitor of varicella zoster virus but also a potent and selective inhibitor against HSV-1 (De Clercq 2005; Grignet-Debrus et al. 2000).

2′,3′-DIDEOXYCYTIDINE
In 1992, the Food and Drug Administration approved the antiretroviral drug 2′,3′-dideoxycytidine, also known as ddC or zalcitabine, as the third drug licensed specifically for use in treating HIV. It was used in combination with AZT as a treatment option for adult patients with advanced HIV infection who showed signs of clinical or immunological deterioration. Early clinical trials have shown that an increase in immune cells, indicating enhanced disease-fighting capability in the body, has been greater and more sustained in patients treated with the combination of ddC and AZT than in those who received AZT alone.

ddC is an analog of the natural nucleoside deoxycytidine. It needs to be phosphorylated, like all nucleoside analogs, to its triphosphate form (ddCTP) to be pharmacologically active. This is mainly done by the nucleoside kinase dCK. It can to some extent also be phosphorylated by the kinase TK2 (Chen and Cheng 1992; Rossi et al. 1999). This limited phosphorylation by TK2 is believed to be due to the presence of dThd and dCyd in the mitochondrial compartment. The TK2 has a higher affinity to the natural nucleosides compared to ddC and therefore prefers them over the analog (Chen and Cheng 1992).

ddC is one of the most potent and severely toxic HIV inhibitor among the nucleoside analogs tested (Chen and Cheng 1992; Lee et al. 2003). The triphosphate form of ddC has been shown to have a much stronger affinity for HIV-1 reverse transcriptase than the natural substrate dCTP (Agarwal et al. 1999; Balzarini 1994; Petit et al. 2003; Rossi et al. 1999).
After several weeks of treatment approximately 30% of the patients develop a peripheral neuropathy and this is a limiting factor when treating with ddC (Dalakas et al. 2001; White 2001). This reversible condition is characterized by a mild muscle weakness and a painful tingling sensation in the feet and toes (Chen and Cheng 1989; Lewis and Dalakas 1995). The mechanism by which ddC causes this neuropathy is still unclear. Although not as pronounced, adverse effects also include granulocytopenia and thrombocytopenia (Rossi et al. 1999).

Mechanism of action
ddC is a hydrophobic molecule and therefore the transport mechanism is important for the passage of the analog into the cell (Balzarini 1994). ddC is transported through the plasma membrane by hENT1, hENT2 and hCNT3 (Yao et al. 2001; Zhang et al. 2007). In the cytosol ddC is rapidly phosphorylated to its active metabolite ddcTP and it interacts with viral polymerases inhibiting viral DNA replication. However, the ddC molecule does not only convert into ddcTP but also into less hydrophilic dideoxyliponucleotides, mainly ddcDP-choline. Neither the transport process of this metabolite, nor its route of biosynthesis or biological significance is known. It has been demonstrated that, under steady state conditions, ddC accumulates preferentially in the cell in the form of ddcDP-choline rather than ddcTP (Rossi et al. 1999).

ddC exhibits an unwanted side effect; severe mitochondrial toxicity. The analog is then either phosphorylated by dCK in the cytosol and thereafter transported into the mitochondria, or phosphorylated by TK2 inside the mitochondria (Chen and Cheng 1992; Magnani et al. 1995a; White 2001). The V_max for ddcTP uptake is four times lower than for ddcDP-choline suggesting the ddcDP-choline is probably the preferred ddC metabolite for entry into the mitochondria (Magnani et al. 1995a; Rossi et al. 1999). It has been suggested that ddcDP-choline might act as a reservoir form of ddcTP inside the mitochondria, thus contributing to mtDNA depletion (Rossi et al. 1999). Once inside the mitochondria, ddcTP inhibits polymerase γ (Chen and Cheng 1989; Chen and Cheng 1992; Dalakas et al. 2001). ddcC is considered the best substrate for incorporation by pol γ to which it has a much higher affinity than the nuclear pol α and β. It is also removed from pol γ with the least efficiency by the proofreading exonucleases (Hanes and Johnson 2008; Martin et al. 1994; White 2001). This polymerase γ inhibition leads to depletion of mtDNA (Medina et al. 1994). According to Brown et al there are five different mechanisms by which ddcTP can inhibit mtDNA replication:

1) incorporation of ddC into the 3' end of a replicating chain which would terminate elongation through the inability to form the subsequent 5'-phosphodiester linkage;
2) ddcTP can directly inhibit the processivity of the polymerase through competitive occupation of the nucleotide binding site;
3) the exonuclease domain of polymerase γ could be inhibited by ddcTP or cause the enzyme to repeatedly proof-read incorporation of the aberrant chain-terminating nucleotide. The inhibition of the exonuclease function has
not yet been demonstrated for ddC but does occur with other chain terminating nucleoside analogs;
4) any inhibition of pol β is expected to cripple the role of this enzyme in other DNA repair mechanisms;
5) the cytoplasmic nucleotide kinases and mitochondrial transporters are saturated or inhibited by ddC, thereby causing a limitation in mitochondrial nucleotides and a slower replication rate (Brown and Clayton 2002).

Cells treated with ddC show signs of changes in the mitochondrial ultrastructure. These changes can be swollen mitochondria, decreased number of cristae, distorted transverse orientation of the cristae and increased amount of vesicles in the mitochondria (Medina et al. 1994). However, all these changes are delayed which suggests that the initial defect caused by ddC, is reduction of the mtDNA content (Lewis et al. 1992; White 2001). Cells treated with various concentrations of ddC shows a concentration-dependent decrease in cell viability, mtDNA content and mitochondrial morphology (Rossi et al. 1999). ddC is incorporated only in the DNA of replicating mitochondria, resulting at any given time in normal and abnormal mtDNA and allowing for partial recovery upon discontinuation of the drug (Balzarini et al. 2001). It has also been shown that the metabolism of ddC is highly dependent on the cell species and striking differences in drug potency and intracellular metabolism have been observed among different examined cell lines (Balzarini et al. 1988). The species related differences in ddC activation suggest that other factors than the different rate limiting steps, specific for that analog, may be of importance.

Resistance

Patients treated with ddC can develop resistance to the drug either by viral or cellular mechanisms. Viral resistance is by far the most common, and is caused by mutations in the virus. These mutations can lead to a decreased binding efficiency of the nucleoside analog to the viral polymerase binding site, making the polymerase prefer the natural nucleosides over the analogs. Several mutations in HIV-RT have been linked to ddC resistance (Fitzgibbon et al. 1992; Schinazi et al. 1993). Resistance to ddC can also occur when treating the patients with other nucleoside analogs, in particular ddI and/or AZT. Three substitution mutations of HIV-RT; Lys65 → Arg, Leu74 → Val and Met184 → Val, have been reported to induce ddC resistance following treatment with ddI (Eron et al. 1993; Gu et al. 1994; St Clair et al. 1991).

Cellular resistance, induced from in vitro long term exposure of cells to therapeutic ddC concentrations, depends solely on cellular factors. The cells exhibits a normal viral RT and resistance may be due to other mechanisms, for example a reduced ability to accumulate ddC phosphorylated derivatives (Magnani et al. 1995b). Deficiency in the deoxyribonucleoside kinase dCK appears to be the most important factor in the case of cellular resistance to ddC (Innoceta et al. 2002; Magnani et al. 1995b; Owens et al. 1992; Zhang et al. 1994). This dCK deficiency can be caused by mutations in the dCK gene or decreased mRNA levels (Han et al.
The dCK gene is highly susceptible to mutations suggesting that this enzyme is not essential for cell survival and is easily inactivated in cells growing in the presence of cytosine analogs (Innoceta et al. 2002). dCK mutated cells do not accumulate phosphorylated ddC and are resistant to its mitochondrial toxicity (Rossi et al. 1999). Deficiency in functional dCK is a common characteristic of cells resistant to anticancer analogs, such as araC, and it is also a mechanism behind resistance in acute myeloid leukemia. The identification of the molecular bases of acquired drug resistance in human cells is therefore of great interest. The understanding of the mechanisms behind drug resistance in leukemia may lead to the possibility to avoid drug failure in antiviral treatments, hence the need for further exploration in this area.
MITOCHONDRIA

The mitochondrion is a maternally inherited organelle within the cell. Its main function is to produce the energy required for the cell to survive and function. This energy is produced as ATP through the process of oxidative phosphorylation (White 2001). It is also responsible for many other important cellular processes such as apoptosis, intracellular signaling and intermediary metabolism of amino acids, lipids, cholesterol, steroids and nucleotides (Chinnery and Schon 2003; White 2001).

The mitochondrion contains a double membrane where the outer membrane is permeable to small molecules up to 10 kDa (Figure 4). The inner membrane is impermeable to passage of ions and molecules and therefore transporter proteins are required for passage in and out of the matrix.

The mitochondrion is unique among the organelles in that it has its own DNA (mtDNA) separate from the nuclear DNA. MtDNA has been referred to as the 24th chromosome in humans and it was the first to be completely sequenced (Scheffler 2001a). The mitochondrial genome is a 16,5 kbp circular molecule with double stranded DNA. This mitochondrial DNA represents approximately 1% of the total cellular DNA (Song et al. 2003). The genome is very compact and does not contain introns or long non-coding segments (Anderson et al. 1981; Graziewicz et al. 2006). MtDNA is highly polymorphic with differences up to 60 base pairs in any two individuals (Chinnery and Schon 2003). MtDNA is present in 2-10 copies per mitochondria and 1000-10 000 copies per cell. Copy number tends to vary according to the oxidative capacity of the cells and can range from several hundreds in some cells to over 100 000 in oocytes (Brown and Clayton 2002; Song et al. 2003).
The mtDNA codes for 13 polypeptides that are a part of complex I, III, IV and ATP synthase in the respiratory chain. Of the approximately 850 polypeptides necessary to maintain mitochondrial function only these 13 polypeptides are encoded by mtDNA (Wang et al. 2005). The rest of the mitochondrial respiratory chain polypeptides are encoded in the nucleus together with other essential factors needed for intramitochondrial transcription and translation (Chinnery and Schon 2003). It also encodes two rRNA and 22 tRNA needed for the intramitochondrial protein synthesis (Chinnery and Schon 2003).

The mtDNA molecules are all identical in a healthy individual at birth, a state called homoplasmy. The mutation rates in human mtDNA are 20- to 100-fold higher than that of the nuclear DNA, leading to the accumulation of mutations over time and the coexistence of wild type and mutated mtDNA molecules in the same individual, known as heteroplasmy (Lee and Johnson 2006). The reason for this higher error rate can be the less efficient repair system compared to the nuclear and the lack of protective histones. It is important to mention that mitochondrial abnormalities also occur as part of normal ageing.

MITOCHONDRIAL DNA REPLICATION

mtDNA are synthesized in both proliferating and non-proliferating cells. Their replication is therefore independent of the cell cycle and not linked to the nuclear DNA replication (Bogenhagen and Clayton 1977). This means that their need for available dNTPs is constant. It was thought that the salvage pathway was the only pathway for dNTP synthesis needed for mtDNA replication in resting cells. Two of the salvaging deoxyribonucleoside kinases, TK2 and dGK, are localized in the mitochondria and they may provide the mtDNA replication with precursors without dependence on the de novo synthesis or on the transport of deoxyribonucleotides across the mitochondrial membrane (Arner and Eriksson 1995; Bogenhagen and Clayton 1977). Recently, however, it has been reported that p53R2 substitutes for the absence of R2 as part of the RR complex in quiescent cells, providing de novo synthesis of dNTPs throughout the cell cycle (Bourdon et al. 2007; Hakansson et al. 2006; Rampazzo et al. 2007).

Accordingly; there are several different pathways by which the dNTP could arise within the mitochondria. They could either be transported over the membrane as ribonucleosides and reduced within the mitochondria by mitochondrial RR, transported from the cytosol with mitochondrial carriers or diffuse into the organelle and phosphorylated to the triphosphate form by mitochondrial kinases (Figure 5).
Although the deoxyribonucleosides can enter the organelle through transport proteins and become phosphorylated to their triphosphate forms by mitochondrial kinases, the membrane is impermeable to nucleotides. Therefore, the exchange of dNTPs between the cytosol and the mitochondria is dependent on specific mitochondrial transporters (Rampazzo et al. 2007). Mitochondrial carriers use the concentration gradient of the solutes and/or the H’ electrochemical potential generated across the inner mitochondrial membrane by the respiratory chain as their driving force. Several carriers have been characterized and the existence of several more have been suggested by functional studies (Palmieri 2004). Among them are a transporter for dTMP that has recently been reported by Ferraro et al (Ferraro et al. 2006). Another transporter reported to carry deoxynucleotides over the mitochondrial membrane is the deoxynucleotide carrier (DNC) reported by Dolce et al (Dolce et al. 2001). This protein has been suggested to transport dNDP and ddNDPs into the mitochondria. Later studies however, performing loss-of-function mutations in DNC, revealed that these mutations did not disrupt mitochondrial deoxynucleotide pools. Instead it was suggested that DNC primarily transports the vitamin thiamine pyrophosphate (Lam et al. 2005; Lindhurst et al. 2006). Several mitochondrial transporters are still to be identified and characterized (Arco and Satrustegui 2005).

MtDNA synthesis requires several nuclear encoded enzymes and among them are the polymerase γ that catalyzes the replication reaction (Taanman 1999). Polymerase γ is the only polymerase found in mitochondria and it is responsible for both replication and repair of the mitochondrial genome. It consists of two polypeptides: a catalytic subunit (pol γA) containing the polymerase and the exonuclease sites, and a noncatalytic protein which improves processivity by increasing the rate of polymerization (Johnson et al. 2000). The exonuclease
activity is necessary for the proofreading of the growing DNA strand (Longley et al. 2001). It also increases the fidelity of replication, which is reported to be 1 error in 440,000 bases incorporated (Lee and Johnson 2006).

Another protein required for mitochondrial function and cellular survival is the mitochondrial transcription factor A (Tfam) (Pohjoismaki et al. 2006). It is a nuclear encoded protein of ~25 kDa needed for the initiation of transcription of mtDNA. Moreover, it has a role in regulating mtDNA replication (Larsson et al. 1998; Rantanen et al. 2001). Tfam is suggested to be associated with mtDNA copy number and increases the copy number by directly binding and stabilizing mtDNA (Ekstrand et al. 2004). The amount of mtDNA is directly proportional to the Tfam level and there is approximately 1 molecule of Tfam per 1000 bp of mtDNA (Ekstrand et al. 2004; Kang and Hamasaki 2005).

**MITOCHONDRIAL DISORDERS**

The manifestations of mitochondrial deficiencies have been implicated in ageing, Alzheimer's disease, Parkinson's disease and diabetes mellitus etc. Common for the pathophysiology of all mtDNA diseases is that they mainly affect postmitotic and energy demanding tissues such as nerve, muscle and pancreatic cells. Disruption of oxidative phosphorylation is central to the disease (James and Murphy 2002). Imbalance of mitochondrial nucleotide pools plays an important role in the pathogenesis of several other diseases, including mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), autosomal dominant progressive external ophthalmpoplegia, and Amish microcephaly (Song et al. 2003). Studies made on patients with mutations in the gene encoding ribonucleotide reductase subunit p53R2, demonstrated severe mtDNA depletion implying that p53R2 has a crucial role in dNTP supply for mtDNA synthesis (Bourdon et al. 2007).

Different organs can be affected not only by mutations in different genes, but even by mutations in different parts of the same gene. This could be affected by the fact that even within a given cell, not all mitochondria may be exactly the same depending on their location within the cytoplasm (Scheffler 2001b). Interestingly one single mutant can lead to several different syndromes and different genetic defects can cause the same phenotype (James and Murphy 2002). Individuals with a mitochondrial disease have either identical mtDNA deletions in all cells in the affected tissue or multiple mtDNA deletions in affected tissues (Krishnan et al. 2008). Deletions or point mutations of mtDNA are the most common defects seen in individuals with mtDNA-associated diseases (Krishnan et al. 2008). Shortened mtDNA may be replicated more quickly than native mtDNA leading to increased proportion of mtDNA mutants (Lewis and Dalakas 1995). Patients with pathogenic mtDNA defects often have a mixture of mutated and wild type mtDNA, i.e. heteroplasmy. The percentage of mutated mtDNA can vary widely among different patients, from organ to organ and even between cells within the same individual (Chinnery and Schon 2003). One possible explanation to the variability among patients carrying the same mutations is that a defect in the
nDNA may impair mtDNA replication in early embryogenesis, leading to variable levels of mtDNA depletion in different stem cells (Hirano et al. 2001). When the stem cells differentiate into different organs, tissue-specific depletion would occur. Heteroplasmy is the main problem when investigating mtDNA disorders (Chinnery and Schon 2003). Most mtDNA mutations are highly recessive and cells are able to tolerate a high percentage of mutated DNA (~80%) before they show signs of mitochondrial dysfunction (Cote et al. 2002; Krishnan et al. 2008; Trifunovic et al. 2004; White 2001). The percentage level of mutated mtDNA in clinically relevant tissues does correlate with the severity of disease and is the main factor in determining clinical expression of the mitochondrial disease.

The management of mitochondrial disease is largely supportive and aimed at identifying, preventing and treating complications whenever possible. The overall aim is to reduce the proportion of mutated mtDNA to subthreshold levels. Clinical trials have been carried out with varying degrees of success, but so far no consistent clinical improvements have been demonstrated.

**Mitochondrial DNA depletion syndrome**

Mitochondrial DNA depletion syndrome (MDS) was first described in 1991 by Moraes et al. (Moraes et al. 1991). It is an autosomally inherited heterogeneous group of mitochondrial disorders that can result from any disturbance in the mtDNA replication machinery. MDS specifically affects resting tissues, such as muscle, liver and central nervous system. Non- or slow-dividing cell with a lower turnover of mtDNA, accumulate a larger number of mtDNA defects, thereby increasing the probability and severity of cell damage (Chinnery and Schon 2003; Foli et al. 2001). MDS is characterized by a reduction in mtDNA copy number in affected tissues (Lewis et al. 2003). The decrease can be up to 5-30% of the normal mtDNA levels, leading to respiratory chain dysfunction and insufficient ATP production for cellular requirements. One of the most striking features of MDS is the remarkable tissue specificity of the molecular and biochemical abnormalities, with still unknown underlying mechanisms (Alberio et al. 2007).

MDS normally presents itself in infancy and early childhood with different combinations of myopathy, cardiomyopathy, encephalopathy and liver failure. Infants die within 12 months in the cases of severe MDS. If the disease appears later in life the symptoms are leukodystrophy and gastroenterological disease (Ducluzeau et al. 1999; Moraes et al. 1991; Taanman et al. 1997; Vu et al. 1998). Mutations in proteins encoded by five different genes have been linked to MDS;

1) TK2; associated with the myopathic form of MDS (Saada et al. 2001)
2) dGK; associated with the hepatocerebral form of the disease (Mandel et al. 2001)
3) pol γA; associated with the hepatocerebral form of MDS, more specifically Alpers disease (Naviaux and Nguyen 2005)
4) SUCLA2; associated with a more generalized encephalomyopathic form of the disease (Elpeleg et al. 2005)
5) MPV17; a new protein with an unknown function, that has lately been associated with hepatocerebral MDS (Spinazzola et al. 2006).

Skeletal muscle is especially sensitive to TK2-associated MDS. This can be explained by its high energetic demand in combination with a relative low TK2 activity in these tissues (Alberio et al. 2007). Mutations in the TK2 gene leading to MDS often leads to death by respiratory failure (Oskoui et al. 2006).

NUCLEOSIDE ANALOG INDUCED MITOCHONDRIAL TOXICITY

Several clinically used antiviral nucleoside analogs have been reported to cause acute and delayed toxicity to proliferating cells. This toxicity is mainly caused by inhibition of nuclear DNA replication and affects tissues such as bone marrow and gastrointestinal tract. Some nucleoside analogs also interfere with mtDNA replication and thereby cause severe side effects correlated to various levels of mitochondrial dysfunction (Agarwal and Olivero 1997; Barile et al. 1998; Chariot et al. 1999; Lewis and Dalakas 1995). The result of this interference may be three interrelated mechanisms which works in a sequential manner beginning with energy decline resulting from (i) mtDNA depletion leading to (ii) oxidative stress causing damage to mtDNA, and ultimately leading to (iii) mutations in the mitochondrial genome (Lee et al. 2003). Although mitochondrial depletion is the primary effect of nucleoside analog toxicity, several other mechanisms have been suggested. These could be the inhibition of endogenous nucleotide kinases, direct inhibition of oxidative phosphorylation, reactive oxygen species generation as well as mtDNA and nDNA mutagenesis (Lund et al. 2007). Clinical forms of toxicity spans from mild reversible neuropathy, myopathy and pancreatitis to severe multi-organ failure (Lewis and Dalakas 1995).

The polymerase γ is unique among the cellular DNA polymerases in that it is highly sensitive to inhibition by NRTIs, such as AZT-TP and ddC-TP (Cossarizza et al. 2003; Lewis et al. 2003). The clinical use of NRTIs have been associated with adverse effects such as neuropathy, lactic acidosis and bone marrow suppression (Foli et al. 2001). MtDNA has been shown to decrease in patients submitted to antiretroviral treatment. However, if the treatment is continued an increase in mtDNA level has been observed. This could suggest some compensatory repair process and/or a resistance-adaptation phenomenon to the mtDNA depletive effect of drugs (Petit et al. 2003). Not all NRTIs are efficiently removed by the exonuclease domain of pol γ contributing to the more severe toxicity of some analogs (Lee et al. 2003). Inhibition of pol γ leads to decreased mtDNA copy number resulting in oxidative stress, increased mtDNA mutations and decreased mitochondrial function (Lee and Johnson 2006; Lewis et al. 2003). This phenomenon is formulated in the “Polymerase γ hypothesis” that describes the mechanism by which nucleoside analogs could cause mitochondrial dysfunction. The hypothesis states that the nucleoside analog toxicity in selected tissues is reflected by the combined effects of four principal factors;

i) the tissue must have some dependence on oxidative phosphorylation;
ii) NRTIs must pass into the tissue itself;
3) NRTIs must be phosphorylated by cellular kinases;
4) the nucleoside analog must inhibit polymerase $\gamma$ activity by competing with the natural substrate or by chain termination.

This hypothesis provides a valuable model to understand the toxicity of several NRTIs in many tissues (Lewis and Dalakas 1995; Lewis et al. 2003; Lund et al. 2007).

The importance of nucleoside analog phosphorylation in the mitochondrial matrix for mitochondrial DNA damage is still unresolved and the subcellular localization of the nucleoside analog is probably of minor importance for mitochondrial toxicity whereas other determinants, such as the affinity for mitochondrial polymerase $\gamma$, are more important.
PRESENT INVESTIGATION

AIM OF PRESENT STUDY

This study was conducted to increase the knowledge about the effects of nucleoside analogs on mtDNA and the importance of deoxyribonucleoside kinases with focus on the mitochondrial kinase TK2. In an attempt to improve antiviral and anticancer therapy we performed site-directed mutagenesis on Dm-dNK aiming to find mutants with improved substrate specificity for application in suicide gene therapy. A strategy that would maintain the antiviral activity of nucleoside analogs but avoiding the mitochondrial toxicity of the drug would be an important therapeutic improvement. We therefore investigated the critical events involved in the mitochondrial toxicity of the antiviral nucleoside analog 2',3'-dideoxycytidine (ddC) by characterizing a cell line resistant to this analog. To increase the knowledge about the deoxynucleoside kinase TK2 and with the aim to investigate the enzyme in cell survival, DNA replication and nucleoside analog cytotoxicity, we generated and characterized a Tk2 deficient mouse line. Finally, in order to elucidate the importance of TK2 for the pharmacological effects of several nucleoside analogs, we investigated a human TK2 deficient human fibroblast cell line with respect to TK2 mediated nucleoside analog toxicity.

SPECIFIC INVESTIGATIONS

- The multisubstrate deoxyribonucleoside kinase from Drosophila melanogaster preferentially phosphorylates pyrimidine compared to purine nucleoside analogs. We used structural data and other comparisons to related nucleoside kinases in an attempt to engineer a Dm-dNK enzyme with improved ability to phosphorylate purine nucleoside analogs. Nine variants of Dm-dNK were genetically engineered by site-directed mutagenesis at amino acids 28 (Asn), 29 (Ile), 81 (Gln) and 114 (Phe). The Dm-dNK mutant enzymes were expressed in E. coli and the recombinant proteins purified. Phosphorylation of both deoxyribonucleosides as well as several purine and pyrimidine nucleoside analogs catalyzed by the Dm-dNK mutants was investigated.

- The nucleoside analog ddC cause delayed toxicity due to mtDNA depletion and mitochondrial dysfunction. We decided to generate cell lines resistant to the delayed toxicity of ddC in order to study the molecular events in cells associated with this phenotype. Two CEM cell lines with different levels of
resistance to the delayed toxicity of ddC were generated and these cells were characterized by:
- Investigation of the ddC resistant cell lines with respect to both the acute toxicity as well as the anti-HIV activity of multiple nucleoside analogs to identify patterns of cross-resistance.
- Quantifications of dNTP and ATP pools
- Expression profiling of the cell lines by RT-PCR quantification of deoxyribonucleoside kinase mRNA levels as well as microarray mRNA expression analysis of enzymes involved in the ddC import and phosphorylation.
- Quantification and sequencing of mtDNA from the resistant cell lines
- Quantification of mtDNA and the mitochondrial transcription factor A in cells treated with ddC or ethidium bromide.

• Inherited TK2 deficiency causes mtDNA depletion in humans. We decided to generate a Tk2 deficient mouse model to study the pathogenesis of MDS. The Tk2 gene was targeted and Tk2−/− mice were bred. The development of the mice and their survival were studied. MtDNA was quantified in tissues from several organs and mtDNA sequencing was performed to identify possible mtDNA mutations. Histology analysis of multiple organs was performed as well as electron microscopy analysis of mitochondria from heart muscle. Expression profiling with mRNA microarray analysis of enzymes involved in pyrimidine deoxyribonucleotide metabolism was also performed.

• TK2 phosphorylates several nucleoside analogs in vitro that also are substrates of other nucleoside kinases. To study the importance of TK2 mediated phosphorylation of nucleoside analogs for their cytotoxicity we obtained a human fibroblast cell line encoding a R130W mutant TK2 protein with decreased ability to phosphorylate nucleoside analogs. A recombinant TK2 protein with the R130W mutation was designed and the kinetic properties of this enzyme were compared the wild type enzyme. The growth rate, dTTP pool and incorporation of dThd into mtDNA in resting cells was determined for the TK2 deficient fibroblasts as well as for two unrelated human fibroblast cell lines with normal TK2 expression. The sensitivity of the cell lines to several cytotoxic nucleoside analogs was also studied.
SUMMARY OF PAPERS

The results on which this thesis is based are presented and discussed in detail in papers I-IV. A summary of each paper is given below.

PAPER I

**Active site mutants of Drosophila melanogaster multisubstrate deoxyribonucleoside**

The deoxyribonucleoside kinase of the fruit fly *Drosophila melanogaster* (Dm-dNK) is sequence related to the human deoxyribonucleoside kinases dCK, dGK and TK2. It is a multisubstrate enzyme that can phosphorylate all the naturally occurring deoxyribonucleosides necessary for DNA replication as well as several anticancer and antiviral nucleoside analogs. This broad substrate specificity in combination with its high catalytic rate makes it an interesting candidate gene for suicide gene therapy which is a method that focuses on making cancer cells more sensitive to chemotherapeutics. HSV-1 TK is the most commonly studied suicide gene. It is used in combination with the nucleoside analog ganciclovir, which is known to have an efficient bystander killing effect. The structure of Dm-dNK, HSV-1 TK and dGK reveal a common folding of these enzymes and in particular the amino acid residues involved in substrate recognition are highly conserved. We wanted to gain further understanding of the catalytic mechanism and the broad substrate specificity of this enzyme and the aim was to create an improved enzyme with increased phosphorylation efficiency for application in suicide gene therapy. Based on this structural information we performed site directed mutagenesis on four residues in the Dm-dNK active site. The four mutants showed partial or complete loss of activity compared with the wild type enzyme in terms of dThd phosphorylation. We also investigated two double and one triple mutant but they also showed complete or near complete loss of activity for all the investigated substrates. These decreased phosphorylation efficiencies support the importance of the investigated amino acid residues for their importance for substrate binding.

PAPER II

**Increased mitochondrial DNA copy-number in CEM cells resistant to delayed toxicity of 2',3'-dideoxycytidine**

2',3'-dideoxycytidine (ddC) is a deoxycytidine analog used in combination with other drugs for treatment of HIV. ddC is phosphorylated by the deoxyribonucleoside kinase dCK to its active form and causes DNA chain termination. The clinical utility of ddC is limited by the toxic side effects, such as peripheral neuropathy, and the development of resistance. The delayed toxicity has been associated with depletion of mitochondrial DNA. This depletion and mitochondrial dysfunction is common for several antiviral nucleoside analogs. The development of strategies to avoid mitochondrial toxicity without affecting the anti-HIV activity of nucleoside analogs should be an important therapeutic
improvement. In this study we wanted to identify critical events involved in mitochondrial toxicity. We did this by generating and investigating two T-lymphoblast cell lines resistant to the delayed toxicity of ddC. We identified multiple changes such as increased mtDNA, altered dNTP pools, increased Tfam-level and altered mRNA expression. We concluded that the resistance phenotype may be caused by a combination of metabolic alterations. Most importantly we concluded that resistance to ddC toxicity is not necessarily linked to an anti-HIV resistance of ddC or cross resistance to other dCK-activated nucleoside analogs.

PAPER III

Progressive loss of mitochondrial DNA in thymidine kinase 2 deficient mice
Mitochondrial replication is independent of the nuclear DNA replication and of the cell cycle, and therefore requires a constant supply of dNTPs. The first and rate limiting step of the intramitochondrial phosphorylation is catalyzed by the two mitochondrial deoxyribonucleoside kinases dGK and TK2. Mitochondrial DNA depletion syndrome (MDS) is a heterogenous group of disorders characterized by a reduction of mitochondrial DNA copy number in affected tissues. Deficiencies in the enzymatic activities of dGK and TK2 have been linked to MDS. In this study we wanted to investigate the molecular mechanisms of mtDNA depletion due to TK2 deficiency. We did this by generating and characterizing a Tk2 deficient mouse strain. The animals appeared normal at birth but exhibited mtDNA depletion in multiple organs. Over time they developed a severe and fatal phenotype and died within 21 days. The Tk2 deficient mice showed growth retardation, severe hypothermia and a reduced life span. They also showed a reduced mtDNA level in several tissues, disruption of cristae structures in heart mitochondria and a progressive loss of the hypodermal fat layer. From this we could conclude that TK2 has a major role in supplying deoxyribonucleotides for mtDNA replication and that other pathways of deoxyribonucleotide synthesis cannot compensate for the loss of this enzyme.

PAPER IV

Retained sensitivity to cytotoxic pyrimidine nucleoside analogs in thymidine kinase 2 deficient human fibroblasts
Nucleoside analogs are used both in the treatments of viral infections as well as chemotherapy of cancer. Phosphorylation of the nucleoside analog within the cells is required for their pharmacological effects. The rate-limiting step in nucleoside analog phosphorylation is often catalyzed by nucleoside kinases. One of these kinases is thymidine kinase 2 (TK2) located in the mitochondrial matrix. TK2 phosphorylates deoxycytidine and deoxythymidine as well as several nucleoside analogs used in antiviral and anticancer therapy, such as 3'-azido-2',3'-dideoxythymidine (AZT) and 2',3'-dideoxycytidine (ddC). These analogs all cause severe adverse effects by interfering with mitochondrial DNA replication. Besides being phosphorylated by TK2 these analogs are also phosphorylated by two other
kinases, thymidine kinase 1 (TK1) and deoxycytidine kinase (dCK), and the role of mitochondrial TK2 for phosphorylation has remained unclear. In order to elucidate the importance of TK2 for the cytotoxic effects of these compounds we investigated a fibroblast cell line with partial TK2 deficiency. We also investigated a recombinant enzyme with the same mutation found in the TK2 deficient cells in order to obtain insights into the mechanisms of TK2 deficiency. The cells showed normal growth rate and a slightly increased dTTP and mtDNA levels compared to control cell lines. We investigated the role of TK2 for the sensitivity against cytotoxic nucleoside analogs. The toxicity was similar in all investigated cell lines and loss of TK2 activity did not alter the cell sensitivity towards these compounds. Our study suggests that nucleoside analog phosphorylation mediated by TK2 may be less important, compared to other deoxyribonucleoside kinases, for the cytotoxic effects of these compounds.
CONCLUSIONS

• Site directed mutagenesis of $Dm$-dNK increases the understanding of the function of the kinase. $Dm$-dNK with altered substrate specificity may be useful as a suicide gene in gene therapy.

• Resistance to ddC toxicity is not necessarily linked to anti-HIV resistance of ddC or cross resistance to other dCK-activated nucleoside analogs.

• TK$_2$ has a major role in supplying deoxyribonucleotides for mtDNA replication and other pathways of deoxyribonucleotide synthesis cannot compensate for the loss of this enzyme.

• Nucleoside analog phosphorylation mediated by TK$_2$ may be less important, compared to other deoxyribonucleoside kinases, for the cytotoxic effects of these compounds.
DNA innehåller all vår genetiska information och är en stor molekyl uppbryggd av nukleotider. En nukleotid består i sin tur av en nukleosid och en eller flera fosfatgrupper. Innan nukleotiderna kan inkorporeras i DNA-kedjan måste de först importeras in i cellen av olika transportörer för att därefter aktiveras (fosforyleras) via två olika vägar, de novo och salvage. Nukleärt DNA replikeras endast i cellcyklens aktiva S-fas och använder då nukleosider fosforylerade via de novo-vägen för sin DNA-syntes. Fosforyleringen katalyseras av en grupp enzym som kallas deoxyribonukleosidkininas. Humana celler innehåller fyra olika nukleosidkinas; TK1 och dCK som finns i cytoplasman samt TK2 och dGK som finns i mitokondrierna.


2’3’dideoxyctydin (ddC) är en nukleosidanalog som används tillsammans med andra nukleosidanaloger, tex AZT, för behandling av HIV-infektioner. Denna skombinationsbehandling ger både bättre effekt och minskar risken för resistensuppkomst. Vanligtvis beror resistens mot ddC på att viruset har muterat och inte längre kan inkorporera nukleosidanalogen i DNA-kedjan. ddC kan även ge en fördröjd toxicitet orsakad av en sänkt halt av mitokondriellt DNA i de behandlade cellerna. Vi har genererat och studerat T-lymfocyter resistenta mot ddC. Målet var att karakterisera de molekylära mekanismer som är associerade med denna nukleosidanalog-resistens. Cellerna var resistenta mot minskningen av mtDNA-halten men visade fortfarande aktivitet mot HIV. Vi drog slutsatsen att den fördröjda ddC-resistensen förmodligen orsakats av minskad transport av ddC in i cellen.

syndrome (MDS). MDS är en ärtlig sjukdom som karakteriseras av låga halter mtDNA framför allt i muskelvävnad, lever och centrala nervsystemet. MDS yttrar sig vanligen under spådbarnsåldern med muskelsvaghet, försvagad hjärtmuskul, defekta nervvävnader och leversvikt. Små barn dör inom 12 månader vid svår MDS. Yttrar sig sjukdomen senare i livet påverkas främst hjärnan samt magtarm-systemet.

För att öka kunskapen om den molekylära mekanismen bakom minskade halter mtDNA orsakad av brist på TK2, genererades och karakteriserades en muslinje med Tk2-brist. Mössen såg normala ut vid födseln men visade snart tillväxthämnning, nedsatt kroppstemperatur och förkortad livslängd. De hade också en sänkt halt av mtDNA i flera organ. Det konkluderades att TK2 har en betydande roll i replikationen av mitokondriellt DNA samt att andra vägar för nukleosidsyntes inte kunde kompensera för förlusten av detta nukleosidinas.


Bananflugan Drosophila melanogaster har bara ett deoxyribonukleosidkinas (Dm-dNK) till skillnad från människans fyra. Det är ett enzym som fosforylerar alla de naturliga nukleosiderna såväl som flera nukleosidanaloger. Den breda substratspecificiteten kombinerat med dess höga katalytiska hastighet gör den till en intressant kandidat för suicidgenterapi. För att öka kunskapen om de avgörande faktorerna för substratspecificiteten hos enzymet och för att hitta Dm-dNK-mutanter med förbättrade kinetiska egenskaper för användning i genterapi, utfördes riktad mutagenes. Mutanterna visade partiell eller total förlust av aktivitet jämfört med det omuterade enzymet vilket understödjer deras betydelse för substratbindningen.

Sammanfattningsvis har vi i denna avhandling studerat humana nukleosidinas och genetiskt modifierade nukleosidinas från andra organismer. Vi har även studerat de molekylära mekanismerna bakom resistens mot nukleosidanaloger, med betoning på ddC-resistens. Förhoppningen är en ökad kunskap om effekten av nukleosidanalogers toxicitet samt vilken roll olika nukleosidinas spelar i fosforyleringen av naturliga nukleosider och nukleosidanaloger.
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