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INVESTIGATION OF ANTIVIRAL AND ANTICANCER NUCLEOSIDE ANALOG SUBSTRATE RECOGNITION OF *DROSOPHILA MELANOGASTER* AND HERPES VIRUS DEOXYRIBONUCLEOSIDE KINASES

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Ai miei genitori
e in parte a *G*

“I have little patience with scientists who take a board of wood, look for its thinnest part, and drill a great number of holes where drilling is easy.”
[Albert Einstein]

"Considerate la vostra semenza:
fatti non foste a viver come bruti,
ma per seguir virtute e canoscenza."
[Dante Alighieri, Inferno, Canto XXVI]

"El niño que no juega no es niño, pero el hombre que no juega perdió para siempre al niño que vivía en él y que le hará mucha falta."
[Pablo Neruda]

LIST OF PUBLICATIONS

I. ***Nicola Solaroli***, Mia Bjerke, Marjan H. Amiri, Magnus Johansson, and Anna Karlsson
Active site mutants of *Drosophila melanogaster* multisubstrate deoxyribonucleoside kinase.
Eur J Biochem. 2003 Jul;270(13):2879-84

II. Jan Balzarini, Sandra Liekens, ***Nicola Solaroli***, Kamel El Omari, David K. Stammers and Anna Karlsson
Engineering of a single conserved amino acid residue of herpes simplex virus type 1 thymidine kinase allows a predominant shift from pyrimidine to purine nucleoside phosphorylation.
J Biol Chem. 2006 Jul 14;281(28):19273-9

III. ***Nicola Solaroli***, Magnus Johansson, Jan Balzarini and Anna Karlsson
Enhanced toxicity of purine nucleoside analogs in cells expressing *Drosophila melanogaster* nucleoside kinase mutants
Gene Ther. 2006 Aug 3; [Epub ahead of print]

IV. ***Nicola Solaroli***, Magnus Johansson, Jan Balzarini and Anna Karlsson
Mitochondrial expression of the *Drosophila melanogaster* multisubstrate deoxyribonucleoside kinase increase thymidine incorporation into DNA.
Manuscript

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LIST OF ABBREVIATIONS

Nucleosides and nucleotides

dAdo, dAMP, dADP, dATP	deoxyadenosine, mono-, di-, tri- phosphate
dCyd, dCMP, dCDP, dCTP	deoxycytidine, mono-, di-, tri- phosphate
dGuo, dGMP, dGDP, dGTP	deoxyguanosine, mono-, di-, tri- phosphate
dThd, dTMP, dTDP, dTTP	deoxythymidine, mono-, di-, tri- phosphate
dN, dNMP, dNDP, dNTP	any deoxyribonucleoside or nucleotide mono-, di-, tri- phosphate
Ado, AMP, ADP, ATP	adenosine, mono-, di-, tri- phosphate
Cyd, CMP, CDP, CTP	cytidine, mono-, di-, tri- phosphate
Guo, GMP, GDP, GTP	guanosine, mono-, di-, tri- phosphate
Urd, UMP, UDP, UTP	uridine, mono-, di-, tri- phosphate
N, NMP, NDP, NTP	any ribonucleoside or nucleotide mono-, di-, tri- phosphate
MP, DP, TP	Mono-, di-, tri phosphate

Nucleoside analogs

araA	9-β-D-arabinofuranosyladenine (vidarabine)
araC	1-β-D-arabinofuranosylcytosine (cytarabine)
araG	9-β-D-arabinofuranosylguanine (nelarabine)
araT	1-β-D-arabinofuranosylthymine
dFdC	2',2'-difluorodeoxycytidine (gemcitabine)
dFdG	2',2'-difluorideoxyguanosine
ddC	2',3'-dideoxycytidine (zalcitabine)
CdA	2-chloro-2'-deoxyadenosine (cladribine)
AZT	3'-azido-2',3'-dideoxythymidine (zidovudine)
ACV	9-(2-hydroxyethoxymethyl)guanine (acyclovir)
GCV	9-(2-dihydroxypropoxymethyl)guanine (ganciclovir)
LBV	(1α,2β,3α)-9-[2,3-bis(hydroxymethyl)cyclobutyl]guanine (lobucavir)
BVDU	(E)-5-(2-bromovinyl)-2'-deoxyuridine (brivudine)
BVaraU	1-β-D-arabinofuranosyl-5-(E)-(2-bromovinyl)uracil (sorivudine)
FIAU	1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil
FMAU	2'-deoxy-2'-flouro-5-methyl-1-β-D-arabinofuranosyluracil
3TC	2'-deoxy-3'-thiacytidine (lamivudine)
HU	Hydroxyurea

Enzymes

TK	thymidine kinase
dGK	deoxyguanosine kinase
dCK	deoxycytidine kinase
dNK	deoxynucleoside kinase
rNK	ribonucleoside kinase
NMPK	nucleoside monophosphate kinase
NDPK	nucleoside diphosphate kinase
AK	adenosine kinase
GUK	guanylate kinases
UCK	uridine-cytidine kinase
RNR	ribonucleotide reductase

Other abbreviations

Dm	*Drosophila melanogaster*
Bm	*Bombyx mori*
Ag	*Anopheles gambiae*

Amino acids table

Ala	Alanine	Glu	Glutamic acid	Leu	Leucine	Ser	Serine
Arg	Arginine	Gln	Glutamine	Lys	Lysine	Thr	Threonine
Asn	Asparagine	Gly	Glycine	Met	Methionine	Trp	Tryptophan
Asp	Aspartic acid	His	Histidine	Phe	Phenylalanine	Tyr	Tyrosine
Cys	Cysteine	Ile	Isoleucine	Pro	Proline	Val	Valine

ABSTRACT

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. *Dm*-dNK is sequence related to the human deoxycytidine kinase (dCK), deoxyguanosine kinase (dGK) and thymidine kinase 2 (TK2), as well as to the herpes simplex virus type-1 thymidine kinase (HSV-1 TK). The human and viral deoxyribonucleoside kinases can phosphorylate multiple deoxyribonucleosides, whereas *Dm*-dNK has the ability to phosphorylate all naturally occurring deoxyribonucleosides required for DNA replication. In addition to its broad substrate specificity, *Dm*-dNK also exhibits higher catalytic rates for nucleoside and nucleoside analog phosphorylation compared to other nucleoside kinases.

Nucleoside kinases are being investigated for possible use as suicide genes in combined gene/chemotherapy of cancer. The most commonly studied nucleoside kinase suicide gene is the HSV-1 TK gene used in combination with the guanosine nucleoside analog ganciclovir. The suicide nucleoside kinase is rate-limiting in the pharmacological activation of the cytotoxic nucleoside analogs, and mutants of HSV-1 TK with improved biochemical properties for nucleoside analog phosphorylation are more efficient suicide genes. The broad substrate specificity of *Dm*-dNK and its high catalytic rate makes it an interesting candidate gene for suicide gene therapy. The possible use of *Dm*-dNK as a suicide gene has been studies and it was shown that over-expression of *Dm*-dNK enhances the sensitivity of cancer cells to several cytotoxic nucleoside analogs.

Although *Dm*-dNK phosphorylates both purine and pyrimidine nucleosides, the enzyme has a preference for pyrimidine nucleosides. The maximal catalytic rate of purine and pyrimidine nucleoside phosphorylation is similar, but the enzyme exhibits higher affinity for pyrimidine nucleosides and nucleoside analogs. For suicide gene therapy application, purine nucleoside analogs may be preferred because these compounds appear to induce a higher bystander cell killing, i.e. killing of untransduced neighboring cells by transfer of phosphorylated nucleoside analogs via gap junctions.

The solved structures of *Dm*-dNK, dGK and HSV-1 TK, reveal a common folding of these enzymes and in particular the amino acid residues involved in substrate interactions are highly conserved. However, the substrate binding site also exhibits

some major differences between *Dm*-dNK and HSV-1 TK. Based on this structural information we performed site directed mutagenesis of the residues Asn28, Ile29, Phe114 and Gln81 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.

It has also been shown that *Dm*-dNK with 20 amino acid C-terminal deletion has even higher catalytic rates for deoxyribonucleosides compared to wild-type, and it was also reported that the mutagenesis of a few amino acids allows to change the substrate specificity from pyrimidines to purines. Based on these previous studies we constructed the reported mutated enzymes and designed new mutations, with and without the 20 amino acid C-terminal deletion. We measured the ability to phosphorylate ganciclovir (GCV). We have finally selected the most efficient enzymes phosphorylating GCV and expressed in an osteosarcoma TK^- cell line and determined the sensitivity to nucleoside analogs. The cells expressing the Met88Arg mutant enzyme showed the highest increased sensitivity to purine nucleoside analogs with 8 to 80-fold decreased IC50 compared to untransduced control cells or cells expressing the wild-type nucleoside kinase.

We have also created a *Dm*-dNK protein targeted to the mitochondrial matrix by fusing a mitochondrial targeting signal to the N-terminus of the protein. We showed that the mitochondrial *Dm*-dNK was enzymatically active and that overexpression of the enzyme in an osteosarcoma TK^-deficient cell line resulted in an increased sensitivity to some nucleoside analogs such as 1-β-D-arabinofuranosylthymine (araT), (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU), 5-bromo-2'deoxyuridine (5-Br-dUrd) and 5-fluoro-2'deoxyuridine (5-F-dUrd). Labeling studies using [^{3}H]-dThd also showed that mitochondrial expression of *Dm*-dNK, compared to nuclear expression, resulted in a higher specific [^{3}H]-dTTP activity in the total dTTP pool and as a result a higher rate of [^{3}H]-dTTP incorporation into nuclear DNA

We conducted structural studies on HSV-1 TK. In particular we performed site-directed mutagenesis on Ala167 and Ala168 of the HSV-1 TK. The mutated Ala168His and Ala167Phe enzymes turned out to have knocked-out the dThd activity while retaining full GCV phosphorylation ability.

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1 INTRODUCTION

The use of structural analogs of nucleosides in therapy is reported since the 1940s (Elion 1989). Antiviral nucleoside analogs inhibit the replication of the viral genome while anticancer nucleoside analogs are responsible for the inhibition of cellular DNA replication and repair. The nucleoside analogs are inactive pro-drugs that need to be activated by the intracellular phosphorylation machinery to become pharmacologically active. The phosphorylation steps are usually catalyzed by the deoxyribonucleoside- and nucleotide kinases, in particular the kinases involved in the first phosphorylation step have been studied intensively since they catalyze the rate limiting step in the pharmacological activation of the pro-drug (Van Rompay *et al.* 2003).

Beside the human, mouse and the herpes simplex virus nucleoside kinases, other kinases from different organisms such as *Drosophila melanogaster*, *Bombyx mori* and *Anopheles gambiae* have also been reported (Munch-Petersen *et al.* 1998; Knecht *et al.* 2002; Knecht *et al.* 2003). These kinases, in contrast to the human and mouse enzymes, are multisubstrate enzymes and thus have the ability to phosphorylate all four natural substrates and also many nucleoside analogs.

In an attempt to improve the antiviral and anticancer therapy, we evaluated the properties of both of the multisubstrate *Drosophila melanogaster* nucleoside kinase (*Dm*-dNK) and of the herpes simplex virus type-1 thymidine kinase (HSV-1 TK).

The investigation of *Dm*-dNK was directed to evaluate different aspects of this multisubstrate kinase: (a) to understand the determinants of the substrate specificity of the enzyme; (b) to find *Dm*-dNK mutants with different/improved substrate specificity for application in suicide gene therapy (with special focus on the nucleoside analog ganciclovir); (c) to study the *in vivo* effects of different sub-cellular localizations of the *Dm*-dNK.

We have also engineered the HSV-1 TK performing site-directed mutagenesis on two specific residues to investigate the role of these specific amino acids for the thymidine (dThd) and ganciclovir (GCV) phosphorylation and to increase the ratio of GCV/dThd phosphorylation. In particular, we aimed to preserve the catalytic activity for GCV and related analogs and, at the same time, to abolish the dThd phosphorylation capacity.

2 GENETICS AND DNA SYNTHESIS

2.1 INTRODUCTION

Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are built by deoxyribonucleotides (dNs) and ribonucleotides (Ns), respectively. Nucleotides are build up by three different components (Figure 1): 1) a nitrogen-ring structured base that can be a purine (adenine or guanine) or a pyrimidine (cytosine, thymine or uracil respectively for DNA or RNA); 2) a pentose sugar (with or without a hydroxyl group in position 2' depending if it is RNA or DNA); 3) a phosphate group in position 5' of the sugar moiety. The phosphate group can be a mono-, di- or triphosphate. The phosphate group closest to the sugar moiety is called the α-phosphate, the second phosphate is the β-phosphate and the third phosphate is the γ-phosphate. Without phosphate the compound is called a nucleoside.

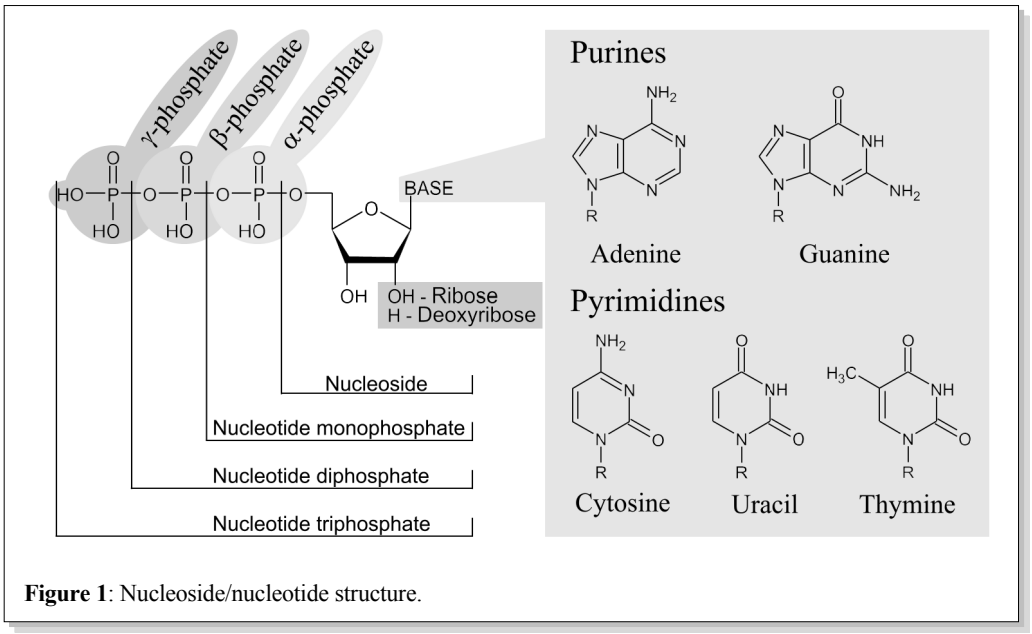


Figure 1: Nucleoside/nucleotide structure.

DNA replication is a semi-conservative process in which each parental strand is the template for the synthesis of a new complementary strand. The central enzyme involved is the DNA polymerase, which catalyze the joining of deoxyribonucleotide triphosphates (dNTPs) to the growing DNA chain. The energy required for this process is derived by the disruption of the phosphodiester bond between the α- and β-phosphates. The product of this reaction is a monophosphate nucleotide that is incorporated into the growing DNA strand by creation of a new bond between the α-phosphate and the 3'-hydroxyl group of a previously added nucleotide (Figure 2).

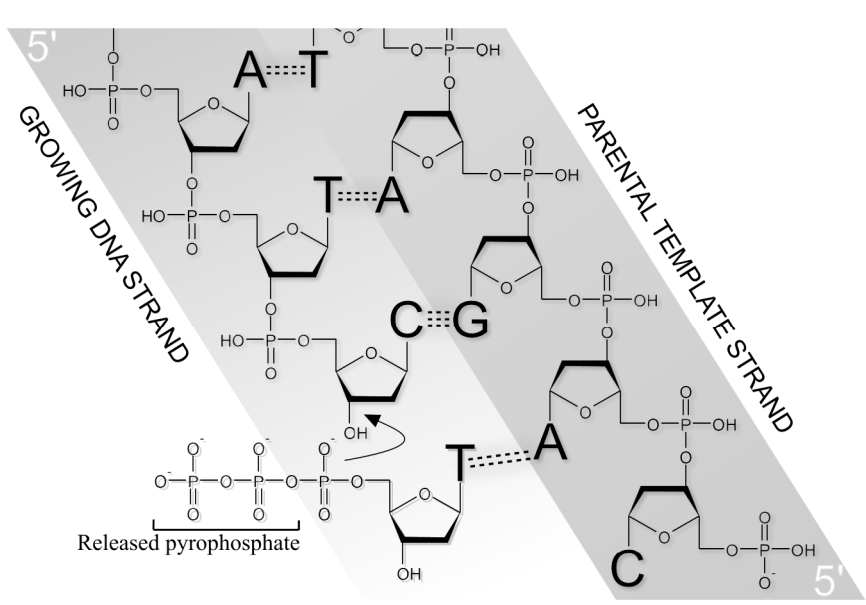


Figure 2: Synthesis of DNA catalyzed by the DNA polymerase.

The principal requirement to obtain a high-fidelity replication is a balanced supply of all four natural deoxyribonucleotide triphosphates (Reichard 1988). Inhibition of the deoxyribonucleotide supply results in error in the duplication of DNA, cell growth arrest and cell death. Therefore all the enzymes involved in the synthesis/recycling of deoxyribonucleotides are natural targets for anti-proliferative drugs. Another way to interfere with the DNA replication aiming to cause the cell death is to provide analogs of nucleosides that can interact with the phosphorylation pathway and then be incorporated by the DNA polymerase into the DNA strand. Three different strategies are possible: (a) to use analogs lacking the 3'-hydroxyl group that can be incorporated into the DNA, but that cannot permit DNA elongation, (b) to use analogs with an intact 3'-hydroxyl group, but with other structural modification that impairs the DNA strand elongation, (c) to use analogs that interfere with the enzymes involved in the supply of nucleotides.

2.2 HUMAN

Man has 46 chromosomes, arranged in pairs, of which 44 (22 pairs) are autosomes, and 2 are sex chromosomes. Although the completion of the Human Genome Project was celebrated in April 2003 and sequencing of the human chromosomes is essentially finished, the exact number of genes encoded by the genome is still unknown. Up to date the number is estimated to be between 20,000 and 25,000 (Stein 2004).

In addition to nuclear DNA there is also mitochondrial DNA (mtDNA), a circular strand of DNA (16.5 kb) which encodes 22 tRNAs, 2 rRNAs and 13 proteins essential for cellular energy production. However, many proteins found in the mitochondria are encoded by nuclear DNA. It has been estimated that 1000-3000 proteins are required for the correct mitochondrial function (Mokranjac and Neupert 2005). Most of the genes are thought to originally have been part of the mtDNA but have since been transferred to the nucleus during evolution (Truscott *et al.* 2003; Lister *et al.* 2005).

2.2.1 Nuclear DNA replication

Nuclear DNA replication occurs in the S-phase of the cell cycle. When the replication machinery is up the majority of the dNTPs necessary for the correct replication are provided by the *de novo* synthesis of nucleotides (Reichard 1988).

DNA polymerases are key enzymes in the replication phase. In human cells four DNA polymerases are present in the nucleus: DNA polymerase α, β, δ and ε. Polymerase α, δ and ε are principally active in dividing cells, suggesting therefore their importance in the nuclear DNA replication system. Polymerase β, is active both in dividing and non dividing cells, suggesting a function in the DNA damage repair (Hubscher *et al.* 2002).

After replication the DNA is associated with proteins to form chromatin. The major proteins involved in chromatin are histone proteins, but other chromosomal proteins are prominent too (Holmquist and Ashley 2006). They have different functions, one of which is to protect the DNA from damage (Roginskaya *et al.* 2006).

2.2.2 Mitochondrial DNA replication

mtDNA is located in the mitochondrial matrix. Mammalian mtDNA is thought to be strictly maternally inherited, although this may not be true for all organisms (Birky 1995; Birky 2001; Schwartz and Vissing 2002).

mtDNA replication is not coordinated with the replication of the nuclear DNA and the mtDNA molecules are not duplicated once every cell cycle, unlike the nuclear DNA (Clayton 1982; Larsson and Clayton 1995).

Replication and maintenance of the mitochondrial genome relies on a relatively modest enzyme pool. As there is only one DNA polymerase found in mitochondria, DNA polymerase γ, it has the sole responsibility for DNA synthesis in all replication, recombination, and repair transactions involving mtDNA (Graziewicz *et al.* 2006). The

importance of this enzyme is supported by the fact that some mutations and/or depletions of mtDNA observed in certain human diseases, can be due to heritable defects in the polymerase γ gene (Longley *et al.* 2005).

For its replication mtDNA requires only a correspondingly small fraction of the total dNTPs of the cell, but this requirement may be particularly critical in resting cells (Bogenhagen and Clayton 1977; Dolce *et al.* 2001; Rampazzo *et al.* 2004). It has recently been shown that most dNTPs are quite evenly distributed between mitochondria and the cytosol in cycling cells. Only the dGTP pool appears to accumulate in mitochondria (Rampazzo *et al.* 2004).

Unlike nuclear DNA, mtDNA is extremely susceptible to damage and mutagenesis, in fact the rate of mutation in mtDNA is up to 20 times higher. This is because mtDNA contains no protective histones, is devoid of introns, and has limited DNA repair capacity (Bogenhagen 1999; Singh 2006).

2.3 HERPES VIRUSES

The herpesviridae is a family of enveloped, double-stranded linear DNA virus that cause disease in humans and animals. More than 100 herpes viruses have been isolated, at least one for each species analyzed. Up to date there are eight known herpes virus that infect humans. The size of the genome can vary from 120kb of the varicella-zoster virus (VZV) to 230kb of the cytomegalovirus (CMV) (Boehmer and Lehman 1997).

2.3.1 Herpes Simplex Virus type 1

Herpes simplex virus type 1 (HSV-1) belong to the alphaherpesviridae sub-family. It is a human neurotropic virus with a viral genome of 152Kb. There are two unique regions, long and short (U_L and U_S), which are linked in either orientation by internal repeat sequences (IR_L and IR_S) (Figure 3). At the non-linker end of the unique regions are terminal repeats (TR_L and TR_S) (Jacob *et al.* 1979).

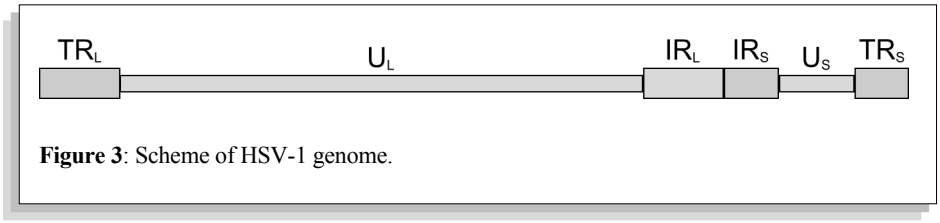


Figure 3: Scheme of HSV-1 genome.

There are up to 81 genes, many of which interact with the host cell or the immune response of the host (Marconi *et al.* 1996). The other genes are involved in the

virus structure or in the virus replication such as: DNA-dependent DNA polymerase, thymidine kinase (TK), ribonucleotide reductase (RNR). All these genes have been classified in three major classes: 1) immediate-early genes (IE or α); 2) early genes (E or β); 3) late genes (L or γ).

After the fusion between the virus and the host cell has occurred the virus releases some proteins such as toxins, protein kinases and transcription initiators into the cytosol. The five immediate-early genes first transcribed promote the early genes. The β proteins include the enzymes required for replication of the viral genome such as: DNA polymerase and the enzymes involved in nucleotide metabolism. Viral DNA synthesis begins shortly after the appearance of the early proteins. The temporal program of viral gene expression ends with the appearance of the late proteins, which constitute the structural proteins of the virus (Boehmer and Lehman 1997).

2.4 *DROSOPHILA MELANOGASTER*

Drosophila melanogaster is one of the most intensively 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. Genetically humans are about 44% similar to flies. About 61% of known human disease genes have a recognizable match in the genetic code of fruit flies, and 50% of fly protein sequences have mammalian analogs.

Drosophila melanogaster has 8 chromosomes, 6 (3 pairs) are autosomes, and 2 are sex chromosomes. The almost complete genome sequence was first released in March 2000, and analysis of the data is now mostly complete (Adams *et al.* 2000). The size of the genome is about 165 million bases and it was estimated to contain about 13,600 genes (Celniker *et al.* 2002).

Drosophila melanogaster mtDNA is a circular strand of DNA of 19.5 kb which encodes 22 tRNAs, 2 rRNAs and 13 proteins necessary for cellular energy production (Lewis *et al.* 1995).

Up to date four different DNA polymerases have been identified. Polymerase α, δ and ε are involved in the nuclear DNA replication (Cotterill *et al.* 1992; Peck *et al.* 1992; Bakkenist and Cotterill 1994; Aoyagi *et al.* 1997). Polymerase γ catalyzes the mtDNA synthesis (Wang *et al.* 1997).

3 NUCLEOTIDE METABOLISM

3.1 INTRODUCTION

Deoxyribonucleotides need to be synthesized inside the cell since there are no protein carriers for them and the negatively charged phosphate group prevents diffusion across the membrane. Therefore the cell has to provide the supply of deoxyribonucleotides. It uses two different pathways for this purpose: the *de novo* pathway and the salvage pathway.

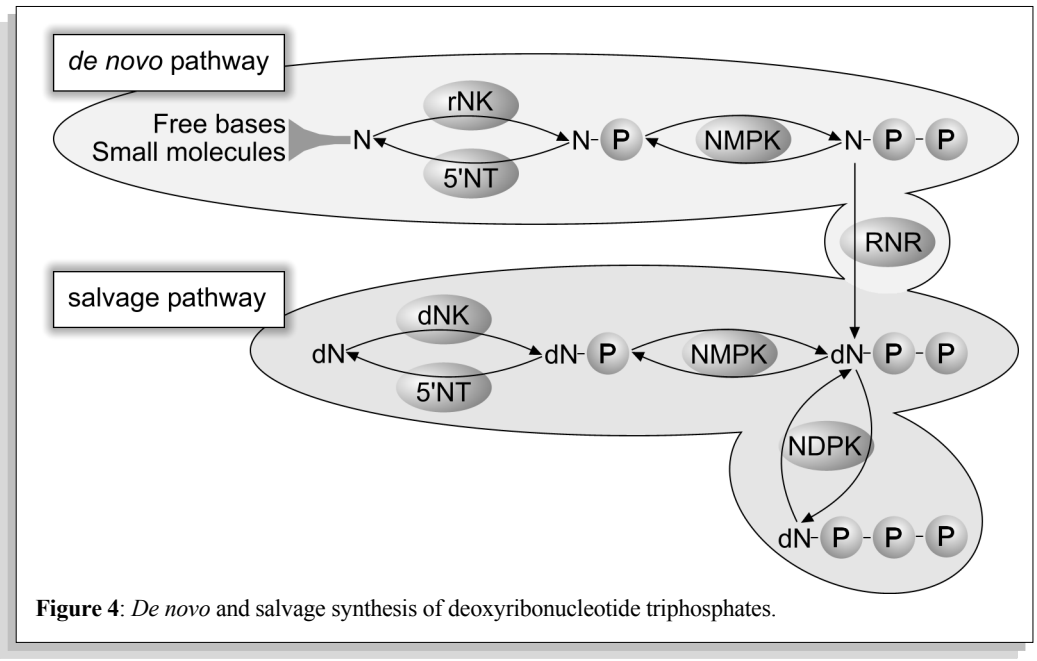


Figure 4: *De novo* and salvage synthesis of deoxyribonucleotide triphosphates.

3.1.1 *De novo* pathway

The *de novo* pathway is the main source of deoxyribonucleotides for nuclear DNA replication, and it is active during the S-phase of the cell cycle (Eriksson *et al.* 1984; Reichard 1988; Wright *et al.* 1990; Xu *et al.* 1995).

Via the *de novo* pathway ribonucleoside monophosphates are synthesized from small molecules (amino acids, ribose, CO_2), ribonucleosides or free bases and then converted to diphosphates (NDP) by nucleoside monophosphate kinases (NMPK) (Figure 4). The ribonucleotide reductase (RNR) catalyzes the irreversible conversion of NDPs to 2'-deoxyribonucleotide diphosphates (dNDP) by the reduction of the 2'-hydroxyl group present on the sugar moiety. The final step, common with the salvage pathway, is the addition of the third phosphate catalyzed by the nucleoside diphosphate kinase (NDPK).

3.1.2 Salvage pathway

The salvage pathway is believed to be important for the DNA repair and for mtDNA synthesis (Xu *et al.* 1995). In the salvage pathway deoxyribonucleotides are synthesized from deoxyribonucleosides. The deoxyribonucleosides are derived from the dephosphorylation of deoxyribonucleotides or are imported into the cell by specific nucleosides transport proteins that facilitate diffusion or actively transport the molecules across the membrane (Belt *et al.* 1993; Griffith and Jarvis 1996; Pastor-Anglada *et al.* 1998).

The first of the three steps leading to deoxyribonucleoside triphosphates (dNTP) is the addition of one phosphate group in position 5' of the pentose ring, and it is catalyzed by a deoxyribonucleonucleoside kinase (dNK). This step is in most cases the rate-limiting step in the formation of dNTPs (Arner and Eriksson 1995) (Figure 4).

Conversion of deoxyribonucleoside monophosphates to their triphosphate forms occurs in two subsequent reversible phosphotransferase reactions catalyzed by the nucleoside monophosphate kinases (NMPKs) and the NDPK.

3.2 HUMAN

3.2.1 *De novo* pathway

3.2.1.1 Ribonucleoside kinases

So far three rNKs have been identified in human cell: adenosine kinase (AK or ADK), uridine-cytidine kinase 1 (UCK1) and uridine-cytidine kinase 2 (UCK2) (Spychala *et al.* 1996; Van Rompay *et al.* 2001). Up to date no guanosine kinase has been reported.

AK catalyzes the phosphorylation of adenosine (Ado) to adenosine monophosphate (AMP). The AK gene is localized on chromosome 10 and the protein is reported as cytosolic (Andres and Fox 1979). Beside the capability to phosphorylate several nucleoside analogs such as ribavirine and mizoribine, it has also been suggested that inhibition of the AK produce an intravascular increase of adenosine concentration and a consequent anti-inflammatory effect (Miller *et al.* 1979; Yamada *et al.* 1981; Cronstein 1992; Cronstein *et al.* 1994; Firestein *et al.* 1994). AK is feedback regulated by its products AMP and ADP (Palella *et al.* 1980).

UCK1 and UCK2 are located on chromosome 9 and 1, respectively, and have a sequence similarity of 72%. They have the ability to phosphorylate uridine (Urd) and cytidine (Cyd) to uridine monophosphate (UMP) and cytidine monophosphate (CMP) (Koizumi *et al.* 2001; Van Rompay *et al.* 2001). UCK1 is a nuclear protein whereas

UCK2 is cytosolic. Their activity is not connected with cell proliferation, and they are widely distributed in the different tissues (Herzfeld and Raper 1979; Shen *et al.* 1998).

Both enzymes have a double regulation system: (a) feedback regulation by CTP and UTP which compete with the phosphate donor (Van Rompay *et al.* 2001) (b) regulation by changes in quaternary structure: ATP stabilizes the protein in an active tetrameric form while UTP and CTP dissociate the enzyme (Cheng *et al.* 1986; Ropp and Traut 1998).

3.2.1.2 Ribonucleotide reductase

Ribonucleotide reductase (RNR) is responsible for the *de novo* conversion of all four ribonucleoside diphosphates to deoxyribonucleoside diphosphates, which are essential for DNA synthesis and repair. RNR is a tetramer composed of two non-identical homodimers, M1 and M2, both necessary for the RNR activity. The subunits are localized on chromosome 11 (M1) and 2 (M2). The large subunit M1 (86-kDa) contains the catalytic site, an allosteric effector-binding site, and redox active disulfides that participate in the reduction of the substrates. Subunit M2 (43-kDa) contains an oxygen-linked di-iron center and one tyrosyl-radical (Kauppi *et al.* 1996; Jordan and Reichard 1998). The expression levels of these two subunits are regulated separately during cell cycle progression. While the level of M1 appears to be constant throughout the cell cycle in proliferating cells, the level of M2 changes with the cell cycle and peaks in the S-phase (Eriksson *et al.* 1984; Engstrom *et al.* 1985; Mann *et al.* 1988; Bjorklund *et al.* 1990). Therefore, RNR is important for cell proliferation, and in particular the level of the M2 subunit plays an essential role in regulating DNA synthesis (Chang and Cheng 1979; Eriksson *et al.* 1984; Thelander *et al.* 1985; Jordan and Reichard 1998).

A new member of the RNR family called p53R2, that is located on chromosome 8, has also been identified (Tanaka *et al.* 2000). p53R2, if compared with M2, lacks 33 amino acid residues in its N-terminus, but contains a p53-binding site (Chabes *et al.* 2003). p53R2 expression, but not M2, is induced by ultraviolet light, γ-irradiation or DNA-damaging agents in a p53-dependent manner (Nakano *et al.* 2000; Tanaka *et al.* 2000). These findings suggest that there are two pathways in human cells that supply dNTPs for DNA synthesis: through the activity of M2, 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).

An inhibitor of RNR is hydroxyurea (HU) that quenches the tyrosyl-radical at the active site of the M2 subunit with the consequent inactivation of the enzyme (Yarbro 1992). This drug is used in anticancer therapy, but also effective in a number of non-neoplastic diseases such as sickle cell anemia and has been in trials for treatment of HIV infection (Bunn 1997; Vila *et al.* 1997; Navarra and Preziosi 1999).

3.2.2 Salvage pathway

In human cells there are four different deoxyribonucleoside kinases: thymidine kinase 1 (TK1), thymidine kinase 2 (TK2), deoxycytidine kinase (dCK) and deoxyguanosine kinase (dGK) (Arner and Eriksson 1995; Van Rompay *et al.* 2003). These enzymes are specific for deoxyribonucleosides, and their nomenclature is based on the preferred deoxyribonucleoside substrate, but they also have the ability to phosphorylate other substrates (Table 1).

Deoxyribonucleoside kinase	Natural substrates
TK1	dThd, dUrd
TK2	dThd, dUrd, dCyd
dCK	dCyd, dAdo, dGuo
dGK	dGuo, dAdo, dIno

Table 1: Natural substrates of the four human deoxyribonucleoside kinases.

3.2.2.1 Thymidine kinase 1

TK1 is a cytosolic enzyme that phosphorylates deoxythymidine (dThd) and deoxyuridine (dUrd) to deoxythymidine monophosphate (dTMP) and deoxyuridine monophosphate (dUMP) (Munch-Petersen *et al.* 1995). The enzyme also can phosphorylate some pyrimidine nucleoside analogs, for instance AZT, FIAU and FMAU (Wang and Eriksson 1996).

The TK1 gene is localized on the long arm on chromosome 17 and has been cloned and extensively characterized (Bradshaw and Deininger 1984; Berenstein *et al.* 2000). The kinetic properties of this enzyme show a Michaelis-Menten constant of 5 μM and the can use most phosphate donors with a preference for ATP (Ellims and Van der Weyden 1981). The protein is located in the cytosol and is widely distributed in all tissues (Kit and Leung 1974; Lee and Cheng 1976).

Recent studies about the phylogenetic relationship among the four human deoxyribonucleoside kinases (dNKs) showed that dCK, dGK and TK2 belong to the

same family, while TK1 seems to have a different origin and to be less related with the other three enzymes (Sandrini and Piskur 2005). This observation is also supported by the comparison of the three dimensional structures of all the dNKs: TK1 only shares sequence similarity in the glycine-rich loop, which binds the phosphate donor, whereas it has a completely different overall monomer structure than the other dNKs (Welin *et al.* 2004, Sandrini, 2005 #73).

The TK1 activity is known to change with the DNA synthesis, and the activity is high in proliferating and malignant cells and low or almost absent in quiescent cells due to a specific S-phase regulated expression (Coppock and Pardee 1987). The rate of transcription of TK1 increases many fold following growth stimulation of non-proliferating cells and during the S-phase of the cell cycle. The half life of the TK1 protein increases during S-phase, but at the end of the S-phase the half life decreases and the protein is rapidly degraded (Sherley and Kelly 1988; Kauffman and Kelly 1991). This regulation is important to provide the correct dTTP pool for DNA synthesis at the right time of the cell cycle (Ke and Chang 2004).

3.2.2.2 Thymidine kinase 2

TK2 is able to phosphorylate dThd and dUrd and, in contrast to TK1, deoxycytidine (dCyd) (Table 1). TK2 also phosphorylates antiviral nucleoside analogs such as AZT, FIAU and FMAU but with lower efficacy than the natural substrates (Wang and Eriksson 1996). The kinetic properties of the enzyme show a K_m value of 16 μM for dThd and 26-36 μM for dCyd (Wang *et al.* 1999).

The TK2 gene is located on chromosome 16, and the TK2 expression is not cell-cycle regulated and the expression is similar in terminally differentiated cells and in growing cells. Therefore, in non-proliferating cells, TK2 is the only thymidine phosphorylating enzyme that is expressed (Van Rompay *et al.* 2003).

TK2 has been shown to be located in the mitochondria, however, some studies suggest also a cytosolic form of TK2 in human cells, but the difference between the cytosolic and mitochondrial enzyme are not known (Soderlund and Arner 1994). The first two published sequences did not include the mitochondrial targeting sequence as part of the cDNA, but in later work the full-length mouse TK2 sequence with N-terminal mitochondrial targeting signal was reported (Johansson and Karlsson 1997; Wang *et al.* 1999; Wang and Eriksson 2000).

Recently, mitochondrial DNA depletion syndrome (MDS) has been linked to mutations in TK2 (Saada *et al.* 2001). In particular, TK2 has been associated with the

myopathic form of MDS (Tulinius *et al.* 2005; Wang *et al.* 2005, Vila, 2003 #81). However, of the many cases with MDS described so far, only a small percentage of patients with the myopathic form of MDS showed TK2 mutations. This suggests that defects in other genes also must be involved in the etiology of myopathic MDS (Mancuso *et al.* 2002).

3.2.2.3 Deoxycytidine kinase

dCK has a broad substrate specificity and phosphorylates the pyrimidine nucleoside dCyd and also the purine nucleosides deoxyguanosine (dGuo) and deoxyadenosine (dAdo) (Table 1) (Bohman and Eriksson 1986). This kinase is constitutively expressed throughout the cell cycle, and is thus also present in resting cells (Arner *et al.* 1988).

The dCK gene is localized on the long arm of chromosome 4, and has been cloned and characterized (Chottiner *et al.* 1991; Eriksson *et al.* 1991). Kinetic studies with a purified enzyme show that dCK catalyzes the phosphorylation of dCyd with a low K_m value (0.4-3.0 μM) and the phosphorylation of the purine dAdo and dGuo at significantly higher K_m values (120-890 μM for dAdo and 150-640 μM for dGuo) (Sarup and Fridland 1987; Bohman and Eriksson 1988; Datta *et al.* 1989; Datta *et al.* 1989).

dCK has been intensively studied since this enzyme phosphorylates several therapeutic nucleoside analogs. In particular the anticancer nucleoside analogs araA, araC, araG, CdA and dFdC are substrates for dCK. Also the anti-retroviral nucleoside analogs ddC and 3TC are phosphorylated by dCK (Balzarini *et al.* 1996).

dCK is reported to be a cytosolic enzyme, however a targeting signal for nuclear import has been identified in the N-terminal sequence of the dCK cDNA. It was shown that the protein could be efficiently imported into the nucleus, but the physiological importance of the nuclear location of dCK is not known, and the intracellular location appears not to be important for the cytotoxicity of nucleoside analogs (Johansson *et al.* 1997).

3.2.2.4 Deoxyguanosine kinase

dGK catalyzes the phosphorylation of purine deoxynucleosides (dNs) and their analogs (Table 1). The dGK gene is localized on the short arm on chromosome 2. The cDNA for dGK has been cloned, and it codes for a 31-kDa protein with an N-terminal mitochondrial leader sequence (Johansson *et al.* 1996; Wang *et al.* 1996). It has been

shown to be ubiquitously distributed in all tissues at approximately equal levels (Arner and Eriksson 1995; Johansson *et al.* 1996; Wang *et al.* 1996). The dGK sequence shows 48% of homology, at the amino acid level, with dCK, but different from the cytosolic enzyme it is not able to phosphorylate pyrimidines (Wang *et al.* 1993). dGK also phosphorylates and thereby activates several anticancer nucleoside analogs such as CdA, dFdG and araG (Zhu *et al.* 1998).

It has been reported three cases in which a one base pair deletion within the dGK gene has caused mitochondrial DNA depletion syndromes (Mandel *et al.* 2001; Salviati *et al.* 2002). Different from TK2, the dGK mutations/deletions cause the hepatocerebral form of MDS (Mandel *et al.* 2001; Mancuso *et al.* 2002). This is a further demonstration that alterations of the mitochondrial dNTPs affect mtDNA maintenance and stability.

3.2.2.5 Nucleoside monophosphate kinases

NMPKs catalyze the reversible phosphorylation of nucleoside monophosphates to nucleoside diphosphates. There are four groups of NMPKs in human cells (Table 2): a dTMP kinase (dTMPK), a UMP-CMP kinase (UMP-CMPK), five isozymes of adenylate kinase (AK), and several guanylate kinases (GUK) (Van Rompay *et al.* 2000). This phosphotransferase reaction is not considered a rate limiting step, however there is evidence that some nucleoside analogs are accumulated in their monophosphate form (Vilpo and Vilpo 1993; Lavie *et al.* 1997; Balzarini *et al.* 1998).

Monophosphate kinase	Natural substrates
dTMPK	dTMP, dUMP
UMP-CMPK	CMP, dCMP, UMP, dUMP, AMP, dAMP
AK1	AMP, dAMP
AK2	AMP
AK3	AMP, dAMP
AK4	AMP
AK5	AMP, dAMP, CMP, dCMP
GUKs	GMP, dGMP

Table 2: Natural substrates of the human nucleoside monophosphate kinases.

3.2.2.6 Nucleoside diphosphate kinases

NDPKs catalyze the last step in the phosphorylation process, the phosphotransferase reaction that transforms nucleoside diphosphates to triphosphates (de la Rosa *et al.* 1995; Lacombe *et al.* 2000). The human NDPK family includes at least eight isozymes, all with the same broad substrate specificity. These enzymes phosphorylate both ribo- and deoxyribonucleotides, and they are not considered a bottleneck in the triphosphate synthesis. However it has been shown that some antiviral nucleoside analogs such as azidothymidine, dideoxyadenosine and dideoxythymidine are very poor substrates for NDPKs (Bourdais *et al.* 1996).

3.3 HERPES SIMPLEX VIRUS TYPE 1

3.3.1 Ribonucleotide reductase

HSV-1 codes for its own viral RNR, which, similar to other RNRs, is formed by the reversible association of two nonidentical homodimers. The sizes of the two subunits are 136-kDa for R1 and 38-kDa for R2 (Frame *et al.* 1985). Different to the human RNR, the HSV-1 R1 subunit lacks the allosteric regulation site (Lankinen *et al.* 1982).

The C-terminus of the HSV-1 RNR small subunit (R2) is critical for the formation of the complex and synthetic peptides containing these amino acid sequences selectively inhibit the viral enzyme by preventing the association of the subunits (Cohen *et al.* 1986; Dutia *et al.* 1986; Filatov *et al.* 1992). This indicates that the HSV-1 RNR is important for virulence and for the reactivation from latency (Brandt *et al.* 1991; Idowu *et al.* 1992).

2',2'-difluorodeoxycytidine (dFdC or gemcitabine) is a potent inhibitor of RNRs and it is clinically used in anticancer applications (Robins 2003). However, up to date none of the compounds that work principally as RNR inhibitors have shown sufficient selectivity to be used as specific anti-HSV drug.

3.3.2 Thymidine kinase

HSV-1 thymidine kinase (HSV-1 TK) is a 41-kDa protein composed of 376 aa. Unlike the human thymidine kinases, HSV-1 TK has a broad substrate specificity including pyrimidines (dThd with a K_m of 0.38-0.9 μM), pyrimidine analogs (zidovudine) as well as acyclic purine analogs (acyclovir, ganciclovir, buciclovir, and penciclovir) (Chen *et al.* 1979; Furman *et al.* 1984; Balzarini *et al.* 1993; Hinds *et al.*

2000; Kokoris and Black 2002). The HSV-1 TK was the first among the deoxyribonucleoside kinases to be crystallized (Wild *et al.* 1995).

The enzyme is required for the activation of the anti-herpes nucleoside analogs such as acyclovir (ACV) and ganciclovir (GCV). ACV is relatively non toxic even at high doses, but HSV-1 TK shows a high K_m towards ACV (417 μM) that make ACV not suitable in gene therapy clinical settings using HSV-1 TK as suicide gene (Kokoris and Black 2002). GCV has a lower K_m, 47.6-69 μM, and it is also active against infections caused by HSV-1, but is potentially more toxic (Hinds *et al.* 2000; Kokoris and Black 2002). (*E*)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU) is also an excellent substrate for HSV-1 TK, whereas it is not at all recognized by the human cytosolic TK1 (De Clercq *et al.* 1979; Zou *et al.* 1984; De Clercq 2005).

HSV-1 TK has also thymidylate kinase (TMPK) activity converting deoxypyrimidine monophosphate substrates, including the 5'-monophosphate of BVDU, to their corresponding 5'-diphosphate derivatives (Chen *et al.* 1979; Fyfe 1982).

3.4 *DROSOPHILA MELANOGASTER*

3.4.1 *De novo* pathway

So far no rNK has been identified, cloned or characterized in *Dm*. Nevertheless searching in the Kyoto encyclopedia of genes and genomes (KEGG) it is possible to find two putative adenosine kinases and two putative uridine-cytidine kinases but no guanosine kinase (Kanehisa *et al.* 2004).

It is also possible to identify the two subunits of the RNR (M1 and M2). They have an identity of 75% and 67% with the respective human subunits, but so far the *Drosophila melanogaster* RNR has not been characterized.

3.4.2 Salvage pathway

In contrast to mammals, which have four different deoxyribonucleoside kinases with distinct but overlapping specificities, *Drosophila melanogaster* has only one multisubstrate deoxyribonucleoside kinase (*Dm*-dNK).

3.4.2.1 Nucleoside kinase

Drosophila melanogaster nucleoside kinase (*Dm*-dNK) is a 29-kDa protein composed of 250 amino acids and localized on chromosome 3. It was purified in 1998 and shown to catalyze the phosphorylation of all the natural pyrimidine and purine deoxyribonucleosides (Munch-Petersen *et al.* 1998).

This enzyme was cloned by Johansson et al. and shown to be closely related to the human dNKs, in particular with TK2 (Figure 5).

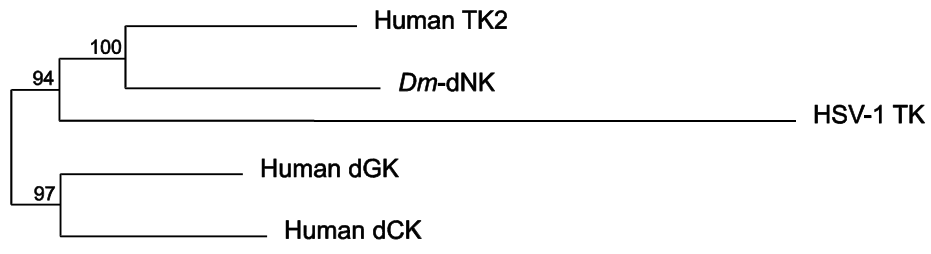


Figure 5: Phylogenetic relation of the deoxyribonucleoside kinase family (Johansson *et al.* 1999).

Dm-dNK recognizes all the natural pyrimidine and purine deoxyribonucleosides, and in addition to its broad substrate specificity, this enzyme also exhibits a high catalytic rate that is 10- to 100- fold higher than reported for the previously studied nucleoside kinases (Table 3).

Substrate	K_m (μM)	V_{max} (nmol/μg/h)
dThd	1.6	240
dCyd	2.6	370
dGuo	2000	220
dAdo	373	910

Table 3: Kinetic properties of *Dm*-dNK (Johansson *et al.* 1999).

It has been shown that *Dm*-dNK efficiently phosphorylates several antiviral and anticancer nucleoside analogs (Johansson *et al.* 1999). The broad substrate specificity of this enzyme, together with its high catalytic rate, makes it a candidate for possible use as a suicide gene in combined gene/chemotherapy of cancer (Zheng *et al.* 2000; Zheng *et al.* 2001; Zheng *et al.* 2001).

A model for the feedback inhibition of the enzyme has been proposed. Apparently dTTP is the only effective inhibitor of *Dm*-dNK and is a competitive inhibitor with respect to ATP (Knecht *et al.* 2002; Mikkelsen *et al.* 2003).

3.4.2.2 Nucleoside monophosphate kinase

In the KEGG database four groups of NMPKs in *Dm* cells has been identified: one putative dTMP kinase (dTMPK), one UMP-CMP kinase (UMP-CMPK), three

isozymes of adenylate kinase (AK) and at least one putative guanylate kinases (GUK) (Noma *et al.* 2000).

It has been shown that the recombinant *Dm* UMP-CMP kinase has a substrate specificity similar to UMP-CMP kinases of mammalian origin. Interestingly, for the first time, it has been reported the presence of a mitochondrial import signal in the N-terminal region (Curbo *et al.* 2003).

The *Dm*-GUK sequence is 58% identical to the human GUKs, and the analysis of *Dm*-GUK showed that the properties of the enzyme are similar to those reported for the mammalian guanylate kinases. In fact, *Dm*-GUK has the ability to phosphorylate GMP and dGMP, using ATP as phosphate donor, with similar kinetic constants as reported for the mammalian GUKs (Johansson *et al.* 2005).

3.4.2.3 Nucleoside diphosphate kinases

The *Drosophila melanogaster* NDPK family comprises at least three isozymes. Some studies have been performed regarding the post-translational modifications and the importance of one of the isozymes of NDPK that plays a critical role in spindle microtubule polymerization (Biggs *et al.* 1990; Stenberg *et al.* 2002).

3.5 OTHER ORGANISMS

Two other multisubstrate deoxyribonucleoside kinases have been discovered. They were found in two different insects: *Bombyx mori* (*Bm*-dNK) and *Anopheles gambiae* (*Ag*-dNK) (Knecht *et al.* 2002; Knecht *et al.* 2003). The *Bm*-dNK enzyme, like *Dm*-dNK, phosphorylates all four natural deoxyribonucleoside substrates with a preference for pyrimidines (Knecht *et al.* 2002). The *Ag*-dNK, instead, phosphorylates all four natural deoxyribonucleosides but with an unexpected higher efficiency for the phosphorylation of purines. In addition *Ag*-dNK can also phosphorylate some nucleoside analogs such as 2-chloro-2'-deoxyadenosine (CdA) and BVDU. Interestingly, it was reported that the differences between these three multisubstrate kinase are very few, and in particular in the active site alteration of only one amino acid results in different substrate specificity (Knecht *et al.* 2003). Probably this amino acid residue is not the only responsible for the observed diversity in the substrate specificity among the three enzymes. Residues outside the active pocket and/or conformational changes taking place during the reaction are suggested to play a major role in the specific properties of each insect multisubstrate kinase.

4 STRUCTURE OF THE dNKs

The comparison between the active site of human, herpes simplex virus and *Drosophila melanogaster* kinases shows few differences in the amino acid sequences. In particular the comparison of the *Dm*-dNK and HSV-1 TK three-dimensional structures and amino acid sequences indicates many common motifs and areas that are well conserved among the different species (Figure 6).

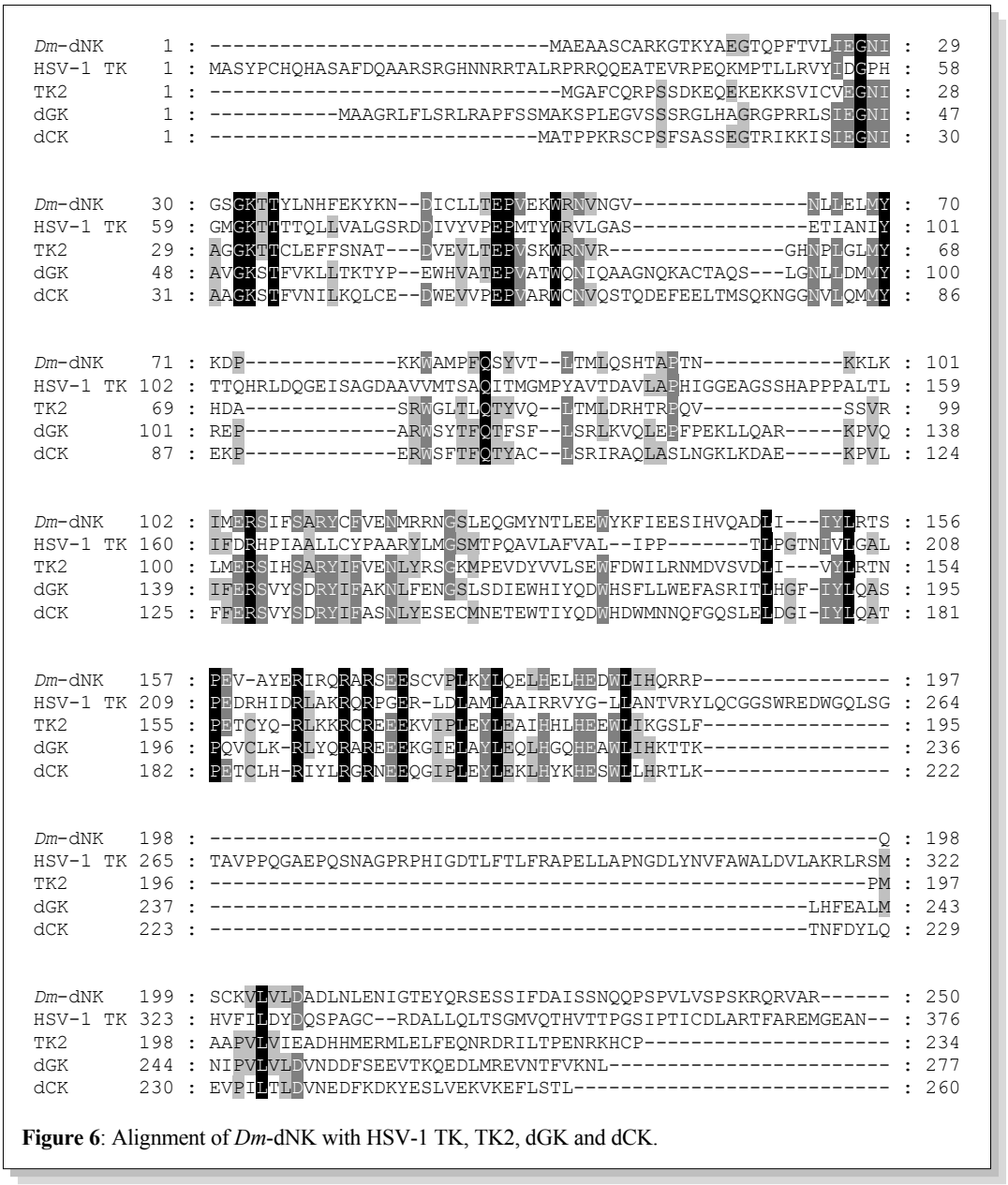


Figure 6: Alignment of *Dm*-dNK with HSV-1 TK, TK2, dGK and dCK.

4.1 SEQUENCE ANALYSIS

4.1.1 Glycine-rich loop (p-loop)

From sequence comparisons and crystallographic data analysis it has been shown that an appreciable proportion of proteins that bind ATP or GTP share a number

of conserved sequence motifs (Saraste *et al.* 1990). The best conserved of these motifs is a glycine-rich region, which typically forms a flexible loop between a beta-strand and an alpha-helix. This loop interacts with one of the phosphate groups of the nucleotide.

Interestingly this motif is very well conserved between humans and *Dm* (but also other organisms and proteins), whereas HSV-1 TK has a different sequence that is not present in other known species (Figure 7).

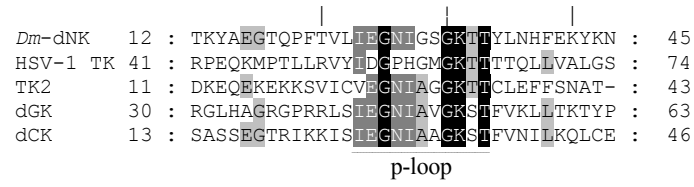


Figure 7: Alignment of the p-loop of *Dm*-dNK, HSV-1 TK, TK2, dGK, and dCK.

4.1.2 Glycine involved in H-bond with the substrate base

Multiple X-ray crystal structures of HSV-1 TK have shown the importance of Gln125 in the hydrogen bonds formation with pyrimidine and purine substrates (Brown *et al.* 1995; Champness *et al.* 1998; Bennett *et al.* 1999). The Gln125 is strictly conserved among HSV-1 TK, *Dm*-dNK and the other human kinases, indicating that this residue should be of crucial importance in the catalytic activity of dNKs (Table 4).

HSV-1 TK	Dm-dNK	TK2	dGK	dCK
Gln125	Gln81	Gln79	Gln111	Gln97

Table 4: Comparison of HSV-1 TK Gln125 with the relative residue in *Dm*-dNK, TK2, dGK and dCK.

4.1.3 Amino acids involved in the definition of the active site

Crystallographic data analysis and sequence comparisons have shown that in addition to the glycine-loop and the Gln125 another four amino acids are important in the definition of the active site (Table 5).

HSV-1 TK	Dm-dNK	TK2	dGK	dCK
Met128	Val84	Val82	Ser114	Ala100
Tyr132	Met88	Met86	Arg118	Arg104
Ala168	Ala110	Ala108	Asp147	Asp133
Tyr172	Phe114	Phe112	Phe151	Phe137

Table 5: Comparison of four important residues in HSV-1 TK, *Dm*-dNK, TK2, dGK and dCK.

4.1.3.1 HSV-1 TK Met128 and Tyr172

The two residues Met128 and Tyr172 form a pocket in the active site of HSV-1 TK which accommodates the thymine ring of the substrate. The nucleoside base is located in a sandwich-like orientation. Met128 and Tyr172 are unique for HSV-1 TK, in the other kinases the Tyr is always replaced by Phe, and the Met has different substitutes. Molecular modeling analysis has suggested that the role of Met128 in HSV-1 TK is mainly hydrophobic and steric (Alber *et al.* 1998).

4.1.3.2 HSV-1 TK Tyr132 and Ala168

Dm-dNK Met88 and Ala110, that correspond respectively to HSV-1 TK Tyr132 and Ala168, have been suggested to be important to alter the *Dm*-dNK substrate specificity from predominantly pyrimidine specific into purine specific (Knecht *et al.* 2002).

4.2 MUTAGENESIS AND ENGINEERING

4.2.1 Glycine-rich loop (p-loop)

In one of the first investigations of the HSV-1 TK p-loop zone, six mutations were analyzed in the sequence 54-65 of HSV-1 TK (Liu and Summers 1988). Five of the mutants retained less than 1% of the wild-type activity. Only when Thr63 was changed to Ser63 (as is present in dCK and dGK) the enzyme was still active. The Ser63 mutation altered not only the affinity for ATP, but also for dThd. This result showed that ATP and dThd are quite close to each other, and that a hydroxyl group is required for the transfer of the phosphoryl group (Liu and Summers 1988).

In another mutagenesis work three amino acids of the HSV-1 TK p-loop were analyzed (Pilger *et al.* 1999). His58 was mutated into His58Leu and this protein retained activity but the K_m for dThd was largely increased (≈ 600-fold) and the reduction of K_{cat} was about 60-fold. It was also reported how an inactive double mutant (Met128Phe / Tyr172Phe) transformed into a triple mutant (His58Leu / Met128Phe / Tyr172Phe) turned out to restore the activity and ability to phosphorylate dThd with about 600-fold increased K_m. To explain this result it was suggested that the triple mutant has a better hydrophobic fit to natural substrates, and in particular residue 58 plays a central role in the formation of a hydrophobic pocket in a catalytically active mutant enzyme. However the functional role of His58 is not yet fully understood.

4.2.2 Glycine involved in H-bond with the substrate base

Three different mutated HSV-1 TK, in which the Gln125 was replaced by Glu, Asn and Asp, have been evaluated. An initial investigation showed that the Glu125 mutant was completely inactive with dThd, dCyd and ACV, and that the Asn125 mutant exhibited decreased binding affinity for dThd about 50-fold (Kussmann-Gerber *et al.* 1998). Afterwards it was demonstrated that these three fairly conservative mutations could significantly alter the substrate specificity and overall enzyme activity. In particular for each mutant there was a marked decrease in its ability to phosphorylate pyrimidine nucleosides, and they also showed minimal TMPK activity. For the metabolism of GCV and ACV only Gln125Asn retained most of the wild-type phosphorylation properties. The mutations Gln125Asp and Gln125Glu instead decreased more than 80% in the ability of HSV-1 TK to phosphorylate GCV and ACV (Hinds *et al.* 2000). The expression of these mutated HSV-1 TK enzymes in two different cell lines confirmed the capability of Gln125Asn to act identically to the wild-type HSV-1 TK, the only major difference was the lower metabolism of deoxypyrimidine substrates. The efficiency of the purified Gln125Asn and Gln125Glu enzymes for GCV is 4-fold and 82-fold lower than the wild-type respectively, but yet both enzymes are still effective in mediating GCV killing of cells (Drake *et al.* 1999).

A further development was the combination of the Gln125Asn mutation and random mutagenesis with the aim to obtain a GCV-specific enzyme by eliminating the competition of dThd and improving GCV phosphorylation specifically (Black and Loeb 1996; Black *et al.* 1996). Of all the mutants evaluated a couple retained minimal thymidine and maximal GCV phosphorylation activities, but they were unable to maintain the wild-type level activities for GCV. Nevertheless an important conclusion was that the surrounding of Ala168 is responsible for loss of deoxypyrimidine substrate binding capacity (Mercer *et al.* 2002).

Molecular modeling studies suggested also that the loss of the hydrogen-bond between thymidine and the Gln125Asp or Gln125Asn mutants effectively contributes to altered activity, while the hydrogen-bond between each mutant and GCV is still retained. Clearly, the replacement of Gln125, that is a hydrogen-bond donor, with Glu or Asp, that are hydrogen acceptors, is an important factor that contributes to change the activity of the HSV-1 TK enzyme.

More recently it was displayed that treatment of HSV-1 TK Gln125Asn gene-transfected tumor cells with BVDU resulted in a marked accumulation of BVDU

monophosphate and this produced a strong inhibition of thymidylate synthase (Degreve *et al.* 2001).

4.2.3 Amino acids involved in the definition of the active site

4.2.3.1 HSV-1 TK Met128 and Tyr172

The non specific role of Met128 has been confirmed by site-directed mutagenesis experiments, which have shown that the binding affinity of dThd is preserved when the Met residue is replaced by another hydrophobic residue such as Ile (Pilger *et al.* 1999). As predicted by the computer model the mutation to Met128Phe produced a completely inactive enzyme. Surprisingly, also the Met128Ala mutation resulted in an almost inactive enzyme, suggesting the importance of a rather bulky residue at position 128. This result is in contrast to the Ala100 present in dCK. The Met128Ile was shown to have an activity comparable with wild-type HSV-1 TK (Pilger *et al.* 1999).

The domain 165-177, and in particular the Tyr172, has been the focus for several random sequence selection studies (Dube *et al.* 1991; Munir *et al.* 1992; Munir *et al.* 1993; Munir *et al.* 1994). The results of random mutagenesis studies suggested that in contrast to the wide spectrum of permissible mutations at position 165-170, the requirement of a Tyr or Phe at position 172 is necessary. Since Tyr and Phe are structurally similar, a change of Tyr to Phe may represent an isosteric replacement that does not lead to conformational instability. If this is the case the hydroxyl side chain of Tyr does not have a dominant role in the conformational stability or in the function of the enzyme (Munir *et al.* 1992). It was also reported that the K_m of the single mutant Tyr172Phe was in the same order of magnitude as the wild-type (Pilger *et al.* 1999).

4.2.3.2 HSV-1 TK Tyr132 and Ala168

The role of Ala168 was not clarified by the random mutagenesis studies. It was shown that many residues could replace the Ala and only the negatively charged residues were not found in any substitution (Munir *et al.* 1992). This data is in contrast with the Asp147 and Asp133 present in dGK and dCK respectively.

The residue Tyr132 has not been considered in any mutagenesis work done on HSV-1 TK. However the corresponding residue in *Dm*-dNK, Met88, has been suggested to be one of the key residues important in the substrate specificity definition (Johansson *et al.* 2001).

dCK and dGK have a conserved Arg corresponding to Met88 in *Dm*-dNK. In particular the Arg118 in dGK has been suggested to be responsible for the high selectivity for purine deoxyribonucleosides (Johansson *et al.* 2001). Mutations of the Met88 showed a dramatic decrease in dThd phosphorylation efficiency, but a higher capacity to phosphorylate purines. Nevertheless dCyd remained the preferred substrate. Position 88 is in the center of the binding site for the methyl group of dThd, which might explain the drastic reduction of dThd phosphorylation (Knecht *et al.* 2002).

4.2.4 C-terminal deletion of amino acids

```
                          |         ¦         |         ¦         |
Dm-dNK    198 : QSCKVLVLDADLNLENIGTEYQRSESSIFDAISSNQQPSPVLVSPSKRQRVAR--- : 250
HSV-1 TK  322 : MHVFILDYDQSPAGC-RDALLQLTSGMVQTHVTTPGSIPTICDLARTFAREMGEAN : 376
TK2       197 : MAAPVLVIEADHHMERMLELFEQNRDRILTPENRKHCP------------------ : 234
dGK       243 : MNIPVLVLDVNDDFSEEVTKQEDLMREVNTFVKNL---------------------- : 277
dCK       229 : QEVPILTLDVNEDFKDKYESLVEKVKEFLSTL------------------------ : 260
```

Figure 8: Alignment of the C-terminal sequences of *Dm*-dNK, HSV-1 TK, TK2, dGK, and dCK.

Between all dNKs there is a general homology distributed over the entire sequence except for the last 30-40 aa (Figure 8). The role of the C-terminal part of the protein has been investigated with the creation of three different truncated versions of *Dm*-dNK. The Δ10 form retained almost the same catalytic efficiency as the wild-type enzyme. The Δ20 protein changed unexpectedly the dThd turnover towards higher rates, supporting the idea that the C-terminal domain of *Dm*-dNK has an inhibitory effect on phosphorylation of deoxyribonucleosides. The Δ30 form decreased the activity to about 1% of wild-type *Dm*-dNK, indicating loss of a domain or a part of a domain essential for catalysis (Munch-Petersen *et al.* 2000).

5 SUICIDE GENE THERAPY

Gene therapy can be defined as the transfer of a new gene into cells of an individual with resulting therapeutic benefit. Although replacement of a single gene is sufficient to treat monogenic diseases, the situation is different for cancer, since cancer is generally a complex system of multi-gene alterations.

Suicide gene therapy is a form of drug delivery system that allows for negative selection of malignant cells using a pro-drug approach. Cancer cells are transduced with a gene encoding an enzyme that can metabolize an otherwise non-toxic pro-drug into a toxic metabolite. The prototype gene of this system is the HSV-1 TK protein used in combination with GCV.

The suicide gene, ideally should be expressed exclusively in the tumor cells and should reach a concentration sufficient to activate enough pro-drug for clinical benefit. Since the expression of foreign enzymes will not occur in all cells of a tumor *in vivo*, a phenomenon referred to as a bystander effect is required to improve the cell killing.

Suicide genes may be introduced into tumor cells either by viral vectors or non viral methods. Much work is currently ongoing to improve both the delivery systems and to optimize the efficacy of the production of the toxic metabolites (Singhal and Kaiser 1998; Wierdl and Potter 2005).

Major advantages of suicide gene therapy are: (a) short-term expression of the suicide gene is often sufficient; (b) the knowledge of the cause of abnormalities is not required/necessary; (c) the toxic metabolite can kill chemotherapy resistant cancer cells: (d) transduction of only a fraction of the tumor cells can be enough due to the bystander effect.

5.1 HSV-1 TK / GCV SUICIDE GENE THERAPY

HSV-1 TK can provide unique activities to cells that express it, and makes those cells susceptible to nucleoside pro-drugs. The triphosphate (TP) product of the pro-drug is the toxic form of the nucleoside analog and it inhibits cellular DNA polymerase by competing with natural nucleotides. It can also be incorporated by the cellular polymerase in elongating DNA, leading to chain termination, which may result in cell death.

The most commonly used genetic pro-drug activation system is the HSV-1 TK / GCV combination. The GCV treatment in association with HSV-1 TK transduced cells has been demonstrated to induce cell cycle arrest and cell death through apoptosis

(Halloran and Fenton 1998; Wei *et al.* 1998; Beltinger *et al.* 1999; Beltinger *et al.* 2000). GCV-TP shows a higher affinity for HSV-1 DNA polymerase than for human polymerases, but initial studies of HSV-1 TK / GCV gene therapy showed that the level of HSV-1 TK obtained after transfection of cancer cells, generated enough phosphorylated GCV to inhibit also mammalian DNA polymerases (Moolten and Wells 1990; Ezzeddine *et al.* 1991).

HSV-1 TK / GCV has been used successful for gene therapy in a wide variety of animal tumor models, and is currently in clinical trials for human cancers (Floeth *et al.* 2001; Hasenburg *et al.* 2002; Voges *et al.* 2003). These trials have indicated the need for optimization of this strategy of combined gene/chemotherapy of cancer (Fillat *et al.* 2003).

In an attempt to enhance the HSV-1 TK suicide gene therapy approach, many routes to improvement have been undertaken: (a) the development of more efficient and less toxic pro-drugs (Balzarini *et al.* 1985; Balzarini *et al.* 1993; Balzarini *et al.* 1994; Shewach *et al.* 1994); (b) three-dimensional studies on crystal structures including different ligands inside the active site; (c) modifications of the wild-type HSV-1 TK (Balzarini *et al.* 2002).

Several of these studies have been focused to improve the efficiency of HSV-1 TK / GCV therapy, for example Black et al. have performed random sequence mutagenesis in the putative nucleoside binding site of HSV-1 TK and identified mutants that, upon transfection in mammalian cells, displayed enhanced sensitivity to GCV and ACV (Black and Loeb 1996). One of these mutants, containing six amino acid substitutions, was later shown to mediate markedly enhanced tumor cell killing *in vitro* and *in vivo* compared with wild-type HSV-1 TK (Kokoris *et al.* 1999).

5.2 BYSTANDER EFFECT

The concept of the previously mentioned bystander effect is that adjacent untransduced cells are killed by the transfer of the toxic metabolite of the pro-drug. It has been shown, that not all cells need to express HSV-1 TK to be susceptible to pro-drug mediated killing and a success of the combined gene/chemotherapeutic approach is believed to be heavily dependent on the bystander effect. Complete tumor eradication has been demonstrated even when as few as 10% of the tumor cells are transfected with the HSV-1 TK (Freeman *et al.* 1993). It has been shown by electron microscopic analysis that non-transfected tumor cells phagocytose apoptotic vesicles containing

phosphorylated GCV metabolites derived from degenerating HSV-1 TK gene-transfected cells.

However, the observation that bystander killing occurs even before the HSV-1 TK gene-transfected cells start to die, suggests that the transfer of phosphorylated GCV metabolites can occur through intercellular gap junctions (Bi *et al.* 1993; Ishii-Morita *et al.* 1997). Bystander insensitive tumor cell lines have been shown to express low levels of connexin-43, a major component of gap junctions. Transfection with the connexin gene rendered these cells more sensitive to bystander killing (Elshami *et al.* 1996; Mesnil *et al.* 1996; Nicholas *et al.* 2003).

Another study showed that the pyrimidine analogs exhibited less bystander effects than the purine analogs (Degreve *et al.* 1999). The reason for this difference in bystander killing potency of the pyrimidine versus purine nucleotides is presently unclear. One speculation suggests that, in contrast to purine analogs depending on viral TK only for monophosphorylation (after which cellular kinases can generate di- and triphosphate form), pyrimidine analogs depend on viral TK for both mono- and diphosphorylation, with cellular kinases adding the third phosphate moiety. Since nucleotide monophosphates are believed to be the predominant form that passes through gap junctions, purine nucleotide monophosphate analogs can be activated by cellular kinases of non-transduced cells.

In general the possibility to have bystander effect is considered essential for a successful suicide gene therapy, due to the limit of actual vector targeting systems that are not able to deliver the suicide gene in every tumor cells.

6 NUCLEOSIDE ANALOGS

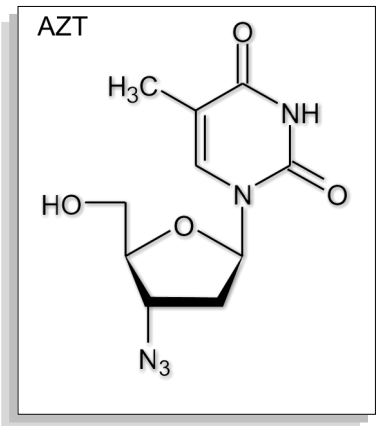
6.1 MECHANISM OF ACTION

Nucleoside analogs are pro-drug that need to be phosphorylated inside the cell to become pharmacologically active and carry on the expected effect. As previously mentioned there are three possible strategies that can be used to block or impair the DNA synthesis: (a) to use analogs lacking in the 3'-hydroxyl group that can be incorporated into the DNA, but that cannot permit DNA elongation, (b) to use analogs with an intact 3'-hydroxyl group, but with other structural modification that can severely impair the DNA strand elongation, (c) to use analogs that interfere with the enzymes involved in the supply of nucleotides.

6.1.1 3'-modified deoxyribonucleoside analogs

Analog triphosphates lacking the 3'-OH group have an inhibitory effect on the DNA synthesis since their incorporation into the DNA chain produce a premature termination of the elongation. They can be substrates and inhibitors for both endogenous DNA polymerases and viral enzymes (like HIV reverse transcriptase and HSV DNA polymerase).

6.1.1.1 AZT



3'-azido-2',3'-dideoxythymidine (AZT or zidovudine) is a thymidine nucleoside analog. AZT was the first drug approved for the treatment of AIDS and HIV infection. It was originally design for the treatment of cancer, but it failed to show efficacy and had severe side effects.

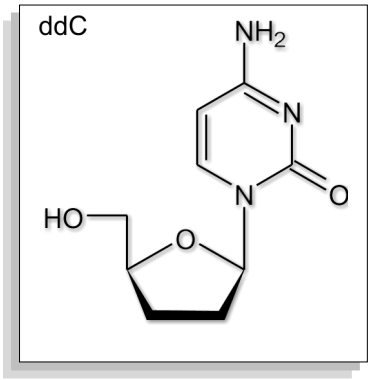
AZT can be phosphorylated by TK1 and TK2 but with different affinity, 50% and 5% respectively using dThd as reference (Eriksson *et al.* 1991; Munch-Petersen *et al.* 1991). Also *Dm*-dNK is able to phosphorylates AZT (Johansson *et al.* 1999).

There is evidence that the conversion from mono to diphosphate, catalyzed by dTMPK, is much slower and becomes the rate limiting step in AZT activation (Balzarini *et al.* 1989; Lavie *et al.* 1997).

Human DNA polymerase γ has a strong interaction with AZT and also DNA polymerase α is able to incorporate AZT into the growing DNA chain (Samuels 2006).

DNA polymerase β can also incorporate AZT, but with less efficacy. Most efficiently AZT can be incorporated by the HIV reverse transcriptase (Copeland *et al.* 1992).

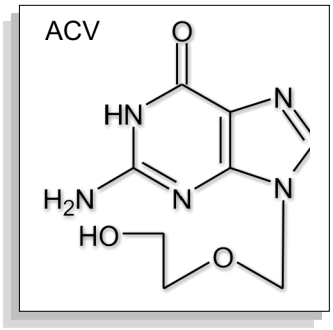
6.1.1.2 ddC



2',3'-dideoxycytidine (ddC or zalcitabine) is a deoxycytidine nucleoside analog and the first phosphorylation step is specifically catalyzed by dCK, followed by UMP-CMP kinase. It is also, but poorly, phosphorylated by TK2 and *Dm*-dNK (Johansson *et al.* 1999).

ddC was approved in 1992 for the treatment of AIDS. However severe toxic side effects limits the use of ddC (Lewis and Dalakas 1995; Dalakas *et al.* 2001). Studies suggest that the severe toxicity was due to the incorporation of the NA into the mtDNA (Lee *et al.* 2003). In particular, the side effects are correlated with the incorporation by DNA polymerase γ into mtDNA. Based on these studies a toxicity index has been proposed in which ddC is the most toxic compound with a value of 160000 (for instance AZT has a value of 0.05) (Lee *et al.* 2003). In contrast to AZT, ddC cannot be incorporated by DNA polymerase α, while the compound has a good affinity for the DNA polymerase β (Copeland *et al.* 1992).

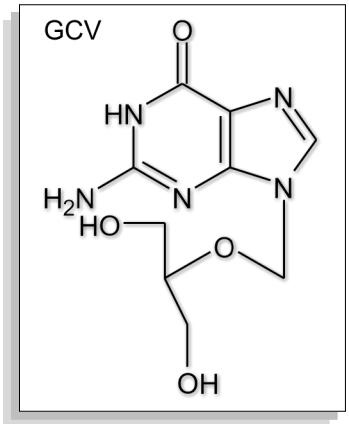
6.1.1.3 ACV and GCV



The two compounds: 9-(2-hydroxy-ethoxymethyl)–guanine (ACV or acyclovir) and 9-(2-dihydroxypropox–methyl)guanine (GCV or ganciclovir) are structurally different from the other NAs since the pentose ring is replaced by an open-chain structure.

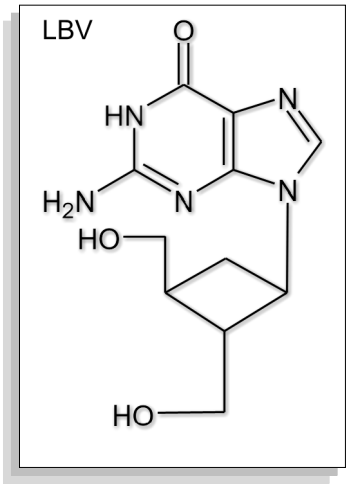
ACV is an extremely selective and specific antiviral compound with low cytotoxicity. The reasons for this are: (a) exclusive phosphorylation by the HSV-1 TK, HSV-2 TK, varicella zoster virus TK (VZV-TK) and Epstein-Barr virus TK (EBV-TK); (b) much higher affinity of ACV-TP for viral polymerases than human polymerases and therefore a specific inactivation of the viral enzymes; (c) termination of chain elongation by the incorporation of ACV into the DNA chain (Elion 1993). ACV is about 10 times more potent against HSV than VZV (Boyd *et al.* 1993; Balzarini *et al.* 1998). Because of its specificity for the viral kinase,

ACV has also been used in cancer gene therapy in association with HSV-1 TK (Hasegawa *et al.* 1995; Hayashi *et al.* 2006).



GCV is a potent inhibitor of viruses of the herpes family, including CMV (Matthews and Boehme 1988). The first phosphorylation step is accomplished by the HSV-1 TK or by the CMV-protein kinase (Sullivan *et al.* 1992). The primary mechanism for GCV activity is a selective and potent inhibition of the viral DNA polymerase. Unlike ACV, GCV is not an absolute chain terminator, and short fragments of viral DNA continue to be synthesized (Hamzeh *et al.* 1990; Hamzeh and Lietman 1991). Therefore the GCV antiviral activity is due to its ability to inhibit the synthesis of viral DNA and to slow down the elongation of viral DNA. As previously mentioned GCV has also been intensively used in cancer suicide gene therapy in combination with the HSV-1 TK.

6.1.1.4 LBV



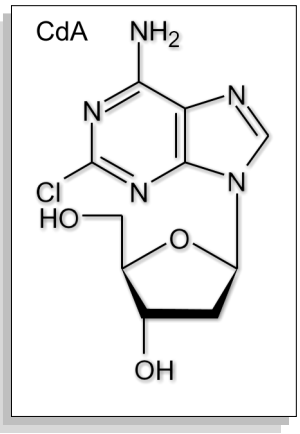
9 - [2,3-bis (hydroxymethyl) cyclobutyl] guanine (LBV or lobucavir) like ACV and GCV, needs to be mono-phosphorylated by the viral kinases.

LBV is used as antiviral against HSV-1, HSV-2, VZV and CMV (Field *et al.* 1990; Braitman *et al.* 1991). LBV has also been studied in application for hepatitis B virus infections, and it has been found to reversibly inhibit HBV production (Innaimo *et al.* 1997; Malik and Lee 2000). In molecular studies, LBV-TP acted as a non-obligate chain terminator of the viral encoded polymerase, inhibiting all three major enzymatic functions in genomic replication: oligodeoxynucleotide primer synthesis, reverse transcriptase activity and DNA-dependent DNA synthesis (Seifer *et al.* 1998).

6.1.2 Other nucleoside analogs

Nucleoside analog triphosphates with an intact 3'-hydroxyl group, but with other structural modifications, can severely impair the DNA strand elongation and/or interfere with other enzymes involved in the metabolism of the nucleosides.

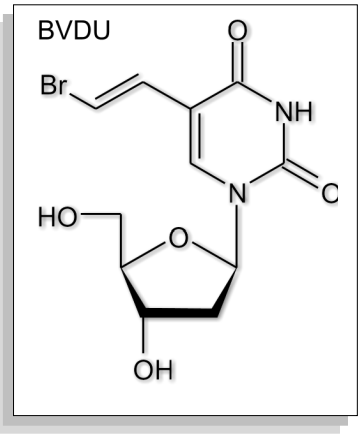
6.1.2.1 CdA



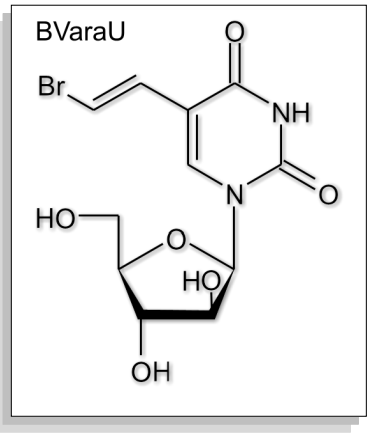
2-chloro-2'-deoxyadenosine (CdA or cladribine) is a deoxyadenosine nucleoside analog. It is phosphorylated to its monophosphate by dCK, dGK and *Dm*-dNK (Wang *et al.* 1993; Zhu *et al.* 1998; Johansson *et al.* 1999). CdA interfere with DNA causing both DNA strand breaks and blocking of RNA synthesis (Tallman and Hakimian 1995). Other effects of this compound may be caused by (a) inhibition of DNA polymerase with consequent reduction of DNA repair and (b) inhibition of RNR by the CdA diphosphate form (Parker *et al.* 1991). It has also recently been demonstrated that incorporation of CdA into the human TATA box sequence interfere with human RNA polymerase II transcriptional processes (Hartman and Hentosh 2004).

CdA is clinically used for the treatment of hairy cell leukemia and other lymphoid malignancies including chronic lymphocytic leukemia (Estey *et al.* 1992; Arner 1996; Tallman *et al.* 1999; Robak 2001).

6.1.2.2 BVDU and BVaraU



(E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU or brivudine) and 1-β-D-arabino furanosyl-5-(E)-(2-bromovinyl)uracil (BVaraU or sorivudine) are phosphorylated by TK2, *Dm*-dNK and viral TKs (Johansson *et al.* 1999; Franzolin *et al.* 2006). After further phosphorylation by cellular enzymes to the triphosphate form, the compounds interfere as competitive inhibitor/alternate substrates with the viral or cellular DNA polymerases (De Clercq 2005).

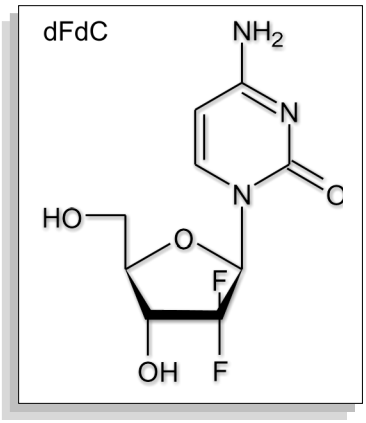


They are both potent inhibitor of HSV-1 as well as VZV. BVDU, compared to ACV, is slightly superior against HSV-1 and much superior against VZV (Naesens and De Clercq 2001). BVaraU is also an excellent anti-VZV compound (Wutzler 1997). In a clinical study patients with stomach cancer that were treated with a combination of fluorouracil and BVaraU developed a serious, and in a few cases lethal, bone

marrow suppression (Diasio 1998). Based on this serious toxicity the development of BVaraU for clinical use was arrested.

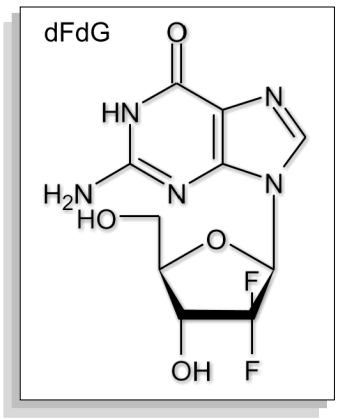
The efficient phosphorylation of BVDU by the viral TKs explains the marked cytostatic activity of the compound against tumor cells that have been transduced by the viral TK genes. Interestingly it has also been shown that a combination of GCV and BVDU in association HSV-1 TK produces an increased cytotoxicity (Hamel *et al.* 2001).

6.1.2.3 dFdC and dFdG



2'-difluorodeoxycytidine (dFdC or gemcitabine) and 2'-difluorodeoxyguanosine (dFdG) belong to the difluoro nucleoside analogs.

dFdC is phosphorylated to its monophosphate by *Dm*-dNK, dCK and poorly by TK2, and to its diphosphate by UMP-CMPK. dFdG is monophosphorylated by dGK, diphosphorylated probably by GUK (as in *Dm*) (Zhu *et al.* 1998; Zhu *et al.* 1998; Johansson *et al.* 2005). For both compounds the last step is catalyzed by NDPK.



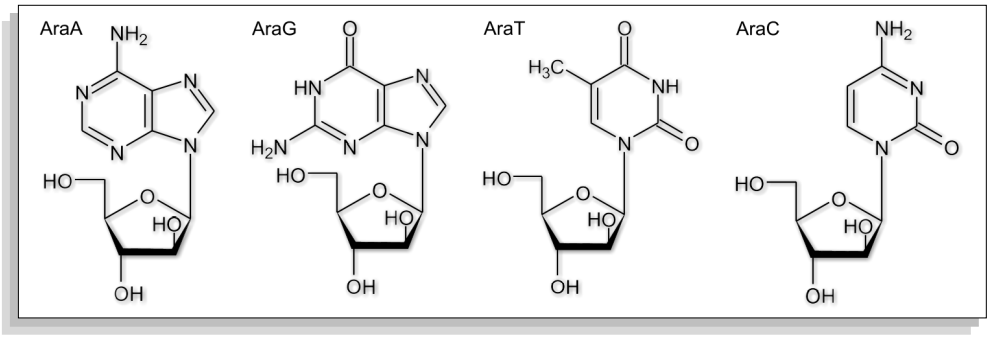
When dFdC or dFdG are incorporated into the growing DNA strand and one more natural nucleotide is added, the DNA polymerases are unable to proceed. This process is called "masked chain termination" and seems to block dFdC/dFdG into the DNA-chain since proof-reading exonucleases are unable to excise it (Huang and Plunkett 1995). dFdC diphosphate also inhibits RNR with a subsequent decrease in the dNTP pool, especially for dCTP. This results in further potentiation of dFdC phosphorylation and increased incorporation of dFdC into DNA (Huang *et al.* 1991). dFdG metabolites affect RNR and lower the dCTP pool, similar to the effects of dFdC.

dFdC was originally investigated for its antiviral effects but has since been developed for anticancer therapy for the treatment of pancreatic, lung, breast and other solid tumors(Bianchi *et al.* 1994; Noble and Goa 1997). dFdG has a narrower spectrum of activity compared to dFdC. Specifically dFdG could inhibit mammary tumor growth in mice, and showed a minor inhibition on ovarian and lymphosarcoma malignancies in

model systems. During preliminary toxicology studies, dFdG was associated with cardiac toxicity and has not been further developed (Andis *et al.* 1995).

6.1.2.4 AraA, araC, araG and araT

9-β-D-arabinofuranosyladenine (araA or vidarabine), 1-β-D-arabinofuranosyl–cytosine (araC or cytarabine), 9-β-D-arabinofuranosylguanine (araG or nelarabine) and 1-β-D-arabinofuranosylthymine (araT), belong all to the class of aranucleoside analogs.



AraA inhibits the DNA polymerase (notably α and β) and RNR with the same "self-potentiation" mechanism show by dFdC and dFdG (York and LePage 1966; Furth and Cohen 1967; Moore and Cohen 1967; Furth and Cohen 1968; Chang and Cheng 1980). When used as a substrate for a viral DNA polymerase, araA-TP competes with dATP leading to the formation of 'faulty' DNA. AraA also inhibits the RNA polyadenylation essential for HIV-1 and other retroviruses (Rose and Jacob 1978; Rose *et al.* 1982). AraA is active against herpes viruses, poxviruses, retrovirus, hepadnarviruses and some RNA tumor viruses, while as an anticancer compound it has been used against monocytic leukemia (Shannon *et al.* 1983; Honma and Niitsu 2000).

AraC is phosphorylated by dCK, UMP-CMPK and NDPK (Jordheim *et al.* 2006). It is also a substrate for *Dm*-dNK (Johansson *et al.* 1999). AraC shares the same mechanisms of action as araA. It is one of the most efficient drugs in the treatment of acute myeloid leukemia, but has also been used as an antiviral in the treatment of herpes virus infection. However since it causes severe side effects, such as bone marrow suppression, araC is mainly used in anticancer therapy.

AraG phosphorylation is catalyzed by dGK and, less efficiently, by dCK (Krenitsky *et al.* 1976; Lewis and Link 1989; Rodriguez *et al.* 2002). The diphosphate reaction is probably catalyzed by GUK (AraGMP has been shown to be a substrate for *Dm*-GUK (Johansson *et al.* 2005)). The triphosphate form, produced by the NDPK, has inhibitory effects on the DNA synthesis but not on RNA synthesis. AraG was

approved, in 2005, for the treatment of patients with T-cell acute lymphoblastic leukemia and T-cell lymphoblastic lymphoma.

AraT is phosphorylated by mammalian nucleoside TK2 and poorly by TK1, *Dm*-dNK and HSV-1 TKs (Arner *et al.* 1992; Johansson *et al.* 1999). It has been shown that araT inhibits of HSV, VZV while CMV was relatively resistant (Gentry and Aswell 1975; Miller *et al.* 1977). Some attempts to use this compound in suicide gene therapy have also been done (Rubsam *et al.* 1998).

7 THE PRESENT INVESTIGATION

7.1 AIM OF THE PROJECT

This study was performed to characterize deoxyribonucleoside kinases involved in the activation of anticancer and antiviral nucleoside analogs. The main focus has been to do site directed mutagenesis of residues involved in substrate recognition with determination of substrate specificity and kinetic properties of the recombinant enzymes and characterization of cells expressing the enzymes with altered substrate specificity.

7.2 SUMMARY OF PAPERS

The results on which this thesis is based are presented and discussed in paper I-II-III-IV. Here are only short summaries of the papers presented.

7.2.1 Paper I

Active site mutants of *Drosophila melanogaster* multisubstrate deoxyribo–nucleoside kinase.

We performed site directed mutagenesis on residues that, based on structural data, are involved in substrate recognition. The aim was to increase the phosphorylation efficiency of purine substrates to create an improved enzyme to be used in suicide gene therapy. A Gln81Asn mutated *Dm*-dNK showed a relative increase in dGuo phosphorylation compared to the wilt-type enzyme although the efficiency of dThd phosphorylation was 10-fold lower for the mutant. In addition to Gln81 the function of amino acid Asn28, Ile29 and Phe114 was investigated by different substitutions. All of the mutated enzymes showed decreased phosphorylation efficiency supporting their importance for substrate binding and/or catalysis as proposed by the solved structure of *Dm*-dNK.

7.2.2 Paper II

Engineering of a single conserved amino acid residue of herpes simplex virus type 1 thymidine kinase allows a predominant shift from pyrimidine to purine nucleoside phosphorylation.

The structure of herpes simplex virus type 1 thymidine kinase (HSV-1 TK) is the most investigated among all the kinases. We performed site-directed mutagenesis of the conserved Ala167 and Ala168 residues in HSV-1 TK based on observations of the possible interference of the side chains of these residues on substrate binding. It is

known that purine and pyrimidine bases bind at different positions of the active site although they are in the same geometric plane. The hypothesis was that the presence of a bulky side chain, such as tyrosine at position 167, could be sterically unfavorable for pyrimidine binding, whereas purine nucleosides would be less affected. The Ala168His- and Ala167Phe-mutated HSV-1 TK enzymes turned out to have a very low dThd kinase activity, maybe due to a steric clash between the mutated amino acid and the dThd ring. The GCV phosphorylating activity was not changed for A168H TK as compared to the wild-type enzyme. The enzyme mutants that lost their binding capacity for dThd also showed a substantially diminished feedback inhibition by thymidine 5'-triphosphate. The side chain size at position 168 seemed to play a less important role regarding GCV or dThd selectivity than at position 167.

7.2.3 Paper III

Enhanced toxicity of purine nucleoside analogs in cells expressing *Drosophila melanogaster* nucleoside kinase mutants

Despite its broad substrate specificity, *Dm*-dNK does not phosphorylate GCV or other guanosine analogs tested. In search for an alternative suicide gene to HSV-1 TK, mutants were constructed based on previous studies on amino acid substitutions that could change the substrate specificity from pyrimidines to purines (Knecht *et al.* 2002). It has also been shown that *Dm*-dNK with 20 amino acids deletion of the C-terminal has even higher catalytic rates for deoxyribonucleosides compared to the wild-type enzyme (Munch-Petersen *et al.* 2000). For this study we constructed three mutants: Met88Arg, Met88Arg+Val84Ala and Val84Ala+Met88Arg+Ala110Asp, with and without the C-terminal deletion. We analyzed the kinetic properties of these enzymes regarding dThd and GCV phosphorylation and substrate specificity. The mutant enzymes were expressed in an osteosarcoma TK^- cell line and the sensitivity of the cell line to nucleoside analogs was determined. The cells expressing the Met88Arg mutant enzyme showed the highest increased sensitivity to purine nucleoside analogs with 8- to 80-fold decreased inhibition constant IC_{50} compared to untransduced control cells or cells expressing the wild-type nucleoside kinase.

7.2.4 Paper IV

Mitochondrial expression of the *Drosophila melanogaster* multisubstrate deoxyribonucleoside kinase.

We have created an engineered *Dm*-dNK nucleoside kinase that is targeted to the mitochondrial matrix and compared it to the wild-type nuclear enzyme. The

osteosarcoma TK^- cells expressing *Dm*-dNK in the mitochondria showed increased sensitivity to several nucleoside analogs (BVDU, 5-BdUrd, 5-FdUrd) compared to the cells expressing the nuclear enzyme. Moreover, labeling studies using [^{3}H]-dThd showed that mitochondrial expression of *Dm*-dNK, compared to nuclear expression, resulted in a higher specific [^{3}H]-dTTP activity in the total dTTP pool and as a result a higher rate of [^{3}H]-dTTP incorporation into nuclear DNA. This study suggests that the mitochondrial targeting of a nucleoside kinase may increase sensitivity to nucleoside analogs.

8 CONCLUSIONS

- The site-directed mutagenesis of *Dm*-dNK contributes to the understanding of the function of the amino acids present in the active site and confirms the critical role of this structure in *Dm*-dNK conformation. The mutated Gln81 highlights the importance of this residue for the binding of the nucleoside base.

- *In vitro* analysis of the three *Dm*-dNK mutants Met88Arg, Val84Ala+Met88Arg and Val84Ala+Met88Arg+Ala110Asp showed an increase in phosphorylation of the purine nucleoside analogs GCV, araG and dFdG. The *in vivo* expression of the Met88Arg mutant enzyme in cell lines resulted in an increased sensitivity to purine nucleoside analogs with 8- to 80-fold decreased inhibition constant IC_{50}.

- Investigations of the amino acids 167 and 168 of HSV-1 TK showed the importance of Ala168 in the substrate recognition. The Ala168His mutant showed unaltered GCV activity but a heavily compromised dThd phosphorylation capacity. This discriminative activity has never previously been observed by one single amino acid mutation in HSV-1 TK.

- Mitochondrial targeting of *Dm*-dNK increased the sensitivity of cells to several nucleoside analogs. The reason for this effect may be related to the finding that the specific [^{3}H]-dTTP activity in the total dTTP pool was higher when *Dm*-dNK was expressed in the mitochondria which resulted in a higher rate of [^{3}H]-dTTP incorporation into nuclear DNA.

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