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**IDENTIFICATION AND
CHARACTERIZATION OF
NOVEL ENZYMES IN
MITOCHONDRIAL AND
CYTOSOLIC NUCLEOTIDE
METABOLISM**

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αξίζει φίλε να υπάρχουν για ένα όνειρο
και ας είναι η φωτιά του να σε κάψει

Intelligent life on a planet comes of age
when it first works out the reason for
its own existence
Richard Dawkins “The selfish gene”

αφιερωμένο στην οικογένεια μου

ABSTRACT

Nucleotides need to be synthesized within the cells since there are no carrier proteins for them in the cell membrane and their negatively charged phosphate groups prevent diffusion across the membrane. There are two main pathways for nucleotide synthesis: the *de novo* pathway and the salvage pathway. Both pathways involve several phosphorylation steps that result in the synthesis of the nucleoside triphosphates. The enzymes that catalyze the conversion of the nucleoside monophosphates to their corresponding diphosphates are called nucleoside monophosphate kinases (NMPKs). A subgroup of NMPKs is the adenylate kinase (AK) family that catalyzes the nucleotide phosphoryl exchange reaction between adenosine monophosphate (AMP) and adenosine triphosphate (ATP) and thus regulates adenine nucleotide ratios in different intracellular compartments.

The previously characterized AK5 was shown to be the second domain of a holoenzyme that we characterized in this thesis. The full-length AK5 exists in two similar transcript variants that differ in a 26 amino acid fragment in the N-terminus. AK5 is cytosolic or both cytosolic and nuclear depending on the transcript variant and it was shown to have two separate functional domains with the same AK activity. Both the full-length AK5 and its first domain phosphorylate AMP, deoxyadenosine monophosphate (dAMP), cytidine monophosphate (CMP) and deoxycytidine monophosphate (dCMP) with ATP or guanosine triphosphate (GTP) as phosphate donors.

Human AK4 was previously characterized as a mitochondrial enzyme but no enzymatic activity was confirmed. In this thesis, AK4 was further characterized and we were able to detect enzymatic activity. AK4 phosphorylates AMP, dAMP, CMP and dCMP with ATP or GTP as phosphate donors and kinetic studies showed that AMP is the preferred substrate. The mitochondrial import sequence of AK4 was found to be located within the first N-terminal 11 amino acid residues, very close to the ATP-binding site of the enzyme. Import analysis suggested that the mitochondrial import sequence is not cleaved and thus AK4 retains its activity upon entering the mitochondria.

In an attempt to complete the picture of the family of human AK isozymes, we characterized the previously identified AK7 and furthermore, we identified and characterized a novel AK which we named AK8. AK8 proved to have two functional domains. AK7, full-length AK8 and the two domains of AK8, all phosphorylate AMP, dAMP, CMP and dCMP with ATP as phosphate donor but also AMP, CMP and dCMP with GTP as phosphate donor. Kinetic studies showed that both enzymes are more efficient in AMP phosphorylation compared with the major cytosolic isoform AK1. Both AK7 and AK8 are located in the cytosol.

In our last study, we used previously generated and characterized CEM cells, resistant to the nucleoside analog 9- β -D-arabinofuranosylguanine (araG). By using microarrays, several genes with different biological functions were found to be down- or up-regulated. The main cytosolic AK isoform, AK1 was shown to be up-regulated, a finding that was further investigated both at the gene expression and the protein expression level. The study suggested that increased AK activity might contribute to araG resistance.

LIST OF PUBLICATIONS

- I. Solaroli N, **Panayiotou C**, Johansson M and Karlsson A
Identification of two active functional domains of human adenylate kinase 5
FEBS Lett. 2009 Sep 3;583(17):2872-6
- II. **Panayiotou C**, Solaroli N, Johansson M and Karlsson A
Evidence of an intact N-terminal translocation sequence of human adenylate kinase 4
Int J Biochem Cell Biol. 2010 Jan;42(1):62-9
- III. **Panayiotou C**, Solaroli N, Xu Y, Johansson M and Karlsson A
The characterization of human adenylate kinases 7 and 8 demonstrates differences in kinetic parameters and structural organization among the family of adenylate kinase isoenzymes
Biochem J. 2011 Jan 14;433(3):527-34
- IV. Curbo S, **Panayiotou C** and Karlsson A
Altered expression of adenylate kinase 1 and other genes in 9- β -D-arabinofuranosylguanine resistant T-lymphoblastic CEM cell lines
Manuscript

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LIST OF ABBREVIATIONS

Bases, nucleosides and nucleotides

A, Ado, AMP, ADP, ATP	adenine, adenosine, mono-, di- and triphosphate
G, Guo, GMP, GDP, GTP	guanine, guanosine, mono-, di- and triphosphate
C, Cyd, CMP, CDP, CTP	cytosine, cytidine, mono-, di- and triphosphate
U, Urd, UMP, UDP, UTP	uracil, uridine, mono-, di- and triphosphate
T, dThd, dTMP, dTDP, dTTP	thymine, deoxythymidine, mono-, di- and triphosphate
dAdo, dAMP, dADP, dATP	deoxyadenosine, mono-, di- and triphosphate
dGuo, dGMP, dGDP, dGTP	deoxyguanosine, mono-, di- and triphosphate
dCyd, dCMP, dCDP, dCTP	deoxycytidine, mono-, di- and triphosphate
dUrd, dUMP, dUDP, dUTP	deoxyuridine, mono-, di- and triphosphate
dIno, Ino, IMP, IDP, ITP	deoxinosine, inosine, mono-, di- and triphosphate
NTPs, dNTPs	ribonucleoside-, deoxyribonucleoside-triphosphates
cAMP, cGMP	cyclic adenosine/guanosine monophosphate

Enzymes

RNR	ribonucleotide reductase
ADK	adenosine kinase
UCK	uridine-cytidine kinase
TK	thymidine kinase
dCK	deoxycytidine kinase
dGK	deoxyguanosine kinase
NMPK	nucleoside monophosphate kinase
AK	adenylate kinase
UMP-CMPK	uridylate-cytidylate kinase
GUK	guanylate kinase
dTMPK	thymidylate kinase
NDPK	nucleoside diphosphate kinase
AMPK	AMP-activated protein kinase

Nucleoside analogs

CdA	2-chloro-2'-deoxyadenosine
FaraA	2-fluoro-9- β -D-arabinofuranosyladenine
araG	9- β -D-arabinofuranosylguanine
araC	1- β -D-arabinofuranosylcytosine
dFdC	2'-2'-difluorodeoxycytidine

Miscellaneous

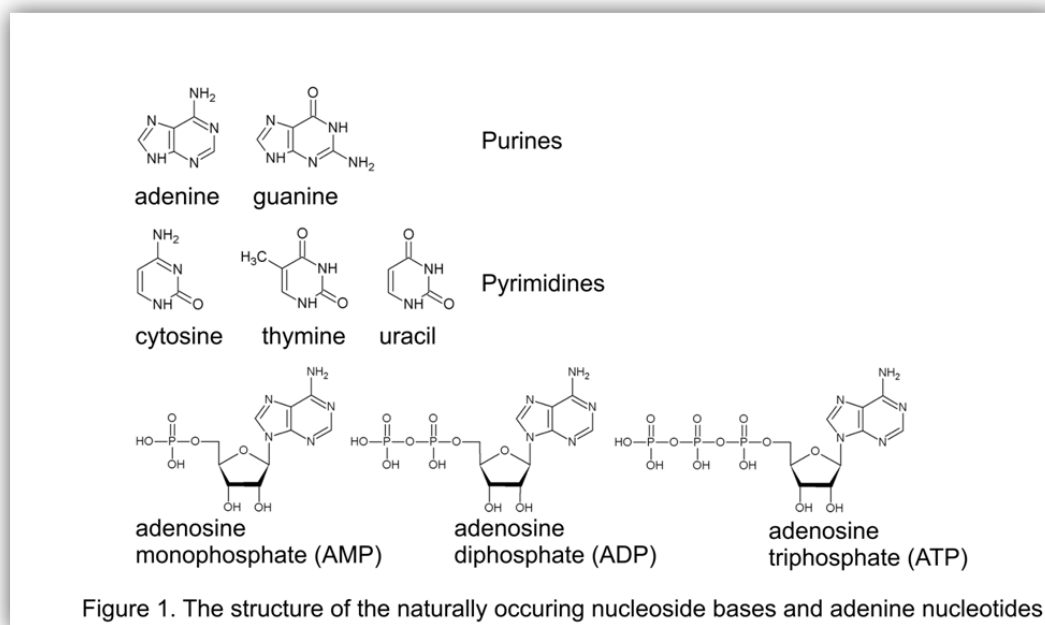
RNA	ribonucleic acid
DNA	deoxyribonucleic acid
mtDNA	mitochondrial DNA
NADH	nicotinamide adenine dinucleotide
FADH ₂	flavin adenine dinucleotide
CoA	coenzyme A
MDS	mtDNA depletion syndrome

1 INTRODUCTION

Nucleotides make up the structure of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) and are also important in cell metabolism as a source of chemical energy, in cell signaling and as cofactors of enzymatic reactions. RNA and DNA are polymeric macromolecules made from ribonucleoside triphosphate (NTPs)- or deoxyribonucleoside triphosphate (dNTPs)-monomers. The different forms of RNA include messenger RNAs that serve as template for protein synthesis, structural ribosomal RNAs, transfer RNAs for amino acid transfer, small nuclear RNAs that participate in RNA processing, small interfering RNAs and micro RNAs that contribute to the regulation of mRNA processing. DNA is made from dNTPs and is the molecule that contains the genetic information and instructions for functions in all living cells. The most important molecule to carry chemical energy is the nucleotide ATP and it is used in most metabolic processes as well as in signal transduction. ATP is continuously recycled in cells and the characterization of some of the enzymes involved in the turnover of ATP is the subject of this thesis. GTP is also an energy carrier formed in the citric acid cycle and used in many cellular processes such as gluconeogenesis and G-protein signaling. However, in mammalian cells the pathways of ATP production have been optimized making ATP the most important energy carrier in cellular processes. All nucleotides are synthesized within the cell since there are no transport systems for these negatively charged molecules in the plasma membrane. The pathways for nucleotide synthesis are the major focus of this thesis and will be presented in detail below. Several diseases are caused by defects in nucleotide synthesis but no or few treatments are available. Increased knowledge about the metabolic pathways for nucleotide synthesis is important for the development of both diagnostic methods and possible novel treatment strategies. Another important area of nucleotides is their use as pharmacologically active agents in anti-viral and anti-cancer therapy. The strategy is to use modified nucleosides as false DNA or RNA precursors. The incorporation of these nucleotide analogs into the growing DNA or RNA chains will interfere with or stop the replication of the targeted nucleic acid. All nucleoside analogs must be phosphorylated in three subsequent steps by cellular or viral enzymes to their corresponding triphosphates to be active as chemotherapeutic compounds. A careful characterization of the enzymes involved in nucleoside analog activation is important for efficient clinical use of these compounds.

2 NUCLEIC ACIDS

RNA and DNA are constructed from simple monomeric ribonucleotides and deoxyribonucleotides respectively, which have a common structure; a phosphate group linked by a phosphodiester bond to a pentose (a five-carbon sugar molecule) that in turn is linked to a nitrogen- and carbon-containing ring structure commonly referred to as a base. In RNA, the pentose is a ribose and in DNA, it is a deoxyribose that at the 2' position has a proton instead of a hydroxyl group present in a ribose. The hydroxyl groups make RNA less stable than DNA because it is more prone to hydrolysis. The bases adenine (A) and guanine (G) are purines, which contain a pair of fused ring structures; the bases cytosine (C), thymine (T) and uracil (U) are pyrimidines, which contain a single ring structure (figure 1). A, G and C are common for both DNA and RNA, whereas T is present in DNA and U in RNA. 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.



2.1 RNA

RNA is generally a single-stranded molecule and its synthesis is catalyzed by RNA polymerase using DNA as a template, a process known as transcription. Initiation of transcription occurs when a DNA helicase unwinds DNA around the initiation site and RNA polymerase binds at specific DNA sequences called promoters in the presence of various transcription factors. RNA polymerase then progresses along the template strand in the 3' to 5' direction, synthesizing a complementary RNA molecule with elongation occurring in the 5' to 3' direction. The DNA sequence also dictates where termination of the RNA sequence will take place. There are several types of RNA molecules including:

- i. Messenger RNAs (mRNAs) serve as templates for protein synthesis conveying the information from DNA to the ribosomes, the machinery for synthesizing proteins by translating mRNA.
- ii. Ribosomal RNAs (rRNAs) are structural components of the ribosomes.
- iii. Transfer RNAs (tRNAs) are small RNA molecules that transfer a specific amino acid at the ribosome. They have sites for amino acid attachment and an anticodon region for codon recognition that binds to a specific sequence on the mRNA through hydrogen bonding.
- iv. Small nuclear RNAs (snRNAs) are RNA transcripts that associate with proteins to form small nuclear ribonucleoprotein particles (snRNPs), which participate in RNA processing.
- v. Small interfering RNAs (siRNAs) are short double-stranded non-coding RNA molecules (usually 20–25 base pairs) with two nucleotide 3' overhangs that guide sequence-specific gene silencing of target mRNAs to which they are perfectly complementary by directing the RNA-induced silencing complex (RISC) to mediate site-specific cleavage, and, hence, destruction of the targeted mRNA. Exogenous siRNAs can also direct transcriptional gene silencing by inducing heterochromatin formation, leading to histone methylation and/or deacetylation, and ultimately DNA methylation (Lares et al., 2010).
- vi. Micro RNAs (miRNAs) are short non-coding RNA molecules, on average only 22 nucleotides long, which are able to control gene expression at the post-transcriptional level by specifically interacting with a target mRNA. Depending on the miRNA and its complementarity, miRNAs lead either to the cleavage of the mRNA and its subsequent degradation due to the presence of unprotected ends or to translational repression (Bartel, 2004).

2.2 DNA

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 enzymes involved are DNA polymerases, which catalyze the joining of dNTPs to the growing DNA chain. The energy required for this process is derived by the disruption of the high-energy pyrophosphate bond between the α - and β -phosphates.

Nuclear DNA replication occurs in the S-phase of the cell cycle. For a double-stranded DNA molecule to replicate, the two strands must be separated from each other, at least locally. Specific enzymes, called helicases, utilize the energy of ATP hydrolysis to catalyze strand separation. To initiate replication, DNA polymerase requires a RNA primer with a free 3'-hydroxyl group already base-paired to the template. Replication begins with the binding of DNA polymerase α , which includes a primase subunit used to synthesize the RNA primer, as well as an active DNA polymerase subunit. After this polymerase has created a stretch of about 20 deoxyribonucleotides to the primer, it is displaced and DNA polymerase δ continues the replication in both directions from the origin of replication until adjacent replicons meet and ligate by DNA ligase. DNA polymerase δ is the main enzyme used by eukaryotes for elongating DNA strands during replication, while DNA polymerase β is active both in dividing and non-dividing cells, suggesting a function in the DNA damage repair. Another member of the polymerase family, DNA polymerase ϵ , also contributes to the synthesis of nuclear DNA, though its exact role is uncertain (Hubscher et al., 2002).

Mitochondria are maternally inherited cytoplasmic double-membraned organelles and their 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. The two membranes that bound a mitochondrion differ in composition and function. The outer membrane, composed of about half lipid and half protein, contains porin proteins that render the membrane permeable to molecules having molecular weights up to 10 kDa. The inner membrane is much less permeable; its surface area is greatly increased by a large number of infoldings or cristae that protrude in the matrix. The mitochondrial DNA (mtDNA), the mitochondrial transcription and translation machinery and the enzymes that are part of the mitochondrial metabolic processes are located in the matrix (Logan, 2006). Although mitochondria contain their own DNA, most of the mitochondrial proteins are encoded by nuclear DNA. DNA polymerase γ is the only DNA polymerase found in

mitochondria, and it has the responsibility for DNA synthesis in all replication, recombination and repair transactions involving mtDNA (Graziewicz et al., 2006). MtDNA is synthesized independently of the cell cycle in both proliferating and non-proliferating cells and therefore, the need for available dNTPs is constant. The human mitochondrial genome consists of 16569 base pairs encoding genes for 13 proteins involved in oxidative phosphorylation, 22 tRNAs and 2 rRNAs.

3 ATP

ATP is one of the monomer subunits of RNA but what makes ATP one of the most important cellular molecules is its function as the molecular unit of intracellular energy transfer. Energy stored in ATP can be released upon hydrolysis of the two high-energy bonds that connect adjacent phosphates. This high-energy molecule is involved in cellular processes that require energy such as muscle contraction, active transport and maintenance of cell membrane integrity, and as a phosphate donor it is utilized to drive metabolic reactions such as the generation of other nucleotides. ATP can be produced by several distinct cellular processes; the three main pathways used to generate energy in eukaryotic cells are glycolysis, the citric acid cycle/oxidative phosphorylation and the β -oxidation.

The first step of the breakdown of carbohydrates is the degradation of glucose in the sequence of reactions known as glycolysis that occurs in the cytosol and produces ATP without the involvement of molecular oxygen. During glycolysis, a glucose molecule with six carbon atoms is converted into two molecules of pyruvate, each of which contains three carbon atoms. For each molecule of glucose, two molecules of ATP are hydrolyzed to provide energy to drive the early steps, but four molecules of ATP are produced in the later steps. At the end of glycolysis, there is consequently a net gain of two molecules of ATP and two molecules of nicotinamide adenine dinucleotide (NADH) for each glucose molecule broken down. Sugars other than glucose are similarly converted to pyruvate after their conversion to one of the sugar intermediates in this glycolytic pathway. The pyruvate then passes from the cytosol into mitochondria, where each pyruvate molecule is converted into CO_2 plus a two-carbon acetyl group—which becomes attached to coenzyme A (CoA), forming acetyl CoA with the gain of one NADH molecule. Acetyl CoA then transfers its two-carbon acetyl group to a four-carbon acceptor compound (oxaloacetate) to form a six-carbon compound (citrate). The final stage in the oxidation of glucose entails a set of nine reactions in which the acetyl group of acetyl CoA is oxidized to CO_2 . These reactions operate in a cycle that is referred to by several names: the citric acid cycle, the tricarboxylic acid cycle, and the Krebs cycle. The oxidation of cycle intermediates generates reduced electron carriers (three NADH molecules and one flavin adenine dinucleotide (FADH_2) molecule) and the hydrolysis of the high-energy thioester bond in succinyl CoA is coupled to synthesis of one GTP by substrate-level

phosphorylation (GTP and ATP are interconvertible). The final reaction regenerates oxaloacetate, so the cycle can begin again (Lodish, 2000).

The breakdown of glucose by glycolysis and the citric acid cycle yields a total of four molecules of ATP, ten molecules of NADH, and two molecules of FADH₂. Electrons from NADH and FADH₂ are then transferred to molecular oxygen, coupled to the formation of an additional 32 to 34 ATP molecules by oxidative phosphorylation. Electron transport and oxidative phosphorylation are critical activities of protein complexes in the inner mitochondrial membrane, which ultimately serve as the major source of cellular energy. During oxidative phosphorylation, electrons derived from NADH and FADH₂ combine with O₂, and the energy released from these oxidation/ reduction reactions is used to drive the synthesis of ATP from ADP. The transfer of electrons from NADH and FADH₂ to O₂ is a very energy-yielding reaction and for this energy to be harvested in usable form, it must be produced gradually by the passage of electrons through a series of carriers, which constitute the electron transport chain. These carriers are organized into four complexes in the inner mitochondrial membrane. A fifth protein complex then serves to couple the energy-yielding reactions of electron transport to ATP synthesis (Cooper, 2000).

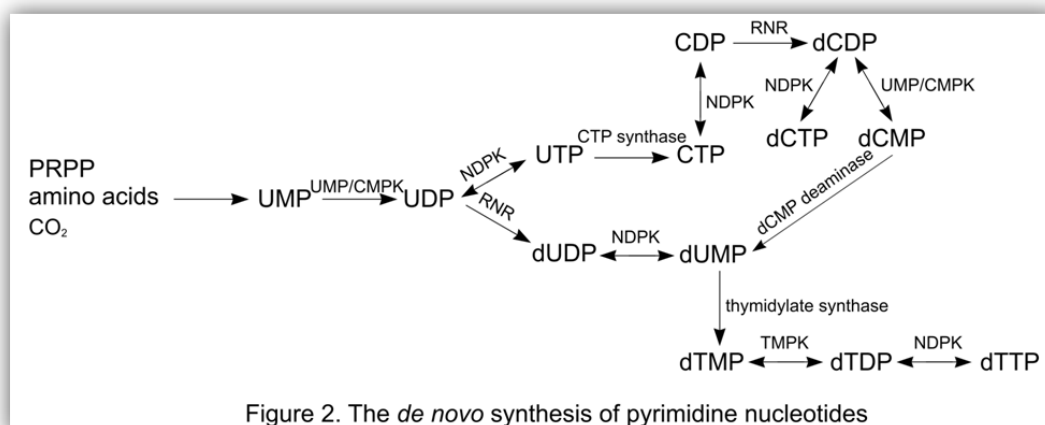
β-oxidation of fatty acids is also a major ATP producing pathway. The first step in oxidation of a fatty acid is its activation to a fatty acyl CoA that occurs in the endoplasmic reticulum or the outer mitochondrial membrane. Fatty acyl CoA is then transferred into the mitochondria by using carnitine as a carrier and there β-oxidation takes place. Two-carbon fragments are removed sequentially from the carboxyl end of the fatty acid and each set of oxidation results in the production of one molecule of acetyl CoA, one molecule of NADH and one molecule of FADH₂. Acetyl CoA enters the Krebs cycle, whereas NADH and FADH₂ enter oxidative phosphorylation (Devlin, 1992).

4 NUCLEOTIDE SYNTHESIS

Ribonucleotides and deoxyribonucleotides need to be synthesized within the cells since there are no carrier proteins for them in the cell membrane and the negatively charged phosphate groups prevent diffusion across the membrane. There are two pathways for nucleotide synthesis in mammalian cells termed the *de novo* and the salvage pathways.

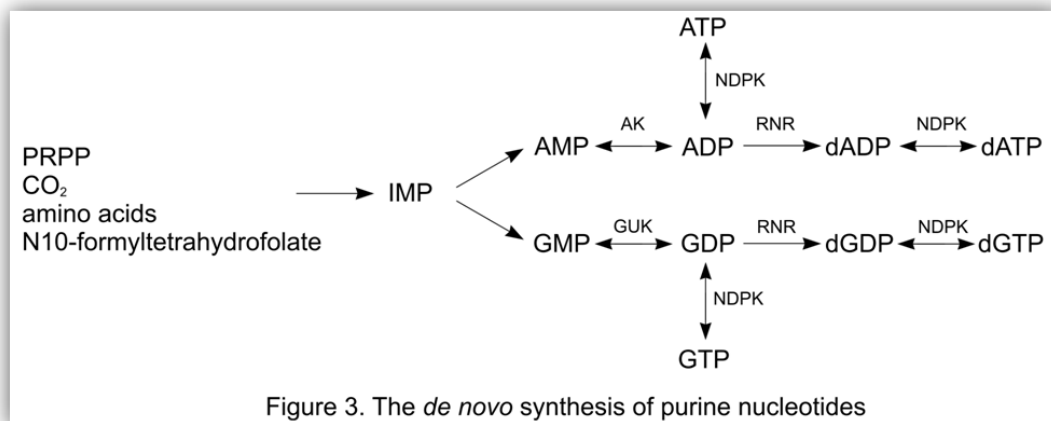
4.1 THE *DE NOVO* PATHWAY

The pyrimidine ring is synthesized utilizing amino acids as carbon and nitrogen donors and CO_2 as a carbon donor. Phosphoribosyl pyrophosphate (PRPP) acts as the donor of ribose 5'-phosphate that is added to the pyrimidine ring and uridine monophosphate (UMP) is created. UMP is phosphorylated by NMPK to uridine diphosphate (UDP) that is either phosphorylated further to uridine triphosphate (UTP) or converted to deoxyuridine diphosphate (dUDP) by ribonucleotide reductase (RNR). UTP can be transformed by cytidine triphosphate (CTP) synthase to CTP, which is dephosphorylated by nucleoside diphosphate kinase (NDPK) to cytidine diphosphate (CDP). CDP is converted to deoxycytidine diphosphate (dCDP) by RNR. Deoxyuridine monophosphate (dUMP) can be produced either by dephosphorylation of dUDP by NDPK or by deamination of dCMP by dCMP deaminase and it can be converted to deoxythymidine monophosphate (dTMP) by thymidylate synthase (figure 2).



Purine nucleotides are synthesized *de novo* by a stepwise building of the ring directly on 5'-phosphoribose to form inosine monophosphate (IMP), which serves as the precursor for AMP and guanosine monophosphate (GMP). The ring is constructed

by amino acids, N10-formyltetrahydrofolate and CO₂. AMP and GMP are then phosphorylated to their diphosphate forms; adenosine diphosphate (ADP) and guanosine diphosphate (GDP) by NMPKs. ADP and GDP are either phosphorylated further to their triphosphate forms or they are converted by RNR to deoxyribonucleoside diphosphates, which in turn are phosphorylated to deoxyadenosine triphosphate (dATP) or deoxyguanosine triphosphate (dGTP) (figure 3)(Berg, 2002).



4.2 SALVAGE PATHWAY

The salvage pathway is believed to supply quiescent or terminally differentiated cells with NTPs and dNTPs necessary for DNA replication, transcription and repair. Ribonucleosides and deoxyribonucleosides that derive either from free bases or dephosphorylation of existing NTPs and dNTPs after degradation of DNA or RNA respectively are imported into the cells by nucleoside carrier proteins that facilitate diffusion or actively transport nucleosides across the membrane. The next step is the phosphorylation of ribo- and deoxyribonucleosides to their monophosphate form by ribonucleoside kinases and deoxyribonucleoside kinases respectively. The nucleoside monophosphates are then phosphorylated to their triphosphate forms in two consecutive phosphorylation steps catalyzed by NMPKs and NDPKs (Reichard, 1988).

4.3 RIBONUCLEOSIDE KINASES

4.3.1 Adenosine kinase (ADK)

Adenosine is toxic to mammalian and bacterial cells. It is associated with inhibition of the immune response, coronary vasodilation and delayed

neurotransmission. It inhibits or stimulates hormone secretion and it is associated with changes in the metabolism of a number of tissues. Adenosine can be removed from cells by direct release to the extracellular environment, by synthesis to adenine nucleotides, or by degradation to purine end products (Andres et al., 1979). Adenosine can alter the intracellular concentration of cyclic AMP (cAMP) and it inhibits pyrimidine synthesis (Kowaluk and Jarvis, 2000). ADK catalyzes the phosphorylation of adenosine (Ado) to AMP. It is ubiquitous but has the highest expression in placenta, liver, muscle and kidneys (Spychala et al., 1996). ADK does not contain the classic N-terminal p-loop sequence motif that binds the phosphate donor, although it has a broad specificity for phosphate donors (Hurley et al., 1985). As previously mentioned, in mammalian cells adenosine is removed either by deamination to inosine (Ino) or by phosphorylation to AMP by ADK which also phosphorylates deoxyadenosine (dAdo). Although ADK has a much higher affinity for Ado than for dAdo, studies with ADK-deficient cell lines showed that it accounts for a significant part of dAdo phosphorylation (Verhoef et al., 1981). Two isoforms of ADK have been identified in mammals and both have the same biochemical and kinetic properties. The two isoforms are identical except in the N-terminal where the long isoform contains an extra 21 amino acids that replace the first 4 amino acids in the short isoform. The short isoform is localized in the cytosol whereas the long isoform has a nuclear localization. The two isoforms derive from the same gene by differential splicing, which introduces a nuclear localization signal to the long isoform (Cui et al., 2009; McNally et al., 1997). The human ADK gene is located on the long arm on chromosome 10 (Klobutcher et al., 1976).

4.3.2 Uridine-Cytidine kinases 1 and 2 (UCK1/UCK2)

UCK1 and 2 catalyze the phosphorylation of uridine (Urd) and cytidine (Cyd) to their monophosphate forms (Cheng et al., 1986). A UCK family with at least two members exists in human cells. UCK1 consists of 277 amino acids with a molecular weight of 31 kDa whereas UCK2 cDNA encodes a 261 amino acid protein with a molecular weight of 29 kDa. Neither UCK1 nor UCK2 phosphorylate Ado, guanosine (Guo) or any deoxyribonucleoside. UCKs can use ATP, GTP and all dNTPs as phosphate donors but not CTP or UTP. UCK1 mRNA is detected in all tissues, with high level of expression in liver, kidney, skeletal muscle and heart, while low levels are present in brain, placenta, small intestine and spleen. UCK2 is only detected in

placenta. UCK1 and 2 genes are localized in chromosomes 9 and 1 respectively (Van Rompay et al., 2001).

The terminal products of the pyrimidine nucleotide synthesis, i.e. CTP and UTP, appear to regulate their own synthesis, acting as a powerful and specific inhibitor at the first step of the enzymatic activity. There are two proposed mechanisms for UCK activity regulation. The first mechanism suggests that UTP and especially CTP are very effective feedback inhibitors, and it has been shown that the inhibition by CTP is mediated by a regulatory site on UCK from Novikoff ascites tumors (Orengo, 1969). The second mechanism proposes that changes in quaternary structure activates or inactivates the enzyme. ATP stabilizes the enzyme in the active tetrameric form, whereas UTP and CTP dissociate the enzyme to an almost inactive monomer (Cheng et al., 1986; Payne and Traut, 1982). In many normal tissues and most tumors, more UMP is synthesized by UCK than by the enzymes of the *de novo* pyrimidine biosynthesis (Denton et al., 1982; Weber et al., 1977). UCK1 and UCK2 have cytosolic localization (Greenberg et al., 1977).

4.4 DEOXYRIBONUCLEOSIDE KINASES

4.4.1 Thymidine kinase 1 (TK1)

The human TK1 gene is located on the long arm of chromosome 17. TK1 is a cytosolic enzyme (Lee and Cheng, 1976) which catalyzes the phosphorylation of deoxythymidine (dThd) and deoxyuridine (dUrd) (Munch-Petersen et al., 1991). Its maximal activity is achieved when ATP is used as a phosphate donor, but in addition to that, it is able to use CTP, UTP and GTP as phosphate donors. The pyrimidine triphosphates dCTP and dTTP were found to be effective inhibitors and among the purine triphosphates, dGTP exhibits a significant inhibitory effect (Ellims and Van der Weyden, 1981). ATP, besides being the best phosphate donor, is a regulator of the enzymatic mechanism as it has a stabilizing effect on the kinase activity. It has been shown *in vitro* that TK1 is a highly efficient tetramer in the presence of ATP, and that complete removal of ATP results in a less active dimer (Munch-Petersen et al., 1993).

TK1 seems to be broadly distributed in all malignant or normally growing tissues (Arner et al., 1992) and its expression is tightly regulated throughout the cell cycle, with transcriptional, translational and post-translational regulatory mechanisms. The activity of TK1 is high in proliferating cells and it is almost undetectable in quiescent cells (Coppock and Pardee, 1987). The TK1 activity increases as cells enter S phase

and decreases after the completion of DNA synthesis. Changes in TK1 activity during the cell cycle are the result of corresponding changes in the level of the TK1 protein. The levels of TK1 protein are not proportional to the levels of TK1 mRNA. At the G1/S border, there is an increase in the rate of synthesis of the enzyme due to an increase in the efficiency of translation of TK1 mRNA. TK1 accumulates because the newly synthesized enzyme is stable. The dramatic decline in the stability of the enzyme at the time of cell division, coupled with a decrease in the rate of TK1 synthesis, accounts for the rapid clearance of the enzyme (Sherley and Kelly, 1988). It has been shown that amino acid residues near the C-terminal of TK1 are responsible for the degradation of the enzyme in the G2 and M phase, and that mutations in this part of the gene allow expression in quiescent cells (Kauffman and Kelly, 1991).

4.4.2 Thymidine kinase 2 (TK2)

The human TK2 gene is located on the long arm of chromosome 16 (Johansson and Karlsson, 1997). TK2 is a mitochondrial enzyme and like the majority of the mitochondrial proteins encoded by the nuclear genome, it contains an N-terminal mitochondrial import signal. This import sequence not only facilitates mitochondrial import, but also regulates the stability of the enzyme (Wang and Eriksson, 2000). TK2 catalyzes the phosphorylation of dThd, dUrd and, in contrast to TK1, deoxycytidine (dCyd) by using ATP or CTP as phosphate donors (Munch-Petersen et al., 1991). The phosphorylation of dThd and dCyd is regulated by the availability of the substrates and by feedback regulation by the end products dTTP and dCTP.

In humans, TK2 is expressed in all tissues with the highest expression in liver, pancreas, muscle and brain and its expression is not cell-cycle regulated. The expression levels are similar in both terminally differentiated cells and growing cells hence; TK2 is the only thymidine phosphorylating enzyme that is expressed in non-proliferating tissues (Johansson and Karlsson, 1997).

4.4.3 Deoxycytidine kinase (dCK)

dCK catalyzes the phosphorylation of dCyd, deoxyguanosine (dGuo) and dAdo to their monophosphate forms (Shewach et al., 1992). The human dCK gene is located on the long arm of chromosome 4. dCK can use several triphosphates as phosphate donors and it can be efficiently inhibited by a number of different nucleotides such as dCTP, dCDP, dCMP, CDP and UDP (Arner and Eriksson, 1995). Overexpressed dCK

fused with GFP is located in the nucleus (Johansson PNAS 1997), whereas, under physiological conditions, native dCK is located in the cytoplasm (Hatzis et al., 1998).

The cell cycle variation in dCK activity is different in different cell lines, while in all cases, the dCK mRNA level is constant at all cell cycle phases (Arner and Eriksson, 1995). The expression profile of the enzyme shows the highest expression in skeletal muscle, fetal liver, thymus, bone marrow and brain and the lowest expression in adult liver and heart muscle (Johansson et al., 1997).

4.4.4 Deoxyguanosine kinase (dGK)

dGK is a nucleoside kinase that phosphorylates the purine deoxyribonucleosides dGuo, dAdo and deoxyinosine (dIno) (Wang et al., 1993). The human dGK gene is located on the short arm of chromosome 2 (Johansson et al., 1996). Although the enzyme can use several phosphate donors, ATP and UTP are the preferred ones (Zhu et al., 1998). dGK is a mitochondrial enzyme and its N-terminal has characteristic properties of a mitochondrial translocation signal.

Several studies have confirmed that dGK is constitutively expressed throughout the cell cycle and is present in all tissues with the highest expression in muscle, liver, brain and lymphoid tissues (Johansson and Karlsson, 1996).

4.5 RIBONUCLEOTIDE REDUCTASE

RNR catalyzes the *de novo* conversion of ribonucleoside diphosphates to deoxyribonucleoside diphosphates and is essential for providing the dNTPs required for DNA synthesis and repair. This rate-limiting step in the synthesis of DNA precursors is regulated in the cells at several different levels, e.g. by allosteric control of activity and specificity of RNR by dNTPs, by enhanced transcription of RNR genes encoding this enzyme during the S phase of the cell cycle and by rapid, proteasome-dependent proteolysis of its small subunit (R2) during the late phase of mitosis. RNR is a tetramer composed of two non-identical homodimeric subunits. The 170 kDa R1 dimer contains a catalytic site, binding sites for allosteric effectors, and a redox-active disulfide that participate in the reduction of substrates, while the 90 kDa R2 dimer contains a tyrosyl free radical generated by an iron center and is essential for catalysis (Chabes and Thelander, 2000; Thelander et al., 1985). Transcription of both R1 and R2 genes occurs exclusively during the S-phase but due to its long half-life, the level of R1 is virtually constant throughout the cell cycle and always exceeds the level of R2. Therefore, the cell cycle-dependent activity of RNR is regulated by the synthesis and degradation of

the R2 protein (Bjorklund et al., 1990). When R2 is not available, R1 associates with p53R2 to form active RNR that supplies quiescent cells with dNTPs for repair of damaged DNA (Tanaka et al., 2000). p53 induces cell-cycle arrest in the G1 and G2 phases, expression and nuclear accumulation of p53R2, and subsequent activation of RNR activity in response to DNA damage. The p53R2 protein is also expressed constitutively at low levels in both non-proliferating and proliferating cells, even in the absence of DNA damage. This suggests that this enzyme plays an essential role in supplying dNTPs for basal repair of DNA and mitochondrial DNA synthesis in G0/G1 cells. Thus, basal levels of p53R2 are expressed regardless of p53 status and p53R2 expression is upregulated by p53 in response to DNA damage (Wang et al., 2009). The central role of p53R2 in mitochondrial DNA synthesis is illustrated by the observation that mutations in p53R2 are associated with severe depletion of mtDNA in both human and mouse cell lines (Bourdon et al., 2007). A possible explanation for the predominant involvement of p53R2 in DNA repair is that p53R2, in contrast to R2, lacks a 33 amino acid fragment in the N-terminus (designated as the KEN box) (Chabes et al., 2003). Therefore, it should be able to evade cell-cycle specific proteolysis, thus allowing it to supply dNTPs during G0/G1. In addition to that, proper mitochondrial DNA synthesis in non-proliferating cells and also in cells in G0/G1 phase requires p53R2-catalyzed ribonucleotide reduction, since salvage of deoxyribonucleosides into mitochondria by dGK and TK2 is not enough (Hakansson et al., 2006; Tanaka et al., 2000).

Mammalian RNRs belong to the class 1 RNRs that are present in aerobic bacteria and in eucaryotes. Most class 1 RNRs are allosterically regulated by binding either the activating ATP or the inhibitory dATP to the active site of R1. They are also using another unique allosteric mechanism that regulates their substrate specificity and ensures that the enzyme produces equal amounts of each dNTP for DNA synthesis. This mechanism involves binding of end products (dATP, dGTP and dTTP) to the specificity site of the enzyme (Jordan and Reichard, 1998).

4.6 NUCLEOSIDE MONOPHOSPHATE KINASES

NMPKs are ubiquitous enzymes, present both in prokaryotes and eukaryotes that catalyze the reversible phosphoryl transfer between various nucleoside mono- and triphosphates both in the salvage and the *de novo* pathways of nucleotide metabolism. This enzyme family is further divided into subgroups consisting of adenylate kinases [EC 2.7.4.3], uridylate-cytidylate kinases (UMP-CMPK) [EC 2.7.4.14], guanylate kinases (GUK) [EC 2.7.4.8], and thymidylate kinases (dTMPK) [EC 2.7.4.9], on the

basis of the differences in substrate specificity. In marked contrast to the unique phosphoryl acceptor specificity of each subgroup, the phosphate donor specificity is not so high, with ATP serving as the general phosphate donor. Three functional domains have been described in the primary structure of NMPKs: the phosphate donor binding glycine rich region (p-loop), the substrate binding site and the lid domain that closes over the substrate upon binding (Scheffzek et al., 1996).

dTMPK phosphorylates dTMP and dUMP to their respective diphosphate forms (Arima et al., 1977; Su and Sclafani, 1991). The best phosphate donors for dTMPK are ATP, dATP, GTP and dGTP and none of the pyrimidine triphosphates tested could act as a phosphate donor. Thymidine was found to inhibit the activity of dTMPK and in addition to that, ADP, dADP, dTDP and dTTP could also exhibit inhibitory actions (Lee and Cheng, 1977). The dTMPK gene is located at 2q37. dTMPK is the only NMPK with a confirmed cell cycle regulation. Both dTMPK mRNA levels and enzymatic activity fluctuate through the cell cycle. In non-proliferating tissues, little or no dTMPK activity has been observed, while in growing tissues the activity increases markedly (Huang et al., 1994).

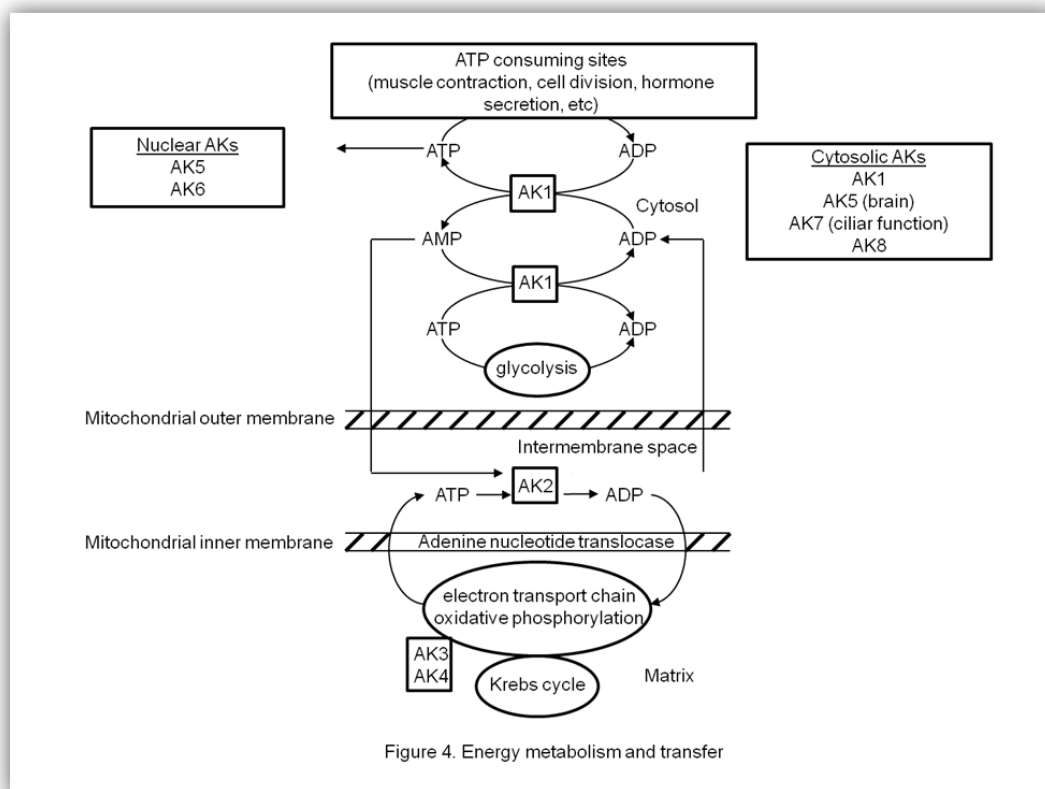
In addition to dTMPK, there are two other enzymes that phosphorylate pyrimidine nucleoside monophosphates; the cytosolic UMP-CMPK1 and the mitochondrial UMP-CMPK2. The chromosome location of human UMP-CMPK1 has been determined to 1q32 and the genomic sequence of UMP-CMPK2 is localized at 2p25. UMP-CMPK1 phosphorylates CMP, dCMP and UMP with highest efficiency and dUMP, AMP and dAMP with lower efficiency. Northern blot analysis has shown that the UMP-CMPK1 mRNA is ubiquitously present in human tissues with highest levels in pancreas, skeletal muscle and liver (Van Rompay et al., 1999b). UMP-CMPK2 phosphorylates dUMP, dCMP, CMP and UMP with ATP as phosphate donor, with higher efficacy for deoxyribonucleotides than for ribonucleotides. The expression profile of UMP-CMPK2 according to the available databases shows highest expression in bone marrow, whereas all other tissues have relatively rare transcripts (Xu et al., 2008).

GUKs catalyze the phosphorylation of GMP and dGMP. In humans, there are several GUK isozymes reported (Jamil et al., 1975) but only GUK1 has been cloned and characterized (Fitzgibbon et al., 1996). GUK mRNA is ubiquitously expressed, as would be anticipated from an essential housekeeping gene, and its chromosome localization was determined to the long arm of chromosome 1. GUKs participate in the

recovery of cGMP and are thought to regulate the supply of guanine nucleotides to signal transduction pathways (Brady et al., 1996).

4.6.1 Adenylate kinases

AKs catalyze the nucleotide phosphoryl exchange reaction $\text{AMP} + \text{ATP} \leftrightarrow 2 \text{ADP}$, and thus regulate adenine nucleotide ratios in different intracellular compartments. In addition to that, AKs enable the transfer and make the energy of the two high-energy phosphoryl bonds of ATP available from generation to utilization sites (figure 4). The presence of several isoforms of AKs in mammalian tissues is already well established. The many isoforms is a specific feature of AKs that is only shared with the GUKs among the nucleotide and nucleoside kinases. Eight different AK isoenzymes have been identified and characterized in human tissues and they have been assigned numbers according to their order of discovery.



AK1 is the major cytosolic AK isoform and its gene is located at 9q32. It is present in most human tissues with the highest expression levels in skeletal muscle, brain and erythrocytes (Khoo and Russell, 1972). AK1 shows no phosphate donor

specificity as it can use all nucleoside triphosphates to phosphorylate AMP and, to a lesser extent, dAMP (Panayiotou et al., 2011; Wilson et al., 1976).

The major mitochondrial isoform AK2 is present in the mitochondrial intermembrane space and its gene is located at 1p34 (Fukami-Kobayashi et al., 1996). Northern blot analysis demonstrated that AK2 mRNA is strongly expressed in liver, heart, skeletal muscle and pancreas, moderately expressed in kidney, placenta and brain and weakly expressed in lung. However, at the protein level, AK2 is detected abundantly in liver, kidney and heart and faintly in lung and skeletal muscle (Khoo and Russell, 1972; Noma et al., 1998). AK2, like AK1, can use all nucleoside triphosphates to phosphorylate AMP (Wilson et al., 1976).

AK3 is localized in the mitochondrial matrix and it has its highest expression levels in liver, brain, heart, skeletal muscle and kidney (Noma et al., 2001). The chromosomal location of human AK3 gene has been determined to be the short arm of chromosome 9. AK3 shows both substrate and phosphate donor specificity, as it can use only GTP and inosine triphosphate (ITP) to phosphorylate only AMP (Wilson et al., 1976).

The gene for human AK4 was identified in 1992 and it was initially named AK3 based on its sequence similarity with bovine AK3 (Xu et al., 1992). However, when the mouse and rat AK4 were identified, human AK3 was renamed to human AK4 as it had a higher sequence similarity with these genes (Yoneda et al., 1998). AK4 is present in the mitochondrial matrix and its gene is located at 1p31 (Noma et al., 2001). The enzyme is expressed mainly in tissues rich in mitochondria like the brain, heart, kidney and liver. AK4 catalyzes the phosphorylation of AMP, dAMP, CMP and dCMP with ATP or GTP as phosphate donors and also AMP with UTP as phosphate donor. The mitochondrial import sequence of AK4 was shown to be located within the first N-terminal 11 amino acid residues, very close to the phosphate donor binding region. Import analysis suggested that the mitochondrial import sequence is not cleaved and thus the enzyme retains its activity upon entering the mitochondria (Panayiotou et al., 2010).

AK5 exists in two main variants which differ in the N-terminus, where one variant has an additional short sequence of 26 amino acids. The enzyme has a cytosolic or both cytosolic and nuclear localization depending on the variant and in contrast to the multi-tissue expression profiles of most other AKs, it is expressed almost exclusively in brain. AK5 has two separate functional domains, named AK5p1 and AK5p2. The full length enzyme and its two domains, when expressed separately, have

similar activity as they phosphorylate AMP, dAMP, CMP and dCMP with ATP or GTP as phosphate donors (Solaroli et al., 2009; Van Rompay et al., 1999a). The chromosomal location of the gene for the human AK5 has been mapped to 1p31.

AK6 is the only nuclear human AK isoform identified so far. AMP and dAMP are the preferred substrates of AK6 but CMP and dCMP are also good substrates. All NTPs and dNTPs can serve as phosphate donors, but CTP and UTP are preferred donors. The nuclear localization of AK6 combined with its broad activity with all phosphate donors, suggest a possible role for the enzyme as a regulator of ATP/ADP and GTP/GDP ratios in the nucleus (Ren et al., 2005). The tissue distribution of AK6 has not yet been determined but according to database analysis there is moderate to strong expression in almost all normal tissues. The gene of AK6 is located at the long arm of chromosome 5.

AK7 is a cytosolic AK isoform, substantially longer than other identified single-domain AKs that have a length between 172 and 239 amino acids. The full length AK7 cDNA was expressed and shown to produce a 656 amino acid protein, while according to the databases, the gene product was expected to be 723 amino acids long. This is due to the presence of a stop codon at the end part of the gene and suggests that there may exist more than one transcript variant of AK7. The enzyme phosphorylates AMP, dAMP, CMP and dCMP with ATP as phosphate donor and in addition to that, it phosphorylates AMP, CMP and dCMP with GTP as phosphate donor (Panayiotou et al., 2011). The chromosomal location of the AK7 gene has been mapped to the long arm of chromosome 14. AK7 seems to have a tissue-restricted expression as it is expressed in tissues rich in epithelium with cilia such as lung, mammary gland, trachea, testis and skeletal muscle and its activity has been associated with cilia function (Fernandez-Gonzalez et al., 2009).

The recently identified and characterized AK8 is another cytosolic AK and its gene is located at the long arm of chromosome 9. Although AK8 has a similar two-domain structure as found in AK5, it is different than AK5 in that it exists only in one transcript variant. When the two AK domains of AK8 were expressed separately they both showed similar AK activity. The AK8 protein may thus harbor two functionally active domains in the same polypeptide chain, something that is suggested by the very high phosphorylation efficiency of the enzyme. The full length AK8 and its two domains use ATP as phosphate donor to phosphorylate AMP, dAMP, CMP and dCMP and GTP to phosphorylate AMP, CMP and dCMP (Panayiotou et al., 2011). According to the databases AK8 is expressed in liver, pancreas, lung, trachea and testis and it has

been identified to be one of the genes that have a negative regulatory role in epithelial cell migration (Simpson et al., 2008).

Adenylate kinase	Subcellular localization	Tissue distribution
AK1	cytosol	all tissues
AK2	mitochondria (inter-membrane space)	liver, heart, skeletal muscle, kidney, lung
AK3	mitochondria (matrix)	liver, heart, skeletal muscle, kidney
AK4	mitochondria (matrix)	liver, heart, brain, kidney
AK5	cytosol / nucleus	brain
AK6	nucleus	all tissues
AK7	cytosol	lung, trachea, testis, mammary gland, skeletal muscle
AK8	cytosol	liver, pancreas, lung, trachea, testis

Table 1. Subcellular localization and tissue distribution of the human AK isoforms

4.7 NUCLEOSIDE DIPHOSPHATE KINASES

NDPKs catalyze the phosphorylation of nucleoside diphosphates to their corresponding triphosphates. In contrast to the nucleoside and nucleotide kinases involved in the first two steps of the nucleotide phosphorylation pathway, NDPKs are not specific for the nucleotide diphosphate as they accept purines and pyrimidines and both ribo- and deoxyribonucleoside diphosphates as substrates. Their ATP-binding site does not involve a p-loop motif. NDPKs are encoded by the *nm23* genes and in humans, ten genes have been identified to be a part of the *nm23*/NDPK family. These genes are named *nm23*-H1-9 and the tenth gene *RP2*. *Nm23*-H1-6 genes and their corresponding proteins have been thoroughly characterized. The activity pathway of the NDPKs is composed of two sequential reactions: (1) the autophosphorylation of a histidine residue, and (2) a phosphotransfer reaction from the phosphohistidine intermediate to the nucleoside diphosphate (Boissan et al., 2009). In addition to their role as housekeeping enzymes, NDPKs are suggested to display a diverse array of biological functions that include roles in cellular proliferation, differentiation, development, apoptosis, protein synthesis and signal transduction by activating GTP-binding proteins (Barraud et al., 2002).

Nm23-H1 and Nm23-H2 are the two main isoforms of human NDPKs and they are 88% identical and show similar kinetic parameters for natural substrates. They can form *in vitro* and *in vivo* homo- and heterohexamers possessing different ratios of the respective subunits (Gilles et al., 1991). Their corresponding genes are located on the long arm of chromosome 17. Nm23-H1 is located in the cytosol and Nm23-H2 is reported to have both a cytosolic and nuclear localization (Kraeft et al., 1996).

The third NDPK isoform, also named DR-nm23 protein, has 66.9% and 64.9% amino acid sequence identity, respectively, to the human nm23-H1 and nm23-H2 proteins. It has a cytosolic localization and its murine counterpart is localized in Golgi and endoplasmic reticulum membranes (Barraud et al., 2002).

Nm23-H4 encodes a catalytically active NDPK that is localized in mitochondria. Submitochondrial fractionation has shown that Nm23-H4 behaves like porin; suggesting that it is associated with contact sites between the inner and the outer mitochondrial membranes. The NDPK activity of the mitochondrial fraction of various tissues represents only a few percent of the total cellular NDPK activity, which is mainly cytosolic due to the abundantly expressed A and B isoforms. The level of nm23-H4 mRNA is at least 10-fold lower than that of nm23-H1 and nm23-H2 (Milon et al., 2000). The genes for the third and the fourth NDPK isoforms are localized at the long and short arm of chromosome 16, respectively.

Nm23-H5, is unique among the nm23 family since its product possesses a C-terminal extension of 55 amino acids and presents an expression which is testis specific (Milon et al., 2000; Munier et al., 1998). The nm23-H5 gene was mapped to chromosome 5q23-31.

Nm23-H6 gene has been mapped at chromosome 3p21 and is highly expressed in heart, placenta, skeletal muscle and several cancer cell lines. Its product has a mitochondrial localization and plays a role in regulation of cell growth and cell cycle progression (Tsuiki et al., 1999).

4.8 FUNCTIONS OF NUCLEOTIDES

All types of cells contain a wide variety of nucleotides and their derivatives, which are involved in many metabolic processes that must be carried out for normal cellular growth and function. ATP is the main form of chemical energy available to the cell and it serves as the driving force of many metabolic reactions and also as a phosphate donor. GTP can also be used as energy carrier. Nucleotides are the monomeric units of nucleic acids and in addition, they are components of coenzymes

such as NAD, FAD and CoA. Other recognized functions of nucleotides involve those in which the nucleotides and their derivatives serve as mediators of key metabolic processes. Cyclic AMP (cAMP) and cyclic GMP (cGMP) are ubiquitous intracellular second messengers, the levels of which are increased in response to a variety of hormonal and chemical stimuli. cAMP can activate the cAMP-dependent protein kinase and thereby activate or inhibit the activity of several enzymes, regulates the expression of specific genes, activates calcium channels and controls glycogenolysis and glycogenesis. cGMP can, through activation of the cGMP-dependent protein kinase, regulate ion channel conductivity, cellular apoptosis and relax smooth muscle tissues that, in blood vessels, results in vasodilation and increased blood flow (Francis and Corbin, 1999). AMP regulates a number of AMP sensitive components, including those in the glycolytic and glycogenolytic pathways and also metabolic sensors and effectors such as the K-ATP channels and AMP-activated protein kinase (AMPK), which in turn can regulate cellular energy balance through managing calcium influx and phosphorylating targeted proteins (Dzeja and Terzic, 2009). ADP plays a key role in normal platelet aggregation and hence blood coagulation (Cattaneo and Gachet, 1999). GTP is required for functions such as the capping of mRNA, microtubule formation and signal transduction through activation of the GTP-binding proteins that regulate metabolic enzymes, ion channels, transporters, and other parts of the cell machinery, controlling transcription, motility, contractility, and secretion, which in turn regulate systemic functions such as embryonic development, learning and memory, and homeostasis (Neves et al., 2002).

4.9 DISORDERS IN NUCLEOTIDE METABOLISM

4.9.1 Adenylate kinase deficiencies

AK1 deficiency in the erythrocytes is a rare disorder associated with haemolytic anaemia. Although several cases have been documented and diagnosed on the basis of low enzymatic activity, only in very few of them was the genetic defect identified. In a Japanese patient with haemolytic anaemia, a single base substitution that caused the change of arginine 128 to tryptophan was identified. This substitution resulted in an enzyme with an activity of 44% of the wild-type enzyme, slight thermal instability and different electrophoretic mobility. The mother and brother of the patient had the same mutation but no symptoms of anaemia. Therefore, it was suggested that, in some cases, the erythrocytes can circumvent severe AK1 deficiency without serious consequences

(Matsuura et al., 1989). In an Italian case of a child with congenital haemolytic anaemia, a substitution of tyrosine 164 by a cysteine resulted to a functionally inadequate enzyme, most probably due to the disulfide bonds formed by the extra cysteine residue (Matsuura et al., 1989; Qualtieri et al., 1997). Furthermore, two cases of mild chronic haemolytic anaemia were reported, where a homozygous nonsense mutation resulted in a truncated enzyme with complete lack of activity. AK1-deficient siblings displayed psychomotor impairment, language retardation and limited learning abilities (Bianchi et al., 1999).

In an AK1 knockout mouse model, the heart displayed accelerated loss of contractile force compared with wild-type controls, indicating reduced tolerance to ischemic stress. The total adenine nucleotide content in postischemic hearts was reduced by 20% compared with the wild-type, providing evidence of larger loss of the adenine nucleotide pool and compromised nucleotide salvage on reperfusion in the absence of AK1 (Pucar et al., 2002). In skeletal muscle of AK1-deficient mice, contraction-mediated AMPK phosphorylation is lower, consistent with limited AMP production. Activation of AMPK is a signal, sensitive to increased energy demands, that is involved in the regulation of glucose uptake and fatty acid oxidation in contracting muscle (Hancock et al., 2006). However, as several studies have shown, the presence of other AK isoforms can compensate, in some cases, for the total loss of AK1 activity as in the case of AK1 knockout muscle, where there is a normal capacity for contraction-mediated glucose uptake due to increased AMP and reactive oxygen species (ROS) production that result in the activation of AMPK. The initial burst of ADP accumulation because of ATP hydrolysis in AK1^{-/-} muscle is followed by the dephosphorylation of ADP to AMP by other AK isoforms (Zhang et al., 2008). In addition to that, under normal conditions, the AK1^{-/-} heart could maintain an apparent energetic homeostasis by adaptive up-regulation of metabolic flux through the remaining AK isoforms and glycolysis (Pucar et al., 2000).

Deficiencies of the mitochondrial AK2 have also been implicated in human disease. It has been shown that the gene encoding AK2 is mutated in individuals with reticular dysgenesis, the most severe form of inborn human combined immunodeficiency. Symptoms of the disease include almost complete absence of granulocytes and lymphocytes, hypoplasia of the thymus and secondary lymphoid organs, and lack of innate and adaptive humoral and cellular immunity. The mitochondrial location of the enzyme suggests a central role in providing the energy required for the proliferation of hematopoietic precursors and in controlling cell

apoptosis (Pannicke et al., 2009). A recent study has shown that in addition to the fatal septicemia that results from reticular dysgenesis within days after birth, sensorineural deafness is also associated with the disease (Lagresle-Peyrou et al., 2009). In support of a role for AK2 in apoptosis, it has also been suggested that AK2 is involved in a novel apoptotic pathway by forming a complex with FADD (Fas-Associated protein with Death Domain) and caspase-10. This complex mediates an amplification loop that ensures the execution of apoptosis, although it is the apoptosome that makes the decision as to whether cells live or die (Lee et al., 2007).

AK4 protein levels are increased in cultured cells exposed to hypoxia and in an animal model of amyotrophic lateral sclerosis (ALS), a neurodegenerative disease in which oxidative stress is implicated. AK4 knock-down in a cell line with high endogenous AK4 levels, resulted in reduced cell proliferation and increased cell death, whereas, over-expression of AK4 in another cell line with low levels of endogenous AK4, offered protection to cells from H₂O₂-induced cell death (Liu et al., 2009).

Autoantibodies against AK5 have been detected in two patients with non-viral immune-mediated limbic encephalitis. The patients had poor prognosis and progressed rapidly to dementia despite treatment with corticosteroids, intravenous immunoglobulin (IVIg) and plasma exchange (Tuzun et al., 2007).

The involvement of AK7 in human disease has been well documented in recent studies and it is related to the fact that the expression pattern of AK7 appears to be restricted to tissues rich in epithelium with (9+2) cilia. Motile cilia require the presence of dynein arms and abundant energy derived by ATP hydrolysis. Ciliary dysfunction causes a disease called primary ciliary dyskinesia, which is a group of heterogeneous disorders characterized by recurrent respiratory infections. The disease typically progresses to bronchiectasis and can cause lung failure in adulthood. Congenital disorders such as hydrocephalus, retinal degeneration, hearing deficits and mental retardation have been reported in primary ciliary dyskinesia patients. Reduced fertility due to sperm dysmotility is frequently observed in male patients and the lack of ciliary movement in the Fallopian tubes may contribute to female subfertility (Badano et al., 2006; Zariwala et al., 2007). Gene expression profiles of bronchial epithelial samples from current or former smokers revealed novel bronchial-enriched genes, including those associated with innate immunity and ciliogenesis, and the AK7 gene was one of the identified genes (Lonergan et al., 2006). An AK7-deficient mouse model presented pathological signs typical of primary ciliary dyskinesia such as microtubular defects, decreased ciliary beat frequency, hydrocephalus, abnormal spermatogenesis, mucus

accumulation and acute respiratory responses upon allergen challenge (Fernandez-Gonzalez et al., 2009).

4.9.2 Cytosolic deoxyribonucleoside kinase deficiencies

4.9.2.1 dCK deficiency

A dCK knock-out mouse model has shown that loss of dCK function has a selective developmental impact. Although dCK knock-out mice developed normally during embryogenesis, organogenesis and other essential developmental processes most probably due to *de novo* dNTP production, dCK inactivation caused significant defects in the central production of T and B cells in the thymus and bone marrow. The mice also showed reduced lymphocyte numbers in secondary lymphoid organs that appeared structurally abnormal (Toy et al., 2009).

4.9.2.2 TK1 deficiency

In a TK1 knock-out mouse model all mice developed sclerosis of kidney glomeruli and died before one year of age of kidney failure. The animals first acquired a hunched posture, which further progressed to the dragging of hind limbs and wasting, until final collapse. Their spleens were small in size and presented with both immunological and hematological abnormalities. Other symptoms included lymphoid atrophy and moderate to marked haemosiderosis because of premature destruction of erythrocytes (Dobrovolsky et al., 2003).

4.9.3 Mitochondrial deoxyribonucleoside kinase deficiencies

The synthesis of mtDNA is not cell cycle regulated and a constant supply of dNTPs is vital for the maintenance of mitochondrial integrity. However, there is no *de novo* nucleotide synthesis in the mitochondria. Nucleotides required for mtDNA replication are either synthesized by the salvage enzymes in the mitochondria or imported from the cytosol. Deficiencies caused by mutations in the nuclear genes encoding proteins responsible for mtDNA replication, maintenance or nucleotide substrate delivery, like TK2 and dGK, impair the synthesis of DNA precursors in the mitochondria and lead to imbalance of the dNTP pools, which eventually causes mtDNA depletion. The decreased copy number of mtDNA results in a reduced amount of mtDNA-encoded enzymes and thereby decreased respiratory activity and ATP yield. Liver, heart, skeletal muscle and brain are among the most energy-dependent tissues of the body and therefore they are vulnerable to mtDNA depletion. MtDNA depletion syndrome (MDS) is a group of severe autosomal recessive disorders of infancy or

childhood, characterized by a quantitative reduction in mtDNA copy number in affected tissues. The myopathic form of MDS has been ascribed to mutations in the TK2 gene, whereas its hepatocerebral form has been ascribed to mutations in the dGK gene (Wang and Eriksson, 2003).

4.9.3.1 *TK2 deficiency*

A TK2-deficient mouse strain was created and the molecular mechanism of mtDNA depletion due to TK2 deficiency was investigated. Growth retardation as well as high-rate of early mortality was observed and several animals died by the second week of life with no animal surviving more than 21 days. The mice were progressively and severely hypothermic from 10 days of age. The hypothermia and shivering was suggested to be due to defective non-shivering thermogenesis caused by abnormal brown adipose tissue together with loss of hypodermal fat. Also a neurological disorder in the brain was suggested as a possible cause since the mice show a marked reduction in mtDNA levels in brain tissue. In fact, all investigated tissues and organs showed reduced mtDNA levels due to the loss of TK2 expression. This deficiency causes a shortage of pyrimidine dNTPs within the mitochondria without any compensatory increase in any enzyme involved in pyrimidine deoxyribonucleotide synthesis (Zhou et al., 2008). Further investigations of TK2-deficient brain and nervous system displayed an ataxic phenotype with severely impaired motor coordination. TK2 deficiency caused a specific reduction of mtDNA-encoded electron transport chain proteins in neurons, while their nuclear DNA-encoded counterparts appeared unaffected. Non-proliferating cells such as neurons are particularly dependent on the mitochondrial salvage pathway due to reduced expression of enzymes involved in cytosolic dNTP salvage and *de novo* nucleotide synthesis. The increasing reduction of mtDNA copy number and the subsequent loss of mtDNA-encoded electron transport chain subunits expression is sufficient to perturb mitochondrial bioenergetics within individual neurons of the cerebellum (Bartesaghi et al., 2010). These results were further confirmed by a H126N homozygous TK2 mutant knockin mouse model of human MDS. Around day 10, the mutant animals stop gaining weight, after which the disease progresses rapidly with reduced spontaneous and locomotor activity, tremor, ataxia, muscle weakness and severe encephalopathy, so that the vast majority of mice die within a few days. They show mtDNA depletion in multiple tissues including the brain, heart and muscle. However, only the brain shows significant deficiency of respiratory chain complexes protein levels and activities (Akman et al., 2008).

In human patients suffering from mitochondrial myopathies due to mutations in the TK2 gene, onset of the disease is always in very early childhood with proximal weakness and hypotonia common to all studied cases, however, the clinical phenotype can include ptosis, progressive external ophthalmoplegia, facial diplegia and epileptic seizures. In the majority of cases the patients die from the disorder within a few years and the cause of death in almost all cases is respiratory failure (Lesko et al., 2010; Oskoui et al., 2006)

4.9.3.2 *dGK deficiency*

Hepatopathy is a prominent feature of MDS and dGK mutations are considered to be the most common genetic background of hepatic MDS. Samples from a number of infants and children, who died due to progressive liver failure, were screened for dGK gene mutations. In some of them known pathogenic dGK mutations were detected as well as one novel molecular variant of unknown pathogenicity. Post-mortem investigation revealed parenchymal injury accompanied by bridging fibrosis that led to micronodular transformation and cirrhosis and a total loss of liver architecture. The children and infants had relatively low birth weight, intrauterine growth retardation or prematurity. Features of iron overload were observed in the affected patients and theoretically, an excess of free iron ions may have deleterious toxic influence on the liver. An impaired function of the mitochondria, associated with dGK deficiency, may aggravate the process of liver damage (Pronicka et al.). A new mutation in the dGK gene was found in two children of North African origin, who died at around the age of 16 months and presented with hepatocerebral syndrome and severe combined respiratory chain deficiency. Respiratory chain complexes showed low levels of activities in the complexes containing mtDNA-encoded subunits in liver and the mtDNA was almost completely depleted (Brahimi et al., 2009). A study that has gathered all available information from all known cases of dGK mutations has revealed that long-term survival is best predicted by the absence of profound hypotonia, significant psychomotor retardation or nystagmus. In the presence of these features, there is increased mortality and liver transplantation does not confer increased survival. In the absence of these neurological features, liver transplantation may be considered as a potential treatment (Dimmock et al., 2008).

5 NUCLEOSIDE ANALOGS

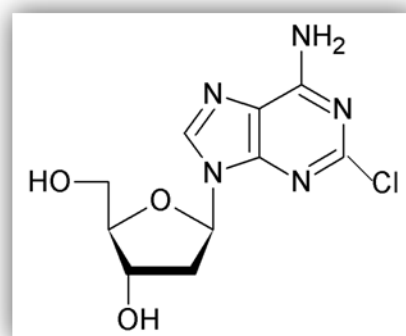
5.1 INTRODUCTION

Nucleoside analogs resemble natural nucleosides in structure and they are used in the clinic for the treatment of certain viral infections and malignant diseases. Nucleoside analogs are activated by phosphorylation to their triphosphate form, and then incorporated into viral or cellular DNA by DNA polymerases or viral reverse transcriptase. Their presence in DNA can cause termination of DNA elongation and offer resistance to proof-reading exonucleases. Furthermore, some analogs inhibit the reactions catalyzed by RNR, thymidylate synthase or dCMP deaminase. Generally the formation of the monophosphate is the rate-limiting step for metabolic activation of nucleoside analogs (Dimmock et al., 2008; Van Rompay et al., 2000). Toxicity can be mediated via inhibition of cellular DNA polymerases and the subcellular toxic target is frequently DNA polymerase γ . Nucleoside analogs that contain a 3'-hydroxyl group enable DNA polymerase γ to extend the mtDNA chain after they are incorporated into mtDNA. On the other hand, analogs that lack the 3'-OH group are incorporated into mtDNA and terminate the mtDNA replication. Inhibition of DNA polymerase γ leads to defective mtDNA replication that causes toxic effects in the oxidative phosphorylation. Long-term therapy with some nucleoside analogs causes multi-organ side-effects that resemble the phenotype of genetic mitochondrial diseases and include myopathy, cardiomyopathy, neuropathy, lactic acidosis, exocrine pancreas, liver and bone marrow failure (Lewis and Dalakas, 1995).

5.2 ANTICANCER NUCLEOSIDE ANALOGS

5.2.1 2-chloro-2'-deoxyadenosine (CdA)

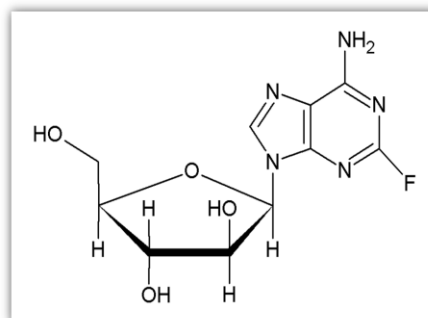
CdA or cladribine is an analog of dAdo, resistant to deamination by adenosine deaminase and it is phosphorylated by dCK and dGK. CdA is commonly used in the treatment of hairy cell leukemia and other lymphoproliferative disorders such as chronic lymphocytic leukemia (Arner, 1996; Galmarini et al., 2001). Once incorporated into DNA,



CdATP is capable of terminating chain elongation and induces an S phase-specific apoptosis. CdA also inhibits DNA replication indirectly through its inhibitory action on RNR, causing a subsequent reduction of the dNTP pools required for DNA synthesis (Lindemalm et al., 2004; Parker et al., 1988).

5.2.2 2-fluoro-9- β -D-arabinofuranosyladenine (FaraA)

FaraA is an analog of dAdo, also used against chronic lymphocytic leukemia and resistant to deamination by adenosine deaminase. It is administered in its monophosphate form FaraAMP (fludarabine) to increase the solubility of the compound. Before entering cells, fludarabine is dephosphorylated to FaraA, which is



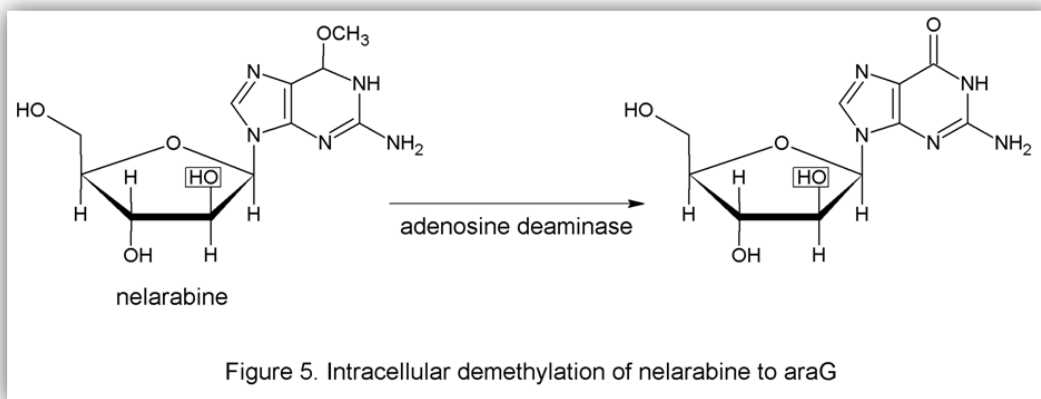
transported inside the cells by nucleoside transporters. Like CdA, once FaraA is incorporated into DNA, it can terminate chain elongation. Other enzyme targets of FaraA in dividing cells include RNR, DNA primase, DNA ligase and topoisomerase II. In non-dividing cells, the inhibition of cellular DNA repair mechanisms appears to be the major mechanism of FaraA cytotoxicity (Huang et al., 1990; Tseng et al., 1982).

5.2.3 9- β -D-arabinofuranosylguanine (araG)

Lymphoid malignancies involving T cells, such as T-cell acute lymphoblastic leukemia and T-cell lymphoblastic lymphoma, are relatively rare but often aggressive. Nelarabine is a pro-drug of the deoxyguanosine analogue 9- β -D-arabinofuranosylguanine (araG) that is activated through demethylation (figure 5). The active compound araG is subsequently phosphorylated in three consecutive phosphorylation steps by the cellular nucleoside and nucleotide kinases to its triphosphate form, araGTP. Nelarabine was given accelerated approval by the US FDA for the treatment of patients with T-cell acute lymphoblastic leukemia and T-cell lymphoblastic lymphoma whose disease has not responded to treatment or has relapsed following treatment with at least two chemotherapy regimens. The leukemic blasts accumulate araGTP allowing for the incorporation into the DNA, which results in inhibition of DNA synthesis and eventual cell death (Gandhi et al., 2006).

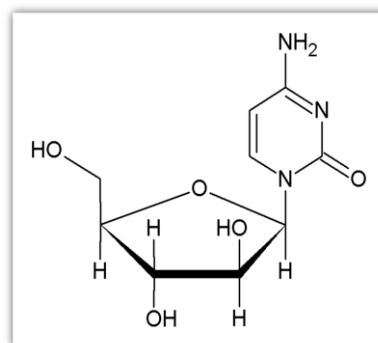
The rate-limiting phosphorylation step is the initial phosphorylation of araG to its monophosphate form, which is catalyzed by dGK at lower araG concentrations

and dCK at higher concentrations (Zhu et al., 1998). The dose-limiting toxicity of araG is neurotoxicity but other adverse effects also occur, like myopathy, myelosuppression and loss of peripheral sensitivity, which resemble symptoms of mitochondrial toxicity (Curbo et al., 2003; Kisor, 2005).



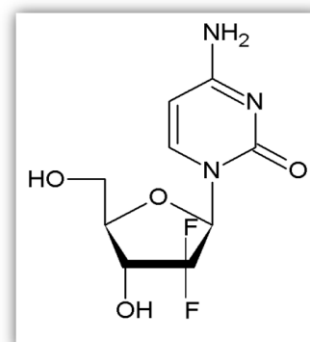
5.2.4 1-β-D-arabinofuranosylcytosine (araC)

AraC or cytarabine is a structural analog of dCyd and it is mainly phosphorylated by dCK. AraC cytotoxicity is caused by a combination of DNA polymerase inhibition and from incorporation of araCTP into DNA. AraC is used mainly in the treatment of acute myeloid leukemia and non-Hodgkin's lymphoma (Wang et al., 1997).



5.2.5 2'-2'-difluorodeoxycytidine (dFdC)

dFdC or gemcitabine is a dCyd analog that is phosphorylated by dCK and TK2. Once incorporated into DNA, it prevents DNA repair by base pair excision. The active diphosphate form of dFdC also inhibits DNA synthesis indirectly through inhibition of RNR. dFdC was originally investigated for its antiviral effects but has developed as an important anticancer compound for the treatment of solid tumors such as lung, ovarian, pancreatic and breast tumors (Noble and Goa, 1997).



6 THE PRESENT INVESTIGATION

6.1 AIM OF THE PROJECT

Since all known nucleoside and nucleotide kinases share common domains such as the P-loop and a common organization of the ATP and substrate binding domains, we used this information to screen databases to identify novel enzymes in nucleotide synthesis. Several unidentified sequences were identified and all of them were suggested to be adenylate kinases. The focus of the project was to further analyze the interesting growing family of adenylate kinases.

6.2 SUMMARY OF PAPERS

6.2.1 Paper I

Identification of two active functional domains of human adenylate kinase 5

A full-length cDNA that partially corresponded to human AK5 was identified and shown to encode for two separate domains. The full-length protein could be divided in two distinct functional domains, a first domain AK5p1 and a second domain that corresponded to the protein characterized as AK5, now called AK5p2. We have characterized the full-length protein and its first domain in terms of enzymatic activity and subcellular localization that proved to be cytosolic or both cytosolic and nuclear depending on the transcript variant. Both AK5 and AK5p1 phosphorylated AMP, CMP, dAMP and dCMP with ATP or GTP as phosphate donors.

6.2.2 Paper II

Evidence of an intact N-terminal translocation sequence of human adenylate kinase 4

Human AK4 was characterized in a previous study as a mitochondrial enzyme but with no confirmed AK activity. The aim our study was: (i) to express AK4 and investigate its activity with a broad range of substrates and phosphate donors in an attempt to determine its enzymatic activity and (ii) to identify and characterize its mitochondrial import sequence. We found that AK4 catalyzes the phosphorylation of AMP, CMP, dAMP and dCMP with ATP or GTP as phosphate donors. The mitochondrial import sequence of AK4 was shown to be located within the first N-terminal eleven amino acid residues, in close proximity with the ATP-binding region.

Import analysis revealed that the import signal is not cleaved and therefore the enzyme retains its activity upon entering the mitochondria.

6.2.3 Paper III

The characterization of human adenylate kinases 7 and 8 demonstrates differences in kinetic parameters and structural organization among the family of adenylate kinase isoenzymes

In this study we have characterized the previously identified human AK7 and in addition to that, we identified and characterized a novel member of the AK family, which we named AK8. AK8 is the second known human AK with two complete and active AK domains within its polypeptide chain, a feature that has previously been shown for AK5. The full-length AK8, its two domains and AK7 have similar enzymatic activity since they all phosphorylate AMP, CMP, dAMP and dCMP with ATP as phosphate donor but also AMP, CMP and dCMP with GTP as phosphate donor. Subcellular localization experiment revealed that both AK7 and AK8 are cytosolic.

6.2.4 Paper IV

Altered expression of adenylate kinase 1 and other genes in 9- β -D-arabinofuranosylguanine resistant T-lymphoblastic CEM cell lines

To determine the mechanism of action of araG, several araG-resistant CEM cell lines were previously generated and characterized. In this study we have used microarrays from Affymetrix to explore further what underlies the araG resistance in the cells. Several genes were found to exhibit altered expression compared to the wild-type cells. However, AK1 gene expression was upregulated in all three araG resistant cell lines. This finding was confirmed by SQ-PCR and the protein expression was also found increased in the araG resistant cells. Total AK activity was found increased in the CEM/araG-1 cells and might contribute to the resistant phenotype in this cell line.

7 CONCLUSSIONS

- All the AKs that were characterized in this thesis, have similar, almost identical, substrate and phosphate donor specificities. The general phosphorylation pattern of these AKs is the phosphorylation of AMP, dAMP, CMP and dCMP with ATP and GTP as phosphate donors.
- AK4 is located in the mitochondria, whereas AK7 and AK8 have a cytosolic localization. AK5 is cytosolic or both cytosolic and nuclear depending on the transcript variant.
- AK5 and AK8 are the only characterized human AKs that harbor two functionally active domains in the same polypeptide chain.
- The mitochondrial import sequence of AK4 is not cleaved, thus the enzyme retains its enzymatic activity after its mitochondrial translocation.
- AK7 and AK8 show a higher catalytic efficiency for AMP phosphorylation, as compared with AK1. This feature could compensate for the low expression levels of these enzymes compared to the ubiquitous and abundant expression of AK1.
- The up-regulation of AK1 may contribute to the development of resistance to araG.

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