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Substrate specificity and phosphorylation of antiviral and anticancer nucleoside analogues by human deoxyribonucleoside kinases and ribonucleoside kinases

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Abstract

Structural analogues of nucleosides, nucleoside analogues (NA), are used in the treatment of cancer and viral infections. Antiviral NAs inhibit replication of the viral genome, whereas anticancer NAs inhibit cellular DNA replication and repair. NAs are inactive prodrugs that are dependent on intracellular phosphorylation to their pharmacologically active triphosphate form. The deoxyribonucleoside kinases (dNK) and ribonucleoside kinases (rNK) catalyze the first phosphorylation step, converting deoxyribonucleosides and ribonucleosides to their corresponding monophosphate form. The dNKs have been studied intensively, whereas the rNKs have not been as thoroughly investigated. This overview is focused on the substrate specificity, tissue distribution, and subcellular location of the mammalian dNKs and rNKs and their role in the activation of NAs.

Keywords: Antiviral therapy; Anticancer therapy; Chemotherapy; Nucleoside analogue; Deoxyribonucleoside kinase; Ribonucleoside kinase

Abbreviations: ADK, adenosine kinase; AIDS, acquired immunodeficiency syndrome; AraC, 1-β-D-arabinofuranosylcytosine (Cytarabine); AraG, 9-β-D-arabinofuranosylguanine (Nelarabine); AZT, 3′-azido-2′,3′-dideoxythymidine (Zidovudine); CAFdA, 2-chloro-2′-fluoro-9-β-D-arabinofuranosyladenine (Clofarabine); CdA, 2-chloro-2′-deoxyadenosine (Cladribine); dCK, deoxycytidine kinase; ddC, 2′,3′-dideoxycytidine (Zalcitabine); ddI, 2′,3′-dideoxyinosine (Didanosine); dGK, deoxyguanosine kinase; dFdC, 2′,2′-difluorodeoxycytidine (Gemcitabine); dNK, deoxyribonucleoside kinase; d4T, 2′,3′-didehydro-3′-deoxythymidine (Stavudine); F-AraA, 2-fluoro-9-β-D-arabinofuranosyladenine (Fludarabine); FDA, Food and Drug Administration; FIAU, 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodouracil (Fialuridine); HBV, hepatitis B virus; mtDNA, mitochondrial DNA; HIV, human immunodeficiency virus; NA, nucleoside analogue; NDPK, nucleoside diphosphate kinase; NMPK, nucleoside monophosphate kinase; 5′-NT, 5′-nucleotidase; rNK, ribonucleoside kinase; RR, ribonucleotide reductase; RT, reverse transcriptase; TK1, thymidine kinase 1; TK2, thymidine kinase 2; UCK1, uridine-cytidine kinase 1; UCK2, uridine-cytidine kinase 2; 3TC, 2′-deoxy-3′-thiacytidine (Lamivudine).

Contents

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1. Introduction

Deoxyribonucleotides and ribonucleotides are the building blocks of DNA and RNA, respectively. Both nucleotides are built up by a nitrogen ring-structured base, a pentose sugar (deoxyribose or ribose), and a phosphate group. DNA is replicated by enzymes known as DNA polymerases. The human DNA polymerases α, β, δ, and ε are located in the cell nucleus and these enzymes replicate and repair nuclear DNA (Zannis-Hadjppoulus & Price, 1999; Hubscher et al., 2000). Mitochondrial DNA (mtDNA) replication is catalyzed by DNA polymerase γ (Taanman, 1999). These enzymes use single-stranded DNA as templates on which they catalyze the synthesis of the complementary strand from deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, and dTTP). The nucleotides are added to the free 3′-OH group of the base-paired polynucleotide so that DNA chains are extended in the 5′ → 3′ direction (Fig. 1).

The accuracy of DNA replication is fundamental for the genetic stability of the cell. From bacteria to higher eukaryotes, error frequencies range from 10^{-3} to $>10^{-6}$ per base replication (Timsit, 1999; Kunkel & Bebenek, 2000). Although errors in the DNA synthesis play a role in aging and diseases like cancer, spontaneous mutations also provide the opportunity for genetic variation and are a primary basis for the evolution. A high fidelity of DNA replication is required to maintain the mammalian genome of 3×10^9 bp and is dependent on a balanced supply of the 4 deoxyribonucleotides (Reichard, 1988). Inhibition of deoxyribonucleotide synthesis results in mutagenic changes, cell growth arrest, or cell death. Two examples of compounds that interfere with the synthesis of deoxyribonucleotides are hydroxyurea that inhibits ribonucleotide reductase (RR) and methotrexate that inhibits dihydrofolate reductase, thymidylate synthase (TS), and also the purine nucleotide de novo pathway (Fig. 2B). Inhibition of the enzymes results in deoxyribonucleotide pool imbalances and thus impaired DNA replication. Another way to interfere with DNA synthesis is by structurally modified nucleosides (i.e., nucleoside analogues [NA]). NAs that lack the 3′-OH group are incorporated into DNA and act as chain terminators because no new deoxyribonucleotide can be attached to the growing DNA strand (Fig. 1). NAs with an intact 3′-OH group are not absolute chain terminators, but there is evidence that they are incorporated into DNA and then severely impair chain elongation (Tan et al., 1999).

2. Deoxyribonucleotide and ribonucleotide synthesis in mammalian cells

Deoxyribonucleotides and ribonucleotides have to be synthesized within the cells because there are no carrier proteins for them in the cell membrane and the negatively

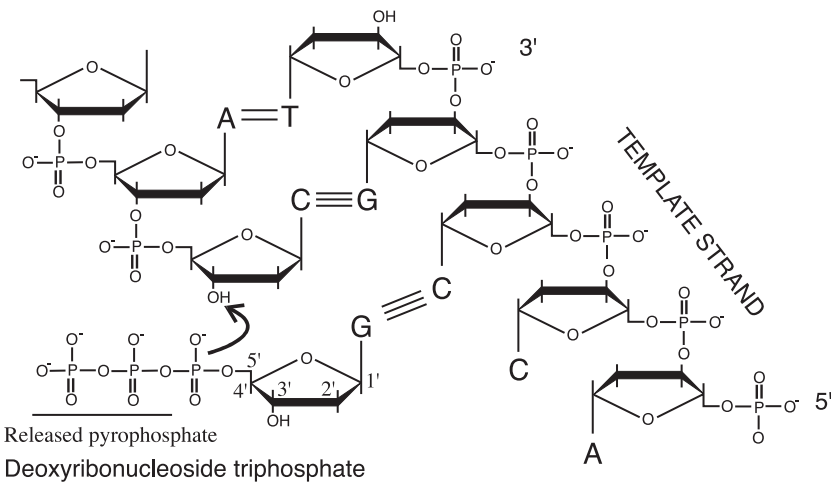

Fig. 1. Deoxyribonucleoside triphosphates are the substrates of DNA polymerase catalyzed replication of DNA. The deoxyribonucleotides are added to the free 3′-OH group of the base-paired polynucleotide so that DNA chains are extended in the 5′ → 3′ direction.

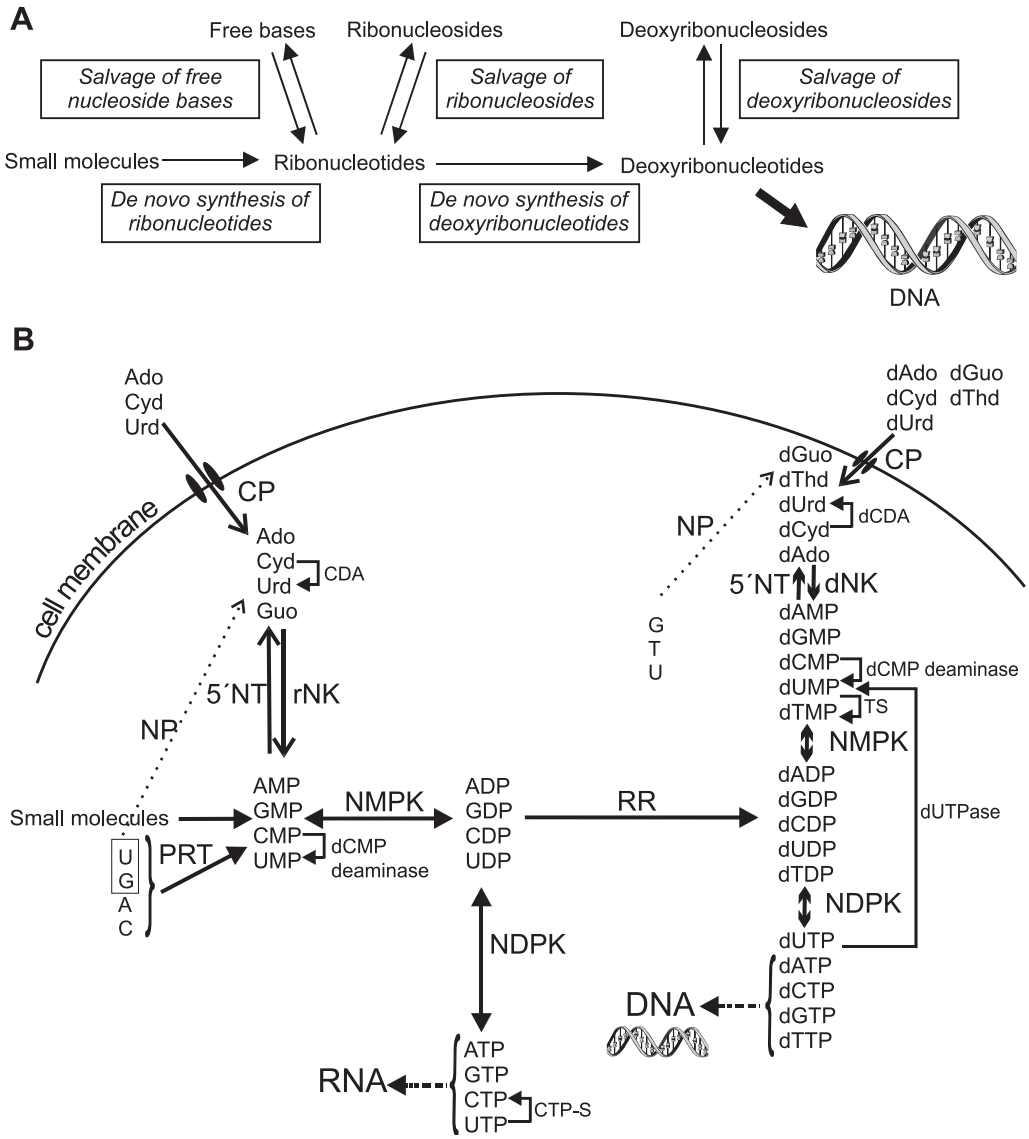

Fig. 2. De novo and salvage synthesis of ribonucleotides and deoxyribonucleotides. (**A**) Major steps in the synthesis of ribonucleoside and deoxyribonucleoside metabolism. (**B**) Detailed steps in the synthesis of ribonucleoside and deoxyribonucleoside metabolism. Abbreviations: A, adenine; C, cytosine; G, guanine; T, thymine; U, uracil; CP, carrier protein; NP, purine/pyrimidine nucleoside phosphorylase; dCDA, deoxycytidine deaminase; CDA, cytidine deaminase; PRT, phosphoribosyl transferase; dCMP deaminase, deoxycytidylate/cytidylate deaminase; CTP-S, CTP synthase.

charged phosphate groups prevent diffusion across the membrane. There are 2 pathways for both deoxyribonucleotide and ribonucleotide synthesis, called the de novo pathway and the salvage pathway (Fig. 2A) (Reichard, 1988). Via the de novo ribonucleotide pathway, ribonucleotides are synthesized from small molecules (e.g., amino acids, ribose-5′-phosphate, and CO_2) to ribonucleoside monophosphates and then further phosphorylated (Fig. 2B). The 2′-OH group of the ribonucleoside diphosphate can also be reduced to the corresponding 2′-deoxyribonucleoside diphosphate via the de novo deoxyribonucleotide pathway. This irreversible reaction is catalyzed by RRs (Reichard, 1988; Greenberg & Hilfinger, 1996; Burton et al., 2003). In proliferating cells, the de novo deoxyribonucleotide synthesis is the main source for nuclear DNA replication (Wright et al., 1990). Deoxyribonucleotides synthesized in the salvage pathway are believed to be important for DNA repair (Xu et al., 1995). The salvage deoxyribonucleotide pathway is derived from deoxyribonucleosides and catalyzed by deoxyribonucleoside kinases (dNK), nucleoside monophosphate kinases (NMPK), and nucleoside diphosphate kinases (NDPK) (Fig. 2B). In addition, there are 2 salvage pathways for ribonucleotides. The first is from free bases and then further phosphorylation through direct sugar phosphate transfer, and the second salvage ribonucleotide pathway is from ribonucleosides with further phosphorylation to their triphosphate form (Fig. 2A and B) (Kornberg & Baker, 1992). Ribonucleosides and deoxyribonucleosides are imported into the cells by nucleoside transport proteins that facilitate diffusion or

actively transport nucleosides across the membrane (Cass et al., 1999). The phosphorylation from ribonucleosides to their ribonucleoside monophosphates is catalyzed by the ribonucleoside kinases (rNK), while the phosphorylation reaction of deoxyribonucleosides is catalyzed by dNK (Fig. 2B). Both dNK and rNK phosphorylation reactions are irreversible. However, the monophosphates can be dephosphorylated to their nucleosides by 5′-nucleotidases (5′-NT) (Reichard, 1988; Rampazzo et al., 2000). Conversion of ribonucleoside monophosphates and deoxyribonucleoside monophosphates to their triphosphate forms occurs in 2 subsequent reversible phosphotransferase reactions catalyzed by the NMPKs and the NDPKs. There are 4 groups of NMPKs in human cells: a dTMP kinase (dTMPK), a UMP-CMP kinase (UMP-CMPK), 5 isozymes of adenylate kinase (AK), and several guanylate kinases (GUK) (Van Rompay et al., 2000). The human NDPK family comprises at least 8 isozymes (Lacombe et al., 2000).

We are interested in enzymes involved in the activation of NAs. The NAs are phosphorylated, via ribonucleotide or deoxyribonucleotide salvage pathways, to their corresponding triphosphates. The initial phosphorylation step by dNKs is in most cases the rate-limiting step in the formation of dNTPs or NA-TPs and has been studied carefully (Arnér & Eriksson, 1995; Johansson & Eriksson, 1996; Johansson et al., 1999; Tan et al., 1999; Galmarini et al., 2001, 2002); however, less is known about rNKs. Both will be the focus of this review. To reconstitute the metabolic pathways of activation and characterize all phosphorylation steps is important for the development and rational use of these compounds in antiviral and anticancer therapy.

3. The salvage pathways of deoxyribonucleotide and ribonucleotide metabolism

The deoxyribonucleosides and ribonucleosides are imported into cells by nucleoside transporters. The deoxyribonucleosides/ribonucleosides are then phosphorylated to their triphosphate forms in 3 subsequent phosphorylation steps that are catalyzed by dNKs/rNKs, NMPKs, and NDPKs.

3.1. Substrate specificity

In human cells, there are 4 dNKs: thymidine kinase 1 (TK1), thymidine kinase 2 (TK2), deoxycytidine kinase (dCK), and deoxyguanosine kinase (dGK) (Bohman & Eriksson, 1990; Arnér et al., 1992; Arnér & Eriksson, 1995). The nomenclature of these dNKs is based on the preferred substrate of the enzyme, but they also accept other substrates (Table 1). TK1 phosphorylates dThd and dUrd (Munch-Petersen et al., 1991). TK1 is able to use most phosphate donors, but ATP shows the highest efficiency (Ellims & Van der Weyden, 1981). TK2 is a pyrimidine dNK and phosphorylates dThd, dUrd, and dCyd (Munch-Petersen et al., 1991). In addition to dCyd, dCK phosphorylates dAdo and dGuo (Sarup & Friland, 1987; Usova & Eriksson, 2002). The enzyme can use several nucleotides as phosphate donors (Shewach et al., 1992), and UTP is suggested to be an important phosphate donor (White & Hines, 1987; Turk et al., 1999). dGK phosphorylates dGuo, dAdo, and dIno (Gower et al., 1979; Wang et al., 1993). dGK can use several phosphate donors, but in vitro UTP is the preferred phosphate donor (Zhu et al., 1998a).

Table 1
Natural substrates of the human dNKs and rNKs

dNK	Natural substrates
TK1	dThd, dUrd
TK2	dThd, dUrd, dCyd
dCK	dCyd, dAdo, dGuo
dGK	dGuo, dAdo, dIno
rNK	**Natural substrates**
ADK	Ado, dAdo
UCK1	Urd, Cyd
UCK2	Urd, Cyd

There are 3 known rNKs in human cells: adenosine kinase (ADK), uridine-cytidine kinase 1 (UCK1), and uridine-cytidine kinase 2 (UCK2) (Spychala et al., 1996; Van Rompay et al., 2001). The existence of a human guanosine kinase has so far not been reported. ADK catalyzes the phosphorylation of Ado to AMP (Table 1) (Spychala et al., 1996). ADK has also been implicated in the phosphorylation of dAdo in vivo (Ullman et al., 1978). Although purified ADK has >1000-fold higher K_m for dAdo than for Ado (Hurley et al., 1985), studies with ADK-deficient cell lines demonstrated that it accounts for a significant part of total dAdo phosphorylation in lymphoblastoid cell lines (Carson et al., 1977; Ullman et al., 1978). ADK might play a major role in the phosphorylation of dAdo at higher concentrations, whereas dCK plays a critical role at low concentrations (Verhoef et al., 1981; Hershfield et al., 1982). ATP and GTP have shown to be effective phosphate donors for ADK (Yamada et al., 1981). ADK is feedback regulated by its products AMP and ADP (Palella et al., 1980). UCK catalyzes the phosphorylation of Urd and Cyd to UMP and CMP (Table 1). The enzyme has first been observed by Canellakis (1957) in mammalian liver and by Reichard and Sköld (1957) in Ehrlich ascites tumor and was then shown to be present in other rapidly proliferating tissues (Reichard & Sköld, 1958; Sköld, 1960a). Purification and properties of tissue-purified UCK have been reviewed (Anderson, 1973; Cihak & Rada, 1976; Traut & Jones, 1996). Different investigators have observed multiple enzyme peaks or bands by chromatography, isoelectric focusing, or electrophoresis (Krystal & Webb, 1971; Krystal & Scholefield, 1973; Fulchignoni-Lataud et al., 1976; Greenberg et al., 1977; Ahmed & Welch, 1979; Absil et al., 1980; Ahmed & Baker, 1980; Dubinina et al., 1982; Fulchignoni-Lataud & Roux, 1984). Recently, 2 human UCKs, named UCK1 and UCK2, have been cloned (Koi-

zumi et al., 2001; Van Rompay et al., 2001). Both enzymes efficiently phosphorylate the ribonucleosides Urd and Cyd, and UCK2 has a higher catalytic rate compared with UCK1. The enzymes do not phosphorylate deoxyribonucleosides or purine ribonucleosides (Van Rompay et al., 2001). The 2 recombinant UCKs resemble the tissue-purified UCKs in exhibiting a broad specificity with regard to the phosphate donor, including dNTPs but not UTP and CTP (Anderson & Brockman, 1964; Orengo, 1969; Van Rompay, 2001). Two modes of regulation have been shown for the UCKs: feedback regulation by the end products CTP and UTP, which exhibit competitive inhibition with respect to the phosphate donor (Anderson & Brockman, 1964; Orengo, 1969; Payne & Traut, 1982), and regulation by changes in quaternary structure caused by orthophosphate, ATP, and CTP (Payne & Traut, 1982; Ropp & Traut, 1998). ATP stabilizes the enzyme in the active tetrameric form, whereas UTP and CTP dissociate the enzyme to an inactive monomer (Payne & Traut, 1982; Cheng et al., 1986).

3.2. Genetics

3.2.1. Sequences

The cDNAs of the 4 human dNKs have been cloned (Bradshaw, 1984; Chottiner et al., 1991; Johansson & Karlsson, 1996, 1997). Alignment of the predicted amino acid sequence of TK2 with the sequences of dCK and dGK show that they are ≈ 33% identical (Fig. 3A). TK1 is related to TKs from *Escherichia coli* and poxviruses and shows little sequence similarity with the other dNKs (Gentry, 1992; Eriksson & Wang, 1997). It is likely that all 4 human dNKs have originated from a common progenitor kinase. It is proposed that the first gene duplication generated the progenitor of the TK1-like kinases and the progenitor of the TK2-like and dCK/dGK-like kinases (Johansson et al., 1999; Knecht et al., 2002). To date, a crystal structure has been determined for dGK and recently for dCK (Johansson et al., 2001; Sabini et al., 2003). For TK1, TK2, dCK, and dGK, the coding region is distributed in 7, 10, 7, and 7 exons, respectively (Fig. 4).

Three cDNAs of the human rNKs have also been cloned. From human tissues, 2 different isoforms of human ADK have been cloned and sequenced (Spychala et al., 1996; McNally et al., 1997). The isoforms are similar in biochemical and functional properties but have a sequence variation in the 5′ end where the first 4 encoded residues of the short form are replaced by 21 residues in the long form (McNally et al., 1997). These 2 mRNA forms are postulated to arise from a single ADK gene by differential splicing (Spychala et al., 1996; McNally et al., 1997). So far, the significance of the 2 forms has not been elucidated. The ADKs show little sequence similarity to other mammalian rNKs. In contrast, 2 regions were identified with significant sequence identity to microbial ribokinases and fructokinases and a bacterial inosine/guanosine kinase (Spychala et al., 1996; Mathews et al., 1998). ADK is grouped with these carbohydrates and other purine kinases into the pfkB family. The crystal structure of ADK has recently been solved from human ADK (Mathews et al., 1998) as well as from *Toxoplasma gondii* ADK (Cook et al., 2000; Schumacher et al., 2000). Structure-activity relationships have been examined for Chinese hamster ADK by making systematic deletions of the N-terminal and C-terminal ends (Maj et al., 2000). For human ADK, the coding region is distributed in 11 exons (Fig. 4).

The longest open reading frame of UCK1 encoded a 277-amino acid protein with a predicted mass of 31 kDa. UCK2 consists of 261 amino acids and has a predicted mass of 29 kDa (Van Rompay et al., 2001). Human UCK1 and UCK2 were 72% similar in amino acid sequence, and the enzymes were ≈ 37 and ≈ 28% similar to UCK of *Caenorhabditis elegans* and *E. coli*, respectively (Fig. 3B). For both human UCK1 and UCK2, the coding region is distributed in 7 exons (Fig. 4).

3.2.2. Chromosomal location

From the human dNKs, human TK1 gene is localized to chromosome 17q25.2–17q25.3 (Kuo et al., 1996; Petty et al., 1996), TK2 to chromosome 16q22–16q23.1 (Johansson & Karlsson, 1996), dCK to chromosome 4q13.3–4q21.1 (Stegmann et al., 1993), and dGK to chromosome 2p13 (Johansson et al., 1996).

From the rNKs, human ADK gene been assigned to the region 10q11–10q24 (Klobutcher et al., 1976; Francke & Thompson, 1979). The human UCK1 gene and the UCK2 gene were localized to chromosome 9q34.2 and 1q22–1q23.2, respectively (Van Rompay et al., 2001).

3.3. Subcellular location

TK1 is a cytosolic enzyme and TK2 is a mitochondrial enzyme with a N-terminal mitochondrial import signal (Fig. 5 and Table 2) (Kit & Leung, 1974; Lee & Cheng, 1976; Wettin et al., 1999; Wang & Eriksson, 2000). In Fig. 5, TK2 green fluorescent protein (GFP) shows mainly a cytosolic fluorescence with a fluorescent border outlining the cell membrane (Johansson et al., 1997). This human TK2 is most likely an alternatively spliced cDNA isoform of human TK2 that lacks the N-terminal mitochondrial targeting sequence. The mitochondrial location of TK2 is supported by the fact that that full-length mouse TK2 has a mitochondrial location (Wettin et al., 1999; Wang & Eriksson, 2000) and that mtDNA depletion syndrome (MDS) has been linked to mutations in TK2 and dGK (Saada et al., 2001; Mancuso et al., 2002). The recombinant dCK has a nuclear import signal and dCK fused with GFP is located in the nucleus (Johansson et al., 1997), whereas the native dCK is reported to be located in the cytosol (Hatzis et al., 1998). The importance of the transport of dCK between subcellular compartments remains unclear. The N-terminal sequence of dGK contains a mitochondrial import signal and the enzyme is located in the mitochondrial matrix (Gower et al., 1979; Jüllig &

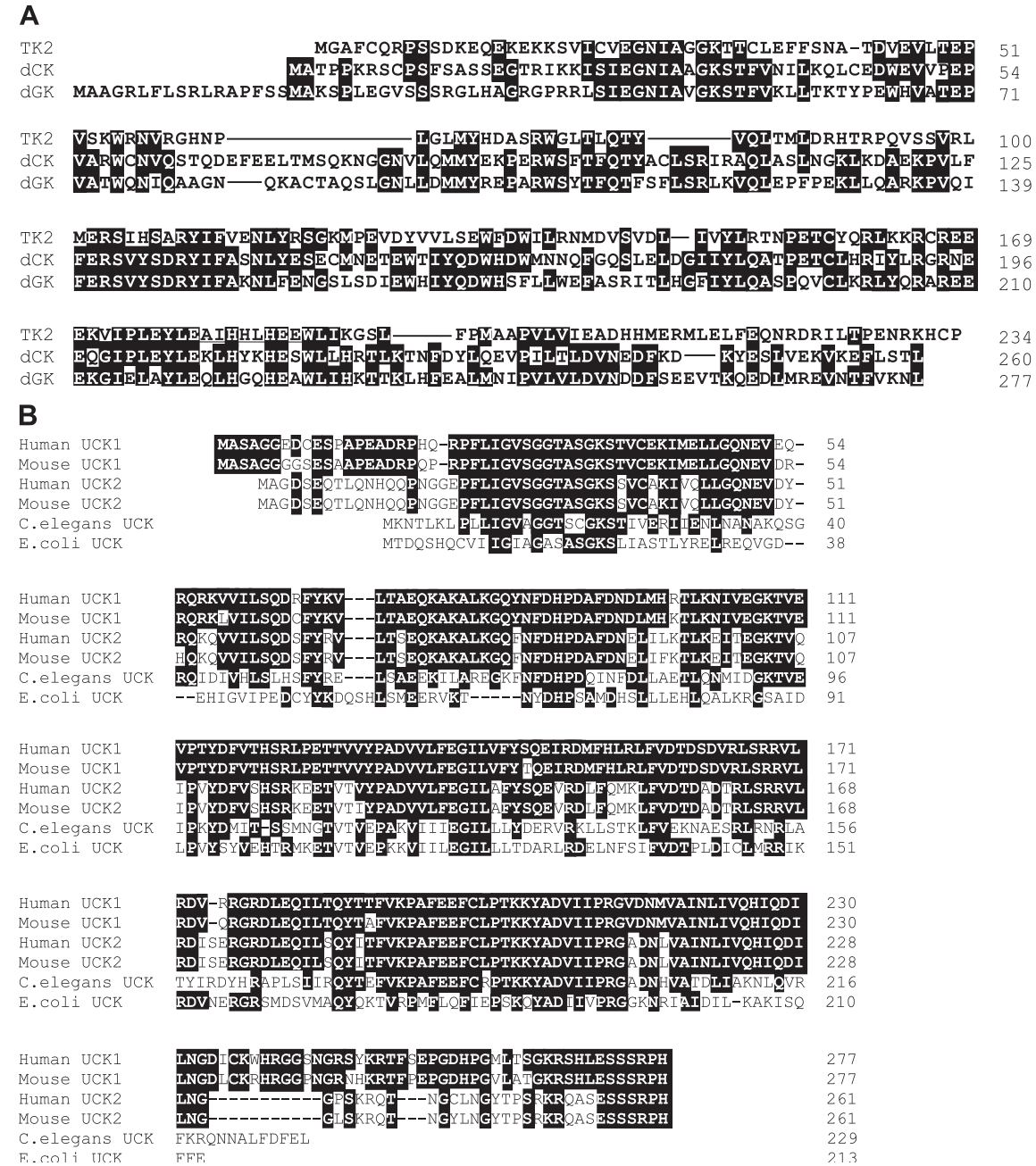

Fig. 3. The human dNK gene family. (**A**) Alignment of the predicted amino acid sequences of human TK2, dCK, and dGK (GenBank database accession nos. NM_004614, NM_000788, and NM_080916). (**B**) Alignment of the predicted amino acid sequences of human UCK1, mouse UCK1, human UCK2, mouse UCK2, *E. coli* UCK, and *Saccharomyces cerevisiae* UCK (GenBank database accession nos. AF237290, BC025146, AF236637, AF23666, X71492, and X53998). Black boxes indicate conserved amino acid residues.

Eriksson, 2000). Surprisingly, dGK might be relocated to the cytosolic compartment during apoptosis (Jüllig & Eriksson, 2001).

ADK is reported to be a cytosolic enzyme (Table 2) (Andres & Fox, 1979). UCK activity, however, has been detected in nuclear, cytosolic, and microsomal fractions in different investigated cells and tissues (Durham & Ives, 1972; Cihak & Rada, 1976; Greenberg et al., 1977). Expression of UCK1 and UCK2 in fusion with GFP showed that UCK1-GFP was predominantly located in the nucleus, whereas UCK2-GFP was located in the cytosol (Fig. 5 and Table 2) (Van Rompay, 2001). The relevance of this finding is probably minimal because the nuclear membrane allows diffusion of small proteins and nucleotides between the cytosol and the nucleus.

3.4. Cell cycle regulation and tissue distribution

TK1 expression is regulated throughout the cell cycle, with transcriptional, translational, and post-translational

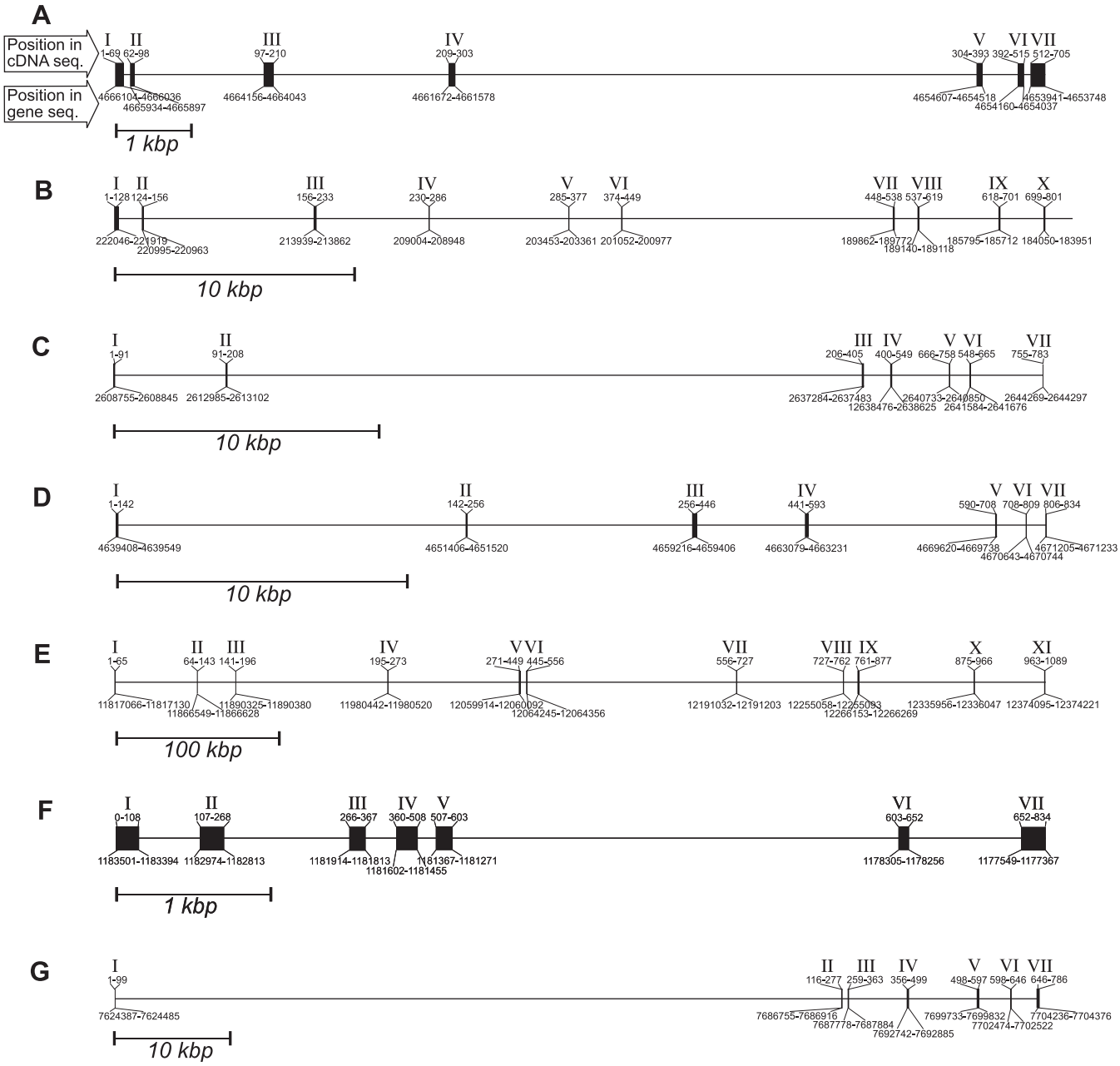

Fig. 4. The human deoxyribonucleoside and ribonucleoside gene family. Human TK1, TK2, dGK, dCK, ADK, UCK1, and UCK2 gene structure. (**A**) For TK1 (BC007872), the genomic sequence is derived from a 12.4-kb DNA fragment on chromosome 17 (NT_010641.11) and the coding region is distributed in 7 exons. (**B**) For TK2 (Y10498), the genomic sequence is derived from a 38.1-kb DNA fragment on chromosome 16 (NT_010478.11) and the coding region is distributed in 10 exons. (**C**) For dCK (NM_000788), the genomic sequence is derived from a 35.5-kb DNA fragment on chromosome 4 (NT_006216.12) and the coding region is distributed in 7 exons. (**D**) For dGK (NM_080916), the genomic sequence is derived from a 31.8-kb DNA fragment on chromosome 2 (NT_022184.10) and the coding region is distributed in 7 exons. (**E**) For ADK (BC003568), the genomic sequence is derived from a 557.2-kb DNA fragment on chromosome 10 (NT_008583.13) and the coding region is distributed in 11 exons. (**F**) For UCK1 (AF237290), the genomic sequence is derived from a 6.1-kb DNA fragment on chromosome 9 (NT_008338.12) and the coding region is distributed in 7 exons. (**G**) For UCK2 (AF236637), the genomic sequence is derived from a 80.0-kb DNA fragment on chromosome 1 (NT_004668.13) and the coding region is distributed in 7 exons.

regulatory mechanisms. Both TK1 mRNA and protein rise near the G_1/S boundary, peak in early S phase, and decline again in the G_2 phase (Sherley & Kelly, 1988; He et al., 1996; Kim et al., 1997). The reason for such a meticulous control has not been fully elucidated, but several regulatory mechanisms have been proposed: (1) a 20-bp inverted CCAAT motif in the promoter of TK1, responsible for transcriptional activation of the TK1 gene; (2) post-transcriptional and post-translational mechanisms such as half-life of TK1 mRNA and TK1 protein that regulate TK1 activity; (3) a 40-amino acid sequence in the carboxyl terminus specific for degradation of TK1 at mitosis; (4)

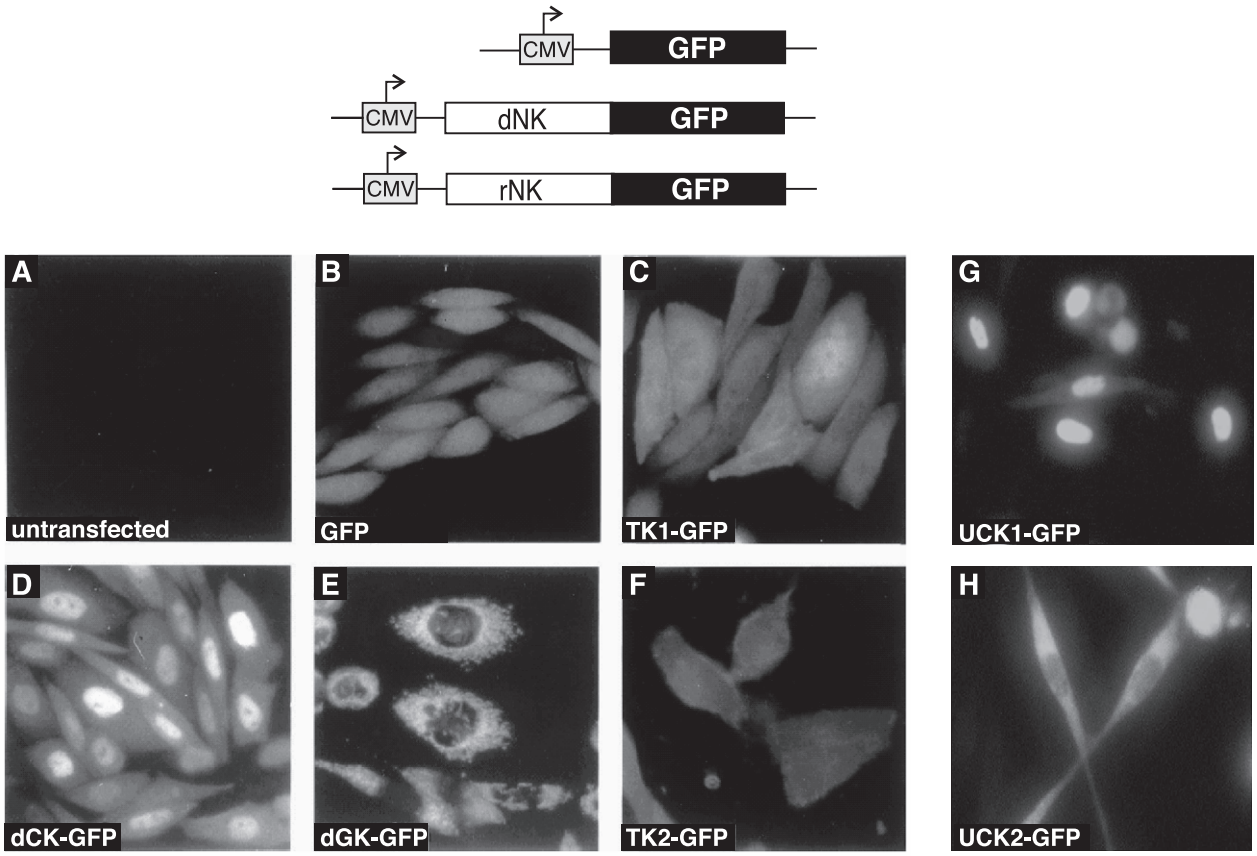

Fig. 5. Fluorescence microscopy images of CHO cells transfected with the plasmids encoding the human dNKs and uridine kinases as fusion protein with the GFP. (**A**) Untransfected cells. (**B**) GFP or the pEGFP-N1 plasmid vector (Clontech). (**C**) TK1-GFP. (**D**) dCK-GFP. (**E**) dGK-GFP. (**F**) TK2-GFP. (**G**) UCK1-GFP. (**H**) UCK2-GFP. CMV, cytomegalovirus promoter.

the extent of phosphorylation of serine residues of the TK1 protein; (5) at the enzymatic level, an ATP-dependent reversible shift between a low and a high affinity form of TK1; and (6) a S phase-dependent mechanism on the transcription factor E2F, in complex with several other factors (Arnér & Eriksson, 1995; Munch-Petersen et al., 1995). TK2, dCK, and dGK are expressed throughout the cell cycle. TK2 represents a rather small fraction of cellular TK levels, compared with TK1 activity, in proliferating cells. However, TK2 corresponds to the predominant fraction of TK activity in resting or terminally differentiated cells, where TK1 activity is virtually undetectable (Johansson & Karlsson, 1997). The TK2 activity in tissue extracts appears to be proportional to the cellular mitochondrial content rather than with the growth state of the cells (Arnér et al., 1992). TK2 has been purified from many tissues such as spleen, liver, heart, and brain (Jansson et al., 1992). Northern blot analyses show TK2 mRNA species with a ubiquitous expression (Johansson & Karlsson, 1997; Wang et al., 1999), with the highest levels in liver, pancreas, muscle, and brain. dCK does not seem to be cell cycle regulated (Richel et al., 1990). dCK is expressed at low levels in most tissues, but high levels are present in lymphoid tissues with particularly high levels in immature T-lymphoblasts (Arnér et al., 1992). Northern blot analysis showed that dCK mRNA had the highest levels in thymus, skeletal muscle, fetal liver, bone marrow, and brain (Johansson & Karlsson, 1996), while liver and brain have been reported to have very low dCK activity (Spasokukotskaja et al., 1995). The molecular mechanism that regulates the tissue-specific expression of dCK is not yet clear. dGK is constitutively expressed throughout the cell cycle and the enzyme level is believed to be proportional to the amount of mitochondria in most tissues (Arnér & Eriksson, 1995). Northern blot analysis of dGK mRNA suggests that dGK is

Table 2
Subcellular location of the dNKs and rNKs

dNKs	Subcellular location
TK1	Cytosol
TK2	Mitochondria
dCK	Nucleus and/or cytosol
dGK	Mitochondria
rNKs	**Subcellular location**
ADK	Cytosol
UCK1	Nucleus
UCK2	Cytosol

expressed in most tissues, with highest levels in muscle, brain, liver, and lymphoid tissues (Johansson & Karlsson, 1996).

ADK plays a key role in the regulation of intracellular and extracellular levels of adenosine (Manfredi & Holmes, 1985; Berne, 1993). Adenosine has widespread effects on cardiovascular, nerve, respiratory, and immune systems (Fox & Kelley, 1978; Manfredi & Holmes, 1985). Inhibitors of ADK might play an important pharmacological role in increasing intravascular adenosine concentrations and act as anti-inflammatory agents (Cronstein, 1992; Firestein et al., 1994). With Northern blot analysis of ADK, the corresponding mRNA species of 1.3 and 1.8 kb were detected in all tissues (placenta, liver, muscle, kidney, heart, pancreas, brain, and lung) investigated (Spychala et al., 1996). UCK activity demonstrates no close correlation with cell proliferation (Sköld, 1960b; Weichsel et al., 1972). UCKs are widely distributed and have been purified from mammalian liver, lung, brain, heart, kidney, pancreas, spleen, thymus, bone marrow, testis, skeletal muscle, and small intestine (Herzfeld & Raper, 1979; Shen et al., 1998). Mouse UCK1 mRNA has previously been shown to be expressed in brain tissue (Ropp & Traut, 1996) and human UCK2 mRNA in human testis (Ozaki et al., 1996). We recently demonstrated that human UCK1 mRNA has been detected as 2 isoforms of ≈ 1.8 and ≈ 2.7 kb in several tissues. The ≈ 2.7-kb band is ubiquitously expressed in the investigated tissues, with high level of expression in liver, kidney, skeletal muscle, and heart, whereas low levels are present in brain, placenta, small intestine, and spleen. The band of ≈ 1.8 kb is detected in skeletal muscle, heart, liver, and kidney. Human UCK2 mRNA was only detected in placenta as 2 transcripts of ≈ 1.2 and ≈ 2.0 kb, because testis was not blotted on this Northern blot (Van Rompay et al., 2001). The molecular mechanisms that regulate the tissue-specific expression of UCK1 and/or UCK2 are not yet clear.

4. Nucleoside analogues

4.1. Deoxyribonucleoside analogues

NAs are used in the clinic for treatment of certain viral infections and tumors (Table 3). Recently, current knowledge concerning the molecular mechanisms of activity and resistance to major NAs in cancer treatment has been reviewed (Galmarini et al., 2001, 2002). Anticancer deoxyribonucleoside analogues used in the clinic include 2-chloro-2′-deoxyadenosine (Cladribine, CdA), 2-fluoro-9-β-D-arabinofuranosyladenine (Fludarabine, F-AraA), 1-β-D-arabinofuranosylcytosine (Cytarabine, AraC), and 2′,2′-difluorodeoxycytidine (Gemcitabine, dFdC). NAs such as 2′,3′-dideoxyinosine (Didanosine, ddI), 2′,3′-dideoxycytidine (Zalcitabine, ddC), 2′-deoxy-3′-thiacytidine (Lamivudine, 3TC), 3′-azido-2′,3′-dideoxythymidine (Zidovudine, AZT), 2′,3′-didehydro-3′-deoxythymidine (Stavudine, d4T), and abacavir (ABC) are used in antiviral treatment. Combination therapy, comprising at least 3 anti-HIV drugs, has become the standard treatment of AIDS or HIV-infected patients. The compounds, currently used or in advanced clinical trials, belong to one of the following classes: (1) nucleoside reverse transcriptase (RT) inhibitors (NRTI) (i.e., AZT, ddI, ddC, d4T, 3TC, ABC, or the nucleotide RT inhibitor [NtRTI] tenofovir [PMPA]), (2) non-NRTIs (NNRTI) (i.e., nevirapine, delavirdine, efavirenz, and emivirine), and (3) protease inhibitors (PI) (i.e., saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, and lopinavir) (De Clercq, 2002). Recently, new developments in anti-HIV chemotherapy have been reviewed (Tan et al., 1999; De Clercq, 2002). Here, we will address the currently used NRTIs or antiviral NAs and also anticancer NAs.

Table 3
Deoxyribonucleoside analogues used in anticancer and antiviral therapy

Drug	dNKs enzyme	Clinical use
Anticancer		
CdA	dCK, dGK[a]	Hairy cell leukemia, CLL, multiple sclerosis, autoimmune disorders
CAFdA[b]	dCK	Pediatric refractory/relapsed acute myeloid and lymphoblastic leukemia
F-AraA	dCK, dGK[a]	CLL
AraC	dCK	Acute leukemia, lymphomas
dFdC	dCK, TK2[a]	Pancreatic cancer, nonsmall cell lung cancer, breast cancer
AraG[b]	dGK, dCK[a]	T-cell acute lymphoblastic leukemia
Antiviral		
ddI	5′-NT	HIV infection
ddC	dCK	HIV infection
3TC	dCK	HIV infection
AZT	TK1, TK2[a]	HIV infection
d4T	TK1[a]	HIV infection

[a] The NA is poorly phosphorylated by this enzyme.
[b] In clinical trial.

Major problems in the treatment of cancer and viral diseases with NAs are acquirement of resistance and side effects such as delayed cytotoxicity. According to Galmarini et al. (2001), there are 3 general mechanisms of resistance to NAs that have been described in cell lines and clinical samples: (1) insufficient intracellular concentrations of NA-TPs, which might be due to inefficient cellular uptake, decreased levels of activating enzymes, increased catabolism by elevated levels of 5′-NT or deaminases, or expansion of dNTP pools; (2) inability to achieve sufficient alterations in DNA strands or dNTP pools, which might result from altered interactions with DNA polymerases, lack of inhibition of RR, or inadequate p53 exonuclease activity; and (3) defective induction of apoptosis. Zhu (2000) reviews this induction of apoptosis by NAs. The key event of apoptosis is caspase activation, which may be a consequence of death receptor triggering, mitochondrial activation, and possibly other not yet defined initiation events. Thus, the cell death pathway may be initiated at different

subcellular compartments, including the plasma membrane, cytosol, mitochondria, and nucleus. A major strategy in the fight against cancers is to induce apoptosis. Incorporation of NAs into nuclear DNA causes DNA strand breaks in S-phase cells and is regarded to be crucial for the initiation of apoptosis. However, there are also other pathways reported that induce apoptosis. The mechanisms that link DNA damage induced by NAs to apoptosis, particularly to mitochondria, are largely unknown. Some studies suggest that mitochondria may be involved in the induction of apoptosis by NAs, and a novel strategy is to selectively target mitochondrial permeabilizing agents to malignant cells (Zhu, 2000; Solary et al., 2003).

NAs are phosphorylated to their triphosphate form and then incorporated into cellular or viral DNA by a cellular or viral DNA polymerase or a RNA polymerase or a viral RT (Fig. 6). The presence of NAs in DNA causes termination of DNA elongation and often also resistance to proofreading exonucleases. While the mechanism of anticancer or antiviral activity of NAs may ultimately be the same, it should be noted that each compound has its own distinct metabolic and pharmacological properties. Some of these analogues also inhibit key enzymes (e.g., RR, TS, or dCMP deaminase) involved in the generation of the purine and pyrimidine nucleotides and RNA synthesis and directly activate the caspase cascade. All of these effects may lead to cell death (Galmarini et al., 2002).

Several studies have provided evidence that dUTP pyrophosphatase (dUTPase) is vital for cellular and, in some cases, viral DNA replication (Fig. 2B) (Ladner, 2001; Payne & Elder, 2001). Aberrant dUTP metabolism plays a critical role in the molecular mechanisms of cell killing induced by inhibitors of dihydrofolate reductase and TS. Under normal conditions, uracil is precluded from DNA by the combined actions of dUTPase and uracil-DNA glycosylase (UDG). However, during TS inhibition, dUTP pools may accumulate and overwhelm dUTPase, resulting in repeated cycles of uracil misincorporation and detrimental repair leading to strand breaks and cell death. Furthermore, herpesviruses (types 1–8), poxviruses, and some nonprimate retroviruses (EIAV, visna, CAEV, and FIV) encode dUTPase, whereas the primate retroviruses (HIV and SIV) do not (Studebaker et al., 2001; Fleischmann et al., 2002). Studies of several virus models have suggested that viral dUTPases may be required for virus replication in resting cells, whereas in proliferating cells cellular dUTPase may substitute for a mutant viral protein (Fleischmann et al., 2002). Cellular and viral dUTPase may be a suitable target for anticancer and antiviral therapy.

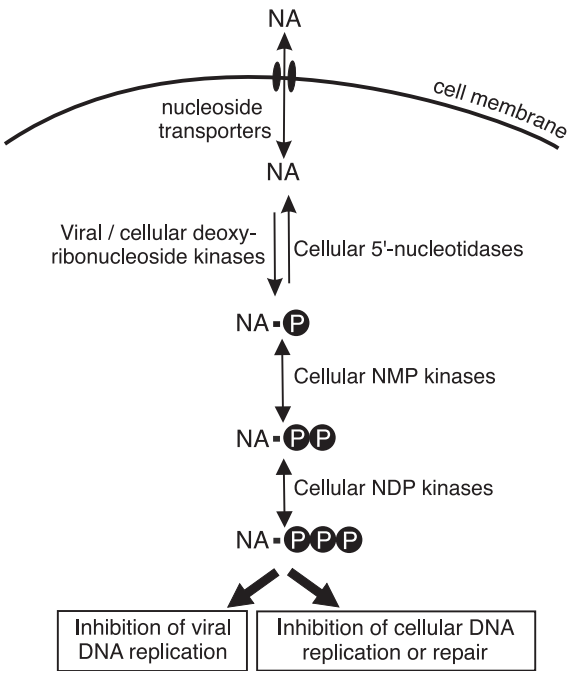

Fig. 6. NAs require intracellular phosphorylation for pharmacological activity. The NAs are transported across the cell membrane and phosphorylated by cellular or viral kinases to their triphosphate form.

Accumulation of the pharmacologically active NA-TP is, however, counteracted by dephosphorylation of NAs by the 5′-NTs and by degradation of the NAs by enzymes involved in nucleoside and nucleotide catabolism. The human dNKs (TK1, dCK, TK2, and dGK), which catalyze the initial and often rate-limiting phosphorylation step, have been investigated in detail (Arnér & Eriksson, 1995; Johansson et al., 1999).

dCK has been studied intensively over the years, because the enzyme is responsible for the initial activation of a number of clinically important anticancer and antiviral drugs, such as AraC, F-AraA, CdA, dFdC, ddC, and 3TC (Fig. 7) (Bohman & Eriksson, 1990; Arnér & Eriksson, 1995; Johansson & Karlsson, 1995). Impaired dCK expression or activity in cells results in resistance to these drugs, indicating that dCK plays a key role in their metabolism and pharmacological activities (Verhoef et al., 1981; Ullman et al., 1988; Owens et al., 1992; Ruiz van Haperen et al., 1994; Dumontet et al., 1999; Månsson et al., 2003). It has also been shown that sensitivity to dCK-activated NAs increases by overexpression of dCK in dCK-deficient tumor cell lines (Hapke et al., 1996). Moreover, in vitro models have shown cross-resistance among CdA, dFdC, F-AraA, and AraC, with reduced dCK activity as the underlying determinant of resistance (Dumontet et al., 1999).

AraC is a structural analogue of dCyd and is extensively used in the treatment of acute leukemia and lymphomas (Fig. 7 and Table 3). Intracellular penetration of AraC happens by a carrier protein or pump-mediated transport (Wiley et al., 1982; Capizzi et al., 1983). Once inside the cell, AraC is phosphorylated by dCK and pyrimidine nucleoside kinases to the active 5′-triphosphate derivative AraC-TP (Kufe et al., 1984; Van Rompay et al., 1999). AraC cytotoxicity is believed to result from a combination of DNA polymerase inhibition and from incorporation of AraC-TP into DNA, in competition with dCTP. This incorporation causes chain termination, resulting in a block of

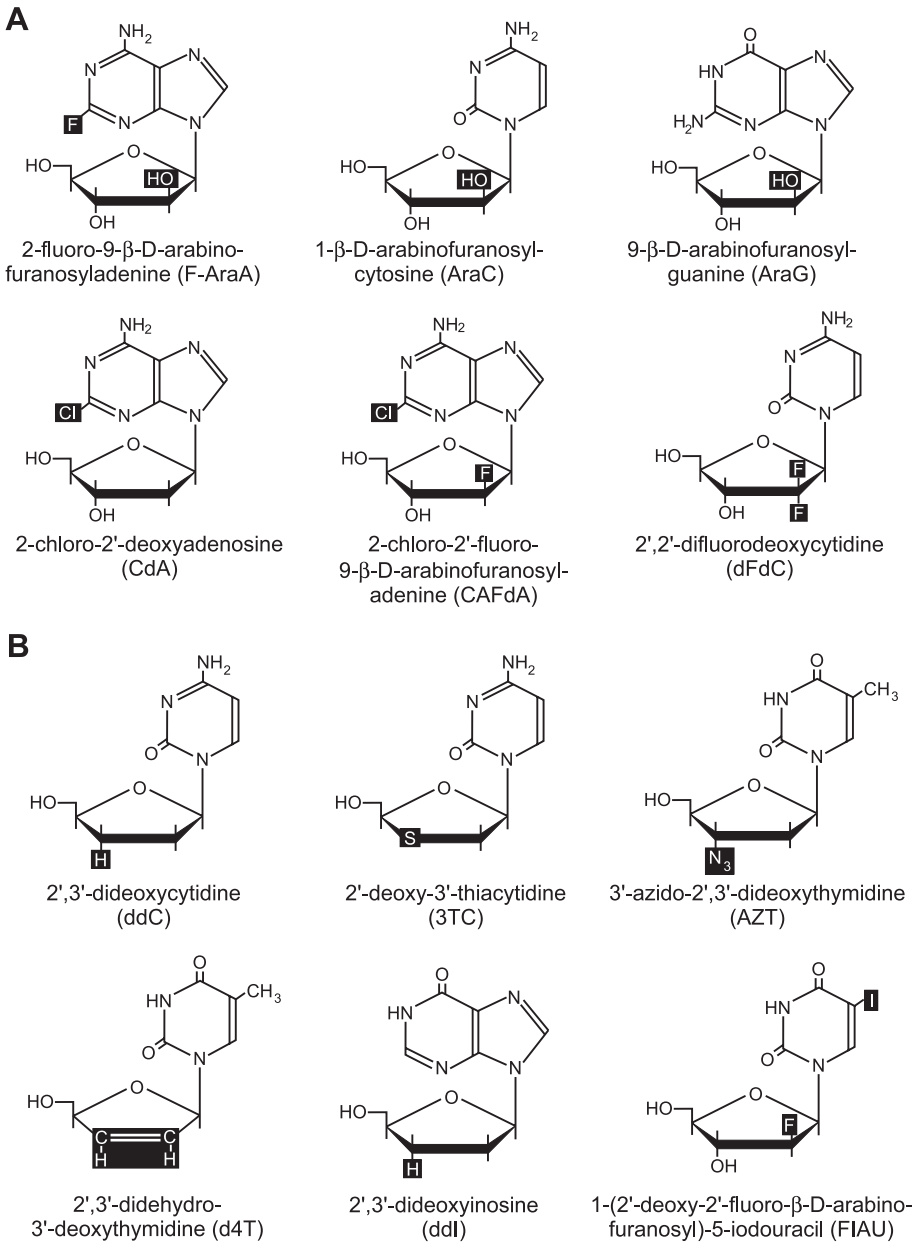

Fig. 7. Deoxyribonucleoside analogues. (**A**) Chemical structures of some anticancer deoxyribonucleoside analogues. (**B**) Chemical structures of some antiviral deoxyribonucleoside analogues. Structural differences compared with the naturally occurring deoxyribonucleosides are shown in black boxes.

DNA synthesis (Kufe et al., 1984). Sustained high cellular concentrations of AraC-TP relative to that of dCTP are thought to favor drug incorporation into replicating DNA, thereby initiating the leukemic cell death associated with therapeutic response. The cytotoxic activity of AraC is limited by characteristics such as metabolic deamination, low affinity for dCK, and rapid elimination of the triphosphate derivative.

The cytostatic agent dFdC is approved for treatment of pancreatic, breast, and nonsmall cell lung cancers (Fig. 7 and Table 3) (Noble & Goa, 1997). It is phosphorylated to its monophosphate by cellular dCK, to the diphosphate form by UMP-CMPK, and to the triphosphate form by NDPKs (Heinemann et al., 1988; Plunkett et al., 1995; Van Rompay et al., 1999). dFdC-MP inhibits deoxycytidylate deaminase, dFdC-DP inhibits RR, and dFdC-TP inhibits DNA synthesis by competitively inhibiting DNA polymerase and by incorporation into replicating DNA (Heinemann et al., 1990). Once the metabolite is incorporated, DNA chain elongation is terminated after the addition of another nucleotide. dFdC does not terminate DNA replication as effectively as AraC, but once dFdC-MP is incorporated, it is more difficult for the cells to excise it from DNA than AraC-MP (Ruiz van Haperen et al., 1993). The reasons that dFdC is and AraC is not active against solid tumors might be multifactorial, including these differences, together with greater lipophi-

licity, masked chain termination, RNA incorporation, and RR inhibition, which are not observed with AraC.

CdA is used for the treatment of lymphoproliferative disorders such as hairy cell leukemia and chronic lymphocytic leukemia (CLL) as well as for therapy of multiple sclerosis and autoimmune disorders (Fig. 7 and Table 3) (Beutler et al., 1996). Its efficacy, however, is compromised by the emergence of resistant cells. Knowledge about the mechanism of CdA cytotoxicity is limited, and most studies have focused on the activation and/or deactivation of the drug (Carson et al., 1983). Among other proposed mechanisms of action of CdA are incorporation of the analogue into DNA, inhibition of RR, inhibition of DNA repair, DNA strand break accumulation, and activation of poly(ADP-ribose) polymerase (Plunkett & Gandhi, 1996). CdA has also been shown to cause direct alterations in mitochondrial function that might trigger apoptosis. It disrupts the integrity of mitochondria, leading to the release of the proapoptotic mitochondrial protein cytochrome *c*. A recent study generated 3 cell lines resistant to CdA, with differing levels of initial phosphorylating enzymes dCK/dGK (Chandra et al., 2002). The 3 cell lines had lowered dCK enzyme activity and heightened dGK activity. Consequently, the resistant cell lines accumulated different levels of the phosphorylated drug, CdA-TP, intracellularly. The mechanisms of resistance to CdA did not solely correlate with the CdA-TP accumulation but also may be dictated by changes in Ca^{2+}-sensitive mitochondrial events (Chandra et al., 2002). 2-Chloro-2′-fluoro-9-β-D-arabinofuranosyladenine (CAFdA, Clofarabine) is a new 2′-arabino-fluoroderivative of CdA (Fig. 7 and Table 3), which has been used in a phase I clinical trial with encouraging preliminary results (Waud et al., 2000) and the phase II clinical trial for pediatric refractory/relapsed acute myeloid and lymphoblastic leukemia has just been finished (Kantarjian et al., 2003). In human leukemia cell lines is CAFdA, a more efficient substrate for dCK, and the active form is more stable due to higher phosphorylation and longer retention time compared with CdA, but the mechanisms leading to acquired resistance to CAFdA seem to be similar to those for CdA (Lotfi et al., 1999; Månsson et al., 2003).

F-AraA is a structural analogue of Ado and is used in the treatment of CLL (Fig. 7 and Table 3) (Keating, 1999). Therapeutic results in advanced CLL are still unsatisfactory in terms of complete remission achievement and duration in spite of the extensive use of purine analogues (Johnson et al., 2003). The standard therapy remains the alkylating agent Chlorambucil (Rai et al., 2000). More recently, the combination of F-AraA with Chlorambucil has emerged as an approach; unfortunately, it might coincide with a significantly higher incidence of major infections and a higher healthcare cost (Rai et al., 2000). F-AraA is administered as F-AraA-MP to make it more water soluble but is rapidly dephosphorylated by serum phosphatases and the membrane-bound 5′-NT, CD73, into F-AraA that is transported inside the cell via a carrier protein (Baldwin et al., 1999). Like other NAs, it requires phosphorylation to its triphosphate form, F-AraA-TP, for cytotoxicity activity (Plunkett & Saunders, 1991). F-AraA, like CdA, is cytotoxic against both dividing and resting cells. In dividing cells, this compound inhibits DNA synthesis and RR, consequently reducing the pool of dNTPs required for DNA synthesis and enhancing their own cytotoxicity by self-potentiation (Plunkett et al., 1993). Other enzyme targets of F-AraA include DNA primase, DNA polymerase α, DNA ligase, and DNA topoisomerase II (Tseng et al., 1982). Thus, the mechanism of action of F-AraA in proliferating cells is mainly cell cycle specific, and incorporation of F-AraA into DNA during S phase is required for the induction of apoptosis (Consoli et al., 1998; Galmarini et al., 2001, 2002). In nondividing cells, the inhibition of cellular DNA repair is the most likely explanation for cytotoxicity of F-AraA and CdA. Incorporation of F-AraA-TP or CdA-TP into DNA by the repair mechanisms leads to the progressive accumulation of DNA single-strand breaks eventually responsible for apoptosis by both p53-dependent and p53-independent pathways (Sandoval et al., 1996). Another consequence of these treatments is the direct activation of the caspase-9 and caspase-3 pathways by F-AraA-TP and CdA-TP, which are nucleotide activators of Apaf-1 (Genini et al., 2000). Moreover, F-AraA and CdA also alter gene transcription, resulting in depletion of proteins required for cell survival. Incorporation of F-AraA-MP into RNA results in premature termination of the RNA transcript, impairing its function as a template for protein synthesis, while F-AraA-TP inhibits RNA synthesis by suppressing the activity of RNA polymerase II (Huang et al., 2000). On the other hand, incorporation of CdA metabolites present in one or both DNA strands reduced the yield of full-length transcripts (Hentosh & Tibudan, 1995). In conclusion, inhibition of DNA repair, termination of mRNA transcription, and consequent depletion of proteins required for cell survival as well as the capacity of F-AraA-TP to activate the apoptotic pathway appear to contribute to the cytotoxicity of F-AraA in nondividing cells.

Among the AIDS drugs approved by the Food and Drug Administration (FDA) for clinical use, 2 are modified cytosine analogues, 3TC and ddC (Fig. 7 and Table 3). Similar to other dideoxynucleosides, these analogues are metabolically activated to the triphosphate form that is incorporated into DNA by HIV-RT, resulting in DNA chain termination and ultimately cessation of viral replication. 3TC is the only FDA-approved NA containing an unnatural β-L-nucleoside conformation. 3TC has been shown to be more potent and less toxic than the D-isomer (Chang et al., 1992a). The mechanistic basis for the sterochemical selectivity and different toxicity of the isomeric 3TC and ddC compounds is not completely understood (Anderson, 2002). The enzyme responsible for the formation of 3TC-MP is dCK (Chang et al., 1992b). 3TC-MP is incorporated into proviral DNA by HIV-RT and thus acts as a chain termina-

tor. It is also incorporated into the cellular DNA chain by polymerase γ; however, polymerase γ retains its 3′,5′-exonuclease activity and is able to excise 3TC-MP from the 3′ end of the chain. This may explain the low mitochondrial toxicity of 3TC (Gray et al., 1995). 3TC is neither intracellularly deaminated nor degraded via phosphorolytic cleavage by pyrimidine nucleoside phosphorylase (Coates et al., 1992). 3TC shows significant anti-HIV activity with less toxicity than AZT, and AZT-resistant HIV strains are not cross-resistant to 3TC (Soudeyns et al., 1991).

(−)FTC (2′,3′-dideoxy-5-fluoro-3′-thiacytidine, Emtricitabine) is in phase III trial for HIV and phase I/II trial for hepatitis B virus (HBV); it is considered for use in the multidrug combination therapy for HIV and HBV infections (De Clercq, 2002). The (−)FTC-TP is a competitive inhibitor of HIV-RT and (−)FTC-MP may be incorporated into proviral DNA as a chain terminator (Furman et al., 1992; Schinazi et al., 1992).

ddC shows potent and selective anti-HIV activity in human cells (Mitsuya & Broder, 1986). In various cell lines, ddC is phosphorylated intracellularly to ddC-MP, ddC-DP, and ddC-TP with concentrations at the same order of magnitude (Cooney et al., 1986). The affinity of ddC-TP to DNA polymerase α is very poor but is intermediate to polymerase β and high to polymerase γ (Starnes & Cheng, 1987). Compared with the other FDA-approved antiviral NAs, ddC has the highest potency of inhibiting mtDNA synthesis (Chen et al., 1991).

dGK has become of interest because the enzyme phosphorylates a number of clinically important anticancer drugs, such as CdA, 9-β-D-arabinofuranosylguanine (Nelarabine, AraG), and 2′, 2′-difluorodeoxyguanosine (dFdG) (Wang et al., 1993; Arnér & Eriksson, 1995; Sjoberg et al., 1998). Zhu et al. (1998a) showed in vitro that dGK is important for AraG and dFdG phosphorylation, whereas dCK is the most important enzyme for activation of CdA and dFdC (Zhu et al., 1998a). Overexpression of dGK in pancreatic cancer cell lines increases the sensitivity to the purine NAs AraG, dFdG, and CdA (Zhu et al., 1998b).

CdA, AraG, and dFdG are efficiently phosphorylated by mitochondrial dGK (Wang et al., 1993; Zhu et al., 1998a, 1998b, 2000) and have early effects on mitochondrial function (Hentosh & Tibudan, 1997; Zhu et al., 2000). However, it is not clear if there is a relationship between cell death and mtDNA incorporation of anticancer NAs. Several clinically used antiviral NAs have been reported to be incorporated into the mitochondria DNA and thereby cause severe side effects (Lewis & Dalakas, 1995; Agarwal & Olivero, 1997; Barile et al., 1998; Chariot et al., 1999). These effects are related to their mtDNA incorporation and described as delayed effects, which occur after several weeks or months of drug exposure (Lewis et al., 1996; Dalakas, 2001). The role of the mitochondrial enzymes for mitochondrial toxicity and the different mechanisms behind delayed and acute toxicity of NAs are not yet clear (Moyle, 2000).

The prodrug of the biologically active AraG has proven very efficient, especially in the treatment of patients with T-cell acute lymphoblastic leukemia (Fig. 7 and Table 3) (Aguayo et al., 1999). AraG is a substrate of both dGK and dCK, and at low substrate concentrations, dGK seems to be the preferred enzyme (Zhu et al., 1998a; Rodriguez et al., 2002). Although the mechanisms of AraG are not fully understood, the accumulation of AraG-TP has been correlated with cytotoxicity both in vitro and in vivo (Scharenberg et al., 1986). Once phosphorylated to its triphosphate derivative, AraG-TP acts as a structural analogue of dGTP and is thereby incorporated into DNA. It has been demonstrated that the accumulation of AraG-TP is independent of the cell cycle, which is not surprising because both dGK and dCK are expressed throughout the cell cycle. Furthermore, it has been suggested that AraG exerts its cytotoxic action by inducing apoptosis and that incorporation of AraG-MP into nuclear DNA is a critical event for triggering apoptosis (Rodriguez & Gandhi, 1999). These data are confirmed in a MOLT-4 leukemia cell line where the results suggest that the resistance to apoptosis in AraG-resistant cells might contribute to the overall insensitivity to a variety of anticancer drugs (Månsson et al., 2002). A recent study has also suggested a role of mitochondria in the cell-specific toxicity of dGTP with intramitochondrial accumulation of dGTP and inhibition of mtDNA (Arpaia et al., 2000). We have shown that AraG can be incorporated into mtDNA, but we do presently not know to what extent the mitochondrial incorporation contributes to the cytotoxic action of the analogue (Curbo et al., 2001). We have also shown that the acute cytotoxicity of AraG is not caused by mtDNA damage (Curbo, 2001). However, it cannot be excluded that long-term exposure to AraG may cause mtDNA alterations with subsequent delayed mitochondrial toxicity. Several in vitro studies have been performed on the molecular mechanisms of resistance to AraG, with partly conflicting results. We recently reported that AraG resistance occurs by 2 separate sequentially mechanisms. The first mechanism is associated with a decrease of AraG incorporation into mtDNA and the second event is associated with loss of dCK activity but not with loss of dGK activity (Curbo et al., 2001). Recent results indicate that mutations in the genes encoding for dGK and TK2 are associated with mtDNA depletion (Mandel et al., 2001; Saada et al., 2001). However, we could demonstrate that AraG did not cause mtDNA depletion or altered translation of mtDNA encoded genes (Curbo, 2001). In conclusion, further studies are necessary to enlighten the many molecular mechanisms associated with AraG resistance.

TK1 phosphorylates the pyrimidine analogues AZT, 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodouracil (Fialuridine, FIAU), and d4T (Fig. 7 and Table 3) (Furman et al., 1986; Munch-Petersen et al., 1991; Ahluwalia et al., 1996; Wang & Eriksson, 1996).

The introduction of AZT into clinical practice in 1987 ushered in the therapeutic era of the AIDS epidemic. AZT is

a thymidine analogue that is mainly phosphorylated by TK1. However, TK2 has been suggested to be important for the activation of AZT in nondividing cells that lack TK1 (Arnér et al., 1992). Following AZT-MP entry into the mitochondria, 2 mechanisms of mitochondrial cytotoxicity are possible. Studies have suggested a hypothesis for mitochondrial toxicity: following phosphorylation to AZT-TP, DNA polymerase γ is inhibited either by AZT-TP serving as a competitive substrate and incorporation into mtDNA or by terminating the mtDNA chain noncompetitively (Lewis & Dalakas, 1995). However, other studies have concluded that AZT-TP is not responsible for mitochondrial toxic effect observed during chronic administration (Szebeni et al., 1991). Secondly, AZT-MP is an alternative substrate inhibitor of dTMP for dTMPK (Furman et al., 1986; Lavie et al., 1997). This may result in decreased dTTP in the mitochondria and thus decreased mtDNA synthesis (Törnevik et al., 1995; Lavie et al., 1997). In conclusion, although the mechanism by which AZT causes mitochondrial toxicity requires full elucidation, recently, there is evidence provided that AZT-MP is primarily responsible for the toxicity and that this anabolite is mainly formed in the cytosol (Sales et al., 2001). 3′-Azido-3′-deoxy-5′-thymidylic acid, mono [3-(dodecylthio)-2-decycloxypropyl] ester, sodium salt (FZD, Fozivudine tidoxil), belonging to the newer AZT generation drugs, is a thioether lipid AZT conjugate that has recently passed phase II clinical trials. Intracellularly, FZD is split into the lipid moiety and AZT-MP, the latter of which is subsequently phosphorylated to the active metabolite AZT-TP. FZD appears to be as effective as and potentially better tolerated than AZT and has the advantage of once daily intake (Girard et al., 2000).

d4T like AZT is an anti-HIV drug. The phosphorylation of d4T to its d4T-MP is the rate-limiting step of the sequential conversion of d4T to d4T-TP, which inhibits HIV-RT equipotently as AZT-TP (Balzarini et al., 1989). d4T-TP is preferentially incorporated into the elongating viral DNA and terminates DNA synthesis at the incorporation site and also inhibits polymerase γ (Ono et al., 1989). Similar to AZT, d4T enters cells by nonfacilitated diffusion (August et al., 1991). d4T shows 10-fold lesser toxicity to human hematopoietic progenitor cells compared with AZT (Mansuri et al., 1989).

TK2 also phosphorylates AZT and FIAU; however, AZT is phosphorylated by TK2 at a much lower rate than by TK1, whereas FIAU and 1-β-D-arabinofuranosylthymine (AraT) are better substrates for TK2 (Munch-Petersen et al., 1991; Arnér et al., 1992; Wang & Eriksson, 1996; Johansson et al., 1999; Sales et al., 2001). dFdC and AraC are also substrates for TK2, but with lower affinity than for dCK.

Recently, MDS has been linked to mutations in dGK and TK2. Mutations in TK2 have been associated with the myopathic form and mutations in dGK with the hepatoencephalopathic form of MDS (Mandel et al., 2001; Saada et al., 2001). The association of mtDNA depletion with mutated dGK and TK2 confirms that these salvage pathway enzymes are involved in the maintenance of balanced mitochondrial dNTP pools. The mutations for both genes are certainly associated with variable phenotypes; however, their low frequencies suggest that TK2 and dGK are not the only genes responsible for mtDNA depletion in muscle and liver, respectively (Mancuso et al., 2002; Salviati et al., 2002).

FIAU was an antiviral agent with potent activity against HBV replication in vitro and in vivo. In a phase II study, 7 of 15 patients experienced severe toxicity due to the drug after 9–13 weeks of treatment. Adverse effects included nausea, vomiting, and painful paraesthesia; subsequently, hepatic failure, pancreatitis, neuropathy, myopathy, and lactic acidosis developed, probably due to multisystem mitochondrial toxicity (McKenzie et al., 1995; Honkoop et al., 1997). Possible mechanisms of FIAU toxicity include mitochondrial injury and pyruvate oxidation inhibition. Indeed, FIAU-TP is efficiently incorporated into DNA, but polymerase γ chain elongation is impaired, possibly leading to increased inhibitory effects of this drug at sites of replication (Johnson et al., 2001).

Recently, novel ribofuranosylnucleosides (*E*)-5-(2-bromovinyl)uridine, 3-spiro-(4-amino-1,2-oxathiole-2,2-dioxide)-5-methyluridine, and 2′-*O*-acyl/alkyl-substituted arabinosyl nucleosides (2′-*O*-Acyl-BVAraU and 2′-*O*-Acyl-AraT) have emerged as potent and selective competitive inhibitors of TK2 (Balzarini et al., 2000, 2001). Very recently, a novel class of potent and highly specific tritylated TK2-inhibitors, 1-[(*Z*)-4-triphenylmethoxy-2-butenyl]thymine and (*E*)-5-(2-bromovinyl)-1-[(*Z*)-4-triphenylmethoxy-2-butenyl]uracil, has been discovered (Balzarini et al., 2003). Such inhibitors will be of great value to enlighten the role of TK2 in mitochondrial homeostasis and might become of practical use when added at concentrations that partially block TK2 in combination with antiviral and anticancer analogues that display toxic side effects due to their activation by TK2 in the mitochondrial environment.

4.2. Ribonucleoside analogues

Most ribonucleoside analogues have so far not been investigated in detail. Adenosine has diverse effects on the cardiovascular, nerve, respiratory, and immune systems (Fox & Kelley, 1978; Manfredi & Holmes, 1985). Inhibitors of ADK play an important pharmacological role in increasing extracellular adenosine concentrations (Cronstein, 1992; Firestein et al., 1994). Examples of ADK inhibitors are 5′-amino-5′-deoxyadenosine, 5-iodotubercidine, and 5′-deoxy-5-iodotubercidine (Kowaluk et al., 1999); recently, many promising new compounds like ABT-702 (Jarvis et al., 2000), GP515 (Siegmund et al., 2001), and a variety of pyrrolo[2,3-D]pyrimidine NAs (Ugarkar et al., 2000) have been synthesized and tested.

ADK exhibits a relatively broad substrate specificity tolerating modifications in both sugar and base moieties (Miller et al., 1979). Accordingly, certain nucleoside antiviral and anticancer drugs are substrates of ADK and consequently undergo rapid phosphorylation in vivo to the 5′-monophosphate. In many cases, the monophosphate is subsequently converted by other kinases to the triphosphate, which functions as the active metabolite. Examples include the antiviral drug ribavirin (Willis et al., 1978) and the immunosuppressive drug mizoribine (Yamada et al., 1981). Ribonucleoside analogues, such as ribavirin, have been demonstrated to be active as anti-RNA virus compounds (Loeb et al., 1999; Crotty et al., 2000). The mechanism of action for ribavirin was recently demonstrated to be through an ''error catastrophe'' induced by mutagenic incorporation of ribavirin triphosphate into the RNA genome of HIV virus (Loeb & Mullins, 2000). Whether other rNKs may be a tool to increase the activation of ribonucleoside analogues in virus-infected cells and thereby suppress the development of resistant virus should be further investigated.

The recent identified UCKs phosphorylate, and thereby pharmacologically activate, several cytotoxic pyrimidine ribonucleoside analogues that may have pharmacological use in chemotherapy of cancer. UCK1 and UCK2 phosphorylated several of the 28 tested cytidine and uridine NAs, such as 6-azauridine, 5-fluorouridine, 5-fluorocytidine, and 2-thiocytidine (Van Rompay et al., 2001). Several pyrimidine ribonucleoside analogues like 6-azauridine (Pasternak et al., 1961; Vesely & Cihak, 1973a), 5-azacytidine (Cihak & Broucek, 1972; Vesely & Cihak, 1973b), 5-hydroxyuridine (Smith & Visser, 1965), and 4-thiouridine (Lindsay & Yu, 1974) exert the pharmacological effects in their monophosphate forms by inhibiting OMP decarboxylase in addition to their incorporation into RNA or DNA (Anderson, 1973). The different mode of actions of ribonucleoside analogues makes this group of compounds interesting for further studies to develop clinically useful drugs. Promising results in animal tumor models have recently been shown for 1-(3-C-ethynyl-β-D-ribo-pentofuranosyl)-cytosine and -uracil (Takatori et al., 1999). Cyclopentenyl cytosine is another interesting compound with antitumor activity that presently is evaluated in clinical trials (Verschuur et al., 2000). Overexpression of human UCK1 and UCK2 in fusion with GFP in a Chinese hamster ovary (CHO) cell line increased sensitivity to certain pyrimidine ribonucleoside analogues (Van Rompay, 2001). Expression of UCK1-GFP increased sensitivity to 6-azacytidine and 5-methylcytidine, while UCK2-GFP overexpression resulted in an increased sensitivity to 3-deazuridine and 6-azauridine and a small increase to 5-methylcytidine. No difference in the cytotoxicity of the base analogue 5-fluorouracil was observed in the UCK-overexpressing cells compared with the parent cancer cell lines, which is in agreement with recent literature (Mascia & Ipata, 2001).

5. Concluding remarks

The cloning, recombinant expression, and characterization of the nucleoside and nucleotide kinases will allow the pathways of nucleoside and NA metabolism to be outlined in more detail. Knowledge of the metabolic pathways will be important for the development of novel antiviral and anticancer ribonucleoside and deoxyribonucleoside analogues and for the characterization of their pharmacological activation in different tissues.

In mammalian cells, DNA replicates in the nucleus and in the mitochondria. The enzymes responsible for the synthesis of deoxyribonucleotides are located in the cytosol, the nucleus, and the mitochondria. The cytosol and the nucleus are separated by the nuclear membrane, which allows diffusion of small proteins and nucleotides. The deoxyribonucleotide pools in the nucleus and cytosol are thereby metabolically related and equilibrated. The deoxyribonucleotide pool in the mitochondria is structurally separated from the pool in the cytosol by the mitochondrial membrane. The mitochondrion contains an inner and an outer membrane; the outer membrane is permeable to small molecules (up to 10 kDa), whereas the inner membrane is almost impermeable to passage of ions and most molecules at physiological conditions. However, the nucleosides can enter the mitochondria by membrane transport proteins. The mitochondrial dNKs dGK and TK2 phosphorylate all 4 deoxyribonucleosides required for DNA synthesis in mitochondria. In addition to the first phosphorylation step, there are also 2 mitochondrial NDPKs identified for the third phosphorylation step. A question remains for the second phosphorylation step, because little is known about mitochondrial NMPKs. Two mitochondrial AKs have been demonstrated, which may be responsible for dAMP phosphorylation, but the enzymes phosphorylating dCMP, dGMP, and dTMP within the mitochondria remain to be identified. Because several clinically used NAs cause severe side effects through mitochondrial toxicity, it is important to identify and characterize the mitochondrial enzymes that may contribute to this toxicity.

An interesting future study is to focus on the identification and use of ribonucleoside analogues to induce mutations of RNA viruses, such as HIV, hepatitis C virus (HCV), and severe acute respiratory syndrome (SARS) (Loeb et al., 1999; Crotty et al., 2000; Cinatl et al., 2003; Nie et al., 2003). Ribonucleoside analogues, such as ribavirin, have been demonstrated to be active as anti-RNA virus compounds (Crotty et al., 2000; Loeb & Mullins, 2000; Nie et al., 2003). RNA viral diseases are responsible for a majority of viral morbidity and mortality of viral diseases, including measles, polio, influenza, AIDS, and hepatitis C. The idea is that RNA NAs will be administered to an infected cell, and the analogue is then incorporated by a polymerase into a RNA copy of the genomic nucleic acid encoding the virus. Subsequent copying of the incorporated analogue will cause an increase in the mutation rate of the virus. Over time, this

results in reduced viability of progeny generations of the virus, thereby inhibiting viral replication. Further investigation of the recently cloned rNKs, which is considered the rate-limiting step, and the next 2 phosphorylation steps (NMPK and NDPK) of cellular and viral RNA polymerases will contribute to develop better RNA incorporated ribonucleoside and ribonucleotide analogues. Purine ribonucleoside analogues should also be investigated as possible RNA virus inhibitors.

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