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QUARTERLY

*This paper is dedicated to Professor David Shugar on the occasion of his 80th birthday*

# Structure-activity relationships for phosphorylation of nucleoside analogs to monophosphates by nucleoside kinases*

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**The mammalian deoxyribonucleoside kinases thymidine kinase 1 and 2, deoxycytidine kinase and deoxyguanosine kinase phosphorylate deoxyribonucleosides and provide an alternative to *de novo* synthesis of DNA precursors. Their activities are essential for activation of several chemotherapeutically important nucleoside analogs. These four salvage kinase enzymes exhibit distinct substrate specificities for nucleoside analogs modified in the base and glycon moieties. In this review their structure-activity relationships are discussed. Alternative routes for phosphorylation of nucleoside analogs are also reviewed, such as the phosphotransfer capacity of 5′-nucleotidase and protein kinases.**

## SALVAGE AND *DE NOVO* SYNTHESIS OF DEOXYRIBONUCLEOSIDES

The salvage pathway is a route supplementary to *de novo* synthesis for providing cells with deoxyribonucleotides as precursors for DNA synthesis. By facilitated diffusion a nucleoside carrier protein with a wide specificity transports deoxyribonucleosides from the extracellular space through the cell membrane (reviewed in [1]). Some lipophilic nucleoside analogs, e.g. the anti-AIDS agent AZT (3′-azido-3′-deoxythymidine) may also bypass the carrier protein [2]. A key step is the phosphorylation of nucleosides to monophosphates thus trapping them inside the cell. Mammalian cells contain four main salvage enzymes: thymidine kinase 1 (TK1, EC 2.7.1.21), thymidine kinase 2 (TK2, no EC number yet), deoxycytidine kinase (dCK, EC 2.7.1.74) and deoxyguanosine kinase (dGK, EC 2.7.1.113). A further enzyme, adenosine kinase (EC 2.7.1.20) phosphorylates deo-

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**Abbreviations:** *Enzymes* — pol α, DNA polymerase α; RT, reverse transcriptase; TK, thymidine kinase; dCK, dGK, deoxycytidine and deoxyguanosine kinase respectively; *Nucleotide analogs* — ACV, acyclovir, 9-(2-hydroxyethoxymethyl)guanine; AZT, 3′-azido-3′deoxythymidine; BVDU, 2′-deoxy-[5-(*E*)-2-bromovinyl]deoxyuridine; CdA, 2-chlo- ro-2′-deoxyadenosine; dd, 2′,3′-dideoxy derivatives; d4, 2′,3′-dideoxydidehydro derivatives; GCV, ganci- clovir, 9-(1,3-dihydroxy-2-propoxymethyl)guanosine; THBG, 9-[1,3-(*S*)-4-trihydroxy-2-(*S*)-butoxyme- thyl]guanine; *Viruses* — HCMV, human cytomegalovirus; HSV, herpes simplex virus; VZV, varicella zoster virus.

phorylates deoxyadenosine with high $K_m$ and low $V_{max}$ values [3, 4] and is probably not of major importance in human cells. The biochemistry and molecular biology of mammalian deoxyribonucleoside kinases have recently been extensively reviewed [5].

*De novo* synthesis can provide all the DNA precursors needed for growth of replicating human cells and the salvage pathway is most likely not essential for cell survival. However, the salvage pathway has important supplementary functions.

In resting or G1 cells, *de novo* synthesis is absent and the salvage pathway is a source of DNA precursors for repair or mitochondrial DNA synthesis. It also provides the major part of pyrimidine deoxynucleotides in S phase thymocytes [6] and in S phase cells dCDP-choline, dCDP-ethanolamine and dCDP diacyl glycerols are also synthesized by the salvage route [7–9]. TK2 and dGK are both mitochondrial enzymes, most likely making mitochondria independent of *de novo* synthesis. Together with *de novo* synthesis the salvage enzymes maintain a balanced pool of deoxyribonucleotides, counteracting disturbances in DNA precursor pools which might be mutagenic (reviewed in [10, 11]). Finally, salvage synthesis is important for activation of antiviral and cytostatic nucleoside analogs.

This latter aspect has been the subject of an earlier general review by D. Shugar [12]. The antimetabolic action of some specific purine nucleoside analogs [13] and the role of dCK in cancer chemotherapy [14] have also been reviewed recently.

The structure activity relationships for various nucleoside analogs as substrates of the four main mammalian salvage enzymes will be the subject of this review. It is confined to nucleosides and nucleoside analogs having β-D-glycon configuration. Apart from mentioning a few examples, acyclic nucleoside analogs, nucleosides having the L-configuration and α-anomers are not discussed here. Studies are ongoing in the authors' laboratories on a number of compounds belonging to these categories. It is clear that they are substrates for phosphorylation by salvage enzymes to a greater extent than previously published reports indicate. D. Shugar long ago drew attention to the antiviral and antitumour effects of α-anomers of some nucleoside analogs [15] and has also discussed this in his recent review [12].

Salvage pathways in microorganisms [16] and viruses (reviewed in [17, 18]) will not be discussed here.

## EXPRESSION OF SALVAGE ENZYMES

The cell cycle regulation and tissue expression of salvage enzymes has been discussed at length in the recent review by Arnér & Eriksson [5]. Most cells and tissues are capable of expressing the cytosolic enzymes TK1 and dCK although not brain, muscle and liver. Lymphoid tissue contains high levels of dCK (reviewed in [19]) and dCK levels are elevated in solid cancer tissues [20, 21].

TK1 is expressed only in proliferating cells, both normal and malignant (reviewed in [17, 19]). The dependence of dCK on cell-cycle regulation is not unambigous, and further studies are needed. For a discussion see [5].

The mitochondrial enzymes TK2 and dGK are present in most tissues. Their activities are independent of cell-cycle regulation, and by and large seem to be correlated to the amount of mitochondria present in the respective cells (reviewed in [19]).

The phosphorylating capacity differs widely between the cells in different tissues. The relative activities of TK2 and dGK for phosphorylating thymidine and deoxyguanosine, respectively, are only 2–5% of the respective capacities of dCK and TK1 for phosphorylating deoxycytidine and thymidine in rapidly growing cells, while in non-profilerating cells such as muscle or brain the low TK2 and dGK activities are the only deoxynucleoside kinases present.

## EXAMPLES OF DIFFERENT ANTIMETABOLIC PROFILES FOR SOME NUCLEOSIDE ANALOG PHOSPHATES

The antimetabolic properties of a few nucleoside analogs are summarily depicted in Scheme 1.

3'-Azido-3'-deoxythymidine (AZT) and 2',3'-dideoxycytidine (ddC) are approved agents for treatment of HIV-infection and AIDS.

They are both activated to monophosphates by salvage enzymes, AZT by TK1 and ddC by dCK, and further phosphorylated by other cellular kinases to di- and triphosphates (Scheme 1A). It is the latter species which inhibit HIV reverse transcriptase (reviewed in [22, 23]).

9-(2-Hydroxyethoxymethyl)guanine (acyclovir, ACV, of Scheme 1B) is widely used for treatment of herpes virus infections. It is selectively phosphorylated to the monophosphate by herpes virus (HSV-1, HSV-2, VZV) thymidine kinases. Cellular kinases catalyse the further stepwise transformation to a triphosphate which is a potent inhibitor of herpes virus DNA polymerase (reviewed in [18, 24]). 2'-Deoxy-[5-(*E*)-2-bromovinyl] deoxyuridine (BVDU, of Scheme 1B) has been much studied for its anti-herpes activity. Herpes simplex virus thymidine kinases (HSV-1 TK and HSV-2 TK) both effect its monophosphorylation. Unlike ACV, cellular kinases do not produce a diphosphate and HSV-1 TK but not HSV-2 TK has the capacity to function as a thymidylate kinase for this compound. Cellular enzymes bring about the final transformation to a triphosphate which inhibits HSV DNA polymerase. As a consequence BVDU shows activity against HSV-1 but not against HSV-2 (reviewed in [18, 25]).

2'-Deoxy-2',2'-difluorocytidine (gemcitabine, of Scheme 1C), a cytostatic agent recently approved for treatment of pancreatic and lung cancers, is phosphorylated by cellular kinases to monophosphate (dCK), di- and triphosphates. It has a complex mode of action. It inhibits ribonucleotide reductase and deoxycytidylate deaminase [26, 27] and its triphosphate accumulates and is incorporated into DNA [28–30].

It is generally assumed that formation of the monophosphate is the key step for metabolic activation of nucleoside analogs, and that once this has been achieved, nucleoside analog triphosphates will eventually be formed and that these will inhibit replication enzymes. The example with BVDU shows that the transformation sequence may stop short of diphosphate formation.

The acyclic guanosine analog 9-[1,3-(*S*)-4-trihydroxy-2-(*S*)-butoxymethyl] guanine (THBG, of Scheme 1D) is a rare example of a compound for which high levels of the triphosphate are formed in HSV-1 infected cells, following activation to the monophosphate by HSV-1 TK, but the triphosphate does not inhibit HSV DNA polymerase and the compound does not show any activity against HSV-1 [24, 31].

Finally, 2'-deoxy-5-(2-thienyl)uridine (of Scheme 1E) is an example of a class of 5-heteroaryl substituted 2'-deoxyuridines, which are substrates for TK2 and phosphorylated to monophosphates [32].

However, as will be discussed below, the further intracellular phosphorylation to di- and triphosphates apparently does not occur.

## GENERAL REMARKS ON THE DATA ASSEMBLED IN THE REVIEW

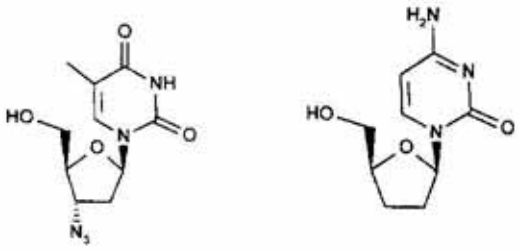
TK1, TK2 and dCK were from human leukemic spleen cells purified to apparent homogeneity: 20 000 fold for TK1 and TK2 and 6 000 fold for dCK [33, 34]. dGK, similarly purified to homogeneity, was from bovine calf brain [35]. The adenosine 5'-triphosphate transfer assays were performed with 100 μM [$\gamma$-$^{32}$P]ATP and 100 mM nucleoside analog, incubation for 30 or 60 min and chromatography of the product mixture on cellulose thin-layer plates. The products of the kinase reactions were detected by autoradiography, the spots were excised and quantified by liquid scintillation.

Tables 1–14 give the relative capacities for phosphorylation of nucleoside and nucleoside analog substrates. The reference standards which have been set to 1.0 are for phosphorylation of thymidine by TK1 or TK2, 2'-deoxycytidine by dCK and 2'-deoxyinosine by dGK [35, 36].

In addition to ATP, other nucleoside triphosphates can function as donors in the phosphotransferase assay. Several studies indicate that UTP [37–39] as well as GTP [40–42] is an efficient donor.

However, it should be born in mind that, within the cell, there is a mixture of the natural ribonucleoside triphosphates at fairly high concentrations, with ATP predominating (about 3 mM). A mixture based on such natural intracellular concentrations has been shown to be an optimal phosphate donor for dCK [37–39, 43].

**A**

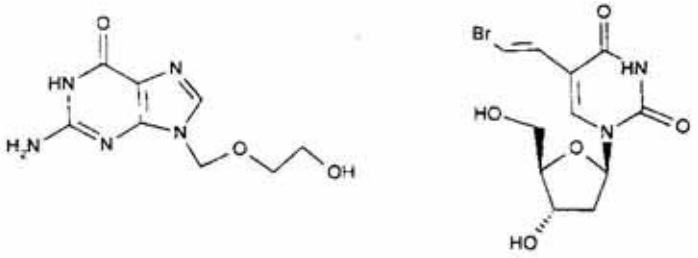


$$NA \rightarrow NA\text{-}MP \rightarrow NA\text{-}DP \rightarrow NA\text{-}TP$$

$$\Downarrow$$

HIV RT

**B**

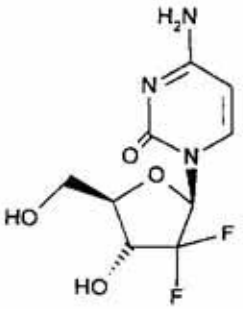


$$NA \rightarrow NA\text{-}MP \rightarrow NA\text{-}DP \rightarrow NA\text{-}TP$$

$$\Downarrow$$

HSV DNA pol.

**C**

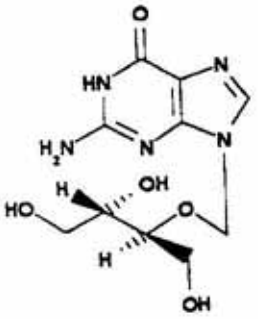


$$NA \rightarrow NA\text{-}MP \rightarrow DNA\text{-}DP \rightarrow NA\text{-}TP$$

$$\Downarrow \qquad \Downarrow \qquad \Downarrow$$

dCMP Deaminase | Ribonucleotide Reductase | DNA

D

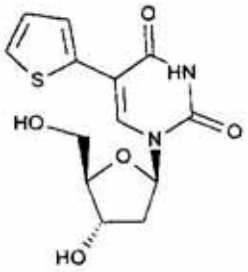


NA → NA-MP → NA-DP → NA-TP

⇓

HSV DNA pol.

E



NA → NA-MP NA-DP NA-TP

⇓

HIV RT

DNA pol α

*Scheme 1. Examples of different antimetabolic profiles for some nucleoside analog phosphates.*
Abbreviations: NA, nucleoside; MP, monophosphate; DP, diphosphate; TP, triphosphate; RT, reverse transcriptase.

## SUBSTRATE SPECIFICITIES OF NUCLEOSIDE KINASES FOR NUCLEOSIDES AND NUCLEOSIDE ANALOGS HAVING THE β-D-GLYCON CONFIGURATION

### Natural bases with ribo-, arabino-, 2′-deoxy- and 2′,3′-dideoxyribofuranosyl glycons

The substrate specificities of the four salvage kinase enzymes for nucleoside analogs containing natural bases with ribo-, arabino-, 2′-deoxy and 2′,3′-dideoxyribofuranosyl glycons are given in Tables 1–3 [35, 36]. Thymidine and 2′-deoxyuridine are good substrates for either TK1 and TK2. 2′-Deoxycytidine and all of the natural 2′-deoxypurines are substrates for dCK. TK2 also has the capacity to phosphorylate 2′-deoxycytidine. The 2′-deoxypurines are good substrates both for cytosolic dCK and mitochondrial dGK.

Table 1
*Substrate specificities of TK1, TK2 and dCK for pyrimidine nucleosides*

| Substrate | Relative activity | | |
|---|---|---|---|
| | TK1 | TK2 | dCK |
| Thd | 1.0 | 1.0 | 0.02 |
| dUrd | 1.0 | 1.0 | 0.06 |
| dCyd | ≤ 0.01 | 0.9 | 1.0 |
| RiboThd | 0.02 | 0.03 | |
| Urd | ≤ 0.01 | 0.04 | 0.02 |
| Cyd | ≤ 0.01 | ≤ 0.01 | 0.2 |
| Ara T | ≤ 0.01 | 0.6 | |
| Ara U | ≤ 0.01 | 0.2 | ≤ 0.01 |
| Ara C | ≤ 0.01 | ≤ 0.01 | 1.2 |

For legend to the Tables see the General Remarks on the Data Assembled in the Review.

Arabinofuranosyl structures are phosphorylated by TK2 when the base is thymine or uracil and by dCK when the base is cytosine or adenine. dGK phosphorylates arabino nucleosides having guanine or hypoxanthine as bases. The ribonucleosides are at best poor substrates for the kinases.

Of the 2′,3′-dideoxynucleosides only ddT and to some extent ddU are substrates for TK1. The anti-HIV compound ddC is phosphorylated by dCK. In early studies with partially purified enzyme, the substrate efficiency was low [44–46]. Later studies show a $K_m$ of 60 μM and the same $V_{max}$ as with deoxycytidine [47].

The 2′,3′-dideoxy-didehydronucleoside analogs d4T and d4C are not phosphorylated by the

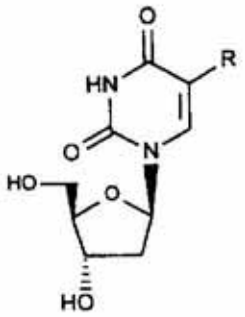
Table 3
*Substrate specificities of TK1, TK2, dCK and dGK for dideoxynucleoside analogs*

| Substrate | Relative activity | | | |
|---|---|---|---|---|
| | TK1 | TK2 | dCK | dGK |
| ddT | 0.4 | 0.04 | ≤ 0.01 | |
| ddU | 0.1 | 0.02 | ≤ 0.01 | |
| ddC | ≤ 0.01 | ≤ 0.01 | 0.3 | |
| ddA | | | ≤ 0.01 | ≤ 0.01 |
| ddG | | | ≤ 0.01 | ≤ 0.01 |
| ddI | | | ≤ 0.01 | ≤ 0.01 |
| d4T | ≤ 0.01 | ≤ 0.01 | ≤ 0.01 | |
| d4C | | ≤ 0.01 | 0.04 | |

Table 2
*Substrate specificities of dCK and dGK for purine nucleosides*

| Substrate | Relative activity | |
|---|---|---|
| | dCK | dGK |
| dAdo | 3.5 | 1.0 |
| dGuo | 3.0 | 0.5 |
| dIno | 1.2 | 1.0 |
| Ado | ≤ 0.01 | |
| Guo | ≤ 0.01 | 0.1 |
| Ino | ≤ 0.01 | 0.02 |
| Ara A | 0.5 | 0.05 |
| Ara G | 0.06 | 0.9 |
| Ara Hx | ≤ 0.01 | 1 |

Table 4
*Substrate specificities of TK1, TK2 and dCK for 5-substituted 2′-deoxyuridine compounds*




| Substrate | Relative activity | | |
|---|---|---|---|
| R | TK1 | TK2 | dCK |
| $CH_3$ (Thd) | 1.0 | 1.0 | 0.02 |
| $CH_2CH_3$ | 0.8 | 1.0 | |
| Br | 0.8 | 1.0 | ≤ 0.01 |
| $NH_2$ | 0.03 | 0.5 | 0.17 |
| $CH_2CH_2Cl$ | ≤ 0.05 | 0.2 | |
| CH=CHBr (BVDU) | ≤ 0.01 | 0.2 | 0.02 |
| $CH=CHCH_3$ | ≤ 0.01 | 0.4 | |
| 2-Furyl | ≤ 0.05 | 0.25 | |
| 2-Thienyl | ≤ 0.01 | 0.12 | |

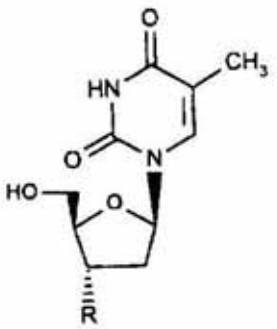
purified pyrimidine kinases. It is not clear which enzyme phosphorylates d4T, which is now approved as an agent for treatment of HIV/AIDS. An early study reported that partially purified TK1 could catalyze phosphorylation with low efficiency [48].

**Pyrimidine bases and 2'-deoxy glycon analogs**

Tables 4–8 and Chart 1 summarize the phosphorylating capacities of TK1, TK2 and dCK for 2'-deoxy- and 2',3'-dideoxy-3'-substituted ribofuranosyl nucleoside analogs with 5-substituted pyrimidine bases [32, 36].

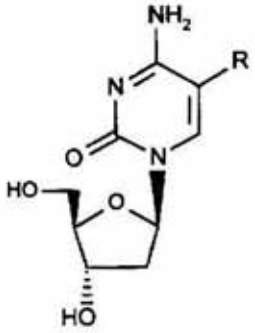
The cytosolic kinases TK1 and dCK exhibit a strict base selectivity. TK1 is discriminative for uracil/thymine bases and dCK for cytosine derived bases. An exception is 5-amino-2'-deoxyuridine which is a weak substrate for dCK. TK1 is the enzyme with the most strict structural requirements. In the 5-position of the uracil base, small alkyl groups and halogens are accepted while in the 3' position of the sugar, fluoro, azido and even methyleneazido are tolerated but not ethyne. Clearly the size of the 3'-substituent is not decisive for activity, ethyne and azido being, respectively, of about the same size and smaller than methyleneazido.

Table 5
*Substrate specificities of TK1, TK2 and dCK for 3'-substituted 2',3'-dideoxythymidine compounds*




| Substrate R | Relative activity TK1 | TK2 | dCK |
|---|---|---|---|
| OH (Thd) | 1.0 | 1.0 | 0.02 |
| F | 0.3 | ≤ 0.01 | ≤ 0.01 |
| $N_3$ | 0.5 | 0.05 | ≤ 0.01 |
| $CH_2N_3$ | 0.15 | 0.03 | |
| C≡CH | ≤ 0.01 | ≤ 0.01 | |

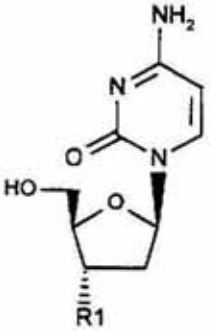
Table 6
*Substrate specificities of TK1, TK2 and dCK for 5-substituted 2'-deoxycytidine compounds*




| Substrate R | Relative activity TK1 | TK2 | dCK |
|---|---|---|---|
| H(dCyd) | ≤ 0.01 | 0.9 | 1.0 |
| $CH_3$ | ≤ 0.01 | 0.4 | 0.6 |
| Cyclopropyl | | ≤ 0.01 | 0.07 |
| Br | ≤ 0.01 | 0.4 | 0.2 |
| F | ≤ 0.01 | 0.9 | 0.2 |
| 5-AzaC | | | 0.2 |
| 2-Thienyl | ≤ 0.01 | 0.3 | 0.02 |

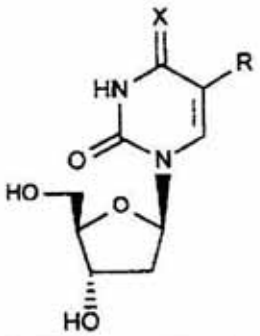
The other cytosolic enzyme dCK behaves similarly to TK1. For acceptance as substrate the 5-substituent can be a small alkyl or halogen

Table 7
*Substrate specificities of TK1, TK2 and dCK for 3'-substituted 2',3'-dideoxycytidine compounds*




| Substrate R1 | Relative activity TK1 | TK2 | dCK |
|---|---|---|---|
| OH (dCyd) | ≤ 0.01 | 0.9 | 1.0 |
| F | ≤ 0.01 | ≤ 0.01 | 0.6 |
| $CH_2F$ | | ≤ 0.01 | 0.3 |
| $CH_2N_3$ | | 0.02 | 0.3 |

Table 8
*Substrate specificities of TK2 and dCK for 5-heteroaryl substituted 2'-deoxypyrimidine compounds*




| Substrate | | Relative activity | |
|---|---|---|---|
| Base | R | TK2 | dCK |
| U | CH= CHBr(BVDU) | 0.2 | 0.02 |
| U | 2-Thienyl | 0.12 | |
| U | 3-Thienyl | 0.04 | |
| U | 2-Furyl | 0.25 | |
| C | 2-Thienyl | 0.3 | 0.02 |
| C | 3-Thienyl | 0.34 | ≤ 0.01 |
| C | 2-Furyl | 0.10 | 0.03 |
| C | 3-Furyl | 0.5 | ≤ 0.01 |
| C | 2-Selenienyl | 0.16 | ≤ 0.01 |
| C | 2-Pyridyl | 0.07 | ≤ 0.01 |
| C | 3-Pyridyl | ≤ 0.01 | 0.04 |
| C | 4-Pyridyl | 0.02 | 0.05 |

group or 5-azacytosine, and the 3'-substituent fluoro or methylenefluoro or methyleneazido.

Other cytidine analogs not shown in the Tables are also substrates. 2',3'-Dideoxy-3'-thiacytidine (3TC) now in clinical trials with interesting anti-HIV and anti-hepatitis B activities, as well as β-L-2',3'-dideoxycytidine and the 5-fluoro analogs of these compounds are phosphorylated by dCK [49, 50]. The acyclic analog cytallene is a substrate [36]. The carbocyclic analog of 2'-deoxyguanosine appears to be a substrate for dCK [51]. Later studies with purified dCK failed to show activity with carbocyclic 2'-deoxyguanosine but dGK was active with this substrate (see the next section [35]). This compound is active against herpes, hepatitis B and cytomegalovirus.

Herpes simplex virus-1 TK and most likely dGK are apparently about equally active on both D- and L-enantiomers of this compound. However, GMP kinase making the further transformation to the diphosphate appeared able to phosphorylate only the D-enantiomer [51]. 2'-Deoxy-2',2'-difluorocytidine (gemcitabine of Scheme 1), is a good substrate for dCK, (reviewed in [14]).

The mitochondrial TK2 enzyme strictly requires a 3'-hydroxyl group in the substrate. No other functional groups are accepted. However, it is very tolerant with respect to the nature of the 5-substituent in both uracil and the cytosine bases. Interestingly, some 5-heteroaryl substituents are compatible with good substrate properties when located on either the uracil or cytosine base (Table 8). The 5-heteroaryl cytosine derivatives are not substrates for dCK. These structure activity relationships can not be simply generalized. The effects on a cytosine base of 2- and 3-thienyls as 5-substituents are about the same. These compounds are better substrates than the corresponding uridine analogs where the

Table 9
*Kinetic parameters for some 5-substituted analogs with TK2, using the phospho-transfer assay*

| | $K_m$(μM) | $V_{max}$ (nmol/min per mg) | $V_{max}/K_m$ |
|---|---|---|---|
| Thd | 1 | 200 | 200 |
| BVDU | 2 | 40 | 20 |
| 5-(2-Thienyl)-2'-deoxyuridine | 9 | 44 | 4.9 |
| 5-(2-Furyl)-2'-deoxyuridine | 6 | 16 | 2.6 |
| 5-(2-Thienyl)-2'-deoxycytidine | 8 | 54 | 6.8 |
| 5-(2-Furyl)-2'-deoxycytidine | 21 | 130 | 6.1 |

S. Eriksson *et al.* [32].

two substituents also differ from each other. However, the 3-furyl group is considerably more favourable than 2-furyl on cytosine, and on uracil 2-furyl is better than on cytosine. The 5-pyridyl substituted 2'-deoxycytidines are very poor substrates.

An alkyl or halogeno substituent in the heteroaryl moiety abolishes all capacity of TK2 to phosphorylate such compounds (Chart 1).

The substrate efficiency, i.e. the apparent $V_{max}/K_m$ values for TK2 with BVDU and 2'-deoxy-5-(2-thienyl)uridine or -cytidine are 10 and 30–40 times, respectively, lower than that with thymidine (Table 9) [32]. The results with 2'-deoxy-5-(2-furyl)uridine and 2'-deoxy-5-(3-furyl)cytidine are similar (Table 9). Results with BVDU and 5-heteroaryl substituted 2'-deoxypyrimidines on HSV-1 TK show a similar pattern of substrate preference [52].

With respect to these compounds, it appears that mitochondrial TK and HSV-1 TK have overlapping specificity. It remains to be deter-

Table 10

*Inhibition of polymerases by triphosphates of 5-(2-thienyl)substituted pyrimidine nucleoside analogs*

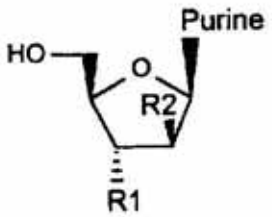
| Compound | $IC_{50}$ μM | | |
|---|---|---|---|
| | HIV-1 RT rAdT | HIV-1 RT Act. CT. DNA | CT.DNA pol α Act. CT. DNA |
| ddT-TP | 0.5 | 0.9 | 100 |
| ddC-TP | | 0.8 | 50 |
| 2'-dU 5-Th-TP | 0.04 | 0.21 | 1.0 |
| Ara U 5-Th-TP | 4.3 | >100 | 6 |
| Ara C 5-Th-TP | | >100 | 380 |
| ddU 5-Th-TP | 0.5 | 6 | 30 |

T. Persson *et al.* [53].

RT, reverse transcriptase.

Table 11

*Substrate specificities of dCK and dGK for 9-β-D-purine-2'-deoxyribose, -arabino- and ribofuranosyl compounds*




| Substrate | | | Relative activity | |
|---|---|---|---|---|
| R1 | R2 | TK2 | dCK | dGK |
| OH | H | A(dAdo) | 3.5 | 1.0 |
| OH | H | G(dGuo) | 3.0 | 0.5 |
| OH | H | Hx(dIno) | 1.2 | 1.0 |
| OH | H | 7-deaza A | 0.5 | 1.0 |
| OH | H | 2-ClA | 2.6 | 1.8 |
| $CH_2OH$ | H | 2-ClA | 0.4 | |
| H | H | 2-ClA | ≤ 0.01 | |
| F | H | A, G, DAP, Hx | ≤ 0.01 | |
| $N_3$ | H | A, G, DAP, Hx | ≤ 0.01 | ≤ 0.01 |
| OH | OH | A(Ara A) | 0.5 | 0.15 |
| OH | OH | G(Ara G) | 0.06 | 1.8 |
| OH | OH | Hx(Ara Hx) | ≤ 0.01 | 2.9 |
| Carbocyclic | | dG | ≤ 0.01 | 0.7 |

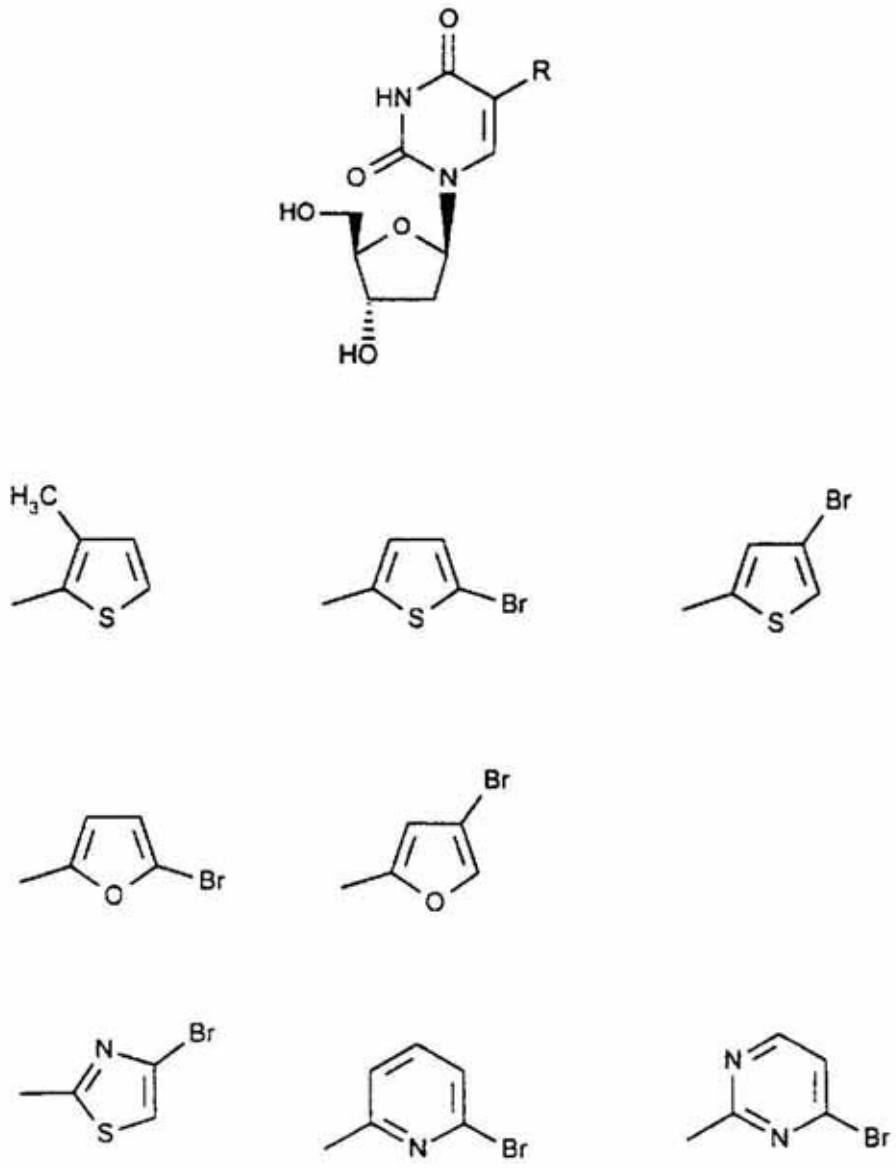



*Chart 1. Some 5-heteroaryl substituted 2'-deoxyuridine non-substrates of TK2.*

mined whether this will have any consequences for mitochondrial or other cellular toxicities.

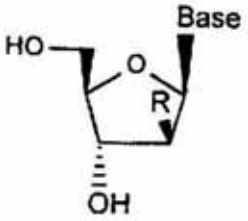
However, as has already been related in the discussion of compounds in Scheme 1, formation of the monophosphate of a nucleoside analog is not necessarily the key event which eventually will lead on to triphosphate formation. This is demonstrated by 2'-deoxy-5-(2-thienyl)uridine triphosphate which is a potent inhibitor of both HIV-1 RT (reverse transcriptase) and of DNA polymerase α (pol α), as shown in Table 10 [53]. The parent nucleoside does not show any activity against HIV in cell culture models, nor any apparent cytotoxicity (CT) against cells used in these assays [53] despite being a substrate for TK2.

A further interesting conclusion to be drawn from the data in Table 10 is that inhibition of HIV-1 RT and DNA pol α by triphosphates of a close by related family of nucleoside analogs is dependent both on the glycon (2'-deoxy *vs* arabino *vs* 2',3'-dideoxy) and on the base (uracil *vs* cytosine).

The potent anti-HSV-1 activities of 2'-deoxy-5-(2-thienyl)uridine and related compounds have also been described (reviewed in [54]). The results refer to tests in human embryonic skin muscle ($E_6$SM) fibroblast cells, and unlike BVDU which is equally potent in different cell lines, the anti-HSV-1 activity of these com-

Table 12

*Substrate specificities of TK1, TK2 and dCK for 2'-substituted 2'-deoxy-β-D-arabinofuranosyl compounds*




| Substrate | | Relative activity | | |
|---|---|---|---|---|
| R | Base | TK1 | TK2 | dCK |
| OH | T (Ara T) | ≤ 0.01 | 0.6 | |
| OH | U (Ara U) | ≤ 0.01 | 0.2 | ≤ 0.01 |
| OH | U 5-cyclopropyl | ≤ 0.01 | 0.15 | |
| OH | U 5 CH=CHCH$_3$ | | 0.5 | |
| OH | U 5 C ≡CCH$_3$ | ≤ 0.05 | 0.3 | |
| OH | U 5-(2-thienyl) | ≤ 0.01 | 0.06 | |
| F | T (FMAU) | 0.45 | 1.0 | |
| F | U 5-I (FIAU) | 0.42 | 0.9 | |
| F | U 5-(2-thienyl) | | 0.5 | |
| OH | C (AraC) | ≤ 0.01 | ≤ 0.01 | 1.2 |
| F | C | | | 1.1 |
| F | C 5-F | | | 1.1 |
| F | C 5-(2-thienyl) | ≤ 0.01 | 1.0 | 0.1 |

pounds is strongly dependent on the cell line and virus strain used in the assay. In vero cells, the concentrations needed for activity are 2–3 logs higher than in $E_6$SM cells (Vrang & Johansson, unpublished). When studied in the HSV-1 TK gene transfected tumor cells these compounds have been shown to inhibit thymidylate synthetase [52].

**Purine bases with 2'-deoxy- and arabinofuranosyl glycons**

Cytosolic dCK is the prime salvage enzyme employed for formation of natural 2'-deoxyribofuranosyl purine nucleoside monophosphates.

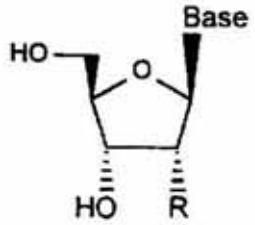
The relative capacity of dCK to synthesize these compounds is higher than it is for 2'-deoxycytidine. Analogs with base modifications, such as 7-deazaadenine and 2-chloroadenine, are also substrates (Table 11) [35, 36]. 2-Chloro-2'-deoxyadenosine (CdA, cladribine) is active against lymphoproliferative diseases, especially hairy-cell leukemia. The properties of CdA and other anti-tumour agents dependent on dCK for activation have recently been reviewed [13, 14].

The homologous CdA analog having a 3'-hydroxymethyl function is also a substrate for dCK. In contrast to pyrimidine analogs, where both dCK and TK1 accept analog substrates having a minor 3' modification like 3'-deoxy, fluoro or azido, this is not the case with dCK and purine 3'-modified 2'-deoxy sugars (except hydroxymethyl) as can be seen from Table 11.

Arabinofuranosyl adenine (AraA) is a substrate for dCK but with the other purine bases guanine (AraG) or hypoxanthine (AraHx) there is very little, or no phosphorylation at all with this enzyme.

dGK is similar to the other mitochondrial enzyme TK2 with regard to substrate properties (Table 11) [35]. It has a broad structural tolerance and accepts changes both in the base and sugar moieties, including arabinofuranosyl sugars but excluding analogs not having a 3'-hydroxyl function. Arabinofu-

Table 13
*Substrate specificities of TK1, TK2, dCK and dGK for 2'-substituted β-D-ribofuranosyl compounds*




| Substrate | | Relative activity | | | |
|---|---|---|---|---|---|
| R | Base | TK1 | TK2 | dCK | dGK |
| OH | C (Cyd) | ≤ 0.01 | ≤ 0.01 | 0.2 | |
| H | C (dCyd) | ≤ 0.01 | 0.9 | 1.0 | |
| $N_3$ | C | | | 0.2 | |
| F | C | ≤ 0.01 | 0.3 | 3.0 | |
| $OCH_3$ | C | | | 0.8 | |
| OH | G (Guo) | | | ≤ 0.01 | 0.1 |
| H | G (dGuo) | | | 1.0 | 1.0 |
| $=CH_2$ | G | | | ≤ 0.01 | 0.4 |
| $N_3$ | G | | | | 0.8 |
| Cyclopropyl | G | | | ≤ 0.01 | 4 |
| Cyclopropyl | Hx | | | ≤ 0.01 | 0.7 |

ranosylguanine and -hypoxanthine are good substrates for this enzyme. It also phosphorylates carbocyclic 2'-deoxyguanosine.

**Arabino- and ribofuranosyl glycon analogs**

Some arabinofuranosyl analogs with both natural and 5-substituted pyrimidine bases are listed in Table 12 [32, 36] and ribofuranosyl analogs with natural pyrimidine and purine bases are collected in Table 13 [35, 36]. Purine arabinofuranosyl compounds are found in Table 11 [35, 36].

TK2 phosphorylates most of arabinofuranosyl pyrimidine analogs, except AraC. Arabino glycons with 2'-hydroxy or 2'-fluoro, as well as a wide range of 5-substituents on the pyrimidine base are accepted.

2'-Fluoroarabino cytidine analogs with no 5-substituent or a 5-halogen substituent (F) are phosphorylated by dCK. Their substrate properties against TK2 remain to be determined. With a heteroaryl substituent in the 5-position this class of compounds is no longer phosphorylated by dCK, but they become efficient substrates for TK2, as in the case of the 5-heteroaryl substituted 2'-deoxycytidine analogs in Tables 6 and 8. The 2'-fluoro, arabino, glycon compounds are better substrates for TK2 than their parent arabino- or 2'-deoxyribofuranosyl counterparts.

FIAU (fialuridine) 1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-5-iodouracil and FMAU, its thymine analog are both good substrates for TK1 as well as for TK2 as shown in Table 12

Table 14
*Phosphorylation by high $K_m$ 5'-nucleotidase*

| Substrate | Relative velocity | | |
|---|---|---|---|
| | $K_m$ (mM) | % of Ino | Ref. |
| Ino | 3.4–5 | 100 | 67–70 |
| Thiazofurin | | 18 | 70, 71 |
| 3-DeazaGuo | | 10 | 70 |
| ddI | 0.5 | 2 | 66, 68 |
| ddG | 0.9 | 1 | 68 |
| (–)Carbovir | 6.1 | 53 | 69 |
| (+)Carbovir | | <0.02 | 69 |
| Acyclovir | 90 | 0.7 | 67 |

[55a]. The results of an early study [55b] with only partially purified enzyme and one high concentration of the compounds can be interpreted as inefficient phosphorylation of FIAU and FMAU by TK1 and TK2. FIAU and FMAU have potent activities against hepatitis B virus *in vitro* and *in vivo* [56–58]. FIAU was tested in 15 patients with chronic hepatitis B but unanticipated liver toxicity was observed and five of the patients died [59]. The multiorgan toxicity of FIAU suggests a mitochondrial dysfunction, although it does not appear to affect mitochondrial DNA replication [60].

Our data show that FIAU, and its metabolite FMAU can be phosphorylated both in proliferating cells by TK1 and in resting tissue by TK2. It is likely that the observed mitochondrial toxicity is related to phosphorylation by TK2.

The natural ribofuranosyl nucleoside compounds are at best poor substrates for the salvage kinase enzymes (Tables 1 and 2). However, with other functional groups replacing the 2'-hydroxyl function, cytidine analogs become good substrates for dCK, and guanosine and inosine for dGK as shown in Table 13 [35, 36].

### 5'-NUCLEOTIDASE

5'-Nucleotidase hydrolyzes the 5'-phosphate ester bond in nucleoside monophosphates. Many different 5'-nucleotidases have been described, and they may be categorized in three major classes as serum, ecto or cytosolic 5'-nucleotidases (reviewed in [5]). Their important function is to serve as regulators of the intracellular deoxyribonucleotide metabolism, helping to maintain a balanced pool of nucleotides. In this feed-back regulation they are stimulated by high nucleoside monophosphate levels [61–63]. An important enzyme in the context of this review is high $K_m$ 5'-nucleotidase [64]. The enzyme prefers deoxy-IMP and deoxy GMP as substrates in its nucleotidase capacity. The $K_m$ values are high, about 300 μM. The enzyme is present in different cell types and does not seem to be dependent on cell cycle regulation. [63, 64]. The enzyme has also been denoted IMP-GMP 5'-nucleotidase and a recent review has been published [65].

High $K_m$ 5'-nucleotidase can serve as a phosphotransferase, phosphorylating primarily purine nucleoside analogs, with IMP as phosphate donor. 2'-Deoxyadenosine [66] and 2'-deoxyguanosine [67] are also phosphorylated by the enzyme but $K_m$ is high (13 mM) for dGuo and $V_{max}$ very low. The enzyme is probably not important in the anabolism of natural nucleoside analogs. Table 14 lists some nucleoside analogs which are phosphorylated by this enzyme. 2',3'-Dideoxyinosine (ddI), which is approved for clinical treatment of HIV/AIDS, is a substrate [66, 68] as well as 2',3'-dideoxyguanosine [68].

The (–)enantiomer of carbovir, which has anti-HIV activity, but not (+) carbovir is phosphorylated by cellular enzymes [69]. Thiazofurin [70, 71] and 3-deazaguanosine [70] which both have antitumour activities in experimental systems are substrates (reviewed in [13]). Acyclovir is a weak substrate [67] and apparently this does not interfere with its properties as a highly selective anti-herpes agent.

### PROTEIN KINASES AND OTHER ENZYMES PHOSPHORYLATING NUCLEOSIDE ANALOGS

The nucleoside analog ganciclovir, GCV [9-(1,3-dihydroxy-2-propoxymethyl)guanine] is a potent inhibitor of human herpes viruses and is an approved agent for treatment of human cytomegalovirus (HCMV) infections (reviewed in [18, 24]). However, unlike herpes simplex virus or varicella zoster virus (reviewed in [17, 18]), HCMV does not encode a thymidine kinase. Accordingly ACV which is active against these latter viruses has no anti-HCMV effect.

It has recently been shown that GCV is phosphorylated by a protein kinase homolog encoded by the HCMV UL97 gene, and resistance to GCV is associated with specific mutations in UL97 [72–74].

This striking finding is the first example of nucleoside phosphorylation carried out by a protein kinase. Many different viruses encode proteins related to cellular serine or threonine protein kinases and also other types of kinases. Other viruses may activate cellular protein kinases. Viral protein kinases and phosphatases have recently been reviewed [75]. The example set by GCV has pinpointed new targets for antiviral chemotherapy and this field is now beginning to develop [76].

In addition to these protein kinases, and to the other kinases and cytosolic 5'-nucleotidase previously discussed, other enzymes have the capacity to phosphorylate nucleoside analogs. 3-Deazaguanosine [77] and tiazofurin [70] are phosphorylated by nicotinamide ribonucleoside kinase, in addition to the 5'-nucleotidase described in the previous section. The adenosine kinase (see the first chapter) mediated phosphorylation of tiazofurin has also been described [71].

## CONCLUSIONS

The results from the foregoing Tables and discussions are summarized in Table 15. The cytosolic salvage enzymes TK1 and dCK phosphorylate thymine and 2'-deoxycytidine, respectively. dCK also phosphorylates 2'-deoxyadenosine and -guanosine.

The two enzymes tolerate some minor changes in the 5-position of the pyrimidine base and/or in the 3'-position of 2'-deoxyribose sugars. dCK accepts 2-halogen substituted adenine bases, and a broad range of cytidine glycon analogs.

The mitochondrial enzymes TK2 and dGK phosphorylate pyrimidine and purine nucleosides and nucleoside analogs, respectively. As long as the glycon has a 3'-hydroxyl function various deoxyribose, ribose and arabinofuranosyl sugar containing nucleoside analogs are phosphorylated by both enzymes. TK2, which has been most extensively studied, phosphorylates uracil and cytosine derived nucleoside analogs containing a large variety of 5-substituents. Studies with dGK are so far limited and indicate a tolerance of the enzyme for at least some substituents or changes in the adenine base.

The recent discovery of a protein kinase phosphorylating a nucleoside analog has added a new dimension to this important class of phosphorylating enzymes and focused interest on new targets for antiviral chemotherapy.

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In contacts and discussions extending over many years, Professor David Shugar has often drawn the attention to the great importance of phosphates and phosphate transfer reactions in molecular biology and biochemistry.

We are indebted to Professors Salo Gronowitz and Bertil Samuelsson and their coworkers named in publications from their laboratories, for productive collaboration on the chemistry of nucleoside analogs (NGJ). We are also indebted to coworkers at Medivir, to Drs Christer Sahlberg and Xiao-Xiong Zhou for synthesis of some compounds in this review, to professor Bo Oberg for discussions, to Mr Jussi Kangasmetsae for making all the data and structures available in a computer database, to Mr Hong Zhang for help in preparing the Tables, to Mr Iain Morrison for improving the English lan-

Table 15
*Substrate specificities of the cytosolic salvage enzymes*

| | TK1 | TK2 | dCK | dGK |
|---|---|---|---|---|
| Base | T | T | C-5 subst. (limited) | G |
| | U-5 subst. (limited) | U-5 subst. (liberal) | A and 2-haloA | A and some 2-and 7 subst. |
| | | C-5 subst. (liberal) | G | Hx |
| Sugar | d-ribo 3'-subst. (limited) | Broad toleration with all bases. | Broad specificity for C base with 2'-and 3'-subst. | 3'-OH and 2'-subst. |
| | | 3'-OH and 2'-subst. | 2'-and 3'-subst. | |
| | | deoxyribose | deoxyribose | deoxyribose |
| | | ribose | ribose | ribose |
| | | arabinofuranosyl | arabinofuranosyl acyclic | arabinofuranosyl |

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