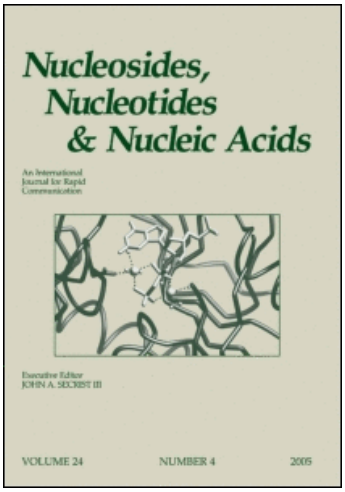

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Substrate/Inhibitor Properties of Human Deoxycytidine Kinase (dCK) and Thymidine Kinases (Tk1 And Tk2) Towards the Sugar Moiety of Nucleosides, Including O'-Alkyl Analogues

Borys Kierdaszuk[a]; Krzysztof Krawiec[a]; Zygmunt Kazimierczuk[ab]; Ulla Jacobsson[c]; Nils G. Johansson[d]; Birgitte Munch-petersen[e]; Staffan Eriksson[f]; David Shugar[ag]
[a] Department of Biophysics, University of Warsaw, Institute of Experimental Physics, Warsaw, Poland [b] Agricultural University, Institute of Chemistry, Warsaw [c] Department of Organic Chemistry, Royal Institute of Technology, Stockholm, Sweden [d] Medivir AB, Huddinge, Sweden [e] Department of Life Sciences and Chemistry, University of Roskilde, Roskilde, Denmark [f] Department of Veterinary Medical Chemistry, Biomedical Centre, Swedish University of Agricultural Sciences, Uppsala, Sweden [g] Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland

To cite this Article Kierdaszuk, Borys , Krawiec, Krzysztof , Kazimierczuk, Zygmunt , Jacobsson, Ulla , Johansson, Nils G. , Munch-petersen, Birgitte , Eriksson, Staffan and Shugar, David(1999) 'Substrate/Inhibitor Properties of Human Deoxycytidine Kinase (dCK) and Thymidine Kinases (Tk1 And Tk2) Towards the Sugar Moiety of Nucleosides, Including O'-Alkyl Analogues', Nucleosides, Nucleotides and Nucleic Acids, 18: 8, 1883 — 1903
To link to this Article: DOI: 10.1080/07328319908044850
URL: http://dx.doi.org/10.1080/07328319908044850

NUCLEOSIDES & NUCLEOTIDES, 18(8), 1883–1903 (1999)

SUBSTRATE/INHIBITOR PROPERTIES OF HUMAN DEOXYCYTIDINE KINASE (dCK) AND THYMIDINE KINASES (TK1 AND TK2) TOWARDS THE SUGAR MOIETY OF NUCLEOSIDES, INCLUDING O′-ALKYL ANALOGUES

Borys Kierdaszuk [a,*], Krzysztof Krawiec [a], Zygmunt Kazimierczuk [a,b], Ulla Jacobsson [c], Nils G. Johansson [d], Birgitte Munch-Petersen [e], Staffan Eriksson [f] and David Shugar [a,g]

[a] University of Warsaw, Institute of Experimental Physics, Department of Biophysics, 93 Żwirki i Wigury St., PL-02089 Warsaw, Poland; [b] Agricultural University, Institute of Chemistry, 26/30 Rakowiecka St., PL-02528 Warsaw; [c] Department of Organic Chemistry, Royal Institute of Technology, S-100 44 Stockholm, Sweden; [d] Medivir AB, Lunastigen 7, S-14144 Huddinge, Sweden; [e] University of Roskilde, Department of Life Sciences and Chemistry, DK-4000 Roskilde, Denmark; [f] Swedish University of Agricultural Sciences, Department of Veterinary Medical Chemistry, Biomedical Centre, S-75123 Uppsala, Sweden; [g] Institute of Biochemistry and Biophysics, Polish Academy of Sciences, 5a Pawińskiego St., PL-02106 Warsaw, Poland.

ABSTRACT: Nucleoside analogues with modified sugar moieties have been examined for their substrate/inhibitor specificities towards highly purified deoxycytidine kinase (dCK) and thymidine kinases (tetrameric high-affinity form of TK1, and TK2) from human leukemic spleen. In particular, the analogues included the mono- and di-O′-methyl derivatives of dC, dU and dA, syntheses of which are described. In general, purine nucleosides with modified sugar rings were feebler substrates than the corresponding cytosine analogues. Sugar-modified analogues of dU were also relatively poor substrates of TK1 and TK2, but were reasonably good inhibitors, with generally lower K_i values *vs* TK2 than TK1. An excellent discriminator between TK1 and TK2 was 3′-hexanoylamino-2′,3′-dideoxythymidine, with a K_i of ~600 μM for TK1 and ~0.1 μM for TK2. 3′-OMe-dC was a superior inhibitor of dCK to its 5′-O-methyl congener, consistent with possible participation of the oxygen of the (3′)-OH or (3′)-OMe as proton acceptor in hydrogen bonding with the enzyme. Surprisingly α-dT was a good substrate of both TK1 and TK2, with K_i values of 120 and 30 μM for TK1 and TK2, respectively; and a 3′-branched α-L-deoxycytidine analogue proved to be as good a substrate as its α-D- counterpart. Several 5′-substituted analogues of dC were

* Corresponding author: Fax: (+48-22)-8220248; e-mail: borys@asp.biogeo.uw.edu.pl

good non-substrate inhibitors of dCK and, to a lesser extent, of TK2. Finally, some ribonucleosides are substrates of the foregoing enzymes; in particular C is a good substrate of dCK, and 2′-OMe-C is an even better substrate than dC.

ABBREVIATIONS: dCK, deoxycytidine kinase; TK1 and TK2, cytoplasmic and mitochondrial thymidine kinases; dGK, deoxyguanosine kinase; N, ribonucleoside; dN, 2'-deoxyribonucleoside; (d)NMP, (d)NDP, (d)NTP, (deoxy)ribonucleoside 5'-monophosphate, -diphosphate, -triphosphate; ddN, 2',3'-dideoxyribonucleoside; ara-N, arabinofuranosides of pyrimidine or purine nucleosides; 2′-OMe-(d)N, 2′-O-methyl-(deoxy)ribonucleoside and related O′-methylated (deoxy)ribonucleosides; ACV, acyclovir, 9-[(2-hydroxyethoxy)methyl]guanine; DHBG, 9-(3,4-dihydroxybutyl)-guanine; DHPG, 9-(1,3-dihydroxy-2-propoxymethyl)guanine; cytallene, 1-(4-hydroxy-1,2-butadienyl)cytosine; adenallene, 9-(4-hydroxy-1,2-butadienyl)adenine; TGdR, 6-thioguanine-2′-deoxyriboside; BSA, bovine serum albumine; HSV, herpes simplex virus; VV, vaccinia virus; VZV, varicella zoster virus; HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome; TLC, thin layer chromatography; HPLC, high pressure liquid chromatography; NMR, nuclear magnetic resonance.

INTRODUCTION

Deoxycytidine kinase (dCK, EC 2:7.1.74), which catalyzes phosphorylation of dC to dCMP in the presence of a nucleoside 5'-triphosphate donor (NTP or dNTP), has been purified to apparent homogeneity from various sources, including human leukemic spleen[1] and leukemic human T-lymphoblasts.[2] It's widely assumed designation as a cytosolic enzyme has, however, been questioned by Johansson et al.,[3] who cloned and expressed the cDNA of the human enzyme and adduced evidence for its localization in the nucleus.

Thymidine kinases (TK, EC 2:7.1.21), which catalyze the phosphorylation of dT to dTMP, exist in eukaryotic cells as TK1, considered a cytosolic enzyme, and TK2, predominantly mitochondrial, although questioned by Johansson et al.[3] TK1 has been purified to apparent homogeneity from HeLa cells[4] and human leukemic spleen,[5] and its cell-cycle regulation studied.[6-8] TK1 can exist in two different forms: a tetrameric high-affinity form with high affinity *vs* dT, and a low-affinity dimeric form.[9] TK2 has been purified to the highest degree from human leukemic spleen,[5] and dCK,[10] TK1[6] and TK2[11] have also been obtained by cloning.

All three enzymes exhibit broad substrate and inhibitor specificities,[5,12,13] and dCK also phosphorylates purine deoxyribonucleosides and some nucleoside analogues. In

particular, the substrate specificity of TK2 partially overlaps that of dCK, consistent with the observed conservation of primary structure motifs between the two enzymes.[11]

During the course of an investigation of the substrate specificities, and potential inhibitors, of human spleen dCK,[14-17] it was found that 5′-OMe-dC is a moderate, and 5′-OEt-dC a more effective, inhibitor of dCK.[18] This prompted us to synthesize and examine the analogous properties of the mono- and di- O′-methyl derivatives of dC, dU and dA, and to extend this investigation to a variety of other nucleosides with modified sugar moieties.

EXPERIMENTAL

Materials

[5-^{3}H]-2'-deoxycytidine (19.3 Ci/mmol), [6-^{3}H]-2'-deoxythymidine (29 Ci/mmol) and [γ-^{32}P]-ATP (~3000 Ci/mmol) were from Amersham, and [2,8-^{3}H]-2'-deoxyadenosine (46 Ci/mmol) from Moravek Biochemicals, Inc. (Brea, California). Unmodified nucleosides (C, U, A, dC, dU, dT, dA, dG), ddC, ddU, ddT, ara-C, ara-U, ara-T, ara-A, ara-G, 3′-deoxy-C, 2′-OMe-C, 2′-fluoro-dC, 2′-azido-dC, 3′-OMe-C, 3′-O-acetyl-dC, 2′,3′-O-isopropylidene-C, 2-chloro-dA, 2′-OMe-A, 3′-O-acetyl-dA, 2′-chloro-dU, 2′-azido-ddU, 3′-azido-ddT, thymine α-D-2′-deoxyriboside (α-dT), ATP and BSA (fraction V, for enzyme stabilization) were obtained from Sigma. 2′,3′-Didehydro-2′,3′-ddC was a gift from Dr. J. Balzarini (Rega Institute, Leuven, Belgium), 1-(4-hydroxy-1,2-butadienyl)-cytosine (cytallene) and 2′-fluoro-2′,3′-dideoxy-ara-A from Dr. D. Johns (NCI, Bethesda, MD, U.S.A.), and 2′,2′-difluoro-dU and 2′,2′-difluoro-dG are from Eli Lilly (Indianapolis, USA). Thymine riboside (T), 2′-fluoro-2′-deoxy-ara-C, 3′-O-acetyl-dC, 3′-fluoro-ddC, 2′-methylene-dT, 3′-fluoro-ddT and 3′-acetylene-ddT were products of Medivir AB. Syntheses of the 3′-branched hydroxymethyl analogues of dC and 2-chloro-dA have been described elsewhere.[19,20] 3'-Hexanoylamino-dT was prepared as described.[21] O′-alkylated analogues of dC, dU and dA were synthesized as described below. All compounds were checked for purity by TLC, HPLC, and UV and NMR spectroscopy.

Enzymes

All three enzymes were highly purified to apparent homogeneity from human

leukemic spleen: dCK 6 000-fold to a specific activity of 260 nmols dCMP formed per min per mg,[1,14] TK1 (low affinity form)[9] 20 000-fold to a specific activity of 9.5 μmols dTMP formed per min per mg, TK2 approximately 20 000-fold to a specific activity of 0.5 μmols dTMP formed per min per mg.[5] Their subunit molecular weights are 30, 25 and 29 kD,[14,5] respectively. The tetrameric high-affinity form of TK1 (ATP-form) was obtained by incubation with ATP,[9] referred to below simply as TK1.

Enzyme assays

Activity of dCK was routinely followed by a radiochemical procedure described originally by Ives and Wang[22] with modifications reported by Kierdaszuk and Eriksson.[14] Activity of thymidine kinases was measured as described by Munch-Petersen.[23] K_i values for competitive inhibition were evaluated from Dixon plots.

The phosphate transfer assay was performed at 37°C in a total volume of 50 μl containing 50 mM Tris-HCl, pH 7.6, 100 μM [γ-^{32}P]ATP (0.7 mCi/mmol), 1 mM $MgCl_2$, 0.1 M KCl, 10-80 ng of pure dCK, TK1 or TK2 and 0.5 mg/ml BSA. Background reactions were performed without the phosphate acceptor. The reaction was terminated after 30 min by boiling for one min, the mixture centrifuged, and 2-4 μl of supernatant applied to a PEI-cellulose F (Merck) thin-layer plate. Chromatography was performed for 8-12 hours using isobutyric acid: 25 % NH_4OH : H_2O (66:1:33, v/v) as the mobile phase. The reaction products were detected by autoradiography, the spots of [γ-^{32}P]-ATP and ^{32}P-labelled nucleotide product excised and eluted with 0.5 ml of 0.2 M KCl/0.1 M HCl (1:1, v/v) and quantitated by liquid scintillation. Fragments with the same R_f and area as the product were excised from the background and subtracted. The amount of enzyme used for a single reaction permitted linear assay for approximately 1 hour, and direct comparison of the amount of product formed during 30 min. The sensitivity of this transfer assay was in the same range as for the usual assay with radiolabelled nucleoside substrates.

SYNTHESIS OF O'-METHYL 2'-DEOXYRIBONUCLEOSIDES

It was long ago shown that dimethylsulphate treatment of ribosides, arabinosides, xylosides and lyxosides of cytosine and adenine in strongly alkaline medium, where the

sugar hydroxyls undergo ionization, led to methylation of the sugar hydroxyls, whereas the base moieties are relatively resistant.[24-26] The use of diethylsulphate gave the corresponding O′-ethyl nucleosides. Attention was directed at that time to the fact that the same procedure was applicable to 2′-deoxyribonucleosides, as well as to nucleoside 5′-phosphates. Actually analogous procedures had earlier been extensively employed for preparation of methylated carbohydrates for studies on linkage analysis in oligo- and polysaccharides, extensively reviewed recently by Jay.[27]

Apparently unaware of the foregoing, several reports have described the preparation of 2,4-diketopyrimidine 3′-O-alkyl-2′-deoxyribonucleosides *via* the more tedious use of protecting groups for the pyrimidine ring N(3) and/or the sugar 5′-hydroxyl.[28-31] The most recent and extensive is that of Cramer and Pfleiderer[32] on both protected and unprotected ribonucleosides with the use of diazomethane and stannous chloride as catalyst, the mechanism of which had earlier been elucidated by Dudycz et al.[33]

General procedures

Chromatographic separation of O'-methyl-2'-deoxyribonucleosides was achieved with a Gin-Dekker column,[34] the order of elution being similar to that for ribonucleosides,[24-26] taking into account the absence of the 2'-hydroxyl group. Thus the first eluted product is 3',5'-di-O-methyl-, followed by 3'-O-methyl- and 5'-O-methyl-nucleosides. The O'-methyl derivatives of dC were then deaminated in aqueous $NaHSO_3$[35,36] to give the corresponding dU derivatives.

The differences in stability of pyrimidine and purine deoxyribonucleosides in acidic medium led to two possible desalting procedures. Desalting of the methylation products was performed using the method applied to ribonucleosides, i.e. cation exchange resin (Dowex 50 W, H^+-form). Because of their lability in acid medium, the O'-methylated derivatives of dA were isolated by continuous extraction with ethyl acetate from the neutralized reaction mixtures.

Melting points (uncorr.) were measured with the aid of a Boetius hot stage microscope. Elementary analyses were performed by the Institute of Organic Chemistry (Polish Academy of Sciences, Warsaw). The fractions obtained from chromatographic separations of O'-methylated nucleosides were monitored by TLC using Merck silica

gel 60 F_{254} plates (Darmstad, Germany) with chloroform-methanol (9:1, v/v) as eluent. UV spectra were run on a Zeiss VS-2 instrument (Jena, Germany) and Kontron (Switzerland) UVIKON 922. ^{1}H and ^{13}C NMR spectra, as well as distortionless enhancement by polarization transfer (DEPT), were recorded on a Bruker AM400 spectrometer in DMSO-d_6 relative to TMS.

Methylation of 2'-deoxycytidine

To a vigorusly stirred solution of 2'-deoxycytidine monohydrate (2.8 g, 11.4 mmol) in 80 ml 2 N KOH was added, stepwise, 10 ml (105 mmol) dimethyl sulphate and 20 ml 10 N KOH, at room temperature. The reaction mixture was diluted with water to 250 ml, and percolated through a 40 x 4,5 cm column of Dowex(H^+) 50W (200-400 mesh). The column was washed with water until the eluate was neutral, and products then eluted with 10% NH_4OH. The eluates were concentrated to small volume and deposited on a 75 x 4 cm column of Dowex (OH^-) 1 x 8 (200-400 mesh). Elution was then performed with water (1.5 l), 95% methanol (1.5 l) and 90% methanol (1.5 l), with collection of 100-ml fractions, and monitoring by UV absorption and TLC. Appropriate fractions were brought to dryness and residues subjected to crystallization as follows:

3',5'-di-O-methyl-2'-deoxycytidine: The residue from fractions 6-11 readily crystallized from water to yield 330 mg (11%) as needles, m.p. 186-188 ^{o}C; R_f 0.36; Anal. (calc. for $C_{11}H_{17}N_3O_4$): C, 51.56 (51.75); H, 6.78 (6.71); N, 16.26 (16.46). UV (pH 1): λ_{max} 279 nm (ε 12500), UV (pH 7): λ_{max} 271 nm (ε 9400).

3'-O-methyl-2'-deoxycytidine: The residue from fractions 13-17 was crystallized from acetone-ethyl acetate to yield 340 mg (12%) as needles, m.p. 174-175 ^{o}C; R_f 0.16; Anal. (calc. for $C_{10}H_{15}N_3O_4$): C, 49.95 (49.78); H, 6.30 (6.26); N, 17.31 (17.42). UV (pH 1): λ_{max} 279 nm (ε 12600), UV (pH 7): λ_{max} 271 nm (ε 9400).

5'-O-methyl-2'-deoxycytidine: The residue from fractions 26-31 was crystallized from acetone to yield 270 mg (10%) as needles, m.p. 179 ^{o}C; R_f 0.19; Anal. (calc. for $C_{10}H_{15}N_3O_4$): C, 49.71 (49.78); H, 6.30 (6.26); N, 17.30 (17.42). UV (pH 1): λ_{max} 279 nm (ε 12400), UV (pH 7): λ_{max} 271 nm (ε 9200).

Unreacted ***2'-deoxycytidine*** (fractions 31-42) eluted as the fourth compound. Total yield of O'-methyl products in crystalline form, 33%.

Deamination to O'-methyl 2'-deoxyuridines

***3',5'-Di-O-methyl-2'-deoxyuridine*:** The stirred solution of 3',5'-di-O-methyl-2'-deoxycytidine (305 mg, 1.2 mmol) in 7 ml 2.5 N $NaHSO_3$ was stored for 2 days at 37 °C. The reaction mixture was adjusted to pH 10 with 10 N NaOH and stirred for 5 hours at room temperature. The solution was then neutralized and applied on a 28 x 2.5-cm column of XAD-4 (200-400 mesh). Elution was performed using a gradient of water-60% isopropanol in water (1.0 l). The fractions containing the nucleoside were brought to dryness and the residue crystallized from a small amount of water to yield 205 mg (67%) of plates; m.p. 89-92 °C; R_f 0.58; Anal. (calc. for $C_{11}H_{16}N_2O_5$): C, 54.70 (54.92); H, 7.03 (7.09); N, 9.96 (9.85). UV (pH 7): λ_{max} 262 nm (ε 9900), UV (pH 12): λ_{max} 262 nm (ε 6600).

3'-O-methyl-2'-deoxyuridine: A solution of 3'-O-methyl-2'-deoxycytidine (360 mg, 1.5 mmol) in 10 mL (11.4 mmol) 2.5 N $NaHSO_3$ was stored for 2 days at 37°C and then brought to pH 10 with 1 N $Ba(OH)_2$. Barium salts were filtered off and the filtrate evaporated to dryness. Crystallization of the residue from water yielded 3'-O-methyl-2'-deoxyuridine (290 mg, 80 %) as platelets, m.p. 164-166 °C; R_f 0.25; Anal. (calc. for $C_{10}H_{14}N_2O_5$): C, 49.45 (49.58); H, 5.81 (5.83); N, 11.44 (11.56). UV (pH 7): λ_{max} 262 nm (ε 10200), UV (pH 12): λ_{max} 261 nm (ε 7000).

***5'-O-Methyl-2'-deoxyuridine*:** Application of the foregoing procedure to 5'-O-methyl-2'-deoxycytidine gave, after crystallization from water, 276 mg (76%) as needles, m.p. 114-116 °C; R_f 0.25; Anal. (calc. for $C_{10}H_{14}N_2O_5$): C, 49.47 (49.58); H, 5.75 (5.83); N, 11.41 (11.56). UV (pH 7): λ_{max} 262 nm (ε 9900), UV (pH 12): λ_{max} 261 nm (ε 6700).

^{1}H chemical shifts for the O'-methylated congeners of dU and dC (± for the last digits): 11.2(1) (*s*, H-N(3) of dU analogues); 7.84(1) (*d*, $J_{5,6}$ = 8.1 Hz, H-C(6) of 3'-OMe-dU); 7.77(1) (*d*, $J_{5,6}$ = 8.1 Hz, H-C(6) of 3'-OMe-dC); 7.65(3) (*d*, $J_{5,6}$ = 8.1 Hz, H-C(6) of 5'-OMe- and 3',5'-diOMe- analogues); 7.17(2) and 7.12(2) (2*s*, NH_2 of dC analogues); 6.12(4) (*m*, H-1'); 5.65(2) and 5.73(1) (*d*, $J_{5,6}$ = 8.1 Hz, H-C(5) of dU and dC analogues, respectively); 5.29(2) (*d*, $J_{3',3'OH}$ = 4.2 Hz, 3'-OH); 5.06(2) (*m*, 5'-OH); 4.05(13) (*m*, H-3'); 3.56(1) (*m*, H-4' of 3'-OMe- analogues); 3.88(4) (*m*, H-4' of 5'-OMe- and 3',5'-diOMe- analogues); 3.51(2) and 3.46(1) (2*m*, H-5' and H5",

respectively); 3.31(2) (*s*, CH_3 of 3′-OMe- and 5′-OMe- analogues); 3.26(1) and 3.31(1) (2*s*, CH_3 groups of 3′,5′-diOMe- analogues); 1.94(1) and 2.37(13) (*m*, H-2′ and H-2″ of dC analogues, respectively); 2.06(4) and 2.17(8) (*m*, H-2′ and H-2″ of dU analogues, respectively).

^{13}C chemical shifts: 165.5(1) and 163.0(1) (C(4) of dC and dU analogues, respectively); 155.0(1) and 150.3(1) (C(2) of dC and dU analogues, respectively); 140.6(2) and 140.2(1) (C(6) of dC and dU analogues, respectively); 94.0(1) and 101.9(1) (C(5) of dC and dU analogues, respectively); 84.7(5) (C-4′); 84.4(4), (C-1′ of 3′-OMe- and 5′-OMe- analogues); 82.3(1) (C-1′ of 3′,5′-diOMe- analogues); 80.6(5) (C-3′ of 3′-OMe- and 3′,5′-diOMe- analogues); 70.5(1) (C-3′ of 5′-OMe- analogues); 72.5(1) (C-5′ of 5′-OMe- and 3′,5′-diOMe- analogues); 61.5(1) (C-5′ of 3′-OMe- analogues); 58.5(1) (5′-CH_3); 56.0(1) (3′-CH_3); 39.5(4) (C-2′ of 5′-OMe- analogues); 36.2(5) (C-2′ of 3′-OMe- and 3′,5′-diOMe- analogues).

Methylation of 2'-deoxyadenosine

A vigorously stirred solution of 2'-deoxyadenosine (4.05 g, 15 mmol) in 70 ml 2 N KOH was treated stepwise with dimethyl sulphate (10 ml, 105.5 mmol) and 20 ml 10 N KOH over a period of 6 hours at room temperature. The reaction mixture was neutralized with 1N HCl and continuously extracted with ethyl acetate. The ethyl acetate extracts were evaporated to an oil, which was dissolved in 40 ml water. The solution was applied on an 85 X 4 cm column of Dowex 1 x 8 (OH^-; 200-400 mesh, and eluted with water (2.0 l) and water-methanol (85:15, 3.0 l), with collection of 100-ml fractions. The fractions were monitored using TLC. Total yield of products, 34 %. Appropriate fractions were then brought to dryness and residues subjected to crystallization as follows:

***3',5'-di-O-methyl-2'-deoxyadenosine*:** The residue from fractions 8-13 was crystallized from water to yield 520 mg (12.5%) as needles, m.p. 174-175 ^oC; R_f 0.53; Anal. (calc. for $C_{12}H_{17}N_5O_3$): C, 51.48 (51.60); H, 6.12 (6.14); N, 25.15 (25.08). UV (pH 1): λ_{max} 257 nm (ε 13900), UV (pH 7): λ_{max} 260 nm (ε 14600).

3'-O-methyl-2'-deoxyadenosine: The residue from fractions 14-19 was crystallized from acetone/ethyl acetate to yield 460 mg (11.5%) as needles, m.p. 101-103 ^oC; R_f

0.31; Anal. (calc. for $C_{11}H_{15}N_5O_3$): C, 49.69 (49.81); H, 5.62 (5.70); N, 26.53 (26.40). UV (pH 1): λ_{max} 275.5 nm (ε 13800), UV (pH 7): λ_{max} 259.5 nm (ε 14900).

5'-O-methyl-2'-deoxyadenosine: The residue from fractions 28-33 was crystallized from ethyl acetate to yield 390 mg (10%) in the form of needles, m.p. 178-180 ^{o}C; R_f 0.40; Anal. (calc. for $C_{11}H_{15}N_5O_3$): C, 49.65 (49.81); H, 5.61 (5.70); N, 26.33 (26.40). UV (pH 1): λ_{max} 257 nm (ε 13700), UV (pH 7): λ_{max} 260 nm (ε 14400).

^{1}H chemical shifts for O'-methylated analogues of 2'-deoxyadenosine (± for the last digits): 8.29(3) (*s*, H-8); 8.15(2) (*s*, H-2); 6.30(5) (*m*, H-1'); 5.39(1) (*d*, $J_{3',3'OH}$ = 2.2 Hz, 3'-OH); 5.36(1) (*m*, 5'-OH); 4.25(15) (*m*, H-3'); 4.03(8) (*m*, H-4'); 3.52(2) and 3.47(1) (2*m*, H-5'and H5", respectively); 3.29(2) (*s*, CH_3 of 3'-OMe-dA and 5'-OMe-dA); 3.28(1) and 3.31(1) (2*s*, CH_3 groups of 3',5'-diOMe-dA); 2.40(10) and 2.83(3) (*m*, H-2' and H-2", respectively).

^{13}C chemical shifts: 152.4(1) (C-2); 156.0(1) (C-6); 149.0(2) (C-4); 139.3(2) (C-8); 119.1(1) (C-5); 85.1(2) (C-4' of 3'-OMe-dA and 5'-OMe-dA); 83.3(1) (C-4' of 3',5'-diOMe-dA); 84.1(1), 83.0(1) and 82.5(1) (C-1' of 3'-OMe-dA, 5'-OMe-dA and 3',5'-diOMe-dA, respectively); 81.2(1) (C-3' of 3'-OMe-dA and 3',5'-diOMe-dA); 70.9(1) (C-3' of 5'-OMe-dA); 72.5(1) (C-5' of 5'-OMe-dA and 3',5'-diOMe-dA); 62.0 (C-5' of 3'-OMe-dA); 58.4(1) (5'-CH_3); 56.0(1) (3'-CH_3); 38.7(1) (C-2' of 5'-OMe-dA); 35.4(2) (C-2' of 3'-OMe-dA and 3',5'-diOMe-dA).

Note that the change in chemical shift (downfield by about 10 ppm) of carbon atoms following methylation (from 70.7(2) to 80.6(5) ppm for C-3', and from 61.7(3) to 72.5(1) ppm for C-5') enables one to differentiate between a 3'-OCH_3 and a 5'-OCH_3.

RESULTS AND DISCUSSION

The relative activities of the enzymes with various nucleosides, often the rate-limiting step in the metabolic activation of nucleoside drugs to their active forms at the triphosphate level,[37] are grouped according to the base moiety, as shown in TABLE 1, which lists the properties of some typical classes of nucleoside analogues examined.(*)

(*) A more complete list of the compounds embraced in this study is available on request to the corresponding author

The enzymes are relatively specific for the pentose moiety of nucleoside substrates, although one cytosine acyclonucleoside, the antiviral (HIV and hepatitis B) 1-(4-hydroxy-1,2-butadienyl)-cytosine (cytallene) exhibited significant substrate activity with dCK, which is also able to phosphorylate cytosine nucleoside analogs with 2′- and 3′-modifications (2′-OMe-dC, 2′-fluoro-dC, 2′-fluoro-2′-deoxy-ara-C, 2′-azido-dC, 3′-deoxy-C, 3′-OMe-dC and 3′-O-acetyl-dC or 3′-fluoro-ddC), although they led to very low (if any) activity with TK2. Similar modifications of the sugar moiety of purine deoxyribonucleosides led to drastically decreased activity *vs* dCK. The dCK activity *vs* ddC was 3-fold lower, and was further reduced *vs* the 2′,3′-O-isopropylidene analogue of C (TABLE 1). Substrate activity of cytallene *vs* dCK is in striking contrast to lack of activity of acyclovir (ACV), which is selectively phosphorylated by herpes viruses (HSV-1, HSV-2, VZV) thymidine kinases.[38] Substrate activities of the various cytosine nucleosides *vs* dCK varied over a broad range, the best being 2′-fluoro-dC, followed by 2′-O-methyl-C, both superior to ara-C, etc. (TABLE 1). Modified purine nucleosides exhibited much lower substrate activities (ara-A > 2′-fluoro-2′,3′-dideoxy-ara-A = 2′-O-methyl-A > 3′-O-acetyl-dA), and 3′-deoxy-A (cordycepin) and 3′-deoxy-ara-A are not phosphorylated at all (data not shown).

Similar sugar-modified analogues of dU and dT were feeble substrates *vs* TK1 and TK2 (TABLE 1). But, surprisingly, α-dT was a remarkably good substrate for both enzymes, as well as an inhibitor, with K_i of 120 ± 20 and 30 ± 3 μM for TK1 and TK2, respectively. Several dU analogues, non-substrates of TK1 and only poor substrates of TK2, proved to be effective inhibitors which readily distinguished between these two enzymes, e.g. K_i values for 2′-chloro-dU *vs* TK1 and TK2 were >5 mM and 25 μM; for 3′-O-methyl-dU, 300 and 15 μM, and for 3′-O-ethyl-dU, 1.1 mM and 10 μM (TABLES 2 and 3). Some analogues of deoxyadenosine with a modified sugar ring (3′-deoxy-, 2′,3′-dideoxy-, 2′-tosyl-2′,3′-dideoxy-, 3′-O-methyl-2′-deoxy-, 3′-fluoro-2′,3′-di-deoxy-, 3′-azido-2′,3′-dideoxy-, 2′-azido-2′-deoxyxylose-), as well as of adenosine (2′,3′-di-O-acetyl-, 2′,3′-O-p-anisylidene-, 2′,3′-O-isopropylidene-, 2′-O-methyl-2′-xylose-), and DHPA, DHPG and adenallene, are inactive (data not shown). Similar modifications of guanosine also led to inactive compounds, but with exceptionally high activity for 2′,3′-difluoro-dG *vs* dCK (TABLE 1).

Among the 2′,3′-dideoxynucleosides, only ddC (TABLE 1), an anti-HIV compound, is phosphorylated by dCK (but not by TK2), albeit 3-fold less effectively than dC, consistent with previous results,[15] bearing in mind that V_{max}/K_m is an order of magnitude lower at the high concentrations of dC [14] (e.g. 100 μM) used for the data in TABLE 1, as compare to the low concentrations of dC. Both ddT and, to a lesser extent, ddU are substrates for TK1 but at much lower efficiency than that observed for dCK *vs* ddC (TABLE 1).

Among the 2′,3′-didehydro-2′,3′-dideoxyribonucleoside analogs, only 2′,3′-didehydro-2′,3′-dideoxycytidine (d4C) and its carbocyclic congeners were slightly phosphorylated by dCK, but not by TK2 (TABLE 1), while 2′,3′-didehydro-2′,3′-dideoxythymidine (d4T) was not phosphorylated by TK1 and TK2 (data not shown). This is in accord with previous results,[5] although Ho and Hitchcock[39] had earlier reported that dT4 is a feeble substrate of partially purified TK1. It remains to be clarified which enzyme(s) phosphorylate dT4, an approved agent for treatment of AIDS.

2′-Deoxyinosine (dI) exhibited 2- and 3-fold lower rates of phosphorylation with dCK than dG and dA, respectively. Its sugar-modified analogues, like ddI, 3′-fluoro-ddI, 3′-azido-ddI, I and ara-I, have no detectable substrate activity *vs* dCK (data not shown).

β-D-2′-Deoxyribonucleosides are more efficient substrates (TABLE 1) or inhibitors (TABLES 2-4) than the corresponding β-D-arabinonucleosides, including those with antitumor activity (ara-C, ara-U, ara-T, ara-A), and ribonucleosides (see also[12,13]), but not arabinonucleosides containing the purine bases guanine (ara-G) and hypoxanthine (ara-Hx). Amongst β-D-ribonucleosides of cytosine, thymine, uracil, adenine and guanine, only 5-azacytidine[12] and cytidine (TABLE 1, *cf.*[13]) exhibited substrate activity *vs* dCK. Furthermore, cytidine is a remarkably good substrate of highly purified leukemic spleen dCK (TABLE 1), as earlier reported with a partially purified enzyme from calf thymus[12] (see, also, third-last section, below).

3′-Branched analogues

The 3′-branched homologue of dC, 3′-hydroxymethyl-2′,3′-dideoxycytidine (3′-hydroxymethyl-ddC), is a better substrate of dCK than the parent ddC (TABLE 1),

TABLE 1. Relative phosphorylation of 100 μM nucleoside analogs by pure human dCK, TK1 and TK2 using 100 μM [γ-^{32}P]ATP as phosphate donor.[a]

Nucleoside analogs		Relative activity		
Base	Pentafuranose ring [b]	dCK	TK1	TK2
Cytosine	2′-deoxy	1.0	0	0.9
	3′-deoxy	0.13	0	0
	2′-OMe-ribose	1.9	n.d.	n.d.
	3′-OMe-ribose	0	n.d.	n.d.
	3′-OMe-2′-deoxy	0.6	0	0.06
	3′-O-acetyl-2′-deoxy	0.18	n.d.	n.d.
	3′-OEt-2′-deoxy	0.08	0	0
	2′-fluoro-2′-deoxy	3.0	0	0.3
	2′-fluoro-2′-deoxyarabinose	1.0	n.d.	n.d.
	3′-fluoro-2′,3′-dideoxy	0.6	0	0
	2′-azido-2′-deoxy	0.2	n.d.	n.d.
	2′,3′-dideoxy	0.3	0	0
	2′,3′-O-isopropylidene	0.09	n.d.	n.d.
	β-D-3'-hydroxymethyl-2', 3'-dideoxy	1.3	n.d.	0
	α-D-3'-hydroxymethyl-2', 3'-dideoxy	0.1	n.d.	n.d.
	β-L-3′-hydroxymethyl-2′,3′-dideoxy	0.4	n.d.	0.07
	α-L-3′-hydroxymethyl-2′,3′-dideoxy	0.09	n.d.	0
	β-D-3'-azidomethyl-2',3'-dideoxy	0.3	n.d.	0.02
	α-D-3'-azidomethyl-2',3'-dideoxy	0	0	0
	β-D-3'-fluoromethyl-2',3'-dideoxy	0.3	n.d.	0
	α-D-3'-fluoromethyl-2',3'-dideoxy	0	0	0
	Carbocyclic 3'-hydroxymethyl-2', 3'-dideoxy	0.02	n.d.	0
	2′,3′-didehydro-2′,3′-dideoxy	0.04	n.d.	0
	ribose	0.2	0	0
	arabinose	1.2	n.d.	0.05
	cytallene	0.2	n.d.	0
Adenine	2′-deoxy	3.5	0	0
	2′-OMe-ribose	0.05	n.d.	n.d.
	3′-O-acetyl-2′-deoxy	0.02	n.d.	n.d.
	2′-fluoro-2′,3′-dideoxyarabinose	0.07	0	0
	ribose	0	0	0
	arabinose	0.5	0	0
2-Cl-Adenine	2'-deoxy	2.6	n.d.	n.d.
	β-D-3'-hydroxymethyl-2', 3'-dideoxy	0.4	n.d.	n.d.
	α-D-3'-hydroxymethyl-2', 3'-dideoxy	0	n.d.	n.d.

TABLE 1. (continued)

Guanine	2′-deoxy	2.5	0	0
	2′,2′-difluoro-2′-deoxy	0.5	n.d.	n.d.
	arabinose	0.06	0	0
Uracil	2′-deoxy	0.06	1.0	0.9
	2′-chloro-2′-deoxy	0	n.d.	0
	2′,2′-difluoro-2′-deoxy	n.d.	0	0.3
	3′-OMe-2′-deoxy	0	0	0.03
	3′-OEt-2′-deoxy	0	0	0.01
	2′,3′-dideoxy	0	0.1	0.02
	2'-azido-2',3'-dideoxy	n.d.	0.7	0.05
	ribose	0	0	0.04
	arabinose	0	0	0.2
Thymine	2′-deoxy	0.02	1.0	1.0
	2′-methylene-2′-deoxy	n.d.	0	0.04
	2′,3′-dideoxy	0	0.4	0.04
	3′-azido-2′,3′-dideoxy	0	0.4	0.05
	3′-acetylene-2′,3′-dideoxy	n.d.	0.03	0
	3′-fluoro-2′,3′-dideoxy	0	0.3	0
	ribose	0	0.02	0.03
	arabinose	0	0	0.6
	α-2′-deoxy	0	0.36	0.35

[a] dC activity of dCK and dT activity of TKs were taken as 1.0; a value of 0 indicates <0.01.
[b] Deoxy and dideoxy refer to deoxyribose and dideoxyribose, unless otherwise indicated.
n.d., not determined.

TABLE 2. Substrate and inhibitor properties of O'-modified analogues of 2'-deoxyuridine (dU), including relative phosphorylation of 100 μM nucleoside substrates by TK1 with 100 μM [γ-^{32}P]ATP. IC_{50} values were determined with 1 μM dT as substrate, and K_i values with variable concentration of dT.

Compound	Relative ^{32}P transfer [a]	IC_{50}, μM — 1 μM dT	K_i (μM)
dU	1.0	56±4	30±8
2'-chloro-dU	n.d.	>10 000 [b]	n.d.
3'-OMe-dU	0	560±40	300±50
3'-OEt-dU	0	2000±100	1100±100
5'-OMe-dU	-	8000±1000 [c]	n.d.
3'-hexanoylamino-dT	n.d.	n.d.	615 [d]

[a] dT activity of TK2 was taken as 1.0
[b] No inhibition up to 2.5 mM.
[c] Result obtained by extrapolation from concentrations below 2.5 mM (85 % of v_o).
[d] From Beck et al.[21]
n.d., not determined.

TABLE 3. Substrate and inhibitor properties of O'-modified analogues of 2'-deoxyuridine (dU), including relative phosphorylation of 100 μM nucleoside substrates by pure TK2 with 100 μM [γ-^{32}P]ATP. IC_{50} values were based on use of 0.5 μM dT as substrate, and K_i values with variable concentration of dT.

Compound	Relative ^{32}P transfer [a]	IC_{50}, μM	K_i
		0.5 μM dT	(μM)
dU	1.0	25±2	9±2
2'-chloro-dU	0	80±4	25±5
3'-OMe-dU	0.03	40±5	15±3
3'-OEt-dU	0.01	30±1	10±2
3'-hexanoylamino-dT	n.d.	n.d.	0.14±0.06
5'-OMe-dU	-	>2000	n.d.

[a] dT activity of TK2 was taken as 1.0
n.d., not determined.

TABLE 4. Substrate and inhibitor properties of O'-modified analogues of C and dC including relative phosphorylation of 100 μM nucleoside substrates by pure dCK with 100 μM [γ-^{32}P]ATP. IC_{50} values were determined with 1 μM dC and 30 μM dA as substrates, and K_i values with variable dC.

Pentafuranose moiety [b]	Relative ^{32}P transfer [a]	IC_{50}, μM		K_i
		1 μM dC	30 μM dA	(μM)
2'-deoxy	1.0	1.0±0.1	0.5±0.03	-
2'-OMe-ribose	1.9	>1000	100±6	300±20
3'-OMe-ribose	0	n.d.	n.d.	n.d.
3'-OMe-2'-deoxy	0.6	75±5	n.d.	20±2
3'-OEt-2'-deoxy	0.08	n.d.	n.d.	n.d.
3',5'-diOMe-2'-deoxy	0	980±50	n.d.	n.d.
5'-OMe-2'-deoxy	-	500±20	100±20	230±8
5'-OEt-2'-deoxy	-	68±2	5.4±0.2	32±1
5'-azido-2',5'-dideoxy	-	104±4	14.0±0.6	n.d.

[a] dC activity of dCK was taken as 1.0
[b] Deoxy and dideoxy refer to deoxyribose and dideoxyribose, unless otherwise indicated.
n.d., not determined.

although similar modifications of dA, dU and dT diminished their substrate activity (data not shown). The α-anomer of 3′-hydroxymethyl-ddC is also a substrate, albeit a poorer one (TABLE 1). This is reminiscent of the earlier finding[40,41] that α-TGdR (6-thioguanine-2′-deoxyriboside) is phosphorylated, although less efficiently than β-TGdR, by extracts of normal tissues, but not by purified dCK from the same tissues, for which the β-anomer was a substrate. However, in an araC-resistant mouse L1210 cell line, both anomers were efficiently phosphorylated, presumably by a putative kinase differing from dCK or a nucleoside phosphotransferase[40] (*cf.* [37]). This presumed kinase cannot be dGK, since Park and Ives[42] subsequently showed that β-TGdR is not an inhibitor of dGK. Its identity remains to be established.

The foregoing led Acton et al.[40] to synthesize the α- and β- anomers of 3′-hydroxymethyl-TGddR. Both of these were effectively phosphorylated in a Mecca lymphosarcoma in mice, and incorporated as terminators of short chains of DNA. The authors pointed to the striking structural similarities between the two anomers, and postulated that the observed intracellular phosphorylation could be accounted for by phosphorylation of the 3′-CH_2OH of one of the anomers, consistent with the behaviour of the two phosphorylated anomers towards 3′- and/or 5′-nucleotidases. From TABLE 1 it will be noted that a similar situation prevails for our α and β anomers of 3′-hydroxymethyl-ddC, as well as for the α and β anomers of the corresponding L-enantiomers. Attempts are under way to determine the site of phosphorylation of each of these by dCK. By contrast, whereas the β anomers of 3′-azidomethyl-ddC and 3′-fluoromethyl-ddC are good substrates for dCK, the α anomers are inactive (TABLE 1). Similarly the β anomer of 2-chloro-3′-hydroxymethyl-ddA is also a good substrate for dCK, but the α anomer is not.

Substrate properties of O'-alkyl-2'-deoxyribonucleosides

In general, blocking of the 3′-OH of the 2′-deoxynucleosides, e.g. 3′-O-acetyl-dC, 3′-OEt-dC, 3′-O-acetyl-dA, 3′-fluoro-ddG, 3′-OMe-dU, 3′-OEt-dU, 3′-azido-ddT, 3′-acetylene-ddT and 3′-fluoro-ddT, leads to marked reduction of substrate activity (TABLE 1) and affinity *vs* dCK and TK2, as shown by higher IC_{50} and K_i values (TABLES 2-4). This may be related to interaction of this hydroxyl with the enzymes, as

in the crystal structure of the complex of dT with HSV-1 TK,[43,44] where the 3′-OH is hydrogen-bonded to Tyr-101.

With the HSV enzyme, the 5′-OH interacts with Arg-163,[43,44] and should also be sensitive to chemical modifications. We have previously shown that 5′-O-methyl- and 5′-O-ethyl analogues of 2′,5′-dideoxy-C are nonsubstrate inhibitors of dCK, like 5′-amino-2′,5′-dideoxy-C.[18] The latter exhibited a pH-dependent inhibition pattern, being more effective at pH 8.6 (≈50 % of neutral form) than at pH 7.2 (≤10 % of neutral form). From this it may be inferred that the neutral from has a higher affinity for the enzyme, as might have been anticipated, since otherwise there would be electrostatic repulsion between the protonated form of the 5′-amino group (pK_a~8.5) and the positively charged arginine in the presumed active site of human dCK.

From TABLE 1 it will be seen that C is a surprisingly good substrate of dCK, and, as for 2′-deoxyribonucleosides with a blocked 3′-OH, described in the preceding section, 3′- OMe-C is no longer a substrate of dCK (TABLE 4). All the more striking, therefore, is the finding that 2′-OMe-C is an excellent substrate of dCK, in fact twice as effective as dC (TABLE 4), but with a much lower affinity for the enzyme (K_i = 300 μM). In this context, it is worth recalling that Krenitsky et al.[12] long ago reported that C is a substrate of calf thymus dCK. However, the enzyme was only partially purified; in addition, assays were based on the use of a spectral method involving enzymatically coupling the formation of ADP to NADH oxidation, and requiring very high substrate concentrations. Nonetheless, the present results fully confirm that C itself is a good substrate of human dCK. Furthermore, Krenitsky et al.[12] also found that K_m of C is much higher than that of dC, but its V_{max} was 5-fold higher, hence qualitatively consistent with our results for 2′-OMe-C.

It should, however, be noted that Verri et al.,[45] apparently unaware of the earlier findings of Krenitsky et al.,[12] reported that a partially purified preparation of dCK from HeLa cells did not phosphorylate C, A, G, dT, U, regarded as evidence that their preparation was not contaminated with ribonucleoside kinases and thymidine kinnases. However, the specific activity of their preparation, the degree of purification of which was not given, was 0.1 nmol/min/mg, hence three orders of magnitude lower than that of our preparation, although relative phosphorylation rates for dA, dG and dC were

similar to those for our preparation. This is perhaps not unexpected, inasmuch as affinity chromatography, not used in their purification scheme, by itself leads to an additional 1000-fold purification.[1]

It should be recalled that both C and U are substrates of the well-known uridine/cytidine kinase.[46] However, as seen from TABLE 1, neither uridine nor thymine riboside are substrates of dCK, but the former is a weak substrate of both TK1 and TK2. As regards C, it is clear from the above that its intracellular phosphorylation is probably controlled not only by uridine/cytidine kinase, but also by dCK. Furthermore, it should be noted that 2′-OMe-A is also a substrate of dCK, albeit a weaker one.

Substrate/Inhibitor discrimination between TK1 and TK2

Discrimination between TK1 and TK2 on the basis of differences in substrate/inhibitor properties of dU, dT and dC would clearly be useful in following the role of these enzymes in intracellular metabolism, and the mechanism of recognition of both substrates and inhibitors. While cytosine nucleosides are, as expected, inactive *vs* TK1 (TABLE 1), several of them (e.g. 2′-fluoro-dC, 3′-OMe-dC and the L-enantiomer of 3′-hydroxymethyl-ddC) are relatively good substrates of TK2. By contrast, several uracil and thymine nucleosides are preferential substrates of TK2 (2′,2′-difluoro-dU, U, 2′-methylene-dT, ara-T and 3′-azido-3′-deoxy-ara-T), whereas others exhibit similar substrate properties towards both enzymes (T and α-dT). Note, in particular, that several of the 2′,3′-dideoxyribonucleosides (ddU, 2′-azido-ddU, ddT, 3′-azido-ddT, 3′-fluoro-ddT, see TABLE 1) exhibit a marked preference for TK1.

From TABLES 2 and 3 it will be seen that 3′-OMe-dU is a poor inhibitor of TK1 (K_i ~ 300 μM), but 20-fold more effective *vs* TK2 (K_i ~ 15 μM). This discrimination between the two enzymes is further accentuated in the case of 3′-OEt-dU with K_i ~ 1100 μM for TK1 and 10 μM for TK2. These results prompted us to follow up previous findings on the development of 3′- and 5′-substituted thymidines for the purification of cytosolic TK1 by affinity chromatography.[21] One of these compounds, 3′-hexanoylamino-3′-deoxythymidine, was found to inhibit TK1, competitively with respect to thymidine, with a K_i ~ 600 μM. Evaluation of this compound with TK2 now showed that it was also a competitive inhibitor of this enzyme, with K_i ~ 0.15 μM,

hence a 4,000-fold higher affinity. This is, therefore, not only an excellent discriminator between TK1 and TK2, but also a good lead compound for development of even more potent and more selective inhibitors. Marked discrimination between the two TK enzymes was also exhibited by 2′-chloro-dU, which was virtually inert *vs* TK1 (TABLE 2), and inhibited TK2 with $K_i \sim 25$ μM (TABLE 3).

Concluding remarks

The foregoing overall results depict the substrate/inhibitor properties of a variety of nucleoside analogues with modified sugar moieties against three key nucleoside kinases, and further underline the broad substrate specificities of theese enzymes, especially dCK. Some of the analogues are good inhibitors, and can serve as lead compounds for developments of more potent and specific inhibitors (e.g. 5′-O-alkyl analogues of dC and 5′-amino-ddC[18]), as well as discriminators, e.g. between cytosolic and mitochondrial TKs (cf. TABLES 2 and 3). The utility of these findings is further enhanced by the fact that they apply to enzymes highly purified from natural, human, sources. We have noted that the analogous enzymes obtained as recombinats expressed in *E. coli* exhibited properties somewhat at variance with those from natural sources, dependent also on the cloning system employed; e.g. for recombinant dCK with the terminal His-tag, $K_m = 0.15$ μM with dC, and 0.5 μM following truncation of the His-tag, as compared to 0.8 μM for the native enzyme. Significant differences between natural and recombinant TK1 have been described by Munch-Petersen et al.[47] For cloned TK2,[11] no results have as yet been reported.

None of the three enzymes examined in this study has yet been obtained in crystalline form suitable for determination of the modes of binding of structurally dissimilar substrates and inhibitors by X-ray diffraction. Such results, reported for crystalline complexes of the TK of HSV-1 with thymidine and Acyclovir should be helpful in this regard. For example, how do the substrate binding pockets of TK1 and TK2 accommodate α-dT almost as well as natural β-dT (cf. TABLES 2 and 3). Furthermore, in view of the current widespread interest in the L-enantiomers of nucleosides and nucleotides as substrates/inhibitors of nucleoside kinases and other enzymes, reviewed by Wang at al.,[48] it is quite striking that not only is our β-L-3′-

hydroxymethyl-2′,3′-dideoxycytidine a good substrate of dCK, but so is its α-anomer (see TABLE 1).

Finally, attention should be drawn to the fact that, for all three enzymes embraced in this study, the phosphate donor employed was ATP. It is of interest, in this context, that for the dCK the L-enantiomer of ATP is as good a donor as the natural D-ATP.[49] However, it has long been known that nucleoside kinases do not exhibit a strict specificity for ATP. [50] In the case of dCK there is now ample evidence that the major physiological phosphate donor is not ATP, but UTP. [50] And with dGK, it was long ago shown that, at physiological pH, UTP and CTP are better donors than ATP. [42,51] As recently shown by Zhu et al.,[52] the antitumour nucleoside analogues araG and 2-chloro-2′-deoxyadenosine are phosphorylated *in vitro* 50-fold more effectively with UTP as compared to ATP. It follows that there is an interrelationship between substrate/inhibitor properties of a nucleoside analogue and the nature of the NTP phosphate donor, which should be taken into consideration in further investigations.

Acknowledgement

Research supported by the Polish State Committee for Scientific Research (KBN, grant no. 6P04A03812), including those for international scientific co-operation projects (UM-756 and UM-757), in part (B.K., K.K., D.S.), by an International Research Scholar's award from the Howard Hughes Medical Institute (HHMI 75195-543401).

REFERENCES

1. Bohman, C.; Eriksson, S. *Biochemistry* **1988, *27*,** 4265-4273.
2. Kim, M.-Y.; Ikeda, S.; Ives, D. H. *Biochem. Biophys. Res. Commun.* **1988, *156*,** 92-97.
3. Johansson, M.; Brismar, S.; Karlsson, A. *Proc. Natl. Acad. Sci. U. S.* **1997, *94*,** 11941-11945.
4. Sherley, J. L.; Kelly, T.J. *J. Biol. Chem.* **1988, *263*,** 375-382.
5. Munch-Petersen, B.; Cloos, L.; Tyrsted, G.; Eriksson, S. *J. Biol. Chem.* **1991, *266*,** 9032-9038.
6. Bradshaw, H.D.,Jr.; Deininger, P.L. *Molec. Cell. Biol.* **1984, *4*,** 2316-2320.
7. Kreidberg, J.A.; Kelly, T.J. *Mol. Cell. Biol.* **1986, *6*,** 2903-2909.
8. Knight, G.B.; Gudas, J.M.; Pardee, A.B. *Proc. Natl. Acad. Sci. U.S.* **1987, *84*,** 8350-8354.

9. Munch-Petersen, B.; Tyrsted, G.; Cloos, L.; Beck, R.A.; Eger, K. *Biochim. Biophys. Acta* **1995**, ***1250***, 158-162.
10. Chottiner, E.G.; Shewach, D.S.; Datta, N.S.; Ashcraft, E.; Gribbin, D.; Ginsgurg, D.; Fox, I.H.; Mitchell, B.S. *Proc. Natl. Acad. Sci. U.S.* **1991**, ***88***, 1531-1535.
11. Johansson, M.; Karlsson, A. *J. Biol. Chem.* **1997**, ***272***, 8454-8458.
12. Krenitsky, T.A.; Tuttle, J.V.; Koszalka, G.W.; Chen, I.S.; Beacham, L.M.; Rideout, J.L.; Elion, G.B. *J. Biol. Chem.* **1976**, ***251***, 4055-4061.
13. Eriksson, S.; Kierdaszuk, B.; Munch-Petersen, B.; Oberg, B.; Johansson, N.G. *Biochem. Biophys. Res. Commun.* **1991**, ***176***, 586-592.
14. Kierdaszuk, B.; Eriksson, S. *Biochemistry* **1990**, ***29***, 4109-4114.
15. Kierdaszuk, B.; Bohman, C.; Ullman, B.; Eriksson, S. *Biochem. Pharmacol.* **1992**, ***43***, 197-206.
16. Bretner, M.; Balinska, M.; Krawiec, K.; Kierdaszuk, B.; Shugar, D.; Kulikowski, T. *Nucleosides & Nucleotides* **1995**, ***14***, 657-660.
17. Johansson, N.G.; Eriksson, S. *Acta Biochim. Polon.* **1996**, ***43***, 143-160.
18. Krawiec, K.; Kierdaszuk, B.; Shugar, D. *Nucleosides & Nucleotides* **1995**, ***14***, 495-499.
19. Kvarnström, I.; Svansson, L.; Svansson, C.; Svansson, S.C.T. *Nucleosides & Nucleotides* **1992**, ***11***, 1367-1370.
20. Svansson, L.; Kvarnström, I. *J. Org. Chem.* **1991**, ***56***, 2993-2997.
21. Beck, R.A.; Munch-Petersen, B.; Dolker, M.; Cloos, L.; Tyrsted, G.; Eger, K. *Pharmaceutica Acta Helv.* **1996**, ***71***, 279-291.
22. Ives, D. H.; Wang, S.-M., *Methods Enzymol.* **1978**, ***51***, 337-345.
23. Munch-Petersen, B. *Mol. Cell. Biochem.* **1984**, ***64***, 173-185.
24. Kuśmierek, J.T.; Giziewicz, J.; Shugar, D. *Biochemistry* **1973**, ***12***, 194-200.
25. Kuśmierek, J.T.; Shugar, D. *Acta Biochim. Polon.* **1973**, ***20***, 365-381.
26. Kazimierczuk, Z.; Darzynkiewicz, E.; Shugar, D. *Biochemistry* **1976**, ***15***, 2735-2740.
27. Jay, A. *Carbohydrate Chem.* **1996**, ***15***, 897-923.
28. Hampton, A.; Chawla, R.R.; Kappler, F. *J. Med. Chem.* **1982**, ***25***, 644-649.
29. Sekine, M.; Peshakova, L.S.; Hata, T. *J. Org. Chem.* **1987**, ***52***, 5060-5064.
30. Krayevsky, A.A.; Kukhanova, M.K.; Atrazhev, A.M.; Chidgeavadze, Z.G.; Beabeakashvilli, R.S. *Mol. Biol. (Russian)* **1987**, ***20***, 33-38.
31. Sekine, M.; Nakanishi,T. *J. Org. Chem.* **1990**, ***55***, 924-928.
32. Cramer, H.; Pfleiderer, W. *Helv. Chim. Acta* **1996**, ***79***, 2114-2136.
33. Dudycz, L.; Kotlicki, A.; Shugar, D. *Carbohydrate Res.* **1981**, ***91***, 31-37.
34. Gin, J.B.; Dekker C.A. *Biochemistry* **1968**, ***7***, 1413-1417.
35. Hayatsu, H.; Wataya, Y.; Kai, K. *J. Am. Chem. Soc.* **1970**, ***92***, 724-729.
36. Shapiro, R.; Servis, R.E.; Welcher, M. *J. Am. Chem. Soc.* **1970**, ***92***, 422-424.
37. Shugar, D. *Molecular aspects of chemotherapy*, Shugar, D.; Rode, W.; Borowski, E. (eds.), Springer Verlag, Brlin, Haidelberg and New York, **1992**, pp. 239-270.
38. Gentry, G.A. *Pharmacol. Ther.* **1992**, ***54***, 319-355.
39. Ho, H.-T.; Hitchcock, M.J.M. *Antimicrob. Agents Chemother.* **1989**, ***33***, 844-849.
40. Acton, E.M.; Goerner, R.N.; Uh, H.S.; Ryan, K.J.; Henry, D.W. *J. Med. Chem.* **1979**, ***22***, 518-525.

41. LePage, G.A.; Banks, P.A.; Noujaim, M.J.; Buzzell, G.R. *Cancer Chemother.* **1980**, *5*, 127-131.
42. Park, I.; Ives, D.H. *Arch. Biochem. Biophys.* **1988**, ***266***, 51-60.
43. Brown, D.G.; Visse, R.; Sandhu, G.; Davies, A.; Rizkallah, P.J.; Melitz, C.; Summers, W.C.; Sanderson, M.R. *Nature Struct. Biol.* **1995**, *2*, 876-880.
44. Wild, K.; Bohner, T.; Aurby, A.; Folkers, G.; Schultz, G.E. *FEBS Lett.* **1995**, ***368***, 289-292.
45. Verri, A.; Focher, F.; Priori, G.; Gosselin, G.; Imbach, J.-L.; Capobianco, M.; Garbesi, A.; Spadari, S. *Mol. Pharmacol.* **1997**, ***51***, 132-138.
46. Payne, R.C.; Cheng, N.; Traut, T.W. *J. Biol. Chem.* **1985**, ***260***, 10242-10247.
47. Munch-Petersen, B.; Cloos, L.; Jensen, H.K.; Tyrsted, G. *Adv. Enzyme Regulation*, **1995**, *35*, 69-89.
48. Wang, P.; Hong, J.H.; Cooperwood J.S.; Chu, C.K. *Antiviral. Res.* **1998**, ***40***, 19-44.
49. Verri, A.; Montecucco, A.; Gosselin, G.; Boudou, V.; Imbach, J. -L.; Spadari, S.; Focher, F. *Biochem. J.* **1999**, ***337***, 585-590.
50. Krawiec, K.; Kierdaszuk, B.; Eriksson, S.; Munch-Petersen, B.; Shugar, D. *Biochem. Biophys. Res. Commun.* **1995**, ***216***, 42-48.
51. Shugar, D. *Acta Biochim. Polon.* **1996**, ***43***, 9-24.
52. Zhu, C.; Johansson, M.; Permert, J.; Karlsson, A. Biochem. Pharmacol. **1998**, ***56***, 1035-1040.

Received 12/23/98
Accepted 4/7/99