Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical Pharmacology 68 (2004) 1879–1888
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Anabolism of amdoxovir: phosphorylation of dioxolane guanosine and its 5′-phosphates by mammalian phosphotransferases

Joy Y. Feng[a,*], William B. Parker[b], Megan L. Krajewski[a], Dominique Deville-Bonne[c], Michel Veron[c], Preethi Krishnan[d], Yung-Chi Cheng[d], Katyna Borroto-Esoda[a]

[a]*Gilead Sciences, 4 University Place, 4611 University Drive, Durham, NC 27707, US*
[b]*Southern Research Institute, 2000 Ninth Avenue South, Birmingham, AL 35205, USA*
[c]*Unité de Régulation enzymatique des Activités cellulaires, Institut Pasteur, CNRS URA 2185, 75724 Paris Cedex 15, France*
[d]*Department of Pharmacology, School of Medicine, Yale University, 333 Cedar Street, New Haven, CT 06520, USA*

Received 16 March 2004; accepted 14 June 2004

Abstract

Amdoxovir [(−)-β-D-2,6-diaminopurine dioxolane, DAPD], the prodrug of dioxolane guanosine (DXG), is currently in Phase I/II clinical development for the treatment of HIV-1 infection. In this study, we examined the phosphorylation pathway of DXG using 15 purified enzymes from human (8), animal (6), and yeast (1) sources, including deoxyguanosine kinase (dGK), deoxycytidine kinase (dCK), high K_m 5′-nucleotidase (5′-NT), guanylate (GMP) kinase, nucleoside monophosphate (NMP) kinase, adenylate (AMP) kinase, nucleoside diphosphate (NDP) kinase, 3-phosphoglycerate (3-PG) kinase, creatine kinase, and pyruvate kinase. In addition, the metabolism of ^{14}C-labeled DXG was studied in CEM cells. DXG was not phosphorylated by human dCK, and was a poor substrate for human dGK with a high K_m (7 mM). Human 5′-NT phosphorylated DXG with relatively high efficiency (4.2% of deoxyguanosine). DXG–MP was a substrate for porcine brain GMP kinase with a substrate specificity that was 1% of dGMP. DXG–DP was phosphorylated by all of the enzymes tested, including NDP kinase, 3-PG kinase, creatine kinase, and pyruvate kinase. The BB-isoform of human creatine kinase showed the highest relative substrate specificity (47% of dGDP) for DXG–DP. In CEM cells incubated with 5 μM DXG for 24 h, 0.015 pmole/10^6 cells (~7.5 nM) of DXG–TP was detected as the primary metabolite. Our study demonstrated that 5′-nucleotidase, GMP kinase, creatine kinase, and NDP kinase could be responsible for the activation of DXG in vivo.

Keywords: Amdoxovir; Anabolism; DXG; Phosphorylation; Phosphotransferase

1. Introduction

Nucleoside analogs play important roles in anti-viral and anti-cancer therapy. Most of these compounds need to be transformed into their active 5′-triphosphate form by cellular or viral kinases to exert their pharmacological activity. Through three consecutive steps, nucleoside analogs are phosphorylated to 5′-monophosphate (MP), 5′-diphosphate (DP), and finally 5′-triphosphate (TP). Studies have shown that an elevated intracellular concentration of nucleotide 5′-triphosphate analogs (ddNTP) correlated with enhanced activity [1]. Amdoxovir [(−)-β-D-2,6-diaminopurine, DAPD], the prodrug of dioxolane guanosine (DXG), is currently in Phase I/II clinical development for the treatment of HIV-1 infection (Fig. 1). Both in vitro and in vivo studies have demonstrated that the anti-HIV activity seen upon treatment with DAPD is almost entirely due to the generation of DXG by the action of adenosine deaminase (ADA) on DAPD [2–4]. DXG is subsequently phosphorylated to its 5′-triphosphate (DXG–TP), which is a strong alternative substrate inhibitor of the HIV reverse transcriptase (RT) [2]. Since DXG is a deoxyguanosine (dGuo) analog, it has been assumed that DXG is activated to DXG–TP by the same phosphorylation pathway used by the natural nucleoside dGuo. In addition, several other

Abbreviations: AMP kinase, Adenylate kinase; BDG, 9-benzyl-9-deazaguanine; DAPD, [(−)-β-D-2,6-diaminopurine dioxolane]; d4T, 2′,3′-didehydro-3′-deoxythymidine; DXG, dioxolane guanosine; dCK, deoxycytidine kinase; dGK, deoxyguanosine kinase; DP, 5′-diphosphate; GMP, guanylate; MP, 5′-monophosphate; MPA, mycophenolic acid; NMP, nucleoside monophospahte; NDP, nucleoside diphosphate kinase; 5′-NT, 5′-nucleotidase; 3-PG, 3-phosphoglycerate; TP, 5′-triphosphate.

* Corresponding author. Tel.: +1 919 294 7647; fax: +1 919 493 5790.
E-mail address: joy.feng@gilead.com (J.Y. Feng).

0006-2952/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2004.06.019

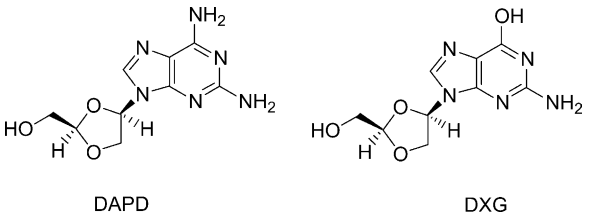

Fig. 1. Structures of DAPD and DXG.

enzymes were known to be involved in the phosphorylation of dGuo analogs (−)carbovir [5,6], acyclovir [7], and ganciclovir [8]. In this study, we examined the phosphorylation pathway of DXG using 15 purified enzymes from human (8), animal (6), and yeast (1) sources (Table 1). The enzymes in this study were divided into three groups: (1) enzymes that phosphorylate deoxynucleoside to dNMP, including dGuo kinase (dGK), deoxycytidine (dCyd) kinase (dCK), and high K_m 5′-nucleotidase (5′-NT); (2) enzymes that phosphorylate deoxynucleoside-MP (dNMP) to dexoynucleoside-DP (dNDP), including guanylate (GMP) kinase, bovine liver nucleoside monophosphate (NMP) kinase, and adenylate (AMP) kinase, which is also known as myokinase; and (3) enzymes that phosphorylate dNDP to deoxynucleoside-TP (dNTP), including nucleoside diphosphate (NDP) kinase, 3-phosphoglycerate (3-PG) kinase, creatine kinase, and pyruvate kinase. In addition, we evaluated the metabolism of ^{14}C-labeled DXG in CEM cells. Previously, we reported the metabolism of [8-^{3}H]-DXG [2], however, the levels of DXG–MP, DP, and TP were found to be significantly lower in later studies [9] (personal communication with Dr. Robert St. Claire III, Gilead Sciences).

dGK is a mitochondrial enzyme constitutively expressed during the cell cycle with dGuo and 2′-deoxyadenosine (dAdo) as natural substrates [10]. It has been shown to phosphorylate anti-cancer nucleoside analogs, including 2-chloro-2′-deoxyadenosine (CdA), 9-β-D-arabinofuranosylguanine (araG), and 2′,2′-difluorodeoxyguanosine (dFdG) [11]. dCK, a cytosolic enzyme constitutively expressed during the cell cycle, phosphorylates dCyd, dAdo, and dGuo with ATP and UTP as phosphate donors, and is known to have very broad substrate specificity [10]. An ubiquitous enzyme, the high K_m 5′-NT is specific for the reversible dephosphorylation reaction of 6-oxypurine nucleoside monophosphate (IMP and GMP) [12,13]. With IMP as the phosphate donor, it also phosphorylates dGuo, dAdo, acyclovir, tiazofurin, didanosine (ddI), and (−)-carbovir [13].

GMP kinase has been purified from many eukaryotic sources including bovine retina, pig brain, Baker's yeast, rat liver, and human erythrocytes [14]. However, there is limited information on the activity of the purified recombinant human enzyme [14,15]. In this study, we chose to use the commercially available GMP kinase purified from pig brain. The high sequence similarity between the pig GMP kinase and human GMP kinase (90.3% identity, 93.8% homology) [14] suggests that the information gained on phosphorylation of dGMP and DXG–MP by pig GMP kinase is likely to be directly transferable to the human enzyme. NMP kinase from bovine liver [16,17] has been shown to phosphorylate UMP, CMP, GMP, AMP, and acyclo-GMP (only to a limited extent) with ATP as the phosphate donor [7]. In this study, we also tested the phosphorylation of DXG–MP using commercially available AMP kinase purified form pig heart, which is genetically similar to the human enzyme [18].

NDP kinase phosphorylates all natural nucleoside diphosphates into triphosphates using ATP as the major phosphate donor. The NDP kinase activity is found to be much higher than many of the other enzymes involved in nucleotide metabolism [19]. Eight isoforms (nM23-H1 to nM23-H8) have been isolated in humans. The cytosolic enzymes nM23-H1 (type A) and nM23-H2 (type B) share high homology and are the most extensively studied [20–23]. In this study, we used recombinant human enzyme (type A, NM23-H1) and commercially available NDP

Table 1
Kinases used in the study of DXG phosphorylation pathway

Kinases	EC #	Sources
dGK	2.7.1.113	Recombinant human enzyme
dCK	2.7.1.74	Recombinant human enzyme
High K_m 5′-NT	3.1.3.5	Recombinant human enzyme
GMP kinase	2.7.4.8	Porcine brain (Sigma #G9385)
NMP kinase	2.7.4.4	Bovine liver (ICN #155962)
AMP kinase	2.7.4.3	Porcine heart (CalBiochem #475945)
NDP kinase	2.7.4.6	Bovine liver (Sigma #2635)
		Recombinant human enzyme, type A
3-PG kinase	2.7.2.3	Baker's yeast (Sigma #P7634)
		Recombinant human enzyme
Creatine kinase	2.7.3.2	Rabbit muscle, type I (Sigma #C3755)
		Recombinant human enzyme (MM) (Sigma #C9858)
		Recombinant human enzyme (BB) (Sigma #C9983)
		Recombinant human enzyme (MB) (Sigma #C0984)
Pyruvate kinase	2.7.1.40	Rabbit muscle, type II (Sigma #P1506)

kinase purified from bovine liver, which was shown to phosphorylate acyclovir-DP [24]. 3-PG kinase is a glycolytic enzyme that catalyzes transfer of a phosphate group from 3-phospho-D-glycerol phosphate to ADP forming 3-phospho-D-glycerate and ATP, and is 96% conserved in mammals [25]. It has been reported that human cellular 3-PG kinase phosphorylated ganciclovir-DP to its triphosphate form [8]. In addition, acyclovir-DP was phosphorylated by the 3-PG kinase partially purified from human erythrocytes [24]. Recently, Krishnan et al. suggested human 3-PG kinase as being the key enzyme for the last step of phosphorylation during the activation of L-nucleoside analogs [23]. Creatine kinase phosphorylates ADP to ATP using creatine phosphate as the phosphate donor [26]. There are four types of creatine kinase subunits that are expressed specifically depending on species, development stage and tissue. The two cytosolic types are M-CK (muscle) and B-CK (brain), which form dimers of three isoenzymes: MM-CK, MB-CK, and BB-CK. The enzyme has been shown to phosphorylate 2′,3′-dideoxycytidine 5′-DP (ddCDP) [23], cidofovir-MP [27], carbovir-DP [5,6], adefovir-DP [28], and 2′,3′-didehydro-3′-deoxythymidine (d4T)-DP [23]. Pyruvate kinase, a key glycolytic enzyme, catalyzes the transfer of a phosphate group from phospho(enol)pyruvate to ADP. In this study, we used commercially available rabbit muscle pyruvate kinase, which was shown to phosphorylate a series of the nucleoside analog diphosphates to a limited extent [6,23,24].

2. Material and methods

2.1. Reagents

[8-^{14}C]-Inosine, [8-^{14}C]-deoxyguanosine, and [8-^{14}C]-DXG were obtained from Moravek Biochemicals. ATP was from Amersham Pharmacia Biotech. Inosine, IMP, dGuo, dGMP, dGDP, dGTP, ADP, phospho(enol)pyruvate, creatine phosphate, NAD^+, DL-glyceraldehyde-3-phosphate, glyceraldehyde-3-phosphate dehydrogenase, and N-acetyl-L-cysteine were from Sigma. All other materials and reagents were of the highest quality available.

2.2. Cell culture

CEM cells, obtained from the American Type Culture Collection were grown in RPMI 1640 medium (Gibco-BRL) containing 10% fetal bovine serum (Gibco-BRL), 10 U/mL penicillin, 10 μg/mL streptomycin, and 50 μg/mL gentamycin [29]. Cells were routinely tested for the presence of mycoplasma by measuring [^{3}H] thymidine uptake [30]. Cells were maintained in a humidified atmosphere of 5% CO_2 in air at 37 °C. All experiments were performed with cells that were proliferating at maximal rates. Cell numbers were determined with a coulter counter.

2.3. Enzymes

The sources of the enzymes are summarized in Table 1. The recombinant human dGK and dCK were generously provided by Eriksson and coworkers [31]. The clone for the high K_m 5′-NT was kindly provided by Spychala et al. [12] and purified in our laboratory based on published methods. Human recombinant NDP kinase (form A, NM23-H1) and human recombinant 3-PG kinase were purified as previously described [23,32]. The molecular weights used to calculate the k_{cat} values for dGK, dCK, and 5′-NT is 58, 60, and 195 kDa, respectively. The molecular weight used for the calculation of k_{cat} is 22 kDa for GMP kinase, 17 kDa for bovine liver and human NDP kinase, 47 kDa for Baker's yeast 3-PG kinase, 46 kDa for human 3-PG kinase, 80 kDa for all of the creatine kinases, and 59 kDa for rabbit pyruvate kinase.

5′-NT was purified based on a published method with modifications [12]. Fifty milliliter overnight growth of *Escherichia coli* (50 mg/L kanamycin) was used to inoculate 2 L volumes of LB media. Cells were grown to a density of OD_{600} = 0.6–0.7 and induced with a final concentration of 1 mg/mL of IPTG for 5 h. Cells were collected by centrifugation at 8000 rpm for 10 min on a Sorvall RC 5B Centrifuge with a SLA-150 rotor. The cell pellets were suspended in 10 volume of homogenization buffer containing 20 mM Tris–HCl, pH 8.0, 20 mM KCl, 1 mM EGTA, 1 mM 2-mercaptolethanol, 0.1 mM phenylmethansulfonyl fluoride (PMSF), 10 μg/mL of pepstatin and leupeptine, and 0.2 mg/mL of lysozyme. The cells were lyzed by stirring at room temperature in the lysis buffer for 30 min, followed by sonication using Branson Sonifier 450 (100% constant, output 3, 3 min × 1 min). The crude lysate was centrifuged at 14,000 rpm for 20 min using a Sorvall RC 5B Centrifuge. The supernatant was removed and subjected to 50% ammonium sulfate precipitation. After 1 h of incubation at 4 °C, protein precipitates were collected by centrifugation at 8000 rpm on a Sorvall RC 5B Centrifuge. The protein pellets were suspended in 13 mL Buffer A (20 mM TrisCl, pH 7.0, 5 mM imidazole, 0.6 M NaCl, and 5 mM 2-mercaptolethanol) and dialyzed in Buffer A overnight (2 × 2 L). The dialyzed protein was loaded onto a pre-equilibrated Ni-NTA Superfolw resin (bed volume = 3 mL), and incubated for 30 min at 4 °C on a shaker. The mixture of the resin and protein solution was loaded on to a column (3 cm × 13 cm). The column was washed with Buffer A (~70 mL) until no protein was detected from the wash (OD_{280} < 0.07 AU). The protein was eluted with Buffer B (20 mM Tris, pH 7.0, 20 mM–1 M imidazole, 0.6 M NaCl, and 5 mM 2-mercaptolethanol). The step gradient of Buffer B was set at a rate of 20 mM per step, 10 mL per step. Five milliliters of fractions was collected, and fractions containing 5′-NT as determined by SDS-PAGE analysis were pooled, exchanged into Buffer C (100 mM imidazole, pH 7.0, 1 M NaCl, 10 mM 2-mercaptolethanol), and then

mixed with an equal volume of glycerol. The final protein concentration was 1.2 mg/mL in 50% glycerol, 50 mM imidazole, pH 7.0, 0.5 M NaCl, and 5 mM 2-mercaptolethanol. The protein concentration was determined using the Bradford assay (Bio-Rad protein assay) with BSA as a standard. The enzyme was stored at −20 °C, under which condition the enzyme is stable for at least two years.

2.4. Enzymatic phosphorylation of nucleosides and nucleotides

The phosphorylation of dGuo and DXG by dGK and dCK was conducted in a direct phosphorylation assay by monitoring the formation of product from ^{14}C-labeled nucleoside substrates using ATP as phosphate donor [33]. A mixture containing 50 mM Tris–HCl (pH 7.6), 2.5 mM ATP, 5 mM $MgCl_2$, 125 mM KCl, 15 mM NaF, 10 mM DTT, 0.5 mg/mL BSA, and 0.5 μM–20 mM of the corresponding nucleoside substrate was pre-incubated at 37 °C. The reaction was initiated by the addition of the enzyme, and at each time interval, 5 μL of the reaction mixture was removed and spotted onto Whatman DE81 filter paper (A). For each reaction, 3 × 5 μL of mixture was spotted onto another piece of Whatman DE81 filter paper (B) to determine total counts. After air-dried, filter paper (A) was washed with 5 mM ammonium formate (×3) and ethanol (×1) and dried. Both filter papers (A) and (B) were transferred to scintillation vials and eluted with 0.5 mL elution buffer (0.2 M KCl, 0.1 M HCl). After the mixture was incubated at room temperature for 30 min on a shaker, 4.5 mL of scintillation fluid was added to each vial, and briefly mixed by vortex. The amount of the radio-labeled reaction product and substrate were quantitated by a liquid scintillation counter.

The phosphorylation of dGuo, inosine, and DXG by high K_m 5′-NT were carried out at 37 °C in a reaction mixture containing 50 mM imidazole–HCl (pH 6.5), 20 mM $MgCl_2$, 0.5 mg/mL BSA, 5 mM IMP (as phosphate donor), 5 mM ATP (as activator), and ^{14}C-labeled nucleoside in a volume of 20 μL [6,12]. The reaction was initiated by adding 5′-NT, and at each time interval 2 μL aliquots were spotted onto polyethylenimine (PEI) thin-layer chromatography plates pre-spotted with 10 nmol of unlabeled nucleoside. Plates were developed in 50% MeOH. Products and substrate were quantitated by cutting out the UV spots and counting the radioactivity by liquid scintillation counter.

The phosphorylation of dGMP and DXG–MP by GMP kinase, NMP kinase, and AMP kinase was initiated by adding enzyme to a pre-incubated 37 °C mixture of Tris acetate (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, and 4 mM ATP. At 2, 4, 6, 8, and 10 min, a portion of the reaction mixture was removed and quenched with 3.5–3.8% (w/w) HCl. The reaction products were subjected to HPLC analysis as described below.

All of the enzymatic phosphorylation of dGDP and DXG–DP reactions were carried out in 50 mM Tris acetate (pH 7.5), 5 mM $MgCl_2$ at 37 °C. At 2, 4, 6, 8, and 10 min, a portion of the reaction mixture was removed and quenched with 50 mM EDTA (final concentration after mixing). In cases where EDTA interfered with the chromotography, 3.5–3.8% (w/w) HCl was used to stop the reaction. The reaction products were subjected to HPLC analysis as described below. NDP kinase reaction mixture contained 5 mM NaF and 4 mM ATP for NDP kinase from bovine liver or 1 mM ATP for recombinant human NDP kinase (form A). 3-PG kinase phosphorylation of dGuo and DXG–DP was measured using coupled reactions in a mixture containing 10 mM sodium phosphate (pH 7.5), 4 mM NAD^+, and 4 mM DL-glyceraldehyde [23]. Prior to the addition of the enzyme, the phosphate donor of this reaction, 1,3-biphosphoglycerate was generated in situ by incubating the reaction mixture with 8 unit/mL glyceraldehyde-3-phosphate dehydrogenase at room temperature for 20 min. For creatine kinase, the reaction mixtures contained 5 mM NaF, 20 mM creatine phosphate, 0.1% (w/w) BSA, and 10 mM N-acetyl-L-cysteine as an activator [34]. Pyruvate kinase reactions were studied in the presence of 100 mM KCl and 4 mM phospho(enol)pyruvate.

2.5. HPLC and data analysis for enzyme assays

Prior to HPLC analysis, each collected quenched mixture was passed through a YM-10 Microcon centrifugal filter unit (Millipore) and analyzed within 48 h using HPLC (HP 1100, Agilent Technologies). The samples were stored at room temperature prior to analysis. No degradation or conversion of the reaction mixture was detected during analysis. The strong-ion exchange HPLC was performed on a BioBasic AX (4.5 mm × 100 mm, 5 μm) column (ThermoHypersil-Keystone). Elution of the nucleotides was accomplished with a 15 min linear gradient from 10 mM ammonium phosphate (pH 5.5, 5% MeOH) to 300 mM ammonium phosphate (pH 5.5, 5% MeOH) buffer followed by 9 min of 300 mM ammonium phosphate (pH 5.5, 5% MeOH) at a flow rate of 1.5 mL/min [35]. The nucleotide substrate and product were detected at 253 nm and quantitated based on peak area.

The chosen enzyme concentration was based on an established linear correlation between the enzyme concentration and the rate of the reaction. In each experiment, at least five time points were taken before 15% of the substrate was consumed. The initial reaction velocities were determined using linear regression. The observed reaction rate (v) was fitted to the Michaelis–Menten equation as a function of substrate concentration ([S])

$$v = \frac{(k_{cat}[E]_0[S])}{(K_m + [S])}$$

The kinetic parameters k_{cat} and K_m were determined using non-linear regression curve fitting (KaleidaGraph, 3.5).

2.6. Measurement of DXG–MP, –DP, and –TP in CEM cells

CEM cells (~500,000 cells/mL) were incubated with 5 μM of [8-^{14}C]-DXG (45 mCi/mmol) for 6 and 24 h at 37 °C. The cells (45 mL) were collected by centrifugation, and the pellets were washed twice with ice-cold Puck's saline G [36] and mixed with 200 μL of ice-cold 0.5 M perchloric acid. The acid-insoluble material was removed by centrifugation at 12,000 × *g* for 5 min, and the supernatant fluid was removed and neutralized with 1 M potassium phosphate (pH 7.5) and 8 M KOH. $KClO_4$ was removed by centrifugation at 12,000 × *g* for 5 min, and 180 μL of the supernatant fluid was injected onto a Partisil SAX strong ion-exchange column (4.6 mm × 250 mm, 10 μm, ThermoHypersil-Keystone). Elution of the nucleotides was accomplished with a 30-min linear gradient from 5 mM $NH_4H_2PO_4$ (pH 2.8) to 750 mM $NH_4H_2PO_4$ (pH 3.7) buffer followed by 10 min of 750 mM $NH_4H_2PO_4$ (pH 3.7) at a flow rate of 2 mL/min. The fractions were collected at 1-min intervals, and radioactivity was determined with a scintillation counter.

3. Results

In this study, we examined the phosphorylation of dGuo, DXG, and their mono- and di-phosphates by a series of phosphotransferases. Kinetic constants, k_{cat} and K_m, were measured using steady state analysis. The catalytic efficiency for each substrate is expressed as k_{cat}/K_m. The relative phosphorylation efficiency for DXG and its nucleotides is expressed as a percentage of that of dGuo and its corresponding nucleotides. The results are summarized in Tables 2–4.

The dGK, dCK, and high K_m 5′-NT were tested for their ability to phosphorylate dGuo and DXG. All three of the enzymes phosphorylate the natural nucleoside dGuo efficiently. DXG did not serve as a substrate for dCK. DXG was phosphorylated by dGK at a rate of 0.025 s^{-1}, similar to the 0.030 s^{-1} rate for dGuo, however, the high K_m (7 mM) value of DXG revealed that the binding affinity of DXG to the enzyme was more than 4000-fold weaker than that of dGuo. As a result, the substrate specificity (k_{cat}/K_m) for DXG was only 0.02% of that of dGuo for this dGK catalyzed reaction. Both dGuo and inosine were shown to be good substrates for the high K_m 5′-NT, with K_m values of 1 and 3.7 mM, and k_{cat} values of 260 and 320 s^{-1}, respectively. Compared to dGuo, DXG was phosphorylated at a rate that was 4.3-fold slower than dGuo and was bound 5.6-fold weaker to the enzyme than dGuo. Overall, DXG was phosphorylated by 5′-NT at a level that was 4.2% of dGuo.

Among the three enzymes tested for the phosphorylation of DXG–MP, GMP kinase was the only enzyme that phosphorylated dGMP and DXG–MP. The K_m value for

Table 2
Phosphorylation of dGuo and DXG to dGMP and DXG–MP

Enzymes	dGuo			DXG			Percentage of dGuo[c]
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)	
dGK[a]	$(1.7 \pm 0.1) \times 10^{-3}$	0.030 ± 0.002	18	7.0 ± 0.5	0.025 ± 0.008	0.0036	0.02
dCK[a]	0.167 ± 0.004	2.7 ± 0.1	16	Not detectable	Not detectable	–	–
5′-NT (high K_m)[b]	1.0 ± 0.1	260 ± 10	260	5.6 ± 0.9	60 ± 10	11	4.2
Enzyme	Inosine			DXG			Percentage of inosine[d]
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)	
5′-NT (high K_m)[b]	3.7 ± 0.7	1040 ± 130	280	5.6 ± 0.9	60 ± 10	11	3.9

[a] ATP (2.5 mM) as the phosphate donor.
[b] IMP (5 mM) as the phosphate donor.
[c] Percentage of dGuo is defined as $(k_{cat}/K_m)_{DXG}/(k_{cat}/K_m)_{dGuo} \times 100\%$.
[d] Percentage of inosine is defined as $(k_{cat}/K_m)_{DXG}/(k_{cat}/K_m)_{inosine} \times 100\%$.

Table 3
Phosphorylation of dGMP and DXG–MP to dGDP and DXG–DP

Enzymes	dGMP			DXG–MP			Percentage of dGMP[b]
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)	
GMP kinase[a]	0.16 ± 0.02	7.3 ± 0.04	46	0.23 ± 0.01	0.108 ± 0.003	0.47	1.0
NMP kinase[a]	Not detectable	Not detectable	–	Not detectable	Not detectable	–	–
AMP kinase[a]	Not detectable	Not detectable	–	Not detectable	Not detectable	–	–

[a] ATP (4 mM) as the phosphate donor.
[b] Percentage of dGMP is defined as $(k_{cat}/K_m)_{DXG-MP}/(k_{cat}/K_m)_{dGMP} \times 100\%$.

Table 4
Phosphorylation of dGDP and DXG–DP to dGTP and DXG–TP

Enzymes	dGDP			DXG–DP			Percentage of dGDP[c]
	K_m (mM)	k_{cat}[a] (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)	K_m (mM)	k_{cat}[a] (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)	
NDP kinase[a] (bovine liver)	0.103 ± 0.013	70 ± 3	680	0.38 ± 0.05	0.31 ± 0.01	0.82	0.12
NDP kinase[b] (human, form A)	0.13 ± 0.02	970 ± 30	7460	1.0 ± 0.1	7.5 ± 0.3	7.5	0.10
3-PG kinase (Baker's yeast)	2.9 ± 0.3	14500 ± 300	5000	3.2 ± 0.3	260 ± 10	81	1.6
3-PG kinase (human)	2.0 ± 0.4	1900 ± 200	950	5.7 ± 1.0	47 ± 7	8.2	0.86
Creatine kinase (rabbit, type I)	6.6 ± 1.6	16 ± 2	2.4	3.1 ± 0.4	0.27 ± 0.02	0.088	3.7
Creatine kinase (human BB)	2.6 ± 0.4	45 ± 3	17	0.74 ± 0.12	5.9 ± 0.5	8.0	47
Creatine kinase (human MM)	3.3 ± 0.5	9.4 ± 0.6	2.8	1.4 ± 0.3	0.13 ± 0.01	0.093	3.3
Creatine kinase (human MB)	3.8 ± 0.7	114 ± 1	30	0.80 ± 0.02	2.0 ± 0.2	2.5	8.3
Pyruvate kinase (rabbit muscle)	7.8 ± 1.0	210 ± 20	27	5.4 ± 1.0	6.4 ± 0.4	1.2	4.4

[a] ATP (4 mM) as the phosphate donor.
[b] ATP (1 mM) as the phosphate donor.
[c] Percentage of dGDP is defined as $(k_{cat}/K_m)_{DXG-DP}/(k_{cat}/K_m)_{dGDP} \times 100\%$.

DXG–MP was 0.23 mM, similar to the 0.16 mM K_m value for dGMP. However, GMP kinase phosphorylated DXG–MP at a rate 67-fold slower and 1% as efficiently as dGMP, the natural substrate.

The phosphorylation of dGDP and DXG–DP were carried out by a series of enzymes including NDP kinase, 3-PG kinase, creatine kinase, and pyruvate kinase. DXG–DP was phosphorylated at efficiencies of 0.12, 0.10, 1.6, 0.86, and 4.4% of dGDP by bovine NDP kinase, human NDP kinase, Baker's yeast 3-PG kinase, human 3-PG kinase, and pyruvate kinase, respectively. For these five enzymes, the K_m values for DXG–DP were 0.7–7.7-fold higher than that of dGDP, where the reaction rate for DXG–DP was 33–226-fold slower than dGDP. Among the four creatine kinases tested, human BB-isoform showed the highest activity towards DXG–DP. Interestingly, even though all of the creatine kinases phosphorylated DXG–DP at a slower rate than dGDP, their apparent binding affinity for DXG–DP was 2.1–4.8-fold higher than dGDP.

The metabolism of [8-^{14}C]-DXG was studied in CEM cells after 6 and 24 h of incubation with DXG at a physiologically relevant concentration of 5 μM. The intracellular levels of DXG–MP, DP, and TP were measured using HPLC analysis. During incubation with 5 μM DXG, a considerable level of GTP was detected due to a minor contamination of [8-^{14}C]-guanine. Even though the [8-^{14}C]-DXG we used was >99% pure, it is not uncommon to observe a significant formation of [8-^{14}C]-GTP from a trace of [8-^{14}C]-guanine during studies of guanosine analogs [37]. Once in the cell, [8-^{14}C]-guanine is converted to GMP by hypoxanthine-guanine phosphoribosyltransferase, and guanine is a far better substrate for this enzyme than DXG is for the nucleoside kinases. As shown in Table 5, no DXG metabolite was detected at 6 h in cells treated with 5 μM DXG, and only very low concentrations of DXG–MP and TP were detected at 24 h, at levels of 0.006 and 0.015 pmole/10^6 cells, respectively. The presence of 5 μM of mycophenolic acid (MPA) significantly increased the DXG–MP, DP, and TP levels to 0.08, 0.23, and 0.28 pmole/10^6 cells, respectively, after 24 h incubation with 5 μM DXG. DXG–TP levels were increased approximately 19-fold higher than the non-MPA-treated control (Table 5). Purine nucleoside phosphorylase (PNP) is a key enzyme involving in the degradation of dGuo in vivo. In order to verify that guanine was not generated from DXG by PNP, we tested the effect of a potent inhibitor of PNP, 9-benzyl-9-deazaguanine (BDG) on the levels of GTP [38]. The levels of GTP in cells treated with 5 μM DXG and 1 μg/mL BDG were similar to the cells treated with 5 μM DXG only (data not shown), indicating that DXG was not degraded to guanine by PNP. In addition, BDG did not affect the metabolism of DXG to DXG–MP

Table 5
Anabolism of DXG in human CEM cells incubated with 5 μM DXG

Treatment of cells	Incubation time (h)	Intracellular concentration (pmole/10^6 cells)		
		DXG–MP	DXG–DP	DXG–TP
5 μM DXG	6	ND[a]	ND	ND
	24	0.006	ND	0.015[b]
5 μM DXG + 5 μM MPA	6	0.08	0.24	0.24
	24	0.08	0.23	0.28
5 μM DXG + 1 μg/mL BDG	6	ND	ND	ND
	24	0.006	ND	0.012

[a] ND: not detectable.
[b] Converted to concentration ≈7.5 nM, based on cell volume of 2.1 ml/10^9 cells for human lymphoblasts [47].

Table 6
Phosphorylation pathways of nucleoside analogues that are currently used for anti-HIV infection

Compounds	Nucleoside to 5′-MP	5′-MP to 5′-DP	5′-DP to 5′-TP
Emtricitabine	dCK	UMP-CMP kinase	NDP kinase?[a]
Lamivudine	dCK	UMP-CMP kinase	3-PG kinase
Zalcitabine	dCK	UMP-CMP kinase?	Creatine kinase
Abacavir	Adenosine phosphotransferase	Cytosolic deaminase and GMP kinase	Creatine kinase/ NDP kinase/ pyruvatekinase/ 3-PG kinase
Tenofovir	Not applicable	AMP kinase	Creatine kinase/ NDP kinase[b]
Didanosine	5′-NT/adenylate synthetase/ adenylate lysase	AMP kinase	NDP kinase?
Stavudine	Thymidine kinase	Thymidylate kinase?	Creatine kinase/ NDP kinase/ 3-PG kinase
Zidovudine	Thymidine kinase	Thymidylate kinase	NDP kinase?

[a] "?" indicated that the enzyme listed is based on speculation.
[b] Data based on the studies of adefovir.

or DXG–TP (Table 5). These data suggested that PNP is not a major player in the catabolism of DXG in vivo.

4. Discussion

The nucleoside reverse transcriptase inhibitors (NRTI) play critical roles in anti-HIV therapy. NRTIs use the cell's nucleoside phosphorylation pathways by sharing the same enzymes, which also phosphorylate their corresponding natural deoxynucleoside counterparts [1]. The phosphorylation of currently clinically used NRTIs to their 5′-MP has been relatively well studied (Table 6) [1]. However, the phosphorylation of NRTI 5′-MP to 5′-DP and then to 5′-TP is less known [38]. Moreover, it has been widely assumed that the last step, catalyzed by NDP kinase, is shared by most NRTIs, partially based on its broad substrate specificity for all four natural dNTPs [38]. However, recent studies showed that 3′-azido-3′-deoxythymidine (AZT)-DP [22], 2′,3′-dideoxyadenosine 5′-diphosphate (ddADP) [22], 2′,3′-dideoxythymidine 5′-diphosphate (ddTDP), and (−)-β-L-2′,3′-dideoxy-3′-thiacytidine (3TC)-DP [38] are all very poor [22] substrates of human NDP kinase. Even d4T-DP, the best ddNDP substrate for the enzyme reported so far, was phosphorylated at efficiency less than 0.09% of that of dTDP [20]. Crystal structure studies suggested that the 3′-hydroxyl group, which is lacking in many NRTIs, was critical for the activity of NDP kinase [22]. In addition, the catalytic efficiency for the phosphorylation of the unnatural L-configured ddNDP is much lower than their D-enantiomers [39]. Since an elevated intracellular concentration of didexoynucleotide 5′-triphosphate analogs (ddNTP) correlated with enhanced activity [1], understanding of the individual phosphorylation pathway of each nucleoside analog will help to predict and explain drug potency and drug interactions, and to modify NRTI structures to facilitate its activation.

dGK is an enzyme with a broad substrate specificity, which can phosphorylate all of the purine deoxyribonucleosides (dGuo, dAdo, and dIno) [10]. Studies have shown that dGK can phosphorylate many pharmacologically important analogs, including the dGuo analogs penciclovir and ganciclovir, which were phosphorylated at levels of 10 and 1% of that of dGuo [13,40]. However, dGK cannot phosphorylate 2′,3′-dideoxyadenosine (ddA), 2′,3′-dideoxyguanosine (ddG) [13,41], or guanosine analogs such as acyclovir, lobucavir, and 3′-fluoro-2′,3′-dideoxyguanosine (FLG) [41]. In this study, we showed that DXG was phosphorylated by human dGK at a similar rate as dGuo. However, the apparent binding affinity of DXG to dGK was over 4000-fold weaker than dGuo. Although the naturally occurring deoxynucleoside, dGuo, can be phosphorylated efficiently by human dCK, DXG does not serve as a substrate for the enzyme. This observation is in partial agreement with an earlier study by Kierdaszuk et al. [33] where human dCK was completely inactive towards dGuo analog 2′,3′-dideoxyguanosine (ddGuo).

Similar to previous reports [6,42–45], inosine was phosphorylated by 5′-NT with a K_m of 3.7 mM. However, contrary to earlier studies, which showed that dGuo was a poor substrate of 5′-NT with a high K_m (~13 mM) and a very low V_{max} [42,45], we found that dGuo was phosphorylated by 5′-NT as efficiently as inosine, with a K_m of 1 mM. These differences could be due to differences in enzyme preparation. The catalytic efficiency for the phosphorylation of dGuo, inosine, and DXG by 5′-NT is much higher than the phosphorylation of dGuo and DXG by dGK. Due to the ubiquitous presence of 5′-NT in human tissues and the relatively high substrate activity of DXG as shown in this study, 5′-NT is likely to play a key role in the activation of DXG both in vitro and in vivo.

Among all of the enzymes tested for the phosphorylation of DXG–DP, the human BB-isoform of creatine kinase showed the highest *relative* substrate specificity (47% of dGDP) for DXG–DP. In addition, 3-PG kinase and NDP kinase had the highest catalytic efficiency for the phosphorylation of the natural dGDP, even though the relative catalytic efficiency for DXG–DP phosphorylation by human 3-PG kinase and NDP kinase is only 0.10 and 0.86% of that of dGDP, which were much less than many of the enzymes tested in Table 4. Bourdais et al. [22] reported that AZT-DP, ddADP, and ddTDP were very poor sub-

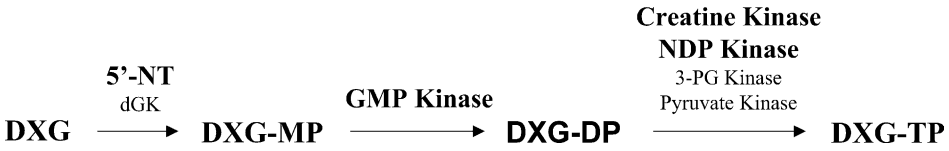

Fig. 2. Phosphorylation pathway for DXG. The most important enzymes are shown in bold.

strates of NDP kinase (type B), mainly due to a significant decrease in rate, instead of binding. Similarly, in this study, there was a 230-fold decrease in the rate (k_{cat}) and only a 3.7-fold decrease in the binding affinity (K_m) for the phosphorylation of DXG–DP by human NDP kinase, compared to the phosphorylation of dGDP. It is worth mentioning that even though DXG–DP is phosphorylated by human NDP kinase at an efficiency that is only 0.1% of dGDP, the inherent high activity of this enzyme warrants its important role in the activation of DXG.

The cellular anabolism profile of a nucleoside analog provides useful information in determining the rate-limiting step(s) involved in its activation pathway. Previously, we reported the intracellular level of DXG–TP to be 4.4 and 1.8 pmole/10^6 cells, respectively, in CEM and stimulated PBMC cells after 24 h of incubation with 5 μM [8-^{3}H]-DXG [2]. However, later studies using a highly sensitive Liquid Chromatography Mass Spectrometry (LC-MS-MS) method indicated that the level of DXG–TP was only 0.016 pmole/10^6 for stimulated PBMC cells after 24 h incubation with 5 μM of DXG (personal communication with Dr. Robert St. Claire III, Gilead Sciences). In this study, we re-investigated the anabolism of DXG using [8-^{14}C]-DXG and found that the levels of DXG metabolites were similar to the levels detected by LC-MS-MS. The identities of the DXG metabolites were further confirmed by their dramatic increase after the cells were treated with MPA, a known stimulator of DXG anabolism [9]. The high level of DXG metabolites reported in our previous study is likely due to contaminant of [8-^{3}H]-guanine and inadequate HPLC resolution of [8-^{3}H]-DXG–TP and [8-^{3}H]-GTP (anabolite of [8-^{3}H]-guanine). In this assay, we also detected radio-labeled guanine nucleotides, but they were sufficiently separated from the DXG-nucleotides. In the presence of 5 μM MPA, the level of DXG–TP was increased 19-fold. This is consistent with the enhancing effect of MPA on DXG anti-viral activity and its stimulatory effect on DXG–TP levels observed in PBMC [9,46]. MPA inhibits IMP dehydrogenase and results in increased intracellular IMP levels and decreased GMP levels. Since IMP is the phosphate donor in the phosphorylation of DXG by high K_m 5′-NT, the observed enhancing effect of MPA further support the hypothesis that high K_m 5′-NT is one of the key enzymes for DXG activation. Furthermore, decreased GMP synthesis by MPA led to reduced dGTP levels and could augment the activation and anti-viral activity of analogs of dGuo [9]. The fact that DXG–TP was the primary metabolite in cell culture and conversion of DXG to DXG–MP was enzymatically inefficient suggests that the rate-limiting step in the DXG phosphorylation pathway is the addition of the first phosphate. A different HPLC profile was observed for cells treated with both DXG and MPA and the level of metabolites followed the order of DXG–TP = DXG–DP > DXG–MP. However, it should be interpreted with caution since cell growth was inhibited by MPA at the level tested.

Direct comparison of the catalytic efficiency (k_{cat}/K_m) between 5′-NT and GMP kinase indicates that the second step of phosphorylation is 23-fold less efficient than the first step, and therefore, the rate-limiting step in the phosphorylation pathway is not the conversion of DXG to DXG–MP. This apparent contradiction between the enzymatic study and the cellular assays could be due to the differences in the concentration of each enzyme and possible involvement of other metabolic enzymes in cell.

Our enzymatic and cellular study demonstrated that high K_m 5′-NT, GMP kinase, creatine kinase, and NDP kinase are likely to play important roles in the phosphorylation pathway of DXG (Fig. 2). Enzymes like dGK, 3-PG kinase, and pyruvate kinase could also contribute to the activation of DXG. This study also provides valuable information on the phosphorylation pathway of dGuo analogs, which is currently limited compared to the data available on the phosphorylation pathway of other nucleoside analogs.

Acknowledgements

We thank Dr. Staffan Eriksson for the generous gifts of human recombinant dGK and dCK enzymes. We also thank Dr. Josef Spychala and Dr. Beverly Mitchell for providing the clone for high K_m 5′-NT. The authors are especially grateful for the insightful discussion with Dr. Josef Spychala, Dr. Wayne Miller, Dr. Robert Hart, and Michelle Brosnan-Cook. DDB was recipient of a grant from ANRS (Agence Nationale de Recherche contre le Sida).

References

[1] Stein DS, Moore KHP. Phosphorylation of nucleoside analog antiretrovirals: a review for clinicians. Pharmacol Ther 2001;21: 11–34.

[2] Furman PA, Jeffery J, Kiefer L, Feng JY, Anderson KS, Borroto-Esoda K, et al. Mechanism of action of 1-b-D-2,6-diaminopurine dioxolane, a prodrug of the human immnuodeficiency virus type 1 inhibitor 1-b-D-dioxolane guanosine. Antimicro Agents Chemother 2001;45:158–65.

[3] Wang LH, Bigley JW, Brosnan-Cook M, Sista ND, Rousseau F. The disposition of DAPD and it active metabolite DXG in therapy naive and experienced HIV-infected subjects (Abstract 752). In: Proceedings of the 8th Conference on Retroviruses and Opportunistic Infections. . Chicago, IL, February 4–8, 2001; Foundation for Retrovirology and Human Health; 2001. p. 272.
[4] Mewshaw JP, Myrick FT, Wakefield DA, Hooper BJ, Harris JL, McCreedy B, et al. Dioxolane guanosine, the active form of the prodrug diaminopurine dioxolane, is a potent inhibitor of drug-resistant HIV-1 isolates from patients failing standard nucleoside therapy. J AIDS 2002;29:11–20.
[5] Faletto MB, Miller WH, Garvey EP, St Clair MH, Daluge SM, Good SS. Unique intracellular activation of the potent anti-human immunodeficiency virus agent 1592U89. Antimicrob Agents Chemother 1997;41:1099–107.
[6] Miller WH, Daluge SM, Garvey EP, Hopkins S, Reardon JE, Boyd FL, et al. Phosphorylation of carbovir enantiomers by cellualr enzymes determines the stereoselectivity of antiviral activity. J Biol Chem 1992;267:21220–4.
[7] Miller WH, Miller LR. Phosphorylation of acyclovir (acycloguanosine) monophosphate by GMP kinase. J Biol Chem 1980;255:7204–7.
[8] Matthews T, Boehme R. Antiviral activity and mechanism of action of ganciclovir. Rev Infect Dis 1988;10:490–4.
[9] Myrick F, St Clair R, Hart R, Borroto-Esoda K. Effect of mycophenolic acid on intracellular dioxolane guanosine triphosphate levels in PBMC (Abstract #43). In: Proceedings of the 16th International Conference on Antiviral Research. . Savannah, GA, April 27–May 1, 2003; 2003.
[10] Eriksson S, Wang L. The role of the cellular deoxynucleoside kinases in activation of nucleoside analogs used in chemotherapy. In: Chu CK, editor. Recent advances in nucleosides: chemistry and chemotherapy. New York: Elsevier; 2002.
[11] Zhu C, Johansson M, Permert J, Karlsson A. Phosphorylation of anticancer nucleoside analogs by human mitochondrial deoxyguanosine kinase. Biochem Pharmacol 1998;56:1035–40.
[12] Spychala J, Chen V, Oka J, Mitchell BS. ATP and phosphate reciprocally affect subunit association of human recombinant high Km 5′-nucleotidase: role for the C-terminal polyglutamic acid tract in subunit association and catalytic activity. Eur J Biochem 1999;259: 851–8.
[13] Arner ESJ. Eriksson S. Mammalian deoxyribonucleoside kinases. Pharmacol Ther 1995;67:155–86.
[14] Brady WA, Kokoris MS, Fitzgibbon M, Black ME. Cloning, characterization, and modeling of mouse and human guanylate kinases. J Biol Chem 1996;271:16734–40.
[15] Sekulic N, Shuvalova L, Spangenberg O, Konrad M, Lavie A. Strutural characterization of the closed conformation of mouse guanylate kinase. J Biol Chem 2002;277:30236–43.
[16] Strominger JL, Heppel LA, Maxwell ES. Nucleoside monophosphate kinases: I. Transphophorylation between adenosine triphosphate and nucleoside monophosphates. Biochim Biophys Acta 1959;32:412.
[17] Noda L. Nucleoside triphosphate-nucleoside monophosphokinases. New York: Academic Press; 1962.
[18] Van Rompay AR, Johansson M, Karlsson A. Identification of a novel human adenylate kinase: cDNA cloning, expression analysis, chromosome localization and characterization of the recombinant protein. Eur J Biochem 1999;261:509–16.
[19] Henderson JF, Paterson ARP. Deoxyribonulceoside metabolism. In: Nucleotide metabolism: an introduction. New York: Academic Press; 1973 (pp. 65–67, Chapter 14).
[20] Schneider B, Biondi R, Sarfati R, Agou F, Guerreiro C, Deville-Bonne D, et al. The mechanism of phosphorylation of anti-HIV D4T by nucleoside diphosphate kinase. Mol Pharmacol 2000;57:948–53.
[21] Gallois-Montbrun S, Schneider B, Chen Y, Giacomoni-Fernandes V, Mulard L, Morera S, et al. Improving nucleoside diphosphate kinase for antiviral nucleotide analogs activation. J Biol Chem 2002;277: 39953–9.
[22] Bourdais J, Biondi R, Sarfati S, Guerreiro C, lascu I, Janin J, et al. Cellular phosphorylation of anti-HIV nucleosides. J Biol Chem 1996;271:7887–90.
[23] Krishnan P, Fu Q, Lam W, Liou J-Y, Dutschman G, Cheng Y-C. Phosphorylation of pyrimidine deoxynucleoside analog diphosphates. J Biol Chem 2002;277:5453–9.
[24] Miller WH, Miller LR. Phosphorylation of acyclovir diphosphate by cellular enzymes. Biochem Pharmacol 1982;31:3879–84.
[25] Mori N, Singer-Sam J, Riggs AD. Evolutionary conservation of the substrate-binding cleft of phosphoglycerate kinases. FEBS Lett 1986;204:313–7.
[26] Wyss M, Kaddurah-Daouk R. Creatine and creatine metabolism. Physiol Rev 2000;80:1107–213.
[27] Cihlar T, Chen MS. Identification of enzymes catalyzing two-step phosphorylation of cidofovir and the effect of cytomegalovirus infection on their activities in host cells. Mol Pharmacol 1996;50: 1502–10.
[28] Merta A, Votruba I, Jindrich J, Holy A, Cihlar T, Rosenberg I, et al. Phosphorylation of 9-(2-phosphonomethoxyethyl)adenine and 9-(S)-(3-hydroxy-2-phosphonomethoxypropyl)adenine by AMP(dAMP) kinase from L1210 cells. Biochem Pharmacol 1992;44:2067–77.
[29] Parker WB, Shaddix SC, Vince R, Bennett LLJ. Lack of mitochondrial toxicity in CEM cells treated with carbovir. Antiviral Res 1997;34: 131–6.
[30] Merkenschlager M, Kardamakis D, Rawle FC, Spurr N, Beverly PCL. Rate of incorporation of radio-labelled nucleosides dose not necessarily reflect the metabolite state of cells in culture: effects of latent mycoplasma contamination. Immunology 1988;63:125–31.
[31] Wang J, Choudhury D, Chattopadhyaya J, Eriksson S. Stereoisomeric selectivity of human deoxyribonucleoside kinases. Biochemistry 1999;38:16993–9.
[32] Schneider B, Xu YW, Sellam O, Sarfati R, Janin J, Veron M, et al. Presteady state of reaction of nucleoside diphosphate kinase with anti-HIV nucleotides. J Biol Chem 1998;273:11491–7.
[33] Kierdaszuk B, Bohman C, Ullman B, Eriksson S. Substrate specificity of human deoxycytidine kinase toward antiviral 2′,3′-dideoxynucleoside analogs. Biochem Pharm 1992;43:197–206.
[34] Gerhardt W. Creatine kinase. Bergmeyer HU, Bergmeyer J, Grabl M, editors. Methods of enzymatic analysis. Deerfield Beach, FL: Verlag Chemie; 1983. p. 509.
[35] Miller HW, Beauchamp LM, Meade E, Reardon JE, Biron KK, Smith AA, et al. Phosphorylation of ganciclovir phosphonate by cellular GMP kinase determines the stereoselectivity of anti-human cytomegalovirus activity. Nucleosides Nucleotides Nucleic Acids 2000;19: 341–56.
[36] Puck TT, Cieciura SJ, Robinson A. Genetics of somatic mammalian cells III long-term cultivation of euploid cells from human and animal subjects. J Exp Med 1958;108:945–56.
[37] Parker WB, Shaddix SC, Bowdon BJ, Rose LM, Vince R, Shannon WM, et al. Metabolism of carbovir, a potent inhibitor of human immunodeficiency virus type 1, and its effects on cellular metabolism. Antimicrob Agents Chemother 1993;37:1004–9.
[38] Parker WB, Allan PW, Niwas S, Montgomery JA, Bennett LLJ. Effect of 9-benzyl-9-deazaguanine, a potent inhibitor of purine nucleoside phosphorylase, on the cytotoxicity and metabolism of 6-thio-2′-deoxyguanosine. Cancer Res 1994;54:1742–5.
[39] Kreimeyer A, Schneider B, Sarfati R, Faraj A, Sommadossi JP, Veron M, et al. NDP kinase reactivity towards 3TC nucleotides. Antiviral Res 2001;2001:147–56.
[40] Sjonerg Herrstrom A, Wang L, Eriksson S. Substrate specificity of human recombinant mitochondrial deoxyguanosine kinase with cytosolic and antiviral purine and pyrimidine analogs. Mol Pharmacol 1998;53:270–3.
[41] Sjonerg Herrstrom A, Wang L, Eriksson S. Antiviral guanosine analogs as substrate for deoxyguanosine kinase: implication for chemotherapy. Antimicrob Agents Chemother 2001;45.

[42] Keller PM, McKee SA, Fyfe JA. Cytoplasmic 5′-nucleotidase catalyzes acyclovir phosphorylation. J Biol Chem 1985;260:8664–7.
[43] Johnson MA, Fridland A. Phosphorylation of 2′,3′-dideoxyinosine by cytosolic 5′-nulceotidase of human lymphoid cells. Mol Pharmacol 1989;36:291–5.
[44] Saunders PP, Spindler CD, Tan MT, Alvarez E, Robins RK. Tiazofurin is phosphorylated by three enzymes from Chinese hamster ovary cells. Cancer Res 1990;50:5269–74.
[45] Pesi R, Turriani M, Allegrini S, Scolozzi C, Camici M, Ipata PL, et al. The bifunctional cytosolic 5′-nucleotidase: regulation of the phosphotransferase and nucleotidase activities. Arch Biochem Biophys 1994; 312:75–80.
[46] Borroto-Esoda K, Myrick F, Feng JF, Jeffrey JL, Furman PA. In vitro combination of amdoxovir (DAPD) and the inosine monophosphate dehydrogenase inhibitors mycophenolic acid and ribavirin demonstrates potent activity against wild type and drug resistant variants of HIV-1. Antimicrob Agents Chemother 2004, in press.
[47] Traut T. Physiological concentrations of purines and pyrimidines. Mol Cell Biochem 1994;140:1–22.