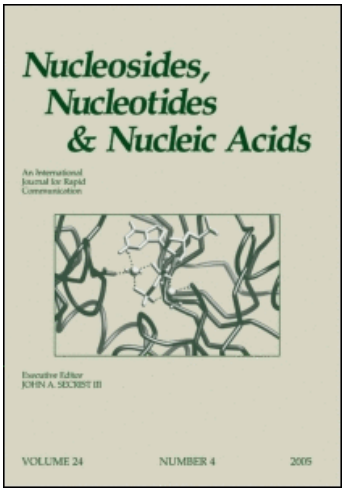

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Nucleoside Diphosphate Kinase and the Activation of Antiviral Phosphonate Analogs of Nucleotides: Binding Mode and Phosphorylation of Tenofovir Derivatives

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NUCLEOSIDE DIPHOSPHATE KINASE AND THE ACTIVATION OF ANTIVIRAL PHOSPHONATE ANALOGS OF NUCLEOTIDES: BINDING MODE AND PHOSPHORYLATION OF TENOFOVIR DERIVATIVES

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□ *Tenofovir is an acyclic phosphonate analog of deoxyadenylate used in AIDS and hepatitis B therapy. We find that tenofovir diphosphate, its active form, can be produced by human nucleoside diphosphate kinase (NDPK), but with low efficiency, and that creatine kinase is significantly more active. The 1.65 Å x-ray structure of NDPK in complex with tenofovir mono- and diphosphate shows that the analogs bind at the same site as natural nucleotides, but in a different conformation, and make only a subset of the Van der Waals and polar interactions made by natural substrates, consistent with their comparatively low affinity for the enzyme.*

Keywords Nucleoside reverse transcriptase inhibitor; nucleotide binding; nucleotide conformation; phosphorylation

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1. INTRODUCTION

Nucleoside reverse transcriptase inhibitors (NRTI) are nucleoside analogs that lack a 3′-hydroxyl group. Compounds such as azidothymidine (AZT) and dideoxynucleosides are potent inhibitors of HIV replication and play an essential part in antiretroviral therapies. However, they are only prodrugs that must be converted to the fully phosphorylated forms in order to be incorporated by reverse transcriptase.[1] The conversion relies on cellular kinases, but the analogs are generally poor substrates for these enzymes, with the consequence that the active forms remain at low concentration relative to the natural deoxynucleoside triphosphates (dNTP) substrates, which greatly affects their antiviral potency. Acyclic nucleoside phosphonate analogs have become a major class of antiviral nucleoside derivatives.[2] Among them, tenofovir (also called PMPA: [R]-9-[2-(phosphonomethoxy)propyl]adenine), administrated clinically as tenofovir disoproxil fumarate to enhance cellular availability, has proved to be well tolerated and efficient in combination with other drugs in large scale HIV studies,[3] and it is also approved for treatment against hepatitis B virus.[4] Remarkably, tenofovir retains potency against HIV strains carrying several of the mutations that cause resistance to other NRTI, and resistance to tenofovir itself appears to be comparatively difficult to select;[5,6] it may even be fully suppressed with an α−borano analog.[7]

Tenofovir is an analog of deoxyadenylate (dAMP) in which the adenine base is linked to an acyclic methoxypropyl group instead of a deoxyribose, and the phosphonate group replaces the α-phosphate (Figure 1). Its diphosphate derivative binds HIV reverse transcriptase like the natural substrate dATP,[8] and is incorporated by the enzyme while base-pairing with uracil or thymine, causing termination of the DNA chain. In the cell, tenofovir behaves as an analog of dAMP, and the monophosphate as an analog of dADP. The active diphosphate form is found in abundance in dendritic and Langherans cells, the first cell types targeted by a primary HIV infection.[9] The activation process requires a kinase that can add a γ-phosphate onto the β-phosphate of dADP. Nucleoside diphosphate kinase (NDPK), a ubiquitous enzyme, efficiently performs that reaction on all natural NDP and dNDP, but it is extremely poor at phosphorylating

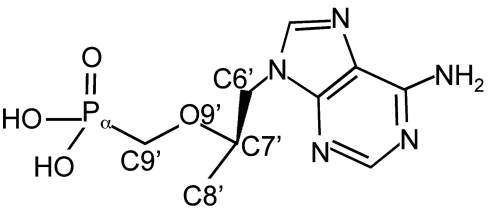

FIGURE 1 Chemical formula of tenofovir.

NRTI derivatives, because the 3′OH of the substrate sugar moiety plays a major role in its catalytic mechanism.[10,11] As a result, the phosphorylation of dideoxyADP and of AZT diphosphate by NDPK is 10^4 or 10^5-time slower than for dADP or TDP,[12,13] which explains the low level of the triphosphate forms found in cells treated with these NRTI. We show here that this is also true for tenofovir monophosphate, which NDPK can convert to the diphosphate form, but with a low efficiency relative to a natural substrate. Furthermore, we demonstrate that other phosphotransferases including creatine kinase and pyruvate kinase, can produce tenofovir diphosphate more efficiently, and may therefore contribute to the activation process.

X-ray structures are available for a number of NDPKs including the two major human isozymes NDPK-A and B, and for the complexes with several natural substrates and phosphorylated NRTI derivatives.[14] All of them show the same protein fold, which assembles into a homohexamer, except in some bacterial enzymes that are tetramers. The substrate nucleotide binding site is highly conserved, and the ligands that have been tested in crystallographic experiments all bind similarly with few exceptions. The present study describes a complex of tenofovir mono- and diphosphate with the enzyme from *Dictyostelium discoideum* (Dd-NDPK). It shows that the analogs also bind at that site, but in a conformation not found with normal substrates, and which may contribute to their inefficient processing by the enzyme.

2. MATERIALS AND METHODS

2.1. Enzyme Expression and Activity

Recombinant human NDP kinase A (NDPK-A/NM23-H1) and the H122G mutant of *Dictyostelium discoideum* NDP kinase (Dd-NDPK) were expressed in *E. coli* and purified to homogeneity as described.[15,16] Natural nucleotides were from Sigma. Tenofovir and its mono- and diphosphate derivatives were synthesized by Trilink Biotechnologies (San Diego, CA, USA). Human BB, MB, and MM creatine kinases (C9983, C0984, and C9858), and rabbit muscle pyruvate kinase type II (PI506) were purchased from Sigma (St. Louis, MO, USA). Human 3-phosphoglycerate kinase was a generous gift from Dr. Preethi Krishnan and Dr. Y.C. Cheng (Yale University, New Haven, CT, USA).

The phosphorylation of tenofovir monophosphate by NDPK-A (40 μg/mL) was carried out in Tris acetate buffer pH 7.5, 5 mM $MgCl_2$, at 37°C. The donor substrate was ATP (1 mM). Aliquots (100 μl) were removed every 2 minutes and quenched with 5 μl 3.5–3.8% HCl (final pH 3–4). The reaction products were subjected to HPLC analysis as previously described.[17] Since dADP and dATP could not be sufficiently separated

on HPLC from the more abundant ADP and ATP, a boronate column was used to remove ATP and ADP from the reaction mixture prior to HPLC analysis.[18] More than 98% of dADP and dATP were recovered, and more than 99% of ATP and ADP were removed from the reaction mixture. All enzymatic reactions involving creatine kinase, pyruvate kinase, and 3-phosphoglycerate kinase were conducted as previously described.[17]

2.2. Crystallization and X-Ray Structure Determination

Crystals of the H122G mutant protein of Dd-NDPK at 8 mg/ml in the presence of 10 mM tenofovir diphosphate were obtained in hanging drops containing 12% (w/v) PEG 1000 and 100 mM Hepes pH 7.5 over pits consisting of 26% PEG 1000 in the same buffer. Crystals were flash-frozen in the mother liquor and 20% glycerol. Data collection was carried out at 100 K on beamline ID14-H1 at the European Synchrotron Radiation Facility (Grenoble, France); 270° of data were collected in 1° frames, with 1 second per frame exposure. Diffraction intensities to 1.65 Å resolution were evaluated with the program MOSFLM[19] and further processed using the CCP4 (Collaborative Computational Project Number 4, 1994) program suite. Data collection and processing statistics are given in Table 1.

The crystals contain a hexamer in their asymmetric unit and, as they are isomorphous to previously solved structures of Dd-NDPK, preliminary phases could be calculated using coordinates from PDB entry 1S5Z.[20]

TABLE 1 Crystallographic data and refinement statistics

Space group	$P2_1$
Unit-cell parameters (Å)	$a = 69.4$, $b = 104.6$, $c = 69.2$, $\beta = 118°$
Resolution (Å)	20–1.65 (1.74–1.65)
No. of observed reflections	424591 (61272)
No. of unique reflections	104433 (15223)
R_{sym} (%)[a]	7.5 (35.7)
Completeness (%)	99.8 (100)
I/σ	7.6 (1.8)
R_{cryst} (%)[b]	18.1
R_{free} (%)[c]	20.5
rms bond deviation (Å)	0.004
rms angle deviation (°)	1.29
Average B (Å^2)	
Protein atoms	16.0
Tenofovir diphosphate	38.2
Tenofovir monophosphate	26.7
Solvent	24.4

Values for the highest resolution shell are in parentheses

[a] $R_{sym} = \Sigma_{hkl}\Sigma_i|I_i(hkl) - <I(hkl)>|/\Sigma_{hkl}\Sigma I_i$ (hkl), where $I_i(hkl)$ is the i th observed amplitude of reflection hkl and $<I(hkl)>$ is the mean amplitude for all observations i of reflection hkl.

[b] Rcryst = Σ ||Fobs|—|Fcalc||/Σ |Fobs|

[c] 5% of the data were set aside for free R-factor calculation.

The resulting electron density map showed the presence of a ligand in all six subunits. After minor modifications of the model, refinement was performed using CNS,[21] leading to the statistics reported in Table 1. The atomic coordinates and structure factors have been deposited in the Protein Data Bank (PDB, http://www.rcsb.org) as entry 3FKB.

2.3. Binding Site Comparisons

We used the MED-SuMo software to compare ligand binding sites in NDPK and other proteins in the Protein Data Bank. This software, developed by MEDIT SA (Palaiseau, France) implements the SuMo algorithm[22,23] to identify sets of surface chemical features (SCF) on a protein. A SCF is a chemical group with a character relevant to ligand binding, such as aromaticity, hydrophobicity, electric charge, or H-bond donor or acceptor capacity. SCFs closest to each other in space are assembled into triangles and a graph is built by connecting adjacent triangles that share at least one edge. A graph matching algorithm can then be used to efficiently compare two protein surfaces or ligand binding sites, and MED-SuMo generates three-dimensional superimpositions based on the match. A score is calculated by giving each matched SCF a weight that depends on its chemical character and its distance to neighbouring SCFs[22] and by summing the weights.

3. RESULTS

3.1. Tenofovir Monophosphate as a Substrate of NDPK and Other Kinases

Purified recombinant human NDPK-A was able to phosphorylate tenofovir monophosphate in vitro with ATP as the phosphate donor. The steady-state kinetic parameters for that reaction are reported in Table 2, and they can be compared to those obtained with dADP, a natural substrate, using the same protein sample. The K_m values are similar, but the turnover is much lower with the analog, and the catalytic efficiency measured by k_{cat}/K_m is three orders of magnitude less. Thus, tenofovir monophosphate is a substrate of NDPK-A, albeit a poor one.

When other kinases were tested, no phosphorylation of tenofovir monophosphate was detected using human 3′-phosphoglycerate kinase, but rabbit muscle pyruvate kinase displayed about the same efficiency as NDPK-A in terms of k_{cat}/K_m, and all three isozymes of human creatine kinase (BB, MB, and MM) proved to be orders of magnitude more active. Table 2 shows that the BB isozyme has a high k_{cat} with both dADP and tenofovir monophosphate, and a k_{cat}/K_m ratio above 3×10^5 $M^{-1}s^{-1}$ for the analog

TABLE 2 Steady-state kinetic parameters for the enzymatic phosphorylation of tenofovir monophosphate

Enzyme Acceptor substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}.s^{-1}$)	Ratio[a]
NDPK-A[b]				
dADP	0.18 ± 0.03	65 ± 3	3.6×10^5	
Tenofovir monophosphate	0.29 ± 0.03	0.12 ± 0.01	410	880
Pyruvate kinase[c]				
dADP	3.0 ± 0.4	280 ± 20	9.3×10^4	
Tenofovir monophosphate	11 ± 3	6.8 ± 2.3	620	150
Phosphoglycerate kinase[d]				
dADP	2.0 ± 0.3	103 ± 7	5.2×10^4	
Tenofovir monophosphate	ND[e]	ND[e]	-	
Creatine kinase[f]				
dADP	0.08 ± 0.01	1030 ± 30	13×10^6	
Tenofovir monophosphate	1.2 ± 0.20	400 ± 30	3.3×10^5	39

[a]Ratio of k_{cat}/K_m for dADP versus the analog.

[b]The donor substrate is ATP (1 mM).

[c]Rabbit muscle pyruvate kinase type II (Sigma PI506); the donor substrate is phospho(enol)pyruvate (4 mM).

[d]Human 3-phosphoglycerate kinase; the donor substrate is DL-glyceraldehyde 3-phosphate (4 mM).

[e]No product was detected.

[f]Human BB isozyme (Sigma C9983); the MM and MB isozymes yielded similar results; the donor substrate is creatine phosphate (20 mM).

in spite of its higher K_m. Similar k_{cat}/K_m ratios were obtained with the MB and BB isozymes with tenofovir monophosphate as substrate.

3.2. NDPK Structure in the Crystalline Complex

The protein used for crystallization was the H122G mutant of *Dictyostelium discoideum* NDPK. Dd-NDPK, the first NDPK to have a crystal structure determined,[24] is easy to overexpress and crystallize, and has over 60% sequence identity with human NDPK-A and NDPK-B, which are the product of the *Nme1/Nm23-H1* and *Nme2/Nm23-H2* genes, and the two major isozymes found in human cells. All three proteins are very close in structure, especially at the substrate binding site, and they have similar enzymatic characteristics.[10] The NDPK catalytic mechanism operates in two steps: the $\gamma-$phosphate group of the donor substrate (usually ATP) is transferred first onto a histidine (His 122 in Dd-NDPK, His 118 in NDPK-A), and then from the phosphohistidine onto the acceptor nucleotide that replaces the donor and occupies the same site. The H122G substitution prevents the transfer altogether, which makes the mutant useful when structural studies are done in the presence of ATP or another donor substrate that would transfer part of its phosphate to the histidine, resulting in a mixture of chemical species at the active site. The substitution has no

observable structural effect, and the H122G protein efficiently phosphorylates externally supplied imidazole.[16]

In the crystals of the complex, the protein structure is well defined by the 1.65 Å resolution electron density for 150 of the 155 residues in the polypeptide chains, except for N-terminal residues 1–5. These residues are disordered like in other crystal structures of Dd-NDPK. The asymmetric unit contains six subunits labeled A to F for convenience. Each provides independent information, and differences that affect the bound ligand are described below. Nevertheless, the subunits have essentially identical structures, with a RMSD (root-mean-square distance after least-square superimposition) of 0.3–0.6 Å over all the 150 Cα. They are also very similar to wildtype Dd-NDPK subunits in complex with natural nucleotides. For instance, the Cα RMSD is 0.4–0.7 Å with subunits of the ADP-BeF_3 complex (PDB entry 2BEF), where BeF_3 (beryllium fluoride) sits in between the β-phosphate of ADP and the imidazole group of His 122, mimicking the γ-phosphate of ATP.[25] Thus, the absence of the H122 side chain and the presence of a nucleotide analog in the present structure have little effect on the protein conformation.

3.3. The Bound Ligand

Electron density indicates that the ligand binding site is occupied in all six subunits. As shown in Figure 2 for subunit A, the density fits tenofovir monophosphate but the expected position of the second phosphate is empty. Thus, we built the ligand as the monophosphate in subunit A and in four other subunits. Well-defined density also indicates the presence of an Mg^{++} ion that ligates oxygens of the phosphonate, of the first phosphate (called below the $\beta-$phosphate by analogy to natural substrates), and of four water molecules in the octahedral geometry that is usual for Mg^{++}. Subunit D contains electron density that extends beyond the $\beta-$phosphate, and in which either a $\gamma-$phosphate or inorganic phosphate can be placed. We chose to build tenofovir diphosphate in that subunit, but the presence of a mixture containing inorganic phosphate and tenofovir mono- and diphosphate cannot be excluded. Thus, the electron density seen at the six ligand binding sites implies that tenofovir diphosphate has been partly hydrolyzed on the time scale of the crystallographic experiment, a plausible event given that the H122G mutant has low, but detectable ATPase activity.[16]

Table 3 reports the polar interactions and Van der Waals contacts that tenofovir monophosphate makes in subunit A, and the diphosphate in subunit D. The protein residues involved are shown in Figure 2. Whereas the base and the linker make only nonpolar contacts, one of the phosphonate oxygens receives two H-bonds from Lys 16 and Asn 119, and the phosphate groups make H-bonds or salt bridges with the side chains of His 59, Arg 92,

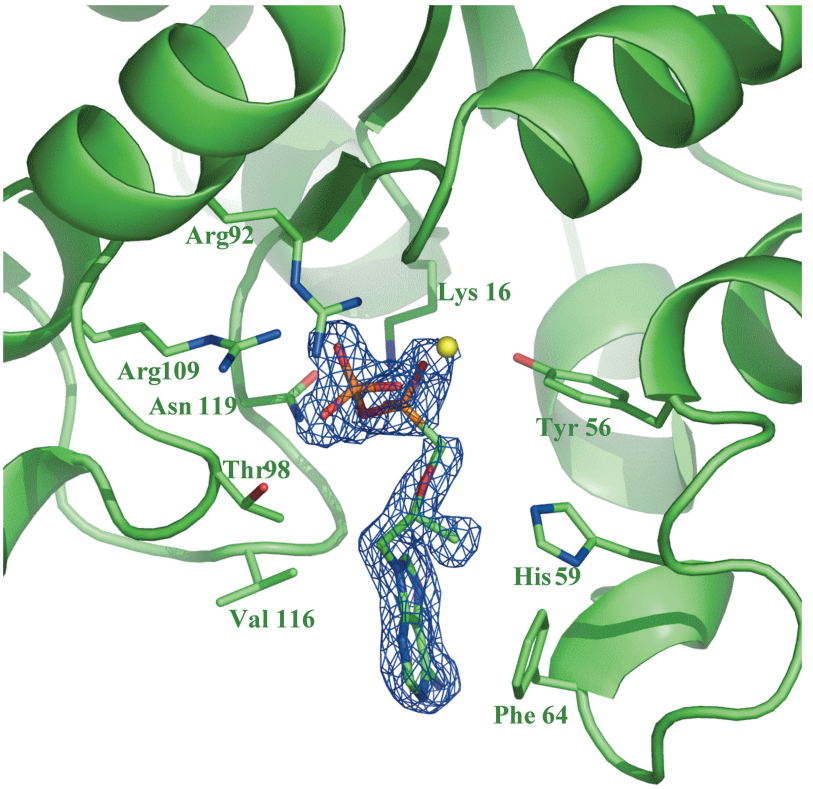

FIGURE 2 Tenofovir monophosphate bound to Dd-NDPK. The 2Fo-Fc electron density for tenofovir monophosphate in subunit A is contoured at 1.5 σ. The ligand and the side chains in contact with it are drawn as sticks colored by atom type, and the Mg^{++} ion, as a yellow ball.

Thr 98, and Arg 109. These residues are the same that interact with the phosphates of ADP in wildtype Dd-NDPK.[26] Thus, the analog occupies essentially the same site. Nevertheless, Figure 3 shows that it binds in a quite different conformation. The adenine base is sandwiched between the side chains of Phe 64 and Val 116 as in NDPK-bound ADP, but it is oriented in the opposite way. The methoxypropyl linker is well defined in the electron density and it follows a different path from the ribose. The Pα atoms of ADP and of the tenofovir phosphonate are 3.5 Å apart, but the Pβ atoms are within 0.3 Å of each other, and Mg^{++} is bound in the same way, offering the possibility for a phosphate group on His 122 to be transferred to the tenofovir β-phosphate. However, the phosphonate group of the bound analog overlaps with the expected position of the phosphohistidine phosphate, which is where BeF_3 is located in the ADP-BeF_3 complex and, therefore, the mode of binding seen in the crystal cannot be productive.

Figure 4 compares tenofovir monophosphate in subunit A to the residual diphosphate seen in subunit D, and to tenofovir diphosphate in HIV reverse transcriptase (PDB entry 1T05), superimposing the

TABLE 3 Interactions made by phosphorylated tenofovir derivatives in Dd-NDPK

Ligand[a]		Protein	Distance[b] (Å)
Adenine base	C6	Phe 64 CB	3.5
	C8	Leu 68 CD2	3.7
	N9	Val 116 CG1	3.6
Methoxypropyl linker	C6′	Thr 98 CG2	3.5
	C8′	His 59 NE2	3.8
		Phe 64 CE1	3.7
	O9′	Leu 68 CD2	3.8
	C9′	Tyr 56 CE1	4.0
Phosphonate	O2A	Lys 16 NZ	2.9
		Asn 119 ND2	2.9
Phosphates	O1B	Arg 109 NH2	2.8
	O3B	Arg 92 NH1	3.6
		Thr 98 OG1	3.0
	O3G	His 59 NE2	2.9

[a]Tenofovir monophosphate in subunit A, γ-phosphate of tenofovir diphosphate in subunit D.

[b]Nonpolar interactions shorter than 4 Å or polar interactions shorter than 3.6 Å.

adenine moiety of the three structures. The conformations of the linker seen in NDPK and reverse transcriptase are remarkably similar, the phosphonate and α-phosphate positions are within 2.5 Å of each other, but the diphosphate moiety of tenofovir diphosphate adopts two orthogonal orientations relative to the acyclic moiety as the result of P-O bond rotations.

3.4. The Ligand Binding Site in NDPK Structures

The present NDPK crystal structure is one of many that have been determined in the presence of a variety of ligands and yield a wealth of information on the substrate binding site. At the end of 2008, 65 entries of the Protein Data Bank described NDPKs from 18 different origins ranging from human to viral. Thirty-three, including the present one, contain natural nucleotides or analogs. Table 4 reports their entry codes, the nature of the ligand and the origin of the protein. The sequence identity relative to Dd-NDPK is in the range 40% to 66%, with the mammalian proteins at the top of the range, and the viral sequence at the bottom. All are hexameric except for the *Myxococcus xanthus* tetramer (1NLK, 1NHK), although they display no cooperative binding and obey Michaelian kinetics.

To analyze the ligand sites, the ADP site in the ADP-BeF3 complex of Dd-NDPK (2BEF) was taken as a reference, and all other structures were systematically compared to it using the MED-SuMo software. For that

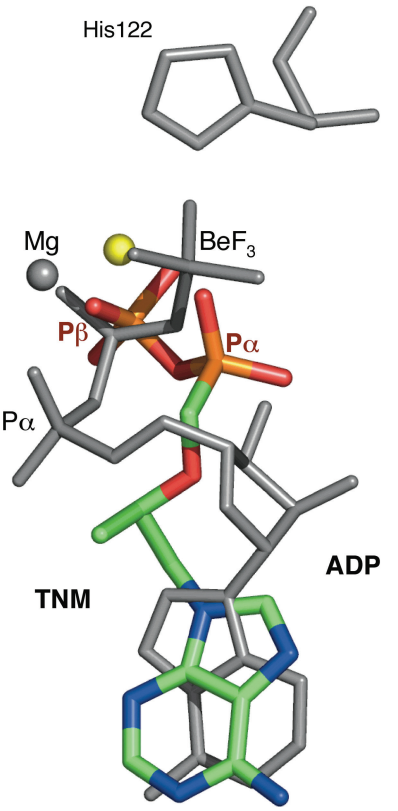

FIGURE 3 The conformation of NDPK-bound ADP and tenofovir diphosphate. ADP (gray sticks) in the ADP-BeF_3 complex with wildtype Dd-NDPK (PDB entry 2BEF) is superimposed with tenofovir monophosphate (TNM, colored by atom type) in subunit A of the present structure. The beryllium fluoride moiety is located between the β-phosphate and the active site His 122, mimicking the γ-phosphate of ATP.[25] The yellow and gray balls are Mg^{++} ions bound to TNM and ADP- BeF_3 respectively.

purpose, a site was defined as the set of SuMo objects located within 6 Å of any ligand atom. The reference ADP site comprises 86 such objects that belong to 19 amino acid residues. Table 4 reports the scores of the comparison to other sites, obtained by summing the weights of matching SuMo objects, and scaling the reference to 100. The Dd-NDPK structures that contain ADP achieve very similar scores in the range of 82 to 91. When other nucleotides or analogs occupy the binding site, the scores are significantly lower: 74–86, and two entries are far below: 1BUX, where the ligand is 3′-phospho-adenosine-5′-phospho-sulfate, a potent inhibitor of NDPK,[27] and 1MN7 where three residues are substituted. The ADP binding site of human NDPK-A (2HVD) scores 73, less than in Dd-NDPK due to the loss of interactions made by His 59, changed to Leu 55 in the human protein. The histidine is present in *Pyrococcus horikoshii* NDPK (2DYA), yielding a score of 84. NDPKs from other sources and

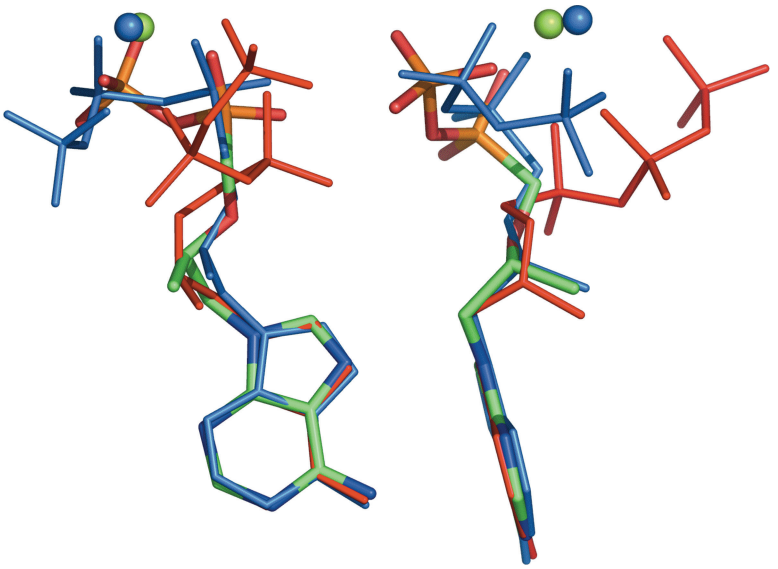

FIGURE 4 Phosphorylated tenofovir derivatives bound to NDPK and HIV reverse transcriptase. Tenofovir monophosphate (colored by atom type) in subunit A of Dd-NDPK is compared to the diphosphate (blue sticks) present in subunit D and in HIV reverse transcriptase (red sticks; PDB entry 1T05[8]) after superimposing the adenine moiety. Two orthogonal orientations are shown. The green and blue balls are Mg^{++} ions bound to the mono- and diphosphate, respectively.

complexes with other ligands than ADP have scores in the range of 53 to 74.

The ligand site of the present complex implicates only 54 of the 86 SuMo objects in the reference ADP site, and its score is 69, below all the natural substrates of Dd-NDPK. The deletion of the His 122 side chain removes only one SuMo object and has a marginal effect on the score, as shown by entry 1B4S, which bears the same H122G mutation. Thus, the score of the tenofovir site reflects its unusual mode of binding. The lowest scores overall are near 60 for wildtype Dd-NDPK binding 3′-phospho-adenosine-5′-phospho-sulfate, a ligand that also binds in a different way than natural substrates,[27] and for mimivirus NDPK in complex with dGDP or TDP. The viral enzyme is highly divergent from the others in terms of its sequence and enzymatic properties.[28] Nevertheless, the SuMo scores reported in Table 4 uniquely identify NDPK, whatever the ligand. We used MED-SuMo to compare all the entries in the PDB to the ADP site in 2BEF. The highest scores were 20 for the coenzyme site in the human glycerol-3-phosphate dehydrogenase 1-like protein (1PLA) and 15 for the site occupied by a coenzyme analog in *E. coli* thymidylate synthase (1DDU). All other entries scored below 13.

TABLE 4 Comparison of the ligand binding sites in NDPK x-ray structures

PDB entry	Ligand		Organism/ Mutation	Seq. Id.[a]	S_{SuMo}[b]
Dictyostelium discoideum					
2BEF	ADP	Adenosine-5′-diphosphate BeF3		100	100
1KDN	ADP	Adenosine-5′-diphosphate AlF3		100	91
1B4S	ADP	Adenosine-5′-diphosphate	H122G	99	90
1MN9	RTP	Ribavirin triphosphate	H122G	99	86
1B99	FUP	2′,3′-dideoxy-3′-fluoro-uridine-5′-diphosphate		100	84
1NDP	ADP	Adenosine-5′-diphosphate		100	82
1F6T	TBD	2′-deoxy-thymidine-5′-α-borano-diphosphate		100	82
1F3F	D4T	2′,3′-dehydro-thymidine-5′-triphosphate	H122G	99	80
1HIY	3AN	3′-amino-adenosine-5′-diphosphate		100	78
1S5Z	SON	Adenosine-5′-phosphonoacetic acid		100	77
1LWX	AZD	3′azidothymidine-5′-diphosphate		100	75
1NDC	TYD	Thymidine-5′-diphosphate		100	74
3FKB[c]	TNM	Tenofovir monophosphate	H122G	99	69
1BUX	PPS	3′-phospho-adenosine-5′-phospho-sulfate		100	59
1MN7	ABT	3′-azidothymidine-5′-α-borano-triphosphate	H122G/N119S/F64W	98	38
Mammalian					
2HVD	ADP	Adenosine-5′-diphosphate	Human A	66	73
2HVE	ADP	Adenosine-5′-diphosphate	Human A S120G	61	69
1NUE	GDP	Guanosine-5′-diphosphate	Human B	64	69
1ZS6	ADP	Adenosine-5′-diphosphate	Human 3	63	58
1UCN	ADP	Adenosine-5′-diphosphate	Human A H118G/F60W	64	57
1BE4	PCG	Cyclic GMP	Bovine retina	64	53
Plant					
1S59	DGI	2′-deoxy-guanosine-5′-diphosphate	*Arabidopsis thaliana*	61	62
Microbial					
2DYA	ADP	Adenosine-5′-diphosphate	*Pyrococcus horikoshii*	56	84
2DXE	GDP	Guanosine-5′-diphosphate	*Pyrococcus horikoshii*	56	74
2DXD	ANP	Phosphoamino-phosphonic acid adenylate ester	*Pyrococcus horikoshii*	56	70
2AZ3	CDP	cytidine-5′-diphosphate	*Halobact. salinarium*	57	69
2DXF	GNP	Phosphoamino-phosphonic acid guanylate ester	*Pyrococcus horikoshii*	56	66
1NLK	ADP	Adenosine-5′-diphosphate	*Myxococcus xanthus*	47	66
3B6B	DGI	2′-deoxy-guanosine-5′-diphosphate	*Mimivirus*	40	61
2B8Q	TYD	Thymidine-5′-diphosphate	*Mimivirus*	40	58
1WKL	ADP	Adenosine-5′-diphosphate	*Thermus thermophilus*	63	57
1NHK	CMP	cyclic AMP	*Myxococcus xanthus*	47	56
1WKK	GDP	Guanosine-5′-diphosphate	*Thermus thermophilus*	63	53

[a]Percentage of identity between aligned sequences.
[b]SuMo score of the comparison to the ADP binding site in entry 2BEF, normalized to 100.
[c]Present structure.

4. DISCUSSION

4.1. NDPK and the Phosphorylation of Tenofovir Monophosphate

NDPK is able to convert tenofovir monophosphate to the diphosphate in vitro, but the value, $k_{cat}/K_m = 410\ M^{-1}s^{-1}$ (cited in Table 2), indicates that the phosphorylation is not very efficient. The phosphorylation of AZT diphosphate by the same human NDPK-A yields a similar value: $192\ M^{-1}s^{-1}$.[15] All NRTI derivatives that lack a 3′OH group are poor substrates of NDPK, which reflects a peculiar feature of that kinase. Natural nucleotides bind in a conformation that allows the 3′OH of the sugar to donate an intramolecular H-bond to the oxygen bridging the β- and the γ-phosphates. This oxygen is the leaving group when a NTP substrate phosphorylates the active site histidine, and the attacking group when a NDP substrate dephosphorylates it. The intramolecular H-bond contributes to catalysis by lowering the energy of the transition state of the reaction.[11] d4T (2′,3′-dehydro-thymidine) diphosphate is a significantly better substrate than other NRTI derivatives ($k_{cat}/K_m = 2650\ M^{-1}s^{-1}$),[15] and this correlates with the observation that, when d4T diphosphate binds, a CH ... O bond implicating the 3′CH group of the dehydro sugar ring replaces the OH ... O bond present in a normal substrate. Albeit weaker, this H-bond also contributes to stabilizing the transition state.[29]

Although the K_m values for tenofovir monophosphate and dADP reported in Table 2 are similar, the affinity of NDPK must be significantly less for the analog than the natural substrate. The analog is a poor substrate for which the histidine dephosphorylation step is rate limiting and slow and, therefore, its dissociation constant K_d should be close to the observed K_m (0.29 mM). This is 50 times the K_d of NDPK-A for ADP (5.8 μM), and 3 times that for d4T diphosphate (0.1 mM),[20] but less than for AZT diphosphate (K_m = 6 mM)[12] and for adenosine phosphonoacetate (K_d = 0.6 mM).[30] AZT diphosphate and d4T diphosphate bind NDPK in the same conformation as natural substrates,[31,29] but tenofovir derivatives display a mode of binding that has never been observed before. The orientation of the adenine base in tenofovir monophosphate corresponds to the *syn* conformation seen in cyclic AMP bound to *Myxococcus xanthus* NDPK;[32] all other nucleotides or analogs have the base in the *anti* conformation when bound to NDPK.[20] Yet, the reversal of the base orientation does not by itself explain the low affinity of tenofovir monophosphate. The base maintains the same nonpolar interactions with protein groups, and NDPK is very tolerant there: It accepts purine or pyrimidine bases in its natural substrates, and even a substituted triazole group in ribavirin diphosphate.[33] On the other hand, the 3′OH of the sugar in a natural substrate makes polar interactions with the side chains of Lys 16 and Asn 119,[26] in addition to the intramolecular H-bond to the phosphate oxygen.

AZT diphosphate, d4T diphosphate, and other analogs that lack a 3′OH, yet bind in the usual conformation, also lose these interactions and show a low affinity. Moreover, the substitution of Asn 115 of human NDPK-A, equivalent to Asn 119 in Dd-NDPK, reduces the catalytic efficiency by an order of magnitude for natural substrates, while it increases it by the same factor for 3′-deoxy analogs.[34] Thus, the absence of a 3′OH in tenofovir and the consequent loss of interactions with protein groups is a likely cause of its low affinity.

4.2. NDPK-Bound Tenofovir Derivatives

The methoxypropyl linker group of tenofovir does not mimic a cyclic sugar, but rather adopts a low-energy conformation that is also observed in the very different environment of reverse transcriptase, placing the phosphonate group in a different position than the α-phosphate of a normal substrate. However, the position seen in the H122G mutant may not be allowed in the wildtype protein, because the phosphonate moiety would be too close to the active site histidine and clash with the phosphate that should be transferred. There is room for the phosphonate to move from the observed position closer to the α-phosphate of ADP, and this can be achieved by rotations around single P-O bonds without displacing the β-phosphate. During the course of the reaction, tenofovir monophosphate may therefore adopt a conformation closer to that of natural substrate. Nevertheless, the present x-ray structure shows that a substrate analog does not necessarily imitate the natural ligand when it binds to a protein. Standard molecular modeling methods tend to optimize the fit between molecules in the same site. Presumably, they would fail to predict the reversal of the base orientation in tenofovir and the path followed by the linker from the base to the phosphonate.

4.3. Alternative Pathways for Tenofovir Activation

The phosphorylated derivatives that are account for the antiviral activity of tenofovir are relatively abundant in human cells.[9] Yet, the first phosphorylation step is very poorly catalyzed by human adenylate kinases,[35,36] and the kinetic and structural data reported here make it unlikely that NDPK is the major player in the second step. Still, the abundance of these housekeeping enzymes counterbalances their low catalytic efficiency, whereas phosphorylated acyclic phosphonate analogs are relatively resistant to hydrolysis by nucleotidases[37] and remarkably stable in the cell with a half-life of days.[38,39] On the other hand, phosphotransferases other than adenylate kinase and NDPK are able to phosphorylate nucleoside mono- and diphosphates. Some have been shown to phosphorylate NRTI derivatives relatively efficiently, for instance phosphoglycerate kinase in

the particular case of the L-nucleoside analogs.[40,41] While this particular enzyme is not active on tenofovir monophosphate, we find that pyruvate kinase is as efficient as NDPK, and creatine kinase much more so. These enzymes, and others that have yet to be tested, may contribute to the activation of tenofovir in cells.

4.3. Conclusion

A prominent outcome of the present study is that a protein site designed to bind natural nucleotides can easily accommodate a ligand that lacks a sugar ring and where a phosphonate replaces the α-phosphate. Nucleotide binding (Gene Oncology ID:166) is one of the most common molecular functions of proteins encountered in nature. The PDB utility *MolecularFunctionTree Search* finds it in about one of seven proteins in the PDB, which offers a wide field for the design of acyclic phosphonate analogs targeting these proteins. Although their nucleotide binding sites may not all be as permissive as in NDPK, many should accept such compounds as ligands, leading to the identification of novel families of substrates, agonists, or inhibitors for the target proteins.

REFERENCES

1. Stein, D.S.; Moore, K.H.P. Phosphorylation of nucleoside analog antiretrovirals: a review for clinicians. *Pharmacol. Ther.* **2001**, 21, 11–34.
2. De Clercq, E.; Holý, A. Acyclic nucleoside phosphonates: a key class of antiviral drugs. *Nat. Rev. Drug Discov.* **2005**, 4, 928–940.
3. Pozniak, A. Tenofovir: what have over 1 million years of patient experience taught us? *Int J. Clin. Pract.* **2008**, 62, 1285–1293.
4. Delaney, W.E.; Borroto-Esoda, K. Therapy of chronic hepatitis B: trends and developments. *Curr. Op. Pharmacol.* **2008**, 8, 532–540.
5. Srinivas, R.V.; Fridland, A. Antiviral activities of 9-R-2-phosphonomethoxypropyl adenine (PMPA) and bis(isopropylmethylcarbonyl)PMPA against various drug-resistant human immunodeficiency virus strains. *Antimicrob. Agents. Chemother.* **1998**, 42, 1484–1487.
6. Wainberg, M.A.; Miller, M.D.; Quan, Y.; Salomon, H.; Mulato, A.S.; Lamy, P.D.; Margot, N.A.; Anton, K.E.; Cherrington, J.M. In vitro selection and characterization of HIV-1 with reduced susceptibility to PMPA. *Antivir Ther.* **1999**, 4, 87–94.
7. Frangeul, A.; Barral, K.; Alvarez, K.; Canard, B. In vitro suppression of K56R reverse transcriptase-mediated tenofovir and adefovir-5′-diphosphate resistance conferred by the boranophosphonate derivatives. *Antimicrob. Agents Chemother.* **2007**, 51, 3162–3167.
8. Tuske, S.; Sarafianos, S.G.; Clark, A.D. Jr.; Ding, J.; Naeger, L.K.; White, K.L.; Miller, M.D.; Gibbs, C.S.; Boyer, P.L.; Clark, P.; Wang, G.; Gaffney, B.L.; Jones, R.A.; Jerina, D.M.; Hughes, S.H.; Arnold, E. Structures of HIV-1 RT-DNA complexes before and after incorporation of the anti-AIDS drug tenofovir. *Nat. Struct. Mol. Biol.* **2004**, 11, 469–474.
9. Balzarini, J.; Van Herrewege, Y.; Vanham, G. Metabolic activation of nucleoside and nucleotide reverse transcriptase inhibitors in dendritic and Langerhans cells. *AIDS* **2002**, 16, 2159–2163.
10. Lascu I.; Gonin, P. The catalytic mechanism of nucleoside diphosphate kinases. *J. Bioenerg. Biomembr.* **2000**, 32, 237–246.
11. Janin, J.; Deville-Bonne, D. Nucleoside-diphosphate kinase: structural and kinetic analysis of reaction pathway and phosphohistidine intermediate. *Methods Enzymol.* **2002**, 354, 118–134.

12. Bourdais, J.; Biondi, R.; Sarfati, S.; Guerreiro, C.; Lascu, I.; Janin, J.; Véron, M. Cellular phosphorylation of anti-HIV nucleosides. Role of nucleoside diphosphate kinase. *J. Biol. Chem.* **1996**, 271, 7887–7890.
13. Schneider, B.; Xu, Y.W.; Sellam, O.; Sarfati, R.; Janin, J.; Veron, M.; Deville-Bonne, D. Pre-steady state of reaction of nucleoside diphosphate kinase with anti-HIV nucleotides. *J. Biol. Chem.* **1998**, 273, 11491–11497.
14. Janin, J.; Dumas, C.; Moréra, S.; Xu, Y.; Meyer, P.; Chiadmi, M.; Cherfils, J. Three-dimensional structure of nucleoside diphosphate kinase. *J. Bioenerg. Biomembr.* **2000**, 32, 215–225.
15. Schneider, B.; Biondi, R.; Sarfati, R.; Agou, F.; Guerreiro, C.; Deville-Bonne, D.; Veron, M. The mechanism of phosphorylation of anti-HIV D4T by nucleoside diphosphate kinase. *Mol. Pharmacol.* **2000**, 57, 948–53.
16. Admiraal, S.J.; Schneider, B.; Meyer, P.; Janin, J.; Véron, M.; Deville-Bonne, D.; Herschlag, D. Nucleophilic activation by positioning in phosphoryl transfer catalyzed by nucleoside diphosphate kinase. *Biochemistry* **1999**, 38, 4701–4711.
17. Feng, J.Y.; Parker, W.B.; Krajewski, M.L.; Deville-Bonne, D.; Veron, M.; Krishnan, P.; Cheng, Y-C.; Borroto-Esoda, K. Anabolism of amdoxovir: phosphorylation of dioxolane guanosine and its 5′-phosphates by mammalian phosphotransferases. *Biochem. Pharmacol.* **2004**, 68, 1879–1888.
18. Shewach, D. Quantitation of deoxyribonucleoside 5′-triphosphates by a sequential boronate and anion-exchange high-pressure liquid chromatographic procedure. *Analytical Biochemistry* **1992**, 206, 178–182.
19. Leslie, A.G. Integration of macromolecular diffraction data. *Acta Cryst. D* **1999**, 55, 1696–1702.
20. Chen, Y.; Gallois-Montbrun, S.; Schneider, B.; Véron, M.; Moréra, S.; Deville-Bonne, D.; Janin, J. Nucleotide binding to nucleoside diphosphate kinases: X-ray structure of human NDPK-A in complex with ADP and comparison to protein kinases. *J. Mol. Biol.* **2003**, 332, 915–926.
21. Brunger, A.T.; Adams, P.D.; Clore, G.M.; Gros, P.; Grosse-Kunstleve, R.W.; Jiang, J.S.; Kuszewski, J.; Nilges, N.; Pannu, N.S.; Read, R.J.; Rice, L.M.; Simonson, T.; Warren, G.L. Crystallography & NMR System (CNS): A new software suite for macromolecular structure determination. *Acta Cryst. D* **1998**, 54, 905–921.
22. Jambon, M.; Imberty, A.; Deléage, G.; Geourjon, C. A new bioinformatic approach to detect common 3D sites in protein structures. *Proteins* **2003**, 52, 137–145.
23. Jambon, M.; Andrieu, O.; Combet, C.; Deléage, G.; Delfaud, F.; Geourjon, C. The SuMo server: 3D search for protein functional sites.*Bioinformatics.* **2005**, 21, 3929–3230.
24. Dumas, C.; Lascu, I.; Moréra, S.; Glaser, P.; Fourme, R.; Wallet, V.; Lacombe, M.L.; Véron, M.; Janin, J. X-ray structure of nucleoside diphosphate kinase. *EMBO J.* **1992**, 11, 3203–3208.
25. Xu, Y.W.; Moréra, S.; Janin, J.; Cherfils, J. AlF3 mimics the transition state of protein phosphorylation in the crystal structure of nucleoside diphosphate kinase and MgADP. *Proc. Natl. Acad. Sci. USA* **1997**, 94, 3579–3583.
26. Moréra, S.; Lascu, I.; Dumas, C.; LeBras, G.; Briozzo, P.; Véron, M.; Janin, J. Adenosine 5′-diphosphate binding and the active site of nucleoside diphosphate kinase. *Biochemistry* **1994**, 33, 459–467.
27. Schneider, B.; Xu, Y.W.; Janin, J.; Véron, M.; Deville-Bonne, D. 3′-Phosphorylated nucleotides are tight binding inhibitors of nucleoside diphosphate kinase activity. *J. Biol. Chem.* **1998**, 273, 28773–28778.
28. Jeudy, S.; Lartigue, A.; Claverie, J.M.; Abergel, C. Dissecting the unique nucleotide specificity of Mimivirus nucleoside diphosphate kinase. *J. Virol.* **2009**, 83, 7142–7150.
29. Meyer, P.; Schneider, B.; Sarfati, S.; Deville-Bonne, D.; Guerreiro, C.; Boretto, J.; Janin, J.; Véron, M.; Canard, B. Structural basis for activation of alpha-boranophosphate nucleotide analogues targeting drug-resistant reverse transcriptase. *EMBO J.* 2000, 19, 3520–3529.
30. Chen, Y.; Morera, S.; Pasti, C.; Angusti, A.; Solaroli, N.; Véron, M.; Janin, J.; Manfredini, S.; Deville-Bonne, D. Adenosine phosphonoacetic acid is slowly metabolized by NDP kinase. *Med. Chem.* **2005**, 1, 529–536.
31. Xu, Y.; Sellam, O.; Moréra, S.; Sarfati, S.; Biondi, R.; Véron, M.; Janin, J. X-ray analysis of azidothymidine diphosphate binding to nucleoside diphosphate kinase. *Proc. Natl. Acad. Sci. USA* **1997**, 94, 7162–7165.
32. Strelkov, S.V.; Perisic, O.; Webb, P.A.; Williams, R.L. The 1.9 A crystal structure of a nucleoside diphosphate kinase complex with adenosine 3′,5′-cyclic monophosphate: evidence for competitive inhibition. *J. Mol. Biol.* **1995**, 249, 665–674.

33. Gallois-Montbrun, S.; Chen, Y.; Dutartre, H.; Sophys, M.; Morera, S.; Guerreiro, C.; Schneider, B.; Mulard, L.; Janin, J.; Veron, M.; Deville-Bonne, D.; Canard, B. Structural analysis of the activation of ribavirin analogs by NDP kinase: comparison with other ribavirin targets. *Mol. Pharmacol.* **2003**, 63, 538–546.
34. Gallois-Montbrun, S.; Schneider, B.; Chen, Y.; Giacomoni-Fernandes, V.; Mulard, L.; Morera, S.; Janin, J.; Deville-Bonne, D.; Veron, M. Improving nucleoside diphosphate kinase for antiviral nucleotide analogs activation. *J. Biol. Chem.* **2002**, 277, 39953–39959.
35. Robbins, B.L.; Greenhaw, J.; Connelly, M.C.; Fridland, A. Metabolic pathways for the activation of the antiviral agent 9-R-2-phosphonomethoxypropyl adenine (PMPA) in human lymphoid cells. *Antimicrob. Agents Chemother.* **1995**, 39, 2304–2308.
36. Topalis, D.; Alvarez, K.; Barral, K.; Munier-Lehmann, H.; Schneider, B.; Véron, M.; Guerreiro, C.; Mulard, L.; El-Amri, C.; Canard, B.; Deville-Bonne, D. Acyclic phosphonate nucleotides and human adenylate kinases: impact of a borano group on alpha-P position. *Nucleosides Nucleotides Nucleic Acids.* **2008**, 27, 319–331.
37. Rinaldo-Matthis, A.; Rampazzo, C.; Balzarini, J.; Reichard, P.; Bianchi, V.; Nordlund, P. Crystal structures of mitochondrial deoxyribonucleotidase in complex with two specific inhibitors. *Mol Pharmacol.* **2004**, 65, 860–967.
38. Robbins, B.L.; Srinivas, R.V.; Bishofberger, N.; Fridland, A. Anti-human immunodeficiency virus activity and cellular metabolism of a potential prodrug of the acyclic nucleoside phosphonate 9-R-2-phosphonomethoxypropyl adenine (PMPA) bis(isopropylmethylcarbonyl)PMPA. *Antimicrob. Agents Chemother.* **1998** 42, 612–617.
39. Pruvost, A.; Negredo, E.; Benech, H.; Theodoro, F.; Puig, J.; Gran, E.; Garcia, E.; Molto, J.; Grassi, J.; Clotet, B. Measurement of intracellular didanosine and tenofovir phosphorylated metabolites and possible interactions of the two drugs in human immunodeficiency virus-infected cells. *Antimicrob. Agents Chemother.* **2005**, 49, 1907–1914.
40. Krishnan, P.; Gullen, E.A.; Lam, W.; Dutschman, G.; Grill, S.P.; Chen, Y.C. Novel role of 3-phosphoglycerate kinase, a glycolytic enzyme, in the activation of L-nucleoside analogs, a new class of anticancer and antiviral agents. *J. Biol. Chem.* **2003**, 278, 36726–36732.
41. Gallois-Montbrun, S.; Faraj, A.; Seclaman, E.; Sommadossi, J.P.; Deville-Bonne, D.; Véron, M. Broad specificity of human phosphoglycerate kinase for antiviral nucleoside analogs. *Biochem. Pharmacol.* **2004**, 68, 1749–1756.