Antiviral Nucleoside Analogs Phosphorylation by Nucleoside Diphosphate Kinase

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Abstract: The reaction of NDP kinase was studied *in vitro* with several antiviral derivatives, using kinetic steady state and presteady state analysis. The enzyme is highly efficient with natural nucleotides but most of the analogs are slow substrates. The catalytic efficiency, also related to the affinity of the analog, is mainly dependent on the presence of a 3'-OH group on the ribose moiety.

Keywords: NDP kinase, antiviral nucleoside analog, dideoxynucleoside, acyclovir, AZT, d4T, 3TC, borano substitution.

INTRODUCTION

Nucleoside diphosphate (NDP) kinases are housekeeping enzymes, responsible for the cellular equilibrium between nucleotides di- and triphosphate. NDP kinases are highly conserved proteins and several isoforms are present in human. They catalyze the phosphotransfer from a NTP (or dNTP) to a NDP (or dNDP) with a broad specificity (reaction1). In general, ATP serves as a donor of phosphate due to its high cellular concentration.

$$ATP\text{-}Mg^{2+} + NDP \rightleftarrows ADP\text{-}Mg^{2+} + NTP \quad (1)$$

NDP kinase was first studied with the yeast enzyme more than 40 years ago [1]. The reaction follows a ping-pong bi bi mechanism with a covalent intermediate due to the phosphorylation of a His residue [2]. Much later, NDP kinase was involved in a variety of other cellular events. Thus *nm23,* a presumed regulator of tumour metastasis in human cancer [3], and *awd*, involved in *Drosophila* development [4], were both identified as genes coding NDP kinase [5, 6]. The possibility of alternative functions for NDP kinase in cellular biology and pathology, independent of its phosphorylation activity, is still controversial and was discussed in a previous review [7]. This review focuses on the enzymatic activity of human NDP kinases towards antiviral nucleoside analogs.

After entry in the cell, antiviral nucleoside analogs are converted into triphosphates by three successive reactions catalyzed by enzymes from the salvage pathway (nucleoside kinases, NMP kinases and NDP kinase). The triphosphate analogs then compete with natural 2'deoxynucleotides (dNTP) for incorporation into viral DNA by reverse transcriptase. As the derivatives lack a 3'-OH group, DNA chain elongation terminates, accounting for the observed antiviral effect [8, 9]. NDP kinase is expressed in high amounts in cells and presents a broad substrate specificity with a high turnover number (k_{cat} = 1000 s^{-1}). NDP kinase was thus supposed to react with antiviral and anticancer analogs also with high efficiency, and the addition of the third phosphate has been long considered as a non rate-limiting step in cells.

After a brief survey of major properties of human NDP kinase isoforms, we shall consider the *in vitro* reactivity of NDP kinase with several antiviral analogs currently used in therapies (Fig. (**1**)) including: (i) nucleosides devoid of the 3'-OH acting as DNA chain terminator like AZT, d4T, ddC used against human immunodeficiency virus (HIV) and acyclovir, an acyclic analog of guanosine used against herpes virus infections, (ii) L-3TC, a L-nucleoside, acting on HIV and on hepatitis B virus (HBV) as a chain terminator due to the low stereospecificity of viral polymerases, (iii) ribavirin, delivered in therapies against hepatitis C virus (HCV), whose mode of action is not yet clear. We show that, although NDP kinase possesses a wide specificity concerning the base, it is very sensitive to the presence of a 3'-OH group on the ribose of the nucleotide.

Human NDP Kinases Sequences

NDP kinases are highly conserved from prokaryotes to higher eukaryotes, and almost all of the residues involved in the active site are identical [10]. Up to eight isoforms of human NDP kinase were found (reviewed in [11]). The genes coding for the major cytosolic forms A and B, respectively *nm23-H1* and *nm23-H2,* map within 4 kb of each other on chromosome segment 17q21.3. The alignment of sequences of NDP kinases A and B (Fig. (**2**)) shows a high similarity with 88% identity for the 152 aminoacids. However, their isoelectric points are quite different (pI_A = 6.1 and pI_B = 8.4). Their expression is ubiquitous, with isoform B being slightly more abundant than the A isoform in normal cells. Both enzymes are cytosolic, with NDP kinase B also found in the nucleus [12, 13]. NDP kinase A was also identified as a constituent of the centrosome [14]. *Nm23-H3,* which is expressed in a small amount, possesses an N-terminus extension of 17 aminoacids and is involved in apoptosis [15]. NDP kinase D, the product of *nm23-H4* gene, presents a N-terminus mitochondrial signal sequence of 33 aminoacids and is expressed in mitochondria [16, 17]. The *nm23-H5* to *H8* cDNA sequences have been identified in the human genome by homology [18-20]. The corresponding proteins are poorly conserved, with only 25% to 34% identity with the major NDP kinases. Some miss critical

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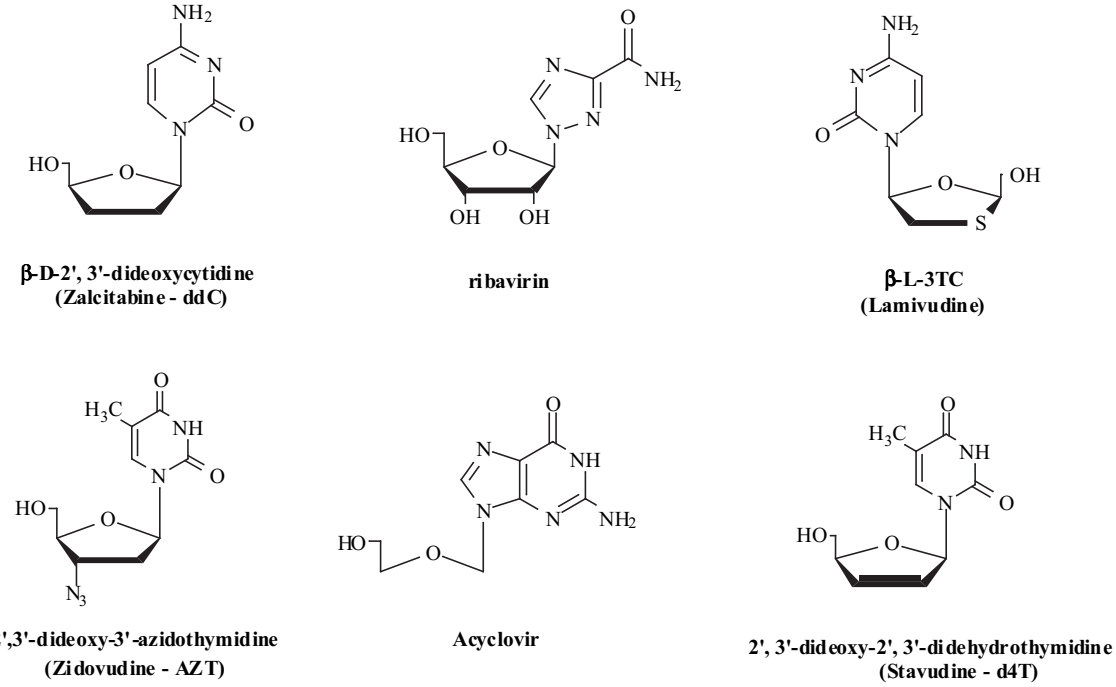

Fig. (1). Chemical formula of some nucleoside analogs currently used in antiviral therapies.

active site residues and are probably devoid of phosphotransfer activity. *Nm23-H7 and H8* possess tandemly repeated NDP kinase domains, also found in the sea urchin axonemal dynein [11].

Our study focuses on the human NDP kinases A and B that contribute to 80% of the total cellular activity. Mitochondria NDP kinase ensures the remaining 20% activity. The enzyme from the amibe *Dictyostelium discoideum* was also used as a faithful model for structural studies. Indeed, *Dictyostelium* enzyme is highly similar to NDP kinase A and B with respectively 60% and 58% sequence identity, as shown in Fig. (**2**). Its catalytic

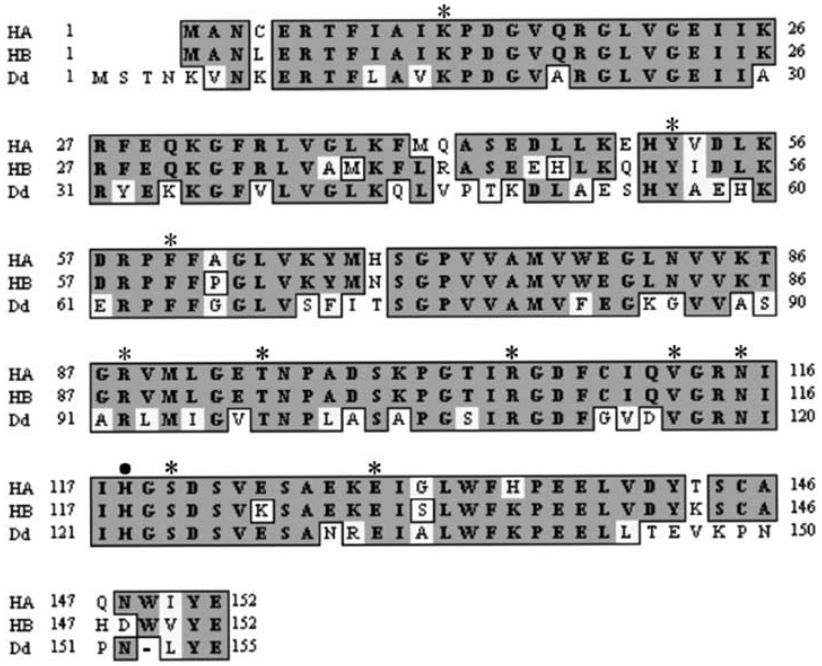

Fig. (2). Primary sequence comparison between the human NDP kinases A and B and *Dictyostelium discoideum* NDP kinase proteins. Gaps are indicated by dashes. The consensus sequence represents identical (dark boxes) and conserved (light grey boxes) residues within all sequences.(*) indicates the residus involved in catalysis.

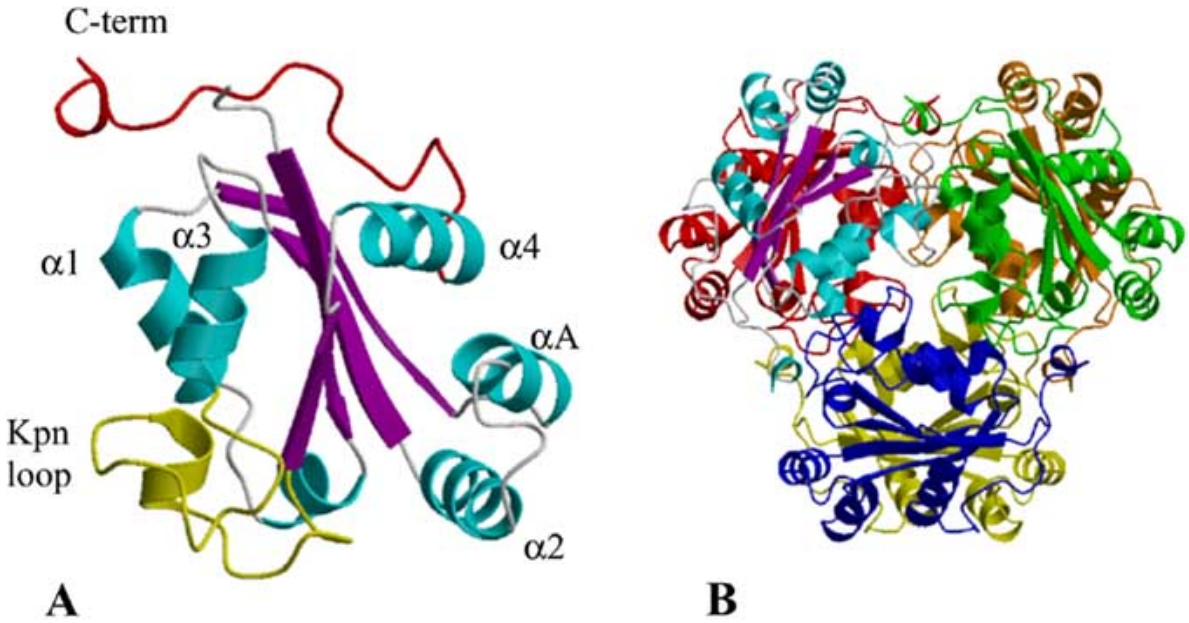

Fig. (3). Fold of the NDP kinase. (A) Ribbon chain tracing of the subunit of *Dictyostelium* NDP kinase with sheets in magenta, helix in blue, Kpn loop in yellow and the C-terminal part in red [68]. (B) Hexameric NDP kinase B [26]. The hexamer is viewed along its threefold axis, with the three twofold axis in the plane of the figure. The Kpn loops are in the center near the three fold axis.

properties were very similar to the human ones in all aspects investigated.

NDP Kinase Structure

The hexameric structure first proposed for the yeast enzyme [21] was confirmed for most eukaryotic NDP kinases by crystallographic and biochemical studies. Some bacterial NDP kinases, however, have a tetrameric structure [22]. The first three-dimensional structure of NDP kinase to be determined by X-ray crystallography was the enzyme of the slime mold *Dictyostelium discoideum* [23]. A series of other NDP kinase structures was later solved including those from *Myxococcus xanthus* [22], *Drosophila melanogaster* [24], bovine retina enzymes [25] and human B [26, 27], mitochondrial D [16], and A [28]. The polypeptide chain adopts a very similar fold in proteins from all origins. The monomer, shown on Fig. (**3A**), reveals an α/β domain comprising a four-stranded antiparallel β-sheet and two connecting α-helixes, according to a common structural motif called αβ sandwich or ferredoxin fold [29]. The packing of helixes α1 and α3 on the β-sheet creates an hydrophobic core. The other face of the β-sheet is covered by the helix α4 and the two helixes α2 and αA, forming an hairpin. The 22 residues long Kpn loop (so-called from *Killer-of-Prune*, a mutation found in *Drosophila)*, participates in the trimer interface and in the active site. The C-terminus peptide appears rather free in the monomer but interacts with the neighbour subunits within a trimer.

In the hexamer, the mobile regions are the α2-αA hairpin and the C-terminus, which correspond to the less conserved sequences [29]. Two monomers associate in a dimer through elongation of the β-sheets resulting in a single, eight-stranded β-sheet. The interface also involves interactions between helixes α1. Eukaryotic and bacterial NDP kinases are both made from the assembly of a similar dimer into homohexamers and homotetramers, respectively. As shown in Fig. (**3B**), the trimer interface involves the Kpn loops which come together near the three fold axis. The monomer, as well as the dimer, is devoid of enzymatic activity [30]. However the presence of ATP promotes reassociation into active hexamers, possibly by stabilizing the Kpn loop [31].

NDP Kinase Active Site

Structures of NDP kinase in complexes with several substrates or inhibitors were determined. They all showed the same ligand binding site. A single nucleotide binding site per subunit accepts both substrates: a nucleoside triphosphate donates the γ-phosphate to the catalytic His 118 and a nucleoside diphosphate receives the phosphate from this His. The nucleotide binding site is located at the surface of the protein, the base stacking with Phe 60 and interacting with Val 112, as shown on Fig. (**4**). The hydrophobic interactions of the base with the enzyme explain the broad specificity for nucleotides: the four common bases are accommodated in a similar way with a shift of only 3-4 Å in the base plane [29]. Unlike the base, the deoxyribose is buried inside the active site. The 3'-OH is involved in a hydrogen bond network with the amino group of Lys 12 and with the amide group of Asn 115, which are two conserved residues. Lys 12 is also involved in catalysis. The phosphates are oriented towards the catalytic His 118, located deeper in the active site. The β-phosphate oxygens interact with Arg 88, Arg 105 and a magnesium ion. The metal ion is bound to oxygens of the α- and β-phosphates and six water molecules complete its coordination shell.

The mode of binding of the γ-phosphate is known from the structure of a NTP with inactive mutants of NDP kinase where the catalytic His was replaced by a Gly [32]. The γ-phosphate adopts the position occupied by AlF_3, a salt

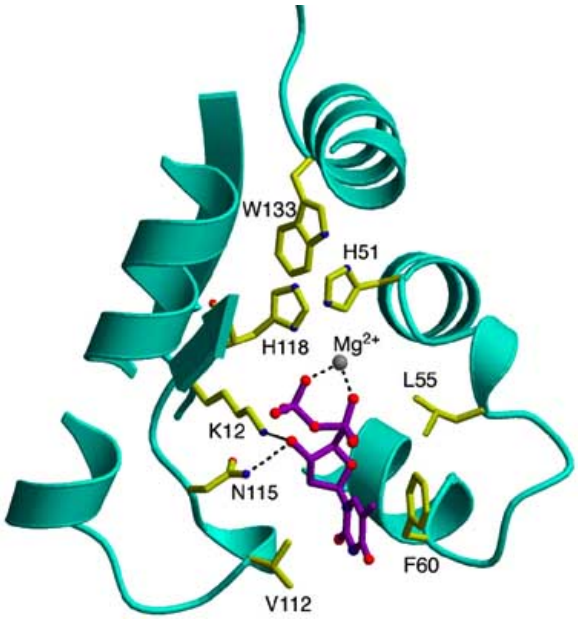

Fig. (4). The nucleotide binding site of NDP kinase. View of human NDP kinase B active site with bound dTDP (ball and sticks) and Mg^{2+} (grey). Some aminoacid side chains are shown. The catalytic His 118 is near Trp 133 whose fluorescence signal is modulated upon phosphorylation of His 118. The 3'-OH of the ribose H-bonds with Lys 12 and Asn 115 side chains and with the oxygen bridging Pα and Pβ. (from Yuxing Chen, LEBS, Gif sur Yvette).

mimicking a phosphate group in the transition state [33]. In the complex with a NTP, the magnesium ion interacts with all three phosphates. The 3'-OH, which receives two hydrogen bonds from Lys 12 and Asn 115, donates one to the oxygen atom bridging the β and γ-phosphates. This latter bond is particularly important in catalysis [29, 34, 35].

Several inhibitors of NDP kinase are known. For example, anionic dyes like Rose Bengal, Cibacron Blue [36] and oligonucleotides [37] were shown to inhibit specifically NDP kinase. The only crystallographic structures of a complex of NDP kinase with an inhibitor are those of cAMP and adenosine 3'-phosphate 5'-phosphosulfate (PAPS) with K_D of 0.5 mM and 10 μM respectively [38, 39].

Properties of the Phosphoenzyme

When NDP kinase is incubated with an excess of ATP-Mg^{2+}, the phosphorylated intermediate is produced in a millisecond time range. The phosphate binds the N-δ of His 118, as shown by ^{31}P-NMR [40]. The phosphohistidine bond is labile at acidic pH, differing from a phosphoserine bond, which is unstable at basic pH. The phosphate–imidazole bond is a high-energy bond, with a standard Gibbs free energy of hydrolysis of about 10-14 kcal/mol, and it rapidly hydrolyses [41]. In the absence of a nucleoside diphosphate, the phosphoenzyme is stable only for a few hours at 4°C and neutral pH [42]. Nevertheless, the X-ray structure of the phosphoenzyme could be solved after incubating the enzyme with small phosphodonors like phosphoramidate. Its structure is similar to the free enzyme except for a slight rotation of the catalytic His side chain allowing the phosphate group to interact with Tyr 52 [43]. In contrast to other kinases like adenylate kinase, phosphoglycerate kinase or hexokinase, NDP kinase may be considered as a rigid protein. Indeed, except for the αA-α2 helix hairpin (Fig. (**3**)), which moves by 1 to 2 Å allowing the nucleotide NDP and/or NTP to bind to or to be released from the active site, no major conformation change is observed during catalysis.

The phosphorylation of NDP kinase on catalytic His 118 can be monitored by fluorescence. Trp 133 (Trp 137 in the *Dictyostelium* enzyme), at the vicinity of the catalytic His, provides a convenient signal for probing its phosphorylation state [44-46]. The fluorescence of the protein is 7% quenched in human enzyme (20% for NDP kinase from *Dictyostelium*) upon phosphorylation of His 118. A time-resolved fluorescence study of NDP kinase confirmed that the Trp close to His118 is tightly bound within the protein core during the time course of the reaction [46].

Kinetic Studies with Analogs Compared to Natural Substrates

a- Steady State Studies

The kinetic parameters of NDP kinases for natural nucleotides were determined at the steady state. The methods include a spectrophotometric assay, measuring the formation of ADP by auxiliary enzymes, pyruvate kinase and lactate dehydrogenase [36] and a radioactive assay with [^{14}C]-ADP or [^{32}P]-GDP as a substrate [47]. The reaction volumes were reduced to 250μL for the microplate spectrometric assay, and to 3μL for the radioactive assay. Natural nucleotides are all excellent substrates for NDP kinases, with Michaelis constants in the 0.1 - 0.3 mM range and high k_{cat} values (1000 s^{-1}). GTP is the best substrate, ATP and dTTP are slightly less efficient and CTP is even lower. The steady state activity of human NDP kinase B with 3'-modified thymidine derivatives (AZT and dideoxy thymidine di-or tri-phosphate) was 5,000 to 10,000 fold lower than with natural substrates [47]. Human NDP kinases A, B, C and D presented a similar drop in activity with AZTDP, d4TDP and ddTDP, as expected from their similarity [34].

b- Pre-Steady State Studies

Rapid mixing experiments of NDP kinase with several analogs di- or triphosphate allowed the determination of presteady state reaction rates. An assay was set up using a fluorescence stopped flow device to follow the reaction of NDP kinase with any nucleoside di- and triphosphate or analog [44]. Each half reaction

$$E + N_1TP \longrightarrow E{\approx}P + N_1DP \quad (2a)$$

$$E{\approx}P + N_2DP \longrightarrow E + N_2TP \quad (2b)$$

follows an exponential time course, the fluorescence decreasing during reaction 2a or increasing for the 2b [44, 46, 48]. The reactions are fast with natural nucleotides and can be monitored with the stopped flow only at subsaturating nucleotide concentrations. In contrast, with less efficient substrates, the reaction can be followed easily after fast mixing in the fluorescence stopped flow device. The rate constants were extracted from the fit to monoexponentials. The rates of phosphotransfer from the

phosphoprotein (reaction 2b) were about five times greater than in the other direction (reaction 2a). This indicates, in agreement with the Haldane equation, that NDP kinase catalyzes the phosphotransfer near the thermodynamic equilibrium with a K_{eq} near 0.2 (equation 3). Given the cellular ATP and ADP concentrations (evaluated to 5mM and 0.5mM, respectively), the enzyme should therefore be present in the cell predominantly in the phosphorylated state (67%). The equilibrium constant of the enzyme with nucleosides di- and tri-phosphate can also be determined by fluorescence measurements according to:

$$K_{eq} = \frac{[E \approx P] \cdot [NDP]}{[E] \cdot [NTP]} \quad (3)$$

In the presence of an excess of Mg^{2+}, K_{eq} was found to be about 0.2 for couples like ADP/ATP, dTDP/dTTP, AZTDP/AZTTP, ddTDP/ddTTP [44, 46, 48]. The equilibrium of NDP kinase with di- and triphosphate was independent of the substrates. In contrast, the reaction rates strongly decreased with most of the analogs.

The presteady state reaction of NDP kinase with several antiviral analogs was studied in our laboratory using the fluorometric assay (reaction 2a) using the di- and triphosphorylated form of the analogs. These studies were made either with the enzyme from *Dictyostelium* [44] or the isoforms A and B of the human enzyme [32, 34, 45, 49, 50, 51]. The reaction rates were analyzed according to the classical scheme involving two coupled reactions where a fast step of nucleotide binding to the enzyme is followed by a slow step of phosphotransfer. The analysis of reaction rates as a function of analog concentration allows to determine both the maximum rate of phosphotransfer (k_2) and the

Table 1. Interaction of Nucleotides and Analogs (Triphosphate) with Human NDP Kinase

	Human NDPK-A catalytic efficiency k2/K_S ($M^{-1}s^{-1}$)[a]	Kd for *Dictyostelium* H122G-F64W inactive NDP kinase (μM)[b]	L55H-N115S NDPK-A catalytic efficiency k2/K_S ($M^{-1}s^{-1}$)[c]
GTP	8.0×10^6	0.15	
dGTP	4.0×10^6		5.0×10^5
ddGTP	190		2600
AcyclovirTP	30	250	4200
ITP	7.6×10^6		
RibavirinTP (RTP)	3.0×10^5	24	
2'deoxyRTP	5.7×10^4	40	
3'deoxyRTP	10	200	
ddRTP	1	2500	
d4RTP	14	1000	
ATP	1.7×10^6	0.2	
dATP	1.4×10^6		
ddATP	65		
UTP	1.3×10^6		
dTTP	1.2×10^6	1.2	4.5×10^5
ddTTP	20	4	2800
AZTTP	75	30	6800
AZTTP-αBH_3^-	375	2	
d4TTP	700	2	55400
d4TTP-αBH_3^-	600	0.25	
CTP	4×10^5		
dCTP	4×10^4		
ddCTP	<10		
L-3TCTP	<1		

(a) Reactivity with human NDP kinase A

(b) equilibrium dissociation constant Kd for the inactive mutant of Dd NDP kinase

(c) reactivity with the improved human double mutant NDP kinase

dissociation constant for the analog K_S [52] , as well as the ratio k_2/K_S that is equivalent to the catalytic efficiency k_{cat}/K_M.

Table 1 shows the catalytic efficiencies of the triphosphate form of several analogs with human NDP kinase A. The values found for natural nucleotides and analogs coincide with the values determined at the steady state. Natural substrates react with a high efficiency, close to that of a diffusion controlled process. The absence of the 2'-OH decreases this efficiency by two-to ten-fold. On the contrary, the absence of the 3'-OH causes a major drop by about 10^4, as seen with the dideoxy series, AZT and d4T derivatives. Removing the Lys 12 or Asn 115 side chains, which interact with the 3'-OH and the γ-phosphate, affects less the activity than the 3'-OH absence, as shown by the activity of the mutants where Lys 12 or Asn 115 have been replaced by Ala [53]. The detailed analysis of the dideoxy series indicates that the phosphotransfer rate decreases by a factor of 500 - 1000 and the affinity by about 10-fold [44]. Most of the analogs tested are very slow substrates for NDP kinase. The less efficient is L-3TCTP, which barely reacted, indicating that the stereospecificity of NDP kinase is restricted to D-analogs [49]. On the contrary, ribavirin (Fig. (**1**)), a broad-spectrum antiviral agent that is an inhibitor or a substrate of several viral RNA polymerases, was very well processed by NDP kinase. A structural interpretation of these results is that ribavirin, which has a natural ribose with a 3'-OH group, adopts the conformation of a normal substrate in the active site of NDP kinase [51], whereas AZTDP, which has a bulky N_3 group in place of the 3'-OH, displaces Lys12 side chain involved in catalysis [54]. Ribavirin is used in therapies against hepatitis C virus but is toxic at relatively low doses. Thus, a series of analogs modified on the sugar moiety was synthesized and tested on several RNA polymerases. 2'deoxy- 3'-deoxy-, 2',3'-dideoxy- and 2',3'dideoxy-2',3'didehydro-ribavirin triphosphate were tested as substrates for NDP kinase. Whilst the absence of the 2'-OH in the 2'-deoxy analog reduces only slightly the catalytic efficiency, all the compounds devoid of the 3'-OH are almost inactive, with catalytic efficiencies lower than 10 $M^{-1}s^{-1}$ (Table 1). As the 3'-OH is required for phosphorylation by NDP kinase and for inhibition of HCV polymerase during the initiation step, 2'-deoxyribavirin appears as the only derivative that is properly activated by NDP kinase and is efficient *in vitro* on HCV polymerase [51].

A strategy for enhancing activation by NDP kinase could be the use of antiviral analogs with a nucleophilic group in 3'-position. However, 3'-amino-3'-deoxythymidine, is an effective antiviral agent *in vitro* but was associated with high renal toxicity [55]. The efficiency of NDP kinase on 3'-amino-3'-deoxy dTDP is only five times higher than on AZTDP [34]. The 3'-fluoro-3'-deoxy-dTDP was found a better substrate, because the fluorine atom is a hydrogen bond acceptor, but the best one among antiviral analogs is clearly d4TDP [34, 45].

c- Borano- Substitution of the α-Phosphate

The comparison of structural data on nucleotides in complex with NDP kinase and with reverse transcriptase reveals some common features. In particular, an oxygen of the phosphate α, which does not interact with protein groups, neither in NDP kinase nor in the reverse transcriptase, could be replaced by a borano group. $-BH_3^-$ is isoelectronic to oxygen and isosteric to a methyl group and makes the α-phosphate chiral [32]. The Rp isomers of α-borano AZTTP and of α-borano d4TTP show a ten-fold improved efficiency with NDP kinase (Table 1) and are also more efficient inhibitors of HIV-reverse transcriptase. Kinetic studies of NDP kinase with α-borano derivatives indicated that the increase in catalytic efficiency mainly results from an improvement in the affinity for the enzyme. In contrast, the Sp isomer derivatives were found to be inactive with both NDP kinase and reverse transcriptase. NDP kinase was crystallised in complex with dTDP-α-boranoP (isomer Rp). The 1.92 Å resolution X-ray structure demonstrated that the α−borano analog binds like dTDP and makes the same interactions with the enzyme and the Mg^{2+} ion [32]. It is remarkable that NDP bound to HIV reverse transcriptase has a similar conformation to that in NDP kinase [32].

A major limitation of AIDS therapies is the emergence of resistant reverse transcriptases. One of the elements making the α-borano nucleotides attractive is its particular efficiency with these resistant reverse transcriptase mutants [32]. Interestingly α-boranophosphate-ddATP, for example, was shown to overcome the resistance of " multidrug resistant" reverse transcriptase while ddATP was inactive [56]. One limit to the development of useful drugs with borano-phosphate-derivatives is the difficulty to cross the plasma membrane. This could possibly be solved by vectorisation of the drug to deliver the borano-derivatives into cells. The α-borano substitution might be of interest *in vivo* with several nucleotide analogs against resistant viruses.

Affinity of Nucleotide Analogs for NDP Kinase

A strategy was developed to measure the affinity of the phosphoanalogs for the NDP kinase active site. For this, several mutants of NDP kinase were designed.

a- Affinity for NDPs

A single mutation where the Phe at the entrance of the active site was substituted by a Trp was made in *Dictyostelium* NDP kinase. The resulting mutant enzyme F64W (F60W in human enzyme numbering) is fully active and the extra Trp constitutes a fluorescent probe sensitive to NDP binding. K_D were found in the 0.015 – 0.20 mM range for natural NDP and AZTDP [44].

b- Affinity for NTPs

NTP affinity for wild type NDP kinase cannot be measured as the phosphotransfer to the catalytic His takes place almost instantaneously upon binding. An inactive NDP kinase was designed with a Gly replacing the catalytic His. The crystal structure of this mutant in a complex with ADP-Pi-Mg^{2+} shows that the mutation does not modify the overall structure of the active site [57]. This mutation was combined with F64W to yield the mutants F64W-H122G and F60W-H118G for *Dictyostelium* and human NDP kinase, respectively. The binding of a NTP to this mutant caused a large fluorescence signal that allows to perform titration curves in the absence of phosphotransfer. As shown in Table 1, the equilibrium dissociation constant (K_D) was found in the micromolar range for normal substrates and

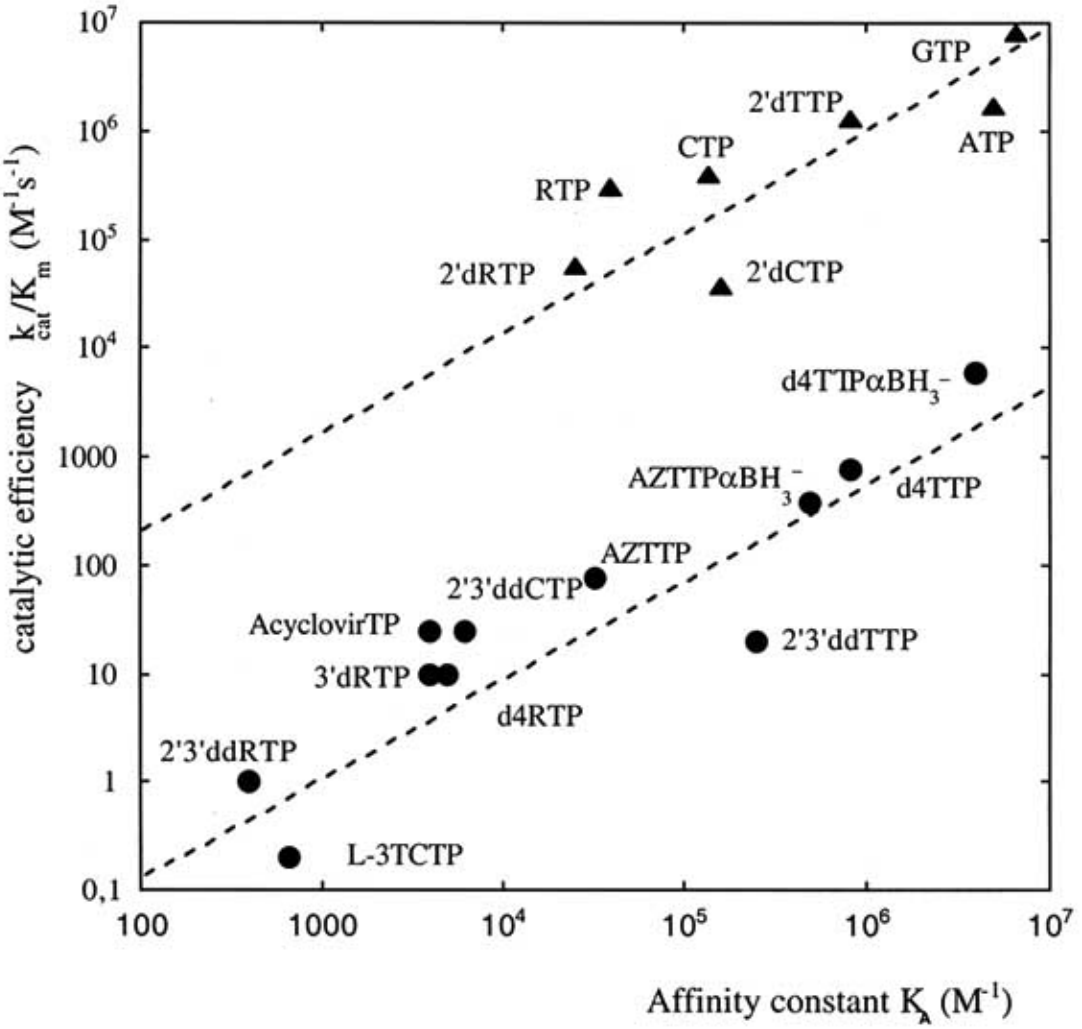

Fig. (5). Correlation of catalytic efficiencies of NDP kinase for several nucleotides triphosphate and analogs and their binding affinities. The catalytic efficiencies are measured in stopped flow experiments with human NDP kinase A. The equilibrium association constants are determined from fluorometric titration curves with the mutant F64W H122C NDP kinase from *Dictyostelium.* The active site of both enzymes is highly conserved [29].

increased slightly with the 3'-OH substituted analog [45, 50]. Only d4TTP was found to bind with a K_D similar to the natural substrate dTTP. The crystal structure provides an explanation for these results. The intramolecular CH…O bond within d4TTP is similar to the H-bond between 3'-OH and the oxygen bridging α- and β-phosphates and confers to d4TTP a dTTP-like conformation [32]. The α-borano substitution caused a ten-fold increase in affinity, in agreement with both the increase in reactivity and the structural data.

c- Affinity for the Phosphorylated Enzyme

A stable analog of phosphorylated NDP kinase was chemically formed by addition of a thiophosphate group on a Cys residue, which was engineered in *Dictyostelium* NDP kinase to replace the catalytic His [58]. The affinity of thiophospho-NDP kinase for NDP was measured by fluorescence titration. The order of affinities found was GDP > ADP > dTDP > CDP, in agreement with the reactivities [48]. The affinity of the phosphoenzyme for NDPs was at least 20 times greater than for NTPs with K_D^{GDP} = 50 μM and K_D^{ATP} > 2 mM. We concluded that the discrimination between di- and triphosphate nucleotides mainly results from affinity changes for the phosphoenzyme.

Correlation between Binding and Catalytic Efficiency

Fig. (**5**) shows a tentative correlation between the catalytic efficiencies and the equilibrium association constant K_A for natural nucleotides and analogs. Substrates are found to belong to two classes according to the presence of a 3'-OH. The two classes show a difference in catalytic efficiency of three orders of magnitude, illustrating the major contribution of the 3'-OH to substrate-assisted catalysis. Within each class, each line indicates that the catalytic efficiency is correlated to the binding affinity *i.e.* that a tighter substrate binding results in a more efficient phosphotransfer. This is probably related to the area of the base, which buries more or less surface upon binding to the enzyme. All complexes of NDP kinases with nucleotide and analogs show a similar mode of binding with slight differences, depending on the presence of the 2'OH and on

the size of the base. Both the nonpolar and the polar interactions made by the 3'-OH (and, to a lesser degree, the 2'OH) contribute to hold the substrate in place. Fig. (**5**) also illustrates that the borano group on the α-phosphate improved both phosphorylation by NDP kinase and binding affinity to the active site.

Improving NDP Kinase Activity with Antiviral Drug Analogs

Using protein engineering, we enhanced the efficiency of human NDP kinase towards antiviral analogs. Our strategy for improving the enzyme specificity, based on structural and catalytic data summarized above, was to introduce an hydroxyl group in the NDP kinase active site, where it could substitute for the missing 3'-OH in the antiviral nucleotide analogs (Asn 115 -> Ser mutation). Another mutation (Leu 55 → His) was inspired from the enzyme from *Dictyostelium* known to have a higher specific activity. The double mutant was found to be about 100-fold more efficient on acyclovirTP, AZTTP and d4TTP, each single mutation contributing about by a factor of about 10 [50] (Table 1). The X-ray structure of the mutant with a bound AZTTP derivative showed an improved geometry of binding and a favourable interaction of the 3'-azido group with the engineered Ser [50]. Improvement of an enzyme by mutagenesis is usually evaluated by the specificity factor, defined as the change in ratio of the catalytic efficiency with a given analog to that of the corresponding natural substrate, between the two enzymes. The gain in activity was accompanied with a larger change in specificity factor as the double mutant lost efficiency for natural substrates. This ratio was in the range of 100-500 for AZT, d4T and acyclovir, more than most of the results obtained by directed evolution [59]. The double mutant was almost as efficient on d4TTP as on dTTP, illustrating a remarkable specificity switch in the mutated enzyme.

CONCLUSION

NDP kinase has long been considered as an efficient enzyme that can convert all diphosphate on nucleoside analogs into triphosphates. We have shown that it phosphorylates with efficacy an analog like ribavirin, which is not a chain terminator. However NDP kinase catalytic efficiency drops to low levels with 3'-OH modified analogs, and the enzyme does not recognize at all L-3TCDP as a substrate. However pharmacokinetic studies of L-3TC phosphorylation in peripheral blood mononuclear cells from patients infected with HIV demonstrate that L-3TC is anabolized to L-3TCTP within cells [60, 61, 62, 63] indicating that other cellular enzymes are probably involved in the activation of L-3TC. Several kinases can phosphorylate natural nucleosides diphosphate and are potential candidates for the activation of analog diphosphates. Phosphoglycerate kinase was recently shown to phosphorylate L-3TCDP and other CDP analogs, although with a relatively low efficiency [64, 65]. Other kinases from basal metabolism may also participate in the synthesis of the analogs triphosphate [64, 66, 67]. On the other hand, the efficiency of NDP kinase on d4T derivatives indicates that it is presumably involved in the *in vivo* activation of d4T analogs. More studies are needed to identify all the enzymes that participate in antiviral nucleosides activation.

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ABBREVIATIONS

AZT	=	2',3'-deoxy-3'-azidothymidine
ddC	=	2'3'-dideoxycytidine
d4T	=	2'3'-dideoxy-2'3'-didehydrothymidine
HCV	=	Hepatitis C virus
HIV	=	Human immunodeficiency virus
ITP	=	Inosine triphosphate
L-3TC	=	β-L-2′,3′-dideoxy-3′-thiacytidine
L-3TCDP	=	β-L-2′,3′-dideoxy-3′-thiacytidine diphosphate
L-3TCTP	=	β-L-2′,3′-dideoxy-3′-thiacytidine triphosphate
NMP	=	Nucleoside monophosphate
NDP	=	Nucleoside diphosphate
NTP	=	Nucleoside triphosphate
RTP	=	Ribavirin triphosphate
2'dR	=	2'-deoxyribavirin
3'dR	=	3'-deoxyribavirin
ddR	=	2′,3′-dideoxy-ibavirin
d4R	=	2′,3′-didehydro-2′,3′-dideoxyribavirin

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