Structural Analysis of the Activation of Ribavirin Analogs by NDP Kinase: Comparison with Other Ribavirin Targets

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ABSTRACT

Ribavirin used in therapies against hepatitis C virus (HCV) is potentially efficient against other viruses but presents a high cytotoxicity. Several ribavirin triphosphate analogs modified on the ribose moiety were synthesized and tested in vitro on the RNA polymerases of HCV, phage T7, and HIV-1 reverse transcriptase. Modified nucleotides with 2′-deoxy, 3′-deoxy, 2′,3′-dideoxy, 2′,3′-dideoxy-2′,3′-dehydro, and 2′,3′-epoxy-ribose inhibited the HCV enzyme but not the other two polymerases. They were also analyzed as substrates for nucleoside diphosphate (NDP) kinase, the enzyme responsible for the last step of the cellular activation of antiviral nucleoside analogs. An X-ray structure of NDP kinase complexed with ribavirin triphosphate was determined. It demonstrates that the analog binds as a normal substrate despite the modified base and confirms the crucial role of the 3′-hydroxyl group in the phosphorylation reaction. The 3′-hydroxyl is required for inhibition of the initiation step of RNA synthesis by HCV polymerase, and both sugar hydroxyls must be present to inhibit elongation. The 2′-deoxyribavirin is the only derivative efficient in vitro against HCV polymerase and properly activated by NDP kinase.

Ribavirin is a broad-spectrum antiviral agent discovered in 1972 (Witkowski et al., 1972). It is used in therapy against chronic hepatitis C virus (HCV) infection in combination with interferon α (McHutchison et al., 1998; Poynard et al., 1998). It is also of potential interest against poliovirus, Lassa fever virus, respiratory syncytial virus, and emerging viruses, such as West Nile virus, but the exact mode of action of ribavirin remains uncertain (Patterson and Fernandez-Larsson, 1990). It may be indirect, through the inhibition of cellular enzymes. Phosphorylated anabolites (ribavirin monophosphate, ribavirin diphosphate, and ribavirin triphosphate) are observed in erythrocytes by RNA polymerases. Phosphorylated anabolites are a substrate for the polymerases of poliovirus (Crotty et al., 2000) and HCV (Maag et al., 2001). These enzymes incorporate ribavirin in the viral RNA facing either cytosine or uracil. Although the efficiency of incorporation is decreased by a factor in the 104 to 105 range compared with natural purine nucleotides, the analog is a powerful mutagen and the accumulation of replicative errors may explain its antiviral effect (Crotty et al., 2000).

Ribavirin transport into cells is probably mediated by nucleoside transporters (Jarvis et al., 1998; Patil et al., 1998). Intracellular phosphorylation is required for antiviral activity and must reach the 5′-triphosphate level for incorporation by RNA polymerases. Phosphorylated anabolites are observed in erythrocytes because of the action of adenosine kinase (Page and Conner, 1990; Homma et al., 1999) and other kinases of the salvage nucleotide pathway. These include nucleoside diphosphate

ABBREVIATIONS: HCV, hepatitis C virus; NDP, nucleoside diphosphate; d4T, 2′,3′-dideoxy-2′,3′-dehydrothymidine; RMP, ribavirin monophosphate; ddR, 2′,3′-dideoxyribavirin; d4R, 2′,3′-dideoxy-2′,3′-dideoxyribavirin; epoxyR, 2′,3′-anhydroribavirin; d2R, 2′-deoxyribavirin; HPLC, high-performance liquid chromatography; DTT, dithiothreitol; PEG, polyethylene glycol; RTP, ribavirin triphosphate; HIV, human immunodeficiency virus; RT, reverse transcriptase; RNAP, RNA polymerase; RMS, root-mean-square; ddRTP, 2′,3′-dideoxyribavirin triphosphate; epoxyRTP, 2′,3′-anhydroribavirin triphosphate.

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(NDP) kinase, which produces the triphosphate form by transferring the γ-phosphate of a nucleoside triphosphate (usually ATP) via a covalent phosphohistidine intermediate (Parks and Agarwal, 1973). NDP kinase has a high catalytic efficiency and a broad specificity, taking as substrates all natural diphosphate nucleosides and deoxynucleosides. It also phosphorylates the diphosphate derivatives of several antiviral nucleoside analogs such as 3′-azidothymidine and 2′,3′-dideoxy-2′,3′-dideoxythymidine (d4T), and with a much lower efficiency (Bourdais et al., 1996). These analogs, which are chain terminators in DNA synthesis, lack a 3′-OH group. Because the latter has been shown to play a major role in catalysis by NDP kinase (Schneider et al., 1998), ribavirin, which contains an unmodified ribose, should be more efficiently activated.

A major problem with ribavirin in therapy is its high cellular toxicity. This is possibly related to the inhibition by ribavirin monophosphate (RMP) of IMP dehydrogenase, which catalyzes the oxidation of IMP into xanthosine monophosphate, the rate-limiting step of the de novo synthesis of guanine nucleotides. The structural and functional properties of IMP dehydrogenase have been studied extensively (Sintchak and Nimmeggen, 2000), and X-ray structures are available (Colby et al., 1999). RMP probably mimics IMP. With the aim of decreasing its toxicity and improving the antiviral properties of ribavirin, we investigate here the action of NDP kinase on phosphorylated ribavirin derivatives.

The enzymatic analysis is supported by an X-ray structure of NDP kinase in complex with ribavirin triphosphate. We also prepared ribavirin analogs bearing modifications on the 2′-OH and/or the 3′-OH of the ribose moiety, study their properties as NDP kinase substrates and their ability to inhibit viral polymerases, HIV-1 reverse transcriptase and T7 RNA polymerase, as well as the HCV polymerase, the RNA polymerase of the hepatitis C virus.

Materials and Methods

Synthesis of Ribavirin Triphosphate Analogs. Analytically pure ribavirin was either a kind gift from Pr. Chris Meier (Hamburg, Germany) or was purchased from ICN Pharmaceuticals. 2′,3′-Dideoxy-2′,3′-dideoxythymidine (d4R), 2′,3′-dideoxyribavirin (d4R), 2′,3′-anhydro-ribavirin (epoxyR) (Fig. 1) were synthesized from d4T, but with a different strategy. The enzymatic analysis is supported by an X-ray structure of the complex ribavirin triphosphate with NDP Kinase and Data Collection. H122G D. discoideum NDP kinase in complex with RTP was crystallized by transferring tiny crystals obtained with a higher concentration of PEG, to a drop containing 13% PEG 550 monomethyl ester, 100 mM Tris-HCl, pH 7.5, 30 mM MgCl₂, 10 mg/ml protein, and 20 mM RTP, over wells containing 26% PEG 550 monomethyl ester in the same buffer. The crystals belong to the tringular space group P3₁2₁ with unit cell a = b = 71.7 Å, c = 153.8 Å. The asymmetric unit contains a trimer.

X-ray diffraction data from a single crystal were collected at λ = 1.542 Å and 18°C on a Rigaku generator with a MARResearch image plate detector. Diffracted intensities were evaluated with the programs DENZO and SCALEPACK (Otwinowski and Minor, 1997) and further processed using the CCP4 program suite (CCP4, 1994). Overall statistics are given in Table 1. Because the crystals were isomorphous to those of H122G NDP kinase-d4T triphosphate, the 1.85-Å model (Meyer et al., 2000) could be used to calculate phases. Electron density maps were examined using Turbo-FRODO (Roussel and Cambillau, 1991). The first 2Fo-Fc electron density showed clear

Fig. 1. Chemical structures of ribavirin and derivatives
density for both the protein and the ligand, needing only minor modifications of the protein structure. An RTP molecule and an Mg\(^{2+}\) ion were bound to each monomer. Water molecules were gradually added during further conjugate gradient refinement with CNS (Brünger et al., 1998). Residues 2 to 5 are missing in the final model for each monomer.

**Fluorometric Binding Studies on NDP Kinase.** The affinity of RTP analogs for NDP kinase was determined by following the variation of the intrinsic fluorescence upon nucleotide binding as described previously (Schneider et al., 1998). The fluorescence of the F64W-H122G mutant in T\(_b\) buffer (50 mM Tris-HCl, 75 mM KCl, and 5 mM MgCl\(_2\), pH 7.5) was measured at 330 nm with excitation at 310 nm (2-nm excitation slit and 4-nm emission slit) (PTI, New Brunswick, NJ). Successive aliquots of nucleotide were added to a 1 \(\mu\)M enzyme solution. The inner filter effect was negligible. Experimental binding curves were fitted to a quadratic equation after correction for dilution.

**Stopped-Flow Kinetic Experiments and Analysis of Kinetic Results.** Stopped-flow kinetic experiments were performed with a Hi-Tech DX2 (Salisbury, UK) microvolume stopped-flow reaction analyzer equipped with a high-intensity xenon lamp as described previously (Schneider et al., 1998). The excitation wavelength was 304 nm, with a 2-nm excitation slit and a 320-nm cutoff filter at the emission. Mixing was achieved in less than 2 ms. After mixing NDPK (1 \(\mu\)M) and NTP (10–500 \(\mu\)M), the intrinsic protein fluorescence was recorded for 10–200 s. In each experiment, 400 pairs of data were recorded, and the data from three or four identical experiments were averaged and fitted to a number of nonlinear analytical equations using the software provided by Hi-Tech. All curves fitted to single exponentials.

As described previously (Schneider et al., 1998), the data were analyzed using the reaction scheme:

\[
E + NTP \xrightarrow{k_{+1}} E \cdot NTP \xrightarrow{k_{+2}} E - P + NDP
\]

The observed single step could be attributed to the phosphotransfer between the nucleotide and the enzyme. Because the product concentration remains very low, the product binding can be neglected. The binding steps are expected to be fast and not detectable in the time range of the stopped-flow experiments. Saturation could not be obtained with the concentrations of nucleotide triphosphate used. Nevertheless, the data are sufficient to determine \(k_1k_2/k_{-1}\) or \(k_2/K_S\), where \(K_S\) is the dissociation constant of the E-NTP complex. This represents the catalytic efficiency of the enzyme phosphorylation step, equivalent to a second-order rate constant and allowing a reliable comparison of different NDP kinase substrates.

**Expression and Purification of HCV 1b Polymerase.** The NS5B gene encoding HCV 1b polymerase was PCR-amplified using

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**TABLE 1**

<table>
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<th>Parameter</th>
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<td>Asymmetric unit</td>
<td>trimer</td>
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<tr>
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<tr>
<td>(R_{ref}(%)^{c})</td>
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</tr>
<tr>
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<td>Bond angle (*)</td>
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\(\sum I_i = \langle I \rangle + \sigma (I)\) where \(I_i\) is the intensity of a reflexion, and \(\langle I \rangle\) is the average intensity of that reflexion.

\(\sum F_{obs} - \sum F_{calc}\)

\(\%\) of the data was set aside for free R-factor calculation.

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**Fig. 2.** RTP binding to NDP kinase. RTP bound to the H122G mutant of D. discoideum NDP kinase (yellow bonds) is superimposed in (A) to d4T triphosphate (green) bound to the same protein in the 1.85 Å resolution structure determined by (Meyer et al., 2000) in (B), to GDP bound to wild type human NDP kinase B (Morera et al., 1995). The active site histidine is His-122 in D. discoideum, His-118 in human. The spheres are Mg\(^{2+}\) ions.
the primers HCV-1b-BamHI forward (5'-GGCGGATCTTAAATGCTGAGATGTTTGATGATGAC-3') and HCV-1b-SalI reverse (5'-GGCGGATCTTAAATGCTGAGATGTTTGATGATGAC-3') from a clinical HCV isolate serotype 1b to yield Δ55 NS5B 1b cDNA, which specifies an NS5B protein truncated at its C terminus by 55 amino acids. The amplified fragment was digested by BamHI and SalI, cloned into PQE-30 (Amerham Biosciences, Orsay, France), which specifies an N-terminal His8 tag, and the resulting vector was used to transform BL21pDNA E. coli cells. Bacteria were cultured at 37°C in Luria broth medium supplemented with 100 μg/ml ampicillin and 25 μg/ml kanamycin until A600 nm reached 0.6. The temperature of the culture was then switched to 27°C, and expression was induced by the addition of 0.3 mM isopropyl β-D-thiogalactoside for 2 h. Bacteria were harvested by centrifugation, washed twice in phosphate-buffered saline, and the pellet was stored at −80°C until use. Bacteria were lysed for 30 min on ice in 3 ml of buffer 1 (50 mM sodium phosphate, pH 7.5, and 20% glycerol) per gram of bacteria supplemented with 1 mg/ml lysozyme, 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine. Lysates were diluted with 3 ml of buffer 2 (50 mM sodium phosphate, pH 7.5, 20% glycerol, 0.6 M NaCl, 10 mM β-mercaptoethanol, 1.6% Igepal, 20 mM imidazole, and 7 μg of DNase) per gram of bacteria, left on ice for 30 min, and sonicated on ice. Lysates were centrifuged for 30 min at 75,000g, and the cleared lysates were loaded on a 1-ml Hi-Trap heparin column (Amersham Biosciences). After extensive washes in buffer 3 (50 mM NaPO4, pH 7.5, 10% glycerol, 0.3 M NaCl, 5 mM (Amersham Biosciences), the cleared lysates were loaded on a 1-ml Hi-Trap heparin column, and 10 mM imidazole), bound proteins were eluted with 3 ml of buffer 3 adjusted to 900 mM NaCl. Eluted proteins were then diluted in T7 buffer (40 mM Tris-HCl, pH 7.5, 6 mM MgCl2, 2 mM spermidine, and 10 mM DTT) at 37°C, using 10 ng of DNA template, 1 or 0.5 mM ATP, CTP, UTP, and [α-32P]GTP (0.1 μCi of [32P]GTP at 10 μCi/μl). Reactions were initiated by the addition of recombinant RT (50 nM). After 30 min, aliquots were withdrawn and spotted onto DE-81 paper discs. Filter paper discs were washed three times for 10 min in 0.3 M ammonium formate, pH 8.0, washed two times in ethanol, and dried. The radioactivity bound to the filter was determined using liquid scintillation counting.

For phage T7 polymerase experiments, reactions were performed in T7 buffer (40 mM Tris-HCl, pH 7.5, 6 mM MgCl2, 2 mM spermidine, and 10 mM DTT) at 37°C, using 10 ng of DNA template, 1 or 0.5 mM ATP, CTP, UTP, and [α-32P]GTP (0.1 μCi of [32P]GTP at 10 μCi/μl). Reactions were initiated by the addition of 1 μg of recombinant T7 RNAP in presence of 0.5 or 1.5 mM RTP or analogs. Aliquots were withdrawn after 30 min and 1 h and spotted onto DE-81 paper discs. Filter papers were processed as described above.

HCV polymerase experiments were performed in Rdp buffer (50 mM HEPES, pH 8.0, 10 mM KCl, 1 mM MgCl2, 5 mM MgCl2, and 5 mM DTT) containing 125 ng/μl of homopolymeric cytosine RNA template (Amersham Biosciences) annealed to a guanosine dinucleotide primer (ESGS/Cybergene, Evry, France), and 0.5 mM [α-32P]GTP (0.1 μCi of [32P]GTP at 10 μCi/μl). Reactions were initiated by the addition of 300 ng of purified recombinant HCV 1b polymerase and incubated at 30°C. Aliquots were withdrawn over time and spotted onto DE-81 paper discs. For elongation inhibition, the reaction was incubated without RTP or analogs at 30°C. After 15 min, 0.5 mM RTP or RTP derivatives were added to the samples, and the reaction was allowed to continue for 80 min. Aliquots were withdrawn over time and spotted onto DE-81 paper discs. The latter were washed and processed as described above. Results shown are representative of three different experiments.

**Results**

**X-Ray Structure of Ribavirin Triphosphate Bound to NDP Kinase.** *D. discoideum* NDP kinase has 60 and 58% sequence identity, respectively, with the major A and B isoforms of the human enzyme, also called Nm23-H1 and Nm23-H2 (Lacombe et al., 2000). All eukaryotic NDP kinases are hexamers, including the *D. discoideum* and human pro-

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**TABLE 2**

<table>
<thead>
<tr>
<th>RTP</th>
<th>Protein</th>
<th>Distance</th>
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<tr>
<td>Ribose</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>O2'</td>
<td>NZ Lys 16</td>
<td>3.5 (0.2)</td>
<td>3.2</td>
</tr>
<tr>
<td>O3'</td>
<td>NZ Lys 16</td>
<td>2.8 (0.1)</td>
<td>2.9</td>
</tr>
<tr>
<td>O3R</td>
<td>ND2 Asn 119</td>
<td>3.0 (0.1)</td>
<td>2.9</td>
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<tr>
<td>O3R</td>
<td>O3R</td>
<td>3.2 (0.4)</td>
<td>2.6</td>
</tr>
<tr>
<td>α-Phosphate O2A</td>
<td>NE His 59</td>
<td>3.2 (0.1)</td>
<td>3.0</td>
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<tr>
<td>β-Phosphate</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>O1B</td>
<td>Mg²⁺</td>
<td>2.1 (0.2)</td>
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<td>γ-Phosphate</td>
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<td>O2G</td>
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<td>O3G</td>
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<td>2.2</td>
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* Oxygen bridging the β- and γ-phosphates in RTP and O7 in ADP-BeF₃.

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**Ribavirin Analogs Activation and Reactivity**

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teins. Their subunit folds are very similar, and their active sites are essentially identical (Janin et al., 2000). Structural data show the same mode of binding for GDP to human NDP kinase B (Morera et al., 1995) and ADP or TDP to wild-type D. discoideum NDP kinase (Cherfils et al., 1994; Morera et al., 1994). Thus, the D. discoideum enzyme is a good model of human NDP kinase for functional studies. In addition, the H122G mutant is useful when studying nucleoside triphosphate binding, because the substitution of the active site histidine prevents transfer of the γ-phosphate to the protein. We previously used the mutant in the analysis of a complex with d4T triphosphate at 1.85-A resolution (Meyer et al., 2000). With the wild-type protein, bound ATP can be mimicked by a complex of ADP and either aluminum or beryllium fluoride (Xu et al., 1997). The present X-ray structure of H122G in complex with ribavirin triphosphate has an R factor of 20.7% (R free = 24.9%) at 2.9 Å resolution and good stereochemistry (Table 1). The protein has essentially the same conformation as in the d4T triphosphate complex, a superposition yielding a RMS distance of 0.26 Å for all equivalent Ca positions. The superposition with GDP-bound human NDP kinase B is almost as good, with an RMS distance of 0.41 Å, excluding nine C-terminal residues that differ in conformation between the D. discoideum and human proteins (Lacombe et al., 2000).

RTP binds in the same site and orientation as other nucleotide substrates or nucleotide analogs (Fig. 2). The substituted triazole that replaces the base is in a slit opening on the protein surface and stacks between the side chains of Val-116 and Phe-64. The 2.9-Å electron density map uniquely defines the plane of the five-membered ring, but it is compatible with a 180° rotation of the ring about the glycosidic bond. Thus, the complex may contain either the syn and anti conformers of the nucleoside analog or a mixture of the two. In addition, the carboxamide group in C3 of the triazole can also flip by 180° relative to the ring. None of these rotations enables the substituted triazole to make polar interactions with the protein. In the GDP complex, guanine makes a hydrogen bond with the side chain carboxylate of the C-terminal glutamate of an adjacent subunit in the hexamer. The distance of the carboxylate to the carboxamide group in C3 of the triazole is more than 5 Å, too long for a hydrogen bond.

Unlike d4T, which has a double bond and no hydroxyl in C2’-C3’, ribavirin carries an unmodified ribose. The sugar in bound RTP has the same 3’-endo ring pucker as in ADP bound to D. discoideum NDP kinase (Morera et al., 1994) or GDP in the human enzyme (Morera et al., 1995). The 2’- and 3’-hydroxyl groups make the same interactions in these two complexes (i.e., with the amide group of Asn-119 and the amino group of Lys-16). All polar interactions that involve the phosphate groups are also conserved (Table 2). The γ-phosphate of RTP, like that of d4T triphosphate (Meyer et al., 2000) interacts with Lys-16, Tyr-56, and the main chain NH of Gly-123. In the wild-type protein, fluoride ions make the same interactions in the ADP-beryllium and aluminum fluoride complexes (Xu et al., 1997).

All the complexes cited above contain a Mg2+ ion. The metal interacts with oxygens of two phosphates if the ligand is a diphosphate and oxygens of three phosphates if it is a triphosphate, which is the case of RTP. In either case, water molecules complete the octahedral coordination. The metal takes two positions approximately 2 Å apart in the complexes with di- and triphosphates, illustrated in Fig. 2, respectively, by the human NDP kinase B-GDP and the H122G-RTP structures. The metal-oxygen distances remain in the range 2.0 to 2.4 Å and the phosphate groups themselves do not move: the α- and β-phosphates of bound RTP and GDP superimpose to within 0.5 Å (that is, within the error in the coordinates of a X-ray structure at 2.9 Å resolution).

**Catalytic Efficiencies of NDP Kinase for Ribavirin Triphosphate and Derivatives.** We followed the time course of the reaction of RTP with human NDP kinase A in fast kinetic experiments by monitoring the intrinsic fluorescence of the protein. The fluorescence is quenched upon phosphorylation of the active site histidine by a NTP substrate.

![Fig. 3](image-url)
and, reciprocally, enhanced upon its dephosphorylation by NDP (Deville-Bonne et al., 1996). The reaction with RTP yields a monoexponential fluorescence decay without a lag, as previously reported for other nucleoside triphosphates (Schaertl et al., 1998; Schneider et al., 1998). The pseudo-first-order constant \( k_{obs} \) of the exponential decay increased linearly with RTP concentrations in the range investigated (5–50 \( \mu \)M) (Fig. 3). The slope of the linear fit, which measures the catalytic efficiency of RTP as a substrate for NDP kinase, was \( K_{S} / K_{M} = 3 \times 10^{5} \text{M}^{-1} \text{s}^{-1} \). This makes RTP a good substrate, comparable with the natural substrate CTP, although inferior to GTP, which is the best known substrate of NDP kinase (Table 3).

The phosphorylation kinetics were also studied with RTP analogs modified on the ribose. The absence of a 2'-hydroxyl group in 2’dRTP led to a 5-fold loss in catalytic efficiency, comparable with the 2-fold change observed between GTP and dGTP (Fig. 3A and Table 2). Removal of the 3'-hydroxyl group had a much larger effect, causing a drop in activity by a factor of \( 10^{4} \) with both 3’dRTP and d4RTP. The factor reached \( 10^{5} \) in the case of the 2’,3’-dideoxy analog (ddRTP) (Fig. 3B and Table 2). Very similar observations have been made with thymidine analogs: the d4T derivative was 10-fold better that the dideoxy derivative when tested as a substrate of NDP kinase (Schneider et al., 2000). The 2’3’-epoxy analog of RTP (epoxyRTP) is also better than the dideoxy compound. Acyclovir triphosphate was also assayed for reaction with NDP kinase. Acyclovir, which is used in herpes virus therapy, is a guanosine analog with a linear connection between the base and 5'-hydroxyl group. The phosphorylation reaction followed in the fluorescence test was \( 10^{5} \) times slower than for GTP and \( 10^{4} \) times slower than for RTP. This confirms the dominant role of the sugar hydroxyl groups and the minor importance of the base in the reaction with NDP kinase.

### Binding Affinities of Ribavirin Triphosphate and Derivatives for NDP Kinase

Although the equilibrium dissociation constant \( K_{D} \) of the NDP kinase-NTP complex is not accessible in these kinetic experiments, it can be estimated by using a mutant NDP kinase, which lacks the catalytic function linear phase occurring after the lag phase (see Inhibition of Viral Polymerase by RTP and Analogs). Mean values of relative activities for each compound are shown as percentage of NS5B activity without inhibitor. S.E. and mean values were calculated from two independent experiments.
DNA polymerase activity of HIV RT. Because 2'-ribavirin is a ribonucleoside analog, RTP may also inhibit the to serious adverse effects (Sim et al., 1998). Indeed, although ity, but its combination with other nucleotide analogs leads
ribavirin has been reported to exhibit some anti-HIV activ-
affinities than the natural substrates.

ratios indicate that all analogs bind NDP kinase with lower
Inhibition of Viral Polymerase by RTP and Analogs. Ribavirin has been reported to exhibit some anti-HIV activity, but its combination with other nucleotide analogs leads to serious adverse effects (Sim et al., 1998). Indeed, although ribavirin is a ribonucleoside analog, RTP may also inhibit the DNA polymerase activity of HIV RT. Because 2',3'-dideoxynucleotides are efficient against HIV RT, it was of interest to test whether 2'- or 3'-deoxy-modified RTP is efficient against HIV RT and retains inhibitory power against HCV polymerase.

We first analyzed the activity of RTP and derivatives against DNA polymerization by HIV RT. Because RMP can be incorporated opposite either cytidine or uridine, we used templates containing all four natural ribonucleotides. HIV RT was not affected by any of the compounds at concentration up to 4 mM (data not shown), suggesting that they are either not incorporated or not chain terminators. We next tested whether ribavirin derivatives inhibit RNA polymerases. Phage T7 RNAP is the best known RNA polymerase and constitutes an interesting model for inhibition. Polymerization was assayed using a DNA template containing the T7 promoter, the four natural nucleotides, and increasing amounts of RTP or derivatives. No significant polymerization inhibition was observed, suggesting that RTP and derivatives are not inhibitors of T7 RNA polymerase (data not shown).

RTP and derivatives were then tested against HCV polymerase. HCV polymerase activity is characterized by a non-processive initiation step, followed after 10 to 15 min by linear incorporation (elongation step). We measured the nu-
cleotide incorporation over time in presence of RTP or RTP analogs. Polymerase activity was determined by calculating the rate of nucleotide incorporation after the initiation step. RTP, 2’dRTP, and epoxyRTP inhibited HCV polymerase; 2’dRTP was the most efficient (Fig. 5, 85% of relative inhibition). The other RTP analogs inhibited HCV polymerase weakly, if at all.

We wanted next to test whether the lower nucleotide incor-
Fig. 6. Inhibition of initiation or elongation of HCV polymerase by RTP and derivatives. Assays were performed in the presence of 125 ng/μl of template/primer [poly(C)/GG] and 0.5 mM of RTP or analogs (○, no inhibitor; ●, RTP; ●, 2’dRTP; ■, 3’dRTP; □, epoxyRTP; ▲, ddRTP; △, d4RTP). For inhibition of initiation, polymerase activity was determined as incorporation of radiolabeled GTP nucleotide during the first 15 min. For inhibition of elongation, polymerase reaction was started without RTP derivatives. After 15 min, 0.5 mM RTP or derivatives were added, and the reaction continued for 80 min. Polymerase activity was determined as incorporation of radiolabeled GTP nucleotide over time.

Fig. 7. Correlation of catalytic efficiencies of NDP kinase for several nucleotides triphosphate and their binding affinities. The equilibrium affinity constant $K_a$ is measured from fluorescence titrations curves on F64W-H122G mutant NDP kinase A as shown on Fig. 4. Catalytic efficiencies are obtained in stopped-flow experiments with human NDP kinase A. The active site of both enzymes is highly conserved (Janin et al., 2000). AZTTP, 3'-azidothymidine triphosphate; d4TTP, 2',3'-dideoxy-2',3'-dideoxyribavirin triphosphate; ddCTP, 2',3'-dideoxy-2',3'-dideoxythymidine triphosphate; d4RTP, 2',3'-dideoxyribavirin triphosphate; 2’dRTP, 2',3'-dideoxyribavirin triphosphate; 3’dRTP, 3'-deoxyribavirin triphosphate; ddRTP, 2',3'-dideoxy-2',3'-dideoxyribavirin triphosphate; d4RTP, 2',3'-dideoxy-2',3'-dideoxyriboavirin triphosphate; ddRTP, 2',3'-dideoxyribavirin triphosphate.

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incorporation induced by RTP, epoxyRTP, and 2′dRTP, whereas 3′dRTP, ddRTP, or d4RTP had no effect or a slight activating effect (Fig. 6A). To measure inhibition of the elongation step, RTP analogs were added after 15 min at the end of the initiation step, and the reaction continued for 80 min. Under these conditions, elongation was inhibited only by RTP, with little effect of the other analogs (Fig. 6B).

In summary, RTP inhibits both initiation and elongation, 2′dRTP and epoxy-RTP act only on initiation, and 3′dRTP, ddRTP, and d4RTP have no effect on nucleotide incorporation. This suggests that both the 3′ and 2′ hydroxyl groups are required for inhibiting elongation but that the presence of an oxygen at the 3′ position is sufficient for inhibiting initiation.

**Discussion**

Unlike nucleoside analogs directed against HIV RT, which lack a 3′OH, ribavirin contains a normal ribose moiety and cannot act as a chain terminator. The derivatives tested here combine the base modification of ribavirin and some of the sugar modifications found in RT inhibitors. We tested the capacity of NDP kinase to produce the triphosphate form of these derivatives and the effect of the triphosphate form on several target viral polymerases.

**Correlation between Binding Affinities and Catalytic Efficiencies of Nucleotides for NDP Kinase.** RTP and its analogs were found to be excellent substrates for NDP kinase on the condition that the 3′hydroxyl group is present. This is in line with previous studies of natural substrates and analogs. Figure 7 shows a 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: those carrying a 3′-OH and those lacking this hydroxyl group. The two lines obtained for the two classes indicate a change in catalytic efficiency by a factor 500 to 1000, illustrating the well-established contribution of the 3′-OH to substrate-assisted catalysis by NDP kinase. The lines themselves show that, within each class, the catalytic efficiency correlates linearly to the binding affinity.

Tighter substrate binding makes for more efficient phosphotransfer. Thus, GTP has both the highest reactivity and the best affinity ($K_a = 0.15 \mu M$). Affinity and reactivity are less for RTP. The structures of GDP bound to the active enzyme and RTP bound to the H122G variant (Fig. 1) suggest a simple reason for the difference: the ribavirin base, which is smaller than guanine or other natural bases, buries less surface upon binding the enzyme. Thus, the nonpolar contribution to the binding energy is less, whereas other interactions are conserved. All complexes of NDP kinase with nucleotides (at least 10 X-ray structures are known) indicate a similar mode of binding for all, except for the presence or absence of the 2′-OH, and the size of the base surface in nonpolar contact with the protein. Both the polar interactions made by the 3′OH and the nonpolar contact contribute to hold the substrate in place.

**The Use of Ribavirin Nucleotide as RNA Polymerization Inhibitors.** Ribavirin is currently used as a therapeutical agent against HCV in combination with interferon (Lauer and Walker, 2001). Although it is a weak antiviral drug when used alone, ribavirin is phosphorylated by cellular kinases up to the triphosphate level, and ribavirin mono-phosphate is incorporated into the viral RNA by the recombinant HCV polymerase (Maag et al., 2001). Templates containing RMP can also block RNA elongation. This makes ribavirin an interesting candidate for chemical modification aiming to increase its antiviral effect.

The RNA replication of the HCV genome goes through two steps relevant to our discussion: the initiation step, comprising the incorporation of the very first nucleotide, up to the 4th or 5th nucleotides, and the elongation step, which consists of processive extension of the neosynthesized RNA. The initiation step, and especially the synthesis of the first phosphodiester bond between the two first nucleotides, is non-processive and rate-limiting (Kao et al., 2001). Moreover, the switch from initiation to elongation requires a conformational change. Therefore, polymerization can be inhibited during the initiation step, by preventing the switch from initiation to elongation, or during processive synthesis. We have observed that 2′dRTP and epoxy-RTP are HCV polymerase inhibitors if they are present at the beginning of the reaction, but not if they are added after 15 min of reaction. This suggests that 2′dRTP and epoxy-RTP are initiation inhibitors only. 3′dRTP, ddRTP, and d4RTP, which were thought to be chain terminators, do not have the expected inhibitory effect, during either initiation or elongation, suggesting that they are not incorporated by the HCV enzyme. In contrast, RTP inhibits both initiation and elongation.

What is the detailed mechanism of this inhibitory effect? HCV polymerase activity is stimulated in the presence of high concentrations of GTP (Lohmann et al., 1998), and kinetic studies revealed two $K_a$ values for GTP, one similar to that of other nucleotides, and a higher one (Luo et al., 2000). Because ribavirin is a guanosine analog, it can compete either for the incorporation of GTP in the nascent RNA or for the stimulating effect of GTP. Recently, an allosteric GTP-binding site has been characterized far away from the catalytic site and is not involved in the polymerization reaction (Bressanelli et al., 2002). This allosteric GTP-binding site might also represent a target for GTP analogs. Indeed, because RTP, epoxyRTP, and 2′dRTP inhibit the initiation step of the polymerization, they may compete for this GTP-binding site and may inhibit the allosteric activation or the switch from initiation to elongation, normally induced by the binding of GTP to this site.

Epoxy-RTP, which does not carry a 3′-OH, is a putative chain terminator, but it does not show the expected effect, at least during the elongation step of polymerization. RTP and 2′dRTP carry a 3′-OH and should not be chain terminators. Nevertheless, they, like epoxy-RTP, inhibit the initiation of synthesis by HCV polymerase. This suggests that a 3′hydroxyl group, or at least the presence of an oxygen at the 3′ position, is required to inhibit this step. Inhibition of elongation requires both hydroxyls in position 2′ and 3′, making RTP the only efficient inhibitor of this step. RTP may act at either step by competing for the NTP binding site or by blocking incorporation per se, as already observed by Maag et al. (2001).

In conclusion, the experiments suggest that 2′-deoxy ribavirin may be an alternative to ribavirin in therapies against C hepatitis. It is efficiently activated to the triphosphate form by NDP kinase and is a potent specific inhibitor of HCV polymerase initiation. The absence of the 2′OH on 2′deoxyribavirin is likely to result in a weaker interaction with
IMP dehydrogenase, the cytotoxic cellular target, because the active site side chains interact with both 2′OH and 3′OH of the nucleotide (Colby et al., 1999). Cellular studies are now needed to validate this in vitro study.

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