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ACCELERATED COMMUNICATION

Ribavirin Is an Inhibitor of Human Immunodeficiency Virus Reverse Transcriptase

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SUMMARY

Ribavirin inhibits the human immunodeficiency virus reverse transcriptase in an *in vitro* reaction. Ribavirin-5′-diphosphate was close to 40% more inhibitory than ribavirin-5′-triphosphate. Unphosphorylated ribavirin had a reduced, but detectable, effect as an inhibitor, compared with the phosphorylated forms. The compounds seem to have a direct effect on the viral polymerase, and no chain termination was observed in the presence of ribavirin-5′-triphosphate. Combination of any of the ribavirin derivatives tested with 3′-azido-3′-deoxythymidine (zidovudine)-5′-triphosphate resulted in an increase of its anti-human immunodeficiency virus reverse transcriptase activity in the *in vitro* assay.

Antiviral compounds have attracted considerable attention as potential therapeutic agents against AIDS almost since the causative agent, now referred to as HIV, was first identified as a retrovirus. To date, interest has focused on selective antiviral compounds targeted against the viral RT, the CD4 cellular receptor molecule for the virus, and, more recently, the protease encoded by HIV (1–4). The most potent inhibitors of HIV RT studied have been the nucleoside analogs. To this group belong the 2′,3′-dideoxynucleosides, which apparently exert their inhibition by chain termination of the growing DNA chain (5, 6). The best known example is AZT, which is the only drug approved for the treatment of HIV infection in the United States.

Ribavirin (1-β-D-ribofuranosyl-1,2,3,-triazole-3-carboxamide) is a nucleoside analog of purines with a broad spectrum antiviral activity against many RNA and DNA viruses (7, 8). It is licensed in the United States for the treatment of pediatric respiratory syncytial virus infection, but it has been used successfully in the treatment of other viral diseases (9). The long history of this drug, as well as its documented toxicity and safety evaluations, has warranted the study of the effects of ribavirin against HIV. Ribavirin inhibits HIV-1 infection of cultures of human adult T lymphocytes (10). It has been reported to delay or prevent development of AIDS in patients with HIV-induced persistent generalized lymphadenopathy (11). The mechanism by which ribavirin might interfere with the replication of the virus has not been studied.

Several theories have been proposed to explain the molecular mode of action of ribavirin. One proposed mechanism states that the monophosphate derivative of ribavirin is a potent competitive inhibitor of IMP dehydrogenase, therefore disrupting the *de novo* synthesis of GMP and causing a depletion of the GTP pools in the cell (12, 13). However, a derivative of ribavirin that depletes GTP pools has no antiviral activity (14). A second proposed mechanism suggests that ribavirin treatment of virally infected cells results in the synthesis of mRNA with absent or abnormal cap formation (15). This theory is supported by the observation that poliovirus, which lacks a cap at the genomic 5′ end, is not significantly inhibited by ribavirin and that the effect of ribavirin can be reversed by the addition of guanosine (12, 13, 16).

The third hypothesis states that ribavirin has a direct suppressive effect on the viral polymerase (17–21). It must be noted that these hypotheses are not mutually exclusive and may be an indication that ribavirin acts in a multiple-site fashion. We have previously reported that ribavirin has a significant direct effect on the viral polymerase in a VSV *in vitro* transcription system (20). This effect did not involve chain termination and was mediated to different degrees by

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ABBREVIATIONS: AIDS, acquired immunodeficiency syndrome; AMV, avian myeloblastosis virus; AZT, 3′-azido-3′-deoxythymidine; HIV, human immunodeficiency virus; ID_{50}, 50% inhibitory dose; RDP, ribavirin-5′-diphosphate; RT, reverse transcriptase; RTP, ribavirin-5′-triphosphate; VSV, vesicular stomatitis virus.

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TABLE 1

Inhibition of AMV RT by ribavirin compounds

The reaction tubes each contained 150 μg/ml levels of the indicated drug and incubation was allowed to proceed for 30 min at 37° for 30 min, at which time 50 μl were removed from each tube for measurement of acid-insoluble radioactivity.

Drug	[^{32}P]dTMP incorporation
	%
None	100
RDP	0.3
RTP	18
Ribavirin	59

RDP and RTP. Inhibition by RDP or RTP could be reversed by addition of GTP, CTP, and UTP, but not by the addition of GDP or ATP (20). Recently, when we examined the kinetics of the effect of phosphorylated ribavirin on the VSV *in vitro* transcription reaction, we found that both RDP and RTP were competitive inhibitors with all four natural nucleoside triphosphates (21).

We report another inhibitory effect of ribavirin against a viral polymerase, the RNA-directed DNA polymerase associated with HIV-1, its probable mechanism of action, and its interaction with AZT.

Materials and Methods

Chemicals. The phosphorylated ribavirin compounds and AZT-5′-triphosphate were kind gifts from Roland K. Robins, Nucleic Acid Research Institute (Costa Mesa, CA), and Phillip A. Furman, Burroughs Wellcome Co. (Research Triangle Park, NC), respectively. Nonphosphorylated ribavirin (Virazole) was obtained from ICN Pharmaceuticals, Inc. (Costa Mesa, CA).

Enzymes. AMV RT was a commercial product purified from virions, obtained from Promega Corporation. Cloned HIV-1 RT (clone BH-10), obtained through the AIDS Research and Reference Reagent Program, AIDS Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health, was used throughout this study.

RT *in vitro* reaction. Reaction mixtures (150 μl) contained 50 mM Tris·HCl (pH 7.3), 100 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 μg of poly(rA)-oligo(dT)$_{10}$, 0.133 mM [α-^{32}P]dTTP (New England Nuclear), 0.5 units of cloned HIV RT, and the indicated amount of RTP or RDP (1 unit of HIV RT was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol of TMP into DNA in 10 min at 37°). For AMV RT experiments, the conditions of the reactions were the same as described above, except the enzyme. Each tube contained 0.5 units of AMV RT (1 unit of AMV RT was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol of dTTP into acid-insoluble form in 10 min at 37°).

Dideoxy chain termination reaction. The dideoxy chain termination sequencing procedure of Sanger *et al.* was used to determine whether RTP could function as a chain terminator in a growing DNA chain (Fig. 2). The template/primer was a pGEM-2 double-stranded DNA plasmid and an 18-mer synthetic oligonucleotide complementary to the T_7 promoter region of pGEM-2. The primer was labeled at its 5′ end with [γ-^{32}P]ATP and T_4 polynucleotide kinase and was preannealed to plasmid template. The reaction mixtures (5 μl) contained 50 mM Tris·HCl (pH 7.3), 100 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 200 μM each of dGTP, dATP, dTTP, and dCTP, 0.7 μg of pGEM-2DNA, 1 μg of oligonucleotide primer, and 1.4 units of AMV RT. Each of the reactions in Fig. 2, *lanes G, A, T, C,* and *RTP*, also contained 40 μM dideoxy-GTP, 40 μM dideoxy-ATP, 40 μM dideoxy-TTP, 40 μM dideoxy-CTP, and 160 μM RTP, respectively. Fig. 2, *Blank*, contained no dideoxy nucleotides or RTP. Incubation was for 20 min at 42°, at which time the reactions were stopped by addition of 5 μl of a formamide loading buffer, heated for 2 min at 100°, and analyzed on a sequencing gel (24). RTP did not cause any base-specific chain termination.

Results

First, studies were conducted with the RT of AMV in an *in vitro* system and it was found that RDP, RTP, and ribavirin were inhibitory to the enzyme (Table 1). These results prompted us to investigate the effect of the drugs on HIV-1 RT.

RTP caused a marked inhibition of cloned HIV RT when added at time zero to an *in vitro* assay with poly(A)$_n$-oligo(dT)$_{10}$ as primer-template (Fig. 1A). The inhibition was almost complete (98%) at a concentration of 309 μM (150 μg/ml), irrespec-

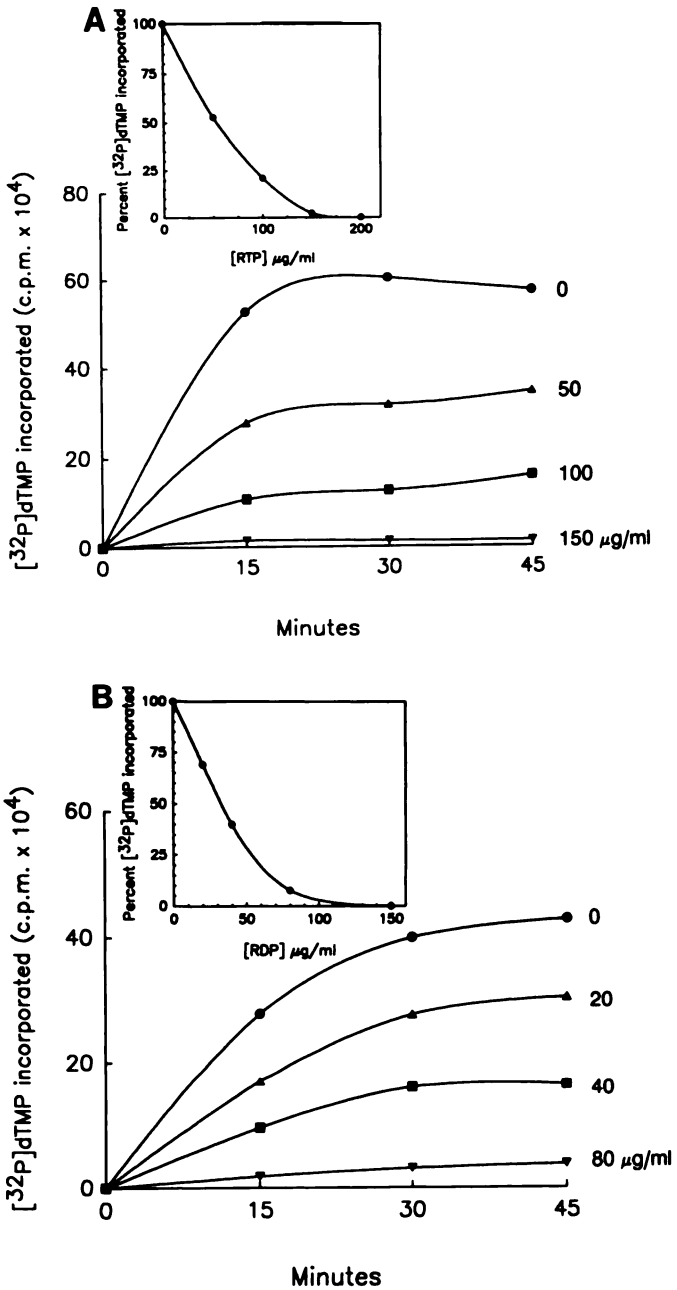

Fig. 1. Inhibition of HIV-1 RT by RTP (A) and RDP (B). The reactions were incubated at 37° for 45 min. Samples (35 μl) of each of the reaction mixtures were removed at 0, 15, 30, and 45 min for measurement of standard trichloroacetic acid-soluble radioactivity.

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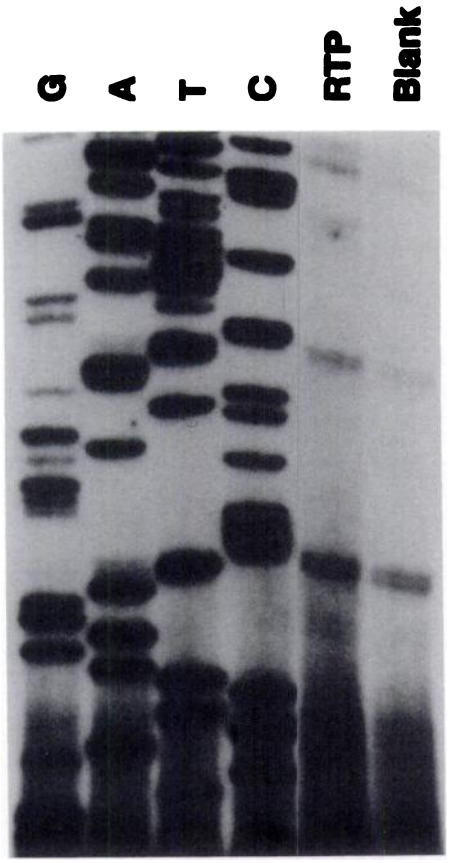

Fig. 2. Dideoxy chain termination reactions using AMV RT. *Lanes G, A, T,* and *C*, 40 μM dideoxy-GTP, 40 μM dideoxy-ATP, 40 μM dideoxy-TTP, and 40 μM dideoxy-CTP, respectively. *Lane RTP*, 160 μM RTP; *Blank*, no dideoxy nucleotides or RTP.

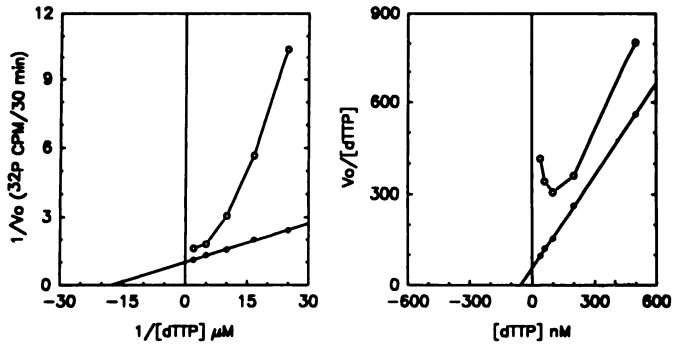

Fig. 3. Lineweaver-Burk (*left*) and a replot of $[S]/V_o$ versus V_o (*right*) presentation of the inhibition of HIV RT. Enzyme activity was determined by measurement of [^{32}P]dTTP incorporation in reaction mixtures (150 μl), as described for Fig. 1, except that the concentration of dTTP was varied. The reactions were incubated for 30 min at 37°, at which time 50-μl samples were removed for measurement. RTP concentration was zero (●) or 27 μM (○).

tive of the time at which the synthesis of DNA was measured. At a concentration of 206 μM (100 μg/ml), RTP effected a 79% inhibition of the RT reaction, and at 103 μM (50 μg/ml) 47% of the reaction was inhibited. From the values obtained for the reaction mixtures that had been incubated for 30 min, a dose-response curve was obtained (Fig. 1A, *inset*), from which the ID_{50} of RTP for the *in vitro* HIV RT reaction was estimated at about 112 μM (54 μg/ml).

The effect of RDP was also studied because it was previously shown that this molecule was more inhibitory to viral RNA-dependent RNA polymerases than RTP (20, 21). RDP caused greater inhibition than RTP in an identical HIV RT *in vitro* assay (Fig. 1B). The inhibition was 100% at 371 μM (150 μg/ml). At 30 min of incubation, 198 μM (80 μg/ml) RDP effected a 92% inhibition of the RT reaction and 99 μM (40 μg/ml), a 60% inhibition; at 49 μM (20 μg/ml) RDP still caused a 31% inhibition. From the dose-response curve (Fig. 1B, *inset*), the ID_{50} was estimated to be about 81 μM (33 μg/ml). RDP was almost 40% more inhibitory than RTP.

Unphosphorylated ribavirin was a much less effective inhibitor of HIV RT and AMV RT. At 30 min of incubation, 615 μM (150 μg/ml) ribavirin caused a 42% inhibition of HIV RT. This percentage of inhibition could be attained with 128 μM RTP, as extrapolated from its dose-response curve. Similarly, it was found that the same concentration of ribavirin (615 μM) caused a 58% inhibition of AMV RT, whereas this same inhibition could be achieved by 82 μM levels of the triphosphate.

Other viral polymerase inhibitors, such as AZT, that are known to be effective when triphosphorylated inhibit by chain termination (25). A Sanger chain termination reaction (22) with RTP was performed and no chain termination was found in the presence of this drug (Fig. 2). This is in agreement with our previous finding, with the VSV polymerase, that transcripts synthesized in the presence of drug were full length and were free of incorporated drug (20).

In order to determine the mechanism of inhibition, we performed a kinetic analysis of the ability of RTP and RDP to inhibit the cloned HIV RT. The K_m for dTTP, without the inhibitor, was 57 ± 4 nmol. It was found that, in several repetitions of the experiment, this value was consistent and significantly lower than K_m values obtained previously with purified HIV RT (25–29). The difference was on the order of 10- to 100-fold lower than published results, depending on the study. The K_m value is also lower than in a previous report where a cloned HIV RT was used (30). The difference between these values could be attributed to the inherent characteristics of our particular cloned HIV RT, to the differences between affinity-purified native HIV RT and cloned HIV RT, or to both.

Even though RTP was an inhibitor of HIV RT, the type of inhibition and the K_i value could not be obtained using standard Lineweaver-Burk plots. The double-reciprocal plot of the inhibition of HIV RT by RTP showed a nonlinear sigmoidal dependence of velocity upon substrate concentration (Fig. 3, *left*), regardless of the different concentrations of RTP used (only one RTP concentration shown). The sigmoidal behavior is also clearly seen in a replot of $[S]/V_o$ against V_o (Fig. 3, *right*).

Discussion

As we have reported previously (for review, see Ref. 8), the structural similarity of ribavirin may include all of the natural substrates of some polymerases. Results of our experiments in which the effects of phosphorylated ribavirin compounds on an *in vitro* VSV polymerase assay indicated that the drug does indeed possess a significant direct suppressive effect on viral polymerase. All three phosphorylated species inhibited VSV transcription. The mono- and diphosphorylated forms of the drug possessed approximately 2 to 3 times the inhibitory activity of the triphosphorylated form. Transcripts synthesized in

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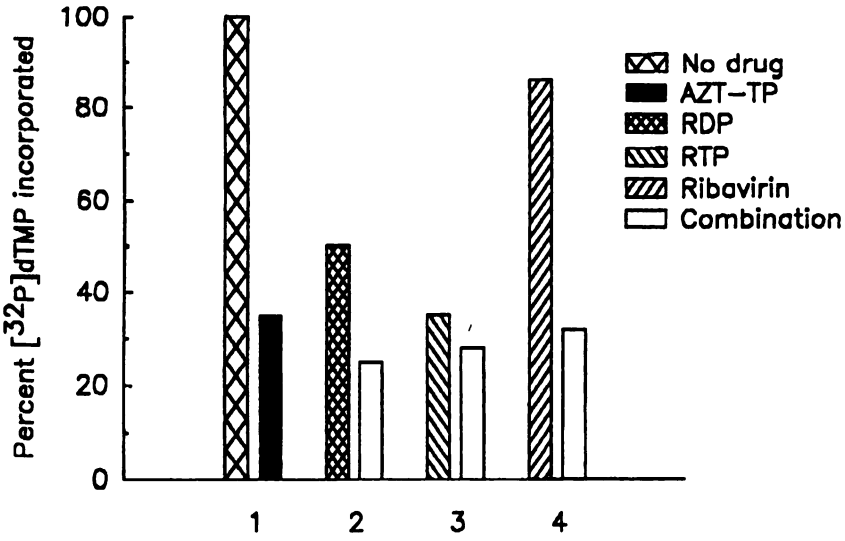

Fig. 4. *In vitro* RT reactions in the presence of various combinations of AZT-5′-triphosphate (*AZT-TP*) and ribavirin compounds. *1*, Control with no drug and AZT-5′-triphosphate (16.6 μM); 2, RDP (49.5 μM) and combination of AZT-5′-triphosphate and RDP (the same concentrations as in the reactions with only one drug); 3, RTP (62 μM) and combination of AZT-5′-triphosphate and RTP; *4*, ribavirin (273 μM) and combination of AZT-5′-triphosphate and ribavirin. The conditions of the reaction mixtures (150 μl) were as for Fig. 1, and incubation was for 30 min at 37°, at which time 50 μl were removed from each tube for measurement of acid-insoluble radioactivity.

the presence of the drug were full length. Inhibition by RDP could be reversed by the addition of UTP, CTP, and GTP, and the addition of GDP to the reaction did not reverse the inhibition. The observation that, besides the triphosphorylated form, both the mono- and diphosphorylated forms of the drug were active against the VSV replicase was surprising. We have used enzyme kinetic procedures and product analysis to further investigate the mechanism of action of ribavirin. When analyzed by double-reciprocal plots, both RDP and RTP gave similar patterns of inhibition, although the K_m values of the nucleoside triphosphates in the presence of the different drugs were not identical. The K_m of RDP reflected its greater inhibitory effect. Both of the phosphorylated forms of ribavirin compete directly with all four of the nucleoside triphosphates, suggesting that they are acting on the polymerase in a similar fashion. Because there appears to be no incorporation of ribavirin into the growing nucleic acid chain and because the transcripts synthesized in the presence of either drug appear full length, it was our hypothesis that RDP and RTP were blocking polymerase initiation at the 3′ terminus of the genome.

Ribavirin and its phosphorylated derivatives inhibit HIV RT in an *in vitro* reaction. The finding that RDP is a better inhibitor than RTP suggests that the modified base of ribavirin and possibly a phosphate charge are the significant factors in this non-chain-terminating interference. The number of phosphates does not seem to be critical, although the presence of the charge might be the potentiating factor when there is no phosphodiester bond involvement. As mentioned already, nonphosphorylated ribavirin had a substantially lower inhibitory effect on both HIV RT and AMV RT, when compared with the phosphorylated compounds.

We were unable to determine the type of inhibition caused by RTP on HIV RT using standard plots. Within the scope of this paper, the results of this graphic analysis are interpreted as cooperativity or allosterism (31). However, it is important to note that we have previously found that phosphorylated ribavirin compounds unmasked an allosteric site, in that case presumably an ATP-binding site, on the transcriptase of a mutant VSV (23). It is, therefore, possible that the abnormal kinetic behavior we see with HIV RT in the presence of RTP is also caused by this molecule acting on a site other than the nucleotide polymerization site, and it is likely that the anti-HIV activity of ribavirin is based on a direct effect on the viral polymerase.

In considering a combination of antiviral drugs that may lead to an increase in the efficacy of available treatments, ribavirin has been tested *in vitro* and found to enhance the effects of 2′,3′-dideoxy purine analogs (32) but to antagonize the inhibitory effects of 2′,3′-dideoxy pyrimidine analogs. In the case of 3′-azido-2,6-diamino purine-2′,3′-dideoxyriboside, combination of this drug with ribavirin also extended the enhancement of its inhibitory activity *in vivo* (33). We were interested to determine the effect of a combination of ribavirin and AZT on the *in vitro* RT reaction, because antagonism of HIV replication has been reported between the two drugs (34). It was found that unphosphorylated ribavirin, RDP, or RTP did not antagonize but rather increased the inhibition by the 5′-triphosphate of AZT of the HIV RT (Fig. 4). This result was not totally unexpected, in view of the very different mechanisms of action of AZT and ribavirin. Also, in the *in vitro* reaction the drugs are stabilized, whereas in the infected cell both drugs undergo similar phosphorylation events that might cause them to antagonize each other. In a clinical situation, that undesirable effect could be avoided if at least one of the drugs is delivered as a stabilized phosphorylated compound. We have previously demonstrated that stabilized RDP derivatives retain antiviral activity (35). These phosphorylated ribavirin compounds could not passively diffuse through the cell membrane but could ostensibly be delivered in a liposome system (36). Our observation that RDP has the highest inhibitory activity of the ribavirin compounds is significant in this context.

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