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Mode of action of ribavirin: effect of nucleotide pool alterations on influenza virus ribonucleoprotein synthesis

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Summary

Ribavirin, a guanosine analogue, is a broad spectrum antiviral agent which is effective in the treatment of influenza. In this study, the effect of ribavirin on influenza virus ribonucleoprotein (RNP) synthesis and nucleotide pool sizes was simultaneously measured. Ribavirin (100 μM) reduced viral RNP synthesis 94% as measured by UTP incorporation. Intracellular GTP pools, measured by high performance liquid chromatography, were reduced approximately 45% in ribavirin treated cells, while other nucleotides remained near control values. Attempts to reverse ribavirin's inhibitory effects on viral RNP synthesis by addition of exogenous guanosine (50 μM) resulted in only a partial restoration of viral RNP synthesis, despite full restoration of the GTP pool. Dose–response experiments indicated that the GTP pool was significantly reduced (65% of control) at 25 μM ribavirin, and increasing concentrations of the drug caused only a small further reduction in the GTP pool (5–10% at 100 μM). In contrast, RNP synthesis was inhibited by 50% at 25 μM ribavirin and was further decreased to 5% of control at 100 μM ribavirin. Thus, ribavirin's antiviral activity may result from a reduction of the GTP pool size combined with direct effects on viral replicative enzymes.

influenza virus; ribonucleoprotein synthesis; nucleotide pools; ribavirin

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Introduction

Ribavirin, 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, is a potent antiviral agent with a broad spectrum of antiviral activity [15]. It resembles guanosine by X-ray crystallography [13] and appears to be a guanosine analogue in several systems [16]. Clinical studies show it to be safe and efficacious when inhaled as a small-particle aerosol for the treatment of influenza A [6] and B [9] virus and respiratory syncytial virus [19] infections.

The mechanism by which ribavirin inhibits influenza virus replication is unclear. Streeter et al. [18] have demonstrated that ribavirin monophosphate inhibits the GMP biosynthetic pathway and that exogenously added guanosine interferes with ribavirin's inhibition of measles virus replication in cell culture. From these results, Streeter et al. proposed that ribavirin inhibits viral replication by reducing the precursor GTP pool in infected cells.

In tests with influenza virus, Oxford [11] and Scholtissek [14] have observed that ribavirin diminished the production of viral macromolecules in infected cells; however, nucleotide pool sizes were not measured. Eriksson et al. [3] tested the effect of ribavirin triphosphate (RTP) on several eukaryotic and prokaryotic polymerases and determined that the influenza viral polymerase was selectively inhibited by RTP.

Previous reports have not simultaneously measured reductions in GTP pool sizes and influenza virus replication. In this study we have examined the effects of ribavirin on viral ribonucleoprotein (RNP) synthesis and nucleotide pools in influenza virus infected Madin Darby Canine Kidney (MDCK) cells to determine if these two phenomena are related. Our observations suggest there are two mechanisms of ribavirin action, one that is dependent on ribavirin's depletion of intracellular GTP pools and another that is independent of the size of the GTP pool.

Materials and Methods

Chemicals

Ribavirin was a gift of Dr. Roberts A. Smith, Viratek, Inc., Covina, CA. Nucleotides and other chemicals were obtained from Sigma Chemical Co., St. Louis, MO. [5,6-^{3}H]Uridine was purchased from New England Nuclear Corp., Boston, MA. High performance liquid chromatography (HPLC) grade reagents were purchased from Fisher Scientific Co.

Virus A/WSN

A/WSN strain of influenza virus pools were obtained by inoculation of 10-day-old embryonated eggs in the allantoic cavity. After 48 h of incubation at 34°C, allantoic fluid was harvested, aliquoted, and frozen at −70°C. The 50% tissue culture infectious dose ($TCID_{50}$) varied from $10^{6.58}$ to $10^{7.18}$.

Tissue culture

MDCK cells were obtained from Flow Laboratories. They were serially passed in

Eagle's minimal essential medium supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, sodium bicarbonate and 5% fetal bovine serum.

Preparation of viral and cellular RNP particles

A modified procedure of Pons [12] and Krug [7] was used. Four plates of confluent monolayers of MDCK cells (3×10^7) were washed twice with phosphate-buffered saline (PBS) and infected with virus at a m.o.i. of 3–7 $TCID_{50}$/cell. After absorption of virus at 4°C, the virus suspension was poured off, and warmed minimal essential medium containing 2 μg/ml trypsin (Worthington Biochem.), 0.2% sodium bicarbonate, 100 U/ml penicillin, 100 μg/ml streptomycin and the appropriate test compound was added. 3 h post-infection, [^{3}H]uridine was added at a concentration of 10 μCi/ml and incubation was continued for an additional 3 h. This period of incubation was found to give optimal labeling of RNP particles. Cultures were washed with cold PBS, harvested with a rubber policeman and divided into two aliquots. One aliquot was reserved for HPLC. In the second aliquot, the cells were pelleted and resuspended in RSB (0.01 M Tris, pH 7.4; 0.01 M NaCl; 0.0015 M $MgCl_2$) containing 0.5% Nonidet P40. The cells were homogenized by 20 strokes in a Dounce homogenizer, and the nuclei were removed by centrifugation (10 min at 2000 rpm). RNP particles were precipitated from the cell homogenate by the addition of a 1/2 volume of 0.2 M sodium acetate, pH 4.5 and incubated at 4°C for 16 h. The sample was centrifuged at 15000 rpm in a Sorvall centrifuge, and the pellet was resuspended in 1 ml of STEU buffer (0.01 M Tris, pH 7.2; 0.1 M NaCl; 0.001 M EDTA; 1 M urea) and dialyzed overnight against the same buffer. The sample was centrifuged at 2000 rpm to remove particulate matter, and the supernatant fraction was loaded onto discontinuous sucrose gradients. The gradients were prepared by layering, in order, 1 ml of 60% sucrose, 2 ml of 30% sucrose, 1 ml of 24% sucrose, 2 ml of 21% sucrose, 1 ml of 19% sucrose, 2 ml of 17% sucrose and 2 ml of 15% sucrose all in sucrose gradient buffer (0.01 M potassium phosphate, pH 7.4; 0.002 M EDTA, 0.075 M NaCl). The gradients were centrifuged for 16 h at 39000 rpm in a SW41 rotor. The gradients were fractionated and analyzed in a liquid scintillation counter after the addition of 3 ml of Aquasol (New England Nuclear Corp.). Samples were not precipitated with trichloroacetic acid prior to counting. The cpm per fraction obtained were multiplied by the measured specific activity of UTP in the cells to determine pmol of UTP incorporated.

High performance liquid chromatography

To measure cellular nucleotide pools, HPLC was employed on an aliquot of each preparation of cells. The technique was that of Hartwick and Brown [4]. Aliquots of cells to be analyzed were pelleted and a 1/2 volume of 25% trichloroacetic acid was added. To monitor recoveries, 20 nmol of dGTP was added as an internal standard. The sample was centrifuged at 2000 rpm for 10 min, and the supernatant fraction was extracted three times with an equal volume of water saturated diethyl ether. The sample was filtered and kept frozen until ready for use. The apparatus consisted of a Waters 6000A solvent delivery system, a Waters model 440 absorbance detector, a U6K injector and a Waters 600 solvent programmer. The system employed a Whatman Partisil 10/SAX column run isocratically at 0.65 M KH_2PO_4, pH 4.3. Standard

preparations of nucleotides were chromatographed to identify characteristic retention times. Peak heights were measured at 254 nm and used for quantification. Fractions from the eluate were collected from the HPLC and analyzed for radioactivity by liquid scintillation counting. Counts eluting with the UTP peak were determined and used to calculate intracellular specific activity after the total mass of UTP was known. Specific activity was expressed as pmol of UTP/cpm. Using this procedure, the amount of ATP, UTP and GTP found intracellularly was measured accurately. CTP was not detected by this system due to the low intracellular concentration of this nucleotide and its relatively poor absorbance at 254 nm.

CsCl gradients

Fractions from the sucrose gradients were mixed 1 : 4 with 7% glutaraldehyde (0.5 ml of 0.2 N KOH, 2 ml of 70% glutaraldehyde, 17.5 ml of sucrose gradient buffer), and incubated at 4°C for 2 h. Samples were loaded onto CsCl density gradients (1.20–1.50 g/ml) containing sucrose gradient buffer. Centrifugation was for 16 h at 32 000 rpm in a SW41 rotor. The gradients were fractionated, analyzed on a refractometer and counted in a liquid scintillation counter.

Results

Separation and quantification of viral and cellular RNP particles

We measured influenza virus RNP particle formation in infected MDCK cells and separated these virus specific RNP particles from cellular particles by sucrose velocity sedimentation. Fig. 1A is a representative profile of a sucrose gradient. Purified viral RNP particles moved to the bottom of the gradient (fraction 2) while RNP particles from uninfected MDCK cells remained near the top of the gradient (fraction 20). RNP particles isolated from influenza virus infected MDCK cells separated into two fractions, one fraction comigrated with viral RNP particles and one fraction comigrated with uninfected cellular RNP particles. Viral RNP particles (fraction 2) and cellular RNP particles (fraction 20) from infected cells were recovered from the sucrose gradients and subjected to CsCl density gradient centrifugation. As shown in Fig. 1B the viral RNP particles were found to have a density of 1.37 g/ml, characteristic of influenza virus RNP particles [7], while cellular RNPs had a density of 1.27 g/ml. Thus the sucrose velocity sedimentation method permits the separation and quantification of viral RNP particles and cellular RNP particles in infected cells.

The effect of ribavirin and guanosine on nucleotide pools and viral RNP synthesis

Infected cells were treated with ribavirin, guanosine, both compounds, or medium alone and subsequently tested for nucleotide pool variation and viral RNP particle synthesis. The results are given in Table 1. At 100 μM ribavirin viral RNP synthesis was reduced by 94% ($P = 0.03$; paired t-test, two-tailed) while cellular RNP was reduced only 37% ($P = 0.019$; paired t-test, two-tailed). Ribavirin treatment reduced the GTP pool size by 44%; however, other nucleotide pool sizes were not affected. Guanosine (50 μM) reduced the UTP pool size 45% and the ATP pool size by 38%, but

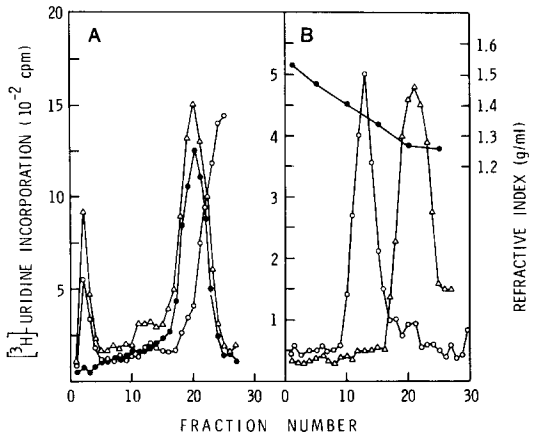

Fig. 1.(A) Sucrose velocity sedimentation of viral and cellular RNP particles. MDCK cells were mock-infected or infected with A/WSN virus at a m.o.i. of 3–7. At 3 h post-infection the cells were labeled with 10 μCi/ml [^{3}H]uridine and incubated for an additional 3 h before lysis of the cells. The RNP particles were precipitated with sodium acetate and centrifuged on discontinuous sucrose gradients. Fractions from the sucrose gradient were counted in a liquid scintillation counter after the addition of 3 ml of Aquasol. RNP particles from purified virus (○), infected cells (△) or uninfected cells (●) were tested. Samples containing the purified virus preparation were not dialyzed, and fractions 18–25 contain unincorporated [^{3}H]uridine. (B) CsCl equilibrium density centrifugation of viral and cellular RNP particles. Viral (○) and cellular (△) RNP particles from infected cells were recovered from the sucrose gradients, fixed with glutaraldehyde and centrifuged on CsCl density gradients.

TABLE 1

Effect of ribavirin and guanosine on nucleotide pool size variation and RNP particle synthesis in infected cells

Treatment	Nucleotide pool size (nmol/3 × 10^7 cells)			UTP incorporated into RNP particles (pmol/3 × 10^7 cells)	
	GTP	ATP	UTP	Viral[a]	Cellular
Medium	15.2 ± 2.1	34.2 ± 2.7	12.2 ± 1.7	631 ± 142	2659 ± 534
Ribavirin (100 μM)	8.6 ± 1.5	30.5 ± 6.1	12.5 ± 3.0	37 ± 43	1668 ± 355
Guanosine (50 μM)	15.1 ± 1.4	21.4 ± 3.0	6.9 ± 1.4	409 ± 101	2429 ± 595
Ribavirin (100 μM) Guanosine (50 μM)	17.4 ± 3.4	21.9 ± 4.7	6.1 ± 1.7	153 ± 86	2450 ± 601

Influenza virus infected MDCK cells were labeled with 10 μCi/ml [^{3}H]uridine 3h post-infection and lysed 3 h later. Each lysate was analyzed for nucleotide pool size variation by HPLC and for RNP synthesis by sucrose velocity sedimentation as described in Materials and Methods. Numbers represent mean values ± S.E.M. of four experiments.

[a] These values have been corrected for the amount of cellular RNPs (140 ± 57 pmol of UTP) in uninfected control cells which co-purify with viral RNPs during sucrose velocity sedimentation.

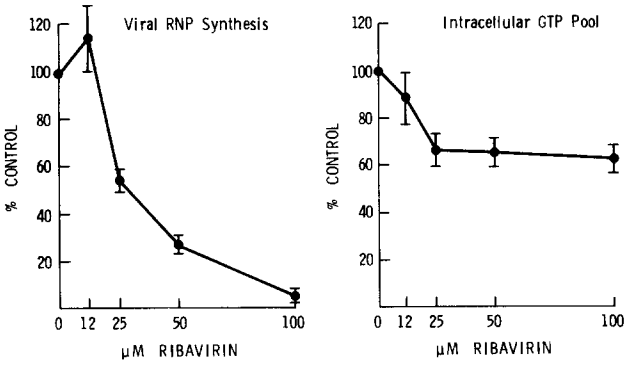

Fig. 2. Quantification of intracellular GTP pool size and viral RNP synthesis. MDCK cells (3×10^7) were infected with influenza virus A/WSN, treated with ribavirin, labeled with 10 μCi/ml of [^{3}H]uridine 3 h post-infection and lysed after an additional 3 h. Lysates were analyzed for viral RNP synthesis by sucrose velocity sedimentation and for GTP pool size variation by HPLC as described in Materials and Methods. Values represent the mean percent inhibition of 5 experiments. Vertical lines represent ± S.E.M.

the GTP pool size was not affected. Guanosine treatment also reduced viral RNP synthesis by 36%. The addition of both ribavirin and guanosine to the medium depleted only the UTP and ATP pools. The GTP pool size did not vary from control values. Treatment with both compounds diminished the production of viral RNP particles by 63% as compared to the guanosine control and by 76% as compared to the medium control. Thus in the presence of both compounds, RNP particle synthesis was reduced despite a normal GTP pool size, demonstrating that guanosine was able to reverse the effect of ribavirin on the GTP pool size but could not completely restore viral RNP synthesis to control levels.

Relationship between GTP pool size and RNP particle synthesis

Fig. 2 shows the effect of ribavirin on viral RNP synthesis and on the size of the GTP pool at various concentrations of the drug. At low concentrations of ribavirin, the GTP pool and RNP synthesis decreased concomitantly. However, at increasing ribavirin concentrations, above 25 μM, RNP synthesis continued to decline, reaching 5% of the control value at 50 μM, while the GTP pool size remained almost constant (i.e., 60–65% of control value).

Discussion

We have described the inhibition of synthesis of influenza virus RNP by ribavirin while concomitantly measuring intracellular pools of GTP, ATP, and UTP. Viral RNP synthesis was nearly completely inhibited by 100 μM ribavirin coincident with a 45% reduction in the GTP pool.

It has been hypothesized that ribavirin inhibits viral replication by interfering with guanosine metabolism, specifically by competitive inhibition of IMP dehydrogenase by ribavirin metabolites [18] shown to exist in cells [20]. Indeed, pools of GTP and

dGTP decrease in L5178Y lymphoma cells in a dose-dependent manner with ribavirin treatment [10].

In this study, GTP pools were measured with increasing doses of ribavirin and it was found that the pool was reduced maximally 35–45% at $\geqslant$ 25 μM ribavirin. Despite this constant amount of GTP in the cells, increasing concentrations of ribavirin ($>$25 μM) caused the level of viral RNP synthesis to continue to decrease, until it was reduced to 5% of control at 100 μM ribavirin. These findings suggested that a mechanism of ribavirin inhibition of influenza viral RNP exists that is distinct from the effect of ribavirin on the GTP pool size.

Several investigators [5,10,1] have reported that guanosine interferes with the action of ribavirin in tissue culture and suggested that restoration of the GTP pool by guanosine is the basis for this interference; however, nucleotide pools were not measured in these studies. To examine this question, we restored the intracellular GTP concentration by the addition of exogenous guanosine and simultaneously measured viral RNP synthesis. We hypothesized that if a limiting GTP pool is the sole mechanism by which ribavirin inhibits virus replication, restoration of the GTP pool by guanosine should also return viral RNP synthesis to control values. However, when interpreting these guanosine reversal experiments, it is important to note that guanosine treatment alone reduced the amount of viral RNP synthesis by 35%, possibly due to the reduced amounts of ATP and UTP in such cells. Similar effects of guanosine on ATP and UTP pools have been observed in *Aedes albopictus* cells, coincident with an inhibitory effect on Sindbis virus replication [17]. Due to this inhibitory effect, the addition of guanosine to ribavirin treated cells was not expected to be followed by an increase in viral RNP synthesis to the medium control level (631 pmol of UTP incorporated). Instead, viral replication in the presence of ribavirin and guanosine should increase to the guanosine control level (409 pmol of UTP incorporated) if a limiting GTP pool is the only mechanism of ribavirin's antiviral action. The results showed that viral RNP synthesis was restored only to 37% of the guanosine control, providing further evidence for a mechanism of inhibition independent of the GTP pool size. In contrast, studies on Sindbis virus replication have shown a complete reversal of ribavirin's antiviral effects by xanthosine, which presumably restores the GTP pool, although this was not shown [8]. Actinomycin D treatment also completely reversed ribavirin's action in these studies and the reversal correlated with both a restoration of the GTP pool and a reduction in the amount of phosphorylated ribavirin. Thus, it is likely that ribavirin has a general inhibitory effect on virus replication by depleting the GTP pool; but ribavirin may also have virus specific effects such as interference with the replicative enzymes of certain viruses such as influenza virus.

In experimental influenza virus infection in mice, ribavirin significantly protected against mortality [5], and this protection was not reduced by guanosine treatment of the animal. A possible explanation for the failure of guanosine to reduce the protective effect of ribavirin in vivo is that homeostatic mechanisms prevent fluctuations in the intracellular concentration of GTP during ribavirin treatment. Thus, the sustained antiviral effect of ribavirin in vivo is likely due to an effect independent of GTP.

We wish to emphasize the importance of determining the specific acticity of nucleic acid precursors when quantitative studies of nucleic acid synthesis are being perform-

ed. The addition of guanosine to reverse antiviral effects in our system altered the specific activity of the radioactive precursor, UTP, and caused artifactually high incorporation values. After guanosine treatment, the intracellular UTP specific activity was 2.3×10^{-2} Ci/mmol, a value more than 2-fold greater than the UTP specific activity in the medium control (1.1×10^{-2} Ci/mmol). Ribavirin has also been shown to alter the specific activity of thymidylate by interfering with phosphorylation of thymidine [2]. Hence, the determination of the specific activity of precursor pools can prevent errors in the quantitation of macromolecular synthesis.

In summary, phosphorylated derivatives of ribavirin probably act at more than one site to inhibit viral replication. At lower concentrations of ribavirin (25 μM and less) some of the in vitro effect of ribavirin may be due to a reduction in the GTP pool size, although inhibition of viral enzymes cannot be disproved. At higher concentrations of ribavirin, where pool effects are no longer evident, or in the intact animal, the effect against influenza virus may be due to another mechanism such as interference with viral polymerases.

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