Vol. 89, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 13, 1979 Pages 830-836

THE BROAD SPECTRUM ANTIVIRAL AGENT RIBAVIRIN INHIBITS CAPPING OF mRNA

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Received June 19, 1979

SUMMARY: Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a broad spectrum antiviral substance active against a wide range of both DNA and RNA viruses. It is, however, virtually inactive against polio virus. Its pharmacological mechanism of action was obscure. A possible common target for a chemotherapeutic agent in both DNA and RNA viruses is the "capping" reaction of mRNAs which inter alia involves the formation of a guanine pyrophosphate structure at the 5' terminus by mRNA guanylyl transferase. We have observed that Ribavirin triphosphate is a potent competitive inhibitor of the capping guanylation of viral mRNA. This finding could account for the antiviral potency of the drug against both DNA and RNA viruses and its ineffectiveness against a virus in which the mRNAs derived from them are not capped.

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a broad spectrum antiviral substance active against a wide range of both DNA and RNA viruses (1). It is, however, inactive against polio virus (2). Its pharmacological mechanism of action was obscure, but almost certainly involves guanine nucleotides since its antiviral effect in cell culture can be totally reversed by guanosine. Ribavirin diminishes the intracellular pool size of guanine nucleotides (3). Ribavirin is phosphorylated in vivo (4,5), and Ribavirin 5'-monophosphate is a potent competitive inhibitor of inosine 5'-phosphate dehydrogenase (4). But if this were its sole mechanism of action (6), it would not account for its selectivity.

0006-291X/79/150830-07$01.00/0

A possible common target for a chemotherapeutic agent in both DNA and RNA viruses is the blocked methylated "cap" structure of mRNAs. This posttranscriptional modification involves: (a) the formation of a guanine pyrophosphate structure by the enzyme GTP: mRNA guanylyltransferase, which transfers GMP from GTP to the 5' terminus of acceptor mRNA, (b) transfer of a methyl group from S-adenosylmethionine to position 7 of the added guanosine by S-adenosylmethionine: mRNA (guanine-7-) methyltransferase, and (c) methylation of a penultimate nucleoside by S-adenosylmethionine: mRNA nucleoside-2'-O-) methyltransferase (7).

We report here that Ribavirin 5'-triphosphate is a potent competitive inhibitor of the 5'-terminal guanylation of vaccinia mRNA. Vaccinia is a large DNA-containing virus which replicates in the cytoplasm of infected cells. Vaccinia mRNAs synthesized *in vitro* in the presence of S-adenosylmethionine contain the cap structures $m^7G(5')pppG^m$ and $m^7G(5')pppA^m$ at the 5'-terminus (8). All enzymes needed for the synthesis of viral mRNAs and posttranscriptional modification are present in the virion (9,10). By manipulating experimental conditions mRNA can be isolated which can serve as substrate for studying the synthesis of blocked methylated structures *in vitro* (11). We therefore examined the effect of Ribavirin on transcription and 5'-terminal modification of vaccinia mRNA.

METHODS

Mouse L929 cells were grown in roller bottles at 37°C in Eagle's minimum essential medium (MEM) containing 5% fetal bovine serum. Confluent monolayers were infected with vaccinia virus in MEM lacking fetal bovine serum. After 30 minutes of absorption, growth medium was added and incubation continued for 18 hours. Infected cells were harvested, washed twice with balanced salt solution and stored frozen at -70°C. Vaccinia virus was isolated and purified by centrifugation through a sucrose cushion and two sucrose gradient centrifugations (12,13). The final virus preparation contained approximately 70 µg protein per A_{260} unit.

Uncapped vaccinia mRNA as a substrate for guanylyltransferase reaction *in vitro* was synthesized in the presence of S-adenosylhomocysteine (11). The incubation system contained in a total volume of 50-100 ml: 4-5 A_{260}/ml of purified virus, 50 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 10 mM DTT, 0.05% Nonidet P-40, 2.5 mM each of ATP, GTP, CTP and UTP, and 100 µM S-adenosylhomocysteine. After 90 minutes of incubation at 37°C, cores

TABLE 1: Effect of Ribavirin and Its Nucleotides on the Incorporation of ^{3}[H] UMP and GMP on *in vitro* Transcription by Vaccinia Core Associated Enzymes

Reaction Mixture	pMole Incorporated UMP	GMP
Experiment 1		
Complete	1000	
-ATP, GTP, CTP	negligible	
Nonidet P-40	negligible	
+Ribavirin, 1 mM	1000	
+Ribavirin 5'-monophosphate, 1 mM	1000	
+Ribavirin 5'-triphosphate, 1 mM	1000	
Experiment 2*		
Complete[a]		50
+Ribavirin 5'-triphosphate, 1 mM		50
Complete[b]		75
+Ribavirin 5'-triphosphate, 1 mM		72
Complete[c]		80
+Ribavirin 5'-triphosphate, 1 mM		80

To study the effect of Ribavirin and its phosphorylated derivatives (5) on RNA synthesis by virion RNA polymerase, the reaction mixture contained in 100 µl: 4-5 A_{260} units/ml of purified virus, 50 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 10 mM DTT, 0.05% Nonidet P-40, 2.5 mM each of ATP, CTP and GTP, 0.5 mM UTP (sigma) and 2 µCi of [^{3}H] UTP (New England Nuclear, Sp. Act. 14.1 Ci/mMole). After 30 minutes of incubation at 37°C, TCA insoluble radioactivity was collected on glass fiber filters, washed with cold 5% TCA and ethanol, dried and counted. *The RNA polymerase reaction mixture was modified to contain 2.5 mM UTP and all unlabelled GTP was replaced with (a) 5µM[8-^{3}H] GTP; (b) 25 µM [8-^{3}H] GTP; and (c) 45 µM [8-^{3}H] GTP (New England Nuclear, Sp. Act. 11.2 Ci/mMole). Reactions were incubated for 10 minutes.

were removed by centrifugation at 30,000 g for 30 min. The supernatant was made 0.1 M in respect to Tris-HCl (pH 8.5) and 0.5% in respect to SDS, and was extracted with water saturated phenol in cold. To the aqueous phase, 2 volumes of ethanol was added and was kept at -20° for several hours. The dense precipitate was dissolved in water and high molecular weight RNA was precipitated with 2 M LiCl. The LiCl precipi-

tation was repeated twice more. The pellet was dissolved in water and RNA was precipitated twice with 2 volumes of ethanol and 0.1 volume of 2 M sodium acetate (14,15).

Vaccinia "capping" enzyme activities were isolated from purified virus (16). After solubilizing the virus cores with 0.1% sodium deoxycholate in 0.3 M Tris-HCl (pH 8.4), 50 mM DTT and 0.25 M NaCl and centrifugation at 140,000 g for 60 minutes to remove insoluble material, the supernatant was adjusted to 0.2M NaCl, 10% glycerol, 0.1% Triton X-100 and 1 mM EDTA. It was then passed through a DEAE-cellulose column equilibrated with 0.15M Tris-HCl (pH 8.4), 0.1% Triton X-100, 2 mM DTT, 10% glycerol and 1 mM EDTA containing 0.2M NaCl. The flow-through from the column which was devoid of most nucleic acid was used for all enzymatic reactions (9, 10).

RESULTS AND DISCUSSION

Ribavirin is a potent inhibitor of both RNA and DNA synthesis *in vivo* (17,18). However, Ribavirin 5'-phosphate does not inhibit either mouse lymphoma cell DNA polymerase or mouse liver RNA polymerase *in vitro* (3). We studied the effect of Ribavirin on DNA-dependent RNA synthesis by vaccinia associated polymerase (Table 1). Ribavirin or its phosphorylated derivatives did not inhibit incorporation of [^{3}H] UMP into TCA insoluble material. Since Ribavirin has molecular conformation similar to guanosine (19), to rule out the possibility that any inhibition of RNA polymerase activity by Ribavirin was overcome by the presence of GTP in the incubation mixture, RNA synthesis was carried out in the presence of limiting amounts of GTP. However, even under these conditions, Ribavirin and its nucleotides did not inhibit RNA polymerase activity.

The effect of Ribavirin on the 5' terminal guanylation of *in vitro* synthesized uncapped mRNA was studied by the soluble enzyme isolated from vaccinia cores (Table 2). Ribavirin 5'-triphosphate was a potent competitive inhibitor of vaccinia mRNA: guanylyltransferase (Figure 1). The Km for guanylyltransferase for GTP was 22 μM and an inhibitor constant (Ki) of 32 μM for Ribavirin 5'-triphosphate was found.

Rat liver poly(A)-containing RNA from which 5'-capped structure was removed chemically by oxidation with $NaIO_4$, followed by excision of the 5'-terminal 7-methylguanosine by β-elimination (20) served as a substrate

TABLE 2: Effect of Ribavirin 5'-Triphosphate on 5'-Guanylation of *in vitro* Synthesized Vaccinia mRNA by GTP: mRNA Vaccinia Guanylyltransferase

Reaction Mixture	^{3}[H] GMP Incorporated*
Complete	(100)
+Ribavirin, 1 mM	100
+Ribavirin 5'-monophosphate, 1 mM	100
+Ribavirin 5'-triphosphate	
50 μM	48
75 μM	45

mRNA guanylyltransferase assay mixture contained in 100 μl: 50 mM Tris-HCl (pH 7.8), 2.5 mM $MgCl_2$, 1 mM DTT, 14 μg of *in vitro* synthesized uncapped vaccinia mRNA, 10 μg of enzyme and 20 μM [8-^{3}H] GTP (Sp. Act. 11.2 Ci/mMole); 10,000 cpm/p mole) (9). After 30 minutes of incubation at 37°C, TCA precipitated material was collected on glass fiber filters (GF/C), washed with 5% TCA and ethanol. The filters were dried and counted in a toluene based scintillation fluid. *Expressed as percentage of incorporation by the complete system, which in this experiment was 12 p moles.

for guanylyltransferase. 5'-Guanylation of chemically modified rat liver mRNA was also competitively inhibited by Ribavirin 5'-triphosphate (Km for GTP = 33 μM, and Ki = 37 μM).

Experiments are in progress to determine whether Ribavirin 5'-phosphate is incorporated at the 5'-terminus of mRNAs.

Ribavirin 5'-triphosphate at higher concentrations (1 mM) inhibited methylation of vaccinia mRNA, when the reaction was carried out in the absence of GTP. Methylation of vaccinia mRNA is inhibited much more effectively by sinefungin, an antifungal antibiotic (21).

The 5'-terminal 7-methylguanosine in mRNAs is required for their efficient translation (11). Inhibition of proper modification of 5'-terminus of mRNAs by Ribavirin would lead to accumulation of mRNAs inert in protein synthesis. This could account for the antiviral potency of this drug

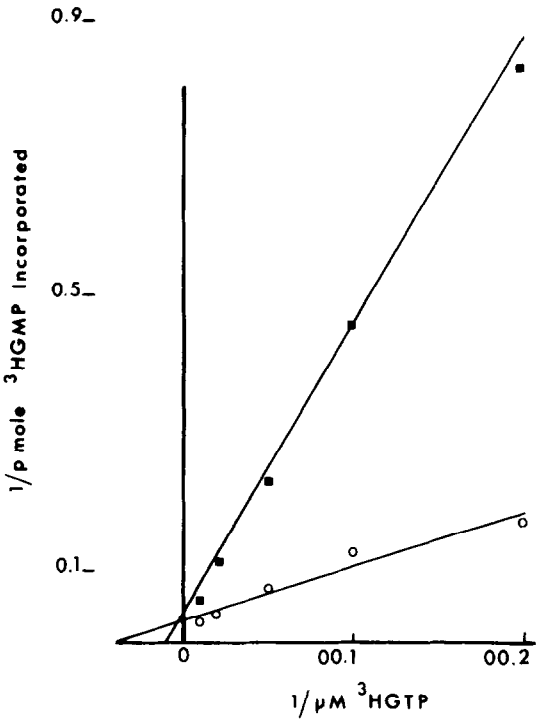

Fig.1 Lineweaver-Burk plots showing competitive inhibition of viral guanylyltransferase by Ribavirin 5'-triphosphate. The incubation mixture in a total volume of 100 µl contained: 50 mM Tris-HCl (pH 7.8), 1 mM DTT, 2.5 mM $MgCl_2$, saturating amounts (30 µg) of RNA substrate, 5 µg of enzyme protein, 5-100 µM [^{3}H] GTP (11.2 Ci/mMole) and 100 µM Ribavirin 5'-triphosphate, where indicated. The reactions were incubated at 37°C for 30 minutes and stopped by the addition of 1 ml ice cold 10% TCA. After 15 minutes in ice, the precipitates were collected on glass fiber filter discs washed with 5% TCA and ethanol, dried and counted. The lines were fitted to the points by the method of least squares using a Smith-Corona Marchant 1016 computer with an IOTA-1 programmer. O—O , no Ribavirin 5'-triphosphate; ■—■ , 100 µM Ribavirin 5'-triphosphate.

against both DNA and RNA viruses and its ineffectiveness against a virus in which mRNAs derived from them are not capped such as polio virus.

Impairment of posttranscriptional modification of mRNA may provide a test system for designing potential antiviral agents.

ACKNOWLEDGEMENTS: We thank Dr. Sylvia J. Kerr for her helpful suggestions. This work has been supported by grants from the National Cancer Institute.

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