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Antiviral activity and mode of action of ribavirin 5′-sulfamate against Semliki Forest virus

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Summary

Ribavirin 5′-sulfamate, a nucleotide analog, inhibited Semliki Forest virus cytopathology by 50% at 10μM, whereas ribavirin was inactive at ≤1 mM. Actinomycin D did not reverse (antagonize) the effect of ribavirin 5′-sulfamate against the virus. The compound inhibited amino acid incorporation into macromolecules of uninfected cells but had no appreciable effect on uridine incorporation. Infected cells treated with actinomycin D and nucleotide analog were inhibited in amino acid and uridine incorporation. The compound blocked the formation of the viral RNA polymerase protein in cells, which could account for the inhibited synthesis of new viral RNA. By electrophoresis, inhibition of the synthesis of viral proteins was more pronounced than the inhibition of cellular polypeptides. The analog inhibited the translation of mRNA to protein. Most animals treated intraperitoneally for 7 days with ribavirin 5′-sulfamate at 20 and 40 mg/kg/day starting 2 h before intraperitoneal Semliki Forest virus inoculation survived the otherwise lethal infection.

Ribavirin nucleotide analog; Protein synthesis inhibition

Introduction

For years investigators have explored the potential of nucleotide analogs as inhibitors of viral infections (Robins, 1984). At present, phosphate analogs of ribofuranosyl- or 2′-deoxyribofuranosylnucleosides have not proven to be potent or effective agents. Since one of the inherent problems with these compounds is their

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poor ability to penetrate into cells, one approach to circumvent the problem is to synthesize phosphate mimics such as 5′-sulfates or sulfamates (Robins, 1984). These non-polar molecules will readily enter cells, and in some cases exert a biological effect. For example, adenosine 5′-sulfamate will inhibit *Trypanosoma rhodesiense* (Jaffe et al., 1970) and protein synthesis in *E. coli* (Bloch and Coutsogeorgopoulos, 1971). Certain uridine 5′-sulfamates are selective inhibitors of herpes simplex virus and African swine fever virus in vitro (Perez et al., 1987).

As part of our antiviral program, ribavirin 5′-sulfamate was synthesized and evaluated. The compound is an inhibitor of Semliki Forest virus, which is surprising since ribavirin itself is inactive against the virus in our Vero cell culture system, or else shows only moderate activity against the virus in other cell lines (Huffman et al., 1973). The present report describes the effects of the novel nucleotide analog against Semliki Forest virus in vitro and in an animal infection model.

Materials and Methods

Compounds

Ribavirin 5′-sulfamate (5′-*O*-sulfamoyl-1-β-*D*-ribofuranosyl-1,2,4-triazole-3-carboxamide, Fig. 1) was synthesized in our laboratory following a procedure similar to that used to produce adenosine 5′-sulfamate (Shuman et al., 1969). Ribavirin was obtained from Viratek, Costa Mesa, CA. Actinomycin D, nucleoside 5′-triphosphates, and puromycin were purchased from ICN Biochemicals, Cleveland, OH.

Cells and virus

African green monkey kidney (Vero) cells and Semliki Forest virus (SFV) (original strain) were purchased from the American Type Culture Collection, Rockville, MD. Cells were cultured in Eagle's medium (EMEM) supplemented with 10% fetal bovine serum. The concentration of serum was reduced to 2% for virus assays.

Antiviral and cytotoxicity experiments

Inhibition of virus-induced cytopathic effect (CPE) was performed in 96-well microplates as previously described (Smee et al., 1987). SFV yield reduction assays were done in two steps. First, Vero cells in 24-well plates were treated with

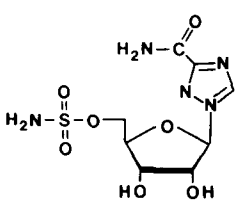

Fig. 1. Structure of ribavirin 5′-sulfamate.

inhibitor concurrently or 18 h before the addition of the infecting virus at varying multiplicities of infection (MOI). When the drug-free control cultures were completely destroyed by the virus (which took 12 h at 10 MOI, 24 h at 1 MOI, 40 h at 0.1 MOI, and 48 h at 0.01 MOI), the plates were frozen at −70°C. Later the extracellular virus from these plates was plaque titrated (Smee et al., 1983).

Cytotoxicity determinations were made by examining 2-day-old stationary monolayers microscopically for morphological abnormalities (which was necessary to determine a virus rating; Sidwell, 1976), and by determining the effects of the inhibitor on the proliferation of actively growing cells (Smee et al., 1987).

Radioactivity labeling studies

Uninfected and infected cells in 24-well plates were exposed to ribavirin 5′-sulfamate prior to and during radiolabeling with 5 μCi/ml of [^{3}H]uridine or [^{3}H]amino acids (ICN Radiochemicals, Irvine, CA). At the end of the labeling periods, the acid-soluble and acid-precipitable fractions of cells were collected as described previously (Smee et al., 1983), but with slight modifications. Cells were precipitated with 0.4 N perchloric acid and resolubilized in 1 N NaOH. Samples were neutralized with an excess of 1 M acetic acid prior to scintillation counting.

Electrophoresis and fluorography

Infected cell monolayers (MOI = 10) treated with inhibitor in 24-well plates were labeled with 50 μCi of [^{3}H]amino acids/ml in amino acid-free medium for 2 h. Cells were aspirated dry and solubilized in buffer (1% sodium dodecyl sulfate or SDS, 2% 2-mercaptoethanol, 8 M urea). Boiled samples were electrophoresed in a 4% stacking/10% running SDS gel prepared according to the method of Laemmli (1970). Pre-stained molecular weight marker proteins (Bio-Rad Labs, Richmond, CA) were run in parallel to help identify viral-specified proteins. After running, the gel was prepared for fluorography (Bonner and Lasky, 1974).

SFV RNA polymerase assay

Approximately 10^7 cells from treated or untreated cultures were infected with 10 MOI of SFV. The P15 fractions (mitochondrial pellet and membranes) from these cells were prepared following a published procedure (Ranki and Kaariainen, 1979). Each P15 fraction was resuspended in 200 μl hypotonic buffer (10 mM Tris-Cl, pH 8.0 and 10 mM NaCl) and vortexed. The RNA polymerase reaction mixture (Ranki and Kaariainen, 1979) contained 20 μl of the above enzyme preparation, 10 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 5 μM actinomycin D, 1 mM ATP, 1 mM CTP, 1 mM GTP, 20 μM UTP, and 3 μCi [^{3}H]UTP (35 Ci/mMole, ICN Radiochemicals, Irvine, CA) in 100 mM Tris-Cl, pH 8.0. The 100 μl reaction volumes were incubated for 1 h at 37°C. The reactions were terminated by adding 3 mg yeast RNA (ICN Biochemicals) and 5% trichloroacetic acid (TCA) to each tube. After 15 min at 4°C, the precipitates were collected on 0.22 micron nitrocellulose filters, which were batch washed 3 × in TCA, dried and counted for radioactivity. A P15 fraction from uninfected, untreated cells served as the background control.

In vitro protein translation

A rabbit reticulocyte lysate preparation (Stratagene, La Jolla, CA) requiring only mRNA and [^{35}S]methionine for protein translation was used. Each 30 μl reaction contained 10 μl lysate, 5 μCi [^{35}S]methionine (ICN Radiochemicals), 0.125 μg rabbit globin mRNA (Bethesda Research Labs, Gaithersburg, MD), and inhibitor. After 1 h at 30°C, 1 ml of water and 0.5 ml of decolorizing agent (1 N NaOH/0.5 M H_2O_2/1 mM methionine) were added for 15 minutes. Proteins were precipitated with 0.5 ml 25% TCA, then captured on nitrocelluiose filters, which were washed, dried and counted as described above for the RNA polymerase assay.

Animal experiments

Swiss Webster female mice (Charles River Labs, Wilmington, MA) weighing approximately 20 grams each were infected intraperitoneally (i.p.) with 10 50% lethal doses of SFV. In this infection the mice died from encephalitis. Twelve mice were in each infected group and 5 mice were in uninfected toxicity control groups. Ribavirin 5′-sulfamate (in a divided daily dose) or saline placebo was administered i.p. twice a day (at 8 a.m. and 4 p.m.) for 7 days starting 2 h pre-virus inoculation. Toxicity control mice were weighed daily, and the amounts of administered compound were adjusted to account for progressive weight loss during the course of therapy, in order to maintain a constant mg/kg dosage. Survival of infected and uninfected mice was recorded for 21 days following virus challenge.

Results

Antiviral and anticellular activities

In a primary screen ribavirin 5′-sulfamate inhibited SFV-induced CPE in Vero cells by 50% at 10 μM (virus rating of 0.8). Complete inhibition of CPE occurred at 32–320 μM. In contrast, ribavirin did not inhibit the virus at ≤1 mM. Uninfected monolayers treated with ribavirin 5′-sulfamate at 10 to 320 μM, although intact, were morphologically different from drug-free cultures. In cell proliferation assays, actively growing uninfected cells were arrested in their growth by 50% at 1–3 μM drug.

After these studies, virus yield assays were employed to evaluate the effects of different multiplicities of virus infection (MOI) on the activity of the nucleotide analog. When the virus and compound were added simultaneously to cell monolayers (Table 1), a reduction of ≥ 1 $\log_{10}$ of virus production was achieved at 50–100 μM concentrations, regardless of the MOI. The degree of inhibition of virus yield decreased with increasing MOI, however. By starting treatment 18 h before infection, a more pronounced inhibition of virus yield occurred (Table 1). Also, actinomycin D did not reverse (antagonize) the anti-SFV activity of the ribavirin 5′-sulfamate at 25–100 μM.

TABLE 1

Antiviral activity of ribavirin 5′-sulfamate under varying multiplicities of SFV infection

Inhibitor concentration (μM)	Log_{10} plaque forming units/ml					
					Pre-treatment[a]	
					Actinomycin D[b] present	
	Simultaneous treatment[c]				None	(5 μM)
	0.01[d]	0.1	1	10	10	10
0	9.3	9.3	9.7	8.3	9.4	9.5
6.25	9.5	9.0	8.6	8.6	9.4	9.0
12.5	9.6	8.9	8.6	8.3	7.7	9.1
25	9.3	8.7	8.3	7.5	6.6	6.9
50	5.6	6.8	6.7	7.3	5.6	5.9
100	3.5	4.6	5.7	6.3	5.2	5.3
Log reduction at 100 μM	5.8	4.7	4.0	2.0	4.2	4.2

[a]Ribavirin 5′-sulfamate present 18 h before and during virus replication.
[b]Actinomycin D applied to cells concurrently with virus.
[c]Ribavirin 5′-sulfamate and virus added simultaneously to cells.
[d]Multiplicity of virus infection (MOI).

Inhibition of macromolecular synthesis

In uninfected cells, ribavirin 5′-sulfamate inhibited amino acid incorporation into protein in a dose-dependent manner (Table 2), without affecting the amount of unincorporated (acid-soluble) counts of [^{3}H]amino acids present inside treated and untreated cells. Roughly a 30% inhibition of uridine incorporation into RNA occurred at each inhibitor concentration. This effect was also evident in acid-soluble

TABLE 2

Effects of ribavirin 5′-sulfamate on incorporation of uridine and amino acids in cells

Inhibitor[a] concentration (μM)	Percent of untreated control					
	Uninfected		Infected			
	Uridine	Amino acids	Uridine	Uridine +Actinomycin D[b]	Amino acids	Amino acids +Actinomycin D[b]
0	107 913[c]	112 689	71 585	36 371	58 502	51 434
6.25	72	37	49	54	65	80
12.5	69	26	42	16	47	39
25	71	21	44	9	38	37
50	69	18	46	10	27	23
100	68	11	54	9	16	9

[a] Treatment with ribavirin 5′-sulfamate before and during radiolabeling with [^{3}H] uridine or [^{3}H]amino acids was as follows: uninfected cells, 22 h treated/2 h labeled; infected cells, 18 h pre-treated/5 h incubation of virus and inhibitor/2 h labeled.
[b] Actinomycin D was added at the time of virus adsorption.
[c] Counts per minute of radioactivity for inhibitor-free controls.

counts, indicating that less [^{3}H]uridine was transported inside treated cells.

In similar studies conducted with virus-infected cells, actinomycin D was used to suppress cellular mRNA synthesis and subsequent protein expression without interfering with the corresponding viral functions (Table 2). The effect of the nucleotide analog on amino acid incorporation was nearly the same with or without actinomycin D present, and was similar to the degree of inhibition observed in uninfected cells. There was a much greater degree of inhibition of uridine incorporation in actinomycin D treated, infected cells than in infected cells not exposed to actinomycin D, indicating that ribavirin 5′-sulfamate interfered with viral RNA synthesis.

To discriminate between viral and cell protein synthesis inhibition, polypeptides from ribavirin 5′-sulfamate treated cells were visualized by electrophoresis/fluorography methods. In Fig. 2, virus-infected cells which were not drug-treated exhibited distinct new polypeptides (lane 2) not present in uninfected cultures (lane 1). These polypeptides were identified as structural (C = capsid; E1, E2, and E3 = glycoproteins; p62 = precursor to E1-E3) and non-structural (ns 86 and ns 97) by comparison with other published works (Keranen and Kaariainen, 1975; Ker-

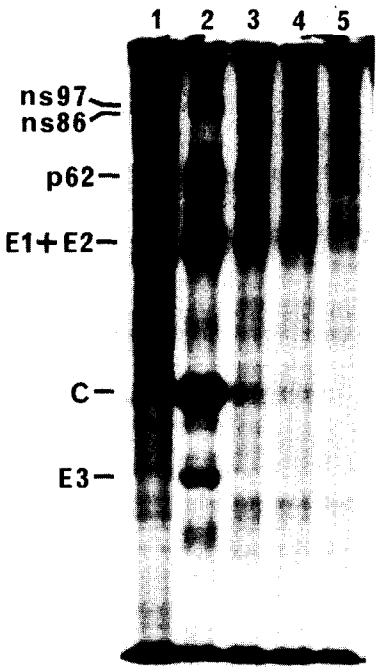

Fig. 2. Effects of ribavirin 5′-sulfamate on viral and cellular polypeptide synthesis. Treatment with inhibitor started 18 h before virus infection. Cells were labeled with [^{3}H]amino acids between 5 and 7 h after applying the virus. Lane 1, uninfected, untreated; lanes 2–5, infected and treated with 0, 25, 50, and 100 μM inhibitor, respectively. Designations at left indicate positions in lane 2 where viral-specified polypeptides occur.

TABLE 3

Effects of ribavirin 5′-sulfamate on the amount of SFV RNA polymerase activity recovered from cells

Inhibitor concentration (μM)[a]	Polymerase activity[b]	Percent of control
0	3698	100
6.25	1673	45
12.5	670	18
25	148	4
50	0	0
100	0	0

[a] Refers to ribavirin 5′-sulfamate concentration present 18 h before and during the infection (1 h virus adsorption/4 h replication) in cell culture. The inhibitor was absent from the RNA polymerase reaction.

[b] Counts per minute per 10^6 infected cells.

anen and Ruohonen, 1983). In lanes 3–5, treatment with 25–100 μM ribavirin 5′-sulfamate completely blocked the expression of these viral proteins. Also, the intensity of cellular polypeptides in these lanes was somewhat diminished relative to the untreated, uninfected control, indicating a partial inhibitory effect.

Inhibition of viral RNA polymerase formation

SFV RNA polymerase activity was partially purified from infected, inhibitor-treated cells (Table 3). The amount of enzyme activity quantified from these cells was a function of the drug concentration present during the virus replication cycle (the inhibitor was not a component of the RNA polymerase reaction mixture). When 1 mM ribavirin 5′-sulfamate was added directly to an RNA polymerase reaction mixture, only a 27% decrease in the rate of polymerization was observed compared to an untreated mixture.

Inhibition of protein translation

Ribavirin 5′-sulfamate and puromycin (a positive control) were tested for their

TABLE 4

Effects of ribavirin 5′-sulfamate and puromycin on mRNA translation[a] in vitro

Inhibitor concentration	(μM)	Percent of control
None	0	67251[b]
Ribavirin 5′-sulfamate	10	102
	15	76
	30	45
	100	14
Puromycin	1	76
	3	21
	10	0

[a] Reactions contained a rabbit reticulocyte lysate system and rabbit globin mRNA.

[b] Counts per minute of [^{35}S]methionine in the inhibitor-free reaction.

TABLE 5

Effects of ribavirin 5′-sulfamate on a lethal Semliki Forest virus infection in mice

Dose[a] (mg/kg)	Survivors/ total (%)	Toxicity controls % weight difference[b]	
		Day 7	Day 14
0	0/12 (0)	0	0
10	7/12 (58)[c]	− 1	+ 2
20	11/12 (92)[c]	−13	− 6
40	10/12 (83)[c]	−32	−13

[a] Compound or saline was administered twice a day in a divided dose for 7 days starting 2 h before virus inoculation.

[b] The percent difference in mouse weight between the untreated and treated uninfected mice.

[c] Statistically significant ($P<0.01$), determined by the two-tailed Fisher exact test.

ability to inhibit protein translation using a rabbit reticulocyte lysate system (Table 4). Ribavirin 5′-sulfamate inhibited protein translation in this system in a dose-dependent manner from 15–100 μM. Puromycin was also active in the assay.

Animal studies

An initial toxicity assay showed that ribavirin 5′-sulfamate could be tolerated by mice at a divided daily dose of ≤40 mg/kg. Higher doses killed the animals. In the experimental infection (Table 5), most mice treated daily with 20 and 40 mg of compound/kg for 7 days survived the illness, whereas the majority of saline-treated mice died. Less of a protective effect was achieved at 10 mg/kg. The compound induced severe weight loss at 40 mg/kg and some weight loss at 20. With each day of treatment the uninfected control mice became more ill due to drug toxicity as judged by weight loss and their overall appearance. Even the mice treated with compound at 10 mg/kg appeared less healthy than saline-treated controls. A subsequent study showed that doses below 10 mg/kg provided no protection to infected animals.

Discussion

In this report we described the antiviral activity of a unique analog of ribavirin that inhibited SFV in cell culture and in mice. The compound was not selective in its antiviral activity in vitro and manifested considerable toxicity to mice at protective doses. Nonetheless, we were interested to explore the potential of the compound because it exhibited properties not possessed by ribavirin itself, namely in its inhibition of Semliki Forest virus, and because it was virus-inhibitory in the presence of actinomycin D (ribavirin is not; Malinoski and Stollar, 1980). Since other nucleoside 5′-sulfamates are also showing antiviral properties (Perez et al., 1987), it is important to elucidate some modes of action of these new agents.

As the multiplicity of virus infection was increased, the potency of the compound decreased. This phenomenon has been reported for other viral inhibitors

(Smee et al., 1981). By pre-treating the cells with ribavirin 5′-sulfamate, a greater degree of antiviral activity was achieved. This most likely occurred because the nucleotide analog needed a certain amount of time to equilibrate into cells to exert its maximum effect. For this reason it was important to pre-treat the cells for the mode of action studies (which required high MOI conditions). We should mention that pre-treatment does not decrease the extracellular infecting virus titer because the compound is non-virucidal.

The studies showed that ribavirin 5′-sulfamate inhibited SFV as a result of inhibiting the translation of mRNA to protein. Its effect on viral RNA synthesis was a consequence of inhibiting the formation of the viral RNA polymerase protein, whereas the analog itself did not significantly affect the function of the enzyme. The inhibition of protein synthesis was accomplished by ribavirin 5′-sulfamate, not a metabolite. To support this conclusion, we detected only the unchanged compound in extracts of treated cells (by reverse phase high pressure liquid chromatography analyses). The site at which ribavirin 5′-sulfamate interferes with protein translation is under current investigation. Adenosine 5′-sulfamate also inhibits protein translation (Bloch and Coutsogeorgopoulos, 1971), but must have other biological activities since it is much more cytotoxic in our cell proliferation assays (50% inhibitory at <0.1 μM) while being only about twice as active as the ribavirin analog in protein translation experiments. The cytotoxic effects of adenosine 5′-sulfamate mask any anti-SFV activity since it is inactive in CPE inhibition experiments. Both of these substances fit into a larger class of protein synthesis inhibitors, which includes puromycin and cycloheximide, that can inhibit positive-stranded viruses such as SFV (Schlesinger and Kaariainen, 1980) and other viruses.

The electrophoresis results in Fig. 2 show that ribavirin 5′-sulfamate caused a marked inhibition of viral polypeptide synthesis without completely shutting off cellular protein synthesis. This differential effect may relate to the greater abundance of cellular compared to viral RNA present in these cells for translation. The few copies of infecting viral genome (mRNA) would not amplify very quickly because of the inhibited synthesis of the viral RNA polymerase protein. In contrast, the cells would continue to produce mRNA in the presence of the inhibitor.

The activity of ribavirin 5′-sulfamate against SFV prompted us to evaluate more carefully the effectiveness of the compound against other viruses, realizing that there may be a narrow concentration window between cytotoxicity and virus inhibition. Indeed, in plaque reduction assays, the compound suppressed the replication of human cytomegalovirus (CMV) (AD 169 strain) by 50% at 1 μM, but was lethal to the host MRC-5 cells at 10 μM. The analog was not protective to mice infected with mouse CMV, however. At 10 and 30 μM it inhibited the cytopathic effects induced by herpes simplex virus type 2 (G strain) and parainfluenza virus type 3 (C243 strain) in Vero cells. Thus, ribavirin 5′-sulfamate inhibits these and probably other viruses, but further investigation is probably unwarranted since the antiviral potential of this substance appears to be low.

Whether nucleoside 5′-sulfamate will find their way into the arsenal of compounds with clinical potential remains to be determined. Clearly, such inhibitors

need to be identified which have virus-specific modes of action, rather than affect cellular processes that viruses also depend on. Along these lines, it will be of interest to learn the mode of action of the recently reported uridine 5′-sulfamate analogs active against herpes simplex virus and African swine fever virus (Perez et al., 1987).

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