Efficacy of 1- β -D-Ribofuranosyl-1,2,4-Triazole-3-carboxamide Against Influenza Virus Infections in Mice

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1- β -D-Ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin) was effective against strains of influenza virus types A and F, whereas amantadine hydrochloride was effective only against strains of influenza virus type A. Dose-related protective effects against lethal influenza infections in mice were obtained with single oral doses of 25 to 400 mg of ribavirin per kg administered at the time of virus inoculation or up to 24 h thereafter. Therapeutic indexes (maximum tolerated dose/median effective dose) against various strains of influenza virus ranged from 5 to 35. With multiple-dose treatment initiated immediately after virus inoculation, oral doses as low as 12 to 25 mg/kg twice daily also afforded significant protection. Treatment with ribavirin inhibited the growth of influenza virus in the lungs of mice and delayed by about 24 h the attainment of maximal viral titers, which in nontreated mice were reached within 24 to 48 h. Inhibition of viral growth was correlated with a suppression of lung consolidation. Ribavirin appears to exert its protective effects against influenza infections by inhibiting virus growth, thereby preventing virus titers from reaching levels that result in massive lung tissue destruction and death of the mice.

Evidence for the efficacy of 1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin—name approved by the U.S. Adopted Names Council for this compound previously identified as Virazole) against infections produced by both ribonucleic acid and deoxyribonucleic acid viruses has been presented (1, 4, 5, 8). Of particular interest is the reported activity of ribavirin against influenza and parainfluenza viruses (1, 2, 5, 6, 7), which are responsible for a considerable proportion of respiratory diseases throughout the world.

This report provides confirmation of the anti-influenza activity of ribavirin and further describes conditions affecting the efficacy of the drug against influenza virus infections and its effect on virus replication and lung consolidation in mice.

MATERIALS AND METHODS

Viruses. Mouse-adapted strains of influenza virus used included: A/Swine/Iowa/15/30 (Hsw 1N1), A/PR/8/34 (H0N1), A/Japan/305/57 (H2N2), A/Hong Kong/1/68 (H3N2), A/England/42/72 (H3N2), B/Lee, B/Maryland, and B/Hong Kong. Virus stocks were clarified supernatant fluids derived from 10% (wt/vol) homogenates in brain heart infusion broth (Difco) of pooled, infected mouse lungs.

Infections. Lethal infections were produced with dilutions of virus calculated, from data obtained in prior infectivity titrations, to produce approximately

95% mortality in nontreated mice. Random-bred female Swiss-Webster mice (Taconic Farms) weighing 18 to 24 g were used in all experiments. Infection was initiated by intranasal instillation of 0.05-ml volumes of appropriately diluted virus preparations in mice under light ether anesthesia. The 95% lethal dose of influenza A/England/42/72, the most frequently used strain, was equivalent to 10^3 plaque-forming units.

Treatment. Ribavirin, was received from the Nucleic Acid Research Institute of ICN Pharmaceuticals, Inc., Irvine, Calif. Amantadine hydrochloride was purchased from the Aldrich Chemical Co., Milwaukee, Wis. Both drugs were dissolved in water, diluted to the required concentration, and administered by gavage in a 1-ml volume. Although infected control mice were not sham treated, it had previously been determined that single and multiple 1-ml doses of water by gavage did not affect the course or mortality of influenza infections.

Evaluation of drug efficacy. Drug efficacy was determined on the basis of survival ratios and the extent of lung consolidation. Statistical significance of the difference between the survival ratios of the treated and control mice at the end of a 14-day test period was determined as described previously (3). Mean effective doses were determined by probit analysis. Significance of the difference between the mean survival times of treated and nontreated animals was determined by the two-sample Student's *t* test. The extent of lung consolidation was assessed by using an index derived from the ratio of the lung weight to body weight $\times 100$. Characteristically, the index in noninfected mice is about 1. Statistical comparisons of lung consolidation in the various

groups were made by applying the Newman-Keuls test to the slopes obtained by plotting lung consolidation indexes against time.

Virus titers in lungs. Virus titers in the lungs of mice were determined by plaque assay of 10% (wt/vol) homogenates in brain heart infusion broth (Difco) of pooled lungs from groups of 6 to 10 mice killed at intervals after virus inoculation. Assays were performed as follows. Monolayers of rhesus monkey kidney cells in 60-mm Falcon dishes were adsorbed with 0.5 ml of 10-fold dilutions of the virus preparations for 1 h at 37 C, after which time the cells were overlaid with an agar medium consisting of Earle salt solution and lactalbumin hydrolysate in 0.4% Noble agar. The infected rhesus monkey kidney cells were incubated for 3 days at 37 C in an atmosphere of 5% CO₂. After removal of the overlay medium, the monolayers were stained with a solution of 0.5% crystal violet in 20% ethanol and the plaques were counted. Virus titers were expressed as log₁₀ plaque-forming units per ml of the 10% lung homogenate.

RESULTS

Efficacy of single and multiple doses. Ribavirin was relatively well tolerated in mice. Single oral doses as high as 1,600 mg/kg were not lethal, and two- to fourfold-higher doses of 3,200 and 6,400 mg/kg, respectively, caused only 10% mortality. Since the mean lethal dose of ribavirin was not established, the therapeutic indexes were determined by the ratio of the maximum tolerated dose (maximum nonlethal dose determined in twofold concentration increments) to the mean effective dose. Oral doses of 400 mg/kg per day for 5 days or 100 mg/kg per day for 10 days were not lethal but caused a 5 to 15% transient loss of body weight.

Single oral doses of ribavirin administered to mice immediately after virus inoculation produced dose-related protective effects against influenza virus infections that caused a 95 to 100% mortality of nontreated control mice (Table 1). The influenza virus strains varied in

their susceptibility to the drug. The mean effective doses against infections by the B/Hong Kong, A/England, A/Hong Kong, and A/Japan strains were 45, 75, 130, and 340 mg/kg, respectively. The therapeutic indexes (maximum tolerated dose/mean effective dose) of ribavirin against infections by these strains thus ranged from 35 to 5.

A comparison of ribavirin with amantadine hydrochloride showed that ribavirin was active against infections in mice by five strains of influenza virus type A, and three strains of influenza type B, whereas amantadine hydrochloride was active only against four strains of influenza virus type A (Table 2).

With multiple-dose treatment initiated immediately after virus inoculation, doses as low

TABLE 2. Activity of ribavirin and amantadine hydrochloride against influenza virus infections in mice

Strain of influenza virus	Activity	
	Ribavirin ^a	Amantadine hydrochloride ^a
A/Swine/Iowa/15/30 (Hsw 1N1) ...	+	+
A/PR/8/34 (H0N1)	+	-
A/Japan/305/57 (H2N2)	+	+
A/Hong Kong/1/68 (H3N2)	+	+
A/England/42/72 (H3N2)	+	+
B/Lee	+	-
B/Maryland	+	-
B/Hong Kong	+	-

^a Single oral dose of 200 mg/kg immediately after virus inoculation. +, Significant protection ($P \leq 0.05$) in groups of at least 40 mice infected with an estimated 95% lethal dose of virus; -, no significant protection.

TABLE 1. Dose response effects of single oral doses of ribavirin against influenza A virus infections in mice

Dose ^a (mg/kg)	% Survivors/40 mice 14 days postinfection			
	A/England	A/Hong Kong	A/J305	B/Hong Kong
400	85 ^b	75 ^b	60 ^b	90 ^b
200	85 ^b	63 ^b	35 ^b	85 ^b
100	58 ^b	40 ^b	20	80 ^b
50	40 ^b	20	13	60 ^b
25	15	25	13	30 ^c
Nontreated infected controls	0 (MST = 6 days) ^a	10 (MST = 6 days)	5 (MST = 6 days)	8 (MST = 6 days)

^a Single oral dose given within 10 min after intranasal instillation of an estimated 95% lethal dose of virus.

^b $P < 0.01$.

^c $P < 0.05$.

^a MST, Mean survival time.

as 12 to 25 mg of ribavirin per kg twice daily afforded a protective effect against a lethal infection with influenza A/England. Higher doses (100 mg/kg) provided greater protection as long as no more than eight doses (a total of 800 mg/kg) were administered. Treatment with 12 to 16 doses of ribavirin at 100 mg/kg was not more protective than with doses of 25 and 50 mg/kg, perhaps because of toxicity at the higher dose levels (Table 3).

Effect of time of treatment. The time of dosing relative to inoculation of virus was critical. Ribavirin was most effective when treatment was initiated immediately after virus inoculation. With a single large oral dose (400 mg/kg), a protective effect was seen when the drug was administered as late as 24 h after virus inoculation. When the drug was not administered until 48 h after virus inoculation, no significant protection was achieved, but there was an extension of the mean survival time (Table 4).

Daily doses of 100 mg/kg for as long as 10 days prior to or 10 days after virus inoculation were protective only if treatment included a dose given immediately after virus inoculation. Best results were obtained when the drug was administered once daily starting 5 days before virus inoculation through 4 days after inoculation or starting on the day of infection and continuing for 9 days (Table 5).

Effect on virus growth and lung consolidation. The effect of ribavirin treatment on virus growth and lung consolidation was examined in mice infected with a 95% lethal dose of influenza A/England. In nontreated mice, virus was first detected in the lungs 6 h postinfection. Virus titers reached a peak of 10^6 plaque-form-

TABLE 4. *Effects of timing of a single large oral dose of ribavirin on efficacy against influenza A/England infections in mice*

Time of dosing (h) relative to virus inoculation ^a	% Survivors/50 mice 14 days postinfection
0	90 ^b
4	62 ^b
8	58 ^b
12	54 ^b
16	62 ^b
24	48 ^b
48	26 ^c
Infected nontreated controls	10 (MST = 6.7 days)

^a Single oral dose of ribavirin, 400 mg/kg, at indicated time after intranasal instillation of virus.

^b $P < 0.01$.

^c Mean survival time (MST) of 7.9 days is significant at $P \leq 0.02$.

TABLE 5. *Effects of timing of oral treatment with ribavirin on efficacy against influenza A/England infections in mice*

Time of dosing (days) relative to virus inoculation ^a	Total no. of doses (100 mg/kg)	% Survivors/20 mice 14 days post-infection
0	1	50 ^b
-1	1	5
-10 through -1	10	15
-9 through 0	10	65 ^b
-5 through ± 4	10	100 ^b
± 1	1	5
± 1 through +10	10	15
0 through +9	10	95 ^b
Infected nontreated controls		5

^a Dosing with drug on day zero was done within 10 min after viral inoculation. Daily dosing with 100 mg/kg resulted in a 0.5- to 1-g loss of body weight during the first 5 days, after which the mice resumed gaining weight.

^b $P \leq 0.01$.

ing units per ml within 24 h and, after remaining at this level for another 48 h, the titers slowly declined over the next 3 days to a level about 2 logs lower. Symptoms of disease appeared first on day 3 or 4. The mice ate and drank less, their breathing became labored, and they became lethargic and huddled. First deaths occurred on day 3 or 4, but most of the mice died between days 5 and 9.

Virus titers in mice treated with single oral doses of ribavirin were consistently lower than those in the nontreated mice. The most striking inhibition of virus growth occurred during the

TABLE 3. *Efficacy of multiple oral doses of ribavirin against influenza A/England infections in mice*

Time of oral dosing (days) relative to virus inoculation ^a	Total no. of doses	% Survivors/20 mice 14 days postinfection				
		100 ^b	50	25	12.5	6.2
0-1	4	100 ^c	70 ^c	35 ^c	15	10
0-3	8	80 ^c	85 ^c	40 ^c	20	5
0-5	12	55 ^c	70 ^c	60 ^c	35 ^c	10
0-7	16	35 ^c		65 ^c	55 ^c	15
Nontreated, infected controls		0 (MST = 6 days) ^d				

^a Indicated oral dose administered twice a day with the initial dose on day zero being given within 10 min of virus inoculation.

^b Ribavirin (milligrams per kilogram).

^c $P \leq 0.01$.

^d MST, Mean survival time.

first 24 h, when the virus titers in the treated mice were 1 to 2 logs less than in the nontreated mice. Maximal virus titers in treated mice were reached 24 h later than in nontreated mice. Evidence of lung consolidation in the nontreated mice was first seen 3 days after virus inoculation, and lung weights increased steadily thereafter. Treatment with ribavirin resulted in a dose-related suppression of lung consolidation (Fig. 1). Treated infected mice also showed signs of disease, but the symptoms were less severe than those in the nontreated mice. The mice appeared to be recovering by day 8 or 9, and 70 to 90% of the mice survived.

When multiple doses of 100, 25, or 6 mg/kg, twice daily for 4 days, were tested, the greatest inhibition in virus growth was found to be achieved with the 100-mg/kg dose. In mice treated with this dose, virus titers remained at levels of about 1 log plaque-forming unit per ml lower than in nontreated mice for about 96 h. By this time the virus titers in the control mice had declined at a faster rate than those in the treated mice and the titers in both groups were about equal. As shown by survival ratios, only treatment with the 100-mg/kg dose provided significant protection. Treatment with doses of 25 mg/kg resulted in survival of 30% of the mice, but doses of 6 mg/kg afforded no protection. Virus titers in the mice treated with the 25- and the 6-mg/kg doses were about 0.5 log plaque-forming unit per ml less than those in the nontreated mice. The degree of lung consolidation in the mice treated with the 25-mg/kg

doses, but not in those treated with the 6-mg/kg doses, was significantly less than in the infected nontreated mice (Fig. 2). Comparison of virus titers and survival ratios in treated and nontreated mice indicated that survival was associated with a degree of virus inhibition that resulted in virus titers not exceeding a threshold level of $10^{5.5}$ plaque-forming units per ml. This inhibition of virus growth was correlated with a marked suppression of lung consolidation (Fig. 1 and 2).

DISCUSSION

The results show that well-tolerated oral doses of ribavirin effectively protected mice against lethal infections produced by strains of influenza virus types A and B. Examination of the dynamics of influenza virus growth in the lungs of nontreated mice indicated that a critical concentration of virus is attained within the first 24 h after virus inoculation. When this critical virus concentration is exceeded, the resulting lung consolidation is apparently so extensive that pulmonary function becomes inadequate, and the mice eventually die. Treatment of the mice with adequate doses of ribavirin administered before peak virus levels are reached results in inhibition of influenza virus replication and concomitant suppression of lung consolidation.

The efficacy of ribavirin against influenza virus infection in mice thus appears to be related to its ability to inhibit virus growth,

FIG. 1. Effect of single oral doses of ribavirin on lung consolidation and viral growth in the lungs of mice infected with influenza virus A/England. The drug was administered immediately after viral inoculation. The percentages of survivors in groups of 10 mice maintained to determine the protective effects of the drug were: 800 mg/kg, 90%; 200 mg/kg, 80%; 50 mg/kg, 70%; no drug, 0%.

FIG. 2. Effect of multiple oral doses of ribavirin on lung consolidation and viral growth in the lungs of mice infected with influenza virus A/England. The drug was administered twice daily with the first dose given immediately after viral inoculation. The percentages of survivors in groups of 10 mice maintained to determine the protective effects of the drug were: 100 mg/kg, 90%; 25 mg/kg, 30%; 6 mg/kg, 0%; no drug, 0%.

thereby preventing virus concentrations from exceeding the critical levels and limiting the extent of pulmonary tissue involvement. In support of this concept is the observation of Khare et al. (2) that the efficacy of ribavirin was apparently dependent on the concentration of virus used to initiate the infection. This suggested a maximal threshold of virus infection beyond which ribavirin was ineffective.

Although the kinetics of natural influenza infections in man is not exactly defined, it is likely to be less precipitous than in the mouse model, where the infection is initiated by the introduction of a large viral inoculum into the lungs. In general, the course of influenza infections in man is milder than in the mouse model and should offer greater opportunity for successful chemotherapeutic intervention. Since riba-

virin has demonstrated efficacy against lethal influenza infections in mice, trial of the drug against influenza infection in man appears warranted.

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