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Suppression by 1-β-D-Ribofuranosyl-1,2,4-Triazole-3-Carboxamide (Virazole, ICN 1229) of Influenza Virus-Induced Infections in Mice

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1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamide (Virazole) was found to possess significant antiviral activity against aerosol-induced or intranasally induced influenza A_0, A_2, and B virus infections in mice. Significant protection was achieved by both oral and intraperitoneal routes of administration. Depending upon the level of virus infection, antiviral activity was best observed at the daily dose of 75 mg/kg. The efficacy of the compound was evidenced by an increase in survivor number, prolongation of mean survival time, suppression of lung consolidation, or decrease in hemagglutinin titer in the infected lung samples. The therapeutic value of this synthetic triazole nucleoside was evident as noted by a significant increase in survivor number even if the treatment was started as late as 24 h after infection with an aerosol of influenza A_2 virus.

1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamide (Virazole, ICN 1229) is a new, synthetic, water-soluble, stable, colorless nucleoside which has been recently synthesized at this Institute (10). Since Virazole has a broad spectrum of in vitro antiviral activity (3, 9), investigations were conducted to elucidate its in vivo activity against epidemiologically significant orthomyxovirus infections. The present paper describes extensive investigations into the anti-influenza virus activity of this nucleoside in mice. (A preliminary report of this work was presented at the 56th Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N.J., 9-14 April 1972.)

MATERIALS AND METHODS

Virus. The following influenza viruses were obtained from F. M. Schabel, Jr. (Southern Research Institute, Birmingham, Ala.): type A_0 (IV/A_0), strain PR8; type A_2 (IV/A_2), strain Japan/305; and type B (IV/B), strain Lee. These viruses were passaged in mice, and lung preparations in Hanks balanced salt solution (HBSS) were subsequently used.

Virus assay. The potency of the virus preparations was determined by use of a mouse infectivity assay. Mice were inoculated with the virus either by instilling intranasally (i.n.) to ether-anesthetized mice 0.03 ml of various dilutions or with the use of an airborne infection apparatus (Tri-R Co., New York, N.Y.) as discussed by Middlebrook (7). In the latter procedure, 2.2 ml of virus suspension in Eagle minimal essential medium (MEM) containing 100 units of penicillin and 100 μg of streptomycin per ml was nebulized into a chamber holding approximately 100 mice for 35 min, followed with a 35-min cloud decay and a 15-min exposure to ultraviolet light. The number of mice that died during a 21-day period was recorded, and the 50% lethal dose (LD_{50}) was determined according to the Reed and Muench (8) procedure. Hemagglutinin (HA) titrations were performed with 0.5% guinea pig red blood cells as described previously (5).

Animals. Random-bred female Swiss Webster mice (9 to 15 g) obtained from either Carworth Laboratories (New York, N.Y.) or Hilltop Laboratory Animals, Inc. (Chatsworth, Calif.), were used in various experiments.

Drug evaluation. A predetermined dose of virus was used for infecting mice either by the aerosol or i.n. route. After virus inoculation, the mice were randomized and divided into different groups of 10, 20, or 25 mice to receive various drug treatments. The drug dissolved in saline was subsequently administered intraperitoneally (i.p.) or by oral gavage, according to various schedules of treatment on a milligram per kilogram basis with the animals being weighed daily. The toxicity control groups, usually comprised of five mice, were sham-infected with the

virus diluent (HBSS or MEM) and were similarly treated with various dosages of the drug. The number of deaths ensuing during a 21-day postinfection period was recorded daily. The mice were also given 0.075% oxytetracycline (Pfizer Inc., New York, N.Y.) every day in drinking water during the experimentation period. The data were evaluated statistically by use of Students' t test to compare the mean survival time (MST) of drug-treated and untreated animals dying during the 21-day experimentation period. If the P value obtained was <0.05, the drug dose was considered to have statistically significant antiviral activity. The survivor number in the virus-infected, drug-treated group was compared with the survivor number of the virus control group and analyzed by the chi-square analysis technique. If the P value was <0.3 (standard error), statistically significant antiviral activity was indicated.

In several experiments, mice were sacrificed on the 5th or 6th day after infection and the lungs of each mouse were scored on a blind basis for the extent of consolidation. A scale of 0 to 4 was used to denote lung consolidation from 0 to 100%. An average lung consolidation was obtained by dividing the total lung grades by the number of lungs examined in each group. In some cases, the lungs from five mice were homogenized after grading for consolidation in 15 ml of phosphate-buffered saline (PBS; pH 7.2; 0.02 M PO_4, 0.15 M NaCl) for 3 min in a Sorvall Omni-Mixer. The homogenized lung preparation was centrifuged at 1,500 rpm for 15 min and the supernatant fluid was used for HA titration (5).

RESULTS

Several experiments with IV/A_2 were performed to determine the antiviral activity of Virazole. The drug dose, virus dose, average weight of the mice used, treatment schedule, survivor number, MST, and extent of lung consolidation from 10 experiments are summarized in Table 1. In experiments 1 and 2, mice weighing 9 g were infected i.n. with 3.2 or 10 LD_{50} of IV/A_2. The drug was administered i.p. twice a day for 7 days, beginning 24 h pre- and 4 h postinfection. The group infected with 3.2 LD_{50} of the virus and treated with 50 mg of Virazole per kg per day had 66.6% survivors as compared with 24% survivors in the virus control, indicating a significant survivor increase. There was also a 68% decrease in lung consolidation at day 5 postinfection. In the second experiment, at a higher virus dose (10 LD_{50}) no increase in survivor number was observed, but significant increases in MST at daily doses of 100, 50, and 25 mg/kg were observed. The variation in the results of experiments 1 and 2 appeared to be due to the fact that the low weight of the animal and a higher challenge virus inoculum in the second experiment might have somewhat affected the outcome. Subsequent experiments were therefore performed with 14- or 15-g mice and various doses of IV/A_2. In experiments 3 and 4, mice were infected i.n. with 3.2 or 32 LD_{50} of the virus and were subsequently treated i.p. with 75 mg of Virazole per kg per day beginning 4 h pre- and 4 h postinfection. The treatment was continued twice daily for nine consecutive days. At this dose, 90 and 70% survivors were observed as compared with 40 and 30% survivors in the virus control group at 3.2 and 32 LD_{50} virus doses, respectively. The MST of those mice dying during the 21-day experimentation period was also significantly increased.

Subsequent experiments to determine the minimal effective concentration, which would still increase the survivor number and the MST or decrease the extent of IV/A_2-induced lung consolidation, were conducted with various virus doses and various treatment schedules. In experiments 5 and 6, 15-g mice were infected i.n. with 3.2 and 10 LD_{50} of IV/A_2 and treated with Virazole in daily doses of 75, 37.5, 18, and 9 mg/kg given twice a day for 9 days, beginning 4 h before and 16 h after virus instillation. In the first study with 3.2 LD_{50} of virus, the mice were sacrificed on day 5 postinfection and the extent of lung consolidation was determined. The group treated with 75 mg of Virazole per kg per day showed up to 55% inhibition in lung consolidation. Little or no inhibition was observed at the lower drug levels. In the second study with 10 LD_{50} of virus, a 20% increase in survivor number was observed at 75 mg per kg per day, whereas a significant increase in MST was obtained at 75, 37.5, and 18 mg per kg per day. The antiviral activity of Virazole was investigated with higher virus doses, 32 and 100 LD_{50} of IV/A_2. The virus was given i.n. and the drug, at 75, 37.5, 18, and 9 mg per kg per day, was administered i.p. twice a day for 9 days, beginning 4 h pre- and 16 h postinfection. The results of the respective investigations are summarized in experiments 7 and 8 of Table 1. There was a significant increase in the survivor number with 75 mg of drug per kg per day at 32 LD_{50} of virus. This was accompanied by a significant increase in MST among mice treated with Virazole doses of 75 and 37.5 mg per kg per day. The mice infected with 100 LD_{50} of IV/A_2 did not show any increase in survivor number or MST at any of the Virazole doses used in this experiment.

The oral efficacy of Virazole was investigated in experiment 9. Mice were infected by the aerosol route with 10 LD_{50} of virus, and the drug was administered orally at 100 and 75 mg per kg per day beginning 4 h pre- and 4 h postinfection with treatment continued twice a day for 9

TABLE 1. *Effect of Virazole on influenza A_2 virus infections in mice*[a]

Expt no.	Daily drug dose (mg/kg)	Virus dose (LD_{50})	Avg mouse wt (g)	Treatment schedule[b]	Survivors/ Total	Survivor increase P[c]	MST (days)	Mean survival increase P[d]	Lung consolidation
1	50	3.2	9	1	8/12	<0.01	8.6	>0.05	0.7
	50	0	9	1	6/6	—	>21.0	—	0
	0	3.2	9	1	6/25		7.8		2.2
2	100	10	9	1	2/10	>0.3	10.0	<0.001	ND
	100	0	9	1	4/4	—	>21.0	—	ND
	50	10	9	1	1/11	>0.3	8.2	<0.001	ND
	50	0	9	1	5/5	—	>21.0	—	ND
	25	10	9	1	0/11	>0.3	7.8	<0.01	ND
	25	0	9	1	4/4	—	>21.0	—	ND
	0	10	9	1	0/19		6.3		ND
3	75	3.2	14	2	9/10	<0.01	9.0	<0.001	ND
	75	0	14	2	5/5	—	>21.0	—	ND
	0	3.2	14	2	4/10		6.0		ND
4	75	32	14	2	7/10	<0.05	7.0	<0.05	ND
	75	0	14	2	5/5	—	>21.0	—	ND
	0	32	14	2	3/10		5.3		ND
5	75	3.2	15	3	—	—	—	—	1.4
	75	0	15	3	—	—	—	—	0
	37.5	3.2	15	3	—	—	—	—	2.9
	37.5	0	15	3	—	—	—	—	0
	18	3.2	15	3	—	—	—	—	2.4
	18	0	15	3	—	—	—	—	0
	9	3.2	15	3	—	—	—	—	2.4
	9	0	15	3	—	—	—	—	0
	0	3.2	15	3	—		—	—	3.1
6	75	10	15	3	6/10	<0.3	8.3	<0.001	ND
	75	0	15	3	5/5	—	>21.0	—	ND
	37.5	10	15	3	4/10	>0.3	7.5	<0.01	ND
	37.5	0	15	3	5/5	—	>21.0	—	ND
	18	10	15	3	3/10	>0.3	7.5	<0.01	ND
	18	0	15	3	5/5	—	>21.0	—	ND
	9	10	15	3	2/10	>0.3	5.9	>0.05	ND
	9	0	15	3	5/5	—	>21.0	—	ND
	0	10	15	3	4/10		5.6		ND
7	75	32	15	3	7/10	<0.01	7.7	<0.001	ND
	75	0	15	3	5/5	—	>21.0	—	ND
	37.5	32	15	3	2/10	>0.3	6.6	<0.01	ND
	37.5	0	15	3	5/5	—	>21.0	—	ND
	18	32	15	3	0/10	>0.3	5.1	>0.005	ND
	18	0	15	3	5/5	—	>21.0	—	ND
	9	32	15	3	1/10	>0.3	4.8	>0.05	ND
	9	0	15	3	5/5	—	>21.0	—	ND
	0	32	15	3	2/10		4.5		ND
8	75	100	15	3	1/10	>0.3	4.4	>0.05	ND
	75	0	15	3	5/5	—	>21.0	—	ND
	37.5	100	15	3	2/10	>0.3	4.9	>0.05	ND
	37.5	0	15	3	5/5	—	>21.0	—	ND
	18	100	15	3	0/10	>0.3	5 9	>0.05	ND
	18	0	15	3	5/5	—	>21.0	—	ND
	9	100	15	3	1/10	>0.3	4.9	>0.05	ND

TABLE 1—*continued*

Expt no.	Daily drug dose (mg/kg)	Virus dose (LD_{50})	Avg mouse wt (g)	Treatment schedule[b]	Survivors/ Total	Survivor increase P[c]	MST (days)	Mean survival increase p[d]	Lung consolidation
	9	0	15	3	5/5	—	>21.0	—	ND
	0	100	15	3	0/10		5.5		ND
9	100	10	15	4	6/9	<0.3	13.0	<0.001	ND
	100	0	15	4	3/3	—	>21.0	—	ND
	75	10	15	4	8/10	<0.05	10.5	>0.05	ND
	75	0	15	4	3/3	—	>21.0	—	
	0	10	15	4	7/20		9.0		ND
10	37.5	3.2	15	5	6/10	<0.05	10.0	>0.05	ND
	37.5	0	15	5	2/2	—	>21.0	—	ND
	0	3.2	15	5	1/20		8.6		ND

[a]Mice were infected with a predetermined virus dose either i.n. (experiments 1-8) or in an aerosol chamber (experiments 9 and 10). The toxicity control mice were sham-infected with sterile minimum essential medium.

[b]Treatment schedules: (1) drug administered i.p. beginning 24 h pre- and 4 h postinfection, twice a day for 7 days. (2) Drug administered i.p. beginning 4 h pre- and 4 h postinfection, twice a day for 9 days. (3) Drug administered i.p. beginning 4 h pre- and 16 h postinfection, twice a day for 9 days. (4) Drug administered orally beginning 4 h pre- and 4 h postinfection, twice a day for 9 days. (5) Drug administered i.p. beginning 4 h pre- and 4 h postinfection, twice a day for 11 days.

[c]Probability value, chi-square analysis.

[d]Probability value, Student's *t* test.

days. Significant increases in survivor number were observed at both doses, with a statistically significant increase in MST seen only at 100 mg per kg per day. Intraperitoneal treatment of IV/A_2 aerosol-infected mice (experiment 10) was also effective at 37.5 mg per kg per day under a similar treatment regimen for 11 days.

The therapeutic value of Virazole administered beginning at different time intervals was subsequently determined. Mice were given 3.2 LD_{50} of IV/A_2 by aerosol, and Virazole, administered i.p. at daily doses of 75 and 37.5 mg/kg, was started 4 h preinfection and 4 and 24 h postinfection. Once initiated, the treatment was given twice daily for 9 days. The highest dose of Virazole was most effective when it was given at 4 h postinfection, whereas the lowest dose was almost equally effective when it was administered beginning 4 h preinfection. However, significant increases in survivor number were observed whether the treatment was started 4 h pre- or 4 or 24 h postinfection (Fig. 1). These results indicate a definite therapeutic value of the drug, even when the drug is administered as late as 24 h after the initiation of infection.

Investigations were extended to other serotypes of influenza virus with 14- to 15-g mice. Mice were infected in with 10 LD_{50} of IV/A_0, and Virazole was administered i.p. at daily doses of 75 and 37.5 mg/kg twice a day for 9

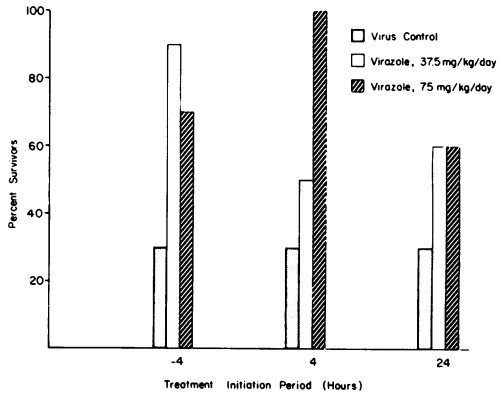

FIG. 1. *Effect of sequential Virazole treatment on course of influenza A_2 virus infection in mice. Results recorded at day 21 postinfection.*

days, beginning 4 h before virus instillation. The dose of 75 mg per kg per day induced a significant protection and also prolonged the MST of mice dying before 21 days (Table 2). Investigations with 3.2 or 32 LD_{50} of IV/B and a similar treatment schedule with 75 mg of Virazole per kg per day resulted in an 85% increase in survivor number, along with prolongation of the life span of the only mouse which died during this experiment (Table 3). In a second experiment (Table 3), the group infected with 32 LD_{50}

of IV/B and treated with Virazole in daily doses of 30 and 15 mg/kg showed 40 and 20% survivor increases at the two doses, respectively (Table 3). In the third experiment (Table 3), mice were inoculated i.n. with 3.2 LD_{50} of the virus under a treatment regimen identical to the previous two experiments. In this experiment, mice were sacrificed on day 6 postinfection, and the extent of lung consolidation and the HA titer in the lungs were determined. There was approximately a 70% inhibition in consolidation along with a fourfold reduction in HA titer in the lungs of mice treated with the daily dose of 30 mg/kg.

The sham-infected toxicity control groups treated with various dosages of the drug, in each experiment, did not show any signs of toxicity. The LD_{50} of Virazole, given in one dose, was approximately 2,000 or 1,200 mg/kg after oral or i.p. administration, respectively. When the drug was given twice daily for 9 days by either route, the LD_{50} was approximately 250 mg per kg per day. The maximal tolerated dose, under a similar treatment schedule, was 150 mg per kg per day. Animals treated with this dose exhibited a 20 to 30% weight loss with some diarrhea.

TABLE 2. *Effect of Virazole on influenza A_0 virus-induced mortality in mice*

Daily Virazole dose (mg/kg)	Virus dose (LD_{50})	Survivors (%)[a]	Survivor increase P	MST (days)	Mean survival increase P
75	10	90	<0.001	12.0	<0.001
75	0	100	—	>21.0	—
37.5	10	40	>0.3	6.7	>0.05
37.5	0	100	—	>21.0	—
0	10	20		7.5	

[a] Percent mice surviving at day 21 postinfection.

DISCUSSION

These studies indicate that Virazole possesses significant activity against three serotypes of influenza virus. This antiviral effect was indicated by an increase in survivor number, prolongation of mean survival time, decrease in lung consolidation, or reduction in HA production. During this study, considerable fluctuation occurred in results when younger mice (8 to 9 g) were used. However, consistently effective antiviral activity of the nucleoside was observed with 14- to 15-g mice. Since younger mice appeared to be more sensitive to virus and drug, the antiviral activity of Virazole might have been masked by using such animals and the higher virus inoculum. The apparent variability seen in viral potency in these experiments was probably a function of both the size of the animal and a lack of efficiency resulting from

TABLE 3. *Effect of Virazole on influenza B virus infections in mice*

Expt no.[a]	Daily drug dose (mg/kg)	Virus dose (LD_{50})	Survivors per total	MST (days)	Lung consolidation[b]	Hemagglutinin titer/ml
1	75	3.2	9/10 ($P < 0.001$)	14 ($P < 0.001$)	ND	ND
	75	0	3/3	>21.0	ND	ND
	0	3.2	1/20	11.2	ND	ND
2	30	32	4/10 ($P < 0.3$)	12.7 ($P < 0.001$)	ND	ND
	30	0	5/5	>21.0	ND	ND
	15	32	2/10 ($P > 0.3$)	10.0 ($P > 0.05$)	ND	ND
	15	0	5/5	>21.0	ND	ND
	0	32	0/10	8.4	ND	ND
3	30	3.2			0.9	1:20
	30	0			0.0	1:0
	15	3.2			2.5	1:80
	15	0			0.0	1:0
	0	3.2			3.0	1:80

[a] Mice were infected with a predetermined virus dose either in an aerosol chamber (experiment 1) or i.n. (experiments 2 and 3). The mice in experiment 3 were sacrificed on day 6 postinfection and the lungs were examined for consolidation and hemagglutinin production.

[b] Lungs were graded from 0 (uninfected) to 4 (100% consolidation). ND = not done.

i.n. administration of the virus. This lack of efficiency was a major reason for the utilization of the aerosol chamber in the later experiments.

Few compounds are at present known that significantly inhibit influenza virus infections in vivo (4), and only adamantanamine·HCl and its various derivatives were found to be effective against influenza A and A_2 virus infections (1, 4, 6). This antiviral action has been principally prophylactic in nature (1, 4). A need would, therefore, seem to exist for other drugs which could be used for the control of such diseases, particularly for therapeutic rather than prophylactic use. In view, also, of the differing strains of virus which cause influenza in man, a chemotherapeutic drug effective against a broad spectrum of such viruses would be of special significance. Besides the prophylactic effect, the therapeutic efficacy of Virazole is in evidence because there is still a significant increase in survivor number, even if the drug is administered as late as 24 h postinfection (Fig. 1).

The protective activity of Virazole in terms of decreased mortality is highly significant against infections due to influenza virus types A_0 and B. Although this activity of the compound was moderately significant against type A_2 influenza virus infection, this was always associated with a significant increase in mean survival time in the drug-treated mice. The therapeutic index (maximal tolerated dose divided by minimal effective dose) of Virazole in influenza virus type A_2 infections in mice appears to be approximately 8 if 150 mg per kg per day is taken as the maximal tolerated dose and 18 mg per kg per day (experiment 6, Table 1) as the minimal effective dose under the aforementioned treatment schedule. The therapeutic indices for influenza virus types A_0 and B infections appear to be 2 and 5, respectively (Tables 2 and 3). The efficacy of Virazole is apparently dependent on both the age of the animal and the concentration of virus used. At virus doses higher than 32 LD_{50}, little or no antiviral activity of the nucleoside was seen; this would suggest a maximal threshold of virus infection beyond which point Virazole is ineffective. Similar observations in regard to effect of virus concentration have been made for the efficacy of the interferon inducer polyinosinic-polycytidylic acid (2). The drug has also been found to be effective after oral gavage in IV/A_2-infected mice; this oral antiviral activity has been confirmed by several independent investigators (R. A. Bucknall, Imperial Chemical Industries, Ltd., Macclesfield, Cheshire, England; F. E. Durr, Lederle Laboratories, Pearl River, N.Y.; R. Faust and E. Grunberg, Hoffman-La Roche, Inc., Nutley, N.J., personal communication). These studies suggest Virazole to be a candidate for clinical trial against viral influenza in man. Preclinical pharmacological studies are currently directed to this end.

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