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Effect of selenazofurin on influenza A and B virus infections of mice*

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Summary

The inhibitory effects of selenazofurin and ribavirin on influenza A and B virus infections in mice were compared. Both compounds, when administered intraperitoneally (i.p.), reduced lung consolidation and prolonged mean day of death, but ribavirin more effectively increased survivor number and lowered lung viral hemagglutinin (HA) titers. Lung HA titers often increased in selenazofurin-treated animals. To determine the most appropriate i.p. treatment schedule, influenza A virus-infected mice were treated once, twice or thrice daily for 7–9 days, or once only. Treatment once daily for 9 days beginning 4 h pre-virus exposure, for 3 days beginning 24 h post-virus exposure, or once only 48 h post-virus exposure was most effective. Body temperature, which usually declined during infection, increased to near-normal levels in animals treated with selenazofurin, especially in animals treated a single time or for 3 days with high dose levels. Selenazofurin was well tolerated at a dose of 50 mg/kg administered twice daily, and at 400 mg/kg administered once only. Rectal temperatures temporarily declined following every other day treatment with 400 mg/kg.

antiviral; selenazofurin; influenza A virus

Introduction

The novel selenazole carboxamide nucleoside, 2-β-D-ribofuranosylselenazole-4-car-

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boxamide (selenazofurin) was previously reported [8] to have in vitro activity against a broad spectrum of viruses. The compound was later shown [11] to have significant activity against influenza A and B viruses in vitro. The structurally related antiviral, ribavirin (1-β-D-ribofuranozyl-1,2,4-triazole-3-carboxamide), has exhibited quite similar antiviral activity [12]. In view of these effects, it seemed appropriate to determine the efficacy of selenazofurin against influenza virus infections of mice, and to compare its activity with that exhibited by ribavirin. These studies included experiments to elucidate the most efficacious therapy schedule for treatment of the disease. These in vivo studies and the use of rectal temperatures to monitor both antiviral and possible toxicological effects are described.

Materials and Methods

Viruses

Influenza A/Japan/305/57 (H2N2), influenza A/PR/8/34 (H1N1) and influenza B/Lee/40 viruses were obtained from the late Dr. Frank M. Schabel, Jr., Southern Research Institute (Birmingham, AL). Each was passaged intranasally (i.n.) through mice, and virus pools were prepared as lung homogenates in Puck's balanced salt solution. Each virus pool was quantified in mice and in Madin Darby canine kidney (MDCK) cells prior to use.

Animals

Female 18–22 g Swiss Webster mice (Simonsen Laboratories, Gilroy, CA) were used. All were quarantined 24 h and maintained on standard mouse food and water containing 0.006% oxytetracycline (Pfizer, New York, NY).

Compounds

Selenazofurin was synthesized by one of us (P.D.C.) as described by others [13]. Ribavirin was obtained from ICN Pharmaceuticals Inc. (Covina, CA). Each was prepared in sterile saline at the concentrations indicated.

Antiviral experiments

In all experiments, ether-anesthetized mice were infected intranasally with an approximate 90% lethal dose (LD_{90}) of virus (0.06 ml). The virus dilutions had titers of approximately 10^5 cell culture 50% infectious dose ($CCID_{50}$)/ml in MDCK cells. In experiments in which the parameters were death pattern and lung consolidation, 20 infected mice were treated with each drug dosage, and 40 infected animals were treated with saline as virus controls. When deaths began to occur in the virus control animals (days 6–9 post-virus exposure), one-half of each infected group was killed. Visual evidence of lung consolidation was scored on a blind basis and the lungs from individual mice were homogenized and assayed for viral hemagglutinin (HA). Lung scores were based on the percentage of the lung displaying signs of consolidation, with 0 being normal and 4 indicative of 100% consolidation. Animals dying prior to sacrifice exhibiting obvious signs of pulmonary distress had their lungs also assigned a

score of 4. Infected animals not killed were observed daily for 21 days. Rectal temperatures were determined in the experiments indicated using a YSI42SC telethermometer (Yellow Springs Instrument Co., Inc., Yellow Springs, OH). Five sham-infected, parallel treated, toxicity control mice were provided for each drug dosage used in treatment of infected animals. These mice, and 5 normal control animals, were held in a room separate from that in which the infected mice were held. The normal control mice and the toxicity control mice were weighed prior to treatment and 18 h after final treatment to determine weight gain or loss. In the indicated experiments, rectal temperatures were measured daily, through day 6, in all mice.

Lung virus titer determinations

Each mouse lung was homogenized to a 10% (w/v) suspension in minimum essential medium (MEM) containing Earles balanced salt solution, 0.1% $NaHCO_3$, 1% sorbitol and 50 μg gentamicin/ml. Each lung homogenate was diluted by serial 2-fold dilutions in round-bottomed 96-well microplates (Nunc, Vangard International, Neptune, NJ) and assayed for viral HA by use of 0.3% guinea pig red blood cells. The titers are expressed as geometric mean $\log_2$. Infectious virus, determined in two experiments using A/PR/18/34, was assayed by adding 10-fold dilutions of each lung homogenate to monolayers of MDCK cells in flat bottomed Nunclon® Delta Microtest plates (Nunc) and the visual virus-induced cytopathic effects were determined 72 h later. The cell culture medium was MEM containing 0.18% $NaHCO_3$, 50 μg gentamicin/ml, 20 μg trypsin/ml and 2.0 μg EDTA/ml. In the same experiments, infectious virus titers were also determined by injecting 10-fold dilutions of lung homogenate into the allantoic cavity of embryonated chicken eggs. Death of the embryo was considered indicative of infectious virus.

Statistical analyses

The increase in the number of survivors was evaluated using chi square analysis with Yate's correction. Mean survival time increases, virus titer and rectal temperature differences were analyzed with the *t*-test. Lung consolidation scores were evaluated using ranked sum analysis.

Results

Multiple dose treatment of influenza A and B virus infections

Separate experiments were run with each type of influenza virus. Treatments were administered intraperitoneally (i.p.) twice daily for 7 days.

Against influenza A/Japan/305/57 virus infections (Table 1), ribavirin treatment, at the single dose used (75 mg/kg per day), resulted in a moderate increase in mean survival time and in highly significant decreases in mean lung score and in mean lung HA titer. Selenazofurin also inhibited mean lung score and reduced lung HA titers.

Against influenza A/PR/8/34 virus (Table 2), selenazofurin prolonged mean survival time and reduced lung consolidation, but lung HA titers increased dramatically compared to those of the placebo-treated virus controls. Day 5 rectal temperatures

TABLE 1

Effect of intraperitoneal treatment[a] with selenazofurin or ribavirin on influenza A/Japan/305/57 virus infections in mice

Compound	Dosage (mg/kg per day)	Infected, treated			
		Surv/ Total	MST[b] (days)	Mean lung score[c]	Mean lung HA titer
Selenazofurin	180	0/10	4.9	2.2	6.1*
Selenazofurin	90	0/10	4.5	1.3**	6.1*
Selenazofurin	45	0/10	5.3	2.0*	2.6**
Ribavirin	75	1/10	5.8	1.2**	1.5**
Saline		0/30	5.2	2.9	17.8
Normal controls				0.0	0

[a] b.i.d. × 7, beginning 4 h pre-virus exposure.
[b] Mean survival time of mice dying on or before day 21.
[c] Lungs removed on day 6 post-virus exposure.
*$P<0.05$; **$P<0.01$.

TABLE 2

Effect of intraperitoneal treatment[a] with selenazofurin or ribavirin on influenza A/PR/8/34 virus infections in mice

Compound	Dosage (mg/kg per day)	Toxicity controls host wt. change[b] (g)	Infected, treated				
			Surv/ Total	MST[c] (days)	Mean lung score[d]	Mean day 5 temp. (°C)	Mean lung HA titer
Selenazofurin	200	- 2.3	0/10	7.4	2.2*	31.5	137
	100	- 0.2	1/10	11.6*	2.8	20.7	294
	50	+2.5	0/10	8.6*	2.1*	31.6	158
Ribavirin	75	+2.8	10/10	>21.0**	0.5*	35.2**	24*
	37.5	+3.2	0/10	8.0	2.1*	34.2**	45*
	18.8	+2.5	0/10	7.0	3.2	34.3**	60
Saline			0/20	7.2	3.4	32.4	119
Normal controls		+3.8			0.0	34.9	7

[a] b.i.d. × 7, beginning 4 h pre-virus exposure.
[b] Weight 18 h after final treatment minus day 0 weight (5/5 mice survived in each group).
[c] Mean survival time of mice dying on or before day 21.
[d] Lungs removed on day 6 post-virus exposure.
*$P<0.05$; **$P<0.01$.

were lower than those of the virus controls. Ribavirin was strongly active using all parameters. The highest dosage of selenazofurin, 200 mg/kg per day, appeared to approach the maximum tolerated dose since the animals experienced a weight loss of >2 g. Ribavirin was well tolerated. The toxicity control data suggest the 50 mg/kg per

day dose of selenazofurin and the 75 mg/kg per day dose of ribavirin are approximately equal in their lack of toxic effects on the host.

In the experiment with influenza B/Lee/40 virus (Table 3), selenazofurin treatment caused moderate increases in mean survival time and markedly reduced lung consolidation scores. Day 5 temperatures were also moderately increased. Lung HA titers again increased, however, and the highest titers were in the animals receiving the highest dosage of the compound. Ribavirin was effective by all of these parameters, particularly at the highest dosage level. This experiment was run in parallel with the study shown in Table 2, with the same toxicity controls used for each.

Effects of varying treatment schedules

Experiments were run to determine the optimal treatment schedule of selenazofurin against influenza A/PR/8/34 virus infections. The various treatment schedules used in an initial experiment are summarized in Table 4. The data, which were the results of a single experiment, are expressed as increases in survivor, mean survival time and temperature increase to simplify comparison. In this study, 1 of 20 virus control animals survived, with a mean survival time of 6.4 days. As seen in Fig. 1, the mean day 6 rectal temperature was 32.5°C. Only survivors, increased mean survival time and increased rectal temperatures taken on infection days 1 through 6 post-infection were used as parameters. Only day 6 temperature differences are indicated in the table. No treatment significantly increased survivor numbers, but mean survival time was prolonged using all schedules. Once-daily treatment for 9 days, and a single treatment 48 h post-virus exposure appeared to be most effective.

TABLE 3

Effect of intraperitoneal treatment[a] with selenazofurin or ribavirin on influenza B/Lee/40 virus infections in mice

Compound	Dosage (mg/kg per day)	Infected, treated				
		Surv/ total	MST[c] (days)	Mean lung score[d]	Mean day 5 temp. (°C)	Mean lung HA titer
Selenazofurin	200	0/10	8.1*	0.9*	30.2	388
	100	0/10	7.9	1.2*	31.7**	588
	50	1/10	7.4	2.1	31.1*	315
Ribavirin	75	2/9	9.9**	0.8*	33.1**	158*
	37.5	1/10	8.4*	2.7	32.2*	208
	18.8	0/10	6.4	1.0**	30.0	137*
Saline		0/20	7.2	3.0	29.7	266
Normal controls				0.0	34.9	7

[a] b.i.d. × 7, beginning 4 h pre-virus exposure.

[b] Weight 18 h after final treatment minus day 0 weight (5/5 mice survived in each group).

[c] Mean survival time of mice dying on or before day 21.

[d] Lungs removed on day 6 post-virus exposure.

*$P<0.05$; **$P<0.01$.

TABLE 4

Effect of varying intraperitoneal treatment schedules with selenazofurin on influenza A/PR/8/34 virus infections in mice

Treatment schedule	Beginning of treatment[a]	Dosage (mg/kg per day)	Tox. controls		Infected, treated		
			Surv/ total	Host wt. change[b] (g)	% survivor increase[c]	MST[d] incr. (days)	Day 6 temp. increase (°C)
bid × 9	4 h pre	100	4/4	+1.7	0	2.2*	1.3*
tid × 7	6 h pre, 1 h pre	100	5/5	+0.6	0	2.8*	−0.5
bid × 9	24 h pre	100	4/4	+1.7	0	2.1*	−1.4
One treatment	24 h post	400	5/5	+0.7	0	0.5	0.0
One treatment	48 h post	400	5/5	+0.7	8	2.1*	2.4**
Once daily × 9	4 h pre	100	5/5	+0.5	0	3.2**	1.1*

[a] Relative to virus exposure.
[b] Weight 18 h after final treatment minus day 0 weight.
[c] No. selenazofurin-treated mice/group: 15; no. placebo-treated mice/group: 20.
[d] Increase in mean survival time of mice dying on or before day 21, as compared to saline-treated controls.
*$P<0.05$; **$P<0.01$.

Fig. 1 shows the rectal temperatures throughout the single treatment experiments. A precipitous temperature drop in virus control mice continued until the animals died of the infection. Single selenazofurin treatments, administered either 24 or 48 h post-virus exposure, resulted in a significant increase in temperature to approximately normal levels. The temperature of the animals treated at 24 h declined after this initial rise until they were the same as those of the virus control mice. The temperatures of the mice treated at 48 h also declined, but the decline was much delayed.

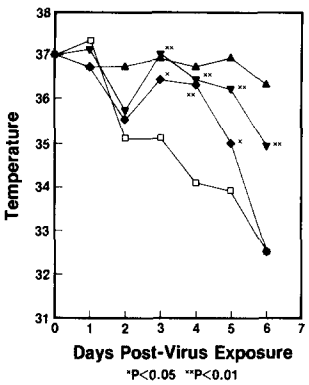
Since a selenazofurin dosage level of 400 mg/kg appeared well tolerated when administered a single time, we attempted to determine the effect of the same dosage level given once daily for three consecutive days. After both the infected and toxicity control animals received the second treatment, however, they immediately became prostrate, and did not appear to fully recover for approximately one hour. Because of this apparent toxicity, the third treatment was reduced to 200 mg/kg. This third treatment at lower dosage did not visibly affect the toxicity control animals. In this experiment, one-half of the infected, treated animals were killed on day 6 and their lungs removed, examined for consolidation, and assayed for both viral HA and infectious virus titer. The results are seen in Table 5. The infected mice treated with selenazofurin exhibited a 2.6 day increase in mean survival time, and their lungs were almost clear of consolidation. Their rectal temperatures were also significantly increased on day 6. In this experiment, the lung HA titers of treated mice were slightly reduced. The titer of infectious virus from the lungs was determined by in vitro assay

TABLE 5

Effect of 3-day[a] or every other day[b] intraperitoneal treatment with selenazofurin on influenza A/PR/8/34 virus infections in mice

Compound	Dosage (mg/kg per day)	Tox. controls		Infected, treated					
		Surv/ total	Host wt. change[c]	Surv/ total	MST[d] (days)	Mean lung score[e]	Day 6 temp. (°C)	Mean lung HA titer	Mean lung virus titer[f]
3-day treatment									
Selenazofurin	400[g]	5/5	0.2	3/10	10**	0.8**	35.2**	34	5.8
Saline	–			6/20	7.4	2.6	33.8	42	6.1
Normal controls			2.0	–	–	0.3	36.6	8	0.0
Every other day treatment									
Selenazofurin	400	5/5	0.1	0/10	9.1*	1.8*	35.6**	79	4.9
Saline	–			0/20	7.4	2.9	32.5	31	5.0
Normal controls			2.1	4/4	>21	0.0	37.0	0	0

[a] Once daily for 3 days beginning 24 h post-virus exposure.
[b] 24, 72 and 120 h post-virus exposure.
[c] Weight 18 h after final treatment minus day 0 weight.
[d] Mean survival time of mice dying on or before day 21.
[e] Lungs removed on day 6 post-virus exposure.
[f] Determined by cytopathic effect produced in MDCK cells ($\log_{10}$ $CCID_{50}$/ml).
[g] Dosage reduced to 200 mg/kg per day on last treatment day because of apparent adverse effects seen on the second day of treatment.
*$P<0.05$; **$P<0.01$.



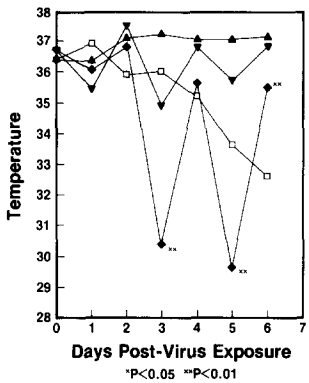


Fig. 1. Effect of single treatment with 400 mg/kg of selenazofurin on rectal temperatures in mice infected with influenza A/PR/8/34 virus. ▲, normal controls. ▼, selenazofurin 48 h post-virus exposure. ◆, selenazofurin 24 h post-virus exposure. □, placebo-treated virus controls.

Fig. 2. Effect of every other day treatment (days 1, 3 and 5) with 400 mg/kg of selenazofurin on rectal temperatures in mice infected with influenza A/PR/8/34 virus. ▲, normal controls. ▼, selenazofurin-treated toxicity controls. ◆, selenazofurin-treated infected mice. □, placebo-treated virus controls.

and by egg inoculation. These infectious titers determined by either method were reduced approximately equally, but the mean titer was only 0.3 $\log_{10}$ ($P > 0.05$) less than those in the saline-treated controls. Only the cell culture assay data, which were found to be more sensitive than egg inoculation, are shown in Table 5.

A final experiment using the 400 mg/kg per day selenazofurin treatment administered every other day was conducted, in order for the treated animals to more fully recover from the adverse drug effects. The animals were thus treated 24, 72, and 120 h after virus exposure. In this experiment (Table 5), the selenazofurin-treated, infected animals had a 1.7 day increase in mean survival time, and their lung consolidation scores were significantly reduced. An increase in lung HA titer similar to that in the initial experiments was again observed, but the infectious virus titers of both treated and control groups were similar. Rectal temperatures were determined late in the afternoon each day through day 6. On the days of drug treatment, these temperatures were determined approximately 2 h after each treatment. The mice did not become prostrate after treatment as had been seen in the previous study, but marked reductions in temperature occurred after the second and third treatment (Fig. 2), particularly in the infected mice. The mean day 6 temperature of the selenazofurin-treated mice increased significantly above that of the saline-treated virus controls.

Discussion

Selenazofurin was previously found to be markedly active against influenza A and B virus infections in vitro, although the activity was associated with some apparent sublethal cytotoxicity and was generally considered of lesser significance than that

exerted by ribavirin [11]. This general effect appears to be confirmed using certain infection parameters in these in vivo experiments.

In addition to its antiviral activity, selenazofurin has significant in vivo activity against P388 and Ridgeway osteogenic sarcoma [1,13]. In the present studies, the inhibitory effect of the compound upon influenza infections in mice appeared quite treatment schedule-dependent. Single treatments timed to provide drug during early stages of the infection or treatments once daily were generally more effective than multiple daily treatments. In the tumor inhibition studies reported by Boritzki et al. [1], a daily dose of selenazofurin for 4 days was effective.

Results of other studies [1,2,10] suggest that selenazofurin is a more potent inhibitor of IMP dehydrogenase than ribavirin, which may explain the broad-spectrum antiviral activity seen in cell culture. This mechanism probably lessens the specific antiviral activity of the compound, however, as seen in the enhanced cytotoxicity we reported previously [11]. Wray et al. [14] have shown that the influenza virus-inhibitory effects of selenazofurin may be because selenazofurin triphosphate inhibits the in vitro elongation of capped primer fragments by the influenza virus transcriptase complex, a mechanism similar to ribavirin. We speculate that the weaker activity of the compound seen in these animal experiments may be due to a guanosine reversal of the activity; guanosine has been shown to more markedly reverse the in vitro activity of selenazofurin than ribavirin [7]. It is also possible that selenazofurin is metabolized to an inactive or to a more toxic material in the mouse, or is inadequately absorbed.

An apparent anomaly was that influenza virus-infected mice treated with selenazofurin exhibited significantly reduced lung consolidation, yet in some instances had increased titers of virus in the lungs than placebo-treated virus controls. An apparent lack of dose-responsiveness also was seen, particularly among the lung scores. This was not due to wide lung score variation, since the scores of killed animals differed little (standard error $\pm$ 1 or less) but was influenced somewhat by animals dying prior to sacrifice. It is also interesting that, despite the markedly reduced lung consolidation, the animals continued to die, although these deaths were usually significantly delayed. Lung consolidation due to virus infection is a result of a combination of events. In man, the virus infection damages the alveolar cells and causes necrosis of the capillary walls leading to hemorrhage in the lungs. In addition, edema and hyperemia occur. The alveolar exudate usually consists of neutrophils and mononuclear cells [3]. Similar observations have been made in influenza virus-infected mice, indicating that the swollen plum-colored lungs are due to both viral infection of the alveolar cell lining, which is subsequently destroyed, and vascular phenomena resulting from immune response to the infection [4]. Secondary bacterial infection was probably not a factor in our study since a broad spectrum antibiotic was administered during the experiment. It is possible that selenazofurin may have exerted an antiviral as well as an immunosuppressive effect in the mouse, thus accounting for the lessened obvious signs of lung consolidation while virus titers increased in the lungs. Other immune response parameters as well as compound bioavailability and histopathology studies should be determined in future experiments with this compound. It would be of interest to determine the effects of treatment on the percent of oxygenation in the blood as a means of demonstrating whether virally inhibited lung function was reversed by therapy.

We are unaware of previous studies illustrating the effect of influenza virus infection on rectal temperatures in mice. A consistent finding in these studies was the almost immediate decline in temperature, continuing until death of the animal. Such hypothermia may have been due to metabolic and respiratory acidosis resulting from underperfusion of the tissues with blood [9]. The inhibition of such temperature suppression appears to be an acceptable additional parameter for studying the effects of antiviral drugs in influenza virus-infected mice. Of particular interest was the marked but reversible drop in rectal temperature which occurred following the second and third selenazofurin treatments. This drop in temperature undoubtedly reflected sublethal toxicity; the animals apparently were capable of recovering from these toxic effects, since the temperatures were at predictable values 24 h later. The temperature declines were particularly pronounced in the infected animals, suggesting that drug toxicity was accentuated in animals, weakened by the infection. Such data indicate that potential drug toxicity should be monitored by means of body temperature.

Selenazofurin has a positive effect when used against murine hepatitis virus infections in mice (Sidwell, Huffman, Call, Alaghamandan, Cook and Robins, manuscript in preparation). The activity against a liver infection seems more pronounced than was seen in the present studies against a lung infection. This observation requires further exploration. Selenazofurin, when used in combination with ribavirin, has a definite enhanced antiviral effect in vitro [5,6] and in vivo (Canonico, P.G., personal communication), suggesting possible additional applications for this substance. The compound is presently undergoing preliminary human toxicity studies in contemplation of anticancer trials (Cook, P.D., personal communication).

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