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In vitro and in vivo influenza virus-inhibitory effects of viramidine

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Abstract

Viramidine, the 3-carboxamidine derivative of ribavirin, was effective against a spectrum of influenza A (H1N1, H3N2 and H5N1) and B viruses in vitro, with the 50% effective concentration (EC_{50}) ranging from 2 to 32 μg/ml. The mean 50% cytotoxic concentration (CC_{50}) in the MDCK cells used in these experiments was 760 μg/ml. Ribavirin, run in parallel, had a similar antiviral spectrum, with EC_{50} values ranging from 0.6 to 5.5 μg/ml; the mean CC_{50} for ribavirin was 560 μg/ml. Oral gavage administrations of viramidine or ribavirin to mice infected with influenza A/NWS/33 (H1N1), A/Victoria/3/75 (H3N2), B/Hong Kong/5/72 or B/Sichuan/379/99 viruses were highly effective in preventing death, lessening decline in arterial oxygen saturation, inhibition of lung consolidation and reducing lung virus titers. The minimum effective dose of viramidine in these studies ranged from 15 to 31 mg/kg/day, depending upon the virus infection, when administered twice daily for 5 days beginning 4 h pre-virus exposure. The LD_{50} of the compound was 610 mg/kg/day. Ribavirin's minimum effective dose varied between 18 and 37.5 mg/kg/day with the LD_{50} determined to be 220 mg/kg/day. Viramidine's efficacy was also seen against an influenza A/NWS/33 (H1N1) virus infection in mice, when the compound was administered in the drinking water, the minimum effective dose being 100 mg/kg/day. Delay of the initiation of either viramidine or ribavirin therapy, using the approximate 1/3 LD_{50} dose of each, was protective as late as 48 h after exposure to the A/NWS/33 virus. While both compounds appear to have similar efficacy against influenza virus infections, when one considers the lesser toxicity, viramidine may warrant further evaluation as a possible therapy for influenza.

Keywords: Viramidine; Ribavirin; Influenza

1. Introduction

The widespread outbreaks of avian influenza occurring throughout eastern Asia, and the ability of those avian viruses to transfer to humans and cause serious infection, have aroused concern that such infections could lead to another pandemic of the disease (Kaye and Pringle, 2005). Antiviral drugs effective against such infections hold great promise as a means to control them but constraints, such as price and sufficient supply may play a major role in their use.

The broad spectrum antiviral, ribavirin, is a recognized inhibitor of influenza A and B virus infections in vitro and in animal models (Sidwell et al., 1972; Huffman et al., 1973; Khare et al., 1973), but mixed results have been obtained in clinical trials with the drug (for review, see Sidwell, 1996). A limitation to the use of ribavirin has been a relatively small therapeutic index, hemolytic anemia induced at high doses, and potential teratogenic effects (Hillyard, 1980; Sidwell, 1996). Viramidine (ribamidine, 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamidine), the carboxamidine analog of ribavirin, has exhibited significant antiviral activity in vitro against a spectrum of viruses, and against an influenza A (H2N2) and a mannan-enhanced influenza B virus infection in mice (Witkowski et al., 1973; Smee et al., 2004). We have previously reported it to also inhibit a *Phlebovirus* infection in vitro and in vivo (Sidwell et al., 1988). Biochemical cytotoxicity experiments have indicated viramidine to have a less pronounced effect on DNA synthesis than ribavirin (Sidwell et al., 1988), and viramidine treatment of rhesus monkeys did not appear to affect the red blood cell parameters usually decreased by similar ribavirin treatment (Pifat et al., 1988). Recent phase I clinical studies have indicated that viramidine is better-tolerated than ribavirin (Hong et al., 2002). The drug is currently being further studied as a

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potential inhibitor of hepatitis C infections (Wu et al., 2004). It was therefore thought of importance to further evaluate viramidine in comparison studies with ribavirin, in regard to its efficacy against influenza virus infections. This report describes the results of these experiments.

2. Materials and methods

2.1. Compounds

Viramidine and ribavirin were provided by ICN Pharmaceuticals Inc. (now Valeant Pharmaceuticals Inc., Costa Mesa, CA).

2.2. Viruses and cells

Influenza A/NWS/33 (H1N1) was obtained from Dr. Kenneth Cochran of the University of Michigan (Ann Arbor, MI). A/PR/8/34 (H1N1), A/Victoria/3/75 (H3N2), B/Lee/40 and B/Hong Kong/5/72 were obtained from the American Type Culture Collection (ATCC; Manassas, VA). A/Beijing/262/95 (H1N1), A/New Caldonia/20/99 (H1N1), A/Sydney/05/97 (H3N2), A/Panama/2007/99 (H3N2), B/Beijing/184/93 and B/Sichuan/379/99 viruses were obtained from the Centers for Disease Control and Prevention (Atlanta, GA). A/Duck/MN/1525/81 and A/Gull/PA/4175/83 (H5N1) viruses were provided by Dr. Robert Webster of St. Jude Children's Research Hospital (Memphis, TN). The viruses used in cell culture experiments were passaged through Madin Darby canine kidney (MDCK) cells (ATCC) at least once to prepare pools. The pools were then titrated in MDCK cells before use. The A/NWS/33 virus was passaged eight times through MDCK cells, and a pool prepared. The A/Victoria/3/75 virus was passaged seven times through mice, after which the recovered virus from the lungs was used to prepare a pool in MDCK cells. A pool of B/Hong Kong/5/72 virus was prepared from embryonated eggs. The B/Sichuan virus was adapted for lethality in mice after 10 passages in the animals. All virus pools were titrated in mice using death as end point. The cells were grown in minimum essential medium (MEM) containing 5% fetal bovine serum (FBS, Hyclone Laboratories, Logan, Utah) and 0.1% sodium bicarbonate with no antibiotics in a 5% CO_2 incubator. Viral propagation and in vitro antiviral assays were run using MDCK cells in MEM, 5% FBS, 0.18% sodium bicarbonate, 10 units of trypsin/ml, 1 ng EDTA/ml and 50 μg gentamicin/ml.

2.3. Animals

Female specific pathogen-free BALB/c mice weighing 18–21 g were obtained from Charles River Laboratories (Wilmington, MA). They were quarantined for 48 h prior to use and fed standard mouse chow and tap water ad libitum.

2.4. Arterial oxygen saturation (SaO_2) determinations

SaO_2 was determined using the Ohmeda Biox 3800 pulse oximeter (Ohmeda, Louisville, OH). The ear probe attachment was used with the probe placed on the thigh of the mouse. Readings were made after a 30 s stabilization time on each animal. This method has been described in detail (Sidwell et al., 1992). All SaO_2 determinations were made on days 3 through 11 after virus exposure.

2.5. Lung virus titer determinations

Each mouse lung was homogenized and varying dilutions assayed in triplicate for infectious virus in MDCK cells grown in 96 well flat bottomed microplates as described previously (Sidwell et al., 2001b). Each lung homogenate was centrifuged at 2000 × *g* for 5 min and the supernatents used in these assays.

2.6. In vitro antiviral evaluations

Three methods were used to assay antiviral activity in vitro: inhibition of virus-induced cytopathic effect (CPE) determined by visual (microscopic) examination of the cells, increases in neutral red (NR) dye uptake into cells and virus yield reduction. These methods have been fully described previously (Smee et al., 2000). Eight concentrations of the test compounds, each varying by one-half $\log_{10}$ from the next, were evaluated in MDCK cells. Standard placebo-treated virus controls, toxicity controls and normal medium controls were included in all assays. CPE inhibition data were expressed as the 50% effective (viral CPE-inhibitory) concentration (EC_{50}), 50% cytotoxicity (cell-inhibitory) concentration (CC_{50}) and selectivity index (SI), determined as the CC_{50}/EC_{50}. Virus yield reduction data were expressed as that concentration inhibiting virus yield by one $\log_{10}$ (EC_{90}); the SI for virus yield results was calculated as CC_{50}/EC_{90}.

2.7. In vivo toxicity determinations

Ribavirin and viramidine were each evaluated for the dose considered lethally toxic to mice. This was done by treating nine mice per dose with each compound. Ribavirin doses studied were 150, 75, 37.5 and 18.8 mg/kg/day and viramidine doses were 500, 250, 125, 62.5 and 31.3 mg/kg/day. The mice were treated by oral gavage (p.o.) twice daily for 5 days. The animal weights were determined prior to the first treatment and again 18 h after the final treatment. They were observed for death daily for 21 days.

2.8. Initial in vivo antiviral studies

The general procedure for initial evaluation of the relative efficacies of viramidine and ribavirin was to infect

groups of mice intranasally with influenza virus. This was done by anesthetizing intraperitoneally (i.p.) with Ketamine (100 mg/kg) and instilling 90 μl of an approximate LD_{100} concentration of virus on the nares. Viruses studied in vivo included A/NWS/33, A/Victoria/3/75, B/Hong Kong/5/72 and B/Sichuan/379/99. The mice were treated with varying dosages of either drug p.o. twice daily for 5 days beginning 4 h pre-virus exposure. Parameters for determining effects of treatment included prevention of death through 21 days, lessening of SaO_2 decline, inhibition of lung consolidation (scores ranging from 0 [normal] to 4 [maximal plum coloration] and lung weight), and lessening of lung virus titers. The lung parameters were assayed on days 3, 6 and 9 of the infection, with 3–5 mice killed at each time point. Ten mice were used per drug-treated group to assay for death and SaO_2. Placebo-treated controls were used in each experiment with twice the numbers of animals used for each disease parameter. Toxicity controls were also run in parallel at each drug dose; three mice were used per dose, with weights taken prior to start of treatment and again 18 h after the last treatment and the animals observed for overt signs of toxicity and death for 21 days. Normal controls were run in parallel; these animals were weighed along with the toxicity controls, and SaO_2 levels ascertained on the same days as the infected animals. Three normal controls were sacrificed along with the infected mice to provide background lung data.

2.9. In vivo antiviral effects of delay in therapy initiation

To determine the relative efficacy of ribavirin and viramidine against an influenza A virus infection when initiation of therapy was delayed, groups of 10 mice infected with the A/NWS/33 virus were treated p.o. twice daily for 5 days with either drug beginning 4, 12 or 20 h post-virus exposure. Effects on prevention of death and lessening of SaO_2 decline were determined, with the numbers of animals per group as outlined above. The doses of each compound were equivalently non-toxic (1/3 LD_{50} dose), for ribavirin, this was 75 mg/kg/day, for viramidine, 200 mg/kg/day. The animals were held for 21 days. When it was determined that all times of treatment were highly effective, the experiment was repeated with treatments delayed until 28, 36 or 48 h post-virus exposure.

2.10. In vivo antiviral effects of viramidine administered in the drinking water

Mice infected with influenza A/NWS/33 virus were treated with viramidine ad libitum in the drinking water at concentrations of 0.93, 0.47 or 0.24 mg/ml, which provided the equivalent doses of 200, 100 or 50 mg/kg/day, respectively, based on approximate volumes consumed daily. Treatment began 4 h pre-virus exposure and continued for 5 days. The numbers of animals per treatment group were as described above.

2.11. Statistical evaluations

Increases in number of survivors were evaluated using χ^2 analyses with Yates' correction. Differences in mean day to death, mean SaO_2 values, and mean lung virus titers were analyzed by *t*-test. The Wilcoxon ranked sum analysis test was used to compare lung scores.

3. Results

3.1. In vitro antiviral effects

Both compounds were inhibitory to all the influenza viruses evaluated, the EC_{50} values for ribavirin ranging from 0.6 to 5.5 μg/ml, depending upon the virus assayed, whereas viramidine was approximately three- to five-fold less potent, the EC_{50} values ranging from 2.1 to 32 μg/ml (Table 1). The efficacies versus each virus were similar utilizing both visual and neutral red assays, so only the results from the latter assays are shown. In general, the influenza B viruses appeared somewhat more sensitive to inhibition than did the various influenza A viruses evaluated. Limited virus yield reduction assays confirmed these same observations. Multiple cytotoxicity assays using neutral red uptake indicated the mean CC_{50} values for ribavirin and viramidine were, respectively, 570 and 760 μg/ml.

3.2. Comparison of murine toxicities

Oral gavage treatment with ribavirin and viramidine using twice daily therapies for 5 days indicated the approximate LD_{50} dose of ribavirin to be 220 mg/kg/day, whereas viramidine was better tolerated, the LD_{50} dose being approximately 610 mg/kg/day (Table 2). It should be noted that some weight loss was seen at dosages below the LD_{50} dose, although all treated mice survived at these dosages. No attempt was made to determine the cause of death in the mice in this range-finding study.

3.3. Comparison of in vivo anti-influenza virus efficacies of ribavirin and viramidine

Utilizing multiple dosages of both ribavirin and viramidine, both compounds were evaluated against influenza A/NWS/33 (H1N1), A/Victoria/3/75 (H3N2), B/Hong Kong/5/72 and B/Sichuan/379/99 viruses in young adult mice. The results using each against influenza A virus infections are summarized in Table 3. The influenza B virus infection data are shown in Table 4. Due to the large amount of data available, only the last day of SaO_2 determination, day 11, is shown in the tables; it is on this day that the values usually reach their lowest levels in placebo-treated animals and thus differences due to therapy are most pronounced. The day 6 lung consolidation data are shown in the tables, since it is at this time lung scores and weights are maximal; day 3

Table 1
In vitro inhibition of influenza viruses by viramidine and ribavirin

Virus	Viramidine		Ribavirin	
	Neutral red EC_{50}[a] (μg/ml)	Virus yield EC_{90}[b] (μg/ml)	Neutral red EC_{50}[a] (μg/ml)	Virus yield EC_{90}[b] (μg/ml)
A/H1N1				
Beijing/262/95/	32	–[c]	3.5	–
New Caledonia/20/99	18	–	5.5	–
NWS/33	18	22	4.5	7.5
PR/8/34	18	–	5.5	–
A/H3N2				
Sydney/05/97	23	–	2.7	–
Panama/2007/99	18	–	5.5	–
Victoria/3/75	11	10	3.0	2.2
Panama/2007/99	16	–	3.0	–
A/H5N1				
Duck/MN/1525/81	18	11	2.3	1.6
Gull/PA/4175/83	18	7	4.3	1.2
B				
Beijing/184/93	7.0	–	1.5	–
Hong Long/5/72	5.0	5.0	1.5	1.5
Lee/40	4.5	–	1.8	–
Sichuan/379/99	7.0	–	4.2	–

Note: IC_{50} values for viramidine were 760 ± 200 μg/ml, IC_{50} values for ribavirin ranged from 570 ± 230 μg/ml.
[a] 50 % virus-inhibitory (effective) concentration.
[b] 90% virus yield-inhibitory (effective) concentration (concentration inhibiting virus yield by 1 $\log_{10}$).
[c] Not run.

lung virus titers are indicated because these titer differences are usually best seen early in the infection. Each compound was significantly inhibitory to these viruses utilizing all disease parameters. Against the A/NWS infection, viramidine significantly prevented deaths down to 62.5 mg/kg/day; the other parameters were primarily inhibited at the minimum dose of 125 mg/kg/day. Ribavirin's minimum effective dose against this same virus infection, using prevention of death, was 37.5 mg/kg/day. Against the A/Victoria virus infection, viramidine was effective in inhibiting death and SaO_2 decline at doses down to 15.6 mg/kg/day, which was the lowest evaluated. Ribavirin's inhibition of this virus infection was seen down to 37.5 mg/kg/day with a 33% survivor rate also seen at 18.8 mg/kg/day. It should be noted, however, that in the viramidine experiment 30% of the placebo-treated controls survived the infection, whereas in the ribavirin experiment, the viral challenge appeared to be higher since all placebo-treated controls died of the infection. Thus, caution should be taken in comparing the relative efficacies of these compounds against the A/Victoria virus infection.

Table 2
Comparison of toxicity of p.o. administered[a] viramidine and ribavirin in mice[b]

Compound	Dosage (mg/kg/day)	Survived/total	MDD[c] ± S.D.	Mean host weight change[d] (g)	Estimated LD_{50}[e] (mg/kg/day)
Viramidine	1000	0/9	5.7 ± 1.3	−1.9	610
	750	0/9	6.4 ± 0.7	−3.4	
	500	9/9	>21.0	−1.7	
	250	9/9	>21.0	−0.3	
	125	9/9	>21.0	0.3	
	62.5	9/9	>21.0	0.7	
Ribavirin	300	3/9	6.8 ± 2.4	−2.8	220
	225	4/9	7.6 ± 0.9	−2.3	
	150	7/9	7.8 ± 0.9	−0.1	
	75	9/9	>21.0	−0.1	
	37.5	9/9	>21.0	0.7	
	18.8	9/9	>21.0	0.8	
Normal controls	–	5/5	>21.0	0.5	

[a] Bid × 5.
[b] Female 18–21 g BALB/c mice.
[c] Mean day to death of mice dying prior to day 21.
[d] Difference between weight prior to start of treatment and weight 18 h after end of therapy.
[e] Determined by line of regression.

Table 3
Inhibition of influenza A virus infections in BALB/c mice treated p.o.[a] with viramidine or ribavirin

Compound, virus	Infected, treated mice						
	Dose (mg/kg/day)	Survived/ total	MDD[b] ± S.D.	Mean day 11 SaO_2 (% ± S.D.)	Mean lung parameters		
					Day 6 score ± S.D.	Day 6 wt (mg ± S.D.)	Day 3 virus titer (log_{10} ± S.D.)
Viramidine							
A/NWS/33 (H1N1)	500	10/10***	>21.0***	83.7 ± 2.6***	0.0 ± 0.0**	147 ± 15**	5.1 ± 1.1*
	250	10/10***	>21.0***	82.7 ± 3.6***	0.0 ± 0.0**	150 ± 0**	5.4 ± 0.5**
	125	10/10***	>21.0***	81.1 ± 4.1***	0.3 ± 0.3*	176 ± 21**	6.3 ± 0.9
	62.5	8/10***	12.0 ± 2.5**	74.6 ± 5.8	2.8 ± 0.3	223 ± 25	6.3 ± 0.8
	31.3	2/10	11.0 ± 1.5***	74.5 ± 4.0	3.0 ± 0.0	227 ± 21	6.4 ± 0.5
	15.6	1/10	9.6 ± 1.4*	74.7 ± 0.9	1.7 ± 1.3	203 ± 66	7.0 ± 2.0
	0	0/20	8.5 ± 1.1	75.0 ± 0.0	2.4 ± 0.7	268 ± 38	6.7 ± 0.4
A/Victoria/3/75 (H3N2)	250	9/9***	>21.0***	84.8 ± 3.7***	0.4 ± 0.4**	200 ± 75*	5.2 ± 0.5*
	125	9/9***	>21.0***	84.3 ± 3.8**	0.9 ± 0.4*	236 ± 27	5.3 ± 0.1*
	62.5	10/10***	>21.0***	85.3 ± 4.3**	0.8 ± 0.3**	262 ± 30	5.3 ± 0.6
	31.3	8/10**	8.5 ± 2.6	84.2 ± 5.2*	1.3 ± 0.3	262 ± 30	5.6 ± 0.4
	15.6	9/10**	12.0 ± 0.0*	85.9 ± 1.8***	1.8 ± 0.4	300 ± 35	5.8 ± 0.4
	0	6/20	9.0 ± 1.5	78.9 ± 5.5	1.8 ± 0.4	286 ± 74	5.9 ± 0.3
Ribavirin							
A/NWS/33 (H1N1)	150	5/10*	6.4 ± 1.1	85.7 ± 2.6***	0.0 ± 0.0*	140 ± 10***	4.9 ± 0.5***
	75	9/10***	7.0 ± 0.0	85.8 ± 2.7***	0.2 ± 0.3*	163 ± 12***	5.8 ± 0.4***
	37.5	10/10**	>21.0***	86.6 ± 3.0***	0.2 ± 0.3*	193 ± 12***	6.5 ± 0.3**
	18.8	4/10	8.3 ± 4.2	82.6 ± 5.6***	0.7 ± 0.8*	220 ± 36.1**	8.0 ± 0.4
	9.4	1/10	6.3 ± 2.0	76.0 ± 3.2	2.3 ± 0.3	256 ± 23*	6.8 ± 0.0*
	0	2/20	5.9 ± 2.2	75.0 ± 0.0	2.9 ± 0.4	320 ± 26	7.4 ± 0.1
A/Victoria/3/75 (H3N2)	150	7/10***	9.3 ± 2.1	80.4 ± 5.0**	2.5 ± 0.9*	203 ± 47	5.8 ± 0.1
	75	10/10***	>21.0***	84.6 ± 3.2***	1.8 ± 0.3*	187 ± 6***	5.8 ± 0.4
	37.5	9/10***	12.0 ± 0.0	82.9 ± 3.9***	3.0 ± 0.5	227 ± 21**	6.0 ± 0.4
	18.8	3/9*	9.2 ± 1.2*	78.2 ± 5.2*	3.3 ± 0.8	793 ± 71	6.0 ± 0.4
	9.4	0/10	7.6 ± 1.5	76.0 ± 3.2	3.2 ± 0.6	323 ± 49	5.9 ± 0.3
	0	0/20	7.2 ± 1.3	75.0 ± 0.0	3.5 ± 0.4	308 ± 26	6.3 ± 0.4
Normal controls	–	5/5	>21.0	88.8 ± 3.7	0.0 ± 0.0	132 ± 12	–

[a] Bid × 5 beginning 4 h pre-virus exposure.
[b] Mean day to death of mice dying prior to day 21.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

Against the influenza B virus infections (Table 4), the minimum effective dose of viramidine against the B/Hong Kong virus infection was 31.3 mg/kg/day and against the B/Sichuan virus challenge it was 80 mg/kg/day, although significant delay of death and inhibition of SaO_2 decline was also seen at 40 mg/kg/day. Ribavirin was inhibitory to the B/Hong Kong virus infection down to 18.8 mg/kg/day; the B/Sichuan infection was inhibited by the lowest dose evaluated, 20 mg/kg/day.

Although the minimum effective dose of ribavirin was lower than seen with viramidine, the lesser toxicity of viramidine displayed in the mice, as described above, resulted in each compound having approximately the same therapeutic index.

3.4. Effects of delay in therapy initiation

The results of delaying initiation of therapy of ribavirin and viramidine on an influenza A virus infection are presented in Table 5. In this experiment, 75 mg/kg/day of ribavirin and 200 mg/kg/day of viramidine were used. These were approximately one-third of the LD_{50} dose of each. Both compounds remained highly protective to the mice despite the delay in start of treatment with highly significant prevention of death seen as late as the 20 h post-virus exposure initiation time, which was the longest delay evaluated in the first experiment. One infected animal treated with ribavirin died at this latest therapy initiation time. All mice survived in the other infected, treated groups. In the second experiment, run with therapy initiation being further delayed, both compounds were highly protective to the mice even when begun 48 h after virus exposure, which was the latest time evaluated.

3.5. Effects of treatment with viramidine in drinking water

The effects of viramidine administered in the drinking water on an infection in mice induced by influenza

Table 4
Inhibition of influenza B virus infections in BALB/c mice treated p.o.[a] with viramidine or ribavirin

Compound, virus	Infected, treated mice						
	Dose (mg/kg/day)	Survived/ total	MDD[b] ± S.D.	Mean day 11 SaO_2 (% ± S.D.)	Mean lung parameters		
					Day 6 score ± S.D.	Day 6 wt (mg ± S.D.)	Day 3 virus titer (log_{10} ± S.D.)
Viramidine							
B/Hong Kong/5/72	125	10/10***	>21.0***	87.9 ± 2.1***	0.2 ± 0.3**	220 ± 10**	4.3 ± 1.1*
	62.5	8/9***	10.0 ± 0.0	84.9 ± 4.6***	1.0 ± 0.5*	277 ± 15*	4.4 ± 1.9
	31.3	7/10***	19.0 ± 0.0***	87.0 ± 2.2***	2.7 ± 1.0	333 ± 75	4.4 ± 0.6*
	15.6	0/9	7.8 ± 3.3	76.7 ± 4.0	3.3 ± 1.2	367 ± 61	4.3 ± 0.7*
	0	2/20	6.1 ± 1.6	76.1 ± 3.4	2.7 ± 1.2	374 ± 71	5.4 ± 1.7
B/Sichuan/379/99	160	9/10***	19.0 ± 0.0***	80.9 ± 3.0***	0.7 ± 0.3***	186 ± 12*	6.2 ± 0.2*
	80	10/10***	>21.0***	81.5 ± 5.0*	0.8 ± 0.3***	180 ± 14*	6.3 ± 0.1*
	40	1/10	10.4 ± 2.6*	77.6 ± 3.0*	3.3 ± 0.6	230 ± 20	6.0 ± 0.1
	0	0/10	8.2 ± 0.9	75.1 ± 0.3	3.7 ± 0.3	263 ± 31	7.2 ± 0.6
Ribavirin							
B/Hong Kong/5/72	150	10/10***	>21.0***	79.9 ± 5.7*	1.8 ± 0.8*	230 ± 27***	4.9 ± 0.5
	75	10/10**	>21.0***	86.0 ± 4.4***	0.7 ± 0.8*	223 ± 21***	5.1 ± 0.5
	37.5	10/10	>21.0***	85.7 ± 3.6***	0.7 ± 0.3	230 ± 10***	5.1 ± 0.5
	18.8	5/10**	11.4 ± 2.5	79.8 ± 5.5*	3.3 ± 0.3	360 ± 36	5.0 ± 1.4
	9.4	0/10	9.7 ± 1.8**	76.0 ± 3.2	3.5 ± 0.5	343 ± 38	5.3 ± 0.4
	0	0/20	8.1 ± 1.0	75.9 ± 2.7	3.7 ± 0.4	400 ± 33	5.1 ± 0.5
B/Sichuan/379/99	80	10/10**	>21.0***	84.3 ± 2.7***	0.0 ± 0.0***	180 ± 17**	6.4 ± 0.4**
	40	9/10**	10.0 ± 0.0	83.8 ± 4.7***	0.4 ± 0.2***	202 ± 20**	6.6 ± 0.4**
	20	3/10*	10.6 ± 3.6*	76.6 ± 2.4	0.9 ± 0.7*	208 ± 22**	7.1 ± 0.1*
	0	0/20	7.8 ± 1.0	75.0 ± 0.0	3.0 ± 0.6	298 ± 50	7.6 ± 0.2
Normal controls	–	5/5	>21.0	88.8 ± 3.7	0.0 ± 0.0	132 ± 12	–

[a] Bid × 5 beginning 4 h pre-virus exposure.
[b] Mean day to death of mice dying prior to day 21.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

Table 5
Effect of delay of p.o. viramidine or ribavirin treatment[a] on an influenza A/NWS/33 (H1N1) virus infection in BALB/c mice

Start of treatment (h post-virus exp.)	Compound	Dosage[b] (mg/kg/day)	Survived/total	MDD[c] ± S.D.	Mean day 11 SaO_2 ± S.D.
4	Viramidine	200	10/10***	>21.0***	84.9 ± 2.9***
	Ribavirin	75	10/10***	>21.0***	84.0 ± 2.0***
12	Viramidine	200	10/10***	>21.0***	84.2 ± 1.9***
	Ribavirin	75	10/10***	>21.0***	85.2 ± 3.2***
20	Viramidine	200	10/10***	>21.0***	83.9 ± 1.6***
	Ribavirin	75	9/10***	9.0 ± 0.0	83.4 ± 3.9***
4	Saline	–	2/20	9.1 ± 1.7	75.9 ± 1.9
28	Viramidine	200	11/11***	>21.0***	83.9 ± 4.0***
	Ribavirin	75	11/11***	>21.0***	82.5 ± 5.8***
36	Viramidine	200	11/11***	>21.0***	84.4 ± 2.6***
	Ribavirin	75	11/11***	>21.0***	84.0 ± 4.5***
48	Viramidine	200	10/11***	19.0 ± 0.0	80.2 ± 3.0***
	Ribavirin	75	11/11***	>21.0***	81.4 ± 3.5***
28	Saline	–	3/21	9.2 ± 2.2	75.9 ± 1.8

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to saline-treated controls.
[a] Bid × 5.
[b] Approximate MTD/2 for each compound.
[c] Mean day to death of animals dying prior to day 21.

Table 6
Effect of viramidine administered in drinking water[a] on an influenza A (H1N1) virus infection in mice

Compound	Dosage (mg/kg/day)	Tox controls		Infected, treated mice					
		Survived/ total	Mean host wt change[b] (g)	Survived/ total	MDD[c] ± S.D.	Mean day 11 SaO_2 (% ± S.D.)	Mean day 6 lung score ± S.D.	Mean day 6 lung wt (mg ± S.D.)	Mean day 3 virus titers (log_{10}/g ± S.D.)
Viramidine	200	3/3	0.4	10/10***	>21.0***	85.3 ± 2.2***	0.0 ± 0.0*	167 ± 6**	5.7 ± 1.2*
	100	3/3	1.1	2/10	11.8 ± 2.4*	81.2 ± 5.6***	0.8 ± 0.3*	227 ± 47*	6.3 ± 0.8
	50	3/3	1.1	1/10	9.2 ± 1.2	76.0 ± 3.2	2.5 ± 0.5	257 ± 81	6.7 ± 1.0
Water	–	–	–	0/20	8.8 ± 1.2	75.5 ± 2.2	3.3 ± 0.3	336 ± 50	7.0 ± 1.3
Normal controls	–	3/3	0.8	–	–	88.8 ± 1.9	0.0 ± 0.0	160 ± 20	–

[a] Ad lib for 5 days beginning 4 h pre-virus exposure.
[b] Difference between initial weight and weight 18 h after final treatment.
[c] Mean day to death of mice dying prior to day 21.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

A/NWS/33 virus are summarized in Table 6. The compound was totally protective to the mice only at the 200 mg/kg/day dose; 20% of the infected animals survived when treated with the 100 mg/kg/day dose, although SaO_2 decline and lung consolidation were significantly inhibited at this dosage. The 50 mg/kg/day dosage administered in the drinking water was essentially not effective in this experiment.

4. Discussion

The purine nucleoside analog, viramidine, was considered highly effective against influenza virus in this study as seen by studies both in vitro and in experimentally infected mice. The spectrum of anti-influenza virus activity appeared to be similar to the related drug, ribavirin, although the in vitro SI values of viramidine were somewhat less than ribavirin due to viramidine being generally less antivirally potent than ribavirin, and the cytotoxicity, while less than ribavirin (CC_{50} values of 760 μg/ml for viramidine versus 570 μg/ml for ribavirin) was not reduced to the extent that the SI values were appreciably affected. Against the B/Beijing/184/93 virus, particularly, the SI was more than 20-fold higher for ribavirin than viramidine. It is probable that the lesser efficacy exhibited by viramidine in vitro is due to the slower conversion of the compound to ribavirin in cells compared to what occurs in animals (Zhi Hong, personal communication, Valeant Pharmaceuticals Inc., Costa Mesa, CA).

Efficacy in infected mice was demonstrated by all disease parameters studied in these experiments, including prevention of death, slowing of the mean day to death, lessening of SaO_2 decline, inhibition of lung consolidation as seen by lessened lung scores and lower lung weights and reducing in titers of infectious virus recovered from the lungs. In some experiments, the lung virus titers were not markedly affected by therapy with either compound, although a strong disease-inhibitory effect was seen. It is probable that in such cases, examination of the lungs at an earlier time, e.g. day 1, would have demonstrated a greater effect. We have shown this to be the case with neuraminidase inhibitors (Sidwell et al., 2001a). This early time period was not selected for the present study because significant lung consolidation is not manifested at this time.

The relative toxicities of ribavirin and viramidine in mice did not correlate well with what was seen in vitro; in the animals, viramidine was approximately three-fold less toxic than ribavirin (LD_{50} of 610 mg/kg/day for viramidine versus 220 mg/kg/day for ribavirin). Thus, the in vivo SI values, determined as LD_{50}/minimum effective dose, of viramidine and ribavirin in the mice were quite similar.

Both compounds appeared to work equally well when initiation of therapy was delayed as late as 48 h after virus exposure. This was the latest time considered. In a previously reported study, ribavirin was not efficacious against the A/NWS/33 virus infection when treatment began 24 h after virus exposure (Sidwell et al., 1998); however, in that study the viral challenge was highly lethal to the placebo-treated animals, causing 100% mortality and a mean day to death of 7.0 ± 1.1 days. It is apparent that when the viral challenge is lessened to kill, in the present experiment, 86% of the placebo-treated mice with a more delayed mean day to death (9.2 ± 2.2 days), treatment can be delayed much longer and still be efficacious.

Viramidine has been shown to act primarily as a prodrug of ribavirin, being converted to ribavirin by adenosine deaminase (Wu et al., 2003b). An important advantage of viramidine is that, in animals, it is not taken up by red blood cells in the efficient manner that is seen with ribavirin (Lin et al., 2003), thus has less potential to cause hemolytic anemia. A recent report (Wu et al., 2004) has shown that viramidine inhibits ribavirin phosphorolysis, thus slowing the degradation of the newly formed ribavirin from viramidine in the cell and delivering more active metabolites for efficacy. The immunomodulatory properties of viramidine appear to be similar to those of ribavirin (Barnard, 2002). Viramidine has been reported to better target the liver than

ribavirin (Wu et al., 2003a), making it a potentially safer alternative to ribavirin for treating patients with hepatitis, and consequently the drug is now in phase 3 clinical trials in combination with pegylated alpha interferon for the treatment of active chronic hepatitis C virus infections.

The present study suggests that viramidine may have potential for treatment of human influenza virus infections. The compound's in vitro efficacy against two strains of influenza H5N1 would suggest that it may be of use against the current outbreaks of avian influenza occurring in human populations in Asia (Li et al., 2004). The paucity of sufficient supplies of active drugs for treatment of such infections, in the eventuality of a pandemic occurring, would justify further studies with viramidine to investigate this possibility.

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