

LIVE-ANIMAL TRIAL

Virutase with Influenza A/Shangdong/09/93 (H3N2)

Virutase exhibited significant in vitro activity against influenza A and B viruses, with IC₅₀ values ranging from 0.4 to 12 µg/mL, and TC₅₀ values of 150 µg/mL or greater. These data prompted a live-animal trial for this compound against influenza A (Shangdong/09/93) (H3N2) in mice. Since no information was known regarding the tolerance of this compound in mice, a preliminary toxicity determination was run using a maximal dose (100 mg/kg/day). It was decided to use an intraperitoneal (i.p.) treatment route and a treatment schedule of twice daily for 5 days beginning 4 h pre-virus exposure in order to maximize any potential antiviral effect.

Methodology

Animals. Female 18-21 g specific pathogen-free BALB/c mice were obtained from Harlan Sprague-Dowley, Inc. (Indianapolis, IN). They were quarantined 24 h prior to use and fed Wayne LabBlox and tap water *ad libitum*.

Compound. Virutase was dissolved in sterile physiological saline for use in this study. Ribavirin, used as a known positive control, was obtained from ICN Pharmaceuticals Inc. (Costa Mesa, CA). It also was dissolved in saline. Both compounds were stored at 4°C until used.

Determination of Arterial Oxygen Saturation (SaO₂). The effects of influenza virus on arterial oxygen saturation (SaO₂) were determined using the Ohmeda Biox 3740 pulse oximeter (Ohmeda, Louisville, OH). The ear probe attachment was used, the probe placed on the thigh of the animal, with the slow instrument mode selected. Readings were made after a 30-sec stabilization time on each animal.

Lung Virus Titer Determinations. Each mouse lung was homogenized and varying dilutions assayed in triplicate for infectious virus in MDCK cells.

Experiment Design, Toxicity Determination. Two mice were injected i.p. with 100 mg/kg/day of Virutase twice daily for 5 days. The animals were weighed daily and observed for death for 10 days.

Experiment Design, Antiviral Experiment. Groups of 19 mice were infected intranasally with virus and treated i.p. with Virutase at dosages of 50, 25, or 12.5 mg/kg/day or with Ribavirin at a dose of 75 mg/kg/day. Treatment was twice daily for 5 days beginning 4 h pre-virus exposure. As controls, 35 infected mice were treated with saline in parallel to the above. Ten mice in each drug-treated group and 20 salinetreated controls were observed for death for 21 days and SaO₂ levels ascertained in days 3-11. An additional 3 mice from each drug-treated group and 5 mice from the saline controls were killed in days 3, 6, and 9 and their lungs removed, assigned a consolidation score ranging from 0 (normal) to 4 (maximal, 100% plum coloration), weighed, and assayed for virus titer. Toxicity controls were included for each treatment group consisting of 3 mice per dosage. These were weighed prior to start of treatment and again 18 h after final treatment, and observed for death for 21 days. A group of 12 normal controls were also included; 3 were weighed in parallel with the toxicity controls and SaO₂ levels determined with the infected animals. Three additional mice were killed on days 3, 6, and 9 to provide background lung data.

Statistical Evaluation. Survivor number differences were analyzed by chi square analysis with Yates' correction. Changes in mean day to death, lung weights, SaO₂ levels, and virus titers were evaluated using the *t* test. Lung scores were analyzed using the Wilcoxon ranked sum analysis.

Results

Toxicity Determination. The preliminary toxicity study is summarized in Table XVIII. No animals died during treatment with 100 mg/kg/day of Virutase, but major weight loss (1.8 g) was seen, indicating the compound was not well tolerated at this dose. In view of these results, the maximum dosage used in the antiviral experiment was 50 mg/kg/day.

Antiviral experiment. The results of this experiment are summarized in Table XIX and in Figs. 1-4. Treatment with Virutase did indeed appear to exhibit some inhibitory effect on this influenza infection at 25 and 12.5 mg/kg/day dosages. This was seen by 30-40% increases in survivors, lessened decline in SaO₂ (Fig. 1), and inhibition of lung scores and weight (Figs. 2-3), and inhibition of lung scores and weight. Interestingly, the highest dosage, 50 mg/kg/day, although not lethal to the toxicity control animals, appeared to enhance the virus infection as seen by a shortened mean day to death (Table XX)

and markedly lowered SaO₂ levels (Fig. 1). It will also be noted that the mice in the group to be sacrificed for lung parameters had all died prior to day 6 when treated with the 50 mg/kg/day dose.

Weight loss was still observed in toxicity control mice receiving both the 50 and 25 mg/kg/day dose of Virutase, but weight gain occurred at the 12.5 mg/kg/day dose (Table XX). Ribavirin appeared well tolerated at the 75 mg/kg/day dose used in this study.

Ribavirin exerted the positive activity expected, preventing any deaths from occurring, markedly lessening SaO₂ decline, inhibiting lung consolidation, and reducing lung virus titers.

As discussed earlier, Virutase was significantly inhibitory to influenza A and B viruses *in vitro*; in anticipation of this experiment the compound was tested *in vitro* also against the influenza A/Shangdong/09/93 (H3N2) virus used in this animal experiment. The IC₅₀ values were 6 and 12 µg/mL using visual and neutral red endpoints, respectively, as presented in the preceding Section. These were up to 10-fold less potent than seen using new clinical isolates, but still indicates the compound was inhibitory to the virus.

Summary and Conclusions

Mice infected with a lethal dose of influenza A/Shangdong/09/93 (H3N2) virus were treated i.p. with 50, 25, or 12.5 mg/kg/day of Virutase. Treatments were twice daily for 5 days beginning 4 h pre-virus exposure. The high dose appeared to enhance the virus infection, presumably due to a sub-lethal toxicity. The lower doses were somewhat inhibitory to the infection as seen by increased numbers of survivors, lessened SaO₂ decline, and inhibition of lung consolidation. Ribavirin, included as a positive control exerted the inhibitory effect expected at the 75 mg/kg/day dose used.

Although only one Virutase was employed against a single influenza strain in this work, the data nevertheless indicate that Virutases do in fact show some promise as potential influenza inhibitors. Using a lower dosage, altering the treatment schedule to once or three times daily, and testing other Virutases against other influenza virus strains might in fact provide quite substantially different and improved drug efficacy.

Table XVIII. Preliminary Toxicity Determination of Intraperitoneally-Administered Virutase CA^a in Young Adult Mice^b

Treatment ^c	Dosage, mg/kg/day	Survivors/ Total	Mean Host Weight Change ^d , g
Virutase CA	100	2/2	-1.8

^a Drug diluent: sterile saline.

^b Female BALB/c mice, 18-21 g.

^c Treatment schedule: bid x 5 beg; 4-h pre-virus exposure. Experiment duration: 10 days.

^d Difference between initial weight and weight 18 hours after final treatment.

Table XIX. Effect of Intraperitoneal Treatment of Virutase CA^a on Influenza Virus Type A (Shangdong/09/93) (H3N2) Infection in Mice^b

Treatment ^c	Dosage, mg/kg/day	Toxicity Controls		Infected Treated Mice		
		Survivors/Total	Mean Host Weight Change ^d ,g	Survivors/Total	Mean Day to Death ^e ± SD	Mean Day 11 SaO ₂ , %±SD
Virutase CA	50	3/3	-0.8	0/10	6.5 ± 4.2	76.4 ± 4.6
	25	3/3	-0.7	3/10 ^f	10.3 ± 4.8	79.8 ± 4.6
	12.5	3/3	0.2	4/10 ^g	10.2 ± 2.8	81.7 ± 4.9 ^f
Ribavirin	75	3/3	0.1	10/10 ^h	>21.0 ± 0.0 ^h	88.2 ± 1.6 ^h
Saline	-	-	-	0/20	9.2 ± 3.5	76.9 ± 3.8
NormalControls	-	3/3	0.6	5/5	>21.0 ± 0.0	89.4 ± 2.2

^a Drug diluent: sterile saline.

^b Female BALB/c mice, 18-21 g.

^c Treatment schedule: bid x 5 beg; 4-h pre-virus exposure. Experiment duration: 21 days.

^d Difference between initial weight and weight 18 hours after final treatment.

^e Mean day to death of mice dying prior to day 21.

^f P<0.05;

^g P<0.01;

^h P<0.001, compared to saline-treated controls.

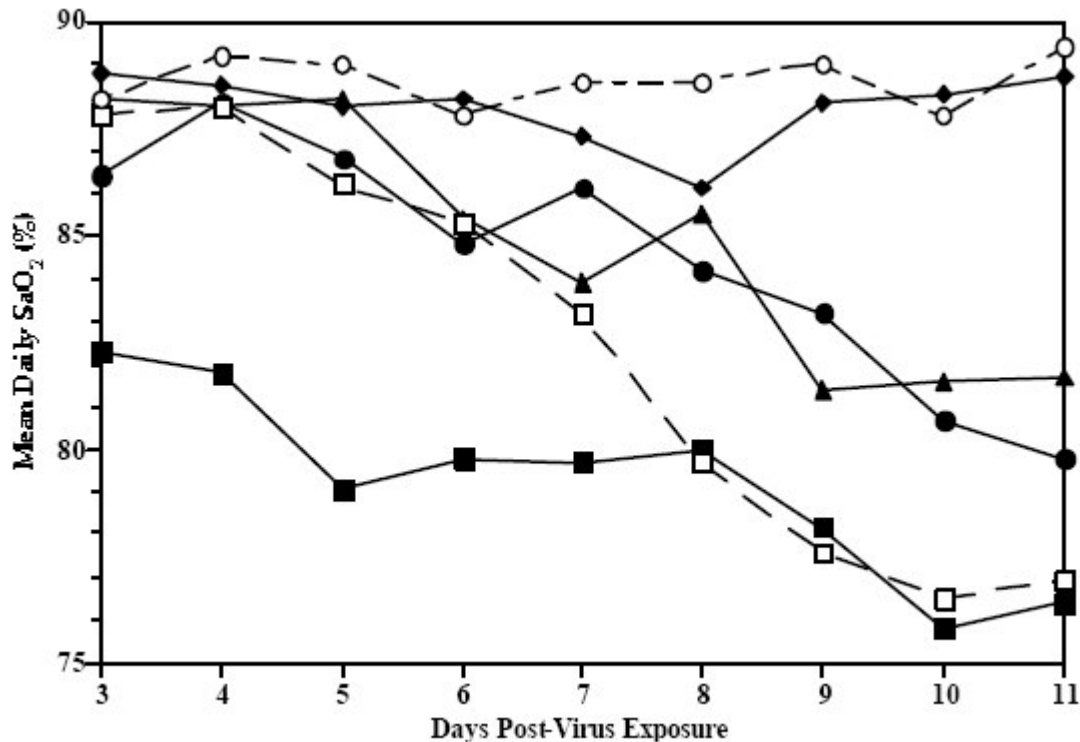


Fig. 1. Effect of intraperitoneal treatment with Virutase CA on arterial oxygen saturation in influenza A (Shangdong/09/93) (H3N2) virus-infected mice. Filled squares: 50 mg/kg/day Virutase CA; filled circles: 25; triangles: 12.5. Diamonds: 75 mg/kg/day Ribavirin. Squares: saline; circles: normal controls.

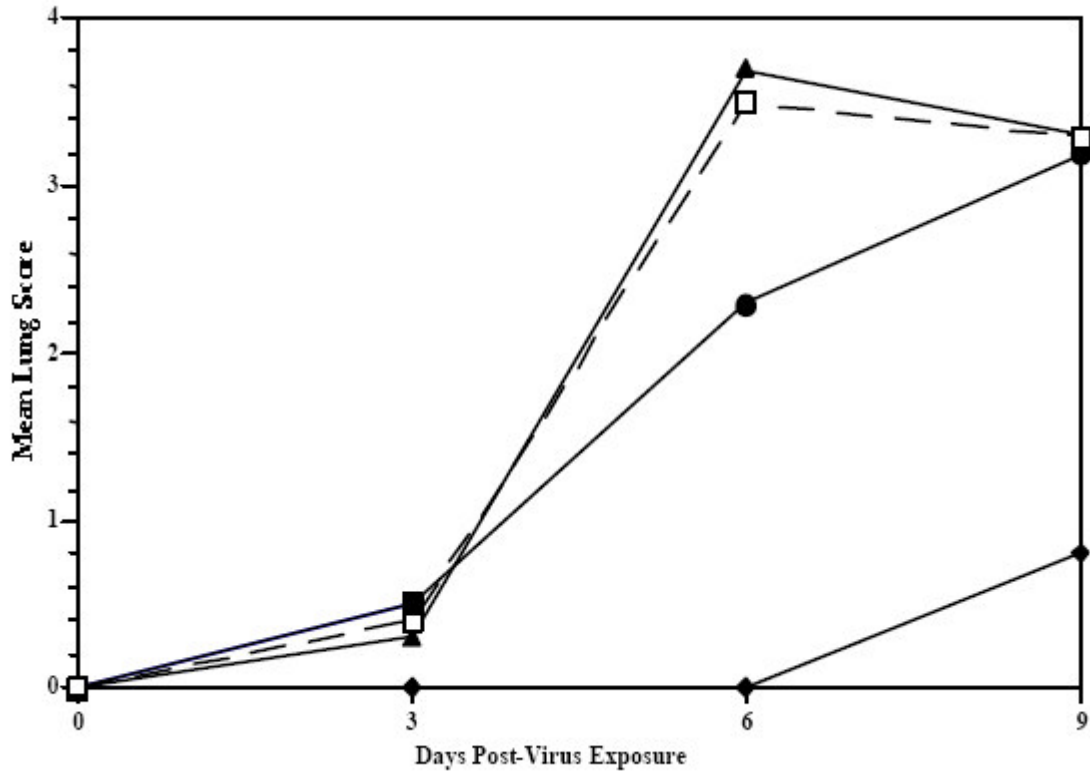


Fig. 2. Effect of intraperitoneal treatment with Virutase CA on mean lung scores in influenza A (Shangdong/09/93) (H3N2) virus-infected mice. Symbols as in Fig. 1.

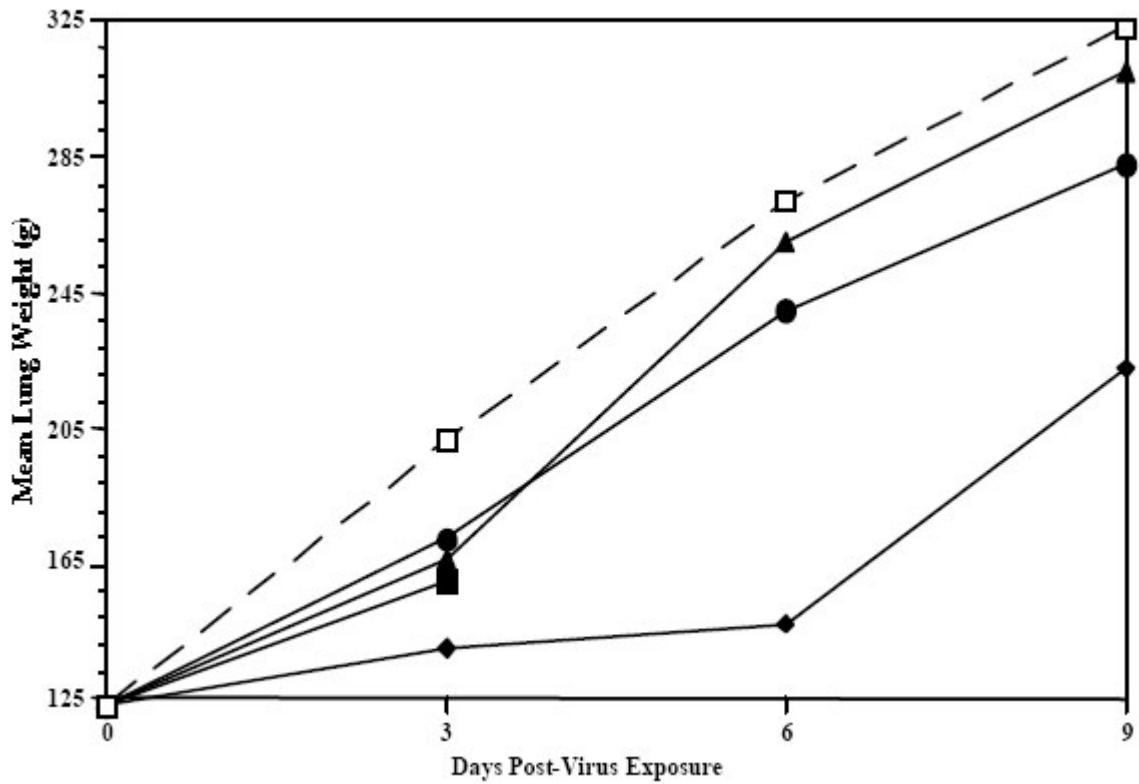


Fig. 3. Effect of intraperitoneal treatment with Virutase CA on mean lung weights in influenza A (Shangdong/09/93) (H3N2) virus-infected mice. Symbols as in Fig. 1.

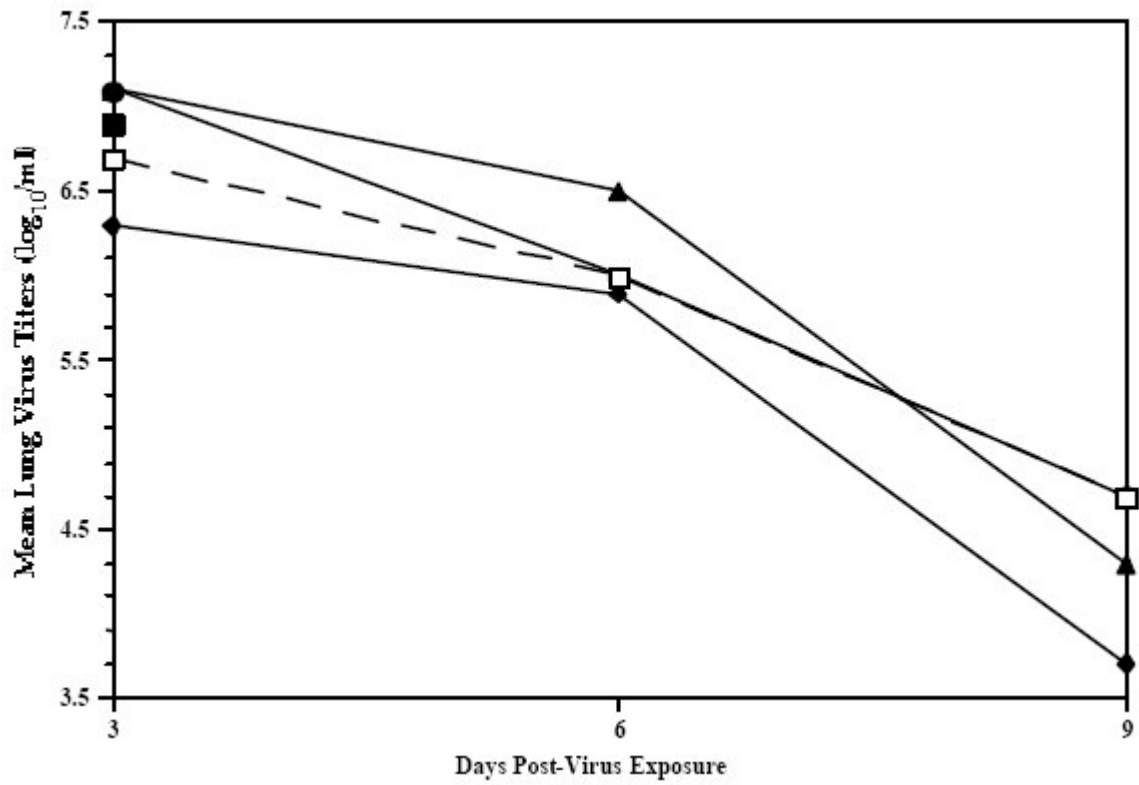


Fig. 4. Effect of intraperitoneal treatment with Virutase CA on mean lung virus titers in influenza A (Shangdong/09/93) (H3N2) virus-infected mice. Symbols as in Fig. 1.