

INFLUENZA VIRUSES

Influenza A/New Caledonia/20/99 (H1N1)

Influenza A/Panama/2007/99 (H3N2)

Influenza A/NWS/33 (H1N1)

Influenza A/PR/8/34 (H1N1)

Influenza A/Shangdong/09/93 (H3N2)

Influenza A/Sydney/05/97 (H3N2)

Influenza B/Beijing/184/93

Influenza B/Harbin/07/94

Influenza B/Hong Kong/5/72

Influenza Viruses

Methodology

Viruses and Cell Line Used in Primary Drug Screening. Influenza A and B were employed in this portion of the work. The virus strains were: A/New Caledonia/20/99(H1N1), A/Panama/2007/99(H3N2), A/NWS/33 (H1N1), A/PR/8/34 (H1N1), A/Shangdong/09/93(H3N2), and A/Sydney/05/97 (H3N2); and B/Beijing/184/93, B/Harbin/07/94, and B/Hong Kong/5/72. (All were tested in the presence of trypsin). The cell line was comprised of Madin Darby canine kidney (MDCK) cells.

Methods for Assay of Antiviral Activity.

Inhibition of Viral Cytopathic Effect (CPE). This test, run in 96-well flat-bottomed microplates, was used for the initial antiviral evaluation of all Virutase test compounds. In this CPE inhibition test, four log₁₀ dilutions of each drug (e.g. 1000, 100, 10, 1 µg/mL) were added to 3 cups, each containing a cell monolayer; within 5 min, the virus was then added and the plate sealed, incubated at 37°C, and the CPE read microscopically when untreated infected controls developed a 3 to 4+ CPE (approximately 72 to 120 h). A known positive control drug (Ribavirin; ICN Pharmaceuticals) was evaluated in parallel with Virutases in each test. Follow-up testing with compounds found active in initial screening tests were run in the same manner except 7 one-half log₁₀ dilutions of each compound were used in 4 cups, each containing a cell monolayer per dilution.

Increase in Neutral Red (NR) Dye Uptake. This test was run to validate the CPE inhibition seen in the initial test, and utilized the same 96-well micro plates after the CPE had been read. Neutral red was added to the medium; cells not damaged by virus take up a greater amount of dye. Color intensity was read on a computerized micro plate autoreader. The method described by McManus (*Appl. Environ. Microbiol.* **1976**, *31*, 35-38) was employed. The IC₅₀ was determined from this dye uptake.

Decrease in Virus Yield (VY). Compounds considered active by CPE inhibition and by NR dye uptake were re-tested using both CPE inhibition and, using the same plate, effect on reduction of virus yield by assaying frozen and thawed eluates from each cup for virus titer by serial dilution onto monolayers of susceptible cells. Development of CPE in these cells was the indication of presence of infectious virus. As in the initial tests, a known active drug was run in parallel as a positive control. The 90% effective concentration (IC₉₀), i.e., a testdrug concentration that inhibits virus yield by 1 log₁₀, was determined from these data.

Secondary Test. Following confirmation of significant antiviral activity in initial testing and in virus yield assays an additional study was performed, consisting of determination of the effect of time of addition of test compounds to virus-infected cells.

Reference Drug. Ribavirin was the reference compound employed in the influenza efficacy testing work.

Results

The efficacy data for all Virutases with the influenza viruses examined in this work are provided in the following tables. As shown, Virutases CA and HA were found to be quite effective against all three influenza viruses. In addition, the potency of Virutase CA exceeded that of Ribavirin in two of the three strains tested. In the time of addition studies (Table XVII), the most efficacious antiviral effect was observed when cells were pre-treated (at time 0) with Virutases, that is, the drugs appeared to prevent infection. In addition, activity was also present with post-infection treatment regimens (Ribavirin lost all its antiviral activity by 24 h). For example, at 100 mg/mL concentration of Virutases CA and HA in infected cells, discrete virus foci were seen that appeared like small plaques (particularly when the drugs were added 24 h after virus exposure). These results suggest that the compounds also inhibited virus adsorption even after the infection process had begun. (Mature influenza virus buds out of the host cell, then goes on to infect new cells during its life cycle. Since the cells were continuously exposed to the Virutase compounds, newly-formed virus exiting cells during the early rounds of virus replication would be blocked from attaching and entering uninfected cells to initiate new infections.)

Table VIII. Effective Inhibitory Concentrations at 50% (IC₅₀) and 90% (IC₉₀) of Virutase Drugs and Ribavirin Reference Compound with Influenza Virus Type A (New Caledonia/20/99) (H1N1) (MDCK Cells)

Virutase	IC ₅₀ , µg/mL			IC ₉₀ µg/mL
	CPE Method	NR Method	VY Method	
CA	1	0.6	3.2	4
CGA	45	40	–	–
HA	2.5	2.5	3.2	5
HGA	3.7	3.2	–	–
Ribavirin	0.55	0.38	0.32	1.4

Table IX. Effective Inhibitory Concentrations at 50% (IC₅₀) and 90% (IC₉₀) of Virutase Drugs and Ribavirin Reference Compound with Influenza Virus Type A (Panama/2007/99) (H3N2) (MDCK Cells)

Virutase	IC ₅₀ , µg/mL			IC ₉₀ µg/mL
	CPE Method	NR Method	VY Method	
CA	<1	<1	0.4	0.5
CGA	6	6.5	–	–
HA	<1	<1	0.22	0.4
HGA	4.5	3.2	–	–
Ribavirin	1.3	1.8	1.9	1.4

Table X. Effective Inhibitory Concentrations at 50% (IC₅₀) of Virutase Drugs and Ribavirin Reference Compound with Influenza Virus Type A (NWS/33) (H1N1) (MDCK Cells)

Virutase	IC ₅₀ , µg/mL		
	CPE Method	NR Method	VY Method
CA	0.65-1	0.55-0.85	–
CGA	–	–	–
HA	1.3	1.3	–
HGA	18	17	–
Ribavirin	5-6.0	4.6-6.5	–

Table XI. Effective Inhibitory Concentrations at 50% (IC₅₀) of Virutase Drugs and Ribavirin Reference Compound with Influenza Virus Type A (PR/8/34) (H1N1) (MDCK Cells)

Virutase	IC ₅₀ , µg/mL		
	CPE Method	NR Method	VY Method
CA	8.5	10	–
CGA	–	–	–
HA	14	18	–
HGA	18	18	–
Ribavirin	9	12	–

Table XII. Effective Inhibitory Concentrations at 50% (IC₅₀) of Virutase Drugs and Ribavirin Reference Compound with Influenza Virus Type A (Shangdong/09/93) (H3N2) (MDCK Cells)

Virutase	IC ₅₀ , µg/mL		
	CPE Method	NR Method	VY Method
CA	4.2-6	4-12	–
CGA	–	–	–
HA	15	18	–
HGA	13	13	–
Ribavirin	1.5-3.2	1.7-3.2	–

Table XIII. Effective Inhibitory Concentrations at 50% (IC₅₀) of Virutase Drugs and Ribavirin Reference Compound with Influenza Virus Type A (Sydney/05/97) (H3N2) (MDCK Cells)

Virutase	IC ₅₀ , µg/mL		
	CPE Method	NR Method	VY Method
CA	0.55	0.55	–
CGA	–	–	–
HA	0.35	0.55	–
HGA	4.2	9	–
Ribavirin	1	2	–

Table XIV. Effective Inhibitory Concentrations at 50% (IC₅₀) and 90% (IC₉₀) of Virutase Drugs and Ribavirin Reference Compound with Influenza Virus Type B (Beijing/184/93) (MDCK Cells)

Virutase	IC ₅₀ , µg/mL			IC ₉₀ µg/mL
	CPE Method	NR Method	VY Method	
CA	<1	<1	0.55	0.75
CGA	5.5	4.7	–	–
HA	<1	<1	0.5	2.5
HGA	3.2	3.2	–	–
Ribavirin	1	1.5	0.5	1

Table XV. Effective Inhibitory Concentrations at 50% (IC₅₀) of Virutase Drugs and Ribavirin Reference Compound with Influenza Virus Type B (Harbin/07/94) (MDCK Cells)

Virutase	IC ₅₀ , µg/mL		
	CPE Method	NR Method	VY Method
CA	1.3	0.85	–
CGA	–	–	–
HA	0.7	0.65	–
HGA	7	7	–
Ribavirin	0.85	1.1	–

Table XVI. Effective Inhibitory Concentrations at 50% (IC₅₀) of Virutase Drugs and Ribavirin Reference Compound with Influenza Virus Type B (Hong Kong/5/72) (MDCK Cells)

Virutase	IC ₅₀ , µg/mL		
	CPE Method	NR Method	VY Method
CA	3.2-23	4.2-19	–
CGA	–	–	–
HA	3.2	5	–
HGA	3.2	3.8	–
Ribavirin	1.2-1.8	1.8-1.8	–

Table XVII. Effect of Time of Addition on Efficacy of Virutase Drugs and Ribavirin Reference Compound against Influenza Virus Type A (New Caledonia/20/99) (H1N1) (MDCK Cells)

Time of Addition, h	IC ₅₀ , µg/mL Visual–Neutral Red Methods		
	CA	HA	Ribavirin
0	6.5–8	5.5–5.5	7.5–6
1	12–15	14–15	6–5.5
2	18–18	16–17	7–8
4	18–18	10–10	7–7
8	16–17	14–14	9–12
24	22–25	48–55	>100–>100