

HERPES VIRUSES

Herpes Simplex Virus Type 1 (HSV-1)

Herpes Simplex Virus Type 2 (HSV-2)

Epstein-Barr Virus (EBV)

Human Cytomegalovirus (HCMV)

Varicella Zoster Virus (VZV)

Methodology

Preparation of Human Foreskin Fibroblast (HFF) Cells. Newborn human foreskins were obtained as soon as possible after circumcision and placed in minimal essential medium (MEM) containing vancomycin, fungizone, penicillin, and gentamicin, at the usual concentrations, for 4 h. The medium was then removed, the foreskin minced into small pieces and washed repeatedly with phosphate buffered saline (PBS) deficient in calcium and magnesium (PD) until red cells were no longer present. The tissue was then trypsinized using trypsin at 0.25% with continuous stirring for 15 min at 37°C in a CO₂ incubator. At the end of each 15-min period the tissue was allowed to settle to the bottom of the flask. The supernatant containing cells was poured through sterile cheesecloth into a flask containing MEM and 10% fetal bovine serum. The flask containing the medium was kept on ice throughout the trypsinizing procedure. After each addition of cells, the cheesecloth was washed with a small amount of MEM containing serum. Fresh trypsin was added each time to the foreskin pieces and the procedure was repeated until all the tissue was digested. The medium was then centrifuged at 1000 rpm at 4°C for 10 min. The supernatant liquid was discarded and the cells resuspended in a small amount of MEM with 10% FBS. The cells were then placed in an appropriate number of 25-mL tissue culture flasks. As cells became confluent and needed trypsinization, they were expanded into larger flasks. The cells were kept on vancomycin and fungizone to passage four, and maintained on penicillin and gentamicin.

Cytopathic Effect Inhibition Assay (CPE) for Herpes Simplex Viruses (HSV), Human Cytomegalovirus (HCMV), and Varicella Zoster Virus (VZV). Low-passage HFF cells were seeded into 96-well tissue culture plates 24 h prior to use at a cell concentration of 2.5×10^5 cells per mL in 0.1 mL of MEM supplemented with 10% FBS. The cells were then incubated for 24 h at 37°C in a CO₂ incubator. After incubation, the medium was removed and 125 µL of experimental drug was added to the first row in triplicate wells, all other wells containing 100 µL of medium. The drug in the first row of wells was then diluted serially 1:5 throughout the remaining wells by transferring 25 µL using the Cetus Liquid Handling Machine. After dilution of drug, 100 µL of the appropriate virus concentration was added to each well excluding cell control wells, which received 100 µL of MEM. For HSV-1 and HSV-2 assays, the virus concentration utilized was 1000 PFU's per well. For CMV and VZV assays, the virus concentration added was 2500 PFU per well. The plates were then incubated at 37°C in a CO₂ incubator for 3 days for HSV-1 and HSV-2, 10 days for VZV, or 14 days for CMV. After the incubation period, the medium was aspirated and the cells stained with a 0.1% crystal violet solution for 4 h. The stain was then removed and the plates rinsed using tap water until all excess stain was removed. The plates were allowed to dry for 24 h and then read on a BioTek Plate Reader at 620 nm.

Efficacy Assay for Epstein-Barr Virus (EBV).

Viruses. There are two prototypes of infectious EBV. One is exemplified by the virus derived from supernatant fluids of the P3HR-1 cell line. This cell line produces nontransforming virus that induces the production of early antigen (EA) and viral capsid antigen (VCA) after primary infection or superinfection of B cell lines. The other prototype is exemplified by the B-95-8 virus. This virus immortalizes cord blood lymphocytes and induces tumors in marmosets. It does not, however, induce an abortive productive infection even in cell lines harboring EBV genome copies. The virus used in the assays of this work was P3HR-1. **Cell Lines.** Daudi is a low level producer that contains 152 EBV genome copies/cell. These cells respond to superinfection by EBV by expressing EA and VCA. This cell line was maintained in RPMI-1640 medium supplemented by 10% FBS, L-glutamine and 100 µg/mL gentamicin. The cultures were fed twice weekly and the cell concentration adjusted to 3×10^5 /mL. The cells were kept at 37°C in a humidified atmosphere with 5% CO₂.

ELISA Assay. Daudi cells were infected and treated with drug as described above. The cultures were incubated for 4 days at 37°C. The cells were counted, washed and brought to the desired final concentration. For each dilution of drug, cells were added to triplicate wells of a 96-well plate and air dried. The cells were then fixed for 20 min in an acetic acid/ethanol solution. A monoclonal antibody to EBV VCA was added and the cells were incubated for 1 h, followed by an incubation with horseradish peroxidase labeled goat antimouse IgG1 for 30 min. Plates were rinsed with PBS/Tween20 between incubations. Substrate containing Ophenylenediamine, citrate buffer and hydrogen peroxide was added to each well, and

the plates were covered and gently shaken for 10 min. The reaction was stopped by adding 3N sulfuric acid, following which the plates were read on a microplate reader at 492 nm.

Reference Drugs. Acyclovir (Glaxo SmithKline) was the reference compound employed in the HSV-1, HSV-2, VZV, and EBV efficacy testing work. Ganciclovir (Roche) was the reference drug used with HCMV.

Results

The efficacy data for all Virutases with the five herpes viruses examined in this work are provided in the following tables. As shown, Virutases CA and HGA were found to be effective against HSV-1 and HSV-2, and their efficacy approached that of Acyclovir. Virutase CA was somewhat effective against human cytomegalovirus, while Virutase HGA was equally so against varicella zoster virus. Virutase CA was very highly effective against Epstein-Barr virus. Table III. Effective Inhibitory Concentrations at 50% (IC₅₀) and 90% (IC₉₀) of Virutase Drugs and Acyclovir (ACV) Reference Compound with Herpes Simplex Virus Type 1 (HSV-1) (HFF Cells)

Table III. Effective Inhibitory Concentrations at 50% (IC₅₀) and 90% (IC₉₀) of Virutase Drugs and Acyclovir (ACV) Reference Compound with Herpes Simplex Virus Type 1 (HSV-1) (HFF Cells)

Virutase	IC ₅₀ , µg/mL	IC ₉₀ , µg/mL
CA	6	17.3
CGA	15.1	–
HA	4.7	13.1
HGA	16.9	51.6
Acyclovir	1.2–1.6	7.9

Table IV. Effective Inhibitory Concentrations at 50% (IC₅₀) and 90% (IC₉₀) of Virutase Drugs and Acyclovir (ACV) Reference Compound with Herpes Simplex Virus Type 2 (HSV-2) (HFF Cells)

Virutase	IC ₅₀ , µg/mL	IC ₉₀ µg/mL
CA	6.2	–
CGA	4.4	–
HA	2.5	6.7
HGA	2.1	19.7
Acyclovir	1.1–1.3	9.5

Table V. Effective Inhibitory Concentrations at 50% (IC₅₀) and 90% (IC₉₀) of Virutase Drugs and Ganciclovir (GCV) Reference Compound with Human Cytomegalovirus (HCMV) (HFF Cells)

Virutase	IC ₅₀ , µg/mL	IC ₉₀ µg/mL
CA	28.2	42
CGA	81.4	>100
HA	32.3	47
HGA	42.6	61
Ganciclovir	0.3–0.76	0.6–1.3

Table VI. Effective Inhibitory Concentrations at 50% (IC₅₀) and 90% (IC₉₀) of Virutase Drugs and Acyclovir (ACV) Reference Compound with Varicella Zoster Virus (VZV) (HFF Cells)

Virutase	IC ₅₀ , μg/mL	IC ₉₀ μg/mL
CA	>100	>100
CGA	>100	>100
HA	53.5	85.8
HGA	24	47.2
Acyclovir	0.23–0.38	16.3

Table VII. Effective Inhibitory Concentrations at 50% (IC₅₀) and 90% (IC₉₀) of Virutase Drugs and Acyclovir (ACV) Reference Compound with Epstein-Barr Virus (EBV) (Daudi Cells)

Virutase	IC ₅₀ , μg/mL	IC ₉₀ μg/mL
CA	>0.4	>0.4
CGA	21.1	33
HA	>50	>50
HGA	16.8	49
Acyclovir	1.8–2.4	16.3