

TOXICITY ASSAYS

HFF Cells MDCK Cells LLC-MK2 Cells

Methodology

The Neutral Red method of assaying for drug toxicity was carried out in roughly the same manner for all cell lines tested; that employed for human foreskin fibroblast (HFF) cells utilized in the herpes work is provided below as a representative example.

Twenty-four hours prior to assay, HFF cells were plated into 96-well plates at a concentration of 2.5×10^4 cells per well. After 24 h, the medium was aspirated and 125 μ L of drug was added to the first row of wells and then diluted serially 1:5 using the Cetus Liquid Handling System in a manner similar to that used in the CPE assay (see p. 7). After drug addition, the plates were incubated for seven days in a CO₂ incubator at 37°C. At this time the medium+drug was aspirated and 200 μ L/well of 0.01% neutral red in PBS was added. This was incubated in the CO₂ incubator for 1 h. The dye was aspirated and the cells were washed using a Nunc Plate Washer. After removing the PBS, 200 μ g/well of 50% EtOH/1% glacial acetic acid (in H₂O) was added. The plates were rotated for 15 min and the optical densities were read at 540 nm on a plate reader.

Visual observation was employed to confirm cell toxicity during the course of influenza and punta toro virus efficacy testing. Thus, during the cytopathic effect (CPE) inhibition tests, two additional wells of uninfected cells treated with each concentration of test compound were run in parallel with the infected, treated wells. At the time CPE was determined microscopically the toxicity control cells were also examined microscopically for any changes in cell appearance compared to normal control cells run in the same plate. These changes became manifest as enlargement, granularity, cells with ragged edges, a filmy appearance, rounding, detachment from the surface of the well, or other changes. The changes were given a designation of T (100% toxic), PVH (partially toxic-very heavy - 80%), PH (partially toxic-heavy - 60%), P (partially toxic - 40%), Ps (partially toxic-slight - 20%), or 0 (no toxicity - 0%), conforming to the degree of cytotoxicity seen. A 50% cytotoxic concentration (TC₅₀) was determined by regression analysis of these data.

Results

All Virutase drugs evaluated were not cytotoxic at levels at least as high as 100 mg/mL, as shown below in Table I. Visual observation of Virutases CA and HA with uninfected MDCK cells in toxicity control wells appeared initially to indicate drug toxicity. However, the drugs were not in fact toxic as revealed by Neutral Red assays. Rather, the Virutase compounds were found to bind to cell surfaces, thereby changing their color and giving them an exanimate appearance. This discoloration was observed in a concentration-dependent manner at levels where antiviral activity was present.

Table I. Toxic Concentrations at 50% (TC₅₀) of Virutase Drugs with Indicated Cell Lines

Virutase	TC ₅₀ , μ g/mL					
	BSC-1 ^a	HFF ^b	MDCK ^c	LLC-MK ₂ ^d		
				Trial 1	Trial 2a ^f	Trial 2b ^g
CA	>100	>100	>100	>100	>1000	>1000
CGA	>100	>100	- ^e	- ^e	>1000	>1000
HA	>100	>100	>100	>100	>1000	>1000
HGA	>100	>100	- ^e	- ^e	700	>1000

^a African green monkey kidney cells. ^b Human foreskin fibroblast cells. ^c Madin Darby canine kidney cells.

^d Adult rhesus monkey kidney cells. ^e Not evaluated. ^f Neutral Red assay. ^g Visual assay.