

Standard Operating Procedure for In Vitro Antiviral Screening
Institute for Antiviral Research, Utah State University
for the
Virology Branch
Division of Microbiology and Infectious Diseases
National Institute of Allergy and Infectious Diseases
National Institutes of Health

Assays for Antiviral Activity Against Respiratory and Biodefense Viruses

1. Viruses used in primary screen

A. *Adenovirus type 1*

Virus strain: 65089/Chicago (Source: M.F. Smaron 1/95 isolate)

Cell line: Human lung carcinoma (A549) cells.

B. *Dengue type 2 virus*

Virus strain: New Guinea C. Clinical isolate (Source: N. Karabatsos of the Division of Vector-Borne Diseases, CDC)

Cell line: African green monkey kidney (Vero) cells.

C. *Influenza types A and B viruses*

Virus strains: A/New Caledonia/20/99 (H1N1). Recent clinical isolate used in latest vaccine (Source: Center for Disease Control and Prevention [CDC]); A/Panama/2007/99 (H3N2). Recent clinical isolate used in latest vaccine (Source: CDC); B/Shanghai/361/02. Recent clinical isolate used in latest vaccine (Source: CDC); Influenza A/Vietnam/1203/04 (H5N1) x Ann Arbor/6/60. Provided by Dr. George Kemble of Medimmune Vaccines, Inc. The Ann Arbor portion of the latter virus contains the PB1, PB2, PA, NP, M and NS gene segments of cold-adapted A/Ann Arbor/6/60, the MedImmune FluMist master donor strain.0 The Vietnam portion has the H5 and N1 segments from that Asian virus. The virus is avirulent and consequently approved for use in our cell systems by federal agencies. A/NWS/33 (H1N1). A well-recognized laboratory strain (Source: K. W. Cochran, Univ. Michigan). (All are tested in the presence of trypsin, although in certain studies the A/NWS/33 virus is used without trypsin when requested)

Cell line: Madin Darby canine kidney (MDCK) cells.

D. *Measles virus*

Virus strain: Chicago. A 1989 clinical isolate (Source: J. Rota, CDC).

Cell line: African green monkey kidney (CV-1) cells.

E. *Parainfluenza type 3 virus*

Virus strain: 14702. Clinical isolate (Source: J. Boivin, Hosp. St. Justine, Montreal Canada, 5/95 isolate).

Cell line: African green monkey kidney (MA-104) cells.

F. *Tacaribe virus*

Virus strain: An 4763. Clinical isolate (Source: Dr. J. D. Gangemi, Univ. of South Carolina School of Medicine, Columbia, SC).

Cell line: African green monkey kidney (BS-C-1) cells

G. *Rift Valley fever virus*

Virus strain: MP-12. Vaccine strain provided by Dr. Robert Tesh, Univ. of Texas Medical Branch, Galveston, TX.

Cell line: Vero 76 cells

H. *Respiratory syncytial virus*

Virus strain: A2. A recognized laboratory strain which also infects cotton rats (Source: ATCC).

Cell line: African green monkey kidney (MA-104) cells.

I. *Rhinovirus type 2*

Virus strain: HGP. Isolated from throat washings of a patient with respiratory illness. (Source: ATCC).

Cell line: Human epidermoid carcinoma of the nasopharynx (KB) cells.

J. *Severe acute respiratory syndrome (SARS) virus*

Virus strain: 200300592. Clinical isolate. (Source: CDC)

Cell line: Vero 76 cells.

K. *Venezuelan equine encephalitis virus*

Virus strain: TC-83. Attenuated strain (Source: ATCC).

Cell line: Vero cells.

L. *West Nile virus*

Virus strain: New York. Isolated from a crow brain in New York (Source: R. Lanciotti, CDC)

Cell line: Vero cells.

M. *Yellow fever virus*

Virus strain: 17D. Attenuated human isolate (Source: ATCC).

Cell line: Vero cells.

2. Methods for assay of antiviral activity

A. *Standard Assay: Inhibition of Viral Cytopathic Effect (CPE)*

This test, run in 96 well flat-bottomed microplates, will be used for the initial antiviral evaluation of all new test compounds. In this CPE inhibition test, four log₁₀ dilutions of each test compound (e.g. 1000, 100, 10, 1 µg/ml) will be added to 3 cups containing the cell monolayer; within 5 min, the virus is then added and the plate sealed, incubated at 37°C and CPE read microscopically when untreated infected controls develop a 3 to 4+ CPE (approximately 72 to 120 hr). A known positive control drug is evaluated in parallel with test drugs in each test. This drug is ribavirin for influenza, measles, respiratory syncytial, parainfluenza, Tacaribe, Rift Valley and Venezuelan equine encephalitis viruses, cidofovir for adenovirus, pirodovir for rhinovirus, infergen for dengue and yellow fever viruses, and M128533, a protease inhibitor, for SARS virus. Follow-up testing with compounds found active in initial screening tests are run in the same manner except 8 one-half log₁₀ dilutions of each compound are used in 4 cups containing the cell monolayer per dilution. The data are expressed as 50% effective concentrations (EC50).

C. *Standard Assay: Increase in Neutral Red (NR) Dye Uptake*

This test is run to validate the CPE inhibition seen in the initial test, and utilizes the same 96-well micro plates after the CPE has been read. Neutral red is added to the medium;

cells not damaged by virus take up a greater amount of dye, which is read on a computerized micro plate autoreader. An EC50 is determined from this dye uptake.

D. *Decrease in Virus Yield Assay*

Compounds considered active by CPE inhibition and by NR dye uptake will be re-tested if additional, fresh material is available, 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 is the indication of presence of infectious virus. As in the initial tests, a known active drug is run in parallel as a positive control. The 90% effective concentration (EC90), which is that test drug concentration that inhibits virus yield by 1 log₁₀, is determined from these data.

3. Methods for assay of cytotoxicity

A. *Visual Observation*

In the CPE inhibition tests, two wells of uninfected cells treated with each concentration of test compound will be run in parallel with the infected, treated wells. At the time CPE is determined microscopically, the toxicity control cells will also be examined microscopically for any changes in cell appearance compared to normal control cells run in the same plate. These changes may be enlargement, granularity, cells with ragged edges, a filmy appearance, rounding, detachment from the surface of the well, or other changes. These changes are 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% cell inhibitory (cytotoxic) concentration (IC50) is determined by regression analysis of these data.

B. *Neutral Red Uptake*

In the neutral red dye uptake phase of the antiviral test described above, the two toxicity control wells also receive neutral red and the degree of color intensity is determined spectrophotometrically. A neutral red IC50 (NR IC50) is subsequently determined.

C. *Viable Cell Count*

Compounds considered to have significant antiviral activity in the initial CPE and NR tests are re-tested for their effects on cell growth. In this test, 96-well tissue culture plates are seeded with cells (sufficient to be approximately 20% confluent in the well) and exposed to varying concentrations of the test drug while the cells are dividing rapidly. The plates are then incubated in a CO₂ incubator at 37°C for 72 hr, at which time neutral red is added and the degree of color intensity indicating viable cell number is determined spectrophotometrically; an IC50 is determined by regression analysis.

4. Data analysis

Each test compound's antiviral activity is expressed as a selectivity index (SI), which is the IC50 or IC90 divided by the EC50. Generally, an SI of 10 or greater is indicative of positive antiviral activity, although other factors, such as a low SI for the positive control, are also taken into consideration.

5. Secondary Testing

Compounds having confirmed SI values of 10 or greater may, at the discretion of the Project Officer, be evaluated against additional strains of the original virus inhibited in order to more fully determined the spectrum of antiviral activity of the compound.

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Figure B-2A. Approach Used to Study Large Numbers of Compounds Against Respiratory and Biodefense Viruses.

The overall approach to this antiviral screening program for large numbers of compounds submitted at one time utilizes an abbreviated treatment protocol that is best represented by the following flow diagram.

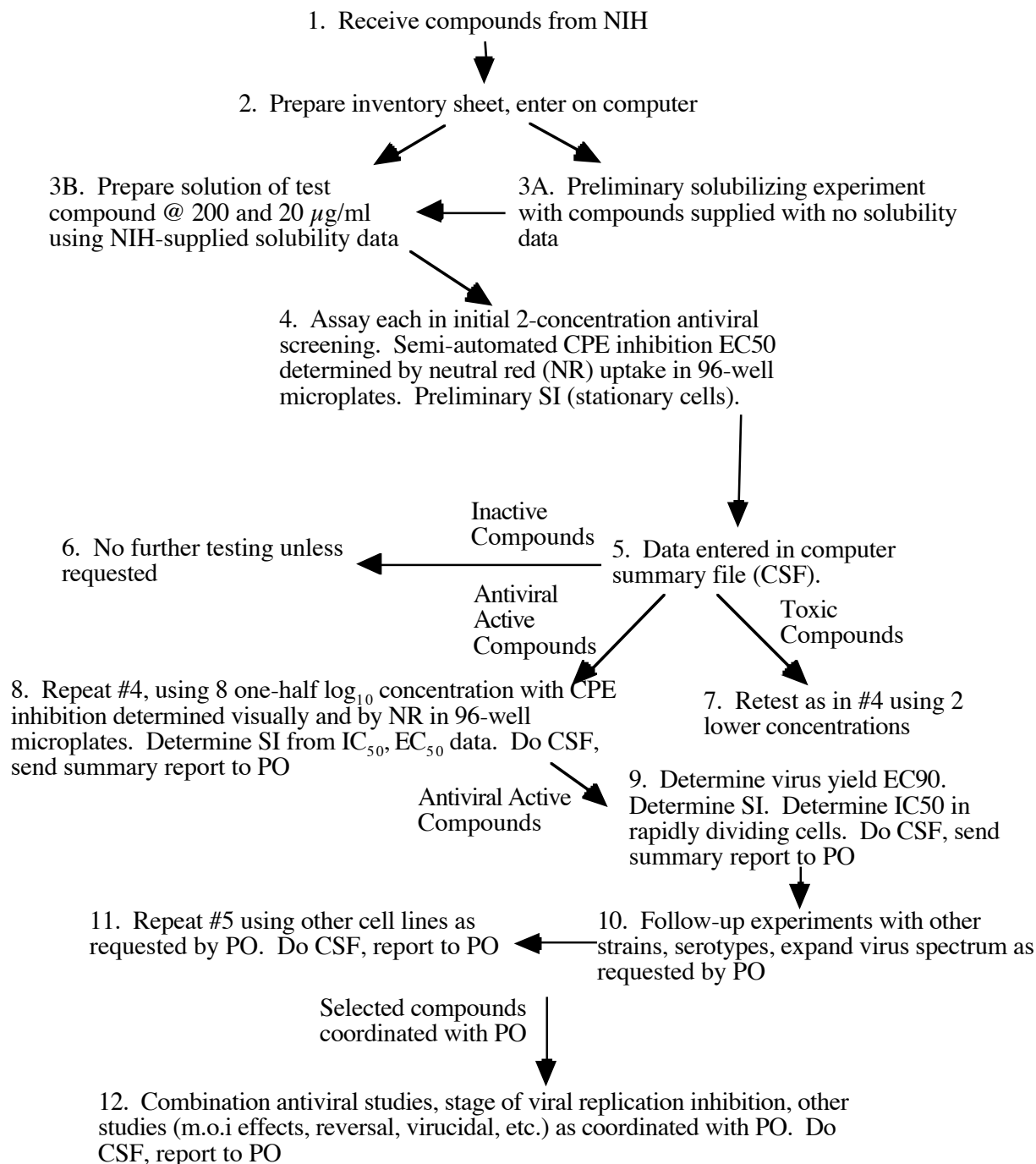


Figure B-2B. Approach Used to Study Small Numbers of Compounds Against Respiratory and Biodefense Viruses.

The following is the approach used when small numbers (< 10) of compounds are submitted for *in vitro* antiviral evaluation.

