USE OF THE MTT CELL VIABILITY ASSAY TO DETERMINE ANTIVIRAL ACTIVITY

THESIS

Presented to the Graduate Council of Southwest Texas State University in partial fulfillment of the requirements for the

Degree of Master of Science

By

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B.S. in Biology

May 2000
San Marcos, Tx.

ACKNOWLEDGMENT

The author wishes to express his gratitude to Dr. Gary M. Aron for his support and guidance throughout my undergraduate and graduate stay at Southwest Texas State University. Also, I would like to extend my appreciation to Dr. R.J.C. McLean and Dr. J. D. Irvin, whose help with my thesis has been valuable. Their instruction and insight has truly been a factor in completing my degree requirements and preparing me for a career in microbiology.

I would also like to thank my family, colleagues, and friends for supporting me throughout my education. I am truly blessed to have such wonderful people in my life.

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INTRODUCTION

1. NEWCASTLE DISEASE VIRUS

Newcastle disease virus (NDV) is a common avian pathogen, generally nonfatal, but results in a decrease in egg production and egg quality in chickens. NDV can infect more than 30 species of birds, along with more than 15 species of mammals, including humans. It first appeared in 1926 near Batavia, Java in Indonesia and also on a farm, in Newcastle-on-Tyne (17). Subsequent emergences in California and Europe occurred in the 1940s. Later, epidemics occurred throughout the 1950s and 1960s in America and overseas caused conditions known as *chicken flu*, *nine-day pneumonia*, and *pneumoencephalitis*.

1.1 STRUCTURE

NDV is a member of the Paramyxovirus family, possessing a non-segmented, negative stranded genome, which contains six genes covalently linked in tandem (5). These genes are denoted as: NP, a nucleocapsid protein; L, a lipoprotein; C and P, which are nucleocapsid associated proteins; Fo, a fusion protein; and M, a nonglycosolated envelope protein (17). The M protein may play a role in nucleocapsid and viral envelope recognition during virus assembly (17). The P gene encodes a viral polymerase. The virion consists of two structural modules:

the first is the internal ribonucleoprotein core containing the genome. The second is the outer spherical envelope consisting of lipoproteins derived from the plasma membrane of the host cell. This envelope consists contains two types of glycoprotein spikes: F which induces cell fusion and hemolysis, and HN which has hemagglutinating and neuraminidase activity, which allows the attachment of the virus to N-acetylneuraminic acid residues on the surface of erythrocytes. The F protein consists of two units, F_1 and F_2, which are disulfide-bonded polypeptide chains. Proteolytic cleavage of these chains is responsible for the biological activity of the F spike in membrane fusion. NDV is generally 150-200 nm long, 18 nm wide, having serrations every 4 nm with an internal opening approximately 2 nm in diameter (5).

1.2 REPLICATION

NDV attaches to permissive cell types via the neuraminic-acid containing receptors on the host cell by the HN glycoprotein on the virus membrane. This attachment is independent of temperature or pH, but, requires cations (18). After adsorption, the F_O protein is cleaved and fusion of the virion with the cell membrane is induced by the F glycoprotein. The virus uncoats and releases the genome into the cytoplasm, where viral transcription, genome replication, viral protein synthesis, and processing of progeny virions occurs (17). The viral RNA-dependent polymerase is activated and transcribes the viral RNA minus genome into RNA plus mRNA (17).

Viral particles are then assembled at the plasma membrane (18). Viral glycoproteins are inserted into the plasma membrane and gather into patches which exclude cellular proteins (5). It has been suggested that the M protein is the scaffold for formation of the prospective virus envelope and that glycoproteins are concentrated into the patches by the interaction between the cytoplasmic portion and the M protein (42). The nucleocapsid aligns underneath these patches in the plasma membrane. The progeny virus is the released by the process of budding, which eventually kills the host cell as the membrane lyses (37, 38). As the virus migrates through the cell membrane, it is coated by the cell membrane, which is coated with viral induced glycoproteins H and N (24).

1.3 EFFECT ON HOST CELL

Following infection by NDV, cell protein synthesis (CPS) gradually decreases in chicken embryo fibroblasts. By 5 hr post-infection, CPS has decreased to only 50% in infected cells, and by 9 hr, only 15% is observed (10) and viral RNA synthesis has peaked. With the increasing inhibition of CPS is a gradual transition from host-specified to virus specified polypeptide synthesis (10).

1.4 PROPHYLAXIS

Prophylaxis and treatment for NDV includes vaccination (7, 53) using both live and inactivated vaccines, eradication by slaughter, and quarantine of infected animals. No antivirals have been commercially developed for use against the

NDV virus, although studies have shown that pokeweed antiviral protein (PAP), ribavirin, and guanidine suppressed viral replication (68). Azauridine, a RNA synthesis inhibitor, along with puromycin, which inhibits host cell shutoff when added at infection, have both proved effective against NDV virus in tissue culture as well.

2. INFLUENZA A VIRUS

Influenza virus is a member of the Orthomyxovirus family, possessing a segmented genome containing eight separate negative-strand RNAs encoding 10 genes surrounded by an enveloped helical nucleocapsid (1, 5). There are two significant glycoproteins on the surface of the virus, hemagglutinin (HA) and neuramidase (NA), which are the major factors in the virulence of this pathogen. Influenza viruses are divided into types A, B, and C. There are three human subtypes for HA glycoprotein (H1, H2, and H3), and two human subtypes for NA (N1 and N2). In types A and B, the HA and NA antigens undergo genetic variation, whereas, type C is antigenically stable (5). Type A influenza viruses are responsible for the rare, severe pandemics that occur. Major changes in these glycoproteins (antigenic shifts) are responsible for the worldwide pandemics seen about every 70 years, with the last one being the outbreak of the Spanish Flu in 1918, which caused 20 million deaths worldwide and 500,000 in the U.S. Minor changes in the surface proteins lead to the yearly epidemics of influenza, which cause more than 20,000 deaths in North America annually.

Complications that arise from influenza infection are characterized as a febrile illness of the upper and lower respiratory tract, characterized by the sudden onset of fever, cough, myalgia, malaise, and other symptoms (5). Pneumonia is the most common serious complication (5). Until recently, only the synthetic drugs amantidine and rimantidine hydrochloride were approved to effectively prevent infection and illness caused by type A influenza strains. When administered early in the course of infection, these antivirals hastened the disappearance of fever along with other symptoms. This past year, the FDA approved the use of Relenza™ (zanamivir), a product created by the pharmaceutical company Glaxo Wellcome. It is approved to treat both A and B strains in children and adults, early in the infection period, within 2 days of symptoms. Applied as an inhalant twice a day for a five day period, it serves as a new approach in combating this disease (16).

2.1 STRUCTURE

Influenza virus is composed of an enveloped helical nucleocapsid which contains eight unique negative-stranded RNA molecules. Seven viral proteins (PB1, PB2, PA, HA,NA, NP, and M1) are transcribed from a complete genome plus three proteins only found in infected cells (M2, NS1, and NS2) (17). The polymerase proteins, PB1, PB2, and PA, are associated with nuclear protein (NP) and viral RNA (18).

2.2 REPLICATION

Influenza A virus binds to sialic acid residues on permissive cells via the hemagglutin glycoproteins on the viral envelope. The virion is endocytosed into clathrin-coated pits and forms vesicles within the cytoplasm (5). The M2 protein regulates the low pH of the endosome which induces fusion of the viral envelope with the vesicle membrane (12). This fusion event is caused by a structural change in the hemagglutinin, causing the hemagglutinin to be cleaved into two disulfide bonded proteins (HA1 and HA2) (17). Hydrophobic interactions caused by HA2 cause the fusion of the viral envelope with the cell membrane and subsequent release of the nucleocapsid into the cytoplasm. The transmembrane protein (M2) forms an ion channel to release the genome from the vesicle in order to enter the nucleus of the cell. The protein M2 is a homotetramer (60 kDa) composed of two 30 kDa dimers stabilized by cysteine-linked disulfide bonds (17). This homotetramer forms a channel which permits the flow of ions from the endosome to the interior of the virion. In the nucleus, the negative-stranded RNA molecules are translated by virion-associated RNA polymerase to form positive-stranded mRNA to serve as template for viral proteins and genomes (5). This requires a 5' terminal region of cell messages as a primer to initiate transcription of the negative-stranded genome segments into mRNAs (18). The virus scavenges cap sequences from nascent mRNA generated in the nucleus by transcription of the host DNA and attaches them to its own viral mRNA (17). These cap sequences allow the viral mRNA to be transported to the cytoplasm, where it is translated by host ribosomes. Seven viral proteins (PB1, PB2, PA,

HA,NA,NP, and M1) are transcribed from a complete genome. Three proteins are found in infected cells (M2, NS1, and NS2) (17, 54). The polymerase proteins, PB1, PB2, and PA, are associated with nuclear protein (NP) and viral RNA (4, 29). PB1 initiates transcription and PB2 acts as a cap-binding protein. The NP protein is associated with all three polymerase proteins and viral RNA and is the major constituent of the nucleocapsid. The matrix proteins, M1 and M2, serve as binding sites for ribonucleoprotein (RNP) segments during virus maturation and associate with new RNPs within the nucleus of the cell (34). RNPs consist of eight RNA segments plus the nucleoprotein (NP) and the three viral-polymerase proteins (PB1,PB2, and PA) (9). Newly synthesized RNPs are escorted through nuclear pores into the cytoplasm by NS2 protein (47).

Following virus infection, primary viral transcription results in the synthesis of the eight mRNA segments at equal rates. Secondary transcription seems to be under temporal control and occurs in both the early phase and late phase. The early phase involves an increase of selective transcription of NP and NS1 mRNAs (43). The late phase involves an increase in the transcription of M1, HA, and NA mRNAs. The synthesis of progeny RNA has been shown to be coupled with the synthesis of viral mRNAs (21).

Viral assembly occurs after replication of progeny RNA and viral protein synthesis and required the presence of the matrix proteins M1 and M2 (5, 64). Once assembled in the nucleus, the nucleocapsid is transported through the trans-golgi apparatus where viral hemagglutinin (57), neuraminidase, and M2 have been inserted. As the nucleocapsid buds from the host cell, an envelope is

obtained from the cell membrane (22, 41). The release of the progeny virus is aided by neuraminidase, which removes neuraminic acid from the surface of new virions and prevents self-agglutination (19, 36).

2.3 EFFECT ON HOST CELL

In infected cells, a "shutoff phenomenon" is observed as cellular protein synthesis (CPS) decreases while viral protein synthesis increases. It has been suggested that either virus-mediated degradation of existing cellular mRNA (27) or a virus specific system the preferentially translates viral mRNAs (31). In eucaryotic infected cells, the "shut-off" of cellular protein synthesis is through the action of eIF2 kinases. It has been shown that influenza A virus is able to prevent the shutdown of all protein synthesis by utilizing a gene product with blocks the phosphorylation of protein kinase P68, which is responsible for phosphorylation of eIF2. Thus, the virus prevents the shutdown of overall protein synthesis, with specificity for viral gene products over host cell proteins.

3. NDV VERSUS INFLUENZA A VIRUS

Both NDV and Influenza A virus have many common characteristics, such as single-stranded RNA genomes and negative polarity (17). Also, both possess helical nucleocapsids, lipid-containing envelopes, and RNA-dependent RNA polymerase (5, 17). But, there are differences between these virions as well. Cell entry is accomplished through fusion with the cell membrane for NDV, whereas

influenza infects via endosomal membrane fusion (5). NDV has a non-segmented genome as opposed to segmented in influenza and their sites of replication differ as well. NDV replication occurs in the cytoplasm, in contrast to the nucleus for influenza.

4. ANTIVIRAL AGENTS

4.1 POKEWEED ANTIVIRAL PROTEIN

Pokeweed antiviral protein (PAP) is a single-chain ribosome-inactivating protein (RIP) that has been proven effective against a wide variety of viruses (65), such as NDV (68), human immunodeficiency virus (51), poliovirus (39), and herpesvirus (3). The 29 k-Da protein is isolated from the American pokeweed plant, *Phytolacca americana* (2). One established mode of action for PAP's antiviral activity is the removal of a single adenine residue from a highly conserved "α-sarcin/ricin (SR)" loop of 28s rRNA in eucaryoktes (25, 49). This elimination results in an irreversible inhibition of protein synthesis during the translocation step, which impairs both the elongation factor (EF)-1-dependent binding of aminoacyl-tRNA and the GTP-dependent binding of EF-2 to the affected ribosome (28, 51). Recent evidence has shown that PAP's antiviral activity is not restricted to the depurination of adenine residues, but PAP is also capable of deguanylating ribosomal and viral RNA (50). A guanine base was shown to be able to fit into the active site pocket of PAP without disturbing its geometry, very much like an adenine base (35). Furthermore, it has been

suggested that PAP and other RIPs might have antiviral effects other than inhibition of host protein synthesis by ribosome inactivation, with possible mechanisms such as depurinating the nucleic acid of the invading pathogens (67).

4.2 RIBAVIRIN

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboximide) is a nucleoside analog which has shown effectiveness against 85% of all virus, including influenza (14, 55), Borna disease virus (30, 44), poliovirus, hepatitis B/C viruses (26), and many others. Two general modes of action occur with ribavirin treatment, a direct reduction in levels of circulating virus, and the promotion of T-cell mediated immunity against viral infection (62). When ribavirin enters the cell, it is phosphorylated by cellular enzymes to form monophosphate, diphosphate, and triphosphate metabolites (56). Ribavirin has been shown to inhibit the production of guanosine triphosphate (GTP) (56). Also, GTP levels are reduced by the competative inhibition of inosine monophosphate (IMP) dehydrogenase conversion to xanthosine monophosphate (XMP) by ribavirin-5'-monophosphate (11, 56). Ribavirin triphosphate inhibits 5'-capping of mRNAs in influenza virus, by interference with guanyl transferase and viral N^7-methyl-transferase.

4.3 GUANIDINE HYDROCHLORIDE

Guanidine hydrochloride has been proven to be an effective antiviral agent against picornaviruses, particularly poliovirus (6, 15, 46). At concentrations of 5 M or higher, guanidine is a protein denaturant, and also inhibits cellular RNA and protein synthesis as well. But, at much less concentration, below 2 μM, guanidine is able to inhibit poliovirus replication. Although studied extensively, the antiviral mechanism for guanidine is still largely unknown. There are four proposed mechanisms: 1) rapid inhibition of the initiation of RNA synthesis 2) interference with newly synthesized membranes from their place of formation (the rough endoplasmic reticulum) to smooth viral specific vesicles 3) inhibition of the release of membrane dependent plus-stranded 35S RNA from the replication complex 4) prevention of the association of procapsids and the poliovirus protein 2C with the replication complex (32).

5. MTT CELL VIABILITY ASSAY

The search for antiviral substances has employed a number of different techniques for the evaluation of antiviral activity. The hemagglutination assay has been used as an indirect method to quantify viral particles as determined by the aggregation of red blood cells by those viruses that exhibit surface hemagglutinin glycoproteins (68). The MTT cell viability assay measures the reduction of the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a blue formazan product by active mitochondrial dehydrogenases in

living cells [Fig.A].

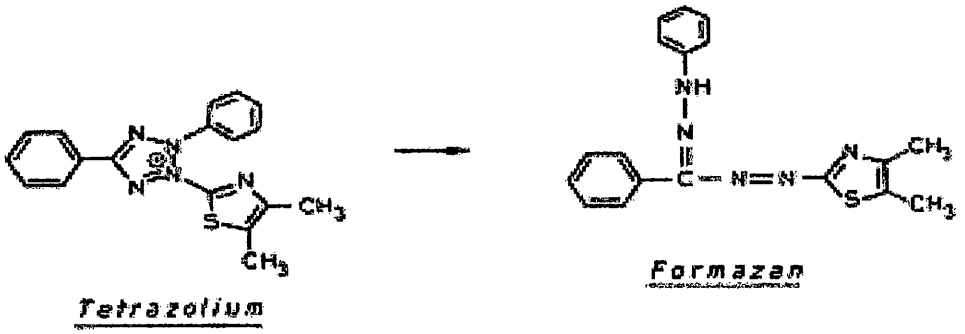

Fig. A – Structural formulae of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and formazan (48)

The MTT assay has been used as an alternative to the trypan-blue exclusion method for the determination of viable cell counts in cytotoxicity studies on antiviral compounds. Recently, the MTT assay has been employed to measure antiviral activity for screening of potential antiviral compounds against such viruses as human immunodeficiency virus (HIV) (8, 48), adenovirus (33), influenza virus (55), and herpes simplex virus type1 (59, 61). The MTT assay has also been utilized in antifungal susceptibility testing for yeasts (13), and detection of rifampin-resistance in vitro-cultured *Mycobacterium tuberculosis* (45).

Studies that utilize the MTT cell viability assay for the determination of antiviral activity employ a tissue culture infective dose of 50% ($TCID_{50}$), which ensures that only half of the susceptible cells are infected. Thus, if the potential antiviral compound prevents the replication of the virus and subsequent passage of progeny virions to neighboring uninfected cells, one would expect a 50% reduction of tetrazolium. On the other hand, if the compound was ineffective as an antiviral, one would expect no reduction of tetrazolium.

In this study a modification of the MTT assay will be used to determine if known antiviral agents act early during the viral replication cycle, prior to viral-induced host cell shutdown, or late in the cycle, following viral-induced host-cell shutdown. The replication cycle for most include: 1] attachment of virus to specific cell receptors on the host cell membrane, 2] penetration of the virus by either endocytosis into cytoplasmic vacuoles or fusion of cell membrane and virion envelope, 3] uncoating of the virion, 4] expression of viral genes, 5] replication of the viral genome, and 7] the assembly and maturation of viral progeny (5). During this cycle, the phenomenon known as viral-induced "host-cell shutdown" occurs as the host cell's replication machinery, key regulatory molecules, and precursors for newly synthesized viral proteins and nucleic acids have been exhausted in producing viral progeny. As a result, programmed cell death (apoptosis) occurs, which coincides with the release of viral progeny.

In this study the investigator plans to use the MTT assay to demonstrate if known antivirals act prior to or following viral-induced host cell shutdown by simply increasing the infective dose to 100% ($TCID_{100}$). Thus, if an antiviral acts prior to viral-induced host cell shutdown, then one would expect a 100% reduction of tetrazolium since none of the cells will be killed and possess active mitochodrial dehydrogenases. If the antiviral acts after viral-induced host cell shutdown, then one would expect zero reduction of tetrazolium since none of the cells would have active mitochodrial dehydrogenases.

Knowledge of the time of action of antiviral activity can be helpful to the virologist in combating viral disease. Determining the time of action of antiviral

activity during the viral replication cycle will help elucidate the antiviral target such as uncoating (early) vs. biosynthesis (late). Knowledge of the target can also be useful in the choice of antivirals for combined therapy of viral disease to achieve synergy, reduced toxicity, and reduced emergence of drug-resistant mutants (17).

MATERIALS AND METHODS

Cell culture. Human cervical carcinoma (HeLa) cells (University of Texas Health Science Center) and primary chicken embryo cells (CEC) from10-12 day old chicken eggs (Ideal Hatchery, Cameron, TX) were grown in 75 cm^2 flasks (Sarstedt, Newton,NC) at 37 °C under 5% CO_2 in Eagle's minimum essential medium (E-MEM) (Sigma- Aldrich Chemical Co., St. Louis, MO) which contained 10% fetal bovine serum(FBS) (Summit Biotechnology, Ft. Collins, CO), glutamine (2 M/ml),1.0% nonessential amino acids, 0.075% $NaHCO_3$, penicillin (100 U/ml), and streptomycin (100 µl/ml) (Sigma-Aldrich Chemical Co., St. Louis, MO). Cells were maintained in E-MEM which contained 5% FBS (Summit Biotechnology, Ft. Collins, CO), glutamine (2 mM/ml), 0.150% NaHCO3, penicillin (100 U/ml), streptomycin (100 µl/ml), 5.0 % tryptose phosphate broth, and 1.0% nonessential amino acids (Sigma-Aldrich Chemical Co., St. Louis, MO). Passage of the cells occurred approximately once every 3 days using 5.0 ml trypsin (2.5 mg/ml) (Sigma-Aldrich Chemical Co., St. Louis, MO) for 5 min to dissociate the cellular monolayer. The cell suspension was then washed with Earle's Balanced Salt Solution (EBSS) (Sigma Aldrich Chemical Co., St. Louis, MO) and centrifuged at 800 rpm for 5 min. The supernatant was discarded and the cell pellet re-suspended in E-MEM maintenance medium. 96-well microtiter assay plates (Baxter, McGaw Park, IL) were then seeded with 100 µl of either HeLa or CEC cell (2-6 X 10^7 cells/well) using an octapippette and incubated for 24 h at 37°C under 5% CO_2 for use in the cytotoxicity and antiviral studies.

Cell storage. HeLa and CEC cells were frozen in solution which contained 15-20% dimethyl sulfoxide (DMSO) and 80-85% FBS. Cells were suspended in the freezing medium and placed in cryogenic vials (Nalgene Company, New York.) in 1.8 ml aliquots and placed directly into liquid nitrogen (-196°C).

Virus production. Newcastle Disease virus strain NDV VR-109 (American Type Culture Collection, Rockville, MD) and Influenza Virus A/PR/8/34 (American Type Culture Collection, Rockville, MD) were grown in the chorioallantoic fluid of 9-10 day old embryonated chicken eggs (Ideal Hatchery, Cameron, TX) for 48 h at 37°C. Harvested chorioallantoic fluid and cell extracts were stored at -80°C and contained approximately 1.8×10^{10} and 3.2×10^{9} hemagglutination units (HAU) per ml for NDV and influenza, respectively.

Hemagglutination Assays. Chorioallantoic fluid and infected-cell extracts were diluted two-fold using phosphate buffered saline in 96-well U bottom microtiter plates (Dynatech Laboratories, Chantilly, VA.). A 0.2%- 0.5% solution of chicken red blood cells (M.D. Anderson Cancer Research Center, University of Texas, Bastrop, TX) which contained approximately $1.6\text{-}4 \times 10^{7}$ cells per ml was added in 0.1 ml aliquots to each well. Chicken red blood cells were washed 1X with EBSS and diluted in a phosphate-buffered saline (PBS) solution pH 7.4 (5.6 g disodium phosphate anhydrous, 2.7 g of potassium dihydrogen phosphate, 4.1 g of sodium chloride, and 1000 ml of purified distilled water or a PBS tablet added to 200 ml of purified distilled water) (Sigma-Aldrich

Chemical Co., St. Louis, MO). Hemagglutination was determined following 45-60 min incubation at 37 or 39°C for Influenza and NDV infected cells, respectively.

Antivirals. Pokeweed Antiviral Protein (PAP) was generously donated by Dr. James D. Irvin (Southwest Texas State University, San Marcos, TX.). PAP was extracted from the spring leaves of the plant *Phytolacca americana*, purified by ammonium sulfate fractionalization followed by ion exchange chromatography. The extract was filtered through a 0.45 μM membrane filter (Type HA, Millipore Corp. Bedford, MA.), neutralized to a pH of 7.2, and stored at -20°C. The molarity of a 1:10 dilution PAP in deionized water was determined by spectroscopy using a wavelength of 280 nm. The absorbance was multiplied by ten and divided by 0.83, the extinction coefficient for PAP, which yielded mg/ml of PAP protein, when divided by 2.9 yielded micromoles (μM) PAP. Guanidine HCl and ribavirin were obtained from Sigma-Aldrich Chemical Co (St. Louis, MO) and ICN Pharmaceuticals, Inc. (Costa Mesa, CA). All antivirals were diluted in E-MEM growth medium and stored in 1.0 ml aliquots at -20°C.

Cytotoxicity. HeLa cells and CEC cell monolayers were grown in 96 well plates for 24 h. Cell monolayers were washed once with EBSS and 100μl of E-MEM growth medium was added to all monolayers. To the first well in each row, 100 μl of the antiviral solution was added and two-fold serial dilutions of the antiviral were performed. Cells were incubated for 24 h under 5% CO_2 at 37°C and 39°C for HeLa and CEC, respectively. Cell viability was determined using the MTT method described by Pauwel et al. (1988). Briefly, 24 h post antiviral treatment, 25 μl of 200 μg/ml of 3(4,5-

dimethyl-thiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) diluted in PBS (Sigma-Aldrich Chemical Co., St. Louis, MO) was added to the monolayers. After incubation in the presence of MTT for 2 hr, 100 µl of acidified isopropanol (0.01 % conc. HCl) with 10% Triton-X 100 (Eastman Kodak Company, Rochester, New York) was added to each monolayer for 20 min. Optical density was determined at a wavelength of 570 nm with a background subtraction of 650 nm using a Biotek EL311 microplate reader. Cytotoxic concentration (CCx) was determined to be the dose at which the absorbency was reduced by (x) percentage.

Effect of antivirals *in vitro*. HeLa or CEC cell monolayers were grown in 24 well microtiter plates (Corning Glass Works, Corning, NY) using E-MEM growth medium. The monolayers were washed once with EBSS, and 1.0×10^9 or 2.8×10^9 HAU of NDV or Influenza A, respectively, was added yielding a MOI of approximately 770 and 2300 HAU per cell together with antiviral. Antivirals diluted in E-MEM which contained 10% FBS (Summit Biotechnology, Ft. Collins, CO), glutamine (2 mM/ml), 0.075% NaHCO3, penicillin (100 U/ml), streptomycin (100 µl/ml), and 1.0% nonessential amino acids (Sigma-Aldrich Chemical Co., St. Louis, MO) plus 5.0% tryptose phosphate broth for the CEC cells. Solutions containing two-fold concentrations of each antiviral were mixed with equal volumes of solutions that contained two-fold concentrations of NDV or influenza. Virus was allowed to adsorb for 1 h at 37°C in 5% CO_2 with shaking every 10 minutes in the presence or absence of antivirals. The infected cell monolayers were washed once with EBSS to remove unadsorbed virus. Medium which contained antiviral was added back to the infected

cells. Monolayers were harvested following 24h incubation at 37°C for influenza and 39°C for NDV. The effect of each antiviral upon viral replication for influenza or NDV-infected HeLa and CEC cells was calculated as percent inhibition of hemagglutinating particles (Hap). Effective concentrations for each antiviral were determined that inhibited 25%, 50%, and 75% of viral replication, or ED^{25}, ED^{50}, and ED^{75}, respectively.

MTT antiviral assay / High MOI. Effective doses (ED_{25}, ED_{50}, and ED_{75}) of antivirals that inhibited replication of NDV and Influenza A virus as determined by hemagglutination assay were used to determine if their antiviral action occurred before of after viral-induced host cell cytotoxicity. HeLa or CEC cells were grown as confluent monolayers in 96 well microtiter plates using E-MEM growth medium. The monolayers (2-3 x 10^3 cells/well) were washed once with EBSS and 0.1 ml solutions containing each antiviral diluted in E-MEM was added to the first well monolayer and the antiviral serially diluted two fold in successive monolayers to create an experimental test set (Tx). Two sets of monolayers served as viral (Vx) and cell controls (Cx). The original concentration of antiviral was prepared at a concentration to allow the full range of effective doses (ED_{25}, ED_{50}, and ED_{75}) for each antiviral to be tested in these studies. Next, either NDV or influenza was diluted in E-MEM at concentrations of 2.25 x 106 HAU/ml and 3 x 106 HAU/ml respectively, were added to all wells except those in the cell controls (Cx), yielding a multiplicity of infection (MOI) of 750-1000 virions/cell. After 1 h incubation in 5% CO_2 with shaking every 10 min at 37°C or 39°C for influenza and NDV, respectively, the infected monolayers were washed gently with EBSS to remove unadsorbed virus. A solution containing the same concentration of each antiviral

was then added to the infected monolayers. After 24-48 h incubation, the viral controls (Vx) were observed microscopically for cytopathic effects. The cells were washed once with EBSS and the MTT assay was performed as previously described. The optical density (OD) was used to calculate percent viable cells (%CV) and percent protection (%P), according to the following equations:

$$\% \text{ Cell Viability} = (Tx / Cx) \times 100 = \%CV$$

$$\% \text{ Protection} = \frac{(Tx - Vx)}{(Cx - Vx)} \times 100 = \% PV$$

Tx = absorbance (optical density) of test sample
Vx = absorbance of virus-infected control
Cx = absorbance of cell control

RESULTS

Effect of antivirals on NDV replication in HeLa and CEC cells. The effective concentrations (ED^x) of each antiviral required to inhibit NDV replication in HeLa or CEC cells by 25, 50, and 75 percent was determined by inhibition of hemagglutinating particles (HAp). Antiviral activity of PAP, guanidine, and ribavirin was nonlinear and followed first order kinetics as indicated in their dose-response curves (Fig.1-3). PAP inhibited NDV replication in HeLa cells with an ED^{25}, ED^{50}, and ED^{75} of 0.03, 0.156, and 1.0 μM, respectively (Table 1). For NDV-infected CEC cells, PAP inhibited virus replication with an ED^{25}, ED^{50}, and ED^{75} of 0.015, 0.025, and 0.0625 μM, respectively (Table 1). The antiviral activity of guanidine was calculated with an ED^{25}, ED^{50}, and ED^{75} of 100, 150, and 1500 μg/ml, respectively, for NDV-infected CEC cells. In NDV-infected HeLa cells, guanidine produced an ED^{25}, ED^{50}, and ED^{75} of 50, 312, and 1300 μg/ml, respectively (Table 2). The antiviral activity of ribavirin on NDV replication in HeLa cells had ED^{25}, ED^{50}, and ED^{75} of 12.5, 18.75, and 25.0 μg/ml, respectively (Table 3). For NDV-infected CEC cells, ribavirin had ED^{25}, ED^{50}, and ED^{75} of 4.75, 6.25, and 50.0 μg/ml, respectively (Table 3). The effective concentrations for each antiviral (ED^{25}, ED^{50}, and ED^{75}) were used for viral-induced cell cytotoxicity studies.

Effect of antivirals on Influenza A replication in HeLa and CEC cells. . The effective concentrations (ED) of each antiviral required to inhibit Influenza A replication in HeLa or CEC cells by 25, 50, and 75 percent was determined. Antiviral activity of PAP, guanidine, and ribavirin were nonlinear as indicated in the dose-response curves (Fig.4-

6). PAP inhibited Influenza A replication in HeLa cells by 25, 50, and 75%, with an ED^{25}, ED^{50}, and ED^{75} of 0.02, 0.05, and 0.42μM, respectively (Table 1). For influenza-infected CEC cells, PAP inhibited an ED^{25}, ED^{50}, and ED^{75} of 0.02, 0.05, and 0.3 μM, respectively (Table 1). Guanidine produced an ED^{25}, ED^{50}, and ED^{75} of 500, 1800, and 3500 μg/ml, respectively, for influenza replication in CEC cells (Table 2). For infected HeLa cells, guanidine produced an ED^{25}, ED^{50}, and ED^{75} of 275, 312, and 500 μg/ml, respectively (Table 2). For influenza-infected HeLa cells, ribavirin had an ED^{25}, ED^{50}, and ED^{75} of 10.0, 12.5, and 22.0 μg/ml, respectively (Table 3). For infected CEC cells, ribavirin had ED^{25}, ED^{50}, and ED^{75} of >75, >100, and >100 μg/ml, respectively (Table 3). The effective concentrations for each antiviral (ED^{25}, ED^{50}, and ED^{75}) were used for viral-induced cell cytotoxicity studies.

Cytotoxicity of Antivirals. In order to determine the effect PAP, ribavirin, and guanidine on the viability of HeLa and CEC cells, the MTT cell viability assay was performed. The percent of cell death for PAP concentrations from 4.63 to 0.01 μM, ribavirin concentrations from 83.0 to 0.08 μg/ml, and guanidine concentrations from 4167 to 4.07μg/ml were determined (Tables 4-6) and used to calculate the cellular cytotoxic concentration (CC^x) for each antiviral. The concentration of PAP required to inhibit 50% (CC^{50}) of cell viability was 5.5 μM and 1.57 μM for HeLa and CEC cells respectively (Table 7). The CC^{50} concentrations for ribavirin were >100 μg/ml for both HeLa and CEC cells (Table 7). The CC^{50} concentrations for guanidine were determined to be 2600 μg/ml and 312 μg/ml for HeLa and CEC cells respectively. All cytotoxic values (CC^{25}, CC^{50}, and CC^{75}) determined were two-fold or greater than the effective

concentrations determined (ED^{25}, ED^{50}, and ED^{75}) for PAP and ribavirin, but not for guanidine. High effective concentrations ED^{50} and ED^{75} of guanidine (Table 2) against both viruses were less than the cytotoxic values determined (Table 7) for HeLa cells. The ED^{50} and ED^{75} of guanidine (Table 2) against both viruses in CEC cells was greater than the highest cellular cytotoxic concentration (CC^{75}) (Table 7) of guanidine. However, at ED^{25} for guanidine against these viruses in CEC cells, the CC^{25} is approximately twice that concentration. The antiviral effect of guanidine at higher concentrations on virus multiplication may be due to its cytotoxicity in chicken embryo fibroblasts.

Effect of antivirals on NDV-induced host cell cytotoxicity. The MTT assay was used to determine the effect of each antiviral upon NDV-induced host cell cytotoxicity. CEC and HeLa cells infected by NDV in the presence of effective concentrations of PAP were not prevented from host-cell cytotoxicity, producing a cell viability range below 55% for HeLa cells, and below 10% for CEC cells (Figure 7). Percent Protection values (%PV) for ED^{25}, ED^{50}, and ED^{75} for PAP in NDV-infected HeLa cells were –58, -69, and –90%, respectively (Table 8), which indicates that the antiviral effect of PAP occurs after host cell shutdown. Percent Protection values (%PV) for ED^{25}, ED^{50}, and ED^{75} for PAP in NDV-infected CEC cells were 1.0, 1.6, and 2.1% respectively (Table 8), which also indicate that PAP was ineffective in preventing viral-induced cytotoxicity and its mechanism for action occurs late in the NDV replication cycle.

CEC and HeLa cells infected by NDV in the presence of effective concentrations of ribavirin were prevented from host-cell cytotoxicity, producing a cell viability range above 75% for HeLa cells and above 65% for CEC cells (Figure 8). Percent Protection

values (%PV) for ED^{25}, ED^{50}, and ED^{75} for ribavirin in NDV-infected HeLa cells were 73, 63, and 90% respectively (Table 8). The data suggests that the effect of ribavirin occurs early in the NDV replication cycle. Percent Protection values (%PV) for ED^{25}, ED^{50}, and ED^{75} for ribavirin in NDV-infected CEC cells were 66, 74, and 74% respectively (Table 8), which also shows that the effect of ribavirin occurs early in the NDV replication cycle, before viral-induced cytotoxicity.

CEC and HeLa cells infected by NDV in the presence of effective concentrations of guanidine were not prevented from host-cell cytotoxicity, producing a cell viability range below 25% for both HeLa and CEC cells (Figure 9), which indicates that the antiviral effect occurs late, after viral-induced host-cell shutdown. Percent Protection values (%PV) for ED^{25}, ED^{50}, and ED^{75} for guanidine in NDV-infected HeLa cells were –220, -650, and –720 respectively (Table 8). Percent Protection values (%PV) for ED^{25}, ED^{50}, and ED^{75} for guanidine in NDV-infected CEC cells were 12, -3.7, and –4.6% respectively (Table 8). Negative percent protection values obtained in both cell hosts indicates that no protection was offered by guanidine against NDV-induced host cell cytotoxicity.

For NDV-infected CEC and HeLa cells, only ribavirin offered protection against viral-induced cytotoxicity, whereas, PAP and guanidine did not. Therefore, the action of ribavirin occurs before this step in the NDV replication cycle, preventing host-cell shutdown. PAP and guanidine were shown to be ineffective in preventing this phenomenon, whose antiviral actions must occur late in the replication cycle for NDV.

Effect of antivirals on Influenza A-induced host cell cytotoxicity using MTT assay and high MOI. The MTT assay was used to determine the effect of each antiviral upon Influenza A-induced host cell cytotoxicity. CEC and HeLa cells infected by influenza in the presence of effective concentrations of PAP were prevented from host-cell cytotoxicity. In the range between the ED^{25} and ED^{50} concentrations, cell viability values were above 60% for CEC cells and above 50% for HeLa cells (Figure 10), which indicate the antiviral effect of PAP occurs early in the replication cycle of influenza. Percent Protection values (%PV) for ED^{25}, ED^{50}, and ED^{75} for PAP in influenza-infected HeLa cells were 102, 80, and –64% respectively (Table 9). Percent Protection values (%PV) for ED^{25}, ED^{50}, and ED^{75} for PAP in influenza virus-infected CEC cells were 77, 27, and –1.8% respectively (Table 9). The ED^{75} for PAP in both CEC and HeLa cell types indicates that these concentrations do not prevent viral-induced host cell cytotoxicity. However, both ED^{25} and ED^{50} of PAP were shown to be effective in preventing viral-induced host cell cytotoxicity in CEC and HeLa cells (Table 9), which suggests that the mechanism for PAP's antiviral action occurs early in the influenza replication cycle for these concentrations.

CEC and HeLa cells infected by influenza in the presence of effective concentrations of ribavirin were prevented from host-cell cytotoxicity, producing a cell viability range above 40% for HeLa cells for CEC cells for all effective concentrations (Figure 11). Percent Protection values (%PV) for ED^{25}, ED^{50}, and ED^{75} for ribavirin treatment against influenza-infected HeLa cells were 25, 30, and 65% respectively (Table 9). Percent Protection values (%PV) for ED^{25}, ED^{50}, and ED^{75} for ribavirin in influenza-infected CEC cells were 57, 65, and 65% respectively (Table 9). These findings indicate

that the effect of ribavirin on influenza multiplication occurs early during the replication cycle of this virus.

HeLa cells infected by influenza in the presence of effective concentrations of guanidine were not prevented from host-cell cytotoxicity, producing a cell viability range below 25% (Figure12), which suggest that it's antiviral effect occurs late in the replication cycle. Percent Protection values (%PV) for ED^{25}, ED^{50}, and ED^{75} for guanidine in influenza-infected HeLa cells were –127, -127, and –172% respectively (Table 9). Effective concentrations of guanidine in CEC cells infected by influenza produced higher cell viability values (Figure 12) than the HeLa cell treatment. However, the percent protection values (%PV) for ED^{25}, ED^{50}, and ED^{75} for guanidine in influenza-infected CEC cells were –5.3, -6.3, and –5.3%, respectively (Table 9), which suggests the antiviral effect of guanidine occurs late in the viral replication cycle, following viral-induced shutdown of either host cell.

For influenza-infected CEC and HeLa cells, ribavirin offered protection against viral-induced cytotoxicity for all effective concentrations, whereas, PAP was effective in preventing host-cell shutdown at ED^{25} and ED^{50}. Therefore, the action of ribavirin and PAP at these concentrations occur before viral-induced host cell cytotoxicity in the influenza replication cycle, preventing host-cell shutdown. Guanidine did not prevent viral-induced host cell cytotoxicity in influenza-infected CEC and HeLa cells, whose antiviral action must occur late in the replication cycle of influenza.

Figure 1. Effect of PAP on NDV multiplication in CEC cells,● ; and in HeLa cells, ■ ; Percent yield of virus multiplication from virus infected cells treated with PAP. Values represent an average of six replicates.

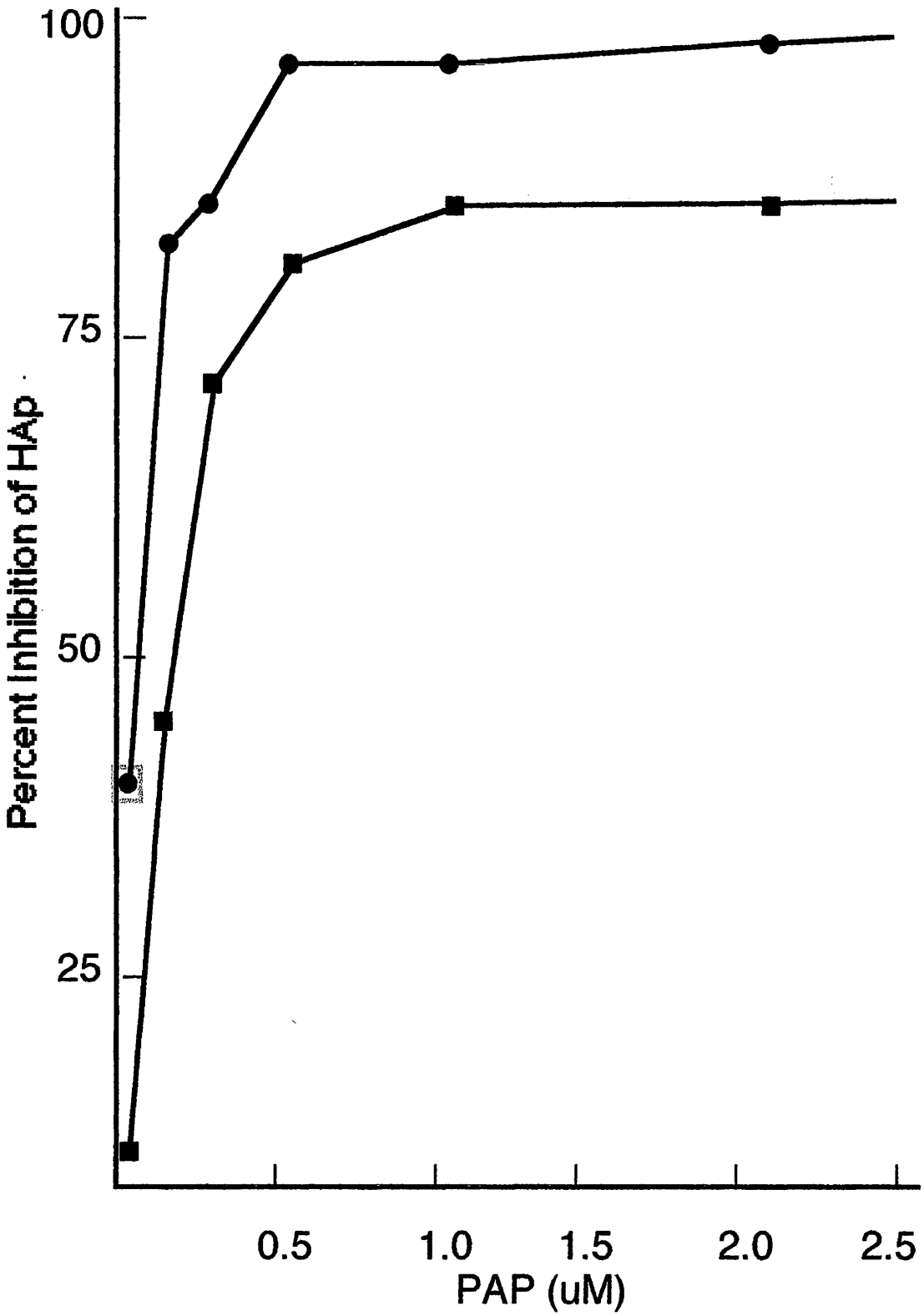
100
75
50
25
Percent Inhibition of HAp
0.5
1.0
1.5
2.0
2.5
PAP (uM)

Figure 2. Effect of PAP on Influenza multiplication in CEC cells, ● ; and in HeLa cells, ■ ; Percent yield of virus multiplication from virus infected cells treated with PAP. Values represent an average of six replicates.

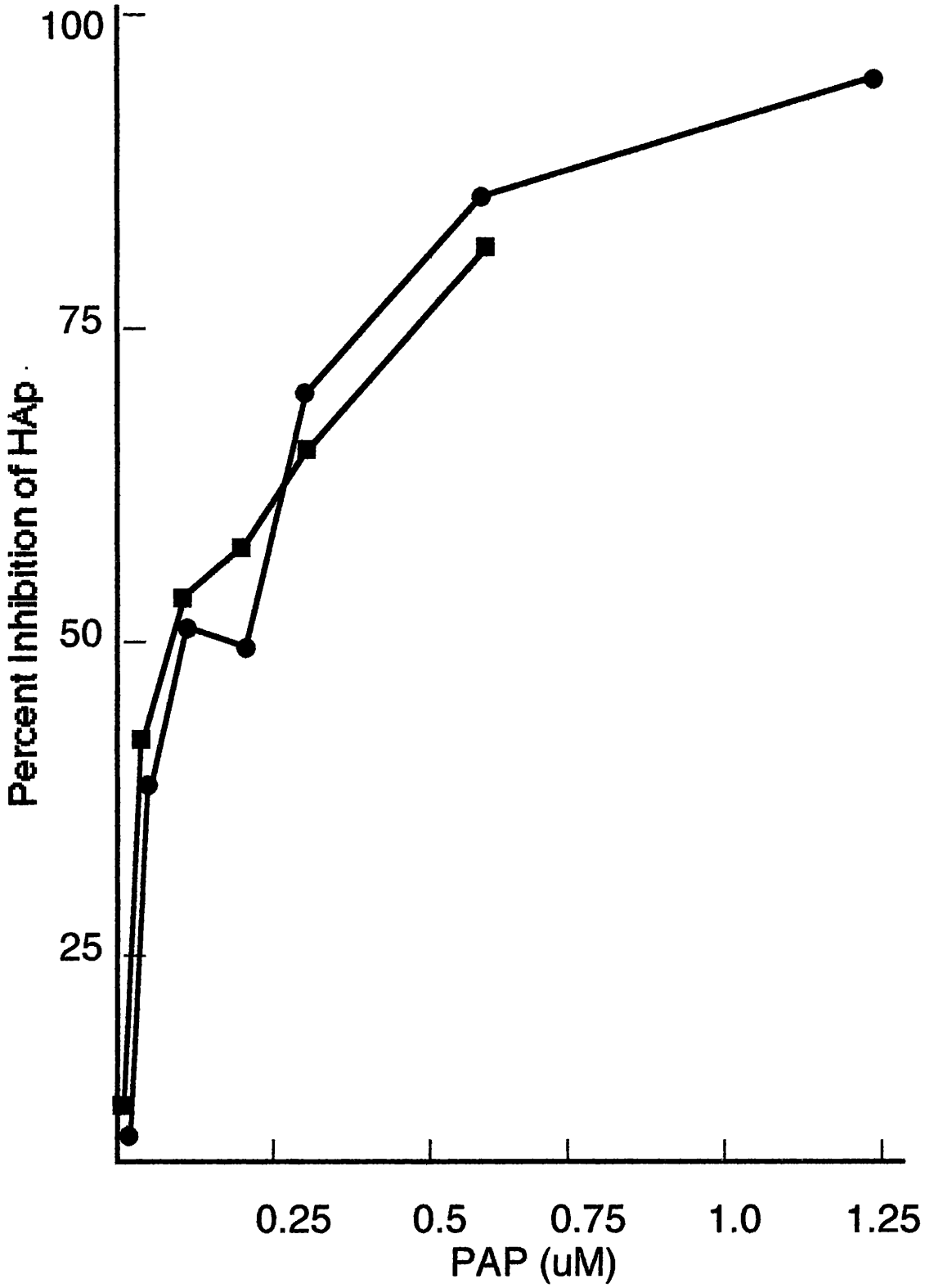
100
75
50
25
Percent Inhibition of HAp
0.25
0.5
0.75
1.0
1.25
PAP (uM)

Table 1. Effective Doses of PAP (μM) vs. NDV or Influenza In CEC and HeLa cells

Cell Type / Virus	ED^{25}	ED^{50}	ED^{75}
CEC / NDV	0.015	0.025	0.0625
HeLa/ NDV	0.03	0.156	1.0
CEC / Inf.A	0.02	0.05	0.3
HeLa / Inf.A	0.02	0.05	0.42

ED^{25} = Effective dose needed to inhibit 25% of viral replication.

ED^{50} = Effective dose needed to inhibit 50% of viral replication.

ED^{75} = Effective dose needed to inhibit 75% of viral replication.

Figure 3. Effect of guanidine on NDV multiplication in CEC cells, ● ; and in HeLa cells, ■ ; Percent yield of virus multiplication from virus infected cells treated with guanidine. Values represent an average of six replicates.

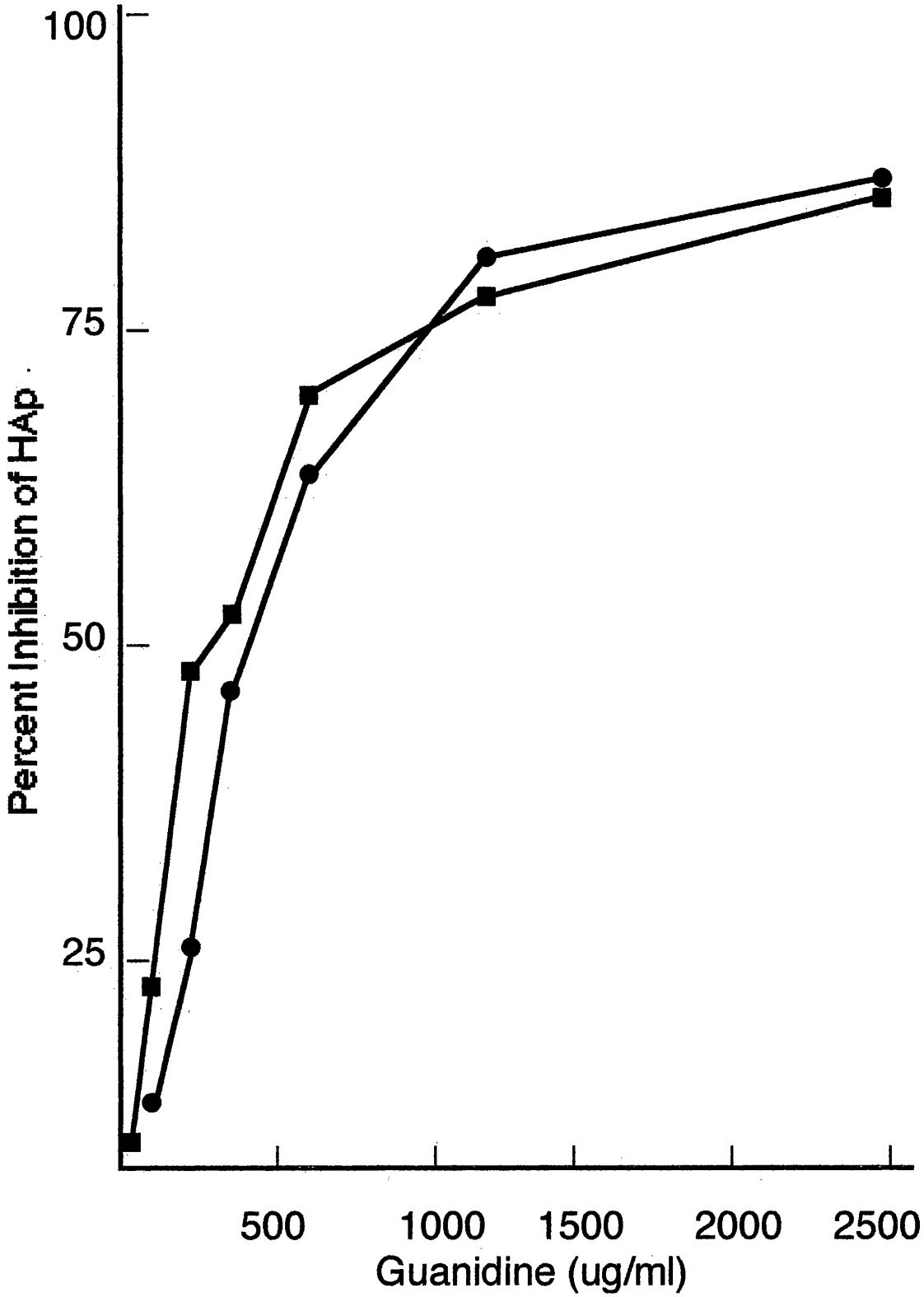
100
75
50
25
Percent Inhibition of HAp
500
1000
1500
2000
2500
Guanidine (ug/ml)

Figure 4. Effect of guanidine on Influenza multiplication in CEC cells, ● ; and in HeLa cells, ■ ; Percent yield of virus multiplication from virus infected cells treated with guanidine. Values represent an average of six replicates.

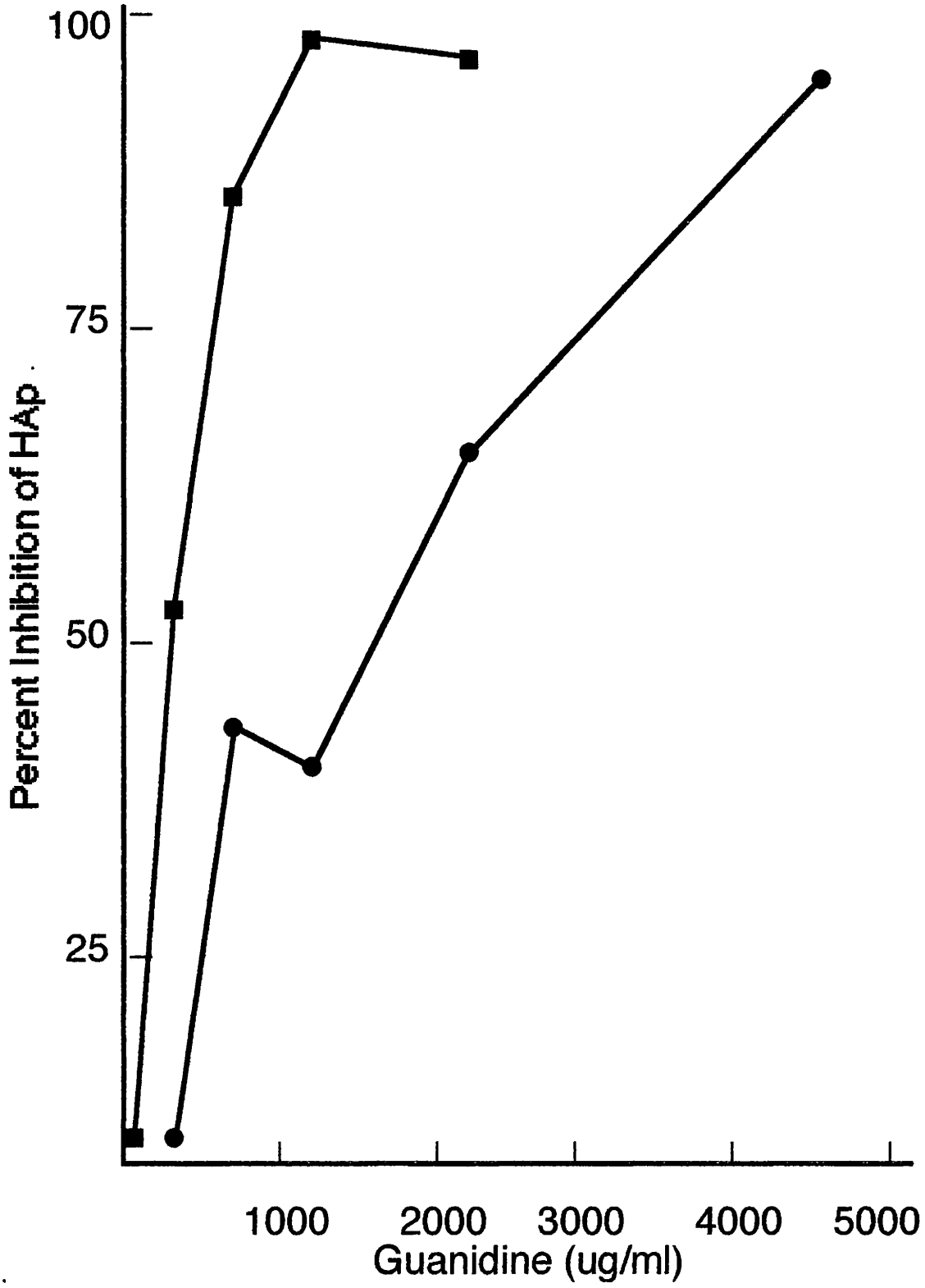
100
75
50
25
Percent Inhibition of HAp
1000
2000
3000
4000
5000
Guanidine (ug/ml)

Table 2. Effective Doses of Guanidine (μg/ml) vs. NDV or Influenza in CEC or HeLa cells

Cell Type / Virus	ED^{25}	ED^{50}	ED^{75}
CEC / NDV	100	150	1500
HeLa/ NDV	50	312	1300
CEC / Inf.A	500	1800	3500
HeLa / Inf.A	275	312	500

ED^{25} = Effective dose needed to inhibit 25% of viral replication.

ED^{50} = Effective dose needed to inhibit 50% of viral replication.

ED^{75} = Effective dose needed to inhibit 75% of viral replication.

Figure 5. Effect of ribavirin on NDV multiplication in CEC cells, ● ; and in HeLa cells, ■ ; Percent yield of virus multiplication from virus infected cells treated with ribavirin. Values represent an average of six replicates.

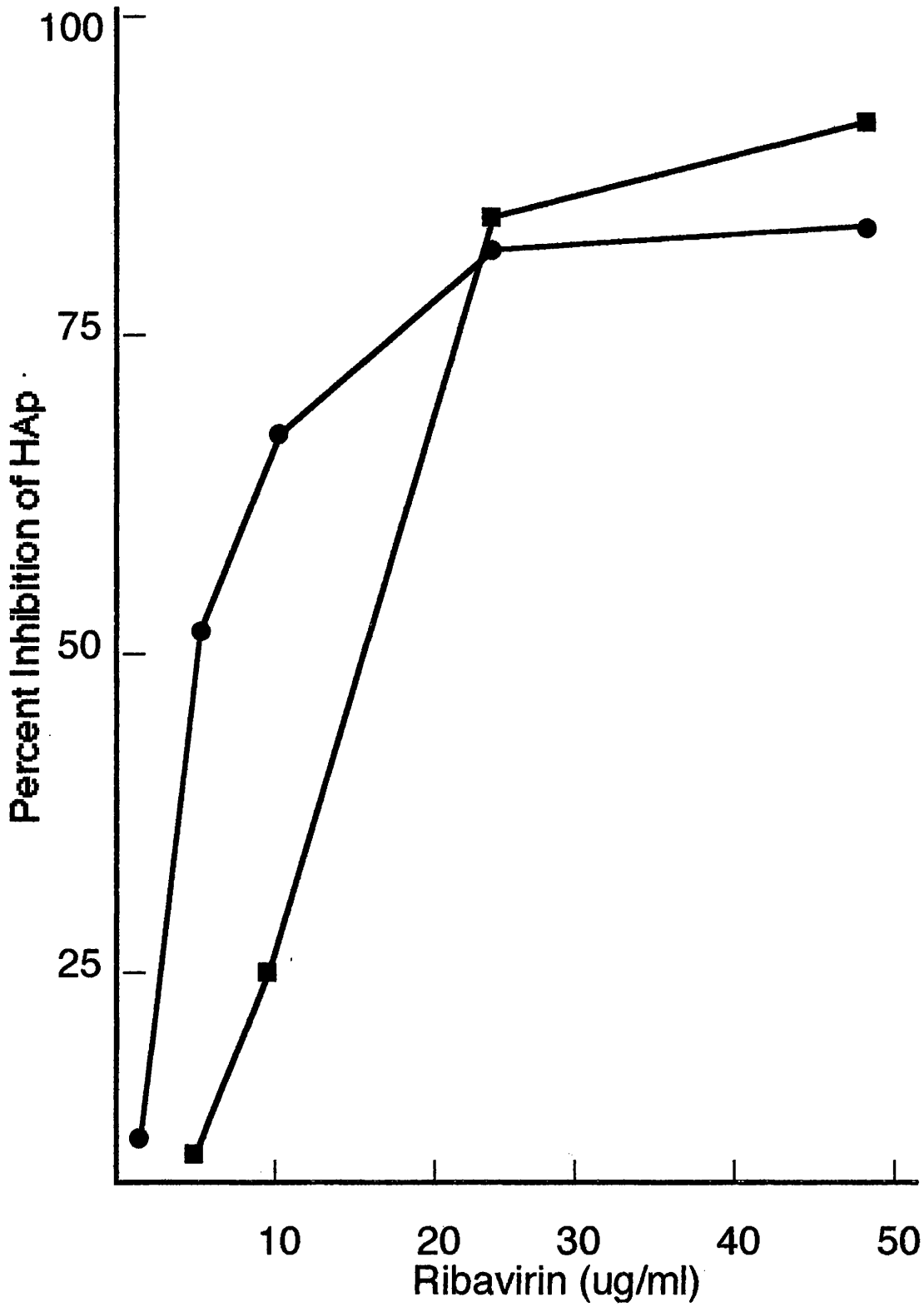
100
75
50
25
Percent Inhibition of HAp
10
20
30
40
50
Ribavirin (ug/ml)

Figure 6. Effect of ribavirin on Influenza multiplication in CEC cells, ● ; and in HeLa cells, ■ ; Percent yield of virus multiplication from virus infected cells treated with ribavirin. Values represent an average of six replicates.

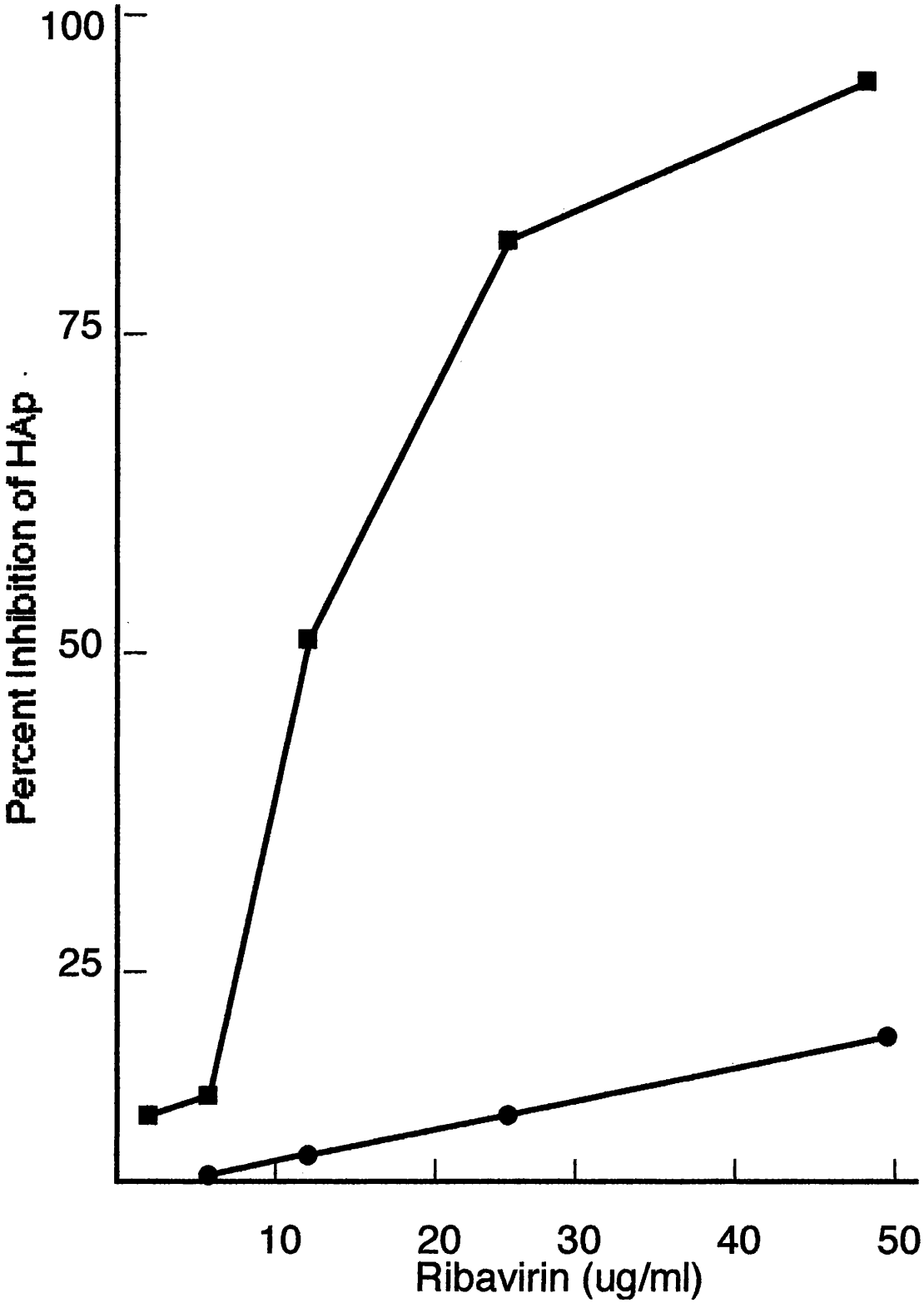
100
75
50
25
Percent Inhibition of HAp
10
20
30
40
50
Ribavirin (ug/ml)

Table 3. Effective Doses of Ribavirin (μg/ml) vs. NDV or Influenza in CEC and HeLa cells

Cell Type / Virus	ED^{25}	ED^{50}	ED^{75}
CEC / NDV	4.75	6.25	50.0
HeLa/ NDV	12.5	18.75	25.0
CEC / Inf.A	>75	>100	>100
HeLa / Inf.A	10.0	12.5	22.0

ED^{25} = Effective dose needed to inhibit 25% of viral replication.

ED^{50} = Effective dose needed to inhibit 50% of viral replication.

ED^{75} = Effective dose needed to inhibit 75% of viral replication.

Table 4. Cytotoxicity of PAP in HeLa and CEC cells

Concentration(μM)	Observed % Nonviable[a] HeLa cells[b]	CEC cells[c]
4.63	9.40 ± 0.83	36.05 ± 0.24
2.31	0.00 ± 1.07	27.82 ± 0.32
1.16	1.25 ± 1.26	6.45 ± 0.12
0.58	0.00 ± 1.21	4.60 ± 0.23
0.29	0.00 ± 0.75	0.00 ± 0.02
0.14	0.00 ± 1.08	0.00 ± 0.03
0.07	0.19 ± 1.16	0.00 ± 0.02
0.04	0.75 ± 1.06	0.00 ± 0.02
0.02	6.78 ± 0.81	0.01 ± 0.03
0.01	5.72 ± 0.96	0.00 ± 0.02
0.00	4.75 ± 0.58	0.00 ± 0.02

[a] The percent of cell death after 24 hours of exposure to antivirals.
[b] Values are average of 16 replicates.
[c] Values are average of 12 replicates.

Table 5. Cytotoxicity of Ribavirin in HeLa and CEC cells

Concentration(μg/ml)	Observed % Nonviable[a]	
	HeLa cells[b]	CEC cells[c]
83.00	14.55 ± 1.42	3.43 ± 0.22
41.50	19.19 ± 1.21	0.78 ± 0.18
20.75	13.17 ± 1.24	0.16 ± 0.08
10.38	9.51 ± 0.79	0.02 ± 0.03
5.19	14.34 ± 1.48	0.00 ± 0.02
2.59	9.82 ± 0.99	0.00 ± 0.03
1.30	9.25 ± 0.87	0.00 ± 0.02
0.65	10.59 ± 0.58	0.00 ± 0.02
0.32	9.27 ± 0.69	0.00 ± 0.01
0.16	8.90 ± 0.68	0.00 ± 0.02
0.08	8.02 ± 0.57	0.00 ± 0.00

[a] The percent of cell death after 24 hours exposure to antivirals.
[b] Values are average of 16 replicates.
[c] Values are average of 12 replicates.

Table 6. Cytotoxicity of Guanidine in HeLa and CEC cells

Concentration(μg/ml)	Observed % Nonviable[a] HeLa cells[b]	CEC cells[c]
4167.00	73.15 ± 0.27	84.76 ± 2.32
2083.50	75.71 ± 0.38	41.29 ± 0.98
1041.75	8.21 ± 0.90	22.45 ± 0.56
520.88	3.63 ± 0.92	12.85 ± 0.79
260.44	2.61 ± 0.86	3.25 ± 0.45
130.22	4.58 ± 1.19	0.00 ± 0.02
65.11	1.24 ± 0.94	0.00 ± 0.04
32.55	0.91 ± 1.13	0.00 ± 0.02
16.28	1.42 ± 0.73	0.00 ± 0.02
8.14	1.95 ± 1.13	0.00 ± 0.03
4.07	0.67 ± 1.35	0.00 ± 0.02
0.00	15.69 ± 0.83	0.00 ± 0.02

[a] The percent of cell death after 24 hours exposure to antiviral.
[b] Values are average of 16 replicates.
[c] Values are average of 12 replicates.

Table 7. Cytotoxic Concentrations of Antivirals for HeLa and CEC cells

Cell Type	Antiviral	CCx^{25}	CCx^{50}	CCx^{75}
HeLa	PAP (μM)	1.5	5.5	8.0
	Ribavirin (μg/ml)	87.5	>100	>100
	Guanidine (μg/ml)	1250	2600	2750
CEC	PAP (μM/ml)	0.93	1.57	2.20
	Ribavirin (μg/ml)	82.5	>100	>100
	Guanidine (μg/ml)	90	312	900

CCx^{25} = The concentration required to kill 25% of cells.
CCx^{50} = The concentration required to kill 50% of cells.
CCx^{75} = The concentration required to kill 75% of cells.

Figure 7. Effect of PAP against viral-induced cytotoxicity in CEC, ● ; and in HeLa cells, ■ ; Percent cell viability of NDV-infected cells treated with PAP as determined by MTT assay. Values represent an average of 12 replicates for CEC, and 24 replicates for HeLa cells.

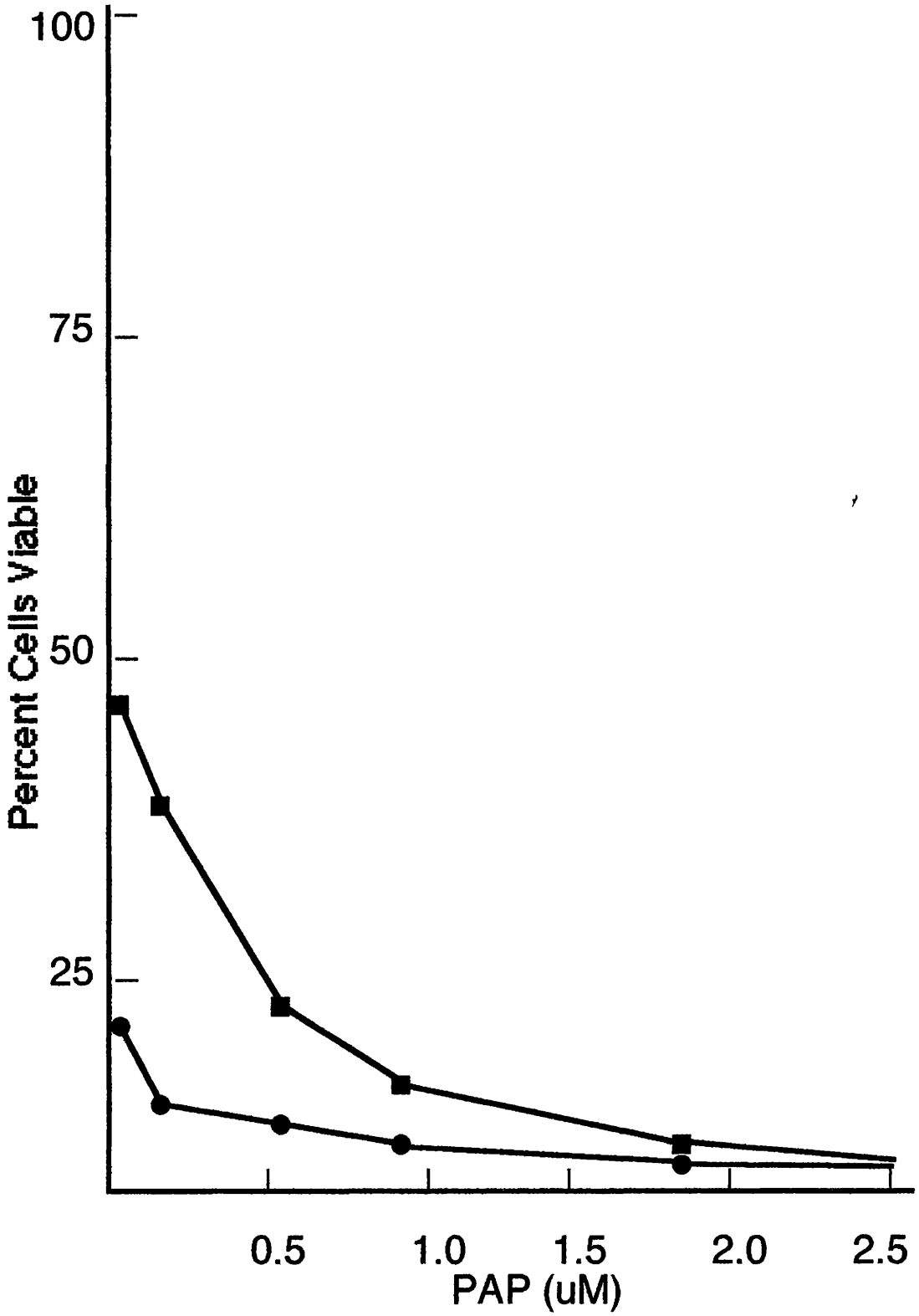
100
75
50
25
Percent Cells Viable
0.5
1.0
1.5
2.0
2.5
PAP (uM)

Figure 8. Effect of ribavirin against viral-induced cytotoxicity in CEC, ● ; and in HeLa cells, ■ ; Percent cell viability of NDV-infected cells treated with ribavirin as determined by MTT assay. Values represent an average of 12 replicates for CEC, and 32 replicates for HeLa cells.

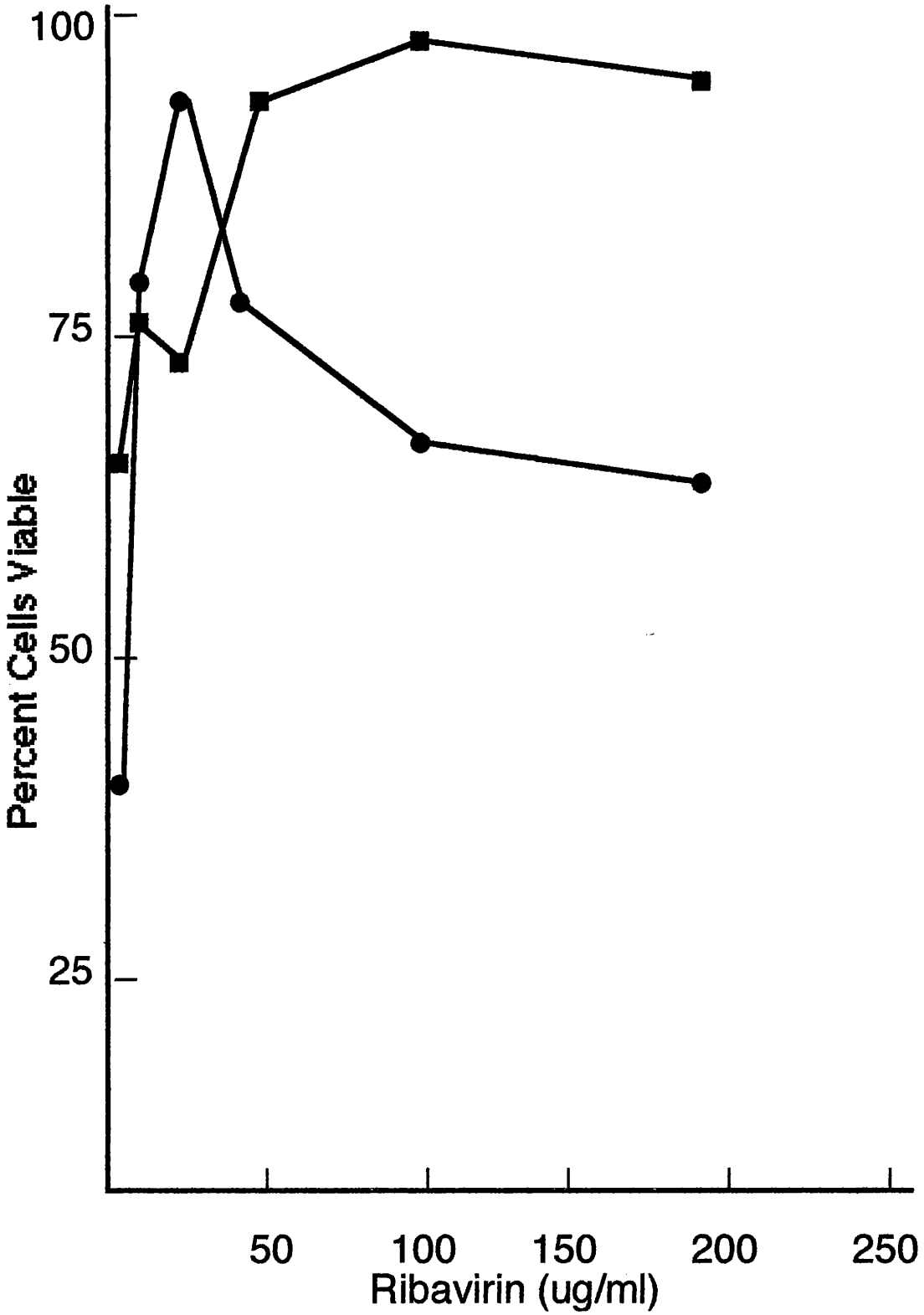
100
75
50
25
Percent Cells Viable
50
100
150
200
250
Ribavirin (ug/ml)

Figure 9. Effect of guanidine against viral-induced cytotoxicity in CEC, ● ; and in HeLa cells, ■ ; Percent cell viability of NDV-infected cells treated with guanidine as determined by MTT assay. Values represent an average of 8 replicates for CEC, and 40 replicates for HeLa cells.

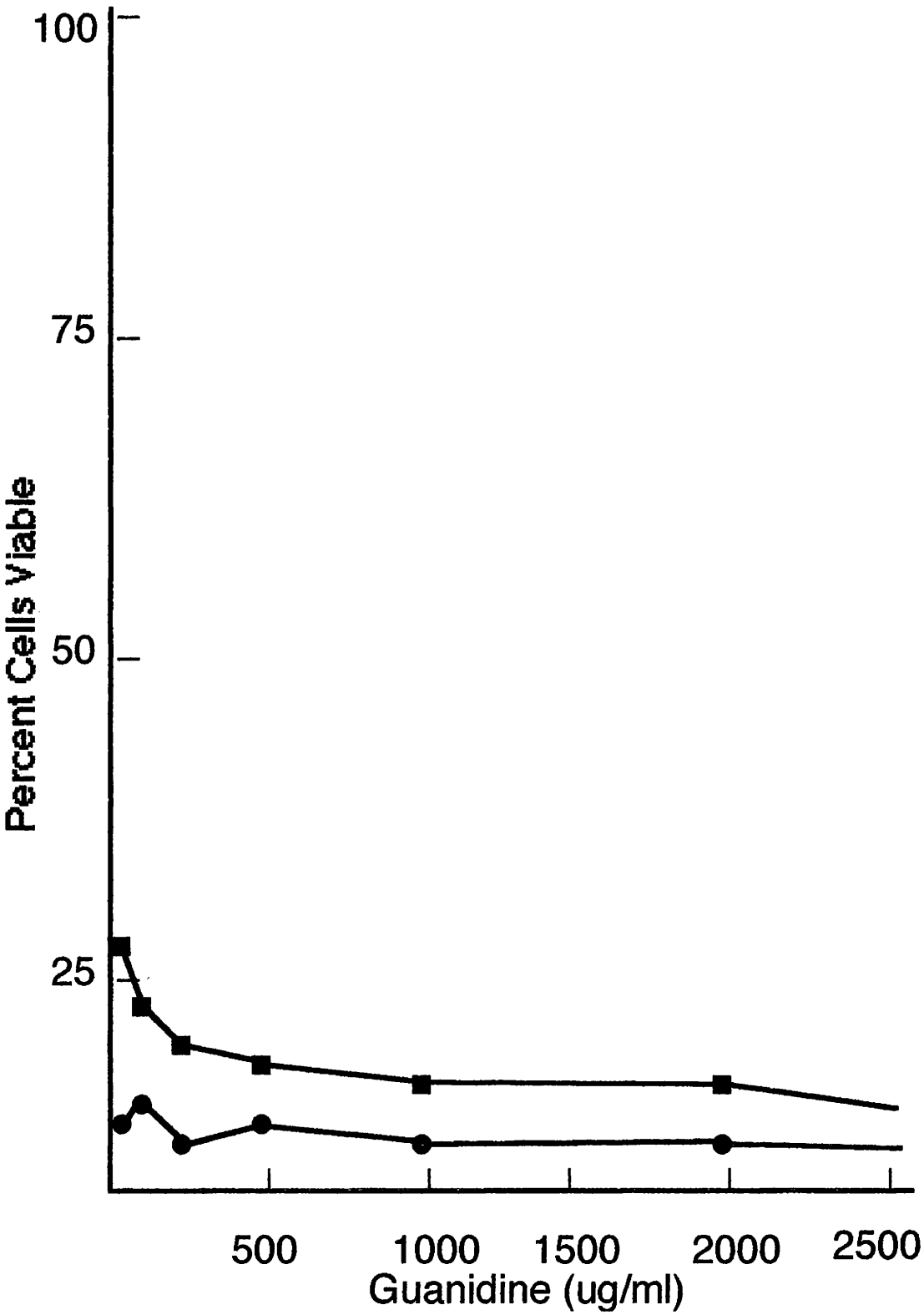
100
75
50
25
Percent Cells Viable
500
1000
1500
2000
2500
Guanidine (ug/ml)

Table 8. % Protection Values for Effective Doses of PAP, Guanidine or Ribavirin vs. NDV in CEC and HeLa cells

Antiviral	Cell type	ED25	ED50	ED75
PAP	CEC	1.0	1.6	2.1
	HeLa	-58	-69	-90
Guanidine	CEC	12	-3.6	-4.6
	HeLa	-220	-650	-720
Ribavirin	CEC	66	74	74
	HeLa	73	63	90

Figure 10. Effect of PAP against viral-induced cytotoxicity in CEC, ●; and in HeLa cells, ■ ; Percent cell viability of influenza-infected cells treated with PAP as determined by MTT assay. Values represent an average of 8 replicates for CEC, and 8 replicates for HeLa cells.

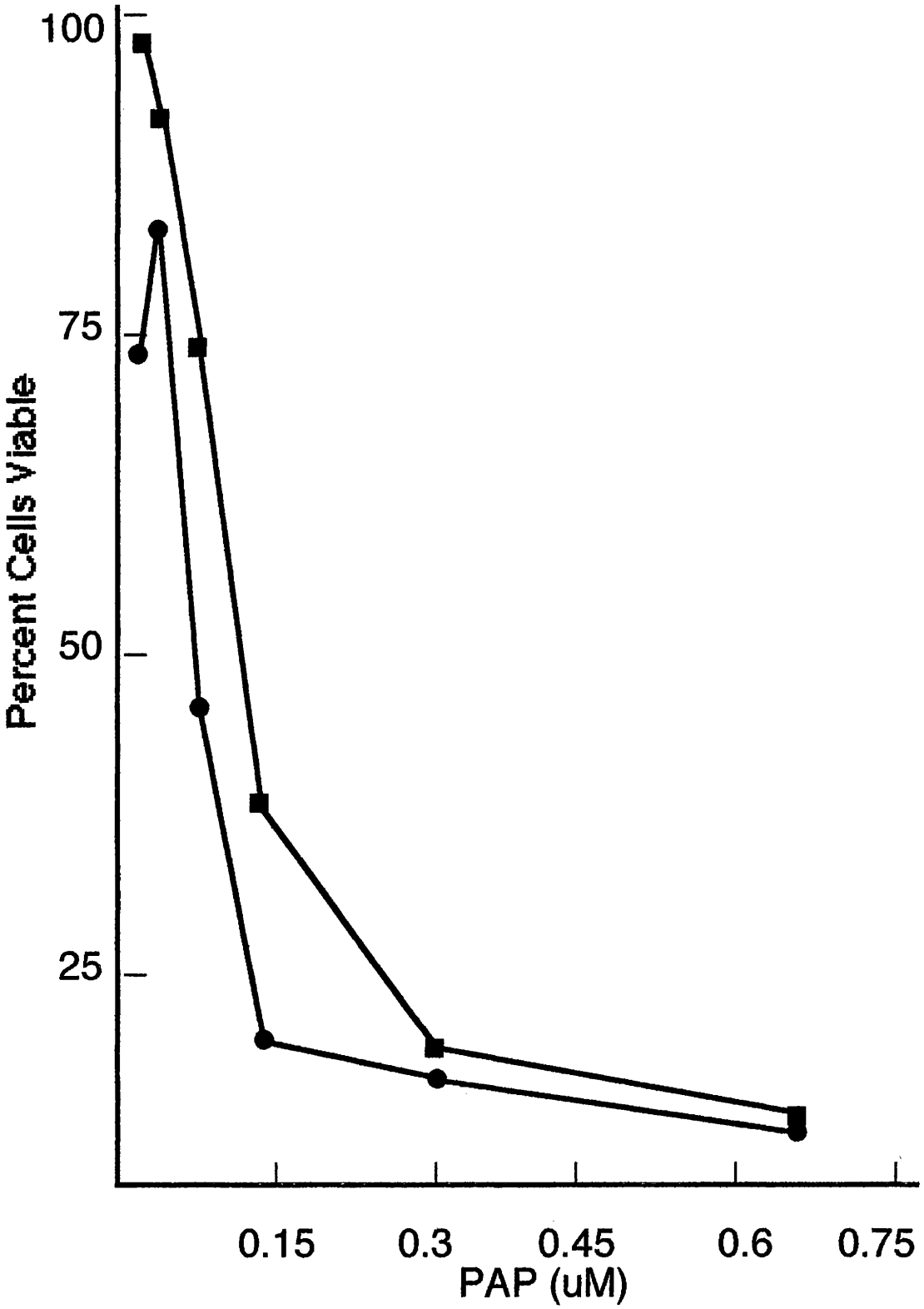
100
75
50
25
Percent Cells Viable
0.15
0.3
0.45
0.6
0.75
PAP (uM)

Figure 11. Effect of ribavirin against viral-induced cytotoxicity in CEC, ● ; and in HeLa cells, ■ ; Percent cell viability of influenza-infected cells treated with ribavirin as determined by MTT assay. Values represent an average of 16 replicates for CEC, and 16 replicates for HeLa cells.

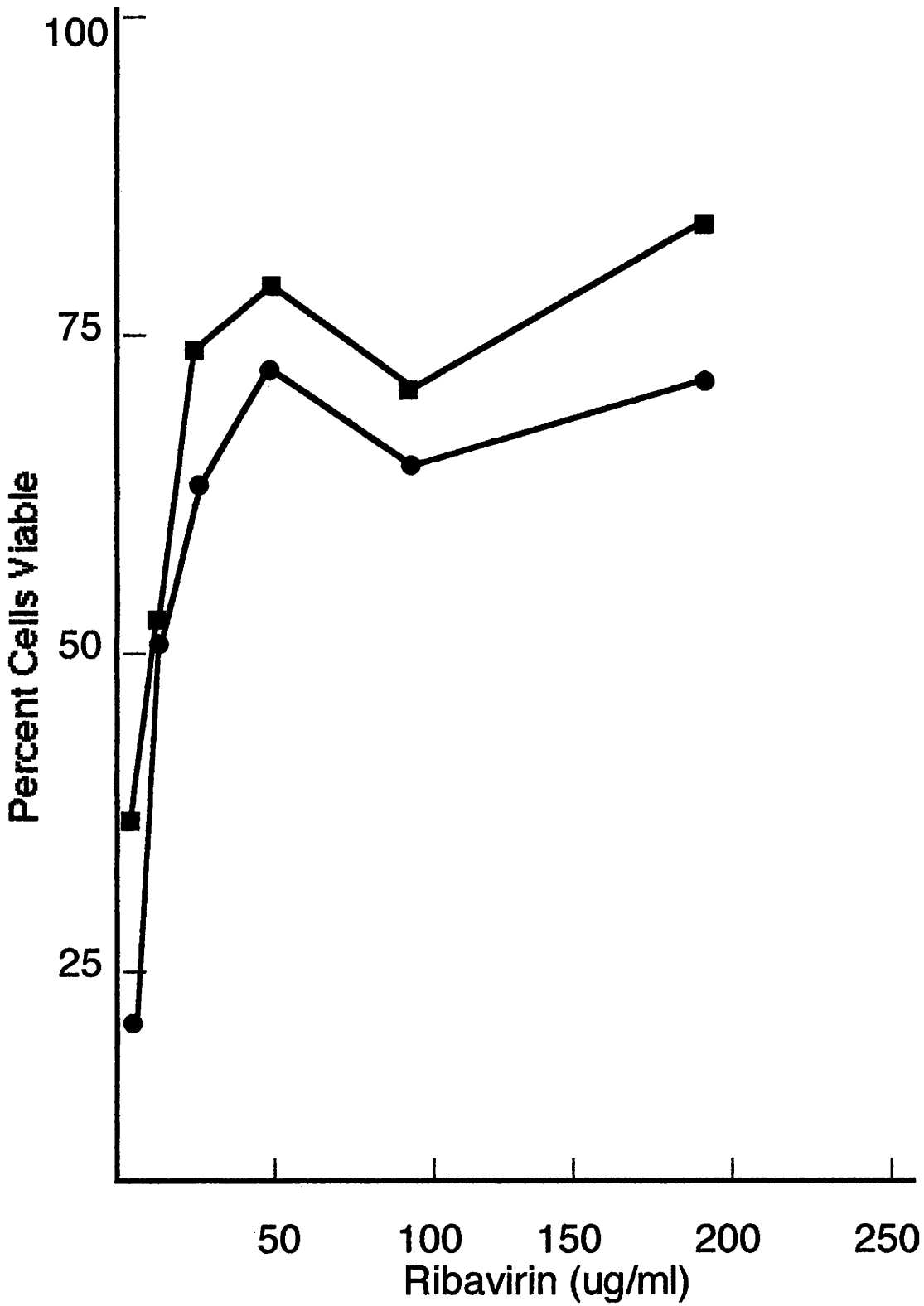
100
75
50
25
Percent Cells Viable
50
100
150
200
250
Ribavirin (ug/ml)

Figure 12. Effect of guanidine against viral-induced cytotoxicity in CEC, ● ; and in HeLa cells, ■ ; Percent cell viability of influenza-infected cells treated with guanidine by MTT assay. Values represent an average of 8 replicates for CEC, and 8 replicates for HeLa cells.

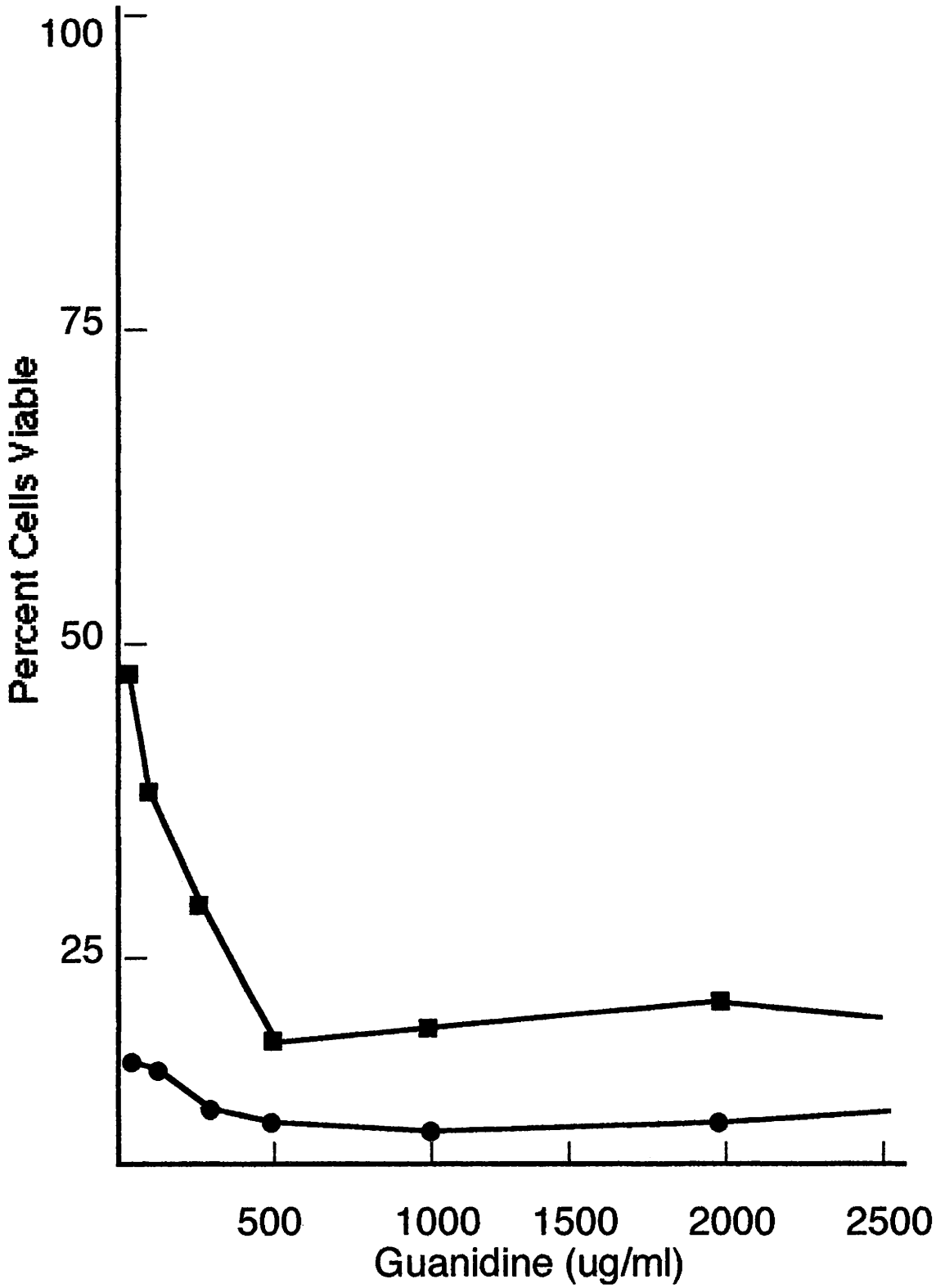
100
75
50
25
Percent Cells Viable
500
1000
1500
2000
2500
Guanidine (ug/ml)

Table 9. % Protection Values for Effective Doses of PAP, Guanidine or Ribavirin vs. Influenza in CEC and HeLa cells

Antiviral	Cell type	ED25	ED50	ED75
PAP	CEC	77	27	-1.8
	HeLa	102	80	-64
Guanidine	CEC	-5.3	-6.3	-5.3
	HeLa	-127	-127	-172
Ribavirin	CEC	57	65	65
	HeLa	25	30	65

DISCUSSION

This study was conducted to determine whether effective concentrations of PAP, ribavirin, and guanidine prevented virus-induced cytotoxicity in NDV and Influenza A virus-infected CEC and HeLa cells. By infecting all cells with virus using a tissue culture infective dose of 100% ($TCID_{100}$) and determining cell viability by the MTT assay, either the antiviral was effective in preventing viral-induced cytotoxicity, which occurs early in the replication cycle, or unable to halt the phenomenon of host-cell shutdown by the virus.

Both PAP and ribavirin were effective in preventing viral-induced cytotoxicity in influenza-infected CEC and HeLa cells as determined by MTT assay, whereas guanidine did not prevent viral-infected host cell death. Ribavirin was also effective in preventing viral-induced cytotoxicity in NDV-infected CEC and HeLa cells, whereas, PAP and guanidine were unable to prevent cell death.

Guanidine was not effective in preventing NDV and influenza viral-induced cytotoxicity for CEC and HeLa cells. Guanidine is a substrate analog for guanosine similar to ribavirin, but has greater cytotoxicity. Although the mechanism of this compound is unknown, guanidine has been demonstrated to inhibit the initiation of negative-strand RNA synthesis (6). For both NDV and influenza virus, their negative-stranded genomes must first be translated in positive-stranded mRNA to serve as templates for progeny genomes (6). The production of progeny RNA genomes occurs during the latter stages of the viral replication cycle, which would explain the

ineffectiveness of gentamicin in preventing viral-induced cytotoxicity. By varying the time of gentamicin addition to infected cells, research on poliovirus has also shown that guanidine acts at a stage late in the replication cycle (66).

Ribavirin was effective in preventing NDV and influenza viral-induced cytotoxicity in chicken embryo fibroblasts (CEC) and HeLa cells. Ribavirin, a substrate analog for the nucleoside guanosine, is rapidly phosphorylated to form mono-, di-, and triphosphate forms once it enters a cell. This antiviral has been shown to be effective in reducing the overall guanosine monophosphate (GTP), diphosphate (GDP), and triphosphate (GTP) levels needed for viral protein synthesis in infected-cells (14). These metabolites are essential for early protein synthesis, involved with the initiation complex (bound to eIF2 to bring intiator tRNA to the ribosomal 40S subunit), elongation factors (EF1α and EF1β), and termination of protein synthesis by GTP-driven single release factor, Erf (58). Thus the prevention of expression of early viral gene products by ribavirin would result in preventing viral-induced host cell cytotoxicity.

A possible early target in NDV multiplication for ribavirin may be during attachment of the virion to the cell by interfering with the proteolytic cleavage of the F^{O} protein during fusion of virus envelope and host cell. F protein cleavage mutants have demonstrated a decrease in syncytia formation of host cells, which is a cytopathic effect commonly observed with this virion (40). Adsorption of the virion only lasts 10 min and early gene products can be detected at low levels within 5 h (20). Ribavirin could also inhibit early expression of the polymerase gene (P), resulting in decreased protein synthesis of early proteins. GMP, which is reduced in cells by ribavirin treatment, is

needed by the NDV viral-polymerase to initiate transcription. By 12 h infection, fragmentation of cellular DNA is observed and apoptotic cells can be observed (37). Therefore, the results indicate the effect of ribavirin must occur before 12h as cell viability was maintained in virus-infected cells after 24h incubation.

In influenza virus-infected cells, transcription has also been shown to be initiated by polymerizing GMP (23). Competitive inhibition between ribavirin and guanosine within the cell at this early stage in the replication cycle may be responsible for the antiviral effect observed. Also, it has been demonstrated that transcription initiation of viral RNA takes place by recognizing and utilizing capped RNA primers from host cell mRNAs. Ribavirin triphosphate has been shown to decrease viral protein synthesis by inhibition of 5'capping of mRNA by interfering with guanyl transferase and viral N7-methyl-transferase (52), which also would interfere in early protein production.

During the influenza replicative cycle, both the non-structural protein (NS1) and nucleprotein (NP) are expressed earlier than other genes (43). The expression of NP eventually leads to a shift in RNA replication by the synthesis of complete positive-polarity RNAs needed as templates for virion RNA synthesis. By interfering with early protein expression, ribavirin could prevent this shift from early to late gene expression that may be responsible for the eventual death of the host cell.

PAP failed to prevent host-cell shutdown for NDV-infected cells. PAP, like other ribosome-inactivating proteins, specifically cleaves an adenine residue in a highly conserved region for the 60S subunit for eucaryotic ribosomes. This target would be effective in reducing expression of early viral genes. However, the data indicates that

PAP did not inhibit viral-induced host cell cytotoxicity by NDV, which agrees with findings involving PAP and Herpes virus replication, where only 40% or early gene product was inhibited by the antiviral and no inhibitory effect was seen against late gene production (63).

PAP prevented influenza-induced host cytotoxicity in HeLa and CEC cells. The extract depurinates adenine residues on eucaryotic ribosomes and is shown to interact with guanine residues as well (35), which would prevent expression of early viral gene products. By working early in the viral multiplication cycle, PAP prevents late gene expression responsible for induction of cytotoxicity and apoptosis of infected cells.

The replication cycle for influenza and NDV are different in the early stages, which may be why PAP prevented influenza virus-cytotoxicity and failed to prevent cell death by NDV infection. The difference in site of multiplication for influenza (nucleus) and NDV (cytoplasm) may be a factor. The influenza genome, once contained within the cytoplasm in a clathrin-coated vesicle, must undergo several steps in order to travel to and from the host cell nucleus. The M2 protein functions during virus uncoating and maturation by modifying the pH in virions as wells as the trans-golgi (12). The M1 protein directs transportation of ribonucleoprotein (RNP) cores into or out of the nucleus (4). Any of these early mechanisms could be a target for PAP. In contrast to influenza, there are minimal steps for NDV replication to begin. NDV, once it has entered the cell, uncoats and releases its genome into the cytoplasm, where transcription can begin and early gene products can be synthesized.

Cytotoxicity studies for the antivirals upon host cell viability as determined by

the MTT assay revealed that the reduction in viral replication observed was due to specific action of the compound rather than toxicity to the host cell, which would decrease viral multiplication as well. Compounds that exhibit antiviral activity must be tested for cytotoxicity *in vitro*, then observed *in vivo* which involves long term effects such as carcinogenesis, pharmokinetic, reproductive, and chronic cytoxicities before approved (60). The cytotoxic concentrations (CC^X) for PAP and ribavirin in both cell hosts were much greater than their respective effective concentrations (ED^X) determined to inhibit both viruses, which suggests that both substances demonstrate antiviral activity against NDV and influenza virus without harming the host cells, CEC and HeLa cells. All effective concentrations (ED^{25}, ED^{50} and ED^{75}) for guanidine against NDV and influenza virus replication were less than the lowest cellular cytotoxic concentration (CC^{25}) for the compound in HeLa cells. This finding suggests that guanidine reduces viral multiplication through antiviral activity rather than by harming this particular host cell. For infected CEC cells, the antiviral effect of guanidine at higher concentrations on virus multiplication may be due to its cytotoxicity in chicken embryo fibroblasts. However, at ED^{25} for guanidine against these viruses in CEC cells, the CC^{25} is approximately twice that concentration, which suggests that guanidine showed antiviral activity at low effective concentration.

SUMMARY

This study investigated whether antiviral effect by Pokeweed antiviral protein (PAP), ribavirin, and guanidine hydrochloride upon Influenza A and Newcastle Disease virus-infected chicken embryo fibroblasts (CEC) and HeLa cells prevented viral-induced host cell cytotoxicity. The MTT cell viability assay was utilized to determine if the antivirals prevented this phenomenon from occuring.

Both PAP and ribavirin were effective in preventing viral-induced cytotoxicity in influenza-infected CEC and HeLa cells as determined by MTT assay, whereas guanidine did not prevent viral-infected host cell death. Therefore, the mode of antiviral action against influenza virus for PAP and ribavirin occurs early in the replication cycle, whereas the effect of guanidine occurs after host-cell death. Ribavirin was also effective in preventing viral-induced cytotoxicity in NDV-infected CEC and HeLa cells, whereas, PAP and guanidine were unable to prevent cell death. Therefore, the antiviral action for ribavirin against NDV-replication occurred early as well, but the effect of PAP and guanidine upon replication occurs later in the cycle for NDV.

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