SYNERGISTIC EFFECTS OF POKEWEED ANTIVIRAL PROTEIN IN COMBINATION WITH GUANIDINE AND RIBAVIRIN ON THE REPLICATION OF NEWCASTLE DISEASE VIRUS

THESIS

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DEDICATION

For Keith, whose love, faith, and encouragement were invaluable throughout this ordeal.

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INTRODUCTION

A major concern in the search for antiviral drugs is toxicity. Since uninfected cells will be exposed to the drug, one must develop compounds that are toxic or preferentially toxic only to the virus-infected cell. One approach to reduce toxicity is through combination therapy. Use of combined antivirals may also result in reduction of resistance, enhanced efficacy, and increased clinical potential of agents (34).

Antiviral therapy is a difficult process due to the virus's intracellular location and its utilization of host cell functions for replication. However, there are several junctures in the virus replicative cycle which could provide targets for antiviral agents, including virus attachment to cell membrane and entry into the cell, virus uncoating, synthesis of viral messenger RNA and viral progeny RNA, viral protein synthesis, and assembly and release of infectious particles (Fig. 1) (51).

Because viral proteins are synthesized sequentially in a coordinated manner, any disruption of this sequence alters protein output dependent upon the point of disruption. Thus, an antiviral agent which targets early protein synthesis, when combined with an antiviral agent that targets late protein synthesis, should negate any antiviral effect of the latter. However, if the combination of the two antiviral agents initiates a new target, a unique protein profile will result.

Pokeweed antiviral protein (PAP) is a broad spectrum antiviral agent which has been shown to act synergistically against the replication of Newcastle disease virus when used in combination with guanidine and ribavirin in HeLa cells (86). Although a wide range of mammalian tissue culture, including human lung, porcine

kidney, and mouse fibroblast, is susceptible to the virus, the natural host of Newcastle disease virus is chicken embryo cells.

The initial objective of this research was to determine the antiviral effect of pokeweed antiviral protein, guanidine, and ribavirin, singularly, on the replication of NDV in chicken embryo cells. Second, based on previous research which showed a synergistic antiviral effect against influenza virus (76) and poliovirus (86), it is hypothesized that pokeweed antiviral protein in combination with guanidine and ribavirin will display a synergistic antiviral effect against replication of NDV.

1.0 NEWCASTLE DISEASE VIRUS

Newcastle disease occurs in every continent of the world, excepting Antarctica. While the origins and epidemiology of the disease are obscure (50), its impact in recent years has resulted in the need for programs using effective vaccines with minimal adverse effects.

Newcastle Disease Virus (NDV) is a single-stranded RNA virus and member of the family *Paramyxoviridae*, genus *Rubulavirus*. It affects both domestic and wild birds and has become a major problem in many countries. Following its recognition, control measures for NDV involving slaughter, sanitary precautions and vaccination were adopted by numerous disease control agencies. These measures have produced mixed results. In 1967-68, the disease became very difficult to control in several countries of the Near East and the Mediterranean, and in 1970, a serious epidemic occurred in Europe (1).

More than 100 strains of NDV have been identified and they are classified according to their relative virulence for chickens (1, 50). Strains are generally designated as belonging to one of three types: lentogenic, mesogenic or velogenic. Lentogenic strains cause slight congestion in young birds and rarely cause death (1, 50). Mesogenic strains cause congestion in adult birds with a resulting mortality rate of less than 10% (1, 50). The velogenic strains are extremely virulent (mortality greater than 90%) and cause severe lesions in birds of all ages (1, 50). All strains grow in developing eggs.

The virus normally enters through the respiratory tract and begins multiplication within 24 hours. Virus multiplication peaks in 3 days, followed by a 3-day lag period, and then curtailment resulting from antibody formation. While the three strains of the virus multiply at similar rates, the more virulent strains of the virus are able to pass through the blood-brain barrier. Additionally, avirulent strains of the virus attach to the epithelial cell surface and allow cell regeneration, while virulent strains destroy epithelial cells (7). Research has also exhibited a tendency for avirulent strains to release virus particles at a slower rate, thus allowing more time for the antibody formation by the host (68).

1.1 STRUCTURE

NDV is generally spherical in shape, although pleomorphic forms are also common. Typical virus particles are between 150-200 nm long and 18 nm wide. The virion is composed of two main structures: the internal nucleocapsid core and the outer envelope (50).

The nucleocapsid core contains a single-stranded, negative-sense, non-segmented RNA genome. The genome codes for six or more proteins which have been identified as: NP, a nucleocapsid protein; P and C, nucleocapsid associated proteins; L, a lipoprotein; F_o, a cell fusion protein; HN, hemagglutinin and neuraminidase protein; and M, a nonglycosolated envelope protein (50). Purified RNA is not infective and is found as a single strand with a molecular weight of about 10^7 daltons (1).

The outer envelope is a spherical lipid bilayer derived from the plasma membrane of the host cell in which the virus is grown. The envelope contains two types of glycoprotein spikes: HN which has hemagglutinating and neuraminidase activity, and F which induces cell fusion and hemolysis. The HN spike is responsible for the attachment of the virus to N-acetylneuraminic acid residues on the surface of erythrocytes. They are also believed to possess enzymatic activity to cleave N-acetylneuraminic acid residues which results in the release of viral particles from the cell surface (44).

The M protein lines the inner surface of the membrane. It is believed to play a role in nucleocapsid and viral envelope recognition during virus assembly (13). The F protein is composed of two disulfide-bonded polypeptide chains, F_1 and F_2. Proteolytic cleavage of these chains is required for biological activity of the F spike in membrane fusion (13). The nucleocapsid also contains copies of the P protein (designation for polymerase) and L protein (large protein). The P protein is closely associated with the template RNA. Both the L and P proteins are involved in the RNA

polymerase activities required for transcription of mRNA and replication of genomic RNA.

1.2 REPLICATION

The virions attach to neuraminic acid-containing receptors on the host cell membrane via the HN glycoprotein on the virus membrane. After adsorption, the F_o protein is cleaved and fusion of the virion with the cell membrane is induced by the F glycoprotein. After entry of the nucleocapsid into the cytoplasm, the virus uncoats and releases the genome from the nucleocapsid (Fig. 1). The viral RNA-dependent RNA polymerase is activated and transcribes the viral RNA minus genome into RNA plus mRNA (65).

Virus assembly takes place at the plasma membrane. Viral glycoproteins are inserted in the plasma membrane and gather into patches which exclude cellular proteins (13). It is suggested that the M protein is the scaffold for formation of the prospective virus envelope and that the glycoproteins are concentrated into the patches by the interaction between the cytoplasmic portion and the M protein (65). The nucleocapsid aligns underneath these patches in the plasma membrane. At completion of assembly the virus buds through this region and is released.

Cells infected with NDV require the presence of arginine in the medium for the synthesis of virions. Culture medium lacking this amino acid allows the synthesis of nucleocapsids, hemagglutinin and neuraminidase; however, budding of the nucleocapsids through the cell membrane is prevented (13).

1.3 EFFECT ON HOST CELL

Following infection of chicken embryo cells by NDV, cell protein synthesis (CPS) gradually decreases. By 6 hr post-infection, it is approximately 50% of that in uninfected cells (65) and by 9 hr post-infection, CPS is only 15% of that in uninfected cells (22). Concurrent with the inhibition of protein synthesis is a gradual transition from host-specified to virus-specified polypeptide synthesis (65).

Research indicates that viral proteins are required to inhibit cell protein synthesis (22). The addition of azauridine, which inhibits RNA synthesis, at the time of infection prevents CPS inhibition. When added 3 hr post infection, inhibition of CPS does occur. The addition of puromycin at the time of infection inhibits shut off of host cell protein synthesis. However, puromycin sensitivity is lost by 4.5 hr post infection, suggesting that viral translation has been achieved (22).

1.4 PROPHYLAXIS

Newcastle disease is highly contagious and attempts to control it by sanitary measures alone are often unsatisfactory. In areas where the disease has become endemic, vaccination programs involving the total poultry population have shown some success. After the Second World War, Hungary almost succeeded in eradicating the disease when all poultry were vaccinated during a three-month period (14). However, viral isolates occur periodically which are unusually virulent and require a different approach. In these situations, satisfactory results have been obtained by adopting measures of isolation and hygiene along with vaccination. This includes

disposal of refuse and infected birds by burial or incineration. The control of the movement of birds in infected areas has also been helpful.

To date, five types of vaccines are available: 1) live lentogenic (egg-adapted), 2) live mesogenic (egg-adapted), 3) live mesogenic (tissue culture-adapted), 4) live vaccine with mineral adjuvant for injection, and 5) inactivated. Generally, the more active vaccines (mesogenic) are used in areas where the disease is severe. While they impart long-lasting immunity, they are not recommended for chickens under 8 weeks of age and can be stressful for adults. Lentogenic vaccine programs are more likely to be used in areas where the disease is less virulent. They are safe to administer to birds of all ages, but do not always produce an immune response strong enough to provide effective protection. The main methods of administering vaccines are by injection, eye or nostril drop, drinking water, and spray or aerosol administration (1).

NDV has been recovered from a wide variety of species. While it is seen most often in domestic poultry, it is also capable of infecting ducks, geese, partridges and quails (1). Human infections are usually manifested as conjunctivitis (50).

2.0 ANTIVIRAL AGENTS

2.1 POKEWEED ANTIVIRAL PROTEIN

Pokeweed antiviral protein (PAP) is a cell wall protein of the pokeweed plant (*Phytolacca americana*) (23). It has a broad spectrum of antiviral activity, being effective against both RNA and DNA viruses, including poliovirus, herpes simplex virus, and influenza virus (3, 58, 63). The antiviral activity of pokeweed was first

reported in 1925 and the specific antiviral agent was identified in 1969 (23). In 1973, PAP was identified as a ribosome-inactivating protein (RIP) (69).

Ribosome-inactivating proteins are a group of related proteins obtained from a wide variety of plant species that have the ability to inactivate ribosomes through an enzymatic mechanism. They are divided into two categories: single-chain RIPs and dimeric RIPs. Single-chain RIPs, which includes PAP, consist of proteins of a single polypeptide chain approximately 30,000 dalton in size. They are relatively non-toxic to most intact cells. Dimeric RIPs, such as ricin and abrin, contain a toxin subunit of similar size plus an additional binding subunit. RIPs of this category are readily internalized by animal cells and are, therefore, extremely toxic (40). RIPs inhibit ribosomal function via an N-glycosidase activity by hydrolytic cleavage of the glycosidic linkage between the ribose and the adenine or guanine at A4234 or G4323 on the 28S rRNA (39).

PAP selectively inactivates the ribosomes of virus-infected cells. Ready et al. (1986) suggested that PAP acts as a 'suicidal' antiviral agent by gaining entrance to cells which have been breached by viral infection. By acting as a cell self-destruct mechanism, PAP is able to prevent transmission of the virus to uninfected cells. Although PAP is cytotoxic to uninfected HeLa and Vero cells at high concentrations (3 μM), low concentrations required to inhibit viral replication (0.25 μM) are not cytotoxic (3).

2.2 GUANIDINE HYDROCHLORIDE

Guanidine hydrochloride selectively inhibits the growth of many picornaviruses, several togaviruses, and several plant viruses (82). At concentrations of 5 M or higher, it is a protein denaturant, but at low concentrations, such as 0.1-1.5 mM, it selectively inhibits the synthesis of viral RNA (11). The initial interest in guanidine as an antiviral agent diminished when it was found to produce a high rate of resistant mutations (60). Research has shown that picornaviruses become completely resistant after only a few passages in the presence of the drug (77). In addition, while it inhibits poliovirus replication at low concentrations *in vitro*, it does not decrease the incidence of disease in polio-infected monkeys due to difficulties in maintaining an adequate blood level (6711).

There are several mechanisms proposed for the antiviral activity of guanidine, including inhibition of initiation of viral RNA replication (31), allosteric inhibition of RNA polymerase(6), and interference between the replication complex and cell membranes (11). Recent research suggests that guanidine inhibits the encapsidation of poliovirus RNA (10, 85).

2.3 RIBAVIRIN (VIRAZOLE)

First synthesized in 1972, ribavirin is a broad spectrum, non-interferon inducing virustatic agent (87, 36). It is structurally related to guanosine, which can reverse its effect (87). Ribavirin inhibits a wide variety of DNA and RNA viruses; however, it is approved in the U.S. only for therapy of severe respiratory infections in

infants and children caused by respiratory syncytial virus (75). It is probably the most thoroughly investigated antiviral imidazole nucleoside analogue.

Although the specific mode of action has not been determined, there are three proposed mechanisms by which ribavirin is believed to inhibit virus replication: inhibition of IMP dehdyrogenase (80), inhibition of mRNA guanyltransferase (47), and inhibition of viral RNA polymerase (71).

Ribavirin is rapidly phosphorylated by cellular adenosine kinase to its 5'-monophosphate (RMP), which competitively inhibits the enzyme inosine monophosphatase (IMP) dehydrogenase. As IMP dehydrogenase is involved in guanosine monophosphate (GMP) synthesis, this results in a decrease of guanosine triphosphate levels (GTP) (87). Within the cell, ribavirin exists mainly as ribavirin-5'-triphosphate (RTP), which has been shown to inhibit mRNA guanyltransferase. This enzyme is responsible for the addition of the 7-methylguanosine 'cap' to the 5'-terminus of viral mRNA. RTP also prevents the elongation of RNA by inhibiting virus-specific RNA polymerase (88).

3.0 COMBINATIONAL THERAPY

Combinational therapy involves the use of two or more antiviral agents with distinct modes of action. This approach has the potential to reduce the probability of drug resistance as well as minimize potential toxic side effects. By acting at different target sites, the combination of two antivirals may produce a synergistic effect. In some instances, increased toxicity has also been observed (antagonistic effect).

There are three recognized interactions involved in combinational therapy (18). The first possible interaction is known as zero interaction. Zero interaction occurs when the combined effect of the drugs reflects a simple summation of their individual effects. The second possible interaction, synergy, occurs when the combined effect of the drugs is greater than zero interaction. The final possible interaction is antagonism, which occurs when their combined effect is less than zero interaction. By varying the concentrations of each antiviral drug, any one of the above interactions can result.

Synergistic effect has been reported for interferon and adenine arabinoside monophosphate (ara-AMP), interferon and acyclovir, and adenine arabinoside (ara-A) and acyclovir against herpes simplex virus (12). Combinations of amantadine with either ribavirin or rimantadine have proven effective against influenza A virus (25, 26).

The determination of zero interaction can be approached using any one of four standard methodologies: fractional product method, median effect principle, fractional inhibitory method and original isobologram method (17, 18). The isobologram method is used in this study as it is considered valid for all combinations of drugs regardless of their mode of action or the nature of their dose response curves (18). Synergy was determined using a modified version of the dissimilar-site additivity equation described by Prichard and Shipman (1990).

Viral replication proceeds in a sequentially regulated fashion (Fig. 1). Thus, the synthesis of viral capsid protein cannot proceed until successful completion of viral RNA synthesis. We propose that a synergistic effect occurs when a drug combination activates a new or unique antiviral mechanism that is unlike the original

individual modes of action. This is reasonable since a drug that inhibits early viral protein synthesis would be expected to negate or cancel out the effect of a drug that inhibits late viral protein synthesis, and the net effect would be indistinguishable from that of the first drug alone. Based on previous research which showed a synergistic antiviral effect on other viruses (76, 86), it is hypothesized that combinations of pokeweed antiviral protein with guanidine and ribavirin will display a synergistic antiviral effect against the replication of Newcastle disease virus.

MATERIALS AND METHODS

Cell Culture. Chicken embryo cells were obtained from 10-12 day old embryonated chicken eggs (Ideal Hatchery, Cameron, TX) and cultivated in 75 cm^2 flasks (Corning Glass Works, Corning, NY) at 37° C in Eagle's minimum essential medium (E-MEM)(Sigma Chemical Co., St. Louis, MO) containing 10% fetal bovine serum (JRH Biosciences, Lenexa, KS), glutamine (2 mM/ml), 0.075% $NaHCO_3$, penicillin (100 U/ml), streptomycin (100 µl/ml), and 1.0% nonessential amino acids (Sigma Chemical Co., St. Louis, MO). Cells were maintained in E-MEM which contained 5% fetal bovine serum, glutamine 92 mM/ml), 0.150% $NaHCO_3$, penicillin (100 U/ml), streptomycin (100 U/ml), 5.0% tryptose phosphate broth, and 1.0% nonessential amino acids (Sigma Chemical Co., St. Louis, MO). Cells were passed approximately once every 3 days using trypsin (2.5 mg/ml). Chicken embryo cells that were propagated in tissue culture plates (Corning Glass Works, Corning, NY) were grown in E-MEM which contained 10% fetal bovine serum, glutamine (2 mM/ml), 0.225% $NaHCO_3$, penicillin (100 U/ml), streptomycin (100 µl/ml), and 1.0% nonessential amino acids (Sigma Chemical Co., St. Louis, MO). Chicken embryo cells were incubated at 37°C in 5% CO_2.

Cell Storage. Chicken embryo cell stocks were frozen in medium which contained 15-20% DMSO and 80-85% FBS. Cells suspended in freezing media were placed in cryogenic vials (Nalgene Company, Rochester, New York). Chicken embryo cells were placed directly into liquid nitrogen for permanent storage.

Virus Production. Newcastle disease virus strain NDV VR-109 (American Type Culture Collection, Rockville, MD) were propagated in chorioallantoic fluid of 9-10-day-old embryonated chicken eggs (Ideal Hatchery, Cameron, TX) and CEF monolayers for 48 h at 37°C. Harvested chorioallantoic fluid and cell extracts were stored at -80°C and contained approximately 1.0×10^{10} hemagglutination units (HAU) per ml for NDV.

Hemagglutination Assays. Chorioallantoic fluid and infected-cell extracts were diluted two-fold using phosphate buffered saline in 96-well microtiter plates (Dynatech Laboratories, Chantilly, VA). A 0.2% - 0.5% solution of chicken red blood cells containing approximately $1.6 - 4 \times 10^7$ cells per ml was added in 0.1 ml aliquots to each well. Chicken red blood cells were washed and diluted in phosphate-buffered saline (PBS), 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 PBS tablets in 200 ml of deionized water (Sigma Chemical Co., St. Louis, MO), pH 7.4. Hemagglutination was determined following 45-60 min. incubation at 37°C.

Antiviral Agents. PAP was extracted from the spring leaves of the plant *Phytolacca americana* and purified by ammonium sulfate fractionization followed by ion exchange chromatography. It was filtered through a 0.45 μM membrane filter (Type HA, Millipore Corp., Bedford, MA), neutralized to pH of 7.2 and stored at 20°C. PAP concentration was determined by spectroscopy. A 1:10 dilution of PAP

was prepared using deionized water. The light absorbance of this dilution was then determined at a wavelength of 280 nm. Absorbance multiplied by ten (1:10 dilution) divided by the extinction coefficient for PAP (0.83) yields mg/mol. Dividing mg/ml by 2.9 yields the quantity of micromoles in solution (57). Guanidine was purchased from Sigma Chemical Co., St. Louis, MO. Ribavirin was obtained from ICN Pharmaceuticals, Inc.

Cytotoxicity. Chicken embryo cells were cultivated in E-MEM for 24 h in 96-well plates (Flow Laboratories Inc., Hamden, CN). Cell monolayers were washed once with Earles balanced salt solution (EBSS) (Sigma Chemical Co., St. Louis, MO) and 0.1 ml of E-MEM containing each antiviral was added. For cytotoxicity studies, E-MEM contained 10% fetal bovine serum (JRH Biosciences, Lenexa, KS), glutamine (2 mM/ml), 0.075% $NaHCO_3$, penicillin (100 U/ml), streptomycin (100 µl/ml), 5.0% and 1.0% nonessential amino acids and 5.0% tryptose phosphate broth (Sigma Chemical Co., St. Louis, MO). Cells were incubated at 39°C in 5% CO_2 for 24 h. and cell viability was determined using the MTT method previously described by Pauwel et. al (1988). After the antiviral treatment, all wells were treated with 0.025 ml of 200 µg/ml of 3(4,5-dimethyl-thiazolyl-2)-2,5 diphenyl tetrazolium bromide (MTT) diluted in PBS (Sigma Chemical Co., St. Louis, MO). MTT is converted to a soluble dye by active mitochondria in living cells, which is then measured spectrophotometrically. After incubation in the presence of MTT for 2 h, the cells were solubilized using 0.1 ml acidified isopropanol (0.01% conc. HCl) with 10% Triton X-100 (Eastman Kodak Company, Rochester, NY) for 10 minutes and optical density was determined. Plates

were read at 570 nm with background subtraction at 650 nm using a Biotek EL311 microplate reader. The cytotoxic concentrations (CCx) were determined as the dosage at which the absorbance was reduced by (x) percentage.

Combinational cytotoxicity was performed in the same manner as previously described with the exception that the cell monolayers were incubated with combinations of the antivirals at different concentrations.

Virus Growth Curve *in vitro*. Cell monolayers (approximately 1.3 x 10^6 cells) were infected with 1.0 x 10^9 hemagglutinating units (HAU) which yielded a multiplicity of infection (MOI) of approximately 770 HAU per cell. Virus was allowed to adsorb for 1 h at 37°C with shaking every 10 min. Cells were washed twice with EBSS to remove unadsorbed virus. E-MEM containing 10% fetal bovine serum (JRH Biosciences, Lenexa, KS), glutamine (2 mM/ml), 0.075% $NaHCO_3$, penicillin (100 U/ml), streptomycin (100 µl/ml), 5.0% tryptose phosphate broth, and 1.0% nonessential amino acids (Sigma Chemical Co., St. Louis, MO) was added. Infected cells were incubated at 37°C and hemagglutination titers were determined at various times post infection.

Effects of Antiviral Agents *in vitro*. Chicken embryo cell monolayers grown in E-MEM were washed once with EBSS and 1.0 x 10^9 of NDV which yielded a MOI of approximately 770 and 2300 HAU per cell together with antiviral was added. Antivirals were diluted in E-MEM which contained 10% fetal bovine serum (JRH Biosciences, Lenexa, KS), glutamine (2 mM/ml), 0.075% $NahCO_3$, penicillin (100

U/ml), streptomycin (100 µl/ml), 1.0% nonessential amino acids (Sigma Chemical Co., St. Louis, MO) and 5.0% tryptose phosphate broth. Virus was diluted in E-MEM which contained 10% fetal bovine serum (JRH Biosciences, Lenexa, KS), glutamine (2mM/ml), 0.075% $NaHCO_3$, penicillin (100 U/ml), streptomycin (100 µl/ml), 1.0% nonessential amino acids (Sigma Chemical Co., St. Louis, MO) and 5.0% tryptose phosphate broth. For single drug assays, solutions with two-fold concentrations of each antiviral were mixed with equal volumes of solutions with two-fold concentrations of NDV. For drug combination assays, an equal volume of four-fold concentrations of each antiviral drug were mixed in equal volumes, resulting in a solution containing a two-fold concentration of each antiviral, before addition to an equal volume of E-MEM containing a two-fold concentration of virus. Virus was allowed to adsorb for 1 h at 37°C in 5% CO_2 and shaken every 10 minutes in the presence or absence of antivirals. Cells were gently washed once with EBSS to remove unadsorbed virus. Medium containing a single antiviral or combination of antivirals was added to infected cell monolayers. The monolayers were harvested following a 24 h incubation at 39°C. Both single drug and drug combination mixtures were tested in duplicate.

Combinational Drug Analysis and Cytotoxicity. The predicted percent inhibition of viral multiplication was characterized using a modification of the dissimilar-site additivity equation described previously by Prichard and Shipman (1990).

$$\% \text{ Predicted} = X + Y(1-X)$$

X and Y represent the inhibitory effect of drugs 1 and 2 alone. Synergy is the percentage at which the combination is more efficient than the antivirals used singularly. Synergy, zero-interaction, and antagonism were determined irrespective of drug dose response or mechanism (82). The data was graphed three-dimensionally using an XYZ surface fill graph on Delta Graph Professional 3.5 program (Delta Point, Monterey, CA).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Drug combinations showing the greatest levels of synergy and antagonism against NDV replication were evaluated for their effect on protein translation. Chicken embryo cell monolayers cultivated in E-MEM were washed once with EBSS and 2.8×10^9 HAU of NDV (MOI = 2300 HAU/cell) along with antiviral were added. The antiviral drugs were diluted in E-MEM containing 10% fetal bovine serum (JRH Biosciences, Lenexa, KS), glutamine (2 mM/ml), 0.075% $NaHCO_3$, penicillin (100 U/ml), streptomycin (100 µl/ml), and 1.0% nonessential amino acids (Sigma Chemical Co., St. Louis, MO). Virus was diluted in E-MEM containing 10% fetal bovine serum (JRH Biosciences, Lenexa, KS), glutamine (2 mM/ml), 0.075% $NaHCO_3$, penicillin (100 U/ml), streptomycin (100 µl/ml), and 1.0% nonessential amino acids (Sigma Chemical Co., St. Louis, MO). For single drug assays, solutions containing two-fold concentrations of each antiviral agent were mixed with equal volumes of solutions containing two-fold concentrations of NDV. Virus was allowed

to adsorb for 1 h at 37°C in 5% CO_2 and shaken every 10 minutes in the presence or absence of antivirals. Cells were gently washed once with EBSS to remove unadsorbed virus. Medium containing either a single antiviral agent or a combination of antiviral agents was added to infected cell monolayers. At 6 h p.i., the media was removed and replaced with antiviral agents diluted in methionine free media (Flow Laboratories, Rockville, MD) containing 20 μCi/ml ^{35}S-methionine (ICN Biomedicals, Inc., Irvine, CA). After 1 h. at 37°C, the monolayers were scraped, removed, and stored at -70°C. Both single drug and drug combination mixtures were tested in duplicate.

Samples were thawed, prepared in a premixed 2X Laemmli sample buffer (BioRad Laboratories, Hercules, CA) and boiled for 3 minutes. SDS-PAGE was performed in a mini-Protean electrophoretic apparatus (BioRad Laboratories, Hercules, CA). A 10-20% Tris-glycine with 4% stacking, 10-well precast Ready Gels (BioRad Laboratories, Hercules, CA) was used and each well was loaded with a 15 μl sample of cellular extracts. The used of a linear gradient gel allows separation of both high and low molecular weight bands. A range of molecule sizes can be separated on linear gradient gels because molecules slow down as they are restricted by the decreasing pore size of the gel. Electrophoresis was applied to the 15μl samples using 10X Tris/Glycine/ SDS Buffer (BioRad Laboratories, Hercules, CA) at 200 V for approximately 45 minutes using a PS500XT DC power supply (Hoefer Scientific Instruments, San Francisco). Kaleidoscope high molecular weight protein standards were used to determine relative molecular mass of the bands in the gel (BioRad Laboratories, Hercules, CA). The gels were stained overnight with 0.25% Coomassie

Blue R (Sigma, St. Louis, MO) in 50% methanol and 10% acetic acid. The gels were then destained in 50% methanol and 10% acetic acid for several changes or until they were completely destained. It was then placed in DI water for 1 to 3 h. for rehydration. The gel was placed in gel drying solution for 1 h. (BioRad Laboratories, Hercules, CA) and dried between two sheets of cellulose acetate using a Gelair Dryer (BioRad Laboratories, Hercules, CA).

Autoradiography. Autoradiographs of ^{35}S-labeled viral proteins were obtained using the Kodak Biomax Transcreen LE intensifying screen system and Biomax MS film (Eastman Kodak Company, Rochester, NY). All handling and exposure of Biomax MS film was done in complete darkness or using a proper safelight. The active gel was placed on the phosphor surface of the folder and the film was inserted in the folder. The film screen combination was then placed into a cassette and stored at -80°C for 24 to 48 hours. Upon removal from the cassette, the exposed film was placed in Kodak GBX developer and replenisher (Eastman Kodak Company, Rochester, NY) for 5 min. at 25°C. It was rinsed for 30 seconds in a water bath and placed in Kodak GBX fixer and replenisher (Eastman Kodak Company, Rochester, NY) for 5 to 10 min with moderate agitation at 25°C. The developed film was washed 5-10 min in continuous running water and dried in a dust-free area at 25°C.

Image Analysis. Autoradiographs were scanned using an Apple Color One Scanner 600/27. Image analysis was performed using NIH Image Video Densitometry Manager, Version 1.61 (National Institutes of Health, USA).

RESULTS

Effect of antivirals on virus replication in Chicken Embryo Cells. The effective concentration (EC) of each antiviral required to inhibit viral replication by25, 50, and 75 percent in chicken embryo cells was determined. The antiviral activity of ribavirin was nonlinear and followed 2nd order kinetics with an EC_{25}, EC_{50}, and EC_{75} at 4, 6.12, and 50 μg/ml (Fig. 4). The effect of guanidine hydrochloride on NDV in chicken embryo cells is shown in Figure 3. Antiviral activity of guanidine hydrochloride was nonlinear with an EC_{25}, EC_{50}, and EC_{75} at 115.21, 312.5, and 1250 μg/ml (Fig. 3). The effect of PAP on NDV replication in chicken embryo cells is shown in Figure 1. Antiviral activity of PAP was nonlinear and followed 2nd order kinetics with an EC_{50}, and EC_{75} at 0.025 and 0.051μM, respectively (Fig. 2).

Cytotoxicity of antivirals. The effect of each antiviral agent singularly on the viability of chicken embryo cells was determined (Tables 1-3). The concentration of PAP required to inhibit 50% (CC_{50}) was 8 μM (Table 1), which was more than eight-fold greater than the ED_{50} concentration of less than 1 μM for NDV. The CC_{50} concentration for guanidine was 2850 μg/ml (Table 2), which was nine-fold greater than the ED_{50} concentration at 312 μg/ml. Ribavirin was not cytotoxic in chicken embryo cells at concentrations up to 100 μg/ml (Table 3) and had an ED_{50} concentration of 6.12 μg/ml.

Combinational drug analysis. Because synergy and antagonism have been found to be concentration dependent, antivirals were tested at low, medium, and high effective doses. The effect of combinations of antivirals against NDV multiplication is shown in Table 4. Inhibition observed was calculated as a percentage of a control and was the average of six replicates. The percent inhibition predicted was derived from individual dose-response curves for the antivirals. Observed minus predicted indicates the percent inhibition which is not due to the individual dose-response curves. A synergistic antiviral effect occurred when the observed percent inhibition exceeded the predicted percent inhibition. Conversely, an antagonistic effect occurred when predicted percent inhibition exceeded observed percent inhibition.

A majority of the combinations of PAP with ribavirin tested were found to be antagonistic (Fig. 6). High (0.0625 μM) and medium (0.031μM) effective concentrations of PAP in combination with low, medium and high concentrations of ribavirin (3.125, 6.25, 25.0 μg/ml) were antagonistic, with the exception of PAP at 0.0625μM with ribavirin at 6.25 μg/ml, which exhibited zero interaction. The low (0.015 μM) effective concentration of PAP in combination with ribavirin at effective concentrations of 3.125 μg/ml and 6.25 μg/ml yielded synergy. The combination of PAP (0.015 μM) and ribavirin (3.125 μg/ml) yielded the highest level of synergy (11%). The combination of 0.015μM of PAP with 25 μg/ml of ribavirin produced the most antagonistic result in the study (27.78%).

The majority of the combinations involving PAP and guanidine were synergistic (Fig. 5). Combinations of medium (0.031 μM) effective concentrations of PAP with guanidine at 78 and 312.5 μg/ml were antagonistic. All combinations of

high (0.0625 μM) and low (0.015) effective concentrations of PAP with guanidine at 78 μg/ml, 312.5 μg/ml, and 1250 μg/ml were synergistic. In addition, the combination of PAP at 0.031 μM with guanidine at 1250 μg/ml was also synergistic.

Autoradiography. The protein profiles resulting from the use of PAP, guanidine and a synergistic combination of PAP and guanidine are shown in Figure 7 and the relative density of each protein band is shown in Table 5. The use of PAP alone resulted in no significant changes in the concentration of viral proteins. The effect of guanidine alone resulted in an increase in L (110%), HN (80%), HN_o (40%), F_o (40%), P (20%) and NP (10%). The synergistic combination of PAP and guanidine also resulted in increased concentrations of all viral proteins: L (120%), HN (140%), HN_o (80%), F_o (80%), P (20%), and NP (10%).

Protein synthesis resulting from the use of PAP, ribavirin and a synergistic combination of PAP and ribavirin are shown in Figure 8 and the relative density of each protein band is shown in Table 6. The use of PAP alone resulted in no significant change in viral protein synthesis. Ribavirin alone resulted in an increase in L (25%), HN (14%), and P (10%) viral proteins, with no significant changes in concentration of the remaining proteins (Table 6). The synergistic combination of PAP with ribavirin resulted in decreased concentrations of HN_o (16%), F_o (15%), P (19%), and NP (35%).

Figure 2. Effect of PAP on NDV multiplication. Percent yield of virus HAp from virus infected chicken embryo cells treated with PAP. Values represent an average of six replicates (n = 6).

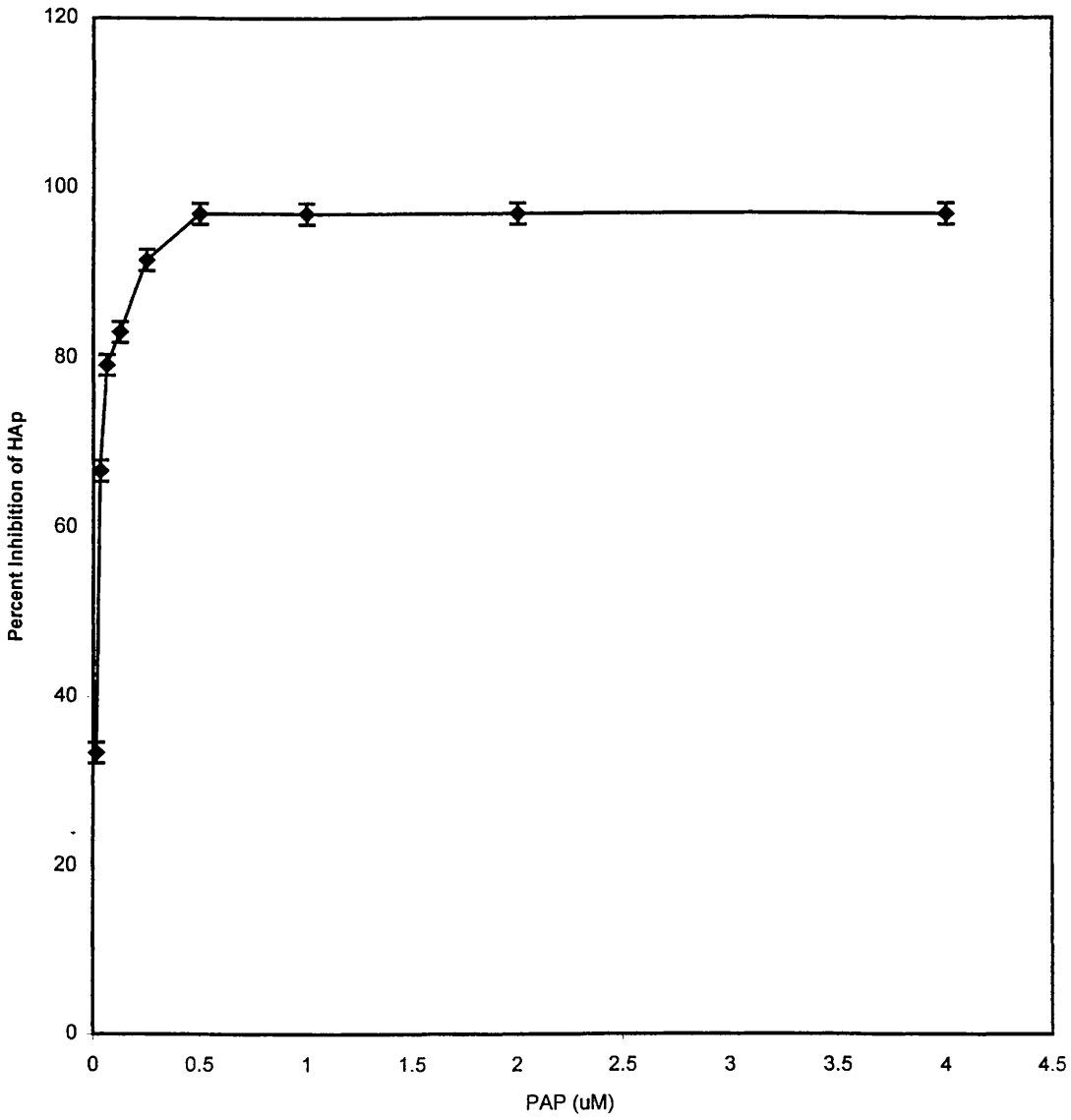
120
100
80
60
40
20
0
Percent Inhibition of HAp
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
PAP (uM)

Figure 3. Effect of guanidine on NDV multiplication. Percent yield of virus HAp from virus infected chicken embryo cells treated with guanidine. Values represent an average of six replicates (n = 6).

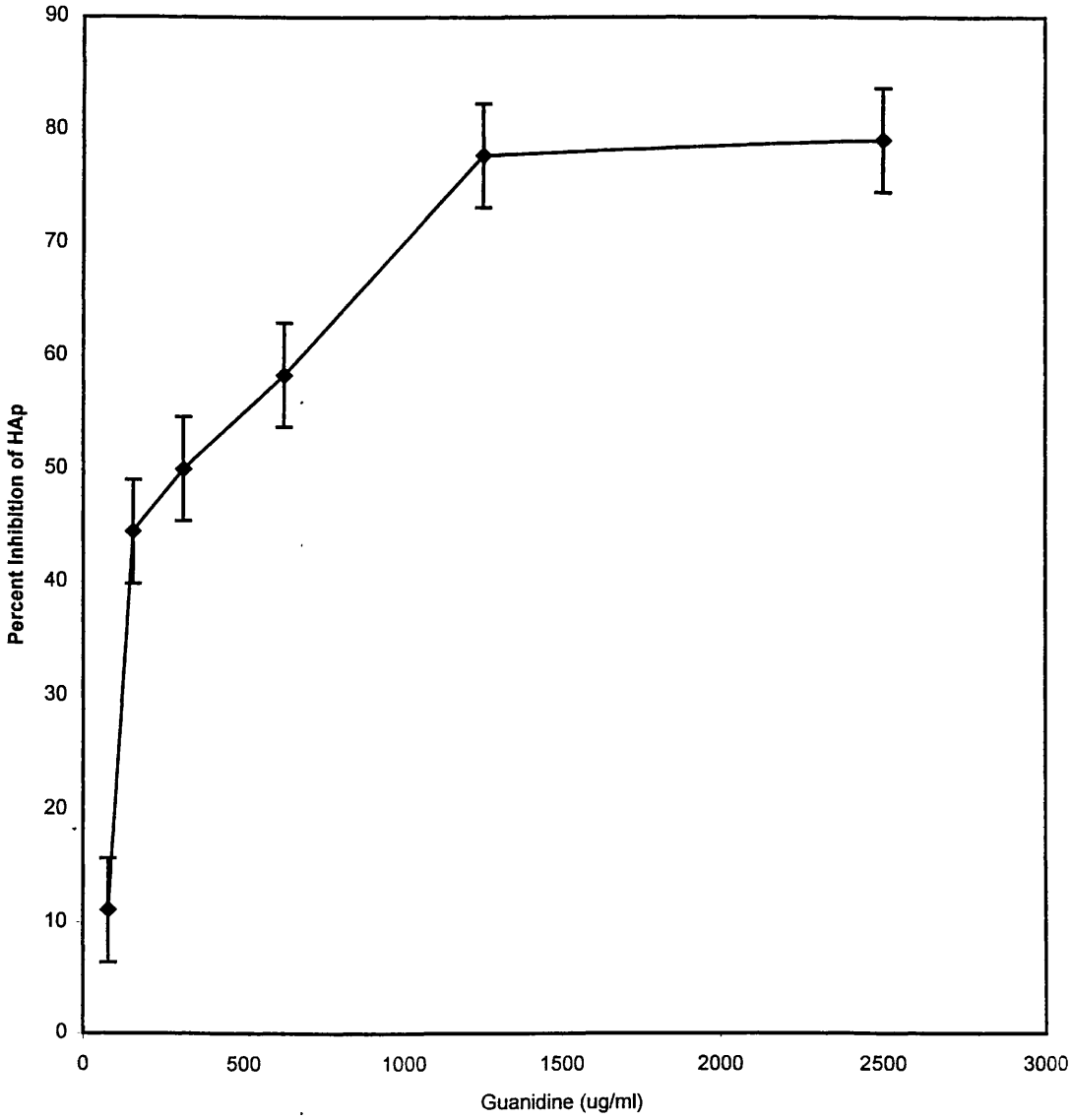
90
80
70
60
50
40
30
20
10
0
Percent Inhibition of HAp
0
500
1000
1500
2000
2500
3000
Guanidine (ug/ml)

Figure 4. Effect of ribavirin on NDV multiplication. Percent yield of virus HAp from virus infected chicken embryo cells treated with ribavirin. Values represent an average of six replicates (n = 6).

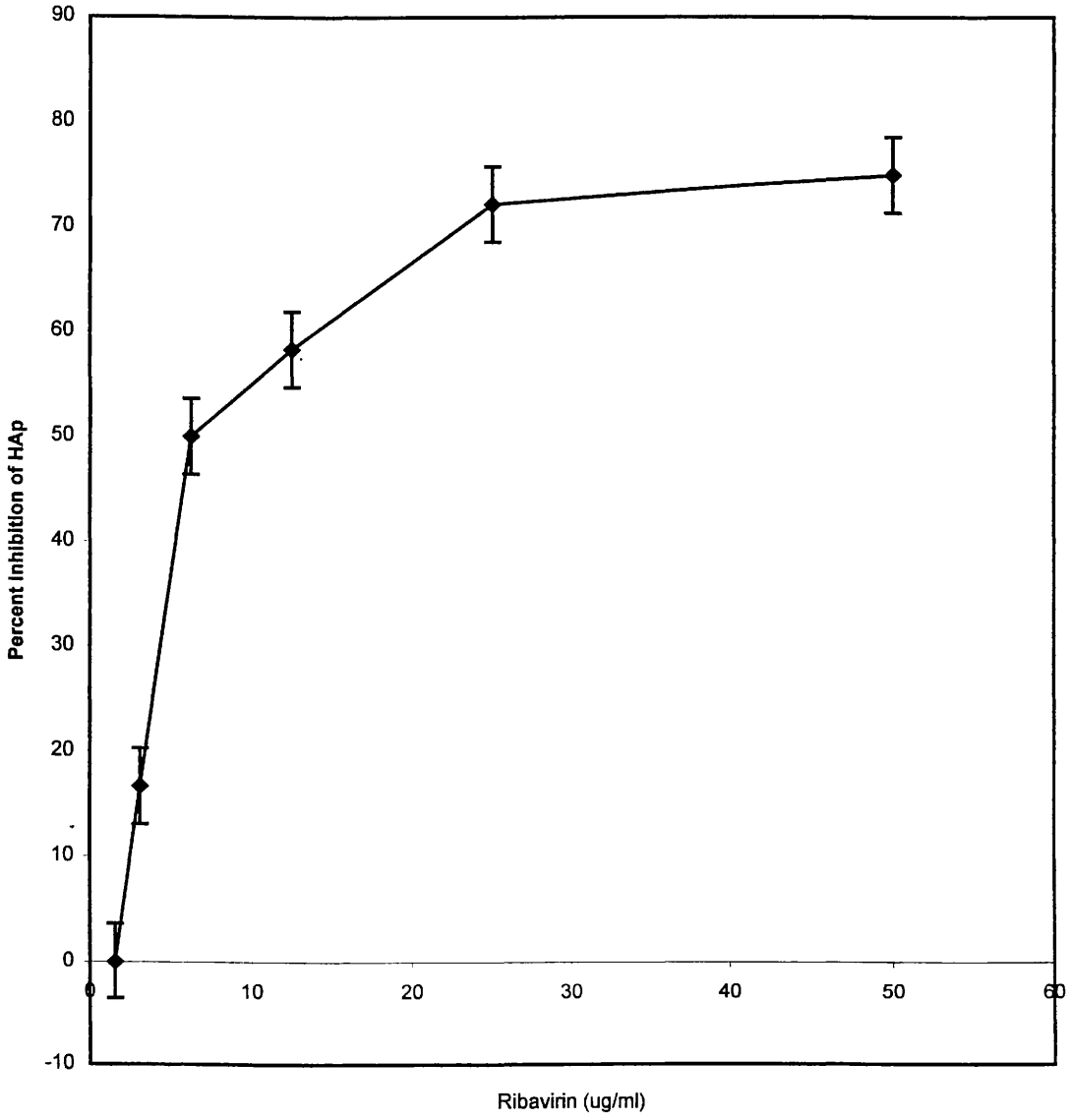
90
80
70
60
50
40
30
20
10
0
-10
Percent Inhibition of HAp
0
10
20
30
40
50
60
Ribavirin (ug/ml)

Figure 5. Effect of PAP and guanidine in combination on NDV virus multiplication in chicken embryo cells. Synergistic values are shaded dark blue for the most synergistic, light blue for the least synergistic. Antagonistic values are shaded dark red for the most antagonistic to light red for the least antagonistic. Values represent an average of two experiments each with three replicates (n = 6).

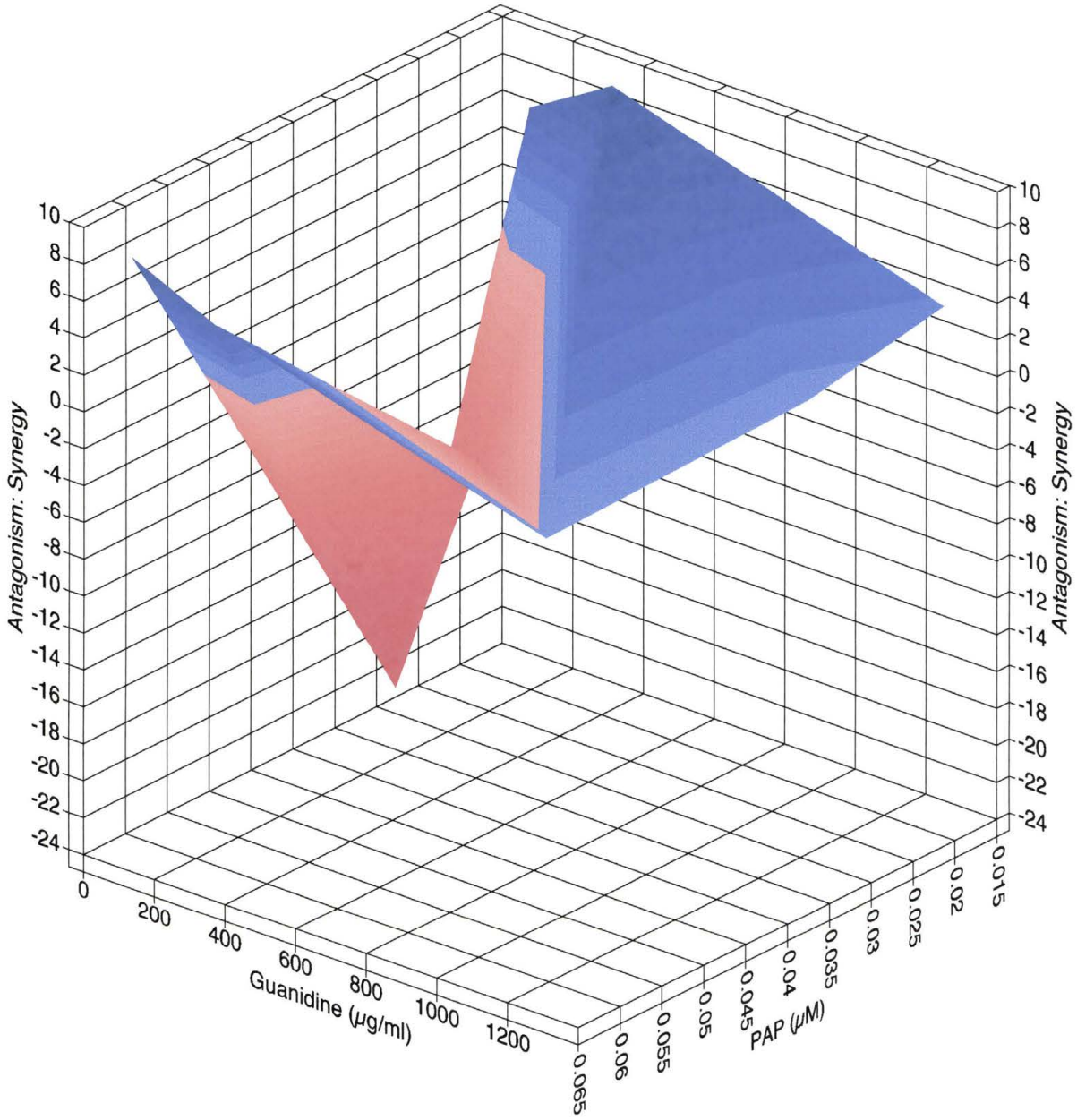
Antagonism: Synergy
10
8
6
4
2
0
-2
-4
-6
-8
-10
-12
-14
-16
-18
-20
-22
-24
0
200
400
600
800
1000
1200
Guanidine (µg/ml)
0.065
0.06
0.055
0.05
0.045
0.04
0.035
0.03
0.025
0.02
0.015
PAP (µM)
Antagonism: Synergy

Figure 6. Effect of PAP and ribavirin in combination on NDV virus multiplication in chicken embryo cells. Synergistic values are shaded dark blue for the most synergistic, light blue for the least synergistic. Antagonistic values are shaded dark red for the most antagonistic to light red for the least antagonistic. Values represent an average of two experiments each with three replicates (n = 6).

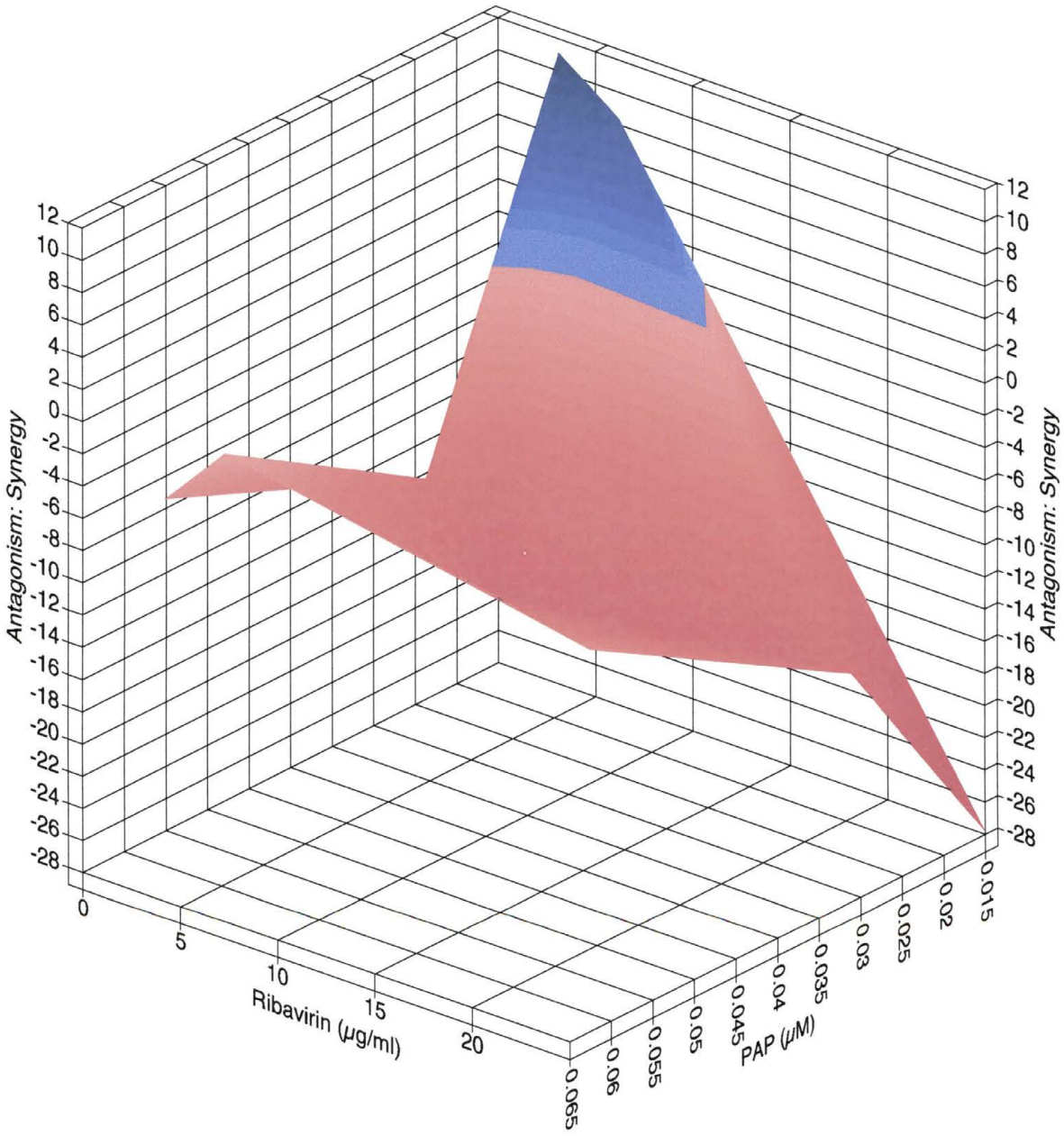
Antagonism: Synergy
12
10
8
6
4
2
0
-2
-4
-6
-8
-10
-12
-14
-16
-18
-20
-22
-24
-26
-28
0
5
10
15
20
Ribavirin (μg/ml)
0.065
0.06
0.055
0.05
0.045
0.04
0.035
0.03
0.025
0.02
0.015
PAP (μM)
Antagonism: Synergy

Figure 7. Autoradiograph image of NDV proteins synthesized in the presence of PAP and guanidine, individually and in combination. CX = Control; SYN = Synergistic combination of PAP (0.015 μM) and Guanidine (78 μg/ml); PAP = pokeweed antiviral protein (0.015 μM); GUA = Guanidine (78μg/ml).

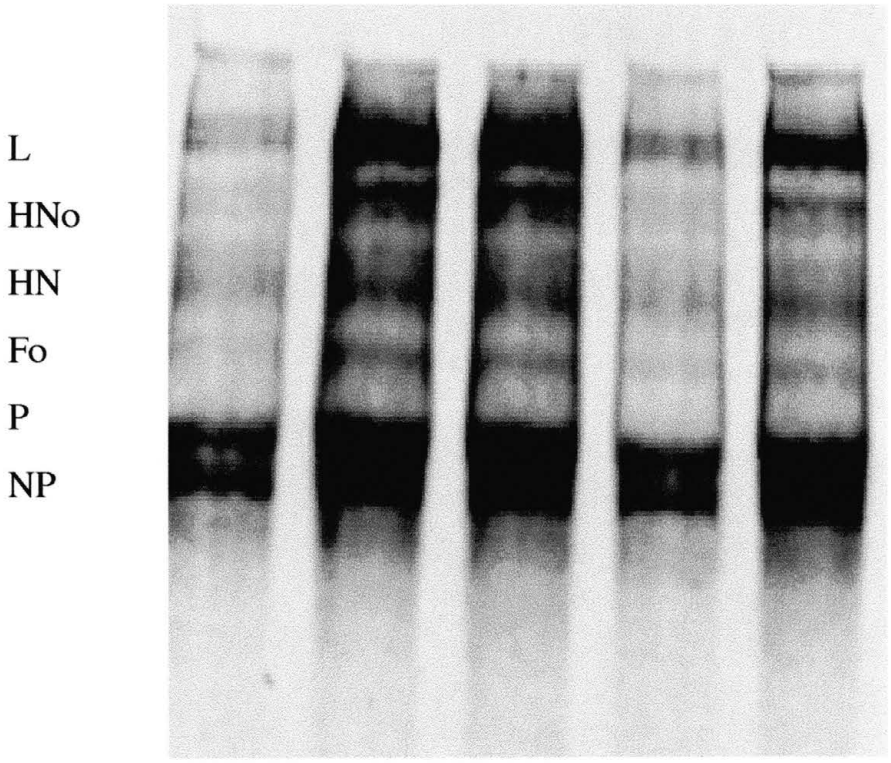
L
HNo
HN
Fo
P
NP
CX
SYN
SYN
PAP
GU

Figure 8. Autoradiograph image of NDV proteins synthesized in the presence of PAP (0.015 μM) and ribavirin (3.125 μg/ml), individually and in combination. CX = Control; SYN = Synergistic combination of PAP (0.015 μM) and Ribavirin (3.125μg/ml); PAP = pokeweed antiviral protein (0.015 μM); RIB = Ribavirin (3.125 μg/ml).

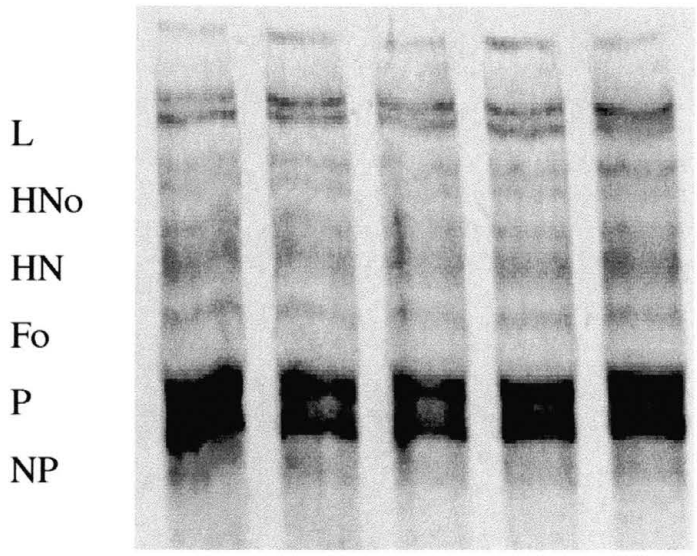
L
HNo
HN
Fo
P
NP
CX
SYN
SYN
PAP
RV

TABLE 1. Cytotoxicity of PAP for CEC cells

Conc. PAP (μM)[a]	Observed % Nonviable[b]
8.00	48.48 ± 0.03[c]
4.00	30.13 ± 0.02
2.00	25.78 ± 0.02
1.00	5.94 ± 0.04
0.50	4.10 ± 0.03
0.25	0 ± 0.03
0.12	0 ± 0.03
0.06	0 ± 0.03
0.03	0 ± 0.02
.05625	0 ± 0.03
0.00	0 ± 0.02

[a] Values are average of 16 replicates (n = 16).
[b] The percent of cell death after 24 hours exposure to antivirals.
[c] Standard error

TABLE 2. Cytotoxicity of Guanidine for CEC cells

Conc. Guanidine (μg/ml)[a]	Observed % Nonviable[b]
5000.00	89.97 ± 0.00[c]
2500.00	43.59 ± 0.03
1250.00	26.40 ± 0.01
625.00	16.79 ± 0.02
312.50	9.28 ± 0.00
156.25	0 ± 0.02
78.125	0 ± 0.02
39.06	0 ± 0.02
19.53	0 ± 0.02
9.75	0 ± 0.02
0.00	0 ± 0.05

[a] Values are average of 16 replicates (n = 16).
[b] The percent of cell death after 24 hours exposure to antivirals.
[c] Standard error

TABLE 3. Cytotoxicity of Ribavirin for CEC cells

Conc. Ribavirin (μg/ml)[a]	Observed % Nonviable[b]
100.00	0.50 ± 0.02[c]
50.00	0.00 ± 0.03
25.00	0.00 ± 0.02
12.5	0.00 ± 0.02
6.25	0.00 ± 0.02
3.12	0.00 ± 0.03
1.56	0.00 ± 0.03
0.78	0.00 ± 0.02
0.39	0.00 ± 0.06
0.19	0.00 ± 0.05
0.00	0 ± 0.02

[a] Values are average of 16 replicates (n = 16).
[b] The percent of cell death after 24 hours exposure to antivirals.
[c] Standard error

TABLE 4. *In vitro* inhibition of NDV replication by PAP in combination with either guanidine or ribavirin

PAP [a] (μM)	Ribavirin [b] (μg/ml)	Guanidine [c] (μg/ml)	% Inhibition Observed	% Inhibition Predicted [d]	% Observed – % Predicted
.0625	3.125	0	79.16	83.33	-4.17
.0625	6.25	0	87.50	87.50	0.00
.0625	25	0	87.50	91.66	-4.16
.0625	0	78	87.50	79.16	8.34
.0625	0	312.5	93.75	87.50	6.25
.0625	0	1250	93.75	92.71	1.04
.031	3.125	0	66.66	77.77	-11.11
.031	6.25	0	66.66	83.33	-16.67
.031	25	0	75.00	88.88	-13.88
.031	0	78	50.00	72.21	-22.21
.031	0	312.5	75.00	83.33	-8.33
.031	0	1250	91.66	90.27	1.39
.015	3.125	0	66.66	55.55	11.11
.015	6.25	0	75.00	66.67	8.33
.015	25	0	50.00	77.78	-27.78
.015	0	78	50.00	44.44	5.56
.015	0	312.5	75.00	66.67	8.33
.015	0	1250	83.33	80.55	2.78

[a] Effective concentration 25, 50, and 75 for PAP are <0.016, 0.025, and 0.051 μM, respectively.
[b] Effective concentration 25, 50, and 75 for ribavirin are 4, 6.12, and 50 μg/ml, respectively.
[c] Effective concentration 25, 50, and 75 for guanidine are 115.21, 312.5, and 1250 μg/ml, respectively.
[d] Derived from individual dose-response curves for PAP, guanidine, and ribavirin. Predicted values are additive effects of two compounds.

Table 5. Effect of pokeweed antiviral protein in combination with guanidine on viral protein synthesis.

Viral Protein	Control[a]	PAP + Guanidine[b]	PAP[c]	Guanidine[d]
L	109[e]	240	146	231
HN	88	214	97	164
HNo	114	202	112	160
HN	95	172	91	134
P	202	244	214	237
NP	225	247	229	244

[a] Virus only
[b] Low effective concentration for PAP (0.015μM)
[c] Synergistic combination of PAP (0.015 μM) and guanidine (78 μg/ml)
[d] Low effective concentration for guanidine (78 μg/ml)
[e] Density of autoradiographic protein bands was determined by analysis with NIH Image Version 1.61.

Table 6. Effect of pokeweed antiviral protein in combination with ribavirin on viral protein synthesis.

Protein	Control[a]	PAP + Ribavirin[f]	PAP[c]	Ribavirin[d]
L	122[e]	125	133	153
HN	106	96	101	121
HNo	136	117	119	137
Fo	122	106	110	118
P	216	182	229	237
NP	232	174	238	241

[a] Virus only
[b] Low effective concentration for PAP (0.015μM)
[c] Synergistic combination of PAP (0.015 μM) and ribavirin (3.125 μg/ml)
[d] Low effective concentration for ribavirin (3.125 μg/ml)
[e] Density of autoradiographic protein bands was determined by analysis with NIH Image Version 1.61.

DISCUSSION

In recent years, several studies have reported the synergistic effect of combinations of antiviral agents. Synergistic inhibition of HIV by the interaction of αA interferon with AZT has been described (9). Combined drug chemotherapy may result in the use of reduced concentrations of antiviral agents while maintaining their antiviral effectiveness. In addition, synergistic drug combinations reduce drug toxicity and prevent the emergence of resistant virus strains. Finally, the study of antiviral synergy provides insight into the process of viral replication. Pokeweed antiviral protein (PAP) in combination with guanidine has been reported to be synergistic against the replication of both wild-type poliovirus and a guanidine-resistant poliovirus mutant (66), which suggests synergy may result in unique antiviral mechanisms.

In this study, PAP in combination with either guanidine or ribavirin was found to be synergistic toward the replication of Newcastle disease virus (NDV) when grown in chicken embryo cells (CEC). The synergistic combination of PAP and guanidine resulted in an increase in the synthesis of specific viral proteins, whereas the synergistic combination of PAP and ribavirin reduced synthesis of some NDV proteins. The data suggests that the synergistic mechanism of PAP plus guanidine inhibits the switch from transcription of viral message to replication of the NDV antigenome, whereas PAP plus ribavirin effectively inhibit translation of viral message.

The antiviral effects of PAP, ribavirin, and guanidine against NDV replication in HeLa cells has been reported (86). PAP and ribavirin were less effective

individually against the replication of NDV in HeLa cells than in CEC, the natural host of the virus. Concentrations of guanidine inhibitory to viral replication in CEC were similar to concentrations found to inhibit NDV in HeLa cells. In both HeLa cells and CEC, most combinations of PAP and guanidine against NDV were found to be synergistic, whereas most combinations of PAP and ribavirin were antagonistic (86). The increased antiviral effectiveness of PAP and ribavirin observed in CEC may be due to the higher surface-to-volume ratio of chicken embryo cells as compared to HeLa cells (50). A larger surface area would allow increased entry of antivirals into the cell, leading to an increased concentration of the drug within the cell.

Guanidine has been shown to interfere with the incorporation of choline into the membranes of infected cells (72) and the observed synergistic activity of PAP in combination with guanidine may be due to increased uptake of PAP into the infected cell. This may result in an increase in cell permeability and enhance the entry of PAP into the cytosol. This seems unlikely since one would expect the increase in PAP to produce no change viral protein concentrations, and in the presence of the synergistic combination of PAP and guanidine, NDV showed a marked increase in viral protein synthesis (Table 5, Fig. 7).

In NDV infected cells, transcription of the negative-stranded viral genome into monocistronic message occurs prior to synthesis of the complete antigenome (34). The monocistronic message is utilized by viral polymerase (P-L) to generate viral proteins. The mechanism that prompts the change in activity from transcription of viral mRNA to replication of viral genome involves the formation of a P-NP complex. This complex causes viral polymerase to ignore junctions and coordinates synthesis of the

complete antigenome (42). Because the synergistic effect of PAP with guanidine resulted in increased viral proteins, it could be postulated that this synergistic combination inhibits the formation or function of the P-NP complex. This would result in all viral replicative activity being directed to protein synthesis. Since PAP inhibits protein synthesis and guanidine inhibits a viral protein involved in the encapsidation of viral RNA, this indicates the exploitation of a new antiviral target.

The synergistic combination of PAP plus ribavirin resulted in a decrease in a number of NDV proteins (HN_o, F_o, P, and NP) (Table 6, Fig. 8). The maintenance of virus protein ratios is essential for efficient virus particle assembly and RNA packaging. Recent research has identified a number of viruses that induce ribosomal frameshifting as a means of regulating viral protein ratios, including some paramyxoviruses (37). The efficiency of the programmed frameshift determines the ratio of proteins available for virus assembly (37). The synergistic effect of PAP with ribavirin resulted in an alteration of normal viral protein ratios, suggesting a disruption of frameshifting efficiencies, ultimately resulting in reduced virus production.

Ribavirin has been shown to inhibit capping of the 5' end of influenza virus mRNA and causes alteration of the 5'-cap formation of vesicular stomatitis virus mRNA (46). Capped mRNAs are central to viral replication as cellular ribosomes initiate translation at the cap. The synergistic effect of ribavirin and PAP may result from enhancement of the antiviral effect of PAP on cellular ribosomes.

The data indicates that the antiviral activity of PAP and ribavirin, both individually and in combination, was not due to cytotoxicity. Although the antiviral effect of guanidine at higher concentrations on virus multiplication may be attributable

to cytotoxicity, the CC_{50} concentration of guanidine in chicken embryo cells (Table 2) was approximately nine times that of the ED_{50} concentration. The concentration of guanidine used for the synergistic combination with PAP showed no cytotoxicity (Table 2).

Virus titers were determined via a hemagglutination assay, which is a rapid method to quantitate virus particles. A major disadvantage of the hemagglutination assay in comparison to the plaque assay is that it measures total virus particles and does not distinguish between infectious and noninfectious particles. However, a measurable decrease in total virus particles as determined by hemagglutination would most likely reflect a decrease in both infectious and noninfectious particles.

Combined drug chemotherapy facilitates the use of reduced concentrations of antiviral agents while maintaining their inhibitory effectiveness. In addition to reducing drug toxicity, combinational chemotherapy also prevents the emergence of resistant virus strains and provides insight into the process of viral replication. This study shows that effective concentrations of PAP, ribavirin and guanidine against Newcastle disease virus in chicken embryo cells are not cytotoxic. In addition, the synergistic effect resulting from the combination of guanidine and PAP may renew interest in guanidine as an antiviral agent by reducing the emergence of guanidine-resistant strains.

In summary, the results suggest that the synergistic combination of PAP and guanidine increases viral protein production by inhibiting the switch from viral translation to genome replication. The synergistic combination of PAP and ribavirin decreases the synthesis of a number of viral proteins and may alter protein ratios

through the disruption of ribosomal frameshift efficiencies. These antiviral combinations provide synergistic treatment for cells infected with Newcastle disease virus, offering increased antiviral efficacy while prohibiting the emergence of drug-resistant mutants.

SUMMARY

Pokeweed antiviral protein (PAP) in combination with either guanidine or ribavirin exhibited both a synergistic effect against virus replication as well as an antagonistic effect against the individual antiviral effect of each antiviral agent. Low and high effective concentrations of PAP in combination with low, medium, and high effective concentrations of guanidine were synergistic. However, combinations of medium effective concentrations of PAP with low and medium effective concentrations of guanidine were antagonistic.

High and medium effective concentrations of PAP in combination with low, medium, and high concentrations of ribavirin were antagonistic. High effective concentrations of ribavirin in combination with PAP were antagonistic. High and medium effective concentrations of ribavirin in combination with low concentrations of PAP were synergistic.

A synergistic combination of PAP and guanidine caused increased viral protein synthesis. The synergistic mechanism may inhibit the switch from translation of the viral genome to replication, resulting in continual synthesis of viral proteins. More specifically, it may inhibit formation or function of the P-NP complex which directs replication of viral genome. A synergistic combination of PAP and ribavirin resulted in decreased NDV protein synthesis and altered the protein ratio. The synergistic combination may affect the efficiency of programmed ribosomal frameshifting, resulting in reduced virus titers. Alternatively, ribavirin in combination with PAP may enhance the effect of PAP on ribosomes by inhibiting initiation of translation.

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