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Combined Effect of Ganciclovir and Vidarabine on the Replication, DNA Synthesis, and Gene Expression of Acyclovir-resistant Herpes Simplex Virus

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ABSTRACT

Combined effects of ganciclovir (GCV) and vidarabine (ara-A) on the replication, DNA synthesis, and gene expression of wild type-1 herpes simplex virus (HSV-1) and three acyclovir (ACV)-resistant HSV-1 mutants were studied. These mutants include a virus expressing no thymidine kinase (ACV^r), a virus expressing thymidine kinase with altered substrate specificity (IUdR^r), and a mutant expressing altered DNA polymerase (PAA^{r}5). GCV, an agent activated by herpesvirus specific thymidine kinase, showed potent antiviral activity against the wild type HSV-1 (KOS) and DNA polymerase mutant (PAA^{r}5).

The ACV-resistant mutants with thymidine kinase gene (ACV^r and IUdR^r) were resistant to GCV. All tested wild type HSV-1 or ACV-resistant HSV-1 mutants did not display resistance to vidarabine (are-A). Combined GCV and ara-A showed potentiating synergistic antiviral activity against wild type KOS and PAA^{r}5, and showed subadditive combnined ativiral activity against thymidine kinase mutants. Combined GCV and ara-A more significantly inhibited the viral DNA synthesis in wild type KOS and PAA^{r}5-infected cells to a greater extent than either agent alone, but the synergism was not determined in ACV^r or IUdR^r-infected cells. These data clearly indicate that combined GCV and ara-A therapy might be useful for the treatment of infections caused by wild type HSV-1 or ACV-resistant HSV-1 with DNA polymerase mutation.

ACV-resistant viruses with the mutation in thymidine kinase gene are also, resistant to GCV, but susecptible to ara-A, indicating that ara-A would the drug of choice for the treatment of ACV-resistant HSV-1 which does not express thymidine kinase or expresses thymidine kinase with altered substrate specificity.

While the synthesis of viral α-proteins of wild type HSV-1 was not affected by ACV, GCV, ara-A, or combined GCV and ara-A, the synthesis of β-proteins was slightly but significantly increased at the later stage of viral infection by the antiviral agents. The synthesis of γ-proteins of wild type HSV-1 was significantly inhibited by ACV, GCV, ara-A, and combined GCV and ara-A.

Combined GCV (5-μM) and ara-A (100-μM) also significantly altered the expression of viral β-and γ-proteins, of which effect was similar to that of GCV (10-μM) alone.

Although ACV at the concentration of 10-μM did not alter the expression of α-, β-, and γ-proteins of ACV-resistant PAA^{r}5, GCV and ara-A significantly alter the epression of β-and γ-proteins, not α-protein, as same manner as they altered the expression of those proteins in cells infected with wild type HSV-1. Combined GCV (5-μM) and ara-A (100-μM) altered the expression β-and γ-proteins in PAA^{r}5 infected cells, and the effect of combined regimen was comparable of that

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of GCV (10-μM). These data indicate that the alteration in the expression of β-and γ-proteins in wild type HSV-1 or PAA^{r}5 infected cells could be more significantly affected by combined GCV and are-A than individual GCV or ara-A.

In view of the fact that (a) viral α-, β-, and γ-proteins are synthesized in a cascade manner; (b) β-proteins are essential for the synthesis of viral DNA; (c) the synthesis of β-proteins are inhibited by γ-proteins; and (d) most γ-proteins are made from the newly synthesized progeny virus, it is suggested that GCV and ara-A, alone or in combination, primarily inhibit the synthesis of viral DNA, and by doing so might exhibit their antiherpetic activity. The alteration in viral protein synthesis in the presence of tested antiviral agents could result from the alteration in viral DNA synthesis. From the present study, it can be concluded that (a) combined GCV and ara-A therapy would be beneficial for the control of infections caused by wild type HSV-1 or ACV-resistant DNA polymerase mutants; (b) the combined synergistic activity of GCV and ara-A is due to further decrease in the viral DNA by the combined regimen; (c) ara-A is the drug of choice for the infection caused by ACV-resistant HSV-1 with thymidine kinase mutation; and (d) the alteration in viral protein synthesis by GCV and ara-A, alone or in combination, is mostly due to the decreased synthesis of viral DNA.

Key Words: Acyclovir, ganciclovir, Vidarabine, Replication, DNA synthesis, Gene expression

INTRODUCTION

Herpes simplex virus (HSV), the most ubiquitous and communicable infectious virus, induces a wide variety of clinical diseases in man: (a) Approximately one-third of the world population has recurrent herpes labialis (RHL) episodes due to HSV and over one-half of these patients have more than one attack each year (NIH, 1973; Spruance *et al.*, 1984), (b) HSV is the most common cause of genital lesions in women, and is second only to syphilis as the cause of genital lesions in men (Hanshaw, 1973).

At least 100,000 cases of herpes genitalis are officially reported each year in the United States (NIH, 1973; Overall, 1979), (c) Ocular herpes simplex is the leading cause of blindness due to corneal infections in the United States (Pavan-Langston, 1979). Up to 500,000 cases of primary or recurrent ocular herpetic infections are reported in a year in the United States (NIH, 1973; Pavan-Langston, 1979), (d) The primary attack of HSV in children and neonates is disastrous leading to encephalitis and infections of other organs. Such infections are associated with a high degree of mortality and morbidity (Nahmias *et al.*, 1970; Nahmias and Roizman, 1973), (e) HSV infections cause extremely serious systemic complications in immunologically compromised patients with acquired immunodeficiency syndrome (AIDS), patients receiving organ transplantation, and patients under cancer chemotherapy (Whitley *et al.*, 1984 and 1986; Wade *et al.*, 1983b and 1984), and finally, (f) Certain human malignancies have been strongly linked to the virus. Type 1 HSV (HSV-1) and type 2 HSV (HSV-2) are associated with squamous cell carcinoma of the orofacial area (Scully *et al.*, 1982; Shillitoe *et al.*, 1983; Park *et al*, 1985 and 1986) and uterine cervix or vulval carcinomas of humans and laboratory animals (Rapp and Reed, 1986; McDougall *et al.*, 1980; Wentz *et al.*, 1981; and Kaufman *et al.*, 1981), respectively.

Numerous antiviral agents with proven efficacy in vitro and in the treatment of ocular endorgan disease, herpetic epithelial keratitis, herpes encephalitis and genital herpes have been introduced. Among those agents, idoxuridine (Kaufman *et al.*, 1962; Itoi *et al.*, 1975; Foster and Pavan-Langston 1977), vidarabine (Pavan-Langston *et al.*, 1972, 1974, and 1976) and trifluridine (Pavan-Langston and Foster, 1977) were approved for the clinical use in the treatment of ocular herpetic diseases by the Food and Durg Administration (FDA) of the United States. However, these drugs are variable in their therapeutic efficacy and not infrequently associated with toxicity and true allergy (Calabresi *et al.*, 1961; Chaube and Murphy, 1968, Heidelberger, 1964; Percy and Albert, 1974).

The major breakthrough in antiherpetic chemotherapy research was the introduction of acyclovir (ACV), which an analog of guanosine with potent and nontoxic antiherpetic activity (Elion *et al.*, 1977; Furman *et al.*, 1979). ACV is phosphorylated to ACV-monophosphate by herpesviral spe-

cific thymidine kinase which is then further phosphorylated to ACV-diphosphate and ACV-triphosphate by cellular guanidylate kinase. ACV-triphosphate inhibits viral DNA polymerase and terminates the elongation of viral DNA chain. Thus, it effects only viral infected cells, leaving noninfected cells essentially untouched (Elion *et al.,* 1977; Furman *et al.,* 1979 and 1984; Hammer *et al.,* 1982; Park and Pavan-Langston, 1982; Furman and Mcguirt, 1983). Numerous laboratory (Elion *et al.,* 1977; Park *et al.,* 1979, 1980a, 1980b, 1980c, 1982; Klein *et al.,* 1979) and clinical studies (Coster *et al.,* 1980; Pavan-Langston *et al.,* 1981; Cupps *et al.,* 1981; Straus *et al.,* 1982 and 1984; Corey *et al.,* 1982; Bryson *et al.,* 1983, Wade *et al.,* 1893b and 1984; Douglas *et al.,* 1984; Whitley *et al.,* 1984 and 1986) have shown a significant therapeutic efficacy of ACV against a wide variety of herpetic diseases. The United States FDA has approved the use of 5% ACV ointment for the treatment of primary herpes genitalis and other cutaneous herpetic infections. Furthermore, the FDA of the United Sates recently approved the oral administration of ACV tablets for the prevention of recurrent herpes genitalis (Straus *et al.,* 1984; Abramowicz, 1985).

Although ACV is now in extensive clinical use for the management of HSV infections, recent reports concerning the facile emergence of ACV-resistant herpes simplex virus (HSV) mutants in laboratory studies (Field and Darby, 1980, Schnipper and Crumpacker, 1980, Coen and Schaffer, 1980; Sibrak *et al.,* 1982; Boijoly *et al.,* 1983; Larder and Darby, 1985) and reports of clinical ACV-resistant HSV infection (Burns *et al.,* 1982; Crumpacker *et al.,* 1982; Paris and Harrington, 1982; Wade *et al.,* 1983a) have received much attention, and gives cause for concern at the paucity of the antiviral armamentarium when resistance can emerge so rapidly against a major nontoxic drug. In view of this fact, the mechanisms of viral resistance to ACV have been investigated extensively. Both biochemical and genetic evidence support the notion that HSV thymidine kinase and DNA polymerase genes as loci, which, when mutated, can confer resistance to ACV in cell culture (Coen and Schaffer, 1980; Schnipper and Crumpacker, 1980; Crumpacker *et al.,* 1980 and 1984; Darby *et al.,* 1981; Furman *et al.,* 1981; Knopf *et al.,* 1981; McLaren *et al.,* 1983; Larder and Darby, 1985 and 1986; Boijoly *et al.,* 1983). So far, five different ACV resistant HSV mutants have been isolated from the patients and laboratories and characterized genetically (Field *et al.,* 1982): (i) mutant with negative or less expression of viral thymidine kinase; (ii) mutant expressing altered thymidine kinase (altered substrate specificity) which does not phosphorylate ACV; (iii) viral mutant expressing altered DNA polymerase which do not take ACV triphosphate as substrate; (iv) mutant expressing negative or less thymidine kinase and altered DNA polymerase; and (v) mutant expressing altered thymidine kinase and DNA polymerase. Because of the rising incidence of oral and genital HSV infections and the increasing use of systemic ACV for the treatment of herpes infections, a rise in human exposure to ACV-resistant HSV is likely. Therefore, searches developing new antiviral agents being safe and effective against ACV-resistant HSV mutants have been focused.

More recently, ganciclovir (9-[1, 3-Dihydroxy-2-propoxymethyl] guanine; GCV), also known as BW759U, BIOLF-62, and 2′nor-2′-deoxyguanosine, was introduced as a second generation drug of ACV. GCV is an analogue of ACV and has been shown to be a potent inhibitor of HSV and cytomegalovirus (CMV) (Field *et al.,* 1983; Marr *et al.,* 1983; Smee *et al.,* 1983; Trousdale *et al.,* 1984; Chun and Park, 1987). Like ACV, GCV is phosphorylated to the monophosphate form by herpesvirus specific thymidine kinase, which is further phosphorylated to its di-and triphosphate by cellular guanylate kinase and other cellular enzymes, respectively (Ashton *et al.,* 1982; Field *et al.,* 1983; Smee *et al.,* 1983). GCV is more rapidly converted to its corresponding triphosphate form in virus-infected cells than is ACV (Cheng *et al.,* 1983). GCV triphosphate inhibits the synthesis of viral DNA by suppressing DNA polymerase (St Clair *et al.,* 1984; Frank *et al.,* 1984; Chun and Park, 1987). Although mechanism of antiherpetic activity of GCV is similar to ACV, a study regarding the susceptibility of ACV-resistant HSV mutants to GCV deserves to be studied.

Vidarabine (ara-A), an analogue of adenosine, has been used for the treatment of ocular herpetic infection (Park and Pavan-Langston, 1982). The mechanism of antiviral action of ara-A is well known: unlike ACV or GCV, ara-A does not have specificity against the virus. It is phosphorylated to its monophosphate form by cellular adenosine kinase, which is further phosphorylated to its di-and triphosphate forms in normal and viral infected cells by cellular

adenylate kinase. ara-A diphosphate inhibits viral and cellular ribonucleotide reductase, while the triphosphate form inhibits viral and cellular DNA polymerase and ribonucleotide reductase (Park and Pavan-Langston, 1982). Unlike ACV or GCV, ara-A can be activated in the absence of herpesvirus-induced thymidine kinase, and thus can produce antiviral activity to ACV-resistant HSV with thymidine kinase mutations (Schnipper and Crumpacker, 1980). Since most ACV-resistant HSVs isolated from patients are thymidine kinase mutants, ara-A has been considered as the drug of choice for the treatment of the infection caused by HSV with thymidine kinase mutation Parris and Harrington, 1982; Boijoly *et al.,* 1983). In the present study, the antiviral activity to ACV-resistant mutants was again confirmed.

As combined chemotherapies have been used successfully against neoplasms (Valeriote and Lin, 1975), combination of antiviral agents with different mechanisms of action has reasonably enhanced therapeutic efficacy while reducing the chances of the development of drug-resistant forms (Bryson and Kronenberg, 1977; Frase-Smith *et al.,* 1984). Furthermore combination would diminish the durg's toxic effects if lower doses of each agent could be used. In fact, Schinazi and Nahmias (1982) and Park *et al.* (1984) found that the combined antiviral effect of ACV and ara-A on HSV infection was additive in vitro and in vivo. In view of the facts that (a) GCV is specifically activated by herpesvirus-encoded enzymes, (b) ACV-resistant HSV with thymidine Kinase mutation is susceptible to ara-A, and (c) the activation mechanisms of GCV and ara-A are different, a study regarding the possible synergistic activity of combined GCV and ara-A against various ACV-resistant HSV-1 mutants deserves to be exploited. Since GCV and ara-A are known to inhibit the viral DNA synthesis, a study regarding the effect of combined GCV and ara-A on the viral DNA synthesis might provide some clue for the understanding of the mechanism of synergistic action, if any, of the combined regimen.

Nearly 50 virus-specific infected cell polypeptides (ICPs) are synthesized from the HSV-1 genome (Honess and Roizman, 1973). The HSV-specific ICPs are classified into three groups: viral α-, β-, and γ-proteins, whose syntheses are coordinately regulated and sequentially ordered in a cascade manner (Honess and Roizman, 1974 and 1975). The γ-proteins are further classified into 2 subgroups, namely γ-1 and γ-2 proteins. When HSV infects cells, the viral DNA immediately begins to synthesize α-proteins (ICPs 0, 4, 22, 27, and 47) in the absence of prior viral proteins (Honess and Roizman, 1975; Powell *et al.,* 1975). The ICP4 is known to act on the viral genome and to turn on the expression of certain viral β-proteins, i.e. ICP8 (DeLuca and Schaffer, 1985). The β-proteins include DNA binding protein (ICP8) and enzymes necessary for the synthesis of viral DNA such as viral thymidine kinase, ribonucleotide reductase (ICP6) and DNA polymerase (Knipe *et al.,* 1982). ICP8 inhibits the expression of ICP4 by the negative feed back mechanism and enhances the expression of most viral γ-genes (Conley *et al.,* 1981; Godowski and Knipe, 1986). Therefore, the expression of β-proteins results in the synthesis of viral DNA, which is followed by the production of most of γ-proteins. The inhibition of viral DNA synthesis results in a moderate reduction in the synthesis of γ-1 proteins and a strong reduction in the production of γ-2 proteins. Some viral γ-proteins also negatively regulate the expression of cretain β-proteins and somy γ-proteins (Godowski and Knipe, 1986). Since the synthesis of viral α-and β-proteins precedes the viral DNA synthesis, a study of combined synergistic effect of GCV and ara-A, if any, on viral protein synthesis might provide insight to the mechanism behind its antiviral action. With this premise in mind, the author also examined the combined effect of GCV and ara-A on the synthesis of viral α-, β-, and γ-proteins in cells infected with wild type HSV-1 or ACV-resistant HSV-1 mutants susceptible to combined GCV and ara-A.

MATERIALS AND METHODS

Viruses and cells

HSV-1 KOS strain and three ACV-resistant HSV-1 mutants including ACV^r (a mutant defective in expressing thymidine kinase), IUdR^r (a mutant expressing thymidine kinase with altered substrate specificity), and PAA^{r}5 (a DNA polymerase mutant) were provided by Professor No-Hee Park, University of Califomia, Los Angeles (UCLA) School of Dentistry, Los Angeles, California, U.S.A. All viruses were propagated in Vero cell monolayers and the viral titers were adjusted to 1×10^9 plaque forming units per milliliter (PFU/ml). The stock virus was stored

at −80℃ until used. Vero cells also provided by Professor No-Hee Park (UCLA School of Dentistry) were grown in monolayers in Eagle's minimum essential medium (E-MEM) supplemented with 5 % fetal bovine serum (FBS) and antibiotic mixture (penicillin G, 100-units/ml; streptomycin, 10-μg/ml; and amphotericin B, 10-μ g/ml). The monolayer cells were cultured in a CO_2 incubator at 37 ℃ with a 5 % carbon dioxide atmosphere.

Antiviral compounds

ACV and GCV powders were provided by Professor No-Hee Park (UCLA School of Dentistry, Los Angeles, California, U.S.A.). ara-A was purchased from the Sigma Chemical Company (St. Louis, MO, U.S.A.). The stock solutions (10-mM) of antiviral compounds were prepared in E-MEM supplemented with 5 % bovine serum.

Yield reduction assay

Confluent Vero cell monolayers were infected with wild HSV-1, KOS-strain or ACV-resistant HSV-1 (PAA^{r}5, ACV^r, and IUdR^r) at a multiplicitity of infection (m.o.i.) of 5 (5PFU/cell) for 1 hour at 37 ℃, with intermittent rocking at 15 minute intervals, and washed twice with phosphate buffered saline (PBS). Medium with or without the antiviral agents (ACV: 0.01, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0-μM; GCV: 0.01, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0-μM; ara-A: 10, 20, 30, 50, 100 or 200-μM ; GCV+ara-A : 0.01+30, 0.05+30, 0.1+30, 0.2+30, 1.0+30, 2.0+30, 5.0+30, or 10.0-μM+30-μM) was then added in triplicate to the appropriate culture dishes. The cultures were incubated at 37 ℃ for 24 hours in a CO_2 incubator. The culture media were then removed and the cells were gently washed with PBS; thereby preventing carry over of the antiviral agents to the plaque assay. The cells were then frozen and thawed three times, collected and centrifuged at 450 g for 5 min. Viral titers in the supernatant were then assayed by an ordinary plaque assay technique (Rapp, 1963). The data obtained from the yield reduction assay were used to calculate the concentration of each antiviral agent which inhibits the replication of the viruses into half (ED_{50}).

Evaluation of combined antiviral efficacy of vidarabine and ganciclovir

The effects of the two antiviral agents combined in culture were analyzed by means of the following criteria (Park *et al.*, 1984): (1) E_A (effect of Drug A)=titer of virus produced in the presence of drug A/titer of virus produced in the absence of the drug. (2) E_B (effect of drug B)= titer of virus produced in the presence of drug B/ titer of virus produced in the absence of the drug. (3) E_{AB} (effect of the combination of drugs A and B)=titer of virus produced in the presence of drug A and B/titer of virus produced in the absence of the drugs. (4) E_C (calculated effect of combined drugs A and B, or additive effect of drugs A and B)=$E_A \times E_B$. Therefore, a potentiation interaction of the drugs can be defined as $E_{AB} < E_C$ and an additive effect as $E_{AB} = E_C$. In addition, if drug A is assumed to be more effective than drug B, a less than additive (subadditive) interaction can be defined as $E_A > E_{AB} > E_C$; antagonistic interaction as $E_{AB} > E_B$; and interference as $E_B > E_{AB} > E_A$.

Isolation and analysis of viral and Cellular DNA

Confluent Vero cells were infected with wild type HSV-1 KOS strain, ACV^r, IUdR^{r}or PAA^{r}5 at a m.o.i. of 10 (10PFU./cell). After adsorption for 1 hour at 37 ℃, the viral inoculum was removed, and the cells were washed with PBS. E-MEM containing 1.0-μM ACV, 1.0-μM of GCV, 30-μM ara-A, or 1.0-μM GCV+30-μM ara-A was then added to the cultures. Four hours postinfection 10-μCi/of ^{3}H-thymidine (specific activity; 23.0 Ci/mM, New England Nuclear, Boston, Massachusetts, U.S.A.) per culture dish (10-μCi/10-ml) was added. After incubation for 24 hours the cells were collected and centrifuged at 600 g for 5 minutes. The cell pellets were washed twice with ice-cold PBS, gently resuspended in TE buffer (10-mM Tris, 10-mM EDTA, pH 7.6) containing 0.4 % sodium dodecyl sulfate (SDS) and 4.0-mg of proteinase, and then incubated at 37 ℃ for 24 hours. Saturated NaI solution at 37 ℃ along with ethidium bromide for a final concentration of 10-μg/ml was then added to the cell digestion to achieve a final density of 1.4 g/ cm^3. Isopycnic equilibrium NaI density gradient centrifugation was performed in a Beckman rotor 50 (Beckman instruments, Fullerton, California,

U.S.A.) at 125,000 g for 48 hours at 20℃. After the confirmation of viral and cellular bands under ultraviolet light, gradients were dripped from the bottom of each tube, and collected in 30 fractions. Fifty-μl portions of each fraction were spotted onto a GF/C filter disk (Whatman, England), and washed 6 times for 1 minute each with ice-cold 0.5 M sodium monophosphate solution. The samples were then washed twice with distilled water and twice with 95% ethyl alcohol. The samples were then dried at 60℃. Radioactivity of the spotted GF/C was measured with a Beckman liquid scintillation counter. An aliquot of each fraction was also spotted onto a refractometer to determine the density of the fraction: the density of HSV-1 DNA is known to be 1.72, while that of Cellular DNA is 1.70.

Labelling of proteins synthesized in infected cells

To understand the mechanism of synergistic antiviral effect of GCV and ara-A, if any, the effect of GCV and ara-A, alone or in combination, on the protein synthesis of susceptible viruses was studied as follows. Confluent Vero cell monolayers were infected with HSV-1 at a m.o.i. of 20 (20PFU/cell) and incubated for 1hour. The monolayer cells were washed with PBS, replenished with fresh medium or medium containing ACV (10-μM), GCV (10-μM), ara-A (200-μM), or GCV plus ara-A (100-μM plus 5-μM), and incubated at 37℃ for additional 3, 7, or 14 hours. After washing the cells with PBS, medium 199 containing ^{35}S-methionine (20-μCi/ml, specific activity: 900 mCi/mM, New England Nuclear, Boston, Massachusetts, U.S.A.), dialysed calf serum and hepes (20-mM) was then added to the cells. The cultures were then continued to be incubated for 1 hour at 37℃. Finally the cells were rinsed with ice-cold PBS and solubilized in sample buffer (60-mM tris [pH 7.6], 2% SDS, 20% glycerol, 0.005% β-mercaptoethanol, and 2% bromophenol blue) for subsequent electrophoresis on polyacrylamide gel[87]. The time dependent synthesis of total cellular and viral protein in infection control or antiviral agents treated cells were monitored by the determination of radioactivity of an aliquot from each group.

Polyacrylamide gel electrophoresis (PAGE), determination of HSV-1 protein bands from the autoradiograms, and scanning of the autoradiogram

The PAGE was done in a discontinuous buffer system with 0.1% SDS. The stacker and separation gels containing 3% and 9.25% acrylamide, respectively, were cross-linked with N-tetramethylethylendiamine. The samples were loaded into the gel with protein size marker (High Molecular Standard®, Bio-Rad Laboratories, Richmond, CA, U.S.A.) to determine the molecular weight of the HSV-1 specific ICPs. The protein size marker was a mixture of proteins with known molecular weights (myosin with molecular weight [M.W.] of 200,000; β-galactosidase of *E. Coli* with M. W. of 116,250; rabbit muscle phosphorylase b with M.W. of 97,000; bovine serum albumin with M.W. 66,200; and ovalbumin with M.W. of 42,699). After the completion of the electrophoresis, the gel was dried and autoradiographed on SB-5 Kodak X-ray film (Eastman Kodak Company, Rochester, New York, U.S.A.) (Morse *et al.*, 1978). After developing and fixing the film, molecular weights of the protein bands found only in the HSV-1 infected cells (infection control or groups with antiviral treatment) were calculated by using the location of known protein size markers. The absorbance measurments of the radiographic images were then made in a Bio-Rad video densitometer model 620 (Bio-Rad Laboratories, Richmond, California, U.S.A.). To calculate the relative rates of representative protein synthesis, the representatives α-, β- and γ-protein peaks of scanned autoradiogram were converted to precentage of the total absorbance. As a representative of the viral α-proteins, the high molecular weight nonstructural polypeptide ICP4 was chosen. ICP8 and ICP5 were selected to represent viral β-and γ-proteins, respectively.

RESULTS

Effects of ACV, GCV, and ara-A on the replication of HSV-1

Fig. 1 shows the effect of ACV on the replication of wild type HSV-1 and ACV-resistant mutants. ACV showed potent antiviral activity against the wild type HSV-1 KOS strain, while the

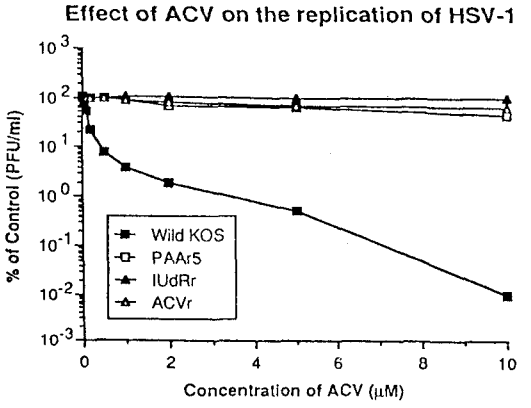

Fig. 1. Effect of ACV on the replication of wild type HSV-1 (KOS) and ACV-resistant mutants (PAA^{r}5, ACV^r and IUdR^r) in Vero cell monolayers. Confluent monolayer cells infected with HSV-1 at the m.o.i. of 5 were cultured in the medium containing ACV (0.0, 0.01, 0.05, 0.1, 0.5, 1.0, 2.0, 5.0 or 10.0-μM) for 24 hours at 37℃. The viral titers of the cultured cells were then determined by plaques assay technique. The viral titers of each group were depicted as % of the infection control (0.0-μM ACV).

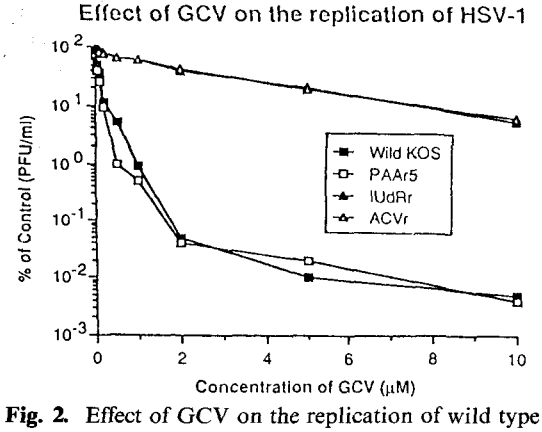

Fig. 2. Effect of GCV on the replication of wild type HSV-1 (KOS) and ACV-resistant mutants (PAA^{r}5, ACV^r and IUdR^r) in Vero cell monolayers. Confluent monolayer cells infected with HSV-1 at the m.o.i. of 5 were cultured in the medium containing GCV (0.0, 0.01, 0.05, 0.1, 0.5, 1.0, 2.0, 5.0, or 10.0-μM) for 24 hours at 37℃. The viral titers of the cultured cells were then determined by plaque assay technique. The viral titers of each group were depicted as % of the infection control (0.0-μM GCV).

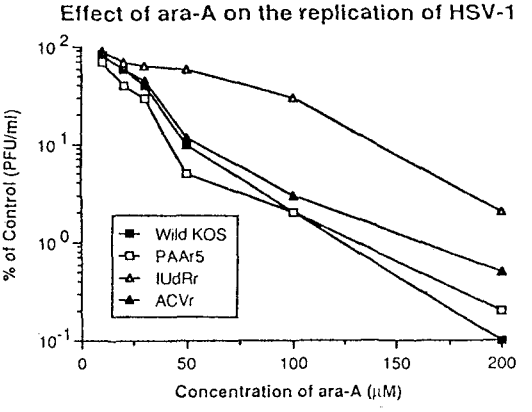

Fig. 3. Effect of ara-A on the replication of wild type HSV-1 (KOS) and ACV-resistant mutants (PAA^{r}5, ACV^r and IUdR^r) in Vero cell monolayers. Confluent Vero cells infected with HSV-1 at the m.o.i. of 5 were cultured in the medium containing ara-A (10, 20, 30, 50, 100, or 200-μM) for 24 hours at 37℃. The viral titers were then determined by plaque assay technique. The viral titers of each group were depicted as % on the infection control (0.0-μM ara-A).

tested ACV-resistant mutants (ACV^r, IUdR^r, and PAA^{r}5) were extremely resistant to ACV: the replication of those viruses were not influenced in the presence of ACV at the concentration of 10 μ g/ml, at which concentration ACV almost completely abolished the replication of wild type HSV-1 (Fig. 1). Fig. 2 shows the antiviral activity of GCV to HSV-1 with or without resistance to ACV. Although GCV did not alter the replication of thymidine kinase mutants such as IUdR^r and ACV^r, it showed strong antiviral activity against wild type HSV-1 KOS and ACV-resistant DNA polymerase mutant PAA^{r}5 (Fig. 2). Ara-A, however, showed moderate inhibitory activity against the replication of all viruses tested.

Wild type HSV-1 KOS and PAA^{r}5 were most sensitive, while IUdR^r was least sensitive to ara-A (Fig. 3). Table 1 shows the concentrations of antiviral agents reducing the viral replication by half (ED_{50}). While wild type HSV-1 KOS showed extreme sensitivity to ACV, DNA polymerase mutant (PAA^{r}5) and thymine kinase mutants (ACV^r and IUdR^r) were 70 or 300 times less susceptible to ACV respectively, as compared to the wild type HSV-1 KOS. GCV showed a potent antiviral activity to the HSV-1 KOS and DNA

Table 1. Concentrations of antiviral agents reducing the viral replication by half (ED_{50})

HSV-1	Antiviral Agents (μM)		
	ACV	GCV	Ara-A
KOS (wild type)	0.1	0.05	25
ACV^r	>30.0	1.6	27
IUdR^r	>30.0	1.6	63
PAA^{r}5	7.0	0.04	15

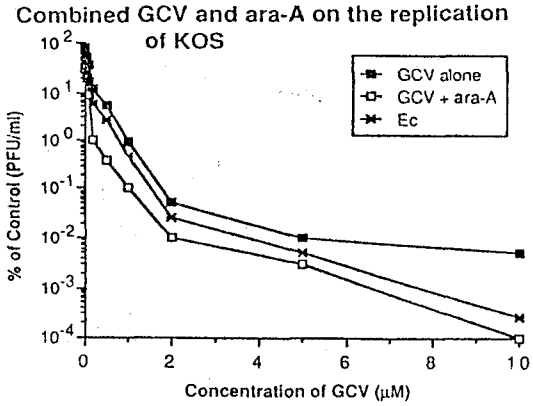

Fig. 4. Combined effect of GCV and ara-A on the replication of wild type HSV-1 (KOS) in Vero cell monolayers. Cells infected with HSV-1 at the m.o.i. of 5 were incubated with different concentrations of GCV (0.0, 0.01, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 or 10.0-μM) in combination with 30-μM of ara-A for 24 hours at 37℃. The viral titers were determined from the cells with plaque assay. The viral titers of each group were depicted as % of control of the infection control group. Ec= calculated additive effect of the two drugs.

polymerase mutant PAA^{r}5 but it elicited rather weak antiviral activity to the thymidine kinase mutants (ACV^r and IUdR^r): ACV^r and IUdR^r were approximately 30 times more resistant to GCV than the wild type HSV-1 (KOS). However, wild type HSV-1 KOS and all ACV-resistant HSV-1 mutants (ACV^r and IUdR^r) were almost equally sensitive to ara-A.

Effect of combined GCV and ara-A on the viral replication

Different doses of GCV (0.01, 0.05, 0.1, 0.5,

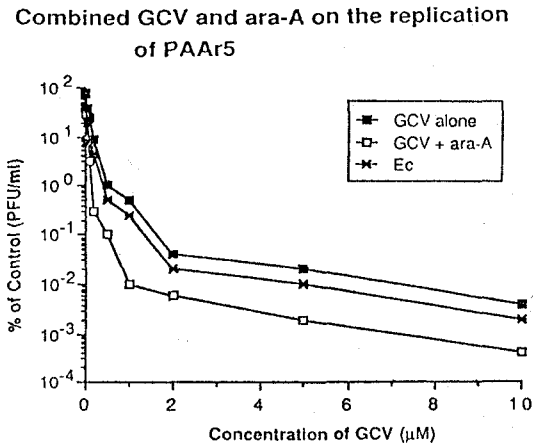

Fig. 5. Combined effect of GCV and ara-A on the replication of ACV-resistant mutant PAA^{r}5. Experimental procedures were similar to those using wild type HSV-1 as described in Fig. 4.

1.0, 2.0, 5.0 or 10-μM) combined with 30-μM ara-A reduced the viral titers of wild type HSV-1 KOS strain more than GCV or ara-A did alone, and the inhibitory effect was in a dose-dependent manner. Moreover, the viral yield was lower than the calculated yield (Ec), thus demonstrating potentiating synergistic activity of GCV and ara-A (Fig. 4). The combined therapy also decreased the yield of PAA^{r}5 more effectively than GCV or ara-A alone. Moreover, the viral yield was lower than the calculated yield reflecting an potentiating synergistic effect (Fig. 5). Although GCV, alone or in combination with ara-A, showed some antiviral activity against thymidine kinase mutants, the antiviral activity of GCV, alone or in combination with ara-A, against these viruses was extremely weaker than that of GCV against HSV-1 KOS strain or PAA^{r}5. Furthermore, it was not able to observe the synergistic combined activity of GCV and ara-A against thymidine kinase mutants. The combined antiviral activity of GCV and ara-A against thymidine kinase mutants was similar to that of GCV alone, indicating the subadditive effect of GCV and ara-A in cells infected with ACV-resistant HSV-1 with thymidine kinase mutation (Fig. 6, 7).

Effect of combined GCV and ara-A on the synthesis of viral and cellular DNA

The viral and cellular DNAs extracted from

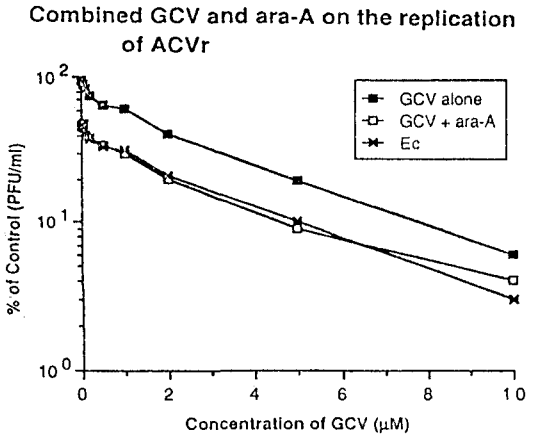

Fig. 6. Combined effect of GCV and ara-A on the replication of ACV-resistant mutant ACV^r. Experimental procedures were similar to those using wild type HSV-1 as described in Fig. 4.

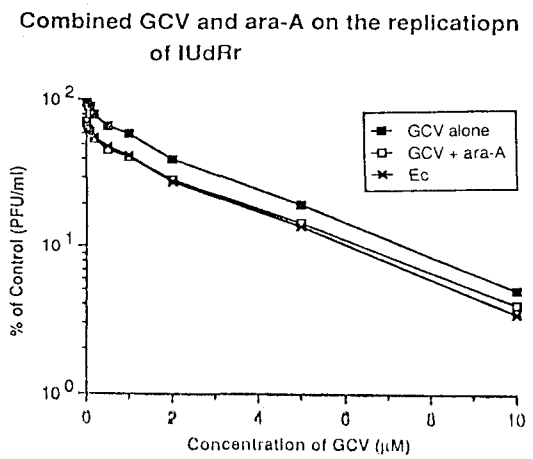

Fig. 7. Combined effect of GCV and ara-A on the replication of ACV-resistant mutant IUdR^r. Experimental procedures were similar to those using wild type HSV-1 as described in Fig. 4.

the HSV-1 infected cells were well separated by the NaI ultracentrifugation density gradient technique. As shown in the figures 8—11, the first and second peaks had the density of 1.72 g/cm^3 and 1.70 g/cm^3, respectively. This indicates that the first peaks from the DNA samples represent the radioactivities of viral DNA, while second peaks depict cellular DNA (Chun and Park, 1987). The effects of ACV, GCV, ara-A, and combined GCV

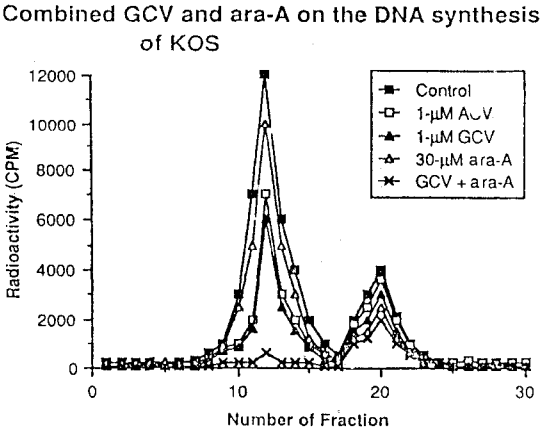

Fig. 8. Isopycnic equilibrium density gradients of DNA from HSV-1 (KOS) infected Vero cell monolayers. The first peak with heavier density (1.72 g/cm^3) represents the viral DNA and the second peak with lighter density (1.70 g/cm^3) depicts the cellular DNA. The DNA synthesis was monitored in tne presence of 1.0-μM of ACV, 1.0-μM of GCV, 30-μM of ara-A, or both GCV (1.0-μM) and ara-A (30-μM). Control indicates the infection only.

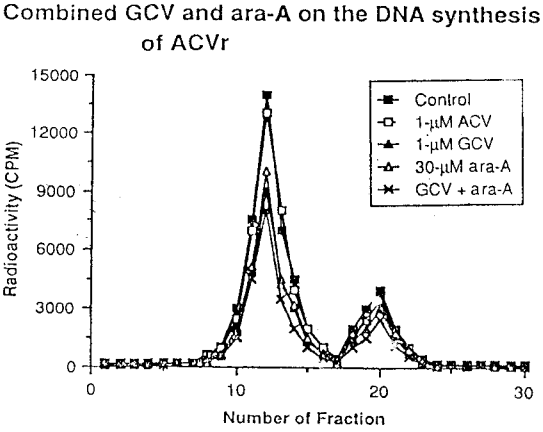

Fig. 9. Isopycnic density gradients of DNA from ACV-resistant PAA^{r}5 infected Vero cell monolayers.

and ara-A on the viral and cellular DNA synthesis in wild type HSV-1 KOS strain infected cells are shown in figure 8. In the presence of 1-μM of ACV or GCV in the culture medium, the viral DNA synthesis was inhibited by

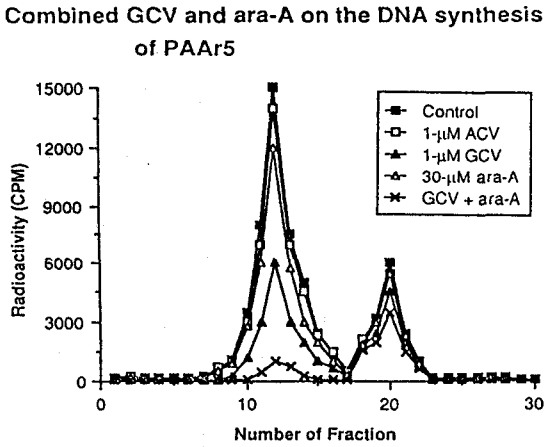

Fig. 10. Isopycnic density gradients of DNA from ACV-resistant ACV^r infected cells.

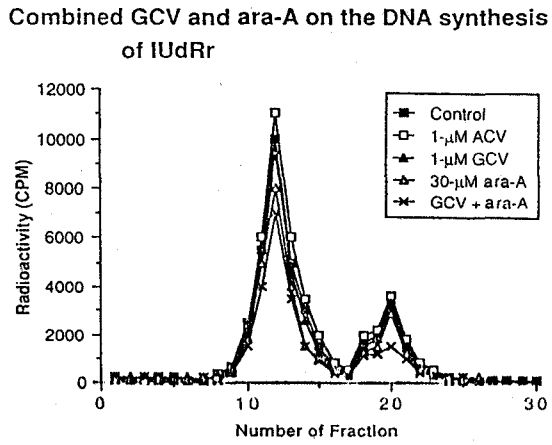

Fig. 11. Isopycnic density gradient of DNA from ACV-resistant IUdR^r infected cells.

approximately 60 % and cellular DNA by 20 %. The viral and cellular DNA synthesis was reduced by 20 % and 30 %, respectively, when the infected cells were cultured in the presence of 30- μM of ara-A, when the viral infected cells were incubated in the presence of both 1-μM GCV and 30-μM ara-A, the viral DNA synthesis was inhibited by 98.5 % and cellular DNA synthesis by 51 % (Fig. 8). The DNA synthesis of ACV-resistant DNA polymerase mutant PAA^{r}5 was significantly reduced (65 %) by GCV, while it was not altered by ACV. In the presence of both GCV and ara-A in the culture medium, the viral DNA synthesis

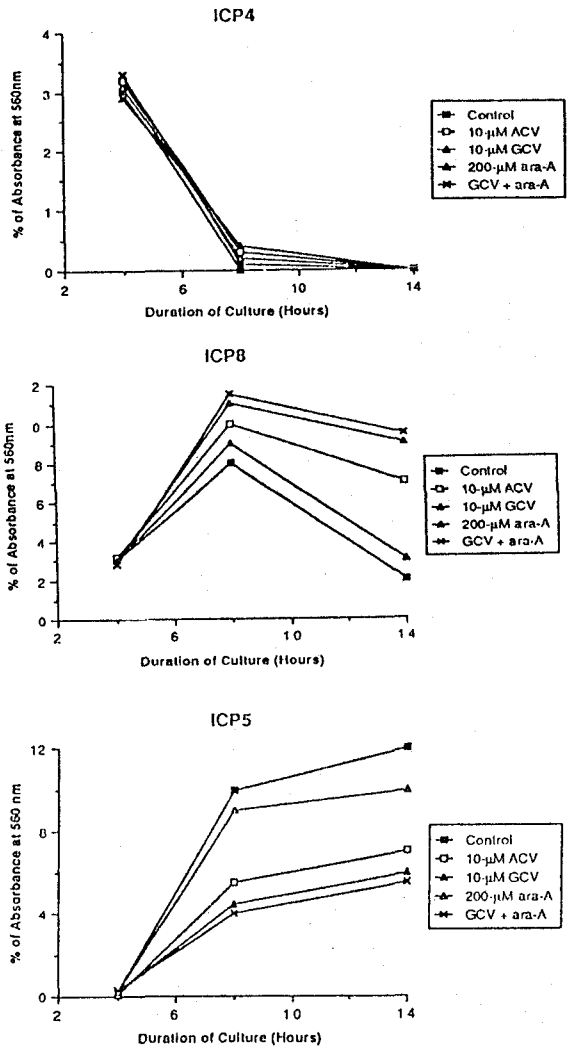

Fig. 12. Relative rates of representative protein synthesis of wild type HSV-1 KOS strain. Vero cell monolayers were infected with wild type HSV-1 KOS strain for 1 hour and cultured in the absence or presence of antiviral agents [ACV (10-μM), GCV (10-μM), ara-A (200-μM), and combined GCV (5-μM)+ara-A (100-μM)] for additional 3,7, or 13 hours. The levels of representative a (ICP4), b(ICP8), and g(ICP5) proteins of the treated cells were calculated from and autoradiograph of polyacrylamide gels. After scanning the autoradiograms with a densitometer, each peak representing viral protein was converted to a percentage on the total absorbance.

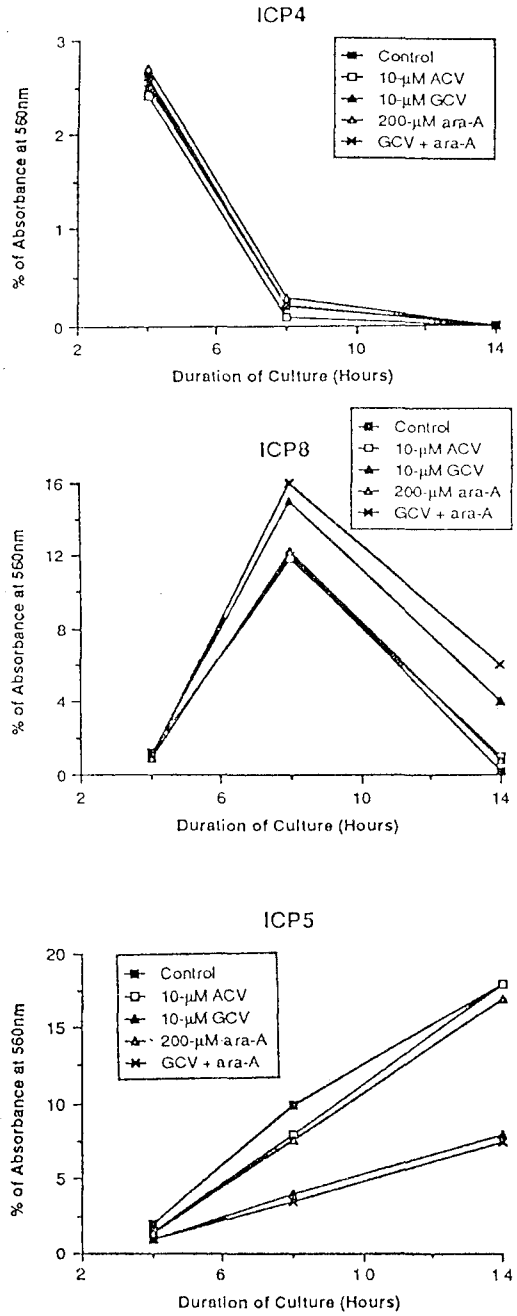

Fig. 13. Relative rates of representative proteins synthesis of ACV-resistant HSV-1, PAA^{r}5 (DNA polymerase mutant).

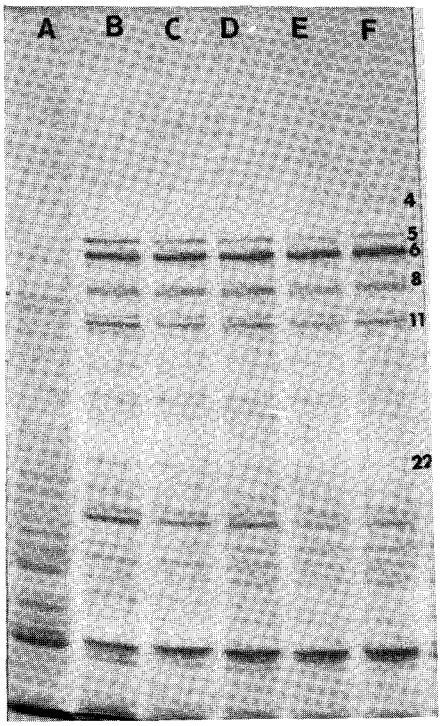

Fig. 14. Autoradiographic images of electrophoretically separated proteins from cells infected with wild type HSV-1 (KOS) and maintained in the absence or presence of antiviral agents from the time of infection until 4 hours postinfection. A: Non-infected cells; B: HSV-1 (KOS) infection only; C: 10-μM of ACV with HSV-1 infection; D: 200-μM of ara-A with viral infection; E: 10-μM of GCV with HSV-1 infection; F: 5-μM of GCV + 100-μM of ara-A with viral infection. The numbers at the left hand side indicate the ICPs.

was more greatly inhibited (approximately 95 %) compared to the conditions where GCV or ara-A alone was present in the culture medium. However, the production of cellular DNA in the viral infected cells was not significantly altered by combined drug therapy (Fig. 10). GCV or ara-A alone or in combination, did not greatly inhibited the viral DNA synthesis in ACV-resistant HSV-1 with thymidine kinase mutation such as ACV^r and IUdR^r (Fig. 9, 11).

Effect of combined GCV and ara-A on the HSV-1 protein synthesis

Fig. 12 and 13 show the effect of ACV (10-μM), GCV (10-μM), ara-A (200-μM), and combined GCV (5-μM) and ara-A (100-μM) on the

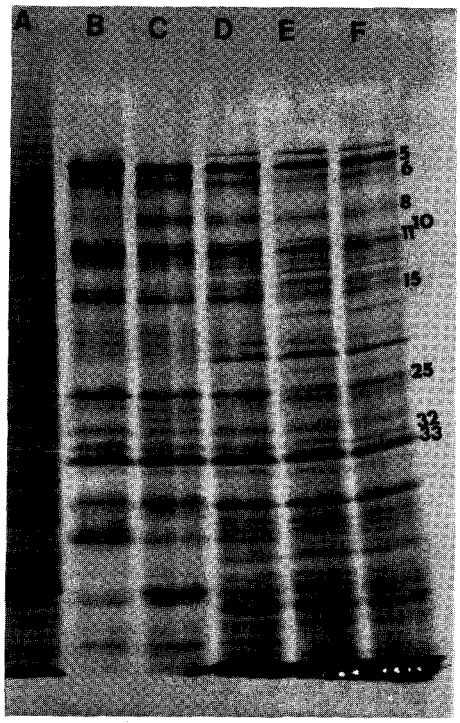

Fig. 15. Autoradiographic images of electrophoretically separated proteins from cells infected with wild type HSV-1 (KOS) and maintained in the absence or presence of antiviral agents from the time of viral infection until 8 hours postinfection.

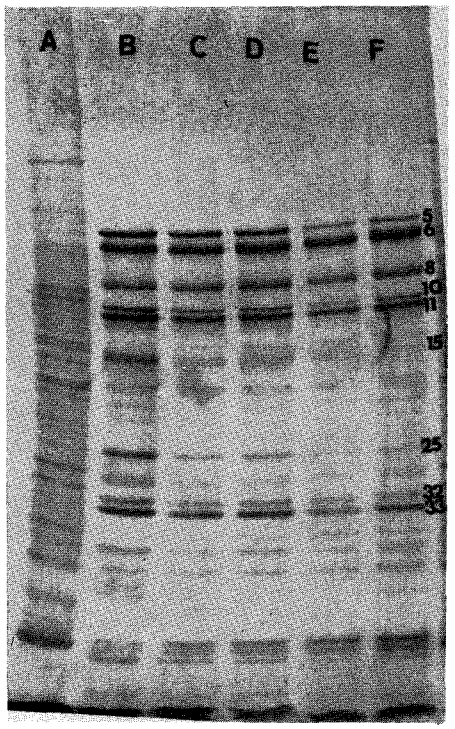

Fig. 16. Autoradiographic images of electrophoretically separated proteins from cells infected with wild type HSV-1 (KOS) and maintained in the absence or presence of antiviral agents from the time of infection until 14 hours postinfection.

synthesis of representative wild type HSV-1 KOS strain and PAA^{r}5 proteins at various postinfection time, respectively. Fig 14-19 represent the autoradiographic images of electrophoretically separated proteins from cells infected with wild type HSV-1 KOS strain or ACV-resistant DNA polymerase mutant (PAA^{r}5), respectively, in the presence of ACV (10-μM), GCV (10-μM), ara-A (200-μM), and combined GCV (5-μM) and ara-A (100-μM) from the time of infection until 4, 8, or 14 hours postinfection. The synthesis of ICP4 (α-protein) in cells infected with HSV-1 KOS strain or PAA^{r}5 was not altered by ACV, GCV, ara-A, or combined GCV and vidarabine. The synthesis rate of ICP8 (β-protein) in cells infected with wild type HSV-1 KOS strain was not changed by those antiviral agents at 4 hours postinfection, but a significant increase in the synthesis rate of ICP8 was noticed at 8 and 14 hours after the viral infection (Fig. 12, 13, 15 and 16). As shown in figure 16, there was a significant increase in the synthesis of ICP8 at 14 hours after viral infection in the presence of antiviral agents, while the synthesis of ICP8 was almost completely shut off in infection control. However, the synthesis of ICP5 (γ-protein) was significantly decreased by antiviral agents, alone or in combination. The inhibitory activity of combined GCV (5-μM) and ara-A (100-μM) on ICP5 in cells infected with wild type HSV-1 KOS strain was comparable to that of GCV (10-μM) alone (Fig. 15 and 16). ACV at concentration of 10-μM did not alter the expression of ICP4, ICP8 or ICP5 in cells infected with ACV-resistant DNA polymerase mutant PAA^{r}5, while GCV (10-μM), ara-A (200-μM), or combined GCV (5-μM) and ara-A (100-μM) altered the expression of ICP8 and ICP5, not ICP4, as similar manner as in cells infected with wild type HSV-1 (Fig. 13, 17~19).

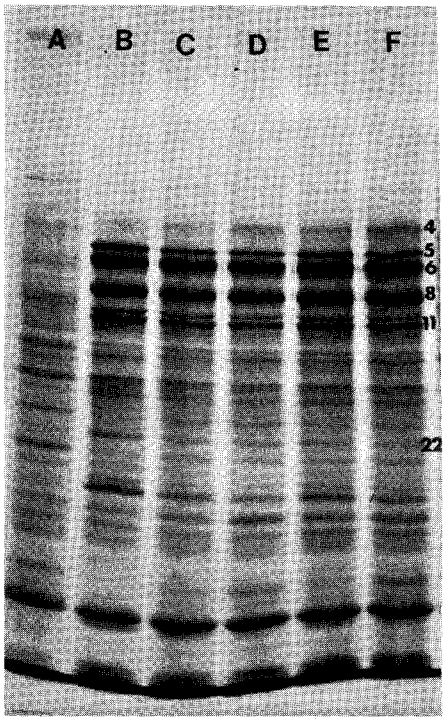

Fig. 17. Autoradiographic images of electrophoretically separated proteins from cells infected with ACV-resistant HSV-1 with DNA polymerase mutation (PAA^{r}5) and maintained in the absence or presence of antiviral agents from the time of infection until 4 hours postinfection. A: Noninfection control; B: HSV-1 (PAA^{r}5) infection only; C: 10-μM of ACV with viral infection; D: 200-μM of ara-A with viral infection; E: 10-μM OF GCV with viral infection; F: 5-μM of GCV + 100-μM of ara-A with viral infection. The numbers at the left hands side indicate the ICPs.

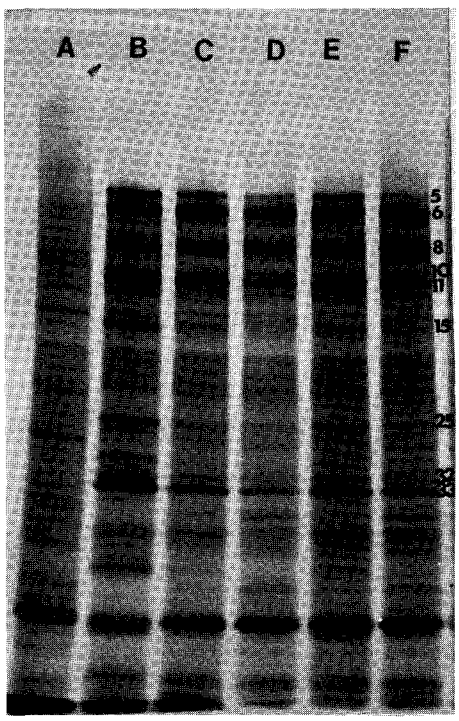

Fig. 18. Autoradiographic images of electrophoretically separated proteins from cells infected with ACV-resistant HSV-1 (PAA^{r}5) and maintained in the absence or presence of antiviral agents from the time of infection until 8 hours postinfection.

DISCUSSION

Although ACV is now in extensive clinical use for the treatment of HSV infections, recent reports of the emergence of ACV-resistant HSV mutants in patients give cause for concern when considering the plausibility of the antiviral therapy if resistance can emerge so rapidly against a major nontoxic drug (Field and Darby, 1980; Field *et al.*, 1982; Boijoly *et al.*, 1983; Larder and Darby, 1985). Therefore, evaluation of the effectiveness of other antiviral agents to combat the ACV-resistant HSV mutants becomes essential. Such studies could also provide guidance to clinicians in the proper selection of antiviral agents for the treatment of ACV-resistant HSV infections. AS expected, ACV-resistant HSV-1 with thymidine kinase mutations were also resistant to GCV. In the light of the fact that GCV is activated by herpesvirus-encoded thymidine kinase, the presence of viral-specific thymidine kinase is essential for the activation of GCV. Therefore, one could expect a weak antiherpetic activity of GCV in cells infected with HSV mutant (ACV^r) which does not express thymidine kinase. Another type of ACV-resistnat HSV-1 (IUdR^r) which expresses thymidine kinase with altered substrate specificity was also resistant to GCV, but it was far less resistant to GCV compared to ACV^r. These data indicate that GCV could be slightly

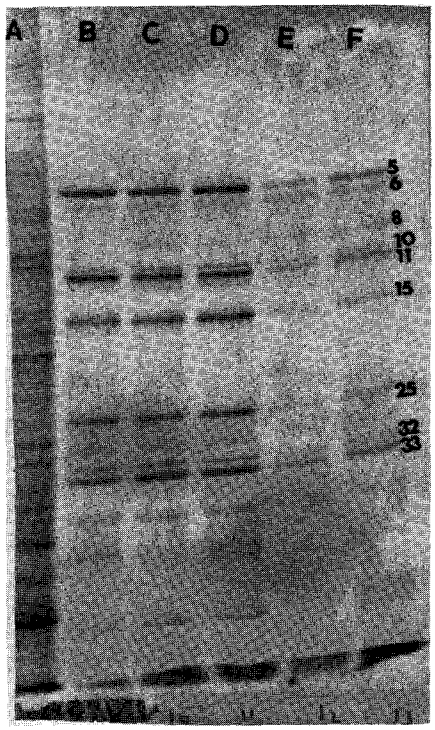

Fig. 19. Autoradiographic images of electrophoretically separated proteins from cells infected with ACV-resistant HSV-1 (PAA^{r}5) and maintained in the absence or presence of antiviral agents from the time of infection until 14 hours postinfection.

activated by the thymidine kinase with altered substrate specificity. Nonetheless, GCV should not be recommended for the control of the infections caused by ACV-resistant HSV-1 with thymidine kinase mutations. However, these thymidine kinase mutants showed the same sensitivity as the wild type HSV-1 to ara-A. As mentioned earlier ara-A is phosphorylated by adenosine kinase and adenylate kinase to be active forms, ara-ADP and ara-ATP (Park and Pavan-Langston, 1982). Since the activation of ara-A is not depend on the presence of herpesvirus-encoded thymidine kinase, ara-A might show similar antiviral activity against the wild type HSV-1 as well as thymidine kinase mutants. Therefore, ara-A must be considered as the drug of choice for infections caused by ACV-resistant HSV with thymidine kinase mutations. Since all tested antiviral agents interact finally with viral specfic DNA polymerase to elicit their antiviral activity, alteration of viral DNA polymerase may confer resistance to these antiviral drugs. However, the DNA polymerase mutant, PAA^{r}5 showed an extreme sensitivity to GCV and to ara-A: alterations at the DNA polymerase locus which gave ACV resistance resulted in no change in susceptibility to the other nucleoside analogs. This result indicates that the binding sites of GCV triphosphate and ara-A triphosphate in viral DNA polymerase might be different from the locus where ACV triphosphate binds.

These results also showed that combined GCV and ara-A showed potentiating synergistic activity in cells infected with wild type HSV-1 or ACV-resistant DNA polymerase mutant. The exact mechanism of this synergism is unknown. However, based on these data that the combination of GCV and ara-A reduced the synthesis of viral DNA more significantly than GCV or ara-A alone in the cells infected with wild type HSV-1 or ACV-resistant DNA polymerase mutant, one would assume that the combined regimen might show synergism by inhibiting the synthesis of viral DNA in the infected cells in a potentiating synergistic manner. Since the mechanisms of activation of GCV and ara-A are different, these drugs would not interfere the each other's activation processes in viral infected cells, which allowed one to predict the synergistic activity of these drugs in combination. However, since GCV and ara-A share the ultimate site of action, viral DNA polymerase, combination of GCV and ara-A can be assumed to produce antagonistic or indifferent antiviral activities in the cells infected with susceptible viruses. In contrast to this assumption, GCV and ara-A showed strong synergistic effect in the inhibition of viral replication and viral DNA synthesis. These results indicate that the binding sites (or locus of action)of GCV and ara-A might be different in the viral DNA polymerase. The mechanisms of failure of additive or potentiation synergism of GCV and ara-A against thymidine kinase mutants is not clear with this experiment. However, weak antiviral and anti-DNA synthesis effects of GCV against the thymidine kinase mutants might be responsible for the lack of combined antiviral effect of GCV and ara-A.

The present study indicates that the tested antiviral agents inhibit the synthesis of viral DNA and alters the expression pattern of some classes of viral proteins in cells infected with the wild type

HSV-1. The synthesis of viral α-and β-proteins in the early stage of infection was not altered by the antivirals, which indicates the expression of α-and β-proteins in the early stage of infection is from the parental viral DNA, not from the newly synthesized progeny viral DNA. The data also shows that the synthesis of β-proteins was increased at the later stage of infection. Based on the facts that (a) The synthesis of 3 classes of HSV proteins is coordinately regulated and sequentially ordered in a cascade manner (Honess and Roizman, 1974 and 1975); (b) the synthesis of viral DNA is known to begins at 3 hours postinfection, coincides with the appearance of β-proteins (Roizman and Honess, 1974); (c) the synthesis of viral β-proteins are negatively regulated by certain viral γ-proteins (Godowski and Knipe, 1986); and (d) most viral γ-proteins are expressed from the newly synthesized progeny viral DNA (Chun and Park, 1987), the increase in the β-proteins in the later stage of viral infection might due to the potent inhibition of viral DNA synthesis by the antiviral agents. Furthermore, since parental viral DNA can serve as template for mRNAs of α-, β-, and γ-proteins (Honess and Roizman, 1973), the apparent stimulation of β-proteins synthesis by the antiviral agents might result from the parental viral DNA to be more available for its transcription for β-proteins rather than any effect of antiviral agents on progeny viral DNA. The electrophoretic profile seen in wild type HSV-1 infected cells treated with antiviral agents, alone or in combination, indicates in the reduction in the synthesis of viral γ-proteins such as IPC5, ICP10, ICP11, ICP15, ICP25, ICP32, and ICP33. Since the synthesis of new progeny viral DNA occurs before the expression of most γ-proteins (Conley, 1981), and the greater portion of viral γ-proteins are made from the newly synthesized progeny DNA (Furman and Mcguirt, 1983; Powell *et al.,* 1975), the inhibition of viral progeny DNA synthesis might be responsible for the decrease in the synthesis of γ-proteins in the infected cells. The reduction of γ-proteins, although a secondary effect of antiviral agents, may also play a role in the antiviral activity. The effect of combined GCV and ara-A also shows somewhat synergistic activity in the inhibition of γ-protein expression. However, the synergism in the inhibition of γ-protein synthesis was not as great as that in viral DNA synthesis. These data suggest that part of viral γ-proteins was synthesized from the parental viral DNA. The effects of GCV and ara-A, alone or in combination, on the viral protein synthesis in cells infected with ACV-resistant HSV-1 with DNA polymerase mutation are similar to those in cells infected with wild type HSV-1. This fact indicates that mode of synergistic antiviral activity of combined GCV and ara-A to ACV-resistant DNA polymerase mutant is similar to that of those combined drugs to wild type HSV-1.

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REFERENCES

Abramowicz M: *Oral acyclovir for genital herpes simplex infection. The Medical Letter on Drugs and Ther 27:1-43, 1985*

Ashton WT, Karkas JD, Field AK and Tolamn RL: *Activation by thymidine kinase and potent antiherpetic activity of 2'-nor-2'-deoxyguanosine (2' NDG). Biochem Biophys Res Commun. 108: 1716-1721, 1982*

Boijoly HM, Park N-H and Pavan-Langston D: *Herpes simplex acyclovir-resistant mutant in experimental keratouveitis. Arch Ophthalmol 101:1728-1786, 1983*

Bryson YJ and Kronenberg LH: *Combined antiviral effects of interferon, adenine, arabinoside, hypoxanthine arabinoside, and adenine arabinoside-5'-monophosphate in human fibroblast cultures. Antimicrob Agents Chemother 11: 299-306, 1977*

Bryson YJ, Dillon M, Lovett M, Acuna G, Taylor S, Cherry JD, Johnson BL, Weismeier E, Growdon W, Creagh-Kirk T and Keeney R: *Treatment of first episodes of genital herpes simplex virus infection with oral acyclovir. N Engl J Med*

308:916-921, 1983

Burns WH, Saral R, Santos GW, Laskin OL, Leithan PS, McLaren C and Barry DW: *Isolation and characterisation of resistant herpes simplex virus after acyclovir therapy. lancet 1:421-423, 1982*

Calabresi P, Ardoso SS, Finch SC, Kingerman NM, Von Essen CF, Chu MY and Welch AD: *Initial clinical studies with 5-iodo-2'-deoxyuridine. Cancer Res 21:550-559, 1961*

Chaube S and Murphy ML: *The teratogenic effects of the recent drugs in cancer chemotherpay. Adv Teratol 3:181-237, 1968*

Cheng Y, Grill SP, Dutschman GE, Nakayama K and Bastow K: *Metabolism of 9-(1,3-dihydroxy-2-propoxymethyl) guanine, a new anti-herpes virus compound, in herpes simplex virus-infected cells, J Biol Chem 258:12460-12464, 1983*

Chun Y-S and park H-H: *Effect of 1-(1,3-dihydroxy-2-propoxymethyl) guanine on the viral DNA and protein synthesis in cells infected with herpes simplex virus. Antimicrob Agents Chemother. 31:249-351, 1987*

Coen DM and Schaffer PA: *Two distinct loci confer resistance to acyeloguanosine in herpes simplex virus type 1. Proc Natl Acad Sci USA 77:144-150, 1980*

Conley AJ, Knipe DM, Jones PC and Roizman B: *Molecular genetics of herpes simplex viurs. VII. Characterization of a temperature-sensitive mutant produced by in vitro mutagenesis and defective in DNA synthesis and accumulation of gamma polypeptides. J Virol 37:191-206, 1981*

Corey L, Nahmias AJ, Guinan ME, Benedetti JK, Critchlow CW and Holmes KK: *A trial of topical acyclovir in genital herpes simplex virus infections. N Engl J Med 306:1313-1319, 1982*

Coster DJ, Wilhelmus KR, Michaub R and Jones BR: *A comparison of acyclovir and idoxuridine as treatment for ulcerative herpes keratitis. Br J Ophthalmol 64:763-765, 1980*

Crumpacker CS, Schnipper LE, Marlow SI, Kowalsky PN, Hershey BJ and Levin MJ: *Resistance to antiviral drugs of herpes simplex virus isolated from a patient treated with acyclovir. N Engl J Med 306:343-346, 1982*

Crumpacker CS, Kowalsky PN, Oliver SA, Schnipper LE and Field AK: *Resistance of herpes simplex virus to 9-{[2-hydroxy-1-(hydroxymethyl)ehtoxy]methyl}guanine: Physical mapping of drug synergism within the viral DNA polymerase locus. Proc Natl Acad Sci USA 81:1556-1560, 1984*

Cupps TR, Straus SE and Waldmann TA: *Successful Treatment with acyclovir of an immunodeficient patient infected simultaneously with multiple herpesviruses. Am J Med 70:882-886, 1981*

Darby G, Field HJ and Salisbury SA: *Altered substrate-specificity of herpes simplex virus thymidine kinase confers acyclovir resistance. Nature (London) 289:81-83, 1981*

DeLuca NA and Schaffer PA: *Activation of immediate-early, early, and late promotors by temperature-sensitive and wild-type forms of herpes simplex virus type 1 protein ICP4. Mol Cellular Biol 8:1997-2008, 1985*

Douglas JM, Critchlow C, Benedetti J, Mertz GJ, Connor JD, Hintz MA, Fahnlander A, Remington M, Winter C and Corey L: *A double-blind study of oral acyclovir for suppression of recurrences of genital herpes simplex virus infection. N Engl J Med 310:1551-1556, 1984*

Elion GP, Furman PA, Fyfe JA, De Miranda P, Beauchamp L and Schaeffer HJ: *Selectivity of action of an antiherpetic agent 9-(2-hydroxyethoxymethyl)guanine. Proc Natl Acad Sci USA. 74:5716-5720, 1977*

Field HJ, Darby G ad Wildy P: *Isolation and characterisation of acyclovir-resistant mutants of herpes simplex virus. J Gen Virol 49:115-124, 1980*

Field HJ, Larder BA and Darby G: *Isolation and characterization of acyclovir-resistant strains of herpes simplex virus. Am J Med 151:369-371, 1982*

Field AK, Daves ME, Dewitt C, Perry HC, Liou R, Germerschausen J, karkas JD, Ashton WT, Johnson BR and Tolman RI: *9-([2-Hydroxy-1-hydroxymethyl]ethoxymethyl)guanine: a selective inhibitor of herpes group virus replication. Proc Natl Acad Sci USA 80:4139-4143, 1983*

Foster CS and Pavan-Langston D: *Corneal wound healing and antiviral medication. Arch Ophthalmol 95:2062-2067, 1977*

Frank KB, Chiou J-F, Cheng Y: *Interaction of herpes simplex virus-induced DNA polymerase with 9-(1,3-dihydroxy-2-propoxymethyl)guanine triphosphate. J Biol Chem 259:1566-1569, 1984*

Fraser-Smith EB, Epstein DA, Marsh YV and Matthew TR: *Enhanced efficacy of acyclic nucleoside 9-(1,3-dihydroxy-2-propoxymethyl)guanine in combination with α-interferon against herpes simplex virus type 2 in mice. Antimicrob Agents Chemother 26:937-938, 1984*

Furman PA and Mcguirt PV: *Effect of acyclovir on viral protein synthesis in cells infected with herpes simplex virus type 1. Antimicrob Agents*

Chemother 23:332-334, 1983

Furman PA, ST Clair MH, Fyfe JA, Rideout JL, Keller PM and Elion GB: *Inhibition of herpes simplex virus-induced DNA polymerase activity and viral DNA replication by 9-(2-hydroxy-ethoxymethyl)guanine and its triphosphate. J Virol 32:72-9, 1979*

Furman PA: *Acyclovir-resistant mutants of hepres simplex virus type 1 express altered DNA polymerase or reduced acyclovir phosphorylating activities. J Virol 40:936-941, 1981*

Furman PA, St Clair MH and Spector T: *Acyclovir triphosphate is a suicide inactivator of the herpes simplex virus DNA polymerase. J Biol Chem 259:9575-9579, 1984*

Godowski PJ and Knipe DM: *Transcriptional control of herpes gene expression: Gene functions required for positive and negative regulation. Proc Natl Acad Sci USA 83:256-260, 1986*

Hammer SM, Kaplan JC, Lowe BR and Hirsch MS: *Alpha interferon and acyclovir treatment of herpes simplex virus in lymphoid cell cultures. Antimicrob Agents Chemother 21:634-640, 1982*

Hanshaw JB: *Herpes simplex hominis infections in the fetus and the new born. Am J Dis Child 126: 546-555, 1973*

Helgstrand E, Eriksson B, Johansson NG, Lanneroe B, Larsson A, Misiorny A, Noren JO, Sjoeberg B, Stenberg K, Stening G, Stridh S and Oeberg B: *Trisodium phosphonoformate, a new antiviral compound. Science 201:819-821, 1978*

Honess RW and Roizman B: *Proteins specified by herpes siplex virus. XI. Identification and relative molar rates polypeptides in the infected cells. J Virol 12:1347-1365, 1973*

Honess RW and Roizman B: *Regulation of herpes simplex macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. J Virol 14:8-19, 1974*

Honess RW and Roizman B: *Regulation of herpesvirus macromolecular synthesis: 1 sequential transition of polypeptide synthesis requires functional viral polypeptides. Proc Natl Acad Sci USA 72:1276-1280, 1975*

Itoi M, Gefter JW, kaneko N, Ishii Y, Ramer RM and Gasset AR: *Teratogenicities of ophthalmic drugs. I. Antiviral ophthalmic drugs. Arch Ophthalmol 93:46-51, 1975*

Kaufman HE, Martola E and Dohlman CH: *Use of 5-iodo-2'deoxyuridine (IDU) in treatment of herpes simplex keratitis. Arch Ophthalmol 68: 235-239, 1962*

Kaufman RH, Dreesman GR, Burek J, Korhonen MO, Matson DO, Melnick JL, Powell KL, Purifoy DJM, Courtney RJ and Adam E: *Herpes virus-induced antigens in squamous-cell carcinoma in situ of the vulva. N Engl J Med 305:483-488, 1981*

Klein RJ, Friedman-Kien AE and DeStefano E: *Latent hepres simplex virus infections in sensory ganglia of hairless mice prevented by acycloguanosine. Antimicrob Agents Chemother 15: 723-729, 1979*

Knipe DM, Quinlan MP and Spang AE: *Characterization of two conformational forms of the major DNA-binding protein encoded by herpes simplex virus 1. J Virol 2:736-741, 1982*

Knopf KW, Kaufman ER and Crumpacker C: *Physical mapping of drug resistance mutations defines an active center of the herpes simplex virus DNA polymerase enzyme. J Virol 39:746 -757, 1981*

Larder BA and Darby G: *Virus drug-resistance: mechanism and consequences. Antiviral Res 4:1 -42, 1984*

Larder BA and Darby G: *Selection and characterisation of acyclovir-resistant herpes simplex virus type 1 mutants inducing altered DNA polymerase activities. Virol 146:262-271, 1985*

Larder BA and Darby G: *Susceptibility to other antiherpes drug of pathogenic variants of herpes simplex virus selected for resistance to acyclovir. Antimicrob Agents Chemother. 29:894 -898, 1986*

Marr EC, Cheng YC and Huang ES: *Effect of 9-(1, 3-dihydroxy-2-propoxymethyl)guanine on human cytomegalovirus replication in vitro. Antimicrob Agents Chemother 24:518-521, 1983*

McDougall JK, Galloway and Fenoglio CM: *cervical carcinoma: detection of herpes simplex virus RNA in cells undergoing neoplastic change. Int J Cancer Res 25:1-8, 1980*

McLaren C, Corey L, Dekker C and Barry DW: *In vitro sensitivity to acyclovir in genital herpes simplex viruses from acyclovir-treated patients. J Infect Dis 148:868-875, 1983*

Morse LS, Pereira L, Roizman B and Schaffer PA: *Anatomy of HSV DNA. XI. Mapping of viral genes by analysis of polypeptides and functions specified by HSV-1X HSV-2 recombinants. J Virol 26:389-410, 1978*

Nahmias AJ and Roizman B: *Infection with herpes simplex viruses 1 and 2. N Engl J Med 289:667 -674, 1973*

Nahmias AJ, Alford CA and Korones SB: *Infection of the newborn with herpes virus hominis. Adv*

Pediatrics 17:185-226, 1970

National Institutes of Health: *Workshop on the treatment and prevention of herpes simplex virus infection. J Infect Dis 127:117-120, 1973*

Overall JC: *Dermatologic disease: In Antiviral Agents and Viral Diseases. Ed by Galasso Gj, Merigan TC and Buchanan RA, Raven Press, NY, pp 305-384, 1979*

Park N-H, Pavan-Langston D, McLean SL and Albert DM: *Therapy of experimental herpes simplex encephalitis with acyclovir in mice. Antimicrob Agents Chemother 15:775-779, 1979*

Park N-H, Pavan-Langston D, McLean SL and Lass JH: *Acyclovir topical therapy of cutaneous herpes simplex virus infection in guinea pigs. Arch Dermatol 116:672-675, 1980a*

Park H-H, pavan-Langston D and Hettinger ME: *Acyclovir therapy for the orofacial and ganglionic HSV infection in hairless mice. J Dent Res 59:2080-2986, 1980b*

Park N-H, Pavan-Langston D, Hettinger ME, McLean SL, Albert DM, Lin T-S and Prusoff WH: *Topical therapeutic efficacy of 9-(2-hydroxyethoxymethyl)guanine and 5-iodo-5'-amino-2',5'-dideoxyuridine on oral infection with herpes simplex virus in mice. J. Infect Dis 141:575-579, 1980*

park N-H, Pavan-Langston D and DeClercq E: *Effect of acyclovir, bromovinyldeoxyuridine, vidarabine and l-lysine on latent ganglionic herpes simplex virus in vitro. Am J Med 73:151-154, 1982*

Park N-H and pavan-Langston D: *Purines: in Chemotherapy of Viral Infections. Handbook of Experimental Pharmacology. Edited by Came PE and Caliguiri LA 61:117-136, 1982*

Park N-H, Callahan J and Pavan-Langston D: *Effect of combined acyclovir and vidarabine on infection with herpes simplex virus in vitro and in vovo. J Infect Dis 149:757-762, 1984*

Park N-H, Niukian K and Shklar G: *Combined effect of herpes simplex virus and tobacco on the histopathologic changes in lips of mice. Oral Surg 59:154-158, 1985*

Park N-H, Sapp JP and Herbosa EG: *Oral cancer induced in hamsters with herpes siplex infection and simulated snuff-dipping. Oral Surg 62:162-268, 1986*

Parris DS and Harrington JE: *Herpes simplex virus variants resistant to high concentrations of acyclovir exist in clinical isolates. Antimicrob Agents Chemother 22:71-77, 1982*

Pavan-Langston D: *Ocular viral diseases. In Antiviral Agents and Viral Diseases in Man. Ed. by Galasso GJ, merigan TC and Buchanan RA, Raven press, NY pp 253-303, 1979*

Pavan-Langston D, North RD, Geary PA and Kinkel A: *Intraocular penetration of the solufle antiviral, ara-AMP. Arch Ophthalmol 94:1585-1588, 1976*

Pavan-Langston D and Foster CS: *Trifluorothymidine and idoxuridine therapy of ocular herpes. Am J Ophthalmol 84:818-825, 1977*

Percy DH and Albert DM: *Developmental defects in rats treated postnatally with 5-iododeoxyuridine (IUdR). Teratol 9:275-286, 1974*

Powell KL, Purifoy DJM and Courtney RJ: *The synthesis of herpes simplex virus proteins in the absence of virus DNA synthesis. Biochem Biophys Res Commun 66:262-271, 1975*

Rapp F: *Variants of herpes simplex virus: Isolation, characterization, and factors influencing plaque formation. J Bacteriol 86:985-991, 1963*

Rapp F and Reed C: *Experimental evidence for the oncogenic potential of herpes simplex virus. Cancer Res 36:800-806, 1976*

Roizman B and Roane PR Jr: *The multiplication of herpes simplex virus. II. The relationship between protein synthesis and the duplication of viral DNA in infected HEp-2 cells. Virol. 22:262-269, 1964.*

St Clair MH, Miller WH, Miller RL, Lambe CU and Furman PA: *Inhibition of cellular alpha DNA polymerase and herpes simplex virus-induced DNA polymerases by the triphosphate of BW759U. Antimicrob Agents Chemother 25:191-194, 1984*

Schinazi RF and Hahmias AJ: *Different in vitro effects of dual combinations of antiherpes simplex virus compounds. Am J Med 73:40-48, 1982*

Schnipper LE and Crumpacker CS: *Resistance of herpes simplex virus to acycloguanosine: Role of viral thymidine kinase and DNA polymerase loci. Proc Natl Acad Sci USA 77:2270-2273, 1980*

Scully C, Eglin RP, Ward-Booth P, McGregor IA and Boyle P: *Human oral squamous cell carcinoma: Evidence for RNA complementary to herpes simplex virus DNA. IRCS Medical Science 10:531, 1982*

Sibrak CD, Gutman LD, Wiffert CM, McLaren C, St Clair MH, Keller PM and Barry DW: *Pathogenicity of acyclovir-resistant herpes simplex virus type 1 from an immunodeficient child. J Infect Dis 146:673-682, 1982*

Smee DF, Martin JC, Verhyden JPH and Matthews

TR: *Anti-herpesvirus activity of the acyclic nucleoside 9-(1,3-dihydroxy-2-propoxymethyl) guanine. Antimicrob. Agents Chemother 23:676-682, 1983*

Spruance SL, Overall JC and Kern ER: *Pathogenesis of herpes simplex labialis: excretion of virus in the oral cavity. J Clin Microb 19:675-679, 1984*

Straus SE, Seidlin M, Takiff H, Jacobs D, Bowen D and Smith H: *Oral acyclovir to suppress herpes simplex virus infections in immunodeficient patients. Ann Internal med 100:522-524, 1982*

Straus SE, Takiff HE, Seidlin M, Bachrach S, Lininger L, DiGiovanna JJ, Western KA, Smith HA, Lehrman SN, Creagh-Kirk T and Alling DW: *Suppression of frequently recurring genital herpes: A placebo-controlled double blind trial of oral acyclovir. N Engl J Med 310:1545-1550, 1984*

Trousdale MD, Nesburn AB, Wiley DE and Taid H: *Efficacy of BW759 (9-([2-hydroxy-1-hydroxymethyl]ethoxy)guanine) against herpes simplex virus type 1 keratitis in rabbits. 3:1007-1015, 1984*

Valeriote F and Lin H-S: *Synergistic interaction of anticancer agents: a cellular perspective. Cancer Chemotherapy Reports 59:895-900, 1975*

Wade JC, McLaren C and meyers JD: *Frequency and significance of acyclovir-resistant herpes simplex virus isolated from marrow transplant patients receiving multiple courses of treatment with acyclovir. J Infect Dis 148:1077-1082, 1983a*

Wade JC, Newton B, Flournoy N and Meyers JD: *Oral acyclovir for prevention of herpes simplex reactivation after marrow transplantation. Ann Int Med 100, 823-828, 1983b*

Wade JC, Day LM, Crowley JJ and Meyers JD: *Recurrent infection with herpes simplex virus after marrow transplantation: Role of the specific immune response and acyclovir treatment. J Infect Dis 149:750-756, 1984*

Wentz WB, Reagan JW, Heggie AD, Fu Y-S and Anthony DD: *Induction of uterine cancer with inactivated herpes simplex virus, types 1 and 2. Cancer 48:1783-1790, 1981*

Whiltley RJ, levin M, Batron N, Hershey BJ, Davis G, keeney RE, Welchel J, Diethelm AG, Kartus P and Soong S-J: *Infections caused by herpes simplex virus in the immunocompromised host: Natural history and topical acyclovir therapty. J Infect Dis 150:323-329, 1984*

Whiltley RJ, Alford CA, Hirsch MS, Schooley RT, Luby JP, Akoi FY, Hanley D, Nahmias Aj, Soong S-J and the NIAID Collaborative Antiviral Study Group: *Vidarabine versus acyclovir therapy in herpes simplex encephalitis. N Engl J Med 314:144-149, 1986*

= 국문초록 =

Acyclovir저항성 Herpes Simplex Virus의 복제, DNA합성 및 형질 발현에 미치는 Ganciclovir 및 Vidarabine의 병용효과에 관한 연구

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Ganciclovir(GCV)와 Vidarabine(ara-A)을 단독으로 또는 동시에 HSV-1에 작용시켰을때 HSV-1의 복제, DNA합성 및 단백질 합성에 미치는 영향을 관찰할 목적으로 본 연구를 시행하였다. 본 실험에서는 4가지의 다른 HSV-1(Wild type KOS, VCV^r, $IUdR^r$, 및 PAA^r5)를 사용하였다. Virus 복제에 미치는 항 virus약의 효과는 Vero 세포단층 배양에서 Yield reduction assay에 의해서 관찰하였다. 항 virus약의 virus DNA 합성에 미치는 영향은 NaI 밀도 구배초원심침전후 H^3-표지 virus DNA의 방사능에 의해서 관찰하였다. Virus 단백질의 합성에 미치는 항 virus약의 효과는 ^{35}S를 표지한 후 polyacrylamide 겔 전기영동법, 자가방사기록법 그리고 virus bands의 음영농도주사법을 이용, 관찰하여 다음과 같은 결과를 얻었다.

1. GCV는 wild trype HSV-1 KOS와 PAA^r5의 복제를 강력하게 억제하였으나 ACV^r와 $IUdR^r$는 GCV에 대해서 중등도의 저항을 보였다. ara-A는 실험대상의 모든 HSV-(KOS, ACV^r, $IUdR^r$ 및 PAA^r5)의 복제에 대해서 거의 비슷하게 억제효과를 보였다. GCV와 ara-A의 동시첨가는 KOS와 PAA^r5의 복제에 대해서 상승적인 억제효과를 보였고 ACV^r와 $IUdR^r5$의 복제에 대해서는 상가작용이하의 억제 효과를 보였다.

2. GCV 또는 ara-A는 HSV-1감염 Vero세포에서 virus DNA의 합성을 유의하게 억제하였다. GCV와 ara-A의 동시첨가는 KOS 또는 PAA^r5 감염세포에서 virus DNA 합성을 GCV 또는 ara-A를 단독 첨가하였을때 보다 현저하게 억제하였다. ACV^r 또는 $IUdR^r$ 감염세포에서는 이런 현상을 관찰할 수 없었다.

3. Wild type HSV-1 감염세포에서 virus-단백질의 합성은 GCV, ara-A의 단독 첨가 또는 GCV와 ara-A의 동시첨가에 의해서 변경되지 않았다. Wild type HSV-1 감염말기에 GCV 또는 ara-A 단독 혹은 동시첨가에 의해서 virus 단백질의 합성은 경미하나마 유의성있게 증가하였다. Wild type HSV-1과 PAA^r5에 의한 단백질의 합성은 GCV 또는 ara-A 단독 첨가에 의해서 유의성있게 억제되었다. GCV 또는 ara-A의 동시첨가는 GCV 또는 ara-A를 단독으로 첨가했을 경우보다 단백질의 합성을 더욱 억제하였다.

이상의 실험결과로 보아 GCV와 ara-A의 동시사용은 HSV-1 혹은 ACV저항 DNA polymerase 변이주인 PAA^r5에 대해서 상승적인 억제작용을 나타냈으며 이 효과는 virus DNA 합성 억제에 의한 것으로 생각된다. ACV저항 thymidine kinase 변이주인 ACV^r 및 $IUdR^r$에 대해서는 ara-A가 유효하였다. 항 virus 약물에 의한 virus 단백질합성의 변화는 virus DNA 합성에 대한 억제효과에 인한 것으로 사려된다.