*Antiviral Research* 15 (1991) 67–76
Elsevier



AVR 00461

# The effect of HSV multiplication rate on antiviral drug efficacy in vitro

J.M. Upadhyay[1], J.M. Hill, M. Jemison, M.F. Helmy[2] and H.E. Kaufman

*Lions Eye Research Laboratories, LSU Eye Center, Department of Ophthalmology, Louisiana State University Medical Center School of Medicine, New Orleans, LA, U.S.A.*

(Received 7 August 1990; accepted 11 October 1990)

## Summary

HSV-1 multiplication rates have been shown to vary in different tissues and the rate of multiplication may correlate with susceptibility to antiviral chemotherapy. Herpetic stromal keratitis is a necrotizing condition refractive to antiviral therapy and this lack of antiviral efficacy in stromal disease may be the result of very low rates of viral replication in the corneal stromal keratocytes. In this study, we investigated the efficacy of antiviral drugs in an in vitro system in which the virus multiplication rate is slow. In this system, the reduced rate of virus multiplication is achieved by a reduction in the incubation temperature. Vero cells were infected at one of several multiplicities of infection with McKrae strain HSV-1 and incubated for 24, 48, or 72 h at 26 or 36.5°C in the presence or absence of trifluridine (50 μg/ml) or acyclovir (20 μg/ml). Both drugs suppressed viral replication at 36.5°C. However, under some specific sets of conditions, trifluridine was not effective in suppressing viral replication in cells incubated at 26°C. At this temperature, viral replication and cell metabolism are slowed to a pace which may be similar to that which occurs in corneal stromal keratocytes in vivo. Acyclovir significantly reduced HSV-1 replication under all conditions at 26°C, indicating that the antiviral activity of this compound is effective in cells whose metabolic rate is slow and in which viral replication is taking place slowly.

Antiviral; HSV-1; In vitro; Stromal disease; Viral replication

*Correspondence to:* Dr. James M. Hill, LSU Eye Center, 2020 Gravier Street, Suite B, New Orleans, LA 70112-2234, U.S.A.

*Present addresses:* [1]Loyola University, Department of Biological Sciences, New Orleans, LA, U.S.A.; [2]Microbiology and Immunology Department, Faculty of Medicine, AIN SHAMS University, Cairo, Egypt.

## Introduction

Ocular epithelial herpetic disease is characterized by cell-to-cell spread of rapidly replicating virus. Trifluridine, an antimetabolite that inhibits virus growth by selective incorporation into viral DNA, is the drug of choice in the USA for epithelial keratitis (Kaufman and Rayfield, 1988). Acyclovir, which exerts part of its antiviral effect by inhibiting viral DNA polymerase, has been shown to be equally effective against herpetic keratitis when topically applied (La Lau et al., 1981, 1982).

The role of replicating virus in stromal herpetic disease is still open to question, although it is generally agreed that if virus replication occurs in stromal keratocytes, the rate is slow. Although antiviral therapy is highly effective against epithelial keratitis, the therapy of stromal herpetic disease is problematic. Stromal herpes is treated with an antiviral such as trifluridine to prevent re-establishment of epithelial keratitis and a corticosteroid to reduce inflammation (Kaufman and Rayfield, 1988). Oral acyclovir has been tested in animal models and some clinical studies, but the results are equivocal (Kaufman and Rayfield, 1988; Kaufman et al., 1984). For the most part, the efficacy of antiviral agents in stromal disease, which lacks the rapidly replicating virus population characteristic of epithelial herpes, has not been conclusively demonstrated. Possible reasons for a lack of effect include: (1) virus strain characteristics, (2) the limitations of the mechanisms of action of the drugs, (3) inadequate dosage and duration of therapy, or (4) some combination of these factors. Alternatively, the problem may be the result of an entirely different pathogenesis, such as a mechanism of disease that does not depend on virus replication.

Metabolic inhibitors that interfere with the synthesis of viral DNA, such as trifluridine and acyclovir, are likely to be therapeutically effective only when DNA turnover is high. Thus, antiviral agents may be relatively ineffective under conditions characterized by limited synthesis of viral DNA, such as low incubation temperatures, non-permissive host tissues, and virus strains with low replication rates (Wheeler and Canby, 1960). Previous studies have shown that, in vitro, eradicating less rapidly multiplying strains of *Toxoplasma gondii* with the metabolic inhibitor pyrimethamine (Daraprim) requires higher drug concentrations and longer exposure times than the elimination of more rapidly multiplying strains (Kaufman et al., 1959; Maloney and Kaufman, 1964). The purpose of this study was to investigate the efficacy of trifluridine and acyclovir under conditions known to reduce the rate of viral replication. To this end, the antiviral activities of trifluridine and acyclovir at 26 and 36.5°C were compared by means of plaque reduction assays against McKrae strain HSV-1 in cell cultures infected at different multiplicities of infection (MOI) and various incubation periods.

## Materials and Methods

*Virus*

Herpes simplex virus type 1 (HSV-1) McKrae strain was used. The HSV-1 was plaque-purified three times and stored in small aliquots at −70°C. Each aliquot was used only once on the day of an experiment.

*Cell cultures*

Vero and CV-1 cells (African Green monkey kidney cells) were purchased from the American Type Culture Collection (Rockville, MD). Both cell lines were grown in Eagle Minimal Essential Medium (EMEM) (Gibco Laboratories, Gaithersburg, MD) with 10% fetal bovine serum (FBS) (Gibco Laboratories) containing 2 ml gentamicin solution (TriBio Laboratories, State College, PA) and 10 ml Fungi-Bact® solution (Irvine Scientific, Santa Ana, CA) per liter of medium. EMEM with 2% FBS containing antibiotics was used in all experiments.

*Antiviral drugs*

Trifluridine 1% (Viroptic®, Burroughs Wellcome Co., Research Triangle Park, N.C.) was purchased. Acyclovir powder was a gift from Dr. G. Elion (Burroughs Wellcome Co.).

*Virus adsorption efficiency*

McKrae strain HSV-1 ($5 \times 10^4$ PFU/ml) was inoculated onto confluent Vero cells in T-25 flasks and incubated at 36.5°C; samples of the medium were taken from triplicate flasks after 30 and 60 min of incubation. The initial inoculum and the virus suspensions were diluted by logs and plaqued on CV-1 monolayers in 24-well plates. The efficiency of adsorption (%) was calculated.

*Antiviral activity of acyclovir and trifluridine at different times of incubation at different temperatures (virus yield reduction assay)*

A stock suspension of McKrae strain HSV-1 ($6 \times 10^7$ PFU/ml) was diluted with EMEM containing 2% FBS to obtain the desired PFU/ml. Three MOIs were used: 0.1, 0.01, and 0.001. Diluted virus (1.0 ml) was adsorbed on a monolayer of Vero cells in 25 $cm^2$ tissue culture flasks for 60 min at 36.5°C. After incubation, the residual virus suspension was aspirated from the flasks. The monolayers were washed once with 1–2 ml of phosphate buffered saline (PBS).

To test the effect of antiviral drugs at different temperatures and incubation times, 3 ml of medium containing either trifluridine (50 $\mu$g/ml) or acyclovir (20 $\mu$g/ml) was added to each of the flasks containing HSV-1-infected cells. Control flasks received 3 ml of medium without drug. The flasks were incubated at 26 or 36.5°C

for 24, 48, or 72 h. After incubation, the flasks were observed for cytopathic effect (CPE) indicative of the presence of replicating HSV. All experiments were done in triplicate.

At the end of the incubation period, the medium was removed from the flasks. The monolayer was washed with PBS and 3 ml of EMEM containing 2% FBS was added. The flasks were stored overnight at −20°C. In flasks incubated for 72 h, microscopic observation revealed that a significant number of infected cells had separated from the monolayer. The medium from these flasks was transferred to 15 ml centrifuge tubes, the monolayer was washed with 1 ml of PBS, and both the medium and the wash were centrifuged at 1000 rpm for 5 min. The supernatant fluid was discarded. The pellet of cells was resuspended in 3 ml of medium, which was transferred back to a flask. The flasks were stored overnight at −20°C.

The next day, the samples were thawed and tenfold serial dilutions of the cell lysate in medium were made. From each dilution, triplicate aliquots (0.2 ml) were transferred onto the confluent CV-1 cell monolayers in 24-well multidishes (2.0 $cm^2$; Becton Dickinson, Oxnard, CA). The plates were incubated at 36.5°C in a humidified atmosphere of 5% $CO_2$ and 95% air. After virus adsorption for 1 h, 0.8 ml of the medium was added to each well and the plates were incubated for 3 days at 36.5°C. Cell monolayers were fixed in formalin and stained with 1% methylene blue, and the PFUs were counted.

*Drug resistance and cytotoxicity*

To test for drug resistance and cytotoxicity, 12 flasks containing cells infected with HSV-1, as described above, were divided into two groups: six flasks received 20 μg/ml acyclovir and six remained as untreated controls. Three of the six flasks in each group were incubated for 48 h and the other three flasks were incubated for 72 h at 36.5°C. All flasks were frozen overnight at −20°C.

The lysate from the three flasks in each group was combined, mixed, and diluted with EMEM with 2% FBS and antibiotics to contain $1 \times 10^3$ PFU per ml. Confluent monolayers of CV-1 cells in 96-well plates were infected by the addition of 0.1 ml of the diluted virus suspension. Following incubation at 36.5°C for 60 min, residual virus was removed. Acyclovir in EMEM plus 2% FBS and antibiotics (0.2 ml) was serially diluted (twofold) to concentrations ranging from 400 μg/ml to 0.39 μg/ml. Each drug concentration was applied to eight wells; uninfected cell control and virus-infected controls with no drug were prepared simultaneously. The plates were incubated for 24 h at 36.5°C. Cell monolayers were fixed in formalin and stained with 1% methylene blue. The observation of plaques in one or more wells was considered positive for CPE. If the entire monolayer appeared disrupted under the microscope, the effect was considered cytotoxic.

*Statistical analysis*

Analysis of variance was used to compare virus yield in the drug-treated and control cultures. A value of $P < 0.05$ was considered statistically significant.

## Results

*Virus adsorption efficiency*

Adsorption of HSV-1 on Vero cells after 60 min was 91.4%, compared to 32.7% after 30 min. Therefore, cultures of Vero cells were incubated for 60 min after HSV-1 inoculation for maximal virus adsorption before drug treatment was initiated.

*Antiviral activity of acyclovir and trifluridine incubated with HSV-1-infected cultures for 24, 48, or 72 h at 26 or 36.5°C*

The results demonstrate that under selected conditions of reduced rates of viral replication, trifluridine is ineffective against HSV-1 in vitro. We tested 36 sets of conditions (Table 1 has 18 sets of conditions) and found that in three of these

TABLE 1
Results of incubating HSV-1 infected cultures with trifluridine (TFT, 50 μg/ml) or acyclovir (ACV, 20 μg/ml) at 26°C

| (MOI) | Drug/length of incubation (h) | | | | | |
|---|---|---|---|---|---|---|
| | TFT | | | ACV | | |
| | 24 | 48 | 72 | 24 | 48 | 72 |
| 0.1 | Not effective[a] | Effective[b] | Effective | Effective | Effective | Effective |
| 0.01 | Effective | Not effective | Effective | Effective | Effective | Effective |
| 0.001 | Effective | Effective | Not effective | Effective | Effective | Effective |

[a]Not effective, no significant difference in virus yield (log PFU), compared to untreated control cultures.
[b]Effective, virus yield (log PFU) significantly reduced compared to untreated control cultures.

TABLE 2
Virus yield (log PFU) under selected conditions in which trifluridine (TFT) was ineffective against HSV-1 in vitro

| Temp. | Treatment | Incubation (h)/MOI | | |
|---|---|---|---|---|
| | | 24/0.1 | 48/0.01 | 72/0.001 |
| 26°C[a] | TFT | 2.81 ± 0.37 | 3.62 ± 0.56 | 1.65 ± 0.45 |
| | ACV | 1.46 ± 0.34 | 1.04 ± 0.56 | 0.50 ± 0.45 |
| | Untreated control | 2.78 ± 0.34 | 3.50 ± 0.56 | 2.50 ± 0.45 |
| 36.5°C[b] | TFT | 1.77 ± 0.34 | 2.34 ± 0.56 | 3.27 ± 0.45 |
| | ACV | 0.63 ± 0.34 | 1.10 ± 0.56 | 0.00 |
| | Untreated control | 6.50 ± 0.34 | 4.79 ± 0.56 | 5.44 ± 0.45 |

[a]At 26°C, the log PFUs for these specific trifluridine-treated cultures were not significantly different from the control values (untreated cultures), indicating lack of antiviral efficacy under these conditions. Log PFUs for the corresponding acyclovir-treated cultures were significantly different from the control values ($P < 0.05$), as well as the trifluridine values ($P < 0.05$), indicating antiviral efficacy for acyclovir under these conditions.
[b]At 36.5°C, both trifluridine and acyclovir values were significantly different from control values ($P < 0.001$), indicating antiviral efficacy under all conditions at this temperature.

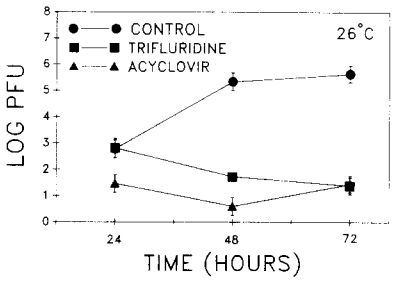


Fig. 1. Effect of trifluridine (50 μg/ml) and acyclovir (20 μg/ml) on HSV-1 at 26°C. Multiplicity of infection = 0.1.

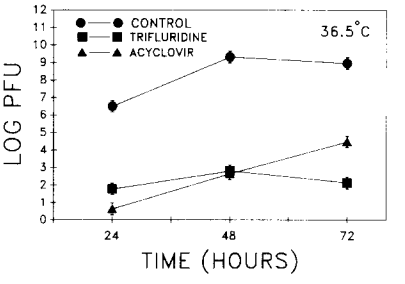


Fig. 2. Effect of trifluridine (50 μg/ml) and acyclovir (20 μg/ml) on HSV-1 at 36.5°C. Multiplicity of infection = 0.1.

(see Table 1: 26°C: MOI of 0.1 and 24 h of incubation; MOI of 0.01 and 48 h of incubation; and MOI of 0.001 and 72 h of incubation; see also Table 2), the virus yield in trifluridine-treated cultures was not significantly different from the virus yield in untreated, control cultures, indicating a lack of antiviral efficacy. All acyclovir-treated cultures demonstrated significantly reduced virus yield compared to untreated controls, indicating that acyclovir was effective under all 18 sets of conditions at this temperature. Both trifluridine and acyclovir inhibited virus growth successfully at 36.5°C at all time periods of incubation and for all MOIs tested.

Fig. 1 illustrates the results of acyclovir and trifluridine treatment at 26°C after 24, 48, and 72 h of incubation in cultures infected at an MOI of 0.1 (data from conditions in Table 1, row 1). At 24 h, there was no difference in the log PFU in trifluridine-treated and untreated control cultures, whereas acyclovir-treated cultures showed a 1.5 log reduction in virus yield compared to controls. At 48 and 72 h both drugs were effective. Fig. 2 demonstrates that at 36.5°C, trifluridine and acyclovir effectively inhibited virus growth at all incubation times (MOI = 0.1). In both figures, the increase in virus yield in the acyclovir-treated cultures between 48 and 72 h may be attributable to the growth of drug-resistant virus strains.

*Drug resistance and cytotoxicity*

To examine the effects of drug resistance, cell lysates from infected cultures incubated at 36.5°C for 48 or 72 h with or without acyclovir (20 μg/ml) were plated on CV-1 monolayers and treated with various concentrations of acyclovir to determine the amount of antiviral that would suppress all CPE. In the monolayers seeded with lysates that had not been treated originally with acyclovir, 6.25 μg/ml acyclovir resulted in no CPE in any of the eight wells after 48 h of incubation; for the cultures incubated for 72 h, 12.5 μg/ml of acyclovir was required to suppress all CPE. In the monolayers seeded with lysates originally incubated with the antiviral, 100 μg/ml acyclovir was needed to suppress CPE in 48 h cultures and 200 μg/ml in 72 h cultures. A concentration of 400 μg/ml of acyclovir was toxic to the cell culture. These results suggest that the cells originally grown in the presence of acyclovir contained highly resistant viral isolates that required high concentrations of acyclovir to inhibit growth. Furthermore, the longer the incubation time, the higher the concentration of drug needed to ensure complete eradication of the virus.

## Discussion

Topically applied trifluridine and acyclovir are equally effective in the treatment of ocular epithelial herpetic infections. La Lau et al. (1981, 1982) showed that when acyclovir (3% ophthalmic ointment) and trifluridine (2% ointment) were compared in a double-blind trial of 38 patients with dendritic keratitis, there was no significant difference in the rate of healing between the two treatment groups. Trousdale et al. (1981) found that topical acyclovir was effective in the treatment of epithelial keratitis caused by the McKrae strain and by strains resistant to idoxuridine and vidarabine.

Most of the antiviral compounds used for the treatment of virus infections are nucleoside analogues that inhibit virus growth by interfering with DNA synthesis (Prusoff et al., 1985; Schaeffer et al., 1978). Such metabolic inhibitors are likely to be most effective when DNA is actively being synthesized. In general, lower temperatures retard the rate of virus multiplication. In our study, in the untreated control cultures, virus yield was lower in the cultures incubated at 26°C than those incubated at 36.5°C, at all MOI and incubation periods. Also, the sets of conditions in which trifluridine was ineffective were found among the cultures incubated at 26°C. The surface temperature of the cornea is about 32°C, compared to the normal body temperature of 37°C in deeper tissues (Efron et al., 1989). Whether temperature is a factor in the reduced rates of virus replication in corneal stromal cells remains to be seen.

Some tissues of the body support more rapid viral replication than other tissues (Abghari et al., 1986; Stulting et al., 1985). In a permissive tissue rapidly replicating viruses may be eradicated more easily because the drug is more effective under these conditions. Conversely, a tissue that permits only slow viral multiplication would render a metabolic antagonist (antiviral) relatively ineffective. Similarly,

some virus strains may replicate less rapidly than others and, therefore, would be less easily inhibited by an antimetabolic agent. Other factors which may be present in vivo are a decrease in the half-life of the antiviral, a decrease in antiviral penetration, or a combination of these factors.

Random development of HSV mutants has been reported (Kaufman et al., 1984; La Lau et al., 1981). Drug-resistant variants of HSV can be isolated relatively easily in tissue culture by exposing sensitive, wild-type virus strains to inhibitors. Viruses resistant to many agents that inhibit HSV in vitro, including acyclovir, idoxuridine, adenine arabinoside, cytosine arabinoside, and others, have been selected through treatment of infected cell cultures with low concentration of drugs (Barry et al., 1985; Elion, 1982; Field, 1983; Kaufman, 1962; Kaufman and Heidelberger, 1964). Our data demonstrate that both acyclovir (20 $\mu$g/ml) and trifluridine (50 $\mu$g/ml) are effective in controlling virus reproduction at 36.5°C for 24 h. However, virus yield increased in the presence of both trifluridine and acyclovir from 24 to 48 h of incubation and continued to increase in the presence of acyclovir through 72 h. This finding suggests the selective replication of drug resistant mutants which were already present at low frequency in the McKrae strain virus stock or which developed in culture after exposure to the drug.

Our results from incubation of cell lysates showed that some acyclovir-resistant particles were already present in the sensitive population of McKrae strain of HSV, since the concentration of acyclovir necessary to inhibit replication of viruses taken from untreated 72 h cultures was higher (12.5 $\mu$g/ml) than that needed to inhibit virus from 48 h cultures (6.25 $\mu$g/ml). It is also likely that additional drug-resistant particles developed when the infected cell cultures were grown in the presence of 20 $\mu$g/ml of acyclovir, since much higher concentrations of acyclovir were required to inhibit growth in the cultures that had been previously treated with the antiviral, compared to the untreated lysates. In the acyclovir-treated lysates, 48 h cultures required 100 $\mu$g/ml and 72 h cultures required 200 $\mu$g/ml of acyclovir to suppress all CPE.

Kaufman and Rayfield (1988) and Kaufman et al. (1984) reported that trifluridine is less subject to the development of drug-resistant strains than acyclovir. However, viruses resistant to IDU and acyclovir are produced relatively easily in vitro and may also develop in man with long-term use (Cobo et al., 1986; Field, 1983; Parris and Harrington, 1982). As suggested by Larder and Darby (1984), the way in which drugs are used could influence the appearance of resistant strains. The protracted use of low doses of a single drug might, for example, be more likely to result in drug resistance. Our experiments showed that, once the resistant strain(s) developed in cultures incubated with low concentrations of acyclovir (20 $\mu$g/ml), higher concentrations (100–200 $\mu$g/ml) of the drug were needed to prevent virus growth.

The route of administration, the time of first application, the frequency of application, and the concentrations of antiviral drugs are critical in achieving the intended therapeutic effect. Concentrations in serum and local areas are markedly different, depending on the drug and route of administration (Balfour, 1983). In topical applications, drug penetration and absorption may be poor and the

concentration in deeper tissues inadequate. The development of drug resistance may be lessened if combination therapy with drugs with independent modes of action are used, as suggested by Larder and Darby (1984). Treatment of viral diseases with lower concentrations of drugs for longer periods, administration of a combination of drugs which allows the use of lower doses of each drug, and more frequent administration of lower doses of drug may all serve to reduce the potential for development of resistant strains (Kaufman et al., 1984).

In summary, conditions which are characterized by slowly replicating virus, such as corneal stromal disease, may be refractory to antiviral therapy because the mechanism of action of the drug may require high rates of DNA turnover to be effective. Larger doses and longer duration of therapy, as well as drugs with different metabolic characteristics, may be required to provide specific and effective treatment for stromal herpes.

## Acknowledgements

This work was supported in part by U.S. Public Health Service grants EY06311, EY02672, EY02580, and EY02377 from the National Eye Institute, National Institutes of Health, Bethesda, MD, and the Egyptian Culture and Educational Bureau.

## References

Abghari, S.Z., Stulting, R.D., Nigida Jr., S.M., Downer, D.N. and Nahmias, A.J. (1986) Comparative replication of HSV-1 in BALB/c and C57BL/6 mouse embryo fibroblasts in vitro. Invest. Ophthalmol. Vis. Sci. 27, 909–914.

Balfour, H.H. (1983) Resistance of herpes simplex to acyclovir. Ann. Intern. Med. 98, 404–406.

Barry, D.W., Nusinoff-Lehrman, S., Ellis, M.N., Biron, K.K. and Furman, P.A. (1985) Viral resistance, clinical experience. Scand. J. Infect. Dis. 47, 155–164.

Cobo, L.M., Foulks, G.N. and Liesegang, T. (1986) Oral acyclovir in the treatment of acute herpes zoster ophthalmicus. Ophthalmology 93, 763–770.

Efron, N., Young, G. and Brennan N.A. (1989) Ocular surface temperature. Curr. Eye Res. 8, 901–906.

Elion, G.B. (1982) Mechanism of action and selectivity of acyclovir. Am. J. Med. 73 (Suppl. 1A), 7–13.

Field, H.J. (1983) The problem of drug-induced resistance in viruses. In: Stuart-Harris, C.H. and Oxford, J. (Eds), Problems of Antiviral Therapy. pp. 71–107. Academic Press, London.

Kaufman, H.E. (1962) Clinical cure of herpes simplex keratitis by 5-iodo-2-deoxyuridine. Proc. Soc. Exp. Biol. Med. 109, 251–252.

Kaufman, H.E. and Heidelberger, C. (1964) Therapeutic antiviral action of 5-trifluoromethyl-2-deoxyuridine in herpes simplex keratitis. Science 145, 585–586.

Kaufman, H.E. and Rayfield M.A. (1988) Viral conjunctivitis and keratitis. In: H.E. Kaufman, B.A. Barron, M.B. McDonald and S.R. Waltman (Eds), The Cornea, pp. 299–331, Churchill Livingstone, New York.

Kaufman, H.E., Remington, J.S., Melton, M.L. and Jacobs, L. (1959) Relative resistance of slow-growing strains of *Toxoplasma gondii* to pyrimethamine (Daraprim). Arch. Ophthalmol. 62, 611–615.

Kaufman, H.E., Varnell, E.D., Centifanto-Fitzgerald, Y.M. and Sanitato, J.G. (1984) Virus chemotherapy: Antiviral drugs and interferon. Antiviral Res. 4, 333–338.

La Lau, C., Oosterhuis, J.A. and Versteeg, J. (1981) Acyclovir and trifluorothymidine in herpetic keratitis. Preliminary report of a multicentered trial. Doc. Ophthalmol. 50, 287–290.

La Lau, C., Oosterhuis, J.A. and Versteeg, J. (1982) Multicenter trial of acyclovir and trifluorothymidine in herpetic keratitis. Am. J. Med. (Acyclovir Symposium) 73, 305–306.

Larder, B.A. and Darby, G. (1984) Virus drug resistance: Mechanisms and consequences. Antiviral Res. 4, 1–42.

Maloney, E.D. and Kaufman, H.E. (1964) Multiplication and therapy of *Toxoplasma gondii* in tissue culture. J. Bacteriol. 88, 319–321.

Parris, D.S. and Harrington, J.E. (1982) Herpes simplex virus variants resistant to high concentrations of acyclovir exist in clinical isolates. Antimicrob. Agents Chemother. 22, 71–77.

Prusoff, W.H., Zucker, M., Mancini, W.R., Otto, M.J., Lin, T. and Lee, J. (1985) Basic biochemical and pharmacological aspects of antiviral agents. Antiviral Res., Suppl. 1, 1–10.

Schaeffer, H.J., Beauchamp, L., DeMiranda, P., Elion, G.B., Bauer, D.J. and Collins, P. (1978) [9-(2-hydroxyethoxy-methyl)] guanine activity against viruses of the herpes group. Nature 272, 583–585.

Stulting, R.D., Kindle, J.C. and Nahmias, A.J. (1985) Patterns of herpes simplex keratitis in inbred mice. Invest. Ophthalmol. Vis. Sci. 26, 1360–1367.

Trousdale, M.D., Nesburn, A.B. and Miller, C.A. (1981) Assessment of acyclovir on acute ocular infection induced by drug-resistant strains of HSV-1. Invest. Ophthalmol. Vis. Sci. 20, 230–235.

Wheeler, C.E. and Canby, C.M. (1960) Relationship of temperature to inapparent infection of Hela cells with herpes simplex virus. J. Bacteriol. 79, 657–660.