ELSEVIER

Antiviral Research 35 (1997) 139–146

Antiviral Research

SCH 43478 and analogs: in vitro activity and in vivo efficacy of novel agents for herpesvirus type 2

Randi Albin [a,*], Robert Chase [a], Christine Risano [a], Melvin Lieberman [a], Eric Ferrari [a], Angela Skelton [a], Peter Buontempo [a], Stuart Cox [a], Jason DeMartino [a], Jacquelyn Wright-Minogue [a], Gilbert Jirau-Lucca [c], Joseph Kelly [b], Adrian Afonso [b], Ann D. Kwong [a], Edward J. Rozhon [a], John F. O'Connell [a]

[a] *Department of Antiviral Chemotherapy, Schering-Plough Research Institute, 2015 Galloping Hill Road, Kenilworth, NJ 07003, USA*

[b] *Department of Chemical Research, Schering-Plough Research Institute, 2015 Galloping Hill Road, Kenilworth, NJ 07003, USA*

[c] *Department of Bioanalytical Research, Schering-Plough Research Institute, 2015 Galloping Hill Road, Kenilworth, NJ 07003, USA*

Received 15 October 1996; accepted 14 April 1997

Abstract

SCH 43478 and analogs are a class of non-nucleoside antiviral agents that have potent and selective activity against herpes simplex virus type 2 (HSV-2). The IC_{50} for these compounds in plaque reduction analysis using Vero cells ranges from 0.8 to 2.0 μg/ml. All compounds have a $LC_{50} > 100$ μg/ml in cytotoxicity analysis. Mechanism of action studies suggest that these molecules have an effect on the transactivation of viral immediate early (α) gene expression. Time of addition studies indicate that antiviral activity of these analogs is limited to the initial 2–3 h after infection and is not due to inhibition of viral adsorption or penetration. Analysis of HSV protein expression demonstrates that SCH 49286 inhibits the accumulation of viral immediate early (α) gene products. SCH 43478 demonstrates statistically significant efficacy ($P < 0.05$) in the guinea pig genital model of HSV infection. Following subcutaneous administration in a therapeutic treatment regimen, SCH 43478 (90 mg/kg/day) is efficacious in reducing the number and severity of lesions and the neurological complications of acute HSV infection. Thus, SCH 43478 and analogs are anti-herpesvirus agents with a unique mechanism of action. © 1997 Elsevier Science B.V.

Keywords: Herpesviruses; Antiviral agent; SCH 43478

1. Introduction

Infection with herpes simplex virus (HSV) is characterized by an acute primary disease fol-

* Corresponding author. Tel.: +1 908 2983025; fax: +1 908 2983918.

PII S0166-3542(97)00023-5

lowed by the development of a latent, lifelong infection. Infected individuals remain a reservoir for further disease episodes which result from reactivation of latent virus harbored in the neurons of the sensory ganglia (Stevens and Cook, 1971; Bastian et al., 1972; Stevens et al., 1972; Baringer and Swoveland, 1973). Current therapies for treatment of HSV infection are predominantly nucleoside analogs which exploit differences between the viral and cellular replication machinery to exert a selective antiviral effect (Coen, 1992). Advances in the understanding of the function and activities of specific herpesvirus proteins have resulted in the identification of novel targets for intervention (Roizman and Sears, 1996).

The parent structure of the series, SCH 43478, was among a series of molecules described by Radl and Zikan (1987), but no antiherpesvirus activity was ascribed to the compound. In this report we describe the in vitro and in vivo activities of SCH 43478 and related derivatives which represent a novel class of anti-herpesvirus agents. The mechanism of action of these molecules is distinct from that of nucleoside analogs in that they inhibit an early stage in the HSV type 2 replicative cycle.

2. Materials and methods

2.1. Compounds

SCH 43478, SCH 46792 (US patent # 5506236) and SCH 49286 (patent pending, Fig. 1) were synthesized at the Schering-Plough Research Institute (SPRI). Acyclovir powder (ACV; Zovirax) was purchased from Neuman Distributors (Ridgefield, NJ).

2.2. Viruses and cells

Vero (African green monkey kidney), WI-38 (human diploid lung) and HeLa (human epithelial) cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). FS-85 cells were a primary culture derived from human foreskin fibroblasts propagated at SPRI. Vero cells were propagated in M199 media (JRH Biosciences) supplemented with 5% fetal calf serum (FCS). FS-85, WI-38 and HeLa cells were propagated in Eagles' minimal essential media (EMEM) supplemented with 10% FCS (Gibco, BRL). Virus stocks were prepared and quantitated by plaque assay on Vero cells and stored in small aliquots at −70°C. Herpes simplex virus type 2 MS strain (HSV-2) was obtained from the ATCC.

2.3. Plaque reduction assay

Confluent monolayers of Vero, FS-85 and WI-38 cells were prepared in 6-well culture dishes (Nunc) at a density of 9×10^5 cells/well. Cells were infected with 100 plaque-forming units (PFU) of HSV-2 in the presence or absence of compound at a final concentration of 20, 10, 2.0, or 0.2 μg/ml in 1% DMSO. Cells were incubated at 37°C with compound for 18 (Vero) or 72 h (FS-85, WI-38), washed, and overlayed with 0.75% methylcellulose in appropriate media for the cells. Monolayers were incubated an additional 24–48 h and plaques were visualized by staining with crystal violet. Antiviral activity was calculated from replicate samples as the concentration of test compound able to reduce the number of plaques by 50% (IC_{50}), as determined by extrapolation of a log plot of the data.

2.4. Cytotoxicity assay

Cellular viability was measured with MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide; Sigma), as previously described by Mossmann (1983), except for solubilizing the dye which was accomplished using 1:1 *N*,*N*-dimethylformamide:water plus 20% sodium lauryl sulfate. Compounds were assayed using four to eight replicates at concentrations of 100, 10, and 1.0 μg/ml using conditions of compound exposure equivalent to that used in plaque assays. Cytotoxicity (LC_{50}) was calculated as the concentration of test compound able to reduce the MTT signal by 50% compared to controls.

2.5. Time course studies

Vero cells were incubated with HSV-2 for 1 h to allow for adsorption and penetration of virus, after which unattached virus was removed by washing. Compound was incubated with cells either concurrent with HSV-2 infection (0 h) and replaced after washing, or at intervals of 1, 2, 3, 4, and 6 h after infection. Virus was quantitated by plaque assay.

2.6. Virus penetration assay

Herpesvirus penetration into Vero cells was performed as described by Huang and Wagner (1964). Vero cells were incubated with HSV-2 for 2 h at 4°C to allow virus adsorption. After adsorption test compound (25 μg/ml) or poly-L-lysine (10 μg/ml) was added and cells were shifted to 25°C to initiate viral penetration. At 10-min intervals cells were washed, treated with PBS at pH 3 for 1 min to inactivate unpenetrated virus and immediately neutralized by the addition of 0.1 N NaOH. Controls, performed in parallel, were washed and treated with PBS at pH 7 to allow penetration of all attached virus. PBS was removed by aspiration and cells were overlayed with 0.75% methylcellulose in M199 media. After incubation at 37°C for 72 h cells were stained and plaques determined by counting. Percent penetration was determined by the ratio of the number of plaques formed following treatment at pH 3 to the pH 7 control.

2.7. Preparation of cell lysates

HeLa cells were infected with HSV-2 at a multiplicity of infection (MOI) = 5 and treated immediately with SCH 49286 (25 μg/ml). Cell lysates were prepared at 3 and 6 h after infection. Cells were washed twice with HBSS (Hanks' Balanced Salt Solution; Gibco, BRL) and lysed in 2 × Laemmli buffer (Laemmli, 1970). Proteins were resolved by electrophoresis on 12.5% polyacrylamide gels and transferred to nitrocellulose membranes (Towbin et al., 1979). Immunoblots were performed with anti-ICP27 monoclonal antibody (H1113-5, gift of Lenore Peirera; University of California School of Dentistry, San Francisco) and anti-β-tubulin monoclonal antibody (Boehringer Mannheim) and detected with alkaline phosphatase-conjugated secondary antibody.

2.8. In vivo evaluations

Studies with guinea pigs were performed as recommended in the *Guide for the Care and Use of Laboratory Animals* (NIH Publication 85-23). Groups of eight to twelve female Hartley guinea pigs (180–200 g) were infected intravaginally with 1.7×10^5 PFU HSV-2 in 0.1 ml (Kern, 1982; Stanberry et al., 1982). Compounds were administered in 1% methylcellulose by the subcutaneous route three times a day for 8 days beginning 24 h after infection. Due to the lack of aqueous solubility SCH 43478 and SCH 46792 were suspensions in the dosing vehicle, while the water-soluble derivative, SCH 49286, was a solution. Genital lesions were scored daily through 10 days according to Kern et al. (1978). The areas under the mean lesion score vs. day curves (AUC) was calculated for each test compound. The activity coefficient for each compound was calculated by determining the ratio of the AUC of the mean lesion score versus day curve for test compound to the AUC of the placebo group (AUC experimental group/ AUC placebo group). Neurological complications associated with HSV infection were evaluated by determining the percent of animals in each group with hindlimb paralysis.

3. Results

3.1. Structures of antiviral molecules

The structures of SCH 43478, SCH 46792, and SCH 49286 are shown in Fig. 1.

3.2. Antiviral activity in cell culture

SCH 43478 and the analogs, SCH 46792 and SCH 49286, demonstrated potent antiviral activity in cell culture (Table 1). The IC_{50} of these compounds in plaque reduction in Vero cells ranges from 0.8 to 1.8 μg/ml. Selective antiviral

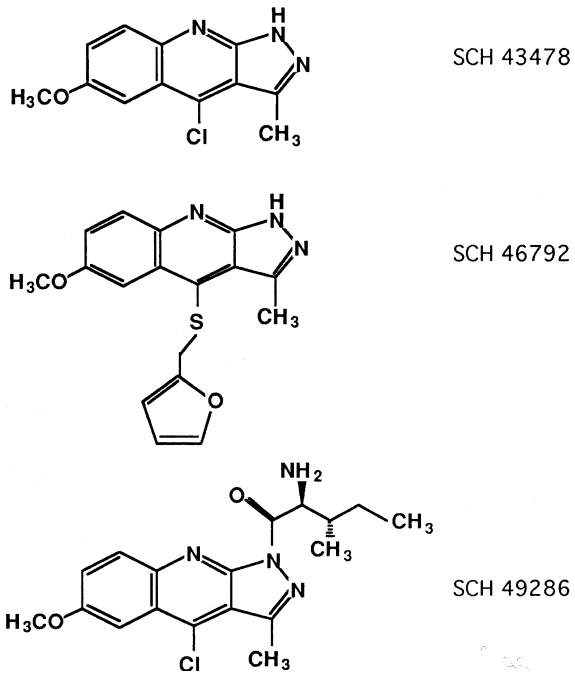

Fig. 1. Structure of SCH 43478 and analogues.

activity was also observed in plaque reduction assays using FS-85 (human foreskin fibroblast) and WI-38 (human diploid lung) cells. As determined by MTT assay, the LC_{50} of these molecules was >100 μg/ml for all cell lines tested. Since the cytotoxicity assay parallels the plaque reduction assay with respect to time of exposure to test compound, the LC_{50} indicates that the observed antiviral activity was not due to adverse effects on cells.

3.3. Time course analysis of SCH 43478 series on HSV-2 infectivity

To determine the stage in the infectious cycle at which SCH 43478 and analogs exert an antiviral affect, HSV infectivity was analyzed over time by plaque reduction (Table 2). As shown in Table 2, antiviral activity for all three compounds was observed only during the initial 2–3 h of infection ($IC_{50} = 0.2–2.0$ μg/ml). No antiviral activity was observed when compound is added at later times ($IC_{50} > 20$ μg/ml). By contrast, ACV was effective in inhibiting viral plaque formation when incubated with cells up to 6 h after infection ($IC_{50} =$ 0.5 μg/ml). These results indicate that SCH 43478 and analogs affect HSV at an early step in the replicative cycle, suggesting a mechanism of action distinct from ACV (Elion, 1982).

3.4. Effect of SCH 49286 on the adsorption and penetration of HSV

The early block in HSV replication is not due to an effect on viral adsorption or penetration as demonstrated by mechanistic studies with SCH 49286, a water-soluble analog. As shown in Fig. 2, the kinetics of viral penetration in the presence of SCH 49286 (25 μg/ml) was essentially indistinguishable from the untreated virus control. By contrast, poly-L-lysine (10 μg/ml), a known penetration inhibitor, effectively blocked this step of virus infection. Incubation of SCH 49286 with HSV-2 during the adsorption phase of infection had no effect on viral penetration (not shown).

Table 1
Inhibition of HSV-2 plaque formation by SCH 43478 and analogs[a,b]

Compound	Antiviral activity, IC_{50} (μg/ml)			Cytotoxicity, LC_{50} (μg/ml)		
	Vero[c]	FS-85	WI-38	Vero	FS-85	WI-38
SCH 43478	1.8 ± 0.5	0.9	1.2	>100	>100	>100
SCH 46792	1.5 ± 0.2	0.7	1.8	>100	>100	>100
SCH 49286	0.8 ± 0.3	5.0	7.0	>100	>100	>100
ACV	2.0 ± 0.3	0.2	0.8	>100	>100	>100

[a]Antiviral activity was determined by plaque reduction assay with HSV-2. Cytotoxicity was determined by MTT assay.
[b]Compounds were incubated for 18 h with Vero and 72 h with FS-85 and WI-38 cells.
[c]Values represent the mean and standard errors of three experiments.

Table 2
Time course analysis of HSV-2 infectivity[a]

Time of addition (h)	Antiviral activity, IC_{50} (μg/ml)			
	SCH 43478	SCH 46792	SCH 49286	ACV
0	0.5	<0.2	0.4	0.3
+1	1.1	0.6	0.7	0.5
+2	0.6	1.1	0.5	0.3
+3	>20	>20	2.0	0.5
+4	>20	>20	>20	0.5
+6	>20	>20	>20	0.5

[a] Vero cells were incubated with HSV-2 for 1 h to allow for virus adsorption and penetration, after which unattached virus was removed by washing. Compounds were added either concurrent with virus (0 h) or at intervals after infection as described in Section 2.

Therefore, the early affect of SCH 49286 on HSV infection must occur at a step following virus entry into cells yet prior to DNA replication.

3.5. Expression of HSV-specific proteins in SCH 49286-treated cells

Analysis of protein synthesis in infected cells indicates that SCH 49286 inhibits HSV replication by an effect on viral immediate early (α) protein expression. As shown in Fig. 3, SCH 49286 inhibited accumulation of the viral immediate early protein ICP27 in HeLa cells harvested 6 h after infection. Inhibition of the accumulation of ICP27 was also detected in cells harvested at 12 h (not shown). By contrast, SCH 49286 had no effect on the accumulation of the cellular protein, β-tubulin (Fig. 3). Similar results were obtained when accumulation of the viral immediate early (α) protein ICP4 was examined by immunoblot (not shown). These results suggest that SCH 43478 and

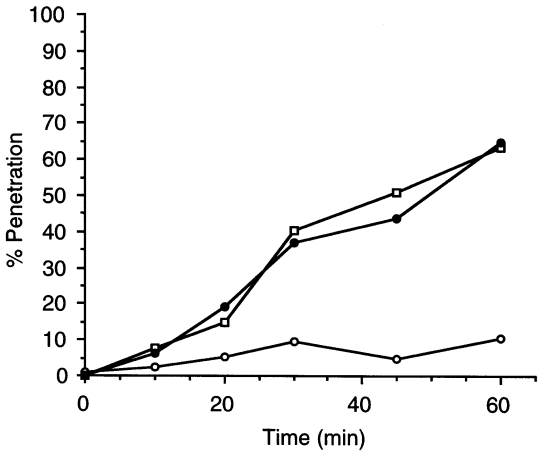

Fig. 2. Effect of SCH 49286 on the kinetics of HSV penetration. HSV-2 penetration was assayed in Vero cells as described in Section 2. Cells were treated with SCH 49286 at 25 μg/ml (●), poly-L-lysine at 10 μg/ml (○), or untreated (□).

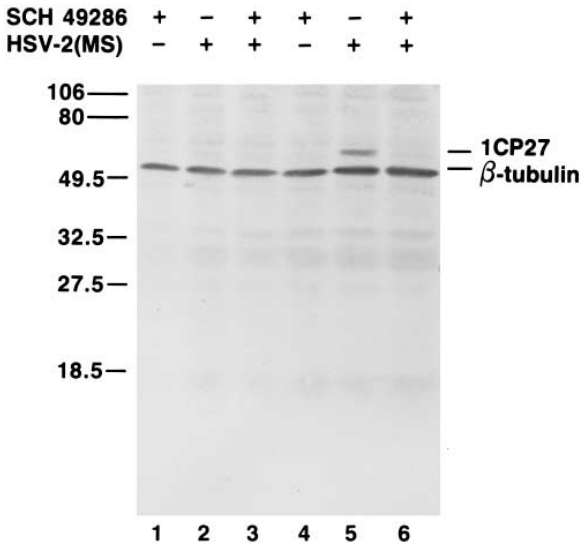

Fig. 3. Effect of SCH 49286 on HSV immediate early protein synthesis. Western blot of total cell lysates of HeLa cells using anti-ICP27 and anti β-tubulin antibodies. Lanes 1–3, HeLa cell lysates harvested at 3 h. Lanes 4–6, HeLa cell lysates harvested at 6 h. Infection was with HSV-2 at an MOI = 5 (lanes 2, 3, 5, 6). SCH 49286 was incubated with cells at a concentration of 25 μg/ml (lanes 1, 3, 4, 6).

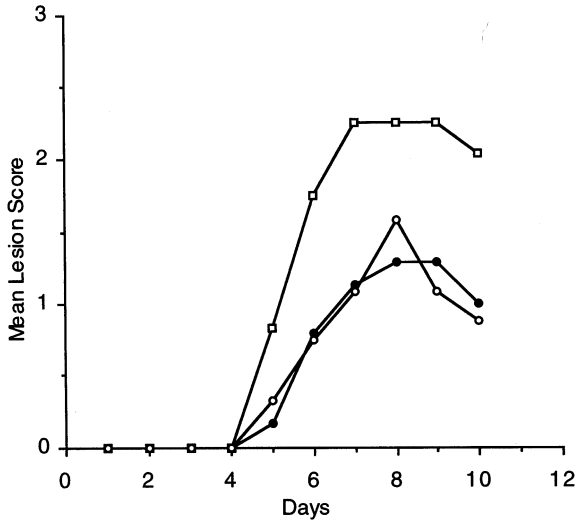

Fig. 4. In vivo efficacy of SCH 43478 in a therapeutic model of acute HSV infection. Female Hartley guinea pigs were infected intravaginally with HSV-2 and treated subcutaneously with 135 mg/kg/day of compound in 1% methylcellulose for 10 days starting 24 h after infection. Treatment was with SCH 43478 (●), ACV (○), or placebo (□).

analogs inhibit HSV infection by affecting the expression of viral immediate early (α) proteins.

3.6. Antiviral efficacy in vivo

SCH 43478 is efficacious in the guinea pig vaginal model of acute HSV infection (Fig. 4 and Table 3). Both the number of lesions and severity of infection was reduced following subcutaneous administration of SCH 43478 in a therapeutic treatment regimen. Efficacy of SCH 43478, expressed as an activity coefficient (0.33), was statistically significant with doses as low as 90 mg/kg/day (30 mg/kg/day, t.i.d.) compared to placebo-treated controls (Table 3).

Efficacy of SCH 43478 at 45, 90, and 135 mg/kg/day was comparable to ACV with respect to activity coefficient and mean lesion score (Table 3). SCH 43478 was efficacious against the neurological effects that accompany acute HSV infection. Hindlimb paralysis was statistically reduced at all doses compared to placebo controls. At doses of 45 and 90 mg/kg/day there was no hindlimb paralysis observed. In the ACV treatment groups 25, 16.7, and 38.8% of animals displayed paralysis in the 45-, 90- and 135-mg/kg/day groups, respectively. Neither SCH 46792 nor SCH 49286 demonstrated efficacy at a dose of 135 mg/kg/day (Table 3). A likely explanation for this lack of efficacy is that the compounds possess different pharmacokinetic profiles than SCH 43478 (i.e. metabolism, tissue distribution) and are not bioavailable at sites of viral replication. No weight loss, morbidity, or mortality was observed in uninfected animals subject to the same treatment regimen, indicating that the drugs did not have adverse effects in vivo (data not shown).

4. Discussion

SCH 43478 and its analogs possess potent in vitro activity against HSV-2 as demonstrated in plaque reduction assays. The IC_{50} for these molecules is comparable to that of ACV and is not the result of cytotoxicity as demonstrated by MTT assay.

Mechanism of action studies are consistent with the compounds having an effect on the transactivation of immediate early (α) gene expression by the viral protein Vmw65 (α-TIF) (Dalrymple et al., 1985; Pellett et al., 1985). SCH 49286 specifically inhibits accumulation of the viral immediate early (α) proteins ICP27 and ICP4 in herpesvirus-infected cells without effecting the accumulation of cellular β-tubulin. Time of addition studies demonstrate that SCH 43478 and analogs are only active during the initial 2–3 h after infection and this effect is not due to inhibition of viral adsorption or penetration. Additional studies have not pinpointed the mechanism of action of the SCH 43478 series. The compounds did not affect the assembly of the transcription complex containing Vmw65 and the cellular factors Oct-1 and C1 (T. Kristie, personal communication). Further experimentation is needed to define the precise mechanism which is the basis of the in vitro activity of these compounds.

SCH 43478 demonstrated statistically significant efficacy in the guinea pig vaginal model of acute HSV infection. When administered subcuta-

Table 3
Effect of SCH 43478 and analogs an HSV infection model[a, b]

Compound	Dosage (mg/kg/day)	Activity coefficient[c]	Mean lesion score[d]	% hindlimb paralysis[e]
Placebo		–	2.82 ± 0.07	62 ± 12.5
SCH 43478	135	0.39 ± 0.09*	1.05 ± 0.25*	2.8 ± 3.9†
	90	0.33*	0.88*	0†
	45	0.63	1.42	0†
SCH 46792	135	0.57	1.50	25
SCH 49286	135	0.94	2.85	60
ACV	135	0.49 ± 0.07*	1.58 ± 0.18*	38 ± 1.3†
	90	0.44*	1.25	17
	45	0.56	1.50	25

[a] Test compound was administered by the subcutaneous route in 1% methylcellulose t.i.d. to obtain total dose indicated for 8 days beginning 24 h after intravaginal infection with HSV-2. Values and statistics for SCH 43478 and ACV administered at 135 mg/kg/day represent the mean and standard error of three experiments. For all other doses the values represent the mean of a single experiment and statistics are based on variation within an experimental treatment group compared to the placebo group for that experiment.
[b] An astersik (*) indicates results statistically different from placebo at $P < 0.05$ by Student's t-test. A '†' indicates results statistically different from placebo at $P < 0.05$ by chi-square test.
[c] The activity coefficient for each compound is the ratio of the AUC of the mean lesion score vs. day curve for test compound to the AUC of the placebo group (AUC experimental group/AUC placebo group).
[d] Mean lesion score is the average of the maximum lesion score for each animal during the course of the study.
[e] The percentage hindlimb paralysis values are cumulative over the course of the study.

neously in a therapeutic treatment regimen, SCH 43478 is comparable to ACV in reducing the number and severity of lesions. In murine models of HSV infection none of the compounds demonstrated efficacy when administered by the oral route (data not shown). Pharmacokinetic profiles indicate the compounds have poor oral adsorption and do not demonstrate serum levels in the inhibitory range for herpesviruses.

In summary, based on in vitro activity, mechanism of action studies, and in vivo efficacy the SCH 43478 series is a novel class of anti-herpesvirus agents. However, the pharmacokinetic profile of the series (i.e. lack of oral adsorption) precludes their current development as clinical candidates. The unique mechanism of action of this series is of scientific interest for defining non-nucleoside targets for herpesviruses.

References

Baringer, R.J., Swoveland, P., 1973. Recovery of herpes-simplex virus from human trigeminal ganglions. New Engl. J. Med. 288, 648–650.

Bastian, F.O., Rabson, A.S., Yee, C.L., Tralka, T.S., 1972. Herpesvirus hominis: isolation from human trigeminal ganglion. Science 178, 306–307.

Coen, D.M., 1992. Molecular aspects of anti-herpesvirus drugs. Semin. Virol. 3, 3–12.

Dalrymple, M.A., McGeoch, D.J., Davison, A.J., Preston, C.M., 1985. DNA sequence of the herpes simplex virus type 1 gene whose product is responsible for transcriptional activation of immediate early promoters. Nucleic Acids Res. 13, 7865–7879.

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

Huang, A.S., Wagner, R.R., 1964. Penetration of herpes simplex virus into human epidermoid cells. Proc. Soc. Exp. Biol. Med. 116, 863–869.

Kern, E.R., Glasgow, L.S., Overall, J.C. Jr., Reno, J.M., Boezi, J.A., 1978. Treatment of experimental herpesvirus infections with phosphonoformate and some comparisons with phosphonoacetate. Antimicrob. Agents Chemother. 14, 817–823.

Kern, E.R., 1982. Acyclovir treatment of experimental genital herpes simplex virus infections. Am. J. Med. 73 (Suppl. 1A), 100–108.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Mossmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Pellett, P.E., McKnight, J.L.C., Jenkins, F.J., Roizman, B., 1985. Nucleotide sequence and predicted amino acid sequence of a protein encoded in a small herpes simplex virus DNA fragment capable of trans-inducing a genes. Proc. Natl. Acad. Sci. USA 82, 5870–5874.

Radl, S., Zikan, V., 1987. Synthesis of 1, 2 and 9-methyl derivatives of 4, 9-dihydro-6- methoxy-3-methyl-4-oxo-1*H* (2*H*)-pyrazolo-[3,4-*b*] quinoline and 4, 9 dihydro-6- hydroxy-3-methyl-4-oxo-1*H* (2*H*)-pyrazolo [3, 4*b*] quinoline and their antiviral activity. Collection Czechoslovak. Chem. Commun. 52, 788–792.

Roizman, B., Sears, A.E., 1996. Herpes simplex viruses and their replication. In: Fields, B.N., Knipe, D.M., Howley, P.M., Chanock, R.M., Melnick, J.L., Monath, T.P., Roizman, B., Straus, S.E. (Eds.), Virology, 3rd ed. Lippincott-Raven, Philadelphia, pp. 2231–2295.

Stanberry, L.R., Kern, E.R., Richards, J.T., Abbott, T.M., Overall, J.C., 1982. Genital herpes in guinea pigs: pathogenesis of the primary infection and description of recurrent disease. J. Infect. Dis. 146, 397–404.

Stevens, J.G., Cook, M.I., 1971. Latent herpes simplex virus in spinal ganglia of mice. Science 173, 843–845.

Stevens, J.G., Nesburn, A.B., Cook, M.L., 1972. Latent herpes simplex virus from trigeminal ganglia of rabbits with recurrent eye infection. Nature (New Biol.) 235, 216–217.

Towbin, H., Staehelin, T., Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulse sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA 76, 4350–4354.