Antiviral Research, 3 (1983) 77–86
Elsevier

Antiherpetic activity and mechanism of action of 9-(4-hydroxybutyl)guanine

A. Larsson*, S. Alenius, N.-G. Johansson and B. Öberg

Department of Antiviral Chemotherapy, Research and Development Laboratories, Astra Läkemedel AB, Södertälje, Sweden

(Received 14 December 1982; accepted 3 March 1983)

9-(4-Hydroxybutyl)guanine was synthesized and tested for antiherpes activity. In cell cultures, different strains of herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) were inhibited by 50% at 2–14 μM of 9-(4-hydroxybutyl)guanine, while a HSV-1 mutant lacking thymidine kinase (HSV-1 TK^-) was resistant. Linear competitive inhibition of purified HSV-1-induced thymidine kinase (TK) with thymidine as a variable substrate was observed for 9-(4-hydroxybutyl)guanine with an apparent K_i value of 2.06 μM while the corresponding K_i value for the cellular TK was >250 μM. By using high performance liquid chromatography, the formation of 9-(4-hydroxybutyl)guanine monophosphate by HSV-1 TK was measured and the rate of product formation was found to be about 10% of that found by using thymidine as a substrate. A selective inhibition of HSV-1 DNA synthesis by 9-(4-hydroxybutyl)guanine was observed in infected Vero cells. 9-(4-Hydroxybutyl)guanine had a low cellular toxicity. A weak therapeutic effect on herpes keratitis in rabbits was observed whereas cutaneous HSV-1 infection in guinea pigs and systemic HSV-2 infection in mice were not affected by this compound.

antiherpetic activity; nucleoside analog; mechanism of action; 9-(4-hydroxybutyl)guanine

Introduction

Herpesvirus infections have been one of the major targets in the search for new antiviral drugs during recent years. The first generation of antiviral compounds with proven clinical value was discovered by random screening or by serendipity. The observation that herpesviruses induce new enzyme activities that differ from the normal cellular enzymes [1,2] made new rational approaches possible. One type of compound utilizing these enzymes is the antiherpes compound acyclovir (ACV) [3]. We have evaluated several newly synthesized hydroxyalkylnucleoside analogs as potential antiherpes drugs. One of these, the guanosine analog 9-(4-hydroxybutyl)-guanine, was found to have antiherpes activity. Preliminary biochemical and cell culture data of this compound were recently published [4]. This paper extends the evidence for a mechanism of action, reports cellular toxicology, and describes the antiherpes effect in animals for this compound.

* To whom correspondence should be addressed.

Materials and methods

Materials

9-(4-Hydroxybutyl)guanine and 9-(4-hydroxybutyl)guanine monophosphate [5] were synthesized at Astra Läkemedel AB. [Methyl-^{3}H]thymidine (77.2 Ci/mmol) and [γ-^{32}P]adenosine 5'-triphosphate (>5000 Ci/mmol) were from New England Nuclear (Boston, MA) and carrier-free [^{32}P]orthophosphate (1 mCi/ml) was from the Radiochemical Centre (Amersham, England). 9-(2-Hydroxyethoxymethyl)guanine (ACV) was synthesized by B. Lindborg and A. Misiorny at Astra Läkemedel AB.

Cells and viruses

Vero cells (CCL81) were grown and used for plaque assays as described earlier [6]. Most of the herpes simplex strains used have been described previously [7] or were patient isolates. The strain HSV-1 C915 TK$^-$ does not induce the formation of any TK polypeptide (Dr. S. Gronowitz, pers. comm.) and the acyclovir-resistant strain (ACV^r) C42 was selected as a strain resistant to at least 100 μM ACV in Vero cells.

Purification of thymidine kinase

Vero cells were grown in roller bottles, 625 cm^2, and infected with HSV-1 strain C42 at a multiplicity of 0.5–1 plaque-forming units/cell (pfu/cell) and cultivated for 16 h (70–90% cytopathic effect). HSV-1 infected and uninfected Vero cells were harvested and treated before the application to a CH-Sepharose-dThd affinity column according to published methods [8]. The application and separation of HSV-1 TK and cellular cytosol TK on the affinity column was as described previously [9]. HSV-1 TK was eluted at about 40–60 μM dThd and the cellular cytosol TK at about 80–100 μM of dThd. The fractions containing TK activities were pooled, dialyzed, and concentrated as described [9].

Thymidine kinase assay

One unit of activity was defined as the amount of enzyme activity that catalyzes the formation of 1 pmol of product/min at 37°C in a standard assay. Thymidine kinase activity was assayed in a 100-μl reaction mixture containing 150 mM Tris-HCl pH 7.5, 2 mM ATP, 2 mM $MgCl_2$, 3 mM dithiothreitol (DTT), 10 μM [methyl-^{3}H]thymidine and TK. After incubation at 37°C for 30 min, 50 μl of the reaction mixture was spotted onto a Whatman DE 81 paper disc (2.3 cm), washed twice with H_2O and three times with ethanol. Each dried paper disc was counted in 3 ml Econofluor scintillation solution (New England Nuclear, Boston, MA).

Kinetic studies were performed using 2 U of HSV-1 TK or Vero cell cytosol TK. The incubation period was 5 min and the reaction mixture was the same as for the standard TK assay with different amounts of [^{3}H]dThd. The reaction rate was linear during the incubation period. The K_m value for dThd and the K_i values for the tested compounds

were determined by linear regression analyses of the data obtained which were plotted in Lineweaver–Burk plots. To determine the ability of the test compound to act as an alternative substrate for the dThd kinases we applied the procedure of Dobersen and Greer [10]. The 100-µl reaction mixture contained 150 mM Tris-HCl pH = 7.5, 0.5 mM [γ-^{32}P]ATP, 2 mM $MgCl_2$, 3 mM DTT, 250 µM of test compound and TK.

Determination of 9-(4-hydroxybutyl)guanine monophosphate by HPLC

To determine the formation of 9-(4-hydroxybutyl)guanine monophosphate by HSV-1 TK the method described above was combined with the HPLC technique. TK from HSV-1-infected Vero cells was prepared as described above, but omitting the purification on the affinity column. 100 or 500 µM of thymidine or 9-(4-hydroxybutyl)-guanine were incubated for 2 h in 200 µl with 50 U of HSV-1 TK, 150 mM Tris-HCl pH = 7.5, 10 mM ATP, 10 mM $MgCl_2$ and 3 mM DTT. After incubation, 60% $HClO_4$ was added to give a final concentration of 0.5 M. After 30 min on ice the precipitates were removed by centrifugation, and the supernatants were neutralized with 4 M KOH containing 0.4 M KH_2PO_4. The neutralized extracts were kept on ice for 5 min before the solutions were cleared by centrifugation. The monophosphate products formed were analysed and identified on a liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.) using a Whatman Partisil 10 sax column. Elution was done with 0.01 M KH_2PO_4 pH 2.80 at a flow rate of 2 ml/min. The UV absorption was monitored at 254 nm and recorded and quantified with a Waters model 730 integrator.

Determination of HSV-1 and cellular DNA synthesis

Determination of viral and cellular DNA synthesis by [^{32}P]orthophosphate labelling and isodensity gradient centrifugation has been described previously [11]

Cellular toxicity

The effect on cell proliferation was as reported previously [12]. The test compound was added to actively growing cells and incubated for 48 h and the cell number and volume were measured by the use of an electronic cell counter (Analysinstrument AB, Stockholm).

Animal experiments

The method to inoculate guinea pigs with HSV-1, strain C42, has been described earlier, as has the scoring system used to determine the therapeutic effect of treatment [13]. The herpes keratitis model using rabbits inoculated with HSV-1 in the cornea has been described previously [14]. In the experiments with systemic herpes infections, 14–15 g NMRI mice were inoculated intraperitoneally with 10^4 pfu of HSV-2 strain 91075 [15].

Results

Antiherpes activity of 9-(4-hydroxybutyl)guanine

The ability of 9-(4-hydroxybutyl)guanine to reduce plaque formation by HSV-1 and HSV-2 strains in cell culture is shown in Table 1. 9-(4-Hydroxybutyl)guanine was an effective inhibitor of both HSV-1 and HSV-2 multiplication. A variation in sensitivity between different HSV strains was observed with 50% inhibition values (ID_{50}) between 2–14 μM of 9-(4-hydroxybutyl)guanine, and a thymidine kinase-negative (TK^-) strain as well as an acyclovir-resistant strain (ACV^r) of HSV-1 were resistant to the inhibitor.

Cellular toxicity of 9-(4-hydroxybutyl)guanine

The effect of 9-(4-hydroxybutyl)guanine on cell proliferation was determined in Vero cells. No effect was observed at concentrations required to inhibit HSV multiplication. A 50% inhibition of cell multiplication was observed at a concentration of 250 μM, while no effect on cell volume was observed (data not shown).

Effect of 9-(4-hydroxybutyl)guanine on cellular and HSV-1 DNA synthesis

As shown in Fig. 1 and Table 2, 9-(4-hydroxybutyl)guanine reduced the HSV-1 DNA synthesis in infected Vero cells by 50% at 0.6 μM while cellular DNA synthesis required 3.6 μM for a 50% inhibition in the infected cells. When viral DNA synthesis was totally inhibited by 10 μM of the inhibitor the cellular DNA synthesis was also inhibited by about 80% in the infected cells. On the other hand, at this concentration the cellular DNA synthesis in the uninfected Vero cells was not inhibited and a 50% inhibition was observed at a concentration >500 μM (Table 2).

TABLE 1

Inhibition of HSV plaque formation by 9-(4-hydroxybutyl)guanine

Virus strain	50% inhibition, μM (ID_{50})
HSV-1 C42	3
HSV-1 KJ502	5
HSV-1 7935-72	2
HSV-1 C915 TK^-	>200
HSV-1 C42, ACV^r	>200
HSV-2 91075	8
HSV-2 72	14
HSV-2 B4327 UR2	13

The plaque assay was carried out as described in Materials and Methods and the ID_{50} was calculated from dose–response curves with 7 different concentrations of 9-(4-hydroxybutyl)-guanine.

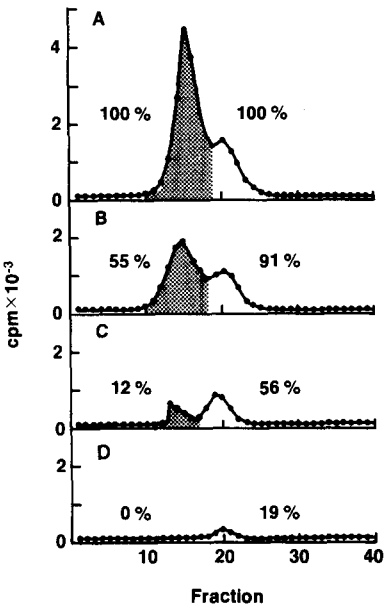

Fig. 1. Inhibition of cellular and HSV-1 DNA synthesis by 9-(4-hydroxybutyl)guanine. Viral and cellular DNA were labelled with [^{32}P]orthophosphate and separated as described in Materials and Methods. The shaded area denotes viral DNA. [^{3}H]Thymidine-labelled DNA from HSV-1 infected and untreated cells was used as internal density marker (not shown). The concentrations used of 9-(4-hydroxybutyl)guanine were: (A) 0 μM, (B) 0.5 μM, (C) 2.5 μM and (D) 10 μM.

Phosphorylation of 9-(4-hydroxybutyl)guanine by thymidine kinases

The affinity for HSV-1 and cytosol TK was determined in enzyme kinetic experiments and compared with that of the structure related antiherpes compound ACV. As shown in Fig. 2(A and B), both 9-(4-hydroxybutyl)guanine and ACV gave linear competitive inhibition of HSV-1 TK with dThd as variable substrate. The K_i values were determined to 2.06 μM for 9-(4-hydroxybutyl)guanine and to 173 μM for ACV

TABLE 2

Inhibition of HSV-1 and cellular DNA synthesis by 9-(4-hydroxybutyl)guanine

Synthesis	Concentrations giving 50% inhibition (μM)
HSV-1 infected cells	
HSV DNA	0.6
Cell DNA	3.6
Uninfected cells	
Cell DNA	>500

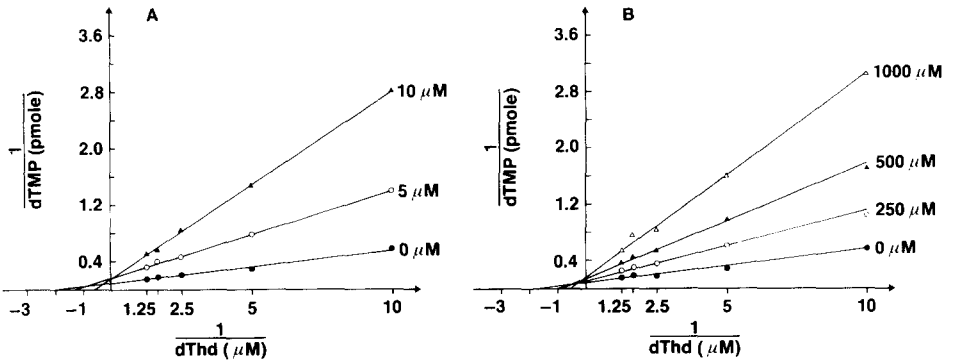

Fig. 2. Inhibition of thymidine phosphorylation by 9-(4-hydroxybutyl)guanine and ACV. Lineweaver–Burk plots with purified HSV-1 TK. [^{3}H]dThd was used as a variable substrate and (A) 9-(4-hydroxybutyl)guanine and (B) ACV as inhibitors. The concentrations used are as indicated in the figures and the assay is described in Materials and Methods.

with HSV-1 TK while K_i values >250 μM were obtained when cytosol TK was used for both these compounds (Table 3). The phosphorylation rates of 9-(4-hydroxybutyl)-guanine and ACV at 250 μM by viral and cellular TK were determined and compared with those of dThd as shown in Table 3. Due to the sensitivity of the assay, by measuring the transfer of γ-^{32}P from [γ-^{32}P]ATP to the tested compound the K_m values could not be determined. 9-(4-Hydroxybutyl)guanine was shown to be phosphorylated to about 10% of dThd and ACV to 27% of dThd by HSV-1 TK while no detectable phosphorylation was detectable for these compounds when cytosol TK was used. These results correspond well with the results obtained by Keller et al. [4].

Product identification by HPLC

The identification and separation of the formed monophosphate after incubation with 9-(4-hydroxybutyl)guanine or thymidine as substrate with cell extracts from HSV-1 infected cells is shown in Fig. 3. 9-(4-Hydroxybutyl)guanine monophosphate

TABLE 3

Cell-free phosphorylation by purified thymidine kinases

Compound	K_i (μM)		Velocity (%) relative to dThd	
	HSV-1 TK	Cytosol TK	HSV-1 TK	Cytosol TK
Thymidine	0.41± 0.11 (K_m)	1.31±0.13 (K_m)	100	100
9-(4-hydroxybutyl)-guanine	2.06± 0.42	>250	9.7±4.2	<5
Acyclovir	173 ±46	>250	27 ±1.7	<5

The data shown are mean values from 5 different determinations as described in Materials and Methods and the SD for these results are included.

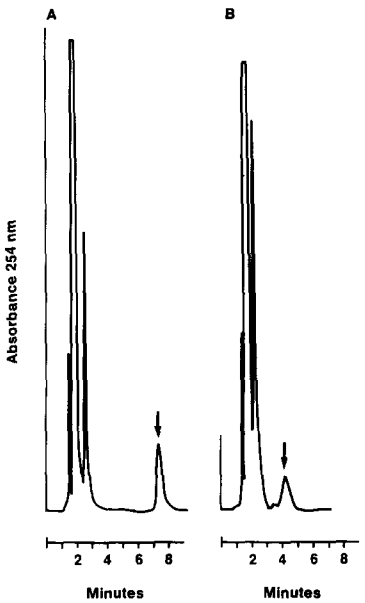

Fig. 3. Phosphorylation of 9-(4-hydroxybutyl)guanine by thymidine kinase. HPLC was used to determine 9-(4-hydroxybutyl)guanine monophosphate or thymidine monophosphate formation after enzymatic phosphorylation by HSV-1 TK as described in Materials and Methods. A. Phosphorylation with 100 μM dThd as substrate. The arrow indicates thymidine monophosphate. B. Phosphorylation with 500 μM 9-(4-hydroxybutyl)guanine as substrate. The arrow indicates 9-(4-hydroxybutyl)guanine monophosphate.

and thymidine monophosphate were used as reference compounds with R_f-values of 4.30 min and 7.40 min, respectively. The products formed were identified by retention times and standard addition. By this method, the amount of 9-(4-hydroxybutyl)guanine monophosphate also was found to be about 10% of that formed with dThd as substrate.

Effect of 9-(4-hydroxybutyl)guanine on herpes keratitis in rabbits

The effect on herpes keratitis in rabbits is shown in Fig. 4. After topical application of 5% 9-(4-hydroxybutyl)guanine in petrolatum base for 5 days a slight but not statistically significant effect on the mean keratitis score was observed. The healing time was reduced from 14 days for the control to 10 days for the compound.

Effect of 9-(4-hydroxybutyl)guanine on cutaneous HSV-1 infection in guinea pigs and systemic HSV-2 infection in mice

Treatment of cutaneous HSV-1 (strain C42) infection on the back of guinea pigs with 5% of the inhibitor in either a water-based ointment or in dimethylsulfoxide was started 2 or 24 h post-inoculation and consisted of 5 daily topical treatments for 4 days. This treatment did not result in any therapeutic effect (data not shown).

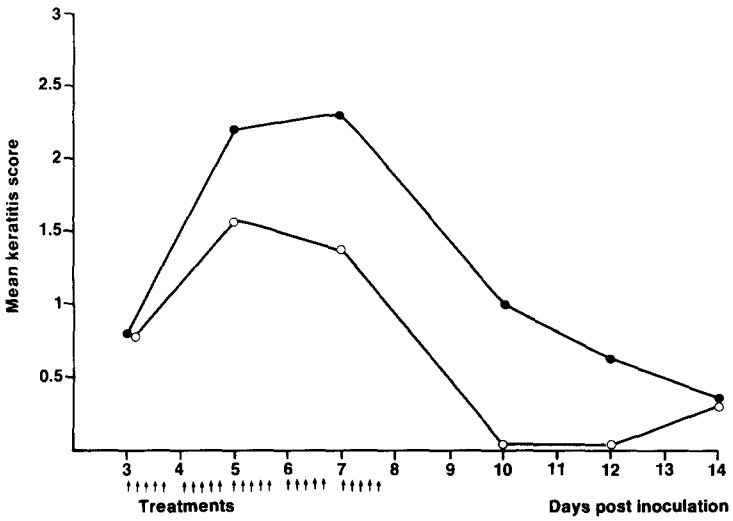

Fig. 4. Effect of 9-(4-hydroxybutyl)guanine on herpes keratitis in rabbits. The rabbits were inoculated with HSV-1, C42, and treated topically with 100 µl petrolatum base containing 5% (w/w) of the finely ground drug. The treatments are indicated by arrows. Each point represents the average of 6 eyes for treatment with the drug and 8 eyes for placebo. ●———● = Placebo ointment and ○———○ = 5% 9-(4-hydroxybutyl)guanine ointment.

The mortality after systemic HSV-2 infection in mice was unaffected after oral treatment with 2 × 75 mg/kg per day of the inhibitor starting 1 h post-inoculation and continued for 5 days. The mortality in both the infected control group and the treated group was 80% at day 12 after inoculation (data not shown).

Discussion

9-(4-Hydroxybutyl)guanine has recently been reported to be phosphorylated by HSV-1 TK but not by cellular TK and to inhibit herpesvirus replication in cell cultures [4]. From the plaque inhibition studies using different HSV-1 and HSV-2 strains and some mutants (Table 1) it is evident that the antiherpes activity is dependent on HSV TK and that both HSV-1 and HSV-2 are inhibited. Both a TK^- strain and a strain resistant to ACV, were resistant to 9-(4-hydroxybutyl)guanine. Kinetic analyses with HSV-1 TK were performed to investigate the mechanism of inhibition for 9-(4-hydroxybutyl)guanine and ACV. Keller et al. [4] have shown that ACV has non-classical kinetics with HSV-1 TK. However, in our experiments, with [^{3}H]dThd as variable substrate against different concentration of ACV or 9-(4-hydroxybutyl)guanine (Fig. 2) both these compounds gave the same type of linear competitive inhibition. This was also shown with the new structure related antiherpes compound 9-(3,4-dihydroxybutyl)guanine (DHBG) [17]. 9-(4-Hydroxybutyl)guanine has higher affinity for HSV-1 TK than for cellular cytosol TK with K_i values of 2.06 and >250 µM, respectively (Table 3). The affinity to HSV-1 TK was lower for ACV, K_i = 173 µM, than for 9-(4-hydroxybutyl)guanine, while the rate of phosphorylation was higher for ACV

than for 9-(4-hydroxybutyl)guanine (Table 3). In HSV-1 infected Vero cells 9-(4-hydroxybutyl)guanine showed a selective inhibition of viral DNA synthesis (Fig. 1, Table 2). This selectivity was less than that found for ACV [16] or foscarnet [11]. On the other hand, while 10 μM of 9-(4-hydroxybutyl)guanine inhibited the cellular DNA synthesis to about 80% in HSV-1 infected cells, the cellular DNA synthesis in uninfected cells was unaffected at this concentration (Table 2). By analogy to ACV it seems likely that 9-(4-hydroxybutyl)guanine acts by first being phosphorylated by HSV TK to a monophosphate and then, as a triphosphate, inhibits the HSV DNA polymerase somewhat better than it inhibits cellular DNA polymerase(s). The selective antiviral effect of 9-(4-hydroxybutyl)guanine seems to be dependent mainly on the selective phosphorylation by HSV TK.

In animal models 9-(4-hydroxybutyl)guanine has a therapeutic effect only on herpes keratitis in rabbits (Fig. 4) but no topical effect on a cutaneous herpes infection in guinea pig or a systemic herpes infection in mice. This is different from the good therapeutic effect recently found for 9-(3,4-dihydroxybutyl)guanine although the cell culture activities were similar [17]. Indeed, a 50% inhibition of HSV-1 C42 plaque formation was obtained at 3 μM 9-(4-hydroxybutyl)guanine and at 6 μM 9-(3,4-dihydroxybutyl)guanine. In the same system ACV gave a 50% inhibition at 0.3 μM (unpubl. obs.).

The reason for the lack of efficacy in the systemic herpes infection in mice or cutaneous herpes infection in guinea pigs is, at present, unclear. A determination of serum levels of the compounds might help to clarify the differences in therapeutic activity. According to these data it is becoming increasingly clear that the antiherpes effect of a compound in cell culture does not necessarily predict its effect in animals [18] and probably not in humans either. To deliniate the properties important for efficacy in infected animals it will be necessary to characterize several inhibitors at both the enzyme and the cell culture level and to correlate these results to the therapeutic activity and pharmacokinetics in (infected) animals.

The present results are one step in the accumulation of data on herpes inhibitors. More inhibitors should be evaluated in this way to determine what parameters govern therapeutic efficacy.

Acknowledgements

The authors wish to thank Dr. C.-E. Hagberg for helpful discussions during this work and kind criticism of the manuscript, Mrs. Gunilla Brännström for expert assistance in cell cultivation, Mr. Kjell Stenberg for cellular toxicity studies, Mr. Mats Berg for expert assistance in animal experiments, Dr. Birgitta Gotthammar for synthesizing *p*-amino-phenyl-thymidine 3'-phosphate used during the purification of the thymidine kinases, and Mrs. Merit Kropp for typing the manuscript.

References

1 Kit, S. (1979) Viral-associated and induced enzymes. Pharmacol. Ther. 4, 501–585.
2 Helgstrand, E. and Öberg, B. (1980) Enzymatic targets in virus chemotherapy. Antibiot. Chemother. (Basel) 27, 22–69.

3 Elion, G.B., Furman, P.A., Fyfe, J.A., De Miranda, P., Beauchamp, L. and Schaeffer, J. (1977). Selectivity of action of an antiherpetic agent, 9-(2-hydroxymethyl)guanine. Proc. Natl. Acad. Sci. U.S.A. 74, 5716–5720.

4 Keller, P.M., Fyfe, J.A., Beauchamp, L., Lubbers, C.M., Furman, P.A., Schaeffer, H.J. and Elion, G.B. (1981) Enzymatic phosphorylation of acyclic nucleoside analogs and correlations with antiherpetic activities. Biochem. Pharmacol. 30, 3071–3077.

5 Yamazaki, A. (1969) Synthesis of 9-(4′-hydroxybutyl)-2-amino-6-hydroxypurine 4′-phosphate. Chem. Pharm. Bull. 17, 1268–1270.

6 Schnürer, J. and Öberg, B. (1981) Inhibitory effects of foscarnet on herpesvirus multiplication in cell culture. Arch. Virol. 68, 203–209.

7 Helgstrand, E., Eriksson, B., Johansson, N.-G., Lannerö, B., Larsson, A., Misiorny, A., Norén, J.-O., Sjöberg, B., Stenberg, K., Stening, G., Stridh, S., Öberg, B., Alenius, S. and Philipson, L. (1978) Trisodium phosphonoformate, a new antiviral compound. Science 201, 819–821.

8 Cheng, Y.-C. and Ostrander, M. (1976) Deoxythymidine kinase induced in Hela TK^- cells by herpes simplex virus type 1 and type 2. J. Biol. Chem. 251, 2605–2610.

9 Fyfe, J.A., Keller, P.M., Furman, P.A., Miller, R.L. and Elion, G.B. (1978) Thymidine kinase from herpes simplex virus phosphorylates the new antiviral compound, 9-(2-hydroxyethoxymethyl)guanine. J. Biol. Chem. 253, 8721–8727.

10 Dobersen, M.J. and Greer, S. (1975) An assay for pyrimidine deoxyribonucleoside kinase using γ-^{32}P-labeled ATP. Anal. Biochem. 67, 602–610.

11 Larsson, A. and Öberg, B. (1981) Selective inhibition of herpesvirus DNA synthesis by foscarnet. Antiviral Res. 1, 55–62.

12 Stenberg, K. (1981) Cellular toxicity of pyrophosphate analogues. Biochem. Pharmacol. 30, 1005–1008.

13 Alenius, S. and Öberg, B. (1978) Comparison of the therapeutic effects of five antiviral agents on cutaneous herpesvirus infection in guinea pigs. Arch. Virol. 58, 227–288.

14 Alenius, S., Laurent, U. and Öberg, B. (1980) Effect of trisodium phosphonoformate and idoxuridine on experimental herpes simplex keratitis in immunized and non-immunized rabbits. Acta Ophthalmol. 58, 167–173.

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

16 Larsson, A. and Öberg, B., (1981) Selective inhibition of herpesvirus deoxyribonucleic acid synthesis by acycloguanosine, 2′-fluoro-5-iodo-aracytosine and (E)-5-(2-bromovinyl)-2′-deoxyuridine. Antimicrob. Agents Chemother. 19, 927–929.

17 Larsson, A., Öberg, B., Alenius, S., Hagberg, C.-E., Johansson, N.-G,, Lindborg, B. and Stening, G. (1983) 9-(3,4-Dihydroxybutyl)guanine, a new inhibitor of herpesvirus multiplication. Antimicrob. Agents Chemother., in press.

18 Alenius, S., Berg, M., Broberg, F., Eklind, K., Lindborg, B. and Öberg, B. (1982) Therapeutic effects of foscarnet sodium and acyclovir on cutaneous infections due to herpes simplex virus type 1 in guinea pigs. J. Infect. Dis. 145, 569–573.