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Inhibiting Effect of (*RS*)-9-[4-Hydroxy-2-(Hydroxymethyl)Butyl] Guanine on Varicella-Zoster Virus Replication in Cell Culture

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The activity and mode of action of the new nucleoside analog (*RS*)-9-[4-hydroxy-2-(hydroxymethyl)butyl]guanine (2HM-HBG) against varicella-zoster virus (VZV) were determined. In cell culture, replication of different strains of VZV was inhibited to 50% by 0.4 to 0.7 μM 2HM-HBG, while 685 μM was required to inhibit 50% of the DNA synthesis in uninfected human lung fibroblasts. A thymidine kinase-negative VZV strain was not inhibited by 100 μM 2HM-HBG. Inhibition of VZV replication was not reversible after 7 to 14 days of incubation, depending on the multiplicity of VZV. 2HM-HBG was shown to be selectively phosphorylated by purified VZV thymidine kinase, with an inhibition constant of 32.5 μM. The antiviral activity of 2HM-HBG in cell culture was decreased by the addition of deoxythymidine and deoxycytidine but not by other ribo- or deoxyribonucleosides.

The new acyclic guanosine analog (*RS*)-9-[4-hydroxy-2-(hydroxymethyl)butyl]guanine (2HM-HBG) exhibits structural similarities to 9-(2-hydroxyethoxymethyl)guanine (acyclovir [ACV] [3]). It was therefore considered that the two compounds may have similar mechanisms of action. These include selective phosphorylation by herpesvirus-induced thymidine kinases (TK) to monophosphates and, after cellular phosphorylation to triphosphates, inhibition of viral DNA synthesis. PP_i analogs, on the other hand, inhibit viral DNA polymerase activity directly (9). They are therefore also active against herpesviruses that do not encode a specific TK (19). The most active PP_i analog is Foscarnet.

The aim of our study was to describe the antiviral activity of 2HM-HBG in cell culture against varicella-zoster virus (VZV), its cellular toxicity, phosphorylation by VZV TK, reversibility of the antiviral activity by exogenous addition of natural nucleosides, and the probable mechanism of action.

MATERIALS AND METHODS

Cells. Human embryonic lung (HL) fibroblasts were cultured in Eagle minimal essential medium with 2% calf serum and antibiotics. These cells were chosen for their low deoxythymidine (dThd) content, which is known to influence the sensitivity to certain antiviral substances (5).

Virus. VZV strains ULF and 9/84 were obtained from vesicles of patients with clinical varicella infections and isolated at the National Bacteriological Laboratory, Stockholm, Sweden. The VZV strain pE, an isolate from a patient with clinical varicella, was obtained from the Department of Medical Virology, Biomedical Center, Uppsala University, Uppsala, Sweden. The mutant strain of VZV obtained in the presence of 5-bromo-2'-deoxyuridine and deficient in viral TK activity was from Fukushima Medical College, Fukushima, Japan (21). All VZV strains were typed by an enzyme-linked immunosorbent assay (ELISA) method previously published (18).

Chemicals. 2HM-HBG (Fig. 1), the (*R*)- and (*S*)-enantiomers of 9-(3,4-dihydroxybutyl)guanine (DHBG), ACV, and phosphonoformic acid (Foscarnet [rINN]) were from Astra Läkemedel AB, Södertälje, Sweden. 9-(1,3-Dihydroxy-2-propoxymethyl)guanine (DHPG) was a gift from Merck, Sharp and Dohme, West Point, N.Y. [*E*]-5-(2-Bromovinyl)-2'-deoxyuridine (BVdU) was a gift from E. De Clercq, University of Leuven, Louvain, Belgium. 5-Iodo-2'-deoxyuridine (IdU) was from Ferring Co., Malmö, Sweden, and 9-β-D-arabinofuranosyladenine (ara-A) was from Parke-Davis, Morris Plains, N.J. Ara-A was suspended in H_2O and dissolved at 800 μM in the tissue culture medium. Natural nucleosides were obtained from Sigma Chemical Co., St. Louis, Mo. 2HM-HBG was synthesized by the reaction of 2-amino-6-chloropurine and dimethyl itaconate in dimethylformamide with a catalytical amount of sodium hydride, followed by reduction with lithium borohydride in *tert*-butanol and hydrolysis by 70% aqueous formic acid. 2HM-HBG was synthesized at Astra Läkemedel AB (N. G. Johansson, B. Lindborg, and J. O. Norén, Eur. Pat. Appl. EP 146,516, 1986; Chem. Abstr. **104**:50735).

VSA. For the viral sensitivity assay (VSA), confluent HL cells were infected with the virus stock solution and a fourfold dilution of the stock solution. The virus dilution giving an A_{410} value of 1.5 to 1.8 (see below) was used to evaluate inhibitory concentrations of the compounds. Following a 1.5-h incubation period, medium containing different concentrations of antiviral substances was added. At 50 to 70% cytopathic effect (CPE), after about 5 to 7 days, the cells were harvested and VZV antigens were quantified by ELISA (18, 20). The sandwich assay for viral antigens was carried out on microplates (M 29AR; Dynatech, Zug, Switzerland) coated with human zoster immunoglobulin (National Bacteriological Laboratory, Stockholm, Sweden). The pooled, lysed infected cell antigen from two to four wells was applied, followed by rabbit anti-VZV and alkaline

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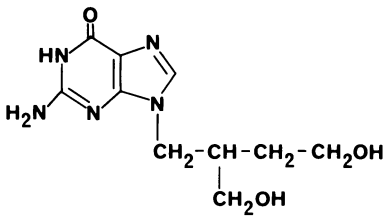

FIG. 1. Structure of 2HM-HBG.

phosphatase-labeled swine anti-rabbit immunoglobulin G (IgG) (Orion Diagnostica, Helsinki, Finland). The substrate was *p*-nitrophenyl phosphate (Sigma Chemical Co.), and the reaction was read at 410 nm. The 50% inhibiting concentration (IC_{50}) of each drug was calculated.

Immunofluorescence. HL cells on cover slides in Leighton tubes were infected with VZV strain ULF at a multiplicity of infection (MOI) of 0.05 to 1.0. After 1.5 h of incubation, medium containing the antiviral substances was added to the tubes. Every third day, slides were fixed in −20°C acetone, stained with fluorescein isothiocyanate-labeled human anti-VZV IgG, and examined in a Zeiss fluorescence microscope.

Attempts to reverse the antiviral activity of 2HM-HBG. Confluent HL cells were infected with two concentrations of each virus strain used. Following a 1.5-h incubation period, medium containing different concentrations of 2HM-HBG and ribo- or deoxyribonucleosides was added. At 50 to 70% CPE in control culture with virus only, all cells were harvested and analyzed by the VSA procedure described above.

Cell toxicity assay. HL cells were grown in 96-well flat-bottomed microtiter plates (Falcon Plastics, Oxnard, Calif.) for 24 h. While the uninfected HL cells were still subconfluent, 0.1 ml of 2HM-HBG solution or diluent alone was added. Three hours later, [^{3}H]dThd (0.25 μCi in 50 μl) was added, and the plates were incubated for 20 h at 37°C. The wells were washed, and 50 μl of a solution of trypsin-versene was added, followed by 50 μl of 0.5% sodium dodecyl sulfate. To harvest the cell lysates, a multiple automated harvester (Skatron, Lierbyen, Norway) was used. The radioactivity was determined in a scintillation spectrophotometer.

Purification of VZV TK and mitochondrial TK. The purification of VZV TK from infected Vero cells (11) and of noninfected human placenta mitochondrial TK was performed by affinity chromatography with *p*-aminophenyl-dThd-3′-phosphate as the ligand (15). The elution was done in a stepwise fashion by addition of IdU. The VZV-specific TK was eluted at 30 μM IdU, and the mitochondrial TK was eluted at 150 μM IdU. The fractions containing enzyme activity were pooled and desalted on a Sephadex G-25 column (Pharmacia, Uppsala, Sweden). Polyacrylamide gel electrophoresis (PAGE) was carried out by the method of Kit et al. (12). The TKs gave profiles with relative R_fs of 0.42 to 0.45 for VZV TK and 0.65 for mitochondrial TK. Both the mitochondrial and VZV TK could use CTP as a phosphate donor (1, 4, 10, 12). The molecular sizes, determined by analytical 4 to 30% PAGE gradient, were approximately 86,000 for the VZV TK and 69,000 for the mitochondrial TK (4, 12, 13).

TK assay. One unit is defined as the amount of enzyme needed to catalyze the formation of 1 pmol of dTMP per min at 37°C. Our kinetic studies were performed with 1 U of VZV or mitochondrial TK. The TK under investigation was added to 1.8 μM [^{3}H]dThd (specific activity, 78 Ci/mmol) in 0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer, pH 7.4, with 15 mM MgCl, 17 mM KCl, 1 mM NaF, 4 mM ATP, dithiothreitol, and bovine serum albumin (10). Samples were taken at 4-min intervals over 20 min. The K_i values for the tested compounds were determined from double-reciprocal plots as described by Lineweaver and Burk, using the relationship for competitive inhibitors that slope I = (slope 0) × (1 + $[I]/K_i$), where slope I = slope for inhibited reaction, slope 0 = slope for uninhibited reaction, and $[I]$ = inhibitor concentration. To determine the suitability of the test compounds as substrates for the TKs, we used the procedure of Dobersen and Greer (2) with 100 μl of reaction mixture containing 0.2 mM [γ-^{32}P]ATP, 0.8 mM $MgCl_2$, 100 μM test compound, and TK. The K_m for dThd with VZV TK was 0.64 ± 0.08 μM, and for dThd with human mitochondrial TK it was 2.7 ± 0.8 μM.

RESULTS

Activity of 2HM-HBG and other antiviral compounds against VZV replication in cell culture. The activity of several compounds against VZV replication was compared (Table 1). All compounds were assayed at similar VZV multiplicities. We chose VZV doses giving an A_{410} of 1.5 to 1.8 in the antigen detection VSA. 2HM-HBG inhibited VZV protein production to 50% (IC_{50}) with a high efficacy, ranging between 0.4 and 0.7 μM (Table 1). The dThd analog BVdU also showed a high inhibitory effect against VZV. ACV and DHPG had similar ranges of antiviral activity. (*R*)-DHBG exhibited a higher activity against VZV IC_{50} than the (S)-DHBG enantiomer. Foscarnet, IdU, and ara-A were also active. With all compounds a range in susceptibility was noted between the three TK$^+$ VZV strains. The influence of 2HM-HBG on cellular DNA synthesis was studied (Fig. 2). A concentration of 685 μM 2HM-HBG inhibited [^{3}H]dThd incorporation in uninfected subconfluent HL cells to 50%. Thus, 2HM-HBG appeared to selectively inhibit VZV production.

A TK$^-$ VZV strain resistant to BVdU was used to assay susceptibility to 2HM-HBG and other metabolic analogs (Table 1). The susceptibility of this mutant VZV strain to 2HM-HBG and several other nucleoside analogs was low. A slight but decreased susceptibility to DHPG and ara-A was retained. Foscarnet, a PP_i analog, showed a similar IC_{50} for the TK$^-$ and TK$^+$ VZV strains.

The two most active guanine analogs and Foscarnet were selected to determine the reversibility of inhibition of VZV replication. A 50% reduction, measured by immunofluores-

TABLE 1. Inhibitory activity of antiviral compounds against three VZV TK$^+$ strains (ULF, pE, and 9/84) and one TK$^-$ strain

Compound	IC_{50} (μM)	
	TK$^+$ strains	TK$^-$ strain
2HM-HBG	0.4–0.7	>100
BVdU	0.6–0.9	>100
ACV	6.5–20	200
DHPG	6.2–30	80
Ara-A	≤20–30	≤50
IdU	30–75	>100
(*R*)-DHBG	40–160	>250
(*S*)-DHBG	425–600	>250
Foscarnet	150–300	250

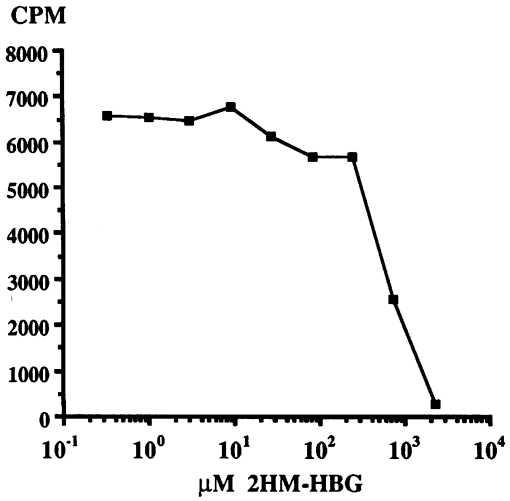

FIG. 2. Cellular toxicity of 2HM-HBG as measured by [^{3}H]dThd incorporation in HL cells.

cence, of VZV antigens was observed after 5 days in cell culture at an MOI of 1.0, with <5 μM 2HM-HBG, 25 μM ACV, and 100 μM Foscarnet. The concentration of drug which irreversibly inhibited virus replication was then determined. The compounds were left for 7 or 14 days in culture together with various MOIs of virus (Table 2). The cultures were then observed for 4 weeks after removal of each compound. With TK$^+$ VZV (MOI, 0.05), <1 to 5 μM 2HM-HBG prevented viral outgrowth after drug removal. To accomplish the same irreversible inhibition, 25 to 50 μM ACV or 100 to 400 μM Foscarnet was needed. The TK$^-$ strain was not inhibited by 2HM-HBG or ACV, but it was inhibited by Foscarnet. With higher MOIs, higher concentrations of compounds were required to obtain complete, irreversible inhibition.

Activity of 2HM-HBG in the presence of deoxyribo- and ribonucleosides. The addition of increasing amounts of dThd or deoxycytidine (dCyd) to VZV-infected cells resulted in a diminished antiviral effect of 2HM-HBG; the normal nucleoside dThd at 100 μM totally eliminated the activity of 6 μM 2HM-HBG, while 50 μM dThd reversed the antiviral activity to 80% (Fig. 3). With dCyd, 100 μM nucleoside could reverse the activity of 6 μM 2HM-HBG to 40% (Fig. 3), while the antiviral activity of 3 μM 2HM-HBG was reversed by 100 μM dCyd to about 87% (data not shown). Addition of deoxyguanosine (dGuo) or deoxyadenosine (dAdo) did not affect 2HM-HBG inhibition of VZV. None of the ribonucleosides (Urd, Cyd, Ado, and Guo) inhibited the antiviral activity of 2HM-HBG. We did not note antiviral activity by the added nucleosides alone; no significant changes were seen in the amount of viral antigen produced by the VZV-infected cells in the presence of nucleosides. These results were similar with the two VZV strains.

TABLE 2. Inhibition of VZV replication after incubation with and removal of antiviral compounds in tissue culture

VZV strain	MOI	Incubation time with antiviral compound (days)	Concn needed for complete inhibition after drug removal (μM)		
			2HM-HBG	ACV	Foscarnet
TK$^+$	1.0	7	25	>50	>400
		14	<1	50	200
TK$^+$	0.2	7	25	50	>400
		14	<1	25	200
TK$^+$	0.05	7	5	50	400
		14	<1	25	100
TK$^-$	0.2	7	>25	>25	300
		14	>25	>25	200

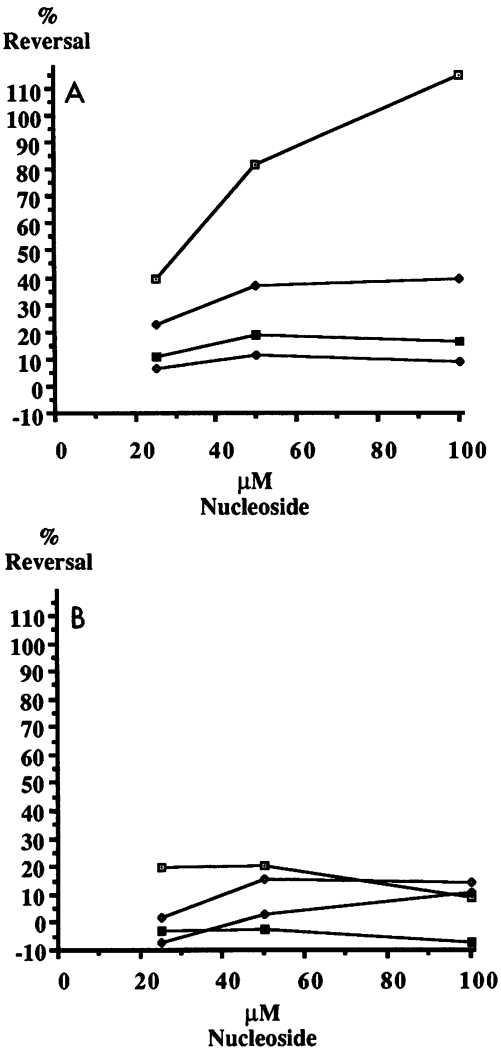

FIG. 3. Reversibility of the antiviral activity of 2HM-HBG (6 μM) in the presence of ribo- and deoxyribonucleosides. Viral (VZV) antigen content measured by ELISA expressed as A_{410}. (A) Symbols: ⊡, dThd; ◆, dCyd; ■, dAdo; ◇, dGuo. (B) Symbols: ⊡, Urd; ◆, Cyd; ■, Ado; ◇, Guo.

Effect of nucleoside analogs on purified VZV TK. In the phosphorylation of dThd, 2HM-HBG acts as a competitive inhibitor (Fig. 4). ACV showed a low affinity to VZV TK compared with the high affinity found for 2HM-HBG (Table 3). The K_i for ACV and 2HM-HBG was 858 and 32.5 μM, respectively. Neither of these two analogs showed any affinity for the mitochondrial TK (K_i, >1,000 μM). The determination of the relative rate of phosphorylation for 2HM-HBG compared with that for dThd showed that 2HM-HBG was phosphorylated to a degree about 247% higher than that of dThd. The relative rate of ACV phosphorylation was 9.5% of the dThd phosphorylation rate.

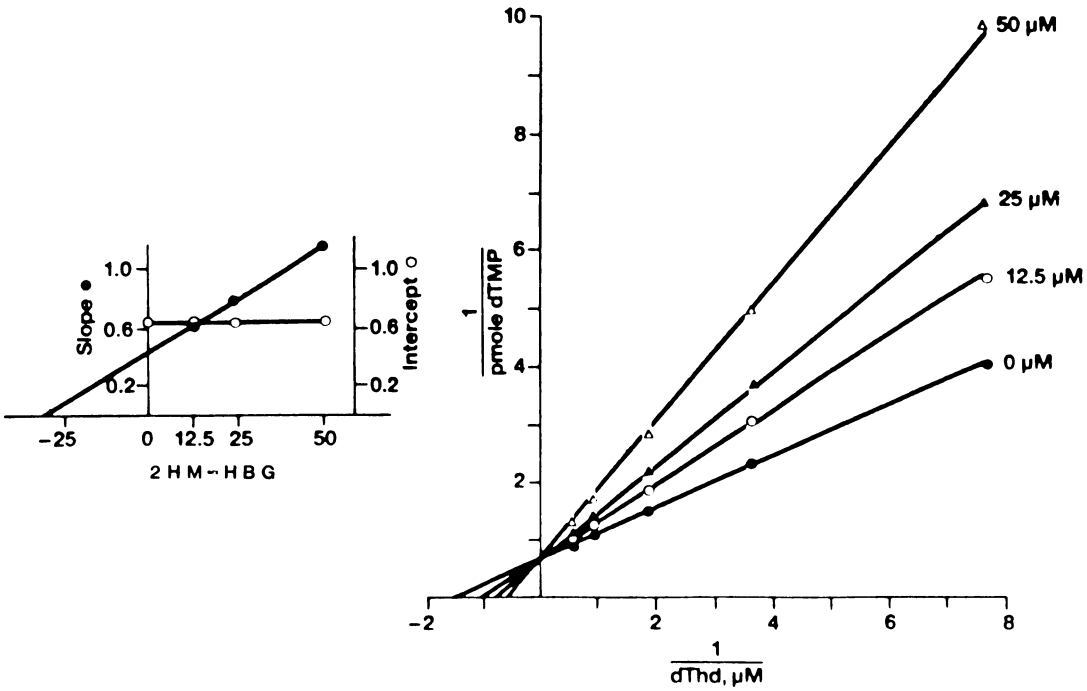

FIG. 4. Inhibition of thymidine phosphorylation by 2HM-HBG with purified VZV TK. Reaction rates were determined at different concentrations of thymidine and three concentrations of 2HM-HBG. The assay conditions are described in the text; the nucleoside concentrations are indicated in the figure.

DISCUSSION

The in vitro selectivity of 2HM-HBG against VZV seems to require the expression of a viral TK. Based on the VSA with different strains of VZV (including a mutant strain deficient in TK-inducing activity), it seems likely that the anti-VZV activity of 2HM-HBG is dependent on VZV TK. We found that 2HM-HBG has a higher affinity to the purified enzyme and a higher phosphorylation rate than ACV.

Many antiherpes substances are active in reactions carried out by viral enzymes, and these substances often have to compete with different cellular metabolites (14). 2HM-HBG, like ACV, acted as a competitive inhibitor of dThd. Our results also showed that 2HM-HBG was less sensitive to reversal of the VZV inhibition by dThd than was ACV. The exogenous addition of dThd and dCyd caused decreased antiviral activity of 2HM-HBG on VZV-infected cells. It is therefore probable that dThd, dCyd, 2HM-HBG, and ACV show affinity to the same or closely related substrate-binding sites on the VZV TK. 2HM-HBG also showed good activity against herpes simplex virus type 1 (HSV-1) and HSV-2, with a 50% plaque reduction obtained at 0.2 and 5.0 μM, respectively (Larsson et al., submitted for publication). In cell culture, VZV multiplication was inhibited by 0.4 to 0.7 μM 2HM-HBG, while 50% of the cellular DNA synthesis was inhibited at 685 μM. This gives an apparently large therapeutic ratio for the compound.

TABLE 3. Affinity nucleoside analogs for purified TKs and rates of phosphorylation[a]

Compound	Mean K_i (μM) ± SD		Phosphorylation velocity relative to Thd (%)	
	VZV TK	Mitochondrial TK	VZV TK	Mitochondrial TK
2HM-HBG	32.5 ± 3.2	>1,000	246.9 ± 57.5	<5
ACV[b]	858 ± 206	>1,000	9.5 ± 2.5	<5

[a] The K_i values are means from at least five different determinations, whereas the relative rates are calculated from three to five experiments.
[b] Data published previously (11).

With another herpesvirus, cytomegalovirus, it was not possible to eradicate the viral genome by prolonged incubation with Foscarnet or guanine analogs in cell culture (19; Wahren et al., submitted for publication). With VZV, however, nonreversible inhibition was obtained after 7 to 14 days of incubation with either 2HM-HBG, ACV, or Foscarnet. Similar to the experience with HSV (7, 16), VZV may be inhibited in vitro. Increased doses of VZV required higher concentrations of 2HM-HBG to obtain a 50% reduction of viral antigen. The effect of 2HM-HBG, like that of several other compounds to other herpesviruses (6, 8), thus appears to be multiplicity dependent.

2HM-HBG is an acyclic guanosine analog and is probably phosphorylated to monophosphate by the viral TK, activated to di- and triphosphate by cellular enzymes, and then likely to interfere selectively with the viral polymerase reaction. An evaluation of the (*R*)- and (*S*)-2HM-HBG triphosphates for their efficacies on purified VZV DNA polymerases is in progress. Intravenous ACV appears to be superior to ara-A in immunocompromised patients with VZV infections (17). 2HM-HBG is more active than ACV in viral TK-dependent phosphorylation and less sensitive to reversal by dThd. It might therefore be considered for clinical application against VZV infections.

LITERATURE CITED

1. **Cheng, Y.-C., T. Y. Tsou, T. Hackstadt, and L. P. Mallavia.** 1979. Induction of thymidine kinase and DNase in varicella-zoster virus-infected cells and kinetic properties of the virus-induced thymidine kinase. J. Virol. **31**:172–177.

2. **Dobersen, M. J., and S. Greer.** 1975. An assay for pyrimidine deoxyribonucleoside kinase using ^{32}P-labeled ATP. Anal. Biochem. **67**:602–610.
3. **Elion, G. B., P. A. Furman, J. A. Fyfe, P. De Miranda, L. Beauchamp, and H. J. Schaeffer.** 1977. Selectivity of action of an antiherpetic agent, 9-(2-hydroxyethoxymethyl)guanine. Proc. Natl. Acad. Sci. USA **74**:5716–5720.
4. **Hackstadt, H., and L. P. Mallvia.** 1978. Deoxypyrimidine nucleoside metabolism in varicella-zoster virus-infected cells. J. Virol. **25**:510–517.
5. **Harmenberg, J.** 1983. Intracellular pools of thymidine reduce the antiviral action of acyclovir. Intervirology **20**:48–51.
6. **Harmenberg, J.** 1984. Interactions between virus and antiviral compounds in the host cell. Med. Biol. **62**:299–303.
7. **Harmenberg, J., and B. Wahren.** 1982. Influence of cell culture conditions on the inhibition of herpes simplex virus type 1 replication by acyclovir. Intervirology **17**:215–221.
8. **Harmenberg, J., B. Wahren, and B. Öberg.** 1980. Influence of cells and virus multiplicity on the inhibition of herpesviruses with acycloguanosine. Intervirology **14**:239–244.
9. **Helgstrand, E., B. Eriksson, N. G. Johansson, B. Lannerö, A. Larsson, A. Misiorny, I. O. Norén, B. Sjöberg, K. Stenberg, G. Stening, B. Öberg, S. Alenius, and L. Philipsson.** 1978. Trisodium phosphonoformate, a new antiviral compound. Science **201**:819–821.
10. **Källander, C. F. R., J. S. Gronowitz, and E. Olding-Stenkvist.** 1983. Rapid diagnosis of varicella-zoster virus by detection of viral deoxythymidine kinase in serum and vesicle fluid. J. Clin. Microbiol. **14**:280–287.
11. **Karlström, A. R., C. F. R. Källander, G. Abele, and A. Larsson.** 1986. Acyclic guanosine analogs as substrates for varicella-zoster thymidine kinase. Antimicrob. Agents Chemother. **29**:171–174.
12. **Kit, S., W.-C. Leung, and D. Trkula.** 1973. Properties of mitochondrial thymidine kinase of parental and enzyme-deficient HeLa cells. Arch. Biochem. Biophys. **158**:503–513.
13. **Kit, S., W.-C. Leung, D. Trkula, and G. Jorgenson.** 1974. Gel electrophoresis and isoelectric focusing of mitochondrial and viral-induced thymidine kinase. Int. J. Cancer **13**:203–218.
14. **Larsson, A., G. Brännström, and B. Öberg.** 1983. Kinetic analysis in cell culture of the reversal of antiherpes activity of nucleoside analogs by thymidine. Antimicrob. Agents Chemother. **22**:55–61.
15. **Lee, L.-S., and Y.-C. Cheng.** 1976. Human deoxythymidine kinase. Purification and general properties of cytoplasmic and mitochondrial isozymes derived from blast cells of acute myelocytic leukemia. J. Biol. Chem. **251**:2600–2604.
16. **Schnürer, J., and B. Öberg.** 1981. Inhibitory effects of Foscarnet on herpesvirus multiplication in cell culture. Arch. Virol. **68**:203–209.
17. **Shepp, D., P. Dandliker, and J. Meyers.** 1986. Treatment of varicella-zoster virus infection in severely immunocompromised patients. N. Engl. J. Med. **314**:208–212.
18. **Sundqvist, V. A., and B. Wahren.** 1982. Development of sensitive and enzyme-linked immunoassays for herpesvirus antigens and applications. Dev. Biol. Stand. **52**:245–253.
19. **Wahren, B., and B. Öberg.** 1980. Reversible inhibition of cytomegalovirus replication by phosphonoformate. Intervirology **14**:7–15.
20. **Wahren, B., P. Wahren, J. Harmenberg, and V.-A. Sundqvist.** 1983. Computer-based virus sensitivity assay and neutralization method. Application for herpesviruses. J. Virol. Methods **6**:271–282.
21. **Yokota, T., M. Ogata, and S. Shigeta.** 1983. A mutant strain of varicella zoster deficient in thymidine kinase inducing activity. Fukushima J. Med. Sci. **29**:101–111.