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Mechanism and Mode of Action of 5-Iodo-2-pyrimidinone 2′-Deoxyribonucleoside, a Potent Anti-Herpes Simplex Virus Compound, in Herpes Simplex Virus-Infected Cells

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SUMMARY

The anti-herpes simplex virus type 2 (-HSV-2) action of 5-iodo-2-pyrimidinone deoxyribonucleoside (IPdR) was found to be exerted through inhibition of HSV DNA synthesis. The inhibition of viral DNA synthesis was not caused by inhibition of the synthesis of HSV-2-specified proteins or HSV-2 mRNA species involved with viral DNA synthesis or by depletion of deoxynucleotides. The inhibition of viral DNA synthesis may be due to damage to the DNA template in the nuclei or to an action at the DNA replication complex, because nuclei isolated from HSV-2-infected cells treated with IPdR could not support DNA synthesis *in vitro*. Moreover, the addition of exogenous template to the reaction enabled nuclear DNA synthesis to occur at the level of control. The major cellular metabolite of IPdR in HeLa S_3 cells infected with HSV-2 was IPdR monophosphate, which was formed through virally specified kinase. Attempts to either identify or synthesize IPdR diphosphate and triphosphate were unsuccessful. The accumulation of IPdR monophosphate was dependent on the extracellular concentration of IPdR. IPdR monophosphate did not have any inhibitory effect on nuclear DNA synthesis, even at 200 μM. Thus, the action of IPdR could be due to an unidentified metabolite of IPdR or the depletion of a cellular metabolite that is essential for viral DNA synthesis.

Recently, a new group of 5-substituted 2-pyrimidinone 2′-deoxyribonucleoside analogues were introduced as anti-HSV compounds (1). IPdR (Fig. 1) has potent activity against HSV-1 and HSV-2 in cell culture and against HSV-2 in mice (1, 2). The antiviral activity of IPdR is dependent on the HSV-specified thymidine kinase, inasmuch as the activity is reduced in HSV-1 variants with altered thymidine kinase (1). The absence of an amino group or oxygen at the 4-position of IPdR suggests that, whereas it is likely that IPdR could be phosphorylated by the HSV thymidine kinase to IPdR-MP, further phosphorylation to IPdR-diphosphate by cellular nucleoside monophosphate kinases and subsequent phosphorylation to IPdR-TP may not be possible. Thus, it is possible that the mechanism of action of IPdR is quite different from that of other compounds whose antiviral action is dependent on viral thymidine kinase and whose activity involves incorporation into DNA (3, 4). In order to better understand the mechanism of the antiviral activity of IPdR, the effect of IPdR on viral macromolecules, the cellular metabolism of IPdR, and the effect of IPdR on dNTP pools in virus-infected cells are investigated in this paper.

Materials and Methods

Chemicals and enzymes. IPdR was produced in and provided by the laboratory of Dr. Thomas Bardos, Department of Medicinal Chemistry, State University of New York, Buffalo (2). The nucleosides and nucleotides were purchased from Sigma Chemical Co. (St. Louis, MO). *Escherichia coli* DNA polymerase was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). *E. coli* dUTPase was prepared and generously provided by Dr. Marshall Williams, Department of Medical Microbiology and Immunology and Comprehensive Cancer Center, Ohio State University (Columbus, OH) (5, 6).

Cells and virus. HeLa S_3 cells were used for all experiments, with the exception of the *in vitro* phosphorylation studies, in which HeLa BU cells were used. HeLa BU cells have been previously described and characterized as thymidine kinase-deficient cells (7). Both cell lines were cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum and 100 μg/ml kanamycin. The cells were maintained in a humidified atmosphere (5% CO_2) at 37°. HSV-2 (strain 333) was maintained as previously described (1, 8). In all experiments, cells were infected with HSV-2 at a multiplicity of infection of 3 plaque-forming units/cell, as previously described (1). After a 1-hr virus absorption,

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ABBREVIATIONS: HSV-1 and HSV-2, herpes simplex virus type 1 and type 2; IPdR, 5-iodo-2-pyrimidinone deoxyribonucleoside; IPdR-MP, 5-iodo-2-pyrimidinone deoxyribonucleoside monophosphate; IPdR-TP, 5-iodo-2-pyrimidinone deoxyribonucleoside triphosphate; dNTP, deoxynucleoside triphosphate; DNase, deoxyribonuclease; dUTPase, deoxyuridine-triphosphate nucleotidohydrolase; IUdR, iododeoxyuridine; ACV, acyclovir; PFA, phosphonoformic acid; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high pressure liquid chromatography.

the medium was removed and fresh medium with IPdR at various concentrations was added. The cells were incubated with IPdR for 9 hr, unless otherwise indicated, at 37°.

Effect of IPdR on viral DNA. Monolayers of HeLa S_3 cells were infected with HSV-2 and incubated in the presence of 30, 60, 100, or 200 μM IPdR for 9 hr or with 100 μM IPdR for various periods of time. The relative amount of HSV-2 DNA in the control and IPdR-treated samples were analyzed by use of *in situ* discontinuous agarose gel electrophoresis, as previously described (1, 9). The DNA was transferred to nitrocellulose paper by means of a vacuum blot apparatus (American Bionetics, Inc., Hayward, CA), according to the manufacturers' instructions, or to Hybond-N nylon paper (Amersham, Arlington Heights, IL) by means of a BIOS blotting unit (BIOS Corporation, New Haven, CT), according to the manufacturer's instructions. Hybridization was then performed with a recombinant plasmid, pKC7*Bgl* N, containing the *Bgl*II N fragment of HSV-2 (strain 333) DNA (10). The probe was labeled with [α-^{32}P]dCTP [labeling was done with a random primed DNA labeling kit (Boerhinger Mannheim), according to the instructions of the manufacturer].

Effect of IPdR on viral protein synthesis. Total cellular proteins were extracted from the HSV-2- or mock-infected cells and subjected to electrophoresis in an 8.5% SDS-polyacrylamide gel by the method of Laemmli and Favre (11). The proteins were transferred to nitrocellulose by diffusion, as previously described (12). The total cellular proteins were visualized on one sheet of nitrocellulose by amido black staining, and HSV-2 viral proteins were detected by immunostaining (13) with a polyclonal antibody to HSV-2 proteins (Dako Immunoglobulins, Santa Barbara, CA).

Enzyme induction assays. Monolayers of HeLa S_3 cells, infected with HSV-2 as described above, were incubated with or without IPdR and other compounds for 9 hr after infection. The cells were harvested by scraping into the growth medium, washed twice with cold phosphate-buffered saline, and resuspended in an extraction buffer (300 mM KH_2PO_4, pH 7.5, 2 mM dithiothreitol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.2% Triton X-100, 10% glycerol). The cells were disrupted by sonication and the protein extracts were examined for HSV-2-specified DNase and DNA polymerase activity (14). The enzyme assays for HSV-2 DNA polymerase and HSV-2 DNase have been previously described (15, 16).

Effect of IPdR on viral RNA synthesis. Total cellular RNA was isolated from HSV-2-infected cells treated with IPdR for 3, 6, and 9 hr, using the guanidinium thiocyanate-phenol-chloroform extraction method described by Chomczynski and Sacchi (17). The samples, containing 40 μg of RNA/lane, were prepared for and analyzed by agarose gel electrophoresis, as previously described (14). The RNA was transferred to Hybond-N nylon paper (Amersham) by use of a BIOS blotting unit (BIOS), according to the manufacturer's instructions. Hybridization was performed with 10^6 cpm/ml HSV-2 DNA (*Bgl*II N fragment) labeled with [α-^{32}P]dCTP, as described above, at 42° with hybridization solution containing 50% formamide (18).

Effect of IPdR on nuclear DNA synthesis. Nuclei were isolated from HeLa S_3 cells that had been infected with HSV-2 and incubated for 12 to 16 hr in the absence or presence of IPdR or other antiviral compounds. An assay for nuclear DNA synthesis was done as previously described (19). The assay mixture contained 6% glycerol, 12.5 mM HEPES, pH 7.8, 5 mM Tris·HCl, pH 7.8, 1.25 mM dithiothreitol, 3 mM 2-mercaptoethanol, 1.5 mM EDTA, 150 μM KCl, 4.5 mM $MgCl_2$, dATP, dCTP, and dGTP (each at 400 μM), 1 μM (20 μCi/ml) [^{3}H]TTP, and 25 μl/100 μl of assay mixture of a 2×10^7 nuclei/ml suspension. The assay was performed at 37°, with or without the addition of exogenous template [100 μg of DNase-activated calf thymus DNA, prepared as previously published (20)].

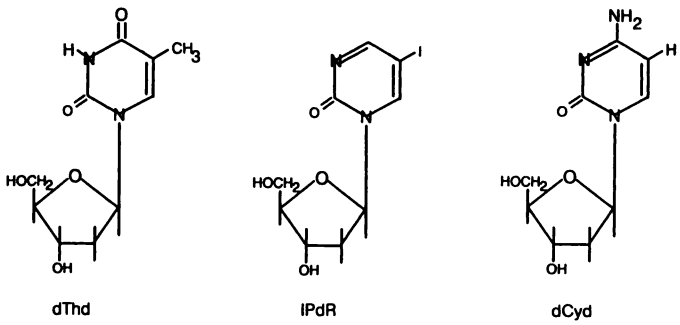

Fig. 1. Structure of IPdR, thymidine (*dThd*), and deoxycytidine (*dCyd*).

HPLC analysis of metabolites of IPdR in HSV-2-infected cells. HeLa S_3 cells infected with HSV-2 were incubated with IPdR for 9 hr, as detailed above. The cell extraction was performed, as previously described, with ice-cold 60% methanol (21). The 60% methanol-soluble fraction was analyzed by anion exchange HPLC with a Partisil 10 SAX column (Whatman, Inc., Clifton, NJ), using an LKB Pharmacia HPLC. The solvent system used was essentially as previously described (22), except that a step gradient of 20 mM potassium phosphate, pH 6.6, for 12 min, followed by 150 mM potassium phosphate, pH 6.6, for 60 min, was used. The flow rate was 1 ml/min for the initial 30 min and 2 ml/min for the remaining time. Because radiolabled IPdR was not available, an alternate method for the detection of IPdR and IPdR phosphorylated metabolites was used. This method is based on the detection of IPdR at 335 nm, a region of the absorption spectrum that is unique to IPdR in comparison with other nucleotides. Elution profiles were obtained at both a wavelength of 335 nm (current time) and a wavelength of 265 nm (memory), using a LC-235 diode array detector and a LCI-100 laboratory computing integrator (both from Perkin-Elmer, Norwalk, CT). The integration of the elution profile at 265 nm was used to estimate the cellular concentration of the ribonucleotides in control and IPdR-treated cells.

Determination of dNTP pool sizes. HeLa S_3 cells were infected with HSV-1 or HSV-2 and treated with IPdR (100 μM) for 9 hr. The cells (1×10^7) were washed twice with ice-cold phosphate-buffered saline and then extracted with 0.5 N perchloric acid for 5 min on ice, as previously described (23). The precipitated proteins were pelleted and the supernatant was removed and neutralized with KOH and potassium phosphate buffer (pH 7.5). The concentrations of the various dNTPs in the cellular extracts were determined by the DNA polymerase method previously published (24). The concentration of dTTP in the cellular extracts was determined as previously published (25). Briefly, the cellular extracts were incubated with 0.3 units of dUTPase for 60 min at 37° before the DNA polymerase reaction mixture was added.

Results

Effect of IPdR on HSV-2 DNA. To determine the target of antiviral activity of IPdR, the effect of the drug on HSV-2 DNA synthesis was examined. HSV-2-infected cells were incubated with 0, 30, 60, 100, and 200 μM IPdR for 9 hr at 37°. The amount of cellular HSV-2 DNA in each sample was determined by Southern blot analysis. The results are shown in Fig. 2. The amount of viral DNA present in HSV-2-infected cells treated with IPdR was greatly reduced, as indicated by the decrease in hybridization with the probe. The percentage of inhibition of cellular HSV-2 DNA synthesis in the treated cells, as compared with the no-drug control, was estimated by cutting out the area of hybridization and counting the radioactivity. By this method, it was estimated that IPdR could inhibit cellular viral DNA synthesis by approximately 50 and 99% at 20 and 100 μM, respectively. Fig. 2, *lower*, shows the correlation between the effect of IPdR on viral DNA synthesis and the effect of IPdR on HSV-2 virus yield. The correlation coefficient of 0.997 suggests that the inhibition of viral DNA synthesis can account for the overall inhibition of HSV-2 virus yield by IPdR. The action of IPdR against viral DNA synthesis over time was also examined. During the 9-hr postinfection period,

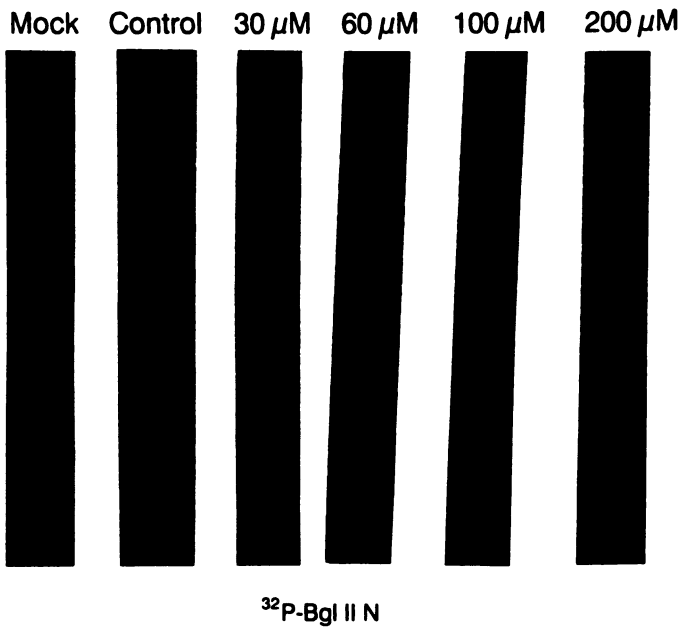

Fig. 2. Effect of various concentrations of IPdR on HSV-2 DNA synthesis. A, Relative amount of HSV-2 DNA in mock-infected, control, and IPdR-treated cells. HSV-2-infected cells were incubated with 30, 60, 100, or 200 μM IPdR for 9 hr, and autoradiography was done as described in detail in Materials and Methods. B, Correlation of the effect of IPdR on HSV-2 viral DNA synthesis with the effect of IPdR on HSV-2 virus yield. The effect of IPdR on HSV-2 DNA synthesis was determined by estimation of the relative radioactivity in the excised hybridization bands from the nitrocellulose paper, in a scintillation counter.

IPdR was present at 0 to 9 hr, 3 to 9 hr, and 6 to 9 hr. The amount of viral DNA present in these samples was compared with the amount of viral DNA in the control samples taken at 0, 3, 6, and 9 hr (Fig. 3). It appears that essentially no DNA synthesis took place until more than 3 hr after infection, because the amount of HSV-2 DNA at 3 hr in the control was approximately equal to that of the input DNA at the 0-hr control. It is evident from the increased intensity of the hybridization band that viral DNA synthesis was taking place between 3 and 6 hr after infection. In the IPdR-treated samples, the presence of IPdR before 6 hr after infection effectively inhibited any further synthesis of HSV-2 DNA. However, if IPdR was not added until 6 hr after infection, viral DNA synthesis did proceed but was significantly reduced.

Effect of IPdR on nuclear DNA synthesis. The IPdR-mediated inhibition of viral DNA synthesis was further examined in nuclei isolated from HSV-2-infected cells incubated

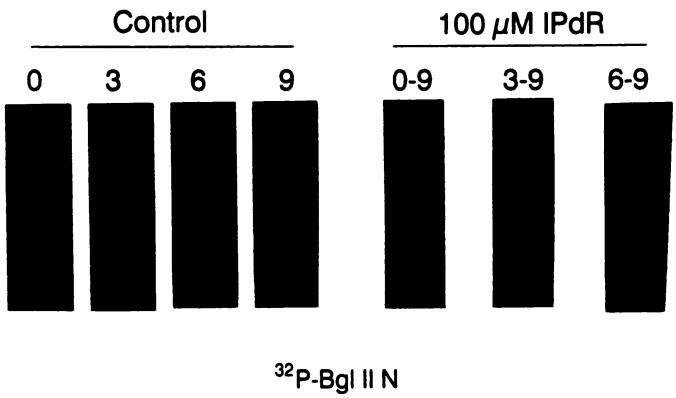

Fig. 3. Time course of effect of IPdR on HSV-2 DNA synthesis. IPdR (100 μM) was added to HSV-2-infected cells at 0, 3, or 6 hr after infection. The total incubation period was 9 hr. The relative amount of HSV-2 DNA in the controls and in the treated cells was visualized by autoradiography with a ^{32}P-labeled recombinant probe containing a portion of HSV-2 DNA, as described in detail in Materials and Methods.

with or without IPdR. The isolated nuclei served as the source of both the DNA polymerase and the DNA template, exogenous nucleotides were added, and DNA elongation was measured by the incorporation of [^{3}H]dTMP into the DNA. In one experiment, HSV-2-infected cells were incubated either with IPdR (32 and 85 μM) or with a known irreversible anti-HSV compound, IUdR (6 and 10 μM) or ACV (38 and 75 μM), for 12–16 hr (Fig. 4A). The concentrations of each compound were selected as concentrations that inhibited the HSV-2 virus yield by approximately 90 and 99% (data not shown). The nuclei were isolated and the capacity to synthesize DNA was measured. All nuclei isolated from the treated cells showed significantly reduced ability to support nuclear DNA synthesis. In a separate experiment, HSV-2-infected cells were treated with a known reversible anti-HSV compound, PFA (100 μM), for 12 to 16 hr and the nuclei were isolated. The nuclei were able to support nuclear DNA synthesis to the same extent as the control nuclei from HSV-2-infected cells. Most likely, the PFA was washed away during the nuclei isolation procedure and, therefore, was not present to interfere with the DNA synthesis reaction. Thus, it appeared that the action of IPdR was an irreversible action, because any remaining IPdR or metabolite of IPdR would also have been washed out during the nuclei isolation procedure. It seemed likely that the most probable sites of action were the viral DNA polymerase and the DNA template. To address this issue, exogenous DNA template (activated calf thymus DNA) was added back to the DNA synthesis reaction. The addition of exogenous template to the reaction resulted in an increase of DNA synthesis back to the level of control in nuclei isolated from IPdR-treated HSV-2-infected cells (Fig. 4B). This result may indicate that IPdR or possibly a metabolite of IPdR can damage the DNA template or disrupt the viral DNA replication complex.

Effect of IPdR on cellular dNTP pools. Because IPdR has the potential to interfere with deoxynucleotide metabolism, the effect of IPdR on cellular dNTP pool sizes was examined. The concentrations of all the dNTPs were measured in perchloric acid extracts from mock-, HSV-1-, or HSV-2-infected cells treated with 25, 50, and 100 μM IPdR (Table 1). At an extracellular concentration of 25 or 50 μM, IPdR had virtually no effect on the dNTP pools in HSV-1-infected cells and only

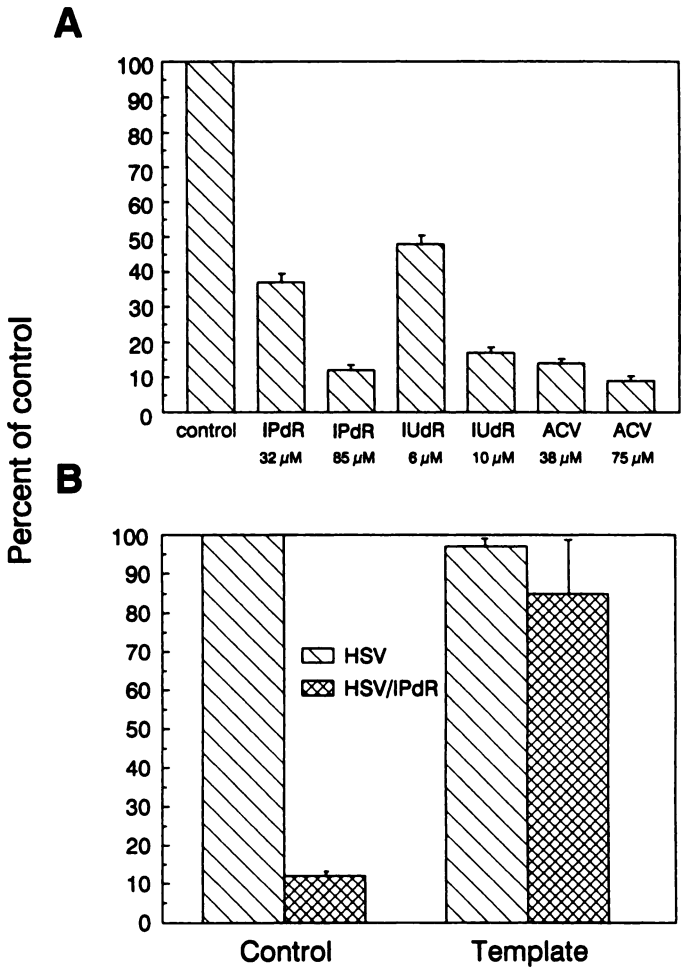

Fig. 4. Effect of antiviral compounds on HSV-2 nuclear DNA synthesis. A, HSV-2-infected cells were incubated with IPdR, IUdR, or ACV for 12 to 16 hr, and the nuclei were then isolated as described in Materials and Methods. The elongation of nuclear DNA was estimated by measurement of the incorporation of [^{3}H]dTMP into the DNA by a standard DNA polymerase assay and is expressed as a percentage of the no-drug control. B, HSV-2 cells were incubated in the absence or presence of 100 μM IPdR for 12 to 16 hr, and the nuclei were isolated. ▧ (*HSV*), nuclei isolated from HSV-2 infected cells; ■ (*HSV/IPdR*), nuclei isolated from IPdR-treated infected cells. The nuclear DNA assay was done in the presence or absence of 0.5 μg/ml exogenous activated calf thymus DNA. *Control*, DNA elongation in the absence of exogenous template; *Template*, DNA elongation in the presence of exogenous template.

TABLE 1

Effect of IPdR on dnTP pools

The experimental details can be found in Materials and Methods. All values were determined from three or more experiments (average ± standard deviation). Significant difference from control was determined by Student's *t* test.

Treatment	IPdR	Pools			
		dTTP	dCTP	dATP	dGTP
	μM	*pmol/10^6 cells*			
Mock	0	33 ± 10	19 ± 3	8 ± 1	33 ± 2
	100	26 ± 7	21 ± 5	7 ± 2	30 ± 7
HSV-1	0	89 ± 21	32 ± 11	5 ± 2	39 ± 5
	100	75 ± 14	68 ± 19[a]	15 ± 2	46 ± 9
HSV-2	0	110 ± 12	70 ± 6	11 ± 2	53 ± 8
	100	41 ± 6[b]	136 ± 15[b]	16 ± 2	59 ± 3

[a] $p < 0.025$.
[b] $p < 0.001$.

a small effect in HSV-2-infected cells (data not shown).[1] At an extracellular concentration of 100 μM IPdR, the dCTP pool was increased to the same extent in both HSV-1- and HSV-2-infected cells (approximately 50%). Although the dTTP pool size was virtually unchanged in HSV-1-infected cells treated with 100 μM IPdR, it decreased 50 to 60% in HSV-2-infected cells under the same condition. Furthermore, the ratio of dCTP to dTTP in HSV-2-infected cells increased significantly, from 0.64 to 3.3, when the infected cells were incubated with IPdR. Similarly, in HSV-1-infected cells the ratio of dCTP to dTTP increased from 0.36 to 0.91 when the infected cells were incubated with IPdR. IPdR did not affect dNTPs pools in mock-infected cells, even at 100 μM. Because the DNA polymerase method for measurement of dNTP pool size cannot discriminate between dTTP and dUTP, the possibility occurred that the relatively small effect of IPdR on the dTTP pool size was due to an increase in the dUTP pool size. To investigate this hypothesis, *E. coli* dUTPase was used to digest the dUTP in the perchloric acid extracts before the DNA polymerase assay was done. The results indicated that the dTTP pool size remained unchanged after dUTPase digestion (data not shown).[1] The effect of IPdR on the ribonucleoside triphosphate pools was also examined, and it was determined that the ribonucleoside triphosphate pools in mock-, HSV-1-, or HSV-2-infected cells were not affected by IPdR at 100 μM (data not shown).[1]

Metabolism of IPdR in HSV-infected cells. The antiviral activity of IPdR was previously shown to be dependent on the HSV-2 thymidine kinase (1), and IPdR was also shown to be a substrate for the viral thymidine kinase (2). To determine the major phosphorylated metabolites of IPdR, HSV-2-infected HeLa S_3 cells were incubated with IPdR for 9 hr. Although several attempts have been made to radiolabel IPdR, the radiolabeled form of IPdR was not available for the metabolism studies; therefore, an alternate method of detection of the phosphorylated metabolites was used. The method of detection is based on the unique absorption spectrum of IPdR and is detailed in Materials and Methods. By anion exchange HPLC (335 nm), a major 60% methanol-soluble metabolite (Fig. 5, peak 1) was identified at an elution time of approximately 8 min. This peak was identified as IPdR-MP by two criteria; 1) it eluted in a position corresponding to the elution position of chemically synthesized IPdR-MP and 2) its absorption spectrum was identical to that of IPdR and IPdR-MP (data not shown). The amount of IPdR-MP formed in the HSV-2-infected cells was dependent on the extracellular concentration of IPdR. The accumulation of IPdR-MP occurred in a dose-dependent manner, accumulating to 18, 25, or 160 μM intracellularly when the extracellular concentration was 25, 50, or 100 μM, respectively. The intracellular concentrations of IPdR-MP were estimated by use of a standard integration curve of known concentrations of IPdR. In HSV-2-infected cells treated with 100 μM IPdR, IPdR-MP represents approximately 80% of the total integrated area of the HPLC profile. No other phosphorylated metabolites of IPdR in treated HSV-2-infected cells, such as the di- or triphosphate derivatives, were detected. The detection of the metabolites is based on the absorption properties of IPdR at 335 nm, and the sensitivity of the detection method is approximately 5 μM. In mock-infected cells, only a small amount of an unidentified substance was formed; it did

[1] G. A. Lewandowski and Y.-C. Cheng, unpublished observation.

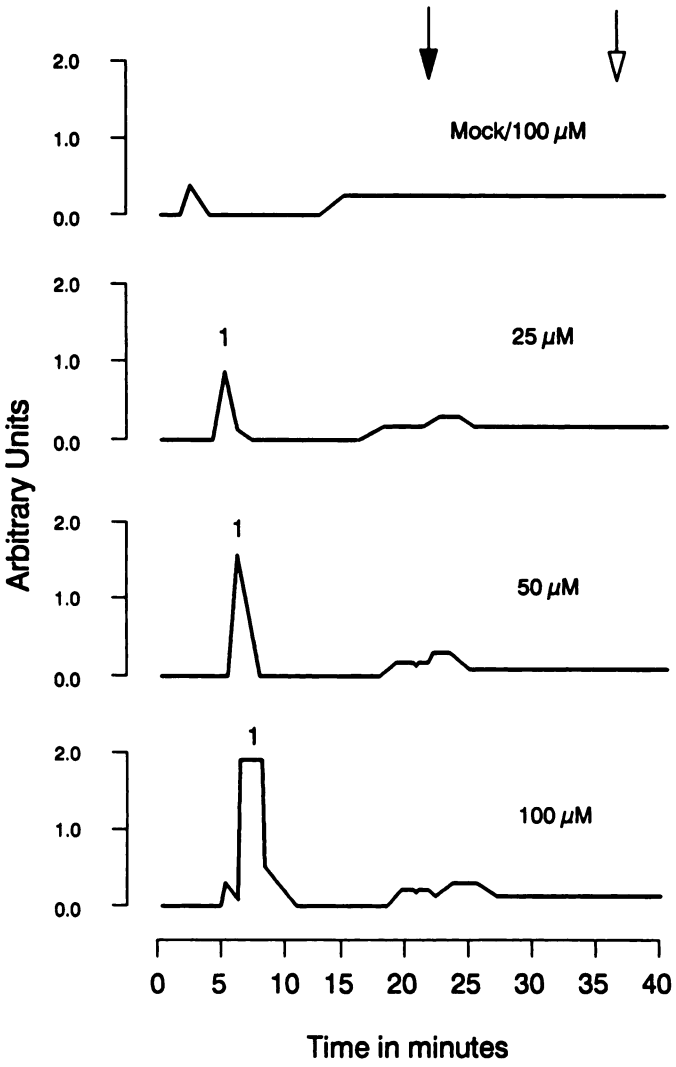

Fig. 5. Cellular metabolism of IPdR in HSV-2-infected cells. HSV-2-infected cells were incubated with various concentrations of IPdR for 9 hr after infection. The cellular metabolites were extracted with 60% methanol. HPLC analysis of the methanol-soluble extracts was done on a Partisil SAX column, as detailed in Materials and Methods. The elution profiles at 335 nm are shown. Peak 1 represents IPdR-MP, as determined by coelution with an IPdR-MP standard. *Solid arrow* and *open arrow*, elution positions of ADP and ATP, respectively.

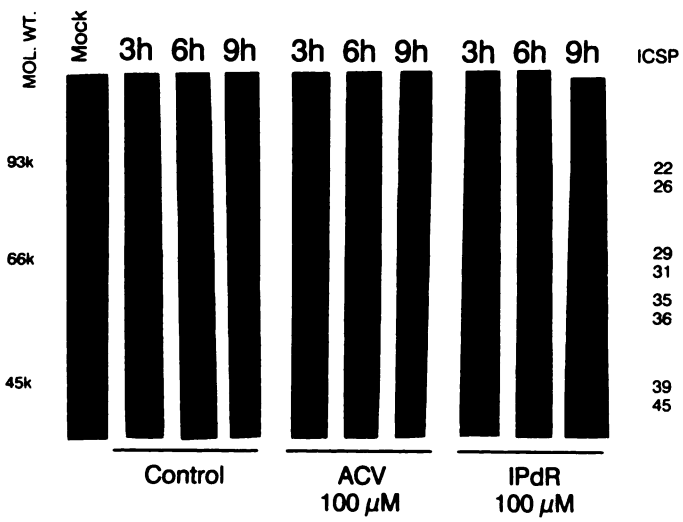

Fig. 6. Effect of IPdR on HSV-2 protein synthesis. HSV-2-infected cells were incubated with or without either ACV (100 μM) or IPdR (100 μM) for 9 hr. Samples were removed at 3, 6, and 9 hr after infection, and the cellular proteins were extracted as described in Materials and Methods. Protein samples of 10 μg each, along with molecular weight markers, were loaded on an 8.5% SDS-polyacrylamide gel, and the proteins were separated by electrophoresis. The proteins were transferred to nitrocellulose paper and HSV-2-specified proteins were visualized by immunological staining. Infected cell-specific protein (*ICSP*) numbers were assigned according to relative electrophoretic mobilities of protein bands reported in previous studies (39, 40).

TABLE 2

Effect of IPdR on HSV-2 enzyme induction

HSV-2-infected cells were incubated with 100 μM IPdR or 100 μM ACV for 9 hr. The cells were extracted and the relative activities of viral DNA polymerase and DNase were determined as described in Materials and Methods. Mock represents the activity of the viral enzymes measured in the mock-infected cells and HSV-2 represents the activity of the viral enzymes measured in the HSV-2-infected cells under each condition. All values were determined in triplicate within each experiment (average ± standard deviation).

	DNase activity		Polymerase activity	
	Mock	HSV-2	Mock	HSV-2
	units/mg		*units/mg* × 10^{-3}	
Control	0	54 ± 4	0.016	2.58 ± 0.20
ACV	0	22 ± 3	0.022	1.61 ± 0.12
IPdR	0	20 ± 3	0.17	1.07 ± 0.10

not have an absorption spectrum similar to that of IPdR-MP or IPdR and it eluted at approximately 4 min. It is not likely, given these dissimilarities, that this substance is IPdR-MP. It may possibly be thiouracil, a RNA degradation product that has an absorption spectrum similar to that of the unidentified substance and an absorption maximum at approximately 335 nm.

Effect of IPdR on HSV-2 protein and enzyme induction. Total cellular proteins were extracted from the HSV-2- or mock-infected cells, incubated with or without IPdR, at various times after infection and were subjected to electrophoresis in an 8.5% SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose paper for immunostaining with the DAKO anti-HSV-2 antibody (Fig. 6). There was no apparent inhibition of the synthesis of HSV-2 proteins in the IPdR-treated cells at any of the time points.

In addition, the induction of viral enzymes, important to viral DNA replication, in the presence of IPdR was investigated (Table 2). The effect of IPdR on the induction of HSV-2 DNA polymerase and DNase was examined, in comparison with the effect of ACV. Both IPdR and ACV could inhibit the induction of the viral polymerase, by approximately 50 and 40%, respectively. The induction of HSV-2 DNase could be inhibited equally by IPdR and ACV, by approximately 60%.

Viral RNA synthesis in IPdR-treated cells. Total cellular RNA was isolated from HSV-2-infected cells incubated with or without IPdR for various periods of time after infection. Northern blot analysis was done with the recombinant DNA probe containing the *Bgl*II N fragment of HSV-2 DNA. This fragment of the HSV-2 genome completely encodes four β group virus gene products (including 38-kDa, 61-kDa, and 56-kDa proteins and a 1.5-kilobase mRNA) (26–30). Four major mRNA species were detected in the control (Fig. 7). There was no significant inhibition of the mRNA species by 100 μM IPdR at any time after HSV-2 infection (9-hr data not shown).

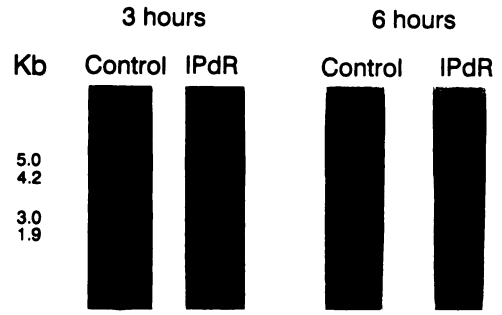

Fig. 7. Effect of IPdR on HSV-2 mRNA synthesis. The preparation of the RNA sample, the RNA agarose gel, electrophoresis, and hybridization are described in the text. HSV-2-infected cells were incubated with 100 μM IPdR for 3, 6, and 9 hr. The results at 3 and 6 hr are shown. Four major mRNA species were detected. The RNA was analyzed as described in the text.

Discussion

For more than 20 years, researchers have been synthesizing compounds with action against HSV. Several of the compounds found to be active have been deoxynucleoside analogues, and the mechanisms of action virtually always involve the inhibition of viral DNA synthesis by the triphosphorylated metabolite (3, 4). However, these compounds share similar toxicities, which arise from low levels of incorporation into host DNA over time. More recently, many groups have been using the rational approach to anti-HSV agent development. The pyrimidinone nucleoside analogues, including IPdR, are an example of a group of rationally developed compounds.

IPdR has been previously demonstrated to exhibit potent anti-HSV-2 activity both in cell culture and *in vivo* (1, 2). The data in this paper indicate that the antiviral action of IPdR is targeted at viral DNA synthesis. The time course of the effect of IPdR suggests that IPdR or a metabolite of IPdR can inhibit HSV DNA synthesis by interfering with both early events, such as initiation of viral DNA synthesis, and late events, such as viral DNA elongation. Most importantly, the dose-dependent inhibition of viral DNA synthesis by IPdR can be correlated with the inhibition of virus yield by IPdR (1), with a correlation coefficient of 0.997. Thus, the action of IPdR against HSV-2 can be accounted for by inhibition of viral DNA synthesis.

The inhibition of HSV-2 DNA synthesis by IPdR was probably not due to a depletion of deoxynucleotides, as determined by measurement of the pool size of the dNTPs, which indicated a level sufficient for DNA synthesis. dTTP alone decreased slightly, but not enough to inhibit DNA synthesis by 99%. IPdR did, however, have an impact on the cellular balance of dCTP and dTTP in HSV-infected cells. This imbalance of nucleoside triphosphates may in turn contribute to the inhibition of viral DNA synthesis. Also, IPdR-MP (150 μM) has been found to inhibit thymidylate synthase activity by approximately 50% when the dUMP concentration is 13.7 μM.[1] This inhibition of thymidylate synthase activity may contribute to the decrease in the dTTP pools and the overall imbalance in the nucleoside triphosphate pools in HSV-2-infected cells.

Likewise, inhibition of HSV-2 DNA synthesis is probably not due to the inhibition of synthesis of viral proteins required for HSV DNA synthesis. Although the presence of IPdR apparently did inhibit the induction of HSV-2-specified DNase and DNA polymerase to some extent, it is likely that the degree of inhibition is not enough to account for the impact of IPdR on viral DNA synthesis. Furthermore, there was no evidence of significant inhibition of protein synthesis in the protein blot. Similarly, IPdR did not significantly alter the synthesis of mRNA species.

The major target of the antiviral action of IPdR or a metabolite of IPdR appears to be the DNA synthesis apparatus, as demonstrated by the significantly reduced ability of nuclei from IPdR-treated HSV-2-infected cells to support nuclear DNA synthesis. During the course of the assay, the nuclei were extensively washed, so that it is unlikely that any drug remained during the actual DNA elongation. All the antiviral compounds tested in this assay, except PFA, exhibited irreversible damage to the nuclei and, as a result, inhibited nuclear DNA synthesis. This is consistent with previous reports on the irreversible antiviral action of IPdR (1) and IUdR (31) and the irreversible inhibition by ACV of DNA synthesis, by termination of DNA elongation (32). Thus, the inability of the nuclei from the IPdR-treated cells to support nuclear DNA synthesis is due to irreversible damage to the template or DNA replication complex. It is unlikely that the target of IPdR is the viral DNA polymerase, because when exogenous template was added DNA synthesis occurred at the level of control.

An attempt was made to determine the active metabolite of IPdR through which the antiviral action is mediated. The major cellular metabolite of IPdR in HSV-2-infected cells is the monophosphate, although, due to the unavailability of the radiolabeled compound and the sensitivity of the current detection method, it is not certain whether the di- and triphosphorylated metabolites are formed. An effort was made to synthesize IPdR-TP chemically, by the modified method of Yoshikawa *et al.* (33), as published by Ruth and Cheng (34). IPdR-MP was successfully synthesized; however, further phosphorylation of the monophosphate proved impossible, because the reaction mixture yielded a gummy product. A second approach to synthesizing the triphosphate, using an enzymatic procedure (22), was then tried. The enzymatic method involved incubating IPdR or IPdR-MP with purified HSV-1 thymidine kinase and human erythrocyte lysate, in the thymidine kinase reaction mixture containing 2 mM ATP instead of thymidine, for 24 hr. The metabolites of IPdR were detected by anion exchange HPLC with a Partisil SAX column. The only IPdR metabolite detected was IPdR-MP. Furthermore, there were no apparent metabolites of IUdR formed.

Even though IPdR-MP was the major metabolite formed, it is not certain that this is the active metabolite. IPdR-MP, at concentrations up to 200 μM, could not inhibit nuclear DNA synthesis when added to the isolated nuclei reaction.[1] Moreover, IPdR-MP, at up to 120 μM, could not significantly inhibit thymidylate kinase activity (when the dTMP concentration was 12.7 μM). The HSV-2 DNA polymerase activity could not be inhibited by 200 μM IPdR.[1] Additionally, a reconstitution study was done to determine whether the soluble fraction of the cytosol contained an active metabolite that could interfere with viral DNA synthesis. Various volumes of the methanol-soluble cytosol (60% methanol), from either IPdR-treated HSV-2-infected cells or from control HSV-2-infected cells, were added to the nuclear DNA synthesis reaction mixtures containing nuclei from HSV-2-infected cells. The addition of the cytosol to the nuclear DNA synthesis reaction did not have any inhibitory effect. Therefore, if the mechanism of action is exerted through IPdR-MP, it is uncertain at this time how this

occurs, although it is possible that IPdR-MP could cause the dissociation or disruption of the DNA synthesis complex during the initiation of DNA synthesis. Other alternative mechanisms also exist. The similarity of the action of IPdR in the nuclear DNA synthesis assay to that of ACV and IUdR, two antiviral compounds that are metabolized to the triphosphate level (35–38), naturally leads one to suspect the possible formation of IPdR-TP. IPdR-TP could inhibit DNA synthesis by incorporation into the DNA or through inhibition of enzymes required for viral DNA synthesis. The interference with DNA synthesis by a triphosphate would most likely involve the HSV viral DNA polymerase; however, as previously shown (1), several HSV-1 variants with altered DNA polymerases are sensitive to IPdR. Additionally, if the triphosphate is the active metabolite (and is chemically stable), one would expect that the reconstitution with the cytosol extract, containing IPdR metabolites, would inhibit nuclear DNA synthesis in the control nuclei, but this was not the case. Thus, several possibilities still exist for the mechanism of antiviral action of IPdR, whether mediated through the monophosphate or through the triphosphate. These possibilities will be investigated when the radiolabled form of IPdR or IPdR-TP becomes available.

References

1. Lewandowski, G. A., S. P. Grill, M. H. Fisher, G. E. Dutschman, S. M. Efange, T. J. Bardos, and Y.-C. Cheng. Anti-herpes simplex virus activity of 5-substituted 2-pyrimidinone nucleosides. *Antimicrob. Agents Chemother.* **33**:340–344 (1989).
2. Efange, S. M. N., E. M. Alessi, H. C. Shih, Y.-C. Cheng, and T. J. Bardos. Synthesis and biological activities of 2-pyrimidinone nucleosides. 2. 5-Halo-2-pyrimidinone 2′-deoxyribonucleosides. *J. Med. Chem.* **28**:904–910 (1985).
3. Frank, K. B., J.-F. Chiou, and Y.-C. Cheng. Interaction of DNA polymerase and nucleotide analog triphosphates. *Adv. Enzyme Regul.* **24**:377–384 (1986).
4. Smee, D. F., J. C. Martin, J. P. H. Verheyden, and T. R. Matthews. Anti-herpes activity of the acyclic nucleoside 9-(1,3-dihydroxy-2-propoxymethyl)guanine. *Antimicrob. Agents Chemother.* **23**:676–682 (1983).
5. Williams, M. V. Effects of mercury (II) compounds on the activity of dUTPases from various sources. *Mol. Pharmacol.* **29**:288–292 (1985).
6. Williams, M. V., and J. D. Pollack. Purification and characterization of a dUTPase from *Acholeplasma laidlawii* B-PG9. *J. Bacteriol.* **159**:278–282 (1984).
7. Kit, S., D. R. Dubbs, and P. M. Frearson. HeLa cells resistant to bromodeoxyuridine and deficient in thymidine kinase activity. *Int. J. Cancer* **1**:19–30 (1966).
8. Cheng, Y.-C., S. Grill, J. Ruth, and D. Bergstrom. Anti-herpes simplex virus and anti-human cell growth activity of *E*-5-propenyl-2′-deoxyuridine and the concept of selective protection in antivirus chemotherapy. *Antimicrob. Agents Chemother.* **18**:957–961 (1980).
9. Nutter, L., S. Grill, J.-S. Li, R.-T. Tan, and Y.-C. Cheng. Induction of virus enzymes by phorbol esters and *n*-butyrate in Epstein-Barr genome carrying Raji cells. *Cancer Res.* **47**:4407–4412 (1987).
10. Galloway, D. A., J. A. Nelson, and J. K. McDougall. Transformation of rodent cells by herpes simplex virus type 2 and cytomegalovirus, in *Acquired Immune Deficiency Syndrome*. Alan R. Liss, Inc., New York, 157–173 (1984).
11. Laemmli, U. K., and M. Favre. Maturation of the head of bacteriophage T_4. 1. DNA packaging events. *J. Mol. Biol.* **80**:575–599 (1971).
12. Towbin, H., T. Staehelin, and J. Gordon. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350–4354 (1979).
13. Domin, B. A., S. P. Grill, and Y.-C. Cheng. Establishment of dihydrofolate reductase-increased human cell lines and relationship between dihydrofolate reductase levels and gene copy. *Cancer Res.* **43**:2155–2158 (1983).
14. Cheng, Y.-C., S. P. Grill, G. E. Dutschman, K. B. Frank, J.-F. Chiou, K. F. Bastow, and K. Nakayama. Effects of 9-(1,3-dihydroxy-2-propoxymethyl)guanine, a new antiherpesvirus compound, on synthesis of macromolecules in herpes simplex virus-infected cells. *Antimicrob. Agents Chemother.* **26**:283–288 (1984).
15. Hoffmann, P. J., and Y.-C. Cheng. The deoxyribonuclease induced after infection of KB cells by herpes simplex virus type 1 or type 2. I. Purification and characterization of the enzyme. *J. Biol. Chem.* **253**:3557–3562 (1978).
16. Ostrander, M., and Y.-C. Cheng. Properties of herpes simplex virus type 1 and type 2 DNA polymerase. *Biochim. Biophys. Acta* **609**:232–245 (1980).
17. Chomczynski, P., and N. Sacchi. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**:156–159 (1987).
18. Maniatis, T., E. F. Fritsch, and J. Sambrook. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 326–327 (1982).
19. Cheng, Y.-C., S. P. Grill, D. Derse, J.-Y. Chen, S. J. Caradonna, and K. Connor. Mode of action of phosphonoformate as an anti-herpes simplex virus agent. *Biochim. Biophys. Acta* **652**:90–98 (1981).
20. Baril, E., J. Mitchner, L. Lee, and B. Baril. Action of pancreatic DNase: requirements for activation of DNA as a template-primer for DNA polymerase α. *Nucleic Acids Res.* **4**:2641–2653 (1977).
21. Townsend, A., J.-M. Leclerc, G. Dutschman, D. Cooney, and Y.-C. Cheng. Metabolism of 1-β-D-arabinofuranosyl-5-azacytosine and incorporation into DNA of human T-lymphoblastic cells (Molt-4). *Cancer Res.* **45**:3522–3528 (1985).
22. Cheng, Y.-C., S. P. Grill, G. E. Dutschman, K. Nakayama, and K. F. Bastow. Metabolism of 9-(1,3-dihydroxy-2-propoxymethyl)guanine, a new anti-herpes virus compound, in herpes simplex virus-infected cells. *J. Biol. Chem.* **258**:12460–12464 (1983).
23. Cheng, Y.-C., K. Nakayama, and S. P. Grill. Roles of thymidylate synthetase activity in herpes simplex virus-infected HeLa cells. *Biochem. Pharmacol.* **32**:1407–1410 (1983).
24. Solter, A. W., and R. E. Handschumacher. A rapid quantitative determination of deoxyribonucleoside triphosphates based on the enzymatic synthesis of DNA. *Biochim. Biophys. Acta* **174**:585–590 (1969).
25. Williams, M. V., C.-H. Chang, and Y.-C. Cheng. An enzymatic method for distinguishing deoxyuridine and deoxythymidine nucleotide pools and its application for determining ribonucleotide reductase activity. *J. Biomed. Biophys. Methods* **1**:153–162 (1979).
26. Docherty, J. J., J. H. Subak-Sharpe, and C. M. Preston. Identification of a virus-specific polypeptide associated with a transforming fragment (*Bgl* II-N) of herpes simplex virus type 2 DNA. *J. Virol.* **40**:126–132 (1981).
27. Galloway, D. A., L. C. Goldstein, and J. B. Lewis. Identification of proteins encoded by a fragment of herpes simplex virus type 2 DNA that has transforming activity. *J. Virol.* **42**:530–537 (1982).
28. McLauchlan, J., and J. B. Clements. DNA sequence homology between two colinear loci on the HSV genome which have different transforming abilities. *EMBO J.* **2**:1953–1961 (1983).
29. Jenkins, F. J., and M. K. Howett. Characterization of mRNAs that map in the *Bgl* II N fragment of the herpes simplex virus type 2 genome. *J. Virol.* **52**:99–107 (1984).
30. Suh, M., C. Chauvin, M. Filion, G. C. Shore, and E. Frost. Localization of the coding region for a 35,000 dalton polypeptide on the genome of herpes simplex virus type 2. *J. Gen. Virol.* **64**:2079–2085 (1983).
31. Roizman, B., L. Aurelian, and P. R. J. R. Roane. The multiplication of herpes simplex virus. I. The programming of viral DNA replication in HEp-2 cells. *Virology* **21**:482–498 (1963).
32. Furman, P. A., M. H. St. Clair, J. A. Fyfe, J. L. Rideout, P. M. Keller, and G. B. Elion. Inhibition of herpes simplex virus-induced DNA polymerase activity and viral DNA replication by 9-(2-hydroxyethoxymethyl)guanine and its triphosphate. *J. Virol.* **32**:72–77 (1979).
33. Yoshikawa, M., T. Kato, and T. Takenishi. Phosphorylation. III. Selective phosphorylation of unprotected nucleoside. *Bull. Chem. Soc. Jpn.* **42**:3505–3508 (1969).
34. Ruth, J. L., and Y.-C. Cheng. Nucleoside analogues with clinical potential in antivirus chemotherapy: the effects of several thymidine and 2′-deoxycytidine analogue 5′-triphosphates on purified human (α,β) and herpes simplex virus (types 1, 2) DNA polymerases. *Mol. Pharmacol.* **20**:415–422 (1981).
35. Prusoff, W. H., and B. Goz. Potential mechanisms of action of antiviral agents. *Fed. Proc.* **32**:1679–1687 (1973).
36. Eidinoff, M. L., L. Cheong, E. G. Gurpide, R. S. Benua, and R. R. Ellison. Incorporation of 5-iodouracil labelled with iodine-131 into the deoxyribonucleic acid of human leukaemic leucocytes following *in vivo* administration of 5-iododeoxyuridine labelled with iodine-131. *Nature (Lond.)* **183**:1686–1687 (1959).
37. Elion, G. B., P. A. Furman, J. A. Fyfe, P. De Miranda, L. Beauchamp, and H. J. Schaeffer. Selectivity of action of an antiherpetic agent, 9-(2-hydroxyethoxymethyl)guanine. *Proc. Natl. Acad. Sci. USA* **74**:5716–5720 (1977).
38. Miller, W. H., and R. L. Miller. Phosphorylation of acyclovir diphosphate by cellular enzymes. *Biochem. Pharmacol.* **31**:3879–3884 (1982).
39. Powell, K. L., and R. J. Courtney. Polypeptides synthesized in herpes simplex virus type 2-infected HEp-2 cells. *Virology* **66**:217–228 (1975).
40. Honess, R. W., and B. Roizman. Proteins specified by herpes simplex virus. XI. Identification and relative molar rates of synthesis of structural and nonstructural herpes virus polypeptides in the infected cell. *J. Virol.* **12**:1347–1365 (1973).

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