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A COMPARISON OF THE ANTIVIRAL AGENTS 2'-NOR-2'-DEOXYGUANOSINE AND ACYCLOVIR: UPTAKE AND PHOSPHORYLATION IN TISSUE CULTURE AND KINETICS OF IN VITRO INHIBITION OF VIRAL AND CELLULAR DNA POLYMERASES BY THEIR RESPECTIVE TRIPHOSPHATES

John Germershausen*, Richard Bostedor*, A. Kirk Field#, Helen Perry#, Richard Liou*, Herbert Bull*, R. L. Tolman* and John D. Karkas*

Merck Sharp & Dohme Research Laboratories
Rahway, New Jersey 07065* and West Point, Pennsylvania 19486#

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A comparative study was conducted between the antiherpetic agents 2'-nor-2'-deoxyguanosine (2'NDG) and acyclovir (ACV) with respect to 1) the relative rates of uptake and phosphorylation to the "active" triphosphate species in tissue culture and 2) the in vitro inhibition of viral and cellular DNA polymerases by their respective triphosphates. The results indicated that a) six hours after HSV1 infection of primary rabbit kidney cells there was seven times more 2'NDG-triphosphate in the cells than ACV triphosphate; b) the relative rate of triphosphate formation in HSV1-infected versus uninfected cells was 4.5 times higher for 2'NDG than for ACV and c) the triphosphate of 2'NDG (2'NDG-TP) was a more selective inhibitor of the viral compared to the cellular DNA α-polymerase than the triphosphate of ACV (ACV-TP). The K_m/K_i ratios for 2'NDG-TP and ACV-TP (in the competitive inhibition of dGTP) were 3.10 and 1.37, respectively, for the highly purified HSV1 polymerase; and 0.05 and 1.11, respectively, for the partially-purified HeLa α-polymerase. Neither triphosphate inhibited the HeLa DNA β-polymerase to any significant extent. These results are in line with the findings [Ashton et al. (1982), Biochem. Biophys. Res. Commun. 108, 1716-1721] that 2'NDG has superior in vivo antiherpetic activity compared to ACV without apparent toxicity.

In two previous communications (1,2) it was reported that the antiherpetic agent 2'NDG (2'-nor-2'-deoxyguanosine; 9-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl]-guanine) exhibited superior in vivo antiherpetic activity to Acyclovir (ACV). Depending on the mode of infection and treatment, 2'-NDG was 6 to 60 times more efficacious than ACV in treating HSV infections in mice. Kinetic in vitro studies with purified enzymes indicated that 2'NDG was a superior substrate for the HSV1-induced thymidine kinase (Vmax/Km 30 times higher for 2'NDG than for ACV) and that the monophosphate of 2'NDG was an even more efficient substrate for GMP kinase (Vmax/Km for 2'NDG-MP

Abbreviations: 2'NDG, 2'nor-2'-deoxyguanosine; 9-[(2-hydroxy-1-(hydroxymethyl)-ethoxy)methyl]guanine. ACV, acyclovir; 9-[2-hydroxy-ethoxymethyl]guanine. 2'NDG-MP, 2'NDG-DP, 2'NDG-TP; ACV-MP, ACV-DP, ACV-TP, the mono-, di- and triphosphates of 2'NDG and ACV, respectively. HSV1, Herpes Simplex Virus 1. TK, thymidine kinase. MOI, multiplicity of infection.

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492 times higher than that for ACV-MP) (1,2). The superior anti-herpetic activity of 2'NDG was attributed to its faster rate of phosphorylation to the "active" triphosphate, which inhibits DNA polymerases. In this study, we compare the uptake and phosphorylation of 2'NDG and ACV in tissue cultures of primary rabbit kidney cells infected with HSV1 and examine the kinetics of inhibition by the respective triphosphates of purified viral and cellular DNA polymerases.

MATERIALS AND METHODS

Bovine serum albumin (crystalline, BSA) was from Miles Laboratories. Salmon testes DNA, phosphocreatine, creatine kinase, sodium pyruvate, glyceraldehyde phosphate, dithiothreitol, dATP, dGTP, dCTP, dTTP and ATP were from Sigma. Glyceraldehyde phosphate dehydrogenase, 3-phosphoglycerate kinase and GMP kinase (hog brain) were from Boeringer/Mannheim. Lactate dehydrogenase was from Worthington. NAD^+ was from Calbiochem. [Methyl-^{3}H]dTTP and Aquasol 2 were from New England Nuclear. [8-^{14}C]guanine was from Amersham. HSV1 (Strain Schooler) was obtained from the Merck Sharp & Dohme stock collection (West Point, Pennsylvania). For enzyme purification, HeLa cells, either uninfected or infected with HSV1 (Strain Patton) at a MOI of 10 and harvested 8 hrs later, were purchased from Bethesda Research Labs. Minimal Essential Medium-Eagle's Salts (EMEM), Fetal Calf Serum (FCS) and 0.25% Trypsin in Hank's salts were from Gibco.

Cell Culture Infection: Primary rabbit kidney (PRK) cells were prepared from 2-3 weeks old New Zealand white rabbits by standard procedures and maintained as monolayers in 75 cm^2 flasks (approx. 10^7 cells/flask) with EMEM + 2% FCS. The medium was removed and the cells infected at a MOI of 10 with HSV1 (Schooler) in a volume of 2.0 ml. After 1 hr at 37°, 5 ml of fresh growth medium was added containing 84 µg of either [8-^{14}C]2'NDG (4.8×10^7 total dpm) or [8-^{14}C]ACV (6.2×10^7 total dpm). This corresponds to 11.5 cpm/pmole of 2'NDG and 14.7 cpm/pmole of ACV at 80% counting efficiency. The incubation was continued for an additional 6 hrs, the medium was removed and the cells washed three times with 10 ml of phosphate-buffered saline (pH 7.0). The cells were treated with 2 ml of 0.25% Trypsin for 10 min at 37° and 5 ml of EMEM containing 10% FCS was added. The cells were transferred to tubes, centrifuged (3,000g, 10 min) and washed with 2 x 5 ml of the same medium. The cell pellets were frozen and stored at -70°.

Nucleotide Extraction and HPLC Analysis: The cell pellets (approx. 10^7 cells) were thawed and extracted with 0.5 ml of 1.5N perchloric acid for 30 min at 0° with occasional mixing. The extract was centrifuged for 5 min at 3,000g and the supernatant neutralized with 165 µl of 2.5N KOH + 10 µl 1M K_2HPO_4. The mixture was again centrifuged for 5 min at 3,000g and the supernatant was analyzed by HPLC; 200 ul of each neutralized extract were chromatographed on a 4 x 300 mm anion-exchange column (Micropak AX-10, Varian) at a flow rate of 3 ml/min. A linear gradient of 0.01-1.0M KH_2PO_4 was applied in the first 7 minutes, the 1M solvent was maintained for an additional 5 minutes and returned to 0.01M 2 min thereafter. The 1M KH_2PO_4 was purified through a Chelex column (3) to reduce background absorbance. The fractions (0.5 min, 1.5 ml) were assayed for radioactivity in 10 ml Aquasol-2. Corrections for quenching at high salt concentrations were made. The retention times of purified acyclic nucleotide standards were used for radioactivity peak identification.

Enzyme preparation: HSV1 thymidine kinase was isolated by affinity chromatography following essentially the procedure of Cheng and Ostrander (4) with minor modifications. The active fractions were concentrated as described by Fyfe et al. (5) and kept in liquid nitrogen.

The viral and cellular DNA polymerases were isolated by DEAE and phosphocellulose chromatography following essentially the methods of Weissbach et al. (6). The HSV1 polymerase was further purified by DNA-cellulose chromatography according to Ostrander and Cheng (7). In all cases the fractionation procedure was adapted to

high pressure liquid chromatography using preparative stainless steel columns and a Varian 5000 analytical HPLC system. The details of this technique, which permitted the simultaneous isolation of several enzymes within 9 hrs will be published elsewhere. The specific activities of the DNA cellulose-purified HSV1 polymerase and the phosphocellulose purified HeLa α-polymerase were 2,000 and 100 units/mg, respectively.

Nucleosides and Nucleotides: 2'NDG was synthesized in the Merck Sharp and Dohme Research Laboratories (MSDRL) by published procedures (1,2). ACV was also synthesized in MSDRL according to Shaeffer (8). The ^{14}C labeled 2'NDG and ACV were prepared by the same methods by Dr. Holly Mertel of MSDRL using [8-^{14}C]guanine as the starting material. The radiochemical purity of the two compounds, determined by reverse phase HPLC, was 99.6% and 99.0%, respectively. The monophosphates of 2'NDG and ACV were synthesized enzymatically with viral TK and purified by HPLC as previously described (1,2). For the synthesis of the diphosphates, the same procedure was followed except that GMP kinase (0.04 mg/ml) was added to the incubation mixtures. For the synthesis of the triphosphates, the purified nucleoside diphosphates (10 mg) were incubated in a mixture containing 50 mM Tris-acetate buffer (pH 8.6), 3 mM $MgCl_2$, 1 mM EDTA, 30 mM $KHPO_4$ (pH 7.6), 5 mM Na-pyruvate, 30 mM glyceraldehyde phosphate, 300 μg lactate dehydrogenase, 300 μg glyceraldehyde phosphate dehydrogenase, 300 μg 3-phosphoglycerate kinase, and 15 mM NAD^+ in a final volume of 10 ml. The progress of the reaction was monitored by HPLC. The mixture was incubated for 4 hrs at 37° and 20 hrs at 30°. The triphosphates were purified by preparative HPLC on an anion-exchange column (Micropak AX-10, Varian) and desalted by HPLC chromatography on DEAE-cellulose with 1.0M triethylammonium carbonate (pH 8.6) as the eluting solvent. The triphosphates, 2'NDG-TP and ACV-TP were >96% pure as determined by analytical HPLC.

Enzyme assays: HSV1 DNA polymerase was assayed in a reaction mixture (0.1 ml) containing 50 mM Tris-Cl (pH 8.0), 5 mM $MgCl_2$, 1.2 mg/ml BSA, 1 mM dithiothreitol, 0.14M $(NH_4)_2SO_4$, 0.38 mg/ml salmon testes DNA (activated as in reference #6) 10 μM each dCTP, dATP, ^3H dTTP (50 μCi/ml) and varying concentrations of dGTP. Identical kinetic constants were obtained when 100 μM each of the 3 dXTPs were used. The reaction was started with enzyme and stopped with 1 ml 10% TCA. TCA-insoluble material was collected on glass fiber filters (Whatman GF/C), washed three times with 5 ml 5% TCA, and once with 5 ml ethanol. The filters were dried and counted in Aquasol-2. HeLa α-polymerase was assayed similarly but with 100 μM each of the three dXTPs and without $(NH_4)_2SO_4$. The HeLa β-polymerase reaction mixture was similar to the α-polymerase mixture, but contained 0.05M KCl in place of $(NH_4)_2SO_4$ and the pH of the Tris buffer was 8.5. In all cases the assays were linear with respect to enzyme concentration and time. All enzymes were assayed at 37° for 40 min. One unit of enzyme catalyzes of incorporation of 1 nmole of dTMP/hr. The amounts of enzyme used in each assay were 0.1 units of highly purified (DNA cellulose) HSV1 DNA polymerase, 0.17 units of partially purified (phosphocellulose) HSV1 DNA polymerase, 0.18 units of partially purified (phosphocellulose) HeLa DNA α-polymerase and 0.1 units of partially-purified HeLa DNA β-polymerase.

RESULTS

Figure 1 shows the HPLC absorbance and radioactivity profiles of perchloric acid extracts of HSV1-infected cells exposed to [^{14}C]2'NDG (Fig. 1A) or [^{14}C]ACV (Fig. 1B). The data from Fig. 1 as well as those from mock-infected cell cultures are summarized in Table 1. Both 2'NDG and ACV were phosphorylated to the respective triphosphates much more efficiently in infected cells than in uninfected cells. The level of 2'NDG-TP in infected cells was 7 times higher than that of ACV-TP six hours post-infection. Additionally, the relative rate of phosphorylation in infected versus

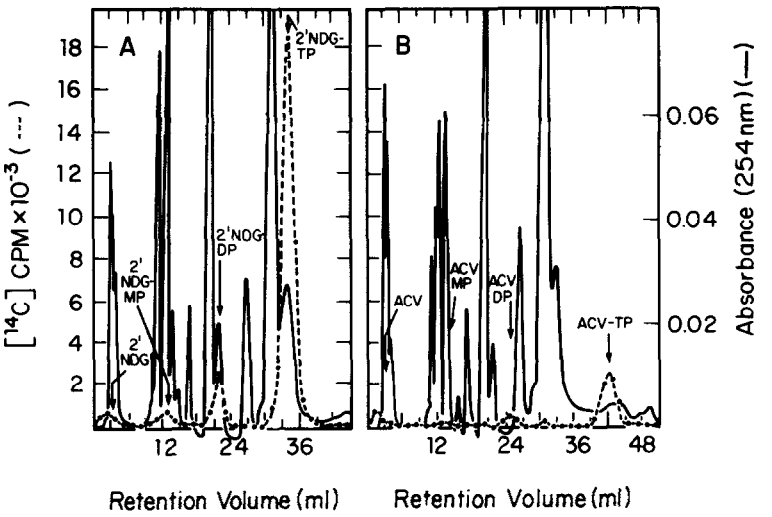

Figure 1. HPLC of Perchloric Acid Extracts of Infected Cells: HSV1 infected PRK cells, treated with [^{14}C]2'NDG (A) or [^{14}C]ACV (B) were harvested 6 hr. post-infection, extracted with perchloric acid and the extract was analyzed by anion exchange HPLC as described in Methods. Fractions (1.5 ml) were assayed for radioactivity (●---●). The positions of authentic acyclic nucleoside and nucleotide standards are indicated by arrows.

uninfected cells was 4.5 times higher for 2'NDG-TP than for ACV-TP (infected/uninfected triphosphate levels = 54.2 and 12.3, respectively).

Enzymatically synthesized, pure triphosphates of 2'NDG and ACV were tested for inhibitory activity against both the highly purified (DNA-cellulose step) and partially purified (phosphocellulose step) HSV1 DNA polymerase and also against the partially purified HeLa α-polymerase. The double reciprocal (Lineweaver Burke) plots are shown in Figure 2. In all cases, the kinetic data, analyzed by a non-linear regression program

Table 1

Summary of Nucleoside Phosphorylation Data

Antiviral	HSV1 Infection	X*	XMP*	XDP*	XTP*	XTP Inf. / XTP Uninf.
		(nmoles/10^7 cells/6 hrs)				
2'NDG	-	0.58	0.10	0.11	0.19	54.2
2'NDG	+	0.34	0.21	1.16	10.29	
ACV	-	0.07	0.07	0.14	0.12	12.3
ACV	+	0.28	0.07	0.20	1.47	

* X = 2'NDG or ACV; XMP, XDP, XTP = mono, di or triphosphates, respectively, of 2'NDG or ACV.

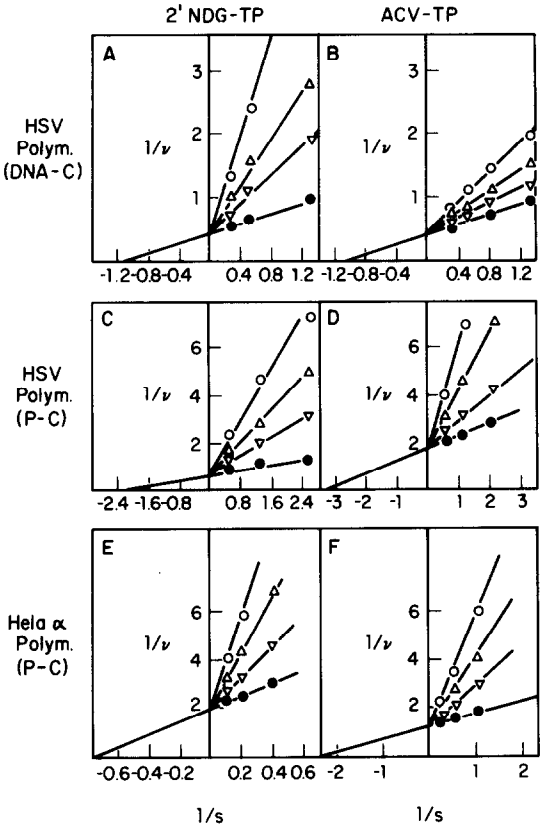

Figure 2. Lineweaver-Burke Plots of the Inhibition of Viral and Cellular DNA Polymerases by 2'NDG-TP and ACV-TP. Inhibition of the highly purified (DNA cellulose) HSV1 DNA polymerase by 2'NDG-TP (A); no inhibitor (●), 0.8 μM (▽), 2 μM (△), 4 μM (○) and by ACV-TP (B); no inhibitor (●), 0.4 μM (▽), 1 μM (△), 2 μM (○). Inhibition of the partially-purified (phosphocellulose) HSV1 DNA polymerase by 2'NDG-TP (C); no inhibitor (●), 0.8 μM (▽), 2 μM (△), 4 μM (○) and by ACV-TP (D); no inhibitor (●), 0.2 μM (▽), 0.5 μM (△), 1 μM (○). Inhibition of the partially-purified (phosphocellulose) HeLa DNA α-polymerase by 2'NDG-TP (E); no inhibitor (●), 50 μM (▽), 100 μM (△), 200 μM (○) and by ACV-TP (F); no inhibitor (○), 2 μM (▽), 5 μM (△), 10 μM (○). 1/v, p moles [^{3}H]dTMP incorporated/min^{-1}; 1/s, μM dGTP^{-1}. Reaction conditions were as described in Methods.

(SAS; ref. 9) fit best a competitive mode of inhibition with respect to dGTP, although a mixed model could not be entirely excluded. The kinetic parameters computed from the data in Fig. 2 are shown in Table 2, along with the data computed for the inhibition of the partially purified HeLa β-polymerase by 2'NDG-TP. For comparison, the kinetic constants reported by other investigators for the inhibition by ACV-TP of HSV1 and HeLa DNA polymerases are also included in Table 2. 2'NDG-TP has somewhat more inhibitory activity than ACV-TP against the highly purified HSV1 polymerase (K_m/K_i ratios 3.10 vs. 1.37, respectively) but somewhat less inhibitory activity against the

Table 2
Kinetic Constants

Inhibitor	Enzyme	Purity	K_m (μM dGTP)	K_i (μM Inh.)	K_m/K_i	Ref.
2'NDG-TP	HSV1 (Patton)	DNA-cell.	0.96 ± .35	0.31 ± .05	3.10	*
ACV-TP	HSV1 (Patton)	DNA-cell.	0.96 ± .35	0.73 ± .31	1.37	*
2'NDG-TP	HSV1 (Patton)	Phos.-cell.	0.45 ± 0.15	0.29 ± 0.09	1.55	*
ACV-TP	HSV1 (Patton)	Phos.-cell.	0.28 ± 0.19	0.07 ± 0.04	4.00	*
ACV-TP	HSV1 (H29)	Phos.-cell.	0.38 ± 0.13	0.08 ± 0.03	4.75	(10)
ACV-TP	HSV1 (KOS)	Phos.-cell.	0.97 ± 0.43	0.55 ± 0.29	1.76	(10)
ACV-TP	HSV1 (C1)	Phos.-cell.	0.63 ± 0.07	0.41 ± 0.05	1.54	(10)
ACV-TP	HSV1 (Mac 1)	Phos.-cell.	1.15 ± 0.09	1.42 ± 0.47	0.81	(10)
ACV-TP	HSV1 (HF)	Phos.-cell.	0.15	0.03	5.00	(11)
2'NDG-TP	HeLa α (TC52)	Phos.-cell.	1.12 ± 0.50	23.8 ± 12.5	0.05	*
ACV-TP	HeLa α (TC52)	Phos.-cell.	0.42 ± 0.15	0.38 ± 0.12	1.11	*
ACV-TP	HeLa α (53)	Phos.-cell.	1.08 ± 0.01	2.32 ± 0.97	0.47	(10)
2'NDG-TP	HeLa β (TC52)	Phos.-cell.	0.33 ± 0.15	338 ± 235	0.001	*
ACV-TP	HeLa β (53)	Phos.-cell.		>50		(10)

* This communication

partially purified enzyme (K_m/K_i ratios 1.55 vs. 4.0, respectively). On the other hand, 2'NDG-TP is considerably less inhibitory to the cellular α-polymerase than ACV-TP (K_m/K_i ratios of 0.05 and 1.11, respectively). Thus, 2'NDG-TP appears to be a more selective inhibitor of the viral as compared to the cellular enzyme. Neither compound inhibits the cellular β-polymerase to any significant extent (Table 2).

It should be mentioned here that the same laboratory that reported most of the K_m and K_i values (other than ours) included in Table 2 (ref. 10), later published another study (12) with considerably lower K_m for dGTP and K_i for ACV-TP with a highly purified HSV1 DNA polymerase. Since the results are not in agreement with those obtained in this work with an enzyme purified in a similar manner (DNA cellulose step), we must assume that the discrepancy is due to the difference in assay conditions; for example, in that study (12) the labeled precursor was [^{3}H]dGTP and not [^{3}H]dTTP as in all other studies.

DISCUSSION

The acyclic nucleosides, ACV and 2'NDG, show potent anti-herpetic activity both in tissue culture and in various animal models (1,2). The relative lack of cellular toxicity (1,2) appears to be due to the selective activation of the nucleoside prodrugs via phosphorylation by the virus-induced thymidine kinase (1,2). Cellular kinases then

convert the acyclic nucleoside monophosphates to the triphosphates which are potent inhibitors of the HSV-induced DNA polymerase (1,2,10,11). Since the triphosphates also inhibit the cellular DNA α-polymerase, and hence could prove cytotoxic to uninfected cells, it was important to examine the steady state levels of the inhibitors in uninfected as well as infected cells and also the relative potencies of the inhibitors against the viral and cellular polymerases. In a previous study (1) it was shown that 2'NDG was phosphorylated much more efficiently than ACV in vitro. Here we demonstrate that the in vitro results correlate with results obtained in tissue culture. In cell cultures, harvested 6 hrs post-infection, the HSV1 thymidine kinase was being expressed (17-fold increase over uninfected control; data not shown). Under these conditions, the level of 2'NDG-TP in the infected cells was 7 times higher than that reached by ACV-TP (Table 1). Additionally, the ratio of triphosphate levels in infected versus uninfected cells was higher for 2'NDG-TP than for ACV-TP (Table 1).

The kinetic advantage of 2'NDG over ACV in the phosphorylation step seems to be accompanied by a selective advantage in the inhibition of DNA polymerases. Comparison of the K_m/K_i ratios of the two triphosphates (Table 2) against viral and cellular DNA α-polymerase (for competitive inhibition with respect to dGTP), demonstrates that ACV-TP and 2'NDG-TP have comparable inhibitory activity against the HSV1 polymerase but ACV-TP is a more potent inhibitor of the HeLa α-polymerase by a factor of 22 (1.1/0.05). The results of the present study, taken together with those of the two previous communications on 2'NDG from these laboratories, indicate that 2'NDG is phosphorylated more efficiently than ACV not only in vitro but also in tissue culture and is more selective than ACV in its action against viral vs. cellular DNA polymerase. These biochemcial findings are in agreement with the in vivo results in animal models which indicate that 2'NDG is a more potent antiviral agent than ACV (1,2).

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