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Comparison of the Modes of Antiviral Action of 2′-Nor-deoxyguanosine and Its Cyclic Phosphate, 2′-Nor-cyclic GMP

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The metabolisms of 9-(1,3-dihydroxy-2-propoxymethyl)guanine (2′NDG) and its cyclic phosphate, 9-[(2-hydroxy-1,3,2-dioxophosphorinan-5-yl)oxymethyl]guanine P-oxide (2′-nor-cGMP), were compared in cultures of primary rabbit kidney cells infected with herpes simplex virus type 1 (HSV-1). 2′-Nor-cGMP was taken up by the cells essentially intact, after which it was opened to the acyclic monophosphate and phosphorylated further, ultimately to the triphosphate. Formation of the triphosphate was independent of HSV thymidine kinase expression, unlike what is observed with 2′NDG. In addition, there was a direct correlation between the antiviral activity of 2′NDG and the level of triphosphate formed in HSV-1-infected cells, whereas such a correlation was absent with 2′-nor-cGMP. In vivo experiments indicated that only a small percentage of free 2′NDG was formed in the bloodstream of mice after oral administration of 2′-nor-cGMP. Incubation of 2′-nor-cGMP with crude extracts of HSV-1-infected or uninfected HeLa cells resulted in the direct production of 2′NDG triphosphate. The possibility that the triphosphate of 2′NDG produced from 2′-nor-cGMP was the enantiomer of the triphosphate made from 2′NDG by viral and cellular kinases was investigated and disproved. Taken together, these data indicate that (i) 2′-nor-cGMP does not act simply as a prodrug of 2′NDG, (ii) 2′-nor-cGMP does not require viral thymidine kinase for its activity, and (iii) 2′-nor-cGMP may have an additional, triphosphate-independent mode of action.

The antiviral agent 9-[(2-hydroxy-1,3,2-dioxophosphorinan-5-yl)oxymethyl]guanine P-oxide (2′-nor-cGMP), the cyclic phosphate of 9-(1,3-dihydroxy-2-propoxymethyl) guanine (2′NDG) (12), has potent, broad-spectrum activity against a number of DNA viruses (14). In contrast to the acyclonucleoside antiviral compounds acyclovir (10), 2′NDG (5, 7, 12), and 9-[(2,3-dihydroxy-1-propoxy) methyl]guanine (iNDG) (1), 2′-nor-cGMP exhibits potent activity not only against the herpes viruses but also against vaccinia virus, simian virus 40, adenovirus, and papillomavirus (14).

The activities of acyclovir, 2′NDG, and iNDG against herpes simplex virus type 1 (HSV-1) and HSV-2 have been shown to depend on the presence of viral thymidine kinase (TK) in the infected cell; the monophosphates produced by its action are further phosphorylated by cellular kinases to the respective triphosphates, which are potent and selective inhibitors of the viral DNA polymerase (2, 4–6, 7, 11, 13; J. D. Karkas, W. T. Ashton, L. F. Canning, R. Liou, J. Germershausen, R. Bostedor, B. Arison, A. K. Field, and R. L. Tolman, J. Med. Chem., in press). The greater in vivo potency of 2′NDG compared with that of acyclovir correlates well with its much more rapid conversion to the triphosphate by the viral and cellular kinases (2, 5, 6).

The antiviral activity of 2′-nor-cGMP, however, is not dependent upon activation by viral TK (A. K. Field, M. E. Davies, C. M. DeWitt, H. C. Perry, T. L. Schofield, J. D. Karkas, J. Germershausen, A. F. Wagner, C. L. Cantone, M. MacCoss, and R. L. Tolman, Antiviral Res., in press). Experiments comparing the activities of 2′NDG and 2′-nor-cGMP against TK-deficient HSV-1 in two strains of 3T3 cells, one lacking TK activity entirely (TK^-) and the other expressing only the HSV-1 TK, indicated that while the effective antiviral concentration of 2′NDG is dramatically increased (100-fold) in the absence of TK, that of 2′-nor-cGMP is essentially independent of TK expression (14). Similarly, the cytotoxicity of 2′NDG was increased in cells expressing viral TK, whereas the cytotoxicity of 2′-nor-cGMP was not affected (9).

We report here the results of studies on the mode of action of 2′-nor-cGMP. The uptake and phosphorylation of 2′-nor-cGMP in cell cultures was compared with that of 2′NDG by two approaches, which permitted a comparison of the levels of 2′NDG triphosphate (2′NDG-TP) produced by the two compounds with the respective antiviral effect. A number of in vitro experiments are also reported which supplement the findings of the in vivo studies. Additionally, the biotransformation of the two compounds is compared in mouse blood after oral administration.

MATERIALS AND METHODS

Phosphocreatine, creatine kinase, ATP, dATP, dTTP, dCTP, and dGTP were obtained from Sigma Chemical Co.; [8-^{14}C]guanine and [methyl-^{3}H]TTP were obtained from Amersham Corp. [γ-^{32}P]ATP was supplied by New England Nuclear Corp.; *N,N′*-dicyclo-hexylcarbodiimide was purchased from Aldrich Chemical Co., Inc.; and GMP kinase (hog brain) was purchased from Boehringer Mannheim Biochemicals.

Preparation of [8-^{14}C/^{32}P]2′-nor-cGMP. [8-^{14}C]2′NDG, prepared as described previously (6), was phosphorylated by HSV TK with [γ-^{32}P]ATP as follows. A 5.2-ml reaction mixture containing 50 mM Tris chloride (pH 7.5); 5 mM $MgCl_2$; 0.5 mg of bovine serum albumin per ml; 2 mM

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dithiothreitol; 2.5 mM NaF; 17 μCi of [8-^{14}C]2′NDG (0.53 mM); 183 μCi of [γ-^{32}P]ATP (1.93 mM); and 97 U of HSV TK (purified as described before [3]) was incubated for 16 h at 30°C. High-pressure liquid chromatography (HPLC) (6) of the mixture indicated that approximately 90% of the 2′NDG was converted to the 2′NDG monophosphate (2′NDG-MP). The [8-^{14}C/^{32}P]2′NDG-MP was purified on a preparative anion-exchange column (Micropack AX-10; Varian Associates) and desalted by DEAE-cellulose chromatography with triethylammonium carbonate as the eluting volatile solvent (6). The desalted material (0.7 mg) was dissolved in 4.0 ml of 50% aqueous pyridine, and 50 mg of *N,N′*-dicyclohexylcarbodiimide was added. The reaction was allowed to proceed at room temperature with daily addition of 50 mg of *N,N′*-dicyclohexylcarbodiimide for 4 days. The progress of the reaction was monitored by HPLC analysis on an analytical AX-10 column eluted at 3 ml/min with 50 mM KH_2PO_4. After 4 days, when the reaction was 75% complete, the mixture was extracted four times with 20 ml of diethyl ether, and the water-soluble product was purified by preparative AX-10 chromatography followed by DEAE-cellulose desalting as before. The product, [8-^{14}C/^{32}P]2′-nor-cGMP, was judged to be 98.6% radiochemically pure by HPLC analysis (AX-10). [8-^{14}C]2′-nor-cGMP was prepared in a similar fashion, except that 5 mM unlabeled ATP was used as the phosphate donor. Unlabeled 2′-nor-cGMP was prepared as described previously (14).

Cell culture infection. Primary rabbit kidney cells were infected with HSV-1, treated with the labeled compounds, collected, and washed as described previously (16). The strains of HSV-1 used were Schooler (5) and HSV-NDG$_{R1}$ (14).

Nucleotide extraction and HPLC analysis. The cell pellets (approximately 10^7 cells) were extracted with 0.5 ml of 1.5 N perchloric acid (PCA) and analyzed by HPLC with an AX-10 column as described previously (6). The specific radioactivities of the labeled compounds are indicated below (see Tables 1 and 2). Because some of the values observed were very low (little more than twice the background counts), duplicate and in many cases triplicate HPLC analyses were performed, and the results were averaged. In only one case (Table 1, 5.8 ± 3.1) was the standard error of the mean more than 50%.

To detect incorporation of labeled compounds into nucleic acids, the PCA-insoluble pellets were treated with proteinase K and extracted with phenol, and the nucleic acids were precipitated twice with ethanol. Total radioactivity as well as alkali-labile and alkali-stable radioactivities were determined.

Determination of plasma levels of 2′-nor-cGMP. Male and female CD-1 mice (20 to 23 g) were fasted overnight and then were given 9-mg/kg doses of either [8-^{14}C]2′NDG or [8-^{14}C]2′-nor-cGMP by oral gavage. Specific activities of [8-^{14}C]2′NDG and [8-^{14}C]2′-nor-cGMP were 100 and 36 cpm/pmol, respectively. The mice were decapitated, and blood was collected into EDTA-treated breakers 1 h after drug administration. Plasma was obtained by centrifugation of the whole blood at 4,000 × *g* for 5 min at room temperature. PCA extraction and HPLC analysis were identical to those mentioned above for the primary rabbit kidney cell pellets.

Inhibition of partially purified HSV-1 DNA polymerase. HSV-1 DNA polymerase, purified according to the method of Weissbach et al. (15) up to the phosphocellulose step from infected HeLa cells, was used for the inhibition studies. The reaction conditions were identical to those described previously (6). (*S*)-2′NDG-MP, -diphosphate (DP), and -TP (analogs of 5′-GMP) and (*R*)-2′NDG-MP, -DP, and -TP (analogs of 3′-GMP) were prepared as described by Karkas et al. (J. D. Karkas, R. Liou, J. Germershausen, and A. F. Wagner, submitted for publication).

Metabolism of 2′-nor-cGMP in HSV-1-infected HeLa cell extracts. HeLa cells were infected with HSV-1 (Schooler) at a multiplicity of infection of 1 at 40°C for 1 h, shifted to 37°C, and harvested 16 h after infection. The crude extract was prepared by suspending 200 mg of cells in 0.7 ml of phosphate-buffered saline (137 mM NaCl, 8.1 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 2.6 mM KCl), sonicating (5 × 10 s at setting 5; Ultrasonics Inc.), and centrifuging the sonicate for 30 min at 100,000 × *g*; the supernatant was used for the in vitro ring opening of 2′-nor-cGMP. The composition of the incubation mixtures is given in the legend to Fig. 1.

Determination of chirality of 2′NDG-MP obtained by enzymatic hydrolysis of 2′-nor-cGMP. A mouse liver extract was prepared by homogenizing a fresh CD-1 mouse liver (1.3 g) with 2 ml of phosphate-buffered saline in a Teflon (E. I. du Pont de Nemours & Co., Inc.) homogenizer; the homogenate was sonicated (5 × 10 s at setting 5; Ultrasonics) and centrifuged for 40 min at 100,000 × *g*. A total of 1 ml of the supernatant was incubated at 37°C with 1 ml of a solution of 2′-nor-cGMP (150 A_{260} U/ml) in phosphate-buffered saline. The progress of the reaction was monitored by analytical HPLC (AX-10). After 65 min at 37°C, when about 70% of the cyclic phosphate had been converted to the acyclic derivative which is the main product under these conditions (absence of ATP), the acyclic monophosphate was isolated free of the cyclic by preparative HPLC on AX-10.

The product (4 A_{260} U/ml) was used directly, without desalting, in the GMP kinase reaction for the determination of chirality, which was based on a comparison of the rate of the reaction to the rates of purified (*R*)- and (*S*)-2′NDG-MPs. The assay couples the GMP kinase reaction through the pyruvate kinase-lactic dehydrogenase system to the oxidation of NADH, which is spectrophotometrically determined (8). The preparation of the monophosphates is described elsewhere (Karkas et al., submitted).

RESULTS

The uptake and metabolism of 2′-nor-cGMP and 2′NDG in cell cultures was examined in two ways. In experiment 1, doubly labeled [8-^{14}C/^{32}P]2′-nor-cGMP was used to investigate primarily the possibility of dephosphorylation to 2′NDG; in experiment 2, singly labeled 2′NDG and 2′-nor-cGMP were used, but the dose was varied to compare triphosphate production by the two compounds and to correlate it with their respective antiviral activities.

Uptake and metabolism of doubly labeled 2′-nor-cGMP. The doubly labeled [^{14}C/^{32}P]2′-nor-cGMP, prepared as described in the Materials and Methods section, was incubated with primary rabbit kidney cells either mock infected or infected with HSV-1 (Schooler) at a multiplicity of infection of 10. The cells were harvested at 1.5 and 6.0 h after infection, as preliminary experiments had indicated that at 6 h, HSV-1 TK expression is near its maximum. The TK levels at 1.5 and 6.0 h were 0.3 and 2.4 U/10^7 cells, respectively. As 2′-nor-cGMP could potentially open to the acyclic monophosphate and subsequently could be dephosphorylated to the nucleoside 2′NDG either before or after entry into the cells, the intracellular ratio of ^{32}P to ^{14}C was examined after PCA extraction of the cells. This ratio, in the total extract, was approximately 1, regardless of time of incubation or virus infection, indicating that 2′-nor-cGMP

TABLE 1. Uptake of 2'NDG and 2'-nor-cGMP into primary rabbit kidney cells and distribution of metabolites[a]

Drug (dose μg/ml)	Infection (HSV-1)	Time after infection (h)	^{14}C in extract (pmol/10^7 cells)	$^{14}C/^{32}P$ ratio (total extract)	Distribution of metabolites (pmol/10^7 cells):				
					2'NDG	2'-Nor-cGMP	2'NDG-MP	2'NDG-DP	2'NDG-TP
2'-Nor-cGMP (24)	+	1.5	31	1.1	1.0	7.1	8.0	4.3	10.9
	−	1.5	30	1.1	2.3	6.5	5.8	2.4	7.8
	+	6.0	122	1.0	7.0	16.6	21.6	21.0	70.1
	−	6.0	62	0.9	12.6	19.6	14.4	8.6	10.3
2'NDG (12)	+	1.5	550		29.0		40.5	81.0	418.5
	−	1.5	37		25.8		1.0	0.5	0.4
	+	6.0	7,066		60.8		1,339.5	1,324.5	4,500.0
	−	6.0	40		29.7		3.2	1.9	1.9

[a] Cell culture conditions and HPLC analyses are described in the text. The specific radioactivity of 2'NDG was 11.5 cpm/pmol, and that of 2'-nor-cGMP was 8.6 cpm/pmol.

was transported into the cells as the intact cyclic phosphate (Table 1).

[8-^{14}C]2'NDG was also included for comparison in this experiment. It is interesting to note the great difference in total uptake between 2'NDG and 2'-nor-cGMP in the infected cells. With 2'-nor-cGMP, a small increase over that of the uninfected cells was seen only after 6 h, whereas with 2'NDG a 15-fold increase was observed after 1.5 h, and a 177-fold increase was observed after 6 h (Table 1). A similar observation with 2'NDG has been reported previously (6).

The metabolites of 2'-nor-cGMP and 2'NDG, determined by anion-exchange HPLC of the PCA extracts, are also included in Table 1. After entry into the cell, most of 2'-nor-cGMP was opened to the acyclic monophosphate, and a large portion (81 to 96%) of this monophosphate was further phosphorylated to the diphosphate and triphosphate derivatives, while only a small portion (4 to 19%) was dephosphorylated to the acyclonucleoside, 2'NDG (see also in vitro experiments below). The $^{32}P/^{14}C$ ratio was approximately 1 for 2'-nor-cGMP and its acyclic mono- and diphosphate derivatives at both time points, regardless of virus infection. However, the ratio in the triphosphate was 0.4 in both infected and uninfected cells and at both time points.

The degree of opening of the cyclic phosphate ring and further phosphorylation or dephosphorylation was somewhat dependent upon virus infection and incubation time. In uninfected cells, in the absence of HSV-1 TK, the amount of 2'NDG-TP formed from 2'-nor-cGMP in 1.5 h (7.8 pmol) was nearly as much as that formed in 6 h (10.3 pmol). In HSV-1-infected cells, the amount of 2'NDG-TP formed was 6.4 times higher at 6 h than at 1.5 h (70.1 versus 10.9 pmol), a slightly higher ratio than that expected merely from the fourfold increase in incubation time. Comparing infected to uninfected cells, the amount of 2'NDG-TP produced was similar at 1.5 h and 6.8 times higher at 6 h in the infected cells (70.1 versus 10.3 pmol).

In contrast, a dramatic dependence of the conversion of 2'NDG to 2'NDG-TP on HSV-1 TK expression is observed. In infected cells, the amount of 2'NDG-TP formed from 2'NDG was 1,000-fold higher than that in mock-infected cells after 1.5 h (418.5 versus 0.4 pmol) and more than 2,000-fold higher after 6 h (4,500 versus 1.9 pmol). The effect of the HSV-1 TK is further illustrated by the comparison of the sum of all phosphorylated derivatives formed at 1.5 and 6 h. This sum was 540 pmol at 1.5 h and 7,164 pmol at 6 h, which represents a 13.3-fold increase in 4.5 h during which TK activity increased eightfold.

The dependence of 2'NDG but not of 2'-nor-cGMP on HSV-1 TK expression for its biotransformation is reflected in the amount of 2'NDG-TP produced from each of the two compounds. 2'-nor-cGMP produced 70.1 pmol of 2'NDG-TP in the infected cells in 6 h, and 2'NDG produced 4,500 pmol (Table 1). This 64-fold difference does not correlate with the 10-fold difference in the effective antiviral concentrations of the two compounds (see 50% effective doses [ED_{50}s], Table 2). This result suggested that the production of 2'NDG-TP might not account for the antiviral activity of 2'-nor-cGMP. A more direct comparison of the metabolism of 2'-nor-cGMP and 2'NDG and its correlation with their respective antiviral potencies was possible in the experiment described below, in which the two drugs were present at various concentrations.

Comparison of 2'-nor-cGMP and 2'NDG at various dose levels. The metabolism of the two antivirals was again compared in another experiment in which the concentration of each was varied and the cells were harvested 6 h after infection, at the peak of HSV-1 TK expression. This experiment provided a direct comparison of the antiviral activity of each compound with its metabolic fate. (Table 2). In addition, the metabolism of the two compounds was compared in cells infected with a mutant virus, HSV-NDG_{R1}, selected for resistance to 2'NDG, which lacked detectable HSV-1 TK activity (14). This mutant virus was 42-fold less sensitive to 2'NDG than was HSV-1 (Schooler), but retained sensitivity to 2'-nor-cGMP (Table 2).

The metabolism of 2'-nor-cGMP was again found to be relatively independent from HSV-1 TK expression. When the compound was present at 36 μg/ml (three times its ED_{50} for HSV-1), the total radiolabel in the PCA extract was similar in cells infected with HSV-1 or with the TK^- virus HSV-NDG_{R1} (Table 2). The amounts of individual metabolites were also similar in HSV-1 and mutant virus-infected cells (Table 2). The amount of 2'NDG-TP produced from 2'-nor-cGMP in HSV-1-infected cells was 5.5 times higher than that in uninfected cells, similar to the 6.8-fold increase seen after 6 h in the experiment shown in Table 1. Moreover, when the concentration of the compound in the medium was increased to 120 μg/ml (10 times the ED_{50} for HSV-1), the difference in the levels of 2'NDG-TP between infected and uninfected cells was only 3.2-fold.

The metabolism of 2'NDG treated in the same manner with either HSV-1 or the TK^- HSV-NDG_{R1} virus is also shown in Table 2. Unlike 2'-nor-cGMP, the uptake of 2'NDG was reduced considerably in the HSV-NDG_{R1}-infected cells. There was a concomitant 33-fold reduction in

TABLE 2. Effect of drug level on uptake and metabolism of 2'-nor-cGMP and 2'NDG in primary rabbit kidney cells infected with HSV-1 or TK⁻ HSV-1[a]

Drug level (μg/ml)	Virus	ED_{50}[b] (μg/ml)	Distribution of metabolites (pmol/10^7 cells per 6 h):					
			Total ^{14}C in extract	2'NDG	2'-Nor-cGMP	2'NDG-MP	2'NDG-DP	2'NDG-TP
2'-Nor-cGMP								
12	WT[c]	12	103	6	30	8	26	33
36	WT	12	241	32	94	26	45	44
36	TK⁻[d]	24	255	34	101	24	65	31
36			143	44	60	14	17	8
120	WT	12	951	113	416	128	204	90
120			572	176	246	61	61	28
2'NDG								
1.2	WT	1.2	515	7		78	89	341
3.6	WT	1.2	1,309	13		161	210	925
12	WT	1.2	5,274	80		902	901	3,391
12	TK⁻	50	800	492		99	105	104
12			283	248		9	15	11
36	TK⁻	50	1,907	1,192		234	234	247
36			875	805		32	28	10

[a] Cell culture conditions and HPLC analyses are described in the text. The specific radioactivity of 2'-nor-cGMP was 60, 23, and 7 cpm/pmol for 12, 36, and 120 μg/ml, respectively. For 2'NDG the values were 126, 114, 93, and 99 cpm/pmol for 1.2, 3.6, 12, and 36 μg/ml, respectively.
[b] ED_{50}s were determined as the concentrations of drugs that gave 100% inhibition of viral cytopathic effect in one-half of quadruplicate treated cultures.
[c] WT, HSV-1 Schooler.
[d] HSV-NDG_{R1}.

the amount of 2'NDG-TP formed in cells infected with this TK⁻ virus compared to cells infected with HSV-1.

The level of 2'NDG-TP produced from 2'NDG correlated well with its antiviral activity. In the cells infected with TK⁻ virus, 104 pmol of 2'NDG-TP were produced at 12 μg of 2'NDG per ml compared with 247 pmol at 36 μg/ml. To estimate the amount of 2'NDG that would produce 341 pmol of triphosphate (the level reached in the HSV-1-infected cells at the ED_{50} of 2'NDG), one can extrapolate from the two values obtained (104 pmol from 12 μg/ml, and 247 pmol from 36 μg/ml) and arrive at a value of approximately 50 μg/ml. This is exactly the observed ED_{50} of 2'NDG against the TK⁻ virus (Table 2).

In the case of 2'-nor-cGMP, however, the level of 2'NDG-TP produced in the HSV-1-infected cells when the drug was present at its ED_{50} (12 μg/ml) was at least 1 order of magnitude lower (33 versus 341 pmol) than that produced by 2'NDG at its ED_{50} (1.2 μg/ml). When the two compounds were present at 10 times their respective ED_{50}s, the difference in triphosphate levels was 38-fold (90 versus 3,391 pmol; Table 2). Moreover, the production of 2'NDG-TP from 2'NDG increased linearly with drug concentration, whereas with 2'-nor-cGMP the increase was not linear. Thus, it is very unlikely that the cells could accumulate 2'NDG-TP to a level comparable to that obtained from 2'NDG under these conditions (341 pmol at its ED_{50}), irrespective of the concentration of 2'-nor-cGMP in the medium.

In the experiments reported in Tables 1 and 2, incorporation of radiolabel into nucleic acids was minimal. In infected cells incubated with 12 μg of 2'NDG per ml (10 times its ED_{50}), 2.8 pmol/10^7 cells per 6 h were incorporated into nucleic acids; of this, 1.2 pmol were alkali-labile material, and 1.6 pmol were alkali stable. In uninfected cells or cells treated with 2'-nor-cGMP, the amount of radiolabel incorporated into nucleic acids was below the level of accurate detection (<0.5 pmol). Since these trace levels of incorporation are insignificant in comparison to the levels of precursor pools inside the cells (3,391 pmol of 2'NDG-TP per 10^7 cells per 6 h in Table 2), we conclude that they have little or no bearing on the mode of action of these drugs.

In vivo metabolism. To determine whether 2'-nor-cGMP is rapidly dephosphorylated in vivo, 2'NDG and 2'-nor-cGMP were administered to mice by oral gavage, and the presence of metabolites in the plasma was investigated. Equal doses of the two compounds were used since they are nearly equally protective in vivo (14; Field et al., in press). The animals were sacrificed 1 h after administration of the drugs. We selected 1 h since upon oral administration, 2'NDG plasma level reaches a peak in 1 h and declines rapidly thereafter (Howard Hucker, Merck, Sharp & Dohme Research Laboratories, personal communication). The biotransformation of 2'NDG and 2'-nor-cGMP was examined by HPLC analysis after PCA extraction of the plasma. Both compounds remained essentially intact (Table 3). No detectable metabolites of 2'NDG were observed, while only approximately 5% of 2'-nor-cGMP was converted to 2'NDG.

TABLE 3. Drug levels in plasma 1 h after oral administration to mice

Drug (9-mg/kg dose)	Sex	Amt of metabolite in plasma:		
		2'NDG (nmol/ml)	2'-Nor-cGMP (nmol/ml)	% 2'NDG
2'NDG	Male	1.45		100
	Male	2.03		100
	Female	1.09		100
		1.52[a]		100
2'-Nor-cGMP	Male	0.04	0.80	5
	Male	0.07	2.35	3
	Female	0.06	0.85	6
		0.06[a]	1.33	5

[a] Average.

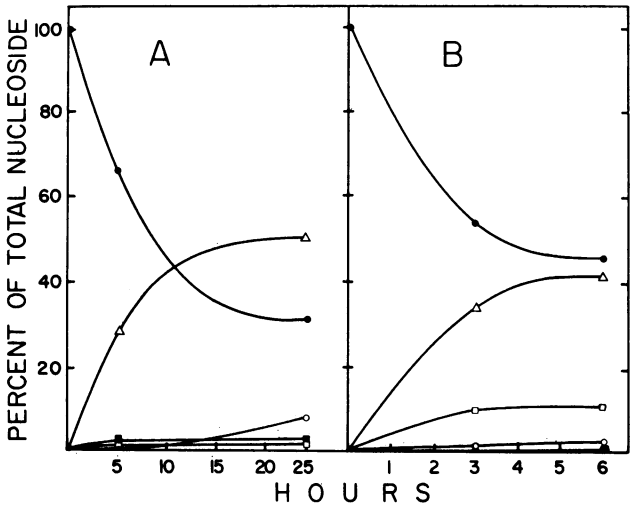

FIG. 1. Opening of the cyclic phosphate ring of 2'-nor-cGMP in crude extracts of HeLa cells. 2'-Nor-cGMP was incubated with crude extracts of HeLa cells, either HSV-1 infected (A) or mock infected (B). The incubation mixtures contained (in a final volume of 200 μl of phosphate-buffered saline) 0.5 mM ATP, 0.5 mM $MgCl_2$, 8 mM phosphocreatine, 10 U of creatine kinase, 100 μl of the crude extract, and 2'-nor-cGMP (0.2 mg/ml). The progress of the reaction was monitored by removing 10-μl samples and analyzing them by HPLC on an AX-10 column; elution started with 30 mM KH_2PO_4 (pH 4.5) for 8 min, which separated the cyclic and acyclic monophosphates, and continued with a gradient from 30 mM KH_2PO_4 (pH 4.5) to 1 M KPO_4 (pH 3.3) in 7 min and isocratic 1 M KPO_4 (pH 3.3) buffer thereafter, which separated 2'NDG-TP from ATP. Symbols: ●, 2'-nor-cGMP; ■, 2'NDG; ○, 2'NDG-MP; □, 2'NDG-DP, △, 2'NDG-TP.

In a separate experiment, 2'-nor-cGMP was incubated for 2 h in vitro with fresh mouse whole blood (EDTA treated). Again, only 5% of the label was found as 2'NDG in the PCA extract (data not shown).

Opening of 2'-nor-cGMP in cell extracts. The cyclic phosphate ring of 2'-nor-cGMP is not hydrolyzed by the action of spleen phosphodiesterase, snake venom phosphodiesterase, or commercial preparations of cyclic AMP (cAMP) or cGMP diesterase. It is also not opened when incubated overnight with mouse plasma (unpublished data). It does open, however, when incubated with extracts obtained by sonication of mouse whole blood, mouse liver, or HeLa cells. The main product obtained from 2'-nor-cGMP upon incubation with cell extracts, as long as ATP and an ATP-generating system are present, is 2'NDG-TP with some acyclic 2'-NDG-MP and very little or no 2'NDG present. This is shown in Fig. 1: when 2'-nor-cGMP was incubated for 24 h at 37°C with a crude extract of HSV-1-infected Hela cells (Fig. 1A), 50% was converted to 2'NDG-TP and 10% was converted to 2'NDG-MP; 2'NDG-DP and 2'NDG were present in very small proportions (3 and 4%, respectively). Similar results were obtained with uninfected HeLa cell extracts (Fig. 1B).

Chirality of 2'NDG-MP produced by opening of the cyclic phosphate ring of 2'-nor-cGMP. To determine whether the opening of the cyclic phosphate ring of 2'-nor-cGMP produces the (*R*)-2'NDG-MP, the (*S*)-2'NDG-MP, or a mixture of both, we isolated a sufficient quantity of the monophosphate obtained by incubation of 2'-nor-cGMP with a mouse liver extract and compared its rate of phosphorylation by GMP kinase to that of purified (*R*)- and (*S*)-2'NDG-MP. As shown in another communication (Karkas et al., submitted), the rate of phosphorylation of the *R* isomer in this reaction is considerably lower than that of the *S* isomer. The results show that the monophosphate obtained by opening of 2'-nor-cGMP is phosphorylated to the diphosphate by GMP kinase at a rate which is, within the limits of accuracy of the experiment, the same as that of the monophosphate obtained by phosphorylation of 2'NDG by HSV-1 TK, namely the (*S*)-2'NDG-MP (Fig. 2).

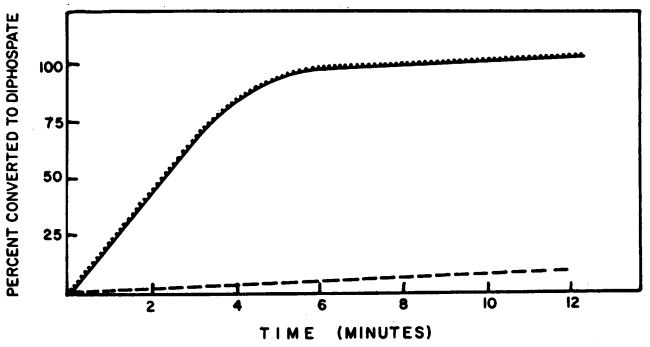

FIG. 2. Reaction kinetics of the phosphorylation of 2'NDG-MP by GMP kinase. The acyclic 2'NDG-MP obtained by opening of the cyclic phosphate ring of 2'-nor-cGMP (——) and the purified (S) and (R) enantiomers of 2'NDG-MP (····· and – – –, respectively) were incubated with GMP kinase in the cuvette of a spectrophotometer at 25°C. The incubation mixtures contained (in a final volume of 1 ml) 100 mM Tris acetate buffer (pH 7.6), 50 mM KCl, 10 mM $MgCl_2$, 5 mM ATP, 100 μg of bovine serum albumin per ml, 0.3 mM NADH, 1 U of lactic dehydrogenase, 4 U of pyruvate kinase, 0.04 U of GMP kinase, and the 2'NDG-MP substrate (1.6 A_{260}/ml, 0.14 mM). The progress of the reaction was monitored by the decrease in A_{340}; 100% conversion to diphosphate corresponds to a decrease of 0.87 absorbance units.

Inhibition of HSV-1 DNA polymerase by other metabolites

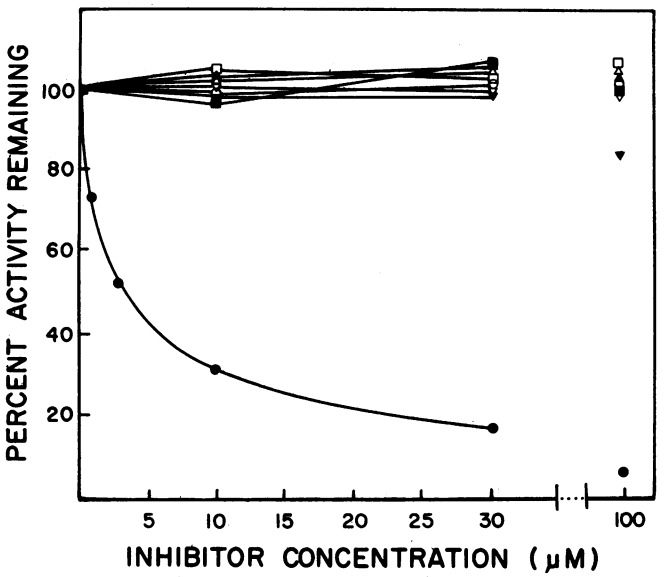

FIG. 3. Inhibition of HSV-1 DNA polymerase by 2'NDG and metabolites. The assays for HSV-1 DNA polymerase reaction were performed in the presence of various amounts of the eight potential inhibitors. Symbols: ●, (*S*)-2'NDG-TP; ▼, (*R*)-2'NDG-TP; ■, 2'NDG; ▲, 2'-nor-cGMP; ▽, (*S*)-2'NDG-MP; △, (*R*)-2'NDG-MP; □, (*S*)-2'NDG-DP; ○, (*R*)-2'NDG-DP.

of 2'-nor-cGMP. The *R* and *S* isomers of 2'NDG-MP, 2'NDG-DP, and 2'NDG-TP, as well as the precursors themselves, 2'NDG and 2'-nor-cGMP, were tested as inhibitors of the partially purified HSV-1 DNA polymerase. Only (*S*)-2'NDG-TP inhibited the polymerase significantly (Fig. 3). 2'NDG, 2'-nor-cGMP, (*S*)- or (*R*)-2'NDG-MP, and (*S*)- or (*R*)-2'NDG-DP showed no measurable inhibition, while (*R*)-2'NDG-TP inhibited slightly. In a separate experiment it was shown that the 50% inhibiting concentration for (*R*)-2'NDG-TP was at least 100 times higher than that for (*S*)-2'NDG-TP. The small inhibition seen with (*R*)-2'NDG-TP could be due to slight contamination with the (*S*) isomer carried over during the isolation.

DISCUSSION

The first conclusion that can be drawn from the results presented above is that 2'-nor-cGMP does not act simply as a prodrug of 2'NDG through ring opening and dephosphorylation. This does not appear to happen to any significant extent in the bloodstream. Although the in vivo biotransformation experiment reported here is preliminary and involves only one time point, it suggests that there is no rapid or massive dephosphorylation as would be expected if 2'-nor-cGMP were simply a prodrug of 2'NDG, in view of the fact that the two compounds have equal potency in vivo (Field et al., in press). Incubation of 2'-nor-cGMP in vitro with mouse whole blood resulted again in a very small proportion of free 2'NDG, while prolonged incubation with mouse plasma did not indicate any measurable opening of the cyclic phosphate ring, suggesting that the 2'NDG found in the blood was dephosphorylated inside the erythrocytes. Thus, the antiviral activity of 2'-nor-cGMP is probably not due to its conversion to 2'NDG in circulation, before presentation to the site of infection.

2'-Nor-cGMP is transported into the cells essentially intact. This is suggested by the fact that the $^{32}P/^{14}C$ ratio, when doubly labeled compound was presented to infected cells in culture, was unchanged in the total PCA extract and in the 2'-nor-cGMP as well as the 2'NDG-MP and 2'NDG-DP inside the cell. The ratio of <1 for the triphosphate is not understood. Had dephosphorylation and rephosphorylation taken place (the latter being difficult to conceive in normal cells, which lack viral TK) one would have expected a lower $^{32}P/^{14}C$ ratio, but this should also be reflected in the mono- and diphosphates, which are intermediates in the path to the triphosphate. It is conceivable that this discrepancy was the result of a complete compartmentalization of the rephosphorylation process, but this cannot be ascertained without further experimentation.

The production of 2'NDG-TP from 2'-nor-cGMP is essentially independent of HSV-1 TK expression. This had been suggested by earlier antiviral (14; Field et al., in press) and cell toxicity (9) experiments. In the present series, the TK independence was shown both in cell-free systems and in cell culture. The cell-free experiments indicated that 2'-nor-cGMP can be opened and further phosphorylated directly to the triphosphate, bypassing the TK step necessary for 2'NDG. It was particularly significant in this respect that this occurred also in extracts of uninfected cells, in which there is no viral TK, and thus, had 2'-nor-cGMP been opened and dephosphorylated, there would be no way for 2'NDG to be phosphorylated again to any significant extent.

The same conclusion is also supported by the cell culture experiments, which indicate that there is little difference in 2'NDG-TP levels between infected and uninfected cells treated with 2'-nor-cGMP (in contrast to the striking differences observed in 2'NDG-treated cells) or between cells infected with HSV-1 and the TK^- mutant virus.

This lack of dependence of 2'NDG-TP production from 2'-nor-cGMP on viral TK expression could explain the broad spectrum of activity of this compound against a variety of DNA viruses (14) some of which do not induce a specific TK. In these cases, significant levels of 2'NDG-TP could still be built up. The situation, however, may be more complex. If TK-independent production of 2'NDG-TP were the only mechanism of antiviral activity of 2'-nor-cGMP, and since phosphate ring-opening and further phosphorylation occur also in uninfected cells, one would expect the compound to be highly toxic. This is not the case: 2'-nor-cGMP is not toxic at therapeutic levels (Field et al., in press). Thus, alternate mechanisms unrelated to the presence of 2'NDG-TP may be involved, in all or in part.

That this may be the case is also suggested by the observed lack of correlation between 2'NDG-TP levels and antiviral activity. Indeed, under a variety of conditions, the levels of 2'NDG-TP reached in cells treated with 2'-nor-cGMP were at least 1 order of magnitude lower than those reached in 2'NDG-treated cells. Of particular significance is the fact that when the two antivirals were present at their ED_{50}s, there was still a 10-fold difference in the amount of 2'NDG-TP produced by the two compounds. It should be noted at this point that the amount of 2'NDG-TP (341 pmol) present in the HSV-1-infected, 2'NDG-treated cells at its ED_{50} does not represent an excess over that required for the observed antiviral effect; as mentioned above, the ED_{50} of 2'NDG for the TK-deficient virus (50 μg/ml) is the concentration required for the production of exactly the same amount of 2'NDG-TP (341 pmol) in the infected cells.

One hypothesis that could explain the lack of correlation of antiviral activity of 2'-nor-cGMP with the level of 2'NDG-TP produced in the cell when the latter is compared to that produced by an equipotent dose of 2'NDG is that different enantiomers of 2'NDG-TP are formed in the two cases. Indeed, 2'NDG is a prochiral molecule; as long as the two hydroxymethyl groups on C-2 of the propoxy moiety are not substituted, the molecule is not optically active; however, phosphorylation creates a chiral center and thus two enantiomers of 2'NDG-MP are possible; this is similarly true for 2'NDG-DP and 2'NDG-TP. When 2'-nor-cGMP is hydrolyzed by strong alkali, equal amounts of the (*R*)- and (*S*)-2'NDG-MP are produced. On the other hand, we have shown that phosphorylation of 2'NDG by HSV-1 TK produces exclusively the (*S*) isomer (Karkas et al., submitted). Yet, the opening of the cyclic phosphate ring of 2'-nor-cGMP in vivo could conceivably produce, in all or in part, the *R* isomer, and this could give rise to the *R* triphosphate, which might be a more potent inhibitor of the viral polymerase than the *S* isomer. Alternatively, (*R*)-2'NDG-MP or (*R*)-2'-NDG-DP could be the inhibitory species.

This hypothesis was tested by two experimental approaches. In the first, the acyclic monophosphate of 2'NDG produced by the enzymatic opening of the cyclic phosphate ring of 2'-nor-cGMP was isolated and examined for the presence of the *R* isomer. The results (Fig. 2) indicated that the 2'NDG-MP produced by incubation with mouse liver extract was exclusively (within the limits of accuracy of the experiment) the *S* isomer, the same as that obtained by the action of HSV-1 TK on 2'NDG (Karkas et al., submitted). It should be noted, however, that the chirality of the monophosphate could conceivably be different if the opening took place in tissues other than liver.

In the second approach, we compared the potency of the

two purified enantiomeric *R* and *S* triphosphates of 2'NDG as in vitro inhibitors of the HSV-1 DNA polymerase. The 50% inhibiting concentration of the *R* isomer was found to be at least 100 times higher than that of the *S* isomer. Thus, even if the *R* isomer were produced in vivo by the opening of the cyclic phosphate ring, it could not have resulted in the dramatic inhibition that this hypothesis predicts. Finally, the remote possibility that the DNA polymerase was inhibited by (*R*)-2'NDG-MP or (*R*)-2'NDG-DP was also shown not to be true since these compounds did not show any inhibitory activity in vitro against HSV-1 DNA polymerase (Fig. 3). Thus, the hypothesis that the 2'NDG *R* phosphate derivatives are responsible for the anti-HSV activity must be rejected.

2'-Nor-cGMP is one of a class of potent antivirals which are effective in animal protection studies (14; Field et al., in press); its mechanism of HSV inhibition remains obscure, although it has been shown not to function effectively as a prodrug for 2'NDG.

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