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Phosphonylmethyl ethers of acyclic nucleoside analogues: inhibitors of HSV-1 induced ribonucleotide reductase

Jaroslav Černý[1], Ivan Votruba[1], Vladimír Vonka[2], Ivan Rosenberg[1], Miroslav Otmar[1] and Antonín Holý[1]

[1]*Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, and* [2]*Department of Experimental Virology, Institute of Sera and Vaccines, Prague, Czechoslovakia*

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Summary

Diphosphates of *N*-(S)-(3-hydroxy-2-phosphonylmethoxypropyl) and *N*-(2-phosphonylmethoxyethyl) derivatives of purine and pyrimidine heterocyclic bases inhibit HSV-1 encoded ribonucleotide reductase. Of the compounds studied, the most efficient inhibitors of CDP reduction (at 5.1 $\mu mol \cdot l^{-1}$) by the HSV-1-encoded enzyme are HPMPApp (IC_{50} = 0.9 $\mu mol \cdot l^{-1}$) and PMEApp (IC_{50} = 8 $\mu mol \cdot l^{-1}$). PMEApp does not inhibit the enzyme isolated from the mutant HSV-1 KOS strain $PMEA^r$ which is resistant to PMEA at a concentration of 100 $\mu g/ml$. The enzyme isolated from the PMEA-resistant virus strain is also insensitive to inhibitory effects of hydroxyurea and HPMPApp. Thus, the inhibitory potency of HPMPApp and PMEApp toward HSV-1 encoded ribonucleotide reductase might be connected with the anti-HSV activity of HPMPA and PMEA.

HSV-1 encoded ribonucleotide reductase; Phosphonylmethyl derivative of acyclic nucleoside analog; HPMPA; PMEA

Introduction

Ribonucleotide reductase (E.C.1.17.4.1) catalyzes the reduction of all four ribonucleoside 5′-diphosphates, and is therefore essential for the synthesis of DNA

Correspondence to: J. Černý, Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6, Czechoslovakia.

in prokaryotes and eukaryotes (Reichard, 1988). Three classes of large genome viruses, the T-even bacteriophages, the herpesviruses and the poxviruses also encode ribonucleotide reductase (Berglund et al., 1969; Cohen, 1972; Cohen et al., 1974, 1977; Henry et al., 1978; Slabaugh et al., 1984; Spector et al., 1987). In contrast to its mammalian counterparts, the HSV-1 encoded enzyme is not sensitive to allosteric regulation by ATP, dATP or dTTP (Averett et al., 1983). It appears to be regulated by competition between substrates for a common catalytic site. Since viral ribonucleotide reductase most probably plays a specific role in the regulatory mechanism of the viral DNA synthesis (Leary et al., 1983; Preston et al., 1984) and differs considerably from the corresponding host cell enzyme, it can be considered as an important target for virus chemotherapy (Turk et al., 1986; Cameron et al., 1988).

Various *N*-(2-phosphonylmethoxyethyl) (PME-) and (*S*)-*N*-(3-hydroxy-2-phosphonylmethoxypropyl) (HPMP-) derivatives of purine and pyrimidine bases are very effective antiherpetic agents (De Clercq et al., 1986, 1987, 1989). The only metabolites of these nucleotide analogues are their mono- and diphosphates, formed from the parent compounds by the action of cellular nucleotide kinases. The antiviral effect of one of the compounds mentioned (HPMPA) is based upon a preferential inhibition of viral DNA synthesis (Votruba et al., 1987) at a concentration which is lower by several orders of magnitude than that required for the inhibition of cellular DNA synthesis. In both the PME- and HPMP-series, the diphosphates (nucleoside-5′-triphosphate analogues) are inhibitors of HSV-1 DNA polymerase (Merta et al., 1990). In this report, we describe the interaction of these compounds with HSV-1-encoded ribonucleotide reductase.

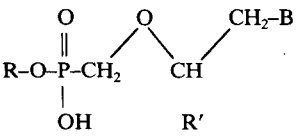
Materials and Methods

Compounds

The phosphoryl and diphosphoryl derivatives of acyclic nucleotide analogues (Fig. 1) were synthesized from the pertinent phosphonylmethoxyalkyl compounds (Holý and Rosenberg, 1987a,b; Rosenberg and Holý, 1987) by the morpholidate (Moffatt, 1964) or imidazolide procedure (Michelson, 1964). The purity of these compounds was checked by HPLC, TLC (PEI cellulose in 0.5 $mol \cdot l^{-1}$ LiCl, 0.125 $mol \cdot l^{-1}$ acetic acid) and paper electrophoresis on Whatman 3 MM paper in 50 $mmol \cdot l^{-1}$ sodium citrate buffer (pH 3.8) at 40 $V \cdot cm^{-1}$.

Chemicals and reagents

[U-^{14}C]CDP (16.9 GBq/mmol), [U-^{14}C]ADP (16.6 GBq/mmol) and [U-^{14}C]GDP (16.4 GBq/mmol) were from UVVVR (Prague, Czechoslovakia). Bacterial alkaline phosphatase, PMSF and hydroxyurea were purchased from Sigma Chemical Co. (U.S.A.). DTT and HEPES were products of Serva (Heidelberg, F.R.G.). All other chemicals used were of the highest purity obtainable.



R	R′	B	Abbreviation
H	H	Adenin-9-yl	PMEA
PO_3H_2	H	Adenin-9-yl	PMEAp
$P_2O_7H_3$	H	Adenin-9-yl	PMEApp
PO_3H_2	H	2-Aminoadenin-9-yl	2-NH_2PMEAp
$P_2O_7H_3$	H	Adenin-9-yl	2-NH_2PMEApp
PO_3H_2	H	7-Deaza-8-azaadenin-9-yl	c^7z^8PMEAp
$P_2O_7H_3$	H	7-Deaza-8-azaadenin-9-yl	c^7z^8PMEApp
PO_3H_2	H	Guanin-9-yl	PMEGp
$P_2O_7H_3$	H	Guanin-9-yl	PMEGpp
PO_3H_2	H	Uracil-1-yl	PMEUp
$P_2O_7H_3$	H	Uracil-1-yl	PMEUpp
PO_3H_2	H	Thymin-1-yl	PMETp
$P_2O_7H_3$	H	Thymin-1-yl	PMETpp
PO_3H_2	H	Cytosin-1-yl	PMECp
$P_2O_7H_3$	H	Cytosin-1-yl	PMECpp
PO_3H_2	(S)-CH_2OH	Adenin-9-yl	HPMPAp
$P_2O_7H_3$	(S)-CH_2OH	Adenin-9-yl	HPMPApp

Fig. 1. Structures and abbreviations of N-(phosphonylmethoxyalkyl) derivatives of heterocyclic bases.

Antiserum

Polyclonal anti-HSV-1 serum was raised by immunizing rabbits with HSV-1 KOS strain. IgG fraction was isolated by precipitation with octanoic acid. IgG fraction (25 μl) was then pipetted to the standard reaction mixture.

Cells and viruses

Monolayers of VERO (green monkey kidney) cells or REF (rabbit embryonic fibroblast) cells were grown to confluency in EPL medium (Sevac, Prague) in the absence of complete serum. REF cells were the same as in previous experiments (Kutinová et al., 1973). VERO cells were obtained fram Dr Rajčani, Institute of Virology, Bratislava. HSV-1 KOS strain or PMEA resistant HSV-1 strain ($PMEA^r$ HSV-1 KOS) was derived from the KOS strain by passaging the virus at gradually increasing concentrations of PMEA (for a total of 33 passages; final concentration 100 $\mu g \cdot ml^{-1}$). The virus stocks were prepared in REF cells.

Preparation of enzyme extract

REF and VERO cells at confluency were infected or mock-infected with virus at an MOI of 20 $PFU \cdot ml^{-1}$. At 16 h p.i., the monolayers were washed twice with

phosphate-buffered saline, the cells were harvested by scraping, pelleted by centrifugation at 2000 × *g* and stored at −80°C until used. In a typical experiment, the cells were then resuspended in 100 mmol·l^{-1} Hepes sodium salt (pH 7.6) containing 2 mmol·l^{-1} DTT, 1 mmol·l^{-1} PMSF and sonicated (10×10 s, on ice, amplitude 14–16 kc, 150 W MSE Ultrasonic desintegrator). The crude extracts were clarified by centrifugation at 10000 × *g* for 20 min, and subsequently for 90 min at 100000 × *g*. The 100000 × *g* supernatant fraction was brought to 45% saturation by the addition of crystalline ammonium sulfate. The precipitate from this fractionation was dissolved in the sonication buffer and desalted on Sephadex G-25 column (PD-10; Pharmacia).

Protein determination was performed using the method described in the literature (Spector, 1978).

Radioactivity measurements were performed on a Beckman liquid scintillation spectrometer (Model LS 5801) and scanning of chromatograms on Radiochromatogram Scanner (Packard 7200).

Enzyme assays

CDP, ADP or GDP reduction by virus ribonucleotide reductase. The standard assay was performed in quadruplicate by modification of a described procedure (Averett et al., 1983): unless otherwise noted, the reaction mixture (50 µl) contained 100 mmol·l^{-1} HEPES sodium salt (pH 7.9), 10 mmol·l^{-1} DTT, varying concentrations of ^{14}C-labelled substrates ([^{14}C]CDP, [^{14}C]ADP, [^{14}C]GDP, respectively) and enzyme (40 µg). The enzyme reaction was stopped at the appropriate time by addition of 10 mmol·l^{-1} hydroxyurea (containing 50 µmol·l^{-1} EDTA) and heating for 3 min at 100°C in a water bath. The mixtures were then incubated for 30 min at 37°C in 300 µmol·l^{-1} Tris-HCl buffer (pH 8.9) containing 1 mmol·l^{-1} $MgCl_2$ and 1 EU of bacterial alkaline phosphatase. The reaction mixtures were separated by descending paper chromatography on Whatman 3 filter paper in the system 2-propanol – conc. aqueous ammonia – water (7:1:2) (Sato et al., 1983). After drying, the distribution of radioactivity was confirmed by scanning, the spots of ribo- and 2′-deoxyribonucleosides were cut out and counted in toluene-based scintillation fluid (10 ml).

Extracts from mock-infected cells were assayed in the reaction mixture (50 µl) consisting of 100 mmol·l^{-1} HEPES sodium salt (pH 7.9), 10 mmol·l^{-1} DTT, 4 mmol·l^{-1} ATP, 6 mmol·l^{-1} $MgCl_2$, varying concentration of [^{14}C]CDP and enzyme (average, 40 µg), and further processed as described above.

The rate of CDP, ADP and GDP cleavage in the absence of ATP was estimated by summation of radioactivity in ribonucleosides and 5′-ribonucleotides, as determined by paper electrophoresis or paper chromatography (see above).

Nucleoside diphosphate kinase was measured in standard reaction mixtures for CDP reduction containing additional 4 mmol·l^{-1} ATP and 6 mmol·l^{-1} $MgCl_2$. The reactions were initiated by addition of enzyme to the reaction mixtures at 37°C. The samples were taken at intervals and the conversion of [^{14}C]CDP to [^{14}C]CTP was evaluated after separation by paper electrophoresis (see above).

Estimation of the cleavage rate of mono- and diphosphoryl derivatives of PME- and HPMP-compounds was performed in the standard reaction mixture for ribonucleotide reductase activity where ATP was omitted. The samples were taken *prior to* the alkaline phosphatase treatment, separated by paper electrophoresis, the spots of the inhibitors and their cleavage products (detection by UV) were eluted by 0.01 M HCl and the absorbancy of the eluates was measured at 260 nm.

Results

Preparation of enzyme extracts

For a reliable determination of ribonucleotide reductase activity levels in the cell-free extracts of virus-infected or mock-infected cells it is necessary to minimize degradative reactions (dephosphorylation) of both the substrates and the acyclic nucleotide analogues. The crude extracts obtained from infected REF and VERO cells which contain high level of phosphomonoesterases are not suitable for the purpose without further purification. In a typical reaction mixture (containing 200 μg protein in 50 μl) the dephosphorylation of ribonucleoside 5′-diphosphates to

TABLE 1

Modulation of CDP reduction[a] by HSV-1 induced ribonucleotide reductase

Compound	IC_{50} ($\mu mol \cdot l^{-1}$)	Ribonucleotide reductase[b] residual activity (%)
PMEA	> 1000	98
PMEAp	96	20
PMEApp	8	19
2-NH_2PMEAp	> 1000	80
2-NH_2PMEApp	19	15
c^7z^8PMEAp	16	52
c^7z^8PMEApp	6	36
PMEGp	> 1000	96
PMEGpp	> 1000	64
PMEUp	> 1000	108
PMEUpp	> 1000	74
PMETp	> 1000	83
PMETpp	> 1000	26
PMECp	> 1000	107
PMECpp	> 1000	68
HPMPAp	480	12
HPMPApp	0.90	c
araC	> 1000	90
araCTP	21	c
ACV-TP	> 1000	61

[a] [CDP] = 5.6 $\mu mol \cdot l^{-1}$.
[b] [I] = 1 $mmol \cdot l^{-1}$.
[c] Below the limit of estimation.

5'-mononucleotides and nucleosides after 10 min incubation amounted to 56% for CDP, 6% for ADP and 9% for GDP. On the other hand, ribonucleoside diphosphate kinase activity which was the main interfering factor in the previous study (Averett et al., 1983) was very low. After 10 min incubation in the presence of 4 $mmol \cdot l^{-1}$ ATP, the radioactivity distribution indicated the following conversion of CDP: 6% CTP, 2% CMP and 4% Cyd.

While the 10000 × *g* supernatant still contained 21% of the original phosphomonoesterase activity, the level of this enzyme in the 100000 × *g* supernatant dropped to 6% of the original value. After the ammonium sulfate precipitation step followed by desalting of the precipitate on a Sephadex G-25 column, the dephosphorylating activity of the preparation amounted to 1.5% of the level originally present in the crude extract. This material was considered satisfactory for further use.

The viral origin of the ribonucleotide reductase measured in the cells at different times p.i. was corroborated by experiments based on inhibition of this enzyme by polyclonal anti-HSV-1 antibodies. No significant activity of ribonucleotide reductase could be detected in mock-infected confluent REF or VERO cells under the optimal conditions for estimation of either viral or eukaryotic enzyme.

Effect of phosphorylated HPMP- and PME-derivatives upon HSV-1 ribonucleotide reductase

The data in Fig. 2A demonstrate significant inhibition of HSV-1 ribonucleotide reductase by HPMPApp in an extended range of very low inhibitor concentrations

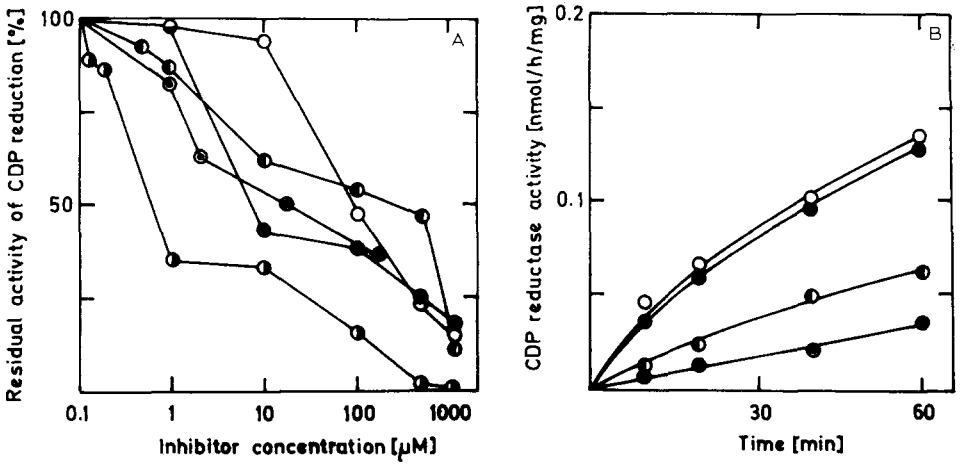


Fig. 2. A. HSV-1 Ribonucleotide reductase inhibition by mono- and diphosphates of acyclic nucleotide analogues and araCTP. The enzyme preparations from HSV-1 KOS infected VERO cells were incubated 10 min at 37°C in the presence of varying concentrations of PMEAp (○), HPMPAp (◐), araCTP (⊙), PMEApp (●) and HPMPApp (◑) plotted on logarithmic scale. B. Time course of CDP reduction in the presence of nucleoside 5'-triphosphate analogues. The enzyme preparation from HSV-1 KOS infected VERO cells was incubated in the absence (○) or presence of 100 μM ACV-TP (●), PMEApp (◐) and HPMPApp (⊙).

TABLE 2

Modulation of GDP and ADP reduction[a] by HSV-1 ribonucleotide reductase by diphosphoryl and monophosphoryl derivatives of acyclic nucleotides

Compound[b]	Residual activity (%)	
	GDP reduction	ADP reduction
PMEAp	97	83
PMEApp	51	56
HPMPAp	76	91
HPMPApp	32	62

[a] [GDP] = 8 $\mu mol \cdot l^{-1}$, [ADP] = 16 $\mu mol \cdot l^{-1}$.
[b] [I] = 10 $\mu mol \cdot l^{-1}$.

(IC_{50} = 0.90 ± 0.15 $\mu mol \cdot l^{-1}$ at 5.1 $mmol \cdot l^{-1}$ CDP). After a plateau (typical for saturation kinetics) between 1 and 10 $\mu mol \cdot l^{-1}$ further increase of inhibition continues and the enzyme is completely inhibited by HPMPApp at a concentration of 1 $mmol \cdot l^{-1}$.

Inhibitory effects weaker than those of HPMPApp were observed with HPMPAp, PMEAp and PMEApp. Our data show that diphosphoryl derivatives of HPMPA and PMEA (analogues of adenosine 5′-triphosphate) are more potent inhibitors of CDP reduction than the corresponding monophosphoryl derivatives (analogues of adenosine 5′-diphosphate).

The effects of HPMPApp and PMEApp on HSV-1 ribonucleotide reductase were compared with the activity of additional base-modified phosphoryl and diphosphoryl derivatives in both the HPMP- and PME-series, as well as with the effects of ACV-TP and araCTP. The data shown in Fig. 2B and Table 1 prove that of the whole series examined HPMPApp and PMEApp are the most efficient inhibitors of CDP reduction. Similar results were obtained for ADP and GDP reduction by HSV-1 ribonucleotide reductase (Table 2).

The extract prepared from the cells infected by the PMEA-resistant strain of HSV-1 ($PMEA^r$ HSV-1 KOS) contained ribonucleotide reductase at a level that was nearly identical to that detected in the cells infected by the parental virus strain. Therefore, we have investigated the sensitivity of viral ribonucleotide reductase from the cells infected by this $PMEA^r$ virus mutant toward selected derivatives of the PME- and HPMP-series as well as additional compounds (ACV-TP, araCTP, hydroxyurea). The concentration-dependent response for CDP reduction inhibition (Fig. 3A) differed significantly from the corresponding response of the enzyme isolated from the parental virus strain (Fig. 2A). In particular, PMEApp did not inhibit CDP reduction even at concentration of 200 $\mu mol \cdot l^{-1}$. The inhibitory effect of HPMPApp on the ribonucleotide reductase from $PMEA^r$ HSV-1 KOS was also lower (IC_{50} = 50 $\mu mol \cdot l^{-1}$) than its inhibitory effect on the enzyme from parental virus. None of the other compounds effected a significant inhibition at a concentration below 5 $\mu mol \cdot l^{-1}$ (Fig. 3A,C). On the contrary, araCTP exerted a slight stimulatory effect on CDP reduction by this enzyme (Fig. 3C). Another interesting difference between the two ribonucleotide reductases was observed in their

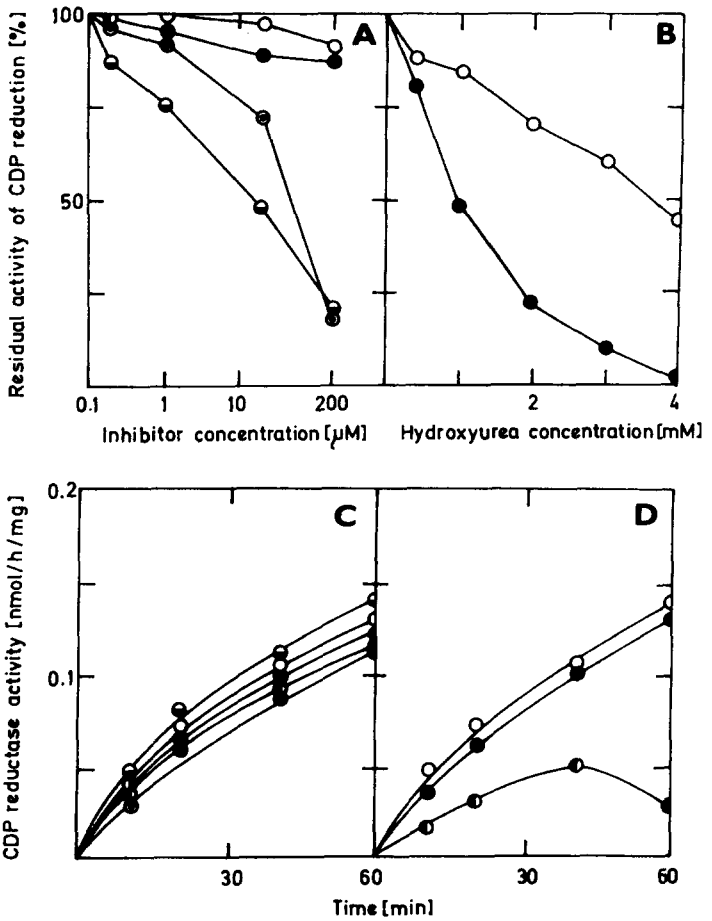


Fig. 3. Effect of nucleoside 5′-triphosphate analogues and hydroxyurea on CDP reduction catalyzed by the enzyme preparation isolated from PMEA^r HSV-1 (KOS)-infected VERO cells. A. Inhibition of CDP reduction by varying concentrations of PMEApp (○), ACV-TP (●), HPMPAp (⊙) and HPMPApp (◒) plotted on a logarithmic scale. The reaction mixture was incubated for 10 min at 37°C. B. Sensitivity of CDP reduction to the presence of hydroxyurea with the enzyme preparations from PMEA^r HSV-1 (KOS)-infected (○) and HSV-1 (KOS)-infected VERO cells (●); incubation, 30 min at 37°C. C. Rate of CDP reduction in the absence (○) or presence of 4 μmol·l^{-1} araCTP (◒), PMEApp (●), HPMPApp (◐) and HPMPAp (⊙). D. CDP reduction in the absence (○) or presence of 0.4 mM hydroxyurea (●) or 4 mM hydroxyurea (◐).

response to hydroxyurea (Fig. 3B, D). While the enzyme from the parental virus strain was inactivated by hydroxyurea at a rather low concentration (IC_{50} = 0.85 ± 0.15 mmol·l^{-1}), the inactivation of the PMEA^r HSV-1 KOS ribonucleotide reductase occurred only at a concentration IC_{50} of 3.50 ± 0.20 mmol·l^{-1} (Fig. 3B). The effect of hydroxyurea at 0.4 mmol·l^{-1} on the enzyme from the PMEA-resistant virus strain was marginal; at 4 mmol·l^{-1} it caused a partial inhibition only. On

the other hand, the ribonucleotide reductase from the parental HSV-1 (KOS) strain was completely inactivated at 4 mmol·l^{-1} hydroxyurea.

Discussion

In this paper, we compared the effects of selected acyclic nucleotide analogues and their derivatives on the activity of partially purified HSV-1 ribonucleotide reductases from the virus-infected cells. The enzymes were encoded by either the HSV-1 KOS strain or the PMEA-resistant mutant, PMEA^r HSV-1 KOS. The latter strain was selected by repeated passages of the KOS strain in the presence of increasing PMEA concentrations (up to 100 μg·ml^{-1}).

The enzyme preparations which were obtained by partial precipitation of the 100000 × *g* supernatants with ammonium sulfate (Averett et al., 1983; Nutter et al., 1985; Spector et al., 1985) contain minimum level of enzyme degrading substrates, products and/or inhibitors. During the optimization experiments related to the enzyme level in the crude extracts and their 100000 × *g* supernatants we observed that the highest level of ribonucleotide reductase activity was achieved between 14 and 16 h p.i. This finding differs substantially from earlier data obtained with other virus strains and cell systems (Dutia, 1983; Huszar and Bacchetti, 1981; Langelier and Buttin, 1981), where the maximum enzyme activity was reached at 6–8 h post infection.

CDP reduction (at 5.1 μmol·l^{-1} CDP) was most efficiently inhibited by HPMPApp (IC_{50} = 0.90 ± 0.15 μmol·l^{-1}) followed by PMEApp (IC_{50} = 8.0 ± 1.1 μmol·l^{-1}), its base-substituted congeners (2-NH_2PMEApp, c^7z^8PMEApp) and araCTP (IC_{50} = 21.0 ± 3.5 μmol·l^{-1}). Our data can be summarized as follows: (a) diphosphoryl derivatives of HPMP- and PME-compounds are more efficient inhibitors of CDP reduction than the corresponding monophosphates (the IC_{50} ratio for monophosphate versus diphosphate is 210 for HPMPA and 5.3 for PMEA derivatives); (b) adenine nucleotide analogues (derived from HPMPA and PMEA) are the most powerful inhibitors of all the compounds tested; (c) concentration-dependent response of the inhibition of CDP reduction has a saturation character (Fig. 2A).

The earlier papers (for a review see Hunting and Henderson, 1982) suggest that ATP, dATP, dTTP and dGTP are involved in the allosteric regulation of the active site both in prokaryotic and eukaryotic ribonucleotide reductases. According to the present knowledge, the HSV-encoded enzyme differs from the host cell reductase by its independence on the presence of both Mg^{2+} ions and any nucleotide. Nevertheless, a partial inhibition of the viral enzyme was observed at high concentrations of the corresponding nucleoside 5′-triphosphates (Averett et al., 1983). We have confirmed that CDP reductase is inhibited by dATP under conditions excluding its dephosphorylation (data not shown). Thus, the loss of allosteric inhibition of HSV-1 ribonucleotide reductase is not absolute.

On the basis of our experiments (Fig. 2A) we suggest that the acyclic analogues of nucleoside 5′-triphosphates possess a strong affinity for the 'residual allosteric

site' at the M1 subunit of the viral enzyme. Consequently, they can induce such conformational change of the HSV-1 ribonucleotide reductase which might simulate the inactive form of the eukaryotic enzyme. This conclusion is corroborated by the fact that acyclic analogues of those natural nucleotides which are lacking the allosteric regulatory activity for eukaryotic enzyme (CTP, UTP and dCTP) are also the least efficient inhibitors of the viral enzyme (Table 1).

This concept of enzyme inhibition by binding of the analogue to a mutation-prone allosteric site is further supported by our data on CDP reduction catalyzed by the ribonucleotide reductase encoded by the PMEA^r HSV-1 KOS virus mutant. In this case, the IC_{50} value for HPMPApp was substantially higher (52 ± 10 $\mu mol \cdot l^{-1}$) than the value for the enzyme of the parental strain (Fig. 3), while PMEApp did not even inhibit CDP reduction in the concentration range of 1-200 $\mu mol \cdot l^{-1}$. Moreover, the enzyme encoded by the PMEA-resistant HSV-1 strain was considerably less sensitive to hydroxyurea. This can be explained by a mutation-caused change of the enzyme conformation in the vicinity of the active site thus diminishing recognition of the hydroxyurea molecule (Lammers and Follmann, 1983).

Our data demonstrate that the diphosphoryl derivatives PMEApp and HPMPApp are strong inhibitors of viral (HSV-1) ribonucleotide reductase. Since these diphosphoryl derivatives are readily formed from PMEA and HPMPA in mock-infected and/or virus-infected cells (Votruba et al., 1987), it is assumed that the anti-HSV effect of the parent drugs (HPMPA, PMEA) may be partly connected with the inhibition of viral ribonucleotide reductase.

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