INTERACTIONS OF 1-[(*S*)-3-HYDROXY-2-(PHOSPHONOMETHOXY)-PROPYL]CYTOSINE (CIDOFOVIR) DIPHOSPHATE WITH DNA POLYMERASES α, δ AND ε*

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The inhibitory and/or substrate activity of 1-[(*S*)-3-hydroxy-2-(phosphonomethoxy)propyl]-cytosine [(*S*)-HPMPC, cidofovir, Vistide™] diphosphate towards eukaryotic DNA polymerases α, δ and ε* was examined. Cidofovir diphosphate is a weak competitive inhibitor of the above enzymes, approximately 3 to 7 times weaker than its adenine analogue (*S*)-HPMPApp. The enzymes also catalyze incorporation of (*S*)-HPMPC into DNA; after insertion of one (*S*)-HPMPC residue into DNA, another dNMP residue may incorporate. DNA polymerase δ and ε* can successively accommodate in the growing chain two (*S*)-HPMPC residues at the maximum, whereas pol α up to three residues.
Keywords: Nucleotides; Acyclic nucleoside phosphonates; Antivirals; Phosphates; (*S*)-HPMPC; Cidofovir; Vistide; (*S*)-HPMPA; Pol α; Pol δ; Pol ε.

(*S*)-HPMPC (1-[(*S*)-3-hydroxy-2-(phosphonomethoxy)propyl]cytosine; cidofovir, Vistide™, Chart 1) is currently used for the treatment of cytomegalovirus (CMV) retinitis in patients with AIDS (for a review, see ref.[1]). In addition, it exhibits antiviral activity against a broad spectrum of other DNA viruses, including polyoma-, papilloma-, adeno-, herpes-, irido- and poxviruses[2–6]. The cellular metabolism of cidofovir begins after its uptake

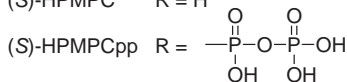(*S*)-HPMPC   R = H
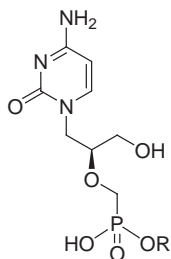
(*S*)-HPMPCpp   R =

Chart 1

into cells presumably *via* an endocytosis-like process[7]. Unlike nucleoside analogues, (*S*)-HPMPC does not require the first phosphorylation step for its activation: it already possesses non-degradable phosphonomethyl ether grouping, which mimics phosphoric ester moiety (Chart 1). The synthesis of (*S*)-HPMPCp (analogue of dCDP) is catalyzed by pyrimidine nucleoside monophosphate kinase[8,9]. Pyruvate kinase, creatine kinase and nucleoside diphosphate kinase can phosphorylate (*S*)-HPMPCp further to (*S*)-HPMPCpp (analogue of dCTP) (ref.[9]). (*S*)-HPMPCpp is also metabolized by CTP:cholinephosphate cytidylyltranferase into HPMPCp-choline which may serve as an intracellular depot form of (*S*)-HPMPCpp (ref.[9]).

Antiviral activity of cidofovir is explained by its interaction with the virus replication apparatus. This hypothesis is supported by the observation that the resistance of human cytomegalovirus and herpes simplex virus against cidofovir is associated with specific mutations in the viral DNA polymerase gene[10,11]. (*S*)-HPMPCpp has been proven to be an inhibitor of HCMV DNA polymerase as well as its substrate[12,13]. The viral enzyme is able to incorporate two consecutive (*S*)-HPMPC molecules into DNA strand. After the incorporation, 3′-5′-exonucleolytic activity of HCMV pol does not excise this substance from DNA (ref.[13]).

In contrast to inhibition of viral polymerases it has been shown that (*S*)-HPMPCpp is a weak inhibitor of cellular DNA polymerases α, β and γ (refs[14,15]).

(*S*)-HPMPC also exhibits an *in vivo* cytostatic effect against virus-induced tumors as demonstrated by regressions of hemangioma in rats and papillomatous lesions in humans (for a review, see ref.[16]).

This paper describes interactions of (*S*)-HPMPCpp with eukaryotic nuclear DNA polymerases α, δ and proteolyzed form of DNA polymerase ε (pol ε*), which probably participate in chromosomal DNA replication (for a review,

Abbreviations: CMV, cytomegalovirus; HCMV, human cytomegalovirus; (*S*)-HPMPA, 9-[(*S*)-3-hydroxy-2-(phosphonomethoxy)propyl]adenine; (*S*)-HPMPApp, 9-[(*S*)-3-hydroxy-2-(phosphonomethoxy)propyl]adenine diphosphate; (*S*)-HPMPC, 1-[(*S*)-3-hydroxy-2-(phosphonomethoxy)propyl]cytosine; (*S*)-HPMPCpp, 1-[(*S*)-3-hydroxy-2-(phosphonomethoxy)propyl]cytosine diphosphate; PCNA, proliferating cell nuclear antigen; PMEA, 9-[2-(phosphonomethoxy)ethyl]adenine; PMEApp, 9-[2-(phosphonomethoxy)ethyl]adenine diphosphate; PMEDAP, 2,6-diamino-9-[2-(phosphonomethoxy)ethyl]purine; PMEDAPpp, 2,6-diamino-9-[2-(phosphonomethoxy)ethyl]purine diphosphate; PMEG, 9-[2-(phosphonomethoxy)ethyl]guanine; PMEGpp, 9-[2-(phosphonomethoxy)ethyl]guanine diphosphate; (*R*)-PMPA, 9-[(*R*)-2-(phosphonomethoxy)propyl]adenine; (*R*)-PMPApp, 9-[(*R*)-2-(phosphonomethoxy)propyl]adenine diphosphate; (*R*)-PMPDAP, 2,6-diamino-9-[(*R*)-2-(phosphonomethoxy)propyl]purine; (*R*)-PMPDAPpp, 2,6-diamino-9-[(*R*)-2-(phosphonomethoxy)propyl]purine diphosphate; pol, DNA polymerase.

see refs[17,18]). We focused on the inhibitory potency of this dCTP analogue and on its substrate activity toward the mentioned enzymes.

RESULTS AND DISCUSSION

Enzyme inhibition experiments revealed that cidofovir diphosphate is a competitive inhibitor (with respect to dCTP) of rat DNA polymerases α, δ and ε*. The $^{ANPpp}K_i/^{dNTP}K_m$ values summarized in Table I show that (*S*)-HPMPCpp is approximately 3–7 times weaker inhibitor of these three enzymes than (*S*)-HPMPApp. (*S*)-HPMPApp also seems to have only a low inhibitory potency against these DNA polymerases. This observation contrasts with the previously published data that (*S*)-HPMPApp strongly inhibits in particular pol ε* on the homopolymeric template-primer poly dT-oligo dA$_{12-18}$ (ref.[19]). Different reaction conditions and/or DNA template-primers used in this study can explain this contrast. It has been shown earlier that also PMEGpp inhibits pol ε about 60 times less efficiently on the short synthetic template-primer than on the long homopolymeric template-primer poly dC-oligo dG$_{12-18}$ (refs[19,20]).

The elongation of template-primer TP3 and TP4 (Table II, Figs 1, 2) in the presence of (*S*)-HPMPCpp down the template regions containing dGMP indicates that this compound not only competes with dCTP for the active site of pol α, δ and ε* but, similar by to other ANPpp (refs[20-23]), also acts as their substrate. (*S*)-HPMPA, PMEDAP and PMEG are also incorporated into synthetic template-primers by pol α, δ and ε* and have been consistently detected in the genomic DNA of cells treated with the respective parent compounds[23-25]. Therefore, one can expect that (*S*)-HPMPC is also incorporated

TABLE I
Inhibition of DNA polymerases α, δ and ε* by (*S*)-HPMPCpp and (*S*)-HPMPApp on the template-primer TP1 and TP2

Enzyme	$^{dCTP}K_m{}^a$ μmol l^{-1}	$^{HPMPCpp}K_i/$ $^{dCTP}K_m{}^a$	$^{dCTP}K_m{}^b$ μmol l^{-1}	$^{HPMPCpp}K_i/$ $^{dCTP}K_m{}^b$	$^{dATP}K_m{}^b$ μmol l^{-1}	$^{HPMPApp}K_i/$ $^{dATP}K_m{}^b$
Pol α	1.7 ± 0.3	35.3	0.8 ± 0.05	44.7	1.5 ± 0.8	6.8
Pol δ	0.31 ± 0.06	2	0.24 ± 0.02	7	1.0 ± 0.1	2.3
Pol ε*	5.8 ± 0.9	2.6	0.9 ± 0.15	5.9	3.5 ± 0.4	1.5

Experiments were carried out on the template-primer: ^{a} TP1 (Table II) and/or ^{b} TP2 (Table II) under reaction conditions described in Experimental. (In all cases, the inhibition showed a competitive character.)

Template-primer symbol	Template-primer sequence
TP1	⁵GATTACGAATTCGAGCTC
	³CTAATGCTTAAGCTCGAGCCAT**GGG**CCCCTA**GGA**G**A**TCTC⁵
TP2	⁵GAGATCTCCTAGGGGCCC
	³CTCTAGAGGATCCCCGGGTACC**GAG**CTC**G**AATTC**G**TAATC⁵
TP3[b]	⁵GAGATCTCCTAGGGG
	³CTCTAGAGGATCCCC**GGGG**TTTAT**GAG**CAA**GAGAG**CAAT**GGG**⁵
TP4[b]	⁵GAGATCTCCTTGGGG
	³CTCTAGAGGAACCCCTTTTACCTATCTC**G**AATTC**G**TAATC⁵

[a] The nucleotides in bold letters denote incorporation sites of (*S*)-HPMPCpp and/or dCTP.
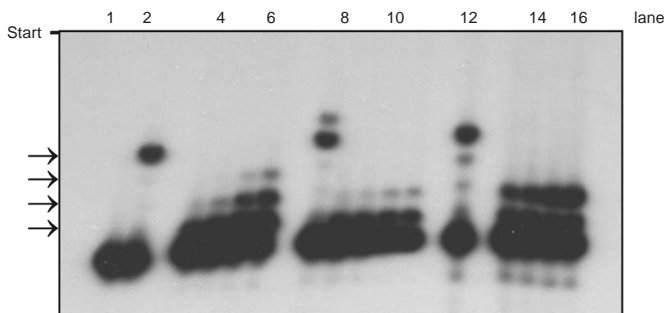[b] Primer was labeled on its 5′-OH end with ³²P.

FIG. 1

Incorporation of (*S*)-HPMPC into template-primer TP3 catalyzed by DNA polymerases α, δ and ε*. Reaction mixture (25 μl) containing template-primer TP3 (molar ratio 1.5 : 1; 1.5 μM primer), 50 μM dGTP, dTTP, dATP and (*S*)-HPMPCpp was incubated for 20, 40, 60 and 120 min at 37 °C in the presence of 4 U ml⁻¹ pol α (lanes 3–6), 4 U ml⁻¹ pol δ (lanes 8–11) or 4 U ml⁻¹ pol ε* (lanes 13–16). Reaction products were analyzed by electrophoresis in 20% polyacrylamide gel under denaturing conditions. Lane 1 – control (³²P labeled primer); lanes 2, 7 and 12 – reaction incubated for 10 min in the presence of 50 μM dCTP and 4 U ml⁻¹ pol α, δ or ε*; → (*S*)-HPMPC incorporation site

into nuclear DNA during DNA replication. The time course of (*S*)-HPMPC accumulation in TP3 revealed that pol δ and ε* can incorporate into the nascent DNA chain two consecutive (*S*)-HPMPC residues at the maximum, whereas pol α can insert a sequence of up to three HPMPC residues (Fig. 1). At 50 μM concentration, the rate of (*S*)-HPMPC incorporation into TP3 by pol α, δ and ε* was 8, 17 and 6 times lower than that for dCMP, respectively.

Figure 2 shows that all the mentioned enzymes can insert more than 14 nucleotides into template-primer TP4 in the presence of (*S*)-HPMPCpp, dGTP, dATP and dTTP indicating that they can overcome the -CGAA- sequence in the template. This experiment proves that the three DNA polymerases can incorporate dNMP into the nascent DNA strand containing (*S*)-HPMPC residue at its 3′-OH end. This fact implies that the genome of proliferating cells treated with cidofovir presumably contains the molecules of (*S*)-HPMPC internally incorporated inside the daughter DNA strands. When they predominate physical damage of nuclear DNA may occur.
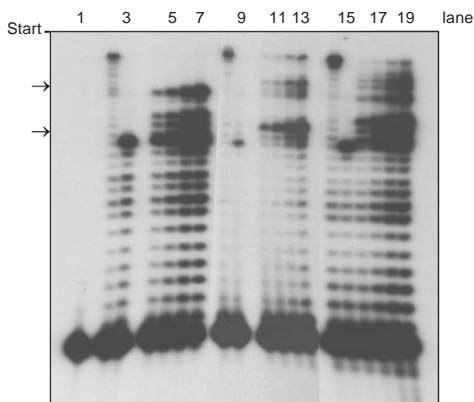

FIG. 2

Time dependence of (*S*)-HPMPC incorporation into template-primer TP4 catalyzed by DNA polymerases α, δ and ε*. Reaction mixture (25 μl) containing template-primer TP4 (molar ratio 1.5 : 1; 1.5 μM primer), 50 μM dGTP, dTTP, dATP and (*S*)-HPMPCpp was incubated for 10, 20, 40 and 80 min at 37 °C in the presence of 4 U ml^{-1} pol α (lanes 4–7), 4 U ml^{-1} pol δ (lanes 10–13) or 4 U ml^{-1} pol ε* (lanes 16–19). Reaction products were analyzed by electrophoresis in 20% polyacrylamide gel under denaturing conditions. Lane 1 – control (^{32}P labeled primer); lanes 2, 8 and 14 – reaction incubated for 10 min in the presence of 50 μM dGTP, dTTP, dATP, dCTP and 4 U ml^{-1} pol α, δ or ε*; lanes 3, 9 and 15 reaction incubated for 10 min in the presence of 50 μM dGTP, dTTP, dATP and 4 U ml^{-1} pol α, δ or ε*; → (*S*)-HPMPC incorporation site, start up

Furthermore, our observations suggest that the insertion of (*S*)-HPMPC into DNA strand results in the decreased processivity of DNA synthesis. In the presence of (*S*)-HPMPCpp, replication products accumulate whose length varies between 29 (the first (*S*)-HPMPC incorporation site) and 40 nucleotides (the full length product) compared to those observed in the presence of dCTP (Fig. 2; lanes 2, 8, 14). This fact indicates a high tendency of DNA polymerases to dissociate from template-primer before and after the insertion of (*S*)-HPMPC into DNA.

By comparing the $^{ANPpp}K_i/^{dNTP}K_m$ ratios determined for pol α, δ and ε*, it is possible to classify the so-far tested ANPpp into three groups according to their decreasing inhibitory efficacy: PMEGpp >> PMEDAPpp, (*S*)-HPMPApp, PMEApp, (*S*)-HPMPCpp >> (*R*)-PMPDAPpp = (*R*)-PMPApp (refs[19,26,27]). The first group contains only one compound, PMEGpp, which is at least 10 times more potent as an inhibitor of the listed enzymes than any member of the second group, comprising PMEDAPpp, (*S*)-HPMPApp, PMEApp and (*S*)-HPMPCpp. The order of inhibitory efficacy within the second group varies depending on the type of DNA polymerase[19,27]. All these antimetabolites are at least 10 times more potent inhibitors of pol α, δ and ε* than (*R*)-PMPDAPpp and (*R*)-PMPApp (ref.[27]).

In vitro cytostatic activity of acyclic nucleotide analogues decreases in the order of PMEG >> PMEDAP, (*S*)-HPMPA, PMEA, (*S*)-HPMPC >> (*R*)-PMPDAP, (*R*)-PMPA; in some cases [PMEDAP, (*S*)-HPMPA, PMEA, (*S*)-HPMPC], it varies with the type of the cell culture[28–33]. Nevertheless, a certain degree of correlation exists between the cytostatic effect of these substances and their ability to inhibit pol α, δ and ε* (ref.[19]). It has been shown that the stalling of DNA replication forks and DNA damage can activate specific signal transduction pathways resulting in slower cell cycle progression and activation of processes aimed at resuming the homeostatic state of the cell (for a review, see refs[34,35]). Stimulation of these pathways due to ANPpp interactions with DNA synthesis could explain the above correlation.

Summing up, we studied the interactions of (*S*)-HPMPCpp with eukaryotic nuclear DNA polymerases α, δ and proteolyzed form of DNA polymerase ε (pol ε*), focusing on the inhibitory potencies of this compound and its ability to be utilized as substrate of the enzymes mentioned.

EXPERIMENTAL

Compounds

The synthesis of (*S*)-HPMPC and its diphosphate was performed according to the published procedures[36,37]. Deoxyribonucleotides (dATP, dGTP, dCTP and dTTP), labeled nucleotides

[8-^{3}H]dATP (888 GBq mmol^{-1}), [^{3}H]dCTP and [γ-^{32}P]ATP (110 TBq mmol^{-1}), MicroSpin G-25 columns, T4 polynucleotide kinase (PNK) and urea were products of Amersham Pharmacia Biotech. Oligodeoxyribonucleotides (Table II) were synthesized by MWG-Biotech (Germany). DEAE Sephacel, EDTA, bovine serum albumine (BSA), glycerol and DTT were purchased from Sigma (Czech Republic).

DNA Polymerases and PCNA

DNA polymerases α, δ and proteolyzed form of DNA polymerase ε (pol ε*) were isolated from Sprague–Dawley rat compact transplantable lymphomas by using the purification procedures described in our previous communications[26,38] except for the last step (glycerol gradient). One unit (U) of the DNA polymerase activity is defined as an amount of enzyme that catalyzes incorporation of 1 nmol of dATP into acid-insoluble precipitate after 30 min under the published conditions[19]. PCNA (proliferating cell nuclear antigen) was purified to homogeneity according to Fien and Stillman[39] from *E. coli* strain BL 1/DE 3 harboring a plasmid encoding the human PCNA. This bacterial strain was kindly provided by Dr B. Stillman, Cold Spring Harbor Laboratory (Cold Spring Harbor (NY), U.S.A.).

DNA Polymerase Assay

The enzymatic activities of DNA polymerases α, δ and ε* on template-primers TP1–TP4 (Table II) were determined under the following reaction conditions: (i) pol α – 40 mM HEPES–KOH (pH 7.0), 25 mM KCl, 10 mM MgCl$_2$; (ii) pol δ – 40 mM HEPES–KOH (pH 7.0), 50 mM KCl, 10 mM MgCl$_2$ and PCNA (18 μg ml^{-1}); (iii) pol ε* – 40 mM HEPES–KOH (pH 7.5), 100 mM KCl, 10 mM MgCl$_2$. All reaction mixtures contained also 1 mM DTT, 200 μg ml^{-1} BSA and 10% glycerol.

Kinetic Experiments

Kinetic constants ($^{(S)\text{-HPMPCpp}}K_i$, $^{(S)\text{-HPMPApp}}K_i$, $^{dCTP}K_m$, $^{dATP}K_m$) for DNA polymerases α, δ and ε* on the template-primer TP1 and/or TP2 (molar ratio 1.5 : 1; 1 μmol l^{-1} primer; Table II) were measured under the reaction conditions described above. The determination of $^{dCTP}K_m$ values was carried out in reaction mixtures which contained 50 μM dGTP, dTTP, dATP and 10 different concentrations of [^{3}H]dCTP (370 kBq ml^{-1}). The Michaelis–Menten constants for dATP ($^{dATP}K_m$) were determined in the presence of 50 μM dGTP, dTTP, dCTP and 10 varying concentrations of [^{3}H]dATP (370 kBq ml^{-1}).

The evaluation of the inhibitory constants for (S)-HPMPCpp ($^{(S)\text{-HPMPCpp}}K_i$) was conducted using 50 μM dGTP, dTTP, dATP, four different concentrations of the substrate ([^{3}H]dCTP; 370 kBq ml^{-1}) and five concentrations of inhibitor. Similarly $^{(S)\text{-HPMPApp}}K_i$ values for pol α, δ and ε* were determined in the presence of 50 μM dGTP, dTTP, dCTP, at four concentrations of [^{3}H]dATP (370 kBq ml^{-1}) and five concentrations of (S)-HPMPApp.

The assays (25 μl) were performed at 37 °C for 10, 20 and 30 min, and terminated by spotting an appropriate aliquot of the sample onto a GF/A fiberglass disk, which was then immersed in cold 20 mM Na$_4$P$_2$O$_7$ in 5% trichloroacetic acid. Disks were extensively washed three times with the same solution, then with 96% ethanol and finally dried. The trichloroacetic acid-insoluble radioactivity was determined in a toluene-based scintillation fluid using scintillation spectrometer (Beckman).

Incorporation of (*S*)-HPMPCpp into DNA

The ability of DNA polymerases α, δ and ε* to insert (*S*)-HPMPCpp into DNA was studied using the template-primer TP3 and/or TP4 (molar ratio 1.5 : 1; 1 μM primer). The primers were labeled on their 5′-OH ends with [32]P. The reaction mixture (30 μl) contained 50 μM dGTP, dTTP, dATP and 50 μM (*S*)-HPMPCpp or 50 μM dCTP in the case of template-primer TP4 or 50 μM (*S*)-HPMPCpp or 50 μM dCTP in the case of template-primer TP3. The mixture was incubated at 37 °C for various time periods. After incubation, the reaction was terminated by addition of an equivalent volume of 98% deionized formamide containing 40 mM EDTA, 0.2% Bromophenol Blue and 0.2% Xylene Cyanole FF. Samples were then heated to 98 °C (5 min), cooled to 4 °C and loaded on 20% polyacrylamide gels containing 89 mM Tris-borate (pH 8.3), 2 mM EDTA and 7 M urea[40]. After electrophoresis (1 800 V, 4 h), the gel was dried and the amount of reaction products was measured using Phosphoimager.

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