Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical Pharmacology 70 (2005) 894–900
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Inhibition of human telomerase by diphosphates of acyclic nucleoside phosphonates

Miroslav Hájek *, Naděžda Matulová, Ivan Votruba, Antonín Holý, Eva Tloušťová

Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, 166 10 Prague 6, Czech Republic

Received 17 March 2005; accepted 7 June 2005

Abstract

Diphosphates of the antiviral acyclic nucleoside phosphonates (ANPs) were evaluated in telomeric repeat amplification protocol (TRAP) for their ability to inhibit the extension of telomeres by human telomerase. Extracts from human leukaemia HL-60 cells were used as a source of the enzyme. Data show that the most effective compound studied was the guanine derivative PMEGpp (IC_{50} 12.7 ± 0.5 μmol L^{-1} at 125 μmol L^{-1} deoxynucleoside triphosphates (dNTPs)). The inhibitory effects of other PME, PMP and HPMP diphosphates on telomerase reverse transcriptase decreased in the order: (*R*)-PMPGpp > (*R*)-HPMPGpp > PMEDAPpp > (*S*)-PMPGpp > (*S*)-HPMPApp >-PMEO-DAPypp > (*R*)-6-cyprPMPDAPpp > (*R*)-PMPApp > (*R*)-PMPDAPpp ≥ PMEApp ≥ PMECpp > PMETpp > (*S*)-PMPApp ~ 6-Me_2PMEDAPpp. These results are consistent with the observed antineoplastic activities of the parental guanine (PMEG) and 2,6-diaminopurine (PMEDAP) PME-derivatives. Moreover, structure–activity relationship indicates enantioselectivity of some of these human telomerase inhibitors: (*R*)-isomers of the PMP-derivatives possess stronger inhibitory potency towards the enzyme than (*S*)-isomers. The data may contribute to the rational design of telomerase inhibitors based on the structure of acyclic nucleotide analogues.

Keywords: Acyclic nucleoside phosphonates; Human telomerase; Telomerase inhibition; Reverse transcriptase; TRAP assay; Repeat addition processivity

Abbreviations: ANP, acyclic nucleoside phosphonate; ANPpp, ANP diphosphate; araGTP, 9-β-D-arabinofuranosylguanine 5′-triphosphate; AZT-TP, 3′-azido-3′-deoxythymidine 5′-triphosphate; CBV-TP, 2′,3′-didehydro-2′,3′-dideoxyguanosine 5′-triphosphate; CdG-TP, carbocyclic 2′-deoxyguanosine 5′-triphosphate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; d4TTP, 2′,3′-didehydro-2′,3′-dideoxythymidine 5′-triphosphate; ddC, 2′,3′-dideoxycytidine; deazadATP, 7-deaza-2′-deoxyadenosine 5′-triphosphate; deazadGTP, 7-deaza-2′-deoxyguanosine 5′-triphosphate; FaraTTP, 2′-fluoro-2′-deoxy-β-arabinofuranosylthymine 5′-triphosphate; GI_{50}, growth inhibition concentration; hTERT, human telomerase reverse transcriptase; 6-Me_2PMEDAP, 2-amino-6-(dimethylamino)-9-[2-(phosphonomethoxy)ethyl]purine; PMEA, 9-[2-(phosphonomethoxy)ethyl]adenine; PMEC, 1-[2-(phosphonomethoxy)ethyl]cytosine; PMEDAP, 2,6-diamino-9-[2-(phosphonomethoxy)ethyl]purine; PMEG, 9-[2-(phosphonomethoxy)ethyl]guanine; PMEO-DAPy, 2,4-diamino-6-[2-(phosphonomethoxy)ethoxy]pyrimidine; PMET, 1-[2-(phosphonomethoxy)ethyl]thymine; (*R*)-6-cyprPMPDAP, (*R*)-2-amino-6-(cyclopropylamino)-9-[2-(phosphonomethoxy)propyl]purine; (*R*)-HPMPG, (*R*)-9-[(3-hydroxy-2-phosphonomethoxy)propyl]guanine; (*R*)-PMPA, (*R*)-9-[2-(phosphonomethoxy)propyl]adenine; (*R*)-PMPDAP, (*R*)-2,6-diamino-9-[2-(phosphonomethoxy)propyl]purine; (*S*)-HPMPA, (*S*)-9-[(3-hydroxy-2-phosphonomethoxy)propyl]adenine; (*S*)-PMPA, (*S*)-9-[2-(phosphonomethoxy)propyl]-adenine; (*S*)-PMPG, (*S*)-9-[2-(phosphonomethoxy)propyl]guanine; thiodGTP, 6-thio-2′-deoxyguanosine 5′-triphosphate; TRAP, telomeric repeat amplification protocol; TS, telomerase substrate (nontelomeric oligonucleotide)

* Corresponding author. Tel.: +420 220 183 393; fax: +420 220 183 560.
E-mail address: hajek@uochb.cas.cz (M. Hájek).

0006-2952/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2005.06.007

1. Introduction

Human telomerase is a large cellular ribonucleoprotein complex [1–3] that is responsible for adding the telomeric repeats to the 3′ ends of chromosomes [4] and thus compensating for the telomere loss that accompanies chromosomal replication and cell division. Telomerase is upregulated in almost 90% of all malignancies [5–7]. Hence, it is thought to be very promising not only as a tumor-specific marker but also as a target for anticancer treatment [8]. The telomerase holoenzyme complex presents multiple potential sites for the development of inhibitors [9].

The identification of the hTERT component of telomerase [10] as a functional catalytic reverse transcriptase [11,12] prompted studies of inhibiting telomerase with established HIV reverse transcriptase inhibitors, such as the chain-terminating 3′-azido-3′-deoxythymidine (AZT) and 2′,3′-dideoxycytidine (ddC). These parent nucleosides are transformed in vivo in the form of triphosphates, which inhibit reverse transcriptases by acting as competitive substrates for the enzyme and terminate DNA synthesis

de novo [13]. AZT-TP inhibits the activity of telomerase to ~50% at 30 μmol L^{-1} [14]. Other nucleotide-based inhibitors, both purine- and pyrimidine-derivatives, have been evaluated against telomerase with effective IC_{50} values in the micromolar range [14]. In a more recent study, Fletcher et al. [15] reported the use of deazadeoxypurines as inhibitors of telomerase with one compound, 6-thio-7-deaza-2′-deoxyguanosine 5′-triphosphate showing an IC_{50} value of 60 nmol L^{-1}. Series of the other nucleotide analogues (ddGTP, ddATP, ddTTP, d4TTP, deazadGTP, deazadATP, thiodGTP, CBV-TP, araGTP, and FaraTTP) have previously been shown to inhibit telomerase activity [1,13,14,16–19].

Numerous acyclic nucleoside phosphonates (ANPs) possess excellent antiviral activities against a broad spectrum of DNA viruses and retroviruses as well as a significant antiproliferative potency [20]. (*S*)-HPMPC (cidofovir, CDV, Vistide™), (*R*)-PMPA (tenofovir, TDV, Viread™), PMEA (adefovir, ADV, Hepsera™) were approved for the treatment of cytomegalovirus retinis in AIDS patients, HIV infection, and chronic hepatitis B, respectively.

In cells, ANPs are phosphorylated to their diphosphates, active antimetabolites, which inhibit viral and/or cellular replicases and terminate nascent DNA chain [20]. ANP diphosphates are analogues of natural nucleoside 5′-triphosphates since their nonphosphorylated parental forms already bear chemically stable *O*-phosphonomethyl ether group resistant to enzymatic degradation [20].

In this work, we studied the interaction of selected ANP diphosphates with telomerase from HL-60 cells to increase our understanding of the substrate requirements of this enzyme. ANP diphosphates were evaluated in the PCR-based telomeric repeat amplification protocol (TRAP) assay [5,21,22] for their ability to inhibit the extension of telomeres by human telomerase, using extracts from human leukaemia HL-60 cells as a source of the enzyme.

2. Materials and methods

2.1. Compounds

The following compounds were used in this study (Fig. 1): (*S*)-HPMPApp, (*R*)-HPMPGpp, PMEApp, PMECpp, PMEGpp, PMETpp, PMEDAPpp, PMEODAPypp, 6-Me_2PMEDAPpp, (*R*)-6-cyprPMPDAPpp, (*R*)-PMPDAPpp, (*R*)-PMPApp, (*S*)-PMPApp, (*R*)-PMPGpp, (*S*)-PMPGpp. (*S*)-HPMPA and (*R*)-HPMPG diphosphates were synthesized according to Otmar et al. [23]. Other ANP diphosphates were synthesized by the modified morpholidate method. In a typical experiment, a mixture of ANP (free acid, 1 mmol), *N*,*N*′-dicyclohexylcarbodiimide (1.3 g) and morpholine (2 mL) in 80% aqueous *tert*-butanol (20 mL) was refluxed under stirring for 6–8 h and evaporated in vacuo. The residue in water (100 mL) was filtered over Celite®, the filtrate extracted with ether (3 × 50 mL) and the aqueous phase was taken down in vacuo. The residue was transferred into 100-mL flask, evaporated, codistilled with ethanol (2 × 20 mL) and dried overnight at 15 Pa over phosphorus pentoxide. Bis(-tributylammonium) monophosphate or tris(tributylammonium) diphosphate solution in dry dimethylsulfoxide (1 mol L^{-1}, 2.5 mL) was added and the mixture was stirred at ambient temperature in a tightly closed flask for 4–6 days. Reaction mixture was then acidified with 6 M HCl to pH 3 and an appropriate amount of activated charcoal was added. After an exhaustive washing of pelleted activated charcoal with HPLC water, desalted nucleotides were eluted by 5% NH_4OH in 50% methanol. The eluate was evaporated at 30 °C, dissolved in 0.05 M triethylammonium bicarbonate and purified by chromatography on POROS® 50HQ anion exchanger (Applied Biosystems, Foster City, CA, USA) in the linear concentration gradient of triethylammonium bicarbonate (0.05–0.4 mol L^{-1}). Peak corresponding to ANPpp (triethylammonium salt) was collected, evaporated in vacuo at room temperature and then converted to the ANPpp sodium salt on DOWEX™ 50X8 (Na^+) (SERVA Electrophoresis GmbH, Heidelberg, Germany).

All other chemicals and materials were commercial products, e.g. activated charcoal, *N*,*N*′-dicyclohexylcarbodiimide, morpholine, *tert*-butanol, Celite®, dimethylsulfoxide, ddGTP, streptomycin, penicillin G, CHAPS, β-mercaptoethanol, RNase A, proteinase K, PBS and RPMI 1640 medium (Sigma–Aldrich, St. Louis, MO, USA), fetal calf serum (PAA Laboratories GmbH, Pasching, Austria), Vitamin B_{12} (Léčiva a.s., Prague, Czech Republic), Pefabloc-SC, Protector RNase inhibitor (Roche Diagnostics GmbH, Mannheim, Germany), [γ-^{32}P]ATP (MP Biomedicals GmbH, Germany), T4 polynucleotide kinase buffer and T4 polynucleotide kinase (TaKaRa Bio Inc., Shiga, Japan), HEPES, deoxynucleoside triphosphates (dNTPs), Taq polymerase reaction buffer, Taq DNA polymerase (Promega, Madison, WI, USA) and TS, ACX, NT, TSNT primers (Invitrogen Ltd., Paisley, UK).

2.2. Cell culture

Human acute promyelocytic leukaemia HL-60 cells (ATCC CCL 240) were cultured in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, antibiotics (200 μg mL^{-1} of streptomycin and 200 units mL^{-1} of penicillin G), 10 mM β-mercaptoethanol, and Vitamin B_{12} at 37 °C in a humidified atmosphere containing 5% CO_2. After harvesting in log-phase growth, cells were pelleted, washed in PBS, and frozen at −70 °C.

2.3. Preparation of cell lysates

Extracts with telomerase activity were prepared and analyzed as described [5] with some modifications. Briefly,

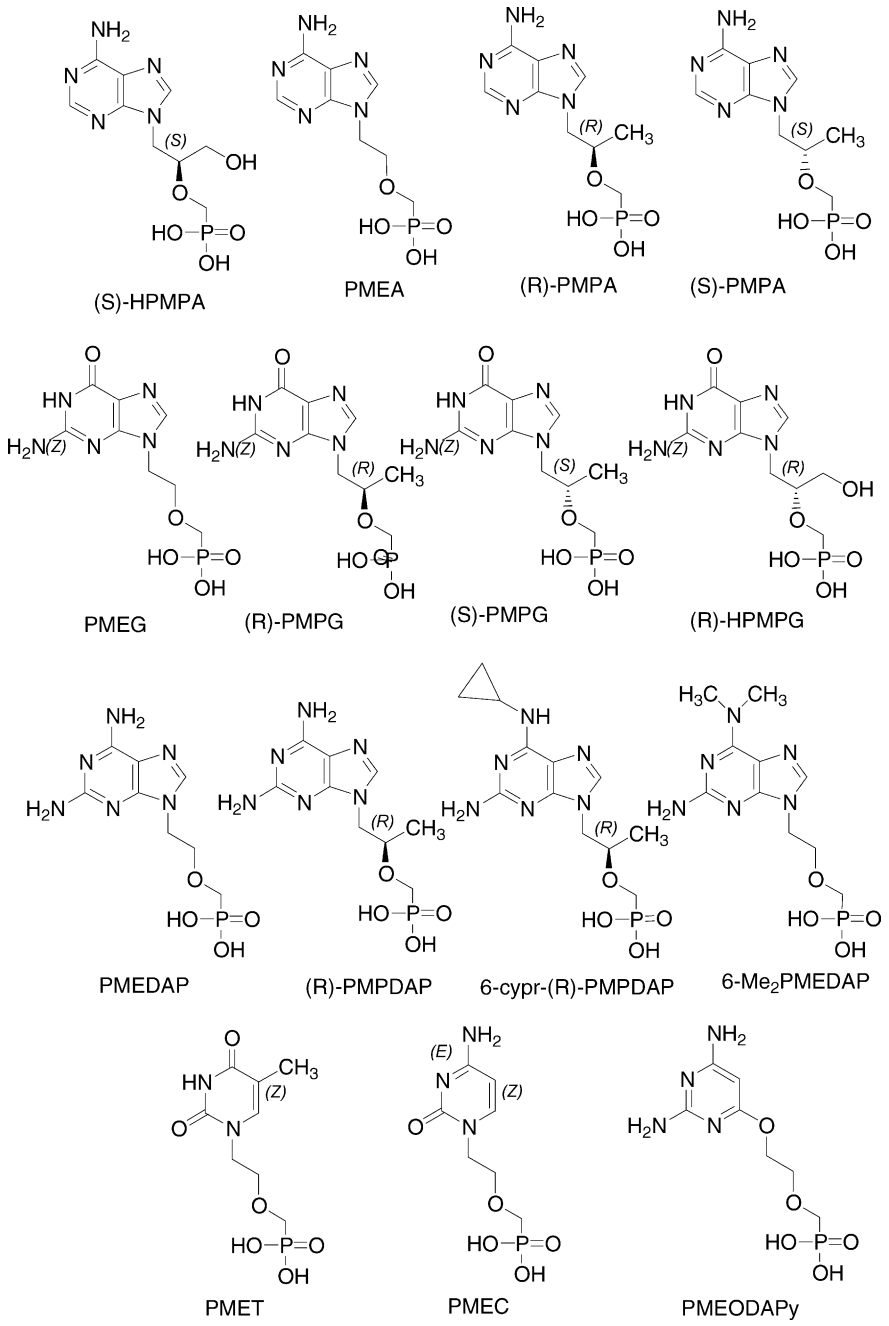

Fig. 1. Structure of PME, PMEO, PMP, and HPMP purines and pyrimidines.

the cells were thawed and incubated on ice for 30 min in CHAPS lysis buffer containing 0.5% CHAPS, 10 mM HEPES–NaOH (pH 7.5), 1 mM $MgCl_2$, 1 mM EGTA, 5 mM β-mercaptoethanol, 2 mM Pefabloc-SC, 10% glycerol plus 1 μL (40 U) of Protector RNase inhibitor. Two hundred microlitres of CHAPS lysis buffer was used to lyse one million cells. In order to ensure lysis of HL-60 cells, these cells were subjected to two freeze/thaw cycles during the incubation. This additional step did not affect telomerase activity in the HL-60 cells. Cell debris was pelleted for 20 min at $16{,}000 \times g$ at 4 °C. The supernatant was removed, aliquoted, frozen on dry ice, and stored at −70 °C. The protein concentration of the supernatant was determined by the Bradford assay.

2.4. TRAP assay

Telomerase activity was determined using the TRAP assay as described by Kim and Wu [22] with the modifications described below. In the present study, the sensitivity of the TRAP assay was increased by the prolongation of incubation time from 10 to 15 min and by increasing the number of PCR cycles from 27 to 33. The dependence of the amount of amplified telomerase product versus number

of PCR cycle was linear in the range of 30–35 cycles (data not shown). An aliquot of 800 pmol of TS substrate primer (5′-AATCCGTCGAGCAGAGTT-3′) was labeled in 100 μL reaction mixture containing 60 μCi [γ-^{32}P]ATP (60 mCi mL^{-1}, 7000 Ci mmol^{-1}), T4 polynucleotide kinase buffer and 40 U T4 polynucleotide kinase. After 30 min incubation at 37 °C and then 2 min at 85 °C, an excess of unincorporated [γ-^{32}P]ATP was removed from reaction mixture on MicroSpin™ G-25 Column (Amersham Biosciences, Piscataway, NJ, USA). Forty microlitre TRAP reactions contained Taq polymerase reaction buffer (50 mM KCl, 10 mM Tris–HCl (pH 9.0 at 25 °C), 1.5 mM $MgCl_2$, 0.1% Triton X®-100), dNTPs (30, 60 and 125 μmol L^{-1}), 18 pmol of end-labeled TS substrate primer, an appropriate amount of studied ANPpp, ANPp and ANP, respectively. Reaction was started by cell extract addition (0.15 μg protein). Each TRAP reaction mixture was placed in a thermocycler block preheated to 30 °C and incubated at 30 °C for 15 min and then heated at 95 °C for 2 min (for one cycle) to stop telomerase reaction. After addition of 10 μL of mixture containing 6 pmol ACX reverse primer (5′-GCGCGG[CTTACC]$_3$CTAACC-3′), 3 pmol NT internal control primer, 0.01 amol TSNT internal control and 1.25 U Taq DNA polymerase, the reaction was cycled 33 times at 94 °C for 20 s, 52 °C for 30 s, 72 °C for 20 s.

To ensure that the observed telomerase activities were really dependent on telomerase, numerous of inactivation experiments were performed. Aliquot of cell lysate was incubated with RNase A (50 μg mL^{-1}) at 37 °C for 30 min. Proteinase K and heat-inactivated cell extracts were prepared by incubating the cell extract with proteinase K (50 μg mL^{-1}) at 37 °C for 30 min and by heating 10 μL extract at 75 °C for 10 min prior to assaying of 3 μL by TRAP assay. HL-60 cell extract showed telomerase activity with the characteristic primer extension-binding pattern on the autoradiographs. We considered the sample being positive for telomerase activity if the signal had disappeared after RNase A treatment and if no signal was detected in the lysis buffer alone (negative control). RNase A and proteinase K treatments abolished the PCR product ladder bands and confirmed both the protein and RNA dependence of the enzyme activity.

2.5. Analysis of reaction products and quantitation of telomerase activity

The amplified telomerase products were analyzed on a denaturating 15% polyacrylamide–7 M urea sequencing gel at 1900 V for 2 h with Tris–borate-EDTA. Dried gels were exposed to a PhosphorImager storage screen and the amount of reaction products was evaluated using TYPHOON™ 9410 imager\ImageQuant™ software (Molecular Dynamics, Sunnyvale, CA, USA). To compare relative telomerase activity in the presence of inhibitors, the TRAP assay signals of the telomerase ladders were normalized to the signal of the corresponding internal standard after background substraction. The signal intensity of the bands from ANPpp, ANPp and ANP-treated samples was expressed as a percentage of the signal intensity detected in the control. Their relative intensities were calculated with the ImageQuant™ software. All results were expressed as means ± S.D. of the four independent determinations.

3. Results

The approximate IC_{50} values for the PME-derivatives are shown in Table 1, where they are listed in the order of their telomerase inhibitory potency: PMEGpp > PMEDAPpp > PMEO-DAPypp > PMEApp > PMECpp ≥ PMETpp > 6-Me$_2$PMEDAPpp.

The guanine derivative PMEGpp is the most potent telomerase inhibitor among all acyclic nucleotide analogues studied with the IC_{50} 12.7 ± 0.5 μmol L^{-1} at 125 μM dNTPs (Table 1, Fig. 2). Its inhibitory potency towards telomerase is comparable to that of ddGTP (IC_{50} 8.1 ± 0.4 μmol L^{-1} at 125 μM dNTPs), which is known to be one of the most effective nucleotide analogue-based telomerase inhibitors. PMEGpp inhibits telomerase activity by 50% when it is present in the dGTP concentration range of 0.08–0.12 only, depending on the dNTPs concentration. The PMEG monophosphate and PMEG itself do not show at 125 μM dNTPs any effect on telomerase activity up to the concentration of 300 μM PMEG and/or PMEGp, respectively.

PMEDAPpp, which selectively inhibits DNA polymerase δ and exerts significant cytostatic effects [20], inhibits the activity of telomerase with IC_{50} 76 ± 13.5 μmol L^{-1} (at 125 μM dNTPs). Surprisingly, its N^6-dimethyl derivative 6-Me$_2$PMEDAPpp increases repeat addition processivity of the enzyme (Fig. 3A). 6-Me$_2$PMEDAP has no effect on telomerase ladder pattern.

Neither PMEApp, PMETpp or PMECpp, which inhibit retroviral reverse transcriptases [20], show any significant inhibitory potency towards telomerase. An inhibition was

Table 1
Inhibition of HL-60 telomerase by PME type of ANPpp (IC_{50})[a]

Compound	dNTPs (μmol L^{-1})		
	125	60	30
PMEGpp	12.7 ± 0.5	7.1 ± 1.7	2.30 ± 0.04
PMEDAPpp	76.0 ± 13.5	41.6 ± 10.1	23.3 ± 3.2
PMEO-DAPypp	133 ± 15	N.D.[b]	N.D.
PMEApp	380 ± 28	N.D.	110 ± 11
PMECpp	N.I.[c]	N.D.	106 ± 16
PMETpp	N.I.	N.D.	N.I.
6-Me$_2$PMEDAPpp	P.E.[d]	N.D.	N.D.

[a] Values are means ± S.D. of the four independent determinations.
[b] Not determined.
[c] No inhibition.
[d] Processivity enhancement.

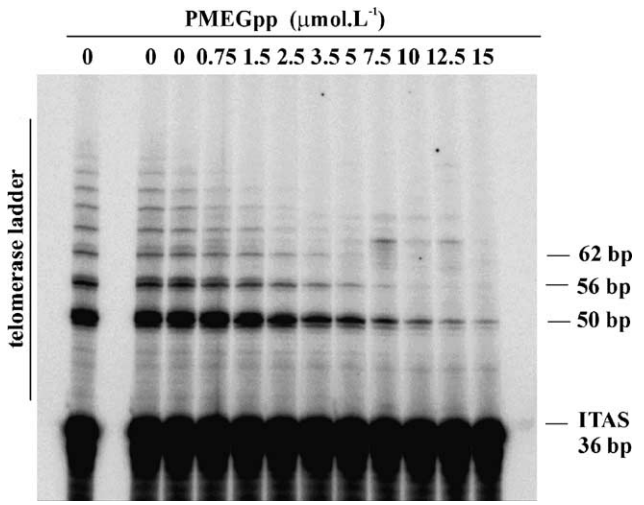

Fig. 2. Inhibition of telomerase by PMEGpp. Telomerase activity was measured in HL-60 cell extract in the presence of 30 μmol L^{-1} dNTPs and increasing concentrations of PMEGpp (0–15 μmol L^{-1}). ITAS—internal standard.

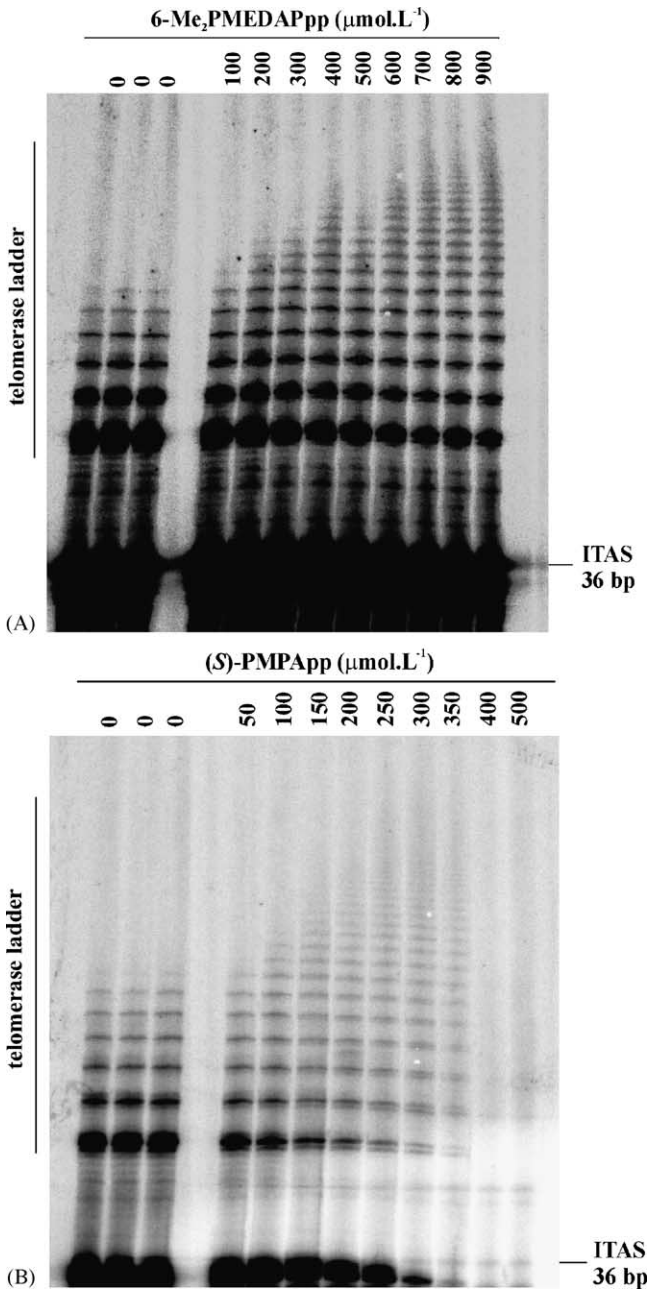

Fig. 3. Enhancement of telomerase processivity by 6-Me_2PMEDAPpp (A) and (*S*)-PMPApp (B). Telomerase activity was measured in HL-60 cell extract in the presence of 125 μmol L^{-1} dNTPs and increasing concentrations of 6-Me_2PMEDAPpp (0–900 μmol L^{-1}) and (*S*)-PMPApp (0–500 μmol L^{-1}). ITAS—internal standard.

observed with PMEO-DAPypp; this open ring ANP is considered to be a PMEDAPpp analogue (Fig. 1).

Inhibitory potency of PMEGpp and PMEDAPpp towards telomerase is consistent with the capability to induce apoptosis, strong cytostatic efficiency, and anticancer activity of their parental compounds PMEG and PMEDAP [20]. The growth of HL-60 cells is inhibited in vitro by PMEG by 50% at the concentration of 2–3 μM, while the growth inhibition concentration (GI_{50}) for PMEDAP is 15–20 μM. The GI_{50} values for (*S*)-PMPG, PMEO-DAPy and PMEA are 22, 30 and 30 μM, respectively. The lymphoid cell line CCRF-CEM is more susceptible to these agents than the myeloid cell line HL-60. Incubation of CCRF-CEM leukaemia cells with PMEG, PMEDAP, PMEO-DAPy, PMEA and (*S*)-PMPG inhibits cell growth at GI_{50} of 1, 6, 14, 25 and 29 μM, respectively [20].

The approximate IC_{50} values for the diphosphates of PMP-derivatives, listed in the order of their efficacy as telomerase inhibitors, are shown in Table 2: (*R*)-PMPGpp > (*S*)-PMPGpp > (*R*)-6-cyprPMPDAPpp > (*R*)-PMPApp > (*R*)-PMPDAPpp > (*S*)-PMPApp.

The most potent inhibitor among the PMP-type analogues is the guanine derivative (*R*)-PMPGpp, which inhibits the enzyme activity with the IC_{50} at five to eight times lower concentration compared to that of the natural substrate dGTP. (*S*)-PMPGpp is less inhibitory compared to (*R*)-enantiomer: its IC_{50} exceeds almost five times that of (*R*)-PMPGpp. This indicates that absolute configuration plays a significant role in the telomerase inhibition and that the enzyme distinguishes between the (*R*)- and (*S*)-enantiomers. (*S*)-PMPApp increases repeat addition processivity of the enzyme (Fig. 3B), while no significant inhibition of telomerase activity is found for (*R*)-PMPApp (IC_{50} 224 ± 30 μmol L^{-1} at 125 μM dNTPs), which is very efficient as a chain-terminating inhibitor of retroviral reverse transcriptases [20]. These results are consistent with the observation of Pai et al. [14] on discrimination between the D and L enantiomers of FaraTTP and Tendian and Parker [16] concerning inhibition efficiency difference in the pair of D and L enantiomers of CdG-TP.

Neither (*S*)-PMPA nor the corresponding monophosphate (*S*)-PMPAp increase repeat addition processivity of telomerase. Similarly to PMEG and 6-Me_2PMEDAP, activity of (*S*)-PMPA on telomerase is limited to its

Table 2
Inhibition of HL-60 telomerase by PMP type of ANPpp (IC_{50})[a]

Compound	dNTPs (μmol L^{-1})		
	125	60	30
(*R*)-PMPGpp	17.3 ± 2.8	11.3 ± 1.6	3.6 ± 1.0
(*S*)-PMPGpp	81.7 ± 19.6	47.6 ± 5.2	16.7 ± 2.9
(*R*)-6-cyprPMPDAPpp	152 ± 3	95 ± 6	41 ± 12.7
(*R*)-PMPApp	224 ± 30	125 ± 24	51.3 ± 6.7
(*R*)-PMPDAPpp	376 ± 24	224 ± 30	103 ± 19
(*S*)-PMPApp	P.E.[b]	N.D.[c]	N.D.

[a] Values are means ± S.D. of the four independent determinations.
[b] Processivity enhancement.
[c] Not determined.

Table 3
Inhibition of HL-60 telomerase by HPMP type of ANPpp (IC_{50})[a]

Compound	dNTPs (μmol L^{-1})		
	125	60	30
(*R*)-HPMPGpp	43.6 ± 5.9	28.3 ± 9.5	23.3 ± 5.9
(*S*)-HPMPApp	104 ± 15	61 ± 25	N.D.[b]

[a] Values are means ± S.D. of the four independent determinations.
[b] Not determined.

diphosphate only. Very high concentration of (*R*)-PMPDAPpp (376 μmol L^{-1}) is required to reach 50% inhibition (at 125 μM dNTPs).

The approximate IC_{50} values of the diphosphates of purine HPMP-derivatives are shown in Table 3. In accordance with the PME and PMP series, the adenine derivative (*S*)-HPMPApp is less inhibitory than the guanine derivative (*R*)-HPMPGpp, however, the effect of stereoisomerism of the aliphatic chain plays the same role as it is in the case of PMP-derivatives and may thus contribute to higher inhibitory efficiency of (*R*)-HPMPGpp.

4. Discussion

Although the telomerase active site was shown to be related to that of other reverse transcriptases [24], the ability of reverse transcriptase inhibitors to interfere with telomerase activity is not general as shown in this work. (*R*)-PMPApp, (*R*)-PMPDAPpp and PME-derivatives PMETpp, PMECpp and PMEApp known to be chain-terminating inhibitors of retroviral reverse transcriptases [20], do not inhibit human telomerase activity as shown in this work.

From these reverse transcriptase inhibitors only the guanine derivative PMEGpp shows strong inhibitory potency towards human telomerase. Although the rate of telomerase inhibition by ANP diphosphates seems to depend on the side-chain structure and absolute configuration of the aliphatic chain in the case of PMP-derivatives, our data imply that the character of the base is probably more important. The data comprised in Tables 1–3 show that the IC_{50} values for inhibition of telomerase activity by all guanine derivatives studied [PMEGpp, (*R*)-PMPGpp, (*S*)-PMPGpp and (*R*)-HPMPGpp] are 2–10 times lower than the dGTP concentration in the assay.

Interestingly, the behavior of two compounds from the above series differs substantially from the other: (*S*)-PMPApp and 6-Me_2PMEDAPpp do not inhibit telomerase, on the contrary, they increase the repeat addition processivity of telomerase in a dose-dependent manner at 125 μM dNTPs concentration (Fig. 3).

The increase of telomerase repeat addition processivity was also observed in the presence of high concentrations of dGTP as previously reported [25]. dGTP seems to mediate its processivity-stimulatory effect by binding at the active site, which harbours a specific increase of dGTP-binding affinity compared to other dNTPs [26]. Previous studies on *Tetrahymena* telomerase revealed that the enzyme requirements for dGTP structure features allowing the enhancement of repeat addition processivity are very strict. Stimulation of telomerase repeat addition processivity was demonstrated to be specific for dGTP base and sugar constituents. Addition of a C-2′ hydroxyl or loss of the C-2 amino group both prevent dGTP from stimulating repeat addition processivity [26]. Reduced repeat addition processivity was also observed for human telomerase when deazadGTP and/or deazadATP were used as substrates [18]. It has been also shown that dGMP promotes as much repeat addition processivity as dGTP [26]. In contrast, (*S*)-PMPA and 6-Me_2PMEDAP, the analogues of nucleoside 5′-phosphates, as well as (*S*)-PMPAp, do not increase repeat addition processivity of telomerase as shown in this study.

Similarly to dGTP, increased repeat addition processivity requires high micromolar concentrations of both (*S*)-PMPApp and 6-Me_2PMEDAPpp. However, due to a very low structural resemblance to dGTP and chain-terminating properties of both (*S*)-PMPApp and 6-Me_2PMEDAPpp, we can speculate that their ability to stimulate repeat addition processivity could be mediated more likely by interaction with a site remote from the active site.

Nevertheless, both (*S*)-PMPApp and 6-Me_2PMEDAPpp may contribute in some way to active site, DNA anchor site or RNA template rearrangements by conformational change in the ribonucleoprotein and prevent the enzyme from dissociation from the growing DNA strand. This ability could stimulate processive repeat addition. According to ‘‘anchor site model’’, the RNA template of one subunit could be used primarily for substrate binding, while the second template would be copied during telomere repeats addition [27]. So-called DNA anchor site, which is distinct from the catalytic site, could be affected by both (*S*)-PMPApp and/or 6-Me_2PMEDAPpp and might thereby facilitate processivity during the synthesis of telomere repeats most likely through enhancing of interactions between DNA product and telomerase. However, these speculations must be confirmed or disapproved by further experimental work.

Acknowledgement

We would like to thank Ludmila Tovcigrecko for her valuable technical assistance. This work was supported by the Project No. 1M6138896301, ID 1M0508 of the Ministry of Education, Youth and Sports of the Czech Republic.

References

[1] Morin GB. The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. Cell 1989;59:521–9.
[2] Wenz C, Enenkel B, Amacker M, Kelleher C, Damm K, Lingner J. Human telomerase contains two cooperating telomerase RNA molecules. EMBO J 2001;20:3526–34.
[3] Collins K, Mitchell JR. Telomerase in the human organism. Oncogene 2002;21:564–79.
[4] Greider CW, Blackburn EH. Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts. Cell 1985;43:405–13.
[5] Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, et al. Specific association of human telomerase activity with immortal cells and cancer. Science 1994;266:2011–5.
[6] Shay JW, Bacchetti S. A survey of telomerase activity in human cancer. Eur J Cancer 1997;33:787–91.
[7] Hiyama E, Hiyama K. Clinical utility of telomerase in cancer. Oncogene 2002;21:643–9.
[8] Lichtsteiner SP, Lebkowski JS, Vasserot AP. Telomerase. A target for anticancer therapy. Ann NY Acad Sci 1999;886:1–11.
[9] White LK, Wright WE, Shay JW. Telomerase inhibitors. Trends Biotechnol 2001;19:114–20.
[10] Nakamura TM, Morin GB, Chapman KB, Weinrich SL, Andrews WH, Lingner J, et al. Telomerase catalytic subunit homologs from fission yeast and human. Science 1997;277:955–9.
[11] Blackburn EH. Telomerases. Annu Rev Biochem 1992;61:113–29.
[12] Cech TR, Nakamura TM, Lingner J. Telomerase is a true reverse transcriptase. A review. Biochemistry (Mosc) 1997;62:1202–5.
[13] Strahl C, Blackburn EH. Effects of reverse transcriptase inhibitors on telomere length and telomerase activity in two immortalized human cell lines. Mol Cell Biol 1996;16:53–65.
[14] Pai RB, Pai SB, Kukhanova M, Dutschman GE, Guo X, Cheng YC. Telomerase from human leukemia cells: properties and its interaction with deoxynucleoside analogues. Cancer Res 1998;58:1909–13.
[15] Fletcher TM, Cathers BE, Ravikumar KS, Mamiya BM, Kerwin SM. Inhibition of human telomerase by 7-deaza-2′-deoxyguanosine nucleoside triphosphate analogs: potent inhibition by 6-thio-7-deaza-2′-deoxyguanosine 5′-triphosphate. Bioorg Chem 2001;29:36–55.
[16] Tendian SW, Parker WB. Interaction of deoxyguanosine nucleotide analogs with human telomerase. Mol Pharmacol 2000;57:695–9.
[17] Strahl C, Blackburn EH. The effects of nucleoside analogs on telomerase and telomeres in *Tetrahymena*. Nucleic Acids Res 1994;22:893–900.
[18] Fletcher TM, Salazar M, Chen SF. Human telomerase inhibition by 7-deaza-2′-deoxypurine nucleoside triphosphates. Biochemistry 1996;35:15611–7.
[19] Raymond E, Sun D, Chen SF, Windle B, Von Hoff DD. Agents that target telomerase and telomeres. Curr Opin Biotechnol 1996;7:583–91.
[20] Holý A. Phosphonomethoxyalkyl analogs of nucleotides. Curr Pharm Des 2003;9:2567–92.
[21] Wright WE, Shay JW, Piatyszek MA. Modifications of a telomeric repeat amplification protocol (TRAP) result in increased reliability, linearity and sensitivity. Nucleic Acids Res 1995;23:3794–5.
[22] Kim NW, Wu F. Advances in quantification and characterization of telomerase activity by the telomeric repeat amplification protocol (TRAP). Nucleic Acids Res 1997;25:2595–7.
[23] Otmar M, Masojídková M, Votruba I, Holý A. An alternative synthesis of HPMPC and HPMPA diphosphoryl derivatives. Collect Czech Chem Commun 2001;66:500–6.
[24] Nugent CI, Lundblad V. The telomerase reverse transcriptase: components and regulation. Genes Dev 1998;12:1073–85.
[25] Hammond PW, Cech TR. dGTP-dependent processivity and possible template switching of euplotes telomerase. Nucleic Acids Res 1997;25:3698–704.
[26] Hardy CD, Schultz CS, Collins K. Requirements for the dGTP-dependent repeat addition processivity of recombinant *Tetrahymena* telomerase. J Biol Chem 2001;276:4863–71.
[27] Kelleher C, Teixeira MT, Forstemann K, Lingner J. Telomerase: biochemical considerations for enzyme and substrate. Trends Biochem Sci 2002;27:572–9.