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Inhibition of thymidine phosphorylase (PD-ECGF) from SD-lymphoma by phosphonomethoxyalkyl thymines

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Abstract

A series of thymine phosphonomethoxyalkyl derivatives were evaluated for their ability to inhibit thymidine phosphorylase (dThdPase) purified from rat spontaneous T-cell lymphoma. A kinetic study of thymidine phosphorolysis catalyzed by dThdPase was performed with thymidine and/or inorganic phosphate as substrates. Data show that the substantial inhibitory effect of these acyclic nucleotide analogues is decreasing in the order of (*R*)-FPMPT > (*S*)-FPMPT ≥ (*R*)-HPMPT > (*S*)-PMPT > (*S*)-HPMPT > PMET ≥ (*R*)-PMPT. The inhibitory potency ($K_i/^{dThd}K_m$) of the most efficient inhibitors from this series against T-cell lymphoma enzyme is 0.0026 for (*R*)-FPMPT and 0.0048 for (*S*)-FPMPT. The studied compounds do not inhibit *Escherichia coli* and human enzyme and possess lower inhibitory potency against rat liver thymidine phosphorylase.

Keywords: Thymidine phosphorylase inhibition; Thymine acyclic nucleoside phosphonates; FPMPT

1. Introduction

Acyclic nucleoside phosphonates (ANPs), exhibit antiviral, cytostatic, antiparasitic and immunomodulatory activities [1]. (*S*)-HPMPC (Vistide™) is approved for the treatment of cytomegalovirus (CMV) retinitis in AIDS patients, tenofovir [(*R*)-PMPA] is an antiretroviral acyclic nucleoside phosphonate, whose lipophilic prodrug, tenofovir disoproxil fumarate (Viread™) is used for treatment of HIV infection and bis(pivaloyloxymethyl) ester of PME derivative of adenine (adefovir dipivoxil, Hepsera™) was recently approved as anti-HBV agent. 2,6-Diaminopurine (PMEDAP) and guanine (PMEG) derivatives display extensive antiviral and antineoplastic activity. These nucleotide analogues are phosphorylated by cellular kinases to their diphosphates (analogues of nucleoside 5′-triphosphates), which inhibit replicative DNA polymerases and/or reverse transctriptase. The chemistry and biochemistry of this very important category of antimetabolites was recently exhaustively reviewed [1]. However, till lately, neither the related cytosine derivative PMEC nor other pyrimidine ANPs derived from uracil and thymine displayed any biological activity, probably due to the poor intracellular transport [2]. Recent study has namely shown that conversion of these compounds to lipophilic alkoxyalkyl esters enhances their antiviral potency [3].

In this paper we report on efficient inhibitors of thymidine phosphorylase based on the structure of specifically modified, metabolically stable [1] thymine ANPs. Thymidine phosphorylase [dThdPase, EC 2.4.2.4, platelet-derived endothelial-cell growth factor (PD-ECGF)] [4–6], an important salvage-pathway enzyme, catalyzes phosphorolysis of thymidine to thymine and 2-deoxy-D-ribose

Abbreviations: (*S*)-HPMPC, 1-(*S*)-[3-hydroxy-2-(phosphonomethoxy)propyl]-cytosine; (*R*)-PMPA, 9-(*R*)-[2-(phosphonomethoxy)propyl]adenine; PME, 9-[2-(phosphonomethoxy)ethyl]; PMEDAP, 9-[2-(phosphonomethoxy)ethyl]-2,6-diamino-purine; PMEG, 9-[2-(phosphonomethoxy)ethyl]guanine; PMET, 1-[2-(phospho-no-methoxy)ethyl]thymine; (*S*)-HPMPT, (*S*)-1-[3-hydroxy-2-(phosphonomethoxy)-propyl]thymine; (*R*)-HPMPT, (*R*)-1-[3-hydroxy-2-(phosphonomethoxy)propyl]-thymine; (*S*)-PMPT, (*S*)-1-[2-(phosphonomethoxy)propyl]thymine; (*R*)-PMPT, (*R*)-1-[2-(phosphonomethoxy)propyl]thymine; (*S*)-FPMPT, (*S*)-1-[3-fluoro-2-(phospho-no-methoxy)propyl]thymine; (*R*)-FPMPT, (*R*)-1-[3-fluoro-2-(phosphonomethoxy)-propyl]thymine; dThd, thymidine (2′-deoxythymidine); dTMP, 2′-deoxythymidine 5′-phosphate; dThdPase, thymidine phosphorylase; Pi, inorganic phosphate; ANP, acyclic nucleoside phosphonate

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1-phosphate [4]. A dephosphorylated product of the latter, 2-deoxy-D-ribose, has a chemotactic activity in vitro and angiogenic activity in vivo stimulating endothelial-cell migration [7–9]. This process is crucial for the formation of new blood vessels within a tumor that overexpress thymidine phosphorylase. Thus the inhibition of 2-deoxy-D-ribose release from the endothelial cells represents a potential anti-angiogenic target in cancer chemotherapy [10–14].

The aim of our work was to investigate the interaction of thymidine phosphorylase with known and newly designed acyclic nucleoside phosphonates of thymine in vitro. The study was performed with the enzyme purified from spontaneous T-cell lymphomas of an inbred Sprague–Dawley rat strain [15] in comparison with recombinant *Escherichia coli* human and rat liver thymidine phosphorylases.

2. Materials and methods

2.1. Chemicals

Thymine ANPs were prepared by the described procedures (for review, see [1]). All other chemicals and materials, e.g. thymidine phosphorylase recombinant, expressed in *E. coli* (Sigma, T-2807), human thymidine phosphorylase recombinant, expressed in Chinese hamster cells (Sigma, T-9319), [^{3}H-methyl]thymidine (740 GBq m mol^{-1}; ICN Biomedicals, Inc.), protease inhibitor cocktail (Sigma Chemical Co.), T-Per^{TM} — tissue protein extraction reagent (PIERCE), HiPrep^{TM} 26/10 Desalting, HiPrep^{TM} 16/10 DEAE FF, PD-10 columns (Amersham Biosciences), Centricon Plus-20 (Millipore), TLC-sheets (Silica gel 60 F_{254}, MERCK), buffers and salts were commercial products.

2.2. SD-lymphoma

Solid lymphomas are formed after subcutaneous inoculation of neoplastic cells obtained from submandibular lymph nodes of primary ill animal into healthy SD/Cub recipients of the Prague inbred subline of Sprague–Dawley rats. Suspension of 10^6 lymphoma cells was injected subcutaneously into the right flank of anaesthetized SD/Cub rats. Twenty days after inoculation of lymphoma cells rats with subcutaneously growing lymphoma were killed and tumor mass was completely taken out. The lymphomas were well vascularized in this period of growth.

2.3. Thymidine phosphorylase

The enzyme was partially purified from SD-lymphoma using a combination of described purification procedures [5,16]. Frozen lymphomas (−70 °C; 46 g wet weight) were sliced, homogenized in a Dounce tissue grinder in the presence of T-Per^{TM} and protease inhibitor cocktail and centrifuged at 100,000 × *g*. Proteins with thymidine phosphorylase activity were salted out from the supernatant with ammonium sulfate (22–50%), desalted (HiPrep^{TM} 26/10 Desalting) and applied onto HiPrep^{TM} 16/10 DEAE FF column. The column was eluted with a linear gradient of 0–200 mM NaCl and active fractions (eluted at 50–70 mM NaCl) were pooled, concentrated (Centricon Plus-20) and rechromatographed on a hydroxyapatite column (1.2 cm × 5.4 cm, linear gradient of 50–500 mM NaCl). The collected fractions (eluted at 200 mM NaCl) with high thymidine phosphorylase activity were concentrated (Centricon Plus-20) and desalted (PD-10 column). All purification steps were carried out in 20 mM bis-Tris–HCl pH 6.4 containing 1 mM EDTA and 2 mM DTT. The resulting enzyme preparation (10.2 nU/mg; one enzyme unit is defined as the amount of enzyme that catalyzes phosphorolysis of 1 μmol of thymidine per min under standard conditions), free of uridine phosphorylase activity, was stored at −70 °C for kinetic measurements. Thymidine phosphorylase from rat liver was purified by the same procedure.

2.4. Enzyme assay

The standard reaction mixture (50 μl) contained 20 mM bis-Tris–HCl pH 6.4, 1 mM EDTA and 2 mM DTT, 100 μM [^{3}H-methyl]thymidine, 200 μM potassium phosphate pH 6,7 and 25.5 pU of enzyme. The reaction was carried out at 37 °C for 10 min and stopped by spotting a 2 μl aliquot onto Silica gel 60 F_{254} plate that had been prespotted with 0.01 μmol of each thymine and thymidine. The plate was developed in the non-aqueous phase of the solvent system ethyl acetate–water–formic acid (60:35:5). The spots were visualized under UV light (254 nm) and cutted out for radioactivity determination in the toluene-based scintillation cocktail.

2.5. Kinetic experiments

Kinetic constants K_m, K_i and V_{max} were determined from the Lineweaver–Burk, Dixon and Eadie–Hofstee plots using various concentrations of thymidine and phosphate (Pi). Data based on results from at least four independent experiments were evaluated by the non-linear regression method (BioSoft EnzFitter, 32 bit version for Windows).

3. Results and discussion

Thymine phosphonomethoxyalkyl derivatives (thymine ANPs, Fig. 1) were studied as potential inhibitors of the thymidine phosphorolysis catalyzed by thymidine phosphorylase from SD-lymphoma. Kinetic analysis of the inhibitory potency (K_i/K_m) of the compounds studied was carried out with respect to the both substrates

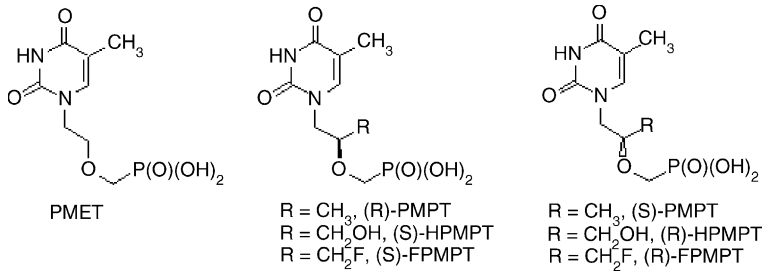

Fig. 1. Structure of phosphonomethoxyalkyl thymines.

Table 1
Inhibition of thymidine phosphorylase by 1-[2-(phosphonomethoxy)alkyl]-thymines (substrate: 2′-deoxythymidine)

Inhibitor	Rat SD-lymphoma[a]		Rat liver[b]	
	K_i (μM)	$K_i/{}^{dThd}K_m$	K_i (μM)	$K_i/{}^{dThd}K_m$
PMET	1.46 ± 0.33	0.0175	n.d.	n.d.
(*S*)-HPMPT	0.93 ± 0.19	0.0110	n.d.	n.d.
(*R*)-HPMPT	0.37 ± 0.09	0.0044	2.32 ± 0.22	0.0122
(*S*)-PMPT	0.68 ± 0.11	0.0082	n.d.	n.d.
(*R*)-PMPT	1.90 ± 0.46	0.0230	n.d.	n.d.
(*S*)-FPMPT	0.40 ± 0.07	0.0048	n.d.	n.d.
(*R*)-FPMPT	0.22 ± 0.06	0.0026	2.55 ± 0.35	0.0135

Data are means ± S.E.M. of the four independent experiments. n.d., not determined.
[a] At 200 μM Pi, ${}^{dThd}K_m \sim 83.2 \pm 8.69$ μM, ${}^{dThd}V_{max} \sim 1.4 \pm 0.07$ μmol l^{-1} min^{-1}.
[b] At 200 μM Pi, ${}^{dThd}K_m \sim 189 \pm 25$ μM, ${}^{dThd}V_{max} \sim 4.4 \pm 0.28$ μmol l^{-1} min^{-1}.

Table 2
Inhibition of thymidine phosphorylase by 1-[2-(phosphonomethoxy)alkyl]-thymines (substrate: inorganic phosphate)

Inhibitor	Rat SD-lymphoma[a]		Rat liver[b]	
	K_i (μM)	$K_i/{}^{Pi}K_m$	K_i (μM)	$K_i/{}^{Pi}K_m$
PMET	1.73 ± 0.35	0.0063	n.d.	n.d.
(*S*)-HPMPT	1.30 ± 0.21	0.0047	n.d.	n.d.
(*R*)-HPMPT	0.78 ± 0.10	0.0028	2.70 ± 2.50	0.017
(*S*)-PMPT	1.00 ± 0.25	0.0036	n.d.	n.d.
(*R*)-PMPT	3.96 ± 0.62	0.0145	n.d.	n.d.
(*S*)-FPMPT	0.64 ± 0.10	0.0023	n.d.	n.d.
(*R*)-FPMPT	0.34 ± 0.13	0.0013	3.44 ± 0.33	0.021

Data are means ± S.E.M. of four independent experiments. n.d., not determined.
[a] At 100 μM dThd, ${}^{Pi}K_m \sim 274 \pm 20.3$ μM, ${}^{Pi}V_{max} \sim 1.31 \pm 0.28$ μmol l^{-1} min^{-1}.
[b] At 100 μM dThd, ${}^{Pi}K_m \sim 161 \pm 19.0$ μM, ${}^{Pi}V_{max} \sim 2.7 \pm 0.29$ μmol l^{-1} min^{-1}.

2′-deoxythymidine (Table 1) and/or inorganic phosphate (Table 2).

Our data demonstrate that the most efficient inhibitor of the enzyme is (*R*)-FPMPT with $K_i \sim 2.2 \times 10^{-7}$ mol l^{-1} with regards to thymidine and/or 3.4×10^{-7} mol l^{-1} with regards to inorganic phosphate, respectively. The affinity of this dTMP analogue towards thymidine phosphorylase is as much as three hundred times higher ($K_m/K_i \sim 320$ for dThd and 769 for Pi) compared to that of the natural substrates (Tables 1 and 2). The character of (*R*)-FPMPT inhibition as well as of all the other thymine ANPs presented in this study is strictly competitive (Fig. 2) with respect to both thymidine and inorganic phosphate as natural substrates. The opposite (*S*)-enantiomer of FPMPT is approximately two times less efficient than its (*R*)-counterpart. The comparable inhibitory potency was found for (*R*)-HPMPT ($K_i \sim 3.7 \times 10^{-7}$ mol l^{-1} for thymidine) and similar to the previous case its (*S*)-counterpart is a weaker inhibitor of the enzyme (Tables 1 and 2). On the whole, from the two mentioned, it is always that of the (*R*)-configuration, which is inhibiting dThdPase more efficiently.[1] These findings indicate certain extent of enantioselectivity upon the interaction of thymine ANPs with thymidine phosphorylase, because (*R*)-FPMPT, (*R*)-HPMPT and (*S*)-PMPT represent molecules with the same absolute configuration.

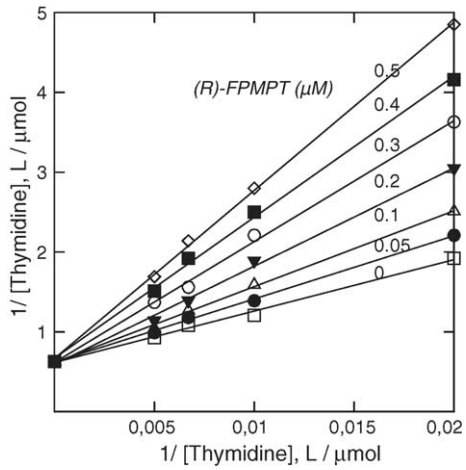

Fig. 2. Lineweaver–Burk plot of rat lymphoma thymidine phosphorylase inhibition by (*R*)-FPMPT (substrate: thymidine). The standard reaction mixture (50 μl) contained 20 mM bis-Tris–HCl pH 6.4, 1 mM EDTA and 2 mM DTT, [^{3}H-methyl]thymidine (50, 100, 150 and 200 μM) 200 μM potassium phosphate pH 6,7 and (*R*)-FPMPT (0.0, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 μM). The reaction was started by 25.5 pU of enzyme and proceeded for 10 min at 37 °C.

[1] The apparent discrepancy in the PMP-series is solely formal, due to altered priority of the substituents of the asymmetry center. In this case, the designation of the absolute configuration of the more active (*S*)-enantiomer is identical with that of (*R*)-HPMPT or (*R*)-FPMPT.

The enantiomeric specificity of dThdPase is not absolute, though the (*R*)- and (*S*)-enantiomers differ considerably in their activity, the inhibition by the less potent (*S*)-enantiomers is still substantial. The interaction of ANPs toward the dThd/Thy-binding site is overwhelmed by the competition with the phosphate-binding site ($K_i^{dThd}K_m > K_i^{Pi}K_m$) by factor of approx. 2 (Tables 1 and 2).

High activity of the pairs of FPMPT and HPMPT cannot be interpreted by the interaction of strongly electronegative fluorine and oxygen atoms linked to the beta-methylene grouping of the side-chain with the protonated amino acids of the peptide chain. The high affinity of the PMP-derivatives to the enzyme (wherein these atoms are replaced by hydrogen in the methyl group) rather suggests that the substrate/inhibitors are primarily bound at the two sites of the molecule: phosphate group and the thymine base while the rest of the molecule between those two sites (chiral aliphatic chain in the ANPs) suitably adapts its configuration to optimize the EI complex formation. The comparatively small difference between the effects of opposite enantiomers fits with the model of such a voluminous cavity in the enzyme binding site.

The activity of PMET, which is not substituted in the position 2 of the aliphatic side-chain (Fig. 1), is comparable with that of (*R*)-PMPT. All these data show that the considerable inhibitory effect of thymine phosphonomethoxyalkyl derivatives on thymidine phosphorolysis catalyzed by thymidine phosphorylase from SD-lymphoma decreases in the order of (*R*)-FPMPT > (*S*)-FPMPT ≥ (*R*)-HPMPT > (*S*)-PMPT > (*S*)-HPMPT > PMET ≥ (*R*)-PMPT.

In the parallel experiments we have also tested the inhibitory efficacy of these compounds on the commercial bacterial (Sigma, thymidine phosphorylase recombinant, expressed in *E. coli*) and human enzymes (Sigma, thymidine phosphorylase human recombinant, expressed in *V79* Chinese hamster cells). Surprisingly, none of the thymine ANPs used in this study including (*R*)-FPMPT was active, at the concentration 100 μmol l^{-1} against bacterial enzyme and at 10 μmol l^{-1} against human enzyme. Therefore we purified thymidine phosphorylase from liver of healthy rat and tested the inhibitory potency of the two compounds (*R*)-FPMPT and (*R*)-HPMPT. Data show (Tables 1 and 2) lower efficiency of these ANPs toward liver enzyme compared with thymidine phosphorylase from SD-lymphoma. Furthermore, liver thymidine phosphorylase possesses different affinity to the both natural substrates (thymidine and inorganic phosphate, Tables 1 and 2) than the enzyme isolated from SD-lymphoma. Kinetic analysis has shown also different character of the inhibition with respect to the inorganic phosphate as substrate, which is competitive for SD-lymphoma enzyme (with the constant $V_{max} \sim 1.3$ μmol min^{-1}, Fig. 3) and noncompetitive (with the variable $V_{max} \sim 2.7$–1.7 μmol min^{-1}, Fig. 4) for rat liver enzyme.

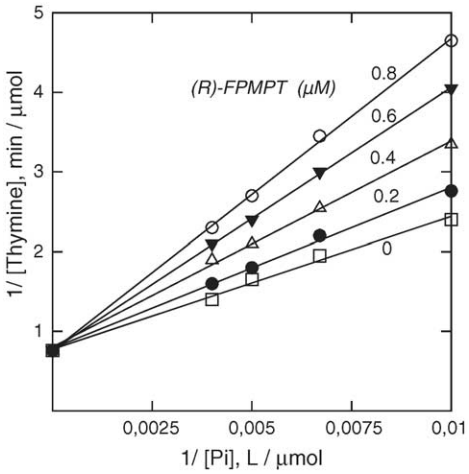

Fig. 3. Lineweaver–Burk plot of rat lymphoma thymidine phosphorylase inhibition by (*R*)-FPMPT (substrate: inorganic phosphate). The standard reaction mixture (50 μl) contained 20 mM bis-Tris–HCl pH 6.4, 1 mM EDTA and 2 mM DTT, 100 μM [^{3}H-methyl]thymidine, potassium phosphate pH 6,7 (100, 150, 200 and 250 μM) and (*R*)-FPMPT (0.0, 0.2, 0.4, 0.6, 0.8 μM). The reaction was started by 25.5 pU of enzyme and proceeded for 10 min at 37 °C.

The competitive interaction of thymine phosphonomethoxyalkyl derivatives of FPMP, HPMP, PMP and PME type at dThd and Pi binding sites of thymidine phosphorylase from SD-lymphoma resembles to the character of interaction of TP-64 and TP-65 towards *E. coli* enzyme published by Balzarini et al. [17]. They postulated that 1-(8-phosphonooctyl)-6-amino-5-bromouracil (TP-64) and 1-(8-phosphonooctyl)-7-deazaxanthine (TP-65) are multisubstrate analogue inhibitors of *E. coli* thymidine phosphorylase [17].

In conclusion, we have found that (*R*)-FPMPT, (*S*)-FPMPT and/or (*R*)-HPMPT possess considerable inhibi-

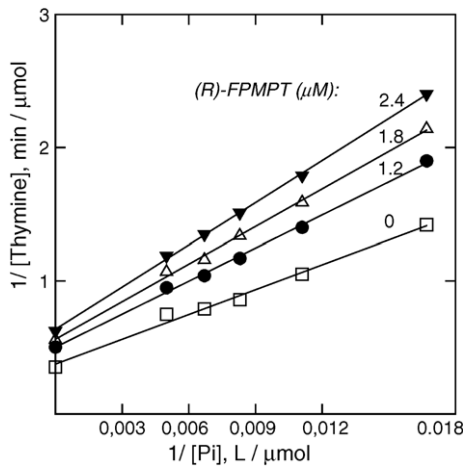

Fig. 4. Lineweaver–Burk plot of rat liver thymidine phosphorylase inhibition by (*R*)-FPMPT (substrate: inorganic phosphate). The standard reaction mixture (50 μl) contained 20 mM bis-Tris–HCl pH 6.4, 1 mM EDTA and 2 mM DTT, 100 μM [^{3}H-methyl]thymidine, potassium phosphate pH 6,7 (60, 90, 120, 150 and 180 μM)and (*R*)-FPMPT (0.0, 1.2, 1.8, 2.4 μM). The reaction was started by 25.5 pU of enzyme and proceeded for 10 min at 37 °C.

tory potency towards thymidine phosphorylase isolated from rat tumor. These compounds do not inhibit *E. coli* and human enzyme and possess lower inhibitory potency against rat liver thymidine phosphorylase, which suggests that the architecture of the catalytic center of the tested enzymes probably is considerably different.

Acknowledgments

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