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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