INHIBITION OF HUMAN PURINE NUCLEOSIDE PHOSPHORYLASE BY TENOFOVIR PHOSPHATE CONGENERS

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The structure-activity study on the phosphates of phosphonomethoxypropyl derivatives of purine bases interacting with human purine nucleoside phosphorylase has shown that the most efficient inhibitors of the enzyme are (*R*)- and (*S*)-PMPGp with K_i ~ 1.9 × 10^{-8} and/or 2.2 × 10^{-8} mol/l. The kinetic experiments have proven, with the exception of both enantiomers of PMP-8-BrDAPp, strictly competitive character of inhibition for all ANP monophosphates tested. Bromine derivatives exhibited uncompetitive and mixed type of inhibition as well. These results were confirmed by docking studies. The substitution of purine moiety with the bromine at the position 8 lead to an allosteric binding of these compounds toward the enzyme.

Keywords: Enzyme inhibitors; Enzyme kinetics; Phosphonates; Purine nucleoside phosphorylase; Phosphates of acyclic nucleoside phosphonates; Tenofovir phosphate congeners.

Abbreviations:

(*R*)-PMPAp: (*R*)-9-[2-(phosphonomethoxy)propyl]adenine monophosphate; (*R*)-PMPDAPp: (*R*)-9-[2-(phosphonomethoxy)propyl]-2,6-diaminopurine monophosphate; (*R*)-PMP-8BrDAPp: (*R*)-9-[2-(phosphonomethoxy)propyl]-8-bromo-2,6-diaminopurine monophosphate; (*R*)-PMPGp: (*R*)-9-[2-(phosphonomethoxy)propyl]guanine monophosphate; (*R*)-PMP-8azGp: (*R*)-9-[2-(phosphonomethoxy)propyl]-8-azaguanine monophosphate; (*R*)-FPMPGp: (*R*)-9-[3-fluoro-2-(phosphonomethoxy)propyl]guanine monophosphate.

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Acyclic nucleoside phosphonates (ANPs) represent a group of very potent antivirals. Moreover, these nucleotide analogues display cytostatic, antiparasitic and immunomodulatory effects[1]. In the eukaryotic cells they are phosphorylated by cellular kinases to their diphosphates (analogues of nucleoside 5′-triphosphates) (ref.[1]), which restrain viral and/or cellular replication polymerases[1]. Further, in early nineties it was shown that phosphates of several ANPs are potent inhibitors of purine nucleoside phosphorylases (PNPase) in vitro[2]. In that time (*S*)-9-[3-hydroxy-(2-phosphonomethoxy)propyl]guanine phosphate has been identified as the acyclic nucleoside phosphonate, which exhibits the strongest affinity (K_i = 2×10^{-8} mol/l) towards PNPase isolated from diverse sources[3].

PNPase is a purine salvage pathway enzyme, which catalyzes the phosphorolysis of guanosine, inosine and 2′-deoxyguanosine to the corresponding purine base and (2′-deoxy)ribose-1-phosphate[4]. Pharmacological aspects of PNPase inhibition are connected with the treatment of human T-cell proliferative disorders leading to the intracellular 2′-deoxyguanosine and consequently dGTP accumulation and finally to the feedback inhibition of nucleotide reductase[5,6]. This process is characteristic for human T-cells because they contain a high level of deoxycytidine kinase capable to phosphorylate the 2′-deoxyguanosine and could be reversed by an excess of 2′-deoxycytidine.

Up to now, it is quite unclear if the ANPs affect the intracellular level of dGTP and subsequently DNA synthesis via independent DNA polymerase inhibition. The only study on tenofovir [9-*R*-[2-(phosphonomethoxy)propyl]adenine] shows that co-administration of tenofovir with 2′,3′-dideoxyinosine (ddI) decreased the amounts of intracellular ddI breakdown products in CEM cells. This finding correlates with proven PNPase inhibition by tenofovir mono- and diphosphate in parallel experiment[7].

On the basis of above mentioned findings, we present the structure-activity study to estimate an inhibition profile of other phosphonomethoxypropyl derivatives of purine bases – tenofovir congeners, to decide if these nucleoside 5′-diphosphate analogues interact with PNPase by the same way as tenofovir does.

RESULTS AND DISCUSSION

Kinetic Experiments

Phosphates of *R*- and *S*-enantiomers of phosphonomethoxypropyl purines – acyclic nucleotide analogues (Fig. 1), were studied as potential inhibitors of

human purine nucleoside phosphorylase purified from CCRF-CEM cells. Kinetic study of the inhibitory potency (K_i/K_m) of the compounds tested was performed with respect to the substrate inosine (Table I). The data demonstrate that (*R*)- and (*S*)-PMPGp are the most efficient inhibitors of the enzyme with $K_i \sim 1.9 \times 10^{-8}$ and/or 2.2×10^{-8} mol/l. Such interaction of both enantiomers of this guanine derivative represents very strong affinity towards the enzyme ($^{Ino}K_m/K_i \sim 3856$). The substantial inhibitory effect of other 9-[2-(phosphonomethoxy)propyl]purine phosphates is decreasing in the order of (*S*)-PMPazGp ≥ (*S*)-FPMPGp = (*R*)-PMPAp = (*R*)-PMPazGp ≥ (*R*)-FPMPGp ≥ (*S*)-PMP-8-BrDAPp = (*R*)-PMP-8-BrDAPp ≥ (*R*)-PMPDAPp ≥ (*S*)-PMPDAPp ≥ (*S*)-PMPAp. The comparable inhibitory activity (in the same order of magnitude) was found for both enantiomers of PMPGp, PMPazGp, PMP-8-BrDAPp and PMPDAPp, while (*R*)-FPMPGp is approximately three times less efficient than its (*S*)-counterpart. Surprisingly, (*R*)-PMPA is about twenty times stronger inhibitor of the enzyme than (*S*)-PMPA. This finding prompts that the presence of adenine moiety in PMPA molecule probably enables the certain extent of enantioselectivity upon the interaction of this compound with purine nucleoside phosphorylase. With the exception of both enantiomers of PMP-8-BrDAPp, the character of the inhibition of all tested purine ANPs is strictly competitive (Table I). On the other hand, for (*R*)-PMP-8-BrDAPp non-competitive and finally for its (*S*)-counterpart mixed type of inhibition was found (Table I). The inhibitory potency (v_i/v_o) of studied ANP monophosphates strictly depends on the concentration of

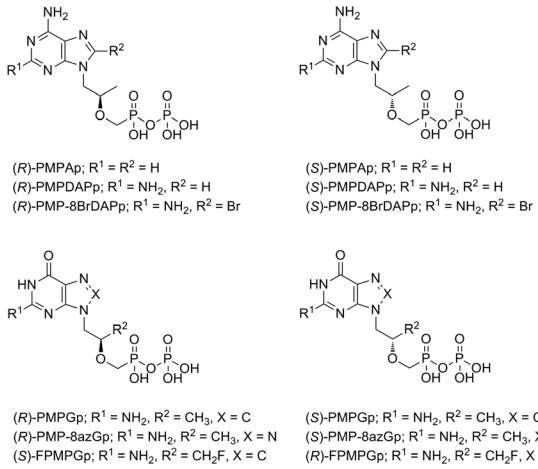

FIG. 1
Structures of phosphonomethoxypropyl purine phosphates

inorganic phosphate in the reaction mixture. We compared the PNPase reaction rate in the presence of individual compounds at 1, 3 and 5 mM P_i (inorganic phosphate) using 75 μM inosine and 50 nM ANPp. The v_i/v_o ratio at 1 mM P_i ranged from 0.25 to 0.6, whereas at 5 mM P_i it was approaching the values from 0.78 to 1.00.

TABLE I
Inhibition of inosine phosphorylysis by 9-[2-(phosphonomethoxy)propyl]purine phosphates

ANPp	K_i, nmol/l	$K_i/^{Ino}K_m$ [a]	Inhibition type[b]
(*R*)-PMPAp	38 ± 7	0.0005	C
(*S*)-PMPAp	710 ± 78	0.0097	C
(*R*)-PMPGp	19 ± 3	0.0003	C
(*S*)-PMPGp	22 ± 3	0.0003	C
(*R*)-PMPDAPp	260 ± 60	0.0036	C
(*S*)-PMPDAPp	340 ± 60	0.0047	C
(*R*)-PMPazGp	40 ± 5	0.0005	C
(*S*)-PMPazGp	25 ± 3	0.0003	C
(*R*)-PMP-8-BrDAPp	128 ± 15	0.0017	NC
(*S*)-PMP-8-BrDAPp	113 ± 6	0.0015	M
(*R*)-FPMPGp	93 ± 12	0.0013	C
(*S*)-FPMPGp	37 ± 7	0.0005	C

[a] $^{Ino}K_m$ = 73.27 ± 2.7 μmol/l at 1 mM P_i (inorganic phosphate). [b] C, competitive; NC, noncompetitive; M, mixed. Data are means ± SEM of four independent experiments (for details, see Experimental).

Docking

All the 9-[2-(phosphonomethoxy)propyl]purine phosphates that do not contain bromine atom at position 8 of the purine base were docked into the active site cavity (data not shown). On the contrary, the first best scored docked poses of both enantiomers of PMP-8-Br-DAPp were docked nonphysically into position of PHE200 (clashing of heavy atoms with this residue). The second best docked modes for both enantiomers of PMP-8-BrDAPp were docked more suitably although for further study of this particular binding pose of *S*-enantiomer, the PHE 200 would had to be artificially moved to cancel hydrogen clash.

The first best docked *R*-enantiomer of PMP-8-BrDAPp has bigger docking rating than the second one differing by 2 kcal/mol. For the second best scored pose the situation is adverse. *S*-enantiomer of PMP-8-BrDAPp is by about 3 kcal/mol more preferable than the *R* one. For more, although the first best scored poses are both in the active site of the protein, the docked

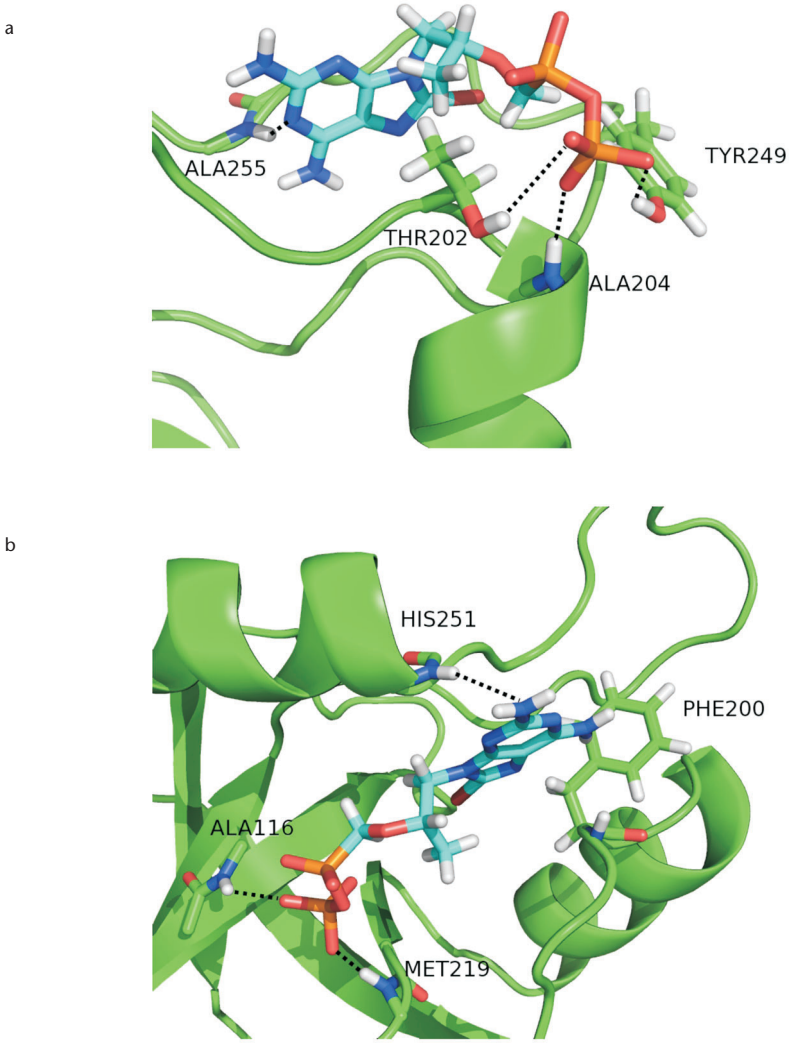

FIG. 2
The binding modes obtained by DOCK for the complexes of PNPase with (*R*)- (a) and (*S*)-PMP-8-BrDAPp (b)

positions of the second best docked pose differ for both enantiomers. Thus *R*-enantiomer has been docked at the surface of the protein to a place among residues Asn256–Val245 and Val203–Ser195 (Fig. 2a). *S*-enantiomer is then bound partly to the active site cavity and partly towards the protein surface (Fig. 2b). These data are consistent with the estimated non-competitive type of inhibition for (*R*)-PMP-8-BrDAPp and/or mixed typed for (*S*)-PMP-8-BrDAPp. Figure 3 shows an overall view on these two binding modes and, for comparison, also on the binding mode of the competitive inhibitor Immucillin-H (crystal code 3BGS (ref.[15])). It should be mentioned again that all the 9-[2-(phosphonomethoxy)propyl]purine phosphates not containing bromine atom at position 8 of purine base were docked into the active site, which means that their binding mode is very similar to the binding mode of Immucillin-H shown in the Fig. 3.

For the same order of docked pose, the score values of both enantiomers do not differ substantially, whereas the opposite holds true for different orders of docked poses. Score values for the first two best docked poses of both enantiomers are summarized in Table II. Since the experiment has proved 8-bromo derivatives bind allosterically and since the first best

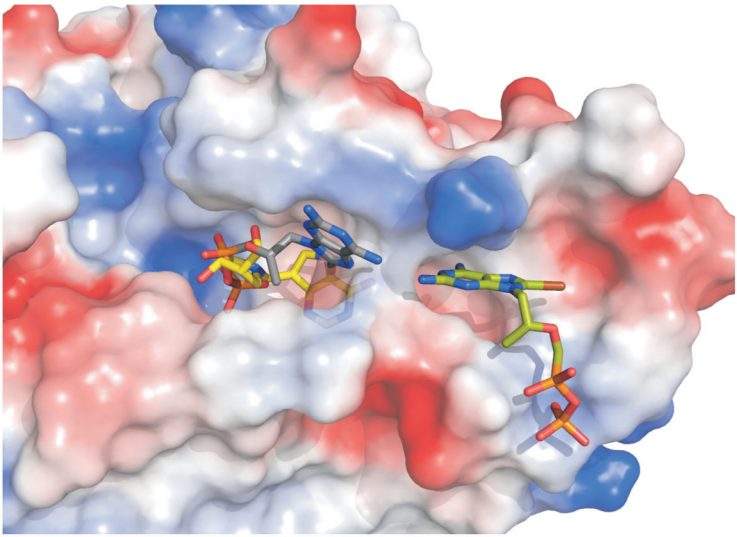

FIG. 3
Superposition of the the crystal binding mode of the competitive inhibitor Immucilin-H (code name 3BGS) (ref.[15]) and of the docked binding modes of both enantiomers of PMP-8-Br-DAPp. Carbon atoms of Immucilin-H and of the *R*- and *S*-enantiomers of PMP-8-Br-DAPp are colored by yellow, green and grey, respectively

scored poses are not physically suitable, the second best docked pose of *R*-isomer seems to represent the binding truth the best.

Nonphosphorylated ANPs are weak inhibitors of PNPase with the efficiency (K_i/K_m) two orders of magnitude higher (data not shown). On the basis of the above discussed findings, we can conclude that phosphates of phosphonomethoxypropyl derivatives of purines – analogues of purine nucleoside 5′-diphosphates, exhibit strong interaction with human PNPase. It is also highly probable that these nucleotide analogues are intracellularly active at the same way as tenofovir {(*R*)-PMPA} does[7]. Our data also show that studied ANPs phosphates act as multisubstrate analogues[3].

TABLE II
Score values for the first two best docked poses of both enantiomers (*R*)- and (*S*)-PMP-8-BrDAPp

Compound	Score	
	1st docked pose	2nd docked pose
(*R*)-PMP-8-BrDAPp	–47.1	–38.9
(*S*)-PMP-8-BrDAPp	–45.8	–41.9

EXPERIMENTAL

Materials

Acyclic nucleoside phosphonates were synthesized by authors P. B. and A. H. by previously described procedures (for details, see the following literature: PMPA, PMPG and PMPDAP (ref.[8]), PMP-8azG (ref.[9]), FPMPG (ref.[10]), PMP-8BrDAP (ref.[11]). The corresponding monophosphates were prepared by modified method[12] originally described by Moffatt and Khorana[13]. The resulting ANP monophosphates were purified by POROS® 50 HQ perfusion chromatography (Applied Biosystems, CA, USA) using linear gradient of triethylammonium bicarbonate (50–400 mmol/l) and their purity was checked by HPLC in a Waters HPLC system (996 PDA Detector, PDA Software Millenium32, version 3.05, 616 Pump with 600S Controller and Waters Fraction Collector II) equipped with 15 cm × 4 mm Supelcosil™ LC 18T 3 μm reverse-phase column. The 30 min nonlinear gradient of acetonitrile against 50 mM potassium dihydrogen phosphate, 3 mM tetrabutylammonium hydrogen sulfate, pH 5.1 at the flow rate 0.75 ml/min was used. An aliquot of every compound was than treated by alkaline phosphatase (37 °C, pH 8.0, 4 h) and analyzed on HPLC. The non-phosphorylated ANPs were identified with the aid of external standards and also with the spectra library.

All other chemicals and materials including cell culture media were commercial products from Sigma–Aldrich (USA). Fetal calf serum was a product of PAA (Austria). [^{3}H]-Inosine was purchased from Moravek Biochemicals (CA, USA).

Cell Culture

CCRF-CEM cells were cultivated in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, antibiotics (200 μg/ml of streptomycin and 200 units/ml of penicillin G) and 3 mM glutamine at 37 °C in a humidified atmosphere containing 5% CO_2.

Human Purine Nucleoside Phosphorylase (PNPase)

Purine nucleoside phosphorylase from human CCRF-CEM cells (*T lymphoblastoid cells*, human acute lymphoblastic leukemia, ATCC CCL 119) was purified to homogeneity by a combination of ion exchange chromatography (DEAE Sephadex A50) and affinity chromatography using AE-Sepharose 4B and 9-(*p*-succinylaminobenzyl)hypoxanthine as the matrix and the ligand, respectively[2]. SDS-PAGE of purified enzyme showed homogenous 31 kDa band.

Enzyme Assay

Kinetic constants K_m, K_i and V_{max} were determined from the Lineweaver–Burk and Dixon plots using various concentrations of [^{3}H]-inosine (10–120 mmol/l at 1 mM P_i) and phosphate P_i (40–350 μmol/l at 50 μM [^{3}H]-inosine) in the presence of 1 mM DTT and BSA 0.2 mg/ml. The enzyme concentration was 91 ng/ml. The reaction was carried out at 37 °C for 10 min and stopped by spotting a 2 μl aliquot onto PEI celulose plate (prespotted with 0.01 μmol of each hypoxanthine and inosine). The plate was developed in the solvent system *n*-butanol–acetic acid–water (10:1:3). The spots were visualised under UV light (254 nm) and cutted out for radioactivity determination in the toluene-based scintillation cocktail. Data based on results from at least four independent experiments were evaluated by the non-linear regression method (GOSA, BioLog, France).

Docking and Modeling

Estimation of the ligands binding positions was carried out using program DOCK 6.2 docking software[14]. As a target molecule human purine nucleoside phosphorylase has been taken under the code name 3BGS (ref.[15]). Both *R*- and *S*-enantiomers have been evaluated in their binding motifs. For each structure, there has been scored 10 conformations and first two of them are taken into account. Settings of the programs were used default, only in the proper input for docking step, max. orient. parameter was changed to 1500 and simple max. iter. parameter was changed to 2500. PHE 200 had to be removed for the docking process in order to make active site accessible.

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