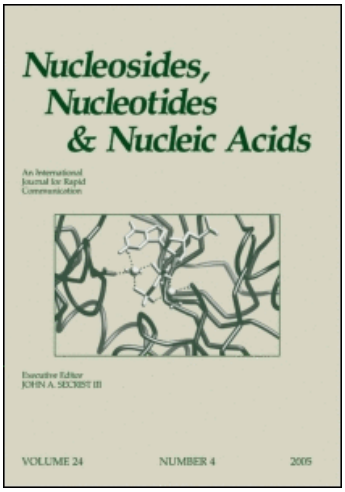

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Acyclic Phosphonate Nucleotides and Human Adenylate Kinases: Impact of a Borano Group on α-P Position

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ACYCLIC PHOSPHONATE NUCLEOTIDES AND HUMAN ADENYLATE KINASES: IMPACT OF A BORANO GROUP ON α-P POSITION

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□ *Adenylate kinases are involved in the activation of antiviral drugs such as the acyclic phosphonates analogs PMEA and (R)PMPA. We examine the in vitro phosphorylation of PMEA and PMPA bearing a borano- or a H- group on the phosphorus atom. The α-borano or α-H on PMEA and PMPA were detrimental to the activity of recombinant human AMP kinases 1 and 2. Docking PMEA to the active site of AMP kinase 1 indicated that the borano group may prevent two conserved critical Arg interactions with the α-phosphate, resulting in substrate bad positioning.*

Keywords Borano analogues; phosphinate; phosphonate; adenylate kinase

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This work is dedicated to the memory of our friend and colleague Dr Simon Robert SARFATI, who died December 12, 2005.

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INTRODUCTION

Nucleos(t)ide analogues are widely used in the treatment of cancers and viral infections and require conversion to their tri-phosphate metabolites before reaching their viral target. Although successful in combination therapies, their long-term efficacy is limited by the emergence of drug-resistant strains of virus. The development of new molecules is thus a permanent challenge. Since a high intracellular concentration of the triphosphate form of the analogues (or diphosphate form of acyclic nucleotide phosphonates) is correlated with enhanced activity, understanding the individual pathways of each nucleoside analogue will help to predict and explain drug potency. Nucleotide analogues with a nonbridging oxygen on the α-phosphate substituted by a borano group (BH_3^-) are promising second-generation molecules.[1] α-Boranophosphates have been proposed as mimics of natural phosphodiesters in DNA, in antisense oligonucleotide strategies or for boron neutron-capture therapy.[2–4] They are also potential antiviral molecules when combined with other chemical modifications.[2] Mechanistic analysis of reverse transcriptase from resistant virus indicates that α-borano derivatives are incorporated into DNA by suppressing reverse-transcriptase mediated resistance when the resistance is due to a poor catalytic rate.[3] α-Borano-derivatives of AZT- and d4T-diphosphate also react faster with NDP kinases, allowing the formation of increasing amounts of the chain terminators.[1] However, the efficacy of AZT- and d4T- borano monophosphate has not yet been demonstrated on HIV-infected cells in culture, perhaps because these charged molecules do not readily enter the cells, or because of problems of phosphorylation in the cells.

Acyclic nucleoside phosphonates (ANPs) have become a key class of antiviral nucleoside derivatives. Prodrugs of tenofovir, (R)PMPA, and adefovir, PMEA, were approved for HIV−1 infections in 2001 and for hepatitis B virus infections in 2002, respectively.[4]. Unlike nucleoside analogues, PMEA and (R)PMPA (further called PMPA), as dAMP mimics, do not require the initial phosphorylation catalyzed by a nucleoside kinase (Figure 1). They rely on cellular adenylate kinases for their activation, resulting in persistent metabolites.[5–7] The NMP kinases catalyze the phosphotransfer “in-line” between nucleotides without the formation of a covalent intermediate. Both the fold and the kinetics are different from NDP kinase, the enzyme involved in the next step of the salvage pathway.[8] The crystal coordinates of human AK1 complexed with the bisubstrate inhibitor Ap5A, and of human AK2 with bound Ap4A, were recently deposited in the Protein Data Bank (code 1Z83 and 2C9Y, respectively).[9] AK1 is one of the most abundant cytosolic proteins in vertebrate muscles with a concentration as high as 0.1 mM.[10] AK2, located in the mitochondrial intermembrane space, plays a major role in heart, liver, and kidney energy metabolism.

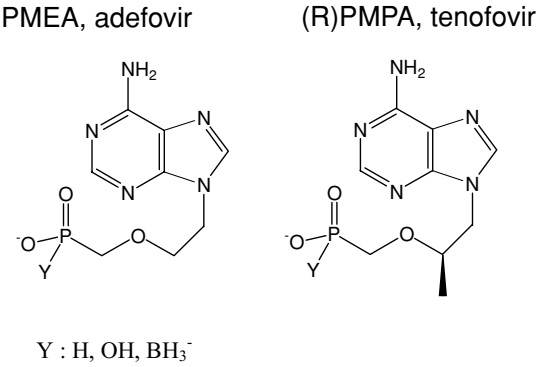

FIGURE 1 Structure of analogues of PMEA (adefovir) and (R)PMPA (tenofovir) bearing a borano group or a hydrogen on the α-phosphorus atom.

We report here the cloning of the gene encoding human AK1 and AK2, the major cytosolic and mitochondrial adenylate kinases of human cells, and their expression in *E. coli*. We used steady-state kinetic analysis to characterize the specificity of the recombinant enzymes for natural substrates, acyclic phosphonates analogues, PMEA and PMPA and for their analogues. The α-boranophosphonate and the H-phosphinates derivated from PMEA and PMPA have been synthesized recently[11] (Figure 1), as well as their diphosphate which presented interesting properties by overcoming resistance of HIV reverse transcriptase.[12] We examined whether the modifications in α-P affected the rate at which a phosphate from ATP is added to PMEA and (R)PMPA, a reaction catalysed by human adenylate kinases 1 and 2 (hAK1 and hAK2).

MATERIALS AND METHODS

Chemistry: α-Borano-phosphonates and H-phosphinates derived of PMEA and PMPA were made according the described procedure.[11]

Cloning of the gene encoding human AMP kinases 1 and 2: The 582 bp and 717 bp fragments corresponding to the AK1 and AK2 genes (coding for the human AK1 and AK2 proteins) were amplified by PCR[13] using the human liver Quick-clone cDNA (BD Bio-sciences Clontech) as a template. The synthetic oligonucleotides used for amplification were

5′-ggaattc**catatg**GaagAGAAGCTGAAGAAAAC-3′ and
5′-ccg**ctcgag**ttacttTAGGGCGTCCAGGTGGGTG-3′ for AK1 and
5′-ggaattc**catatg**GCTCCCAGCGTGCCAGC-3′ and
5′-ccg**ctcgag**ttaGATAAACATAACCAAGTCTTTACA-3′ for AK2.

The NdeI and XhoI restriction sites (in bold letters in the oligonucleotide sequences) were created at both ends of the amplified fragments. The

amplified fragments were digested with NdeI and XhoI and inserted into the pET28a plasmid (Novagen, Inc., Madison, WI, USA) digested with the same enzymes. The DNA inserts were sequenced using the double-stranded dideoxynucleotide sequencing technique[14] in order to verify the absence of any mutation. The plasmids harboring the hAK1 and hAK2 genes were named pHL20-11 and pHL20-21.

Expression and purification of recombinant hAK1 and hAK2: Recombinant proteins were produced in *E. coli* BL21 (DE3) strain (Novagen, Inc.) transformed with the pDIA17[15] and the corresponding expression plasmids. The same procedure was followed for both hAKs. Cultures were grown at 37°C in a rich medium (2YT) containing 30 μg/mL chloramphenicol and 70 μg/mL kanamycine until an absorbance A^{600} of 1 was reached. After induction with 1 mM isopropylthio-β-D-galactoside (IPTG) and continued incubation for 3 hours at 30°C, cells were harvested. A cell pellet corresponding to 1 L of culture was suspended in 40 mL lysis buffer (50 mM phosphate buffer pH 8, 300 mM NaCl, 10 mM imidazol, protease inhibitors (Complete EDTA-free, Roche Applied Science, Indianapolis, IN, USA) and 1 mM DTT) and stored at −80°C. Cells were broken by sonication and centrifuged at 10,000 rpm for 30 minutes at 4°C. The supernatant was placed on a 20 mL Ni-NTA column (Quiagen, Germany) equilibrated with lysis buffer. The column was washed with lysis buffer (100 mL) and the protein was eluted with a linear gradient of imidazol (10-250 mM) pH 8. Fractions containing the enzymatic activity were pooled and dialysed against 50 mM Tris, 20 mM NaCl, 1 mM DTT, 50% glycerol, pH 7.5 and stored at –20°C. The protein appeared to be >95% pure as judged by SDS–PAGE gels (Figure 2). There was no loss of activity after 3 months storage at –20°C. The molar extinction coefficients at 278 nm were calculated from the absorbance of individual amino acids as 9925 M^{-1} cm^{-1} for hAK1 and 7250 M^{-1} cm^{-1} for hAK2. Mass spectra were obtained on a Voyager-DE PRO MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a pulsed nitrogen laser (337 nm, 3 ns pulse). Operating parameters for reflection were: accelerating voltage (20 kV), grid voltage (75%), guidewire voltage (0.005%) and 100 laser shots per spectrum. The ions of des-Arg^1-bradykinin, angiotensin I, Glu^1-fibrinopeptide B and neurotensine were used for external calibration. Monoisotopic masses were used with deviation for mass assignment within +/− 0.5 Da.

Kinetic measurements: The catalytic activity of the NMP kinases was followed in a spectrophotometer by measuring ADP formation.[16,17] The assay mix contained 50 mM Tris-HCl pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, 1 mM ATP, 0.2 mM NADH, 10 mM DTT, 1 mM phosphoenolpyruvate and the auxiliary enzymes: pyruvate kinase (4 U) and lactate dehydrogenase (4 U). The reaction was started at 37°C by adding AMP or another phosphate acceptor and enzyme at the indicated concentration. The magnesium concentration in the hAK2 experiments was 2 mM, *as a slight inhibition was observed for*

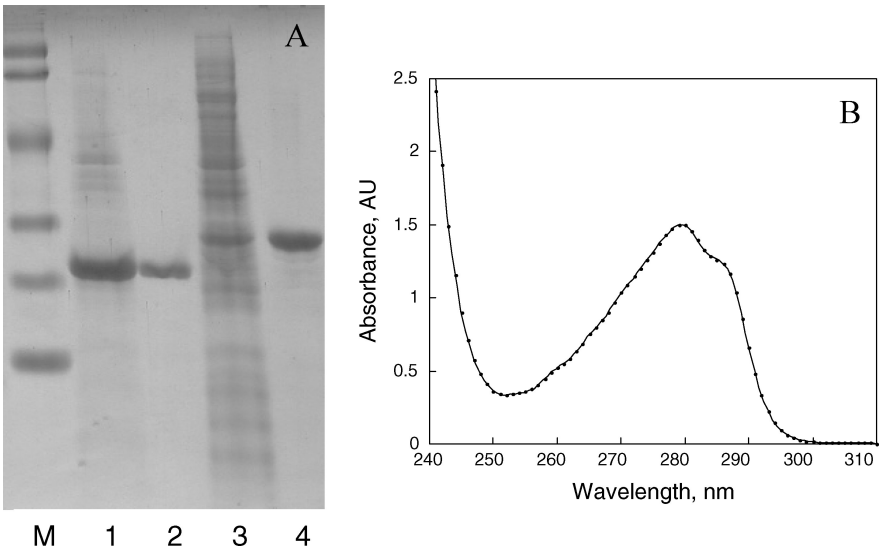

FIGURE 2 Purification of recombinant human adenylate kinases 1 and 2. (A) hAK purifications SDS/PAGE stained with Coomassie Blue: (1) hAK1 expressing cell extract, (2) pooled active hAK1, (3) hAK2 expressing cell extract, (4) pooled active hAK2, (M) markers. (B): Absorption spectrum of the native purified hAK1 enzyme.

Mg2+ concentration above 3 mM. The rates were kept below 0.2 ΔA^{340}/minute to avoid limitation by the coupled system. The rates for AMP kinases were calculated on the basis of two ADPs generated during the reaction with AMP. When dAMP, PMEA, or PMPA were the phosphate acceptors, only one ADP was produced as in the AMP kinase assay. One unit of enzyme activity is defined as 1 μm of NMP transformed per min at 37°C. Curve-fitting was performed using Kaleidagraph (Abelbeck Software, Reading, PA, USA) for a hyperbolic equation.

Structural models: Structural models were built and visualized using Insight II softwares package (Accelrys, San Diego, CA, USA). The coordinate of the human adenylate kinase hAK1 (1Z83.pdb) was used for building our complexes. The pdb file was corrected from some inconstancies, for example multiple positions for some residues. Lacking atoms were reintroduced to be able to accommodate CHARMM force field for minimisation steps. PMEA was built in SMILES format using JME Molecular Editor (Novartis, Inc.) and translated in PDB format using a online SMILES translator (http://cactus.nci.nih.gov/ services/translate). PMEA was then docked to the active pocket of hAK1, and superimposed to AMP ligand for comparison of potent interactions expected for these configurations. Since PMEA minimized structure displayed a certain conformational flexiblity, rotamers were generated using Biopolymer Insight II module functionalities to provide a linear version of PMEA phosphonate chain. Linear PMEA acyclic chain allows a better illustration of interactions network disturbance compared to AMP.

RESULTS AND DISCUSSION

Characterization of Recombinant Human Adenylate Kinases 1 and 2

The recombinant enzymes, hAK1 and hAK2, expressed as a His-tag protein fusion in *E. coli*, were purified in a single step on a Ni-nitrilotriacetic acid agarose column yielding 90 mg hAK1 protein and 16 mg hAK2 per liter of growth medium. The proteins were at least 95% pure by SDS-PAGE (Figure 2A). The purified AMP kinases were analyzed by mass spectrometry (MALDI-TOF): The measured mass of the hAK1 was 25,620 (predicted mass of the recombinant enzyme: 25,929). The measured and expected masses of hAK2 were 28,704 and 28,640. Both recombinant enzymes had extra 19 amino acid residues, including the 6 His-tag, at the N-terminus. The slight difference between the masses for hAK1 could be due to some degradation. The absorbance spectrum of native hAK1 (Figure 2B) is similar to that of native hAK2, but hAK2 tended to aggregate in absence of glycerol.

The catalytic properties of recombinant hAK1 and hAK2 are presented on Table 1. At a saturating concentration of ATP (1 mM), AMP phosphorylation followed a Michaelis-Menten mechanism, with a maximum rate of (1240 +/− 50) U/mg for hAK1 and (170 +/− 25) U/mg for hAK2, and turnover numbers of 500 $s^{-1}$and 80 s^{-1}, respectively. Excess substrate ([AMP] > 0.25 mM) was inhibitory with hAK2 (K_I^{AMP} = 0.5 mM). The magnesium ions (1−10 mM) did not influence the activity of hAK1, but hAK2 activity was optimum at [Mg2+] = 2 mM. AMP was a better substrate than dAMP for both kinases (Table 1), and the maximum rates for AMP and hAK1 gave excellent catalytic efficiencies of 10^5-10^6 $M^{-1}s^{-1}$. The saturation curves for [ATP] in the presence of 1 mM AMP indicated a K_M of 0.060 mM for both enzymes. Among NTPs, ATP only acted as a phosphodonor for human AK1 while GTP, CTP and UTP could replaced ATP for hAK2 with about 15% efficiency (at 0.1 mM NTP and 0.05 mM AMP, results not shown), as previously observed with cytosolic and mitochondrial extracts from CEM cells.[7] The values of the catalytic parameters for the two kinases and their natural substrates (Table 1) are comparable to the kinetic parameters of hAK1 found in previous studies[18,19]and of enzymes purified from cattle heart.[20,21] The absence of any post-translational modification and the presence of the His-tag did not significantly change the enzyme activity, as expected from the N-acetylation of natural hAK1.

Phosphorylation of PMEA, PMPA and Their Analogues

PMEA was phosphorylated by hAK1 at a maximum turnover of 0.1 s^{-1} and PMPA at 0.2 s^{-1}, while hAK2 presented higher rates (1.5 and 2.5 s^{-1}, respectively) (Figure 3). The K_M values for the two enzymes were identical

TABLE 1 Catalytic parameters of recombinant human AK1 and AK2 with several adenylate analogues

	Human AK1				Human AK2			
	Max rate (U/mg)	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($M^{-1}s^{-1}$)	Max rate (U/mg)	k_{cat}(s^{-1})	K_M (mM)	k_{cat}/K_M ($M^{-1}s^{-1}$)
AMP	1240 ± 20	500	0.14 ± 0.02	3×10^6	170 ± 25	80	0.08 ± 0.02	10^6
dAMP	600 ± 30	240	1.5 ± 0.3	1.6×10^5	230 ± 30	110	0.21 ± 0.05	5×10^5
PMPA	0.60 ± 0.03	0.22	3.0 ± 0.3	75 ± 10	5.0 ± 0.1	2.4	3.0 ± 0.3	800
PMEA	0.21 ± 0.03	0.08	6 ± 1	14 ± 5	3.2 ± 0.1	1.5	6.0 ± 0.5	250

Human AK1 and AK2 activities were measured in standard buffer containing 1 mM ATP and 5 mM or 2 mM magnesium ions. $K_M^{ATP} = 0.06$ mM for both enzymes. Human AK2 is inhibited by AMP with a K_I of (0.5 ± 0.1) mM. All experiments were performed at 37°C. Data correspond to at least three independent experiments.

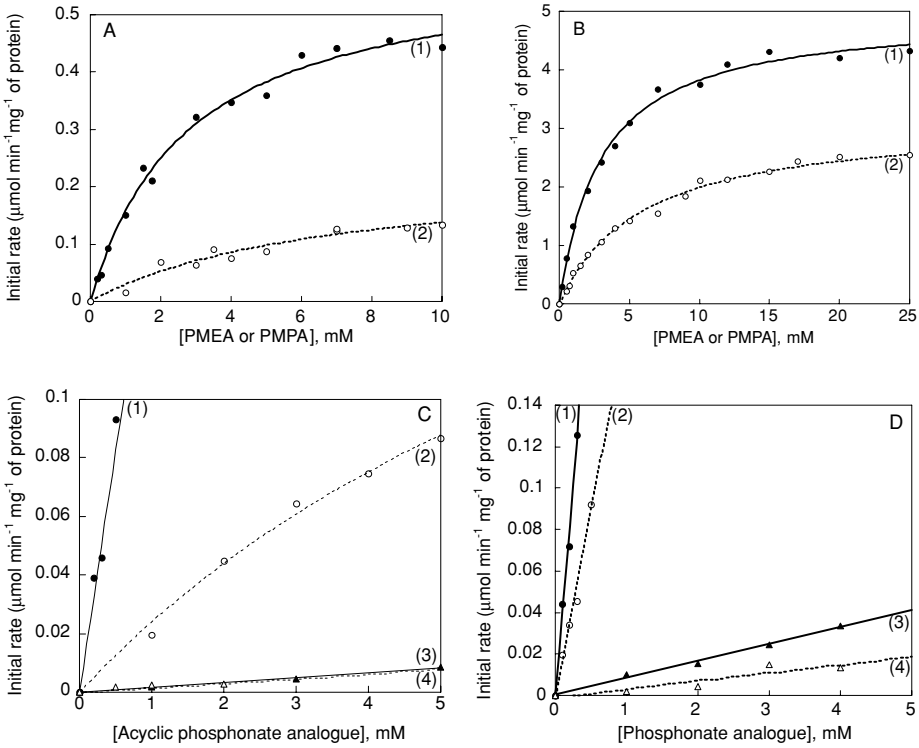

FIGURE 3 Saturation curves of recombinant human adenylate kinases 1 and 2 with PMEA and PMPA in 1 mM ATP and 5 mM Mg2+ (hAK1) or 2 mM (hAK2). (A) and (C) hAK1 activity with (1) PMPA (●) and (2) PMEA (○); (B) and (D) hAK2 activity with (1) PMPA (●) and (2) PMEA (○); (C) hAK1 activity with (3) α-BH_3^- -PMPA (▲) and (4) α-BH_3^- -PMEA (△) derivatives; (D) hAK2 activity with (3) α-BH_3^- -PMPA (▲) and (4) α-BH_3^- -PMEA (△) derivatives. (Parameters are shown in Table 2).

($K_M^{PMEA} = 6$ mM and ($K_M^{PMPA} = 3$ mM, Table 1), revealing lower binding affinities than for dAMP. As a result, the substrate specificity constants (k_{cat}/K_M) for the phosphonates were 0.2–0.01% of that of dAMP in the hAKs catalyzed reactions: that is, 800 $M^{-1}s^{-1}$ and 250 $M^{-1}s^{-1}$ for PMPA and PMEA with hAK2, and 75 and 14 $M^{-1}s^{-1}$ with hAK1 (Table 1). Thus, although phosphonates are thus poor substrates for both hAK1 and hAK2, they are phosphorylated with a ten times higher efficiency by the mitochondrial enzyme hAK2 as observed in previous studies.[7] The K_M values determined in these studies were found slightly higher, which is not surprising for results obtained with cell extracts, i.e. in the presence of contaminants.[7]

Modifications of PMEA and PMPA by replacing an oxygen with a BH_3^- group resulted in α-boranophosphonates (Figure 1). The modification drastically reduced phosphorylation by both hAK1 and hAK2: Figures 3C and 3D show that the α-borano derivatives up 5 mM are phosphorylated extremely slowly. The resulting catalytic efficiencies (k_{cat}/K_M) for hAK2 and α-BH_3-PMPA is 10 $M^{-1}s^{-1}$ (Table 2). The presence of one hydrogen instead

TABLE 2 Catalytic efficiencies of (R)PMPA and PMEA derivatives with hAK1 and hAK2 (Data correspond to at least three independent experiments)

	k_{cat}/K_M (M^{-1} s^{-1})	
	hAK1	hAK2
PMPA	75 ± 5	800 ± 50
PMEA	14 ± 2	250 ± 20
α-BH_3^--PMPA	1	10 ± 2
α-BH_3^--PMEA	<1	4.3 ± 1
α-H-PMPA	<1	<1
α-H-PMEA	<1	<1

of a borano group, resulting in H-phosphinates, is even more deleterious as these compounds are not substrates at all for AMP kinases 1 and 2. These analogs are not either substrates for human UMP-CMP kinase (not shown).

Structural Analysis and Molecular Modeling

The structural basis of AMP kinases activity has been investigated in several studies. AMP kinases, like all NMP kinases, share a highly conserved structure comprising an AMP binding domain, a central CORE domain that carries the ATP binding loop (P-loop) and a LID domain providing catalytic residues necessary for the reaction. The LID domain is larger in hAK2. The AMP and LID domains are extremely mobile and undergo closure upon binding of substrates.[22] Protein dynamics studies correlated the lid-opening rate with the catalytic turnover.[23,24]

In the published structure of hAK2 in a complex with Ap4A, both adenines were located near the ATP site, providing no information on the acceptor site. On the contrary, hAK1 complexed to Ap5A (1Z83) showed the interactions of both ATP and AMP in the active site in a closed conformation.[9] The structure of PMEA bound to hAK1 was then modelized using this structure (Figure 4). The adenosine plus the first phosphate from Ap5A is likely to mimic AMP in the active site of hAK1 in closed conformation where helices α3 and α4 close up on AMP. As shown in Figure 4, the 2′ hydroxyl of AMP interacted with the Gln65 main chain carbonyl belonging to the mobile α4 helix of the NMP binding domain, locking the nucleotide in the closed binding site. Replacement of AMP by dAMP suppressed the H-bond between 2′-OH and the main chain oxygen of Gln65, resulting in looser binding. AMP was readily replaced by PMEA because the volume of the acyclic moiety of the analogue is smaller than a ribose (Figure 4). Thus, both PMPA and PMEA, less bulky than dAMP and lacking "handles,"[25] fitted snugly into the active site cleft. PMPA is slightly bulkier with the extra methyl group, and it may fit better into

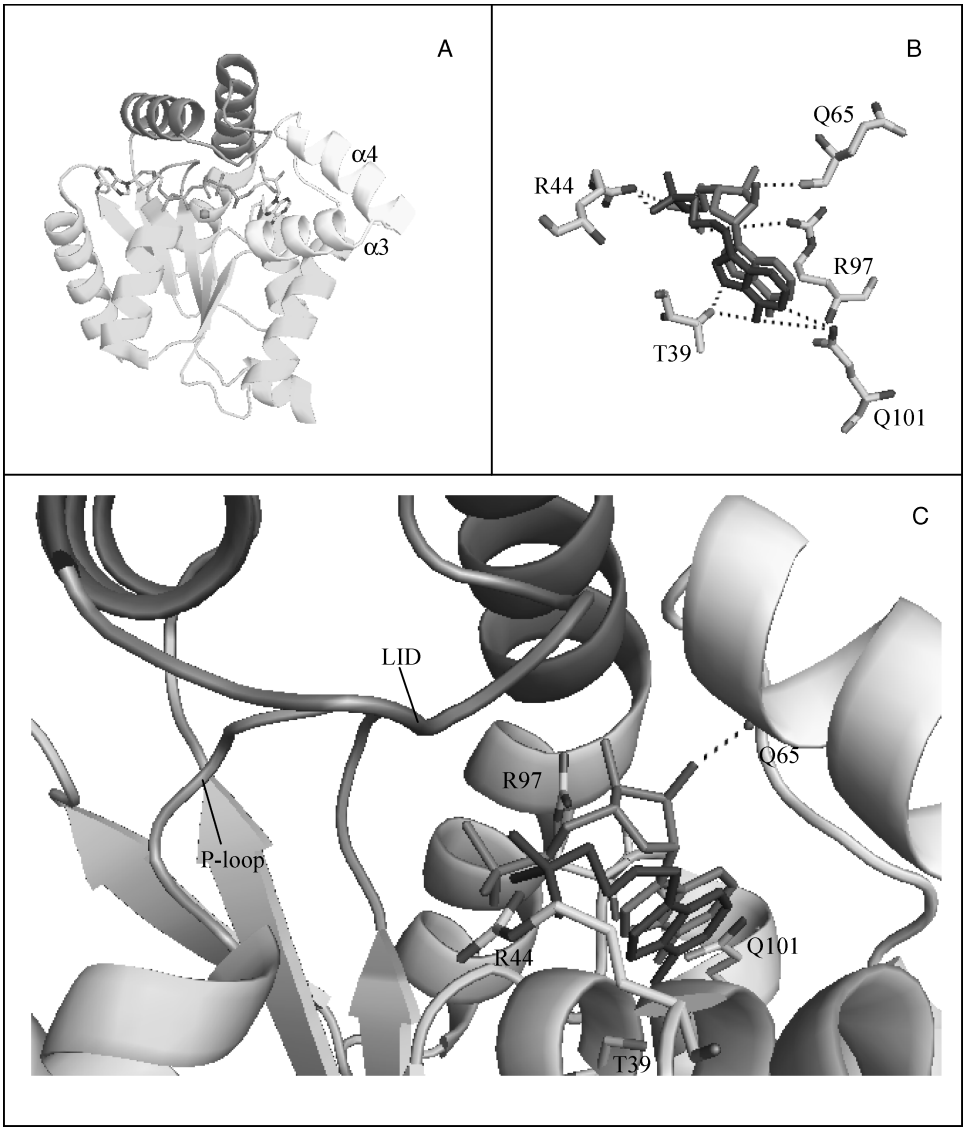

FIGURE 4 Docking of PMEA in the active site of hAK1. (A) Overall structure of hAK1 (1Z83.pdb) showing the CORE domain (green), the NMP domain (yellow), the LID domain (dark cyan), the P loop (orange), the zinc atom (grey) and Ap5A in sticks and CPK colors. (B) Superimposition of PMEA (blue) and AMP (red) docked in hAK1 AMP binding site. Only the residues interacting with AMP are shown with the main interactions in dashed lines. (C) Close up of AMP/PMEA binding site of hAK1 (same colors as in A). The superimposition of AMP and PMEA illustrates the disturbance of the AMP interactions network.

the AMP binding site. The structure of another acyclic analog, ganciclovir-monophosphate in the active site of GMP kinase from *E. coli* showed that the absence of the 2′hydroxyl of the ribose moiety was critical and resulted in the incomplete closure of the NMP domain.[26] Similarly, we propose that

despites the good fit of PMEA in the AMP binding site, the closing of NMP domain is difficult upon PMEA binding.

In the complex hAK1-Ap5A complex, the phosphate equivalent to the α-P of AMP is tightly bound to Arg44 from helix α3 and Arg97 belonging to the CORE domain. These two Arg residues are well conserved in the other NMP kinases, ensuring an efficient nucleophilic attack on γ-P of ATP by the equatorial nonbridging oxygen positioned in line. It seems unlikely that the borano group occupies the equatorial position on the phosphorus but rather it lies in one of the two other positions, interacting with the kinase Arg residues. The borano group in boranophosphate is similar in size to a methyl group in a methylphosphonate and slightly larger than the oxygen in a natural phosphate.[27] The negative charge on the natural phosphate oxygen is retained in the boranophosphonate derivatives but the polarity is affected as the negative charge is polarized away from the borano.[27] Further, there is no lone pair of electrons in the $-BH_3$ group, as with oxygen. As a consequence, interaction of the borano group with the critical Arg residues must be different. A theoretical study using quantum molecular mechanics calculations proposed a concerted phosphoryl transfer for the related enzyme UMP-CMP kinase.[28] According to these authors, the synchronous shift of a proton from NMP to the γ-phosphate should accompany the phosphate transfer. Such a proton shift may be difficult (or impossible) with borano-modified NMP or ANP, resulting in the observed decreased reaction rate.

Similar results were observed with α-boranoP-TMP and TMP kinase. The reaction of thymidine analogs with TMP kinase was described as a bottleneck in the activation of AZT and d4T.[29] The substitution of a BH_3^- group for an oxygen in TMP was found to slow down the phosphotransfer reaction catalyzed by yeast TMP kinase by nearly two orders of magnitude (result not shown). Previous data indicated that the 5′-H-phosphonates and 5′-fluorophosphonates derived from dAMP were apparently not phosphorylated by partly purified hAK from placenta, also suggesting that the nature of the phosphate residue is crucial for the phosphorylation catalyzed by adenylate kinases.[30]

CONCLUSION

The lack of activity of NMP kinases with the derivatives modified on the α-P is in complete contrast with the reported improvement (X 10) in the phosphorylation of α-(*Rp*)-boranotriphosphate derivatives of AZT and d4T by NDP kinase.[1] The BH_3^- group is beneficial for the HIV reverse transcriptase even for enzymes from resistant viruses.[3] The therapeutic potential of PMEA and PMPA with a α-borano group could be demonstrated, in future studies, by vectorising or encapsulating the mono or fully

phosphorylated active form: this should facilitate the delivery of the active derivative to cells. Nanogel formulations of AZT-triphosphate in cross-linked polymers have already been shown to be particularly cytotoxic for breast cancer cell lines.[31]

ABBREVIATIONS

ANP, acyclic nucleotide phosphonates; AZT, 2′,3′-dideoxy-3′-azido thymidine; cidofovir, (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl) cytosine; d4T, 2′,3′-dideoxy-2′,3′-didehydrothymidine; PMEA, 9-[2-(phosphonomethoxy)ethyladenine; (R)PMPA, (*R*)-9-[2-(phosphonomethoxy)propyl]adenine; hAK1, human adenylate kinase 1 (cytosolic); hAK2, human adenylate kinase 2 (mitochondrial); U, enzyme activity unit (1 micromole of substrate transformed per minute).

REFERENCES

1. Meyer, P.; Schneider, B.; Sarfati, S.; Deville-Bonne, D.; Guerreiro, C.; Boretto, J.; Janin, J.; Véron, M.; Canard, B. Structural basis for activation of α-boranophosphate nucleotide analogues targeting drug-resistant reverse transcriptase. *EMBO J.* **2000**, 19, 3520–3529.
2. Boyle, N.A.; Rajwanshi, V.K.; Prhavc, M.; Wang, G.; Fagan, P.; Chen, F.; Ewing, G.J.; Brooks, J.L.; Hurd, T.; Leeds, J.M.; Bruice, T.W.; Cook, P.D. Synthesis of 2′,3′-dideoxynucleoside 5′-alpha -P-borano-*beta, gamma* (difluoromethylene)triphosphates and their inhibition of HIV-1 reverse transcriptase. *J. Med. Chem.* **2005**, 48, 2695–2700.
3. Deval, J.; Alvarez, K.; Selmi, B.; Bermond, M.; Boretto, J.; Guerreiro, C.; Mulard, L.; Canard, B. Mechanistic insights into the suppression of drug resistance by human immunodeficiency virus type 1 reverse transcriptase using α-boranophosphate nucleoside analogs. *J. Biol. Chem.* **2005**, 280, 3838–3846.
4. De Clercq, E.; Holy, A. Acyclic nucleoside phosphonates: a key class of antiviral drugs. *Nat. Rev. Drug Discov.* **2005**, 4, 928–940.
5. Votruba, I.; Bernaerts, R.; Sakuma, T.; De Clercq, E.; Merta, A.; Rosenberg, I.; Holy, A. Intracellular phosphorylation of broad-spectrum anti-DNA virus agent (S)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine and inhibition of viral DNA synthesis. *Mol. Pharmacol.* **1987**, 32, 524–529.
6. Balzarini, J.; Hao, Z.; Herdewijn, P.; Johns, D.G.; De Clercq, E. Intracellular metabolism and mechanism of anti-retrovirus action of 9-(2-phosphonylmethoxyethyl)adenine, a potent anti-human immunodeficiency virus compound. *Proc. Natl. Acad. Sci.* **1991**, 88, 1499–1503.
7. Robbins, B.L.; Greenhaw, J.; Connelly, M.C.; Fridland, A. Metabolic pathways for activation of the antiviral agent 9-(2-phosphonylmethoxyethyl)adenine in human lymphoid cells. *Antimicrob. Agents Chemother.* **1995**, 39, 2304–2308.
8. Morera, S.; Lacombe, M.-L.; Xu, Y.; LeBras, G.; Janin, J. X-ray structure of nm23 human Nucleoside Diphosphate kinase B at 1.8A resolution. *Structure* **1995**, 3, 1307–1314.
9. Filippakopoulos, P.; Bunkoczi, G.; Jansson, A.; Schreurs, A.; Knapp, S.; Edwards, A.; Von Delft, F.; Sundstrom, M. Crystal structure of human AK1A in complex with AP5A. *Structural Genomics Consortium Oxford. PDB code 1Z83*, 2005.
10. Maughan, D.W.; Henkin, J.A.; Vigoreaux, J.O. Concentrations of glycolytic enzymes and other cytosolic proteins in the diffusible fraction of a vertabrate muscle proteome. *Mol. Cell Prot.* **2005**, 4, 1541–1549.
11. Barral, K.; Priet, S.; Sire, J.; Neyts, J.; Balzarini, B.; Canard, B.; Alvarez, K. Synthesis, *in vitro* antiviral evaluation and stability studies of novel alpha-borano-nucleotide analogues of 9-(2-

(phosphonylmethoxy)ethyl)adenine (PMEA) and (R-9-(2-(phosphonomethoxy)propyl)adenine (PMPA). *J. Med. Chem.* **2006**, 49, 7799–7806.
12. Frangeul, A.; Barral.; Alvarez, K.; Canard, B. In vitro suppression of K65R reverse transcriptase-mediated tenofovir- and adefovir-5′-diphosphate resistance conferred by the boranophosphonate derivatives. *Antimicrob. Agents Chemother.* **2007**, 51, 3162–3167.
13. Sambrook, J.; Fritsch, E.F.; Manniati, S.T. *Molecular cloning: A laboratory manual*. Cold Spring Harbor: NY, 1989.
14. Sanger, F.; Nicklen, S.; Coulson, A.R. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **1977**, 74, 5463–5467.
15. Munier, H.; Gilles, A.-M.; Glaser, P.; Krin, P.; Danchin, A.; Sarfati, R.; Barzu, O. Isolation and characterization of catalytic and calmodulin-binding domains of *Bordetella pertussis* adenylate cyclase. *FEBs. J.* **1991**, 196, 469–474.
16. Blondin, C.; Serina, L.; Wiesmuller, L.; Gilles, A.-M.; Barzu, O. Improved spectrophotometric assay of nucleoside monophosphate kinase activity using the pyruvate kinase/lactate dehydrogenase coupling system. *Anal. Biochem.* **1994**, 220, 219–221.
17. Pasti, C.; Gallois-Montbrun, S.; Munier-Lehmann, H.; Veron, M.; Gilles, A.-M.; Deville-Bonne, D. Reaction of human UMP-CMP kinase with natural and analog substrates. *FEBs. J.* **2003**, 270, 1784–1790.
18. Ayabe, T.; Takenaka, H.; Takenaka, O.; Sumida, M.; Maruyama, H.; Onitsuka, T.; Shibata, K.; Uesugi, S.; Hamada, M. Essential lysine residues in the N-terminal and the C-terminal domain of hyman adenylate kinase interact with adenine nucleotides as found by site-directed random mutagenesis. *Biochemistry* **1997**, 36, 4027–4033.
19. Yan, H.; Tsai, M.-D. Nucleoside monophosphate kinases: structure, mechanism and substrate specificity. *Adv. Enzymol.* **1999**, 73, 103–134.
20. Font, B.; Gautheron, D. General and kinetic properties of pig heart mitochondrial adenylate kinase. *Biochem. Biophys. Acta* **1980**, 611, 299–308.
21. Walker, E.J.; Dow, J.W. Location and properties of two isoenzymes of cardiac adenylate kinase. *Biochem. J.* **1982**, 203, 361–369.
22. Vonrheim, C.; Schlauderer, G.J.; Schulz, G.E. Movie of the structural changes during a catalytic cycle of nucleoside monophosphate kinases. *Structure* **1995**, 3, 483–490.
23. Wolf-Watz, M.; Thai, V.; Henzler-Wildman, K.; Hadjipavlou, G.; Eisenmesser, E.Z.; Kern, D. Linkage between dynamics and catalysis in a thermophilic-mesophilic enzyme pair. *Nat. Struc. Mol. Biol.* **2004**, 11, 945–949.
24. Bae, E.; Phillips, G.N.Jr. Roles of static and dynamic domains in stability and catalysis of adenylate kinase. *Proc. Natl. Acad. Sci.* **2006**, 103, 2132–2137.
25. Tuske, S.; Sarafanios, S.G.; Clark Jr, A.D.; Ding, J.; Naeger, L.K.; White, K.L.; Miller, M.D.; Gibbs, C.S.; Boyer, P.L.; Clark, P.; Wang, G.; Gaffney, B.L.; Jones, R.A.; Jerina, D.M.; Hughes, S.H.; Arnold, E. Structures of HIV-1 RT-DNA complexes before and after incorporation of the anti-AIDS drug tenofovir. *Nat. Struc. Mol. Biol.* **2004**, 11, 469–474.
26. Hible, G.; Daalova, P.; Gilles, A.-M.; Cherfils, J. Crystal structures of GMP kinase in complex with ganciclovir monophosphate and Ap5G. *Biochimie* **2006**, 88, 1157–1164.
27. Summers, J.S.; Shaw, B.R. Boranophosphates as mimics of natural phosphodiesters in DNA. *Cur. Med. Chem.* **2001**, 8, 1147–1155.
28. Hutter, M.C.; Helms, V. Phosphoryl transfer by a concerted reaction mechanism in UMP-CMP kinase. *Prot. Sci.* **2000**, 9, 2225–2231.
29. Lavie, A.; Schlichting, I.; Vetter, I.R.; Konrad, M.; Reinstein, J.; Goody, R.S. The bottleneck in AZT activation. *Nat. Med.* **1997**, 3, 922–924.
30. Skoblov, J.S.; Frank-Kamenetskaya, M.D.; Chernov, D.N.; Krayevsky, A.A. Modified nucleotides as substrates and inhibitors of adenylate kinase from different sources. *FEBS. Let.* **1996**, 395, 283–285.
31. Vinogradov, S.V.; Kohli, E.; Zeman, A.D. Cross-linked polymeric nanogel formulations of 5′-triphosphates of nucleosides analogues: role of a cellular membrane in drug release. *Mol. Pharmacol.* **2005**, 2, 449–461.