HUMAN N^6-METHYL-AMP/DAMP AMINOHYDROLASE (ABACAVIR 5′-MONOPHOSPHATE DEAMINASE) IS CAPABLE OF METABOLIZING N^6-SUBSTITUTED PURINE ACYCLIC NUCLEOSIDE PHOSPHONATES

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Recombinant human abacavir monophosphate deaminase (hABC-MP deaminase) was compared with the recently described rat N^6-methyl-AMP (meAMP) aminohydrolase. hABC-MP deaminase, a 42 kDa polypeptide, exists predominantly as a monomer under non-denaturing conditions. Similar to the rat enzyme, hABC-MP deaminase efficiently catalyzes the hydrolytic deamination of natural substrates meAMP (**5**), N^6,N^6-dimethyl-AMP (**13**) and medAMP (**6**). Acyclic nucleoside phosphonate (ANP) N^6-cyclopropyl-2,6-diamino-9-[2-(phosphonomethoxy)ethyl]purine (cPrPMEDAP) (**1**), an intermediate intracellular metabolite of antileukemic agent GS-9219, was effectively converted to the corresponding active guanine analog by hABC-MP deaminase. In addition to cPrPMEDAP (**1**), a number of other biologically active N^6-substituted purine ANPs are alternative substrates for hABC-MP deaminase. The efficiency of their deamination depends on the character of N^6-substitution in the adenine and/or 2,6-diaminopurine ring. ANPs with N^6-cyclic substituents are deaminated more readily than corresponding compounds with aliphatic substituents of the same length. The deamination of ANPs is also influenced by modifications at the phosphonoalkyl side chain. Among 9-[2-(phosphonomethoxy)propyl] ANPs, (*S*)-enantiomers are preferred to (*R*)-enantiomers. Alternatively, the presence of extended 9-[2-(phosphonoethoxy)ethyl] moiety leads to a moderate increase in the reaction velocity compared to cPrPMEDAP (**1**). Comparison of hABC-MP deaminase and the rat meAMP aminohydrolase across a broad spectrum of N^6-substituted substrates revealed a strong correlation of their substrate specificities. Similar to the rat meAMP aminohydrolase, hABC-MP deaminase was highly sensitive to deoxycoformycin monophosphate, but not to the guanine product of cPrPMEDAP (**1**) deamination. Together, these data demonstrate that hABC-MP deaminase is human meAMP aminohydrolase involved in the intracellular activation of biologically active N^6-substituted nucleotide analogs.

Keywords: Abacavir 5′-phosphate; N^6-Methyl-AMP aminohydrolase; 2,6-Diaminopurine; Guanine; Acyclic nucleoside phosphonates; Prodrugs; cPrPMEDAP.

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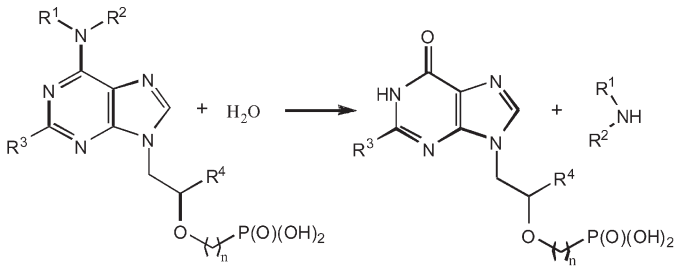
Recently, we reported the isolation and characterization of a rat liver enzyme involved in the deamination of pharmacologically important N^6-substituted purine nucleotide analogs (Scheme 1)[1]. The isolated enzyme is capable of converting an acyclic nucleoside phosphonate (ANP) N^6-cyclopropyl-2,6-diamino-9-[2-(phosphonomethoxy)ethyl]purine (cPrPMEDAP; **1**) to a biologically active nucleotide 9-[2-(phosphonomethoxy)ethyl]guanine (PMEG; **2**). In addition, the enzyme catalyzes the deamination of abacavir 5′-monophosphate (ABC-MP; **3**) to carbovir 5′-monophosphate (CBV-MP; **4**)[1], an essential step in the intracellular activation pathway of HIV reverse transcriptase inhibitor abacavir[2]. We also demonstrated that this enzyme is N^6-methyl-AMP/dAMP aminohydrolase[1] and proposed that its primary natural function in cells is to catabolize N^6-methylated AMPs (meAMP; **5**) and dAMPs (medAMP; **6**) originating from the degradation of nucleic acids[3,4]. Independently described human abacavir monophosphate deaminase (hABC-MP deaminase) exhibits properties similar to those of the meAMP aminohydrolase isolated from rat liver as it can effectively convert ABC-MP (**3**) to CBV-MP (**4**)[5].

The product of cPrPMEDAP (**1**) deamination, PMEG (**2**), possesses potent antiproliferative activity, inhibiting the *in vitro* growth of various transformed cell lines[6,7]. In addition, PMEG exhibits *in vivo* antitumor activity in a Sprague–Dawley rat experimental model for T-cell lymphoblastic leukemia/lymphoma[7,8]. PMEG is intracellularly phosphorylated to its diphosphate PMEGpp (**7**)[9,10], which acts as a potent inhibitor of replicative DNA



SCHEME 1

Reaction catalyzed by N^6-meAMP aminohydrolase. R^1, R^2 = H, alkyl or cycloalkyl; R^3 = H (N^6-substituted adenine nucleotide analogs) or NH_2 (N^6-substituted diaminopurine nucleotide analogs); R^4 = H (9-[2-(phosphonomethoxy)ethyl]purine nucleotide analogs), CH_3 (9-[2-(phosphonomethoxy)propyl]purine nucleotide analogs) or $HOCH_2$ (9-[3-hydroxy-2-(phosphonomethoxy)propyl]purine nucleotide analogs); n = 1 (9-[2-(phosphonomethoxy)]-purine nucleotide analogs) or n = 2 (9-[2-(phosphonoethoxy)]purine nucleotide analogs)

polymerases α, δ and ε (refs[11,12]). It is evident from our prior studies, as well as from studies by others, that cPrPMEDAP (**1**) acts as an intracellular prodrug of PMEG (**2**)[13–15]. Both cPrPMEDAP (**1**) and other N^6-substituted derivatives of PMEDAP (**8**)[15] that can undergo efficient intracellular conversion to PMEG (**2**) via deamination may be considered as potential agents for the treatment of various malignancies. GS-9219 is an example of a novel lipophilic prodrug of PMEG (**2**) that has been recently shown to exhibit potent therapeutic effect in pet dogs with spontaneous non-Hodgkin's lymphomas[16,17]. Given that cPrPMEDAP (**1**) is a key intermediate in the intracellular metabolic activation of GS-9219 (ref.[18]), it is important to understand the metabolic pathway leading to the release of PMEG (**2**) from cPrPMEDAP (**1**). Therefore, we characterized the substrate preference of hABC-MP deaminase including its capability of converting cPrPMEDAP (**1**) to PMEG (**2**). As a part of this study, we conducted substrate specificity experiments with hABC-MP deaminase and a broad set of both natural N^6-methylated adenine nucleotides and purine ANPs containing various N^6-alkyl substituents[15]. Our results demonstrate that hABC-MP deaminase functions as meAMP/dAMP aminohydrolase and is capable of efficiently metabolizing various N^6-substituted ANPs including cPrPMEDAP (**1**).

EXPERIMENTAL

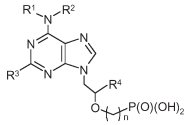
Materials

N^6-Substituted derivatives of AMP (**5**, **11**–**14**), N^6-medAMP (**6**), ABC-MP (**3**), dCF-MP (**9**) and acyclic nucleoside phosphonates (Table I) were synthesized according to previously published procedures[1,15,19]. Rat meAMP/dAMP aminohydrolase was isolated and characterized as described in previous study[1]. IMP, GMP, PIPES (piperazine-1,4-bis(2-ethanesulfonic acid)), imidazole, glycerol, DTT (dithiothreitol), tri-*n*-octylamine, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, tetrabutylammonium hydrogensulfate and protease inhibitor cocktail were purchased from Sigma–Aldrich (St. Louis, U.S.A.). 1,1,2-Trichloro-1,2,2-trifluoroethane was obtained from Merck (Darmstadt, Germany) and acetonitrile from Fluka (St. Louis, U.S.A.). Nonidet P40 (NP-40) was purchased from GS Healthcare and Tris (tris(hydroxymethyl)-aminomethane), H_3BO_3, NaCl, TCA (trichloroacetic acid), 2-mercaptoethanol, SDS, Coomassie Brilliant Blue G-250, acrylamide and *N*,*N*′-methylene bisacrylamide were reagents of Serva (Heidelberg, Germany). LMW and HMW standards for electrophoresis were from Pharmacia Fine Chemicals AB (Uppsala, Sweden).

Human Abacavir Monophosphate Deaminase

To clone the coding sequence of hABC-MP deaminase, total RNA was extracted from SiHa cells (ATCC, Manassas, U.S.A.) using RNeasy Mini Kit (Qiagen, Valencia, U.S.A.). cDNA was synthesized using First Strand cDNA Synthesis Kit (Roche Applied Science, Indianapolis, U.S.A.) and a mixture of random hexamers and oligo-dT according to manufacturer's proto-

TABLE I
N^6-Substituted acyclic nucleoside phosphonates



Compound	R^1	R^2	R^3	R^4	*n*
cPrPMEDAP (**1**)		H	NH_2	H	1
cPrCH$_2$PMEDAP (**21**)		H	NH_2	H	1
cPr(CH$_2$)$_2$PMEDAP (**22**)		H	NH_2	H	1
(cPr)$_2$PMEDAP (**20**)			NH_2	H	1
cButPMEDAP (**15**)		H	NH_2	H	1
cPtylPMEDAP (**16**)		H	NH_2	H	1
cHxPMEDAP (**17**)		H	NH_2	H	1
cHptPMEDAP (**18**)		H	NH_2	H	1
cOctPMEDAP (**19**)		H	NH_2	H	1
mePMEDAP (**28**)	CH_3	H	NH_2	H	1
me$_2$PMEDAP (**29**)	CH_3	CH_3	NH_2	H	1
me-etPMEDAP (**30**)	CH_3	CH_3CH_2	NH_2	H	1
HO(CH$_2$)$_2$PMEDAP (**31**)	HO(CH$_2$)$_2$	H	NH_2	H	1
propylPMEDAP (**23**)	$CH_3(CH_2)_2$	H	NH_2	H	1
allylPMEDAP (**10**)	CH_2=$CHCH_2$	H	NH_2	H	1
propargylPMEDAP (**24**)	CH≡CCH_2	H	NH_2	H	1
butylPMEDAP (**25**)	$CH_3(CH_2)_3$	H	NH_2	H	1
sec-butylPMEDAP (**26**)	$CH_3CH_2CH(CH_3)$	H	NH_2	H	1
isobutylPMEDAP (**27**)	$(CH_3)_2CHCH_2$	H	NH_2	H	1
me$_2$NetPMEDAP (**32**)	$(CH_3)_2N(CH_2)_2$	H	NH_2	H	1
cPrPMEA (**38**)		H	H	H	1
cPrPEEDAP (**39**)		H	NH_2	H	2
(*R*)-cPrPMPA (**34**)		H	H	CH_3	1
(*S*)-cPrPMPA (**33**)		H	H	CH_3	1
(*R*)-cPrPMPDAP (**36**)		H	NH_2	CH_3	1
(*S*)-cPrPMPDAP (**35**)		H	NH_2	CH_3	1
(*S*)-cPrHPMPDAP (**37**)		H	NH_2	$HOCH_2$	1

col. Full-length cDNA fragment encoding hABC-MP deaminase was amplified using High Fidelity PCR Master Kit (Roche Applied Science) and oligonucleotides 5′-ATGATAGAGGCAGAAGAGCAACAGCCTTGC-3′ (5′-end primer) and 5′-TTAAATATGTAACACTCTGGGCTTCAGGTG-3′ (3′-end primer). The amplified PCR fragment was cloned into a baculovirus expression shuttle vector pFASTBacHT (Invitrogen, Carlsbad, U.S.A.) and subjected to DNA sequencing. Expression of hABC-MP deaminase was performed using Bac-to-Bac® Baculovirus Expression System (Invitrogen, Carlsbad, U.S.A.) according to manufacturer's protocol. To generate recombinant baculovirus genomic DNA (bacmid), *E. coli* strain DH10Bac was transformed with the pFASTBacHT plasmid carrying the hABC-MP deaminase cDNA. The isolated bacmid was transfected into SF9 insect cells and the amplification of recombinant baculovirus strain was achieved by two rounds of re-infection of SF9 cells with the initial seed stock. Final stock of recombinant baculovirus was used to infect 10^9 Hi5 insect cells. Cells were harvested by centrifugation 48 h after infection and resuspended in buffer A containing 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 0.1% NP-40, 5 mM 2-mercaptoethanol, 10% glycerol, and a standard cocktail of protease inhibitors. Cell pellet was lysed by microfluidization (three rounds at 124 MPa). The homogenate was centrifuged for 40 min at 30,000 *g* and the supernatant was loaded onto a 5-ml Ni-NTA column (Qiagen, Valencia, U.S.A.). The column was washed with 60 ml buffer A containing 20 mM imidazole. The bound proteins were eluted with linear gradient from 0 to 100% buffer B (buffer A with 400 mM imidazole) in 25 column volumes. Fractions were collected and subjected to SDS-PAGE analysis. Fractions containing the enzyme were pooled and diluted (8×) with buffer C (20 mM Tris-HCl, pH 8.0; 10% glycerol; 1 mM DTT), and loaded onto a 1-ml Q HP column (Amersham Biosciences AB, Uppsala, Sweden) at the flow rate of 1 ml min^{-1}. After washing with buffer C, the column was eluted with a 25-ml linear gradient of 0 to 1 M NaCl in buffer C. 1-ml Fractions were collected and analyzed by SDS-PAGE. Fractions containing the protein were pooled and adjusted to the concentration 2 mg ml^{-1} according to the Bradford assay. The final protein isolate showed the purity > 95% based on SDS-PAGE analysis. Native gel filtration confirmed the presence of a 42 kDa protein, suggesting the presence of a predominantly monomeric form.

Electrophoresis

Denaturing electrophoresis was run on 13% polyacrylamide gel according to Laemmli[20] with subsequent staining using Coomassie Brilliant Blue G-250.

Native gel electrophoresis was performed on a gradient gel (4–25%) in Tris-borate buffer (124 mM Tris-H_3BO_3, pH 8.3). After reaching equilibrium (2500 V h), one part of the gel was stained with silver and the other was cut into 2-mm slices and N^6-methyl-AMP aminohydrolase activity was determined. Each slice was extracted with a buffer (150 µl) containing 50 mM PIPES (pH 6.8), 2 mM DTT and 50 µM meAMP (**5**), and incubated at 37 °C. Concentration of IMP formed in each fraction was determined by HPLC as described below.

Enzyme Assays

The reaction mixtures for K_m and V_{max} determination (50 or 100 µl) contained 50 mM PIPES (pH 6.8), 2 mM DTT, 100 µg ml^{-1} BSA and the tested compound (meAMP, **5**; cPrPMEDAP, **1**; allylPMEDAP, **10** or ABC-MP, **3**) at 8 different concentrations. The reactions were started by addition of appropriate aliquots of the enzyme, carried out at 37 °C for various time intervals to achieve optimal degree of conversion and stopped by addition of 10% TCA. After

10-min incubation on ice, the samples were centrifuged and TCA was removed from the supernatant by extraction with tri-*n*-octylamine/1,1,2-trichloro-1,2,2-trifluoroethane mixture (1:4; v/v). Aqueous phase was then separated by centrifugation and an aliquot was used for HPLC analysis. The kinetic data were evaluated using software GOSA (Bio-Log, Labege Cedex, France).

The inhibition constants K_i were measured under the same reaction conditions as described in the previous section. The reaction mixture contained cPrPMEDAP (**1**) at 8 different concentrations and the inhibitor tested (dCF-MP, **9** or GMP) at 4 different concentrations. After incubation the samples were processed and analyzed as already mentioned. The kinetic data were evaluated using software GOSA (Bio-Log, Labege Cedex, France).

Experiments with other alternative substrates (Table I) were also performed under the conditions as those described above except that substrates were tested only at two concentrations (5 and 50 μmol l^{-1}). The enzyme activity with each substrate was expressed as the rate of the product formation (pmol min^{-1} mg^{-1}) at the selected substrate concentration (either v_5 for 5 or v_{50} for 50 μM substrate).

HPLC Analysis

Samples were analyzed using Waters Alliance System 2795 (2996 PDA Detector, PDA Software Millenium[32]). The analytical separation was performed on Supelcosil™ LC-18T column (4 mm × 15 cm, 3 μm) (Supelco, St. Louis, U.S.A.) at the flow rate of 0.75 ml min^{-1}. Mobile phase consisted of acetonitrile in 50 mM potassium phosphate buffer (pH 3.1 or 5.1) and 3 mM tetrabutylammonium hydrogensulfate. Various optimized non-linear gradients of acetonitrile in phosphate buffer were used for the separation of substrates and products of the deamination reaction. Compounds were identified on the basis of their retention times and comparison of UV spectra with a library of external standards.

RESULTS

Subunit Structure of Human Abacavir Monophosphate Deaminase

To estimate the subunit structure of hABC-MP deaminase and compare it with the previously described rat meAMP aminohydrolase[1], denatured and native gradient electrophoretic separations were carried out. Under denaturing conditions, hABC-MP deaminase migrates as a 42 kDa polypeptide (Fig. 1). Enzymatic detection of meAMP aminohydrolase activity in gel slices following separation by native gradient electrophoresis showed that the enzyme exists predominantly as a monomer with dimer, trimer, and hexamer forms being detected only as minor species (Fig. 2).

Kinetics of Selected Substrates of hABC-MP Deaminase

We determined kinetic constants K_m and V_{max} for selected substrates including meAMP (**5**) as the putative natural substrate, ABC-MP (**3**) and

cPrPMEDAP (**1**) as two key therapeutically relevant N^6-substituted purine nucleotide analogs[2,15], and N^6-allylPMEDAP (**10**), another acyclic nucleoside phosphonate with a potent cytostatic activity[15].

Out of these tested substrates, hABC-MP deaminase displays the highest affinity for ABC-MP (**10**). According to K_m values, the enzyme exhibits approximately two-fold higher binding affinity for ABC-MP (**10**) compared to the putative natural substrate meAMP (**5**) (Table II). In contrast, the deaminase has nine-fold lower affinity for cPrPMEDAP (**1**) compared with meAMP (**5**) (Table II). Replacement of N^6-cyclopropyl by N^6-allyl group in-

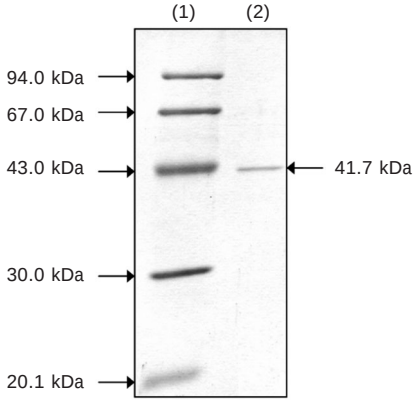


FIG. 1
SDS-PAGE of hABC-MP deaminase. (1) LMW standards; (2) recombinant purified hABC-MP deaminase (0.3 μg)

TABLE II
Kinetic parameters of meAMP, cPrPMEDAP, allylPMEDAP and ABC-MP deamination[a]

Compound	K_m μmol l^{-1}	V_{max} mmol min^{-1} mg^{-1}	V_{max}/K_m ml min^{-1} mg^{-1}	k_{cat} s^{-1}
meAMP (**5**)	0.8 ± 0.2	2540 ± 113	3173	1.76
cPrPMEDAP (**1**)	7.4 ± 1.2	330 ± 19	45	0.23
allylPMEDAP (**10**)	2.8 ± 0.4	9.9 ± 0.3	3.6	0.07
ABC-MP (**3**)	0.4 ± 0.1	1030 ± 25	2715	0.72

[a] The values are means ± S.E.M. of three independent experiments.

creases the enzyme affinity for the ANP substrate as shown by the K_m value of allylPMEDAP (**10**) being 2.6-fold lower than that of cPrPMEDAP (**1**) (Table II). Similar to the substrate affinity, the reaction rate is also strongly affected by the N^6-substitution of the purine ring. The presence of N^6-allyl is associated with thirty-fold reduced V_{max} value compared to the corresponding cyclopropyl derivative as demonstrated by the comparison of allylPMEDAP (**10**) with cPrPMEDAP (**1**) (Table II). In addition, the presence of acyclic PME moiety decreases the rate of deamination approximately three-fold compared to cyclopentene 5′-phosphate moiety (Table II). Furthermore, it is evident from the calculated k_{cat} values that meAMP (**5**) is more efficient substrate for hABC-MP deaminase than ABC-MP (**3**), cPrPMEDAP (**1**), and allylPMEDAP (**10**) (Table II).

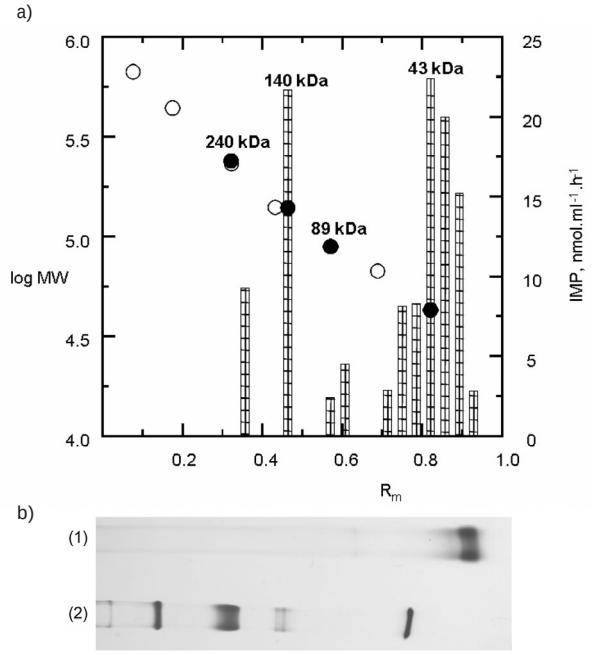


FIG. 2

Native gradient PAGE of hABC-MP deaminase. a) Activity of the enzyme detected in the gel: ○ HMW standards (from left to right: 669, 440, 232, 140, 67 kDa); ● positions of the monomer, dimer, trimer and hexamer of hABC-MP deaminase (from right to left). b) Silver staining of: (1) recombinant purified hABC-MP deaminase, (2) HMW standards

Other Alternative Substrates of hABC-MP Deaminase

In subsequent experiments, the characterization of substrate specificity was expanded to include additional N^6-substituted purine nucleotide analogs. In the series of N^6-substituted AMP and dAMP derivatives, the deamination kinetics of medAMP (**6**) and cPr-, me_2-, allyl-, and propylAMP (**11**–**14**) were characterized. N^6-Cyclopropyl derivative (**12**) was converted to IMP with the highest rate whereas allylAMP (**11**) and propylAMP (**14**) were deaminated least effectively (Table III). The rate of me_2AMP (**13**) deamination was higher than that of medAMP (**6**) and meAMP (**5**) (Table III).

Among the ANP substrates, a broad spectrum of N^6-substitutions of 2,6-diaminopurine analogs were studied (Tables I and IV) including both aliphatic (linear and branched) and cyclic groups of different length and polarity. Of ANPs with cyclic N^6-substituents, hABC-MP deaminase accepts as substrates not only cPrPMEDAP (**1**), but also compounds with bulkier cyclic N^6-groups. Conversion to PMEG (**2**) was observed in the case of cyclobutyl-, cyclopentyl-, cyclohexyl-, cycloheptyl- and cyclooctylPMEDAP derivatives (**15**–**19**). In this series, the efficiency of deamination to PMEG (**2**) decreased with the expansion of the N^6-substituent ring (Table IV). Similarly, in the case of substrates with aliphatic N^6-substituents, the rate of conversion to PMEG (**2**) decreased with increasing length and size of the substituent as evidenced by slower deamination of substrates with three- and four-carbon substituents (propyl, allyl, propargyl, butyl, *sec*-butyl and

TABLE III
Substrate specificity of hABC-MP deaminase: N^6-substituted adenine nucleotides[a]

Compound	Reaction rate, nmol min^{-1} mg^{-1}	
	v_5	v_{50}
medAMP (**6**)	1690 ± 90	2570 ± 70
cPrAMP (**12**)	2830 ± 530	4410 ± 360
meAMP (**5**)	1880 ± 70	2500 ± 90
me_2AMP (**13**)	2000 ± 140	2630 ± 330
allylAMP (**11**)	1190 ± 40	2340 ± 330
propylAMP (**14**)	1230 ± 140	2460 ± 220

[a] The values are means ± S.E.M. of three independent experiments.

isobutyl; **23**, **10** and **24**–**27**) compared with those with one- and two-carbon substituents (methyl, dimethyl and methyl-ethyl; **28**–**30**) (Table IV). Surprisingly, me_2NetPMEDAP (**32**) was not deaminated despite having significant cytostatic activity against several transformed cell lines[15,21] (Table IV). This indicates potential existence of other nucleotide deaminases in cells.

TABLE IV
Substrate specificity of hABC-MP deaminase: N^6-substituted derivatives of 9-[2-(phosphonomethoxy)ethyl]-2,6-diaminopurine (PMEDAP)[a]

Compound	Reaction rate, nmol min^{-1} mg^{-1}	
	v_5	v_{50}
cPrPMEDAP (**1**)	140 ± 20	220 ± 10
$cPrCH_2$PMEDAP (**21**)	1.98 ± 0.41	2.61 ± 0.08
$cPr(CH_2)_2$PMEDAP (**22**)	1.45 ± 0.05	10.4 ± 0.5
$(cPr)_2$PMEDAP (**20**)	0.06 ± 0.01	1.00 ± 0.14
cButPMEDAP (**15**)	13.9 ± 0.6	55.4 ± 5.0
cPtylPMEDAP (**16**)	3.9 ± 0.4	29.6 ± 2.7
cHxPMEDAP (**17**)	NP[b]	0.12 ± 0.01
cHptPMEDAP (**18**)	NP[b]	0.12 ± 0.01
cOctPMEDAP (**19**)	NP[b]	0.11 ± 0.01
mePMEDAP (**28**)	11 ± 2	31 ± 6
me_2PMEDAP (**29**)	36 ± 3	109 ± 8
me-etPMEDAP (**30**)	16 ± 2	27 ± 4
$HO(CH_2)_2$PMEDAP (**31**)	0.38 ± 0.05	2.19 ± 0.04
propylPMEDAP (**23**)	5.6 ± 0.7	8.2 ± 0.6
allylPMEDAP (**10**)	5.3 ± 0.1	9.5 ± 1.0
propargylPMEDAP (**24**)	3.9 ± 0.5	13.3 ± 1.8
butylPMEDAP (**25**)	2.1 ± 0.2	4.6 ± 0.5
sec-butylPMEDAP (**26**)	1.03 ± 0.01	7.1 ± 1.1
isobutylPMEDAP (**27**)	3.6 ± 0.2	7.0 ± 0.8
me_2NetPMEDAP (**32**)	NP[b]	NP[b]

[a] The values are means ± S.E.M. of three independent experiments. [b] No product detected.

Introduction of a 2-methyl group into the PME moiety results in (*S*)- and (*R*)-enantiomers of 9-[2-(phosphonomethoxy)propyl] (PMP) derivatives (**33**–**36**) (Table I). Among these, hABC-MP deaminase metabolizes (*S*)-enantiomers better than (*R*)-enantiomers (Table V). Consistently, (*S*)-cPrHPMPDAP (N^6-cyclopropyl-2,6-diamino-9-[(S)-3-hydroxy-2-(phosphonomethoxy)propyl]purine; **37**) (Table I), which corresponds to absolute configuration of (*R*)-PMP derivatives, is a very poor substrate of the enzyme (Table V), indicating somewhat narrow structural preference of the enzyme for the substrate sugar-mimicking moiety. On the other hand, the efficiency of deamination of ANP derivative with 9-[2-(phosphonoethoxy)-ethyl] moiety (cPrPEEDAP; **39**) (Table I) to the corresponding guanine product (**40**) was comparable to that of cPrPMEDAP (**1**) (Tables IV and V), indicating that the enzyme can also accept substrates with extended length of the acyclic sugar-mimicking moiety.

cPrPMEA (**38**), an ANP containing N^6-substituted adenine and so more similar to N^6-substituted AMP derivatives, was also found to be a substrate for hABC-MP deaminase (Table V). The deamination rate of this compound was comparable with that of cPrPMEDAP (**1**) at the 5 μM substrate concentration, but was more than three-fold faster at the 50 μM concentration (Tables IV and V), suggesting higher V_{max} for N^6-substituted adenine ANPs

TABLE V
Substrate specificity of hABC-MP deaminase: acyclic nucleoside phosphonates of N^6-cyclopropylpurines[a]

Compound	Reaction rate, nmol min^{-1} mg^{-1}	
	v_5	v_{50}
cPrPMEA (**38**)	109 ± 5	721 ± 67
cPrPEEDAP (**39**)	81 ± 2	324 ± 11
(*R*)-cPrPMPA (**34**)	4.8 ± 0.6	32.9 ± 1.2
(*S*)-cPrPMPA (**33**)	41 ± 4	410 ± 22
(*R*)-cPrPMPDAP (**36**)	2.8 ± 0.2	8.5 ± 1.7
(*S*)-cPrPMPDAP (**35**)	57 ± 4	374 ± 29
(*S*)-cPrHPMPDAP (**37**)	0.93 ± 0.05	2.16 ± 0.22

[a] The values are means ± S.E.M. of three independent experiments.

compared to the corresponding 2,6-diaminopurine ANPs. This trend was confirmed with several other pairs of adenine/diaminopurine ANPs (Table V).

Inhibition of hABC-MP Deaminase

cPrPMEDAP (**1**) deamination catalyzed by hABC-MP deaminase was strongly inhibited in the presence of dCF-MP (**9**) that acted as a competitive inhibitor of the reaction with a K_i value of 5.4 nmol l^{-1} (Table VI, Fig. 3). Inhibition of the reaction by GMP was also observed, but it was three orders of magnitude weaker compared to dCF-MP (**9**) (Table VI). PMEG (**2**), an

TABLE VI
Inhibition of hABC-MP deaminase: cPrPMEDAP deamination in the presence of dCF-MP or GMP[a]

Inhibitor	K_i nmol l^{-1}	K_i/K_m
dCF-MP (**9**)	5.4 ± 1.0	0.00073
GMP	4500 ± 620	0.61

[a] The values are means ± S.E.M. of three independent experiments.

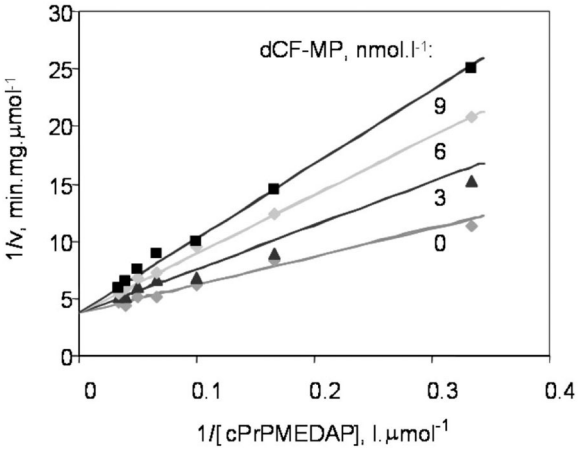


FIG. 3
Lineweaver–Burk plot of hABC-MP deaminase inhibition by dCF-MP (substrate: 3–30 µM cPrPMEDAP)

acyclic analogue of GMP and/or dGMP, and the product of cPrPMEDAP (**1**) deamination did not significantly inhibit the hABC-MP deaminase activity at concentrations as high as 300 μmol l^{-1} when 2 μM meAMP (**5**) was used as a substrate (data not shown), indicating that the intracellular accumulation of PMEG (**2**) is unlikely to substantially reduce the deamination of cPrPMEDAP (**1**).

DISCUSSION

Experiments with hABC-MP deaminase demonstrated its ability to effectively metabolize meAMP (**5**) to IMP, indicating that this enzyme likely represents the human form of meAMP aminohydrolase homologous to the previously reported rat meAMP aminohydrolase[1]. This conclusion is further supported by almost identical substrate specificity observed when the rat and human enzymes were compared for their ability to deaminate various N^6-substituted purine nucleotides and their analogs. Comparison of reaction rates for 18 structurally diverse substrates yielded a highly statistically significant correlation between the two enzymes (Fig. 4). Similar to the rat meAMP aminohydrolase, hABC-MP deaminase is a 42 kDa polypeptide that behaves predominantly as a monomer under non-denaturing conditions. This differs somewhat from the rat enzyme that forms a hexamer and its

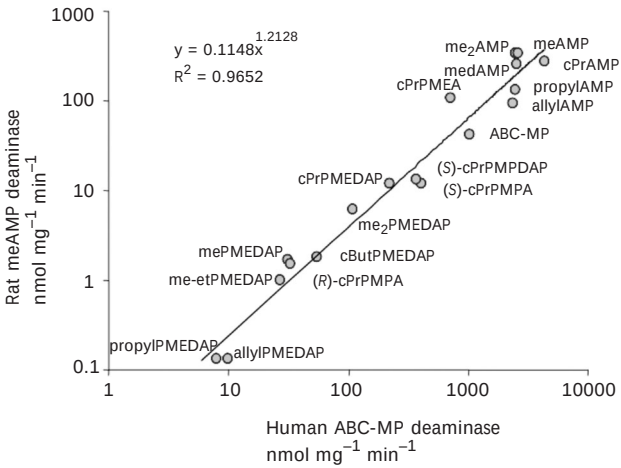


FIG. 4
Correlation of the substrate specificity of the purified rat meAMP aminohydrolase and the recombinant human ABC-MP deaminase

dimer under the same conditions. This may be related to differences in primary sequence of both enzymes or the method of enzyme production, as the hABC-MP deaminase is a recombinant protein expressed in baculovirus whereas the rat enzyme was isolated from native tissue[1].

The described characterization of hABC-MP deaminase indicates that the enzyme accepts not only ABC-MP (**3**), but also a wide range of N^6-substituted ANPs as alternative substrates. Of these, cPrPMEDAP (**1**) and allylPMEDAP (**10**) exhibit cytostatic activities[15], most likely because of their conversion, catalyzed by hABC-MP deaminase, to the potent antiproliferative nucleotide PMEG. Importantly, cPrPMEDAP (**1**) is a key intermediate metabolite in the activation pathway of GS-9219 (ref.[18]), a novel PMEG (**2**) prodrug that has shown potent *in vivo* therapeutic effect in pet dogs with both naive and refractory spontaneous non-Hodgkin's lymphomas[16,17]. In some of the treated dogs, a single intravenous dose of GS-9219 was sufficient to cause a significant regression of diagnosed lymphoid malignancies[17]. Our data indicate that ABC-MP deaminase may play an important role in the activation pathway of GS-9219. Interestingly, GS-9219 exhibits reduced toxicity and improved selectivity *in vivo* compared with the parent nucleotide PMEG (**2**)[16]. Although additional follow-up studies are required to further understand the improved therapeutic profile of GS-9219, it is possible that the activation step catalyzed by ABC-MP deaminase may contribute to this improvement.

The presented characterization of ABC-MP deaminase helped thoroughly understand its substrate specificity. The enzyme is able to convert to corresponding products a variety of N^6-substituted nucleotides and their analogs containing substituents larger than methyl (**5**, **28**), dimethyl (**13**, **29**), or cyclopropyl (**1**, **12**, **33**–**39**), albeit with lower efficiency. In the series of N^6-substituted PMEDAP analogs, the reaction rate decreased with increasing size of the substituent both among compounds with a cyclic moiety (cyclopropyl, **1** > cyclobutyl, **15** > cyclopentyl, **16** >> cyclohexyl, **17** ~ cycloheptyl, **18** ~ cyclooctyl, **19**) and those with an acyclic moiety (dimethyl, **29** > methyl-ethyl, **30** > propargyl, **24** > allyl, **10** ~ propyl, **23** > isobutyl, **27** ~ *sec*-butyl, **26** > butyl, **25**). Cyclic substituents are hydrolyzed, with elimination of an amine, better than linear with the same number of carbon atoms (cyclopropyl, **1** > propargyl, **24**, propyl, **23** and allyl, **10**; cyclobutyl, **15** > isobutyl, **27**, *sec*-butyl, **26** and butyl, **25**). The replacement of the PME moiety with the PMP moiety leads to a decreased affinity of hABC-MP deaminase to the analogs, with (*S*)-PMP enantiomers (**33**, **35**) being better substrates than (*R*)-PMP enantiomers (**34**, **36**).

With respect to the preference of heterocyclic base, it is evident from the comparison of several corresponding ANP pairs that the enzyme possesses lower affinity to N^6-substituted adenine compared with N^6-substituted diaminopurine but the ability to convert N^6-substituted adenine to corresponding products is higher.

Similar to rat meAMP aminohydrolase[1], hABC-MP deaminase is strongly inhibited by dCF-MP (**9**). This effect explains the significant reduction of cPrPMEDAP (**1**) metabolism and, consequently, its antiproliferative activity in cells co-incubated in the presence of dCF (**41**)[14], which is readily phosphorylated to dCF-MP (**9**)[22]. Importantly, hABC-MP deaminase is not affected by the products of deamination reaction as it is insensitive to inhibition by PMEG (**2**).

In conclusion, data presented in this study demonstrate that hABC-MP deaminase is a meAMP aminohydrolase and likely represents a human homolog of the previously isolated and characterized rat meAMP aminohydrolase[1]. As such, this enzyme is primarily involved in the catabolism of N^6-methylated adenine ribo- and 2′-deoxyribonucleotides generated by the intracellular degradation of nucleic acids[3,4]. Although it is not clear at this point whether any other forms of human meAMP aminohydrolases may exist in cells, the characterized hABC-MP deaminase is likely to play an important role in the intracellular metabolic activation of N^6-substituted ANPs including the therapeutically relevant antiproliferative metabolite cPrPMEDAP (**1**).

ABBREVIATIONS

cPrPMEDAP (**1**), N^6-cyclopropyl-2,6-diamino-9-[2-(phosphonomethoxy)-ethyl]purine; PMEG (**2**), 9-[2-(phosphonomethoxy)ethyl]guanine; ABC-MP (**3**), abacavir 5′-phosphate; CBV-MP (**4**), carbovir 5′-phosphate; meAMP aminohydrolase, N^6-methyl-AMP/N^6-methyl-dAMP aminohydrolase; meAMP (**5**), N^6-methyladenosine 5′-monophosphate; medAMP (**6**), N^6-methyl-2′-deoxyadenosine 5′-monophosphate; hABC-MP deaminase, human abacavir monophosphate deaminase; PMEGpp (**7**), 9-[2-(phosphonomethoxy)ethyl]guanine diphosphate; PMEDAP (**8**), 9-[2-(phosphonomethoxy)ethyl]-2,6-diaminopurine; dCF-MP (**9**), 2′-deoxycoformycin 5′-monophosphate; allylPMEDAP (**10**), N^6-allyl-2,6-diamino-9-[2-(phosphonomethoxy)ethyl]purine; PME, 9-[2-(phosphonomethoxy)ethyl]; allylAMP (**11**), N^6-allyladenosine 5′-monophosphate; cPrAMP (**12**), N^6-cyclopropyladenosine 5′-monophosphate; me_2AMP (**13**), N^6,N^6-dimethyladenosine 5′-monophosphate; propylAMP (**14**), N^6-propyladenosine

5′-monophosphate; cButPMEDAP (**15**), N^6-cyclobutyl-2,6-diamino-9-[2-(phosphonomethoxy)ethyl]purine; cPtylPMEDAP (**16**), N^6-cyclopentyl-2,6-diamino-9-[2-(phosphonomethoxy)ethyl]purine; cHxPMEDAP (**17**), N^6-cyclohexyl-2,6-diamino-9-[2-(phosphonomethoxy)ethyl]purine; cHptPMEDAP (**18**), N^6-cycloheptyl-2,6-diamino-9-[2-(phosphonomethoxy)-ethyl]purine; cOctPMEDAP (**19**), N^6-cyclooctyl-2,6-diamino-9-[2-(phosphonomethoxy)ethyl]purine; $(cPr)_2$PMEDAP (**20**), N^6,N^6-dicyclopropyl-2,6-diamino-9-[2-(phosphonomethoxy)ethyl]purine; $cPrCH_2$PMEDAP (**21**), N^6-cyclopropyl-N^6-methyl-2,6-diamino-9-[2-(phosphonomethoxy)ethyl]-purine; $cPr(CH_2)_2$PMEDAP (**22**), N^6-[1-(cyclopropyl)ethyl]-2,6-diamino-9-[2-(phosphonomethoxy)ethyl]purine; propylPMEDAP (**23**), N^6-propyl-2,6-diamino-9-[2-(phosphonomethoxy)ethyl]purine; propargylPMEDAP (**24**), N^6-propagyl-2,6-diamino-9-[2-(phosphonomethoxy)ethyl]purine; butylPMEDAP (**25**), N^6-butyl-2,6-diamino-9-[2-(phosphonomethoxy)ethyl]-purine; *sec*-butylPMEDAP (**26**), N^6-*sec*-butyl-2,6-diamino-9-[2-(phosphonomethoxy)ethyl]purine; isobutylPMEDAP (**27**), N^6-isobutyl-2,6-diamino-9-[2-(phosphonomethoxy)ethyl]purine; mePMEDAP (**28**), N^6-methyl-2,6-diamino-9-[2-(phosphonomethoxy)ethyl]purine; me_2PMEDAP (**29**), N^6,N^6-dimethyl-2,6-diamino-9-[2-(phosphonomethoxy)ethyl]purine; me-etPMEDAP (**30**), N^6-ethyl-N^6-methyl-2,6-diamino-9-[2-(phosphonomethoxy)ethyl]purine; $HO(CH_2)_2$PMEDAP (**31**), N^6-(2-hydroxyethyl)-2,6-diamino-9-[2-(phosphonomethoxy)ethyl]purine; me_2NetPMEDAP (**32**), N^6-[2-(dimethylamino)ethyl]-2,6-diamino-9-[2-(phosphonomethoxy)-ethyl]purine; PMP, 9-[2-(phosphonomethoxy)propyl]; (*S*)-cPrPMPA (**33**), N^6-cyclopropyl-9-[(*S*)-2-(phosphonomethoxy)propyl]adenine; (*R*)-cPrPMPA (**34**), N^6-cyclopropyl-9-[(*R*)-2-(phosphonomethoxy)propyl]adenine; (*S*)-cPrPMPDAP (**35**), N^6-cyclopropyl-2,6-diamino-9-[(*S*)-2-(phosphonomethoxy)propyl]purine; (*R*)-cPrPMPDAP (**36**), N^6-cyclopropyl-2,6-diamino-9-[(*R*)-2-(phosphonomethoxy)propyl]purine; (*S*)-cPrHPMPDAP (**37**), N^6-cyclopropyl-2,6-diamino-9-[(S)-3-hydroxy-2-(phosphonomethoxy)propyl]purine; cPrPMEA (**38**), N^6-cyclopropyl-9-[2-(phosphonomethoxy)ethyl]adenine; cPrPEEDAP (**39**), N^6-cyclopropyl-2,6-diamino-9-[2-(phosphonoethoxy)ethyl]purine; PEEG (**40**), 9-[2-(phosphonoethoxy)ethyl]guanine; dCF (**41**), 2′-deoxy-coformycin.

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