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N^6-Methyl-AMP aminohydrolase activates N^6-substituted purine acyclic nucleoside phosphonates

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N^6-Cyclopropyl-2,6-diamino-9-[2-(phosphonomethoxy)ethyl]purine
9-[2-(Phosphonomethoxy)ethyl]-guanine
Abacavir 5′-monophosphate
Carbovir 5′-monophosphate

Abbreviations:
PMEDAP, 9-[2-(phosphonomethoxy)-ethyl]-2,6-diaminopurine
cypr-PMEDAP, N^6-cyclopropyl-2,6-diamino-9-[2-(phosphonomethoxy)-ethyl]purine
me-PMEDAP, N^6-methyl-2,6-diamino-9-[2-(phosphonomethoxy)-ethyl]purine
me_2-PMEDAP, N^6-dimethyl-2,6-diamino-9-[2-(phosphonomethoxy)-ethyl]purine
cypr-PMEA, N^6-cyclopropyl-9-[2-(phosphonomethoxy)ethyl]adenine
me_2-PMEA, N^6-dimethyl-9-[2-(phosphonomethoxy)ethyl]adenine
PMEG, 9-[2-(phosphonomethoxy)-ethyl]guanine

ABSTRACT

In this study we present the identification and characterization of the enzyme involved in the N^6-cyclopropyl-2,6-diamino-9-[2-(phosphonomethoxy)ethyl]purine (N^6-cyclopropyl-PMEDAP) conversion to biologically active 9-[2-(phosphonomethoxy)ethyl]guanine (PMEG) as well as abacavir 5′-phosphate to carbovir 5′-phosphate. This enzyme was purified from rat liver to homogeneity; it appears to be composed from six 42 kDa subunits and its native form has the molecular weight 260 kDa. This so far unknown enzyme catalyzes conversion of both N^6-methyl-AMP and N^6-methyl-dAMP to IMP and/or dIMP, respectively. The enzyme acts as 6-(*N*-substituted amino)purine 5′-nucleotide aminohydrolase with the reaction mechanism very similar to AMP deaminase. The enzyme does not deaminate AMP and dAMP, or the corresponding nucleosides. It is inhibited by deoxycoformycin 5′-phosphate but not by deoxycoformycin or *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA).

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ABC-MP, abacavir 5′-monophosphate
CBV-MP, carbovir 5′-monophosphate
me-AMP, N^6-methyl-AMP
me_2-AMP, N^6-dimethyl-AMP
me-dAMP, N^6-methyl-dAMP
EHNA, *erythro*-9-(2-hydroxy-3-nonyl)adenine
dCF, deoxycoformycin
dCF-MP, deoxycoformycin 5′-monophosphate

1. Introduction

N^6-Substituted derivatives of PMEDAP {9-[2-(phosphonomethoxy)ethyl]-2,6-diaminopurine} are acyclic nucleoside phosphonates (ANPs; Fig. 1) with significant cytostatic and/or antiviral activities [1]. The most promising compound of this series is N^6-cypr-PMEDAP {N^6-cyclopropyl-2,6-diamino-9-[2-(phosphonomethoxy)ethyl]-purine}. N^6-cypr-PMEDAP is converted to PMEG [2–4] and subsequently phosphorylated by cellular kinases to PMEGpp [5–7], which inhibits DNA polymerases α and ε [8,9]. PMEG possesses potent antiproliferative activity inhibiting the growth of various cell lines [1,6] and increases the life-span of experimental animals suffering from neoplasias [1,5]. Higher concentrations of PMEG induce apoptosis while its low concentrations cause a reversible cytostatic effect [10]. The antiproliferative effect of PMEG in mouse hybridoma cells is accompanied by the enhancement of the antibody production; the cell cycle analysis profile showed a higher proportion of cells in the S and G_2/M phase [11]. Embryotoxic and genotoxic potency of this compound is comparable with mitomycin C [12] and it is also active against herpes simplex viruses (HSV-1, HSV-2), measles virus and parainfluenza virus 3 [1]. Our recent experimental data show that PMEGpp efficiently inhibits telomerase of human leukaemia HL-60 cells [13].

Undoubtedly, the intracellular transformation of N^6-cypr-PMEDAP to PMEG proceeds by a mechanism, which warrants a slower supply of the drug to its intracellular targets in order to reach its optimum antiproliferative and/or antiviral effect. On the basis of in vitro experiments with several cell lines and their cellular extracts it has been postulated that the formation of PMEG from N^6-cypr-PMEDAP is catalyzed by AMP deaminase or AMP deaminase-like enzyme sensitive to 2′-deoxycoformycin [3,4]. Very similar results were obtained with abacavir, the cyclopentene C-nucleoside analogue with antiretroviral potency, which contains N^6-cyclopropyl-2,6-diaminopurine moiety and after the intracellular phosphorylation is converted to guanine nucleotide analogue carbovir 5′-phosphate (CBV-MP). Adenylate deaminase does not catalyze the formation of CBV-MP from abacavir 5′-phosphate (ABC-MP). The process is inhibited by 2′-deoxycoformycin 5′-phosphate while the adenosine deaminase inhibitor EHNA is ineffective [14]. These data indicate the existence of cytosolic enzyme different from adenylate deaminase [14].

In this paper we describe new adenylate deaminase like enzyme purified from rat liver that catabolizes N^6-substituted aminopurine 5′-nucleotides and their analogues.

2. Materials and methods

2.1. Materials

N^6-me-AMP, N^6-me_2-AMP and ABC-MP were synthesized from the corresponding nucleosides [15] and purified by chromatography on POROS® 50HQ (Applied Biosystems). ANPs were prepared by the aforementioned procedures [4]. dCF in form of Nipent® (pentostatin for injection) was from Parkedale Pharmaceuticals Inc. (Rochester). dCF-MP was prepared using ribonuclease T1 from *Aspergillus oryzae*, 2′,3′-cGMP (both from

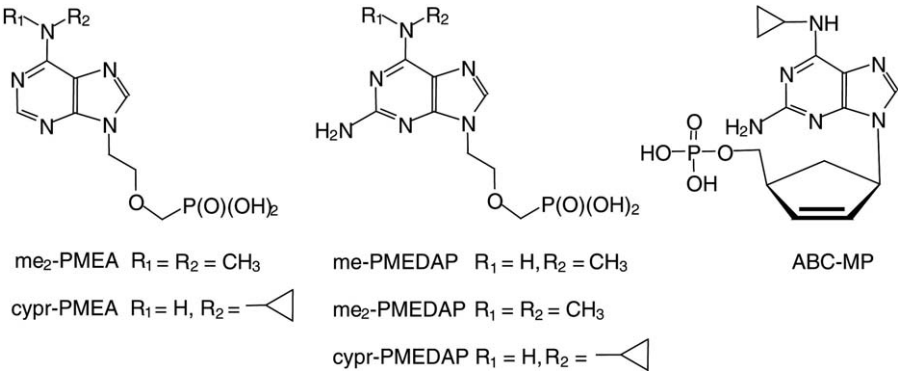


Fig. 1 – Structures of N^6-substituted acyclic nucleotide analogs and ABC-MP.

Sigma–Aldrich) and phosphodiesterase I from *Crotalus adamanteus venom* (Amersham Biosciences) according to the procedure described by Holý and Kowollik [16]. ABC was kindly received from Susanne Daluge (Glaxo Wellcome Inc.). N^6-me-dAMP was synthesized by methylation of dAMP (Calbiochem) with trimethyl phosphate according to Tanabe et al. [17].

All other chemicals were commercial products, e.g. Nonidet P40 (LKB Bromma), 1,1,2-trichlorotrifluoroethane (Merck), protease inhibitor cocktail, DTT, PIPES, TOA and TBHS (Sigma–Aldrich), acetonitrile (Fluka), EHNA (Burroughs Wellcome Co., Research Triangle Park), 2-mercaptoethanol, TCA and other salts (Serva).

HiPrep™ 26/10 desalting and HiPrep 16/10 DEAE FF columns were purchased from Amersham Biosciences, Cellulose Phosphate (P11) was a product from Whatman®, POROS®HS 20 column was from PerSeptive Biosystems and Reactive blue 2 immobilised on Sepharose CL-6B from Sigma–Aldrich. CENTRICON®PLUS 20/PL-30 concentrators were obtained from Millipore.

2.2. Enzyme purification

Frozen liver of Sprague–Dawley rats (−70 °C; 50 g wet weight) were sliced, homogenized in a Dounce tissue grinder in extraction buffer (10 mM Tris–HCl, pH 7.8, 20% glycerol), Nonidet P40 (final concentration 0.1%) and protease inhibitor cocktail and centrifuged 20 min at 32,000 × *g* and then 100 min at 105,000 × *g*. Proteins were salted out from the supernatant with ammonium sulfate (33–55%) and the precipitate desalted (HiPrep™ 26/10 Desalting). The desalted material was applied on P11-cellulose column (2.5 cm × 25 cm, equilibrated in buffer A: 10 mM Tris–HCl, pH 7.4, 9 mM NaCl, 10 mM 2-mercaptoethanol). The column was washed with three column volumes (381 mL) of the same buffer and then with linear concentration and pH gradient (318 mL) mixing 0.1 M KCl in buffer B (10 mM Tris–HCl pH 7.6, 9 mM NaCl, 10 mM 2-mercaptoethanol) with 0.3 M KCl in buffer C (10 mM Tris–HCl pH 8.0, 9 mM NaCl, 10 mM 2-mercaptoethanol). Eluted fractions containing enzyme activity converting cypr-PMEDAP to PMEG were collected and concentrated using CENTRICON®PLUS 20/PL-30 concentrators to the final volume 400 μL, diluted with buffer A to the final volume 10.5 mL and applied onto HiPrep™ 16/10 DEAE FF column. The column was washed with 2.5 column volumes of the same buffer and the active fractions (detected in flow-through) were collected, concentrated, and applied on POROS®HS 20 column (4.6 mm × 100 mm) equilibrated in buffer D (20 mM potassium phosphate, pH 6.8). The active fractions, which were detected in the flow-through were transferred into buffer E (25 mM Tris–HCl, pH 7.4, 2 mM DTT, 3 mM NaN_3) by gel filtration and half of this material was mixed with an appropriate aliquot of Blue-Sepharose equilibrated in buffer F (10 mM Tris–HCl, pH 7.4, 9 mM NaCl, 2 mM DTT). The suspension was, after incubation on ice for 20 min, packed into a column (0.7 cm × 1.3 cm). The column was washed with 2.5 mL of buffer F, 2.5 mL of 0.5 M KCl in buffer F, 2.5 mL of 1 M KCl in buffer F and the enzyme was eluted with 2.5 mL of 1 mM N^6-me-AMP in buffer F containing 1 M KCl. Collected active fractions from specific elution that convert N^6-me-AMP to IMP, represent a pure enzyme.

2.3. Enzyme assay

During the purification procedure the reaction mixture (50 μL) was composed of 100 μM N^6-cypr-PMEDAP, 10 mM Tris–HCl (pH 7.4), 9 mM NaCl and 10 mM 2-mercaptoethanol. The reaction was carried out 16 h at 37 °C and stopped by the addition of 50 μL 10% TCA. After 10 min incubation on ice the samples were centrifuged and TCA was removed from the supernatant with tri-*n*-octylamine-1,1,2-trichlorotrifluoroethane mixture (1:4, v/v). The aqueous phase was then separated by centrifugation and an appropriate aliquot was used for HPLC analysis.

For kinetic experiments the reaction mixture (50 μL) was composed of an appropriate concentration of substrate tested, 50 mM PIPES (pH 6.8) and 2 mM DTT. The reactions were carried out at 37 °C at different time intervals to achieve the optimum degree of conversion and processed as described above.

2.4. HPLC analysis

The acid-soluble extract was analysed in a Waters HPLC system (996 PDA Detector, PDA Software Millenium^{32}, version 3.05, 616 Pump with 600S Controller) equipped with 15 cm × 4 mm Supelcosil™ LC-18T 3 μm reverse-phase column. The nonlinear gradient (curve 4) at a flow rate 0.75 mL/min was used: 10–100% B, 16 min (solvent A, 50 mM potassium dihydrogen phosphate, 3 mM tetrabutylammonium hydrogen sulfate, pH 5.1; solvent B, 50 mM potassium dihydrogen phosphate, 3 mM tetrabutylammonium hydrogen sulfate, 30% acetonitrile, pH 5.1). Peaks of N^6-me-AMP, N^6-me_2-AMP,

Table 1 – Purification of N^6-methyl-AMP aminohydrolase[a]

Purification step	Protein (mg)	Specific activity[b] (U mg^{-1})	Total activity (U)
105,000 × g supernatant	1469	0.41	36,000
33–55% $(NH_4)_2SO_4$ cut	465	0.81	22,520
P-11 cellulose	39	6.24	14,610
DEAE Sepharose FF	6.3	10.58	4,000
POROS®HS	0.540	89.29	2,890
Blue Sepharose	0.010	3362.00	2,020

[a] Purification from 50 g of Sprague–Dawley rat liver.
[b] One enzyme unit is defined as the amount of enzyme that catalyzes conversion of 1 pmol of N^6-cyprPMEDAP to PMEG per min under the assay conditions (see Section 2).

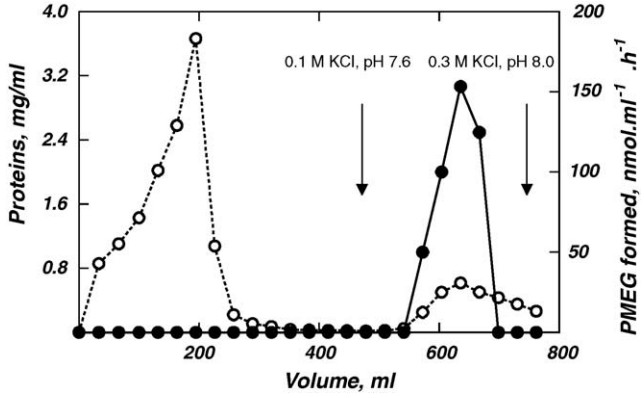


Fig. 2 – N^6-Methyl-AMP aminohydrolase on P11-cellulose. N^6-Methyl-AMP aminohydrolase was eluted with a linear concentration and pH gradient mixing 0.1 M KCl in buffer B (pH 7.6) with 0.3 M KCl in buffer C (pH 8.0): (●) enzyme activity; (○) protein concentration.

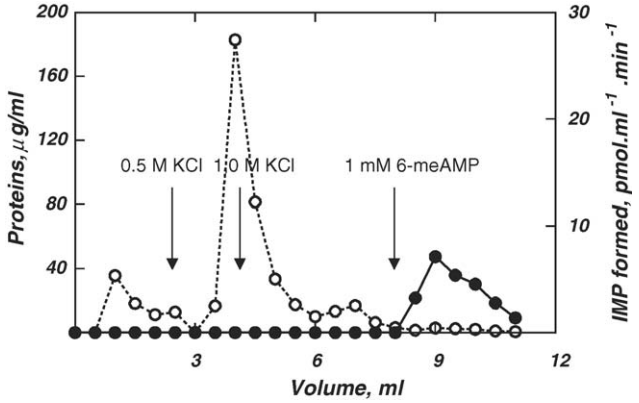


Fig. 3 – Elution profile of N^6-methyl-AMP aminohydrolase from Blue-Sepharose. N^6-Methyl-AMP aminohydrolase was eluted with 1 mM N^6-me-AMP in buffer E containing 1 M KCl: (●) enzyme activity; (○) protein concentration.

N^6-me-dAMP, N^6-cypr-PMEDAP, N^6-me-PMEDAP, N^6-me_2-PMEDAP, N^6-cypr-PMEA, N^6-me_2-PMEA, ABC-MP, CBV-MP, IMP, dIMP and PMEG, were identified with the aid of external standards spectra library.

2.5. *Denaturing gel electrophoresis of proteins*

SDS-PAGE system according to Laemli [18] (13% polyacrylamide) was used for the analysis of the purified N^6-me-AMP aminohydrolase. Samples were heated at 95 °C for 15 min (in some cases after concentration by evaporation in vacuum) in 30 mM Tris–HCl, pH 6.8, 5% SDS, 5% 2-mercaptoethanol. LMW calibration kit was used as a protein standard. The gels were stained with silver.

2.6. *Native gel electrophoresis of proteins*

Native gradient slab gels (4–25%) were run to equilibration (2500 Vh) on a 2050 MIDGET-Electrophoresis Unit (LKB); the gels had been pre-electrophoresed for 30 min in Tris-borate buffer, pH 8.3. HMW calibration kits were used as protein standards. The gel was cut into 2-mm slices. N^6-me-AMP aminohydrolase activity was determined after extraction and incubation of each slice with an assay mixture which contained (150 μL): 50 μM N^6-me-AMP in 100 mM Tris–HCl (pH 7.4), 9 mM NaCl and 2 mM DTT.

2.7. *Protein assay*

The concentration of protein was determined by the method of Bradford [19] with bovine serum albumin as standard.

3. Results

An enzyme that catalyzes the conversion N^6-cypr-PMEDAP to PMEG was purified from liver of Sprague–Dawley rats. The six-step purification scheme is shown in Table 1. The enzyme activity during the whole purification procedure was followed by formation of PMEG, which was determined by HPLC (for details see Section 2). The enzyme activity was eluted from phosphocelullose by pH gradient from 7.7 to 7.8 at 0.15–0.20 M KCl (Fig. 2) and subsequently found in the flow-through eluate from DEAE Sepharose FF at pH 7.4 (data not shown). The same elution profile was recorded in the course of perfusion chromatography on cationic exchanger POROS®HS 20 at pH 6.8 (data not shown). Also in this case the enzyme activity had no binding potency and was eluted from the column in a

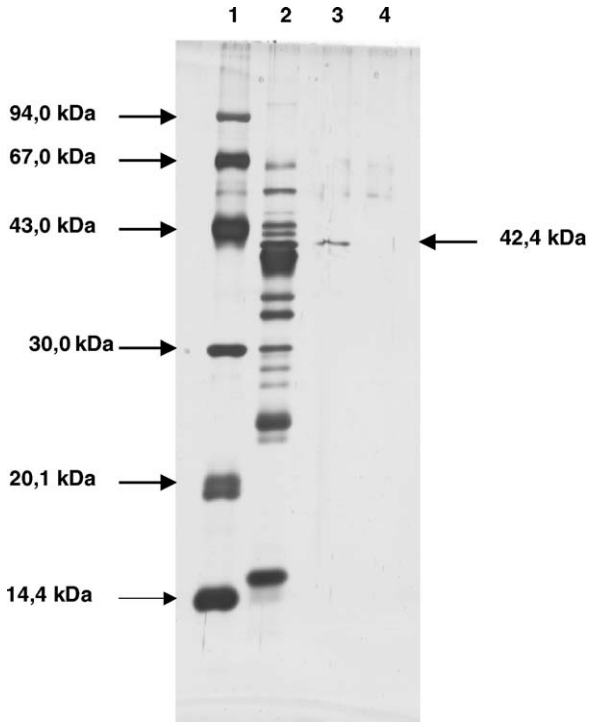


Fig. 4 – SDS-PAGE of N^6-methyl-AMP aminohydrolase in 13% polyacrylamide slab gel. Lines: (1) low-molecular-weight standards; (2) POROS® HS 20 eluate; (3) purified enzyme; (4) no protein added.

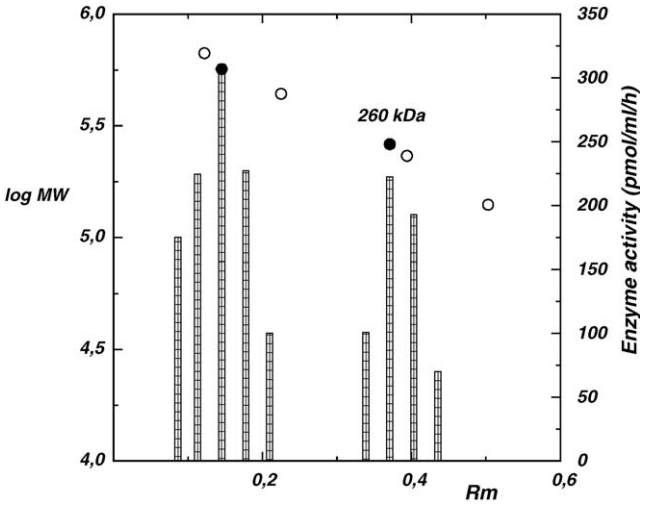


Fig. 5 – Native gradient PAGE of N^6-methyl-AMP aminohydrolase. The purified enzyme was run on polyacrylamide gradient (4–25%) slab gel. Activity of the enzyme was detected after an extraction and incubation of 2 mm gel slices in 50 μM N^6-me-AMP, 100 mM Tris–HCl (7.4), 9 mM NaCl and 2 mM DTT: (○) high-molecular-weight standards (from the left to the right: 669 kDa, 440 kDa, 232 kDa, 140 kDa); (●) positions of the dimer and monomer of N^6-methyl-AMP aminohydrolase.

flow-through. During this procedure the enzyme was purified approximately 200 times compared to the 105,000 × *g* supernatant. The last purification step on Blue-Sepharose was very efficient (Fig. 3) and resulted in pure enzyme preparation (Fig. 4). The specific elution from Blue-Sepharose was achieved by various N^6-substituted 6-aminopurine 5′-nucleotides (e.g. N^6-me-AMP, N^6-cypr-AMP). The yield of the pure enzyme is low; about 10 μg proteins from 50 g of tissue wet weight. It was shown that the enzyme is composed from 42 kDa polypeptide (Fig. 4).

The native gradient PAGE analysis showed that the purified enzyme migrates as hexamer and as its dimer with an apparent molecular weight 260 kDa or 560 kDa, respectively (Fig. 5).

Due to the extremely low yield of N^6-cypr-PMEDAP activating enzyme, we have used a purified enzyme stabilized with bovine serum albumin (100 μg/mL) for the kinetic experiments. With this preparation, we have estimated substrate activity of a series of natural N^6-methyl substituted adenine nucleotides and also of their N^6-substituted 6-aminopurine and 2,6-diaminopurine nucleotide analogues. The results of these experiments are presented in Table 2. Kinetic data (V_{max}/K_m) show that the substrate activity of the studied nucleotides and their analogues is decreasing in the order: 6-me-dAMP > 6-me-AMP > ABC-MP ≥ 6-me_2-AMP > 6-cypr-PMEA > 6-cypr-PMEDAP > 6-me_2-PMEDAP ≥ 6-me_2-PMEA > 6-me-PMEDAP. The interaction of studied nucleotides and their analogues with the enzyme, except for 6-me_2-PMEA and 6-cypr-PMEA, is characterized by very low values of K_m, which indicates high substrate specifity of the enzyme. The enzyme does not catalyze deamination of AMP, dAMP, nucleosides adenosine and 2′-deoxyadenosine or their corresponding N^6-substituted derivatives (data not shown). It is strongly inhibited by deoxycoformycin 5′-phosphate (Table 3). No inhibitory effect was noted for deoxycoformycin and EHNA. Abacavir 5′-phosphate acts as substrate-inhibitor because it weakly inhibits (33%) deamination of 6-me-AMP, probably due to the extremely low value of K_m (Tables 2 and 3).

Table 2 – Substrate activity of N^6-derivatives of AMP, dAMP, PMEA and PMEDAP towards N^6-methyl-AMP aminohydrolase[a]

Substrate	K_m (μmol L^{-1})	V_{max} (nmol min^{-1} mg^{-1})	10^3 V_{max}/K_m (L min^{-1} mg^{-1})
me-AMP	2.5 ± 0.6	336.0 ± 15.3	134.4
me_2-AMP	4.7 ± 1.6	339.3 ± 27.3	72.2
me-dAMP	0.9 ± 0.2	253.7 ± 10.1	281.9
me_2-PMEA	28.9 ± 3.3	32.8 ± 1.3	1.1
cypr-PMEA	70.6 ± 11.5	237.1 ± 20.5	3.4
me-PMEDAP	3.4 ± 0.7	1.8 ± 0.1	0.5
me_2-PMEDAP	5.3 ± 1.3	7.1 ± 0.4	1.3
cypr-PMEDAP	4.5 ± 0.7	14.7 ± 0.6	3.3
ABC-MP	0.5 ± 0.1	39.8 ± 1.0	79.6

[a] 50 mM PIPES, pH 6.8; 2 mM DTT, 37 °C; data are means ± S.E.M. of the three independent experiments.

Table 3 – Inhibition of N^6-methyl-AMP aminohydrolase[a]

Substrat	Deaminated product formed (nmol min^{-1} mg^{-1})				
	Control	1 μM dCF-MP	5 μM dCF	5 μM EHNA	2.5 μM ABC-MP
me-AMP	36.5 ± 0.1	i[b]	35.5 ± 1.5	37.2 ± 0.4	24.0 ± 0.7
ABC-MP	9.8 ± 1.4	i	10.5 ± 0.8	11.3 ± 1.1	–[c]
cypr-PMEDAP	0.8 ± 0.1	i	0.8 ± 0.002	0.9 ± 0.1	–

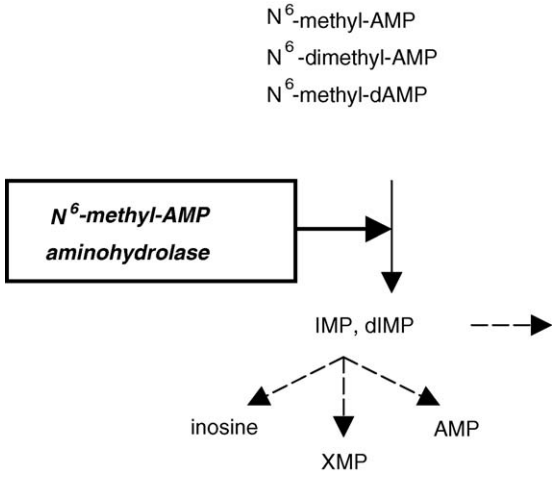
[a] 50 mM PIPES, pH 6.8; 2 mM DTT, 37 °C; data are means ± S.E.M. of the three independent experiments.
[b] 100% inhibition.
[c] Not determined.

4. Discussion

Our results show that enzyme activating N^6-cypr-PMEDAP [2–4], prodrug of PMEG is most probably identical with the AMP deaminase like enzyme, which is able to catalyze the intracellular conversion of abacavir 5′-monophosphate to carbovir 5′-monophosphate [14]. We confirmed that this enzyme accepts natural N^6-methyl-, N^6-dimethyl-AMP and N^6-methyl-dAMP as substrates and catalyzes their conversion to IMP and dIMP, respectively, so that it acts as N^6-methyl-AMP/N^6-methyl-dAMP aminohydrolase. N^6-Methylated AMPs originate from RNAs degradation; therefore we believe that the enzyme participates in the salvage pathway of purine nucleotides (Scheme 1). In the same way, N^6-methyl-dAMP formed after the DNA degradation during the apoptosis could be also catabolized.

The described N^6-methyl-AMP aminohydrolase is highly specific for 5′-phosphates or their structural analogues, e.g. acyclic nucleosides phosphonates and to the presence of aliphatic substitution at the position N^6 of the adenine moiety. Surprisingly, the presence of amino group of purine at the position 2 increases the affinity of the enzyme towards the substrate but decreases the rate of the reaction turnover (Table 2). The substitution of adenine amino function at the position N^6 by cyclopropyl group (cypr-PMEA, Table 2) increases value of V_{max} to the level comparable with the natural substrate. Abacavir 5′-phosphate (ABC-MP) is the best substrate from all the nucleotide analogues tested so far; it indicates that the replacement of the pentose ring of natural substrates by cyclopentene ring results in a more efficient interaction with the enzyme active site than in the case of the aliphatic chain of acyclic nucleoside phosphonates.

Our results are fully consistent with the previous findings of Faletto et al. [14] and Hatse et al. [3], who postulated that pro-drugs ABC-MP and cypr-PMEDAP should be metabolized to the active antivirals CBV-MP and/or PMEG by the catalytic action of an unidentified cellular enzyme, which is sensitive to dCF-MP inhibition. In this study we demonstrate that this unknown enzyme is N^6-methyl-AMP aminohydrolase.



Scheme 1 – Putative role of N^6-methyl-AMP aminohydrolase in the intracellular purine metabolism.

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