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Adenosine Deaminase-like Protein 1 (ADAL1): Characterization and Substrate Specificity in the Hydrolysis of N^6- or O^6-Substituted Purine or 2-Aminopurine Nucleoside Monophosphates†

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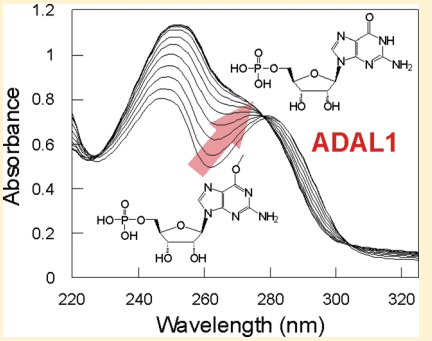
ABSTRACT: Human N^6-methyl-AMP/dAMP aminohydrolase has been shown to be involved in metabolism of pharmacologically important N^6-substituted purine nucleosides and 5′-monophosphate prodrugs thereof. This enzyme was cloned and expressed in *E. coli*, and mass spectroscopic analysis followed by amino acid sequence analyses indicated that the protein was adenosine deaminase-like protein isoform 1 (ADAL1). An extensive structure−activity relationship study showed that ADAL1 was able to catalyze removal of different alkyl groups not only from N^6-substituted purine or 2-aminopurine nucleoside monophosphates but also from O^6-substituted compounds. The ADAL1 activity was susceptible to modifications in the phosphate moiety but not to changes in the sugar moiety. Overall, our data indicated that ADAL1 specifically acts at the 6-position of purine and 2-aminopurine nucleoside monophosphates. Our results may help designing of new therapeutic nucleoside/nucleotide prodrugs with desired metabolic profiles. Furthermore, amino acid sequence analysis in conjunction with crystallographic data and metal analysis suggested that ADAL1 contains a catalytic zinc ion. Finally, a potential physiological role of ADAL1 is discussed.



INTRODUCTION

N^6-Methyladenosine 5′-monophosphate (AMP)/2′-deoxyadenosine 5′-monophosphate (dAMP) aminohydrolase has been shown to be involved in the metabolism of pharmacologically important N^6-substituted purine nucleosides and 5′-monophosphate prodrugs thereof.[1,2] Such compounds include an approved anti-HIV agent, (−)-(1*S*,4*R*)-4-[2-amino-6-(cyclopropylamino)-9*H*-purin-9-yl]-2-cyclopentene-1-methanol (abacavir), and a cytotoxic agent, diethyl *N*,*N*′-[({2-[2-amino-6-(cyclopropylamino)-9*H*-purin-9-yl]ethoxy}methyl)phosphonoyl]di-L-alaninate (GS-9219) (Figure 1) that targets lymphoid cells that is currently in early phase clinical trials.[3,4] Both **1** (abacavir) and **2** (GS-9219) contain a N^6-cyclopropyl-2,6-diaminopurine base. Metabolism studies reported by Faletto et al. identified an adenosine monophosphate deaminase-like activity in CEM cells and human peripheral blood mononuclear cells that specifically removes the cyclopropylamino group of 5′-monophosphate form of **1** (**28**).[5] Examining subcellular fractions of rat liver, Faletto et al. demonstrated that the activity was associated with the cytoplasmic fraction.[5] Schinkmanova et al. isolated and purified an enzyme from rat liver that they named N^6-methyl-AMP aminohydrolase.[2] This enzyme was able to efficiently convert **28** to its deaminated metabolite carbovir 5′-monophosphate. In their study they showed that this enzyme was able to use various N^6-substituted purine nucleoside 5′-monophosphates, 9-[2-(phosphonomethoxy)ethyl]adenine (PMEA), and 9-[2-(phosphonomethoxy)ethyl]-2,6-diaminopurine (PMEDAP) as substrates with the following decreasing order of activity: N^6-methyldeoxy-AMP > N^6-methyl-AMP > **28** ≥ N^6-dimethyl-AMP > N^6-cyclopropyl-PMEA > N^6-cyclopropyl-PMEDAP (a metabolite of **2**) > N^6-dimethyl-PMEDAP ≥ N^6-dimethyl-PMEA > N^6-methyl-PMEDAP. More recently Schinkmanová and colleagues cloned and expressed the human enzyme using the baculovirus expression system and the human enzyme demonstrated almost identical substrate specificity as the rat enzyme.[1]

Currently, there are several O^6-substituted guanosine 5′-monophosphate prodrugs in clinical or preclinical development as anti-HCV agents including a 3′,5′-cyclic phosphate prodrug, 6-ethoxy-9-((2*R*,4a*R*,6*R*,7*R*,7a*R*)-7-fluoro-2-isopropoxy-7-methyl-2-oxotetrahydro-2λ^5-furo[3,2-*d*][1,3,2]dioxaphosphinin-6-yl)-9*H*-purine-2-ylamine (PSI-352938) (**3**), and phosphoamidate prodrugs, (*S*)-2-{(*S*)-[(1*R*,4*R*,5*R*)-5-(2-amino-6-methoxypurin-9-yl)-4-(*R*)-fluoro-3-hydroxy-4-methyltetrahydrofuran-2-ylmethoxy]-phenoxyphosphorylamino}propionic acid isopropyl ester (PSI-353661) (**4**) and (*S*)-2-[[(2*R*,3*R*,4*R*,5*R*)-5-(2-amino-6-methoxypurin-9-yl)-3,4-dihydroxy-4-methyltetrahydrofuran-2-ylmethoxy](naphthalen-1-yloxy)phosphorylamino]propionic acid 2,2-dimethylpropyl ester (INX-189) (**5**) (Figure 1).[6−8] These compounds require removal of the O^6 alkyl group from the guanine base prior to metabolism to the active 5′-triphosphate. Parker et al. demonstrated that O^6-propyl carbovir can be metabolized to carbovir 5′-triphosphate in CEM cells.[9] Therefore, we assessed the ability of purified recombinant human N^6-methyl-AMP aminohydrolase to use O^6-substituted purine 5′-monophosphates as substrates. Further characterization of the enzyme identified it as adenosine deaminase-like protein isoform 1 (ADAL1). Here we present the results of an extensive

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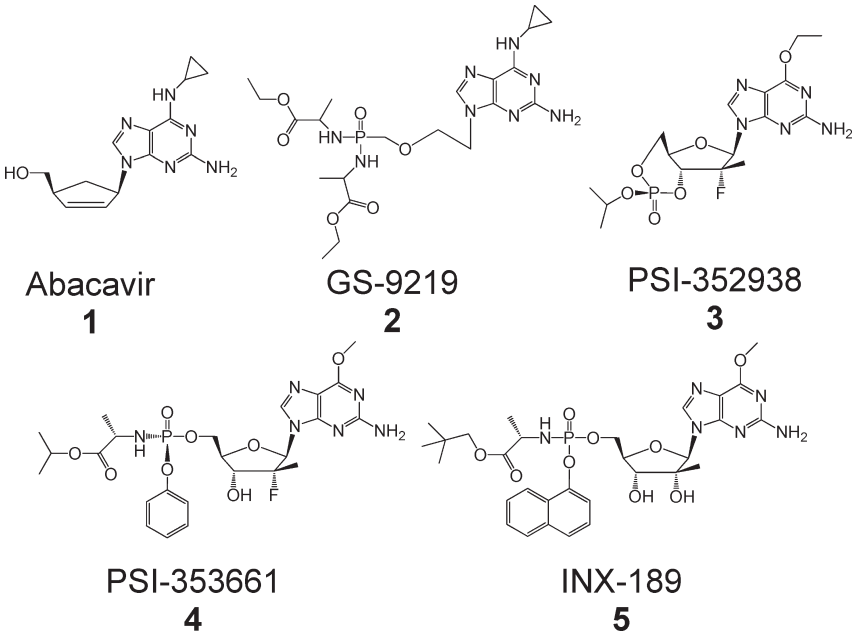


Figure 1. Structures of **1**−**5**.

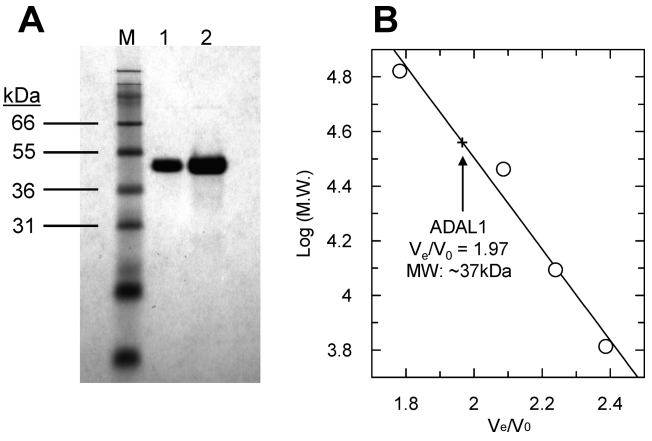


Figure 2. SDS−PAGE and gel filtration analyses of purified human recombinant ADAL1. (A) 1.6 and 4.8 μg of purified ADAL1 were loaded on lanes 1 and 2, respectively. Lane M indicates molecular weight marker. (B) Molecular weight determination of the nondenatured enzyme by a gel filtration column chromatography. Open circles are molecular weight standards (see Experimental Section for details).

structure−activity relationship study (SAR) demonstrating that ADAL1 was capable of effectively metabolizing various substitutions at the 6 position of the purine base. Furthermore, amino acid sequence analyses of ADAL1 and related enzymes were performed to help delineate the interactions between the substrate and ADAL1 on the basis of crystallographic data for the related enzymes.

RESULTS AND DISCUSSION

Enzyme Characterization. Human N^6-methyl-AMP aminohydrolase was cloned using the primer set described by Schinkmanová et al. A NCBI Blast search using the sequence of the primer set aligned with the sequence of human ADAL1.[10] The protein was expressed in *E. coli* and purified as described in Experimental Section, and the purity was demonstrated to be >95% by SDS−polyacrylamide gel electrophoresis (PAGE) (Figure 2A). To further identify the protein, the protein band was excised from the gel and mass spectrometric peptide mapping and sequence analysis were performed. The sequence coverage was 38%, and results from a database search indicated that the protein was adenosine deaminase-like protein isoform 1 (ADAL1). The molecular weight of the protein based on SDS−PAGE was similar to that calculated based on ADAL1 amino acid sequence (40.26 kDa). The subunit composition of ADAL1 was examined by gel filtration column chromatography using known molecular weight proteins as standards (Figure 2B). The purified ADAL1 eluted as a single peak, and on the basis of the elution volume, the molecular weight of the native form of the enzyme was calculated to be approximately 37 kDa, indicating that ADAL1 was a monomer with a molecular weight similar to that calculated based on the amino acid sequence of ADAL1. Schinkmanová et al. also reported that human N^6-methyl-AMP aminohydrolase was a monomer protein.[1]

N^6-Methyl-AMP aminohydrolase has also been referred to as abacavir 5′-monophosphate deaminase based on the ability of the enzyme to metabolize **28** to carbovir 5′-monophosphate.[1] Additionally it was reported that the enzyme could utilize other N^6-sustituted purine nucleoside 5′-monophosphate analogs as substrates. To ensure that our bacterially expressed ADAL1 possesses the same activities as previously reported for N^6-methyl-AMP aminohydrolase, which was expressed in insect cells using the bacluovirus system, we tested the ability of the recombinant ADAL1 to use **14** and **28** as substrates, both of which were substrates for N^6-methyl-AMP aminohydrolase. Because the UV spectra of **14** and **28** and their deaminated products were significantly different, the change in the UV spectrum was followed to monitor the reaction. After addition of ADAL1 the spectra of **14** and **28** were collected every 5 min for 1 h (Figure 3A and Figure 3B). The spectra for both compounds shifted in a time-dependent manner (Figure 3A and Figure 3B).

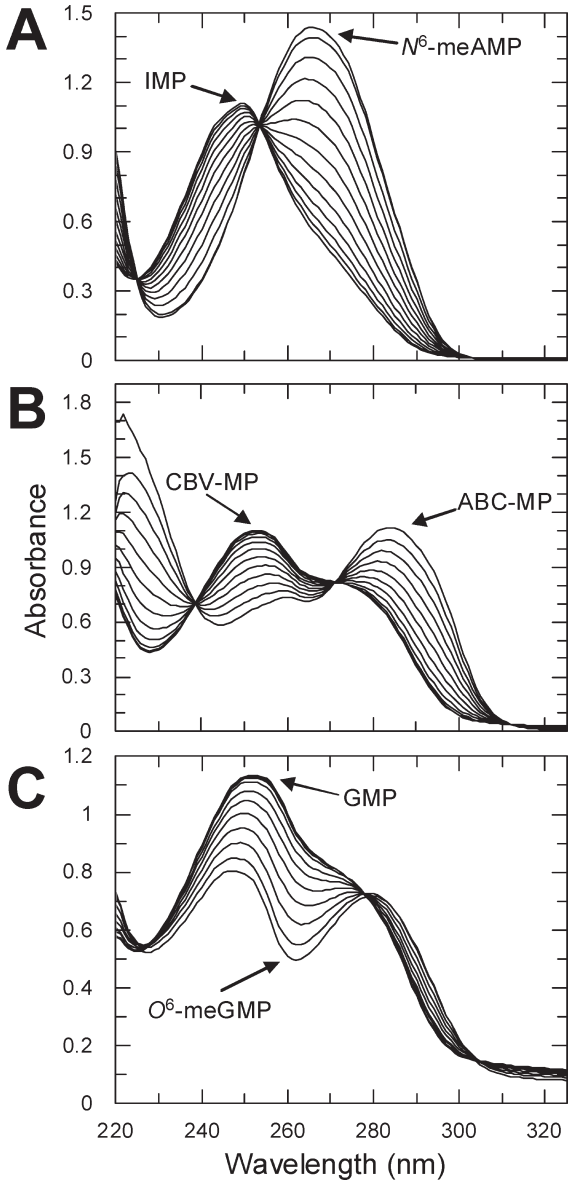


Figure 3. Spectral change during ADAL1-mediated hydrolytic removal of the alkyl group from **14** (A), **28** (B), and **15** (C).

Therefore, both **14** and **28** were substrates for the enzyme. Taken together, our data indicated that our ADAL1 expressed in *E. coli* showed the same activity as previously reported for N^6-methyl-AMP aminohydrolase/abacavir 5′-monophosphate deaminase.

We next conducted the same experiment using an O^6-methylated compound **15** as a substrate for ADAL1. As shown in Figure 3C, the spectrum of **15** shifted in a time-dependent manner. At the end of the reaction with **15**, the UV spectrum gave a λ_{max} of 252 nm, which indicated that **15** was converted to guanosine 5′-monophosphate (GMP). The identity of the product was also confirmed to be GMP by high pressure liquid chromatography (HPLC) analysis (data not shown).

Substrate Specificity of ADAL1. The substrate specificity of ADAL1 was explored by testing various modified nucleosides and nucleotides. A structure−activity relationship (SAR) for ADAL1 was developed based on the following criteria: (i) phosphorylation state and modification to the phosphate moiety, (ii) modifications to the sugar moiety, and (iii) modifications to the base.

Effect of the State of Phosphorylation and Modifications to the Phosphate Moiety on Enzyme Activity. The effect of the phosphorylation state at the 5′-position of 6-substituted purines on their ability to serve as a substrate for ADAL1 was tested (Table 1). Eight natural and unnatural nucleosides **1**, **6**−**12** were tested with ADAL1, but none of them served as substrates for the enzyme. Likewise, a diphosphate **18** and a triphosphate **19** were not substrates for ADAL1. Only 5′-monophosphate derivatives were substrates for ADAL1. Although **13** was deaminated to inosine 5′-monophosphate (IMP) by ADAL1, the catalytic efficiency (k_{cat}/K_m) for AMP was 76-fold less than that for **14**. Because AMP demonstrated only a 2.6-fold increase in K_m and approximately a 29-fold decrease in k_{cat} compared to N^6-methyl-AMP, the lower catalytic efficiency of **13** was due to inefficient conversion rather than weak binding. Since adenylate deaminase utilizes **13** as its natural substrate, it is unlikely that ADAL1 has a significant physiological role in the deamination of **13**. Catalytic efficiencies of removal of alkyl groups from compounds **15**−**17** were similar and were somewhat better than that for **14**.

We then tested compounds that have modification in the phosphate moiety. Cyclic phosphate compounds **3** and **20** were not substrates for ADAL1. Phosphoramidate pronucleotides **4**, **5**, **21** also did not act as substrates. Although **13** was poorly deaminated to IMP, it still acted as a substrate for ADAL1, but **22** was not deaminated to IMP-NH_2, indicating that the amino group on the phosphate affected the activity of ADAL1. Taken together, ADAL1 specifically acts on 5′-monophosphate derivatives of N^6- or O^6-substituted purine/2-aminopurine nucleosides and the activity is sensitive to modifications in the phosphate moiety.

Effect of Modifications to the Sugar Moiety on Enzyme Activity. O^6-Methylated compounds **15** and **23** were found to be good substrates for ADAL1 with similar k_{cat}/K_m values of 0.45 and 0.49 s^{-1} μM^{-1}, respectively (Table 2). Slightly reduced activity was observed when the substitution on the ribose was 2′-β-*C*-Me (**24**). Compared to **15** and **23**, somewhat higher K_m and k_{cat} values were observed with the 2′-F-2′-*C*-Me substitution (**16**), but the overall catalytic efficiency was very similar. Compounds **25** and **26**, which are both acyclic phosphonates with adenine as the base, were not deaminated by ADAL1. This is probably not due to the acyclic phosphonate moiety, since it was previously shown that ADAL1 deaminated various N^6-substituted acyclic phosphonates, PMEAs, and PMEDAPs.[1,2] Instead it is likely that ADAL1 acts poorly on substrates containing the unsubstituted adenine base because **13** was deaminated by ADAL1 76-fold less efficiently than **14** as described above. Overall, the ADAL1 activity is not susceptible to modifications in the sugar moiety.

Effect of Modifications to the Base on Enzyme Activity. As described above, ADAL1 hydrolyzes N^6- or O^6-substituents from purine/2-aminopurine nucleoside 5′-monophosphates. Here, ADAL1-mediated removal of *N*-alkyl groups was evaluated using compounds that have various substitutions at the N^6-position (Table 3). Compound **27** was hydrolyzed to IMP slightly more efficiently than **14**. Compounds **28** and **29** were also good substrates with similar k_{cat}/K_m values as **14**. These results suggest that the active site pocket of ADAL1 can accommodate modest sized lipophilic N^6-substitutions. The K_m for **28** in our experiment (7.37 μM) was significantly higher than the one reported

Table 1. ADAL1 Activity against Compounds with Different Phosphorylation States

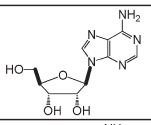
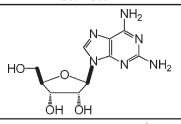
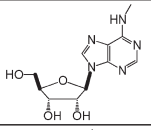
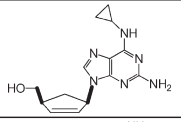
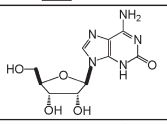
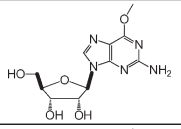
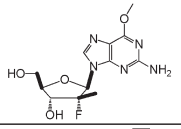
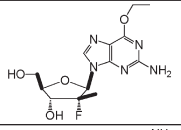
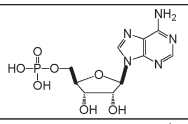
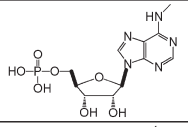
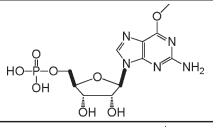
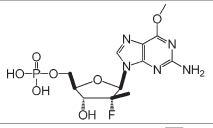
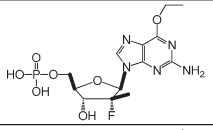
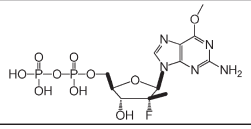
Compound No.	Structure	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\mu M^{-1}$)	W.L. (nm)	Δε ($mM^{-1}cm^{-1}$)
6		No activity			N.A.	N.A.
7		No activity			N.A.	N.A.
8		No activity			N.A.	N.A.
1		No activity			N. A.	N. A.
9		No activity			N.A.	N.A.
10		No activity			N.A.	N.A.
11		No activity			N.A.	N.A.
12		No activity			N.A.	N.A.
13		32.8 ± 7.5	0.055 ± 0.004	0.0017	264	-9.00
14		12.5 ± 1.7	1.58 ± 0.12	0.13	270	-11.1
15		5.2 ± 0.9	2.33 ± 0.07	0.45	262	4.44
16		8.96 ± 2.1	5.85 ± 0.40	0.65	258	6.11
17		25.1 ± 2.6	8.14 ± 0.08	0.32	258	6.11
18		No activity			N.A.	N.A.

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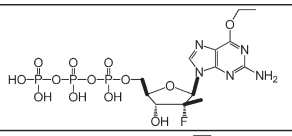
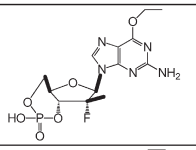
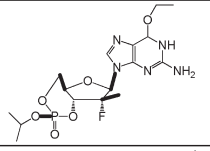
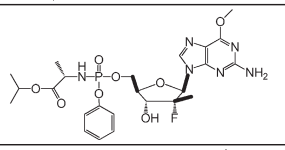
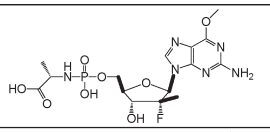
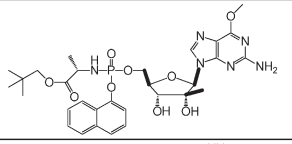
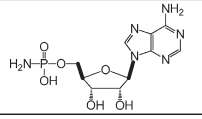
Table 1. Continued

Compound No.	Structure	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\mu M^{-1}$)	W.L. (nm)	Δε ($mM^{-1}cm^{-1}$)
19		No activity			N.A.	N.A.
20		No activity			N.A.	N.A.
3		No activity			N.A.	N.A.
4		No activity			N.A.	N.A.
21		No activity			N.A.	N.A.
5		No activity			N.A.	N.A.
22		No activity			N.A.	N.A.

previously (0.4 μM).[1] We believe this discrepancy is due to differences in the preparation of the enzyme and the assay used; we overexpressed the enzyme in *E. coli* and used a continuous spectroscopic assay, while Schinkmanová et al. expressed the enzyme in insect cells using the baculovirus expression system and the activity assay was performed using an HPLC. Compound **30** was not a substrate for ADAL1 because the C-6 position is part of a fused hetercyclic ring that cannot be hydrolyzed. Tricyclic nucleoside 5′-phosphate **31** was not deaminated possibly because the base portion of the molecule is too large to be accommodated in the active site of ADAL1 or because the heteroatom array at C-6 is not compatible with the mechanism of hydrolysis. Compound **32** was also not a substrate, possibly because of the size and/or the charge of the succinyl group.

The hydrolytic activity of ADAL1 was also tested with several O^6-substituted compounds (Table 3). We tested O^6-methyl- (**16**), O^6-ethyl- (**17**), and O^6-isopropyl- (**33**), 2-aminopurine-2′-F-2′-*C*-methyl nucleoside 5′-monophosphates, and all were substrates for ADAL1 with similar kinetic parameters. These results agree with our view of the nature of the active site binding pocket described above. No activity was observed with **34** possibly because the thiol may directly coordinate to the catalytic metal ion that may perturb the activation of a water molecule. A weak activity was observed with **35**. This may be because the thioether substituent at C-6 changes the electronic character of the C-6 carbon atom, reducing its electrophilicity relative to a nitrogen or an oxygen containing substituent, resulting in reduced susceptibility to a nucleophilic attack by a water molecule. Consistent with this mechanistic rationale, the halogenated compound **36** was a substrate for ADAL1. A similar dehalogenation activity by adenosine deaminase (ADA) has been reported with 6-halogenated 2′,3′-dideoxypurine ribofuranosides.[11] We assayed two cytidine analogues **37** and **38** and found that neither was able to serve as substrates for ADAL1.

Since **16**, **24**, and **17** are metabolic intermediates for **4**, **5**, and **3**, respectively, ADAL1 is involved in activation of these drugs. In addition, it was shown that ADAL1 deaminates abacavir-MP and cPrPMEDAP, which are metabolites of **1** and **2**, respectively.[3,5] Therefore, ADAL1 plays a significant role in activation of N^6- or O^6-substituted purine or 2-aminopurine nucleoside/nucleotide analogues and further SAR studies may potentially help to design a compound that is metabolized at a desired rate.

ADAL1 Activity on Methylated DNA Substrate. To further study substrate specificity of ADAL1, double-stranded DNA containing O^6-methylguanosine in one strand at a Pst1 restriction site was used as a substrate. The DNA substrate was treated with either O^6-methylguanine DNA methyltransferease (MGMT) or ADAL1 followed by digestion with Pst1. The unreacted DNA

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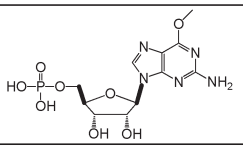
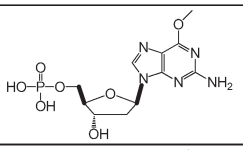
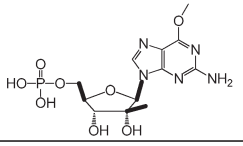
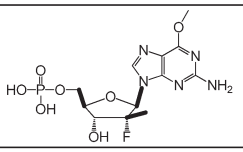
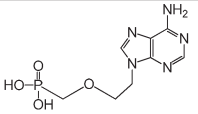
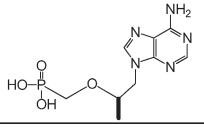
Table 2. ADAL1 Activity against Compounds with Modifications in the Sugar Moiety

Compound No.	Structure	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\mu M^{-1}$)	W.L. (nm)	Δε ($mM^{-1}cm^{-1}$)
15		5.2 ± 0.9	2.33 ± 0.07	0.45	262	4.44
23		6.0 ± 1.0	2.92 ± 0.14	0.49	262	4.79
24		10.7 ± 1.5	2.36 ± 0.09	0.22	258	6.11
16		8.96 ± 2.1	5.85 ± 0.40	0.65	258	6.11
25		No activity			N.A.	N.A.
26		No activity			N.A.	N.A.

substrate and the cleavage product (8-mer) were separated on a DNA sequencing gel (Figure 4). Pst1-mediated cleavage of the DNA was observed when the DNA substrate was treated with MGMT, whereas no cleavage was observed with ADAL1-treated DNA because of protection of the restriction site by the methyl group (Figure 4). In the presence of O^6-benzylguanine, a specific inhibitor of MGMT, the amount of the cleavage product was significantly reduced. This result indicated that a methyl group cannot be removed from a methylated DNA strand by ADAL1.

Sequence Analysis. Our SAR study indicated that ADAL1 hydrolyzes different N^6- and O^6-substituted purine/2-aminopurine nucleoside 5′-monophosphates. In addition, it hydrolyzed the 6-chloro compound (**36**). A similar SAR profile has been reported for adenosine deaminase (ADA) which removes various substituents at the 6-position of purine/2-aminopurine nucleosides including N^6-methyl, O^6-methyl, O^6-ethyl, and 6-halogen groups.[11−13] This similarity in substrate specificity suggests that ADAL1 and ADA may share a similar active site architecture. However, ADA utilizes nucleosides as substrates while ADAL1 specifically utilizes 5′-monophosphates, suggesting that ADAL1 may contain amino acids involved in binding to the phosphate moiety. AMP deaminase (AMPD) has a similar catalytic function to ADA and shares half of the evolutionarily conserved sequence elements in adenosine deaminases including the highly conserved motif "SLN(S)TDDP".[14] Since AMPD utilizes a 5′-monophosphate substrate, AMPD and ADAL1 may contain a similar phosphate binding site. Therefore, the amino acid sequence of ADAL1 was compared with that for ADA and AMPD.

A BLASTp search using the human ADAL1 (*h*ADAL1) sequence (accession number Q6DHV7) over the UniProtKB using default parameters and taxonomy filtered for *Homo sapiens* retrieves 39 entries.[10,15,16] When sorted by score, *h*ADA1 (accession number P00813) is the first entry after various isoforms of ADAL with a sequence identity of 21% and score of 171. The CLUSTALW sequence alignment using default parameters is depicted in Figure 5.[17] A crystallographic complex of *h*ADA and 2′-deoxyadenosine became available in 2009 with a resolution of 1.52 Å (PDB entry 3IAR). The sequence of *h*ADA1 has been colored to highlight residues in direct contact with the catalytic Zn^{2+} (cyan), the adenine component of the nucleoside (green), and the 5′-OH of the ribose (yellow).

Unfortunately, there is no crystallographic complex of *h*AMPD available. However, there is a structure available for the AMPD domain of embryonic factor 1 (FAC1) derived from thale cress (*Arabidopsis thaliana*) cocrystallized with coformycin 5′-phosphate (PDB entry 2A3L) with a resolution of 3.34 Å.[18] Full length *a*AMPD (839aa, accession number O80452) has 40% sequence identity with *h*AMPD2 (liver isoform, accession number Q01433) and 57% homology. The sequence alignment of *h*ADAL1 to the C-terminus of *a*AMPD (701 aa found in the 2A3L PDB entry) and *h*AMPD2 is depicted in Figure 6. With this alignment, *h*AMPD2 and *h*ADAL1 have a sequence identity of ∼7% and homology of ∼18%.

In the alignment to *h*ADA1, *h*ADAL1 has the identical residues necessary to interact with a putative catalytic Zn^{2+}. This is not the case with *a*AMPD/*h*AMPD2. His659/His687 is not

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Table 3. ADAL1 Activity against Compounds with Modifications in the Base[a]

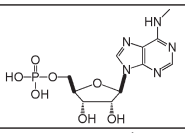
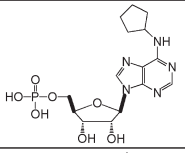
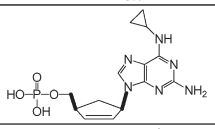
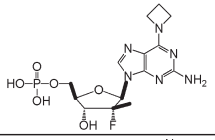
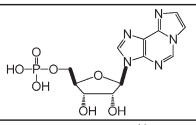
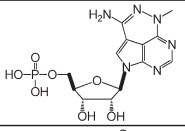
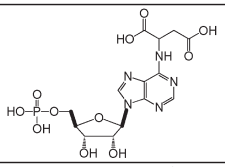
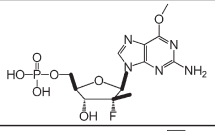
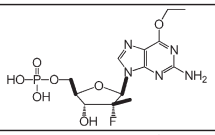
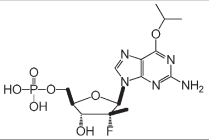
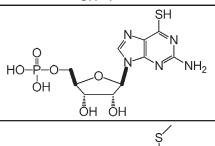
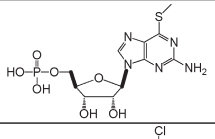
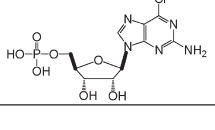
Compound No.	Structure	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\mu M^{-1}$)	W.L. (nm)	Δε ($mM^{-1}cm^{-1}$)
14		12.5 ± 1.7	1.58 ± 0.12	0.13	270	-11.1
27		14.3 ± 5.9	4.24 ± 0.43	0.30	235	5.92
28		7.37 ± 1.11	1.63 ± 0.08	0.22	292	-6.79
29		8.63 ± 0.98	1.55 ± 0.05	0.18	295	-10.0
30		No activity			N.A.	N.A.
31		No activity			N.A.	N.A.
32		No activity			N.A.	N.A.
16		8.96 ± 2.1	5.85 ± 0.40	0.65	258	6.11
17		25.1 ± 2.6	8.14 ± 0.08	0.32	258	6.11
33		25.9 ± 3.5	7.2 ± 0.3	0.28	260	5.62
34		No activity			N.A.	N.A.
35		Slow[l]			N.A.	N.A.
36		16.3 ± 3.3	3.64 ± 0.18	0.223	271	8.23

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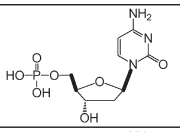
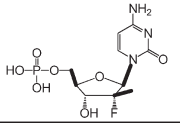
Table 3. Continued

Compound No.	Structure	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\mu M^{-1}$)	W.L. (nm)	Δε ($mM^{-1}cm^{-1}$)
37		No activity			N.A.	N.A.
38		No activity			N.A.	N.A.

[a] [1]Only approximately 20% of the product formation observed by HPLC after 6 h of incubation at 37 °C in the presence of 124 nM ADAL1.

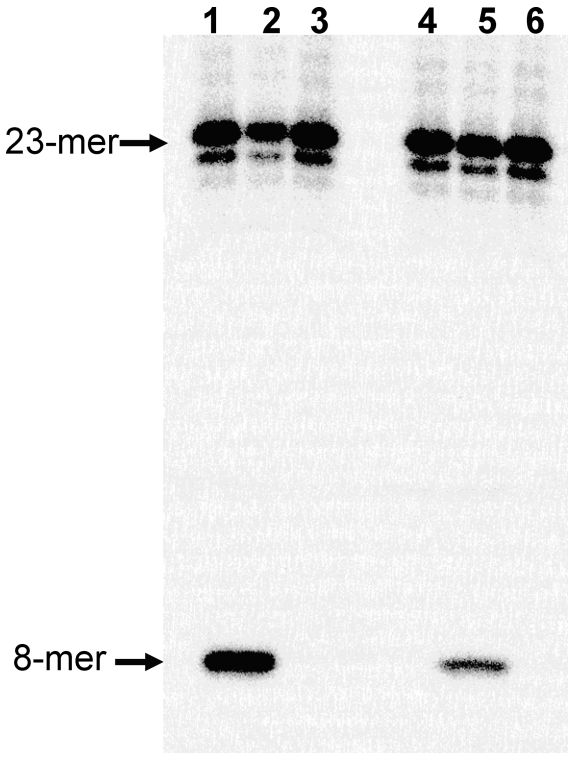


Figure 4. ADAL1 does not remove a methyl group from O^6-methylated guanine in DNA strand. Experiment was performed in the absence (lanes 1−3) or in the presence (lanes 4−6) of benzylguanine, an inhibitor of MGMT. No enzyme controls are shown in lanes 1 and 4. The methylated DNA was treated with either MGMT (lanes 2 and 5) or ADAL1 (lanes 3 and 6). All the samples were treated with Pst1 restriction enzyme after the demethylation reaction. See Experimental Section for details.

mapped by *h*ADAL1 in this alignment. However, it is not unreasonable to expect that Asn237 in *h*ADAL1 could fill this role if the alignment is tweaked to superpose "NSGE" with "HSGE". The highly conserved motif found in the adenosine deaminase family, "SLN(S)TDDP", is not completely found in *h*ADAL1. The "TDD" core is present, however, with *h*ADAL1's Asp293 potentially acting as one of the Zn^{2+} chelators and Asp294 interacting directly with the nucleotide. In the available ADA crystallographic complexes, this Asp (D296) is conjectured to have a much higher pK_a due to the hydrophobic environment and thus able to provide an H-bond interaction with the N-7 of the purine.[19] This is also found in the structure of *h*ADA2 complexed with coformycin (PDB entry 3LGG); its homologous Asp (D416) also interacts with the coformycin N-1 (analogous to adenosine N-7).[20] Interestingly, in the much lower resolution complex of FAC1 and coformycin phosphate, the homologous Asp (D737) appears to interact with the ribose oxygen if not the phosphate group itself rather than the heterocycle. In fact, the presentation of coformycin phosphate bound to FAC1 when compared to coformycin bound to *h*ADA2 or to 2′-deoxyadenosine bound to *h*ADA1 is rotated about the bond to the Zn ion such that interactions with the heterocycles vary widely between the two closely related structure classes. This is evident in Figure 6 in which residues homologous to those in *h*ADA1 are highlighted according to the interactions observed in the *h*ADA1 complex. For example, the hydrophobic residues Leu63, Phe67, and Phe70 in *h*ADAL1 are highly homologous to the same residues present in *h*ADA which provide hydrophobic stabilization to the nucleoside. The same homologous residues are present in FAC1, Leu429, Phe433, and Leu436, but the presentation of coformycin phosphate is such that they do not fall within 5 Å of the inhibitor. If this difference in presentation of the coformycin phosphate in FAC1 relative to that seen in the ADA family is due to the presence of the phosphate, then it suggests that compounds processed by AMPD may have a different leaving group profile. Indeed, unlike ADAL1 or ADA, human AMPD1 demonstrated little or no activity with **14** or **15**, respectively (unpublished results), suggesting that AMPD is not a good model for the phosphate interaction.

Finally, the presentation of the phosphate "anchor" is completely different between the two crystal structures. As a consequence of the rotation of the heterocycle about its bond to Zn^{2+}, the ribose of each substrate explores an adjacent but different binding region. Since a nucleoside is bound to *h*ADA1, we approximate that residues within 5 Å of the 5′-OH will provide some insight into where to expect differences between ADAL1 and ADA1 which could stabilize a phosphate group over just the hydroxyl group. One such difference is Asn28 in the place of the Asp found in *h*ADAL1. Conversely, there are a number of residues in ADAL1 that could interact with a phosphate on the basis of the sequence alignment to AMPD. These include Lys100 and Lys295 (with another tweak of the alignment) as matches to Lys466 and Gln740, respectively, which stabilize the phosphate group found in the FAC1 complex.

In lieu of crystallographic information being available for *h*ADAL1 in the near term, it may be necessary to use mutagenesis in conjunction with SAR and/or homology model building to

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hADAL1     MIEAEEQQPCKTDFYSELPKVELHAHLNGSISSHTMKKLIAQK----PDLKIHDQMTVID 56
hADA1      MAQTPAFD---------KPKVELHVHLDGSIKPETILYYGRRRGIALPANTAEGLLNVIG 51
           * ::   :          ******.**:***...*:     ::    *  . .. :.**.

hADAL1     KGKKRTLEECFQMFQT-IHQLTSSPEDILMVTKDVIKEFADDGVKYLELRST-------- 107
hADA1      MDKPLTLPDFLAKFDYYMPAIAGCREAIKRIAYEFVEMKAKEGVVYVEVRYSPHLLANSK 111
            .*  ** : :  *:  :  ::.. * *  :: :.::  *.:** *:*:* :

hADAL1     ----PRRENATGMTKKTYVESILEGIKQSKQENLDIDVRYLIAVDRRGGPLVAKETVKLA 163
hADA1      VEPIPWNQAEGDLTPDEVVALVGQGLQEGERD-FGVKARSILCCMR-HQPNWSPKVVELC 169
               * .:   .:* .  *  : :*:::.::: :.:..* ::.  *   *  : :.*:*.

hADAL1     EEFFLSTEGTVLGLDLSGDPTVG---QAKDFLEPLLEAKKAGLKLALHLSEIPNQKKETQ 220
hADA1      KKY---QQQTVVAIDLAGDETIPGSSLLPGHVQAYQEAVKSGIHRTVHAGEVGSAEVVKE 226
           :::    : **:.:**:** *:       ..::.  ** *:*:: ::* .*: . :  .:

hADAL1     ILLDLLPDRIGHGTFLNSGEGGSLDLVDFVRQHRIPLELCLTSNVKSQTVPSYDQHHFGF 280
hADA1      AVDILKTERLGHGYHTLEDQA----LYNRLRQENMHFEICPWSSYLTGAWKPDTEHAVIR 282
            :  * .:*:*** .  ..:.    * : :**..: :*:*  *.  : :  .  :* .

hADAL1     WYSIAHPSVICTDDKGVFATHLSQEYQLAAETFNLTQSQVWDLSYESINYIFASDSTRSE 340
hADA1      LKNDQANYSLNTDDPLIFKSTLDTDYQMTKRDMGFTEEEFKRLNINAAKSSFLPEDEKRE 342
             .      : ***  :* : *. :**:: . :.:*:.:.  *. :: :  * .:. : *

hADAL1     LRKKWNHLKPRVLHI------ 355
hADA1      LLDLLYKAYGMPPSASAGQNL 363
           * .   :
```

Figure 5. ClustalW (2.1) sequence alignment for *h*ADAL1 and *h*ADA: sequence identity of 21% and 42% similarity. Gray segment is not seen in 3IAR. Cyan highlighting indicates contact with catalytic Zn. Green are residues within 5 Å of the adenine (necessarily includes the metal binding residues). Yellow indicates residues within 5 Å of the ribose 5′-OH. Residues in bold/purple fall within multiple distance criteria. The asterisk (∗) signifies identity. The colon (:) signifies high similarity, and the period (.) signifies low similarity.

further clarify which binding mode, based on this structural analysis, is more relevant: that derived from the reasonably homologous *h*ADA1 or that derived from FAC1 with low homology but containing key substrate features.

Metal Analysis. Other deaminases such as adenosine deaminase, AMP deaminase, and cytidine deaminase contain a catalytic metal ion in their active sites.[18,19,21] Either of the two sequence alignments in Figures 5 and 6 suggests that the appropriate residues are present in *h*ADAL1 to act as chelators for a putative catalytic metal ion. Therefore, a metal analysis was performed to identify which metal ion is used by ADAL1. As shown in Table 4, concentrations of different divalent metals were determined, and only calcium and zinc were detected in ADAL1. Approximately 0.4 and 1.1 mol of calcium and zinc were found per mole of ADAL1, respectively. Since other deaminases function via a catalytic zinc ion, it is likely that ADAL1 also utilizes zinc ion for catalysis.

Potential Physiological Role of ADAL1. We showed that ADAL1 mRNA was expressed in various cell types but the physiological role of this enzyme is unclear. Schinkmanová et al. proposed that ADAL1 may be involved in the catabolism of N^6-methylated adenosine nucleotides produced by degradation of nucleic acids; however, there is no direct evidence for the presence of N^6-methylated adenosine in mammalian DNA.[1,2,22,23] Here we reported that ADAL1 efficiently catalyzes not only the hydrolysis of N^6-substituted purine/2-aminopurine nucleotides but also the hydrolysis of O^6-substituted nucleoside 5′-monophosphates. Since methylation of DNA at the O^6-position of guanine base is known to occur because of endogenous and environmental alkylating agents, methylation of guanine could occur at nucleoside or nucleotide levels. Previously, Topal and Baker reported that nucleotides in the precursor pool are more susceptible to methylation by a potent methylating agent, *N*-methyl-*N*-nitrosourea (MNU), than the residues in the DNA in mouse embryo fibroblast C3H/10$T_{1/2}$ clone 8 cells.[24−26] They have estimated that the O^6-position of dGTP is approximately 400-fold more susceptible to methylation by MNU than the same position in the guanosine residues in DNA. Therefore, it is likely that GMP and dGMP can be intracellularly methylated at the O^6-position by alkylating agents. Then what is the physiological role of the enzyme? Since it has been demonstrated that O^6-CH_3-dGTP is a substrate for DNA polymerases, one assumption is that removal of the O^6-CH_3 group by ADAL1 would prevent the formation of O^6-CH_3-dGTP and/or O^6-CH_3-GTP to their triphosphate forms consequently preventing the incorporation of the nucleotides into DNA and/or RNA.[27−30] Although it is not known if **23** and **15** can be phosphorylated to their respective triphosphates and act as substrates for DNA or RNA polymerases, it is clear that ADAL1 is capable of converting **23** and **15** to dGMP and GMP, respectively, which are then metabolized to their triphosphate forms for nucleic acid synthesis. Therefore, based on our in vitro data, we propose the physiological role of ADAL1 to be nucleotide salvage and the enzyme may play a significant role in purine nucleotide metabolism. It would be interesting to examine effects of MNU on nucleotide pools in ADAL1 depleted cells, and such an experiment is ongoing in our laboratory.

CONCLUSION

Human recombinant ADAL1 demonstrated the ability to remove different alkyl groups from O^6-substituted purine or 2-aminopurine nucleoside monophosphates as well as removal of alkyl groups from N^6-substituted compounds. This enzyme plays a significant role in activation of known nucleoside/nucleotide analogues currently approved or in clinical trials. Therefore, our SAR study described here would help design new therapeutic nucleoside/nucleotide prodrugs with N^6- or O^6-substituted purine or 2-aminopurine. Amino acid sequence alignment of ADAL1 with human adenosine deaminase and human and *Arabidopsis thaliana* adenylate deaminase and comparison to crystallographic complexes of these and other adenosine deaminase family members identified residues that are potentially part of the active

```
hADAL1      -------------------------------------------MIEAEE 6
aAMPD_2A3L  DATAFFTDLHHVLKVIAAGNIRTLCHRRLVLLEQKFNLHLMLNADKEFLA 373
hAMPD2      DLQEFVADVNVLMALIINGPIKSFCYRRLQYLSSKFQMHVLLNEMKELAA 400
                                                          *

hADAL1      QQPCKTDFYSELPKVELHAHLNGSISSHTMKKLIAQKPDLKIHDQMTVID 56
aAMPD_2A3L  QKSAPHRDFYNVRKVDTHVHHSACMNQKHLLRFIKSKLRKEP-DEVVIFR 422
hAMPD2      QKKVPHRDFYNIRKVDTHIHASSCMNQKHLLRFIKRAMKRHL-EEIVHVE 449
            *:      : :: **: * * ...:..: : ::*      .  :::. .

hADAL1      KGKKRTLEECFQMFQTIHQLTSSPEDILMVTKDVIKEFADDGVKYLELRS 106
aAMPD_2A3L  DGTYLTLREVFESLDLTGYDLNVDLLDVHADKSTFHRFDLFENLKYNPCGQ 472
hAMPD2      QGREQTLREVFESMNLTAYDLSVDTLDVHADRNTFHRFDKFNAKYNPIGE 499
            .*   **.* *: ::      .     : . :..::.* . . **    .

hADAL1      TPRRE---NATGMTKKTYVESILEGIKQSKQENLDIDVRYLIAVDRRG-- 151
aAMPD_2A3L  SRLREIFLKQDNLIQGRFLGEITKQVFSDLEASKYQMAEYRISIYGRKMS 522
hAMPD2      SVLREIFIKTDNRVSGKYFAHIIKEVMSDLEESKYQNAELRLSIYGRSRD 549
            :  **   :  .  .  :.  * : : .. : .    ..  :::  *

hADAL1      -----GPLVAKETVKLAEEFFLSTEGTVLGLDLSG---DPTVGQAKDFLE 193
aAMPD_2A3L  EWDQLASWIVNNDLYSENVVWLIQLPRLYNIYKDMGIVTSFQNILDNIFI 572
hAMPD2      EWDKLARWAVMHRVHSPNVRWLVQVPRLFDVYRTKGQLANFQEMLENIFL 599
                 .   . . :   :  :*     : .:              .:::

hADAL1      PLLEAKKAG---LKLALHLSEIPNQKKE---------------------- 218
aAMPD_2A3L  PLFEATVDPDSHPQLHVFLKQVVGFDLVDDESKPE-RRPTKHMPTPAQWT 621
hAMPD2      PLFEATVHPASHPELHLFLEHVDGFDSVDDESKPENHVFNLESPLPEAWV 649
            **:**.       :* :.*..: . .

hADAL1      ----------------TQILLDLLPDRIGHGTFLN---SGEGGSLD--LV 247
aAMPD_2A3L  NAFNPAFSYYVYYCYANLYVLNKLRESKGMTTITLRPHSGEAGDIDHLAA 671
hAMPD2      EEDNPPYAYYLYYTFANMAMLNHLRRQRGFHTFVLRPHCGEAGPIHHLVS 699
                        .  :*: *    *  *:     .**.* :.

hADAL1      DFVRQHRIPLELCLTSN------------------VKSQTVPSYDQHHF 278
aAMPD_2A3L  TFLTCHSIAHGINLRKSPVLQYLYYLAQIGLAMSPLSNNSLFLDYHRNPF 721
hAMPD2      AFMLAENISHGLLLRKAPVLQYLYYLAQIGIAMSPLSNNSLFLSYHRNPL 749
             *:  . *.  : * .                    :.. . .*.:: :

hADAL1      GFWYSIAHPSVICTDD---KGVFATHLSQEYQLAAETFNLTQSQVWDLSY 325
aAMPD_2A3L  PVFFLRGLNVSLSTDPLQIHLTKEPLVEEYSIAASVWKLSACDLCEIAR 771
hAMPD2      PEYLSRGLMVSLSTDDPLQFHFTKEPLMEEYSIATQVWKLSSCDMCELAR 799
              :   .    :.***     .    * :**.:*:..::*: .:: :::

hADAL1      ESINYIFASDSTRSELR-KKWNHLKPRVLHI------------------- 355
aAMPD_2A3L  NSVYQSGFSHALKSHWIGKDYYKRGPDGNDIHKTNVPHIRVEFRDTIWKE 821
hAMPD2      NSVLMSGFSHKVKSHWLGPNYTKEGPEGNDIRRTNVPDIRVGYRYETLCQ 849
            :*:     *.  :*.    .: :  *   .*

hADAL1      ------------------------------
aAMPD_2A3L  EMQQVYLGKAVISDEVVP------------ 839
hAMPD2      ELALITQAVQSEMLETIPEEAGITMSPGPQ 879
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Figure 6. ClustalW (2.1) sequence alignment for *h*ADAL1, *a*AMPD, and *h*AMPD2: sequence identity of ∼6% and ∼14% similarity for all three sequences. Cyan highlight indicates contact with catalytic Zn. Green are residues within 5 Å of the adenine (necessarily includes the metal binding residues). Yellow indicates residues within 5 Å of the ribose 5′-phosphate. Residues in bold/purple fall within multiple distance criteria. Residues in hADAL1 are highlighted to represent contacts based on homology to hADA (Figure 5). The asterisk (∗) signifies identity. The colon (:) signifies high similarity, and the period (.) signifies low similarity.

site. Furthermore, the amino acid sequence and metal content analyses of ADAL1 indicated that the enzyme, like ADA and AMPD, contains one catalytic zinc ion per monomer enzyme. It is our assumption that the physiological role of ADAL1 is to salvage O^6-substituted purine or 2-aminopurine nucleoside monophosphates and it may be involved in nucleoside/nucleotide metabolism.

EXPERIMENTAL SECTION

Compounds. Compounds **6**−**10**, **13**−**15**, **22**, **23**, **25**−**28**, **30**, **31**, **32**, and **34**−**37** were purchased from Sigma (St. Louis, MO), Berry & Associates (Dexter, MI), Jena Bioscience (Jena, Germany), and Biolog Life Science Institute (Bremen, Germany). Novel nucleoside 5′-monophosphates, diphosphates, and triphosphates **16**−**19**, **24**, **29**, **33**, and **38** were synthesized by NuBlocks LLC (Vista, CA). Structures of the synthesized compounds were confirmed by mass spectral analysis, and their purities were determined to be greater than 98% by high performance liquid chromatography (HPLC). 2′-Fluoro-2′-*C*-methyl-2′-deoxynucleosides (**11** and **12**), starting nucleosides for their 5′-phosphates (**16**−**21**, **31**, and **33**) and 3′,5′-cyclophosphate prodrugs (**3** and **20**), were prepared according to methods developed by our laboratory.[6] Phosphoramidate analogues (**4** and **21**) of **11** were also prepared at Pharmasset, Inc.[8]

Cloning, Expression, and Purification. For cloning of N^6-methyl-AMP aminohydrolase (subsequently identified as adenosine deaminase-like protein isoform 1), cDNA from Huh7 cells was used. The full length cDNA fragment encoding N^6-methyl-AMP aminohydrolase was amplified using platinum PCR supermix HF from Invitrogen (Carlsbad, CA) with the following primers described by Schinkmanová et al.: forward primer 5′-ATG ATA GAG GCA GAA GAG CAA CAG

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Table 4. Metal Analysis[a]

element	ADAL1 (ppm)	buffer (ppm)	concn in ADAL1 (μM)	concn in buffer (μM)	mol/mol of ADAL1[b]
Ca	0.1551	0.0695	3.87	1.73	0.43
Cd	BDL	BDL	BDL	BDL	BDL
Co	BDL	BDL	BDL	BDL	BDL
Cr	BDL	BDL	BDL	BDL	BDL
Cu	0.215	0.3958	3.38	6.23	0[c]
Fe	BDL	BDL	BDL	BDL	BDL
Mg	BDL	BDL	BDL	BDL	BDL
Mn	BDL	BDL	BDL	BDL	BDL
Mo	0.0121	0.0266	0.126	0.277	0
Ni	BDL	BDL	BDL	BDL	BDL
Pb	0.0248	0.0598	0.120	0.289	0
Zn	0.4553	0.0923	6.96	1.41	1.12

[a] BDL: below detection limit. [b] Calculated as [(concn in ADAL1) − (concn in buffer)]/[ADAL1 concentration (4.9 μM)]. [c] Reported as "0" when metal concentration was higher in buffer than in ADAL1.

CCT TGC-3′ (T_m = 63.4 °C) and reverse primer 5′-TTA AAT ATG TAA CAC TCT GGG CTT CAG GTG-3′ (T_m = 58.4 °C).[1] The amplified PCR product was inserted into pCR4-TOPO TA (Invitrogen, Carlsbad, CA), and the DNA sequence was confirmed (Agencourt, Beverly, MA). This construct was used as a template for ADAL1 cloning into the gene expression vector pET28a+ (Novagen, La Jolla, CA) which introduced a 6× histidine tag at the amino terminus, with the forward primer containing a Nde1 restriction site (5′-GCA GCC ATA TGA TAG AGG CAG AAG AGC AAC-3′) and the reverse primer containing a HindIII site (5′-CCG CAA GCT TAA ATA TGT AAC ACT CTG GGC TTC-3′). After the DNA sequence was confirmed (Agencourt, Beverly, MA), the pET28a+ construct was transformed into BL21-gold (DE3) competent cells (Stratagene, La Jolla, CA). The cells were grown in LB medium at 37 °C until the OD reached 0.6, at which time protein expression was induced by adding IPTG to a final concentration of 0.5 mM. The cells were incubated at 25 °C for an additional 22 h. The cells were harvested by centrifugation at 4000 rpm for 12 min. The cell pellet was resuspended in buffer A (50 mM Tris/HCl, pH 8.0, 300 mM NaCl, 0.1% NP-40, 5 mM β-mercaptoethanol, and 10% glycerol) containing Roche complete protease inhibitor cooktail (Roche Applied Science, Indianapolis, IN), and the cells were lysed by sonication. The extract was clarified by centrifugation at 15 000 rpm for 30 min. The protein was purified using a 1 mL Ni affinity column (GE, Piscataway, NJ) by a linear gradient from 20 to 400 mM imidazole in buffer A. The fractions containing N^6-methyl-AMP aminohydrolase were identified by both SDS−PAGE analysis and activity using **14** as substrate. Those fractions containing the enzyme were pooled, and the protein was concentrated using an Amicon Ultra-4 centrifugal filter unit with an Ultracel-10 membrane (Millipore,Billerica, MA). To reduce the salt concentration prior to the next purification step, the protein was diluted in a buffer containing 20 mM Tris/HCl, pH8.0, 10% glycerol, and 1 mM DTT. The protein solution was then applied to a MonoQ 5/50GL column (1 mL) (GE, Piscataway, NJ), and the protein was eluted using a linear gradient of 0−1 M NaCl in 20 mM Tris/HCl, pH 8.0, 10% glycerol, and 1 mM DTT. The purified protein showed >95% purity based on SDS−PAGE. The concentration of the protein was determined based on the extinction coefficient (30 160 M^{-1} cm^{-1}) and the molecular weight of 40 263 Da of the protein using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE). The purified protein was stored at −80 °C.

Protein Identification. The protein band with a molecular mass of approximately 40 kDa was excised from the SDS gel and submitted to Alphalyse, Inc. (Palo Alto, CA) for protein identification. Protein identification was performed by matrix-assisted laser desorption ionization time of fight (MALDI-TOF) peptide mass fingerprinting after trypsin digestion. A database search was performed using the Mascot software (Matrix Science, Inc., Boston, MA).

Subunit Composition Determination. Molecular weight of the native protein was determined by size exclusion column chromatography using a Superdex 200 10/300GL column (GE Healthcare, Piscataway, NJ) and a cocktail of molecular weight standards containing aprotinin (~6.5 kDa), cytochrome *c* (~12.4 kDa), carbonic anhydrase (~29 kDa), albumin (~66.2 kDa), and dextran blue (~2000 kDa) obtained from Sigma (St. Louis, MO). The elution volume for dextran blue was considered as the void volume (V_0). The elution volumes (V_e) for the other standards were determined, and a standard curve was created by plotting the V_e/V_0 values against the logarithm of the molecular weight. ADAL1 molecular weight was determined based on the standard curve.

Enzyme Activity Assay. The ADAL1 assay was conducted in a 1 mL reaction mixture containing 50 mM potassium phosphate buffer, pH 6.7, 2 mM DTT, 100 μg/mL BSA, and varying concentrations of the test compound. The reactions were started by addition of an appropriate amount of the enzyme, and the reaction was followed by measuring the UV absorbance change at 37 °C in a Lambda 35 UV−visible spectrophotometer (Perkin-Elmer, Waltham, MA). The wavelength and difference extinction coefficient ($\Delta\varepsilon$) used in the assay varied depending upon the substrate, and the values are shown in Results and Discussion. For determination of steady-state kinetic parameters, initial rates were plotted against the substrate concentration and nonlinear regression was performed using GraphFit (Erithacus Software, Horley, Surrey, U.K.). The results were reported as "value ± standard error of regression".

Removal of a methyl group from methylated DNA was performed as described in Sigma Technical Bulletin for MGMT assay kit (St. Louis, MO). An oligonucleotide containing O^6-methylguanine residue (5′-GAA CT[O^6-MeG] CAG CTC CGT GCT GGC CC-3′) and its complementary strand (5′-GGG CCA GCA CGG AGC TGC AGT TC-3′) were synthesized by Gene Link (Hawthorne, NY). The methylated strand was radiolabeled at the 5′-end using [γ-^{32}P]ATP and polynucleotide kinase (New England Biolabs) as described previously, and it was annealed to the complementary strand.[31] This double stranded DNA substrate was designed so that a Pst1 restriction site is created by removal of the methyl group. The labeled double stranded DNA substrate (200 nM) was incubated with either 4 μM ADAL1 or 4 μM O^6-methylguanine-DNA methyltransferase (MGMT) (ProSpec, Rehovot, Israel) in a buffer containing 50 mM Hepes, pH 7.5, 50 mM KCl, 0.025% Triton X-100, and 10 μg/mL BSA at 37 °C for 30 min. The experiment was performed in the presence or in the absence of 100 μM O^6-benzylguanine, a known inhibitor of MGMT. The reaction was quenched by incubating at 65 °C for 5 min followed by incubation on ice. Pst1 (20 units) was added to the quenched reaction mixture and incubated at 37 °C for additional 60 min. The Pst1 reaction was stopped by adding 5 μL of a dye solution containing 95% formamide and 0.1% each of bromophenol blue and xylene cyanol. The product was separated on a 20% polyacrylamide sequencing gel and visualized on a phosphorimager.

Metal Analysis. Purified ADAL1 (1 mL) was dialyzed against 1 L of 50 mM Tris buffer, pH 7.5, for 2 h followed by an additional 2 h of dialysis in 1 L of the same fresh buffer. After dialysis, the protein was diluted to 2 mL using the dialysis buffer and the protein concentration was measured using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE). Both the dialysis buffer and ADAL1 sample were sent to University of Georgia Chemical Analysis Laboratory (Athens, GA) for an inductively coupled plasma optical emission spectrometry (ICP-OES) analysis.

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ABBREVIATIONS USED

AMP, adenosine 5′-monophosphate; dAMP, 2′-deoxyadenosine 5′-monophosphate; ABC-MP, abacavir 5′-monophosphate; CBV-MP, carbovir 5′-monophosphate; PMEA, 9-[2-(phosphonomethoxy)-ethyl]adenine; PMEDAP, 9-[2-(phosphonomethoxy)ethyl]-2,6-diaminopurine; ADAL1, adenosine deaminase-like protein isoform 1; PAGE, polyacrylamide gel electrophoresis; GMP, guanosine 5′-monophosphate; HPLC, high pressure liquid chromatography; IMP, inosine 5′-monophosphate; MGMT, O^6-methylguanine DNA methyltransferease; ADA, adenosine deaminase; AMPD, adenosine 5′-monophosphate deaminase; FAC1, adenosine 5′-monophosphate deaminase domain of embryonic factor 1; MNU, *N*-methyl-*N*-nitrosourea

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