Adenosine-5'-O-phosphorylated and adenosine-5'-O-phosphorothioylated polyols as strong inhibitors of (symmetrical) and (asymmetrical) dinucleoside tetraphosphatases

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Dinucleoside 5',5'''-P¹,Pⁿ-polyphosphates, and particularly the diadenosine compounds, have been implicated in extracellular purinergic signalling and in various intracellular processes, including DNA metabolism, tumour suppression and stress responses. If permitted to accumulate, they may also be toxic. One approach to understanding their function is through the various specific degradative enzymes that regulate their levels. Eight adenosine-5'-O-phosphorylated polyols (derivatives of glycerol, erythritol and pentaerythritol) and 11 adenosine-5'-O-phosphorothioylated polyols (derivatives of glycerol, erythritol, pentaerythritol, butanediol and pentanediol) have been tested as inhibitors of specific diadenosine tetraphosphate (Ap₄A) hydrolases. Of these two groups of novel nucleotides, the adenosine-5'-O-phosphorothioylated polyols were generally stronger inhibitors than their adenosine-5'-O-phosphorylated counterparts. 1,4-Di(adenosine-5'-O-phosphorothio) erythritol appeared to be the strongest inhibitor of (asymmetrical) Ap₄A hydrolases (EC 3.6.1.17) from both lupin and human, with K_i values of

0.15 μM and 1.5 μM respectively. Of eight adenosine-5'-O-phosphorylated polyols, 1,4-di(adenosine-5'-O-phospho) erythritol was the only compound that inhibited the lupin enzyme. Two derivatives of pentaerythritol, di(adenosine-5'-O-phosphorothio)-di(phosphorothio) pentaerythritol and tri(adenosine-5'-O-phosphorothio)-phosphorothio-pentaerythritol, proved to be the strongest inhibitors of the prokaryotic (symmetrical) Ap₄A hydrolase (EC 3.6.1.41) so far reported. The estimated K_i values were 0.04 μM and 0.08 μM respectively. All of these inhibitors were competitive with respect to Ap₄A. These new selectively acting Ap₄A analogues should prove to be valuable tools for further studies of Ap₄A function and of the enzymes involved in its metabolism.

Key words: adenylylated polyol, diadenosine tetraphosphate analogue (Ap₄A analogue), diadenosine tetraphosphatase (Ap₄Aase), dinucleoside tetraphosphate hydrolase, (phosphorothio)adenylylated polyol.

INTRODUCTION

Dinucleoside 5',5'''-P¹,Pⁿ-polyphosphates (Np_nN's, where N and N' are 5'-O-nucleosides and n is the number of phosphate residues in the polyphosphate chain that links the two 5'-esterified nucleosides) occur in both prokaryotes and eukaryotes [1]. Most predominant are the dinucleoside tri- and tetra-phosphates, but diadenosine penta- [2], hexa- [3] as well as di- [4] and heptaphosphates [5] have been found in some mammalian cells. The biological roles of Np_nN's are still fairly obscure, with data suggesting a variety of possible functions in addition to toxic properties [6]. Several ligases and transferases [7–10] have the ability to synthesize Np_nN's, while various non-specific and specific lytic enzymes control their degradation [11]. Studies on the metabolism of Np_nN's have included investigations of different types of substrate analogues prepared by either chemical or enzymic synthesis (for reviews, see [11,12]). Numerous analogues have been tested as alternative substrates and/or inhibitors. Among the former are fluorogenic compounds, such as N¹,N⁶-etheno-Ap₃A and diadenosine tetraphosphate (Ap₄A) [13], and analogues of Ap₃A (diadenosine triphosphate) such as Appp-S-[7-diethylamino-4-methyl-3-(4-succinimidylphenyl)] coumarin, Appp-S-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacine-3-yl) methylaminoacetyl and Gppp-S-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacine-3-yl) methylaminoacetyl [14]. The

application of these analogues has increased the sensitivity of enzyme assays, and they can be used as alternatives to radio-labelled substrates. Some analogues, such as (S_p,S_p)-P¹,P⁴-bis-(5'-O-adenosyl)-1[thio-¹⁸O₂],4[thio-¹⁸O₂] tetraphosphate [15], P¹-chiral phosphorothioate analogues of Ap₄A [16,17] and γ-(m-nitrobenzyl) ATP as an analogue of Ap₃A [18], have allowed the reaction mechanisms of (asymmetrical) Ap₄A hydrolase, Ap₄A phosphorylase and Ap₃A hydrolase respectively to be established. Others, such as the non-hydrolysable Ap₃A analogue P¹-thio-P²,P³-methylene diadenosine 5',5'''-P¹,P³-triphosphate (ApCH₂pp_sA) [19] and the Ap₄A analogue P¹,P⁴-dithio-P²,P³-monochloromethylene diadenosine 5',5'''-P¹,P⁴-tetraphosphate (Ap_pCHClpp_sA) [20], have facilitated studies of the three-dimensional structures of the human Fhit protein (Ap₃A hydrolase) and of Ap₄A hydrolase from narrow-leaved lupin respectively.

More recently, new non-isopolar analogues of Ap₄A and Ap₃A have been synthesized, consisting of di-, tri- or tetra-(adenosine-5'-O-phosphorylated) or -(adenosine-5'-O-phosphorothioylated) derivatives of glycerol, erythritol or pentaerythritol [21]. Some of these have been described as powerful inhibitors of the Fhit protein [22], a dinucleoside triphosphate hydrolase belonging to the HIT protein family with tumour suppressor and anti-apoptotic functions [23]. Further to these studies of Fhit inhibition, it was of interest to check whether these same compounds

Abbreviations used: Ap₄A, diadenosine tetraphosphate; Np_nN', dinucleoside 5',5'''-P¹,Pⁿ-polyphosphate (where N and N' are 5'-O-nucleosides and n is the number of phosphate residues in the polyphosphate chain that links the two 5'-esterified nucleosides).

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Figure 1 Structures of adenosine-5'-O-phosphorylated and adenosine-5'-O-phosphorothioylated polyols

could be useful for the study of other N_pN' -metabolizing enzymes. Qualitative screening performed with various specific Ap_3A - and/or Ap_4A -degrading enzymes (both hydrolases and phosphorylases) revealed that the strongest inhibitory effects were exerted by certain adenosine-5'-O-phosphorothioylated polyols on the Ap_4A hydrolases. The present paper summarizes the results of a detailed quantitative study performed with both (*asymmetrical*) and (*symmetrical*) Ap_4A hydrolases derived from various organisms. The former enzymes are members of the Nudix hydrolase family and generate ATP + AMP as products, while the latter, exclusively prokaryotic, enzymes are structurally related to serine/threonine protein phosphatases and cleave Ap_4A to form 2 mol of ADP [11].

EXPERIMENTAL

Enzymes

Homogeneous recombinant (*asymmetrical*) Ap_4A hydrolases (EC 3.6.1.17) from human [24], narrow-leafed lupin (*Lupinus angustifolius*) [25] and *Caenorhabditis elegans* [26] were obtained as described. The lupin enzyme was kindly donated by Dr D. Maksel and Dr K. Gayler (University of Melbourne, Melbourne, Australia). (*Symmetrical*) Ap_4A hydrolase (EC 3.6.1.41) was partially purified from *Escherichia coli* K12 as described previously [27]. The equivalent enzyme from *Salmonella typhimurium* LT2 was expressed in *E. coli* as a His-tagged protein from the vector pET-15b and purified on NiCAMTM-HC resin (Sigma) (T. Ismail, C. A. Hart and A. G. McLennan, unpublished work).

Chemicals

Unlabelled mono- and di-nucleotides were from Sigma (St. Louis, MO, U.S.A.), and [³H] Ap_4A (740 TBq/mol) was purchased from Moravsek Biochemicals (Brea, CA, U.S.A.). The following adenosine-5'-O-phosphorylated (**a**) and adenosine-5'-O-phosphorothioylated (**b**) polyalcohols were synthesized as described previously [20], and their structures are presented in Figure 1: 1,3-di(adenosine-5'-O-phospho) glycerol (**1a**), 1,3-di(adenosine-5'-O-phosphorothio) glycerol (**1b**), 1,2,3-tri(adenosine-5'-O-phospho) glycerol (**2a**), tri(adenosine-5'-O-phosphorothio) glycerol (**2b**), 1,3-di(adenosine-5'-O-phospho)-2-phospho-glycerol (**3a**),

1,3-di(adenosine-5'-O-phosphorothio)-2-phosphorothio-glycerol (**3b**), 1,4-di(adenosine-5'-O-phospho) erythritol (**4a**), 1,4-di(adenosine-5'-O-phosphorothio) erythritol (**4b**), 1,2,3,4-tetra(adenosine-5'-O-phospho) erythritol (**5a**), 1,2,3,4-tetra(adenosine-5'-O-phosphorothio) erythritol (**5b**), di(adenosine-5'-O-phospho)-di(phospho) pentaerythritol (**6a**), di(adenosine-5'-O-phosphorothio)-di(phosphorothio) pentaerythritol (**6b**), tri(adenosine-5'-O-phospho)-phospho pentaerythritol (**7a**), tri(adenosine-5'-O-phosphorothio)-phosphorothio pentaerythritol (**7b**), tetra(adenosine-5'-O-phospho) pentaerythritol (**8a**), tetra(adenosine-5'-O-phosphorothio) pentaerythritol (**8b**), as well as 1-(adenosine-5'-O-phosphorothio) erythritol (**9b**), 1,4-di(adenosine-5'-O-phosphorothio) butanediol (**10b**) and 1,5-di(adenosine-5'-O-phosphorothio) pentanediol (**11b**). All phosphorothioylated derivatives were prepared and used as a mixture of *P*-diastereomers. *meso*-Erythritol has been used as the starting material for the synthesis of compounds (**4**) and (**5**).

Enzyme assays

Asymmetrically acting Ap_4A hydrolases were assayed in an incubation mixture (0.05 ml total volume) containing 50 mM Hepes/KOH (pH 7.6), 0.02 mM dithiothreitol, 5 mM $MgCl_2$, 0.05 mM [³H] Ap_4A (300 000 c.p.m.), various concentration of nucleotide analogue and rate-limiting amounts of enzyme. For assaying the (*symmetrical*) Ap_4A hydrolases, 5 mM $MgCl_2$ was replaced with 0.1 mM $CoCl_2$. Incubations were carried out at 30 °C. To estimate reaction rates, 5 µl aliquots were spotted on to TLC plates (aluminium plates precoated with silica gel containing fluorescent indicator; Merck cat. no. 5554), usually after 6, 12, 18 and 24 min of incubation. Unlabelled standards of the products [ATP for (*asymmetrical*) Ap_4A hydrolases and ADP for (*symmetrical*) Ap_4A hydrolases] were applied at the origins, and the plates were developed for 90 min in dioxan/ammonia/water (6:1:4, by vol.). Spots of the products, visualized under short-wave UV light, were cut out, immersed in scintillation cocktail and the radioactivity measured. An example of the separation of the Ap_4A substrate from the products (ATP plus AMP, or ADP) in this system is shown in Figure 2. K_i values were calculated according to the method of Dixon and Webb [28] from the slopes of plots of v/v_i against $[I]$ (where v and v_i are velocities in the absence and presence of inhibitor respectively, and $[I]$ is the inhibitor concentration), where $slope = K_m/K_i(1/K_m + S)$.

Figure 2 Time course of Ap_4A hydrolysis catalysed by (*asymmetrical*) Ap_4A hydrolase (A, B) and (*symmetrical*) Ap_4A hydrolase (C, D) in the absence (A, C) and presence (B, D) of inhibitors

The incubation mixture (50 μl final volume) contained 50 mM Hepes/KOH (pH 7.6), 0.02 mM dithiothreitol, 1 mM Ap_4A , 5 mM MgCl_2 (for the lupin Ap_4A hydrolase) or 0.1 mM CoCl_2 (for the *E. coli* Ap_4A hydrolase), the appropriate enzyme (in amounts that completed the reaction in less than 60 min; *asym.*, *asymmetrical*; *sym.*, *symmetrical*) and the inhibitor where indicated. In (B) this was 100 μM 1,4-di(adenosine-5'-*O*-phosphorothio) erythritol (**4b**), and in (D) it was 40 μM tri(adenosine-5'-*O*-phosphorothio)-phosphorothio pentaerythritol (**7b**). Incubations were carried out at 30 $^\circ\text{C}$. At the times indicated, 5 μl aliquots were spotted on a TLC plate. The chromatogram was developed in dioxan/ammonia/water (6:1:4, by vol.) and photographed under short-wave UV light.

RESULTS AND DISCUSSION

Lack of recognition of analogues as substrates by the (*asymmetrical*) and (*symmetrical*) Ap_4A hydrolases

Each compound was checked as a potential substrate for two (*asymmetrical*) Ap_4A hydrolases, from lupin and human, and for the (*symmetrical*) Ap_4A hydrolase from *E. coli*. Typical incubation mixtures (see the Experimental section) contained 1 mM analogue and an excess of enzyme, i.e. an amount that, under the same conditions, completely hydrolysed 1 mM Ap_4A in less than 15 min. Incubation was carried out for up to 16 h and the progress of potential hydrolysis was analysed by TLC. None of the eight adenosine-5'-*O*-phosphorylated or the eight adenosine-5'-*O*-phosphorothioylated polyols was measurably degraded by any of the specific Ap_4A hydrolases. This result was not unexpected, since these hydrolases cleave pyrophosphoryl bonds, while compounds (**1**)–(**8**) comprise phosphodiester in which P–O–P bonds have been replaced by a P–O–C structural motif.

Analogues as inhibitors of four specific Ap_4A hydrolases

Each of the analogues (**1a**)–(**8a**) and (**1b**)–(**8b**) was assayed as a potential inhibitor of the lupin and human (*asymmetrical*) Ap_4A hydrolases and of the (*symmetrical*) Ap_4A hydrolase from *E. coli*. Pilot experiments allowed an appropriate concentration range of the compound under study to be chosen that permitted measurements of reaction rates at given concentrations of labelled [^3H] Ap_4A (usually 0.05 mM) and enzyme. As qualitative examples, the effects exerted by compound (**4b**) on Ap_4A hydrolysis catalysed by the (*asymmetrical*) Ap_4A hydrolase from lupin, and by compound (**7b**) on the reaction catalysed by the (*symmetrical*) Ap_4A hydrolase from *E. coli*, are shown in Figure 2. The adenosine-5'-*O*-phosphorylated polyols (**1a**–**8a**) were much poorer inhibitors of the (*asymmetrical*) Ap_4A hydrolases than their adenosine-5'-*O*-phosphorothioylated counterparts (**1b**–**8b**) (compare the K_i values in Table 1). The lupin enzyme was inhibited by only one adenosine-5'-*O*-phosphorylated polyol, i.e. 1,4-di(adenosine-5'-*O*-phospho) erythritol (**4a**). The K_i value for this compound was over 8-fold higher than the K_m for Ap_4A . The human (*asymmetrical*) Ap_4A hydrolase was not inhibited by the two fully esterified polyols, 1,2,3-tri(adenosine-5'-*O*-

Table 1 Inhibition of (*asymmetrical*) Ap_4A hydrolases (EC 3.6.1.17) from lupin (*Lupinus angustifolius*) and human by adenosine-5'-*O*-phosphorylated (suffix a) and adenosine-5'-*O*-phosphorothioylated (suffix b) polyols

G, E, PE, B-ol and P-ol represent the polyolic skeletons of glycerol, erythritol, pentaerythritol, butanediol and pentanediol respectively. pA and p_sA represent adenosine 5'-phosphate and adenosine 5'-phosphorothioate residues respectively. '–' indicates that no inhibition was observed ($K_i > 1$ mM). K_m values for Ap_4A estimated for the lupin and human enzymes were 1 μM [30] and 2 μM (the present study) respectively. K_i values are means of three independent estimations; standard errors did not exceed 20 %.

Compound (formula; no.)	K_i (μM)	
	Lupin	Human
Adenosine-5'- <i>O</i> -phosphorylated polyols		
1,3 (pA) ₂ G; 1a	–	109
1,2,3 (pA) ₃ G; 2a	–	–
1,3 (pA) ₂ ,2p G; 3a	–	66
1,4 (pA) ₂ E; 4a	8.5	142
1,2,3,4 (pA) ₄ E; 5a	–	31
(pA) ₂ ,p ₂ PE; 6a	–	16
(pA) ₃ ,p PE; 7a	–	28
(pA) ₄ PE; 8a	–	–
Adenosine-5'- <i>O</i> -phosphorothioylated polyols		
1,3 (p _s A) ₂ G; 1b	–	–
1,2,3 (p _s A) ₃ G; 2b	17	37
1,3 (p _s A) ₂ ,2p G; 3b	25	32
1,4 (p _s A) ₂ E; 4b	0.15	1.5
1,2,3,4 (p _s A) ₄ E; 5b	13	18
(p _s A) ₂ ,p ₂ PE; 6b	–	3.7
(p _s A) ₃ ,p PE; 7b	–	10
(p _s A) ₄ PE; 8b	28	12
1 (p _s A) E; 9b	20	–
1,4 (p _s A) ₂ B-ol; 10b	–	–
1,5 (p _s A) ₂ P-ol; 11b	–	–

phospho) glycerol (**2a**) and tetra(adenosine-5'-*O*-phospho) pentaerythritol (**8a**). Six other adenosine-5'-*O*-phosphorylated polyols acted as moderate (compound **6a**; $K_i = 16$ μM) or rather poor (compound **5a**; $K_i = 142$ μM) inhibitors of this enzyme. The estimated K_m for Ap_4A was 2 μM .

Of the eight adenosine-5'-*O*-phosphorothioylated polyols (**1b**–**8b**), six compounds inhibited the lupin (*asymmetrical*) Ap_4A hydrolase and seven inhibited the human hydrolase.

1,4-Di(adenosine-5'-*O*-phosphorothio) erythritol (**4b**) was the strongest inhibitor of these two enzymes; corresponding K_i values were lower than the K_m values for Ap_4A . Compounds (**2b**), (**3b**) and (**5b**) were moderate inhibitors, while derivatives of pentaerythritol (compounds **6b** and **7b**) inhibited only the human (*asymmetrical*) Ap_4A hydrolase, and 1,3-di(adenosine-5'-*O*-phosphorothio) glycerol (**1b**) inhibited neither the lupin nor the human enzyme.

The recently established three-dimensional structures of the lupin Ap_4A hydrolase [20] and the hydrolase from *C. elegans* [29], which is closely related to the human enzyme [26], show a number of structural differences within the substrate-binding sites of the plant and animal enzymes. Therefore it is not surprising that recognition of other potential ligands (inhibitors) by these hydrolases differs too. No or poor inhibition of the plant enzyme by many analogues that affect the corresponding human enzyme can be explained by the more restricted space in the substrate-binding cleft in the former enzyme. Because compound (**4b**) is the strongest inhibitor of both of these (*asymmetrical*) Ap_4A hydrolases, it may most resemble the natural substrate, Ap_4A .

The additional compound, mono(adenosine-5'-*O*-phosphorothio) erythritol (**9b**) was a 130-fold weaker inhibitor of the lupin enzyme than was compound (**4b**), and did not inhibit the human enzyme. Overall, the adenosine-5'-*O*-phosphorylated (**1a–8a**) and adenosine-5'-*O*-phosphorothioylated (**1b–8b**) polyols appeared to be more effective inhibitors of the (*symmetrical*) Ap_4A hydrolase from *E. coli* than of the (*asymmetrical*) Ap_4A hydrolases. Two derivatives of pentaerythritol, the adenosine-5'-*O*-phosphorylated polyols (**6a**) and (**7a**) and the adenosine-5'-*O*-phosphorothioylated polyols (**6b**) and (**7b**), were particularly strong inhibitors. The K_i values estimated for (**6b**) and (**7b**) were 0.04 μM and 0.08 μM respectively, i.e. 625- and 312-fold lower than the K_m value for Ap_4A of 25 μM [27]. Until now, the lowest K_i values known were for phosphonate analogues of Ap_4A . For example, AppCH_2ppA inhibited the (*asymmetrical*) Ap_4A hydrolases from lupin [31] and *Artemia* [32] with K_i values of 0.25 μM and 0.4 μM respectively, and the (*symmetrical*) Ap_4A hydrolase from *E. coli* with a K_i of 7 μM [32]. Comparable effects were also observed with halomethylene analogues. For example, $\text{AppCCl}_2\text{ppA}$ and AppCF_2ppA inhibited the lupin hydrolase with K_i values in the range 0.2–0.3 μM [33]. However, the methylene and halomethylene analogues of Ap_4A were susceptible to slow *asymmetrical* hydrolysis by the lupin and *Artemia* Ap_4A hydrolases. Therefore these analogues are not ideal inhibitors of these enzymes. Bis(phosphorothioylated) analogues of Ap_4A , including $\text{Ap}_5\text{ppp}_5\text{A}$ and diastereoisomers of $\text{Ap}_5\text{pCH}_2\text{pp}_5\text{A}$, inhibited the *Artemia* (*asymmetrical*) Ap_4A hydrolase with K_i values ranging from 2.5 μM to 1.0 μM [34]. Finally, another family of $\text{Ap}_3\text{A}/\text{Ap}_4\text{A}$ analogues, tripodal adenylated derivatives of methanetrissphosphonic acid [35], although quite good inhibitors of Ap_3A hydrolases (K_i values in the micromolar range), were barely inhibitory towards the lupin or human (*asymmetrical*) Ap_4A hydrolases [36].

Nature of inhibition by the strongest inhibitors

To answer the question of whether these new analogues act as competitive or non-competitive inhibitors with respect to Ap_4A , a more detailed analysis of the two strongest inhibitors of each enzyme was performed. Relevant measurements were performed at three substrate concentrations, usually 50, 100 and 200 μM [^3H] Ap_4A . Inhibition by all the compounds [(**2b**) and (**4b**) assayed with the lupin enzyme, (**4b**) and (**6b**) assayed with the human

Table 2 Inhibition of (*symmetrical*) Ap_4A hydrolases (EC 3.6.1.41) from *Escherichia coli* and *Salmonella typhimurium* by adenosine-5'-*O*-phosphorylated (suffix a) and adenosine-5'-*O*-phosphorothioylated (suffix b) polyols

G, E, PE, B-ol and P-ol represent the polyolic skeletons of glycerol, erythritol, pentaerythritol, butanediol and pentanediol respectively. pA and p_sA represent adenosine 5'-phosphate and adenosine 5'-phosphorothioate residues respectively. K_i values are means of three independent measurements; standard errors did not exceed 15%. K_m values for Ap_4A estimated for the *E. coli* and *S. typhimurium* enzymes were 25 μM [27] and 5 μM (the present study) respectively. n.d., not determined.

Compound (formula; no.)	K_i (μM)	
	<i>E. coli</i>	<i>S. typhimurium</i>
Adenosine-5'- <i>O</i> -phosphorylated polyols		
1,3 (pA) ₂ G; 1a	215	
1,2,3 (pA) ₃ G; 2a	4080	
1,3 (pA) ₂ ,2p G; 3a	5	
1,4 (pA) ₂ E; 4a	n.d.	
1,2,3,4 (pA) ₄ E; 5a	6	
(pA) ₂ ,p ₂ PE; 6a	1.1	
(pA) ₃ ,p PE; 7a	3	
(pA) ₄ PE; 8a	28	
Adenosine-5'- <i>O</i> -phosphorothioylated polyols		
1,3 (p _s A) ₂ G; 1b	157	n.d.
1,2,3 (p _s A) ₃ G; 2b	7	n.d.
1,3 (p _s A) ₂ ,2p G; 3b	1.4	3.9
1,4 (p _s A) ₂ E; 4b	78	n.d.
1,2,3,4 (p _s A) ₄ E; 5b	1.3	2.9
(p _s A) ₂ ,p ₂ PE; 6b	0.04	0.2
(p _s A) ₃ ,p PE; 7b	0.08	1.7
(p _s A) ₄ PE; 8b	1.4	1.8
1,4 (p _s A) ₂ B-ol; 10b	89	n.d.
1,5 (p _s A) ₂ P-ol; 11b	109	n.d.

enzyme, and (**6b**) and (**5b**) assayed with the *E. coli* enzyme] proved to be competitive with respect to Ap_4A . This result is not surprising if one compares the structures of the analogues and of the natural substrate Ap_4A . Nucleoside moieties in all of these compounds are appropriately separated, and the linker (the polyalcoholic scaffold or tetrapolyphosphate chain) is flexible enough to allow stacking of the adenine rings as in Ap_4A , as judged by CD (results not shown).

Comparison of the susceptibility of other specific Ap_4A hydrolases to the most effective inhibitors

The strong inhibitors of the lupin and human (*asymmetrical*) Ap_4A hydrolases (compounds **4b**, **6b** and **4a**) were assayed with the orthologous enzyme from *C. elegans* [26]. The resulting K_i values for this enzyme were 12, 3 and 8.5 μM respectively. Five of the most potent inhibitors of the (*symmetrical*) Ap_4A hydrolase from *E. coli* were also studied with the corresponding enzyme from *S. typhimurium*. The K_i values determined for these compounds are included in Table 2. The general conclusion is that the compounds that inhibit one type of enzyme are inhibitory regardless of the origin of that enzyme. However, the strength of inhibition does differ. In the case of the *E. coli* and *S. typhimurium* hydrolases, differences may reflect the presence of a histidine tag in the latter protein. Interestingly, the powerful inhibitors of the (*symmetrical*) Ap_4A hydrolase from *E. coli* (compounds **6b** and **7b**) did not inhibit the lupin (*asymmetrical*) Ap_4A hydrolase, and compound (**4b**), the strongest inhibitor of the (*asymmetrical*) Ap_4A hydrolases, was only a moderate inhibitor of the *E. coli* hydrolase.

Which part of the molecule contributes most to the inhibitory effect exerted on the (*asymmetrical*) Ap₄A hydrolases?

In order to determine which structural element of the 1,4-di(adenosine-5'-*O*-phosphorothio) erythritol molecule (**4b**) contributes most to inhibition, three additional compounds were synthesized and tested: (**9b**), which has only one adenosine-5'-*O*-phosphorothio- group attached to erythritol, and (**10b**) and (**11b**), in which two such groups are attached to butanediol and pentanediol respectively. Compared with (**4b**), which exerts the strongest inhibitory effect, the monosubstituted erythritol (**9b**) was over two orders of magnitudes less effective. Compounds (**10b**) and (**11b**) did not inhibit the (*asymmetrical*) Ap₄A hydrolases. This result indicates that the hydroxy groups attached to β -carbon atoms of the erythritol scaffold connecting both adenosine-5'-*O*-phosphorothio- functions are indispensable for recognition (compound **4b**).

Conclusion

The data presented here show the potential usefulness of certain adenosine-5'-*O*-phosphorylated and adenosine-5'-*O*-phosphorothioylated polyols as tools for the further study of Np_nN' metabolism and function. In contrast with the (*asymmetrical*) Ap₄A hydrolases [20,29], the three-dimensional structure of a (*symmetrical*) Ap₄A hydrolase has not yet been established. Two adenosine-5'-*O*-phosphorothioylated pentaerythritols (**6b** and **7b**) that very strongly inhibit the *E. coli* enzyme may help to solve the structure of this hydrolase and to identify amino acid residues involved in substrate–ligand binding.

Substitution of one of the oxygens surrounding phosphorus by sulphur markedly enhanced the inhibitory effects of the compounds. With regard to the stereoselectivity of recognition of any particular adenosine-5'-*O*-phosphorothioylated polyol by a given enzyme, separation of diastereoisomers will be required to determine which isomers of (**6b**) or (**7b**) are the most effective inhibitors of the (*symmetrical*) Ap₄A hydrolases, and which isomer of (**4b**) is most effective with the (*asymmetrical*) Ap₄A hydrolases. Finally, it has been demonstrated that phosphodiesterases from snake venom [37] and rat liver [38] are able to adenylate alcohols, including glycerol. Thus it is plausible that such reactions take place *in vivo*. Therefore investigation of the interaction of adenylated polyols should be extended to other nucleotide-metabolizing enzymes.

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