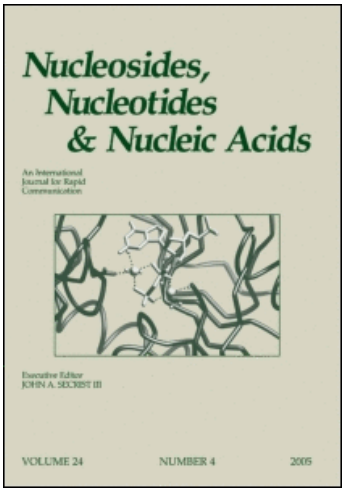

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Methylene and Halomethylene Analogues of Diadenosine 5',5''-P¹, P³-Triphosphate (ApppA) as Substrates or Inhibitors of ApppA-Degrading Enzymes

Andrzej Guranowski[a]; Elżibieta Starzyńska[a]; Lidia Gzik[a]; Steve P. Langston[b]; Paul Brown[c]; G. Michael Blackburn[b]
[a] Katedra Biochemii i Biotechnologii, Akademia Rolnicza, Poznań, Poland [b] Departments of [2]Chemistry and [3]Molecular Biology and Biotechnology, Krebs Institute, University of Sheffield, Sheffield, U.K. [c] Moleatlar Biology and Biotechnoiogv, krebs Institute, University of Sheffield, Sheffield, U. K

To cite this Article Guranowski, Andrzej , Starzyńska, Elżibieta , Gzik, Lidia , Langston, Steve P. , Brown, Paul and Blackburn, G. Michael(1995) 'Methylene and Halomethylene Analogues of Diadenosine 5',5''-P¹, P³-Triphosphate (ApppA) as Substrates or Inhibitors of ApppA-Degrading Enzymes', Nucleosides, Nucleotides and Nucleic Acids, 14: 3, 731 — 734
To link to this Article: DOI: 10.1080/15257779508012460
URL: http://dx.doi.org/10.1080/15257779508012460

NUCLEOSIDES & NUCLEOTIDES, 14(3-5), 731-734 (1995)

METHYLENE AND HALOMETHYLENE ANALOGUES OF DIADENOSINE 5',5'''-P^1,P^3-TRIPHOSPHATE (ApppA) AS SUBSTRATES OR INHIBITORS OF ApppA-DEGRADING ENZYMES

Andrzej Guranowski,[1]* Elżbieta Starzyńska,[1] Lidia Gzik,[1] Steve P. Langston,[2] Paul Brown,[3] and G. Michael Blackburn[2]

[1]Katedra Biochemii i Biotechnologii, Akademia Rolnicza, ul. Wołyńska 35, 60-637 Poznań, Poland, and Departments of [2]Chemistry and [3]Molecular Biology and Biotechnology, Krebs Institute, University of Sheffield, Sheffield S3 7HF, U.K.

Abstract: Relative velocities and inhibition constants have been determined for three ApppA-degrading enzymes using six chemically synthesized analogues of ApppA.

Dinucleoside polyphosphates (Np_nN's) in which common nucleosides, N and N', are linked at their C5' *via* a polyphosphate chain (n=3-6) occur in both prokaryotic and eukaryotic cells. (For recent reviews see 1-3). The most common Np_nN's seem to be dinucleoside tri- and tetraphosphates (Np_3N's and Np_4N's). Adenylylated compounds, Ap_3Ns and Ap_4Ns, can be synthesized chiefly but not exclusively by some aminoacyl-tRNA synthetases which transfer an adenylate moiety from a mixed anhydride intermediate back onto NDP or NTP respectively[4,5]. Catabolism of Ap_3Ns and Ap_4Ns commences usually with cleavage at their polyphosphate chains catalyzed either by hydrolases or phosphorylases, depending on the organism[6,7]. Several Np_4N-degrading enzymes have been purified to homogeneity and characterized. The characterization also comprised studies on the interaction of these enzymes with analogues of natural substrates; mainly analogues of Ap_4A[8]. Our knowledge about specific enzymes that degrade Np_3N's is much poorer. So far, none of them has been purified to homogeneity. Analogues of Ap_3A, synthesized in one of our laboratories[9], allowed us to perform studies presented in this communication on the recognition of those compounds by the following three enzymes which degrade the naturally occurring Ap_3A: (a) Ap_3A hydrolase (EC 3.6.1.29) from yellow lupin seeds which cleaves its substrates (Np_3N's>Np_4N's) at the phosphate proximate to the bound nucleoside (adenosine) moiety to give always NMP (AMP) and corresponding NDP or NTP[3]; (b) *(symmetrical)* Ap_4A hydrolase (EC 3.6.1.41) from *Escherichia coli* which hydrolyzes its substrates (Np_4N's>Np_5N's>Np_3N's) at the second (β) phosphate to give always NDP (ADP) and the corresponding NDP, NTP or NMP, respectively[3]; and (c) Ap_3A/Ap_4A phosphorylase (EC 2.7.7.53) from *Acanthamoeba castellanii* which,

in contrast to other Np_nN'-phosphorylases from yeast *Saccharomyces cerevisiae*[10], *Euglena gracilis*[11] and *Scenedesmus obliquus*[7], exhibits slightly higher specificity for Ap_5A than for Ap_4A and splits the anhydride bond between the α and β phosphates by phosphorolysis.

MATERIALS AND METHODS

Chemicals AMP, ADP, ATP, Ap_3A and Ap_4A were from Sigma. Ap_3A analogues and respective analogues of ADP were synthesized as described[9]. Labelled Ap_3A was synthesized enzymatically[12] from [2,5,8-^{3}H]-ATP (45 Ci/mmol, Amersham) with yeast lysyl-tRNA synthetase kindly supplied by Dr. Pierre Kerjan (CNRS, Gif-sur-Yvette).

Enzymes Yellow lupin Ap_3A hydrolase[12] and *(symmetrical)* Ap_4A hydrolase from *E. coli*[13] were isolated as described previously. Specific phosphorylase which splits both Ap_3A and Ap_4A has been purified from *Acanthamoeba castellanii*. The cells grown for 48 h at 28°C in a medium containing yeast extract, peptone and glucose were collected by centrifugation, suspended in 50 mM potassium phosphate (pH 6.8) and homogenized in a glass/teflon homogenizer.
The phosphorylase was precipitated from the crude extract with ammonium sulfate (30-50% saturation). Resolubilized proteins were chromatographed on Sephadex G-75 superfine. Active fractions which eluted at $V_e/V_o=1.8$ were applied onto DEAE-Sephacel column equlibrated with the same buffer. The enzyme was not adsorbed under these conditions. It was concentrated and kept frozen for further work.

Enzyme Assays Inhibitory properties of the Ap_3A analogues were estimated in the incubation mixtures, 50 μl final volume, containing 50 mM Hepes/KOH (pH 8.0), 0.1 mM dithiothreitol, 0.1 mM [^{3}H]-Ap_3A (about 100,000 cpm), 5 mM $MgCl_2$ [or 1 mM $MnCl_2$ in case of the *(symmetrical)* Ap_4A hydrolase], varied concentrations of an Ap_3A analogue, (5 mM K_2HPO_4 in case of the Ap_3A/Ap_4A phosphorylase) and rate-limiting amounts of the enzyme tested. The incubation was carried out at 30°C. Rates were determined by taking 5-μl aliquots at set times and spotting them onto thin layer aluminium plates precoated with silica gel (Merck). An ADP standard was added and the chromatogram developed in dioxane: ammonia:water (6;1:4, v/v). ADP spots were cut out and the radioactivity counted. Assaying of the Ap_3A analogues as the enzyme substrates was performed by ion-exchange chromatography on a MonoQ column as described earlier[3]. Velocities of the degradation reactions were calculated based on the linearity between concentration of the appearing product, AMP or ADP, and peak area.

RESULTS AND DISCUSSION

As shown in Table 1, the Ap_3A analogues in which one of the P-O-P bridging oxygens was substituted with $-CH_2-$, -CHF- or $-CF_2-$ were substrates of all three Ap_3A-degrading enzymes studied. Slight cleavage was also observed for two Ap_3A analogues with bulkier substituents, -CHCl- or $-CCl_2-$, when tested with the *(symmetrical)* Ap_4A hydrolase from *E. coli*. In the latter system, the relative degradation rates for three more susceptible Ap_3A analogues exhibited much lower values than those obtained in the lupin or *Acanthamoeba* systems. It can be explained by the fact that Ap_3A itself is much poorer substrate for the *E. coli* hydrolase than is Ap_4A. In the presence of Mn^{2+}, Ap_3A is hydrolyzed 7-fold slower than Ap_4A and in the presence of Co^{2+}, 50-fold slower. The K_m value of Ap_3A estimated in the presence of Mn^{2+} is 50 times higher than that for Ap_4A[14]. So, any modification in the structure of such a poor substrate as Ap_3A additionally decreases the effectiveness of catalysis. In line with this conclusion we observed that the *(symmetrical)* Ap_4A hydrolase preferentially recognizes unmodified ADP-moiety of potential substrates and cleaves

TABLE 1. Kinetics for ApppA and some ApppA analogues with ApppA-degrading enzymes.

Dinucleotide	Ap_3A hydrolase *Lupinus luteus*		Ap_4A hydrolase *Escherichia coli*		Ap_3A/Ap_4A phosphorylase *Acanthamoeba castellanii*	
	V_{rel}	K_m or K_i (μM)	V_{rel}	K_m or K_i (μM)	V_{rel}	K_m or K_i (μM)
ApppA	100	1.2	100	600	100	9
$ApCH_2ppA$	20	117	4	25	4	12
ApCHFppA	37	90	1.5	88	10	3.5
$ApCF_2ppA$	23	212	1.8	71	13	4
ApCHClppA	0	260	0.5	211	0	8
$ApCCl_2ppA$	0	126	0.5	266	0	14
$ApCH_2pCH_2pA$	0	>2000	0	32	0	n.d.

the polyphosphate chain at the second phosphate from the bound adenosine moiety[3].

The compounds tested here have modifications precisely at that crucial phosphorus. The Ap_3A hydrolase, which cleaves its substrates at the phosphate proximate to the bound adenosine moiety[3], tolerates the $-CH_2-$, -CHF- or $-CF_2-$ substitutions and hydrolyzes the respective analogues only 3-5-fold slower than the natural substrate, Ap_3A. From the analysis of the V_{rel} values, one can conclude that the *Acanthamoeba* enzyme exhibits moderate tolerance with respect to the structure of its substrates. The susceptible Ap_3A analogues were degraded 8-25-fold slower than Ap_3A itself.

It should be mentioned here that, in contrast to the *Euglena* counterpart[11], the *Acanthamoeba* enzyme degrades Ap_3A slightly faster than Ap_4A (about 1.5-fold). Thus, the *Acanthamoeba* phosphorylase is clearly less strict than the yeast Ap_4A phosphorylase which degrades neither Ap_3A nor the Ap_4A analogues bearing a methylene group between the β and β' phosphorus atoms[15].

As concerns the K_i *versus* K_m values, it is clear that the largest difference was demonstrated for the Ap_3A hydrolase system. All the Ap_3A analogues were poorly recognized by this hydrolase and the analogue with two methylene groups was not recognized at all. The K_i values for the *Acanthamoeba* phosphorylase are within a factor of three of the K_m for Ap_3A. ApCHClppA and $ApCCl_2ppA$ seem to be the best candidates for being true inhibitors of the phosphorylase.

Acknowledgements

This study was supported in part by the State Committee for Scientific Research (KBN) within Project 4 0164 91 01, by grant GR/E 3521 from the SERC and by assistance from the British

Council/KBN Joint Research Collaboration Programme. We thank Mrs Margaret Bew for technical assistance.

REFERENCES

1. Garrison, P.N. and Barnes, L.D. in *Ap$_4$A and Other Dinucleoside Polyphosphates* (McLennan, A.G., Ed.) **1992**, pp 29-51, CRC Press, Boca Raton, FL.
2. Plateau, P. and Blanquet, S. *Adv. Microbiol. Physiol.* **1994**, *36*, 81-109.
3. Guranowski, A., Brown, P., Ashton, P.A. and Blackburn, G.M. *Biochemistry* **1994**, *33*, 235-240.
4. Randerath, K., Janeway, C.M., Stephenson, M.L. and Zamecnick, P.C. *Biochem.Biophys. Res. Commun.* **1966**, *24*, 98-105.
5. Plateau, P. and Blanquet, S. in *Ap$_4$A and Other Dinucleoside Polyphosphates* (McLennan, A.G., Ed.) **1992**, pp 63-79, CRC Press, Boca Raton, FL.
6. Guranowski, A. and Sillero, A. *ibid* **1992**, pp 81-133.
7. McLennan, A.G., Mayers, E., Hankin, S., Thorne, N.M.H., Prescott, M. and Powls, R. *Biochem. J.* **1994**, *300*, 183-189.
8. Blackburn, G.M., Guo, M-J. and McLennan, A.G. in *Ap$_4$A and Other Dinucleoside Polyphosphates* (McLennan, A.G., Ed.) **1992**, pp 305-342, CRC Press, Boca Raton, FL.
9. Blackburn, G.M., Guo, M-J., Langston, S.P. and Taylor, G.E. *Tetrahedron Lett.* **1990**, *31*, 5637-5640.
10. Guranowski, A. and Blanquet, S. *J. Biol. Chem.* **1985**, *260*, 3542-3547.
11. Guranowski, A., Starzyńska, E. and Wasternack, C. *Int. J. Biochem.* **1988**, *20*, 449-455.
12. Jakubowski, H. and Guranowski, A. *J. Biol. Chem.* **1983**, *258*, 9982-9989.
13. Guranowski, A., Jakubowski, H. and Holler, E. *J. Biol. Chem.* **1983**, *258*, 14784-14789.
14. Plateau, P., Fromant, M., Brevet, A., Gesquière, A. and Blanquet, S. *Biochemistry* **1985**, *24*, 914-922.
15. Guranowski, A., Biryukov, A., Tarussova, N.B., Khomutov, R.M. and Jakubowski, H. *Biochemistry* **1987**, *26*, 3425-3429.