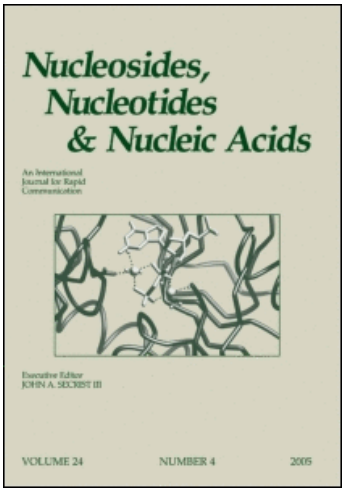

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The Inhibition of Adenosine Kinase by α,ω-Di (Adenosin - N⁶- YL) Alkanes[1]

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THE INHIBITION OF ADENOSINE KINASE BY α,ω-DI (ADENOSIN - N^6- YL) ALKANES[1]

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Abstract: Members of a series of α,ω-di(adenosin- N^6-yl)alkanes, comprising two adenosine residues linked with alkyl bridges from 1 to 14 methylene units in length , were found to be inhibitors of rat liver and BHK cell adenosine kinase. The inhibition was competitive with respect to adenosine and non-competitive with respect to ATP. The corresponding α,ω-di(cytidin-N^4-yl) alkanes were not inhibitors and N^6-alkyladenosines inhibited only weakly.

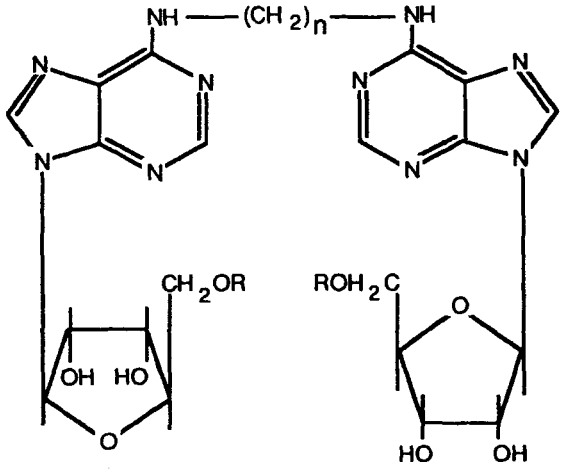
INTRODUCTION

Bis(5'-nucleosidyl) oligophosphates in which two nucleoside moieties are joined 5' to 5' via an oligophosphate bridge are powerful inhibitors of nucleoside and nucleotide kinases. Thus, bis(5'-adenosyl) pentaphosphate, Ap_5A, and its homologues Ap_4A and Ap_6A inhibit adenylate kinase[2-4], Ap_4A and Ap_5A inhibit adenosine kinase[5], Ap_ndT (n=3-6) inhibit thymidine and thymidylate kinases[5,6] and Ap_4U inhibits uridine kinase[7]. Bis (5'-adenosyl) oligophosphates are also inhibitors of terminal deoxynucleotidyl transferase[8,9]. In all cases, these compounds have been suggested to act as bisubstrate analogues with the two nucleoside residues adopting a spatial relationship similar to that of the proposed transition states for phosphate or nucleotide transfer.

An alternative method for satisfying this spatial requirement is to link the nucleosides via the bases[10-11]. We have recently described the synthesis of a series of α,ω-di(adenosin-N^6-yl) alkanes in which two adenosines are linked via their N^6 positions by an alkyl bridge containing from 1 to 14 methylene units (1a - 1n)[12]. These compounds have the advantage of being able readily to cross cell membranes and so to act as potential enzyme inhibitors *in vivo*. Here we report the inhibition of mammalian adenosine kinases by these compounds.

MATERIALS AND METHODS

Synthesis of α,ω-di(adenosin-$\underline{N}^6$-yl)alkanes, $\underline{N}^6$-alkyladenosines and α,ω-di(cytidin-$\underline{N}^4$-yl) alkanes was as previously described[12]. Briefly, 6-chloro-9-β-*D*-(2,3,5-tri-*O*-acetyl) ribofuranosylpurine was treated with a 0.6 mole equivalent of the appropriate diaminoalkane in dry pyridine. The resulting protected intermediates were isolated by flash chromatography on silica gel and characterised by ^{1}H n.m.r. and FAB (positive ion) mass spectrometry. Deacetylation was accomplished by treatment overnight with saturated methanolic ammonia. Using this procedure the α,ω-di(adenosin-N^6-yl) alkanes precipitated from the reaction mixture and were generally in excess of 95% pure as determined by h.p.l.c. The homologues were characterised chiefly by FAB (positive ion) mass spectrometry. $(M+H)^+$ ions were observed for all compounds in the series together with (M-264+H) which results from the loss of both ribose sugars. Structural confirmation was also obtained from ^{1}H and ^{13}C n.m.r. and u.v. spectroscopy.



1a-1n: R = H, n = 1 to 14

2a-2n: R = PO_3^{2-}, n = 1 to 14

Phosphorylation of 1a - 1n to yield the bis-monophosphates 2a - 2n was accomplished using $POCl_3$ under standard Yoshikawa conditions[12,13] and the isopropylidene groups removed by hydrolysis at pH 1.5. After purification on reverse phase silica, products appeared as single peaks by h.p.l.c. (4 x 250mmLiChrosorb C-18 column run with a gradient of 0-100%B where A = 50mM tri-ethylammonium acetate and B = 100% acetonitrile). ^{31}P n.m.r. showed a singlet at 4ppm in each case. Further structural confirmation was obtained from FAB (negative ion) mass spectrometry which showed the expected $(M-H)^-$ ion signals.

Purification of adenosine kinases: All operations were performed at 4°C. Fresh rat livers (25g) were homogenised in 50ml 10mM Tris-HCl pH7.5, 0.1mM EDTA with a Potter-Elvehejm homogeniser and the homogenate centrifuged for 1h at 135,000g. The supernatant was

adsorbed on to a column of Q-Sepharose (2.5 x 10cm, Pharmacia) and the unbound protein washed through at 50ml/h with 20mM Tris-HCl pH7.5, 1mM dithiothreitol, 10% glycerol. A 250ml gradient of 0 to 0.5M NaCl in washing buffer was applied and the adenosine kinase activity, which eluted at 0.1M NaCl, concentrated by ammonium sulphate precipitation (0-100% cut), redissolved in 2ml 50mM potassium phosphate buffer pH7.4, 1mM dithiothreitol, 10% glycerol and applied to a column (100cm x 1.6cm) of Ultrogel AcA44. Fractions containing adenosine kinase activity were pooled (30ml), dialysed against 100vol Tris-maleate pH 5.4, 1mM dithiothreitol, 20% glycerol and applied to a column of 5'-AMP-Sepharose (5cm x 1cm, Sigma). Enzyme activity was eluted with 20mM Tris-HCl pH 9.0, 1mM dithiothreitol, 20% glycerol and stored routinely at -70°C at which temperature it was stable for at least six months.

Adenosine kinase was prepared from 5.4g of log phase baby hamster kidney fibroblasts (BHK 21/C13 cells) by a similar method omitting the AcA44 step.

Adenosine kinase assay: Unless otherwise stated, standard assays contained 20mM Hepes-NaOH pH7.0, 25mM NaCl, 0.1mM Mg acetate, 0.5mM ATP, 20μM [2,5',8-^{3}H]adenosine (0.1Ci/mmol, Amersham International), 0.5mg/ml BSA and 0.2mU rat liver or 0.4mU BHK adenosine kinase in a final volume of 100μl. After 20min incubation at 37°C, mixtures were cooled on ice and 60μl portions transferred to 2.5cm DE81 discs (Whatman). Discs were washed successively in 1mM $HCOONH_4$ (2 x 5ml), H_2O (5ml), 95% ethanol (5ml), dried, eluted with 1ml 0.2M NaCl, 0.2M HCl and counted in 6ml Optiphase MP scintillant (LKB). One unit of adenosine kinase activity is defined as that amount which will convert 1nmol of adenosine to AMP per min under standard conditions.

RESULTS AND DISCUSSION

The inhibition of rat liver adenosine kinase by members of the α,ω-di(adenosin-N^6-yl)alkane series is shown in Figure 1a. Compounds containing 1, 4 or 6 carbon atoms in the alkyl bridge show roughly the same degree of inhibition, about 60-70%. However as n is increased from 8 to 14, the degree of inhibition is greatly enhanced, reaching 95% with compounds 1l and 1n under the conditions described. That the inhibition requires the presence of two adenosine residues is shown by the lack of inhibition by simple N^6-alkyladenosines (Figure1b), in agreement with previous findings[14]. The specificity for adenosine is further indicated by the lack of effect of the corresponding α,ω-di(cytidin-N^4-yl)alkanes (Figure 1c). Both these results also show that the inhibition is not simply due to the presence of a long, hydrophobic alkyl chain. Very similar results were obtained with the BHK cell adenosine kinase (not shown).

The type of inhibition was determined by analysing rate versus substrate and inhibitor concentrations with a non-linear curve fitting algorithm and was found to be competitive with respect to adenosine and, in the case of 1,12-di(adenosin-N^6-yl)dodecane (1l), non-competitive

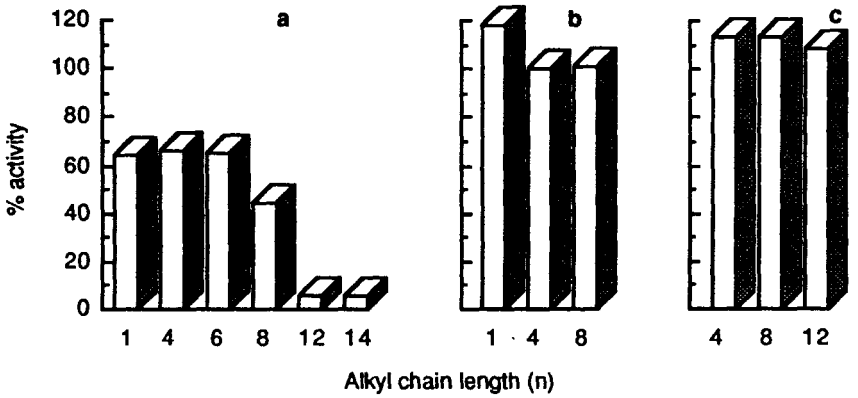


FIGURE 1: Effect on rat liver adenosine kinase of (a) α,ω-di(adenosin-N^6-yl)alkanes, (b) N^6-alkyl-adenosines and (c) α,ω-di(cytidin-N^4-yl)alkanes. Alkylated nucleosides were dissolved in DMSO and added to assays to give a final concentration of 5% DMSO. Adenosine kinase (0.2mU) was assayed as described in Materials and Methods with 0.5μM adenosine and 1μM inhibitors.

with respect to ATP. Kinetic constants are shown in Table 1. 1,12-di(adenosin-N^6-yl)dodecane was the most potent of the inhibitors examined in detail,with a K_i of 75nM with adenosine as the variable substrate. Comparison of this data to that of Miller *et al.* [14] who surveyed 119 nucleosides and nucleoside analogues with respect to their substrate and inhibitor specificity shows that 1,12-di(adenosin-N^6-yl)dodecane is the most potent N^6-substituted adenosine and the second most powerful of all adenosine analogue inhibitors of adenosine kinase yet described.

In order to determine whether product inhibition might contribute to the observed results, the ability of the α,ω-di(adenosin-N^6-yl)alkanes to act as substrates for adenosine kinase was also investigated. These compounds proved to be very poor substrates for the kinase. Only when incubated with a high concentration of adenosine kinase and [γ-^{32}P] ATP for 18h, were products corresponding to the respective authentic bis-monophosphates (2a -2n) detected by reversed phase hplc (Figure 2); no products with a single phosphorylated ribose were seen.

Therefore under standard assay conditions it can be estimated that, for example, the phosphorylated product of 1,12-di(adenosin-N^6-yl)dodecane would attain a concentration of only 10-50pM which is unlikely to be responsible for the observed inhibition. Furthemore, the general trend of efficiency as substrates is opposite to that of efficiency as inhibitors. It is likely therefore that the inhibition is due to the nucleosides themselves.

Inhibition of adenosine kinase by 1,12-di(adenosin-N^6-yl)dodecane was found to be competitive with respect to adenosine but non-competitive with respect to ATP. Therefore it may

TABLE 1: Kinetic constants for the inhibition of adenosine kinase by α,ω-di(adenosin-$\underline{N}^6$-yl)alkanes. C = competitive; NC = non-competitive; nd = not determined. When ATP was the variable substrate, assays contained 100μM adenosine and 30mU adenosine kinase.

Substrate/ Inhibitor	K_m (μM)	Variable substrate	
		adenosine	ATP
		K_i (C) (μM)	K_i (NC) (μM)
adenosine	0.95	-	-
ATP	340	-	-
$\underline{N}^6$-octyladenosine	-	2.0	nd
1d (n=4)	-	0.43	nd
1h (n=8)	-	0.20	nd
1 l (n=12)	-	0.075	1.0

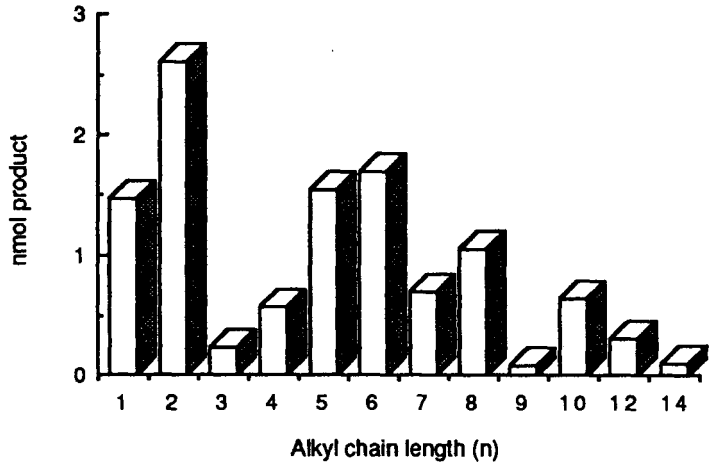


FIGURE 2: Phosphorylation of α,ω-di(adenosin-$\underline{N}^6$-yl)alkanes by adenosine kinase. 100μM of each substrate was incubated with 1 U rat liver adenosine kinase for 18h with 0.5mM [γ-^{32}P] ATP (Amersham International) at 20mCi/mmol. 10μl of each reaction mixture were mixed with 2μl of 0.25M tri-ethylammonium acetate and 1μl authentic bis-monophosphates and chromatographed on a Lichrosorb C-18 column as described in Materials and Methods. 1ml fractions were collected and counted in Optiphase scintillant (LKB).

not be acting as a bisubstrate analogue occupying both the adenosine and ATP catalytic binding centres: competition with both substrates might then be expected as has been shown for AMP and ATP with adenylate kinase and TMP and ATP with thymidylate kinase[5]. Interestingly, although Ap_4A has been regarded as a bisubstrate analogue inhibitor of adenosine kinase, the inhibition is competitive only with respect to ATP, being non-competitive with respect to adenosine[5]. Furthermore, Ap_4A is as effective an inhibitor of uridine kinase as is Ap_4U even though the nucleoside substrate specificity of uridine kinase does not extend to adenosine[7]. Hence when one substrate is a nucleoside, alternative interactions may be possible.

Several nucleoside kinases are known to have regulatory sites in addition to the catalytic sites where nucleosides and nucleotides may bind. Adenosine kinase has at least two distinct adenosine binding sites, one catalytic and the other required for substrate inhibition[15]. The increased effectiveness of the α,ω-di(adenosin-N^6-yl)alkanes with longer alkyl chains and the requirement for two adenosine moieties may therefore indicate binding of these compounds to both adenosine binding sites.

In vivo, the α,ω-di(adenosin-N^6-yl)alkanes have effects on cultured cells which are probably unrelated to the inhibition of adenosine kinase and which depend on the length (n) of the alkyl chain. 1,2- di(adenosin-N^6-yl)ethane and 1,4- di(adenosin-N^6-yl)butane have previously been reported to have no effect on cell growth or DNA synthesis[10] while 1,1-di(adenosin-N^6-yl)methane has been reported to be cytostatic towards cultured BHK cells[16]. In our hands, compounds with $n<4$ have no discernible effect on cells; however, those with $n>4$ severely impair nucleoside uptake and utilisation while compounds with $n>8$ are additionally cytotoxic (Prescott and McLennan, unpublished observations). The usefulness of these compounds as pharmacologically active adenosine analogues is currently under investigation.

Acknowledgements

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