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6-Ureido derivatives of 9-[β-D-ribofuranosyl]purine as inhibitors of adenosine kinase*

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N^6-(Δ^2-ISOPENTENYL)adenine and N-(purin-6-ylcarbamoyl)threonine (PCT) are known to occupy strategic positions adjacent to the 3′-end of the presumed anticodon triplet of certain tRNA species.[1] The former compound and its 9-(β-D-ribofuranosyl) derivative display strong cytokinin activity in plant systems[2] but only the ribosyl derivative exhibits significant growth inhibitory activity against a variety of mammalian cell types, including Sarcoma 180 (S-180) cells in culture.[3] Earlier studies in this laboratory demonstrated that N^6-(Δ^2-isopentenyl)adenosine and several other cytotoxic adenosine analogues were substrates of adenosine kinase (ATP: adenosine 5′-phosphotransferase, EC 2.7.1.20) of S-180 cells, while most of the N^6-substituted analogues inactive in cell culture were not phosphorylated by this enzyme.[4] However, many of the latter were potent inhibitors of adenosine kinase. The amount of this enzyme was drastically reduced in S-180/KR (an S-180 subline which is resistant to the cytotoxic analogues of adenosine[5]), suggesting that the phosphorylation of adenosine analogues by adenosine kinase was a prerequisite for their cytotoxic activity.

Recently Dyson *et al.*[6] studied the cytokinin activity of PCT and several related compounds. Although PCT itself was inactive, several related analogues, lacking polar groups in the 6-substituent, displayed good cytokinin activity. The authors suggested that the inactivity of PCT, as a cytokinin, may be due to poor uptake of this compound by plant cells. 9-(β-D-ribofuranosyl) derivatives of these cytokinins exhibited only a mild growth inhibitory activity against cultured human cells, normal (NC 37) and leukemic (6410), both of hematopoietic origin.[7] In the light of our previous findings the study reported here was undertaken to determine whether these analogues of purine ribonucleoside could serve as substrates and/or inhibitors of adenosine kinase partially purified from S-810 cells. An abstract on this subject has been published.[8]

The sources of the compounds used in the present study, the preparation of partially purified adenosine kinase from S-180 cells (which was completely free of adenosine deaminase but contained significant amounts of adenylate kinase), the enzyme assay and the conditions for testing of inhibitors have been described in an earlier report.[4] The specific activity of the enzyme preparation was 63 nm of adenosine phosphorylated/min/mg of protein at 4·4 μM adenosine. 6-Ureidopurine ribonucleosides [6-ureido-9-(β-D-ribonfuranosyl)purines], their 5′-monophosphate derivatives and 8-^{14}C-labeled analogues were synthesized according to published procedures.[9,10]

6-*Ureidopurine ribonucleosides as substrates of adenosine kinase.* The ribonucleosides listed in Tables 1 and 2 were tested as substrates of adenosine kinase by incubating for 30 min at 35° the unlabeled compounds at 0·1 mM with 2·5 mM ATP-γ-^{32}P (1 μCi/μmole), 0·25 mM $MgCl_2$ and 15 μg of protein in a total volume of 0·4 ml in 0·05 M potassium phosphate buffer, pH 7·0. An aliquot of the reaction mixture was subjected to descending chromatography on a Whatman 3 MM paper strip for 16 hr using isobutyric acid–NH_4OH–water (66:1:33) as the solvent. The strip was cut in 1-cm sections which were counted for ^{32}P. The relative order of migration in this solvent system is ATP < ADP < AMP < adenosine. Monophosphates may be formed from N^6-adenosines but these appear not to be phosphorylated further.[4] Thus, if these nucleoside analogues were phosphorylated, two distinct radioactive peaks would be expected to appear on the chromatogram. The first major peak (R_f about 0·2) would represent unreacted ATP together with inorganic phosphate (due to ATP degradation). A second peak (R_f about 0·4) would represent a monophosphate. Using this method, no phosphorylation of any of the analogues listed in Tables 1 and 2 was observed. However, the monophosphates of those analogues in Table 1, which contained a negative charge in the N^6-substituent (NH-R, an amino acid residue) could have migrated more slowly than monophosphates with a neutral N^6-substituent and thereby escaped detection. This, indeed, was the case for an authentic, synthetically prepared sample of PCT ribonucleoside-5′-phosphate, which migrated faster than ATP, but slower than ADP. For this reason, two of the compounds, PCT ribonucleoside and N-[9-β-D-ribofuranosyl-(purin-6-ylcarbamoyl)]glycine, were labeled with ^{14}C at 8-position. These ^{14}C-labeled compounds at 0·1 and 0·4 mM were then tested as substrates of adenosine kinase by incubating as above but with unlabeled ATP for 60 min. By using this method no phosphorylation of either compound was detected. These results together with those above provide evidence for suggesting that none of the compounds in Table 1 were substrates of adenosine kinase. It is interesting to note that N^6-furfuryl and -allyladenosines were found to be substrates of adenosine kinase[4] while the corresponding ureido derivatives were not (Table 2). It therefore appears that the introduction

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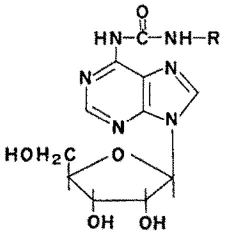

TABLE 1. 6-UREIDOPURINE RIBONUCLEOSIDES AS INHIBITORS OF ADENOSINE KINASE

—NH—R	Per cent inhibition* of 0·82 μM ado phosphorylation at 75 μM
Threonine	NS
Glycinamide	NS
Threoninamide	NS
Alanine	22
Valine	25
Methionine	25
Tyrosine	28
Isoleucinamide	32
Phenylalanine	36
Phenylalaninamide	39
Histamine	40
Leucine	42
Glycine	46
β-Alanine	51
Isoleucine	52

* The average of duplicates; maximum variation ± 15 per cent of the mean; in controls the adenine nucleotides contained 970 ± 70 counts/min (average of triplicates); NS, not significant.

of —CO—NH— bridge between these groups and N^6 of adenosine completely abolished the substrate activity. The slight growth inhibitory activity of these analogues[7] in cell culture does not appear to depend on phosphorylation by adenosine kinase.

6-*Ureidopurine ribonucleosides as inhibitors of adenosine kinase.* Many of the N^6-substituted adenosines, even though not substrates of adenosine kinase, were shown to be good inhibitors of the enzyme.[4] Most of the 6-ureidopurine ribonucleosides in this study were also found to be inhibitors of adenosine kinase (Tables 1 and 2). The inhibitory activity of these compounds at 75 μM was tested in the presence of 0·82 μM adenosine-8-^{14}C (47 μCi/μmole), 2·5 mM ATP, 0·25 mM $MgCl_2$, 50 mM potassium phosphate buffer, pH 7·0, and the enzyme (1·6 μg of protein) in a total volume of 0·4 ml by incubating for 2 min at 35°. In these conditions the conversion of adenosine to nucleotides was about 40 per cent in the controls. The limit of detection of inhibitory potency was 10 per cent. Since this degree of inhibition could not be determined with accuracy, it was considered not significant. With respect to their inhibitory potency the ribonucleosides could be conveniently grouped into three categories. Those described in Table 1, which possessed the general structure N-[9-(β-D-ribofuranosyl)-purin-6-ylcarbamoyl]amino acid or the corresponding amide, were poor or no inhibitors of adenosine kinase causing less than 55 per cent inhibition of adenosine phosphorylation at almost one hundred times the substrate concentration. The compounds in which R was an alkyl group having no net charge were inhibitors of an intermediate potency (upper part of Table 2) and the compounds in which R was $(CH_2)_n$-phenyl (n varied from 0 to 4) were still more potent inhibitors. At equimolar concentration the latter inhibited the phosphorylation of adenosine by 14–44 per cent (Table 2). For the most potent inhibitor, 2-phenylethylureidopurine ribonucleoside, the type of inhibition was determined and was found to be competitive with a K_i value of 0·4 μM, slightly lower than the K_m for adenosine, 0·5 μM.[4] The inhibitory potency of phenylureidopurine ribonucleoside was similar to that of N^6-phenyladenosine, another competitive inhibitor with a K_i of 0·6 μM.[4] Thus,

TABLE 2. 6-UREIDOPURINE RIBONUCLEOSIDES AS INHIBITORS OF ADENOSINE KINASE

R*	Per cent inhibition† of 0·82 μM ado phosphorylation at 75 μM	Per cent inhibition‡ of 4·4 μM ado phosphorylation at 4·4 μM
Allyl—	55	
3-Hydroxypropyl—	56	
Isopropyl—	63	
Furfuryl—	67	
Isoamyl—	69	
4-Hydroxybutyl—	79	
3-Phenylpropyl—	73	14
Phenylmethyl—	85	21
4-Phenylbutyl—	86	27
Phenyl—	92	43
2-Phenylethyl—	94	44 K_i 0·4 μM

* For R refer to the structural formula given in the heading of Table 1.

† See footnotes to Table 1.

‡ Average of duplicates ± 5 per cent; the adenine nucleotides in controls contained 1240 ± 20 counts/min (average of duplicates).

the introduction of —CO—NH— bridge at N^6 of adenosine had no profound effect on the inhibitory activity of these molecules, although such introduction abolished the substrate activity of some of the compounds (see above).

Schnebli *et al.*[11] have previously described several inhibitors of adenosine kinase of H. Ep. No. 2 cells, most potent being 6-mercapto-9-(β-D-ribofuranosyl)purine, 2-fluoro-6-mercapto-9-(β-D-ribofuranosyl)purine and 6,6′-dithiobis-(9-β-D-ribofuranosyl)purine. More recently Caldwell and Henderson[12] reported the inhibitory activity of 149 purine and pyrimidine derivatives on adenosine kinase in crude extracts of Ehrlich ascites cells. The substrate used in their study was 6-methylmercapto-9-(β-D-ribofuranosyl)purine. Since the K_m of this compound for adenosine kinase is about twenty times that of adenosine,[11] it is possible that compounds which appear as potent inhibitors of 6-methylmercapto-9-(β-D-ribofuranosyl)purine phosphorylation may be less potent inhibitors of the phosphorylation of adenosine, the natural substrate of the enzyme.

Most mammalian tissues possess the capacity to synthesize purine nucleotides *de novo* and the functional significance of adenosine kinase in such conditions remains unexplained. The multiplication of S-180/KR cells, deficient in adenosine kinase,[5] has been shown to be equal to or faster than that of the parent cells, when grown in cell culture in Eagle's medium.[13] In cell culture, adenosine kinase becomes critical for cell survival only in unusual circumstances. This may occur in media supplemented with methotrexate.[14,15] In such conditions it was shown that N^6-phenyladenosine prevented the growth of S-180 cells which was dependent on extracellular adenosine.[16] Whether this inhibition of adenosine utilization was due to inhibition of adenosine kinase remains to be determined in view of the fact that N^6-phenyladenosine also happens to be a potent inhibitor of the uptake of purine and pyrimidine nucleosides.[17]

Adenosine kinase could be critical for human erythrocytes since these cells cannot synthesize purine nucleotides *de novo* nor carry out the conversion of IMP to AMP.[18] It has been recently shown that the parasitic organism *Schistosoma mansoni*, which feeds on erythrocytes, is also devoid of *de novo* capacity to synthesize purine nucleotides and consequently depends on adenine or adenosine for growth.[19–21] In view of this, inhibitors of adenosine kinase described here, which are only mildly cytotoxic to cells in culture,[7] may be potentially useful, e.g. in the treatment of schistosomiasis.

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