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REACTION OF 5'-DEOXYADENOSINE AND RELATED ANALOGS WITH THE 5'-METHYLTHIOADENOSINE CLEAVING ENZYME OF SARCOMA 180 CELLS, A POSSIBLE CHEMOTHERAPEUTIC TARGET ENZYME

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Because adenosine analogs such as 5'-deoxyadenosine (5'-dAdo, Figure 1) lack a hydroxyl group on the 5' carbon of the ribose moiety, they are assumed incapable of direct formation of 5'-nucleotides. Also, since many of these compounds, including 5'-dAdo, are not substrates for purine nucleoside phosphorylase (PNP) and have very weak activity with mammalian adenosine deaminases, they have been believed to be metabolically inert. For these reasons, analogs of this type have been considered as potential biochemical tools for study of direct effects of adenosine in phenomena such as nucleoside transport, stimulation or inhibition of adenylate cyclases, etc. [1,2]. However, incubation of Ehrlich ascites cells with 5'-dAdo causes major metabolic perturbations, which resemble those encountered when intracellular ATP or ATP analog levels are greatly increased, i.e. marked depletion of 5-phosphoribosyl-1-pyrophosphate (PRPP), inhibition of purine biosynthesis de novo, and inhibition of glycolysis with a "crossover" involving the phosphofructokinase reaction [3]. It was postulated that the latter effects resulted from direct actions of 5'-dAdo (as the unmodifed nucleoside) on tumor cell metabolism.

We now wish to report that 5'-dAdo is a highly active substrate for the 5'-MTA cleaving enzyme which frees adenine from 5'-deoxy-5'-methylthioadenosine (5'-MTA, Figure 1), a byproduct in the biosynthesis of spermidine and spermine from S-adenosyl-L-methionine (SAM) and 1,4-diaminobutane (putrescine) [4]. The presence of the 5'-MTA cleaving enzyme, previously identified in rat ventral prostate [5], has now been demonstrated in the cytosol of Sarcoma 180 (S-180) and L5178Y cells. Partial purification of the cytosolic enzyme of S-180 cells has been accomplished by ammonium sulfate fractionation, thus enabling preliminary studies of substrate specificity with various 5'-modified adenosines and determination of kinetic parameters. Hopefully, such investigations will permit the rational design of new analogs of chemotherapeutic value. In any case, these studies demonstrate that the metabolic effects of 5'-dAdo result from the metabolism of this compound, causing an accumulation of ATP, rather than from effects produced by the unaltered nucleoside.

The results described in this preliminary report will be presented at a national meeting and will be documented in detail elsewhere.

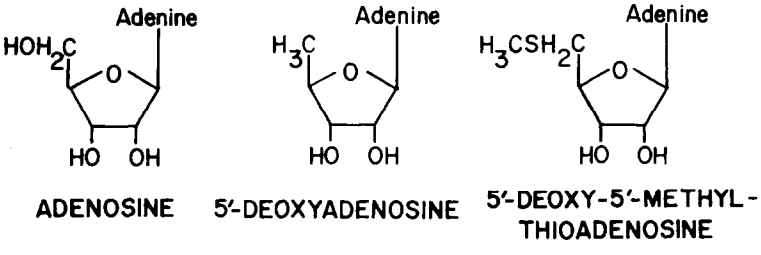

Figure 1. Structural Formulae.

In accord with a prior report [3], when S-180 ascites cells were incubated with 5'-dAdo, PRPP accumulation was inhibited in a dose-dependent manner (ID_{50}=0.02mM). Among the adenosine

analogs examined to date by this laboratory, 5'-dAdo is the most potent inhibitor of PRPP accumulation in S-180 cells. When extracts of S-180 cells incubated with 5'-dAdo (0.5mM) were subjected to HPLC analysis for determinaton of nucleotide profiles [6], 2-3 fold increases were observed in the concentrations of the adenine nucleotides, ATP and ADP, in comparison with controls. This suggested that 5'-dAdo is cleaved intracellularly, liberating free adenine, which is salvaged by adenine phosphoribosyltransferase (APRT) and PRPP to form 5'-AMP (see below), which is then converted metabolically to polyphosphate nucleotides.

$$\text{5'-dAdo} \xrightarrow{P_i(?)} \text{Adenine + 5-deoxyribose product}$$

$$\text{Adenine + PRPP} \xrightarrow{\text{APRT}} \text{5'-AMP + PPi}$$

We now have evidence demonstrating that S-180 cells perform the enzymic cleavage of adenine from 5'-Ado, as well as from several other adenosine analogs modified in the 5'-position. Initial studies were performed with the dialyzed high-speed (105,000g) supernatant fluids of S-180 homogenates (Fr I) and later studies with 40-65% ammonium sulfate precipitates of Fr I (Fr II). The specific activity of Fr II was approximately 3-fold greater than Fr I. The formation of adenine from 5'-dAdo was shown by three different methods: (a) A time-dependent formation of free adenine from 5'-dAdo was detected by reversed-phase HPLC (Waters Assoc. μBondapak C-18 column and a methanol-phosphate buffer gradient system) after an aliquot of Fr I was incubated with 5'-dAdo (0.8mM) in potassium phosphate buffer (pH 7.4, 15mM). Adenine was identified by comparison of its retention time with that of an authentic adenine standard. (b) When Fr II was incubated with 5'-dAdo (0.37mM) and PRPP (1.0mM), the time-dependent formation of 5'-AMP was detected by anion-exchange HPLC. Fr II was shown to contain APRT activity. The identity of 5'-AMP was established by the use of a specific enzymic peak-shift method as described elsewhere [7]. (c) When an aliquot of Fr II was incubated with 5'-Ado and xanthine oxidase (Sigma Grade III, 1.1 units/mg, 0.8 units/ml final reaction volume) in 15mM phosphate buffer (pH 7.4), a progressive increase in absorbancy at 305nm was seen consistent with the formation of 2,8-dihydroxyadenine from free adenine [5,8]. The rate of change in absorbancy at 305nm was directly proportional to the concentration of Fr II. This method was employed in the kinetic studies described below.

It seemed possible that the enzyme in S-180 cells responsible for the formation of adenine from 5'-dAdo might be similar or identical to an enzyme described previously [5] in rat ventral prostate which cleaves adenine from 5'-MTA. This enzyme, which is essential for the recovery of the adenine moiety of this metabolite, to date, has been examined in rat prostatic tissue and several micro-organisms [9-11]. In order to test for this enzyme in S-180 cells, as well as other mammalian tissues, various experiments were performed. As with 5'-dAdo, when S-180 cells were incubated with 5'-MTA there was a 2-3 fold increase in intracellular adenine nucleotides and a marked depletion of PRPP levels. Also, when Fr II was incubated with 5'-MTA, the liberation of adenine was demonstrated by the three methods described above. To establish that the 5'-dAdo and 5'-MTA cleaving enzymes are identical, three types of experiments have been performed. (1) When a mixture of 5'-dAdo and 5'-MTA (at saturating concentrations) was incubated with an aliquot of Fr II, the rate of adenine formation (xanthine oxidase assay) was intermediate between the V_{max} values of the two competing substrates. (2) Application of methods [12,13] for study of competing alternative substrates with substantially different V_{max} values (see Table 1) yielded the predicted Lineweaver-Burk plots. (3) The analog, 5'-deoxy-5'-chloroformycin (7-amino-3-(5-deoxy-5-chloro-β-D-ribofuranosyl)pyrazolo[4,3-d]pyrimidine), a C-nucleoside incapable of enzymic cleavage, is an unusually potent inhibitor of both 5'-dAdo and 5'-MTA cleaving activity (Table I). Significantly, K_i values measured in the presence of each substrate incubated with Fr II were essentially identical. Further studies to establish the identity of these enzymic activities will include monitoring of the relative substrate activities throughout enzymic purification and during enzymic inactivation procedures, the performance of competing substrate studies in which only one of the substrates is radiolabeled in the adenine moiety, the monitoring of relative substrate activities during possible enzymic inductions as postulated below, etc.

In addition to 5'-dAdo and 5'-MTA, several other 5'-modified adenosines liberate adenine upon incubation with Fr II. Several have been subjected to kinetic analyses with the coupled xanthine oxidase procedure enabling determinations of K_m and V_{max} values (Table 1). Interestingly, a relatively wide range of substituents on the 5'-carbon of the ribose is consistent with retention of good substrate activity. None of the substrates listed show activity with PNP from calf spleen or human erythrocytes (an important consideration since Fr II contains PNP activity). No adenine liberation was detected with arabinosyladenine, 3'-deoxyadenosine (cordycepin), or 2'-deoxyadenosine. In the presence of the adenosine deaminase inhibitor, 2'-deoxycoformycin, slow liberation of adenine from adenosine was observed upon incubation with aliquots of Fr II. However, because adenosine has weak, but

definite substrate activity with PNP [14], it will be necessary to obtain the 5'-MTA cleaving enzyme free of PNP in order to clarify this matter.

Montgomery and colleagues [15] have synthesized a variety of analogs of 5'-MTA and report a low order of toxicity in mice. The 2-fluoroadenine analog of 5'-ethylthioadenosine was highly toxic to KB and H.Ep. 2 cells in culture, but had no effect on H.Ep. 2 cells lacking APRT. They postulated that the toxicity was due to cleavage to 2-fluoroadenine which was then converted to nucleotides by APRT.

The 5'-MTA cleaving activity in terms of μmoles of adenine liberated/min/ml of packed S-180 cells was about 0.025. In addition to S-180 cells, we have identified the 5'-MTA cleaving enzyme in L5178Y murine lymphoblastic leukemia cells and isolated rat hepatocytes.

TABLE I. KINETIC CONSTANTS OF VARIOUS 5'-SUBSTITUTED ADENOSINE ANALOGS FOR THE 5'-MTA CLEAVING ENZYME FROM SARCOMA 180 CELLS*

	K_m(μM)	Rel. V_{max}	K_i(μM)
5'-deoxy-5'-methylthioadenosine (5'-MTA)	9	100	----
5'-deoxyadenosine (5'-dAdo)	23	180	----
5'-deoxy-5'-chloroadenosine	21	46	----
5'-deoxy-5'-S-isobutyladenosine	8	89	----
5'-deoxy-5'-chloroformycin	----	----	0.3

*All assays were performed on a Gilford recording spectrophotometer at 37°, 305nm. Each reaction mixture (1 ml) contained 15mM potassium phosphate, pH 7.42, 0.8 units xanthine oxidase (Grade III, Sigma Chemical Co.) and partially purified enzyme preparation (450-675 μg protein) pretreated with 2'-deoxycoformycin, 5 μg/ml.

The present studies provide evidence that the action of 5'-MTA cleaving enzyme on 5'-dAdo results in the liberation of adenine in murine tumor tissue treated with this analog. The adenine produced and its subsequent salvage to adenine nucleotides can account for many of the metabolic effects (see above) previously ascribed to the nucleoside itself. Specifically, the salvage of each mole of free adenine by APRT would consume a mole of PRPP, leading to a depletion of the steady-state level of PRPP; furthermore, elevation of ATP levels, resulting from the salvage of adenine from 5'-dAdo, could inhibit the synthesis of PRPP, as has been shown in the Ehrlich ascites cell system [16]. The reduced availability of PRPP could in turn account for the previously observed 5'-dAdo-induced inhibition of purine biosynthesis de novo [3], a PRPP-dependent process. Finally, the 5'-dAdo-induced elevation of ATP may account for the "crossover" at the phosphofructokinase step observed in the Ehrlich system [3], ATP being a well-known regulator of this enzyme.

These studies may also provide answers to questions that have long puzzled investigators of purine biochemistry, e.g. the reason for the ubiquitous distribution in animal tissues of APRT, an enzyme with a very low K_m for adenine, and the source of free adenine in these tissues. Polyamine synthesis is required for cells to enter the S-phase of the cell cycle [17] and polyamines can achieve millimolar concentrations intracellularly. Two moles of SAM are consumed in the synthesis of one mole of spermine from 1,4-diaminobutane. Thus, in the absence of an efficient adenine salvaging mechanism (the 5'-MTA cleavage enzyme plus APRT), cells would rapidly be depleted of essential adenine-containing compounds, e.g. ATP. Since the product of the reaction of adenine with xanthine oxidase, 2,8-dihydroxyadenine, is highly insoluble and can crystallize in the kidneys with occasionally fatal anuria, the physiologic need for APRT in relatively high activity and ubiquitous tissue distribution is readily appreciated.

Enzymes of polyamine synthesis, ornithine decarboxylase and SAM decarboxylase, are normally at very low activity in mammalian tissues but increase markedly in response to a variety of stimuli of cellular division. These enzymes also have very short half-lives in comparison with other mammalian enzymes [18]. Thus, an important question to be answered is whether the 5'-MTA cleaving enzyme is also subject to marked fluctuations in activity at

various stages of the cell cycle, during partial hepatectomy, blastogenesis of lymphocytes, etc. If this activity does, in fact, increase in rapidly growing tissues, e.g. neoplasms, this could be exploited chemotherapeutically. A potent inhibitor of the enzyme, e.g. 5'-deoxy-5'-chloroformycin (Table I), might cause major disruptions of the metabolism of these cells by preventing adenine salvage or by interfering with polyamine synthesis or function. Also, substrates might be designed to liberate toxic adenine analogs, e.g. 2-fluoroadenine, 4-aminopyrazolo[3,4-d]pyrimidine, or 8-azaadenine, specifically in tissues that have high 5'-MTA cleaving enzyme activity. Although we have not yet identified the ribose products formed during this enzymic reaction, earlier work with the prostate enzyme [5] suggests that they are analogs of ribose-1-phosphate; therefore, one must also consider the possible metabolic consequences of the intracellular formation of 5'-modified sugar phosphates, e.g. 5'-deoxyribose-1-phosphate, 5'-halogen-substituted ribose-1-phosphate, etc. Another consideration is the design of analogs that liberate nonsalvagable adenine analogs, i.e. not substrates for APRT, such as 6-methylmercaptopurine, which should be eliminated in the urine and readily detected by its special spectral characteristics. Such an analog might be employed as a marker for the detection of high 5'-MTA cleaving enzyme activity, as might occur in patients with certain malignancies. Since the 5'-MTA cleaving enzyme is known to occur in high activity in prostatic tissue, certain of the concepts discussed above could find application in prostatic disease, e.g. hypertrophy, neoplasia. The validity of the above speculations, of course, will depend upon the outcome of investigations of the distribution of this enzyme in normal and malignant mammalian tissues and of the possible fluctuations of the activity in response to the many stimulators of polyamine biosynthesis, such as partial hepatectomy and various mitogens.

If the activity of the 5'-MTA cleaving enzyme fluctuates during the cell cycle, perhaps the use of cell cycle phase-specific inhibitors could either increase or decrease this activity and offer logical chemotherapeutic drug combinations. Of special interest will be the effect of methylglyoxal bis(guanylhydrazone), an agent that blocks SAM decarboxylase and thus the formation of spermine and spermidine from 1,4-diaminobutane [19].

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