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Characteristics of the enzyme uridine–cytidine kinase isolated from a cultured human cell line

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Uridine–cytidine (U–C) kinase catalyzes the phosphorylation of the nucleosides uridine and cytidine to their nucleotide forms. This enzyme has been previously isolated from rat liver [1], murine tumor cells [2, 3], 5-azacytidine resistant murine leukemic cells [4], and calf thymus [5]. Previous studies [6] concluded that U–C kinase was responsible for the phosphorylation of the antileukemic agent 5-azacytidine and that partial deletion of this enzyme was responsible for resistance to 5-azacytidine in the murine leukemia line [4]. Because of the important role of this enzyme in the activation of 5-azacytidine, and in resistance to this drug, we have undertaken its isolation and kinetic characterization from human leukemia cells.

5-Azacytidine[4-^{14}C] (204 mCi/mg), 5-azacytidine (NSC 102816) and tetrahydrouridine (NSC 112907) were obtained from the Drug Research and Development Program, National Cancer Institute. 5-Azacytidine[4-^{14}C] was judged to be 96 per cent pure by thin-layer chromatography on cellulose plates with water–ethanol (7:3) as solvent. Cytidine[2-^{14}C] (25 mCi/m-mole) and uridine [^{3}H] (4000 mCi/m-mole) were obtained from Schwarz/Mann, Orangeburg, N.Y. Non-radiolabeled nucleotides, pig muscle myokinase (2250 units/mg), pyruvate kinase (450 units/mg), alkaline phosphatase (43 units/mg), and the sodium salt of phosphoenolpyruvate were from the Sigma Chemical Co., St. Louis, Mo. AG-1-X2 ion exchange resin was purchased from BioRad, Richmond, Calif. DEAE cellulose discs (Whatman 52) were purchased from H. Reeve Angle, Clifton, N.J. Disposable plastic columns, 0.8 × 20 cm, were from Kontes Glass Co., Vineland, N.J. Thin-layer PEI and DEAE cellulose chromatography plates were obtained from Analtech Inc., Newark, Del.

A human lymphoblastic leukemia cell line, CEM [7], previously isolated at the Children's Cancer Research Center in Boston, Mass. and maintained in continuous cell culture, was obtained from Dr. Ivor Royston formerly of the Bureau of Biological Standards, Bethesda, Md. The cell line was grown in spinner culutre in RPMI series 1640 medium containing penicillin, streptomycin, and glutamine plus 5% heat inactivated fetal calf serum, with a starting concentration of 3×10^5 cells and a final concentration of 3×10^6 cells/ml. Approximately 2×10^9 cells were harvested by centrifugation at 500 *g* at 5° for 10 min, and washed free of media with two changes of phosphate-buffered saline. The cell button was then diluted with 6–10 ml of 0.05 M Tris–HCl buffer, pH 8, freeze-thawed twice and homogenized with 20 strokes of a Dounce homogenizer. After centrifugation at 20,000 *g* for 30 min, the supernatant fraction containing U–C kinase activity of 900–5000 units*/ml and a specific activity of 285–1580 units/mg of supernatant protein was removed and stored at 4° until further use. This crude cell supernatant fraction also contained CMP kinase activity in the range of 2000 units/mg of protein.

Phosphorylation of uridine and cytidine was assayed as previously described [8], separating the product from the substrate by adherence of the nucleotide to DEAE discs. Phosphorylation of 5-azacytidine[4-^{14}C] was assayed using the previously described incubation mixture (ATP, 10 mM; $MgCl_2$, 10 mM; Tris–HCl, pH 8, 50 mM; NaF, 15 mM; 5-azacytidine[4-^{14}C], 0.01 μCi in a final substrate concentration of 0.05 to 6.0 mM; and 5–40 μl of enzyme preparation in a total volume of 120 μl). The reaction was stopped with 1 ml of ice-cold water, and the solution was immediately passed onto a disposable plastic column (0.8 × 20 cm) containing 2 ml of AG-1-X2 ion exchange resin (previously washed with 15 vol. of distilled water). The substrate was selectively eluted with 25 ml of distilled water and the nucleotide products with 6 ml of 1 M ammonium chloride (see Fig. 1). Two ml of the 1 M ammonium chloride wash, containing the phosphorylated product, was then added to 18 ml of Aquasol and counted in a Packard Tri Carb liquid scintillation counter. By using this method, the phosphorylation of 5-azacytidine was shown to be linear with supernatant protein concentrations of 50–200 μg/0.120 ml of reaction volume and was linear with respect to time for a period of 60 min.

In control experiments, 96 per cent of the substrate, 5-azacytidine[4-^{14}C], was recovered from the AG-1-X2 columns in the distilled water fraction, while 4 per cent eluted in the 1 M ammonium chloride fraction (corresponding to the background rate of the assay). Cytidine mono-, di-, and triphosphates were used to assess the likely migration pattern of 5-azacytidine nucleotides, and the results are shown in Fig. 1. There was complete recovery of the cytidine nucleotides in the 1 M NH_4Cl wash. In the disc assay, 77 per cent of nucleotide standards added directly to the disc was recovered.

The phosphorylated product of 5-azacytidine was chromatographed on DEAE cellulose plates with a water solvent and on PEI cellulose plates with a 1 M LiCl solvent along with the known standards: 5-azacytidine, cytidine, CMP, CDP and CTP. As a second proof of the product, the previously described reaction mixture, containing 1.7×10^{-3} M 5-azacytidine as the substrate and 120 units of enzyme, was preincubated at 37° for 2 hr to form the nucleotide product. Nucleotide formation was confirmed by elution from an AG-1-X2 column. In a duplicate tube, prior to passage over a column, the reaction mixture was incubated for an additional 15 min at 37° in the presence of 2 units of alkaline phosphatase and then passed onto an AG-1-X2 column to determine if the nucleoside could be reformed from the product by phosphatase activity.

In order to be certain that the CEM cell line was sensitive to the antineoplastic activity of 5-azacytidine, a series of 20 ml cultures containing 1×10^6 cells/ml in RPMI 1630 medium with 5% fetal calf serum was incubated in the presence of 5-azacytidine, 10^{-5} to 10^{-7} M, for a period of 48 hr, and inhibition of growth in treated cultures as compared to controls lacking 5-azacytidine was determined.

Our initial experiments were directed at characterizing the affinity and relative velocity of 5-azacytidine as a substrate for human U–C kinase. A double reciprocal plot of substrate concentration vs product formation (Fig. 2) yielded a K_m value of 75 μM for cytidine, 140 μM for uridine, and 11 mM for 5-azacytidine. Table 1 presents approximate relative velocities for the three substrates and shows that the maximum velocity for the analog, 5-azacytidine, was only 26 per cent that of the physiologic substrate cytidine. The limited solubility of 5-azacytidine prevented our obtaining substrate concentrations above the K_m of 11 mM; thus the maximum velocity for this substrate was

*Units of U–C kinase = nmoles of nucleotide product formed/hr with uridine as the substrate.

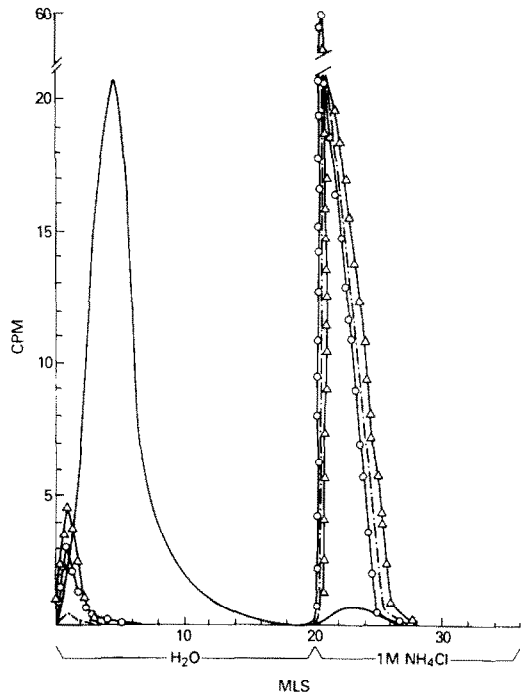

Fig. 1. Elution of 3×10^{-4} M (80,000 cpm ^{14}C) 5-azacytidine (——), cytidine monophosphate (O——O), cytidine diphosphate (·——·), and cytidine triphosphate (△——△) from a 0.8 × 20 cm column containing 2 ml AG-1-X2 resin.

estimated from the Lineweaver–Burk plot. The K_i value for 5-azacytidine was 17 mM with cytidine as the substrate, a value in good agreement with the previously determined K_m for 5-azacytidine.

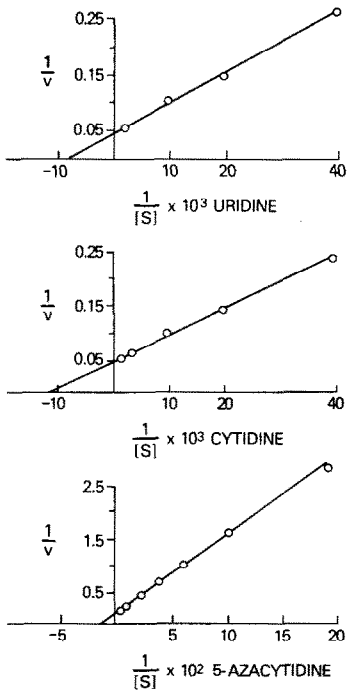

Fig. 2. Lineweaver–Burk plots of the reciprocals of substrate concentration vs reaction velocity, with uridine, cytidine and 5-azacytidine as substrates, as noted.

Table 1. Relative velocities for 5-azacytidine, cytidine and uridine

Substrate	K_m (mM)	Relative velocity
5-Azacytidine	11	0.26
Cytidine	0.075	1.00
Uridine	0.140	0.92

It was considered important to determine whether the uridine–cytidine kinase from CEM cells had the same affinity for 5-azacytidine as did enzyme from cells obtained from patients with acute myelocytic leukemia (AML). Circulating myeloblasts were obtained by leukapheresis from a previously untreated patient with AML, and cells were disrupted by Dounce homogenization in 0.05 M Tris, pH 7.5. K_m values of 16 mM for 5-azacytidine phosphorylation and 183 μM for uridine phosphorylation were determined by double reciprocal plots of substrate concentration vs nucleotide formation. These results using enzyme from AML cells were in good agreement with the previously determined K_m values for the uridine cytidine kinase from CEM cells.

The reaction product of 5-azacytidine, when chromatographed on DEAE cellulose in a water solvent, remained at the origin as a nucleotide, while 5-azacytidine itself migrated to the solvent front. This same product, when chromatographed on PEI cellulose in a 1 M LiCl solvent, gave two u.v. absorbing spots with R_f values of 0.29 and 0.14 which corresponded to standards of cytidine diphosphate and cytidine triphosphate respectively. In this latter system, 5-azacytidine migrated with cytidine (R_f 0.80). When the reaction product was incubated with 2 units of alkaline phosphatase for 15 min at 37° and passed over AG–1–X2 resin, no radioactivity above background was found to elute in the 1 M ammonium chloride wash, indicating that the PO_4 groups added during the reaction had been removed and the parent nucleoside reformed.

Studies of the cytotoxic effects of 5-azacytidine on CEM cells in tissue culture were undertaken to determine the sensitivity of this cell line to this agent. A drug concentration of 10^{-6} M produced 50 per cent inhibition of cell growth at 48 hr, a concentration comparable to the 50 per cent inhibitory level of this drug for the sensitive L1210 leukemia cell line (0.6×10^{-6} M) [9] (Fig. 3).

The foregoing studies suggest that 5-azacytidine has a low affinity for U–C kinase as indicated by its high K_m (11 mM) and K_i (17 mM) values. This enzyme is present in levels comparable to those of deoxycytidine kinase [8],

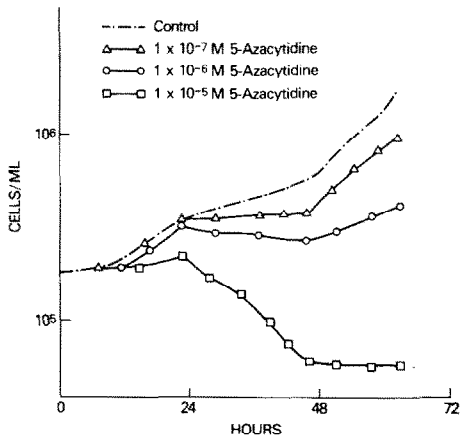

Fig. 3. Growth curves of CEM cell cultures containing various concentrations of 5-azacytidine.

but considerably lower than those of the primary catabolic enzyme cytidine deaminase in the leukemic cell. High levels of 5-azacytidine would have to be achieved in order to approach the K_m of U–C kinase, and in view of its rapid metabolism by cytidine deaminase [10] and its chemical lability, a high dose infusion schedule would seem justified. However, recent data [11] indicate that high dose therapy with 5-azacytidine may be associated with neurotoxicity as well as the extreme nausea and vomiting often seen at lower doses of this agent.

The affinity of U–C kinase from human leukemic cells for 5-azacytidine appears to be substantially lower than that previously reported by Liacouras and Anderson [2] for the murine mast cell tumor enzyme and by Lee *et al.* (K_m = 200 μM) for the calf thymus enzyme. The Michaelis constants for cytidine and uridine using all three sources of enzyme, mast cell tumor, calf thymus and CEM cell line, were quite similar, in the 10^{-4} to 10^{-5} M range. Lee *et al.* [5], in their studies of the calf thymus U–C kinase, estimated the relative maximum velocities for 5-azacytidine, cytidine, and uridine were approximately 0.25:0.75:1.0, respectively, values which are similar to those of the CEM enzyme except for reversal of the relative velocities of cytidine and uridine. Further studies will be required to establish whether the enzyme from human tissues has other properties differing from those of the murine and calf thymus enzymes.

It is noteworthy that despite the poor affinity of substrate for this enzyme in the human leukemic cells, the CEM cell line remained responsive to 5-azacytidine *in vitro*.

U–C kinase levels have been measured in extracts of myeloblasts obtained from previously untreated patients with acute non-lymphocytic leukemia [12], and averaged 16.4 ± 19.4 units/mg of protein (median value of 11.1 units/mg of protein), with variation over a three log range (0–75 units/mg of protein). The levels of U–C kinase in myeloblasts were considerably lower than the levels of the degradative enzyme, cytidine deaminase, in the human myeloblastic cells (mean concentration, 377 ± 530 units/mg of protein).

The poor affinity of U–C kinase for 5-azacytidine as a substrate and the low activity of the enzyme in leukemic cells in comparison to cytidine deaminase raise the possibility that metabolism of 5-azacytidine to the nucleotide form and subsequent incorporation into RNA [13] may not be the only mechanism responsible for its cytotoxicity. Other possible routes of metabolism, such as deamination to 5-azauridine [10] or ring cleavage, might be responsible for formation of an active antimetabolite. Further studies of the metabolism of 5-azacytidine in intact cells and in the whole animal are needed to determine the relative importance of nucleotide formation as compared to alternate transformations of this agent under physiologic conditions.

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Effects of luteinizing hormone and follicle stimulating hormone on hepatic drug metabolism in gonadectomized male and female rats

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Many hormones participate in the regulation of drug and steroid metabolism by rat liver microsomes [1–3]. The actions of gonadal hormones have been particularly well investigated [4–10]. Androgens increase the oxidative metabolism of various substrates including ethylmorphine, hexobarbital and testosterone [4–7]. As a result, hepatic oxidation of many substances proceeds far more rapidly in male than female rats. In contrast, reductive steroid metabolism (Δ^4-hydrogenase activity) is inhibited by testosterone and enhanced by estradiol, producing a sex difference in Δ^4-hydrogenase activity opposite that in oxidative metabolism [8–10].

Until recently, gonadal hormones were thought to act directly and independently on the liver to alter the activities of drug- and steroid-metabolizing enzymes. However, Colby *et al.* [11] demonstrated that the actions of both testosterone and estradiol on hepatic corticosteroid metabolism in rats and hamsters were not demonstrable in hypophysectomized animals, indicating a dependence on the pituitary gland. Subsequently, other investigators