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CYTIDINE AND DEOXYCYTIDYLATE DEAMINASE INHIBITION BY URIDINE ANALOGS

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Abstract—Cytidine deaminase, an enzyme found in the supernatant fluid of hepatocytes, granulocytes and tumor cells, and in plasma, degrades the antitumor agents cytosine arabinoside and 5-azacytidine. Uridine and its analogs, 3-deazauridine, 5-bromodeoxyuridine, 5-fluorodeoxyuridine and 6-azauridine, were found to competitively inhibit cytidine deaminase; the most potent inhibitor was 3-deazauridine ($K_i = 1.9 \times 10^{-5}$ M). In addition, deoxycytidylate deaminase, which degrades cytosine arabinoside monophosphate to the inactive uracil arabinoside monophosphate ($K_m = 9 \times 10^{-4}$ M), was competitively inhibited by 3-deazauridine monophosphate, as well as by the nucleotides of other uridine analogs. These results suggest that uridine analogs such as 3-deazauridine may have value in protecting cytosine arabinoside, 5-azacytidine and their monophosphate nucleotides from degration by neucleoside and nucleotide deaminases.

1-β-*d*-Arabinofuranosylcytosine (cytosine arabinoside, ara-C)† has been in clinical use for more than a decade and is firmly established as a primary agent for treating acute myelogenous leukemia. The intracellular metabolism of this agent in tumor cells is known to proceed by the pathway shown in Fig. 1, with activation by successive phosphorylation steps and degradation at the level of nucleoside and nucleotide by cytidine and deoxycytidylate deaminases respectively [1]. The relative importance of the deamination processes in determining tumor cell response has not been established, although it is known that both enzymes are found in concentrations higher than that of the initial activating enzyme, deoxycytidine kinase, in human leukemic cells. Steuart and Burke [2] found a correlation between responsiveness to ara-C and intracellular levels of cytidine deaminase, a finding which has not been confirmed by subsequent studies [3, 4].

In an effort to define a biochemical basis for combination therapy with ara-C and other antimetabolites, we have examined the effects of various uridine analogs and their nucleotides on the deamination of ara-C and ara-CMP. In the present study, it is shown that 3-deazauridine and 5-fluorodeoxyuridine, as well as other antineoplastic pyrimidine analogs and their monophosphate derivatives, competitively inhibit ara-C degradation *in vitro*.

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† Abbreviations used are: ara-C, cytosine arabinoside; ara-CMP, cytosine arabinoside monophosphate; 3-deazaUMP, 3-deazauridine-5′-monophosphate; 5-FdUMP, 5-fluorodeoxyuridine monophosphate; 5-BdUMP, 5-bromodeoxyuridine monophosphate; and dCMP, deoxycytidine monophosphate.

MATERIALS AND METHODS

Materials. Unless otherwise mentioned, all non-radioactive nucleosides and nucleotides were obtained from the Sigma Chemical Co., St. Louis, MO. 3-Deazauridine, 5-bromodeoxyuridine, 5-fluorodeoxyuridine and 6-azauridine were obtained from Dr. Robert Engle, Drug Research and Development Branch, National Cancer Institute. The initial sample of 3-deazauridine monophosphate (3-deazaUMP) was supplied by Dr. Robert Brockman, Southern Research Institute, Birmingham, AL. A second sample of 3-deazaUMP was synthesized as described below. All radioactively labeled nucleosides and nucleotides were purchased as ^{14}C-labeled compounds from the New England Nuclear Corp., Boston, MA.

Enzyme assays. Assays for cytidine deaminase [5] and deoxycytidine monophosphate (dCMP) kinase [8] were performed using previously published assay techniques listed, and dCMP deaminase was assayed by modification of the cytidine deaminase assay, as described in the legend to Fig. 4. For uniformity, enzyme units are listed as nmoles converted per hr per mg protein.

Protein assay. Protein determinations were performed by the method of Lowry *et al.* [9].

Enzyme kinetic studies. Cytidine deaminase for enzyme inhibition studies was obtained from human granulocytes which were procured from normal donors by leukopheresis. The granulocytes were disrupted by three cycles of freeze thawing, followed by Dounce homogenization. The supernatant fraction containing the enzyme was purified 65-fold to a specific activity of 210 units/mg protein by heating to 70° for 6 min, ammonium sulfate precipitation (saving the 30–60% precipitate), and Sephadex G-150 gel filtration, as described previously [5]. Deoxycytidylate deaminase was prepared from a super-

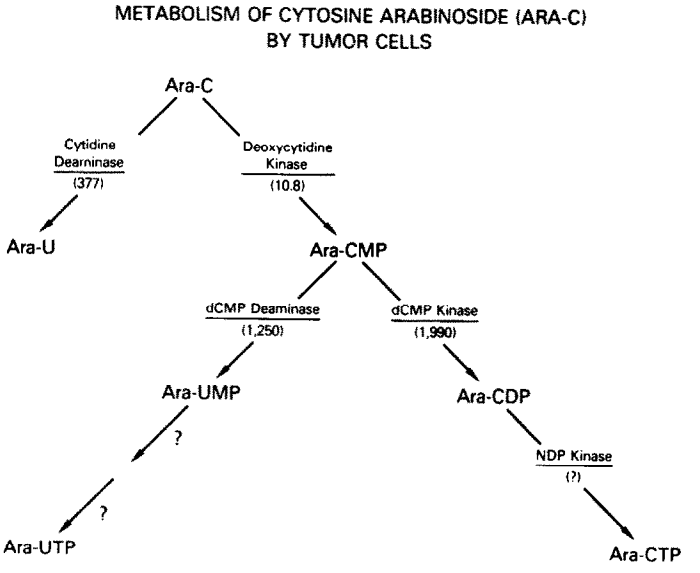

Fig. 1. Intracellular metabolism of cytosine arabinoside in tumor cells. The approximate concentrations of enzymes in human acute leukemia cells are indicated in parentheses [5–7]. Enzyme units are nmoles of product formed per hr per mg protein.

natant fraction of the CCRF-CEM human lymphoblastic leukemia cell line grown in continuous tissue culture. Cells were disrupted by freeze thawing and Dounce homogenization, cell debris was removed by centrifugation at 105,000 *g* for 60 min, and the supernatant fraction capable of deaminating 380 μmoles dCMP/mg protein under standard assay conditions, was saved.

Michaelis–Menten constants were determined with cytidine and ara-C as substrates for cytidine deaminase, and with dCMP and ara-CMP as substrates for deoxycytidylate deaminase. The K_m values were calculated by weighted linear regression analysis of the data by the method of Wilkinson [10]. The K_i values for inhibition by pyrimidine analogs were calculated according to the formula

$$K_i = \frac{[\text{Inhibitor}]}{\dfrac{1 - K_m/K_p}{K_m/K_p}},$$

where K_p is the apparent dissociation constant of the substrate in the presence of inhibitor.

Synthesis of 3-deazauridine-5'-monophosphate (3-deazaUMP). Although the chemical preparation of 3-deazaUMP has been reported, neither the experimental conditions nor the nucleotide properties have been described [11]. Our synthetic procedure employed the general method of Yoshikawa *et al.* [12, 13] for the phosphorylation of unprotected nucleosides, as modified by Dawson *et al.* [14].

To a suspension of 3-deazauridine (0.25 g, 1.02 nmoles) in 1.25 ml triethyl phosphate at 0° was added phosphorus oxychloride (0.2 ml, 2.14 mmoles) pretreated with 0.018 ml H_2O (1.0 mmole). This mixture was stirred at 0° for 5 hr and then hydrolysed with 2 ml H_2O at room temperature for 2 hr.

The hydrolysis mixture was then placed on a cation exchange column (Dowex 50W 8-X, H^+ form, 2 cm × 17 cm) and eluted with water. Five milliliter fractions were collected. The peaks of u.v.-absorbing eluate and neighboring fractions were combined and applied to an anion exchange column (IR-400 $CO_3^=$ form, 2 × 17 cm), which was then washed with 300 ml water. The column was then washed with 1.0 M $(NH_4)_2CO_3$, eluting the desired product. Five milliliter fractions were taken of the u.v.-absorbing material, and those with maximum absorbance were

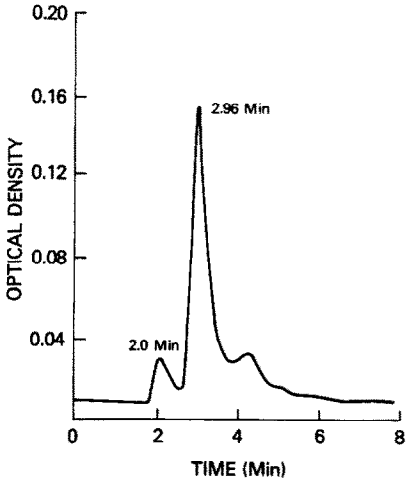

Fig. 2. High pressure, liquid chromatography analysis of synthetic 3-deazaUMP. The reaction mixture and all column chromatography fractions were monitored by high pressure liquid chromatography on a Waters liquid chromatograph with a u.v. detector (254 nm), using a Whatman SAX anion exchange column (4.6 mm, 10 × 25 cm). Mobile phase was a linear gradient from 0.1 M NaH_2PO_4 (pH 3.3) to 0.5 M NaH_2PO_4 (pH 4.5) over 4 minutes at 1.5 ml/min. The elution times of 3-deazauridine and its 5'-monophosphate were 2.0 and 2.96 min, respectively, under these conditions.

combined and evaporated; ethanol was added to the residue. A precipitate formed [$(NH_4)_2CO_3$], which was filtered and washed with 10 ml ethanol. The combined ethanol extracts were evaporated, leaving 75 mg of the desired product as a white powder. High pressure liquid chromatography analysis of this material indicated a single pure compound (Fig. 2). Mass spectral analysis of the penta-TMS derivative of the free acid (M^+ 683) showed it to be the desired compound.

RESULTS

As reported previously [5], cytidine deaminase from human granulocytes had less affinity for ara-C as a substrate ($K_m = 8.8 \times 10^{-5}$ M) than for its physiological substrate cytidine ($K_m = 1.9 \times 10^{-5}$ M). In competition inhibition studies with cytidine or deoxycytidine as substrate, the reaction products, uridine, deoxyuridine and three uridine analogs (3-deazauridine, 5-fluorodeoxyuridine and 5-bromodeoxyuridine), competitively inhibited cytidine deaminase. The K_i values, calculated on the basis of these experiments, are given in Table 1, and indicated that 3-deazauridine was the most potent inhibitor, with a K_i of 1.5×10^{-5} M (Fig. 3). The product of ara-C deamination, uracil arabinoside, was also inhibitory, with a K_i of 2.4×10^{-5} M.

The monophosphate derivatives of uridine analogs were tested as inhibitors of human leukemic cell deoxycytidylate deaminase, the second degradative enzyme in the ara-C metabolic pathway. The 5-halogenated deoxyuridine monophosphates as well as 3-deazaUMP inhibited this enzyme (Figs. 4 and 5), with K_i values of 1.7×10^{-4} M for 3-deazaUMP, 2.3×10^{-5} M for 5-fluorodeoxyuridine monophosphate (5-FdUMP) and 4.9×10^{-5} M for 5-bromodeoxyuridine monophosphate (5-FdUMP) and 4.9×10^{-5} M for 5-bromodeoxyuridine monophosphate (5-BdUMP). These dissociation constant values exceeded the affinity of ara-CMP for the same enzyme ($K_m = 9 \times 10^{-4}$ M), and equalled or exceeded that of the physiological substrate dCMP ($K_m = 1.2 \times 10^{-4}$ M). Competitive inhibition of ara-CMP deamination by 3-deazaUMP was confirmed by incubation of enzyme with [^{3}H] ara-CMP in the presence of 3-deazaUMP.

Table 1. Inhibition of cytidine deaminase by uridine analogs*

Analog	K_i (μM)
3-Deazauridine	15
5-Bromodeoxyuridine	70
5-Fluorodeoxyuridine	134
6-Azauridine	1400
Uracil arabinoside	24
Uridine	30
Deoxyuridine	44

* Analogs in concentrations of 0.5 to 1.0 mM were added to the standard cytidine deaminase reaction mixture (see Materials and Methods) in the presence of varying concentrations of the substrate, deoxycytidine or cytidine. The K_i values were calculated as described in Materials and Methods.

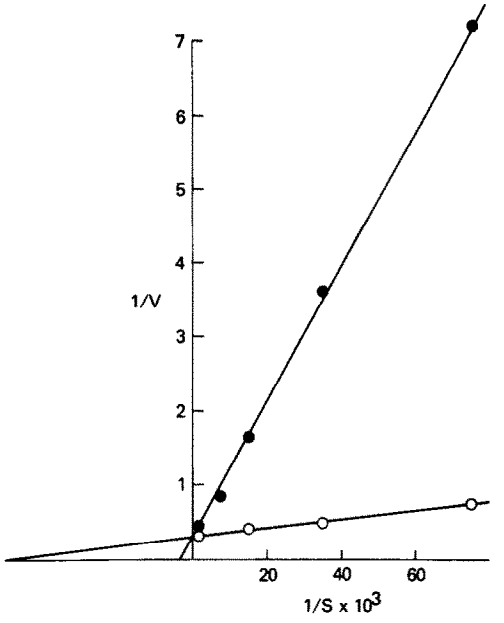

Fig. 3. Inhibition of deamination of cytidine by 3-deazauridine. The assay solution contained the indicated substrate concentrations at a specific activity of 0.04 to 0.4 μCi/μmole, 82 units of partially purified cytidine deaminase and Tris-Cl, 0.05 M, pH 7.5, all in the presence (●——●) or absence (○——○) of 3-deazauridine, 3×10^{-4} M. The assay procedure was as described previously [5].

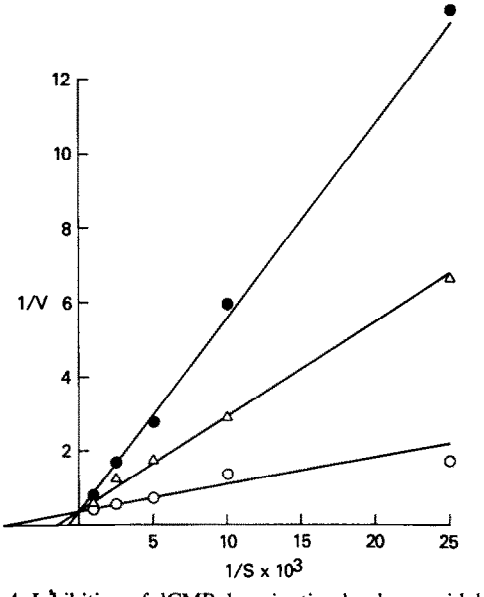

Fig. 4. Inhibition of dCMP deamination by deoxyuridylate analogs. The assay solution contained the indicated substrate concentration at a specific activity of 0.4 to 0.33 μCi/μmole, 114 units of enzyme, 4.5 mM dCTP, and 0.05 M Tris-Cl, pH 7.5. Assays also contained (○——○) no inhibitor; (△——△) BdUMP, 1.6×10^{-4} M; and (●——●) FdUMP, 1.6×10^{-4} M. Assay solutions were incubated for 15 min at 37°. The reaction was terminated by addition of 0.02 ml of 4 N HCl. The reaction contents were applied to a 0.75 × 5 cm Dowex 50 H^+ cation exchange column, and the product, [^{14}C] dUMP, was eluted with 2 ml of distilled water and counted in 18 ml Aquasol (New England Nuclear).

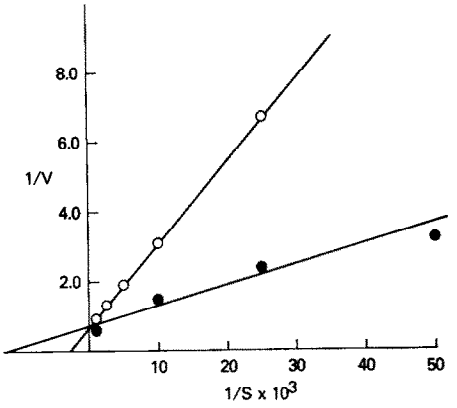

Fig. 5. Inhibition of dCMP deaminase by 3-deazaUMP. The incubation solution was the same as in Fig. 3, except for the presence of 3-deazaUMP, 4×10^{-4} M (○——○). For the curve indicated by (●——●), no inhibitor was present.

3-deazaUMP, synthesized as described in Materials and Methods, was also examined as a substrate for dCMP kinase, the enzyme which phosphorylates the natually occurring uridine and cytidine monophosphates, as well as ara-CMP. dCMP kinase affinity for 3-deazaUMP was considerably less ($K_m = 1.95 \times 10^{-3}$ M) than the affinity for ara-CMP ($K_m = 6.8 \times 10^{-4}$ M), indicating that 3-deazaUMP, at equimolar concentrations, would not compete effectively with ara-CMP at this activation step.

DISCUSSION

Cytosine arabinoside is believed to exert its cytotoxic effects through the formation of a triphosphate, ara-CTP, which inhibits DNA polymerase, and, to a limited extent, is incorporated into DNA [15]. The ability of malignant cells to form and retain the triphosphate is believed to determine the degree of ara-C cytotoxicity observed [16], although the specific enzymatic steps responsible for resistance have not been elucidated in human tumors. In human leukemic cells, the activity of cytidine deaminase, the initial degradative enzyme in the ara-C pathway, was shown to vary widely among patients [4] but the median value in 24 patients (372 units/mg protein) greatly exceeded the activity of the initial activating enzyme, deoxycytidine kinase (15 units/mg protein) [1, 6]. At the nucleotide level, enzymes which activate (dCMP kinase) and degrade (dCMP deaminase) ara-CMP are found; both occur in high concentrations in leukemic cells [7, 8], usually in excess of 1000 units/mg protein, the deaminase having a higher affinity for ara-CMP. Thus, degradative enzymes constitute an important limiting factor for leukemic cell activation of ara-C at both the nucleoside and nucleotide levels.

In the present study we have found that several uridine analogs of interest for combination therapy regimens exert inhibitory effects on the degradation of ara-C and ara-CMP. Particular attention was drawn to 3-deazauridine, an agent currently undergoing initial clinical trials. 3-Deazauridine has multiple sites of favourable biochemical interaction with ara-C, in addition to those demonstrated by the present study. It inhibits CTP synthetase [11] and thus decreases intracellular pools of dCTP [17], the substrate with which ara-CTP competes for an active site position on DNA polymerase. Recent work by Mills-Yamamoto *et al.* [18] has demonstrated synergistic interaction of ara-C and 3-deazauridine in tissue culture vs HeLa and RPMI 6410 myeloblastoid cells. Further studies showed that 3-deazauridine enhanced the formation of ara-CTP in RPMI 6410 and L1210 cells, but not in HeLa cells or in two human leukemic cell samples [19]. The explanation for this enhanced formation of ara-CTP was not apparent, although an RPMI 6410 mutant, deficient in uridine kinase, failed to show enhancement [18], suggesting that the effects were caused by a 3-deazauridine nucleotide. Considering the results of the present study, it is possible that increased ara-CTP pools found in the previous study in the presence of 3-deazauridine could be the result of inhibition of dCMP deaminase. In order to clarify the role of deaminase inhibition in the interaction of cytosine arabinoside and 3-deazauridine, it will be necessary to examine the relationship of synergism to several factors, including cytidine deaminase and dCMP deaminase activities and intracellular metabolic products derived from ara-C, in the presence and absence of 3-deazauridine.

Previous workers have noted inhibition of cytidine deaminase activity by uridine and uracil arabinoside [20], although this inhibition was not quantitated in usual kinetic terms. A more potent cytidine deaminase inhibitor, tertahydrouridine, was described by Camiener [21]. This compound is currently undergoing clinical trial in combination with ara-C and appears to potentiate markedly the toxicity of ara-C [22]. The therapeutic benefits of this combination remain to be determined. Maley and Maley [23] reported that dUMP inhibits dCMP deaminase; deoxytetrahydrouridine was found to inhibit the same enzyme in tissue culture experiments [24], presumably through formation of the active product, deoxytetrahydrouridine monophosphate. Deoxytetrahydrouridine has not been tested in combination with ara-C in chemotherapy trials. The present study demonstrates that the nucleoside monophosphates of several uridine analogs, including the deoxynucleosides of the commonly used agent, 5-fluorouracil, also inhibit this degradative step in ara-C metabolism, an interaction of potential importance in combination chemotherapy.

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