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Kinetics of deamination of 5-aza-2′-deoxycytidine and cytosine arabinoside by human liver cytidine deaminase and its inhibition by 3-deazauridine, thymidine or uracil arabinoside*

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5-Aza-2′-deoxycytidine (5-aza-dCyd) was shown to be a potent antineoplastic agent against tumor cells *in vitro* [1] and against leukemic cells in mice [2, 3]. This agent is now undergoing clinical trial at this institution in leukemic patients resistant to conventional chemotherapy [4]. Because of the rapid plasma elimination half-life seen in man [4], we were interested to study the interaction of 5-aza-dCyd with human liver cytidine deaminase and to compare it with 5-aza-cytidine (5-aza-Cyd) and cytosine arabinoside (ara-C), nucleoside analogs which are used currently for the clinical treatment of leukemias. Also, we tested the potential interference of various substances with the deamination of 5-aza-dCyd, 5-aza-Cyd or ara-C. The potential inhibitors chosen were 3-deazauridine (3-DU), thymidine (Thd), and uracil arabinoside (ara-U). 3-DU and Thd have been shown to enhance the antileukemic action of 5-aza-dCyd [5, 6] and ara-C [7, 8]. Ara-U is the deaminated catabolite of ara-C and may attain higher plasma concentrations than the parent compound, especially when high dose ara-C therapy is administered [9].

5-Aza-dCyd was obtained from Chemapol (Prague, Czechoslovakia); 5-aza-Cyd and ara-C were obtained from the Drug Development Branch, National Cancer Institute (Bethesda, MD); cytidine (Cyd), deoxyuridine and uridine were purchased from P-L Biochemicals (Milwaukee, WI); and deoxycytidine (dCyd) and Thd were purchased from Boehringer Mannheim (West Germany). 3-DU was obtained from Ash Stevens Inc. (Detroit, MI), and uracil arabinoside was purchased from Calbiochem (San Diego, CA).

Human cytidine deaminase was prepared from freshly frozen liver which was obtained at autopsy from a normal adult. The enzyme purification was done according to Wentworth and Wolfenden [10]. The final purification step yielded a specific activity of 8.2×10^{-3} units/mg protein. Protein concentrations were determined by the method of Lowry *et al.* [11]. One unit of activity was defined as that amount of enzyme required to deaminate 1 μmole cytidine/min under the following conditions. The deamination of the substrates was followed by measuring the decrease in absorbance at 291 nm for Cyd, dCyd and ara-C, whereas the deamination of 5-aza-Cyd and 5-aza-dCyd was followed at 272 nm. Kinetic measurements were also done at 247 nm for 5-aza-dCyd and gave similar results as at 272 nm. A recording Gilford DU-2 spectrophotometer was used with a thermally regulated block. Assays were conducted at 25° in 20 mM potassium phosphate buffer, pH 7.4. Changes in extinction coefficients corresponding to complete deamination of substrates were determined enzymatically from the change in absorbance after prolonged incubation with the enzyme for all substrates. In the case of Cyd, dCyd, and ara-C, a similar molar extinction coefficient was found enzymatically and with pure substrates and products. For the two 5-aza-analogues the deaminated products did not have any detectable absorbance at 272 nm. The changes in molar extinction coefficients using a 10 mm light path were: Cyd, −1297; dCyd, −1169; ara-C, −1350; 5-aza-Cyd, −904; and 5-aza-dCyd, −750.

The reaction products of the 5-aza-dCyd deamination were analyzed by TLC and high pressure liquid chromatography (HPLC). Excess enzyme and substrate were incubated at 8° during 8 hr at pH 7.4. TLC separation was achieved on Avicel cellulose plates (1000 μm thick) using the solvent system *n*-butanol–acetic acid–water (100:10:30) [12] at 4° and gave an R_f value of 0.24 for 5-aza-dCyd. HPLC separations were made after a trichloroacetic acid (TCA) precipitation of a similar reaction mixture followed by a neutralization with a concentrated phosphate buffer. An aliquot (20 μl) was injected on a C_{18} μBondapak (10 μm) reverse-phase column using a 10 mM potassium phosphate buffer (pH 6.8) as the eluent at a flow rate of 1.5 ml/min. A variable wavelength u.v. detector was used. The retention time for 5-aza-dCyd was 5 min.

The different K_m and V_{max} values for the substrates studied are shown in Table 1. It can be seen that the two natural substrates, Cyd and dCyd, showed the lowest K_m for the enzyme. Ara-C showed an intermediate K_m as compared to the natural substrates and the two 5-aza-analogues, which had the highest K_m for the human liver cytidine deaminase. On the other hand, the enzyme had a similar V_{max} for all the substrates studied, and this would indicate that, once

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Table 1. Kinetics of deamination and inhibition of pyrimidine nucleosides by cytidine deaminase from human liver*

Substrate	K_m (μM)	V_{max} (μmoles·min^{-1}·mg^{-1})	K_i (μM)		
			3-DU	Thd	Ara-U
Cyd†	12 ± 0.9‡	16.1 ± 3.0	146 ± 20	680 ± 200	1040 ± 300
dCyd	19 ± 4	14.4 ± 2.4	100 ± 20	290 ± 170	930 ± 140
Ara-C	87 ± 10	14.5 ± 3.3	80 ± 10	540 ± 270	480 ± 50
5-Aza-Cyd	216 ± 51	13.3 ± 5.1	170 ± 50	130 ± 40	180 ± 90
5-Aza-dCyd	250 ± 33	15.2 ± 3.8	210 ± 70	290 ± 110	770 ± 300

* Deamination of the substrates was followed spectrophotometrically at various wavelengths. Assays were done at 25° in 20 mM potassium phosphate buffer, pH 7.4.

† Abbreviations: Cyd, cytidine; dCyd, deoxycytidine; ara-C, cytosine arabinoside; 5-aza-Cyd, 5-aza-cytidine; 5-aza-dCyd, 5-aza-2′-deoxycytidine; 3-DU, 3-deazauridine; Thd, thymidine; and ara-U, uracil arabinoside.

‡ Mean ± S.E.; N = 3 or more determinations.

the enzyme–substrate complex is formed, the rate at which the deamination takes place is similar for the different substrates.

The kinetic values for some of the substrates were different from those reported by others [10, 13]. This may have been due to the differences in the assay conditions used and the source of the enzyme. We used a lower pH and a lower temperature in our enzyme assay to minimize the chemical decomposition of the 5-aza-cytosine nucleoside analogues. 5-aza-dCyd is very unstable at alkaline pH, especially at elevated temperatures [14].

An attempt to isolate 5-aza-2′-deoxyuridine, the reaction product of the deamination of 5-aza-dCyd, was made using excess enzyme and substrate at low temperature and neutral pH. No highly u.v. absorbing compound in the region of 230–272 nm was found, suggesting that the triazine ring structure was readily lost after deamination. The instability of the deaminated intermediate is probably due to a nucleophilic attack at the carbon 6 which is presumably facilitated by the presence of an oxygen atom on carbon 4 as it was already reported for 5-aza-uridine [15].

3-DU which may be used to enhance the effectiveness of ara-C [7] or 5-aza-dCyd [5] was shown to inhibit competitively their deamination; the K_i values for these two analogs were 80 and 210 μM respectively (Table 1). These concentrations of 3-DU are attainable *in vivo* [16] and could prolong the half-life of these drugs, thereby increasing efficacy and/or toxicity. Thd may also be used as a biochemical modulator with these drugs [6, 8]. The K_i values of Thd were 540 and 290 μM for ara-C and 5-aza-dCyd, respectively (Table 1), showing that it is a weak inhibitor of deamination. Ara-U, the catabolite product of ara-C, may attain plasma levels in the range of 350 μM when high doses of ara-C are given and its biological half-life in man is in the order of 6 hr [9]. Therefore, it was of interest to test if ara-U is inhibitory to the deamination of ara-C by human liver cytidine deaminase at these concentrations. The K_i of ara-U for the deamination of ara-C was 480 μM (Table 1). The K_i found in this study is intermediate between the one reported by Capizzi *et al.* [9] and Drake *et al.* [17], probably due to different assay systems and the tissue used to isolate cytidine deaminase.

This study demonstrated that Cyd deaminase from human liver has less affinity for 5-aza-dCyd and 5-aza-Cyd than for ara-C. Also significant inhibition of the deamination of the 5-aza-analogues and ara-C may occur *in vitro* when used in combination with high concentrations of 3-DU or Thd. These interactions are of potential clinical importance for combination chemotherapy since the effectiveness of one drug may be modulated by the inhibition of its catabolism by another drug or by its own catabolite.

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