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Resistance of Cyclocytidine to Cytidine Deaminase[1]

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Deamination of cytidine derivatives especially of cyclocytidine by mouse kidney cytidine deaminase was examined. Cyclocytidine was not deaminated at either pH 6.5 or pH 7.3 by the enzyme. Furthermore, cyclocytidine did not inhibit the deamination of aracytidine and $([I]/[S])_{0.5}$ value for cyclocytidine was over 100. As a result, cyclocytidine is found to be markedly active compound with resistance against cytidine deaminase.

Aracytidine (1-β-D-arabinofuranosylcytosine) has been widely used for acute leukemias and lymphomas,[3,4] but the compound disappears very rapidly from the blood of cancer patients, due probably to the deamination of aracytidine to arauridine (1-β-D-arabinofuranosyluracil), its inactive metabolite.[5,6] Furthermore, distribution of the nucleoside deaminase activity is different among tissues and animal species. The enzyme activity is especially high in man[5] and resistance to aracytidine in human leukemic cells is reported to be associated with increased tumor deaminase activity.[7]

In the course of studies on the antitumor activity of the derivatives of aracytidine which would be less easily deaminated and therefore possibly more effective than the mother compound, cyclocytidine (2,2'-O-cyclocytidine: 2,2'-O-anhydro-1-β-D-arabinofuranosylcytosine hydrochloride) was found as a potent antitumor agent.[8,9] In the present study, properties of cyclocytidine was enzymatically examined. Preliminary results were reported previously.[8]

Experimental

Materials——Aracytidine (1-β-D-arabinofuranosylcytosine hydrochloride), arauridine (1-β-D-arabinofuranosyluracil), araCMP (aracytidine 5'-monophosphate), araUMP (arauridine 5'-monophosphate), cyclocytidine (2,2'-O-cyclocytidine hydrochloride), cycloCMP (cyclocytidine 5'-monophosphate hydrochloride), and cyclouridine (2,2'-O-cyclouridine) were supplied by Kohjin Co., Ltd. Deoxycytidine (Sigma), deoxyuridine (Sigma), cytidine (Sigma), and uridine (Sigma) were purchased commercially.

Preparation of Mouse Kidney Cytidine Deaminase——Cytidine deaminase was prepared by the method of Tomchick, *et al.*[10] Kidneys derived from ddN mice weighing about 20 g were decapsulated and homogenized with 3 volumes of 1.15% KCl. The homogenate was then centrifuged for 60 min at $78000 \times g$ and the resulting supernatant was heated for 7 min at 60°. After cooling, the heat-denatured mixture was centrifuged for 15 min at $17300 \times g$. The supernatant was treated with solid ammonium sulfate. The precipitate obtained between 40 to 50% saturation of ammonium sulfate was centrifuged and the resulting

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pellet was dispersed in 0.02M sodium acetate buffer (pH 4.5). The suspension was stored at −80° and after thawing diluted with water prior to use. The protein concentration was determined by the method of Lowry, *el at.*[11]

Assay of Deamination——Deaminase activity was measured by the decrease of substrate remaining in the reaction mixture. Incubation mixture contained 0.6 μmoles of one of the substrates to be tested, 1.0 ml of 0.25M glycylglycine–NaOH buffer (pH 6.5) or 1.0 ml of 0.05M Na–K phosphate buffer (pH 7.3) and enzyme preparation (containing 0.87 mg protein and water to make a final volume of 3.0 ml). After incubation at 37° for 5, 10, 20, or 40 min, the reaction was terminated by the addition of 0.2 ml of 30% perchloric acid and centrifuged for 10 min at 3000 rpm. Ultraviolet (UV) spectrum was measured by a Cary 14 recording spectrophotometer and substrate remaining in the solution was determined by the shift of wave length showing absorption maximum (λ_{max}) or by the decrease of UV absorption at 290 mμ.[5] One of the calibration curves for the former method was shown in Fig. 1. Values of λ_{max} are 263, 249, 280, 263, 280, 262, 280, 262, 280, 262, and 262 for cyclocytidine, cyclouridine, aracytidine, arauridine, cytidine, uridine, deoxycytidine, deoxyuridine, araCMP, araUMP, and cycloCMP respectively.

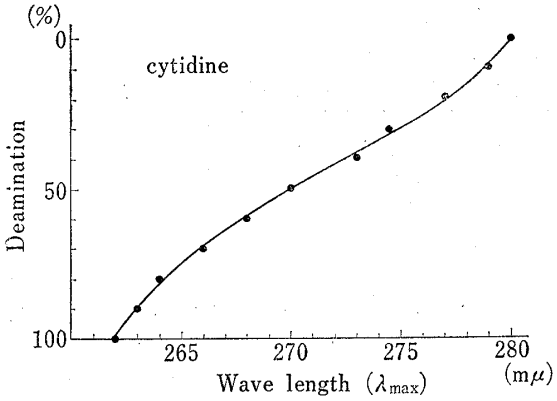

Fig. 1. Calibration Curve for the Determination of Deamination

Assay of Cytidine Deaminase Inhibition——Inhibition of the deaminase activity by each compound was measured by the decrease of substrate remaining in the reaction mixture. Incubation mixture contained 0.6 μmoles of aracytidine or other substrate, 1.0 ml of 0.25M glycylglycine–NaOH buffer (pH 6.5), one of the inhibitors to be tested at various concentrations and enzyme preparation (containing 0.87 mg protein and water to make a final volume of 3.0 ml). After incubation for 50 min (aracytidine) or 10 min (other substrate) at 37°, the reaction was terminated by the addition of 0.2 ml of 30% perchloric acid and substrate remaining in the solution was determined by the decrease of UV absorption at 290 mμ[5] using a Hitachi-Parkin Elmer 139 spectrophotometer.

Result and Discussion

Deamination of Cytidine Derivatives

Since aracytidine was deaminated rapidly to arauridine by cytidine deaminase,[5] many trials have been made for the potentiation of the activity of aracytidine by rational design of a structural modification of aracytidine which would be less easily deaminated and therefore possibly more effective.[9,12] Deamination of cytidine analogs especially of cyclocytidine which was an antitumor compound,[8,9] was examined. As shown in Fig. 2 (a), cyclocytidine was not deaminated by cytidine deaminase at the optimal pH (6.5) of the enzyme, while reference compounds were deaminated. Cyclocytidine was reported to be rather unstable in aqueous basic solution,[13,14] thus deamination of the compound under a physiological condition (pH 7.3 and 37°) was examined and it was found that cyclocytidine was also not deaminated at this pH (Fig. 2 (b)). Rate of deamination of these compounds were calculated and compared (Table I). Cyclocytidine and cycloCMP were not deaminated by the enzyme, while aracytidine and araCMP were deaminated under the same condition. Deamination of the latter compounds were one-fifth of that of cytidine for aracytidine and one-twentyth for araCMP.

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TABLE I. Deamination of Cytidine Derivatives by Cytidine Deaminase

Compound	pH 6.5 μmole/10 min (ratio, %)[a]		pH 7.3 μmole/10 min (ratio, %)[a]	
Cytidine	0.52	(100)	0.42	(100)
Deoxycytidine	0.33	(63)	0.25	(60)
Aracytidine	0.12	(23)	0.07	(17)
AraCMP	0.02	(4)	0.02	(5)
Cyclocytidine	0	(0)	0	(0)
CycloCMP	0	(0)	0	(0)

a) Deaminase activity is expressed in terms of the most active compound, cytidine, which is arbitrarily assigned a value of 100%.

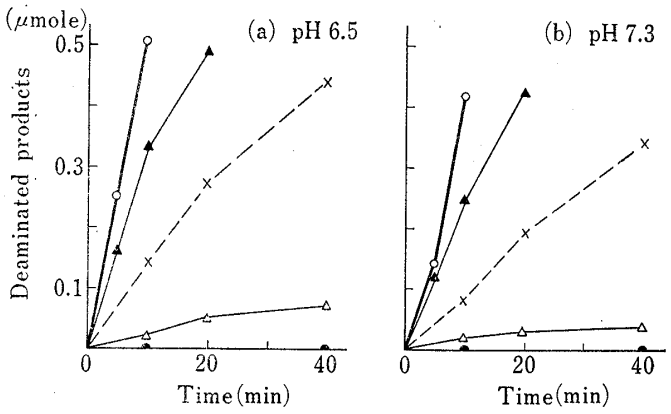

Fig. 2. Time Course of Deamination of Cytidine Derivatives by Cytidine Deaninase

: cytidine
: deoxycytidine
: aracytidine
: araCMP
: cyclocytidine
: cycloCMP

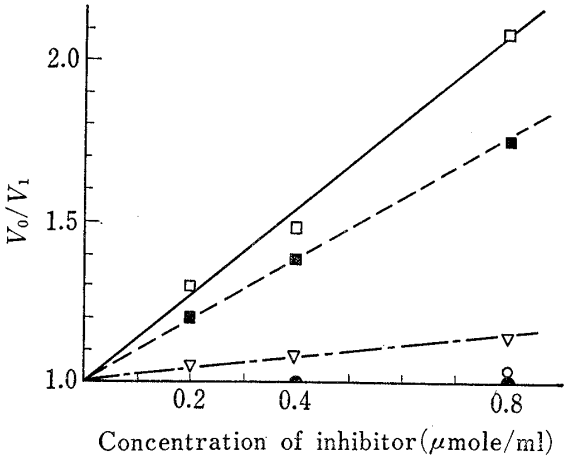

Fig. 3. Inhibition of Aracytidine Deamination

: deoxyuridine
: uridine
: arauridine
: cyclouridine
: cyclocytidine

TABLE II. Inhibition of Aracytidine Deamination by Some of the Nucleosides

Compound	$([I]/[S])_{0.5}$[a]
Deoxyuridine	3.7
Uridine	5.1
Arauridine	32
Cyclouridine	over 100
Cyclocytidine	over 100

a) Ratio of concentration of inhibitor to substrate given 50% inhibition.

Effect on Aracytidine Deamination of Cyclocytidine

Inhibition of aracytidine deamination by the compound was examined. As shown in Fig. 3, cyclocytidine did not inhibit the aracytidine deamination within 50 min incubation, while reference compounds inhibited the deamination in the same condition. Ratio of concentration of inhibitor to substrate given 50% inhibition, $([I]/[S])_{0.5}$ value, was calculated from the data in Fig. 3 as shown in Table II. That was over 100 for cyclocytidine. The compound also did not inhibit the deamination of cytidine and deoxycytidine after 10 min incubation at 37°.

As a result, cyclocytidine is found to be the desired compound with marked antitumor activity and resistance against cytidine deaminase. This compound would be active against the aracytidine resistant leukemia having high deaminase activity in man. Studies on mechanism of action and metabolism of cyclocytidine are now in progress.

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