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Inhibition by Cyclocytidine of Nucleic Acid Biosynthesis in Cultured Cells (L5178Y)[1]

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Mechanism of action of cyclocytidine was examined in cultured cells (L5178Y). Cyclocytidine was active against the leukemia *in vitro* as well as *in vivo* and its IC_{50} was 0.041 μg/ml, whereas that for aracytidine was 0.023 μg/ml. This compound was also inhibited the thymidine incorporation into DNA and its IC_{50} was 110 μg/ml, while that was 1.1 μg/ml for aracytidine. Cyclocytidine itself was therefore considered to have no direct inhibitory effect. Since cyclocytidine seems to act *in vitro* as aracytidine after transformation, the factor influencing the transformation was examined and revealed to be only pH of the aqueous solution. Ratio of the transformation under physiological conditions (pH 7.3, 37°) was about 30 and 70% after 4 and 24 hr incubations, respectively.

Cyclocytidine (2,2'-O-cyclocytidine hydrochloride) is one of the synthetic cytidine analogs, being similar to aracytidine (1-β-D-arabinofuranosylcytosine hydrochloride).[3] This compound was markedly active against L1210[4] and various mouse tumors.[5] Mechanism of actions especially concerning effects of DNA biosynthesis of cyclocytidine and transformation of the compound to aracytidine was examined in cultured cells. Preliminary results were reported previously.[6]

Experimental

Tumor System——A mouse leukemia cell line L5178Y was used. The cells were cultured in RPMI 1640 medium supplemented with 10% calf serum in a CO_2 incubator at 37°.

Determination of Antitumor Activity——Antitumor activity was determined by the ratio of cell number in treated and control groups (T/C%) after 48 hr incubation of 5×10^4 cells/ml at various concentrations of cyclocytidine or aracytidine and IC_{50} (50% inhibiting concentration) was calculated.

Determination of DNA, RNA, and Protein Synthesis——^{14}C-Labeled thymidine (0.2 μCi/ml: specific activity (s.a.) was 58 mCi/mmole), uridine (0.5 μCi/ml: s.a. was 507 mCi/mmole) and L-leucine (0.5 μCi/ml: s.a. was 331 mCi/mmole) were used as precursors. Biosynthesis was determined by the respective incorporation of ^{14}C-labeled precursors into the cold trichloroacetic acid insoluble fraction of the cells after incubation for 30 min of 3×10^5 cells/ml at various concentrations of cyclocytidine or aracytidine and IC_{50} was calculated. Radioactivity in the fraction was measured as follows: reaction mixture was chilled in an ice cold water-bath and cells in 0.4 ml medium were collected on a glass fiber disc and followed by twice washings with each 10 ml of ice cold 5% trichloroacetic acid and by drying in a oven at about 100° for 30 min. Radioactivity on the fiber disc was counted in a Packard 3320 liquid scintillation counter using toluene-PPO-dimethylPOPOP scintillator system.

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3) T. Kanai, T. Kojima, O. Maruyama, and M. Ichino, *Chem. Pharm. Bull.* (Tokyo), **18**, 2569 (1970); K. Kikugawa and M. Ichino, *J. Org. Chem.*, **37**, 284 (1972).
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Detection of Metabolites by Thin-Layer Chromatography——Silicagel F_{254} precoated plates "Merck" were used and a solvent system, *n*-propanol–tetrahydrofurfuryl alcohol–acetic acid (40: 20: 1), was applied. The sample of 5 μl was spotted on the plate and developed ascendingly with the solvent. Reference compounds and metabolites on the plate were detected by ultraviolet (UV) absorption for the measurement of *Rf* values. λ_{max} in UV spectrum of the compound was determined by Cary-14 spectrophotometer after eluting the spot with 3 ml of 0.1N HCl.

Determination of Stability in Aqueous Solution of Cyclocytidine——Cyclocytidine was dissolved in 0.1N HCl and diluted with 50 volumes of 1/15N phosphate buffer at pH 6.8 to 8.0 and incubated for 4 or 24 hr at 37°. Concentrations of cyclocytidine and aracytidine were measured by the shift of λ_{max} in acidic condition after dilution with 10 volumes of 0.1N HCl. λ_{max} of the compounds were 280 mμ for cyclocytidine and 263 mμ for aracytidine.

Result

Antitumor Activity of Cyclocytidine *in Vitro*

Since cyclocytidine was considered to be a transport form of aracytidine in chemical nature, antitumor activity of the compound *in vitro* will furnish a key for the solution of problems in the studies on the mechanism of action and on the metabolism of the compound. Cyclocytidine inhibited the growth of L5178Y leukemia *in vitro* as well as *in vivo* and its IC_{50} was 0.041 μg/ml, whereas that for aracytidine was 0.023 μg/ml. Growth inhibition at various concentrations of the compounds was shown in Fig. 1. The inhibition lines are parallel to each other. Potency of cyclocytidine *in vitro* was almost one-half of that of aracytidine and similarity of the mechanism of action in the two compounds will be suggested from this parallelism.

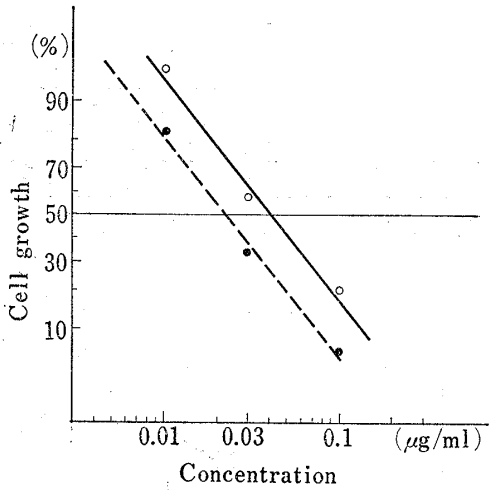

Fig. 1. Growth Inhibition by Cyclocytidine and Aracytidine

——: cyclocytidine
------: aracytidine

Inhibition of Biosynthesis of DNA, RNA, and Protein

Thymidine incorporation into acid insoluble fraction was inhibited by cyclocytidine at the concentration of over 10 μg/ml, whereas incorporation of other two precursors was not inhibited under the same conditions (Table I). Incorporation of uridine was inhibited at 1000 μg/ml and that of L-leucine was still not inhibited at the high concentration. Aracytidine also inhibited the incorporation of thymidine. Inhibition of thymidine incorporation of the two compounds was shown in Fig. 2. Potency of the inhibition of cyclocytidine was one-handredth of that of aracytidine, being

Table I. Effect of Cyclocytidine on Incorporation of ^{14}C-Labeled Precursors

	Concentration (μg/ml)	Incorporation ratio (%)		
		Thymidine	Uridine	L-Leucine
Cyclocytidine	1000	23	67	99
	100	46	99	97
	10	83	101	100
	1	93	95	103
	0.1	101	102	103
Aracytidine	1000	—	39	97
	100	12	82	96
	10	23	87	97
	1	47	85	95
	0.1	79	91	86

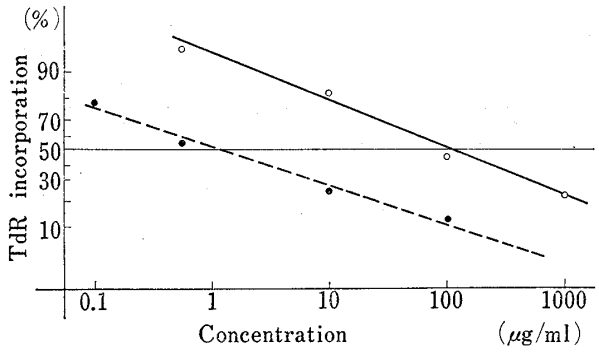

Fig. 2. Effect of Cyclocytidine on Thymidine Incorporation

——: cyclocytidine ------: aracytidine

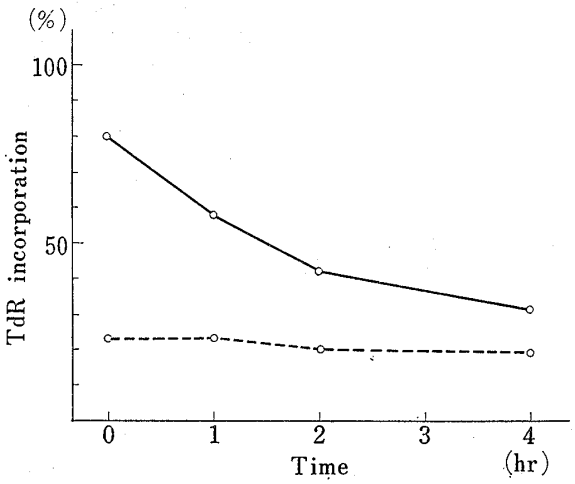

Fig. 3. Changes of Activity of Cyclocytidine as a Function of Time

—○—: cyclocytidine ---○---: aracytidine

110 μg/ml of IC_{50} for cyclocytidine and 1.1 μg/ml for aracytidine. According to the difference in these two values, cyclocytidine itself was considered to have no direct inhibitory effect.

Transformation of Cyclocytidine to Aracytidine

In view of the above facts, it seemed that cyclocytidine acts as aracytidine after transformation. The inhibitory activity of cyclocytidine dissolved in RPMI 1640 medium increased with time, while the activity of aracytidine did not change under the same conditions as shown in Fig. 3. The ratio of transformation to aracytidine was 32% after 4 hr incubation (pH 7.3, 37°) when determined by inhibition of thymidine incorporation.

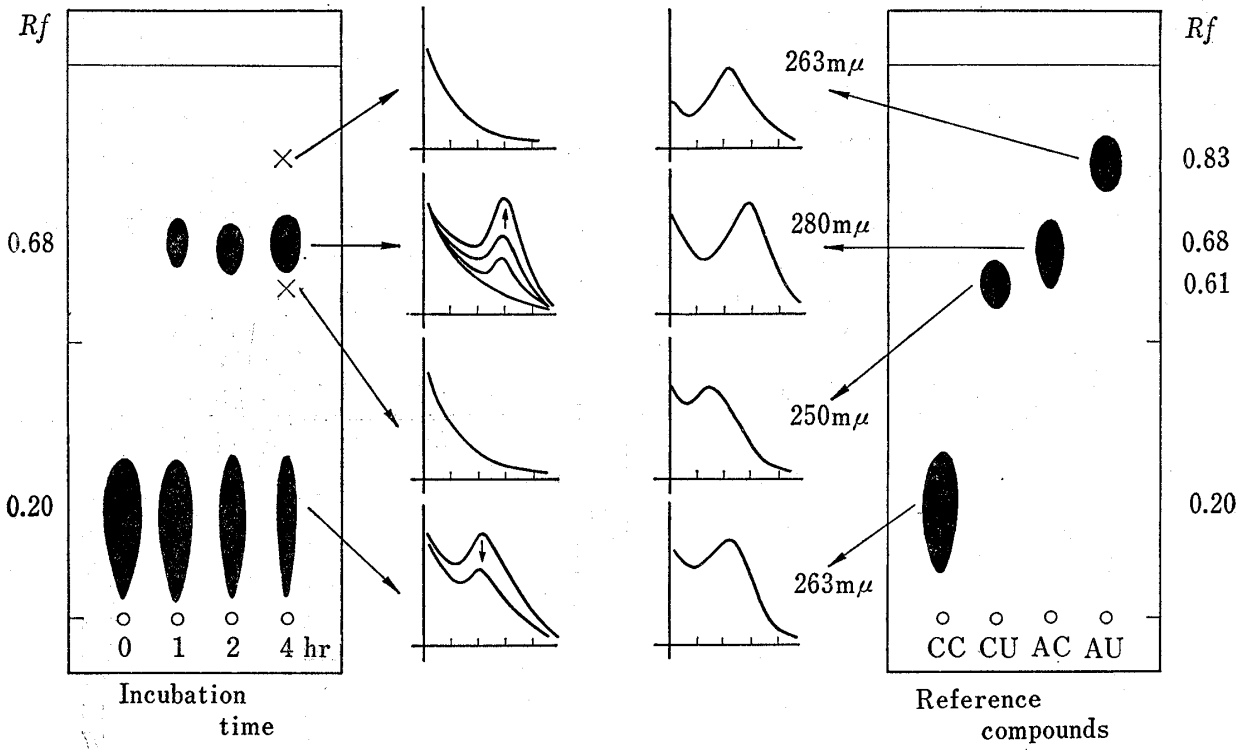

Fig. 4. Chromatograms and UV Spectra of the Metabolites of Cyclocytidine and Reference Compounds

CC: cyclocytidine; CU: cyclouridine; AC: aracytidine; AU: arauridine

Metabolites of Cyclocytidine

Metabolites of cyclocytidine after incubation for 4 hr at 37° in the medium without leukemic cells were examined by thin-layer chromatography. As shown in Fig. 4, a spot was appeared on the chromatogram at *Rf* 0.68 after 1 hr and UV absorption of the spot was increased with time and other spots were not appeared. *Rf* values of the reference compounds were 0.20 for cyclocytidine, 0.61 for cyclouridine, 0.68 for aracytidine, and 0.83 for arauridine. λ_{max} in UV spectrum of the reference compounds in acidic condition was also determined, and those were 263 mμ for cyclocytidine, 280 mμ for aracytidine, 250 mμ for cyclouridine, and 263 mμ for arauridine.

The spot of *Rf* 0.68 was identified by UV spectrum as aracytidine. As the result, cyclocytidine seems to be transformed to only aracytidine in medium. Ratio of transformation were 27 and 76% after 4 and 24 hr incubation respectively. Cyclouridine and arauridine were not found on the chromatograms.

Effect of pH on Transformation of Cyclocytidine

Stability of cyclocytidine in aqueous solution at 37° was examined for detection of the factors influencing transformation. As shown in Fig. 5, the ratio of transformation to aracytidine was increased as a function of pH and about 50% of the compound was transformed for 4 hr at pH 7.8 or 24 hr at pH 7.0. Under physiological conditions (37°, pH 7.3), ratio of transformation was 27 and 71% after 4 and 24 hr incubation respectively.

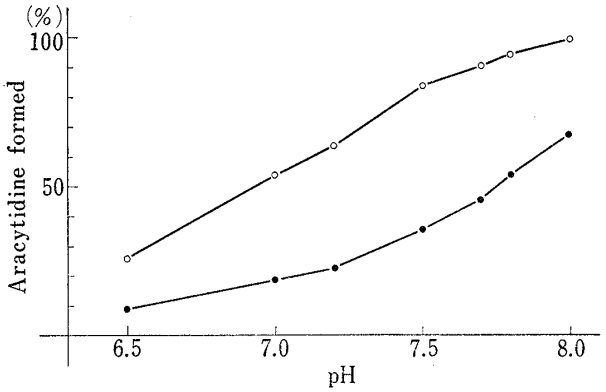

Fig. 5. Effect of pH on Transformation of Cyclocytidine to Aracytidine

—●—: after 4 hr; —○—: after 24 hr

It is concluded that cyclocytidine shows a marked antitumor activity *in vitro* when determined by growth rate of cells, as well as *in vivo* through the specific depression of DNA biosynthesis similar to aracytidine. However, this inhibition seems to be due to aracytidine transformed from cyclocytidine instead of cyclocytidine itself. Furthermore, ratio of the transformation in physiological conditions (pH 7.3, 37°) was about 30 and 70% after 4 and 24 hr respectively.

Discussion

Cyclocytidine exhibited a marked activity against L1210 leukemia with low toxicity and resistance to the nucleoside deaminase.[4] This compound was active not only against leukemias but also various tumors.[5] However, it was inactive against an aracytidine-resistant subline of L1210.[7] Influence of route of administration on the activity was less in cyclocytidine than in aracytidine, thus cyclocytidine was markedly active against L1210 by oral treatment.[7,8]

As for the mechanism of action of cyclocytidine, the following possibilities are suggested: (a) cyclocytidine itself acts similarly to aracytidine, (b) cyclocytidine acts after transformation to aracytidine, and (c) both of them. Activity of cyclocytidine itself *in vitro* will furnish a key for the solution of problems in the studies on the mechanism of action.

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In the present experiment, cyclocytidine scarcely inhibits the thymidine incorparation into DNA, though it is active against leukemic cells *in vitro* when determined by growth rate of the cells. Both experiments were performed under the same conditions except duration of incubation. The possibility (b) seems to be the most reasonable from the results obtained by the experiments *in vitro*. The factors influencing the transformation is examined, but the components of medium such as calf serum, amino acids, sugars, salts *etc.* are not the factors. The factor may be only pH of aqueous solution. In other words, transformation of cyclocytidine to aracytidine is influenced by physicochemical conditions and not by biochemical conditions including enzymes.

Site and rate of transformation to active form *in vivo* are the next problems and studies on inhibition of enzymes such as DNA polymerase in cell free systems and on metabolites in urine are in progress.