Biochemical Pharmacology, Vol. 22, pp. 2829–2834. Pergamon Press, 1973. Printed in Great Britain.

EFFECT OF PHOSPHORYLATED CYCLOCYTIDINE ON DEOXYRIBONUCLEIC ACID POLYMERASE*

AKIO HOSHI, FUMIHIKO KANZAWA, KAZUO KURETANI, TADASHI KANAI† and MOTONOBU ICHINO†

Pharmacology Division, National Cancer Center Research Institute, Tsukiji 5-1-1, Chuo-ku, Tokyo 104, Japan

(*Received* 13 *February* 1973; *accepted* 4 *May* 1973)

Abstract—Since cyclocytidine primarily inhibits the incorporation of thymidine into DNA of cultured cells, the inhibition of DNA polymerase by some cyclocytidine derivatives was examined. Cyclocytidine 5′-monophosphate (cycloCMP) and aracytidine 5′-monophosphate (araCMP) markedly inhibited DNA polymerase, whereas cyclocytidine and aracytidine did not inhibit the enzyme in cell-free systems. Inhibitory potency of cycloCMP was about 1/15th that of araCMP. The possibility of phosphorylation of cyclocytidine to cyclo CMP was also examined, and it was revealed that cyclocytidine was phosphorylated at a rate 1/7th that of aracytidine or 1/10th that of deoxycytidine.

CYCLOCYTIDINE‡ is markedly active against various mouse tumors,[1–4] and is also active against the L1210 leukemia by oral treatment.[4,5] Investigation of the mechanism of action of cyclocytidine indicated that the compound inhibits primarily DNA biosynthesis after transformation to aracytidine *in vitro*.[6] The sites of action of aracytidine have been reported to be DNA polymerase[7–9] and ribonucleotide reductase,[10] with the former being considered the most important for antitumor activity.[7,8] In the present work the mechanism of inhibition of DNA biosynthesis by cyclocytidine was examined in cell-free systems.

MATERIALS AND METHODS

Materials. Cyclocytidine,[11] aracytidine,[11] cycloCMP,[12] araCMP[12] and araU[13] were synthesized by the authors. CdR, dATP, dGTP, dCTP, creatine phosphate, phosphoenol pyruvate, creatine kinase and pyruvate kinase were purchased from Boehringer Manheim; calf-thymus DNA was obtained from Worthington Biochemical Corp. and ^{3}H-TTP (98·5% pure) from New England Nuclear.

Preparation of DNA polymerase. Crude enzyme was prepared by the method of Kimball *et al.*[14] Seven-day implants of L1210 leukemia cells were suspended in ice-

* A preliminary report of this investigation was presented at the 31st Annual Meeting of the Japanese Cancer Association, Nagoya, October, 1972.

† On leave of absence from the Research Laboratory, Kohjin Co., Ltd., Saiki, Oita 876, Japan.

‡ Abbreviations used: cyclocytidine (cyclo-C) = 2,2′-*O*-cyclocytidine hydrochloride or 2,2′-anhydro-1-β-D-arabinofuranosylcytosine hydrochloride; aracytidine (ara-C) = 1-β-D-arabinofuranosylcytosine hydrochloride; araCMP = aracytidine 5′-monophosphate; araCDP = aracytidine 5′-diphosphate; araCTP = aracytidine 5′-triphosphate; cycloCMP = cyclocytidine 5′-monophosphate; cycloCDP = cyclocytidine 5′-diphosphate; cycloCTP = cyclocytidine 5′-triphosphate; araU = 1-β-D-arabinofuranosyluracil; CdR = 2′-deoxycytidine.

cold saline containing heparin (1000 i.u./100 ml), centrifuged for 5 min at 1000 *g*, and the supernatant was removed. The cells were resuspended in 3 vol of cold 0·1 M Tris–HCl buffer (pH 7·0); leukemic cells were then collected by centrifugation for 5 min at 1000 *g*. The washed cells were suspended in 4 vol. of cold distilled water, allowed to swell for 10 min, and homogenized in a Teflon homogenizer with 20 passes of the pestle. The mixture was immediately made 1 mM with respect to mercaptoethanol and 5 mM Tris-HCl buffer (pH 7·0). The nuclei and cell debris were removed by centrifugation for 60 min at 100,000 *g* and the supernatant fluid was stored at −80°. The protein concentration was determined by the method of Lowry *et al.*[15]

Assay of DNA polymerase activity. Enzyme activity was measured by the incorporation of ^{3}H-TTP into an acid-insoluble product, presumably DNA, according to the method of York and LePage.[16] The incubation mixture (0·75 ml) contained 0·05 ml of 1 M Tris–HCl buffer (pH 7·0), 0·05 ml of 0·04 M mercaptoethanol, 0·05 ml of 0·12 M $MgCl_2$, 0·05 ml of 0·1 M creatine phosphate, 0·04 ml creatine kinase (1·5 mg/ml), 0·025 ml of 0·05 M ATP, 0·05 ml of an 0·0004 M mixture of equivalent amounts of the deoxyriboside triphosphates of guanine, adenine and cytosine, 0·1 ml of heat-denatured calf-thymus DNA (1 mg/ml), 0·1 ml of 0·0001 M ^{3}H-TTP (0·1 μmole/ml), 0·1 ml of an inhibitor (0·0001 to 0·5 M) and 0·1 ml of cell-free extract (containing 1·8 mg protein) and water to make 0·75 ml. After incubation for 20 min at 37°, 5·0 ml of cold 5% perchloric acid was added. After standing for 10 min in an ice-cold water bath, the precipitate was collected on a glass-fiber disk, which was washed twice with 10 ml each of 5% perchloric acid and twice with 10 ml each of distilled water. Radioactivity on the disk was counted in a Packard 3320 liquid scintillation counter using toluene–PPO (2,5-diphenyloxazole)–dimethylPOPOP [1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene] as the scintillator system.

Preparation of nucleoside kinase. Crude enzyme was prepared by the method of Schrecker.[17] The washed cells, collected in a manner similar to that used for the preparation of DNA polymerase, were suspended in an equal volume of 0·02 M mercaptoethanol, and kept in an ice-cold water bath for 10 min. After addition of 1 M Tris–HCl buffer, pH 7·0 (5 per cent of the total volume), the mixture was frozen (−60°) and thawed rapidly three times, and then centrifuged at 100,000 *g* for 45 min. The supernatant fraction was used immediately in the enzyme assays or stored at −80°. The protein concentration was determined by the method of Lowry *et al.*[15]

Assay of nucleoside kinase activity. Enzyme activity was measured by the production of radioactive nucleotides from ^{14}C-labeled nucleosides (CdR, cyclocytidine and aracytidine).[18] The reaction mixture (0·5 ml) contained 3 mM ATP, 3 mM $MgCl_2$, 9 mM KF, 3 mM phosphoenol pyruvate, 0·05 mg pyruvate kinase, 10 mM mercaptoethanol, 50 mM Tris–HCl buffer (pH 7·0), 0·1 mM radioactive nucleoside, and cell-free extract (containing 6·7 mg protein) and water to make 0·5 ml. After incubating the mixture at 37° for 15 min, the reaction was terminated by heating at about 100° for 2·5 min. The reaction mixture was diluted with water to 5 ml and centrifuged at 1000 *g* for 10 min to remove the denatured protein. The supernatant was permitted to flow by gravity through a 2·5-cm disk of DEAE cellulose that had been previously washed twice with 2 ml of 0·1 N HCl and 15 ml water.[19] The disk was washed twice with water (15 ml each), dried, placed in a scintillation vial containing 10 ml of the scintillator, and radioactivity was determined in the same way as for the assay of DNA polymerase.

RESULTS

Inhibition of DNA polymerase. Cyclocytidine was active against L5178Y leukemia *in vitro*[6] as well as against L1210 leukemia *in vivo*.[1] The compound inhibited specifically the incorporation of thymidine into DNA in a manner similar to aracytidine. The effect of cyclocytidine in comparison with aracytidine on DNA polymerase was therefore examined.

As shown in Table 1, cyclocytidine and aracytidine scarcely inhibited the ^{3}H-TTP incorporation into the acid-insoluble fraction. The ratio of inhibitor to substrate

TABLE 1. INHIBITION OF DNA POLYMERASE BY CYCLOCYTIDINE DERIVATIVES

Concn of inhibitor (μmoles/0·75 ml)	Inhibitor			
	Cyclocytidine	Aracytidine	CycloCMP	AraCMP
50	67*	49		
25	73	58		
10	97	68		
2·5		78		
0·50			50	
0·25			62	
0·10			72	
0·050				35
0·025				49
0·010				69
0	100	100	100	100

* Per cent incorporation of ^{3}H-TTP by L1210 DNA polymerase into acid-insoluble product. Concentration of substrate (^{3}H-TTP) was 0·010 μmole/0·75 ml.

concentration giving 50 per cent inhibition, the $([I]/[S])_{0.5}$ value, was over 100 for each compound (Table 2). Both compounds were therefore considered to have no

TABLE 2. INHIBITION OF DNA POLYMERASE BY CYTIDINE DERIVATIVES

Compound	$([I]/[S])_{0.5}$*
Cyclocytidine	over 100 (ca 8500)
Aracytidine	over 100 (ca 4000)
CycloCMP	40
AraCMP	2·6

* Ratio of inhibitor [I] to substrate [S] concentrations giving 50 per cent inhibition. Reaction mixture was incubated for 20 min at 37°. Concentration of substrate (^{3}H-TTP) was 0·010 μmole/0·75 ml.

inhibiting activity against the enzyme, although they inhibited cell growth. Since nucleoside kinase is the rate-limiting enzyme in the phosphorylation of nucleosides such as uridine in cell-free systems,[20] the effect of monophosphates of the two compounds cycloCMP and araCMP, on the enzyme was examined. As shown in Fig. 1,

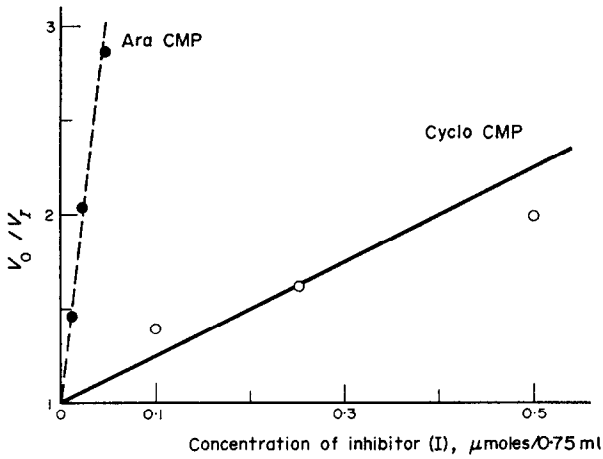

FIG. 1. Inhibition of DNA polymerase by nucleotides. The reaction mixture was incubated for 20 min at 37°. The concentration of substrate (^{3}H-TTP) was 0·010 μmole/0·75 ml.

both of them markedly inhibited DNA polymerase. The $([I]/[S])_{0.5}$ values were 40 for cycloCMP and 2·6 for araCMP (Table 2). Inhibitory potency of cycloCMP was about 1/15th that of araCMP.

Phosphorylation of cyclocytidine. Since cycloCMP was a potent inhibitor of DNA polymerase, while cyclocytidine itself was not in a cell-free system, phosphorylation of cyclocytidine by nucleoside kinase was examined in comparison with that of aracytidine. As shown in Table 3, cyclocytidine was phosphorylated at 1/7th the rate of aracytidine and 1/10th that of CdR.

TABLE 3. RATE OF PHOSPHORYLATION OF CYCLOCYTIDINE*

Compound	Nucleotide formed (nmoles/15 min)
Cyclocytidine	0·50
Aracytidine	3·51
CdR	5·66

* Reaction mixture was incubated for 15 min at 37°. Concentration of substrate (^{14}C-labeled nucleoside) was 50 nmoles/0·5 ml. Nucleotide formed was determined after adsorption to the DEAE-cellulose disk.

DISCUSSION

In previous reports the mechanism of action of cyclocytidine was examined in intact cells. It was found that cyclocytidine was active against L5178Y leukemia cells *in vitro*, as well as against L1210 leukemia *in vivo*, and that cyclocytidine seemed to act like aracytidine after transformation.[6] Furthermore, cyclocytidine primarily inhibited the incorporation of thymidine into DNA in a manner similar to aracytidine.[6] In the present work, cycloCMP was found to be a potent inhibitor of DNA polymerase, but cyclocytidine itself was not; therefore, phosphorylation of the nucleoside was considered to be the most important event for the biochemical action of this inhibitor to be expressed.

Though the rate of phosphorylation of nucleosides was relatively slow in cell-free systems, that of the conversion of the mononucleotide to the triphosphate was rapid.[21] Furthermore, the triphosphate of acacytidine was accumulated in the cells during incubation of intact cells,[22] and the triphosphate was considered to be the proximate active form of aracytidine.[9,23] Cyclocytidine and cycloCMP are chemically rather unstable in aqueous basic solution and they are transformed to their corresponding aracytidine derivatives. The triphosphate level of the pyrimidine nucleoside is, in contrast, unstable in aqueous acidic solution and is transformed to the corresponding monophosphate. The proximate active form of cyclocytidine could not be determined in the present experiments; however, it is considered to be either araCTP or cycloCTP. The latter compound is extremely labile and, therefore, was not available for testing. The rate of transformation of cyclocytidine to aracytidine or of cycloCMP to araCMP* was very slow; thus, about 30 per cent conversion occurred in both cases after 4 hr of incubation under physiological conditions (pH 7·3 and 37°).[6]

On the other hand, the biological half-life of the plasma concentration of cyclocytidine in mice was about 20 min.* Further, cyclocytidine was excreted intact mainly in urine when administered intraperitoneally[1] or intraveneously* in mice. Acute toxicity and cumulative toxicity of cyclocytidine were lower than those of aracytidine.[1,2,24] In view of the above findings, the possible primary pathway of the metabolism of cyclocytidine in tumor cells may be phosphorylation to cycloCMP and not transformation to aracytidine *in vivo*, unlike *in vitro* (Fig. 2). The difference in the activity and toxicity between cyclocytidine and aracytidine may be due to such

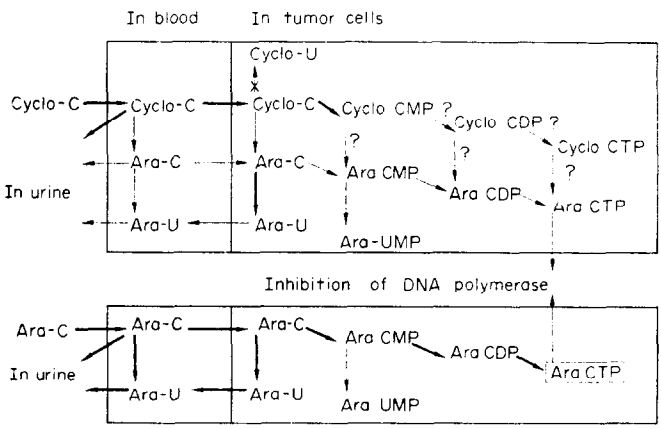

FIG. 2. Possible main pathways of metabolism of the compounds.

a difference in their metabolism as well as in their stability under physiological conditions. Cyclocytidine is therefore considered to be not a simple transport form of aracytidine but a transport form of araCTP with low toxicity and resistance to cytidine deaminase.

* Unpublished data.

REFERENCES

1. A. HOSHI, F. KANZAWA, K. KURETANI, M. SANEYOSHI and Y. ARAI, *Gann* **62,** 145 (1971).
2. A. HOSHI, F. KANZAWA and K. KURETANI, *Gann* **63,** 353 (1972).
3. W. NAKAHARA and R. TOKUZEN, *Gann* **63,** 379 (1972).
4. J. M. VENDITTI, M. C. BARATTA, N. H. GREENBERG, B. J. ABBOTT and I. KLINE, *Cancer Chemother. Rep.* **56,** 483 (1972).
5. A. HOSHI, F. KANZAWA and K. KURETANI, *Gann* **63,** 279 (1972).
6. A. HOSHI, M. YOSHIDA, F. KANZAWA, K. KURETANI, T. KANAI and M. ICHINO, *Chem. pharm. Bull., Tokyo* **20,** 2286 (1972).
7. A. INAGAKI, T. NAKAMURA and G. WAKISAKA, *Cancer Res.* **29,** 2169 (1969).
8. J. J. FURTH and S. S. COHEN, *Cancer Res.* **28,** 2061 (1968).
9. F. L. GRAHAM and G. F. WHITMORE, *Cancer Res.* **30,** 2636 (1970).
10. M. Y. CHU and G. A. FISCHER, *Biochem. Pharmac.* **11,** 423 (1962).
11. T. KANAI, T. KOJIMA, O. MARUYAMA and M. ICHINO, *Chem. pharm. Bull., Tokyo* **18,** 2569 (1970).
12. T. KANAI, M. ICHINO, A. HOSHI and K. KURETANI, *Tetrahedron Lett.* **1971,** 1965 (1971).
13. Y. FURUKAWA and M. HONJO, *Chem. pharm. Bull., Tokyo* **16,** 2286 (1968).
14. A. P. KIMBALL, B. BOWMAN, P. S. BUSH, J. HERRIOT and G. A. LEPAGE, *Cancer Res.* **26,** 1337 (1966).
15. O. H. LOWRY, N. J. ROSENBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193,** 265 (1951).
16. J. L. YORK and G. A. LEPAGE, *Can. J. Biochem. Physiol.* **44,** 19 (1966).
17. A. W. SCHRECKER, *Cancer Res.* **30,** 632 (1970).
18. N. B. FURLONG, *Analyt. Biochem.* **5,** 515 (1963).
19. R. L. MOMPARLER and G. A. FISCHER, *J. biol. Chem.* **243,** 4298 (1968).
20. E. P. ANDERSON and R. W. BROCKMAN, *Biochim. biophys. Acta* **91,** 380 (1964).
21. Y. KOZAI and Y. SUGINO, *Cancer Res.* **31,** 1376 (1971).
22. A. W. SCHRECKER and M. J. URSHEL, *Cancer Res.* **28,** 793 (1968).
23. R. L. MOMPARLER, *Biochem. biophys. Res. Commun.* **34,** 465 (1969).
24. H. HIRAYAMA, K. SUGIHARA, K. WAKIGAWA, M. IWAMURA, J. HIKITA and H. OHKUMA, *Pharmacometrics* **6,** 1255 (1972).