[Chem. Pharm. Bull. 23(3) 586—591 (1975)]

UDC 547.853.3.09 : 615.27.015.4

Metabolites of 5-^{3}H-Cyclocytidine in Monkeys Given Single Intravenous Injection

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(Received August 17, 1974)

Newly synthesized 5-^{3}H-cyclocytidine was injected intravenously in rhesus monkeys having a high level of cytidine deaminase which inactivates aracytidine, an antitumor substance analogous to cyclocytidine, in human plasma and tissues. After rapid distribution as intact molecule in the liver, kidney, spleen, and other organs, 46.5% of the administered radioactivity was excreted *via* the renal pathway within 160 min. Metabolite analysis of 5-^{3}H-cyclocytidine in plasma, tissues, and urine of the monkeys revealed that extensive or rapid degradation of cyclocytidine did not occur, and confirmed the resistance of cyclocytidine against the deaminase activity and its stability in biological condition *in vivo*. Phosphorylated derivatives of cyclocytidine were also detected in the monkey liver after injection.

The new antileukemic agent, cyclocytidine (2,2′-anhydro-1-β-D-arabinofuranosylcytosine hydrochloride) was synthesized from cytosine in a good yield by Kanai, *et al.*[2] and by Kikugawa and Ichino.[3] It has been reported that the compound had a marked antitumor activity against various experimental tumors.[4] Its clinical activity and low toxicity was ascertained by phase I study.[5] Cyclocytidine has a very low toxicity in experimental animals compared to aracytidine (1-β-D-arabinofuranosylcytosine hydrochloride).[6] Aracytidine disappears very rapidly from the blood of cancer patients,[7] due to its deamination to a biologically inactive product, arauridine, whereas cyclocytidine is substantially resistant to cytidine deaminase and other metabolic enzymes, both *in vitro*[8] and *in vivo*.[9]

Distribution of the cytidine deaminase activity is different among tissues and animal species,[7] and especially high level of plasma deaminase in macaca monkeys are compared favorably with that of man. Metabolic fate of cyclocytidine in macaca monkeys and other

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animals was previously examined by spectrophotometric assay, but minor metabolites in plasma and organs could not be detected because of its limit of sensitivity.[9]

In the present experiment we have investigated the metabolites of cyclocytidine in plasma and tissues of rhesus monkeys, incorporation of cyclocytidine into cellular components and phosphorylated compounds in tissues, using newly synthesized 5-^{3}H-cyclocytidine.

Experimental

Animals——Rhesus monkeys (*Macaca mulatta*) weighing 1.6—2.2 kg were used. Animals were purchased from Kyudo Co., Ltd. (Kumamoto) and commercial diet and water were given freely.

Materials——5-^{3}H-Cyclocytidine was prepared at Daiichi Chemical Co., Ltd. (Tokyo) by the method of Kanai and Ichino (unpublished method). The material obtained had a specific activity of 52.3 mCi/mmole and its radiochemical purity was over 97%. The specific activity was adjusted to 2 μCi/mg cyclocytidine immediately before injection.

Unlabelled cyclocytidine, aracytidine, arauridine, aracytidine 5′-monophosphate (ara-CMP), and cyclocytidine 5′-monophosphate (Cyclo-CMP) were prepared by one of the authors (T.K.).

Dowex 50 W×4, Dowex 1×8 (Dow Chemical, U.S.A.) and Toyo Roshi No. 51A filter paper were used for chromatography. 5′-Nucleotidase (from *Crotulus adamanteus venum*) and alkaline phosphatase (from calf intestine) were purchased from The British Drug House (England) and Boehringer Mannheim (West Germany), respectively.

Administration of 5-^{3}H-Cyclocytidine and Preparation of Samples——Rhesus monkeys were anesthetized with ether and a glass canula was inserted into urinary bladder for continuous collection of urine. Then, 5-^{3}H-cyclocytidine was injected into the median antebrachial vein at a dose of 200 μCi/100 mg/kg. At 10, 20, 80, and 320 min after the administration, Ringer solution was perfused *via* junglar vein to remove circulating blood. The abdomen was opened immediately after clarification of perfusate. Then, visceral organs and tissues were removed and weighed individually. Urine and heparinized blood were collected at appropriate intervals before perfusion. These samples were treated as described below and subjected to assay of metabolites.

Plasma was separated from the heparinized blood by centrifugation at 3000 rpm for 15 min in a cold room (4—6°) and deproteinized with cold trichloroacetic acid at the final concentration of 4%.

A portion of tissues was minced and homogenized with 3—4 volumes of 4% cold trichloroacetic acid by a cutting-blade homogenizer in an ice-cold bath and the supernatant was centrifuged off. Urine was also treated with 4% trichloroacetic acid.

Determination of Metabolites——Metabolites were analyzed as described by Hirayama, *et al.*[9] Metabolites were separated by ion exchange resin chromatography, followed by paper chromatography. Samples of 10^5—10^6 dpm equivalent of the extracts were applied to each column.

Preparation of Subcellular Fractions of Liver and Kidney——A portion of the liver and kidney dissected 40 min after injection was homogenized with 10 volumes of 0.25 M sucrose and 0.4 M sucrose containing 0.1 mM EDTA, respectively. The $1000\times g$ precipitate of the homogenate was referred to as the nuclear fraction. Crude mitochondrial fraction was obtained by centrifugation ($1000\times g$, 20 min) of the supernatant from low-speed centrifugation. The radioactivity in these three fractions was measured.

Separation of Nucleosides and Nucleotides——Concentrated arauridine fraction obtained from column chromatography was adjusted to pH 6.0 with 0.1 N NaOH and passed through a column (1.2×14 cm) of Dowex 1×8 (100—200 mesh, formate form) after addition of ara-CMP and cyclo-CMP as the marker . Cyclo-CMP fraction was eluted with 50 ml of distilled water. Then ara-CMP fraction was eluted with 80 ml of 0.1 M formic acid. After concentration *in vacuo* these nucleotide fractions were subjected to an ascending paper chromatography with a solvent system of 95% ethanol and 1 M NH_4OCOCH_3 containing 0.1% EDTA (70: 30, v/v) pH 5.0, for 16 hr. These nucleotide fractions from column chromatography were digested with alkaline phosphatase and 5′-nucleotidase as follows: the incubation mixture contained 0.1 ml of the condensate, 0.4 ml of 50 mM Tris buffer (pH 7.2), and 0.1 ml of the enzyme solution (100 μg/ml). After incubation at 37° for 30 min, the reaction was terminated with 0.1 ml of 0.1 N AcOH. After centrifugation, 0.4 ml of the supernatant was applied to paper chromatography.

Determination of Radioactivity——Radioactivity of all the samples was counted in a Horiba Model LSC-501 liquid scintillation counter. Aliquots of 0.1 or 0.2 ml of the extract were diluted with a scintillator consisting of 0.4% PPO (2,5-diphenyloxazole), 0.02% POPOP (1,4-bis[2-(4-methyl-5-phenyl-oxazolyl)]-benzene), 10% MeOH, 6% naphthalene, and 2% ethylene glycol in dioxane. Section of 1×2 cm of the paper chromatogram was placed in the counting vial and its radioactivity was counted using a scintillator consisted of 0.4% PPO and 0.01% POPOP in toluene.

Results and Discussion

Metabolites in Plasma

As shown in Table I, about 90% of radioactive compounds in acid-soluble fraction of the plasma was intact cyclocytidine, and small amounts of aracytidine and arauridine were detected. Stability of cyclocytidine in plasma, as reported previously,[9] was confirmed by the present examination, and the amount of the metabolites was only 2 to 8%, which could not be detected by photometric analysis because of their minor quantities. Ratio of metabolites to cyclocytidine increased with time and aracytidine was still detected 160 min after the administration. Therefore, concentration of aracytidine in the plasma was considered to be maintained for a long period.

TABLE I. Radioactivity of Cyclocytidine and Its Metabolites in Plasma of Monkeys After Intravenous Administration of ^{3}H-Cyclocytidine

Time after injection (min)	Count per ml-plasma ($\times 10^5$ dpm)	Cyclocytidine (%)	Aracytidine (%)	Arauridine (%)
5	1626	93.4	2.8	3.8
10	1066	94.7	3.1	2.2
20	549	90.3	4.6	5.1
40	759	91.2	4.5	4.3
80	321	86.2	7.9	5.9
160	109	84.5	9.1	6.4

Metabolites in Tissues

Focke, *et al.*[10] has reported that the acid-soluble metabolites prepared from the mouse after intraperitoneal injection of ^{14}C-cyclocytidine 3′-monophosphate comprised only 50% of the total radioactivity found in the liver and spleen, presumably due to the direct binding of cyclo-CMP and/or cyclocytidine to proteins through a covalent bonding. Therefore, the extraction efficiency was estimated by calculation of the proportions of the specific activities of the extracts and that obtained from direct combustion method.[11] As indicated in Table II, almost of all the radioactivity in the liver and kidney was transferred to the acid fraction. In contrast, more than half amount of total radioactivity in other tissues, especially in brain, remained in acid-insoluble fraction. Because in other experiment (data not presented here), sufficient amount of radioactivity was extracted from brain by trichloroacetic acid precipitation after homogenization with distilled water, the binding of the nucleoside to proteins could not be ascertained in the present study.

Most of the extractable radioactive compounds in tissues except in brain was 5-^{3}H-cyclocytidine. It is characteristic in brain that arauridine was detected in equal amount or more than cyclocytidine, and that cyclocytidine was permeable to the blood-brain barrier of monkey in contrast with our previous reports[9,12] on the distribution of this compound in mice and other animal species.

Thus in spite of the high level of cytidine deaminase activity in plasma and tissues of rhesus monkeys, low but relatively constant level of aracytidine was found in the plasma and tissues after injection of 5-^{3}H-cyclocytidine. As a result, deamination of aracytidine is considered not to be much more rapid than chemical hydrolysis of cyclocytidine, and a small amount of aracytidine remains in the steady state.

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TABLE II. Relative Amounts of Cyclocytidine and Its Metabolites Extracted from Monkey Organs after Intravenous Administration of ^{3}H-Cyclocytidine (100 mg/200 μCi/kg)

Tissue	Time (min)	Extraction ratio[a] (%)	dpm/g wet tissue[b] (×10^5 dpm)	Cyclocytidine (%)	Fraction aracytidine (%)	Arauridine (%)
Liver	20		43	91	8	1
	40		23	95	4	1
	80	95—110	10	89	6	4
	160		8	88	9	3
	320		2	67	10	20
Kidney	20		27	86	10	3
	40		36	85	10	4
	80	93—104	16	90	5	4
	160		22	88	6	5
	320		11	65	14	9
Spleen	20		2	76	5	19
	40		2	78	3	19
	80	30—64	4	87	8	6
	160		3	82	2	16
	320		1	91	3	6
Brain	20		0.23	52	6	41
	40		0.26	50	5	45
	80	8.9—3.5	0.09	36	6	58
	160		0.38	15	2	83
	320		0.18	32	4	64
Submaxillary gland	20		7.8	82	11	7
	40		3.0	84	5	11
	80	75—45	3.5	93	2	5
	160		0.53	91	3	7
	320		2.2	94	1	5
Small intestine	20		2.2	83	3	15
	40		1.2	87	4	11
	80	------[c]	1.3	86	3	11
	160		1.3	82	4	15
	320		0.46	81	0	19

a) calculated as $\frac{\text{radioactivity obtained by trichloroacetic acid extraction}}{\text{radioactivity obtained by combustion method}}$
b) acid-soluble radioactivity
c) did not examine

TABLE III. Distribution of Radioactivity in Crude Subcellular Fractions of Monkey Liver and Kidney After Intravenous Administration of ^{3}H-Cyclocytidine

Fraction[a]	Liver time after injection				Kidney time after injection	
	20 min	40 min	80 min	160 min	20 min	80 min
	% of total count				% of total count	
sup.	92	91	91	92	87	87
mit.	4	4	4	4	6	5
nuc.	5	5	5	5	8	9

a) sup.=final supernatant (10000×*g* supernatant)
mit.=crude mitochondrial fraction (10000×*g* precipitate)
nuc.=crude nucleus fraction (10000×*g* precipitate)

Incorporation in Subcellular Fractions of Liver and Kidney

As shown in Table III, almost of all radioactivity was distributed in the supernatant. About 4 and 5% of the labeled material were respectively distributed in the mitochondrial and nuclear fractions of the liver 20 to 160 min after injection. Similar distribution pattern was observed in the kidney.

Nucleotide Fraction

The biological activity of cyclocytidine is obviously due to its ultimate transformation into aracytidine 5′-diphosphate (ara-CDP) and aracytidine 5′-triphosphate (ara-CTP),[13] which are also considered to be crucial intermediates in the action of aracytidine.[14]

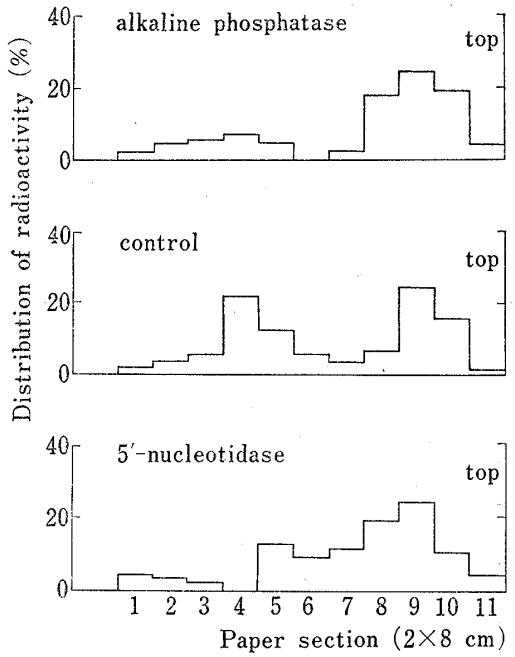

Fig. 1. Chromatogram of Enzymic Digest of Nucleotide Fraction isolated from Monkey Liver after Intravenous Administration of ^{3}H-Cyclocytidine

In order to examine whether the ara-CMP and cyclo-CMP fractions contain radioactive nucleotide(s), enzymic digestion by alkaline phosphatase and 5′-nucleotidase was carried out with the fractions isolated from the monkey liver dissected 40 min after the injection. As indicated in Fig. 1, a peak having the same *Rf* value (*Rf*=0.31) of ara-CMP and probably with other mononucleotides was detected on paperchromatogram, and lowered after enzymic digestion. Cyclo-CMP fraction was also treated but nucleotide peak was not detected.

Those results auggested that a part of 5-^{3}H-cyclocytidine (calculated as about 0.1% of dose) was converted to some nucleotide(s) in liver at relatively early stage after injection.

Metabolites in Urine

After intravenous injection of 100 mg/kg of 5-^{3}H-cyclocytidine, 46.5% of the administered dose was recovered in urine within 160 min. The relative amount of the excreted metabolites is summarized in Table IV. Rapid changes of cyclocytidine did not occur in either plasma

Table IV. Urinary Excretion of Radioactivity of Cyclocytidine and Its Metabolites in Monkey After Intravenous Administration of ^{3}H-Cyclocytidine

Time after injection (min)	Count ($\times 10^{5}$ dpm)	Recovery (%)	Cyclocytidine (%)	Aracytidine (%)	Arauridine (%)
10	361	3.8	95.4	3.0	1.6
20	413	4.2	94.1	3.8	2.1
40	1129	11.5	93.7	3.0	3.3
80	1476	15.1	87.1	8.5	4.4
160	1169	11.9	72.7	18.7	8.6
Total	4549	46.5			

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or urine, and relatively low but constant levels of aracytidine and arauridine were detected. The ratios of metabolites in urine were quite similar to those in plasma. By determination of distillable counts derived from tritium water in urine, it was revealed that more than 95% of the radioactivity was in tritiated compounds. This result indicated that prototropic degradation of the nucleotides did not occur after intravenous injection of 5-^{3}H-cyclocytidine.

Acknowledgement The authors thank Kohichi Wakigawa for his expert technical aid.