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Mode of Action of 5-Fluorouridine 5'-Phosphate *in Vivo* and *in Vitro*

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5-Fluorouridine and 1-β-D-arabinofuranosylcytosine are active against various tumors as antimetabolites. Antitumor activity and mode of action of their nucleotides, 5-fluorouridine 5'-phosphate and 1-β-D-arabinofuranosylcytosine 5'-phosphate, were examined. 5-Fluorouridine 5'-phosphate was active similar to the nucleoside against L1210 leukemia, but the nucleotide showed higher therapeutic ratio than 5-fluorouracil and 5-fluorouridine. The mode of action of these nucleotides was examined by the incorporation of labeled precursors into acid-insoluble fraction of the cells for 30 min. Inhibition patterns of these nucleotides were the same as those of the nucleosides, but the potency of the nucleotides was very weak, and increased with time. Analysis showed that these nucleotides were dephosphorylated on the cell surface and the dephosphorylation was greater than that of phenolphthalein monophosphate. 5-Fluorouridine 5'-phosphate was considered to be a good candidate for an antitumor agent.

Keywords——L1210 leukemia; L5178Y cells; arabinofuranosylcytosine; fluorouridine; fluorouridine monophosphate; dephosphorylation; arabinofuranosylcytosine monophosphate; 50% inhibiting concentration; phenolphthalein monophosphate

5-Fluorouridine is active against various tumors,[2)] but antitumor property of its 5'-phosphate has not been examined. Phosphorylated drug metabolites do not generally pass through the outer cell membrane readily. 1-β-D-Arabinofuranosylcytosine 5'-phosphate is active against L1210 leukemia comparable to related nucleoside, though the rate of passage of the nucleotide seems to be about 1% or less of that of the free nucleoside.[3)] In the present work, antitumor activity and mode of action of the nucleotides were examined and compared with those of 5-fluorouridine and 1-β-D-arabinofuranosylcytosine.

Materials and Methods

Chemicals——5-Fluorouracil (5FU), 5-fluorouridine (FUR), and 5-fluorouridine 5'-phosphate (FUMP) barium salt were supplied from Mitsui Pharmaceuticals, Inc., Tokyo. 1-β-D-Arabinofuranocylcytosine (Ara-C) and 1-β-D-arabinofuranosylcytosine 5'-phosphate (Ara-CMP) were supplied from Kohjin Co., Ltd., Tokyo. Phenolphthalein monophosphate was obtained from Sigma Chem. Co., U.S.A. RPMI 1640 and Ham F 12 medium were obtained from Nissui Seiyaku Co., Ltd., Tokyo. ^{14}C-labeled thymidine (specific activity, 58 mCi/mmol), uridine (spec. act., 491 mCi/mmol), L-leucine (spec. act. 331 mCi/mmol) and ^{3}H-deoxycytidine (spec. act., 2.0 Ci/mmol) were obtained from The Radiochemical Centre Ltd., U.K.

Determination of Antitumor Activity *in Vivo*——A mouse leukemia L1210 and male BDF_1 mice weighing 20 ± 2 g were used. Six mice for each group, either test or control, were inoculated intraperitoneally with 1×10^5 cells of L1210 Leukemia. The compound to be tested was injected intraperitoneally once daily for 5 days, starting 24 hr after the transplantation. Antitumor activity was evaluated by the increase in life-span over controls (ILS$=T/C\%-100$) as reported previously.[4)] ILS_{30}, ILS_{max}, and maximum ILS were also determined.

Determination of Antitumor Activity *in Vitro*——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°. L5178Y cells (5×10^4 cells/ml) were cultured for 48 hr with the test compound at various concentrations.

1) Location: *Tsukiji 5-chome, Chuo-ku, Tokyo 104, Japan.*
2) M. Umeda and C. Heidelberger, *Cancer Res.*, **28**, 2529 (1968); D. Kessel, R. Bruns, and T.C. Hall, *Mol. Pharmacol.*, **7**, 117 (1971).
3) A.M. Schrecker and A. Goldin, *Cancer Res.*, **28**, 802 (1968).
4) A. Hoshi and K. Kuretani, *Farumashia*, **9**, 464 (1973).

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Antitumor activity was determined by the ratio of cell number in treated and control groups (T/C%), and IC_{50} (50% inhibiting concentration) was calculated.

Determination of DNA, RNA, and Protein Syntheses——Labeled thymidine (0.2 μCi/ml), deoxycytidine (1 μCi/ml), uridine (0.5 μCi/ml), and L-leucine (0.5 μCi/ml) were used as precursors. Biosynthesis was determined by the incorporation of respective precursor into the cold trichloroacetic acid-insoluble fraction of the cells. L5178Y cells (2.5×10^5 cells/ml) were incubated for 30 min with the precursor at various concentrations of the test compound. A 0.4-ml aliquot of the cell suspension was collected on Whatman GF/C filter paper after being chilled to 0° and washed with ice-cold 5% trichloroacetic acid, and radioactivity was counted with a Packard Tri-Carb 3320 liquid scintillation spectrometer, using a toluene scintillator consisting of 5 g PPO, 0.5 g dimethyl-POPOP, and 1000 ml toluene.

Detection of Nucleosides——Nucleosides in the culture medium were detected with a high-pressure liquid chromatograph (JASCO FLC-A700) using an ion-exchange column (JASCOREX AV-02). L5178Y cells in exponential growth phase were collected and mixed with a nucleotide. After incubation for 0.5, 1, and 2 hr, the mixture was filtered over a Whatman GF/C filter, and 2—3 μl of the filtrate was applied to chromatography. Concentration of NH_4HCO_3 for elution was 0.025 M for Ara–C and 0.07 M for FUR.

Determination of Phosphatase Activity——Phosphatase activity was determined by dephosphorylation of phenolphthalein monophosphate. Final concentration of the phenolphthalein monophosphate added was 0.054 mM. The collected cells were cultured for 2 hr with Ham F 12 medium containing phenolphthalein monophosphate. After incubation for 0.5, 1, and 2 hr, the mixture was adjusted to 0.7% with 28% NH_4OH and then filtered over a Whatman GF/C filter paper. Phenolphthalein formed was determined from absorption at 555 nm.

Results and Discussion

Antitumor Activity of 5-Fluorouridine 5′-Phosphate *in Vivo*

Antitumor activity of FUMP against L1210 leukemia and relationship between the nucleotide and corresponding nucleoside in activity were first examined. As shown in Fig. 1, FUMP was markedly active against the leukemia. ILS_{30} and ILS_{max} of FUMP, FUR, and 5FU were 0.25 and 10, 0.15, and 3.0, and 1.9 and 20 mg/kg/day, respectively. FUMP was active similar to FUR and they were more active than 5FU on a molar basis. Therapeutic ratio (ILS_{max}/ILS_{30}) was 40 for FUMP, 20 for FUR, and 11 for 5 FU. The nucleotide showed the highest therapeutic ratio among them. This result suggested that the nucleotide was better than the rest of compounds.

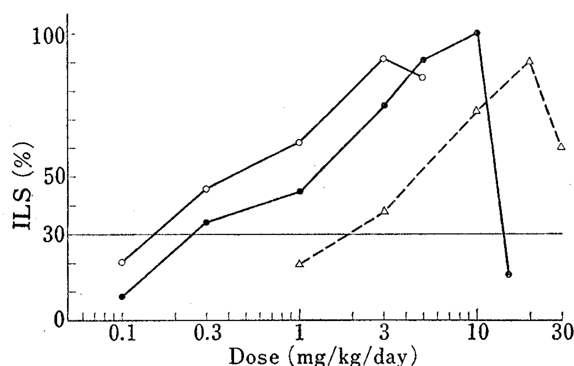

Fig. 1. Dose-Response Curves of 5FU, FUR, and FUMP

1×10^5 Cells of L1210 leukemia were inplanted intraperitoneally in mice (BDF_1). The compound was given intraperitoneally once daily for 5 days, starting 24 hr after transplantation.

---△---: 5FU, —○—: FUR, —●—: FUMP.

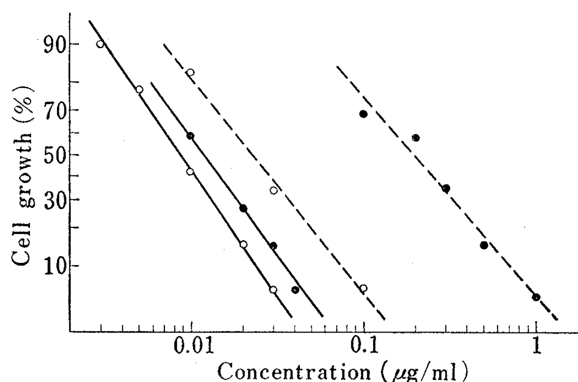

Fig. 2. Cell Growth Inhibition by FUR, FUMP, Ara-C, and Ara-CMP

—○—: FUR, —●—: FUMP, ---○--- :Ara-C, ---●--- :Ara-CMP.

L 5178Y cells (5×10^4 cells/ml) were cultured for 48 hr in RPMI 1640 medium supplemented with 10% calf serum in a CO_2 incubator at 37°. The results were plotted on a probit scale against the concentration on a log scale.

Antitumor Activity of Nucleotides *in Vitro*

Antitumor activity of FUMP against cultured L5178Y cells *in vitro* was determined. IC_{50} of the test compounds was calculated from the graph of Fig. 2. All the compounds were

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markedly active against L5178Y cells *in vitro* and FUR was the most active on a density basis but FUMP was more active than FUR on a molar basis, as shown in Table I. Ara-C was about 7-fold more active than the corresponding nucleotide. These results suggested that the mode of action of FUMP was different from that of Ara-CMP.

TABLE I. Inhibition of Cell Growth by FUR, Ara-C, and Their Nucleotides

Compound	Molecular weight	50% inhibiting concentration (IC_{50})	
		(μg/ml)	(M)
FUR	262.2	0.0085	3.2×10^{-8}
FUMP·Ba	477.5	0.012	2.5×10^{-8}
Ara-C	243.2	0.023	9.5×10^{-8}
Ara-CMP	323.2	0.20	6.2×10^{-7}

Effect of Nucleotides on Biosyntheses of Macromolecules

Effect of FUMP on the biosyntheses of macromolecules in L5178Y cells was compared with those of FUR and Ara-CMP. As shown in Fig. 3, FUMP inhibited the incorporation of both deoxycytidine and uridine to almost a similar extent, but did not affect that of leucine.

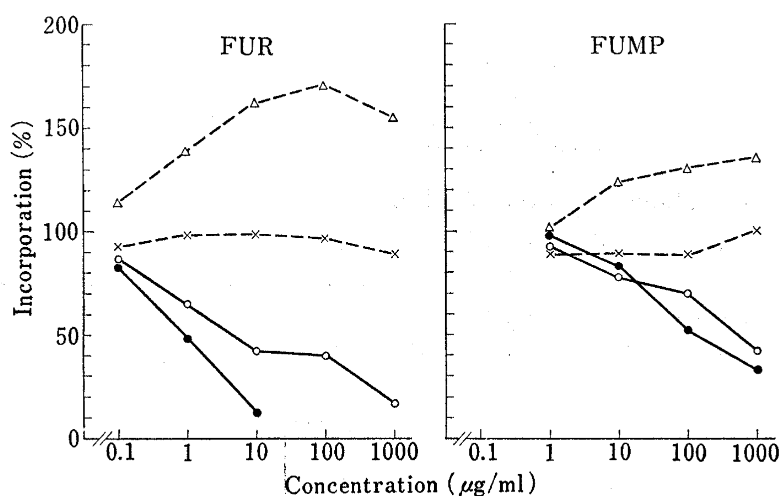

Fig. 3. Effect of FUR and FUMP on Incorporation of Precursors in L5178Y

---△---: ^{14}C-thymidine, ---×---: ^{14}C-L-leucine,
—○—: ^{3}H-deoxycytidine, —●—: ^{14}C-uridine.
L5178Y cells (2.5×10^{5} cells/ml) were incubated with precursor in the presence of varying concentrations of FUR or FUMP for 30 min.

TABLE II. Inhibition of the Incorporation of Precursors by FUR, Ara-C, and Their Nucleotides

Compound	50% inhibiting concentration (IC_{50})		
	^{14}C-Thymidine (M)	^{3}H-Deoxycytidine (M)	^{14}C-Uridine (M)
FUR	Increase	1.8×10^{-5}	3.2×10^{-6}
FUMP·Ba	Increase	1.1×10^{-3}	2.9×10^{-4}
Ara-C	4.1×10^{-6}	4.9×10^{-5}	2.4×10^{-3}
Ara-CMP	3.4×10^{-4}	1.4×10^{-3}	—[a]

a) Data not obtained.

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Incorporation of thymidine was increased by the nucleotide. FUR also inhibited the incorporation of the precursors similar to the case of FUMP but potency of the nucleoside was about 100 times higher than that of FUMP, as shown in Table II. These results suggested that FUMP itself was not active against L5178Y cells, though the pattern of inhibition was similar.

Fig. 4 shows that Ara-CMP inhibited the incorporation of both thymidine and deoxycytidine, but the potency was about one-hundredth and one-thirtieth of that of Ara-C, respectively as shown in Table II. These cytidine analogs did not affect the incorporation of either uridine or leucine at the concentration less than 100 μg/ml.

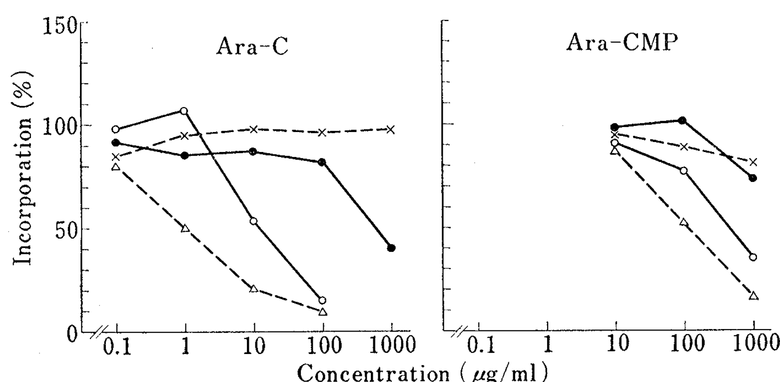

Fig. 4. Effect of Ara-C and Ara-CMP on Incorporation of Precursors in L5178Y

---△---: ^{14}C-thymidine, ---×---: ^{14}C-L-leucine,
—○—: ^{3}H-deoxycytidine, —●—: ^{14}C-uridine.
The method was performed as described in Fig. 3.

Transformation of Nucleotides in Culture Medium

Relationship between FUMP and FUR in antitumor activity *in vitro* was very similar to that between cyclocytidine and Ara-C.[5] Cyclocytidine is a masked form of Ara-C.[5] FUMP at the concentration less than 10 μg/ml scarcely inhibited the incorporation of uridine into RNA (Fig. 3) but, as Fig. 5 shows, uridine incorporation progressively decreased with

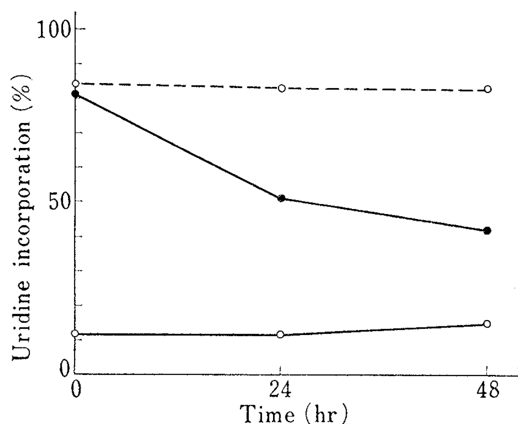

Fig. 5. Changes of Activity of FUMP as a Function of Time

---○---: FUMP in physiological saline (10 μg/ml).
—●—: FUMP in RPMI 1640 medium supplemented with 10% calf serum (10 μg/ml).
—○—: FUR in RPMI 1640 medium supplemented with 10% calf serum (10 μg/ml).

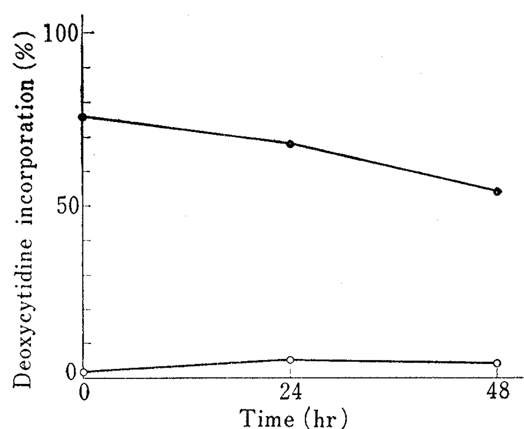

Fig. 6. Changes of Activity of Ara-CMP as a Function of Time

—●—: Ara-CMP in RPMI 1640 medium supplemented with 10% calf serum (100 μg/ml).
—○—: Ara-C in RPMI 1640 medium supplemented with 10% calf serum (100 μg/ml).

5) A. Hoshi, M. Yoshida, F. Kanzawa, K. Kuretani, T. Kanai, and M. Ichino, *Chem. Pharm. Bull.* (Tokyo), **21**, 1446 (1973).

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preincubation period of FUMP in the medium added with serum but not in physiological saline. Effect of FUR on the incorporation of the precursor did not change during preincubation for 48 hr. A similar decrease in the incorporation of thymidine into DNA with time was also observed in Ara-CMP but not in Ara-C, as shown in Fig. 6. These results suggested that FUMP changed to an active metabolite in the medium. This metabolite was not considered to be 5FU, because the nucleobase was less active than either FUMP or FUR in this system.[6] The active metabolite would be FUR.

Dephosphorylation of Nucleotides

In order to clarify the cause of this higher activity of FUMP and increased inhibition of RNA biosynthesis, dephosphorylation of FUMP was examined and was compared with that of Ara-CMP. FUR appeared in the medium by incubation with 3×10^6 cells/ml at the concentration of 600 μg/ml of FUMP as shown in Fig. 7.

The rate of dephosphorylation of FUMP was 1.83×10^{-9} μmol/cell/min during the initial 30 min. Similarly 400 μg/ml of Ara-CMP was also dephosphorylated and its rate was 1.31×10^{-9} μmol/cell/min during 30 min. Both nucleotides were dephosphorylated at a similar rate.

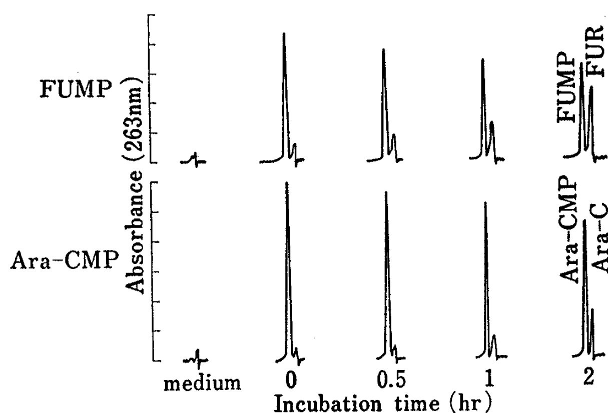

Fig. 7. Changes of Chromatograms of FUMP and Ara-CMP in Medium with Incubation Time

Incubation and analysis were performed as described in the text.

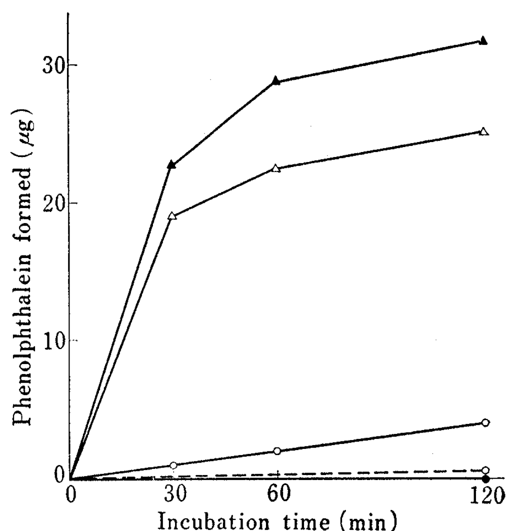

Fig. 8. Enzymic Formation of Phenolphthalein with Incubation Time

Phenolphthalein monophosphate (25 μg/ml) was incubated in the following Ham F 12 medium supplemented with 10% calf serum.
—▲—: in the medium in the presence of L5178Y cells (1.4×10^6 cells/ml).
—△—: in the medium in the presence of L5178Y cells (9.2×10^5 cells/ml).
—○—: in cultured fluid separated after 48-hr cultivation.
---○---: in the fresh medium.
—●—: in the Ham F 12 medium without serum.

Mode of Dephosphorylation

To examine the mode of dephosphorylation using spectrophotometer, phenolphthalein monophosphate, whose concentration was about one-twentieth of FUMP and Ara-CMP, was used. Medium used was changed to Ham F 12 from RPMI 1640, because the latter contained phenolsulfonphthalein which interfered with the determination of phenolphthalein. Other factors in the medium affecting dephosphorylation were also examined.

6) M. Yoshida, A. Hoshi, K. Kuretani, T. Kanai, and M. Ichino, *Gann*, **66**, 561 (1975).

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Calf serum added to the medium showed a weak phosphatase activity, and cultured fluid separated after a 48-hr cultivation showed a stronger activity than the fresh medium as shown in Fig. 8. The most important factor was the number of cells present. When the number of cells was increased, dephosphorylation of phenolphthalein monophosphate also increased. The rate of dephosphorylation was 0 for Ham F 12, 4.58×10^{-6} for the fresh medium (Ham F 12 supplemented with 10% calf serum), and 2.61×10^{-5} μmol/ml/min for cultured fluid (Fig. 8). On the other hand, L5178Y cells dephosphorylated phenolphthalein monophosphate in a higher rate than the medium. The rate of that was 4.26×10^{-10} for 1.4×10^{6} cells and 5.49×10^{-10} μmol/cell/min for 9.2×10^{5} cells during the initial 30 min. These rates were almost the same and suggested that dephosphorylation of FUMP was faster than that of phenolphthalein monophosphate and that both compounds were mainly dephosphorylated by L-5178Y cells.

As a rule, intact nucleotides are unable to pass through a cell membranes[3,7] but, in the present experiments, FUMP was active similar to FUR *in vitro* and it was therapeutically better than FUR *in vivo*. One of the principles of the antitumor activity of FUMP was considered to be dephosphorylation at the cell surface. FUMP is considered to be a masked form of FUR from pharmacological viewpoint, though the nucleotide is an enzymically active form of FUR. FUMP was considered to be a good candidate for an antitumor agent for clinical treatment because FUR was reported to be active clinically.[8]

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