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DIFFERENTIAL EFFECTS OF 2,2′-ANHYDRO-5-ETHYLURIDINE, A URIDINE PHOSPHORYLASE INHIBITOR, ON THE ANTITUMOR ACTIVITY OF 5-FLUOROURIDINE AND 5-FLUORO-2′-DEOXYURIDINE

MASAAKI IIGO,*† KEN-ICHI NISHIKATA,* YOKO NAKAJIMA,* ISTVAN SZINAI,‡ ZSUZSA VERES,‡ ANNA SZABOLCS‡ and ERIK DE CLERCQ§

* Chemotherapy Division, National Cancer Center Research Institute, Tokyo 104, Japan; ‡ Central Research Institute for Chemistry of the Hungarian Academy of Sciences, H-1525 Budapest, Hungary; and § Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium

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Abstract—2,2′-Anhydro-5-ethyluridine (ANEUR), a potent inhibitor of uridine phosphorylase, markedly potentiated the antitumor activity of fluorouridine (FUR) against murine mammary adenocarcinoma 755 in BDF_1 mice and human colon adenocarcinoma LS174T in athymic-nude mice. Whereas ANEUR annihilated the antitumor activity of 5-fluoro-2′-deoxyuridine (FUdR) and 5′-deoxy-5-fluorouridine (DFUR) in the murine adenocarcinoma 755 system, it did not alter the antitumor activity of FUdR in the human adenocarcinoma LS174T system. *In vitro*, ANEUR proved inhibitory to the phosphorolytic cleavage of both FUR and FUdR by uridine phosphorylase, and this could explain why *in vivo* conversion of FUR and FUdR to 5-fluorouracil was suppressed. FUR can be held directly responsible for the antitumor effects observed in the murine adenocarcinoma 755 system, whereas in the activity against human adenocarcinoma LS174T may be mediated by both FUR and FUdR.

The 5-fluorouracil derivatives 5-fluorouridine (FUR) and 5-fluoro-2′-deoxyuridine (FUdR) are converted to 5-fluorouracil (5-FU) by pyrimidine nucleoside phosphorylases. Two distinct pyrimidine nucleoside phosphorylases are widely distributed in both prokaryotes and eukaryotes. One is uridine nucleoside phosphorylase (EC 2.4.2.3), which phosphorolyses both uridine and thymidine [1, 2]. The other is thymidine phosphorylase (EC 2.4.2.4), which is assumed to be specific for pyrimidine 2′-deoxynucleosides such as thymidine and 2′-deoxyuridine [3]. Uridine phosphorylase would be responsible for the phosphorolysis of FUdR in murine tumors, while in human tumors phosphorolysis of FUdR would be achieved by thymidine phosphorylase [4]. Degradation of FUR or FUdR to 5-FU by the pyrimidine nucleoside phosphorylases is viewed as a limiting factor in their antitumor activity. In fact, combination of FUR with 2,2′-anhydro-5-ethyluridine (ANEUR), which is a potent inhibitor of uridine phosphorylase [5], markedly enhances the antitumor activity and toxicity of FUR [6]. We have now examined the effect of ANEUR on the antitumor activity of various 5-fluorouracil derivatives in mice bearing murine adenocarcinoma 755 or human colon adenocarcinoma LS174T. Plasma levels of FUdR, FUR, 5-FU and ANEUR were determined following administration of FUdR, FUR and ANEUR. *In vitro*, ANEUR was evaluated for its effect on the phosphorolysis of FUR and FUdR by uridine phosphorylase and/or thymidine phosphorylase.

† Correspondence: Dr Masaaki Iigo, Chemotherapy Division, National Cancer Center Research Institute, Tsukiji 5-chome, Chuo-ku, Tokyo 104, Japan.

MATERIALS AND METHODS

Drugs. 5-FU, FUR and FUdR were obtained from the Sigma Chemical Co. (St Louis, MO). 5′-Deoxy-5-fluorouridine (DFUR) was generously provided by Nippon Roche (Kamakura, Japan). ANEUR was synthesized essentially as described previously [5]. [2-^{14}C]ANEUR (101.7 MBq/mmol) was prepared according to the methods of Szabolcs *et al.* [7] and Nishimura *et al.* [8]. [6-^{14}C]-5-FUR (106.6 MBq/mmol) was prepared from [6-^{14}C]FU (2.07 GBq/mmol; Amersham) by the method of Niedballa and Vorbrüggen [9]. [6-^{14}C]-5-FUdR (84.7 MBq/mmol) was also prepared from [6-^{14}C]FU by the method of Szabolcs *et al.* [7].

Enzyme preparation and partial purification. Uridine phosphorylase was prepared and partially purified from (Wistar male) rat (160–180 g) intestinal mucosa; thymidine phosphorylase was isolated from (NMRI male) mouse (20–25 g) liver, as previously described [5].

Protein determination. Protein concentration was determined by the method of Lowry *et al.* [10].

Enzyme assay. The phosphorolysis of [6-^{14}C]FUR and FUdR by uridine phosphorylase was examined in a 125 μL incubation mixture which contained 0.04 M potassium phosphate buffer (pH 7.4), 2 mM 2-mercaptoethanol, 0.9–1.8 μg protein, 20–100 μM FUR or 60–600 μM FUdR and an appropriate

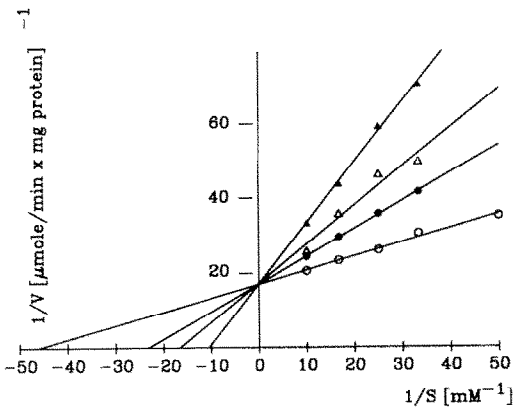

Fig. 1. Double reciprocal plot for inhibition of uridine phosphorylase by ANEUR. Key: (○) FUR alone; (●) 75 nM ANEUR; (△) 125 nM ANEUR; (▲) 250 nM ANEUR.

amount of inhibitor. The phosphorolysis of the 5-fluorouracil derivatives by thymidine phosphorylase was also examined in a 125 μL incubation mixture which contained potassium phosphate buffer and 2-mercaptoethanol as indicated above, 0.3–1.5 mg protein, 1 mM FUR or 150–500 μM FUdR, and inhibitor. K_i values were determined from the Lineweaver–Burk plots of the data by least-squares fitting. Incubations were carried out at 37°. The enzyme reactions were stopped with ice-cold methanol (125 μL). The amount of radioactive product was determined by thin-layer chromatography as described elsewhere [5]. The solvent system for FUR-FU and FUdR-FU was chloroform–methanol–acetic acid (8:2:0.1, v/v/v and 30:10:5, v/v/v, respectively).

Animals and treatment. Groups of six male BDF_1 mice (Shizuoka Laboratory Animal Center, Hamamatsu, Japan) or six male athymic-nude mice (CLEA Japan, Tokyo, Japan) weighing 20–23 g were used. Murine mammary adenocarcinoma 755 (5×10^5 cells/BDF_1 mouse) or human colon adenocarcinoma LS174T (30 mg/nude mouse) were implanted subcutaneously (s.c.) on day 0 into the right thigh. All mice were housed under SPF conditions in plastic cages in an air-conditioned room (25°) kept in the light for 12 hr per day. BDF_1 mice received CA-1 pellets (CLEA Japan, Inc., Tokyo, Japan) and nude mice received CMF pellets (Oriental Yeast Co. Ltd, Tokyo, Japan). The mice were given sterilized water *ad lib.*

Twenty-four hours after implantation of adenocarcinoma 755, the mice were treated intravenously (i.v.) with FUR, FUdR or DFUR, or any of these 5-FU derivatives combined with ANEUR. Treatment was continued for 4 consecutive days. The tumors of treated and untreated mice were weighed on day 11, and the ratio of average tumor weight in the treated group to that of the control group (T/C %) was determined. For the LS174T tumors, i.v. treatment with FUR, FUdR, FUR + ANEUR or FUdR + ANEUR was started on day 7 and continued for 4 days. The growth of s.c. implanted LS174T cells was monitored by measurement of the perpendicular diameters with calipers, and tumor volume size (mm) was calculated as the square root of long diameter (mm) × short diameter (mm).

HPLC assay of plasma concentrations of 5-FU, FUR and FUdR. Single dose of FUR (3 mg/kg) or FUdR (100 mg/kg), whether or not combined with ANEUR (100 or 300 mg/kg) were given i.v. to the (BDF_1 or nude) mice. Blood samples were collected from the inferior vena cava under light anaesthesia with ether at the indicated times after administration of the drugs. Plasma concentrations of FUR, FUdR and 5-FU were determined as reported previously [11].

Clearance of ANEUR from plasma. Male NMRI mice (24–25 g) were given [2-^{14}C]ANEUR (100 mg/kg; dissolved in 0.9% NaCl; 0.28 MBq/0.2 mL/animal) by injection into the tail vein. At the indicated times mice were decapitated, blood was collected in heparinized tubes and centrifuged. Plasma was pooled per group (3 samples, 50 μL), dried at 60° and then burnt in an automatic sample analyser prior to scintillation counting [12].

Measurement of intratumor FdUMP concentration following FUdR administration. Single doses of FUdR (100 mg/kg) were given i.v. to BDF_1 or nude mice, 7 or 14 days after the transplantation of murine mammary adenocarcinoma 755 or human colon adenocarcinoma LS174T, respectively. The tumors were removed under light anaesthesia with ether at half an hour or 3 hr after administration of FUdR

Table 1. Kinetic parameters for the phosphorolysis of FUR and FUdR by pyrimidine nucleoside phosphorylases and for the inhibitory effects of ANEUR on these enzymatic reactions

Compound	K_m (μM)	V_{max} (nmol/min mg protein)	K_i (nM)
Uridine phosphorylase			
FUR	22	77.5	77.7 ± 5.5
FUdR	190	157.0	158.0 ± 36.0
Thymidine phosphorylase			
FUR	*	*	*
FUdR	237	0.4	>10^6

* Could not be determined.

Table 2. Plasma concentrations of ANEUR following i.v. administration of a dose of 100 mg/kg

Time (min)	Plasma concentration of ANEUR (μg/mL)
2.5	115.7 ± 17.0
5	79.4 ± 7.1
15	44.4 ± 7.7
30	20.8 ± 2.2
60	6.4 ± 0.7
120	1.5 ± 0.2
240	0.4 ± 0.03
360	0.3 ± 0.05
480	ND
24 hours	ND

Values are mean ± SD of five mice.
ND, not detectable.

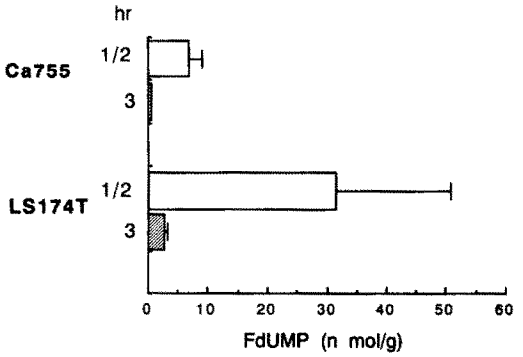

Fig. 2. FdUMP concentration in murine mammary adenocarcinoma 755 and human colon adenocarcinoma LS174T at 1/2 hr and 3 hr following i.v. administration of FUdR (100 mg/kg) to the host mice (BDF_1 and nudes, respectively).

and chilled in dry ice–acetone as rapidly as possible. Concentrations of FdUMP in the tumors were determined according to the procedure of Moran *et al.* [13].

RESULTS AND DISCUSSION

Effect of ANEUR on the phosphorolysis of FUR and FUdR by pyrimidine nucleoside phosphorylases

FUR is not a substrate for thymidine phosphorylase (Table 1). This is consistent with the concept that this enzyme is specific for 2'-deoxyribonucleosides [3]. In contrast, FUR is a good substrate for uridine phosphorylase. Its K_m value is similar to that of the natural substrate, uridine [14]. This is in agreement with data reported by Niedzwicki *et al.* [15]. ANEUR is a competitive inhibitor of uridine phosphorylase (Fig. 1). Its K_i is 77.7 nM with FUR as the substrate.

FUdR is a substrate for both uridine phosphorylase and thymidine phosphorylase. The K_m value of thymidine phosphorylase for FUdR is similar to that for thymidine [14]. According to the data of Niedzwicki *et al.* [15], the apparent K_i of thymidine phosphorylase for FUdR is about two-fold higher than for thymidine. ANEUR does not inhibit the cleavage of FUdR by thymidine phosphorylase up to an inhibitor concentration of 1 mM (Table 1). The K_m value of uridine phosphorylase for FUdR is one order of magnitude higher than the K_m for uridine. ANEUR inhibits the phosphorolysis of FUdR by uridine phosphorylase with a K_i value of 158 nM.

Table 3. Inhibition of the growth of murine mammary adenocarcinoma 755 in BDF_1 mice by combination of FUR with ANEUR

Dose (mg/kg/day)		Tumor weight (mg)*	*T/C* (%)
FUR	ANEUR		
Expt. I			
0	0	3784 ± 372	100
1	0	1675 ± 238	44
3	0	1479 ± 567	39
5	0	481 ± 135	13
1	300	584 ± 120‡	15
3	300	78 ± 26†	2
5	300	0 (2/6)	
Expt. II			
0	0	3401 ± 360	100
1	0	2588 ± 267	76
3	0	630 ± 131	19
5	0	384 ± 142	11
10	0	18 ± 4 (2/6)	
1	100	904 ± 248§	27
3	100	133 ± 17‡	4
5	100	0 (4/6)	

* Values are means ± SE.
† $P < 0.05$, ‡ $P < 0.01$, § $P < 0.001$, as compared to FUR alone. In parentheses: ratio of dead mice to total number of mice.

Clearance of ANEUR from plasma

Following i.v. injection of [2-^{14}C]ANEUR at a dose of 100 mg/kg (0.28 MBq/mouse), ANEUR is rapidly cleared from the plasma (half-life: <15 min, Table 2). This suggests that ANEUR must be rapidly taken up by the tissues and/or rapidly eliminated (i.e. by urinary excretion [6]).

Effect of ANEUR on the antitumor effect of various 5-FU derivatives on murine mammary adenocarcinoma 755

ANEUR markedly potentiated the antitumor activity, and host toxicity, of FUR in mice bearing murine mammary adenocarcinoma 755 (Table 3). However, the antitumor effects of FUdR or DFUR on the growth of murine adenocarcinoma 755 were abolished in the presence of ANEUR (Table 4). Apparently, conversion of DFUR and FUdR to 5-FU by uridine phosphorylase is essential for the antitumor activity of DFUR and FUdR in murine tumor systems, and, as demonstrated in Table 1, uridine phosphorylase is blocked by ANEUR. ANEUR is not inhibitory to thymidine phosphorylase, and, hence, should not interfere with the conversion of DFUR to 5-FU by thymidine phospho-

Table 6. Plasma concentrations of FUdR and 5-FU after administration of FUdR with or without ANEUR to BDF_1 mice

Time (min)	FUdR alone		FUdR + ANEUR	
	Plasma concentrations of FUdR (μg/mL)	Plasma concentrations of 5-FU (μg/mL)	Plasma concentrations of FUdR (μg/mL)	Plasma concentrations of 5-FU (μg/mL)
15	6.37 ± 0.15	0.196 ± 0.011	10.72 ± 2.40	0.055 ± 0.005
30	0.58 ± 0.33	0.039 ± 0.006	0.89 ± 0.17	0.008 ± 0.005
60	0.05 ± 0.02	<0.005	0.18 ± 0.04	<0.005

Both FUdR and ANEUR were administered i.v. at 100 mg/kg.
Values are means ± SE for three mice per group.

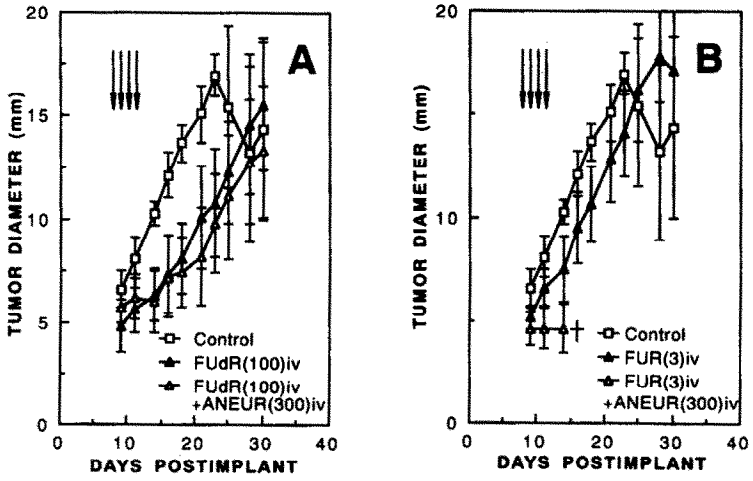

Fig. 3. Effects of FUR and FUdR, alone and in combination with ANEUR, on growth of human colon adenocarcinoma LS174T in athymic-nude mice. Values are means ± SE for six mice per group.

Effect of ANEUR on the antitumor effect of FUdR on human colon adenocarcinoma LS174T

A markedly higher level of FdUMP was found in human adenocarcinoma LS174T as compared to murine adenocarcinoma 755 tumors after FUdR had been administered to the host mice (BDF_1 and nude mice, respectively) (Fig. 2).

When ANEUR treatment was combined with FUdR or FUR treatment in the LS174T-nude mouse system, ANEUR markedly potentiated the antitumor activity and host toxicity of FUR but did not alter the antitumor activity of FUdR (Fig. 3). Yet, ANEUR blocked the metabolism of FUdR to 5-FU in athymic-nude mice (Table 7) in the same fashion as in BDF_1 mice (Table 6). The fact that, on the one hand, FUdR remained active against LS174T when its conversion to 5-FU was impeded by ANEUR and that, on the other hand, high levels of FdUMP were

Table 7. Plasma concentrations of FUdR and 5-FU after administration of FUdR with or without ANEUR in athymic-nude mice

Time (min)	FUdR alone		FUdR + ANEUR	
	Plasma concentrations of FUdR (μg/mL)	Plasma concentrations of 5-FU (μg/mL)	Plasma concentrations of FUdR (μg/mL)	Plasma concentrations of 5-FU (μg/mL)
15	3.14 ± 0.47	0.226 ± 0.060	6.66 ± 1.37	0.060 ± 0.040
30	0.79 ± 0.14	0.093 ± 0.016	0.86 ± 0.21	0.016 ± 0.002
60	0.12 ± 0.01	0.006 ± 0.006	0.08 ± 0.01	0.008 ± 0.004

FUdR and ANEUR were administered i.v. at 100 and 30 mg/kg, respectively.
Values are means ± SE for three nude mice per group.

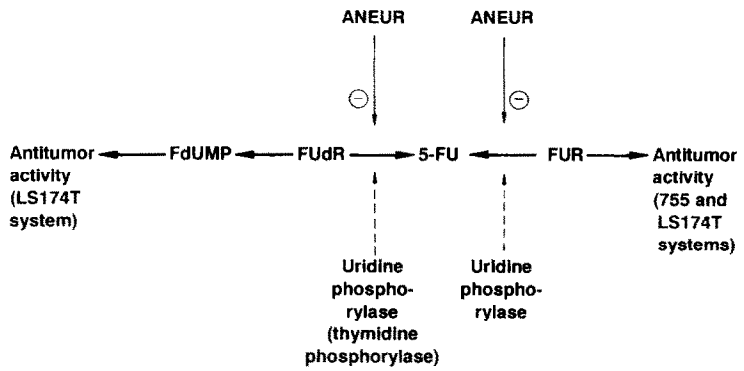

Fig. 4. Synoptic scheme of modulatory effect of ANEUR on antitumor activity of FUR and FUdR in the murine mammary adenocarcinoma 755 and human colon adenocarcinoma LS174T systems.

generated in LS174T tumor cells, suggests that in the adenocarcinoma LS174T system FUdR achieves its antitumor activity *via* FdUMP, that is, most probably with thymidylate synthase as the target enzyme. For FUR, however, the mechanism of action in the adenocarcinoma LS174T system would be similar to that in the adenocarcinoma 755 system: i.e. FUR is directly responsible for the antitumor activity, and if its degradation to 5-FU is blocked by ANEUR, its antitumor activity is increased.

CONCLUSION

The sequence of events that lead to the antitumor effects of FUR and FUdR in both the murine adenocarcinoma 755 and human adenocarcinoma LS174T systems can be presented in a synoptic scheme (Fig. 4). By blocking uridine phosphorylase, ANEUR prevents the conversion of FUR to 5-FU. This leads to a higher antitumor activity of FUR in both adenocarcinoma systems. ANEUR also prevents the conversion of FUdR to 5-FU. This shuts off its antitumor activity in the murine adenocarcinoma system, but still permits FUdR to act against the human tumor cells.

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REFERENCES

1. Razzel WE, Preparation of oligoribonucleotides with RNase I. *Methods Enzymol* **12A**: 255–257, 1967.
2. Reichard P and Sköld O, Pyrimidine synthesis and breakdown. *Methods Enzymol* **6**: 177–197, 1963.
3. Razzel WE, Pyrimidine nucleoside and deoxynucleoside phosphorylases. *Methods Enzymol* **12A**: 118–125, 1967.
4. Miwa M, Cook A and Ishitsuka H, Enzymatic cleavage of various fluorinated pyrimidine nucleosides to 5-fluorouracil and their antiproliferative activities in human and murine tumor cells. *Chem Pharm Bull* **34**: 4225–4232, 1986.
5. Veres Z, Szabolcs A, Szinai I, Dénes G, Kajtár-Peredy M and Ötvös L, 5-Substituted-2.2′-anhydrouridines, potent inhibitors of uridine phosphorylase. *Biochem Pharmacol* **34**: 1737–1740, 1985.
6. Veres Z, Szinai I, Szabolcs A, Ujszászy K and Dénes G, Biological activity of the potent uridine phosphorylase inhibitor 5-ethyl-2,2′-anhydrouridine. *Drugs Exptl Clin Res* **13**: 615–621, 1987.
7. Szabolcs A, Kruppa G, Sági J and Ötvös L, Unnatural nucleosides and nucleotides. III. Preparation of 2-^{14}C and 4-^{14}C labelled 5-alkyluracils and 5-alkyl-2′-deoxyuridines. *J Lab Comp Radiopharm* **14**: 713–721, 1978.
8. Nishimura T, Shimizu B and Iwai I, A new synthetic method for nucleotides. *Chem Pharm Bull* **11**: 1470–1477, 1963.
9. Niedballa U and Vorbrüggen H, A general synthesis of *N*-glycosides. I. Synthesis of pyrimidine nucleosides. *J Org Chem* **39**: 3654–3660, 1974.
10. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
11. Iigo M, Yamaizumi Z, Nakajima Y, Nishimura S and Hoshi A, Mechanisms of potentiation of antitumor activity of 5-fluoro-2′-deoxyuridine in the adenocarcinoma 755 system by guanosine 5′-monophosphate. *Drugs Exptl Clin Res* **14**: 257–263, 1988.
12. Gács I, Vargai Z, Dobis E, Dombi S, Payer K and Ötvös L, A new automatic combustion method for the liquid scintillation assay of tritium and carbon-14 in singly or doubly labeled organic materials. *J Radioanal Chem* **68**: 93–105, 1982.
13. Moran RG, Spears CP and Heidelberger C, Biochemical determinants of tumor sensitivity to 5-fluorouracil: ultrasensitive methods for the determination of 5-fluoro-2′-deoxyuridylate, 2′-deoxyuridylate, and thymidylate synthetase. *Proc Natl Acad Sci USA* **76**: 1456–1460, 1979.
14. Veres Z, Szabolcs A, Szinai I, Dénes G and Jeney A, Enzymatic cleavage of 5-substituted-2′-deoxyuridines by pyrimidine nucleoside phosphorylases. *Biochem Pharmacol* **35**: 1057–1059, 1986.
15. Niedzwicki JG, el Kouni MH, Chu SH and Cha S, Structure–activity relationship of ligands of the pyrimidine nucleoside phosphorylases. *Biochem Pharmacol* **32**: 399–415, 1983.
16. Kono A, Hara Y and Matsushima Y, Enzymatic formation of 5-fluorouracil from 1-(tetrahydro-2-furanyl)-5-fluorouracil (Tegafur) in human tumor tissues. *Chem Pharm Bull* **29**: 1486–1488, 1981.

17. Kono A, Hara Y, Sugata S, Karube Y, Matsushima Y and Ishitsuka H, Activation of 5'-deoxy-5-fluorouridine by thymidine phosphorylase in human tumors. *Chem Pharm Bull* **31**: 175–178, 1983.
18. Darnowski JW and Handschumacher RE, Tissue uridine pools: evidence *in vivo* of a concentrative mechanism for uridine uptake. *Cancer Res* **46**: 3490–3494, 1986.
19. Darnowski JW and Handschumacher RE, Tissue-specific enhancement of uridine utilization and 5-fluorouracil therapy in mice by benzylacyclouridine. *Cancer Res* **45**: 5364–5368, 1985.
20. Iigo M, Ando N, Hoshi A and Kuretani K, Effect of pyrimidines, purines and their nucleosides on antitumor activity of 5-fluorouracil against L-1210 leukemia. *J Pharm Dyn* **5**: 515–520, 1982.
21. Iigo M and Hoshi A, Effect of guanosine on antitumor activity of fluorinated pyrimidines against P388 leukemia. *Cancer Chemother Pharmacol* **13**: 86–90, 1984.