Jpn. J. Cancer Res. 82, 476-482, April 1991

Antitumor Activity of Fluoropyrimidines and Thymidylate Synthetase Inhibition

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Experimental chemotherapy with 5-fluorouracil (5-FU; 60 mg/kg), 1-hexylcarbamoyl-5-fluorouracil (HCFU; 70 mg/kg), 3-(3-(6-benzoyloxy-3-cyano-2-pyridyloxycarbonyl)benzoyl)-1-ethoxymethyl-5-fluorouracil (BOF-A2; 30 mg/kg) and UFT (20 mg/kg as tegafur with uracil at a molar ratio of 1:4) was performed using human gastric (H-111) and colon (Co-4) carcinoma strains in nude mice. 5-FU was administered ip with a q4d×3 schedule and the other agents were given po daily for three weeks. Concentrations of 5-FU in the serum and the tumor were assessed by gas chromatography-mass fragmentography, two hours or 12 days (5-FU) after the last treatment, and thymidylate synthetase (TS) was assayed according to the method of Spears *et al.* using the same schedule. The antitumor activity of the agents was assessed in terms of the actual tumor weight at the end of the experiment. HCFU was effective against both strains and 5-FU was effective against Co-4, although the other agents were ineffective against either strain. Statistically significant correlations were found between the serum and tumor concentrations of 5-FU and antitumor activity. Statistically significant correlations were also observed between the antitumor activity and TS inhibition rate (TSIR) and the activity of free thymidylate synthetase (TSfree), with higher TSIR and lower TSfree resulting in higher antitumor activity. Therefore, TSIR and TSfree were thought to be promising indicators for predicting the antitumor activity of fluoropyrimidines.

Key words: Fluoropyrimidine — Human tumor xenograft — Nude mouse — Thymidylate synthetase — Drug concentration

5-Fluorouracil (5-FU), along with other fluoropyrimidine compounds, is one of the key drugs for chemotherapy of solid tumors in adults, including gastric, colon and breast carcinomas. The mechanism of action of fluoropyrimidines has been explained in terms of inhibition of thymidylate synthetase (TS) by an active metabolite, 5-fluorodeoxyuridine 5′-monophosphate (FdUMP), or incorporation of 5-FU into RNA resulting in the distortion of gene expression.[1-5] However, it is still controversial whether a clear correlation exists between the antitumor activity of fluoropyrimidines and changes in TS. This may be partly due to the differences in fluoropyrimidine metabolism between rodents and humans and also in the pharmacodynamics of rodent and human tumors. This paper describes the antitumor activity of four fluoropyrimidines on two human tumor xenografts in nude mice with reference to the serum and tumor concentrations of 5-FU, and the activity of TS in the tumor tissue.

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MATERIALS AND METHODS

Mice Male nude mice with a BALB/cA genetic background were purchased from CLEA Japan Inc., Tokyo. The mice were maintained under specific pathogen-free conditions using an Isorack, and fed on sterile food and water *ad libitum* in our experimental animal center. Six- to eight-week-old mice weighing 20–22 g were used for the experiments.

Tumors One gastric carcinoma (H-111, well differentiated adenocarcinoma) and one colon carcinoma (Co-4, poorly differentiated adenocarcinoma) were used.[6,7] H-111 was kindly supplied by Dr. M. Fujita, Osaka University. Co-4 was established at the Pathology Division, National Cancer Center Research Institute,[3] and was maintained in our institute.

Agents Commercially available 5-FU was purchased from Kyowa Hakko Kogyo, Co. Ltd., Tokyo. 1-Hexylcarbamoyl-5-fluorouracil (HCFU) was prepared at the Institute of Biological Science, Mitsui Pharmaceuticals Inc. 3-(3-(6-Benzoyloxy-3-cyano-2-pyridyloxycarbonyl) benzoyl)-1-ethoxymethyl-5-fluorouracil (BOF-A2)[8] and UFT were kindly supplied by Otsuka Pharmaceutical Co. Ltd., Osaka and Taiho Pharmaceutical Co. Ltd., Tokyo, respectively.

A 60 mg/kg dose of 5-FU was administered intraperitoneally (ip) using a q4d×3 schedule. HCFU at a dose of 70 mg/kg dissolved in 0.2 ml of 1% hydroxymethyl cellulose was administered perorally (po) daily for three weeks except on Sundays. Thirty mg of BOF-A2 per kg dissolved in 0.2 ml of 1% hydroxymethyl cellulose was administered po daily for three weeks except on Sundays. Tegafur and uracil were mixed in 5% gum arabic solution at a molar concentration of 1/4 (UFT), and 20 mg of UFT as tegafur per kg was administered po daily for three weeks except on Sundays.

The dose, route and schedule of administration of 5-FU had been estimated in our previous report[9] to constitute the maximum tolerable drug load producing maximum antitumor activity. The administration method for the other drugs was determined from the previous animal experiments.

Tumor inoculation, measurement of tumor size and evaluation of agent activity Two tumor tissue fragments, each approximately 3×3×3 mm in size, were inoculated into the subcutaneous tissue of the bilateral dorsum of ether-anesthetized nude mice, using a trocar needle. The tumors were measured (length and width) with sliding calipers three times weekly by the same observer.

The tumor weight was calculated according to the method of Geran *et al.*[10] from linear measurements using the formula: tumor weight (mg) = length (mm) × (width (mm))^2/2. When the tumors reached 100–300 mg, the tumor-bearing mice were allocated randomly to test groups each consisting of between 4 and 6 mice. The relative mean tumor weight (RW) was calculated as $RW = W_i/W_0$, where W_i is the mean tumor weight at any given time and W_0 is the mean tumor weight at the time of initial treatment. The antitumor effects of the agents were evaluated in terms of the lowest T/C value (%) during the experiment, where T is the relative mean tumor weight of the treated group and C the relative mean tumor weight of the control group at any given time. The antitumor activity was evaluated as positive when the lowest T/C was equal to or less than 42%, which was calculated from $(0.75)^3$, corresponding to a 25% reduction of each diameter.[8]

At the end of the experiments, all the mice were killed 2 h after the last administration of HCFU, BOF-A2 and UFT, and 12 days after the last treatment with 5-FU, the tumors were weighed aseptically and the differences were analyzed statistically using Student's *t* test. The tumors were stored in a deep freeze for the thymidylate synthetase assay. The concentration of 5-FU in tumors and collected serum was also assessed by gas chromatography-mass fragmentography (GC-MF) according to the method of Marunaka *et al.*[11]

Change in the activity of thymidylate synthetase with time The change in the activity of thymidylate synthetase with time was assessed in Co-4 tumors treated with 5-FU and HCFU. At 2, 6 or 24 h after administration of 50 mg of 5-FU or 100 mg of HCFU per kg ip or po, respectively, to nude mice bearing Co-4 tumors with an estimated weight of 500–1,000 mg, the tumors were resected. The specimens were immediately stored in a deep freeze for assay of thymidylate synthetase. Each group consisted of 6 tumors, except for the control group in which 10 tumors were used.

Assay of thymidylate synthetase and its degree of inhibition Thymidylate synthetase (TS) was assessed according to the method of Spears *et al.*[1,2] with some modifications which have been reported elsewhere.[4] In brief, an excess amount of [^{3}H]-fluorodeoxyuridine monophosphate ([^{3}H]FdUMP) was added to the samples to determine TS by binding assay. Approximately 200 mg of tumor tissue in 200 m*M* Tris-HCl, 10 m*M* 2-mercaptoethanol, 100 m*M* NaF and 15 m*M* cytidine monophosphate (pH 7.4) was homogenized with a Polytron, sonicated five times for 20 s, and centrifuged at 25,000*g* for 60 min. Part of the cytosol fraction was stored at 4°C for the assay of TSfree and the rest was incubated with 600 m*M* NH_4HCO_3, 100 m*M* 2-mercaptoethanol, 100 m*M* NaF and 15 m*M* cytidine monophosphate (pH 8, Buffer B) at 25°C for 4 h to release FdUMP from the TS for assay of total TS.

A volume of 0.05 ml of the cytosol fraction was incubated with 0.05 ml of Buffer B, 0.05 ml of 10 mg/ml bovine serum albumin, 0.01 ml of coenzyme solution (10 m*M* tetrahydrofolate, 67 m*M* formaldehyde, 15 m*M* sodium ascorbate) and 0.05 ml of [^{3}H]-FdUMP (in 5 m*M* potassium phosphate buffer; pH 7.4) at 30°C for 20 min. After 1 ml of charcoal suspension (3% activated charcoal, 0.5% BSA, 0.05% dextran T-70 in 0.1 *N* HCl) had been added to the samples, they were centrifuged at 2,000*g* for 20 min and 0.8 ml of the supernatant was used to estimate radioactivity employing a liquid scintillation counter (LSC-903, Aloka).

Free TS was calculated from the measurement as $TSfree = (TSfree^{app} - 0.13TStot)/0.87$, where TSfree is free TS, $TSfree^{app}$ is apparent free TS and TStot is total TS. This equation was used because 13% of TS combined with FdUMP would be dissociated and re-combined with [^{3}H]FdUMP during the incubation at 30°C for 20 min.[4] TS inhibition rate (TSIR) was calculated from the measurement as TSIR = (TStot − TSfree)/TStot. TSIR was calculated for each tumor as the mean and standard deviation for statistical analysis, and consequently the mean value of TSIR could not be obtained directly from the mean of TStot and TSfree.

Statistical analysis To clarify the correlation between the antitumor activity and concentration of 5-FU or TS, the coefficients of correlation were calculated between the lowest T/C ratio of the actual tumor weight and the

concentrations of 5-FU in the serum and tumor, or TStot, TSfree and TSIR. The coefficients of correlation were examined statistically by using the t test.

RESULTS

The antitumor activity of fluoropyrimidine derivatives is shown in Table I. Statistically significant reductions in the size of the treated tumors were observed in H-111 treated with HCFU and in Co-4 treated with 5-FU and HCFU. The antitumor activity against Co-4 was evaluated as positive for 5-FU and HCFU according to our criteria. UFT suppressed the growth of both H-111 and Co-4 slightly, but the antitumor activity of this agent was evaluated as negative for both strains. The antitumor activity of BOF-A2 on these strains was limited. Co-4 seemed to be more sensitive to fluoropyrimidines in comparison with H-111.

The concentrations of 5-FU in the serum and tumor are shown in Table II. No 5-FU was detected in the serum or tumors in the 5-FU group, since the assay was performed 12 days after the last treatment. Higher concentrations of 5-FU were observed in the serum and tumor after treatment with HCFU, whereas the 5-FU concentration in the serum of mice in the BOF-A2 and UFT groups was less than 0.1 μg/ml. The concentration of 5-FU in the tumors of mice treated with BOF-A2 and UFT ranged from 0.125 to 0.230 μg/g, although the 5-FU concentrations in the tumors of mice treated with HCFU were 0.468 μg/g for H-111 and 4.427 μg/g for Co-4. When the control and 5-FU groups were eliminated, there was a statistically significant correlation between the serum and tumor concentrations of 5-FU with a coefficient of correlation of 0.904.

The change in the concentration of TS with time is shown in Table III. Total TS (TStot) was increased in Co-4 treated with 5-FU and HCFU to a statistically significant extent at each point tested, and TStot values were highest 24 h after the treatment. No statistically significant differences were observed in the changes in TStot between the 5-FU and HCFU treatment. The activity of TSfree was suppressed by 5-FU and HCFU, and no TSfree was detected 2 h after treatment with 5-FU and HCFU. TSfree recovered to the control levels after the significant suppression at 6 h after the treatment with both drugs. TS inhibition rates (TSIR) were 0.75 and 0.85 at 2 h after the treatment with 5-FU and HCFU, respectively, and this suppression continued until 6 h after treatment. Although TSIR values recovered to 0.68 for 5-FU and 0.73 for HCFU, the inhibition of TS seemed to continue even after the recovery of TSfree.

Table I. Antitumor Activity of Fluoropyrimidine-related Compounds

Tumor	Group	n	Actual TW[a]	T/C (TW)[b]	T/C (RW)[c]
H-111	Control	7	1843±791	—	—
	5-FU[d]	10	1224±814	66.3	58.9
	HCFU[e]	11	814±312*	44.1	52.6
	BOF-A2[f]	3	1138±244	61.7	108.8
	UFT[g]	9	1262±711	68.4	78.3
Co-4	Control	8	1392±554	—	—
	5-FU	8	689±272*	49.5	35.4
	HCFU	5	525±189*	37.7	23.6
	BOF-A2	7	1214±1000	87.2	92.2
	UFT	6	981±472	70.5	56.6

a) Actual tumor weight in mg at end of experiment (mean± SD).
b) T/C ratio of actual tumor weight (%).
c) The lowest T/C of relative mean tumor weight (%) during the experiment. Underlining indicates positive antitumor activity.
d) 5-FU at a dose of 60 mg/kg was administered ip in a q4d×3 schedule.
e) HCFU at a dose of 70 mg/kg was administered po daily for 3 wk.
f) BOF-A2 at a dose of 30 mg/kg was administered po daily for 3 wk.
g) UFT at a dose of 20 mg/kg (as tegafur) was administered po daily for 3 wk.
* $P<0.01$ relative to control.

Table II. Concentrations of 5-Fluorouracil in Serum and Tumor of Mice Treated with Fluoropyrimidine-related Compounds

Tumor	Group	n	Serum[a]	Tumor[b]
H-111	Control	9	—	—
	HCFU[c]	12	0.45±0.28	0.468±0.369
	BOF-A2[d]	4	0.02±0.04	0.125±0.018
	UFT[e]	5	0.04±0.023	0.136±0.057
Co-4	Control	10	—	—
	HCFU	6	0.84±0.65	4.427±3.639
	BOF-A2	8	0.07±0.06	0.230±0.065
	UFT	6	0.02±0.005	0.148±0.099

Tumors were resected 2 h after the last treatment.
a) Serum concentration of 5-FU in μg/ml.
b) Tumor concentration of 5-FU in μg/g.
c) HCFU at a dose of 70 mg/kg was administered po daily for 3 wk.
d) BOF-A2 at a dose of 30 mg/kg was administered po daily for 3 wk.
e) UFT at a dose of 20 mg/kg (as tegafur) was administered po daily for 3 wk.
A statistically significant correlation was found between serum and tumor concentrations with a coefficient of correlation of 0.904 ($t=5.846$, $P<0.01$).

TS and TSIR values for each fluoropyrimidine treatment are shown in Table IV. TStot in control tumors was 0.88 pmol/g for H-111 and 0.93 pmol/g for Co-4 without any statistically significant difference (t=0.446), and no difference was observed between TSfree in H-111 and Co-4 tumors (t=0.439). No TS inhibition was observed in the control Co-4 tumors, and TSIR in the control H-111 tumors was limited to 0.06. TStot was slightly increased in the treated H-111 and Co-4 groups, whereas TSfree was suppressed in the H-111 groups treated with HCFU and UFT, and in the Co-4 groups treated with 5-FU, HCFU and UFT, with statistical significance. TSIR in the treated Co-4 groups was more apparent than that in the treated H-111 groups except for those given UFT. However, a statistical comparison was impossible, because no TSIR in the control Co-4 group was calculated.

The statistical correlations between antitumor activity in terms of the actual tumor weights and the pharmacokinetics of 5-FU or TS are shown in Table V. A statistically significant correlation was observed between the 5-FU concentration in serum and tumor, and actual tumor weight at the end of the experiment when the control and 5-FU-treated groups were eliminated from the calculations. These correlations indicated that higher 5-FU concentrations in the serum and tumor resulted in higher antitumor activity. TSIR and antitumor effect were also significantly correlated when the control tumors were included, whereas TStot was not correlated with antitumor activity. Suppression of TSfree also resulted in higher antitumor activity when the control tumors were included. From these results, higher concen-

Table III. Changes in Thymidylate Synthetase Activity with Time in Co-4 Tumor Treated with 5-FU and HCFU

Drug	Time[a] (h)	n[b]	TStot[c]	TSfree[d]	TSIR[e]
Control	0	10	0.9±0.2	1.0±0.4	0
5-FU[f]	2	6	1.7±0.3***	UD	0.75±0.13
	6	6	1.9±0.8**	0.4±0.2**	0.73±0.08
	24	6	3.4±0.7***	1.0±0.2	0.68±0.04
HCFU[g]	2	6	2.5±0.5***	UD[h]	0.85±0.08
	6	6	2.3±0.4***	0.5±0.1*	0.85±0.05
	24	6	3.1±0.8***	0.8±0.2	0.73±0.08

a) Time after drug administration in hours.
b) Number of tumors.
c) Total thymidylate synthetase activity in pmol/g.
d) TSfree in pmol/g.
e) Inhibition rate of thymidylate synthetase TSIR=(TStot−TSfree)/TStot.
f) 5-Fluorouracil at a dose of 50 mg/kg was administered po once.
g) HCFU at a dose of 100 mg/kg was administered po once.
h) Undetectable.
* $P<0.02$, ** $P<0.01$, *** $P<0.001$ relative to control.

Table IV. Inhibition of Thymidylate Synthetase by Fluoropyrimidine-related Compounds

Tumor	Group	n	TStot[a]	TSfree[b]	TSIR[c]
H-111	Control	9	0.88±0.30	0.92±0.32	0.06±0.17
	5-FU[d]	10	1.36±0.28**	0.96±0.22	0.24±0.33
	HCFU[e]	12	1.31±0.12**	0.34±0.32***	0.75±0.24***
	BOF-A2[f]	2	1.00±0.14	0.80±0.00	0
	UFT[g]	5	1.90±0.38***	0.32±0.19**	0.80±0.07***
Co-4	Control	10	0.93±0.18	0.99±0.37	0
	5-FU	8	1.30±0.11***	0.54±0.11**	0.60±0.08
	HCFU	6	1.05±0.27	0.18±0.20***	0.85±0.16
	BOF-A2	8	1.52±0.31***	0.74±0.11	0.49±0.10
	UFT	6	1.25±0.38	0.60±0.20*	0.52±0.04

Tumors were resected 2 h after the last treatment with HCFU, BOF-A2 and UFT, or 12 days after the last treatment with 5-FU.
a) Total thymidylate synthetase activity in tumor in pmol/g.
b) TSfree in tumor in pmol/g.
c) Inhibition rate of thymidylate synthetase TSIR=(TStot−TSfree)/TStot.
d) 5-FU at a dose of 60 mg/kg was administered ip in a q4d×3 schedule.
e) HCFU at a dose of 70 mg/kg was administered po daily for 3 wk.
f) BOF-A2 at a dose of 30 mg/kg was administered po daily for 3 wk.
g) UFT at a dose of 20 mg/kg (as tegafur) was administered po daily for 3 wk.
* $P<0.05$, ** $P<0.01$, *** $P<0.001$ relative to control. Control tumor of H-111 vs. Co-4.
TStot, t=0.446; TSfree, t=0.439: not significant.

Table V. Correlation between Antitumor Activity and Concentration of 5-FU or Thymidylate Synthetase Inhibition

Parameter of concentration or thymidylate synthetase	Coefficient of correlation with actual tumor weight[a)]
Concentration of 5-FU in serum[b)]	−0.927***
Concentration of 5-FU in tumor[c)]	−0.841*
Thymidylate synthetase inhibition rate	−0.694*
Total thymidylate synthetase	−0.144
Free thymidylate synthetase	0.722**

a) Coefficient of correlation with actual tumor weight at the end of the experiment.
b) Concentration of 5-fluorouracil in serum in μg/ml.
c) Concentration of 5-fluorouracil in tumor in μg/g.
* $P<0.05$, ** $P<0.02$, *** $P<0.01$.

trations of 5-FU in the serum and tumor, and higher TSIR and lower TSfree values seemed to be important factors for higher antitumor activity of fluoropyrimidine-related compounds.

DISCUSSION

Although 5-FU along with its related fluoropyrimidines is one of the most widely available antitumor agents for treatment of solid tumors, the mechanism of its action is still controversial in terms of the blocking of DNA phosphorylation through inhibition of thymidylate synthetase or the distortion of gene expressions after incorporation of fluoropyrimidines into RNA.[1-5] However, it has been reported that the main mechanism of action of fluoropyrimidines involves blocking of phosphorylation by inhibiting the activity of TS at drug doses equivalent to those used clinically and in *in vivo* experiments.[3, 12] The clinically successful chemical modulation of fluoropyrimidines by leucovorin[13, 14] via the ternary complex of TS, FdUMP and 5,10-methylene tetrahydrofolate, a metabolite of leucovorin, also supports this main mechanism of action of fluoropyrimidines in TS inhibition. Since this chemical modulation of fluoropyrimidines and the development of fluoropyrimidine derivatives are widely applicable to cancer patients, clarification of the mechanism of action of these agents is important.

This paper describes the antitumor activity of four fluoropyrimidines against two human carcinoma xenografts in nude mice with reference to the pharmacodynamics of 5-FU and changes in TS and TS inhibition. Co-4 is a poorly differentiated adenocarcinoma of the colon which was established from a cultured cell line, C-1, derived from a pulmonary metastatic lesion of colon adenocarcinoma. We have already reported that Co-4 is highly sensitive to various kinds of antitumor agent including 5-FU,[15] and that the chemosensitivity of this strain has remained stable through serial passages over more than 15 years. In fact, Co-4 was sensitive to 5-FU and HCFU in this study and its sensitivity to fluoropyrimidines in general seemed to be higher than that of H-111, which is a well differentiated adenocarcinoma of the stomach. H-111 is reported to be less sensitive to many antitumor agents other than 5-FU, which was most effective on this strain when administered ip at a dose of 60 mg/kg using a q4d×3 schedule. Since the AUC-dose curve showed a turning point at a dose of 50–60 mg/kg 5-FU because of metabolic saturation in the mouse, 5-FU was thought to be most effective when given as a bolus three times.[9]

Because 5-FU was administered using a q4d×3 schedule to elicit its maximum antitumor activity, no 5-FU was detected in the serum or tumor at the end of the experiment 12 days after the last administration. With the other three masked compounds of 5-FU, however, 5-FU still remained in the serum and tumor at 2 h after the last administration in each case. It was apparent that the concentration of 5-FU in the tumor was 3 to 7 times higher than that in the serum except for the HCFU-H-111 pair match, for which the serum and tumor concentrations were approximately the same. This might be due to the fact that these masked compounds were developed to produce a lower serum concentration in order to reduce any adverse effects and to increase the tumor concentration, thus improving the antitumor activity. However, there was a statistically significant correlation between the serum and tumor concentrations of 5-FU, suggesting that a higher serum concentration resulted in a higher tumor concentration, which would be necessary for better antitumor activity.

When the changes in TS with time were observed in the Co-4 tumor treated with 5-FU and HCFU, the patterns of change induced by 5-FU and HCFU were essentially identical, manifested by increased TStot and suppressed TSfree, resulting in increased TSIR. TSfree was not detectable 2 h after the treatment, but had recovered by 24 h. In contrast, the TSIR continued up to 24 h after the treatment, because the increased TStot values were stable for up to 24 h. These changes with time indicated that the most remarkable change in TS would be observed 2 h after the treatment; in particular, complete suppression of TSfree. These results were comparable with data from our previous study[4] in which the maximum TSIRs were observed at 3 h after the po administration of the fluoropyrimidines. Since the maximum concentrations of 5-FU in the serum and tumor are also observed at 2 h after the po administration of the fluoropyrimidines,[16] the concentrations of 5-FU in the serum and tumor were assessed at 2 h after the treatment for comparison with TS in the present study.

The changes in TS activity would continue until 24 h after the treatment in terms of increased TStot and TSIR. This continued inhibition of TS was also supported by the observation that TS in H-111 and Co-4 tumors was inhibited even 12 days after the last administration of 5-FU. It was noteworthy that TSIR continued when no 5-FU was detectable in the serum or tumor. This suggested that TSfree and TSIR could be promising markers for evaluation of antitumor activity as distinct from the pharmacodynamics of 5-FU. This was also supported by the statistically significant correlation between antitumor activity in terms of actual tumor weights, and TSfree and TSIR. Greater inhibition of TSfree and higher TSIR resulted in higher antitumor activity in the present study. Although the concentrations of 5-FU in the serum and tumor were also significantly correlated with antitumor activity, this high correlation was influenced by the result for HCFU, for which the high 5-FU concentration and its resulting effect were markedly different from those in the cases of the other two masked compounds. On the other hand, the distributions of TSfree and TSIR were close to a standard distribution, indicating that the correlation between the two parameters and antitumor activity was more significant than would be expected from the pharmacodynamics of 5-FU.

This study has demonstrated that the antitumor activity of fluoropyrimidines is related to the inhibition of thymidylate synthetase in human tumor xenografts in nude mice. However, it should be mentioned that some discrepancies were also observed in the experiments. For example, the antitumor activity of UFT on H-111 was limited, although the TSIR by UFT was similar to that of HCFU, which was effective on H-111. This discrepancy may be partly explained by the RNA-directed antitumor activity of HCFU but not UFT. However, since an overall correlation between the antitumor activity and TSIR was found, the findings suggested that assay of TSfree and TSIR would be useful for estimating the antitumor activity of fluoropyrimidines. It is also suggested that the enhancement of TSIR using *l*-leucovorin through the formation of a ternary complex of TS, FdUMP and a derivative of *l*-leucovorin is a promising method for increasing the antitumor activity of fluoropyrimidines.[13, 14] Further trials to increase TSIR in tumor tissues while minimizing the serum concentration of 5-FU to overcome the severe side effects of fluoropyrimidine will be important for clinical application.

(Received October 3, 1990/Accepted January 25, 1991)

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