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Experimental report

Development of a novel form of an oral 5-fluorouracil derivative (S-1) directed to the potentiation of the tumor selective cytotoxicity of 5-fluorouracil by two biochemical modulators

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We have focused our attention on the development of a novel form of a tegafur-based [FT; a prodrug of 5-fluorouracil (5-FU)] antitumor agent. We have used two biochemical and pharmacological modulators of 5-FU to improve its overall activity. To potentiate the antitumor activity of FT, 5-chloro-2,4-dihydroxypyridine (CDHP) was used as a potent reversible inhibitor of 5-FU degradation. The reduction of gastrointestinal (GI) toxicity, induced in the host by 5-FU, was modulated by potassium oxonate (Oxo), an inhibitor of orotate phosphoribosyltransferase that catalyzes the phosphorylation of 5-FU, a process believed to be responsible for the toxic effects of 5-FU. When CDHP and FT were simultaneously given orally to Yoshida sarcoma-bearing rats in various molar ratios, the antitumor effect of FT was significantly potentiated by the combination consisting of at least a 0.2 versus 1 molar ratio of CDHP to FT, respectively. This augmentation of an antitumor activity was supported by potent and prolonged inhibition of dihydrouracil dehydrogenase activity (5-FU degrading activities) in the liver of tumor-bearing rats after oral CDHP (0.2:0.8 molar ratio) and furthermore by elevation and over 12 h retention of 5-FU levels in the tumors following combined administration of FT and CDHP at a molar ratio of 1:0.4, respectively. Moreover, to reduce the severe GI injury and subsequent loss of body weight, observed in parallel with an increased antitumor efficacy, Oxo was given orally to Yoshida sarcoma-bearing rats and nude rats xenografted with H-81 human gastric carcinoma, during consecutive administration of the FT–CDHP mixture. Combined treatment with Oxo and FT (1:2 molar ratio) supplemented with 0.4 molar CDHP resulted in protection of body weight loss without affecting the high antitumor efficacy of the FT–CDHP mixture. When [2-^{14}C]FT plus CDHP was administered with Oxo, the ^{14}C-labeled fluoronucleotide content was objectively decreased in the GI tract of the tumor-bearing rats but not in the tumor and bone marrow, which supports our initial hypothesis. Based on these promising data, we propose a suitable formulation of a FT-based anticancer drug, called S-1, and consisting of FT, CDHP and Oxo at a 1:0.4:1 molar ratio and showing tumor-selective cytotoxicity of 5-FU.

Key words: 5-Fluorouracil, cytotoxicity, gastrointestinal toxicity, novel, oral, S-1, tegafur.

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Introduction

The fluorinated pyrimidine 5-fluorouracil (5-FU) is an antimetabolite that is frequently used in the treatment of patients suffering from breast, head and neck, stomach, and colorectoral cancers. However, the clinical effect of 5-FU alone is not satisfying from the viewpoint of antitumor response, survival and quality of life. To improve the clinical response, various biochemical and pharmacokinetic modulations of 5-FU have been tried in clinics and, recently, continuous venous infusion (CVI) of 5-FU is though to be a useful method to gain a relatively higher response rate.

In 1989, Rokich *et al.*[1] reported that a long-term CVI of 5-FU at 300 mg/m^2 day resulted in a higher response rate than i.v. bolus 5-FU at 500 mg/m^2/5 weeks in a randomized study on adjuvant chemotherapy of metastatic colorectal cancers. CVI of 5-FU has also been tried extensively in patients with gastric, colorectal and breast cancers, and resulted in about 30% response rates.[2–9] However, gastrointestinal (GI) toxicity, but not myelosuppression, appeared to be a dose-limiting factor during CVI of 5-FU. Caballero[10] also reported that myelosuppres-

sion, alopecia, nausea and vomiting were not observed during long-term CVI of 5-FU for 54–324 days.

It has been generally recognized that GI toxicity is caused by the phosphorylation of 5-FU in the digestive tract.[11] This prompted us to search for an inhibitor of 5-FU phosphorylation catalyzed by orotate phosphoribosyltransferase (OPRTase) (EC 2.4.2.10) and to apply it to protection against GI toxicity induced in tumor-bearing animals during 5-FU therapy. We have found that oxonic acid (Oxo), when given orally, markedly inhibited the conversion of 5-FU to 5-fluorouridine 5′-monophosphate (FUMP) in GI tissues and protected against 5-FU-induced injury of the GI tract in rats.[12]

Another complication of therapy with 5-FU given in CVI is the variation of 5-FU levels in the blood of cancer patients, as indicated by several reports.[13–23] Erlichman *et al.*[20] and Fox *et al.*[15] reported marked variation in the plasma levels of 5-FU in different patients on a day to day basis. Also Petit *et al.*,[21] Harris *et al.*[22] and Metzger *et al.*[23] demonstrated circadian variation in the plasma concentration of 5-FU during CVI in cancer patients. These variation are probably related to circadian changes in the catabolism of 5-FU.[24,25]

5-FU is rapidly degraded, mainly in the liver by dihydropyrimidine dehydrogenase (DPDase) (EC 1.3.1.2), and is excreted into the urine as 2-fluoro-β-alanine.[26] Therefore, inhibition of the activity of DPDase seems to lead to potentiation of antitumor activity of 5-FU *in vivo*. Considering such a possibility, we found potent reversible inhibitors of DPDase such as 5-chloro-2,4-dihydroxypyridine (CDHP) and 3-cyano-2,6-dihydroxypyridine, showing about a 200- to 300-fold higher inhibitory effect than uracil.[27] Thus, we demonstrated that 5-FU levels in the blood of rodents during CVI were significantly increased and remained stable during co-infusion with these inhibitors.[25]

In a further investigation on the tumor-selective cytotoxicity of fluorinated pyrimidines, we have attempted to develop a novel anticancer drug possessing high antitumor activity and low toxicity. The present paper reports a desirable effect of the combination of two biochemical and pharmacological modulators, CDHP (a potent inhibitor of 5-FU degradation) and Oxo (an inhibitor of 5-FU phosphorylation) with tegafur (FT), a prodrug of 5-FU, synthesized by Hiller *et al.*[28] that is gradually converted to 5-FU in the microsomal fraction of the liver after oral administration.

Materials and methods

Chemicals

5-FU was purchased from Sigma (St Louis, MO). [6-^{14}C]tegafur (2.0 GBq/mmol) was obtained from Amersham. [6-^{3}H]5-FU (654.9 GBq/mmol) was from DuPont (Boston, MA). FT CDHP, Oxo and uracil were products of Taiho Pharmaceutical (Tokyo, Japan). All other chemicals used were commercially available products.

Animals

Four-week old male donryu strain rats were purchased from Charles River Japan (Tokyo, Japan) and supplied *ad libitum* with a commercial diet and autoclaved water until use. Male BALB/cA Jcl-nu nude mice (5 weeks old), and male and female F344/N Jcl-rnu nude rats (each 5 weeks old) were obtained from CLEA Japan (Tokyo, Japan) and maintained in clean cabinets until the end of the therapeutic experiments.

Tumor cells

Yoshida sarcoma cells were supplied by Sasaki Research Institute (Tokyo, Japan) and passaged in donryu rats weighing about 150 g by i.p. inoculation of 1×10^4 cells at weekly intervals. Human stomach cancer, H-81, was kindly provided by Dr Fujita (Institute for Microbial Disease, Osaka University, Suita, Japan) and passaged in nude mice weighing 25–35 g by s.c. implantation of approximate 1–2 mm^3 cubed fragments into the back of mice.

Antitumor experiments

Groups consisting of 7 to 10 rats were used. Solid-type Yoshida sarcoma was prepared by s.c. transplantation of 1×10^4 cells into the back of rats on day 0. Drugs, suspended in 0.5% hydroxypropylmethyl cellulose (HPMC) solution, were administered orally daily for seven consecutive days, starting 24 h after implantation of tumor cells. Control rats were given 0.5% HPMC solution only by the same schedules. On day 8, the rats were killed and their tumors were removed and weighed for evaluation of antitumor activity.

In separate experiments, Yoshida sarcoma cells (1×10^7 cells/20 μl of saline) were implanted into the colon tissue of rats under ethyl ether anesthesia on day 0.

After drug administration for 7 days, the tumors in colon and pancreas were removed and weighed.

Human stomach cancer, H-81 was prepared by s.c. implantation of about 2 mm^3 cubed fragment into the right armpit of nude rats. When tumor volume [1/2 × (the major axis) × (a minor axis)^2] reached about 200 mm^3 were administered for 8 days.

On day 9, the antitumor activity and toxicity of drugs were evaluated as the relative inhibition rates of tumor growth of drug-treated rats to that of control rats and the body weight change (rats of gain or loss) of rats in each group, respectively.

Pathological evaluation of injury of the digestive tract

The degree of GI injury of drug-treated rats was evaluated pathologically as described in a previous paper.[12]

Measurement of DPDase activities in the liver

Rats were decapitated and the liver of each rat was rapidly perfused with ice-cold saline and removed. All subsequent procedures were carried out at 4°C. The livers were homogenized in 4 volumes of 50 mM Tris–HCl (pH 8.0) containing 5 mM 2-mercaptoethanol, 25 mM KCl and 5 mM $MgCl_2$. The homogenate was centrifuged at 105 000 *g* for 60 min and the supernatant was used for the measurement of DPDase activity as described before.[27] The assay mixture, in a final volume of 0.25 ml, consisted of 50 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 25 mM NaF, 50 mM nicotinamide, 5 mM ATP, 1 mM NADPH, 10 μM [6-^{3}H]5-FU (74 Bq) and 0.1 ml of the enzyme extract. The mixture was incubated for 5 min at 37°C and the reaction was stopped by adding 0.025 ml of 2 M perchloric acid. After centrifugation at 3000 r.p.m., the supernatant (0.1 ml) was neutralized with 0.03 ml of 2 M potassium hydroxide and an aliquot of the supernatant was spotted onto a thin layer chromatography plate (Merck silica gel 60 F254 precoated plate, 2.5 × 10 cm, thickness 0.25 mm) and developed with a mixture of chloroform, methanol and acetic acid (17:3:1, v/v/v). The spots of 2-fluro-β-alanine and 2-fluoro-β-ureidopropionic acid, which are degradative products of 5-FU, were scraped into vials and mixed with 10 ml of ACS-II scintillation fluid (Amersham) and the radioactivity was measured in a Wallac 1410 liquid scintillation counter (Pharmacia).

Extraction and determination of FT and 5-FU in blood and tumors

Drugs were administered orally to Yoshida sarcoma-bearing rats on day 8 after tumor implantation. Then rats were sacrificed at various times and their blood and tumors were rapidly removed. The tumors were homogenized with 3 volumes of ice-cold saline, followed by centrifugation at 10 000 *g* for 20 min, and the resultant supernatant was used as crude extracts containing FT and 5-FU. Then 1 ml of the serum or the crude extract was added to 0.1 ml of the known amount of the internal standard (IS) solution (5-bromouracil for 5-FU and 1-ethoxymethyl thymine for FT) and shaken with 5 ml of chloroform.

The mixture was centrifuged at 3000 r.p.m. for 10 min and the organic layer containing FT and IS was removed. Next, the remaining aqueous layers containing 5-FU and 5-bromouracil were extracted with 4 ml of ethyl-acetate twice and the two ethyl-acetate layers were combined and evaporated at 40°C under a gentle stream of nitrogen gas. The residue was dissolved in distilled water, passed through a 0.45 μm filter and the 5-FU content of the filtrates was determined by reverse-phase high performance liquid chromatography (Gulliver HPLC System, JASCO Co., Tokyo, Japan). An aliquot of the sample was applied to a Chemcosorb (4.6 ID × 250 mm) column (Chemco Co., Osaka, Japan), under the following chromatographic conditions: monitoring wavelength, 270 nm; flow rate, 1 ml/min; mobile phase, 20 mM monopotassium phosphate solution (pH 4.5).

Extraction and determination of radiolabeled 5-fluoronucleotides in tissues

Drugs containing [2-^{14}C]FT were orally given to tumor-bearing rats. At the indicated time, rats were sacrificed, and the tumor and normal tissues were rapidly removed and promptly frozen and kept at − 80°C until analysis. The tissues were homogenized with 3 volumes of 5% trichloroacetic acid and centrifuged at 3000 r.p.m. for 10 min. Subsequent procedures were the same as described in a previous paper.[12]

Results

Effects of CDHP on the antitumor activity of FT

One of the objectives of our recent research was the potentiation of antitumor activity of 5-FU by bio-

chemical and pharmacological modulation. To achieve a desirable effect, we used FT, a masked form of 5-FU which was maintained for a long time in the blood following oral administration and CDHP, a potent inhibitor of the 5-FU degradation process mainly occurring in the liver.

Table 1 shows the antitumor effect of FT, CDHP and their combination on the growth of Yoshida sarcoma in rats. The combination of CDHP and FT was markedly effective against Yoshida sarcoma, while both FT and CDHP given alone had no activity at the dose range used. Moreover, it was found that maximal antitumor efficacy of FT (10–20 mg/kg) had been observed with the supplementation of at least 0.4 molar equivalents of CDHP in Yoshida sarcoma system. Similar effects of the combination of FT and CDHP were observed in sarcoma-180-bearing mice (data not shown).

However, 5-FU-induced toxicities such as diarrhea and loss of body weight universally accompanied almost complete tumor growth inhibition.

Effect of administration of CDHP on DPDase activity in the liver of tumor-bearing rats

In vivo inhibitory effect of oral CDHP on DPDase activity in the liver was investigated using tumor-bearing rats. Rats received 0, 2.91, 5.82 and 11.63 mg/kg CDHP corresponding to the following molar ratios of CDHP, i.e. 0, 0.2, 0.4 and 0.8, to FT that was used at a constant dose of 20 mg/kg. Control (no CDHP) values of DPDase activity ranged from of 1.18 to 1.67 nmol/mg/5 min, suggesting possible circadian changes of DPDase in the liver (Figure 1A). When 2.91–11.63 mg/kg CDHP was given to the rats, DPDase activities in the liver markedly decreased to about 20% of the control values at 1–2 h after administration and these inhibitory effects were maintained for at least 12 h. However, no significant differences of the inhibition of 5-FU degradation by CDHP were observed between 0.2 and 0.8 molar CDHP as shown in Figure 1(b).

5-FU levels in the blood and tumors after administration of FT plus CDHP

Suitable molar composition of CDHP and FT was also examined from the point of view of 5-FU levels in the blood and tumor tissue in rats: 20 mg/kg FT combined with 2.91, 5.82 and 11.63 mg/kg CDHP was orally administered to Yoshida Sarcoma-bearing rats. 5-FU levels in the blood and tumors of rats following administration of FT alone were almost not detected (data not shown).When CDHP was co-administered with FT, 5-FU levels in the blood were markedly elevated and reached levels of about 600–900 ng/ml at

Table 1. Antitumor effects of FT, CDHP and their respective combinations against Yoshida sarcoma in rats

Experiment	Drug	Dose (mg/kg)		*n*	Tumor weight (g ± SD)	IR[a] (%)	Body weight (g)[b]		BWC[c] (% control)
		FT	CDHP				day 0	day 8	
I	control	—	—	14	2.533 ± 0.437	—	142.9	201.9	—
	FT alone	10		7	2.164 ± 0.511	15	140.3	203.6	107.2
		20		7	2.015 ± 0.441	20	145.3	204.5	100.4
	CDHP alone	—	11.7	7	2.406 ± 0.652	5	141.0	194.4	90.5
		—	23.4	7	2.400 ± 0.718	5	145.2	208.8	107.8
II	control	—	—	14	1.479 ± 0.646	—	160.5	219.2	—
	FT+CDHP	5	0.73	6	1.346 ± 0.418	9	160.5	217.6	97.4
	(1:0.2)[d]	10	1.45	7	0.784 ± 0.269	47	159.7	185.1	43.2
		20	2.91	7	0.020 ± 0.049	99	157.0	162.2	8.8
	FT + CDHP	5	1.54	7	1.318 ± 0.374	11	156.5	200.0	74.1
	(1:0.4)	10	2.91	7	0.352 ± 0.292	76	158.8	193.7	59.5
		20	5.82	7	0.000 ± 0.000	100	164.8	140.9	− 40.5
	FT + CDHP	5	2.18	7	1.103 ± 0.304	25	154.7	204.2	84.4
	(1:0.6)	10	4.36	7	0.106 ± 0.088	93	160.7	179.8	32.6
		20	8.73	7	0.000 ± 0.000	100	157.6	126.6	− 52.9
	FT + CDHP	5	2.91	7	1.263 ± 0.241	15	157.3	201.3	75.0
	(1:0.8)	10	5.82	7	0.210 ± 0.201	86	155.3	182.5	46.0
		20	11.63	7	0.000 ± 0.000	100	155.5	112.5	− 75.8

[a] Inhibition rate (IR) of tumor growth. [b] Mean body weight of seven or 14 rats. [c] Body weight change was calculated as a percentage of weight gain or loss in drug-treated groups against that of the control group. [d] Molar ratio of FT and CDHP.

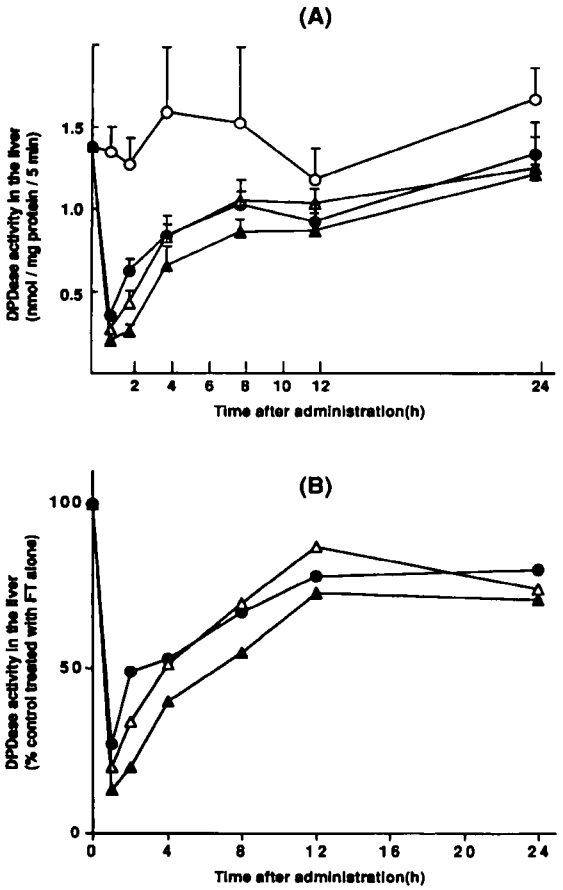

Figure 1. Changes in DPDase activities in the liver of rats following administration of the mixture of FT and CDHP: 20 mg/kg FT combined with 0 (○), 0.2 (●) 0.4 (△) and 0.8 (▲) molar CDHP was orally administered to Yoshida sarcoma-bearing rats on day 8 after tumor implantation. Rats were decapitated at indicated times, and the livers were perfused with saline and used for a measurement of DPDase activities as described in Materials and methods. (A) Activity of DPDase (means ± SD for three rats). (B) Percentage of DPDase activity in the rat liver treated with 1 molar FT plus 0.2 (●), 0.4 (△) or 0.8 (▲) molar CDHP to those treated with FT alone.

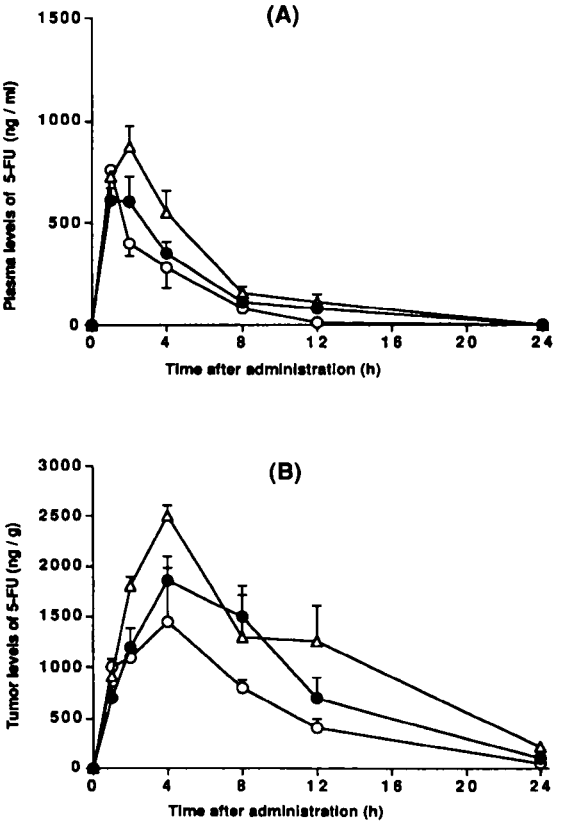

Figure 2. 5-FU levels in the blood and tumor after oral administration of the mixture of FT and CDHP to Yoshida sarcoma-bearing rats: 20 mg/ FT combined with 0.2 (○), 0.4 (●) and 0.8 (△) molar CDHP was orally administered to Yoshida sarcoma-bearing rats, and the blood and tumor were removed at indicated times. 5-FU concentrations in the plasma (A) and tumor (B) were determined as described in Materials and methods. Values are means ± SD for three rats.

1–2 h after administration. It should be stressed that increased levels of 5-FU declined very slowly from the blood within 12 h (Figure 2A).

The 5-FU level in the tumor tissue is closely related to its antitumor effect. As shown in Figure 2(B), the increase of 5-FU levels in the tumor was more dramatic than that in the blood, reaching maximal concentrations of about 1400–2500 ng/g tissues at 4 h after administration of 20 mg/kg FT combined with CDHP at a molar ratio of 0.2 to 0.8.

Moreover, elevated 5-FU levels were maintained and persisted for a long time (over 12 h). Namely, 5-FU concentrations in the tumor were 750 and 1162 ng/g at 12 h following administration of FT at a constant dose of 20 mg/kg and CDHP at a molar ratio of 1:0.4 and 1:0.8, respectively.

These results indicate that co-administration of 20 mg/kg FT with CDHP at a molar ratio 1:0.4 and more resulted in reaching high levels persisting for 24 h of 5-FU in the tumor tissue.

Effect of Oxo on antitumor activity and toxicity of FT plus CDHP in tumor-bearing rats

As mentioned before, the combination of FT and CDHP causes severe toxicities such as marked loss of body weight and GI injury in tumor-bearing rats achieving almost complete reduction of the tumor mass. Therefore, we have investigated the effect of

co-administration of Oxo, an agent predominantly distributed at higher levels in GI tissues but not in the plasma and tumor and selectively inhibiting a phosphorylation of 5-FU,[10] on antitumor activity and toxicity of the combination of FT and CDHP in Yoshida sarcoma-bearing rats. In this experiment, 0, 0.5, 1 and 2 molar ratios of Oxo to FT, given over the dose range of 5–20 mg/kg, were administered orally to rats.

As shown in Table 2, Oxo alone (39 and 78 mg/kg) exerted neither antitumor nor toxic effects to rats. Co-administration of Oxo at molar ratios of 0.5 and 1.0 decreased the incidence of 5-FU-induced diarrhea and loss of body weight caused by FT plus CDHP without affecting the antitumor activity of those.

However, when the molar ratio of Oxo was increased up to 2 in the combination of 1 molar FT plus 0.4 molar CDHP, there was a significant decrease in both antitumor activity and toxicity in the tumor-bearing rats. Table 3 shows the change of the grade of pathological injury of the GI tract such as ileum, cecum and colon in rats following 7 day administration of 1 molar FT (20 mg/kg) plus 0.4 molar CDHP with or without Oxo; 20 mg/kg FT combined with 5.82 mg/kg CDHP induced an injury of the ileum, cecum and colon (grade ± to + +). However this injury was significantly reduced by co-administration of 9.76–19.5 mg/kg of Oxo and was almost completely prevented by the combination with 39.0 mg/kg of Oxo (2:1 Oxo:FT molar ratio).

In another experiment presented in Table 4, 1 molar equivalent of Oxo (19.5 mg/kg) was given before, simultaneously or after oral FT (20 mg/kg) plus CDHP (5.82 mg/kg) administration to Yoshida sarcoma-bearing rats.

As a result, simultaneous administration of Oxo with FT plus CDHP was found to exert the most suitable balance between antitumor activity and toxicity.

The above finding prompted us to evaluate the effect of Oxo on the antitumor activity and toxicity of the FT–CDHP combination in nude rats bearing xenografts of human stomach cancer, H-18. In this case, 20 mg/kg FT plus 5.82 mg/kg CDHP in combination with 0, 3.90, 9.75, 19.50 and 39.00 mg/kg Oxo were administered for 8 days. As shown in Figure 3, co-administration of the FT–CDHP combination (1:0.4 molar ratio) with 0 and 0.2 molar Oxo resulted in a potent antitumor efficacy but caused toxic death to two and three of six rats, respectively. However, co-administration of a 0.5 to 2 molar ratio of Oxo with the FT–CDHP combination reduced the toxicity (toxic death and loss of body weight) without affecting antitumor activity of the latter. This result is fairly in agreement with that observed in Yoshida sarcoma-bearing rats.

On the basis of these results, we recommend that the combination of FT:CDHP:Oxo at a molar ratio of 1:0.4:1, respectively, is suitable to achieve higher antitumor activity and lower toxicity in tumor-bearing rats.

Table 2. Effect of Oxo on antitumor activity and toxicity of FT plus CDHP in Yoshida sarcoma-bearing rats

FT:CDHP:Oxo (molar ratio)	Dose[a] (mg/kg)	*n*	Tumor weight (g ± SD)	IR[b] (%)	Diarrhea (*n*)	Body weight (g)[c]		BWC[d] (% control)
						day 0	day 8	
Control	—	13	1.330 ± 0.283	—	0	143.0	199.0	—
1:0.4:0	10	7	1.059 ± 0.379	20	1	143.8	173.1	52.3
	20	7	0.000 ± 0.000	100	6	146.0	120.7	−45.1
1:0.4:0.5	10	7	0.398 ± 0.302	70	0	147.5	186.0	68.8
	20	7	0.000 ± 0.000	100	1	144.2	167.0	40.8
1:0.4:1	10	7	0.370 ± 0.224	72	0	146.7	185.4	69.0
	20	7	0.009 ± 0.020	99	0	147.1	177.8	54.9
1:0.4:2	10	6	0.538 ± 0.217	60	0	149.8	187.7	67.7
	20	7	0.551 ± 0.295	59	0	150.2	181.7	56.2
Control	—	14	2.533 ± 0.437	—	0	142.9	201.9	-
Oxo alone	39[e]	7	2.164 ± 0.536	15	0	142.2	199.0	96.3
	78[e]	7	2.321 ± 0.914	8	0	143.7	204.9	103.9

[a] Dose of FT. [b] Inhibition rate of tumor growth. [c] Mean body weight of rats in each group. [d] Body weight change was calculated as a percentage of weight gain or loss in the drug-treated group against that of the control group. [e] Dose of Oxo corresponding to 2 and 4

Table 3. Effect of co-administration of Oxo on 5-FU induced GI injury in Yoshida sarcoma-bearing rats during FT plus CDHP therapy

FT:CDHP:Oxo (molar ratio)	Dose[a] (mg/kg)	*n*	Injury in											
			ileum				cecum				colon			
			−[b]	±	+	+ +	−	±	+	+ +	−	±	+	+ +
Control	—	7	7[c]	0	0	0	7	0	0	0	7	0	0	0
1:0.4:0	20	7	0	**2**	**5**	0	0	0	**2**	**5**	0	**1**	**3**	**3**
1:0.4:0.5	20	7	**6**	**1**	0	0	0	**4**	**2**	**1**	**6**	**0**	**1**	0
1:0.4:1	20	7	**3**	**2**	**2**	0	**3**	**3**	**1**	0	**4**	**3**	0	0
1:0.4:2	20	7	**7**	0	0	0	**6**	0	1	0	**7**	0	0	0

[a] Dose of FT. [b] Degree of injury scored under light microscope: (−) none, (±) very slight, (+) slight, (+ +) mild and (+ + +) severe. [c] Number of rats with pathological injury.

Table 4. Effect of administration schedule of Oxo on antitumor activity and toxicity of FT plus CDHP in Yoshida sarcoma-bearing rats

Method	Administration time			*n*	Tumor weight (g ± SD)	IR[a] (%)	Diarrhea (*n*)	Body weight[b] (g)
	10:00	13:00	16:00					
			HPMC[c]	8	2.328 ± 0.514	—	0	+ 58.5
A	Oxo[d] ———	———	→ FT[4] + CDHP[f]	8	0.000 ± 0.000	100	4	− 15.3
		Oxo ———	→ FT + CDHP	8	0.000 ± 0.000	100	3	− 6.2
			FT + CDHP + Oxo	8	0.280 ± 0.196	88	0	+ 18.2
	HPMC			8	1.913 ± 0.571	—	0	+ 53.6
B	FT + CDHP + Oxo			8	0.029 ± 0.027	98	0	+ 14.0
	FT + CDHP ———	→ Oxo		8	0.000 ± 0.000	100	4	− 11.8
	FT + CDHP ———	———	→ Oxo	8	0.000 ± 0.000	100	3	− 14.5

[a] Inhibition rate of tumor growth. [b] Mean body weight gained or lost throughout 7-day therapy. [c] 0.5% HMPC solution was administered to control rats. [d] Oxo administered at a constant dose of 19.5 mg/kg. [e] FT administered at a dose of 20 mg/kg. [f] CDHP administered at the dose of 5.82 mg/kg.

Effect of co-administration of Oxo on 5-FU phosphorylation in various tissues of tumor-bearing rats following combined administration of FT and CDHP

To provide biochemical evidence for the promising results described above, 5.7 mg/kg (6.4 MBq/kg) [6-^{3}H]FT plus 1.7 mg/kg CDHP combined with or without 5.6 mg/kg Oxo were given orally to Yoshida sarcoma-bearing rats, and the levels of radiolabeled 5-fluoronucleotide in the tumor and normal tissues were determined. As shown in Figure 4, 5-fluoronucleotide levels in the tumor, small and large intestine, and bone marrow were 0.26, 0.36, 0.19 and 0.16 nmol/g tissue, respectively, at 2 h after administration of the FT–CDHP combination without Oxo. On the other hand, when Oxo was included in the combination, 5-fluoronucleotides in GI tissues decreased to 0.2–0.1 nmol/g tissue, while in the tumor and bone marrow tissues remained unchanged.

Antitumor effect of the FT–CDHP combination alone or with Oxo on the growth of tumors implanted into the colon of rats

Since Oxo is well distributed in the GI tracts of rats after oral administration, it is important to clarify whether oral Oxo reduces the antitumor efficacy of the FT–CDHP combination on the tumor growing locally in the colon or rectum of rats. Therefore, Yoshida sarcoma cells were experimentally implanted into the colon of rats and drugs were given orally for 7 days. It is essential to mention that tumor cells also showed an ability to spread to the pancreas. As seen in Table 5, 1 molar FT (20–30 mg/kg) plus 0.4 molar CDHP (5.8–8.7 mg/kg) without Oxo resulted both in strong antitumor efficacy and severe toxicity. However, severe symptoms of toxicity, such as diarrhea and loss of body weight, were significantly reduced by co-administration of the FT–CDHP combination with Oxo

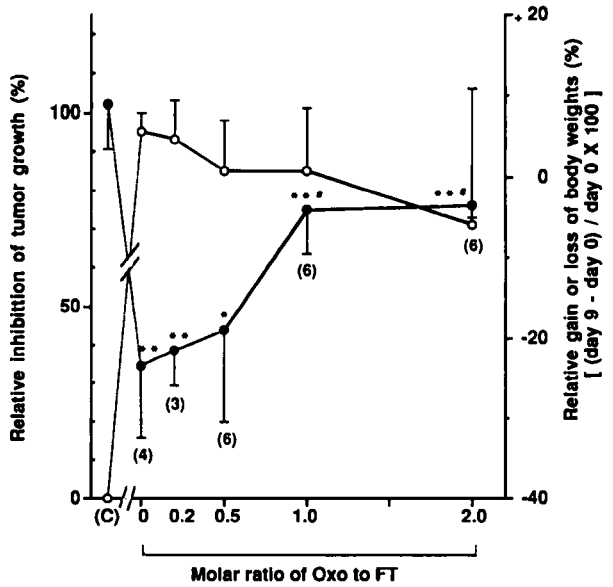

Figure 3. Effect of Oxo on antitumor activity and toxicity of the combination of FT and CDHP in nude rats xenografted with human stomach cancer: 20 mg/kg FT combined with 0.4 molar equivalent amount of CDHP was orally administered to H-81-bearing rats for 8 days. Oxo corresponding to a 0 to 2 molar ratio of FT was simultaneously administered. On day 9, the antitumor activity (○) and body weight change as the toxicity (●) was evaluated. (C) indicates antitumor activity and body weight change in control rats and the parentheses represent survival number of tested rats ($n = 6$) on day 9. Values are means ± SD for all rats in each group. $^{*}p < 0.05$, $^{**}p < 0.01$; significantly different from the control group by Welch's *t*-test. $\#p < 0.05$; significantly different from the FT–CDHP (no Oxo) group by the same test.

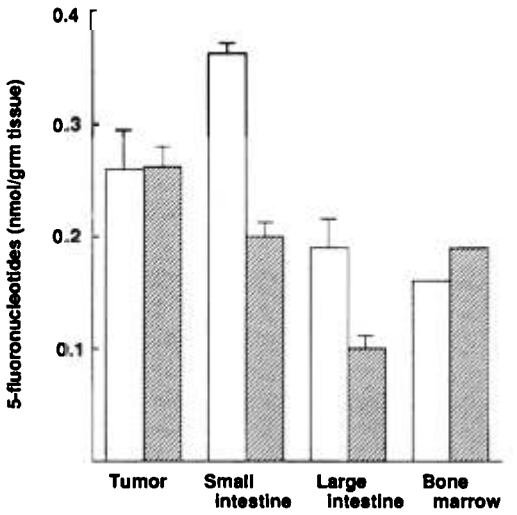

Figure 4. Effect of co-administration of Oxo on 5-FU phosphorylation in tissues of Yoshida sarcoma-bearing rats following administration of [6-^{3}H]FT plus CDHP: 5.7 mg/kg (6.4 MBq/kg) [6-^{3}H]FT plus 1.7 mg/kg CDHP combined with (▨) or without (□) Oxo were given orally to Yoshida sarcoma-bearing rats at 8 days after tumor implantation, and 2 h later radiolabeled 5-fluoronucleotide contents in the tumor, small and large intestine and bone marrow tissues were determined as described in Materials and methods. Values are means ± SD for three rats. Bone marrow cells were collected and combined from three rats.

while their potent antitumor activity was almost completely maintained.

Discussion

5-FU has been frequently used in the treatment of patients suffering from breast, head and neck, stomach, and colorectal cancers. However, the reported clinical response rates of 5-FU alone accounted for only 10–30%. It is postulated that this clinical inefficiency of 5-FU may be related to several problems such as a rapid catabolism of 5-FU, low intracellular concentration of cofactors that are utilized in the anabolic reaction of 5-FU, over-expression or low levels of enzyme proteins modulating 5-FU cytotoxicity and appearance of 5-FU-induced toxicities. Therefore, a number of approaches were undertaken to increase the efficacy of 5-FU, e.g. biochemical modulators such as leucovorine, methotrexate, cisplatin and interferons, etc., were evaluated both in basic and clinical studies.

On the other hand, CVI of 5-FU has been evaluated as an optimal dosing method to reach a suitable balance between efficacy and toxicity in a number of clinical studies. Up to date, the combination of CVI of 5-FU with biochemical modulators has resulted in relatively higher response rates, although 5-FU-induced toxicity was not well balanced.

This prompted us to develop an inhibitor of DPDase, an enzyme catalyzing rapid inactivation of 5-FU, to increase the efficacy of 5-FU. A dramatic improvement of antitumor activity of 5-FU, or 5-FU released from FT, was obtained after applying uracil as an inhibitor of hepatic degradation of 5-FU.[29,30] In 1987, we also found a new pyridine-based inhibitor of DPDase, i.e. CDHP, that was approximately 200-fold more potent *in vitro* than uracil.[27] This finding was well documented in *in vivo* conditions by providing a constant and efficient concentration of 5-FU in the plasma of rats treated with continuous infusion of the combination of 5-FU with the inhibitor.[25] As mentioned previously, the improved activity of 5-FU had been accompanied by more pronounced toxicity.

Table 5. Antitumor effect and toxicity of 1 molar FT plus 0.4 molar CDHP with or without 1 molar Oxo in rats with Yoshida sarcoma implanted in the rectum.

Drug	Dose[a] (mg/kg)	*n*	Tumor weight in rectum (g ± SD)	IR[b] (%)	Tumor weight in pancreas (g ± SD)	IR[b] (%)	Toxic death (n)	Diarrhea (n)	Mean body weight (g)		BWC[c] (% initial)
									start	end	
Control	—	18	1.108 ± 0.993	—	1.683 ± 1.503	—	0	1	215.0	237.0	+10.2
FT + CDHP	20	10	0.012 ± 0.023	98.9	0.000 ± 0.000	100.0	0	9	209.9	167.3	− 20.3
(1:0.4)	30	10	0.000 ± 0.000	100.0	0.000 ± 0.000	100.0	2	8	215.7	155.6	− 27.9
FT + CDHP + Oxo	20	10	0.333 ± 0.525	69.9	0.087 ± 0.275	94.8	0	0	214.4	232.8	+5.1
(1:0.4:1)	30	10	0.000 ± 0.000	100.0	0.000 ± 0.000	100.0	0	0	209.6	194.2	− 7.3

[a] Dose of FT. [b] Inhibition rate of tumor growth. [c] Body weight change expressed as percentages of weight gain or loss to initial body weights of rats in each group.

However, our previous investigation showed that oral Oxo, a potent inhibitor of OPRTase, was able to reduce GI toxicity induced by 5-FU without affecting its antitumor effect.[12] Therefore, we assumed that tumor selective cytotoxicity of 5-FU could be established by co-administration of 5-FU or its oral prodrug, FT, and these two inhibitors, i.e. CDHP and Oxo. As shown in this recent study, oral co-administration of FT and CDHP, at a respective molar ratio of 1:0.4, markedly augmented the antitumor activity of FT in murine tumor models. Also, the addition of Oxo to this combination significantly reduced 5-FU-induced GI toxicity and body weight loss without influencing the high antitumor activity of the FT–CDHP combination against animal tumors implanted s.c. or orthotopically in the colon of rats and against human tumors xenografted to nude rats.

As the result of this promising study, a novel formulation, called S-1, consisting of a mixture of FT, CDHP and Oxo, at a respective molar ratio of 1:0.4:1, retaining potent antitumor efficacy and lacking severe toxicity, was developed.

Recent reports indicated that DPDase activity may play a potential role in controlling 5-FU effectiveness in cancer patients.[31–33] Based on this suggestion, Keizer *et al.*[34] have tried to inhibit the catabolism of 5-FU in cancer patients by the antiviral agent (*E*)-5-(2-bromovinyl)-2′-deoxyuridine. Also, Burris *et al.*[35] and Adjei *et al.*[36] have reported strong inhibition of DPDase activity in patients with refractory tumors by 5-ethynyluracil, a compound discovered by Porter *et al.*[37]

As to the reduction of 5-FU-induced toxicity, allopurinol was suggested to reduce the lethal toxicity of high-dose 5-FU and to delay tumor growth by 100% more than the optimal administration schedule of 5-FU in colon 38-bearing mice.[38] However, neither decreased toxicity nor an increased response rate was observed in cancer patients.[39–40]

S-1, composed of a suitable combination of an effector compound FT and of CDHP and Oxo as biological modulators, possesses potent antitumor efficacy and low toxicity as shown in this report and it is suggested that these properties will contribute to extensive treatment of cancer patients. Further comparative studies on antitumor efficacy and toxicity, such as diarrhea, of S-1 in comparison with continuous infusion of 5-FU are now in progress using murine and human tumor models in rats.

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