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Effects of the Plasma Concentration of 5-Fluorouracil and the Duration of Continuous Venous Infusion of 5-Fluorouracil with an Inhibitor of 5-Fluorouracil Degradation on Yoshida Sarcomas in Rats

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The correlations of the 5-fluorouracil (5-FU) level in the plasma and the duration of continuous 5-FU infusion with the antitumor activity of 5-FU on Yoshida sarcomas in rats were examined. The circadian variation in the plasma level of 5-FU during continuous infusion was prevented by treatment with 3-cyano-2,6-dihydroxypyridine (CNDP), which strongly inhibits 5-FU degradation. On continuous venous infusion of 2 to 30 mg/kg of 5-FU over 24 h with CNDP at a molar ratio of 1:10 into normal rats, the 5-FU level in the blood was linearly proportional to the dose of 5-FU. The optimum schedule for antitumor activity on Yoshida sarcomas in rats was found to be infusion of 5-FU at 5 mg/kg over 24 h for 6 consecutive days, which gave a plasma 5-FU level of 176 ng/ml. Continuous infusion of 5-FU to give a plasma level of 300 ng/ml for 6 consecutive days from day 5 after implantation of tumor cells, when the tumors weighed about 1.0 g, resulted in complete regression of the tumors in all rats.

Key words: Continuous venous infusion — 5-Fluorouracil — Antitumor activity — Yoshida sarcoma — 5-Fluorouracil degradation

The fluorinated pyrimidine 5-fluorouracil (5-FU) is an antimetabolite that is frequently used in the treatment of gastrointestinal, breast, head, neck and genitourinary cancers. A number of schedules of drug administration have been evaluated, but there is no consensus of opinion on the optimal dose-schedule. Consequently the clinical response rate to single treatment with 5-FU is variable. Two schedules of venous infusion of 5-FU have often been used: (a) short-term infusion of high doses of 5-FU,[1,2] and (b) long-term infusion (over 30 days) of low doses.[3–5] These schedules are based on findings that the cytotoxicity of 5-FU to murine and rat tumors *in vitro* depends not only on the concentration of 5-FU, but also on the duration of treatment. Drewinko and Yang[6] also recently demonstrated the importance of both the dose of 5-FU and the duration of exposure for its activity against established human colon carcinoma cell lines.

Despite these *in vitro* findings, the optimal 5-FU level in the blood and optimal duration of drug infusion to attain a complete response *in vivo* are not yet clear. There are several reports on the 5-FU levels in the blood of patients during its prolonged venous infusion.[7–16] Erlichman *et al.*[15] and Fox *et al.*[10] reported marked variation in the plasma levels of 5-FU in different patients and from day to day. Moreover Petit *et al.*[16] recently reported circadian variation in the plasma concentration of 5-FU during its continuous venous infusion in cancer patients for 5 days. These variations are probably related to circadian variations in the catabolism of 5-FU[17,18] or its total clearance from the body.[19]

Recently, we found that 3-cyano-2,6-dihydroxypyridine (CNDP) was a potent inhibitor of dihydrouracil dehydrogenase, which catalyzes 5-FU degradation; its IC_{50} value was $6.0\times10^{-8}M$.[20] Thus, continuous venous infusion of this inhibitor with 5-FU should result in stable maintenance of the blood 5-FU level.

In the present work, we examined the 5-FU level in the blood and the duration of drug infusion required to achieve complete regression of Yoshida sarcomas in rats by continuous infusion of 5-FU plus CNDP.

MATERIALS AND METHODS

Chemicals 5-FU was purchased from Sigma Chemical Co., MO. CNDP was synthesized in Otsuka Laboratories.

Animals Male Donryu-strain rats weighing 120–150 g were obtained from Laboric Service Co., Shiga, and supplied *ad libitum* with commercial diet and autoclaved water until use.

Tumor cells Yoshida sarcoma cells were obtained from Sasaki Research Institute, Tokyo, and passaged in male Donryu rats by intraperitoneal inoculation at weekly intervals.

Chemotherapy Solid-type Yoshida sarcoma was prepared by implantation of 2×10^5 cells into subepidermal tissues of the back of rats on day 0, and the rats were subjected to the operation described below within 8 h thereafter. Various concentrations of 5-FU with CNDP at a molar ratio of 1:10 were normally administered as a

solution in autoclaved saline by continuous venous infusion for 6 days, starting 24 h after tumor implantation. Control rats received saline alone by the same schedule. On day 7, rat were sacrificed, and tumors were removed and weighed. The antitumor activity of the drug was evaluated as the T/C (%) value:

$$T/C\ (\%) = \frac{\text{mean tumor weight in drug-treated rats}}{\text{mean tumor weight in control rats}} \times 100.$$

In another experiment, 5-FU plus CNDP was infused for 6 consecutive days, starting 5 days after implantation of tumor cells.

Continuous venous infusion A standard safelet catheter kit from Nipro Co., Osaka was used. The 16-gauge, 60 cm, silicon catheter was inserted into the right cardiac vein from the right carotid of rats under anesthesia with ethylether. Then, the catheter was tunneled through the subepidermal tissue to the back of the neck, and passed through a harness with a 30 cm spring. The rats were placed in metabolic cages and the other end of the catheter attached to an infusion pump (Terumo Co., Tokyo). In this system, the rats were free to move and they were supplied with commercial diet and autoclaved water *ad libitum*. Saline alone was infused until the start of drug infusion.

Extraction and determination of 5-FU in the blood Blood samples obtained from tumor-bearing rats were promptly centrifuged (3000 rpm 10 min) at 4°C and stored at −20°C until use. For measurement of 5-FU, samples of 1 ml of the serum were acidified with 0.1 ml of 1 *M* HCl, and vigorously shaken with 4 ml of ethyl acetate for 10 min. The mixture was centrifuged at 3000 rpm and the organic layer was removed. The remaining aqueous layer was again extracted with 4 ml of ethyl acetate, followed by centrifugation, and the two organic layers were combined, evaporated at 40°C under a stream of nitrogen gas, and filtered through a 0.45 μm filter. Then the 5-FU contents of the samples were determined by high-performance liquid choromatography with an 871 pump and 873 spectrophotometer from IRICA Co., Kyoto. For this, aliquots of the sample were applied to an ULTRON N-C_{18}·L column (4.6 ID ×150 mm) from Shinwa-Kako Co., Kyoto, under the following chromatographic conditions: monitoring wavelength, 265 nm; flow rate, 1 ml/min; mobile phase, 5 m*M* tetrabutylammonium hydrooxide containing 2% methanol adjusted to about pH 5 with dilute formic acid.

RESULTS

Gudauskas and Goldie,[9] and Petit *et al.*[16] reported that during infusion of 5-FU at a constant rate into cancer patients the plasma concentrations of 5-FU seemed to be higher at night than during the day. There are many reports of circadian rhythms of activity of various liver enzymes involved in drug metabolism, such as dehydrouracil dehydrogenase.[21] Therefore, it was expected that the level of 5-FU in the blood would remain constant during continuous venous infusion of 5-FU combined with CNDP, a potent inhibitor of 5-FU degradation.

To test this possibility, we measured the 5-FU levels in the blood of normal rats at 13:00 h and 23:00 h for 3 days during continuous infusion of 5-FU with or without CNDP. As shown in Table I, during continuous infusion of 5-FU (50 mg/kg per 24 h for 3 days) alone, the plasma 5-FU levels in the day and at night were significantly different, the level at night being about half that in the day. Moreover, although 5-FU was infused continuously at a constant rate during the 3-day period, the plasma 5-FU levels in the day and at night were not constant. In contrast, during continuous venous infusion of 5-FU (5 mg/kg over 24 h for 3 days) plus CNDP (52.3 mg/kg over 24 h for 3 days) the plasma 5-FU concentrations in the day and at night were similar. A molar ratio of CNDP to 5-FU of 10:1 was required to maintain a constant level of 5-FU (data not shown). A plasma level of 5-FU of about 200 ng/ml was attained on continuous infusion of 5-FU at 5 mg/kg with CNDP (52.3 mg/kg). These results suggest that the circadian variation in the plasma concentration of 5-FU during its continuous infusion is completely blocked by simultaneous infusion of CNDP, which is a potent inhibitor of 5-FU degradation.

Next, various doses of 5-FU plus CNDP (molar ratio, 1:10) were infused continuously into normal rats and the plasma 5-FU concentrations were determined. As shown in Fig. 1, at doses in the range of 2 to 30 mg/kg/day of 5-FU the level of 5-FU in the blood was linearly proportional to the dose of 5-FU.

Effects of various schedules of continuous infusion of 5-FU plus CNDP on the antitumor activity and toxicity of 5-FU to Yoshida sarcoma in rats Doses of 2, 3, 5, 10 and 30 mg/kg/24 h of 5-FU combined with a 10-fold molar excess of CNDP were infused for 1 to 6 days, and a total dose of 30 mg/kg was infused for 6 consecutive days in infusion times of 4, 8, 12 and 24 h. Also, a dose of 20 mg/kg/24 h of 5-FU alone was infused for 6 consecutive days. The 5-FU levels in the blood, the antitumor activities (T/C), and the toxicities achieved with these schedules are shown in Table II. The greatest antitumor activity was obtained with a total dose of 30 mg/kg of 5-FU in combination with CNDP (schedules D, E, G, H and I), while the continuous infusion of 5-FU alone gave little antitumor activity (schedule A). However, administration of this dose in 1 day or over 3 consecutive days, which gave a 5-FU concentration in the blood of about 1400 or 700 ng/ml, respectively, resulted in loss of body

Table I. Circadian Variation in Serum Concentration of 5-FU during Continuous Venous Infusion of 5-FU alone or with CNDP

Drug	Doses (mg/kg/day)	Rat No.	5-FU level (ng/ml)		Day/Night
			day (13:00 h)	night (23:00 h)	
5-FU	50	1	344	104	3.31
		2	292	171	1.71
		3	316	194	1.63
		4	210	92	2.28
		5	332	107	3.10
		6	213	153	1.39
		7	189	153	1.24
		8	172	142	1.21
		9	168	142	1.18
		10	239	112	2.13
		mean±SE	248±21	137±10	1.92
5-FU plus CNDP (mol. ratio 1:10)	5	1	267	256	1.04
		2	219	222	0.99
		3	244	248	0.98
		4	227	219	1.04
		5	233	263	0.89
		6	168	179	0.94
		7	211	200	1.06
		8	179	174	1.03
		9	181	205	0.88
		10	214	189	1.13
		mean±SE	214±10	216±10	1.00

The drugs, dissolved in saline, were infused continuously into rats for 3 days. Blood samples were taken on day 3 at 13:00 h (day) and 23:00 h (night), and their 5-FU levels were determined as described in "Materials and Methods."

weight or severe decrease in the white blood cell count (schedules E, F and G). Its administration in a period of 4 to 12 h for 6 consecutive days also gave high levels of 5-FU in the blood and either marked loss in body weight or severe decrease in the WBC count, or both (schedules G, H and I).

Overall, the most effective schedule was infusion of 5-FU combined with CNDP at 5 mg/kg over 24 h for 6 consecutive days, which gave a 5-FU level in the blood of 176 ng/ml.

Antitumor activity of continuous infusion of 5-FU plus CNDP from 5 days after implantation of Yoshida sarcoma in rats Solid-type Yoshida sarcoma was prepared by transplantation of 2×10^5 ascites cells into the subepidermal tissues of the back of rats on day 0. Continuous infusion of 5-FU at 0, 3, 4, 5, 6 and 7 mg/kg over 24 h for 6 consecutive days was started 5 days after implantation of tumor cells, when the tumors weighed about 1.0 g. Without 5-FU (0 mg/kg) all rats died within 10 days after tumor implantation.

But as shown in Table III, infusion of 5-FU at 5 mg/kg over 24 h for 6 days caused slight decrease in tumor weight, and infusion of 5-FU at 6 and 7 mg/kg over 24 h for 6 days caused complete tumor regression. These results suggested that tumor regression was achieved with 5-FU levels of about 300 ng/ml of blood over 24 h for 6 consecutive days. In groups of rats receiving continuous infusions of 5-FU at 6 and 7 mg/kg during 24 h for 6 days the death rates were 1/6 and 2/6, respectively.

DISCUSSION

In recent studies on treatment with 5-FU, continuous infusion has been used to decrease toxicity and increase antitumor efficacy. With improvement in infusion pumps and techniques for intravenous access, continuous intravenous infusion has become practicable on an out-patient basis. However, even on continuous infusion of 5-FU at a constant rate for 5 consecutive days, constant plasma concentrations of 5-FU were not achieved. There are

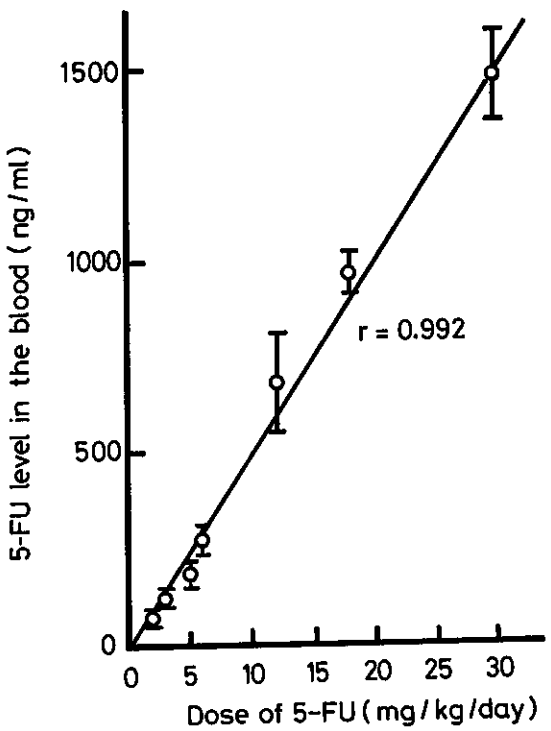

Fig. 1. Relationship between steady-state 5-FU levels in blood and dose of 5-FU with CNDP during their continuous venous infusion into rats. Blood samples were taken from the cardiac vein of rats on day 3 during the continuous infusion, and their 5-FU levels were determined as described in "Materials and Methods." Values are means±SE for 4 to 27 rats.

several reports of fluctuation in the circulating level of 5-FU,[7-16] and 5-FU clearance has been shown to result mainly from its metabolism in the liver.[22]

In the present study we found that this fluctuation in the plasma 5-FU level could be prevented by co-infusion of a potent inhibitor of 5-FU degradation. Howell *et al.*[11] reported that a plot of the steady-state 5-FU serum concentration was not linear at higher doses, and that clearance of 5-FU from the blood was also not linearly proportional to the dose. Moreover, Petit *et al.*[16] found that the mean values of lowest and highest 5-FU plasma concentrations in seven patients receiving 5-FU continuous venous infusion at a constant rate for 5 days were 254±33 ng/ml at 1 p.m. and 584±160 ng/ml at 1 a.m.

Many reports have established that circadian rhythms govern the activity of numerous liver enzymes involved in drug metabolism, such as dihydrouracil dehydrogenase involved in 5-FU metabolism.[21]

In our experiments, the circadian variation in the plasma level of 5-FU during its continuous venous infusion for 3 days was the opposite of that in humans, probably due to the difference in the circadian variation of dihydrouracil dehydrogenase between humans and rodents. But we found that the 5-FU level in the plasma of rats could be maintained at a constant level by continuous infusion of 5-FU in combination with CNDP and that long-term infusion of 5-FU in combination with CNDP could greatly potentiate the antitumor activity compared with that of 5-FU alone.

Seifert *et al.*[23] reported that continuous infusion of 5-FU for 120 h was clinically more effective than intrave-

Table II. Effect of Various Conditions of Infusion of 5-FU alone and 5-FU plus CNDP on Antitumor Activity and Toxicity to Yoshida Sarcoma-bearing Rats

Schedule No.	CNDP (+or−)	Infusion time (hr/day)	Infusion time (days)	Dose of 5-FU (mg/kg/day)	Total dose of 5-FU (mg/kg)	No. of rats (n)	T/C (%)	Body wt. change (g)	WBC[b] count ($\times 10^{-2}$/mm^3)	No. of deaths	No. of tumor-free rats	5-FU level in blood (ng/ml)
A	−	24	6	20	120	8	66±3	−1±6	104±9	0	0	58±8
B	+	24	6	2	12	8	69±5	0±4	108±13	0	0	46±3
C	+	24	6	3	18	30	40±5	−5±2	54±4	0	0	98±9
D	+	24	6	5	30	20	2±1	−25±3	28±2	0	12	176±18
E	+	24	3	10	30	6	1[a]	−46[a]	6[a]	4	0	717±83
F	+	24	1	30	30	6	17±3	−18±4	15±4	0	0	1444±118
G	+	4	6	5	30	4	3[a]	−63[a]	6[a]	2	1	1380±28
H	+	8	6	5	30	3	1±1	−46±4	33±11	0	2	995±16
I	+	12	6	5	30	4	0.3±0.3	−46±8	25±4	0	3	695±63

Yoshida sarcoma (2×10^5 cells) was implanted into rats. 5-FU alone or 5-FU combined with a 10-fold molar excess of CNDP, dissolved in autoclaved saline, was administered by continuous venous infusion starting 24 h after tumor implantation in each schedule. Blood samples were immediately taken from the cardiac vein of rats at the end of infusion on day 6, and their 5-FU levels were determined as described in "Materials and Methods." Values are means±SE for 3 to 30 rats. *a*) Mean value for surviving rats (n=2). *b*) WBC; white blood cell.

Table III. Effect of Dose of 5-FU plus CNDP Infused and Blood Level of 5-FU on Tumor Regression

Dose of 5-FU (mg/kg/24 h)	Infusion time (days)	Total dose of 5-FU (mg/kg)	No. of rats (n)	Tumor weight (g±SE)	No. of deaths	No. of tumor-free rats	5-FU level in blood (ng/ml±SE)
Non (saline alone)	6	0	6	—	6	—	—
3	6	18	5	4.84±0.33	0	0	142±4
4	6	24	5	0.77±0.30	0	0	178±19
5	6	30	9	0.29±0.11	0	3	236±19
6	6	36	6	0.00±0.00	1	5	321±42
7	6	42	6	0.00±0.00	2	4	396±36

Inocula of 2×10^5 cells of Yoshida sarcoma were implanted into rats, and from 5 days after tumor implantation, various doses of 5-FU combined with a 10-fold molar excess of CNDP were infused for 6 consecutive days. Tumor weights 5 days after implantation were 1.08±0.06 g (n=13). On day 11 after tumor implantation, rats were killed and their tumor and blood were removed. 5-FU levels in their blood (serum) were determined as described in "Materials and Methods." Values are means±SE for 4 to 9 rats.

nous bolus injection daily for 5 days, particularly with respect to myelotoxicity. Shah *et al.*[2] treated 94 patients with advanced colorectal adenocarcinoma by continuous iv 5-FU infusion on three different dose schedules (every 3 weeks, every 2 weeks and every week), and found that the schedule of 48 h infusion of 5-FU every week resulted in a significant response and prolongation of the median survival time. Moreover, Caballero *et al.*[5] reported that nausea, vomiting, myelosuppression, and alopecia were not observed on long-term continuous iv infusion of 5-FU for 54–324 days.

These clinical data suggest that long-term infusion of 5-FU at a low dose is more effective than bolus injection or infusion at a high dose over a short period. Similar results were obtained in this study, in which CNDP, a potent inhibitor of 5-FU degradation, was co-infused with 5-FU. Further studies on the most effective total dose of 5-FU and duration of administration for anti-tumor activity are in progress.

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