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Enhanced Antitumor Effect of 5′-Deoxy-5-fluorouridine by Oral Administration with L-Cysteine

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When given orally in combination with L-cysteine, 5′-deoxy-5-fluorouridine (DFUR) brought about a significant reduction in the growth of adenocarcinoma 755 and a significant prolongation of life-span in mice bearing Lewis lung carcinoma without increased toxicity to the host as compared with DFUR alone, though L-cysteine alone did not show an appreciable antitumor activity. Moreover, the combination of DFUR and L-cysteine resulted in a marked retardation of growth of human colon tumor LS174T transplanted into nude mice. Thus, the potency of DFUR was increased by L-cysteine. Pharmacokinetic studies revealed that after DFUR administration, plasma DFUR and 5-fluorouracil (5-FU) levels rapidly declined, but that, in the combination with L-cysteine, the plasma clearances of DFUR and 5-FU were slowed down considerably. In the tumor, DFUR and 5-FU levels were similar to those in the plasma. Such a prolongation of DFUR and 5-FU levels in plasma and tumor may produce the enhancement of antitumor effect seen with the combination of DFUR and L-cysteine.

Key words: 5′-Deoxy-5-fluorouridine — L-Cysteine — Antitumor activity — Combination therapy

L-Cysteine potentiates the antitumor effect of 5-fluorouracil (5-FU) against adenocarcinoma 755 when given by simultaneous intravenous injection (iv)[1,2] without increasing the toxicity to the host. The labeled 5-FU concentration in the plasma and tumor following administration of labeled 5-FU and L-cysteine was higher than that after 5-FU alone, but incorporation of 5-FU into RNA in the tumor was markedly decreased.[1] This suggests that 5-fluoro-2′-deoxyuridylate (FdUMP) is important to potentiate the antitumor effect in the combination of 5-FU and L-cysteine.

The combination of 5-FU and L-cysteine, however, when given orally (po) did not show a potentiated antitumor effect.[3] When 5-FU is administered po, 5-FU is very rapidly degraded to inactive metabolites.[4] 5′-Deoxy-5-fluorouridine (DFUR) is a prodrug of 5-FU and releases 5-FU slowly in the body; it is currently used clinically as an oral medicament in Japan.

The present studies were undertaken to determine whether oral L-cysteine potentiated the antitumor effect of oral DFUR, and to provide a pharmacological basis for the enhancing effect of L-cysteine on the antitumor effect of DFUR.

MATERIALS AND METHODS

Drugs DFUR was obtained from Nippon Roche (Kamakura) and L-cysteine was purchased from Sigma Chemical Co. (St. Louis, MO). L-Cysteine was homogenized with 0.5% carboxymethyl cellulose in physiological saline and administered orally in a volume of 0.01 ml/g of body weight. Other compounds were dissolved in physiological saline.

Animals Groups of six or more male BDF_1 mice with body weights of 20–23 g (Shizuoka Laboratory Animal Center, Hamamatsu) and five athymic mice (*nu/nu*) with body weights of 19–21 g (CLEA Japan, Inc., Tokyo) were housed in plastic cages with wood chip bedding are received a CA-1 diet (CLEA Japan, Inc.) and CMF diet (Oriental Yeast Co., Ltd., Chiba), respectively. Water was given *ad libitum*. All experiments were performed under specific-pathogen-free conditions in our animal laboratory.

Evaluation of antitumor effect on adenocarcinoma 755, Lewis lung carcinoma and LS174T human colon tumor Mice were inoculated subcutaneously (sc) on day 0 with adenocarcinoma 755 (20 mg), Lewis lung carcinoma (4×10^5) and LS174T colon tumor (30 mg). Each tumor has been maintained by sc transfer every 12, 14 and 28 days into C57BL/6, C57BL/6 and athymic mice, respectively. Beginning 24 h after tumor inoculation of adenocarcinoma 755 or Lewis lung carcinoma, the drugs were administered po daily for five consecutive days. For evaluation of the antitumor effect on adenocarcinoma 755, tumor weight was determined on day 11 and the ratio of the average tumor weight in the treated groups to that in the untreated control group (T/C, %) was calculated. In Lewis lung carcinoma, antitumor effect was evaluated by calculating the increase in life-span. In LS174T colon tumor, the drugs were administered po when the tumors became palpable (day 7), and continued daily for a period of 5 days. The longest (*a*) and shortest (*b*) tumor diameters were measured twice each week

using calipers, and volume was calculated using the formula: $ab^2/2$ (mm^3).

HPLC assay of DFUR and 5-FU in plasma Groups of three BDF_1 mice or adenocarcinoma 755-bearing BDF_1 mice were used. Blood samples were collected under diethylether anesthesia from the descending vena cava at specified times after oral administration of the drugs, and were immediately chilled on ice. The samples were then centrifuged for 10 min at 3000 rpm at 4°C and plasma was separated immediately.

Plasma samples (0.3–0.5 ml) from individual mice were adjusted with distilled water to a total volume of 1 ml, and 0.2 ml of 0.5 *M* KH_2PO_4 buffer and 8 ml of ethyl acetate were added.[5] After extraction and centrifugation, the organic layer was evaporated *in vacuo* at 35°C. The residue was dissolved in water, in the same volume as the original, and applied to a Bond Elut (bonded phase: SAX) column (Analytichem International, Harbor City, CA), and then 25–100 μl of the eluted extract was injected into an HPLC apparatus (CCPM, Toso, Tokyo).

HPLC assay of DFUR and 5-FU in tumor After blood was taken, the tumor was removed as soon as possible, and extraneous normal and necrotic tissues were removed. The tumor (1 g) was minced with scissors in a Petri dish and homogenized in 4 ml of distilled water with a Polytron homogenizer (Kinematica, Lucerne, Switzerland) and then 0.8 ml of 0.5 *M* KH_2PO_4 buffer and 32 ml of ethyl acetate were added. After extraction and centrifugation, the organic layer was removed and evaporated *in vacuo* at 35°C. The residue was dissolved in water (1 ml), and applied to a Bond Elut, SAX, column and then 20 μl of the eluted extract was injected into an HPLC apparatus.

Measurement of intratumor FdUMP pools following DFUR and L-cysteine A single dose of DFUR (500 mg/kg) or DFUR plus L-cysteine (1000 mg/kg) was given po to mice bearing an 8-day-old tumor. The mice (three mice/group) were killed 1, 3 and 6 h later by decapitation and the tumor was removed and chilled as quickly as possible. Free FdUMP in the tumor was determined as reported by Murinson *et al.*[6]

Statistical analysis The *t* test for small samples was used to determine the statistical significance of differences; $P \leqq 0.05$ was considered significant.

RESULTS

Antitumor activity following combined oral DFUR and L-cysteine administration against adenocarcinoma 755, Lewis lung carcinoma, and LS174T human colon tumor The combination of oral DFUR plus oral L-cysteine was investigated to evaluate its inhibitory effect on the growth of adenocarcinoma 755 in BDF_1 mice (Fig. 1). L-Cysteine itself, at the dose of 1000 mg/kg, had no effect on tumor growth but, when combined with DFUR, it markedly enhanced the antitumor effect of the latter. DFUR alone at 100 mg/kg did not affect the growth of adenocarcinoma 755 (T/C=71%). If, however, DFUR was combined with L-cysteine, DFUR at 100 mg/kg caused a marked reduction of tumor growth (T/C=14%), so

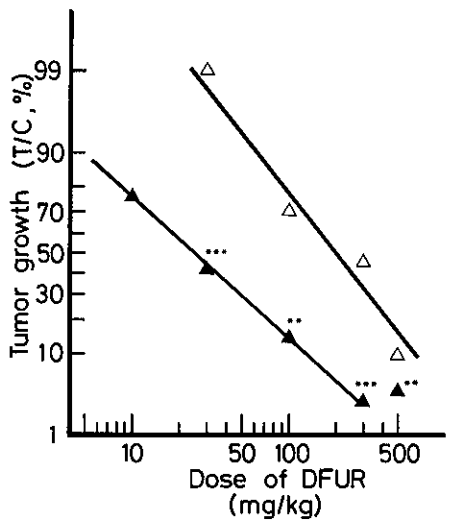

Fig. 1. Antitumor activity of the combination of DFUR with L-cysteine, both administered po to tumor-bearing mice (adenocarcinoma 755). Percent growth ratio (T/C) is plotted on the ordinate. The dose of L-cysteine was 1000 mg/kg. Treatment was started one day after tumor inoculation and continued for five consecutive days. The T/C ratio was determined on day 11. There were six mice/group. DFUR alone (△), DFUR + L-cysteine (▲). ＊＊, Different from DFUR alone ($P<0.01$). ＊＊＊, Different from DFUR alone ($P<0.001$).

Table I. Effect of Time Interval between the Drugs on Antitumor Activity of DFUR-L-Cysteine Combination against Ad-enocarcinoma 755

Sequence of administration[a)]	Time interval (h)	Tumor weight[b)] (mg)	T/C (%)
DFUR		1670±860	48
DFUR+L-cysteine	0	300±110[c)]	9
DFUR→L-cysteine	1	1440±730	41
L-Cysteine→DFUR	1	930±610	26
L-Cysteine→DFUR	6	1340±930	38
L-Cysteine		2830±1640	81

a) Drugs were administered po on days 1–5 to groups of 6 mice. Doses of DFUR and L-cysteine were 100 and 1000 mg/kg/day, respectively.
b) The untreated control was 3510±640 mg (mean±SD). Tumor weight was determined on day 11.
c) $P<0.01$, different from DFUR alone.

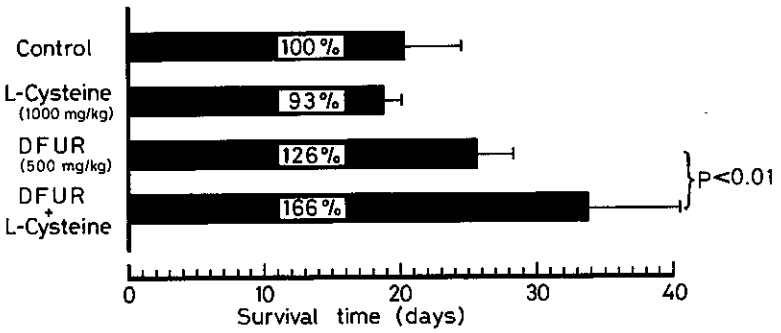

Fig. 2. Increase in survival time of Lewis lung carcinoma-bearing mice treated with the combination of DFUR and L-cysteine, which were administered po. Doses of DFUR and L-cysteine were 500 and 1000 mg/kg, respectively. Treatment was started one day after tumor transplantation and continued for five consecutive days. Figures in bars mean T/C (%).

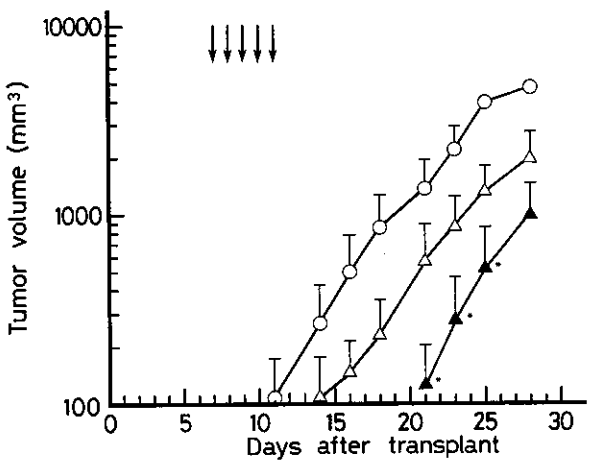

Fig. 3. Effect of the combination of DFUR and L-cysteine on growth of human colon carcinoma LS174T. Treatment was started on day 7 and continued for five consecutive days. Control (○), DFUR (300 mg/kg, △) and DFUR + L-cysteine (300 mg/kg + 1000 mg/kg, ▲) were administered po to tumor-bearing nude mice (5 mice/group). Mean±SD of 5 mice. *, Different from DFUR alone ($P<0.05$).

Table II. Effect of iv L-Cysteine on Inhibition of Growth of Adenocarcinoma 755 by iv DFUR

Dose[a] (mg/kg/day)		Tumor weight[b] mean±SD	T/C (%)
DFUR	L-Cysteine	(mg)	
—	—	4080±650	100
200	—	3450±890	84
200	100	2930±1130	72
200	500	2960±1260	72

a) DFUR and L-cysteine were administered iv simultaneously on days 1–4.
b) Tumor weight was determined on day 11.

that 100 mg DFUR/kg when combined with 1000 mg L-cysteine/kg was as effective as 500 mg DFUR/kg alone. If the maximum nontoxic dose (500 mg/kg) of DFUR was used, it brought about an approximately 90% reduction in tumor growth. When combined with L-cysteine at 1000 mg/kg, 500 mg DFUR/kg effected a 96% reduction in tumor growth ($P<0.01$) without any death from toxicity.

To determine the most effective treatment schedule, the effect of different time intervals between the administrations of DFUR and L-cysteine was examined (Table I). DFUR (100 mg/kg) and L-cysteine (1000 mg/kg) were administered po at definite intervals via separate syringes on days 1–5. The antitumor activity of DFUR against adenocarcinoma 755 was significantly potentiated only when DFUR and L-cysteine were administered simultaneously.

In order to compare the effect of DFUR alone and in combination with L-cysteine on other tumor systems, Lewis lung carcinoma and human colon carcinoma LS174T were used. Using a schedule of five consecutive treatments against Lewis lung carcinoma, the combination of DFUR and L-cysteine was more effective than DFUR alone (Fig. 2). Mice receiving 500 mg DFUR/kg together with L-cysteine (1000 mg/kg) showed a significant increase in life-span compared with those given DFUR alone (66 and 26%, respectively, $P<0.01$).

Furthermore, the combination of DFUR and L-cysteine also produced a markedly antitumor effect against human colon carcinoma LS174T. The tumor growth was significantly retarded by the combination of DFUR and L-cysteine during days 14–25 as compared with that after DFUR alone (Fig. 3).

Effect of L-cysteine on antitumor activity of DFUR by intravenous injection The combination of DFUR and L-cysteine showed a marked antitumor effect after oral administration. If L-cysteine activates the antitumor effect of DFUR in the tumor or liver, this combination should also give enhancement of the antitumor effect

after intravenous injection. However, intravenous injection of L-cysteine (500 mg/kg) had little effect on the antitumor activity of intravenous injection of DFUR (200 mg/kg) (Table II), in contrast to the combination of 20 mg 5-FU/kg and 100 mg L-cysteine/kg.[1]

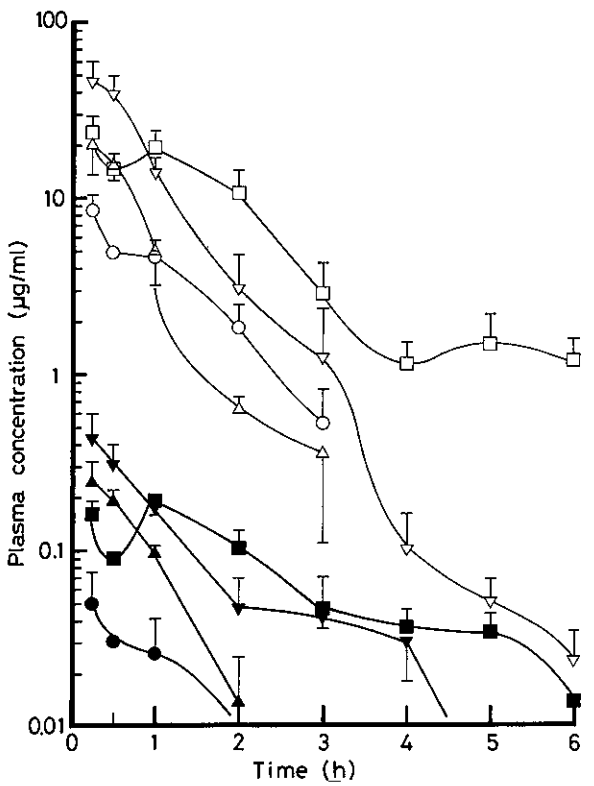

Fig. 4. Plasma levels of DFUR (open symbols) and 5-FU (closed symbols) following po administration of DFUR (100 mg/kg, △ ▲; 300 mg/kg, ▽ ▼) and DFUR + L-cysteine (100 mg/kg + 1000 mg/kg, ○ ●; 300 mg/kg + 1000 mg/kg, □ ■). Mean±SD of 3 mice.

Plasma DFUR and 5-FU levels after treatment with DFUR plus L-cysteine To examine the absorption of DFUR from the gastro-intestinal tract, we measured the plasma levels of DFUR and 5-FU in non-tumor-bearing BDF_1 mice during 6 h after the treatment. Peak concentrations of DFUR in plasma after administration of DFUR alone (300 mg/kg) and in combination with L-cysteine (1000 mg/kg) were 46.2 and 23.9 μg/ml, respectively (Fig. 4), and those of 5-FU were 0.43 and 0.16 μg/ml, respectively. Thus, the DFUR and 5-FU levels in plasma after administration in combination with L-cysteine were significantly lower than after DFUR alone during the first 30 min. In contrast, at 5 h after DFUR administration or later, the plasma DFUR and 5-FU levels were significantly higher if DFUR had been combined with L-cysteine than if it had been given alone. Moreover, in 8-day-old tumor-bearing mice, plasma levels of DFUR and 5-FU after the combination of DFUR (500 mg/kg) and L-cysteine (1000 mg/kg) were considerably lower than those after DFUR alone at 1 h after treatment, but the DFUR level was higher than after DFUR alone at 3 h later, similar to the result in non-tumor-bearing mice (Table III).

DFUR and 5-FU levels in tumor tissue after treatment with DFUR plus L-cysteine To determine the concentrations of DFUR and 5-FU in the tumor, we excised the tumor 1 or 3 h after treatment and homogenized it in a Polytron homogenizer, and then extracted the homogenate with ethyl acetate as in the case of the plasma samples. DFUR and 5-FU levels in the tumor 1 h after treatment of DFUR plus L-cysteine were markedly lower than after DFUR alone, whereas those 3 h after the combination were considerably higher than after DFUR alone (Table III). These data were very similar to those for plasma.

Table III. Plasma and Tumor Levels of DFUR and 5-FU in Adenocarcinoma 755-bearing Mice after Administration of DFUR Alone or in Combination with L-Cysteine

Treatment[a]	Time (min)	Plasma (μg/ml)		Tumor (μg/g)	
		DFUR	5-FU	DFUR	5-FU
DFUR	60	92.0±9.0[b]	1.45±0.38	104.3[c]	3.27
	180	5.6±2.0	0.17±0.05	8.5	0.18
DFUR+	60	42.6±6.4[d]	0.87±0.26	45.4	0.99
L-cysteine	180	10.1±2.6	0.18±0.02	14.7	0.18

a) Doses of DFUR and L-cysteine were 500 and 1000 mg/kg, respectively.
b) Values are expressed as mean±SD of 3 or 4 mice.
c) The tumors of three mice were pooled and analyzed.
d) $P<0.001$, different from DFUR alone (60 min).

Table IV. Free FdUMP in the Tumor following Oral Administration of DFUR and DFUR plus L-Cysteine

	Free FdUMP[a] (pmol/g)		
	1 h	3 h	6 h
DFUR[b]	47±12[c]	44±22	23±11
DFUR+L-cysteine[b]	29±6	63±20	23±6

a) Free FdUMP in the tumor was assayed 1, 3 and 6 h after administration of DFUR alone or in combination with L-cysteine.
b) DFUR and L-cysteine were administered po to adenocarcinoma 755-bearing mice at 500 and 1000 mg/kg, respectively.
c) Values represent the mean±SD of three mice.

Free FdUMP levels in tumor after treatment with DFUR plus L-cysteine Incorporation of 5-FU into RNA was markedly low after the combination of 5-FU and L-cysteine.[1] The FdUMP level in the tumor may be important in the treatment with fluoropyrimidine plus L-cysteine. Therefore, we measured the FdUMP level in the tumor after treatment with DFUR plus L-cysteine by the method reported by Murinson *et al.*[6] However, the free FdUMP level in the tumor after the combination of DFUR and L-cysteine was not as high as after DFUR alone (Table IV).

DISCUSSION

The ability of L-cysteine to increase the antitumor effect of 5-FU on adenocarcinoma 755 has been reported by us.[1] L-Cysteine administered with 5-FU decreases its acute and subacute toxicity in mice.[7] L-Cysteine shows marked potentiation of the antitumor effect of 5-FU only by iv, but not by po administration.[3] In contrast, L-cysteine potentiated the antitumor effect of po DFUR, but not iv DFUR. These differences may be due to the plasma levels of 5-FU and L-cysteine. The plasma L-cysteine level is markedly increased 15–30 min after oral administration of L-cysteine.[8] Maximum plasma DFUR and 5-FU levels were observed within 15 min after oral administration of DFUR. The different results of iv and po treatments with DFUR plus L-cysteine suggest that the mechanism of the potentiation does not depend on activation of DFUR in the tumor and liver by L-cysteine, or on inhibition of degradation of 5-FU. In the case of 5-FU plus L-cysteine, a high level of 5-FU is produced in the plasma and tumor, but incorporation of 5-FU into tumor RNA is markedly decreased by addition of L-cysteine.[1] In the case of the combination of DFUR and L-cysteine, peak levels of DFUR and 5-FU in plasma and tumor were markedly lower than after DFUR alone, but those levels in the plasma and tumor 3 h after treatment with DFUR plus L-cysteine were higher than after DFUR alone. The prolongation of 5-FU levels in the plasma and tumor is very important for potentiation of the antitumor effect.[4, 5] The rather constant levels of DFUR and 5-FU in plasma may result from control of absorption from the gastro-intestinal tract by L-cysteine. In spite of the low level of 5-FU in the plasma and tumor during the first 3 h after treatment with DFUR plus L-cysteine, a rather high level of free FdUMP was produced in the tumor. The FdUMP level may be enough to inhibit thymidylate synthetase. Furthermore, L-cysteine effectively inhibits dihydroorotase[9, 10] and inhibits *de novo* synthesis of pyrimidine. Thus, the dUMP level will be decreased. The result may be to enhance the activity of FdUMP against thymidylate synthetase.

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