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Optimal Treatment Regimens for 5′-Deoxy-5-fluorouridine, with or without (*E*)-5-(2-Bromovinyl)-2′-deoxyuridine, against Various Tumors in Mice

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The antitumor activity of 5′-deoxy-5-fluorouridine (DFUR), a prodrug of 5-fluorouracil (5-FU), is markedly enhanced if DFUR treatment is combined with (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU). Combined oral administration of DFUR (10 mg/kg) and BVDU (10 mg/kg) three times (every 3 h) per day for 5 days afforded greater antitumor activity than a single dose of DFUR (300 mg/kg/day) for 5 days in mice bearing either adenocarcinoma 755 or Lewis lung carcinoma, while in the colon 26 system the antitumor effects of both treatment regimens were equivalent. Thus, a low-dose regimen of DFUR when combined with BVDU provides a similar or greater antitumor activity than a high-dose regimen of DFUR that is not combined with BVDU. The area under the curve of plasma 5-FU following a treatment with the combination of DFUR (10 mg/kg) and BVDU (10 mg/kg) was equal to that following DFUR (300 mg/kg) treatment.

Key words: 5′-Deoxy-5-fluorouridine — (*E*)-5-(2-Bromovinyl)-2′-deoxyuridine — Antitumor activity — Combination therapy

The antitumor activity of 5-fluorouracil (5-FU) against adenocarcinoma 755 is closely correlated with the area under the curve (AUC) of plasma 5-FU.[1)] 5′-Deoxy-5-fluorouridine (DFUR), a prodrug of 5-FU, is currently used as an oral anticancer agent in Japan. This drug is metabolized by uridine phosphorylase to 5-FU[2–4)] and, again, the antitumor activity of DFUR is closely correlated with the AUC of 5-FU.[5)]

5-FU is rapidly catabolized through the pyrimidine degradation pathway.[6)] The first step in the catabolism of 5-FU is the formation of 5,6-dihydro-5-fluorouracil by dihydrouracil dehydrogenase.[6)] (*E*)-5-(2-Bromovinyl)-uracil (BVU) is a specific inhibitor of this enzyme[7)]; it prolongs 5-FU level in the plasma and enhances the antitumor activity of DFUR.[5)] In this study, we investigated various treatment regimens to optimize the antitumor activity of DFUR against adenocarcinoma 755, Lewis lung carcinoma and colon 26. We also measured plasma levels of 5-FU following treatment of DFUR in combination with (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU), a prodrug of BVU. Furthermore, we measured pyrimidine nucleoside phosphorylase (PyNPase) activity in the tumors, as the efficiency of DFUR therapy may depend on these PyNPase levels.

MATERIALS AND METHODS

Drugs DFUR was obtained from Nippon Roche (Kamakura). BVDU was synthesized essentially as described by Jones *et al.*[8)]

Animals Groups of six (for antitumor activity determinations) or three (for plasma drug level determinations) male BDF_1 or BALB/c mice with a body weight of 21–23 g (Japan SLC, Inc., Hamamatsu) were housed in plastic cages with woodchip bedding, and received a CA-1 pellet diet (CLEA Japan, Inc., Tokyo) and water *ad libitum*. All experiments were performed in the animal laboratory at a controlled temperature of 25°C.

Antitumor activity Adenocarcinoma 755 tumor cells (20 mg/mouse), Lewis lung carcinoma (5×10^5 cells/mouse) and colon 26 (1×10^6 cells/mouse) were implanted subcutaneously (sc) on day 0. The tumors had been maintained by sc transfer every 10–12 days into syngeneic C57BL/6 or BALB/c mice kept in our laboratory at the National Cancer Center Research Institute (Tokyo). Beginning on day 5 after tumor implatation, the drugs were administered orally (po) daily for five consecutive days. Tumor weight of adenocarcinoma 755 was determined on day 11. Antitumor activity was evaluated by calculating the ratio of the average tumor weight in the treated groups to that in the control group (T/C, %). In Lewis lung carcinoma and colon 26, antitumor effect was evaluated by calculating the increase in life-span.

Determination of plasma drug concentration Groups of three mice were given DFUR and BVDU at the indicated doses in 0.1 ml of physiological saline by the oral route (po). Blood samples (0.5–1.0 ml) were collected in heparinized tubes at 0.5, 1, 2 and 3 h following treatment, and centrifuged immediately after collection. The plasma supernatants were harvested and frozen at −20°C until

assayed. Plasma samples were adjusted with distilled water to a total volume of 1 ml, and 0.2 ml of 0.5 *M* NaH_2PO_4 buffer and 8 ml of ethyl acetate were added. After extraction and centrifugation, the organic layer was transferred to other tubes and evaporated *in vacuo* at 35°C. The residue was dissolved in distilled water, in the same volume as had originally been present as plasma, and applied to a Bond Elut SAX column (Analytichem International, Harbor City, CA), and then 100 μl of the eluted materials was injected into an HPLC instrument (Tosoh, Tokyo) equipped with a Hibar prepacked column, LiChrosorb RP-18 (5 μl; Cica-Merck, Tokyo). Uracil, 5-FU, DFUR, BVU and BVDU were separated by using 2% methanol for 10 min, a linear gradient from 2 to 30% methanol-sodium acetate buffer (10 m*M*, pH 4.0) for 5 min and finally, 30% methanol-sodium acetate buffer for 25 min. The retention times of 5-FU, DFUR, BVU and BVDU were 7.9, 21.0, 29.7 and 33.5, respectively (0.7 ml/min).

Preparation of PyNPase from tumors Solid tumors were excised from tumor-bearing mice (three mice/group) on day 14, and homogenized in a Potter homogenizer in 10 m*M* Tris buffer (pH 7.4) containing 15 m*M* NaCl, 1.5 m*M* $MgCl_2$ and 50 μM potassium phosphate. All operations were carried out at 0–4°C. Then the homogenates were centrifuged at 100,000*g* for 90 min and the supernatants were dialyzed overnight against 20 m*M* potassium phosphate buffer (pH 7.4) containing 1 m*M* 2-mercaptoethanol and stored at −70°C.

Assay of PyNPase DFUR (1.2 μmol) was incubated at 37°C for 60 min with the enzyme extract and 22 μmol of potassium phosphate (pH 7.4). The amount of 5-FU generated from DFUR was estimated based on the activity of the reaction mixture against *Micrococcus flavus* BP1202 DR-1. This bacterium is deficient in PyNPase and, consequently, is resistant to DFUR but susceptible to 5-FU.[9]

Experimental procedure for inducing liver metastases Lewis lung carcinoma was maintained sc in C57BL/6 mice. The tumor was rapidly removed, minced with scissors in sterile Petri dishes containing 5 ml of Cytotoxicity Medium with 0.3% fetal calf serum (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) and pressed through a wire mesh (120 mesh). The cell suspension was washed twice with saline and adjusted to 5×10^5 cells/ml. Multiple hepatic metastases were produced according to the method of Kopper *et al.*[10] BDF_1 mice were anesthetized with ether, a left subcostal incision (5–10 mm) was made, and the spleen was externalized. A 27-gauge needle (Terumo Japan, Tokyo) was used to puncture the splenic capsule and 5×10^4 viable tumor cells in 0.1 ml of saline solution were injected directly into the upper pole of the spleen. Gentle pressure was applied for a period of 10 s to prevent hemorrhage and tumor cell extravasation. The arteria and vena lienalis were then clamped with a medium hemoclip (Edward Weck & Co., Inc., NC) and the spleen was removed. The abdominal incision was stitched with surgical suture and the skin was closed with disposable skin clip applicators (Avlox 12, Medi Plast, Sweden). The mice were allowed to recover and randomized before being distributed to cages. Histologically well-circumscribed tumors developed from the 4th day following intrasplenic injection of Lewis lung carcinoma.[10] Drugs were administered po daily for 5 consecutive days starting from the 4th and 7th days after tumor cell inoculation. The anti-metastatic effect was evaluated by determining the ILS (%). At death, the mice were examined for the presence of liver metastases.

RESULTS

Antitumor activity of the combination of DFUR and BVDU Antitumor activity of DFUR against adenocarcinoma 755 is closely correlated with the AUC of plasma 5-FU.[2] The AUC of plasma 5-FU following DFUR treatment at 100 mg/kg is about three-fold higher than plasma 5-FU AUC following DFUR treatment at 30 mg/kg.[5] If DFUR is administered at 30 mg/kg three times every 3 h, the total plasma 5-FU AUC is almost the same as the plasma 5-FU AUC following DFUR treatment at 100 mg/kg. However, the fractionated dosage regimen (DFUR 30 mg/kg, 3 times per day, every 3 h) had a greater inhibitory effect on the growth of advanced adenocarcinoma 755 than did a single dose of 90 mg/kg/

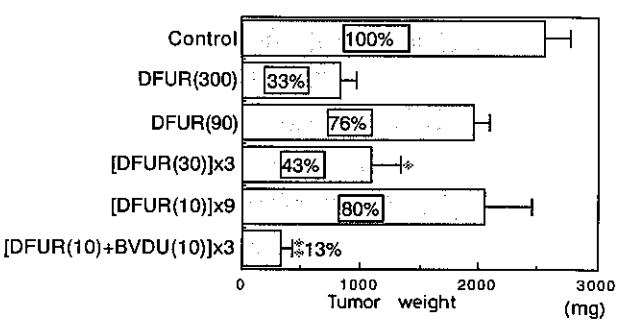

Fig. 1. Antitumor activity of DFUR, combined with or without BVDU, against adenocarcinoma 755. Numbers in parentheses refer to doses in mg/kg. DFUR (300 or 90 mg/kg/day) was administered as a single daily dose. DFUR (30 or 10 mg/kg) was administered three times (every 3 h) or nine times (every 1 h), respectively. When combined, DFUR (10 mg/kg) and BVDU (10 mg/kg) were administered three times per day (every 3 h). * Different from the DFUR (90) group, $P<0.05$. ** Different from the DFUR (300) group, $P<0.01$.

Table I. Effect of DFUR Combined with or without BVDU in Lewis Lung Carcinoma- or Colon 26-bearing Mice

Tumor	Treatment[a]	Survival time Mean±SE (days)	ILS(%)
Lewis lung carcinoma			
	None	20.0±1.0	
	DFUR alone	23.2±1.8	16
	DFUR+BVDU	26.8±2.8[b]	34
Colon 26			
	None	16.7±1.7	
	DFUR alone	24.4±0.9[c]	46
	DFUR+BVDU	25.2±3.0[b]	51

a) DFUR alone (single dose of 300 mg/kg/day) or DFUR (10 mg/kg, three times per day) combined with BVDU (10 mg/kg, three times per day) were administered po for five consecutive days, beginning on day 5.
b) Different from untreated control ($P<0.05$).
c) Different from untreated control ($P<0.01$).

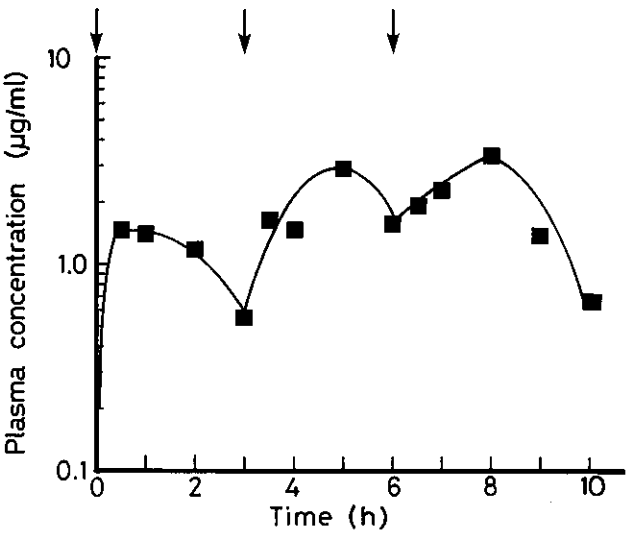

Fig. 3. Plasma level of BVU following administration of DFUR (10 mg/kg) combined with BVDU (10 mg/kg). Arrows represent times of po administration of drugs.

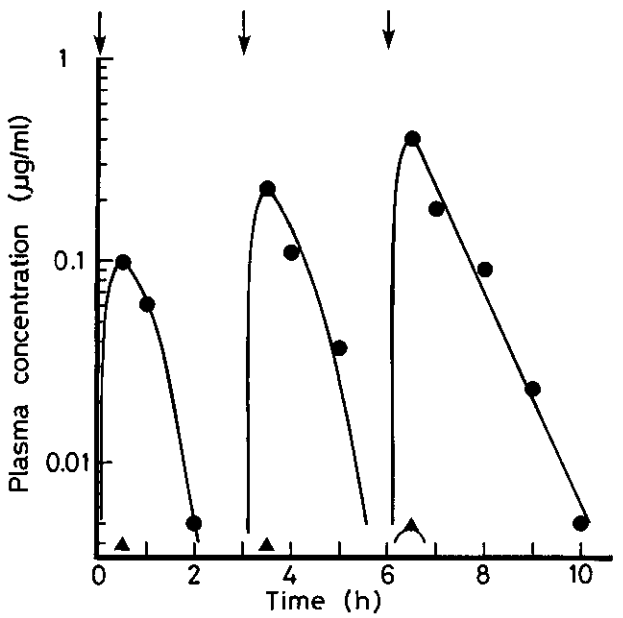

Fig. 2. Plasma level of 5-FU following administration of DFUR (10 mg/kg) combined with (●) or without (▲) BVDU (10 mg/kg). Arrows represent times of po administration of drugs.

day of DFUR; tumor reductions of 57% and 24% were achieved, respectively ($P<0.05$) (Fig. 1).

The antitumor activity achieved by the thrice-daily doses of 30 mg DFUR/kg (every 3 h) was almost equal to that of a single dose of DFUR at 300 mg/kg/day (67% inhibition). However, a more fractionated dosage regimen of DFUR (10 mg/kg, 9 times per day, every 1 h) had virtually no effect on the growth of adenocarcinoma 755 tumors (Fig. 1). Apparently, the peak plasma 5-FU levels (<0.005 μg/ml) reached with the 10 mg/kg dosage regimen did not suffice to afford antitumor activity. However, when the 10 mg/kg doses of DFUR were combined with 10 mg/kg of BVDU, and these combined doses were administered 3 times per day (every 3 h) for 5 days, a marked antitumor activity was observed: 87% inhibition of tumor weight.

In the Lewis lung carcinoma system, the combination of DFUR and BVDU was superior to DFUR alone, but in the colon 26 tumor system, the high-dose regimen of DFUR showed similar activity to the combination of DFUR plus BVDU (Table I).

Plasma levels of 5-FU after treatment with DFUR and BVDU Plasma levels of 5-FU following combined treatment of DFUR (10 mg/kg) with BVDU (10 mg/kg) are shown in Fig. 2. Thus, plasma 5-FU levels following combined DFUR and BVDU treatment were markedly increased as compared to 5-FU levels following DFUR treatment alone, so that the AUC after the third administration of the combination of DFUR (10 mg/kg) and BVDU (10 mg/kg) was equal to that of DFUR administered alone at 300 mg/kg. The BVDU-derived BVU maintained a high plasma level (0.5–3 μg/ml) over a 9 h period (Fig. 3).

Activity of PyNPase in tumors DFUR is known to be metabolized to 5-FU by PyNPase.[4] When the tumors were analyzed for PyNPase activity (Table II), it appeared that the PyNPase levels in colon 26 were 6- or 71-fold higher than those found in Lewis lung carcinoma and adenocarcinoma 755, respectively.

Effect of treatment of DFUR and BVDU on the mortality of mice bearing liver metastases If untreated, mice bearing liver metastases die within approximately 20 days after tumor cell inoculation. When Lewis lung carcinoma was implanted sc, the combination of DFUR and BVDU (3×10 mg/kg/day) was superior to the high dose of DFUR (300 mg/kg/day). However, if the Lewis tumor cells were inoculated through the spleen, the high-dose regimen of DFUR (300 mg/kg/day) was equivalent to the combination of low-dose DFUR (3×10 mg/kg/day) and BVDU (3×10 mg/kg/day) in increasing the life-span of the mice (Table III).

Table II. Pyrimidine Nucleoside Phosphorylase Activities in Various Tumors

Tumor	Enzyme activity in tumor (μmol 5-FU/mg protein/h)
Colon 26	920
Lewis lung carcinoma	154
Adenocarcinoma 755	13

DISCUSSION

Oral DFUR has a marked antitumor activity in various murine tumor systems (i.e. colon 26[11]), but has no strong activity against adenocarcinoma 755 or Lewis lung carcinoma. BVDU is known to increase the antitumor effect of fluoropyrimidine derivatives.[1, 5]

In this study, we examined several treatment regimens aimed at optimizing the antitumor activity of DFUR. From a number of treatment regimens that were compared in the adenocarcinoma 755 system, the combination of 10 mg/kg DFUR and 10 mg/kg BVDU (3 times per day, every 3 h) appeared to be the optimal regimen. This regimen also proved effective in Lewis lung carcinoma-bearing mice.

The marked antitumor activity achieved by this regimen can be readily attributed to the much higher plasma 5-FU levels that were generated following administration of the combination of DFUR with BVDU than with DFUR alone. These higher 5-FU levels achieved following co-administration of BVDU may be accounted for by the inhibitory effect of BVDU-derived BVU on the degradation of 5-FU by dihydrouracil dehydrogenase.[7, 12]

DFUR is rapidly converted to 5-FU by PyNPase.[2-4] PyNPase activity levels differed markedly from one tumor to another. The highest PyNPase level was found in colon 26, and this tumor system responds as well to the high-dose DFUR (300 mg/kg/day) regimen as to the combination of low-dose DFUR (3×10 mg/kg/day)

Table III. Effect of DFUR Combined with or without BVDU in Mice Bearing Hepatic Metastases of Lewis Lung Carcinoma

	Treatment[a]	Survival time[b] Mean±SE (days)	ILS (%)	Survivors[c]
I.	Control	19.9±1.1		0/12
	DFUR (100)[d]	19.0±4.2	−5	0/6
	DFUR (300)	26.3±1.1	32	0/6
	[DFUR(10)+BVDU(10)]×3	27.7±1.6	39	0/6
II.	Control	19.7±1.6		0/7
	DFUR (300)	29.8±2.5	51	0/6
	[DFUR(10)]×3	21.3±1.8	8	0/6
	[DFUR(10)+BVDU(10)]×3	26.0±2.0	32	0/6
	[DFUR(20)+BVDU(10)]×3	24.4±3.1	24	1/6

a) Drugs were administered po daily for 5 consecutive days starting from the 4th (I) or 7th (II) day after tumor cell inoculation.
b) Mean survival time of dead mice (cured mice were excluded from the calculations).
c) Sixty-day survivors/number of treated mice.
d) Numbers in parentheses refer to doses in mg/kg. The 100 or 300 mg/kg dose of DFUR was administered as a single daily dose. The 10 mg/kg dose of DFUR was administered three times per day (every 3 h). When combined, DFUR (10 or 20 mg/kg) and BVDU (10 mg/kg) were administered three times per day (every 3 h).

and BVDU (3×10 mg/kg/day). Adenocarcinoma 755, where the PyNPase level was lowest, responded better to the combination of low-dose DFUR and BVDU than to the high-dose DFUR regimen. The antitumor efficiency that could be expected from the combination of DFUR with BVDU, relative to DFUR alone, may obviously vary from one tumor system to another and may be related at least in part to the PyNPase activity levels of the tumors.

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