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1-Hexylcarbamoyl-5-fluorouracil is More Cytostatic than 5-Fluorouracil Against Human Tumors *in vitro*

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Abstract—*The sensitivity of HeLa cells and 15 human tumors, including eight gastric cancers, five colorectal cancers and two lung cancers to 1-hexylcarbamoyl-5-fluorouracil (HCFU) was compared with that to 5-fluorouracil (5-FU) in vitro. HeLa cells were doubly sensitive to HCFU, as compared to 5-FU. After the HeLa cells had been treated with 5-FU or HCFU at 77 μM for 1–5 h, the intracellular levels of 5-FU and HCFU were determined, using gas chromatographic–mass spectrometric methods. The level of HCFU plus 5-FU in the HCFU-treated cells was twice as high as the level of 5-FU in the 5-FU-treated cells. The sensitivity to HCFU in 15 tumor tissues varied with the tissue; however, all tissues tested were more sensitive to HCFU than to 5-FU, assessed using the succinate dehydrogenase inhibition test. These results suggest that the hexylcarbamoyl structure facilitates the rapid uptake of HCFU through the cell membrane. HCFU may prove to be more effective for treating each individual patient with a malignant lesion.*

INTRODUCTION

1-HEXYLCARBAMOYL-5-FLUOROURACIL (HCFU), a lipophilic masked compound of 5-fluorouracil (5-FU) [1], is converted to 5-FU enzymatically or nonenzymatically, and has an antineoplastic effect [2–4]. It was noted that oral HCFU has a higher therapeutic ratio and a wider tumor spectrum than 5-FU, in a variety of experimentally induced tumors [1, 2]. HCFU is used to treat patients with solid tumors, such as breast and gastrointestinal carcinomas [5, 6]. While the sensitivity to 5-FU of human tumor tissues has been determined, using various chemosensitivity tests [7–9], the sensitivity to HCFU has apparently not been documented. We examined the *in vitro* sensitivity to HCFU of various human tumor cells, in comparison with that to 5-FU, to evaluate the effectiveness of HCFU, as therapy for patients with cancer.

MATERIALS AND METHODS

Antitumor drugs

5-FU came from Kyowa Hakko Co., Ltd., Tokyo and HCFU came from Mitsui Pharmaceutical Inc., Ltd., Tokyo.

Cells

HeLa cells were routinely cultured in monolayer on plastic dishes, using minimal essential medium (MEM) (Nissui Seiyaku Co., Tokyo) with L-glutamine (292 mg/ml), 10% fetal calf serum (Difco Laboratories, U.S.A.), penicillin (100 U/ml), streptomycin (100 μg/ml) and gentamycin (40 μg/ml). Two × 10^5 HeLa cells were plated in 60 mm plastic dishes in the absence of the drug and were incubated at 37°C in a humidified 5% CO_2 atmosphere for 2 days. The cells in each dish were exposed to various concentrations of 5-FU or HCFU (0.77, 2.3, 7.7, 23, 77, 230 or 770 μM) in the medium containing 1% dimethylsulfoxide (DMSO) and then were incubated for 1, 2, 3 or 4 days. The number of attached cells was determined by using the dye exclusion method [10].

Tumor tissues

Solid tumor specimens from 15 Japanese (eight gastric cancers, five colorectal cancers and two lung cancers) were obtained at surgery and placed in Hanks' solution.

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Uptake of the drug in HeLa cells

HeLa cells were exposed to 5-FU or HCFU at a concentration of 77 μM for 1–5 h and the intracellular levels of 5-FU and HCFU were determined by the method of Mori *et al.* [11]. One × 10^7 cells were dissolved in 1 ml of distilled water. A suspension of 0.5 ml was put into a test tube containing 5-chlorouracil, as an internal standard. Next, 0.5 ml of 0.5 N HCl and 6 ml of $CHCl_3$ were added and the aqueous layer was used to determine the level of 5-FU. The level of HCFU was assessed by determining the level of total 5-FU, using the following method, then subtracting the level of 5-FU from this amount of total 5-FU. To extract total 5-FU, 0.3 ml of 6 N NaOH was added, the mixture was incubated for 10 min in a 50°C water bath and all substances with the structure of HCFU or 5-FU were converted to 5-FU. The hydrolyzed solution was acidifed to a maximum pH of 2 using HCl, then $CHCl_3$ was added and the preparation centrifuged. XAD-2 resin, 0.5 ml, was added to the upper layer and the aqueous layer separated and evaporated. The residue was dissolved in 0.5 ml of 1 M phosphate buffer solution (pH 7.0). Ten ml of ethyl acetate was added, the preparation centrifuged to separate the ethyl acetate layer and then evaporated. The level of total 5-FU in the residue was analyzed using gas chromatographic–mass spectrometric determinations (GC-CI-NS system, model JMS-DX 300, manufactured by JEOL).

SDI test

To procure a tumor cell suspension, the tumor tissue in Hanks' solution was minced with scissors, the fragments were put into a sterile flask containing Hank's solution and 0.25% trypsin, 0.25% collagenase (type I–A) and 0.1% DNase (type I) (Sigma Chemical Co., U.S.A.), the preparation was stirred with a magnetic stirrer for 20 min and passed through a No. 100 stainless steel mesh. The cells were washed with Hanks' solution, pelleted and re-suspended in 30 ml MEM, as described above. These cells were placed in plastic dishes, incubated for 30 min and the adherent cells removed. A discontinuous gradient of 10 ml each of 25, 15 and 10% Percoll in MEM was layered, and 10 ml of the tumor cell suspension was laid on the top. The gradient was centrifuged at room temperature for 7 min at 25 ***g***. The bottom layer (tumor cell rich fraction) was collected, washed twice with Hanks' solution and re-suspended in MEM [12, 13]. The SDI test was done as described in Refs [14, 15]. One × 10^6 tumor cells were plated in each of 35 mm plastic dishes, exposed to 5-FU or HCFU, and the preparation incubated for 3 days. These cells were then tested for succinate dehydrogenase (SD) activity. 3(4,5-Dimethyl-2-thiazolyl)2,5-diphenyl-2*H* tetrazolium bromide (MTT) [16] was used as a hydrogen acceptor for the SD activity. The formazan formed from MTT was extracted with acetone containing 0.5% trichloroacetic acid and the absorption of formazan was measured at 565 nm. The SD activity was presented as the optical density. The chemosensitivity was estimated by the percentage of SD activity of the drug-treated cells, compared to that of control cells.

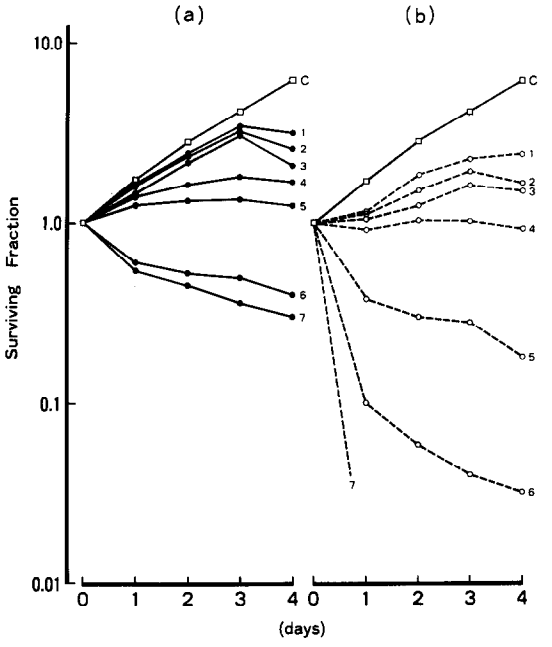

Fig. 1. Changes in the number of HeLa cells exposed to 5-FU or HCFU. Two × 10^5 HeLa cells were placed in plastic dishes, incubated for 2 days and exposed to various concentrations of 5-FU (a) or HCFU (b) for 1, 2, 3 or 4 days. C, Control; 1, 0.77 μM; 2, 2.3 μM; 3, 7.7 μM; 4, 23 μM; 5, 77 μM; 6, 230 μM; 7, 770 μM.

RESULTS

Sensitivity of HeLa cells to 5-FU and HCFU

Changes of the cell numbers, following exposure to 5-FU or HCFU for 1, 2, 3, or 4 days, are shown in Fig. 1. The cell numbers decreased when exposed to HCFU, with each concentration. The percentage decrease in the number of cells exposed to 5-FU or HCFU is shown in Fig. 2. HCFU was approximately twice as active as 5-FU against HeLa cells. After HeLa cells had been exposed to 5-FU or HCFU at 77 μM for 1, 2, 3, 4 or 5 h, the intracellular levels of 5-FU and total 5-FU (HCFU fraction plus 5-FU) were determined, using gas chromatographic–mass spectrometric methods. The level of total 5-FU in the HCFU-treated cells was twice as high as the 5-FU level in the 5-FU-treated cells, as shown in Fig. 3. The intracellular levels in the HCFU fraction plus 5-FU correlated with the cytostatic activity of HCFU against HeLa cells. HCFU was taken up through the membrane and rapidly converted to 5-FU.

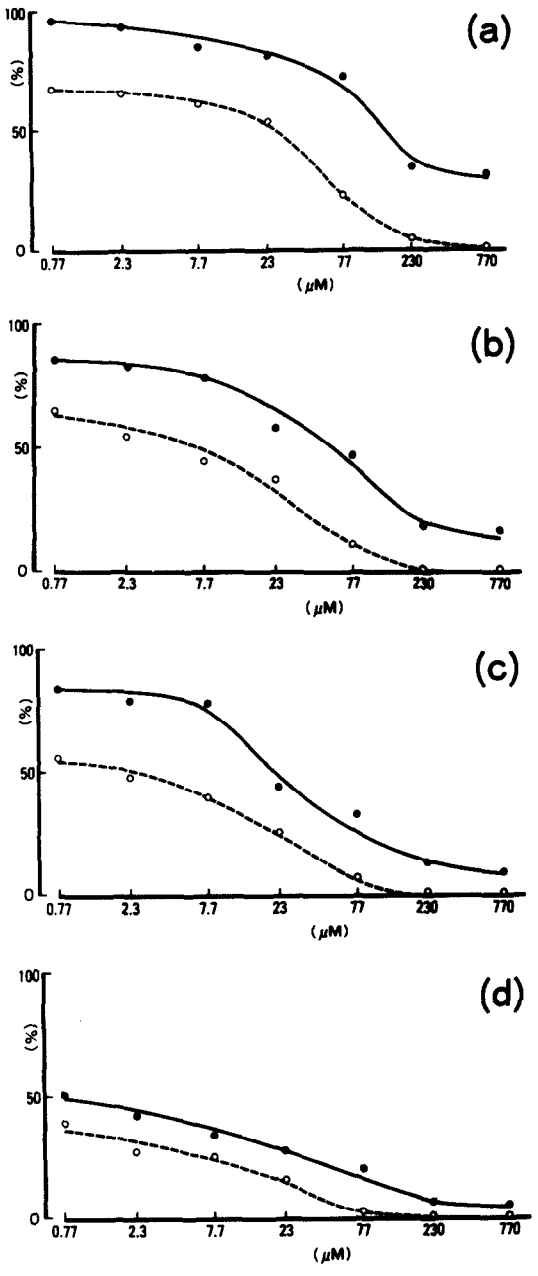

Fig. 2. Percentage decreases in cell numbers of 5-FU- or HCFU-treated HeLa cells compared to control cells. Details are shown in Fig. 1(a), Day 1; (b), day 2; (c), day 3; (d), day 4. (●), 5-FU-treated cells; (○), HCFU-treated cells.

Sensitivity of human tumors to 5-FU and HCFU

The rates of tumor cells were 75–93% in 15 tissues determined using Papanicolaou's staining (Table 1). Non-tumor cells were lymphocytes, neutrophils and macrophages; however, the fraction of macrophages was removed as the adherent cells.

As shown in Table 1, the SD activity decreased little at 77 μM (the peak plasma concentration) of 5-FU, but decreased individually at 770 μM (the 10 times peak plasma concentration). Exposed to HCFU, the SD activity decreased, even at 77 μM. The decrease in the SD activity in the HCFU-treated cells was prominent, at each concentration as compared to the 5-FU-treated cells. The correlation rates of the SD activities in the each tissue between the 5-FU-treated and the HCFU-treated cells were $r = 0.695$ at 77 μM, $r = 0.759$ at 770 μM in the two drugs, and $r = 0.732$ at 770 μM in 5-FU and at 77 μM in HCFU.

DISCUSSION

It was reported that the sensitivity to 5-FU of human tumor tissue varied with the individual tumor [8, 15]. This result prompted us to examine the sensitivity to HCFU, a synthetic masked compound of 5-FU [1]. HCFU is clinically prescribed to treat solid tumors [5, 6]. We found that HCFU *in vitro* was approximately twice as active as 5-FU against HeLa cells. As the tumor tissues have a higher phosphorylating activity of 5-FU than do the normal tissues [17], the higher intracellular level of HCFU plus 5-FU in HCFU-treated HeLa cells can explain the higher sensitivity of HeLa cells to HCFU, as compared to 5-FU. Iigo *et al.* [18] reported that the hexylcarbamoyl structure facilitates rapid absorption through the gastrointestinal tract and blood–ascites barrier. It is thus considered that the chemical structure of HCFU relates to the rapid uptake of HCFU, through the cell membrane, compared to 5-FU, and that HCFU is converted to 5-FU within a short time. As this effect of HCFU in HeLa cells was also noted in Chinese hamster lung V79 cells, human blood buffy coat containing mainly lymphocytes and pleural fluid (data not shown), our *in vitro* data may not be specific for tumor cells. However, HCFU given orally had a higher therapeutic ratio and a higher therapeutic index than did 5-FU against murine tumors [19, 20]. 5-FU is toxic to cerebellar tissues [21, 22] and HCFU may have an increaed cerebellar toxicity compared to 5-FU. The phase I study of HCFU revealed that the toxic effect specific for this drug are a transient hot sensation and pollakisuria [23].

We also determined the sensitivities of various human tumors to 5-FU and HCFU, using the SDI test. Although the level of the sensitivity to HCFU varied in each tissue, all tissues tested were more sensitive to HCFU than to 5-FU. As HCFU must be changed to 5-FU for cytostatic action [2–4], a positive correlation is evident between the SD activity in 5-FU-treated cells and that in HCFU-treated cells, in each tissue, as determined using the SDI test. This test is advantageous for predicting the chemosensitivity of various human tumors [14, 15].

Our observations suggest that HCFU is more cytostatic than 5-FU against various human tumors *in vitro* and may be more effective for treating individual patients with a malignant lesion.

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Table 1. Percentages of SD activity of the drug-related cells compared to control cells, in 15 tumor tissues

Tissue	5-FU 77 μM	5-FU 770 μM	HCFU 77 μM	HCFU 770 μM	Percentage of tumor cells in each case
Gastric cancer	97.5	71.6	62.6	15.2	91
Gastric cancer	99.6	73.2	54.6	23.9	88
Gastric cancer	96.3	67.9	46.5	4.1	86
Gastric cancer	99.1	87.7	73.0	55.6	81
Gastric cancer	82.7	55.9	30.2	6.5	87
Gastric cancer	80.2	58.3	53.6	21.6	85
Gastric cancer	78.2	44.6	27.3	9.5	93
Gastric cancer	97.7	92.3	80.9	61.5	83
Colorectal cancer	75.6	47.2	44.9	18.0	75
Colorectal cancer	91.6	62.3	63.4	17.6	91
Colorectal cancer	93.1	63.1	76.5	14.2	88
Colorectal cancer	99.8	80.5	59.8	27.6	78
Colorectal cancer	89.0	60.7	50.0	12.6	86
Lung cancer	95.4	83.2	71.5	38.4	75
Lung cancer	97.7	67.4	78.2	45.3	79
Mean	91.6 ± 8.1	67.7 ± 13.5	58.2 ± 16.0	24.8 ± 17.1	84 ± 6

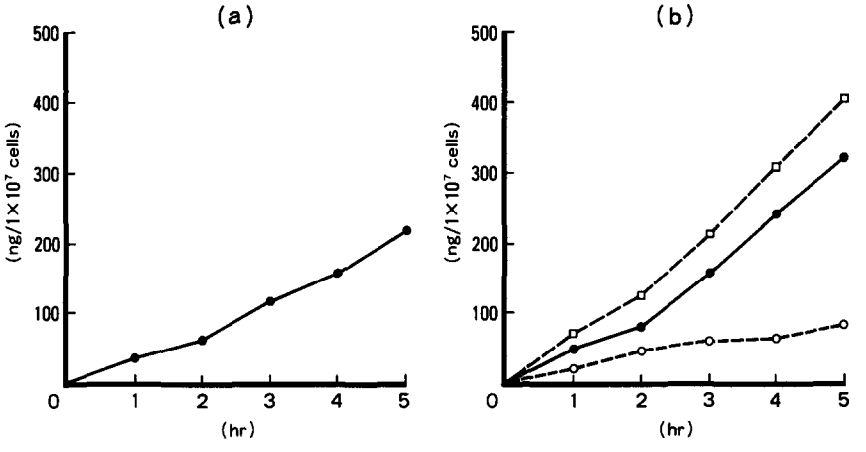

Fig. 3. Intracellular levels of 5-FU and HCFU in HeLa cells exposed to 5-FU or HCFU. After HeLa cells had been exposed to 5-FU or HCFU at a concentration of 77 μM for 1, 2, 3, 4, or 5 h, the intracellular levels of 5-FU and total 5-FU (HCFU plus 5-FU) were determined, using gas chromatographic–mass spectrometric methods [11]. (a), 5-FU-treated cells; (b), HCFU-treated cells. (●) Intracellular levels of 5-FU; (○) HCFU fraction; (□) total 5-FU (5-FU plus HCFU).

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