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Abstract

We investigated the antitumor activities of 5-fluorouracil (5-FU), 5'-deoxy-5-fluorouridine (5'-DFUR), 1-hexylcarbomoyl-5-fluorouracil (HCFU) and 1-(tetrahydro-2-furanyl)-5-fluorouracil (FT-207) in combination with hyperthermia in vitro. The antitumor effect of 5-FU (10^{-4} M) was slightly enhanced by combination with hyperthermia (42 degrees C) for 2h, and the effect was determined to be additive. Synergistic enhancement of antitumor activity was obtained by the concurrent use of hyperthermia (42 degrees C, 2h) and 5'-DFUR (10^{-4} M) or HCFU (10^{-5} M). However, the antitumor effect of FT-207 (10^{-4} M) in combination with hyperthermia was comparable that of hyperthermia alone. The synergistic enhancement of antitumor activity was not obtained for all drugs when the cells were preheated at 42 degrees C for 2h. On the other hand, when cells were pretreated with drugs before heat exposure, weak interactions were obtained after 5-FU and 5'-DFUR treatment, and a synergistic interaction was obtained after HCFU treatment. It is speculated that the metabolites of 5'-DFUR and HCFU enhance the cytotoxicity of 5-FU, or might change the threshold concentration for a cytotoxic effect of 5-FU in cancer cells.

KEYWORDS: hyperthermia, 5-fluorouridine, 5'-deoxy-5-fluorouridine, 1-hexylcarbomoyl-5-fluorouracil, FT-207

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Comparative Antitumor Activity of 5-Fluorouracil and its Prodrugs in Combination with Hyperthermia *in Vitro*

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We investigated the antitumor activities of 5-fluorouracil (5-FU), 5'-deoxy-5-fluorouridine (5'-DFUR), 1-hexylcarbamoyl-5-fluorouracil (HCFU) and 1-(tetrahydro-2-furanyl)-5-fluorouracil (FT-207) in combination with hyperthermia *in vitro*. The antitumor effect of 5-FU (10^{-4} M) was slightly enhanced by combination with hyperthermia (42°C) for 2h, and the effect was determined to be additive. Synergistic enhancement of antitumor activity was obtained by the concurrent use of hyperthermia (42°C, 2h) and 5'-DFUR (10^{-4} M) or HCFU (10^{-5} M). However, the antitumor effect of FT-207 (10^{-4} M) in combination with hyperthermia was comparable that of hyperthermia alone. The synergistic enhancement of antitumor activity was not obtained for all drugs when the cells were preheated at 42°C for 2h. On the other hand, when cells were pretreated with drugs before heat exposure, weak interactions were obtained after 5-FU and 5'-DFUR treatment, and a synergistic interaction was obtained after HCFU treatment. It is speculated that the metabolites of 5'-DFUR and HCFU enhance the cytotoxicity of 5-FU, or might change the threshold concentration for a cytotoxic effect of 5-FU in cancer cells.

Key words : hyperthermia, 5-fluorouridine, 5'-deoxy-5-fluorouridine, 1-hexylcarbamoyl-5-fluorouracil, FT-207

Hyperthermia potentiates the cytotoxicity of several chemotherapeutic agents including bleomycin(1), cyclophosphamide(2), cisplatin(3, 4), mitomycin C (5), 1, 3-bis (2-chloroethyl)-1-nitrosourea (6), and Adriamycin (7, 8). Several investigators have reported combined hyperthermia and chemotherapy for cancer patients.

5-Fluorouracil (5-FU) is an established cytostatic drug for the treatment of a variety of neoplastic diseases, particularly for cancers of the

breast and gastrointestinal organs. 1-Hexylcarbamoyl-5-fluorouracil (HCFU) is a lipophilic masked compound of 5-FU and is known to release 5-FU enzymatically or nonenzymatically, and has an antitumor effect (9). 5'-Deoxy-5-fluorouridine (5'-DFUR) is a prodrug from which 5-FU is generated by pyrimidine nucleoside phosphorylase(10), mainly by thymidine nucleoside phosphorylase in human tumors (11, 12). 1-(tetrahydro-2-furanyl)-5-fluorouracil (FT-207) is also a prodrug of 5-FU, from which 5-FU is thought to be generated *in vivo* by hepatic

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metabolism involving cytochrome P-450 (13). Recently, it has been reported that the cleavage of FT-207 to 5-FU is assumed to be catalyzed by thymidine phosphorylase in human tumors (14). In combination with hyperthermia, 5-FU was classified in the group that showed no change in cytotoxicity between 37 and 45 °C (15). However, several investigators have reported clinical trials of 5-FU in combination with hyperthermia (16, 17). Moreover, a synergistic cytotoxic effect between hyperthermia and HCFU has been reported (18, 19). These observations prompted us to assess the comparative antitumor effects of 5-FU, 5'-DFUR, HCFU, and FT-207 in combination with hyperthermia *in vitro*.

Materials and Methods

Cell lines. RPMI4788 (a human colon cancer cell line), MKN-28 (a human gastric cancer cell line) and ZR-75-1 (a human breast cancer cell line) were maintained in RPMI1640 medium (Nissui Pharmaceutical Co., Tokyo) supplemented with 10 % fetal calf serum (Gibco, NY), penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37 °C in a humidified 5 % CO₂ atmosphere. Cells in the exponential growth phase were harvested for the experiments.

Chemicals. 5-FU and FT-207 were supplied by Taiho Yakuhin (Tokyo, Japan), HCFU was supplied by Mitsui Seiyaku (Tokyo, Japan) and 5'-DFUR was supplied by Nippon Roche Pharmaceutical Inc. Ltd. (Tokyo, Japan).

Treatment and antiproliferative assay. 5-FU and 5'-DFUR were dissolved in methanol, while HCFU and FT-207 were dissolved in phosphate buffered saline (PBS), and further diluted with PBS. Cells were suspended in 4 ml of growth medium (1×10^5 cells) with 1 ml of drugs and incubated in polystyrene tubes (Corning, NY) at various temperatures in a water bath (Thermomix Co. Ltd., Tokyo). Under these conditions, 5 ml of aqueous solution reached within 0.1 °C of the water bath temperature within 5 min. Then, the cells were chilled, centrifuged, and washed twice with 5 ml of cold PBS. The surviving cells were counted again, suspended in fresh growth medium at 5,000 cells/well, and plated in 96-well microculture plates (Falcon, Calif.). They were then incubated at 37 °C in a 5 % CO₂ incubator for

3 days. After incubation, the adherent viable cells were fixed with 95 % methanol and stained with crystal violet. The dye was eluted with Sorenson's buffer (20), and the absorbance (A) at 590 nm was determined with a plate analyser (Toyo Instrument Co. Ltd., Tokyo). The percentage of survival was calculated from the following formula:

$$\% \text{ survival} = 100 \times A \text{ of the experiment} / A \text{ of the control at } 37^\circ\text{C}$$

The % survival of the cells treated with each drug and hyperthermia was compared to the expected value calculated by multiplication of the % survival after treatment with the drug alone by that of hyperthermia alone. The interactive effect was considered as synergistic if the difference between the experimental value and the expected value was more than 2 standard deviations (SD), and as additive if the difference was between -2SD and +2SD (21).

Results

Effect of concurrent treatment with drugs and hyperthermia. Fig. 1 shows the sensitivity of RPMI4788 cells exposed to various temperatures for 2 h. The growth inhibition of the cells at 42 °C for 2 h was 41 %. The sensitivity of RPMI4788 cells to drugs at 37 °C for 2 h is shown in Fig. 2. The cells were highly sensitive to HCFU, and only 24 % survival was observed in cells treated with 10^{-4} M HCFU at 37 °C for 2 h. Based on these results, further examinations were conducted with hyperthermia at 42 °C for 2 h and drug concentrations of 10^{-4} M for 5-FU, 5'-DFUR, and FT-207, and 10^{-5} M for HCFU. The results of concurrent treatment of RPMI4788, MKN-28, and ZR-75-1 cells with drugs and hyperthermia are shown in Figs. 2, 3 (A) and (B). The survival of these cells after exposure to 5-FU in combination with hyperthermia was slightly lower than that after heat exposure alone, and the interactive effect of hyperthermia and 5-FU was determined to be additive. The effects of FT-207 (up to 10^{-3} M) in combination with hyperthermia were comparable to that of hyperthermia alone in RPMI4788 cells, and the antitumor effect was not enhanced by hyperther-

mia in these three cells. On the other hand, the antitumor effects of 5'-DFUR and HCFU on

RPMI4788 were markedly enhanced by hyperthermia, and the interactive effect of hyperthermia and 5'-DFUR or HCFU was determined to be synergistic(Fig. 2). Also the synergistic effects of hyperthermia and 5'-DFUR or HCFU were obtained in MKN-28 and ZR-75-1 cells (Fig. 3).

Effects of sequential treatment with drugs and hyperthermia. The effect of sequence on the interaction of drugs and hyperthermia were determined in RPMI4788 cells. When the cells were heated at 42°C for 2h and then exposed to drugs at 37°C for 2h, the antitumor effect was not enhanced compared to that of hyperthermia alone (Fig. 4(A)). However, when the cells were treated with drugs at 37°C for 2h, washed twice with PBS, and then exposed to heat at 42°C for 2h, the antitumor effect was slightly enhanced for 5-FU and 5'-DFUR and markedly enhanced for HCFU compared with hyperthermia alone (Fig. 4(B)). The enhanced effect obtained by sequential treatment with HCFU and hyperthermia was determined as synergistic.

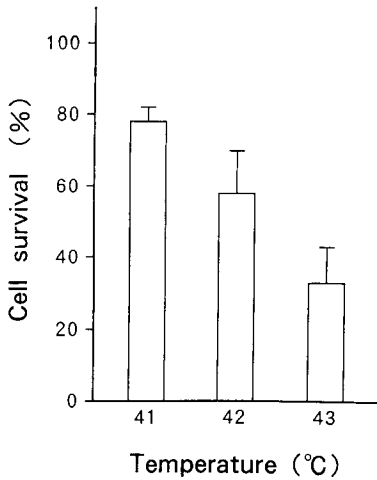


Fig. 1 Effect of hyperthermia on RPMI4788 cells. Cells were incubated at various temperatures for 2h.

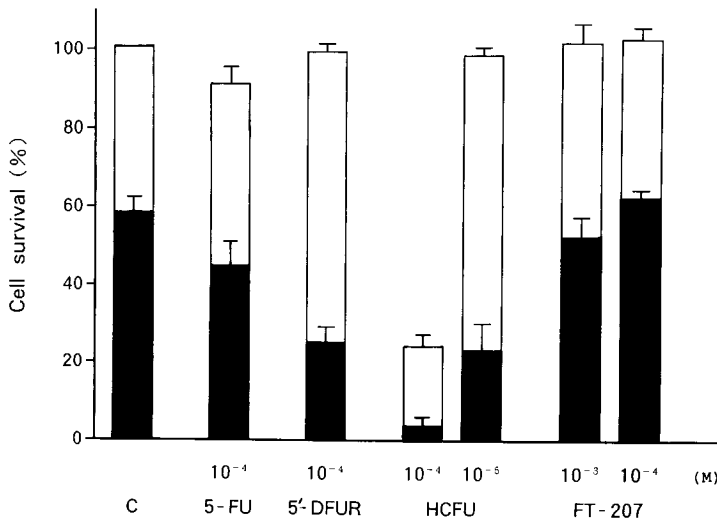


Fig. 2 Effect of 5-FU, 5'-DFUR, HCFU or FT-207 on RPMI4788 cells in combination with hyperthermia. Cells were exposed to medium (C) or various concentrations of drugs (5-FU, 5'-DFUR, HCFU and FT-207) with (■) or without (□) hyperthermia at 42°C for 2h.

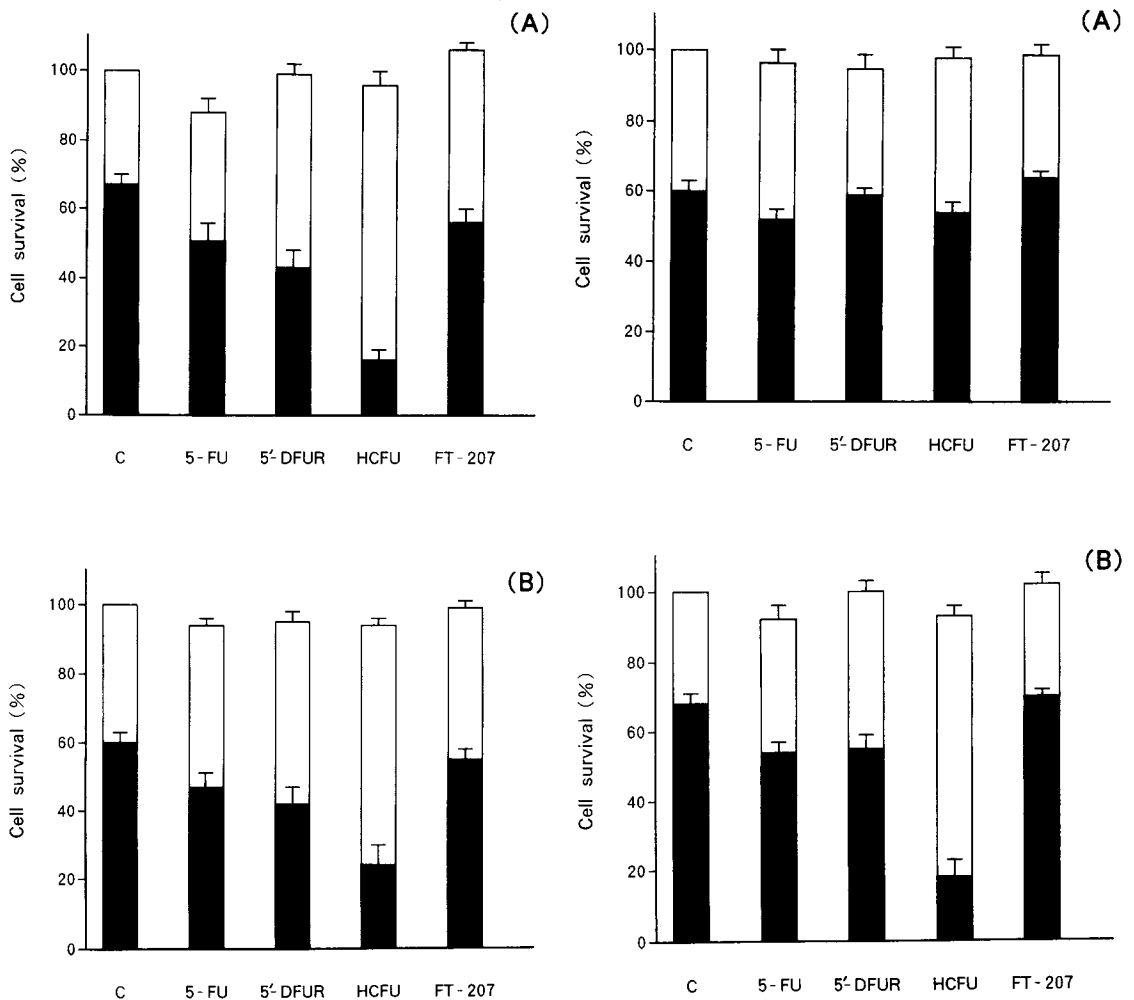


Fig. 3 (left) Effect of 5-FU (10^{-4} M), 5'-DFUR (10^{-4} M), HCFU (10^{-5} M), or FT-207 (10^{-4}) on MKN-28 cells (A) and ZR-75-1 cells (B) in combination with hyperthermia.

Cells were exposed to medium (C) or drugs with (■) or without (□) hyperthermia at 42°C for 2h.

Fig. 4 (right) Effect of 5-FU (10^{-4} M), 5'-DFUR (10^{-4} M), HCFU (10^{-5} M), or FT-207 (10^{-4} M) in sequential combination with hyperthermia on RPMI4788 cells.

(A) Cells were exposed (□) or not (■) to hyperthermia at 42°C for 2h, and then treated with medium (C) or drugs at 37°C for 2h. (B) Cells were treated with medium (C) or drugs at 37°C for 2h, and then exposed (■) or not (□) to hyperthermia at 42°C for 2h.

Discussion

Hyperthermia enhances the cytotoxic effect of some, but not all, antitumor drugs including bleomycin (1), cyclophosphamide (2), cisplatin

(3, 4), mitomycin C (5), 1, 3-bis (2-chloroethyl)-1-nitrosourea (6), and adriamycin (7, 8). 5-FU is one of the most effective drugs against human neoplasms, particularly for cancers of the breast and gastrointestinal organs, and is either used

alone or in combination with other anticancer agents. There are contrastive reports on the combined effect of 5-FU and hyperthermia. Some investigators have reported that no enhanced cytotoxicity was noted between 5-FU and hyperthermia (22, 23). However, other investigators have reported better clinical results for combined therapy with 5-FU and hyperthermia (16, 17). Recently, the synergistic effect of HCFU, one of the prodrugs of 5-FU, and hyperthermia has also been reported (18, 19).

The mechanism underlying the synergism between hyperthermia and these agents is probably multifarious, and may involve changes in drug accumulation (24), drug activation (5), or alterations in DNA repair (25). There is limited information on the role of various postulated mechanisms. Indeed, the mechanism of tumor regression due to hyperthermia alone is unknown, although some evidence suggests that hyperthermic cell killing involves membrane damage.

In general, the intracellular potassium ion level is about twenty times higher than the extracellular. On the other hand, the intracellular calcium ion level is normally low, and the ionic gradient of calcium shows a more than one thousand-fold difference (26). One of the important functions of the plasma membrane is to keep these ionic gradients constant. Previously, we reported that hyperthermia progressively inhibited the membrane potential (27) and increased the influx of calcium ions into the cytoplasm (28). It is conceivable that heat-induced membrane damage could also increase the membrane permeability of drugs and affect their cytotoxicity when the cells were treated with the various agents in combination with hyperthermia.

HCFU is known to release 5-FU spontaneously (9) and is hydrolyzed to 5-FU in a temperature-dependent manner (18). Therefore, it seems likely that HCFU would be almost completely converted to 5-FU in combination with hyperthermia at 42°C for 2h. 5'-DFUR is a prodrug which releases 5-FU when it is cleaved by thymidine phosphorylase. These enzymes are

preferentially localized in tumors and the levels in human cancer tissues are higher than in normal tissues (11). FT-207 is also a prodrug of 5-FU and is considered to be converted to 5-FU by cytochrome P-450 in microsomes of the liver (13). Recently, it has been reported that FT-207 is also converted to 5-FU in cancer cells, and this effect is thought to be attributable to thymidine phosphorylase in the cytoplasm (14).

We report that the antitumor effect of 5-FU in combination with heat was additive for RPMI4788, MKN-28 and ZR-75-1 cells, while the antitumor effect of 5'-DFUR or HCFU plus heat was synergistic for these cells. HCFU was used at a concentration that was one-tenth of the level of other drugs, but, with hyperthermia, HCFU was more cytotoxic than the other treatments. However, the antitumor effect of FT-207 was not enhanced by hyperthermia in these three cell lines.

The effects of sequence on the interaction of hyperthermia and drugs were also examined. Our results showed that the interactive effects of 5-FU, 5'-DFUR, and HCFU were increased when the cells were treated with drugs and hyperthermia concurrently. When the cells were preheated at 42°C for 2h and then exposed to drugs at 37°C for 2h, the antitumor effect was not enhanced compared to hyperthermia alone. This suggests that the membrane damage due to heat recovered during the following 2h at 37°C, or that the 2h-heat exposure induced some form of protection in cancer cells against the chemotherapeutic agents. When the cells were pretreated with drugs at 37°C for 2h and then exposed to heat at 42°C for 2h, the antitumor effects of 5-FU and 5'-DFUR were slightly enhanced compared to heat alone, however the effect of HCFU was synergistically enhanced. These results suggest that the cell damage caused by drugs was enhanced by heat exposure.

In addition, it has been reported that the chemical structure of HCFU allows it more rapid uptake through the cell membrane, and that HCFU is rapidly converted to 5-FU after uptake

(29). Therefore, the chemical structure of the drug may be one of the factors in obtaining synergism with hyperthermia. If the antitumor effect was only due to the drug concentration, the cytotoxic effect on the cell lines of 5'-DFUR in combination with hyperthermia should be the same as that of 5-FU with hyperthermia, and also the cytotoxicity of HCFU in combination with hyperthermia should be one tenth of that of 5-FU with hyperthermia. (Therefore, it is speculated that the metabolites of 5'-DFUR and HCFU enhance the cytotoxicity of 5-FU or change the threshold concentration for a cytotoxic effect of 5-FU in these cancer cells.) It is also possible to speculate that the structure of the drug may alter the architecture of cell membrane (19) and affect the uptake of drug through the cell membrane. Such membrane modification could be major factor in the synergistic interaction between hyperthermia and 5'-DFUR or HCFU.

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