*Eur J Cancer Clin Oncol*, Vol. 25, No. 3, pp. 477–481, 1989.
Printed in Great Britain

0277-5379/89$3.00 + 0.00


# Hyperthermia Enhances the *In Vitro* Activity of 1-Hexylcarbamoyl-5-fluorouracil Compared to that of 5-Fluorouracil

TETSUYA KUSUMOTO,* YOSHIHIKO MAEHARA,* YOSHIHISA SAKAGUCHI* and KEIZO SUGIMACHI*†

*Cancer Center of Kyushu University Hospital, †Second Department of Surgery, Faculty of Medicine, Kyushu University, Fukuoka, Japan*

**Abstract**—*The synergic effects on HeLa cells between 1-hexylcarbamoyl-5-fluorouracil (HCFU), a lipophilic masked compound of 5-fluorouracil (5-FU), and hyperthermia were investigated, with the following results:* (1) *After the cells had been exposed to the drug at* 77 μM *for* 1, 2 *or* 3 *days, with or without heat* (43°C) *for* 2 h, *treatment with HCFU and heat inhibited cell growth by* 9.2% *on day* 3, *compared to control cells, HCFU alone, heat alone or the combined treatment with 5-FU plus heat.* (2) *The cells were exposed to the drugs at* 77 μM *or heat* (43°C) *for* 15–45 *min and the colonies counted on day* 14. *Combined treatment with HCFU plus heat inhibited the clonogenicity of HeLa cells to* 22.4% *of findings in the control cells and as compared to findings with other treatments. The combination of* 5-*FU*, n-*hexylamine and heat had much the same effect as the combined effect of the treatment of HCFU plus heat.* (3) *Intracellular levels of the drugs did not increase with hyperthermic treatment.*

*Our findings suggest that it is the hexylcarbamoyl structure of HCFU that relates to the synergic effect between HCFU and hyperthermia. This combination is expected to have positive effects for treating patients with malignancy.*

## INTRODUCTION

HYPERTHERMIA enhances the cytotoxic effect of some, but not all, antitumour drugs and the magnitude of the enhancement varies with the drugs [1]. Hence, the combined effect of chemotherapy agents and hyperthermia for treating clinical cancer has gained much attention [1–5].

The pyrimidine analogue 5-fluorouracil (5-FU) is one of the few drugs with definite positive effects in the treatment of solid tumours. There are reports on the combined effect of 5-FU plus hyperthermia [6, 7]. No synergy in cytotoxic effect was noted between 5-FU and hyperthermia [1, 3–5]. Mizuno *et al.* [3] and Rose *et al.* [4] found no enhancement of the cytotoxic effect of 5-FU at temperatures of 42°C and 38.9°C. 1-Hexylcarbamoyl-5-fluorouracil (HCFU), a lipophilic masked compound of 5-FU given orally, has a higher therapeutic ratio and a wider tumour spectrum than does 5-FU, in a variety of experimentally induced tumours [6–11]. In Japan, this drug is prescribed for treatment of patients with gastrointestinal and breast cancers [12–14]. The sensitivity to HCFU under conditions of an elevated temperature has apparently not been documented. We report here the synergy in cytotoxic effect between recombinant HCFU and heat in HeLa cells.

Accepted 20 October 1988.
Correspondence should be addressed to Dr. Tetsuya Kusumoto, Cancer Center of Kyushu University Hospital, Faculty of Medicine, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812, Japan.

## MATERIALS AND METHODS

### *Chemicals*

5-FU was from Kyowa Hakko Co. Ltd. (Japan), HCFU was from Mitsui Pharmaceutical Inc. Ltd. (Japan) and *n*-hexylamine was from Sigma Chemical Co. (U.S.A.).

### *Cell cultures and treatment*

HeLa cells were grown in Eagle's minimal essential medium (MEM) (Nissui Pharmaceutical Co., Japan) containing L-glutamine (292 mg/ml), 10% foetal calf serum (Gibco Laboratories, U.S.A.), penicillin (100 U/ml), streptomycin (100 μg/ml) and gentamycin (40 μg/ml). Stock cultures were maintained in a humidified 5% $CO_2$ atmosphere at 37°C. These cells were exposed to 5-FU or HCFU at 77 μM [10] for the indicated time or heat (43°C)

for 2 h in a model 7100 incubator (Napco Scientific Co., U.S.A.), following exchange of the fresh medium and warmed at the indicated temperature. For clonogenicity, $3 \times 10^2$ cells were plated into 60 mm plastic dishes in the absence of the drugs and were incubated at 37°C in a humidified 5% $CO_2$ atmosphere for 2 days. The cells were then exposed to various concentrations of 5-FU or HCFU (with or without the same concentration of *n*-hexylamine, which is similar to the hexylcarbamoyl side-chain of HCFU) at 43°C for 15, 30, 45 min. After washing three times with phosphate buffered saline (PBS), the cells were incubated and maintained under the conditions described above. The colonies were determined on day 14. Clusters over 0.5 mm in diameter were counted as one. The control dishes usually contained 130–150 colonies.

*Uptake of the drug*

HeLa cells were exposed to 5-FU, HCFU, 5-FU plus *n*-hexylamine or HCFU plus *n*-hexylamine, either alone or in combination with hyperthermia, at a concentration of 77 μM for 60 min and the intracellular levels of 5-FU and HCFU were determined by the following method [10, 15]. $1 \times 10^7$ 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, 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 hydrolysed solution was acidified 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 PBS (pH 7.0). Ten millilitres of ethyl acetate were added, the preparation centrifuged to separate the ethyl acetate layer and then evaporated. The level of total 5-FU in the residue was analysed using gas chromatographic–mass spectrometric determinations (GC-CI-NS system, model JMX-DX 300, manufactured by JEOL).

## RESULTS

*Inhibition of cell growth*

The cytotoxic effect of HCFU combined with hyperthermia against HeLa cells was assessed using the dye exclusion method [16]. 5-FU, HCFU and hyperthermia individually affected cell growth. However, HCFU combined with hyperthermia greatly inhibited the cell growth. At 77 μM of HCFU, the surviving fraction of HeLa cells decreased after 72 h to 9.2% of that in the control cells with a 2-h heat treatment (Fig. 1). On the other hand, at the same concentration of 5-FU, the surviving fraction decreased to 18.3% of the findings in the control cells.

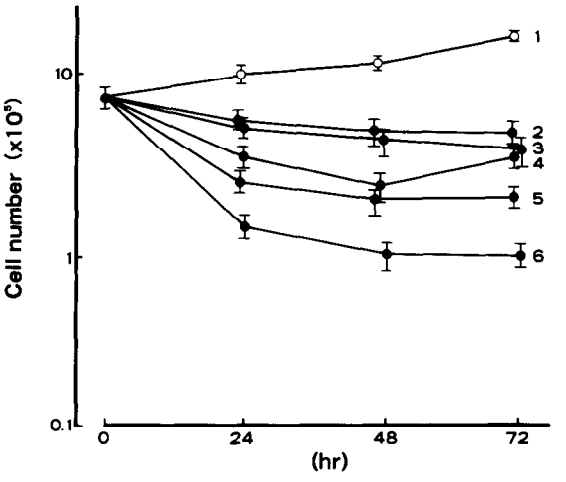


*Fig. 1. Changes in the number of HeLa cells exposed to 5-FU or HCFU, either alone or in combination with heat. $8 \times 10^5$ HeLa cells were incubated for 48 h and then exposed to the drug at 77 μM for the indicated time or heat (43°C) simultaneously for 2 h. The cell number was determined at 24, 48 or 72 h. 1, Control; 2, 5-FU; 3, HCFU; 4, heat; 5, 5-FU plus heat; 6, HCFU plus heat; bars, S.E.*

*Clonogenic assay*

HeLa cells treated with 5-FU, HCFU or both plus *n*-hexylamine, either alone or in combination with hyperthermia (43°C), led to the survival response shown in Fig. 2. The interactive effect was maximal with HCFU and heat given simultaneously. The combination of 5-FU, *n*-hexylamine and heat also showed a considerable cytotoxicity while HCFU plus *n*-hexylamine combined with heat showed no difference from the results with combination of HCFU and heat.

*Intracellular levels of 5-FU and HCFU in HeLa cells*

The uptake of 5-FU into HeLa cells was determined and the levels of each drug are shown in Table 1. This total level of 5-FU in HCFU-treated cells was about 2.5 times that in the 5-FU-treated cells. However, hyperthermia-related increases in the intracellular levels of drugs were never evident.

## DISCUSSION

HCFU is a lipophilic masked compound of 5-FU with high antineoplastic activity against various murine tumours [11] and a low toxicity to the host, as compared with the other pyrimidine analogues [17]. We reported that HCFU was twice as active as 5-FU in the case of HeLa cells and human cancer

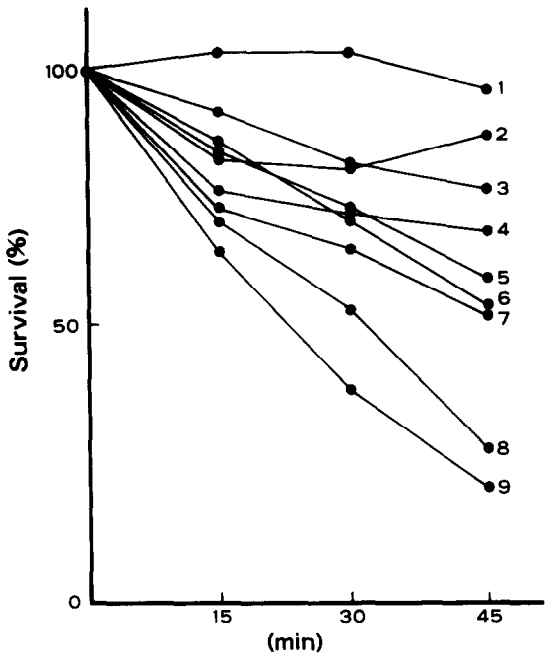


*Fig. 2. Effects of 5-FU, HCFU and/or n-hexylamine, either alone or in combination of heat, on colony formation of HeLa cells.* 3 × $10^2$ *HeLa cells were exposed to the drug at* 77 μM *with heat for the indicated times and maintained for* 14 *days at* 37°C. 1, *Heat*; 2, *5-FU*; 3, *n-hexylamine*; 4, *5-FU plus heat*; 5, *HCFU*; 6, *5-FU plus n-hexylamine*; 7, *n-hexylamine plus heat*; 8, *5-FU plus n-hexylamine plus heat*; 9, *HCFU plus heat.*

*Table 1. Changes in intracellular levels of 5-FU and HCFU following exposure of HeLa cells to the drug at* 77 μM *with or without heat*

| Treatment | Concentration (μg/$10^8$ cells) | |
|---|---|---|
| | 5-FU | HCFU fraction |
| 5-FU* | 0.171 | |
| 5-FU* + heat† | 0.150 | |
| HCFU* | 0.158 | 0.363 |
| HCFU* + heat | 0.150 | 0.279 |

*77 μM of 5-FU or HCFU, 1 h.
†43°C, 1 h.

tissue, *in vitro* [10]. Hyperthermia enhances the targeting of chemotherapeutic agents, since cell killing within the region of elevated temperature is accelerated but systemic toxicity is minimal [6, 9, 18]. We found no evidence of synergy in the cytotoxicity of 5-FU and hyperthermia, yet hyperthermia did enhance the activity of HCFU against HeLa cells as compared to findings with 5-FU.

Putative mechanisms of this synergic effect are: (1) enhancement of cytotoxicity of HCFU with increased uptake of the drug by hyperthermia, (2) the influence of the hexylcarbamoyl structure of HCFU.

(1) Other workers reported that hyperthermia increases the uptake of adriamycin [19–21], daunorubicin [22], actinomycin D [23], bleomycin [20, 24] and peplomycin [25]. We reported that the higher intracellular level of HCFU plus 5-FU would explain the higher sensitivity of HCFU, compared to 5-FU [10]. To support this hypothesis, the intracellular levels of HCFU plus 5-FU in the HCFU- and heat-treated cells and that of 5-FU in the 5-FU- and heat-treated cells were measured and the findings were compared with levels of these drugs in cells not exposed to hyperthermia. The level of total 5-FU in the HCFU-treated cells was twice as high as that of 5-FU in the 5-FU-treated cells [10]. However, the levels in the hyperthermic state and at a normal temperature (37°C) showed little difference. Therefore, this hypothesis was not tenable.

(2) Secondly, the lipophilic side-chain of HCFU was given attention. The increase in intracellular levels of HCFU and 5-FU in the HCFU-treated cells could be explained if the hexylcarbamoyl structure relates to the rapid uptake of HCFU, through the lipophilic cell membrane, compared to 5-FU, and if HCFU is converted to 5-FU within a short period. Iigo *et al.* [26] reported that the structure facilitates rapid absorption through the gastrointestinal tract and blood–ascites barrier. *n*-Hexylamine has a similar structure to the hexylcarbamoyl side-chain of HCFU. The combination of 5-FU and *n*-hexylamine at an elevated temperature had a cytotoxic effect comparable to that of HCFU plus heat. Therefore, the lipophilic hexylcarbamoyl structure may alter the authentic architecture of cell membranes, under conditions of high temperature, and if so, then this event would be an essential factor in the synergy recombinant between HCFU and hyperthermia.

Clinical colorectal cancer has remained resistant to chemotherapy [27]. Niimoto *et al.* [28] found that patients with a non-curatively resected colorectal cancer responded to the post-operative chemotherapy of mitomycin C and HCFU, compared to the effectiveness of mitomycin C. We obtained evidence that HCFU was more effective for colorectal cancer than was 5-FU and five other drugs, determined by using the chemosensitivity test [29, 30]. The combined effect of HCFU and hyperthermia is expected to show beneficial effects for patients with colorectal cancer.

**Acknowledgements**—This work was supported in part by Grants-in-Aid for General Scientific Research (63480291) from the Ministry of Education, Science and Culture, and the Fukuoka Cancer Society. We thank K. Miyamoto and K. Fukuchi for technical assistance and M. Ohara for comments.

## REFERENCES


1. Engelhardt R. Hyperthermia and drugs. In: Streffer C, ed. *Hyperthermia and the Therapy of Malignant Tumors*. Berlin, Springer, 1987, 136–203.
2. Komatsu K, Miller RC, Hall EJ. The oncogenic potential of a combination of hyperthermia and chemotherapy agents. *Br J Cancer* 1988, **57**, 59–63.
3. Mizuno S, Amagai M, Ishida A. Systemic cell killing by antitumor agents and hyperthermia in cultured cells. *Gann* 1980, **71**, 471–478.
4. Rose WC, Veras GH, Laster WR Jr, Schabel FM Jr. Evaluation of whole-body hyperthermia as an adjunct to chemotherapy in murine tumors. *Cancer Treat Rep* 1979, **63**, 1311–1325.
5. Miyazaki M, Makowka L, Falk RE *et al.* Hyperthermochemotherapeutic *in vitro* isolated perfusion of the rat liver. *Cancer* 1983, **51**, 1254–1260.
6. Adwanker MK, Chitnis MP. Effect of hyperthermia alone and in combination with anticancer drugs on the viability of P388 leukemic cells. *Tumori* 1984, **70**, 231–234.
7. Cange I, Zänker KS, Sievert JR, Blümel G, Eisler K, Kolb E. The effect of whole body by hyperthermia on 5-fluorouracil pharmacokinetics *in vitro* and clonogenicity of mammalian colon cancer cells. *Anticancer Res* 1984, **4**, 27–32.
8. Iigo M, Hoshi A, Nakamura A, Koretani K. Antitumor activity of 1-alkylcarbamoyl-derivatives of 5-fluorouracil in a variety of mouse tumors. *Cancer Chemother Pharmacol* 1978, **1**, 203–208.
9. Hoshi A, Yoshida M, Inomata M, Iigo M, Ando N, Kuretani K. Antitumor activity of metabolites of 1-hexylcarbamoyl-5-fluorouracil and related compounds against L1210 leukemia *in vivo* and L5178Y lymphoma cells *in vitro*. *J Pharm Dyn* 1980, **3**, 478–481.
10. Maehara Y, Kusumoto H, Anai H, Kusumoto T, Hiramoto Y, Sugimachi K. 1-Hexylcarbamoyl-5-fluorouracil is more cytostatic than 5-fluorouracil against human tumors *in vitro*. *Eur J Cancer Clin Oncol* 1987, **23**, 1511–1515.
11. Hoshi A, Iigo M, Nakamura A, Yoshida M, Kuretani K. Antitumor activity of 1-hexylcarbamoyl-5-fluorouracil in a variety of experimental tumors. *Gann* 1976, **67**, 725–731.
12. Okabayashi K, Koyama Y, Maruyama K, Okazaki N, Sakamo T, Ise T. Clinical trials of a new anticancer drug, 1-hexylcarbamoyl-5-fluorouracil. *Jpn J Clin Oncol* 1979, **9**, 35–40.
13. Yoshida M, Murai H, Miura S. Effects of 1-hexylcarbamoyl-5-fluorouracil (HCFU), a new antitumor agent for oral use, against recurrent cases of breast cancer. *Jpn J Cancer Chemother* 1980, **7**, 262–266.
14. Higashi H, Takenaka K, Kanematsu T, Matsumata T, Yoshida Y, Sugimachi K. 1-Hexylcarbamoyl-5-fluorouracil (HCFU) effective for hepatocellular carcinoma metastatic to the lungs—a case report. *Jpn J Cancer Chemother* 1987, **14**, 2956–2959.
15. Mori K, Fuse M, Kobari T. Gas chromatographic–mass spectrometric determination of drugs in organ tissues after administration of 1-hexylcarbamoyl-5-fluorouracil (Carmofur). *Jpn Pharmacol Ther* 1983, **11**, 4655–4662.
16. Weisenthal LM, Maesden JA, Dill PL, Macaluso CK. A novel dye exclusion method for testing *in vitro* chemosensitivity of human tumors. *Cancer Res* 1983, **43**, 749–757.
17. Ozaki S, Ike Y, Mizuno H, Ishikawa K, Mori H. 5-Fluorouracil derivatives I. The synthesis of 1-hexylcarbamoyl-5-fluorouracils. *Bull Chem Soc Jpn* 1977, **50**, 2406–2412.
18. Kobashi T, Iguro Y, Ujiic A, Namekawa H. Metabolism of 1-hexylcarbamoyl-5-fluorouracil, a new antitumor agent, in rats, rabbits, and dogs. *Am Soc Microbiol* 1980, 1584–1586.
19. Magin RL, Cysyk RL, Litterst CL. Distribution of adriamycin in mice under conditions of local hyperthermia which improves systemic drug therapy. *Cancer Treat Rep* 1980, **64**, 203–210.
20. Hahn GM, Braun J, Har-Kedar I. Thermochemotherapy: synergism between hyperthermia (42–43°C) and adriamycin (or bleomycin) in mammalian cell inactivation (cancer chemotherapy/cell membrane). *Proc Natl Acad Sci USA* 1975, **72**, 937–940.
21. Hahn GM, Strande DP. Cytotoxic effects of hyperthermia and adriamycin on Chinese hamster cells. *J Natl Cancer Inst* 1976, **57**, 1063–1067.
22. Klein ME, Froyer K, Gangji D. Hyperthermic enhancement of chemotherapeutic agents in L1210 leukemia. *Blood* 1977, **50**, 223.
23. Donaldson SS, Gordon LF, Hahn GM. Protective effect of hyperthermia against the cytotoxicity of actinomycin D on Chinese hamster cells. *Cancer Treat Rep* 1978, **62**, 1489–1495.
24. Lin PS, Hefter K, Jones M. Hyperthermia and bleomycin schedule on V79 Chinese hamster cell cytotoxicity *in vitro*. *Cancer Res* 1983, **43**, 4557–4561.
25. Mizuno S, Ishida A. Selective enhancement of the cytotoxicity of the bleomycin derivate, pepleomycin by local anesthetics alone and combined with hyperthermia. *Cancer Res* 1982, **42**, 4726–4729.
26. Iigo M, Hoshi A, Kuretani K. Pharmacokinetics of 1-alkylcarbamoyl-5-fluorouracil in plasma and ascites fluid after oral administration in mice. *Cancer Chemother Pharmacol* 1980, **4**, 189–193.
27. Danis HL. Chemotherapy of large bowel cancer. *Cancer* 1982, **50**, 2638–2646.

28. Niimoto M, Hattori T, Tamada R, Sugimachi K, Inokuchi K, Ogawa N. Mitomycin C plus HCFU adjuvant chemotherapy for noncuratively resected cases of colorectal carcinoma (first report). *Jpn J Surg* 1978, **17**, 354–361.
29. Maehara Y, Anai H, Kusumoto H, Kusumoto T, Sugimachi K. Colorectal carcinoma *in vitro* is more sensitive to 1-hexylcarbamoyl-5-fluorouracil compared with six other antitumor drugs: carboquone, adriamycin, mitomycin C, aclacinomycin A, cisplatin, 5-fluorouracil. *Dis Colon Rectum* 1988, **31**, 62–67.
30. Kusumoto T, Maehara Y, Anai H *et al.* Human colorectal carcinoma is more sensitive than 5-FU and Tegafur in *in vitro* and *in vivo* drug sensitivity tests. *Jpn J Cancer Chemother* 1988, **15**, 257–261.