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# Microcalorimetric study on the enhanced antitumor effects of 1-hexylcarbamoyl-5-fluorouracil by combination with hyperthermia on K-562 cell line

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**Abstract**

The antitumor effects of 1-hexylcarbamoyl-5-fluorouracil (Carmofur) combined with hyperthermia were studied on K-562 cell line by microcalorimetry and fluorescence microscopy. The effect was evaluated by the maximal thermal power of the cell line K-562 and the total energy $Q$ released during the measurement period (44 h). The results indicate that the combination of Carmofur and hyperthermia has a synergetic effect. Hence, microcalorimetry is a powerful tool in fields of clinical cases and Carmofur is clinically useful in thermochemotherapy. The result of fluorescence microscope observations show that apoptosis occurs under the action of Carmofur combined with hyperthermia. 

*Keywords:* Microcalorimetry; Hyperthermia; Metabolism; Apoptosis; Fluorescence microscopy

## 1. Introduction

Hyperthermia is found to greatly enhance cancer cell killing and potentiate the action of several antitumor agents, including bleomycin [1], cyclophosphamide [2], mitomycin C [3], cisplatin [4], and Adriamycin [5]. The use of long duration, mild temperature hyperthermia in combination with chemotherapy or irradiation in the treatment of cancer is recently developed [6–8]. Successful application of this form of hyperthermia in clinical cases requires a detailed understanding of the role of exposure conditions such as temperature, length of heating time and the dose of antitumor drugs, etc. Microcalorimetry is a powerful tool to solve these problems. It provides a continuous measurement of heat production, thus gives useful information about the activity of the total metabolism of the sample under examination in a both qualitative and quantitative way [9].

During the past decades, our group has applied microcalorimetry to the study of the metabolism in microorganisms [10,11], cultured tissue cells [12–14] and organelles such as mitochondria [15–17] to obtain useful information of the metabolic processes. The first calorimentric measurement of the metabolic activity of tumor cells was carried out by a Swedish team in 1986 [18]. In our previous work, we have studied the heat sensitivity of tumor cells by means of microcalorimetry combined with other methods [19]. The results demonstrated that temperature has a

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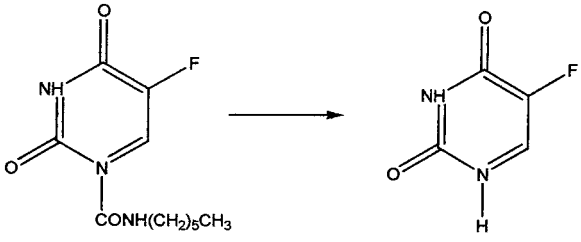
remarkably selective destructing effect of heat against tumor cells.

This paper describes the application of the microcalorimetric method to a clinical field. It is a fast, quantitative, inexpensive and versatile method for determining the clinical efficiency of drugs. Heat production of cell line K-562 under the action of Carmofur combined with hyperthermia were studied by an LKB 2277 Bioactivity Monitor. The antitumor effect was evaluated by the maximal thermal power $P_{max}$, the mean thermal power $P_{mean}$ and the total energy $Q$ released during the measurement period (44 h). We have also used fluorescent microscopy in this study. The morphology of cell line K-562 indicates that cells died through apoptosis.

Carmofur is a masked compound of 5-fluorouracil (5-FU) and is well known to release 5-FU spontaneously [20] as following:




Carmofur 5-FU

## 2. Experimental

### 2.1. Materials

The human chronic myelogenous leukemia cell line K-562 (ATCC CCL-243) was provided by China Center for Type Culture Collection, Wuhan University.

Exponentially growing cultures are obtained by seeding $10^5$ cell $ml^{-1}$ into T-25 plastic flasks containing 8 ml of the medium at 37°C, 5% $CO_2$ for 3 days. The medium consisted of 90% RPMI 1640 medium (GIBCO Co., USA), 10% heat-inactivated fetal calf serum (GIBCO Co., USA) with 100 IU $ml^{-1}$ penicillin and 100 μg $ml^{-1}$ streptomycin added (pH = 7.2−7.3). The same medium was used for the calorimetric experiments.

Carmofur was obtained from Mitsui Seiyaku (Japan), purified by recrystallization twice in acetone (analytical pure).

The fluorescent dye Hoechst 33258 was obtained from Sigma Chemical Co., USA.

### 2.2. Equipments

A microcalorimeter, LKB 2277 Bioactivity Monitor, was used to obtain the thermogenesis curves. The signal was recorded by means of an LKB-2210 recorder (1000 mV range). For more details of the performance and construction of the instrument, see [21,22].

A fluorescence Microscope (BH-2, Olympus Co., Japan) was applied in observation with a fluorescein filter set at a wavelength of 520 ± 20 μm.

### 2.3. Experimental determination

The exponentially growing cells were counted using a Thoma's hemocytometer and cell number was set to $10^5$ cell $ml^{-1}$ by adding the medium. The 1 ml suspension of the exponentially growing cells with different concentrations of Carmofur was moved into a 3 ml glass ampoule and then was put into the microcalorimeter to monitor the growth of the cell.

The measurement was carried out at 37°C, which is corresponding to the physiological body temperature, and 40, 43°C, i.e. hyperthermia, respectively. Carmofur was added to the sample at the beginning of the experiment.

### 2.4. Sample preparation for fluorescence microscope observation

To obtain information on nuclear structure, control and experimental cells were collected when cultured for 48 h, fixed with a 1:3 mixture of acetic acid: methanol, stained with Hoechst 33258 fluorescent dye, and examined in a BH-2 fluorescence microscope using a standard fluorescein filter set (520 ± 20 nm). The magnification was set at 200×. The photographs are shown in Fig. 7.

## 3. Results and discussions

### 3.1. The thermogenesis curves for cell line K-562 at different temperatures

The thermogenesis curves for K-562 cell line at 37, 40, and 43°C are shown in Fig. 1.

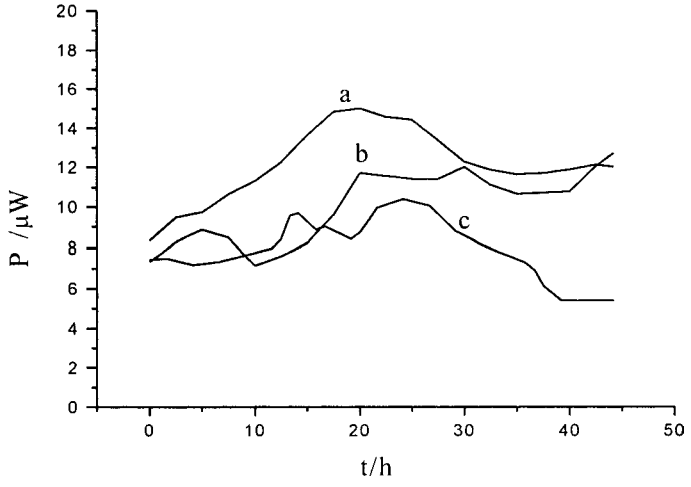



Fig. 1. Thermogenesis curves of cell line K-562 with an initial cell number of $10^5$ cell $ml^{-1}$ in 1 ml RPMI 1640 medium containing 10% calf serum. (a) Thermogenesis curve at 37°C; (b) thermogenesis curve at 40°C, and (c) thermogenesis curve at 43°C.

Fig. 1 shows that the metabolic activity of cell line K-562 decreased when the temperature increased. The maximal thermal power of cell line K-562 declined from 15.0 μW at 37°C to 12.7 μW at 40°C and 10.4 μW at 43°C and the total heat released per ml during the experimental period is as follows:

$$Q(37^\circ\mathrm{C}) = 1.83\,\mathrm{J}\cdot\mathrm{ml}^{-1},$$
$$Q(40^\circ\mathrm{C}) = 1.60\,\mathrm{J}\cdot\mathrm{ml}^{-1},$$
$$Q(43^\circ\mathrm{C}) = 1.22\,\mathrm{J}\cdot\mathrm{ml}^{-1}.$$

The mean thermal powers per ml are:

$$P_{\mathrm{mean}}(37^\circ\mathrm{C}) = 11.6\,\mu\mathrm{W}\cdot\mathrm{ml}^{-1},$$
$$P_{\mathrm{mean}}(40^\circ\mathrm{C}) = 10.1\,\mu\mathrm{W}\cdot\mathrm{ml}^{-1},$$
$$P_{\mathrm{mean}}(43^\circ\mathrm{C}) = 7.7\,\mu\mathrm{W}\cdot\mathrm{ml}^{-1}.$$

The results indicate that cell line K-562 was sensitive to temperature. Higher temperature caused a decrease of the metabolic activity of K-562 cells. And the inhibitory action of hyperthermia is stronger when the temperature is higher.

### *3.2. The thermogenesis curves for cell line K-562 under the action of Carmofur combined with hyperthermia*

The thermogenesis curves for cell line K-562 with 5 μg $ml^{-1}$ Carmofur at 37, 40, and 43°C are shown in Figs. 2–4, respectively. The total energy $Q$ released during the measurement period (44 h) are shown in Fig. 5.

When in presence of 5.0 μg $ml^{-1}$ Carmofur, the maximal thermal power of the K-562 cell line per ml is as follows:

$$P_{\max}(37^\circ\mathrm{C} + \mathrm{Carmofur}) = 11.5\,\mu\mathrm{W}\cdot\mathrm{ml}^{-1},$$
$$P_{\max}(40^\circ\mathrm{C} + \mathrm{Carmofur}) = 3.9\,\mu\mathrm{W}\cdot\mathrm{ml}^{-1},$$
$$P_{\max}(43^\circ\mathrm{C} + \mathrm{Carmofur}) = 3.3\,\mu\mathrm{W}\cdot\mathrm{ml}^{-1}.$$

And the total energy released during the experimental period(44 h) per ml are:

$$Q(37^\circ\mathrm{C} + \mathrm{Carmofur}) = 1.58\,\mathrm{J}\cdot\mathrm{ml}^{-1},$$
$$Q(40^\circ\mathrm{C} + \mathrm{Carmofur}) = 0.48\,\mathrm{J}\cdot\mathrm{ml}^{-1},$$
$$Q(43^\circ\mathrm{C} + \mathrm{Carmofur}) = 0.29\,\mathrm{J}\cdot\mathrm{ml}^{-1}.$$

The mean thermal powers per ml are:

$$P_{\mathrm{mean}}(37^\circ\mathrm{C} + \mathrm{Carmofur}) = 9.98\,\mu\mathrm{W}\cdot\mathrm{ml}^{-1},$$
$$P_{\mathrm{mean}}(40^\circ\mathrm{C} + \mathrm{Carmofur}) = 3.03\,\mu\mathrm{W}\cdot\mathrm{ml}^{-1},$$
$$P_{\mathrm{mean}}(43^\circ\mathrm{C} + \mathrm{Carmofur}) = 1.83\,\mu\mathrm{W}\cdot\mathrm{ml}^{-1}.$$

Analysis of the metabolic heat of the cells growing for 44 h is shown in Table 1.

Define the inhibitory ratio of temperature as: $I_1\% = ((Q - Q_1)/Q)\%$; the inhibitory ratio of Carmofur combined with hyperthermia as: $I_2\% = ((Q - Q_2)/Q)\%$. $Q$, $Q_1$ and $Q_2$ stand for the total heat released of K-562 cells at 37°C without the presence of Carmofur ($Q = 1.83$ J), at certain

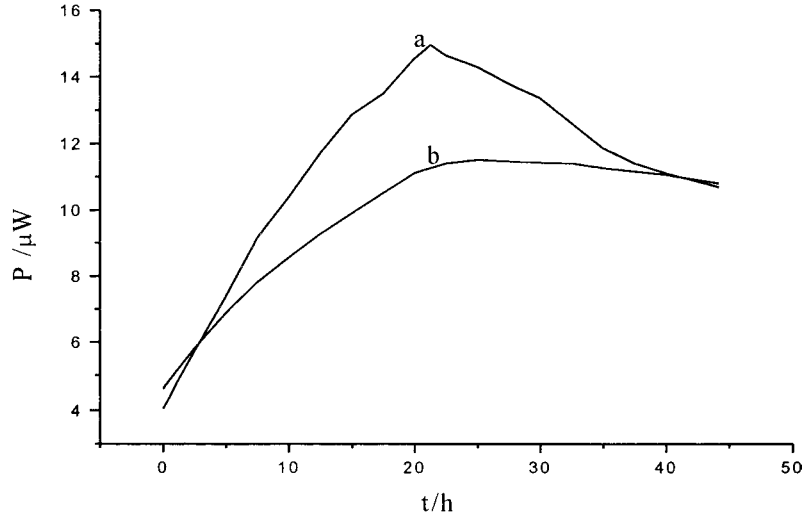



Fig. 2. Thermogenesis curves of K-562 cell line at 37°C. (a) Control, and (b) +5 μg Carmofur.

temperature without Carmofur and at certain temperature with Carmofur, respectively.

Plots of the inhibitory ratio versus temperature are shown in Fig. 6.

We can see from Figs. 2–6 that at a certain temperature, the thermogenesis curves of cell line K-562 in presence of Carmofur are below that without Carmofur, and they are below the thermogenesis curve of control group at 37°C. The thermogenesis curves, the metabolic heat released and the inhibitory ratio demonstrate that Carmofur has strong antitumor effects on cell line K-562. A synergistic effect appeared when using Carmofur combined with hyperthermia. The results indicated that hyperthermia can potentiate the cytotoxicity of Carmofur. We believe that this is because: (1) higher temperatures can increase the permeability of cell membranes so that the antitumor drugs can enter into the cellular

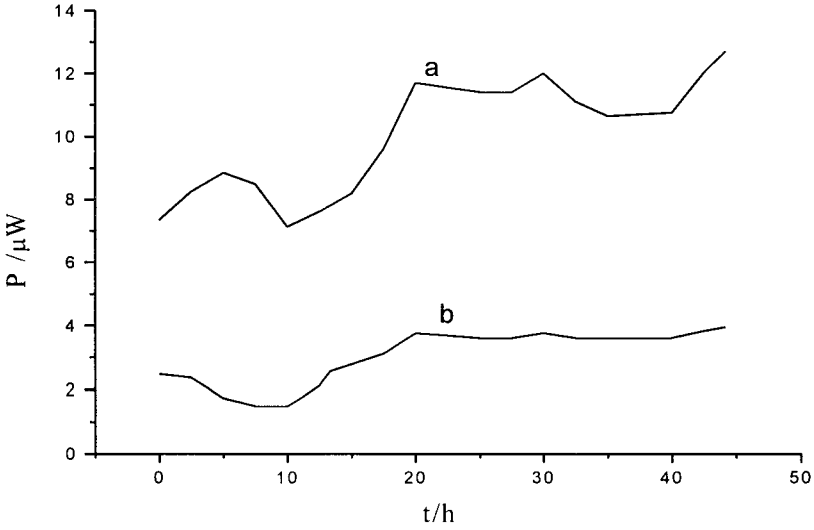



Fig. 3. Thermogenesis curves of K-562 cell line at 40°C. (a) Control, and (b) +5 μg Carmofur.

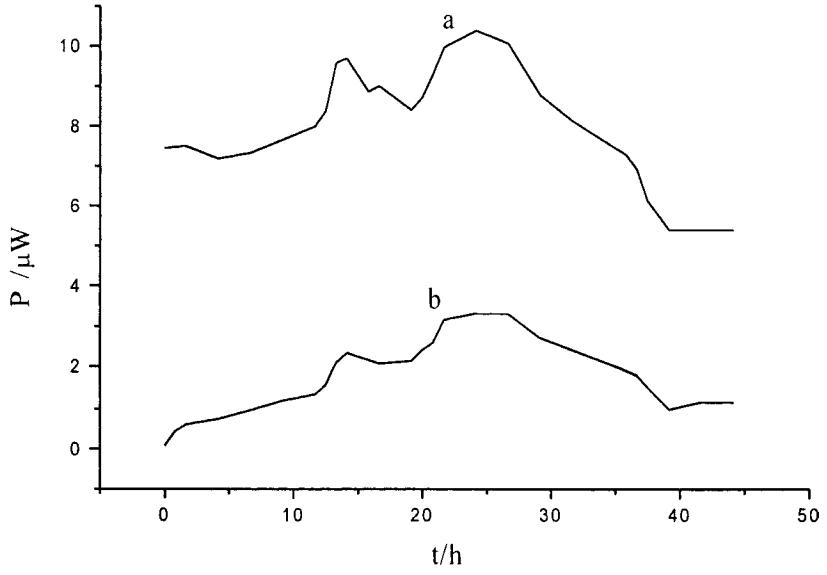



Fig. 4. Thermogenesis curves of K-562 cell line at 43°C. (a) Control, and (b) +5 μg Carmofur.

matrix easier [5,19]; (2) the chemical reactions in cells proceed faster at higher temperatures thus hyperthermia can cause an increase in $O_2$ consumption and $O_2$ content in the tissue [23] which is more crucial to tumor cells than to normal cells; (3) higher temperatures can weaken the capability of self-repair systems in cells by stimulating the production of interferon (antiviral and antitumor proteins) [24]. It is obvious that the combination of hyperthermia and chemotherapy can reduce the dose of drugs used in chemotherapy, which may reduce the side effects of the drugs on patients. Our results indicate that Carmofur is clinically useful in thermochemotherapy.

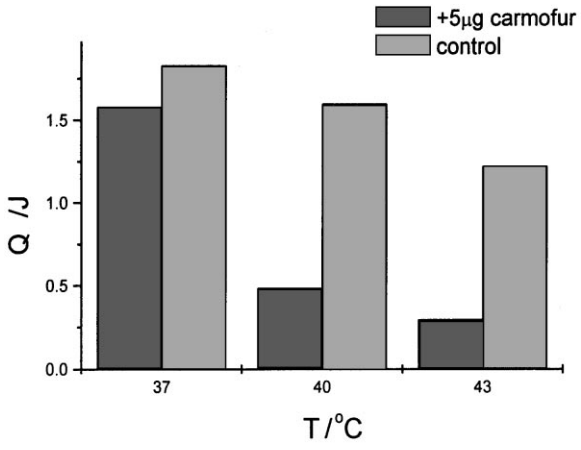



Fig. 5. Total heat released during the measurement period of 44 h as function of temperature with and without Carmofur.

### 3.3. Result of fluorescence microscope observation

The photographs of K-562 cells by means of a fluorescence microscope are shown in Fig. 7. The concept of apoptosis has relied on morphological distinctions between necrosis and apoptosis [25], and morphological observation may be the most reliable method to characterize apoptosis [26]. The K-562 cells which were treated with the combination of hyperthermia and Carmofur exhibited characteristics

Table 1
Comparison of metabolic heat production of cell line K-562 cells growing for 44 h[a]

| $T$ (°C) | $Q_1$ (J) | $Q_2$ (J) | $I_1$ (%) | $I_2$ (%) |
|---|---|---|---|---|
| 37 | 1.83 (control) | 1.58 | 0 | 13.7 |
| 40 | 1.60 | 0.48 | 12.6 | 73.8 |
| 43 | 1.22 | 0.29 | 33.3 | 84.2 |

[a] $Q_1$: total heat released without the presence of Carmofur; $Q_2$: total heat released with the presence of Carmofur; $I_1$%: inhibitory ratio without Carmofur; $I_2$%: inhibitory ratio in the presence of Carmofur.

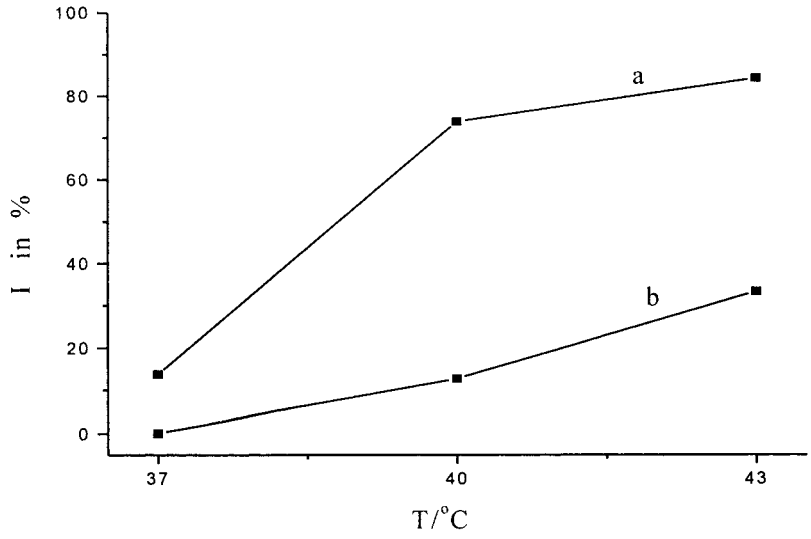



Fig. 6. Comparison of the inhibitory ratios at various conditions. (a) Inhibitory ratio of higher temperatures in the presence of Carmofur; (b) inhibitory ratio without Carmofur.

of apoptosis including nuclear heterochromatin condensation and fragmentation into membrane-bound apoptotic bodies (Fig. 7B) in contrast to normal cells (Fig. 7A). It indicates that the combination of chemotherapy and hyperthermia can induce apoptosis of K-562 cells. We believe it is the mechanism of the antitumor effects of Carmofur combined with hyperthermia.

### *3.4. Relation between metabolic activity and apoptosis*

Apoptosis is a physiological or 'programmed' form of cell death, distinct from 'accidental' cell death or necrosis. A large number of biochemical changes have been observed in cells undergoing apoptosis [27]. Many of the biochemical changes are related to the degradation of cellular DNA into 180–200 bp integer-fold sized pieces. The induction of this process requires RNA and protein synthesis, and was proved by Sellins and Cohen to be an active metabolic process using protein and RNA synthesis inhibitors [28]. The impairment of adenosine triphosphate synthesis may play an important role in apoptosis [27]. Necrosis, in contrast with apoptosis, is a non-active process that needs no energy. So less heat will be produced by cells undergoing apoptosis than by normal cells, while there is no heat produced or absorbed by cells undergoing

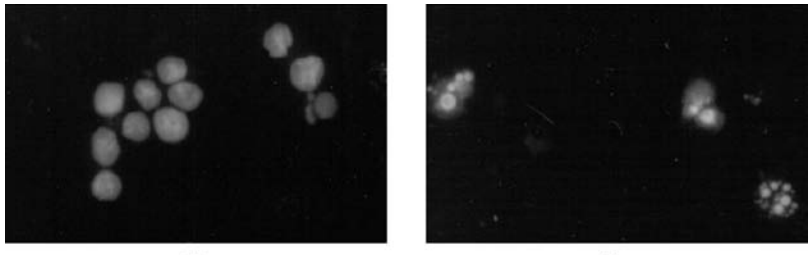



Fig. 7. Fluorescence microscopic photographs. (A) K-562 cell line at 37°C (after 48 h of cultivation), normal cells. (B) K-562 cell line at 43°C in the presence of 5.0 μg $ml^{-1}$ Carmofur (after 48 h of cultivation), cells undergoing apoptosis.

necrosis. Our study confirmed the difference between the metabolic energy of apoptotic cells and that of normal and necrotic cells.

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