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Curcumin Reverses Adriamycin-resistance of Thermotolerant Hepatocarcinoma Cells by Down-regulating P-glycoprotein and Heat Shock Protein 70*

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Abstract The aim of this present work was to investigate whether curcumin reverses the adriamycin-resistance of thermotolerant hepatocarcinoma cell line and the underlying mechanisms. Cytotoxicity was evaluated by MTT assay. Apoptosis was determined by flow cytometer using propidium iodide staining. The accumulation of intracellular adriamycin was measured by high-performance liquid chromatography. The expressions of P-glycoprotein (P-gp), heat shock protein 70 (Hsp70), and caspase-3 were analyzed by Western blotting. The thermotolerant hepatocarcinoma cell line HepG2/TT was resistant to adriamycin-induced cytotoxicity and apoptosis. Curcumin, at 5 μmol/L, 10 μmol/L, and 20 μmol/L, decreased the IC_{50} of adriamycin to thermotolerant HepG2/TT cells and enhanced adriamycin-induced apoptosis in HepG2/TT cells in a concentration-dependent manner. The levels of P-gp and Hsp70 in HepG2/TT cells were obviously higher than that in HepG2 cells. Treatment with curcumin (10 μmol/L) for 24 h significantly reduced the levels of P-gp and Hsp70 in HepG2/TT cells. The accumulation of intracellular adriamycin in HepG2/TT cells was markedly lower than that in HepG2 cells and curcumin (10 μmol/L for 3 h) significantly increased the level of intracellular adriamycin accumulation in HepG2/TT cells. HepG2/TT cells obviously inhibited caspase-3 activation triggered by adriamycin, but co-treatment with 10 μmol/L of curcumin for 24 h significantly augmented the activation of caspase-3 in HepG2/TT cells treated with adriamycin. Curcumin overcomes the adriamycin-resistance of thermotolerant HepG2/TT cells by promoting adriamycin-triggered caspase-3 activation through down-regulating the activity and expression of P-gp and the expression of Hsp70.

Key words curcumin, thermotolerance, adriamycin-resistance, P-glycoprotein, heat shock protein 70
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Hyperthermia has recently attained attraction as a potentially clinically important co-adjuvant in cancer treatment. In phase Ⅰ / Ⅱ / Ⅲ clinical trials, hyperthermia has shown positive results [1–3]. Heat treatment not only facilitates diffusion of drugs into tumor but also delays clearance of drugs and enhances cytotoxicity of chemotherapeutic agents [4]. However, the major complication associated with the use of hyperthermia in cancer treatment is the phenomenon of thermotolerance [5–7], first described by Gerner and Schneider [8], which is the transient resistant state of cells to a (second) heat treatment after being pre-exposed to hyperthermia. Thermotolerance has been displayed to be a major influence on the response of tumor cells in culture, tumors *in situ*, and normal tissues in virtually all of the biological systems studied to date. While thermotolerance appears to be a universal nature, differences exist in its degree of development and time course under various conditions and in differing tumor cells and normal tissues [9–10].

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Furthermore, thermotolerance has been shown to diminish the cytotoxicity of a number of chemotherapeutic drugs [11-13]. In 1988, Hever-Szabo *et al*.[14] reported that acquired stable heat resistance of hepatocarcinoma cells, isolated by ten repeated cycles of heat exposure at 45℃ for 80 min, display an enhanced resistance to some anticancer drugs, such as colchicine and adriamycin, resulting in multidrug resistant (MDR). Hence, hindrance of the MDR of thermotolerant cancer cells would sensitize cancer cells to chemotherapies that use hyperthermia as a complement of treatment.

Curcumin, a natural phenolic coloring compound that is found in the rhizomes of *Curcuma longa* L., commonly called turmeric, has a wide range of biological and pharmacological activities, including antioxidant properties[15], anti-inflammatory properties[16], anti-mutagenic activity [17], and anti-carcinogenic [18], hypocholesterolemic [19] and hypoglycemic effects [20]. Interestingly, *in vitro* studies have demonstrated that curcumin is able to overcome MDR[21-22]. It is not clear whether curcumin can reverse the MDR of thermotolerant cancer cells. This led us to evaluate the effect of curcumin on the MDR of acquired stable heat resistant cancer cells.

In the present work, we investigated the effect of curcumin on adriamycin resistance in thermotolerant hepatocarcinoma cell line HepG2/TT and the underlying mechanisms. We found that co-treatment of thermotolerant HepG2/TT cells with curcumin increased their sensitivity to adriamycin. Curcumin decreased the expressions of P-glycoprotein (P-gp) and heat shock protein 70 (Hsp70) and enhanced the accumulation of intracellular adriamycin in thermotolerant HepG2/TT cells. In addition, curcumin was able to promote adriamycin-stimulated caspase-3 activation in HepG2/TT cells. It is possible that curcumin could be useful in the treatment of drug-resistance in thermotolerant hepatocarcinoma cells as a MDR modulator.

1 Materials and methods

1.1 Materials

Curcumin, propidium iodide (PI), RNase, and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical (St Louis, MO, USA); Verapamil was supplied by Baiyunshan Pharmaceutical Co., Ltd. (Guangzhou, China); Adriamycin was supplied by Pharmacia & Upjohn Co., Ltd (Shanghai, China); Antibodies for detecting P-gp, Hsp70, and cleaved caspase-3 were obtained from Abcam Technology (Cambridge, CB, UK); RPMI-1640 medium, horse serum and fetal bovine serum were supplied by Gibico BRL (Ground Island, NY, USA).

1.2 Cell cultures and *in vitro* heating procedure

HepG2 cells were maintained on tissue culture plastic in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/L glutamine, and antibiotics at 37℃ under an atmosphere of 5% CO_2 and 95% air.

For heating procedure, after 12 h incubation at 37℃, HepG2 cells were subjected to a heat shock challenge at 43℃ for 80 min, and then incubated at 37℃. HepG2 cells were performed under the above condition of 10 cycles and this thermotolerant hepatocarcinoma cell line was named as HepG2/TT cell line.

1.3 Cytotoxicity assay by MTT

Cells were seeded into 96-well plates at densities of 1×10^4 HepG2/TT cells per well and 0.5×10^4 HepG2 cells per well, and incubated in a humidified atmosphere of 5% CO_2+95% air overnight, then normal cell medium containing either test compounds or solvents at the desired concentration were added. After 24 h incubation, 20 μl MTT (5 g/L in phosphate buffered saline, PBS) were added. The plates were incubated for 4 h and the blue dye formed was dissolved in 100 μl dimethyl sulfoxide (DMSO). The absorbance at 570 nm was recorded using an ELISA reader. The survival rate was calculated as follows: Survival rate $=(T-B)/(U-B)\times100\%$, where T is the absorbance of treated tumor cells when exposed to drugs, U is the absorbance of untreated cells, B is the absorbance when neither the drug nor MTT was added (blank).

The 50% inhibitory concentration (IC_{50}) of 24 h exposure for a particular agent was defined as the drug concentration that causes in a 50% reduction in the number of cells compared with the untreated control. The IC_{50} values were determined by Bliss software.

1.4 Apoptosis assay by flow cytometer using propidium iodide staining

Cells were treated with various concentrations of adriamycin and curcumin for 24 h, and then harvested with 0.25% trypsin and washed with PBS. Cells(2×10^5) were fixed in 70% ice-cold EtOH/PBS for 20 min on ice, and then washed with PBS and incubated

in propidium iodide (PI) solution (69 mmol/L PI, 388 mmol/L sodium citrate, 100 mg/L RNase A) for 15 min at 37℃. Cells were immediately analyzed by FAC scan flow cytometry (Becton Dickinson, San Jose, CA).

1.5 Adriamycin accumulation assay by high-performance liquid chromatography (HPLC)

Cells (2×10^6 cell/well) were plated in a 6-well plate. After a 3 h incubation with 10 μmol/L curcumin and/or 10 μmol/L adriamycin, cells were washed thrice with ice-cold PBS, lysed in 250 μl MilliQ water, and homogenized by ultrasonification Labsonic P (Braun; cycle 0.2 min an Amplitude 60%). Hundred microliters of the homogenate was processed for HPLC analysis as described by Harmsen *et al.*[23] In brief, samples were eluted isocratically with a mobile phase that consisted of 28% acetonitrile in 60 mmol/L phosphoric acid. A Waters Symmetry C18 3.5 μmol/L, 4.6 mm × 100 mm reverse-phase column was used. Fluorescence was detected using a JASCO FP-920 fluorometer (excitation: 455 nm; emission: 550 nm), Shimadzu LC10AT-VP pumps and a Shimadzu SCL10A-VP controller. The flow rate was set at 1.0 ml/min. Adriamycin retention times were 2.38 min. Adriamycin was used as an external standard. A standard curve of Adriamycin(0.05, 0.5, 2, 10 and 50 μg/L)was prepared in milliQ water.

1.6 Western blot analysis for expressions of P-gp, Hsp70, and caspase-3

SDS-polyacrylamide gel electrophoresis (PAGE) was carried out on 5% stacking and 12% resolving gel with low range molecular mass standards (Solarbio, China). Equal amounts of protein were loaded in each lane with loading buffer (Beyotime, China) containing 0.1 mol/L Tris (pH6.8), 20% glycerol, 10% mercaptoethanol, 4% SDS and 0.2% Bromophenol Blue. Samples were heated at 100℃ for 5 min before gel loading. Following electrophoresis, the proteins were transferred to a PVDF transfer membrane (Solarbio, China). After this, the membranes were blocked with TBST (50 mmol/L Tris-HCl, pH 7.4, 0.15 mol/L NaCl, 0.1% Tween-20) containing 5% BSA (Sigma, USA) for 2 h. Following this, the membranes were incubated with primary antibodies diluted at 1∶1 000 at 4℃ over night. After washing with TBST, the membranes were incubated with anti-rabbit IgG labeled with horseradish peroxidase (Zsbio, China)diluted at 1∶1 000 at room temperature for 2 h. The membranes were washed again and developed with an enhanced chemiluminescence system (ECL, Zsbio, China) followed by apposition of the membranes with autoradiographic films (Kodak, China). The optical density of the protein band on Western blots was calculated by Image-J software.

1.7 Statistical analysis

Data were expressed as $\bar{x} \pm s$ and the significance of inter-group differences was evaluated by one-way analyses of variance (ANOVA: Least-significant difference's test for post hoc comparisons). Differences with *P*-value less than 0.05 were considered significant.

2 Results

2.1 Heat resistance of thermotolerant hepatocarcinoma cell line HepG2/TT

To evaluate the responses of HepG2 and HepG2/TT cells to hyperthermia, HepG2 and HepG2/TT cells were exposed to 43℃ for 24 h and cell viability and apoptosis were detected. As shown in Figure 1, exposed to 43℃ for 24 h, the viability of HepG2/TT cells was about 5 times higher than that of HepG2 cells (Figure 1a); however, the apoptotic rate

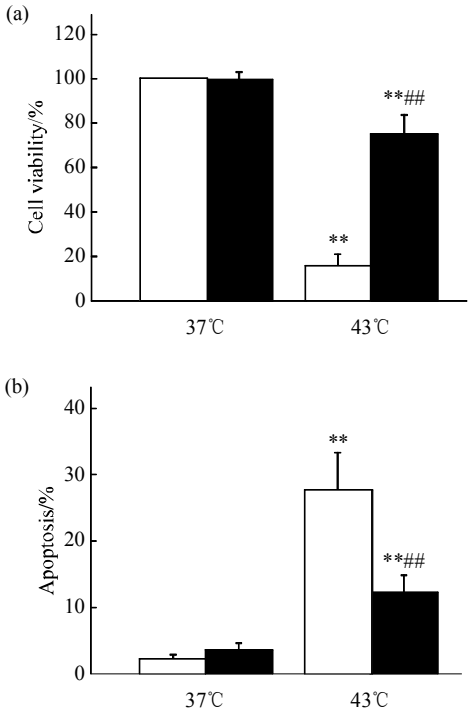

Fig. 1　Responses of HepG2 and HepG2/TT cells to a heat treatment of 24 h at 43℃

HepG2 and HepG2/TT cells were exposed to 37℃ or 43℃ for 24 h, respectively. Cell viability was detected by MTT assay (a) and apoptosis was determined by flow cytometry analysis after PI staining (b). Values are the $\bar{x} \pm s$. □: HepG2; ■: HepG2/TT. $^{**}P < 0.01$, compared with exposure to 37℃; $^{\#\#}P < 0.01$, compared with HepG2 cells exposed to 43℃.

of HepG2/TT cells was about 1/2 fold less than that of HepG2 cells (Figure 1b). These data indicated that thermotolerant hepatocarcinoma cell line HepG2/TT is resistant to heat.

2.2 Adriamycin resistance of thermotolerant hepatocarcinoma cell line HepG2/TT

Next we investigated whether the thermotolerant hepatocarcinoma cell line HepG2/TT resists to adriamycin treatment. As shown in Figure 2a, the IC_{50} values of adriamycin at 37℃ were 18.56 μmol/L and 198.80 μmol/L in the heat sensitive hepatocarcinoma cell line (HepG2 cells) and the thermotolerant hepatocarcinoma cell line (HepG2/TT), respectively, which indicated that HepG2/TT cell line was 11 times more resistant to adriamycin than the parent HepG2 cell line. After treatment with 1, 10 or 100 μmol/L of adriamycin for 24 h at 37℃, the apoptotic rate of HepG2 cells is 17.8%, 36.8%, or 62.7%, respectively; however, the apoptotic rate of HepG2/TT cells is 3.9%, 11.8%, or 21.3%, respectively, indicating that the thermotolerant hepatocarcinoma cell line HepG2/TT possesses apoptotic resistance to adriamycin. These data suggested that HepG2/TT is resistant to adriamycin.

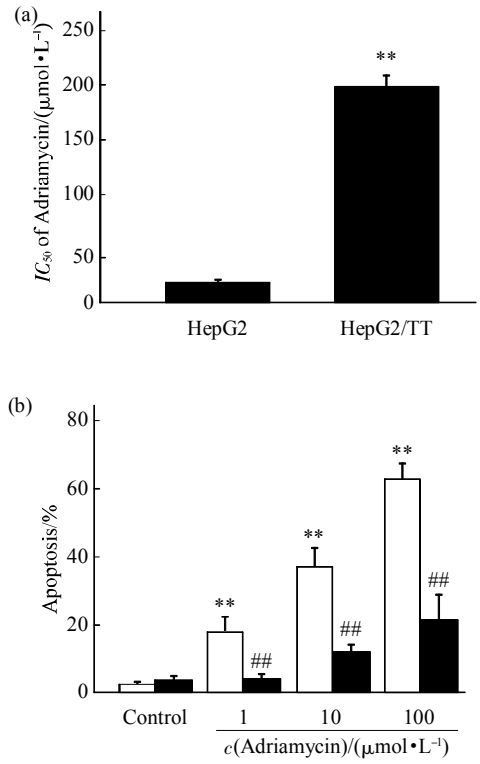

Fig. 2 Responses of HepG2 and HepG2/TT cells to adriamycin treatment

(a) HepG2 and HepG2/TT cells were treated with various concentration of adriamycin for 24 h at 37℃. Cell viability was detected by MTT assay and the IC_{50} of adriamycin was analyzed by Bliss software. Values are the $\bar{x} \pm s$. $^{**}P < 0.01$, compared with HepG2 cells. (b) HepG2 and HepG2/TT cells were treated with 1, 10 or 100 μmol/L of adriamycin for 24 h at 37℃ and the percentage of apoptotic cells was determined by flow cytometry analysis after PI staining. Values are the $\bar{x} \pm s$. □: HepG2; ■: HepG2/TT. $^{**}P < 0.01$, compared with control group; $^{\#\#}P < 0.01$, compared with HepG2 cells.

2.3 Curcumin reversed adriamycin resistance of thermotolerant hepatocarcinoma cell line HepG2/TT

To investigate whether curcumin reverses the adriamycin resistance of HepG2/TT cells, we explored the effects of curcumin on the IC_{50} and apoptosis of HepG2/TT cells treated with adriamycin. As shown in Figure 3a, when 5 μmol/L, 10 μmol/L and 20 μmol/L

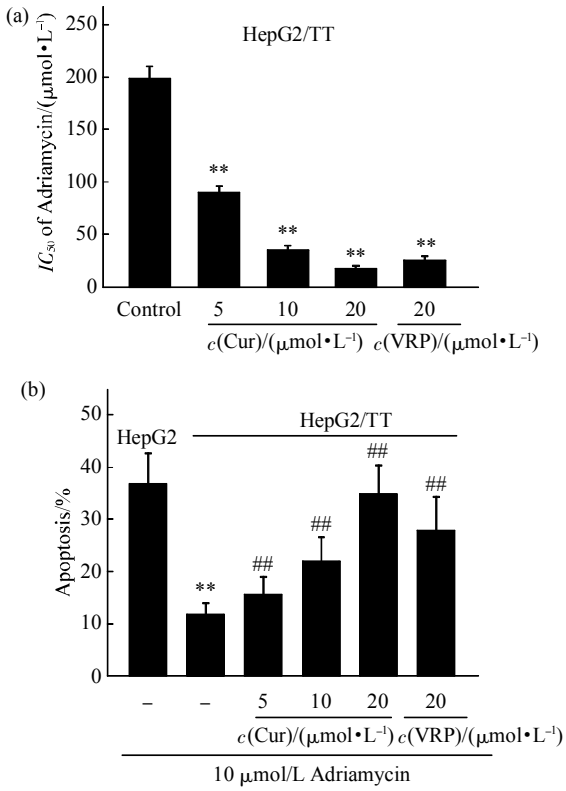

Fig. 3 Effect of curcumin on adriamycin resistance of thermotolerant hepatocarcinoma cell line HepG2/TT

(a) HepG2/TT cells were treated with various concentration of adriamycin in the presence of curcumin (Cur, 5, 10, 20 μmol/L) or verapamil (VRP, 20 μmol/L) for 24 h at 37℃. Cell viability was detected by MTT assay and the IC_{50} of adriamycin was analyzed by Bliss software. Values are the $\bar{x} \pm s$. $^{**}P < 0.01$, compared with HepG2/TT cells without curcumin treatment. (b) HepG2 cells were treated with 10 μmol/L of adriamycin for 24 h at 37℃ and HepG2/TT cells were treated with 10 μmol/L of adriamycin in the presence or absence of curcumin (Cur, 5, 10, 20 μmol/L) or verapamil (VRP, 20 μmol/L) for 24 h at 37℃. The percentage of apoptotic cells was determined by flow cytometry analysis after PI staining. Values are the $\bar{x} \pm s$. $^{**}P < 0.01$, compared with HepG2 cells treated with 10 μmol/L of adriamycin; $^{\#\#}P < 0.01$, compared with HepG2/TT cells only treated with 10 μmol/L of adriamycin.

of curcumin was added, the IC_{50} value of adriamycin in the HepG2/TT cell line significantly decreased from 199.8 μmol/L to 89.7 μmol/L, 34.5 μmol/L and 16.8 μmol/L, respectively, in a dose-dependent manner. With treatment with 20 μmol/L verapamil (positive control), the IC_{50} value of adriamycin in the HepG2/TT cell line only decreased from 199.8 μmol/L to 24.5 μmol/L. Curcumin, at concentrations of 5 μmol/L, 10 μmol/L, and 20 μmol/L for 24 h, promoted the adriamycin-mediated apoptosis of HepG2/TT cells from 11.8% to 15.6%, 21.9% and 34.8%, respectively, in a dose-dependent manner. In addition, co-treatment with 20 μmol/L of verapamil for 24 h only enhanced the adriamycin-mediated apoptosis of HepG2/TT cells from 11.8% to 27.8%. These data suggested that curcumin overcomes the adriamycin resistance of thermotolerant hepatocarcinoma cell line HepG2/TT.

2.4 Curcumin down-regulated P-gp protein expression in thermotolerant hepatocarcinoma cell line HepG2/TT

To study the mechanisms of the reversal effect of curcumin, we firstly detected the effect of curcumin on the expression of P-gp protein measured by Western blot analysis in HepG2/TT cells. As shown in Figure 4,

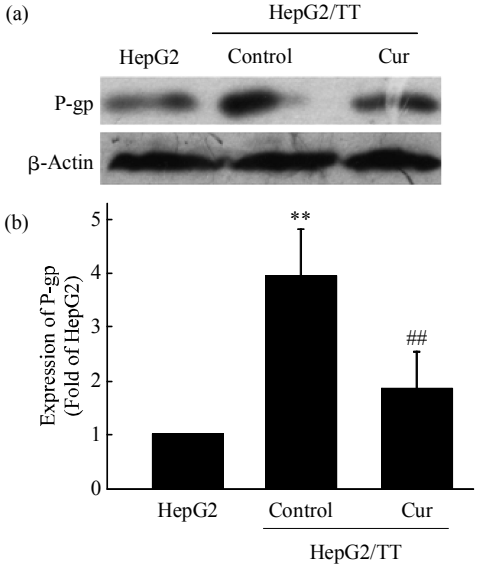

Fig. 4　Effect of curcumin on P-gp protein expression in the thermotolerant hepatocarcinoma cell line HepG2/TT

After exposure of HepG2/TT cells to curcumin (Cur, 10 μmol/L) for 24 h at 37℃, P-gp protein expressions in HepG2 cells, HepG2/TT cells, and curcumin-treated HepG2/TT cells at 37℃ were determined by Western blot using an anti-P-gp antibody. Western blot images show representative results from three independent experiments. In all blots, staining for β-actin was used as a loading control. The level of P-gp expression obtained in each experimental condition was calculated as a fold of HepG2 cells. Values are the $\bar{x} \pm s$ (n=3). **P < 0.01, versus HepG2 cells; ##P<0.01, versus HepG2/TT cells treated without curcumin (Control).

the level of P-gp in HepG2/TT cells was obviously more than that in HepG2 cells, which indicated that over-expression of P-gp is associated with the adriamycin resistance of HepG2/TT cells. On the other hand, treatment with 10 μmol/L of curcumin for 24 h significantly reduced the level of P-gp in HepG2/TT cells, suggesting that curcumin overcomes the adriamycin resistance of HepG2/TT cells by down-regulating P-gp protein expression.

2.5 Curcumin enhances the concentration of adriamycin in thermotolerant hepatocarcinoma cell line HepG2/TT

P-gp plays an important role in drug-resistance by removing drugs from cell. We next determine the concentration of adriamycin in cells to study the mechanisms of the reversal effect of curcumin. As shown in Figure 5, after exposure to 10 μmol/L of adriamycin for 3 h, the level of intracellular adriamycin accumulation in HepG2/TT cells was obviously lower than that in HepG2 cells, which indicated that the lower level of intracellular adriamycin accumulation is associated with the adriamycin resistance of HepG2/TT cells. On the other hand, co-treatment with 10 μmol/L of curcumin for 3 h significantly increased the level of intracellular adriamycin accumulation in HepG2/TT cells, suggesting that curcumin overcomes the adriamycin resistance of HepG2/TT cells by enhancing the accumulation of intracellular adriamycin.

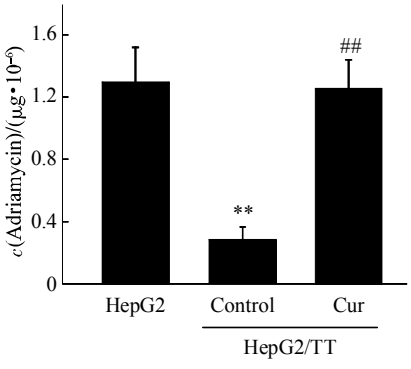

Fig. 5　Effect of curcumin on intracellular adriamycin accumulation in the thermotolerant hepatocarcinoma cell line HepG2/TT

After exposure to 10 μmol/L of adriamycin for 3 h at 37℃, the accumulation of intracellular adriamycin in HepG2 cells, HepG2/TT cells, and curcumin-cotreated HepG2/TT cells at 37℃ were determined. Values are the $\bar{x} \pm s$ (n=3). **P < 0.01, versus HepG2 cells; ##P <0.01, versus HepG2/TT cells treated without curcumin (Control).

2.6 Curcumin down-regulated Hsp70 protein expression in thermotolerant hepatocarcinoma cell line HepG2/TT

Next we determined the effect of curcumin on the expression of Hsp70 protein measured by Western blot analysis in HepG2/TT cells. As shown in Figure 6, the level of Hsp70 in HepG2/TT cells at 37℃ was obviously more than that in HepG2 cells, which indicated that over-expression of Hsp70 is involved in the adriamycin resistance of HepG2/TT cells. On the other hand, treatment with 10 μmol/L of curcumin for 24 h at 37℃ significantly reduced the level of Hsp70 in HepG2/TT cells, suggesting that curcumin overcomes the adriamycin resistance of HepG2/TT cells is associated with down-regulation of Hsp70 protein expression.

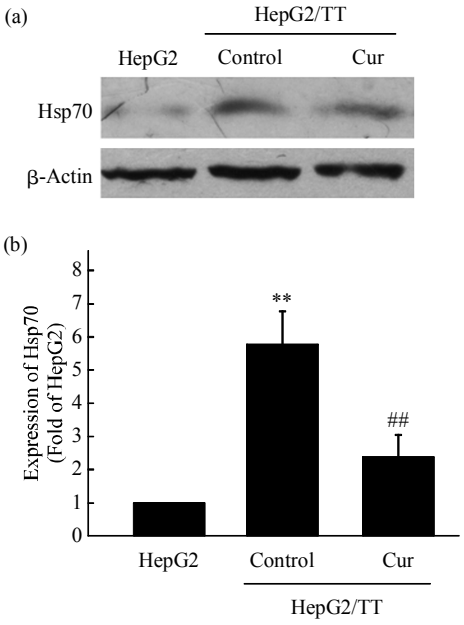

Fig. 6 Effect of curcumin on Hsp70 protein expression in the thermotolerant hepatocarcinoma cell line HepG2/TT

After exposure of HepG2/TT cells to curcumin (Cur, 10 μmol/L) for 24 h at 37℃, Hsp70 protein expressions in HepG2 cells, HepG2/TT cells, and curcumin-treated HepG2/TT cells at 37℃ were determined by Western blot using an anti-Hsp70 antibody. Western blot images show representative results from three independent experiments. In all blots, staining for β-actin was used as a loading control. The level of Hsp70 expression obtained in each experimental condition was calculated as a fold of HepG2 cells. Values are the $\bar{x} \pm s$ (n=3). $^{**}P < 0.01$, versus HepG2 cells; $^{\#\#}P < 0.01$, versus HepG2/TT cells treated without curcumin (control).

2.7 Curcumin promotes adriamycin to activate caspase-3 in thermotolerant hepatocarcinoma cell line HepG2/TT

We also investigated the effect of curcumin on caspase-3 activation induced by adriamycin in HepG2/TT cells by Western blot analysis. As shown in Figure 7, after exposed to 10 μmol/L adriamycin for 24 h at 37℃, the level of cleaved caspase-3 in HepG2/TT cells was obviously less than in HepG2 cells, which indicated that a decrease in caspase-3 activation is involved in the adriamycin resistance of HepG2/TT cells. On the other hand, co-treatment with 10 μmol/L of curcumin for 24 h at 37℃ significantly augmented the activation of caspase-3 in HepG2/TT cells treated with adriamycin, suggesting that curcumin overcomes the adriamycin resistance of HepG2/TT cells is contributed to enhancing the activation of caspase-3.

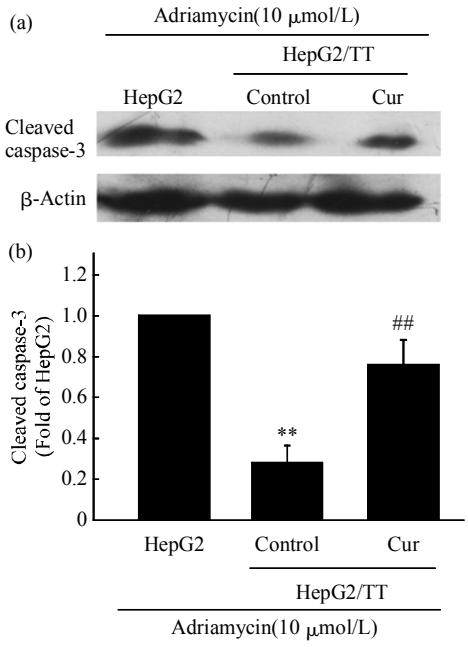

Fig. 7 Effect of curcumin on cleaved caspase-3 expressions in the thermotolerant hepatocarcinoma cell line HepG2/TT

After exposure to adriamycin (10 μmol/L) for 24 h at 37℃, the expressions of cleaved caspase-3 protein in HepG2 cells, HepG2/TT cells, and curcumin-cotreated HepG2/TT cells at 37℃ were determined by Western blot using anti-cleaved caspase-3 antibodies. Western blot images show representative results from three independent experiments. In all blots, staining for β-actin was used as a loading control. The level of cleaved caspase-3 expression obtained in each experimental condition was calculated as a fold of HepG2 cells. Values are the $\bar{x} \pm s$ (n=3). $^{**}P<0.01$, versus HepG2 cells; $^{\#\#}P<0.01$, versus HepG2/TT cells treated without curcumin (control).

3 Discussion

Hyperthermia, alone or in combination with radiotherapy or chemotherapy, is now recognized and used as an effective form of treatment of certain types of cancer [1–3]. However, thermotolerance [5–7] and hyperthermia-associated chemotherapeutic drugs resistance[11–13] in tumor cells are the major concerns in the treatment of human cancer with hyperthermia. In the present work, our data demonstrated that the thermotolerant hepatocarcinoma cell line HepG2/TT cells, isolated by ten repeated cycles of heat exposure at 43 ℃ for 80 min per 12 h, are resistant to both hyperthermia- and adriamycin-induced cytotoxicity and apoptosis, confirming that acquired stable heat resistance of cancer cells can prevent not only the efficacy of hyperthermic treatment, but also the success of chemotherapy.

Because of the developments of thermotolerance and multidrug resistance (MDR) in tumor cells are the major limiting factors in the treatment of human cancer with hyperthermia, the scientific attention has focused recently on the molecular mechanisms responsible for those phenomenons and exploiting the reversing agents to circumvent these shortcomings. P-glycoprotein (P-gp), a 170 ku protein, is an ATP-dependent drug efflux proteins belonging to the superfamily of ATP binding cassette transporters [24]. It binds to and transports various structurally unrelated compounds to maintain their intracellular concentrations below cytotoxic levels [25–26]. MDR is a major obstacle in cancer treatment and is often the result of overexpression of the drug efflux protein, P-gp [27–28]. In this work, the thermotolerant hepatocarcinoma cell line HepG2/TT cells have been shown to express P-gp at high level, but P-gp was not markedly expressed in the heat-sensitive HepG2 cells. These data suggested that P-gp is involved in the adriamycin-resistance of thermotolerant HepG2/TT cells. It has been shown that modulation of P-gp expression and activity overcomes MDR in cancer cells [29–30] and that curcumin is a modulator of P-gp [22, 31–32]. These lead us to determine the role of curcumin in adriamycin-resistance of thermotolerant HepG2/TT cells.

We showed that curcumin enhanced the toxicity of adriamycin in the thermotolerant hepatocarcinoma cell line HepG2/TT. The IC_{50} of adriamycin to the thermotolerant HepG2/TT cell line is 11 times higher than the parent HepG2 cell line. Curcumin, at concentrations of 5.0 μmol/L, 10.0 μmol/L and 20.0 μmol/L, decreases significantly the IC_{50} of adriamycin to thermotolerant HepG2/TT cells in a dose-dependent manner, indicating that curcumin reverses the adriamycin-resistance of thermotolerant HepG2/TT cell line. The potent adriamycin-resistance-reversing capacity of curcumin in tumor cells was further confirmed by its ability to enhance adriamycin-induced apoptosis in the thermotolerant HepG2/TT cells. Apoptotic and anti-apoptotic character are also strongly related to drug sensitivity and resistance. Adriamycin, at a concentration of 1 μmol/L, did not induce apoptosis in thermotolerant HepG2/TT cells, but significantly induced apoptosis in the parental HepG2 cells, which indicated that the thermotolerant HepG2/TT cell line is apoptosis-resistant. In our study, we found that curcumin, at concentrations of 5.0 μmol/L, 10.0 μmol/L and 20.0 μmol/L, promotes the adriamycin-mediated apoptosis of HepG2/TT cells, which suggests that curcumin could overcome the apoptosis-resistance of thermotolerant HepG2/TT cells.

The experiment for evaluating the effect of curcumin treatment on P-gp expression determined that curcumin clearly inhibited P-gp expression in the thermotolerant HepG2/TT cell line. In the present study, we also investigated the effects of curcumin on the P-gp function of HepG2/TT cells by determining the accumulation of intracellular adriamycin. Curcumin caused a substantial increase in the accumulation of intracellular adriamycin in HepG2/TT cells, but had no effect on heat-sensitive HepG2 cells (data not shown), which do not overexpress P-gp. Because adriamycin is known to be a substrate for P-gp [33], we suppose that curcumin modulates intracellular adriamycin level by inhibiting P-gp function. It is unlikely that curcumin acts by downregulating P-gp expression because the time of exposure of cells to curcumin in these experiments was short (3 h). Taken together, our data indicate that treatment of thermotolerant HepG2/TT cells with curcumin increased their sensitivity to adriamycin by increasing in intracellular drug concentration through down-regulation of P-gp function and expression.

At the cellular level, hyperthermic stress induces the synthesis of a class of proteins termed "heat shock proteins" (Hsp)[34]. The Hsps are acidic proteins which range in molecular mass from 8 to 110 ku [35]. Hsps

have been reported to be cytoprotective and this function has been attributed to Hsp70 [36-37], a member of the 70 ku family of Hsps. Hsp70 has been shown to confer resistance to hyperthermic treatment for prostate cancer [38]. Proteomic analysis revealed that Hsp70 correlates directly with the adriamycin-resistant in the poorly differentiated squamous cell lung carcinoma DLKP [39]. Our data showed that Hsp70 over-expressed in the thermotolerant HepG2/TT cells, which indicated that the adriamycin- and heat-resistance of thermotolerant HepG2/TT cells is attributed to the overexpression of Hsp70. On the other hand, we found that curcumin markedly downregulated the expression of Hsp70 in the thermotolerant HepG2/TT cells. The study thus puts forth the notion that downregulated Hsp70 expression is involved in the role of curcumin in reversing the adriamycin-resistance of thermotolerant HepG2/TT cells.

In the present work, we found that the thermotolerant HepG2/TT cells are resistant to apoptosis induced by adriamycin and curcumin overcomes this apoptosis-resistance. It has been shown that caspase-3 executes adriamycin-triggered apoptosis. Therefore, we further investigated the role of caspase-3 activation in the adriamycin-resistance of thermotolerant HepG2/TT cells and the effect of curcumin on the activation of caspase-3 in the adriamycin-treated thermotolerant HepG2/TT cells. We demonstrated that thermotolerant HepG2/TT cells inhibited adriamycin-induced caspase-3 activation. However, curcumin promoted adriamycin to activate caspase-3. These results indicate that the adriamycin-resistance reversing function of curcumin is associated with promoting adriamycin-induced caspase-3 activation. In addition, it has been reported that P-gp plays a role in regulating drug-resistance not only by removing drugs from cell, but also by inhibiting the activation of caspase-3 [40]. Recently, the results form Ribeil *et al* [41]. reveal the inhibitory effect of Hsp70 on the activation of caspase-3. Taken together, our data suggest that the adriamycin-resistance of thermotolerant HepG2/TT cells is attributed to the up-regulations of P-gp and Hsp70, which prevent active caspase-3, and that curcumin overcomes the adriamycin-resistance of thermotolerant HepG2/TT cells by down-regulating P-gp and Hsp70, which promotes adriamycin-triggered caspase-3 activation.

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姜黄素通过下调 P-糖蛋白和热休克蛋白 70 逆转耐热肝癌细胞的阿霉素耐受性 *

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摘要　探讨姜黄素对耐热肝癌细胞 (HepG2/TT) 阿霉素耐受性的逆转作用及其机制．用 MTT 检测细胞活力，PI 染色流式细胞术检测细胞凋亡，高效液相色谱法检测细胞内阿霉素的积累，Western blot 检测细胞 P- 糖蛋白 (P-glycoprotein，P-gp)、热休克蛋白 70 (heat shock protein 70, Hsp70) 和 caspase-3 的表达．耐热肝癌细胞 HepG2/TT 能耐受阿霉素引起的细胞毒性和凋亡；姜黄素在 5、10 和 20 μmol/L 时，能浓度依赖性地降低阿霉素对 HepG2/TT 细胞的 IC_{50}，增强阿霉素对 HepG2/TT 细胞的凋亡诱导作用．耐热肝癌细胞 HepG2/TT 与非耐热肝癌细胞 HepG2 比较，其 P-gp 和 Hsp70 的表达水平明显增高；10 μmol/L 姜黄素处理 24 h 后，HepG2/TT 细胞 P-gp 和 Hsp70 的表达水平显著下降．HepG2/TT 细胞内阿霉素的积累低于 HepG2 细胞；10 μmol/L 姜黄素处理 3 h 后，HepG2/TT 细胞内阿霉素的积累明显增加．HepG2/TT 细胞能抑制阿霉素激活 caspase-3；10 μmol/L 姜黄素处理 24 h 后，阿霉素对 HepG2/TT 细胞 caspase-3 的激活作用增强．上述结果表明，姜黄素能逆转耐热肝癌细胞 HepG2/TT 的阿霉素耐受性，其机制可能与其下调 P-gp 和 Hsp70 的表达，进而促进阿霉素激活 caspase-3 有关.

关键词　姜黄素，热耐受，阿霉素耐受性，P- 糖蛋白，热休克蛋白 70

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