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ORIGINAL ARTICLE

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In vivo antitumor activity by 2′,4′-dihydroxy-6′-methoxy-3′, 5′-dimethylchalcone in a solid human carcinoma xenograft model

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Abstract Previously we have shown that 2′,4′-dihydroxy-6′-methoxy-3′,5′-dimethylchalcone (DMC), which is isolated from the buds of *Cleistocalyx operculatus*, significantly inhibits the growth of human liver cancer SMMC-7721 cells and is able to induce apoptosis of SMMC-7721 cells in vitro. Here we report the antitumor effects of DMC in vivo, using a solid human tumor xenograft mouse model using human liver cancer SMMC-7721 cells. The average tumor weights in the control group and in mice injected with 150 mg/kg DMC were 1.42 ± 0.11 g and 0.59 ± 0.12 g, respectively. Flow cytometric analysis of the tumor cell population demonstrated an aneuploid peak (representing $33.60 \pm 0.80\%$ of the total in mice injected with 150 mg/kg DMC). To our knowledge, this is the first time that chalcone compounds have been applied to a human tumor xenograft model.

Keywords *Cleistocalyx operculatus* · Flavonoids · Tumor · Acute toxicity · Xenograft

Introduction

Over the past 10 years, research for new drugs to be used in oncology has refocused on natural products. The discovery of natural products has yielded promising compounds such as taxanes and camptothecins. In particular, interest has intensified in the class of flavonoids present in normal human diet and in many folk medicines still in use. Various pharmacological activities of flavonoids have been studied extensively [1–4].

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Cleistocalyx operculatus (Roxb.) Merr. et Perry (Myrtaceae) is a well-known medicinal plant whose buds are commonly used as an ingredient in tonic drinks in southern China. It has been reported that the water extract of the buds of *C. operculatus* increases the contractility and decreases the frequency of contraction in an isolated rat heart perfusion system [5]. Our previous phytochemical attention to the species has led to the characterization of sterol, flavanone, chalone and triterpene acid from its buds [6]. Chalcones, considered as the precursors of flavonoids and isoflavonoids, are abundant in edible plants. A number of chalcones have demonstrated cytotoxic [7, 8] and anticancer properties [9, 10]. To evaluate the effectiveness of 2′,4′-dihydroxy-6′-methoxy-3′,5′-dimethylchalcone (DMC), one of the main compounds from the buds of *C. operculatus*, as an anticancer agent, we have performed antitumor screening using six human cell lines. Both the MTT assay and colony-forming assay indicated that SMMC-7721 cells are the most sensitive of six cell lines tested. Staining with Hoechst 33258 and flow cytometric analysis have indicated that DMC is able to induce apoptosis of SMMC-7721 cells in vitro [11].

The major objective of this study was to investigate whether DMC has antitumor activity in vivo using a solid human tumor xenograft model with human liver cancer SMMC-7721 cells. The percentage of hypodiploid cells in the tumors was determined by flow cytometric analysis and acute toxicity of DMC was also evaluated using ICR mice.

Materials and methods

Materials

DMC was isolated from *C. operculatus* in our laboratory as described by Ye et al. [6]. Previous experiments have shown that the purity of DMC purity is above 96%. The structure of the compound is shown in Fig. 1. Mitomycin-C was purchased from Sigma (St. Louis, Mo.).

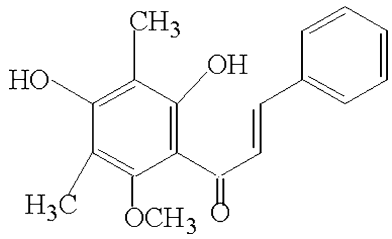

Fig. 1 Structure of 2′,4′-dihydroxy-6′-methoxy-3′,5′-dimethylchalcone

DMC and mitomycin-C were dissolved in ethanol/PBS (1:20). Control mice received the same amount of vehicle. RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Life Technologies (Rockville, Md.).

Cell line and animals

The human liver cancer SMMC-7721 cell line was purchased from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cell line was cultured in RPMI 1640 medium with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml), and was incubated at 37°C within an atmosphere of air containing 5% CO_2.

Male athymic (BALB/c nu/nu) nude mice at 4–6 weeks of age were used for the SMMC-7721 xenograft model. The mice were provided with sterilized food and water. Male ICR mice at 4–6 weeks of age were used for investigation of the acute toxicity of DMC. Both nude mice and ICR mice were purchased from Shanghai Center of Experimental Animals, Chinese Academy of Sciences.

Acute toxicity of DMC

The index generally used to evaluate acute toxicity is the LD_{50}. In this study, the LD_{50} was determined as described by Lorke [12]. Acute toxicity is tested in two steps: (1) in an initial investigation the range of doses producing the toxic effects is established; (2) based on these results, further specific doses are administered to calculate the LD_{50}. Briefly, in the initial investigation, ICR mice at 4–6 weeks of age were randomized into three groups each comprising three mice. These groups were treated with DMC by intragastric infusion at doses of 10, 100, and 1000 mg/kg, respectively. The mortality of the treated mice was recorded for up to 14 days in each group. Based on the results of the initial investigation, new dosages were administered to the animals in the second test (Table 1). In the second test each group comprised one animal.

DMC plasma levels

Male nude mice at 4–6 weeks of age were injected intraperitoneally (i.p.) once with DMC (150 mg/kg). At 3, 4, 5, 6, 7, 8, 12 and 24 h, the mice were anesthetized with ether and blood samples were taken via cardiac puncture. First, one mouse was tested at each sampling time point, and then two other mice were tested at the time point of maximal value. DMC was extracted from the blood sample by the method described by Liu et al. [13], with slight modification. Briefly, serum was obtained from the coagulated blood by centrifugation, and 400 μl serum was incubated with 20 μl 5 *N* HCl for 30 s, then extracted three times with 500 μl ethyl acetate. The extract was evaporated to dryness and dissolved in methanol.

Plasma levels of free form DMC were determined by HPLC with a reversed-phase column (ZORBAX, Eclipse XDB-C_{18}, 5 μm, 4.6×250 mm; Agilent, Palo Alto, Calif.). Chromatography was carried out using an isocratic flow program. The flow rate of the mobile phase (A, methanol; B, 0.2% H_3PO_4 aqueous; A:B = 93:7, v/v) was kept constant at 1.0 ml/min and the peaks were detected at 220 nm.

Table 1 Doses used in the initial investigation and the second test. The results of the initial investigation are presented as number of animals that died/number of animals in the group

Initial investigation			Doses for the second test (mg/kg)			
10 mg/kg	100 mg/kg	1000 mg/kg				
0/3	0/3	0/3		1600	2900	5000
0/3	0/3	1/3	600	1000	1600	2900
0/3	0/3	2/3	200	400	800	1600
0/3	0/3	3/3	140	225	370	600
0/3	1/3	3/3	50	100	200	400
0/3	2/3	3/3	20	40	80	160
0/3	3/3	3/3	15	25	40	60
1/3	3/3	3/3	5	10	20	40
2/3	3/3	3/3	2	4	8	16
3/3	3/3	3/3	1	2	4	8

Human carcinoma xenograft model

SMMC-7721 cells were established as a xenograft in male athymic nude mice. Monolayer cultures were harvested with trypsin and resuspended in PBS. About 5×10^6 cells were injected subcutaneously (s.c.) into the right flank of the mice. After 4 weeks the tumors were aseptically dissected and a tumor slurry was prepared to provide a single-cell suspension, which was then injected s.c. (2×10^6 cells in 0.1 ml) into the mice. The animals were used for experiments when the tumors were about 5×5 mm in size.

Tumor-bearing mice were randomized into five groups each comprising six mice. The study groups included: (1) control; (2) animals injected with mitomycin-C (2 mg/kg, i.p.) on days 1, 3, 5, 7, 9, 11 and 13; (3) animals injected with DMC (150 mg/kg, i.p.) on days 1–14; (4) animals injected with DMC (100 mg/kg, i.p.) on days 1–14; (5) animals injected with DMC (50 mg/kg, i.p.) on days 1–14. Tumor growth was monitored starting on the first day of treatment and the volume of

the xenografts and the weight of the animals were measured every 4 days. Tumor volumes were measured in two perpendicular diameters (A and B). Tumor volume (V) was estimated according to the formula [14]:

$$V = \frac{\pi}{6}\left(\frac{A+B}{2}\right)^3.$$

Tumor growth curves was drawn according to tumor volume and time of implantation. The mice were anesthetized and killed when the mean tumor weight was over 1 g in the control group. Tumor tissue was excised from the mice and weighed.

Flow cytometry assay

The tumor tissue was rapidly removed, weighed, and placed into 10 ml ice-cold PBS containing 0.2% bovine serum albumin (BSA) (Sigma), 0.01 mol/l EDTA, and 10 mg/ml deoxyribonuclease I (Sigma). Then the tissue was disrupted in a glass homogenizer and passed through a 40 μm nylon cell strainer (Becton Dickinson, San Diego, Calif.). The suspension was centrifuged at 500 *g* for 10 min at room temperature. The pellet was resuspended in 500 μl PBS with BSA and transferred into a fresh tube. The cells obtained by the above method were fixed with ice-cold 70% ethanol in PBS at 4°C for 8 h, then incubated with RNase (20 μg/ml) for 30 min at 37°C and labeled with propidium iodide (50 μg/ml). DNA contents were measured using a FACSCalibur cytometer (Becton Dickinson). Multicycle software (CELLQUEST, Becton Dickinson) was used to produce histograms of DNA content frequency. Subdiploid DNA peaks were quantified from the DNA content data.

Statistical analysis

Each experimental value is expressed as the mean ± SD. The scientific statistical software GraphPad Instat version 3.05 was used to evaluate the significance of differences between groups. Comparisons between groups were done using one-way ANOVA followed by the Student-Newman-Keul's test, and the criterion of statistical significance was taken as $P < 0.01$ or $P < 0.001$.

Results

Acute toxicity of DMC in ICR mice

In the initial investigation, the mortality of the mice was 0/3 in the three treated groups. Based on the results of the initial investigation, the new dosages (1600, 2900 and 5000 mg/kg) were administered to the animals in the second test according to Table 1. The mortality of the mice was 0/1, 0/1 and 1/1 at the doses of 1600, 2900 and 5000 mg/kg, respectively. The LD_{50} was estimated by geometric mean at the doses of 2900 and 5000 mg/kg. The LD_{50} value of DMC was 3800 mg/kg, indicating that DMC was slightly toxic.

Plasma pharmacokinetics of DMC in nude mice

Figure 2 shows the plasma levels of free DMC in samples taken at various times from nude mice treated with a single i.p. injection of DMC (150 mg/kg). During a period of 24 h, the plasma levels of free form DMC rose to a peak at about 3 h, then declined rapidly to a low level at 12 h, and up to 24 h the level was 2.749 μg/ml. This result suggests that DMC is quite stable in the blood of mice, and continuous administration of DMC should be beneficial to maximizing the effect of DMC in vivo, because at 24 h the DMC in blood had dropped to a relatively low level.

In vivo antitumor activity of DMC in the human tumor xenograft model

The antitumor activity of DMC in vivo was evaluated using a xenograft model of human liver carcinoma. When tumors were treated with mitomycin-C and DMC at different doses, tumor growth suppression was observed. Figure 3a shows the curves of tumor growth in the nude mouse xenografts of liver cancer cells. The effects of mitomycin-C and DMC on the weights of SMMC-7721 solid tumors are shown in Fig. 3b. The average tumor weights were 1.42 ± 0.11, 1.12 ± 0.18, 0.74 ± 0.15 and 0.59 ± 0.12 g in the control group, and the groups receiving 50, 100 and 150 mg/kg DMC, respectively. Furthermore, there was no substantial decrease in body weights in mice treated with DMC

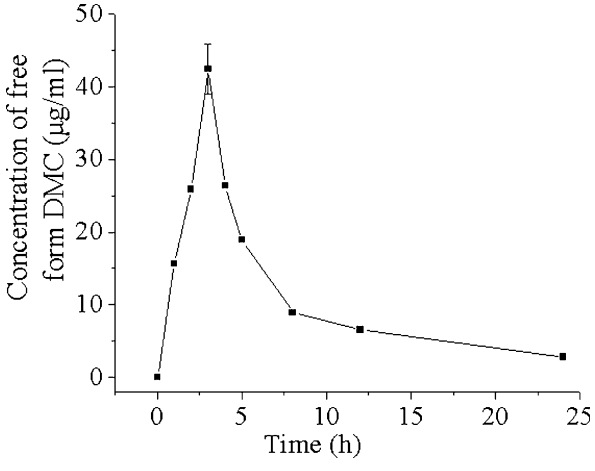

Fig. 2 Plasma levels of DMC in nude mice. Mice were injected i.p. once with DMC at a dose of 150 mg/kg and samples were taken at various times. Plasma levels of free DMC were determined by HPLC. The maximal value shown at 3 h after injection is the mean ± SD of three mice

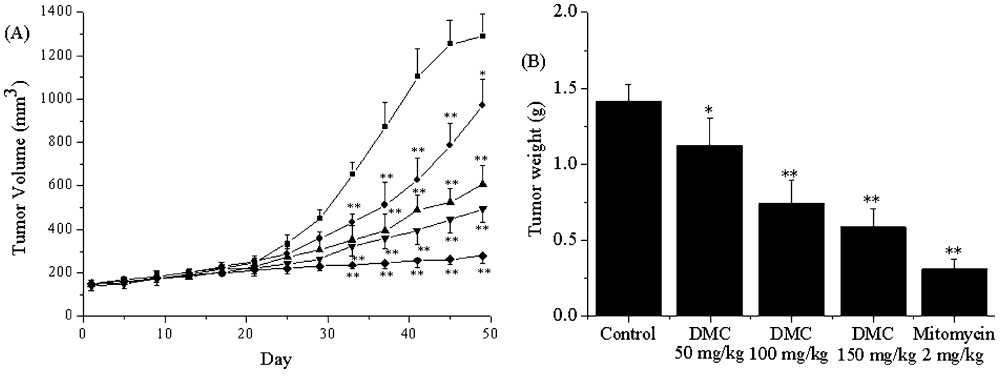

Fig. 3 Antitumor activity of DMC in vivo. **a** Effect of DMC on tumor volume. Groups were control (*filled squares*), animals injected with DMC (50 mg/kg, i.p.) on days 1–14 (*filled circles*), animals injected with DMC (100 mg/kg, i.p.) on days 1–14 (*filled triangles*), animals injected with DMC (150 mg/kg, i.p.) on days 1–14 (*filled stars*), and animals injected with mitomycin-C (2 mg/kg, i.p.) on days 1, 3, 5, 7, 9, 11 and 13 (*filled diamonds*). The data presented are means ± SD of six samples. $*P < 0.01$, $**P < 0.001$, vs control. **b** Effect of DMC on tumor weight. Each

compared with the control mice (data not shown). This finding suggests that DMC has low toxic side effects.

Effects of DMC on the population of hypodiploid cells in the tumors in mice

DMC induced the appearance of hypodiploid peak in the tumors in mice. As shown in Fig. 4, the sub-G_0/G_1 population in drug-treated groups increased with the drug dose: 4.80 ± 0.61, 16.42 ± 0.91, 20.80 ± 1.45 and 33.60 ± 0.80% of the tumor cells were hypodiploid in the control group, and the groups treated with DMC at 50,

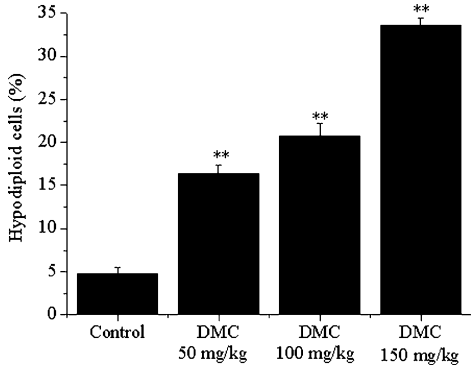

Fig. 4 The percentage of hypodiploid cells after staining with propidium iodide. Tumor tissues were processed to isolate cells, and then the cells obtained were stained with propidium iodide, and analyzed in a FACScan flow cytometer. Groups were control, animals injected with DMC (50 mg/kg, i.p.) on days 1–14, animals injected with DMC (100 mg/kg, i.p.) on days 1–14, and animals injected with DMC (150 mg/kg, i.p.) on days 1–14. The data presented are the means ± SD of three tumors per group. $**P < 0.001$, vs control

100 and 150 mg/kg, respectively. The treatment resulted in the appearance of a hypodiploid peak (A_0 region) [15], probably due to the presence of apoptosing cells and/or apoptotic bodies with DNA content less than 2*n*.

Discussion

Among the number of substances identified from plants, flavonoids are one of the most important and interesting classes of biologically active compounds. The common synthone of the flavonoid family are the chalcones, which in the cyclized form generate flavanones, flavones, isoflavones and flavonols. Chalcones have demonstrated cytotoxic [7, 8] and anticancer properties [9, 10]. In the last decade, various pharmacological activities of chalcone, such as anticancer and antioxidant activity [16], anti-invasive activity [17], aromatase inhibition, 17β-hydroxysteroid dehydrogenase activity [18], cytotoxic and inhibitory effects on proliferation of leukemic [19] and antiangiogenic activity [20] have been described. However, there have been few reports on the in vivo antitumor activity of chalcone compounds. In this study, we attempted to determine whether DMC could inhibit growth of tumor in vivo.

Human tumor xenografts are now well-established tools for the preclinical screening of anticancer drugs and an integral part of the current NCI and EORTC disease-orientated strategies for drug screening [21]. The average tumor weights were 1.42 ± 0.11, 1.12 ± 0.18, 0.74 ± 0.15 and 0.59 ± 0.12 g in the control group, and in animals treated with 50, 100 and 150 mg/kg DMC, respectively. Furthermore, there were no substantial decreases in body weight in mice treated with DMC compared with those treated with the vehicle. These results suggested that DMC, at a dose of 150 mg/kg, could markedly inhibit the growth of tumor, with low side effects, in a solid human carcinoma xenograft model.

Previously we have shown that DMC can induce apoptosis in SMMC-7721 cells in vitro [11]. In the present study, flow cytometry indicated that the sub-G_0/G_1 population in drug-treated groups increased with drug dose, and 33.60 ± 0.80% of the cells in tumors were hypodiploid in the group treated with 150 mg/kg DMC.

These findings demonstrate that DMC can induce apoptosis in SMMC-7721 cells both in vitro and in vivo. The mechanism of the antitumor activity of chalcones remains to be fully clarified. Further studies are now in progress in our laboratory to clarify the mechanisms of induction of apoptosis by DMC, such as bcl-2 gene expression, myc gene expression and decreased activation of the NF-kB transcription factor.

In this study, the plasma level of free DMC 3 h after DMC administration was 42.47 ± 3.47 μg/ml, which is relatively high. Consecutive administrations of DMC over 14 days possibly resulted in relatively high levels of DMC.

Evaluation of the acute toxicity is the first step in the toxicological investigation of a substance. The LD_{50} of DMC was 3800 mg/kg, which means DMC is slightly toxic. This result is consistent with the report of Havsteen [22]. It is not possible for humans to suffer acute toxic effects from the consumption of DMC, with the exception of a rare occurrence of allergy. The margin of safety for the therapeutic use of DMC in humans, therefore, is very large and probably not surpassed by any other drug in current use.

In conclusion, DMC markedly inhibited the growth of tumor in a solid human carcinoma xenograft model. To our knowledge, this is the first time that a chalcone compound has been investigated in a human tumor xenograft model. Our results suggest that DMC is a promising chemotherapeutic agent with low toxicity and high efficacy and further studies are warranted.

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