General gambogic acids inhibited growth of human hepatoma SMMC-7721 cells *in vitro* and in nude mice[1]

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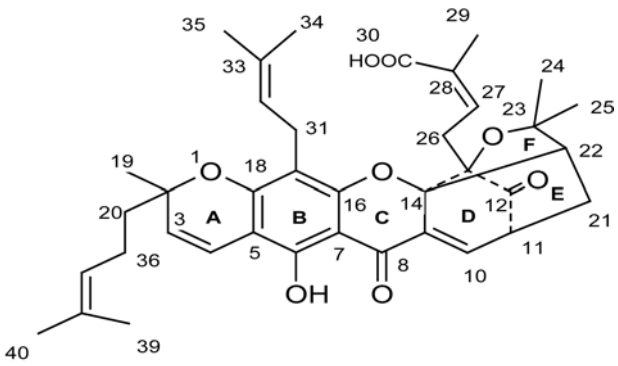
KEY WORDS gamboge; gambogic acid; hepatoma; SMMC-7721 cells; telomerase; nude mice

ABSTRACT

AIM: To study the inhibitory effect of general gambogic acids (GGA) on transplantation tumor SMMC-7721 in experimental animal model and SMMC-7721 cells *in vitro*. **METHODS:** Anti-tumor activity of GGA in the experimental transplantation tumor SMMC-7721 was evaluated by relative tumor growth ratio. Cell morphology was observed with inverted microscope and electron microscope. Cell proliferation was measured by MTT assay and the telomerase activity was determined by PCR. **RESULTS:** *In vivo* study indicated that GGA (2, 4, and 8 mg/kg, iv, 3 times per week for 3 weeks) displayed an inhibitory effect on the growth of transplantation tumor SMMC-7721 in nude mice compared with the normal saline group ($P<0.01$). At the concentrations of 0.625-5.0 mg/L, GGA remarkably inhibited the proliferation of SMMC-7721 cells *in vitro*. GGA 2 mg/L dramatically changed morphology of SMMC-7721 cells and inhibited the telomerase activity in SMMC-7721 cells. **CONCLUSION:** GGA had inhibitory effect on the growth of SMMC-7721, which might be related to its inhibition of telomerase activity.

INTRODUCTION

Gamboge is the dry resin obtained from *Garcinia hanburryi*. It was reported that gamboge had inhibitory effects on some experimental tumors[1-4]. The main active compounds of gamboge are gambogic acid, neogambogic acid, morellic acid, and isomorellic acid[5,6]. The general gambogic acids (GGA) were extracted from gamboge and purified, in which the main component is gambogic acid (GA) ($C_{38}H_{44}O_8$)[7]. In 1980s, the Cambogia anticancer investigation group separated GA and identified its chemical structure and studied the antitu-



Chemical structure of gambogic acid

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mor activity of the crude extract of Gamboge *in vitro* and *in vivo*, as well as its absorption, distribution, and excretion[8,9]. In 1990s, Kong *et al* studied its activity and components from various separation[10]. We previously studied the growth inhibition of GGA on the experimental transplantation tumors such as Heps, EC, and S_{180} in mice[11]. This study was aimed to investigate the effect of GGA on the experimental tumor SMMC-7721 in nude mice and *in vitro* and explore its possible mechanism.

MATERIALS AND METHODS

Chemicals GGA injection (25 mg per ampoule) was supplied by the School of Pharmacy, China Pharmaceutical University. The GGA was dissolved in 0.9 % NaCl before experimental use. Hydroxycamptothecin (OPT, 2.5 g/L) was supplied by Feiyun Pharmaceutical Ltd Co, Huangshi, China.

Animals BALB/c nude mice (half female and half male), 35-40 d old, weighing 18-22 g, were supplied by Shanghai Institute of Materia Medica, Chinese Academy of Sciences (Certificate No 122). Animals were kept at room temperature of 18-22 ºC and a relative humidity of 70.

Tumor cell Human hepatoma cell line SMMC-7721 was supplied by Cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Cells were cultured in RPMI-1640 medium.

Experimental transplantation tumor in mice According to the protocol of transplantation tumor research[12], SMMC-7721 tumor tissues were chopped into 1.5 mm^3, which was subcutaneously transplanted into right axillary fossa of nude mice. The diameter of transplantation tumor was measured with vernier caliper and the 24 tumor-transplanted mice were randomly divided into 4 groups (n=6) when tumor size grew to 100-300 mm^3, ie, positive control group (OPT, 3 mg/kg) and GGA-treated groups (2, 4, and 8 mg/kg, iv, 3 times per week). Mice in the negative control group (n=12) were treated with normal saline.

The tumor volume (TV) and relative tumor volume (RTV) was calculated by the following formula:

$TV=1/2\times a\times b^2$ (in which a is the length and b is the width of tumor).

$RTV=Vt/V_0$ (in which V_0 is the TV at the day when the chemicals were given and Vt is the TV of succedent measurement).

The evaluation index of anti-tumor activity was relative tumor growth ratio T/C (%), which was calculated by the following formula:

$T/C\ (\%)=T_{RTV}/C_{RTV}\times 100\ \%$ (T_{RTV}: test group's and positive control group's RTV; C_{RTV}: common negative control group's RTV).

The data were then subjected to a statistical analysis (*t*-test) for actual efficiency of the material tested.

SMMC-7721 morphology The logarithm cells were dispersed with 0.02 % edetic acid to prepare the suspension of a cell density of 1×10^9/L in a 25-mL cell culture bottle. When cells grew to a mono-layer, GGA 2 mg/L and OPT 12.5 mg/L were added, respectively. The control group was treated with diluted solution without the test drugs. After an incubation for 48 h, the cells were collected and fixed with 3 % glutaraldehyde and washed with PBS 0.1 mol/L. The cells were fixed with 1 % osmic acid and dehydrated by ethanol in gradient concentration. Embedded with EPOr812 and divided to ultraslice, cells were double-stained with uranium acetate and plumbum citrate. The changes were observed with inverted microscope and electron microscope. The common human hepatocyte L_{02} was used as the control.

MTT assay According to protocol tetramethyl azo salt MTT colorimetric analysis, the logarithm cells were dispersed with 0.02 % edetic acid to prepare the suspension of a cell density of 3×10^7/L. Then cell suspension was partitioned into a 40-well plate in a total volume of 100 mL per well and incubated in a 5 % CO_2 atmosphere at 37 ºC for 4 h. Then, the cells were added with different concentrations of GGA (100 μL per well), respectively. After the 24-h, 48-h, and 72-h incubation, cells were added with MTT solution 5 g/L (20 μL per well). After another 4-h incubation, the supernatant were discarded and isopropanol hychloride 0.04 mol/L were added (100 μL per well). Then the suspension was vibrated on a micro-vibrator for 5 min and the absorbance (A) was measured at 570 nm with an Enzyme Immunoassay Instrument. The cell inhibitory ratio was calculated with the following formula: Inhibitory ratio= $(1-A_{\text{Treated group}}/A_{\text{Control group}})\times 100\ \%$.

Inhibitory effects of medicine on telomerase of SMMC-7721 The logarithm cells were dispersed with 0.02 % EDTA to prepare the 1×10^9/L cell suspension in a cell culture bottle. After formation of a monolayer, cells were treated with GGA 2 mg/L and incubated for 1, 3, and 5 d. The cell suspension was centrifuged and washed with PBS 0.1 mol/L twice and the cell sediment was stored in Eppendorf tubes. The dried

cells were mixed with cell splitting solution, put into ice bath for 30 min. and then centrifuged at 1600 r/min for 30 min. The supernatant was stored at -20 ºC. Extraction solution was mixed with TRAP solution at 30 ºC for 30 min. The above TRAP solution 50 μL was added 1 μL of Cx primer 0.1 mg/L. Then PCR (at 94 ºC for 30 s; 56 ºC for 30 s; 72 ºC for 30 s, 35 cycles) was performed and 25 μL of PCR production was electrophoresed vertically in a 12 % PAGE-gel at 150-200 V for 2 h. The gel was washed with double-distilled water twice, fixed with 10 % ethyl alcohol for 15 min, decolorized with 1 % nitric acid for 10 min, stained with 0.2 % silver nitrate for 30 min, developed with 3 % sodium carbonate solution, and finally fixed with 3 % glacial acetic acid.

RESULTS

Inhibitory effects of GGA on transplantation tumor SMMC-7721 in nude mice The *in vivo* experiment indicated that iv injection of GGA 2, 4, and 8 mg/kg inhibited dramatically the growth of SMMC-7721 tumor in nude mice from the early administration (Fig 1, Tab 1).

Cell morphology GGA-treated cells appeared round, and scaled off from each other, rather than polygon-shaped as under normal conditions with the inverted microscope (Fig 2). Their nuclei became smaller and pyknotic. Cell shrinkage, loss of microvilli, chromatin clumps and reduction of the nuclear/cytoplasmic ratio were noted under the electron microscope. The

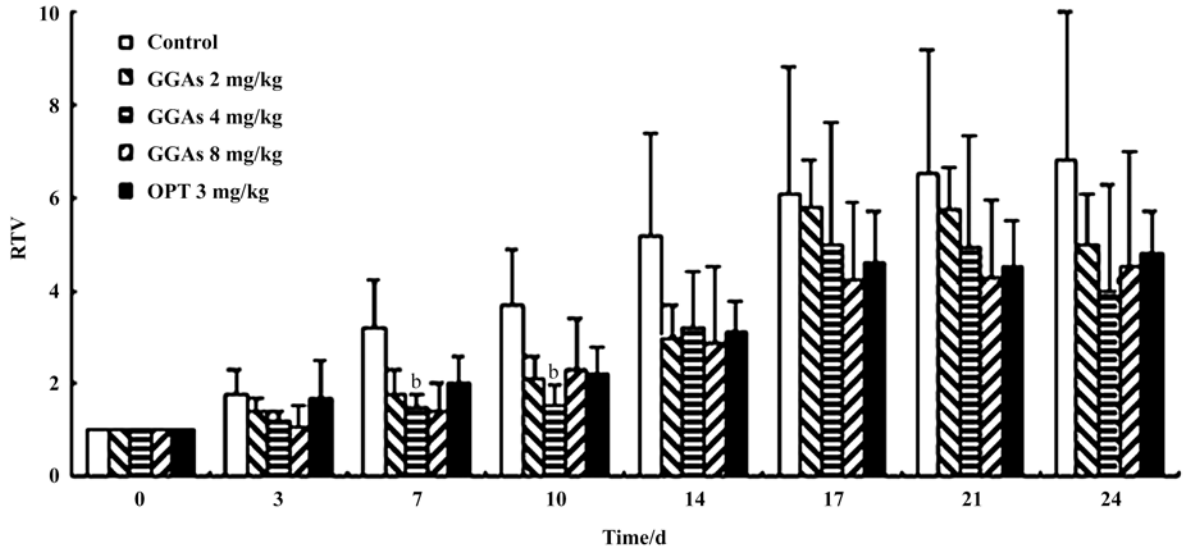

Fig 1. Inhibitory effects of general gambogic acids (GGA) on transplantation tumor SMMC-7721 in nude mice. *n*=3. Mean±SD. $^{b}P<0.05$ *vs* control group.

Tab 1. The experimental therapeutic efficacy of iv injection of GGA on human hepatocellular cell line SMMC-7721 in nude mice.

Group	Doses	Animal Number		Weight (g)		TV		RTV	T/C (%)	*P*
		D_0	D_n	D_0	D_n	D_0	D_n			
NS	0.2 mL/kg	16	16	20.0	23.3	290±149	922±565	3.2±1.0		
OPT	3 mg/kg	6	6	19.0	22.0	307±184	611±243	2.2±0.6	69.5	>0.05
GGA	2 mg/kg	6	5	18.3	22.0	270±216	517±462	2.0±0.5	61.6	>0.05
GGA	4 mg/kg	6	6	18.0	21.5	251±111	411±206	1.6±0.3	49.9	<0.05
GGA	8 mg/kg	6	5	18.0	19.7	249±177	359±310	1.5±0.6	47.0	<0.05

D_0: The day of the start of treatment. D_n: The day of the optimal treatment, which was the 7th day in the experiments.

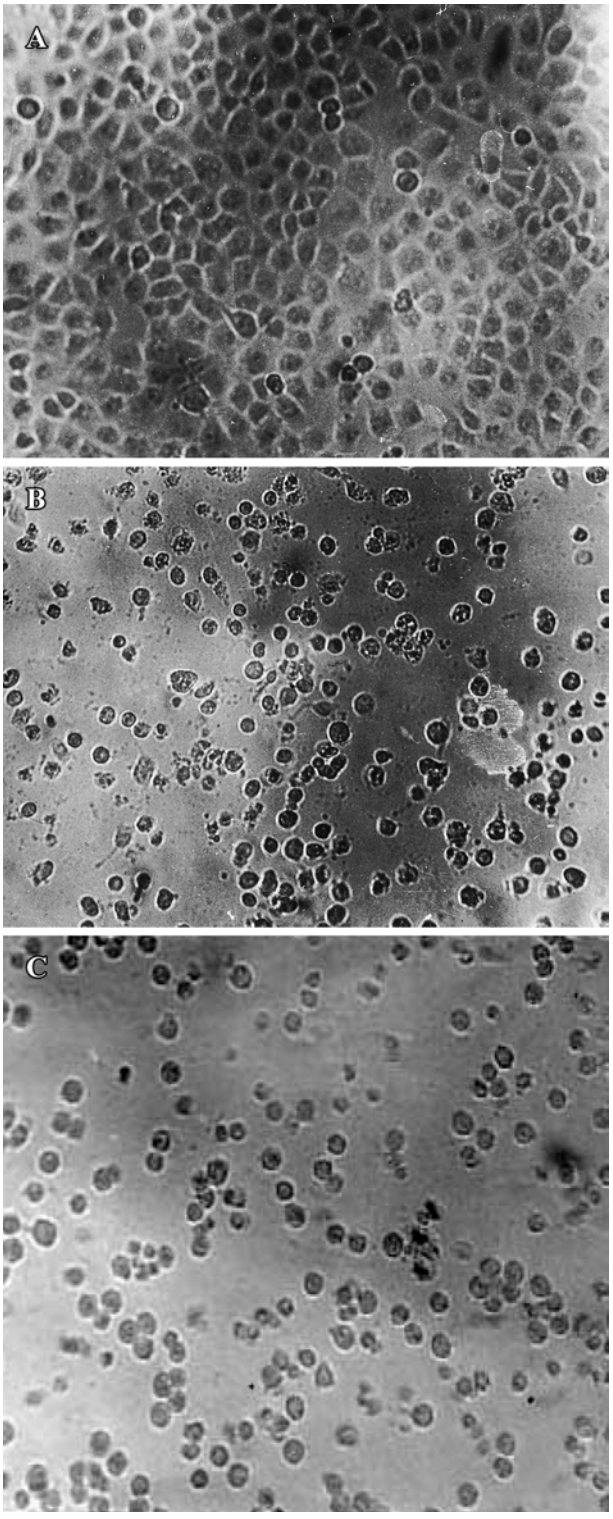

Fig 2. The morphology changes of human hepatocarcinoma cell SMMC-7721 observed with inverted microscope. A: Control; B: Treated with GGA 2 mg/L; C: Treated with OPT 12.5 mg/L. ×562.

pycnosis, smashed nucleus, cytoplasmic vacuolation , and heterochromatin were observed (Fig 3).

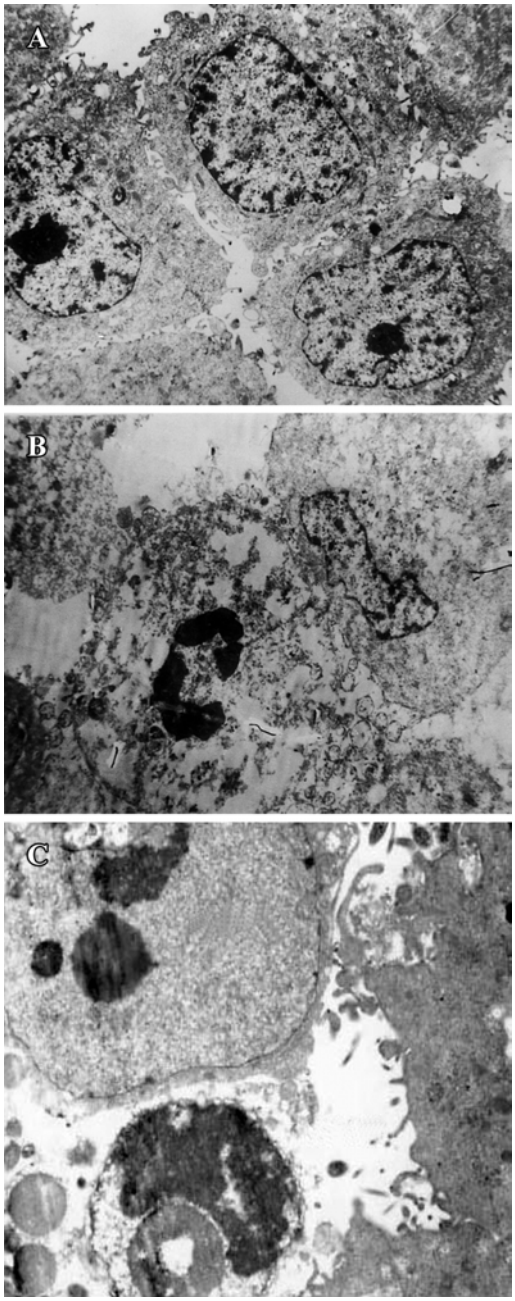

Fig 3. The morphology change of human hepatocarcinoma cell SMMS-7721 observed with electron microscope. A: Control; B: Treated with GGA 2 mg/L; C: Treated with OPT 12.5 mg/L. ×7500.

Inhibitory effects of GGA on proliferation of SMMC-7721 Treated with different concentrations of GGA for 24, 48, and 72 h, the proliferation of SMMC-7721 tumor cells was remarkably inhibited. The higher the concentration of GGA was, the higher the cell inhibitory ratio was. But GGA had no remarkable effect on the common human liver cell L_{02}. OPT also had the inhibitory effect on SMMC-7721, but the effect was

lower than that of GGA (Fig 4).

Inhibitory effects of GGA on telomerase of SMMC-7721 In the negative control group (the common human liver cell L_{02}), there was no strip of telomerase from PCR production. In SMMC-7721 and positive control groups, strip of telomerase could be observed at d 1 after GGA treatment, and the strip of telomerase disappeared at d 3 and d 5 after GGA treatment. The experimental results showed that GGA could inhibit the telomerase activity in SMMC-7721 cells (Fig 5).

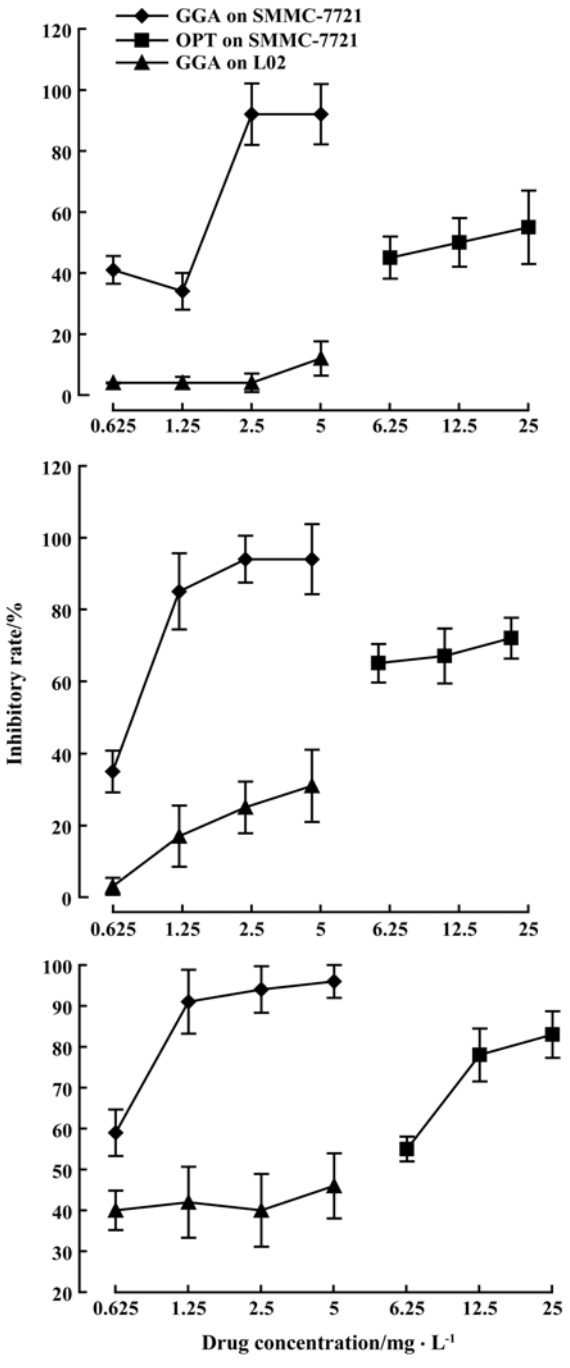

Fig 4. The inhibitory ratio of GGA on tumor cell SMMC-7721. A: 24 h; B: 48 h; C: 72 h. *n*=3. Mean±SD.

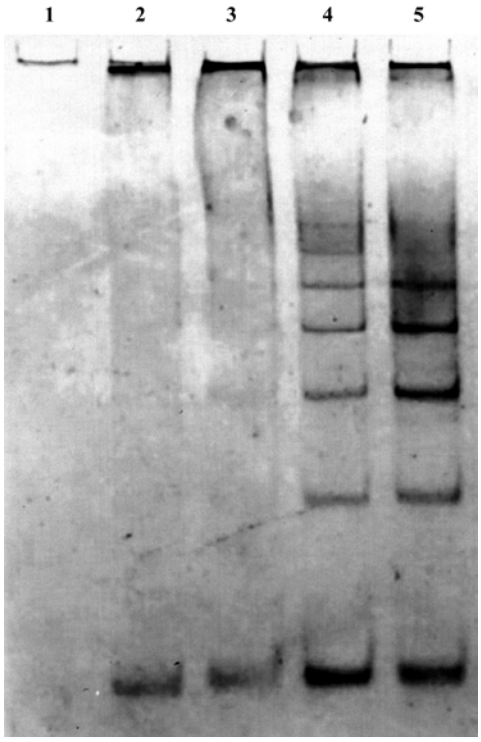

Fig 5. The inhibitory effect of GGA 2 mg/L on telomerase activity in SMMC-7721 cells. Lane 1: Negative control (killed by heated); Lane 2-4: 5, 3, 1 d after GGA treatment, respectively; Lane 5: positive control (untreated).

DISCUSSION

It is reported that Cambogia had significant antitumor activities and it was different from common chemotherapeutical drugs. It can kill cancer cells selectively, but has no influence on normal hematopoietic system and leucocytes[1,6]. So it provids prospects in finding new antitumor drugs. Since there was little evidence about the antitumor action and mechanisms of Cambogia, the primary study was performed to give some information about pharmacodynamic and action mechanism of omni-cambogic acid (mainly involved cambogic acid and new-cambogic acid) which is the active component of Cambogia.

Our *in vivo* study indicated that GGA significantly inhibited the growth of hepatoma cell SMMC-7721 and improved the morphology of SMMC-7721 cells, such as necrosis, cell size and scale, pycnosis of nuclei, and heterochromatin. At the higher concentration of GGA,

the proliferation ratio of SMMC-7721 was reduced and the inhibitory ratio was increased. But GGA had little effect on the normal liver cells, indicating that GGA possessed selectivity on tumor cells. Therefore, the inhibitory effect of GGA on SMMC-7721 telomerase activity was studied subsequently. The results showed that GGA could dramatically inhibit the telomerase activity of SMMC-7721.

Telomerase is one of the important special enzymes for the replication of RNA and it is one kind of ribonucleoprotein[13-18]. Telomerase uses itself RNA as a model to synthesize telomere DNA and adds to gene terminal.

There are high concentration of telomerase in malignant tumor cells and telomerase could be a new potential target of anticancer agents. The proliferation of tumor cells could be reduced or stopped by inhibiting the activity of telomerase. The results of this work suggested that the accelerated tumor cell death and inhibitory effect of GGA on transplantation tumor SMMC-7721 might be accomplished through inhibition of telomerase activity.

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