



Ginkgol C17:1 inhibits tumor growth by blunting the EGF-PI3K/Akt signaling pathway

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Abstract

Ginkgol C17:1 has been shown to inhibit apoptosis and migration of cancer cells, but the underlying mechanisms are not fully elucidated. In this study, we explored whether the inhibitory effects of Ginkgol C17:1 were associated with epidermal growth factor receptor (EGFR) and PI3K/Akt signaling. The results showed that EGF treatment increased the phosphorylation of EGFR, PI3K, Akt, mTOR and NF- κ B, and also enhanced the proliferation, migration and invasion of HepG2 cells. Ginkgol C17:1 dose-dependently inhibited EGF-induced phosphorylation/activation of all the key components including EGFR, PI3K, Akt, mTOR and NF- κ B, leading to a significant reduction either of proliferation or migration and invasion of HepG2 cells. Notably, treatment with Ginkgol C17:1 in mice suppressed the growth of tumor mass *in vivo*, and expression of EGFR in the tumor tissue. The results suggest that Ginkgol C17:1 is a potent tumor inhibiting compound that acts on EGF-induced signal transduction of the PI3K/Akt signaling pathways, and may represent a clinically interesting candidate for cancer therapy.

Keywords: Ginkgol C17:1, epidermal growth factor, PI3K/Akt, HepG2

Introduction

Epidermal growth factor receptor (EGFR) is a 170-kDa trans-membrane protein that belongs to the ErbB receptor tyrosine kinase subfamily, including ErbB1 (EGFR), ErbB2 (HER2), ErbB3 (HER3) and ErbB4 (HER4)^[1]. EGFR is present on cell surface and is activated by binding of its specific ligands, including epidermal growth factor (EGF). Upon activation, EGFR undergoes a transition from an inactive monomeric form to an active homodimer. EGFR dimerization subsequently stimulates its intrinsic intracellular protein tyrosine kinase activity that elicits several signal transduction cascades, principally the MAPK, Akt and JNK pathways, leading to DNA synthesis and cell proliferation.

EGFR is implicated in tumorigenesis. EGFR over-expression has been observed in various types of cancer^[2]. Clinically, patients with over-activity of EGFR are usually associated with poor prognosis^[3]. Furthermore, pharmacological inactivation of the EGFR pathway has been proven to inhibit the proliferation of certain types of tumor cells and thus leads to several successful therapeutical strategies^[4].

Ginkgols are natural phytochemicals found in *Ginkgo biloba* L. and chemically named as 3-alkylphenols. Ginkgols are biologically active compounds and have been reported to have a wide range of pharmacological activities, including anti-bacterial^[5-6], antifeeding^[7], antioxidant^[8], anti-tumor activity as well as apoptotic effects of tumor cells *in vitro*^[9-10].

Ginkgol C17:1 is one of the Ginkgol monomers

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Received 21 March 2016, Revised 28 April 2016, Accepted 10 November 2016, Epub 16 December 2016

CLC number: R730.52, Document code: A

The authors reported no conflict of interests.

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which has been shown to yield the strongest inhibitory effect on various human cancer cells among the monomers^[11–13]. Ginkgol C17:1 has been separated, purified and identified by our group. We have reported the anti-cancer effects of Ginkgol C17:1 in our previous studies^[13–14]; however, the mechanistic targets by which Ginkgol C17:1 suppresses the proliferation of cancer cells remain undisclosed.

In the present study, we hypothesized that the inhibitory action of Ginkgol C17:1 on tumor cells may be involved in the regulation of EGFR activity as well as its downstream cascade. Our results showed that Ginkgol C17:1 suppressed the proliferation, migration and invasion of hepatocellular carcinoma cells (HepG2), and at the same time inhibited the EGF/EGFR-induced downstream phosphorylation of the PI3K/Akt-mediated signaling pathway. Therefore, the results strongly suggest that Ginkgol C17:1 is a potent candidate for EGFR-mediated cancer therapy.

Materials and methods

Cancer cell lines and experimental animals

Human hepatoma carcinoma cells HepG2, and murine H22 tumor cells were obtained from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China), and cryopreserved at School of Medicine, Jiangsu University (Zhenjiang, Jiangsu, China).

Forty SPF Kunming mice aged between 6 and 8 weeks, weighing approximately 20 g, were provided by the Laboratory Animal Center of Jiangsu University (SCXK(Su)2009-0002). The animals were housed in laboratory conditions (20°C±2°C, 55%-65% humidity, a 12-hour light/dark cycle with the light cycle from 6:00 to 18:00 and the dark cycle from 18:00 to 6:00) with *ad libitum* access to standard laboratory chow and water. The study protocol was approved by the local institutional review board at the authors' affiliated institutions and animal studies were carried out in accordance with the established institutional guidelines regarding animal care and use. Animal welfare and the experimental procedures were carried out strictly in accordance with the Guide for Care and Use of Laboratory Animals (National Research Council of USA, 1996).

Reagents

Dulbecco's modified eagle media (DMEM), fetal bovine serum (FBS) and trypsin-EDTA solution were purchased from Gibco Life Technologies (Grand Island, NY, USA). Horseradish peroxidase conjugated secondary antibody (HRP-goat anti-rabbit polyclonal IgG,

A0562) was purchased from Beyotime Institute of Biotechnology (Haimen, Jiangsu, China). Electrochemiluminescence (ECL) reagents were bought from Amersham Biosciences (Buckinghamshire, UK). LDH-cytotoxicity colorimetric assay kit II (Cat. #K313-500) was obtained from BioVision, Inc. (Milpitas, CA, USA). EGF and 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbit anti-mTOR polyclonal antibody (ab2732) was purchased from Abcom (Cambridge, MA, USA). Rabbit anti-NF-κB p65 (sc-114) and β-actin antibody (sc-47778) polyclonal IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-p-EGFR (Tyr1068, 3777), anti-p-EGFR (Tyr1173, 4407), anti-p-PI3Kp55 (Tyr199, 4228), PI3K p85 (4292) and anti-p-mTOR (Ser2448, 5536) polyclonal antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Rabbit anti-EGFR (IM001-0377), rabbit anti-Akt (IM001-0359) and rabbit anti-p-Akt1/2/3 (Tyr315/316/312, IM001-0270) polyclonal antibodies were purchased from ExCell Biology Co., (Shanghai, China). Ginkgol C17:1 (HPLC purity > 96.5%) was kindly provided from Dr. Yang at the Laboratory of Food and Biological Engineering School of Jiangsu University^[12–13].

Western blotting assays

The protein samples were derived from the cell lysis in the presence of cocktail of protease inhibitors. After quantification of protein content using Bradford, 50 μg of proteins per each lane was loaded on 8% or 10.0% SDS polyacrylamide gel. The separated proteins were subsequently transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). PVDF membranes were initially blocked with 5% milk in TBS-T (NaCl 80 g/L; KCl 2 g/L; Tris 30 g/L; Tween-20 0.1%; pH 7.4) at room temperature for 1 hour and then incubated with the primary antibodies (p-EGFR, p-PI3K, p-Akt, p-mTOR, NF-κB) (dilution of 1:1,000) at 4°C over night. After washing, the membranes were further incubated with HRP-conjugated secondary antibodies (dilution of 1:1,000) for additional 1 hour at room temperature. To visualize the target proteins, ECL reagents were applied according to the manufacturer' instructions and positive protein bands were detected using Typhoon 9400 imager (GE Healthcare Life Science, Piscataway, NJ, USA).

MTT assays

HepG2 cells were diluted to a density of 5×10^4

cells/mL and seeded on a 96-well plate with final culture medium of 100 μ L per well. After incubation for 12 hours at 37°C in 5% CO₂, EGF (100 ng/mL) and various concentrations of Ginkgol C17:1 (20 and 40 μ g/mL) were applied to the cells with identical volume medium and incubated for further 24 hours before 10 μ L of the MTT solution (5 mg/mL) was added in each well. After additional 4-hour incubation, the culture medium was removed and replaced by 100 μ L DMSO. Cell survival was measured at an absorbance of OD490 nm using a microplate reader (Bio-Rad, USA).

Migration assay

The migration activity of HepG2 was assessed using Transwell Boyden chambers (Corning, Acton, MA, USA) as published previously. Briefly, the bottom chambers contained 500 μ L DMEM with 10% FBS, while the upper chambers were seeded 10⁴ cells of the exponential phase in 300 μ L of serum-free medium containing EGF and various concentrations of Ginkgol C17:1. Transwell chambers were incubated for 24 hours at 37°C in 5% CO₂ to allow the cells on the upper chamber to migrate through the polyethylene terephthalate membrane to the lower chamber. The cells remaining on the upper membranes were removed using cotton swabs, and only those reached the other side of the membranes were fixed in 4% paraformaldehyde for microscopic determination of cell numbers. The average number of migrated cells in the five randomly selected fields was taken as the mean of cell-migration number. All experiments were performed in triplicate.

In vitro invasion assays

Preparation of peridium basement membrane: Matrigel (50 μ g/mL, Corning Matrigel basement membrane matrix) was prepared in serum-free DMEM medium at the ratio of one to ten and then 50 μ L of mixture was gently paved to the bottom of Transwell chamber. The Matrigel was kept in 37°C for 2 hours to allow solidification. The gelled Matrigel was gently washed with pre-warmed serum free-culture medium and 50 μ L serum free DMEM was added to Transwell insert to hydrate the basement membrane in 37°C for 30 minutes.

The ability of HepG2 cells to invade through Matrigel membrane was assessed by using the identic protocol to the migration assays as described above.

Inhibitory effects of Ginkgol C17:1 *in vivo*

Ascitic H22 tumor cells were obtained from the ascites of SPF Kunming mice. The collected ascites were diluted with saline to a cell density of 4 \times 10⁷ cells/mL. A total of 0.2 mL of H22 tumor suspension

was injected subcutaneously into the axillary area of mice. The growth of implanted cells in the host mice was monitored every 3 days by imaging and caliper measurements (L \times W \times D). Mice were randomly divided into the groups that either received intraperitoneal injection of DMSO as control, or Ginkgol C17:1 at various concentrations (20 mg/kg, G20; 40 mg/kg, G40; and 80 mg/kg, G80) on every other day for 2 weeks. At the end of experiments, the tumor mass was excised, weighed and fixed for further immunohistochemical analysis.

Immunohistochemistry

The expression of EGFR in the tumor samples was analyzed by immunohistochemical staining. In brief, the paraffin embedded tissues were sliced in sections of 5 μ m thickness and, after deparaffinization and rehydration, was blocked with 3% H₂O₂ in methanol for 15-minute at 37°C to remove endogenous peroxidase activity. Antigen retrieval was performed by heating the sections in EDTA buffer (pH 8.0) using a 700-W microwave oven on full power for 5 minutes and half power for 10 minutes. After cooling down to room temperature, the sections were blocked in goat serum for 1 hour and incubated with primary antibody (anti-EGFR, 1:500) at 4°C overnight. After wash with PBS for 3 times, the sections were further incubated with HRP-conjugated secondary antibody (goat anti-rabbit IgG, 1:1,000) for 1 hour at 37°C. Finally, DAB substrate solution was applied to visualize the color of antibody staining. EGFR expression was evaluated by staining intensity using microscope.

Statistical analysis

All data were represented as mean \pm standard deviation (SD). Inhibitory effects of different concentrations of Ginkgol C17:1 on tumor growth, migration, invasion and tumor inhibitory rate *in vivo* were evaluated by analysis of variables (one way ANOVA) using the SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). A statistically significant difference was considered at $P < 0.05$.

Results

Ginkgol C17:1 inhibits EGF-induced EGFR phosphorylation

Given that Tyr1173 and Tyr1068 in EGFR are two important auto-phosphorylation sites leading to the activation of MAPK/PI3K-mediated signaling, we examined the effects of Ginkgol C17:1 on phosphorylation of both sites using Western blotting assays. Our results demonstrated a weak but clear phosphorylation

in HepG2 cells at the basal condition. When HepG2 cells were primed with EGF for 10 minutes, the phosphorylation at either site of Tyr1173 or Tyr1068 was promptly induced by 5 folds, suggesting that they are two downstream targets upon EGF stimulation. Interestingly, EGF-induced phosphorylation at both residues were diminished in the presence of Ginkgol C17:1 in a dose-dependent manner and almost abolished at the concentration of 40 $\mu\text{g}/\text{mL}$, while total EGFR was not altered (**Fig. 1**). This data indicated that Ginkgol C17:1 suppressed EGFR-mediated activity by inhibiting phosphorylation of Tyr1173 and Tyr1068.

Ginkgol C17:1 blocks EGF-induced downstream phosphorylation/activation

To unmask the downstream cascade that may mediate the inhibitory effects of Ginkgol C17:1 on tumor growth, we further investigated several protein kinases that have been previously demonstrated to be involved in EGFR signaling. To this end, the phosphorylation of PI3K, Akt, mTOR and NF- κB was analyzed in cells stimulated with EGF and in the absence or presence of Ginkgol C17:1 at the concentrations of 10, 20 and 40 $\mu\text{g}/\text{mL}$, respectively. Our data showed that, as expected, phosphorylation level of PI3K, Akt and mTOR was all significantly induced by EGF stimulation, with a relatively strong effect on Akt and mTOR while a weak effect on PI3K (**Fig. 2A-C**). The stimulation of EGF also led to activation of NF- κB by nuclear translocation (**Fig. 2D**). Most importantly, EGF-triggered phosphorylation of its downstream kinases was

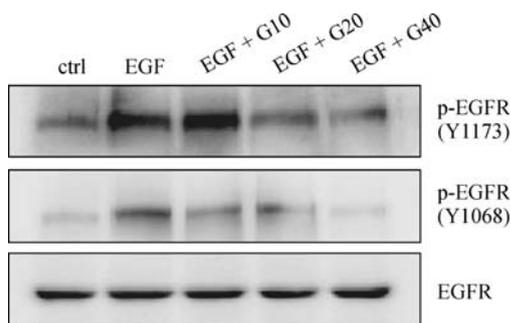


Fig. 1 Ginkgol C17:1 inhibits EGF-induced phosphorylation of EGFR in HepG2 cells. In the EGF group, cells were only incubated with EGF (100 ng/mL) for 10 minutes. In the EGF + G10 group, cells were treated with Ginkgol C17:1 10 $\mu\text{g}/\text{mL}$ for 24 hours and then with EGF (100 ng/mL) for 10 minutes. In the EGF + G20 group, cells were treated with Ginkgol C17:1 20 $\mu\text{g}/\text{mL}$ for 24 hours and then with EGF (100 ng/mL) for 10 minutes. In the EGF + G40 group, cells were treated with Ginkgol C17:1 40 $\mu\text{g}/\text{mL}$ for 24 hours and then with EGF (100 ng/mL) for 10 minutes. The cells were harvested and lysed as described in Materials and Methods and the cell lysate was subjected to Western blotting. Results are representative of three separate experiments. Ctrl: control; G10: Ginkgol C17:1 10 $\mu\text{g}/\text{mL}$; G20: Ginkgol C17:1 20 $\mu\text{g}/\text{mL}$; G40: Ginkgol C17:1 40 $\mu\text{g}/\text{mL}$.

dose-dependently diminished by the administration of Ginkgol C17:1. As a transcriptional effector, NF- κB can translocate into the nucleus upon activation and bind to the corresponding promoter to initiate an array of transcriptional responses. In the present study, we found that the nuclear translocation of p65 phosphorylated NF- κB occurred in response to EGF stimulation in HepG2 cells. Likewise, Ginkgol C17:1 effectively prevented the activation and translocation, indicated by dose-dependent reduction of p65 phosphorylated NF- κB in the nuclei (**Fig. 2D**).

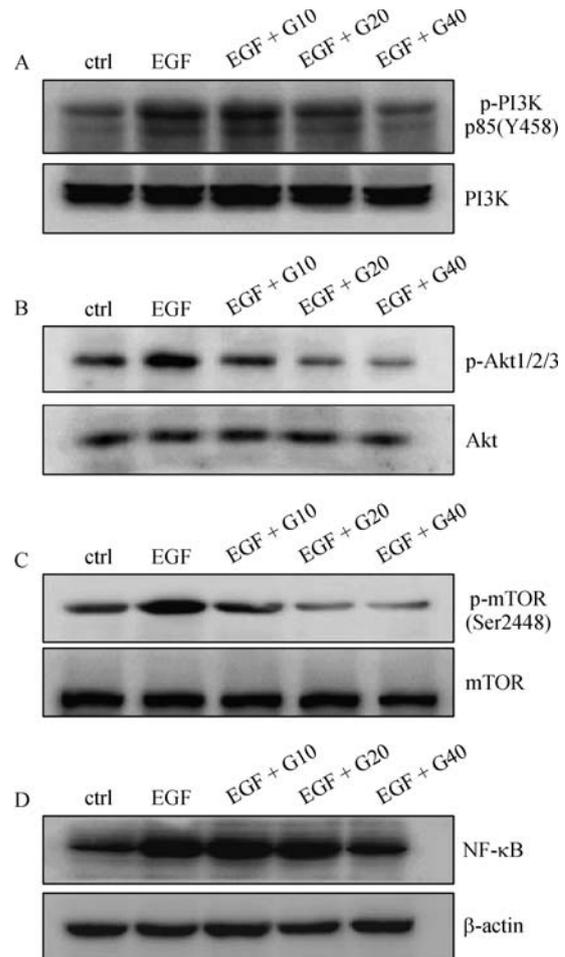


Fig. 2 Ginkgol C17:1 inhibits EGF-induced PI3k/Akt signaling activities. Western blotting assay was performed to detect proteins related to PI3k/Akt signaling. HepG2 cells were treated the same as **Fig. 1** in **Fig. 2A, 2B** and **2C**; however, the difference is treatment with EGF (100 ng/mL) for 24 hours in **Fig. 2D**. A: the expression of p-PI3K p85 and PI3K; B: the expression of p-Akt1/2/3 and Akt; C: the expression of p-mTOR and m-TOR; D: the expression of NF- κB . The results indicated that 100 ng/mL EGF treatment induced a prominent increase in Tyr458 phosphorylation of p-PI3K p85, Tyr315/316/312 phosphorylation of p-Akt, Ser2448 phosphorylation of p-mTOR, and NF- κB , but except the expression of PI3K, Akt and mTOR. Ginkgol C17:1 of various concentration efficiently inhibited EGF-induced phosphorylation of PI3K, Akt and mTOR. The results are representative of three independent experiments. Ctrl: control; G10: Ginkgol C17:1 10 $\mu\text{g}/\text{mL}$; G20: Ginkgol C17:1 20 $\mu\text{g}/\text{mL}$; G40: Ginkgol C17:1 40 $\mu\text{g}/\text{mL}$.

Ginkgol C17:1 suppresses EGF-induced cell proliferation, migration and invasion

The signal transduction of EGF/PI3K/Akt has been shown to functionally relate to cell proliferation, transformation and apoptosis. Our data showed that addition of EGF led to an enhanced activity of proliferation, migration and invasion of HepG2 cells (**Fig. 3**). This effect, however, was massively counteracted by the presence of Ginkgol C17:1 in the culture medium, evidenced by the reduced transmembrane mobility. Remarkably, Ginkgol C17:1 seemingly also acted on endogenous EGF activity, as the both tested concentrations at 20 and 40 $\mu\text{g/mL}$ Ginkgol C17:1 suppressed the replication as well as transmembrane activity of HepG2 cells to the level below the condition without EGF boosting (**Fig. 3**).

Anti-tumor effect of Ginkgol C17:1 has inverse relation with EGFR expression

To test whether the inhibitory effects of Ginkgol C17:1 on tumor cell could be functionally translated into *in vivo* condition, we used H22 tumor mouse models and treated the mice with various concentrations of Ginkgol C17:1. We found that, after 2 weeks treatment, tumor masses in mice received 40 mg/kg (G40) and 80 mg/kg (G80) were significantly less than that in the control group as well as than the mice that received 20 mg/kg (G20) (**Fig. 4A, B**, $P < 0.05$). Interestingly, Ginkgol C17:1 treatment also led to a trend of downregulation of EGFR expression inside the tumor tissues (**Fig. 4C**), suggesting that, in addition to the inhibition of phosphorylation/activation of the PI3K/Akt pathway, Ginkgol C17:1 transcriptionally regulated the key protein at the upstream of signal transduction. The dual-inhibitory effects may synergistically attenuate the activity of EGF cascade and thus suppress tumor growth *in vivo*.

Discussion

Our previous studies have shown that Ginkgol C17:1 could inhibit the apoptosis and migration of SMMC7721 cells^[13-14], but those experiments were very preliminary and the underlying mechanism of Ginkgol C17:1 induced inhibition has not been intensively explored. Specifically, the question regarding to the potential cellular targets and the associated signal pathways activated by Ginkgol C17:1 treatment remains unanswered. Therefore, the present study aimed to determine whether Ginkgol C17:1 induced inhibitory effect is associated with the activation of

EGFR and its downstream targets of the PI3K/Akt-mediated pathway.

EGFR is a receptor tyrosine kinase that belongs to the RTK superfamily. Upon activation by ligand binding with EGF, for instance, autophosphorylation of the trans-membrane domain at the tyrosine sites occurs, which subsequently recruits PI3K to the its C-terminal and further triggers the phosphorylation/activation of PI3K. Therefore, signal transduction into intracellular compartment is initiated^[15-16]. PI3K is also known to be able to phosphorylate the third OH group on the inositol ring of phosphatidylinositol (PI), leading to the formation of phosphatidylinositol-3,4,5-triphosphate (PIP3). PIP3 subsequently binds to Akt and causes the translocation of Akt from the cytoplasm to the cell membrane^[17-18]. Akt, also known as protein kinase B, is a serine/threonine-specific protein kinase that plays a crucial role in the regulation of multiple cellular functions including proliferation, differentiation, apoptosis and migration. Additionally, PI3K/Akt-mediated signal transduction may be initiated by interacting with receptor tyrosine kinases (RTKs)^[19] and $\text{NF-}\kappa\text{B}$ and mTOR serve as substrates of PI3K/Akt pathway. Notably, the EGF/PI3K/Akt-mediated signal transduction was found to be critical to tumorigenesis and has been targeted for cancer therapy^[20].

In the present study, we confirmed that, in the hepatocellular carcinoma HepG2 cell line, treatment with EGF rapidly caused Tyr1068 and Tyr1173 phosphorylation of EGFR, and the subsequent activation of the key components of the PI3K/Akt-mediated cascade, including PI3K, Akt, mTOR and $\text{NF-}\kappa\text{B}$. Most importantly, the natural product of Ginkgol C17:1 that has been previously identified and purified in our laboratory was also found to dose-dependently suppress the phosphorylation at the key residues in the entire cascade of EGF-induced signal transduction. At the concentration of 40 $\mu\text{g/mL}$, Ginkgol C17:1 was found to completely abolish the EGF-induced response of several key kinases of this cascade, suggesting that Ginkgol C17:1 effectively blocks the signal transduction of the EGFR pathway.

EGF signaling is known to associate with cellular function such as proliferation, migration and invasion of tumor cells. We demonstrated that, both *in vitro* and *in vivo* conditions, Ginkgol C17:1 refrained the growth of implanted H22 cells in a dose-dependence manner and negatively regulated the expression of EGFR inside the tumor mass. This finding indicates a feedback loop on the transcriptional regulation of upstream proteins that may desensitize the EGF pathway after Ginkgol C17:1 treatment. Therefore, this may explain that Ginkgol

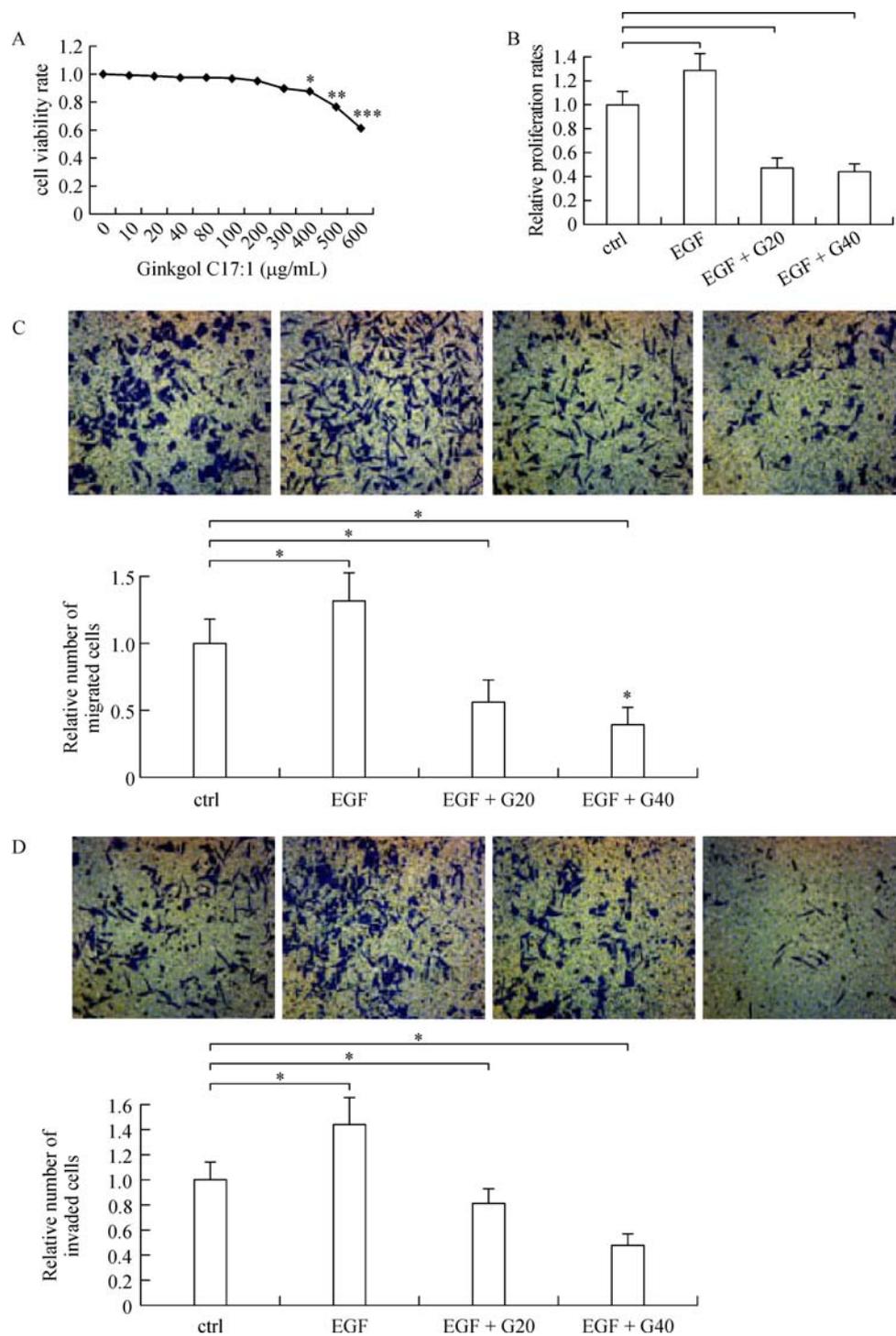


Fig. 3 Ginkgol C17:1 inhibits EGF-induced cell proliferation, migration and invasion. Cytotoxicity assay of Ginkgol C17:1 (A) represents the cytotoxicity of Ginkgol C17:1 from the LDH colorimetric assay to HepG2 cells 24 hours since Ginkgol C17:1 incubation. The spontaneous release of LDH from the Ginkgol C17:1-free group (0 μg/mL) was considered the control group ($n = 12$). The proliferation of HepG2 cells was detected by MTT assay (B), the migratory activity was analyzed with a Transwell assay (C), and cell invasion was examined by Matrigel invasion assay *in vitro*, using a modified Transwell Boyden chamber (D). The transmembrane migrated and invaded cells were examined under a light microscope (images below each graph, magnification, $\times 200$). In the EGF group, cells were only incubated with EGF (100 ng/mL) for 24 hours. In the EGF + G20 group, cells were treated with Ginkgol C17:1 20 μg/mL and then EGF (100 ng/mL) for 24 hours. In the EGF + G40 group, cells were treated with Ginkgol C17:1 40 μg/mL and EGF (100 ng/mL) for 24 hours. Data are shown as mean \pm SD from four independent experiments, each performed in duplicate (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Ctrl: control; G20: Ginkgol C17:1 20 μg/mL; G40: Ginkgol C17:1 40 μg/mL.

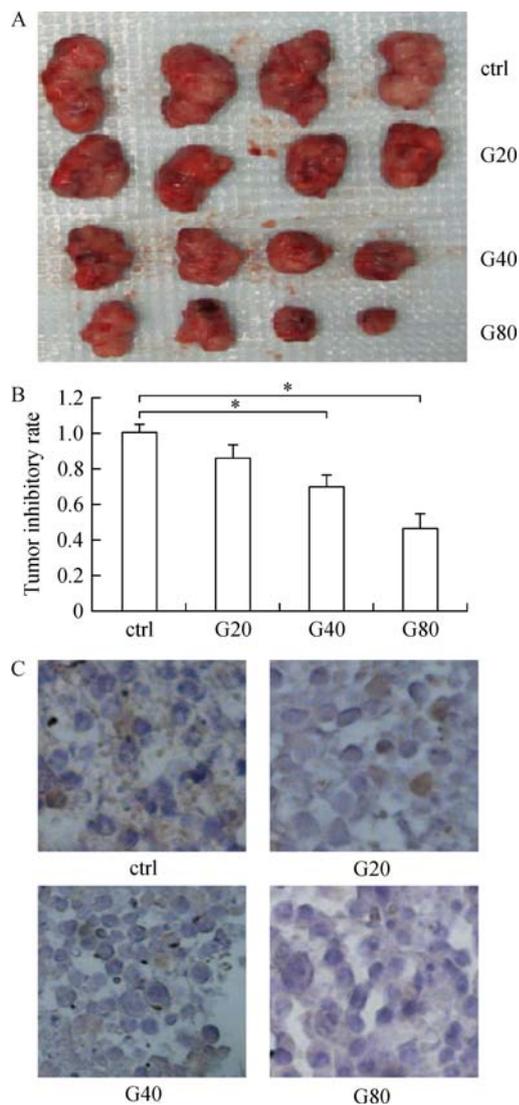


Fig. 4 The size of tumor mass and the immunostaining of EGFR inside the tumor tissue. H22 tumor mouse models were treated by various concentrations of Ginkgol C17:1. A: Tumor mass from individual mice 2 weeks after implantation *in vivo*. B: Relative tumor inhibitory rate (* $P < 0.05$). C: Immunohistochemical staining of EGFR in the sections from tumor biopsies ($\times 400$). Ctrl: control; G20: Ginkgol C17:1 20 mg/(kg·2 days), i.p.; G40: Ginkgol C17:1 40 mg/(kg·2 days), i.p.; G80: Ginkgol C17:1 80 mg/(kg·2 days), i.p.

C17:1 efficiently conferred a significant inhibition on tumor growth *in vivo*.

In summary, our results showed that Ginkgol C17:1 dose-dependently inhibited the EGF-induced the phosphorylation/activation of all the key components, including EGFR, PI3K, Akt, mTOR and NF- κ B, leading to a significant reduction either of proliferation or migration and invasion of HepG2 cells. Notably, treatment with Ginkgol C17:1 in mice refrained the growth of tumor mass *in vivo*, and the expression of EGFR inside the tissue. The results suggest that Ginkgol C17:1 is a potent tumor inhibiting compound acting on EGF-induced signal transduction of PI3K/Akt-mediated

pathways, and may represent a clinically interesting candidate for cancer therapy.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (grant no. 81372404), and the Postdoctoral Foundation of China (grant no. 2012M521018) to Yueying Li; the Zhenjiang Social Development Project (No. SH2015072) to Yaxiang Shi.

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