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Geranylgeranylacetone inhibits ovarian cancer progression *in vitro* and *in vivo* ☆

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Abstract

Geranylgeranylacetone (GGA), an isoprenoid compound, is an anti-ulcer drug developed in Japan. In our previous study, GGA was shown to inhibit ovarian cancer invasion by attenuating Rho activation [K. Hashimoto, K. Morishige, K. Sawada, M. Tahara, S. Shimizu, M. Sakata, K. Tasaka, Y. Murata, Geranylgeranylacetone inhibits lysophosphatidic acid-induced invasion of human ovarian carcinoma cells in vitro. Cancer 103 (2005) 1529–1536]. In the present study, GGA treatment inhibited ovarian cancer progression *in vitro* and suppressed the tumor growth and ascites in the *in vivo* ovarian cancer model. *In vitro* analysis, treatment of cancer cells by GGA resulted in the inhibition of cancer cell proliferation, the inactivation of Ras, and the suppression of tyrosine phosphorylation of mitogen-activated protein kinase (MAPK). In conclusion, this is the first report that GGA inhibited ovarian cancer progression and the antitumor effect by GGA is, at least in part, derived not only from the suppression of Rho activation but also Ras–MAPK activation.

Keywords: Geranylgeranylacetone; Lysophosphatidic acid; Rho; Ras; Geranylgeranylation; Ovarian cancer

Among many growth-promoting factors known to be present in ovarian cancer ascites, lysophosphatidic acid (LPA) is found at significant levels (~10 μM) and may play an important role in the development or progression of ovarian cancer [1]. LPA has been reported to induce many cellular effects, including mitogenesis, the secretion of proteolytic enzymes [2] and migration activity [3].

Protein isoprenylation, such as geranylgeranylation and farnesylation, is a post-translational modification that is essential for the membrane localization and full function of small GTP-binding proteins (G proteins), such as Rho, Rac, and Ras [4,5]. Since small G proteins play crucial roles in signal transduction, isoprenoids are of fundamental importance in the control of various cellular functions [6]. Recently, several isoprenyl compounds such as farnesol (FOH), geranylgeraniol (GGOH), and geranylgeranoic acid have been shown to induce apoptotic cell death [7,8] and modulate cell motility [9,10]. Accordingly, isoprenyl compounds might influence cancer cell activity.

Geranylgeranylacetone (GGA), an isoprenoid compound developed in Japan, has been used orally as an anti-ulcer drug. GGA protects the gastric mucosa from various stresses without affecting the gastric acid secretion [11]. Moreover, the chemical structure of GGA is similar to that of geranylgeranylpyrophosphate, which is a component in the metabolic pathway of Rho and is essential for geranylgeranylation of Rho [12].

Abbreviations: LPA, lysophosphatidic acid; GGA, geranylgeranylacetone; GGOH, geranylgeraniol; FOH, farnesol; ROCK, Rho-associated kinase.

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In our previous study [13] GGA inhibited Rho activity, cell motility and the invasion of cancer cells, in the same way as do HMG-CoA reductase inhibitors [9,14,15] and bisphosphonates [10,16]. GGA has also been demonstrated to suppress cell growth and induce differentiation or apoptosis by modulating small G protein activation in leukemia cell lines [17,18].

The Ras–Raf–MAP kinase/Erk kinase (MEK)-extracellular signal regulated kinase (Erk) cascade has a central role in regulating tumor cell growth, survival, and has been targeted for therapeutic intervention in the past [19]. LPA is also the prototypic receptor agonist that stimulates Ras through pertussis toxin-sensitive G*i* [20,21]. As such, LPA acts like epidermal growth factor with respect to down stream Ras signaling, particularly MAP kinase. In this study, we analyzed the effects of GGA on Ras/MEK/Erk signaling *in vitro*, and showed that GGA markedly inhibited LPA-induced proliferation of human ovarian cancer cells by attenuating the activation of Ras signaling. Thus, GGA inhibited not only the cell motility [13], but also the proliferation of ovarian cancer cells.

Advanced ovarian cancer cases respond well to platinum-containing chemotherapy after debulking surgery [22]. However, most cases (60–80%) relapse after the remission status [23,24]. Therefore, preventive or consolidation therapy after complete remission is clinically important to avoid recurrence. Here, we tested GGA in the preventive protocol using an intraperitoneal dissemination model [25] of ovarian cancer.

Materials and methods

Materials. GGA was supplied by Eisai Co. (Tokyo, Japan). Bovine serum albumin (BSA), collagen (type I) and LPA were purchased from Sigma (St. Louis, MO). Anti-phospho-p44/p42 MAPK (Thr 202/Tyr 204) (Erk) polyclonal antibody and anti-p44/p42 MAPK (Erk) polyclonal antibody were purchased from Cell Signaling Technology (Beberly, MA). Anti-MEK1 monoclonal antibody and the Ras activation assay Kit were obtained from Upstate Biotechnology (Lake Placid, NY). Anti-phospho-MEK-1/2 (Ser 218/Ser 222) polyclonal antibody, horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyvinylidene difluoride membrane (Hybond-P) and enhanced chemiluminescence (ECL) Western blotting detection reagents were obtained from Amersham (Arlington Heights, IL). The Cell Titer 96AQ kit to monitor cell proliferation was purchased from Promega (Madison, WI). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco-BRL (Gaithersburg, MD).

Cell culture. The human ovarian cancer cell lines, Caov-3 and SKOV-3 were purchased from American Type Culture Collection (Rockville, MD). Both cell lines were grown in DMEM, supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (10 U/ml)-streptomycin (10 U/ml) in 95% air, and 5% CO_2 at 37 °C, and were used within 15 passages after the initiation of culture.

Cell proliferation assessment. Cell proliferation was assessed by the MTT assay using a Cell Titer 96AQ kit according to the manufacturer's instructions. Briefly, the cells (3×10^3/well) were plated in 96-well plates and allowed to attach for 4.5 h, and then cultured with 25 μM LPA and various concentrations of GGA for 24–72 h in DMEM supplemented with 2% FBS. The number of surviving cells was determined by measuring the absorbance at 590 nm (A 590 nm) of the dissolved formazan product formed after addition of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt for 1 h as described by the manufacturer. All of the experiments were carried out in quadruplicate, and proliferation was expressed as the ratio of the absorbance of GGA treated and control cells to the absorbance before treatment.

Ras pull-down assay. The Ras pull-down assay was performed using Ras activation assay kit according to the manufacturer's instructions. Briefly, cells were cultured under serum-free conditions for 24 h, and then pretreated with GGA and GGOH for 24 h. After pretreatment, cells were stimulated with 25 μM LPA for 2 min, washed with cold PBS, and lysed. Cell lysates were clarified by centrifugation, and equal volumes of lysates were incubated with Raf-1 RBD-agarose beads. Bound Ras proteins were detected by Western blotting using a monoclonal antibody against Ras. Western blotting to estimate the total amount of Ras in cell lysates was performed for the comparison of Ras activity (level of GTP-bound Ras) in the same samples.

Western blot analysis. The cells were allowed to attach and cultured in serum-free conditions with or without GGA and GGOH for 24 h. After the treatment, cells were stimulated with 25 μM LPA for 30 min, washed and lysed in sample buffer. Equal amounts of samples were resolved by SDS–PAGE and transferred to Hybond-P membranes. The transferred samples were incubated with the antibody indicated in the text and then incubated with HRP-conjugated IgG. The immunoblotted proteins were visualized with ECL reagents.

Animal study. Six-week-old female nude mice (BALB-c nu/nu, Japan SLC, Inc., Hamamatsu, Japan) were housed in filtered-air laminar-flow cabinets and were manipulated using aseptic procedures. Procedures involving animals and their care were conducted in conformity with the guidelines of our university. The *in vivo* ovarian cancer models using Caov-3 cells were prepared as previously described [26]. Caov-3 cells were injected i.p. as a cell suspension into 10 nude mice for each experimental group. Mice were assigned to one of three treatment regimens, which started the day after tumor inoculation and continued for 7 weeks. GGA as an emulsion with 5% gum arabic was given orally every 2 days. The daily doses of GGA used were as follows: 0, 50, and 100 mg/kg/2 days. The daily dosage levels were determined, according to the GGA doses of previous experiments [27]. At the end of the treatment period, the mice were sacrificed. The volume of ascites was measured, and tumor tissue was weighed.

Statistical analysis. Results are presented as means ± SD. Data were analyzed using one-way ANOVA followed by an unpaired Student's *t*-test for comparison between groups. Differences between groups were considered statistically significant at $p < 0.05$.

Results

LPA-induced cancer cell proliferation was inhibited by GGA

We recently demonstrated that GGA inhibited LPA-induced ovarian cancer cell migration *in vitro* [13]. We also examined the effect of GGA on cancer cell proliferation, based on the findings of previous studies [17,18]. The effects of GGA on the LPA-induced proliferation of Caov-3 cells were studied by performing the MTT assay in the absence or presence of various concentrations of GGA for 3 days incubation. Although GGA did not alter the proliferation or apoptosis after 24 h of treatment [13], the effect of longer treatments (48 or 72 h) with GGA were examined in Caov-3 cells *in vitro*. LPA stimulated Caov-3 cell proliferation by ~30% compared with non-stimulated cells after 48–72 h incubation. GGA significantly inhibited LPA-induced cancer cell proliferation at more than 30 μM and exhibited a dose-dependent inhibition (Fig. 1). Higher concentration (more than 50 μM) of GGA inhibited cell proliferation

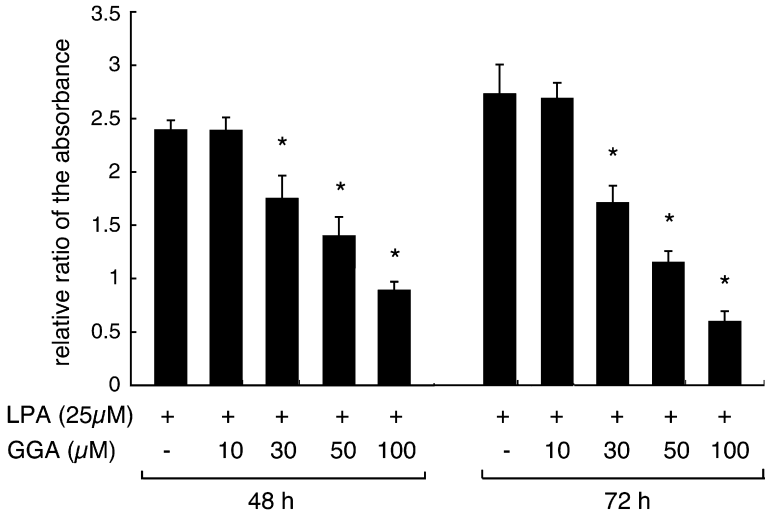

Fig. 1. GGA inhibits the growth of Caov-3 cells. Caov-3 cells were incubated with various concentration of GGA (1–100 μM) in the presence of 25 μM LPA. After 48 and 72 h of incubation, cell proliferation was assessed by the MTT assay. Cell growth was expressed as the ratio of the absorbance of GGA-treated and control cells to that of the cells at the time the medium was replaced. Each point is the mean value of at least four replicate experiments ± SD. $^{*}p < 0.01$ relative to LPA treated cells.

below the non-stimulated level. These results suggest that GGA has not only an anti-invasive effect, but also an anti-proliferative effect on ovarian cancer cells *in vitro*.

LPA-induced Ras activation was inhibited by GGA

Since GGA exerted an anti-proliferative effect on Caov-3 cells, we hypothesized that the mechanism of this inhibitory effect of GGA on LPA-induced cancer cell proliferation is due to the inactivation of Ras, which is a potent molecule controlling proliferation and apoptosis. To confirm this, we measured the intracellular levels of the GTP-bound, active form of Ras using the pull-down assay system. Because the activation of Ras was reported to reach a peak after 2 min of LPA treatment (our preliminary data and Ref. [27]), we examined the inhibitory effect of GGA on the activation of Ras after 2-min treatment with LPA. As shown in Fig. 2, the level of the active form of Ras was elevated after the addition of LPA, and GGA inhibited the

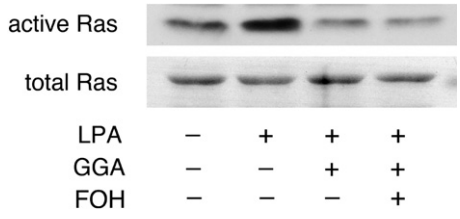

Fig. 2. The activation of Ras by LPA is attenuated by GGA. Cells were pretreated with GGA and GGOH for 24 h. And then cells were stimulated with 25 μM LPA for 2 min. After lysis, cell lysates were incubated with Raf-1 RBD-agarose beads. The beads were washed, and bound Rho proteins were detected by Western blotting using monoclonal antibody against Ras (top panel). Western blotting of the total amount of Ras in cell lysates (bottom panel) was performed for the comparison of Ras activity (level of GTP-bound Ras) in the same lysates. Blots are representative of three experiments. The concentrations of agents that were used in this figure are as follows: LPA 25 μM; GGA 100 μM; GGOH 10 μM.

Ras activation induced by LPA. Because GGA has the similar structure to GGPP, which is important in the prenylation of Ras, it seemed possible that GGOH or FOH might modulate the GGA-induced inhibition of Ras activation. However, the addition of GGOH and/or FOH in the presence of GGA did not restore the activation of Ras (data not shown). The profiles of the responses to LPA and the modulation by GGA and FOH were also the same as in SKOV-3 cells (data not shown). These results suggest that the anti-proliferative effect of GGA is derived from the inhibitory effect of GGA on Ras activation *via* other mechanisms than the inhibition of prenylation.

The inhibitory effect of GGA on LPA-stimulated tyrosine phosphorylation of MEK and Erk

The MEK-Erk pathway is downstream of Ras activation, and tyrosine phosphorylation of these proteins is essential for cancer cell proliferation [19]. Therefore, we evaluated the effect of GGA on the phosphorylation of these proteins by Western blotting (Fig. 3). First we assessed the time course of the LPA-induced tyrosine phosphorylation of these proteins and found that tyrosine phosphorylation of these proteins reached a peak after 30 min of treatment (data not shown), as previously reported [28]. Therefore, we compared the phosphorylation of these proteins in cells treated with various concentrations of GGA after treatment with LPA for 30 min. The results of Western blotting showed that GGA inhibited the tyrosine phosphorylation of MEK and Erk dose-dependently and that the phosphorylation was not restored by FOH or GGOH (Fig. 3).

Effects of GGA in in vivo ovarian cancer model

Thus, GGA has anti-invasive [13] and anti-proliferative effects on Caov-3 cells *in vitro*. Therefore, questions remain

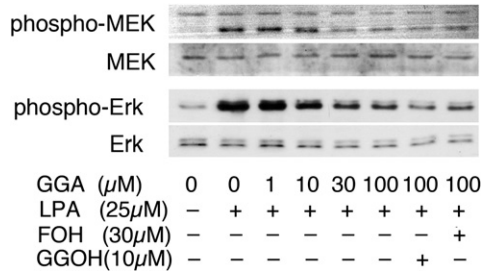

Fig. 3. Western blot analysis of the effect of GGA on LPA-stimulated tyrosine-phosphorylation of MEK and Erk. Caov-3 cells were cultured under serum-free conditions with or without various agents described in the figure for 24 h. After incubation, cells were stimulated with 25 μM LPA for the indicated times described in the text. After cell lysis, lysates were resolved by 10% SDS–PAGE, and immunoblotted with anti-phosphospecific MEK, or anti-phosphospecific Erk antibody. To demonstrate specificity and equal loading, the membranes were stripped and reprobed with anti-MEK or anti-Erk antibody. Blots are representative of three experiments.

regarding the ability of GGA to inhibit ovarian tumor growth *in vivo*. To assess the effect of GGA on tumor development *in vivo*, we utilized a model of intraperitoneal ovarian carcinoma in athymic immunodeficient mice [25]. We started the oral administration of the drug from the day after cancer cell inoculation in the GGA-treated group, and treated the control group animals with vehicle orally. We sacrificed and examined the mice 7 weeks after cancer cell inoculation, because preliminary experiments revealed that tumor-bearing mice began to exhibit abdominal swelling with ascites around 4 weeks after the cancer cell inoculation, and died with cachexia after 8 weeks without any treatment. At postmortem examination, tumors were found on the surface of the peritoneum, diaphragm, intestines, omentum and uterus, with massive ascites in the control group. Body weight, intraperitoneal tumor burden and ascites were quantified. As shown in Fig. 4B, mean tumor burden in the control group (2.36 ± 0.33 g) was significantly reduced (by ~75%) in the 100 mg/kg administered group (0.52 ± 0.52 g; $p < 0.001$). Ascites formation was similarly reduced in the 100 mg/kg administered group (2.13 ± 2.11 ml), compared with the control group (6.35 ± 1.35 ml) (Fig. 4C). The inhibitory effects of GGA on ascites formation and tumor burden were dose-dependent. GGA in this dose range did not impair the viability of mice and did not change the actual body weight, because the difference of body weight between the control group and treated group was accounted by the tumor burden and ascites (Fig. 4D). These data suggest that GGA has anti-tumor effects *in vivo*, and tested doses of GGA did not induce pathological features and are appropriate for *in vivo* studies.

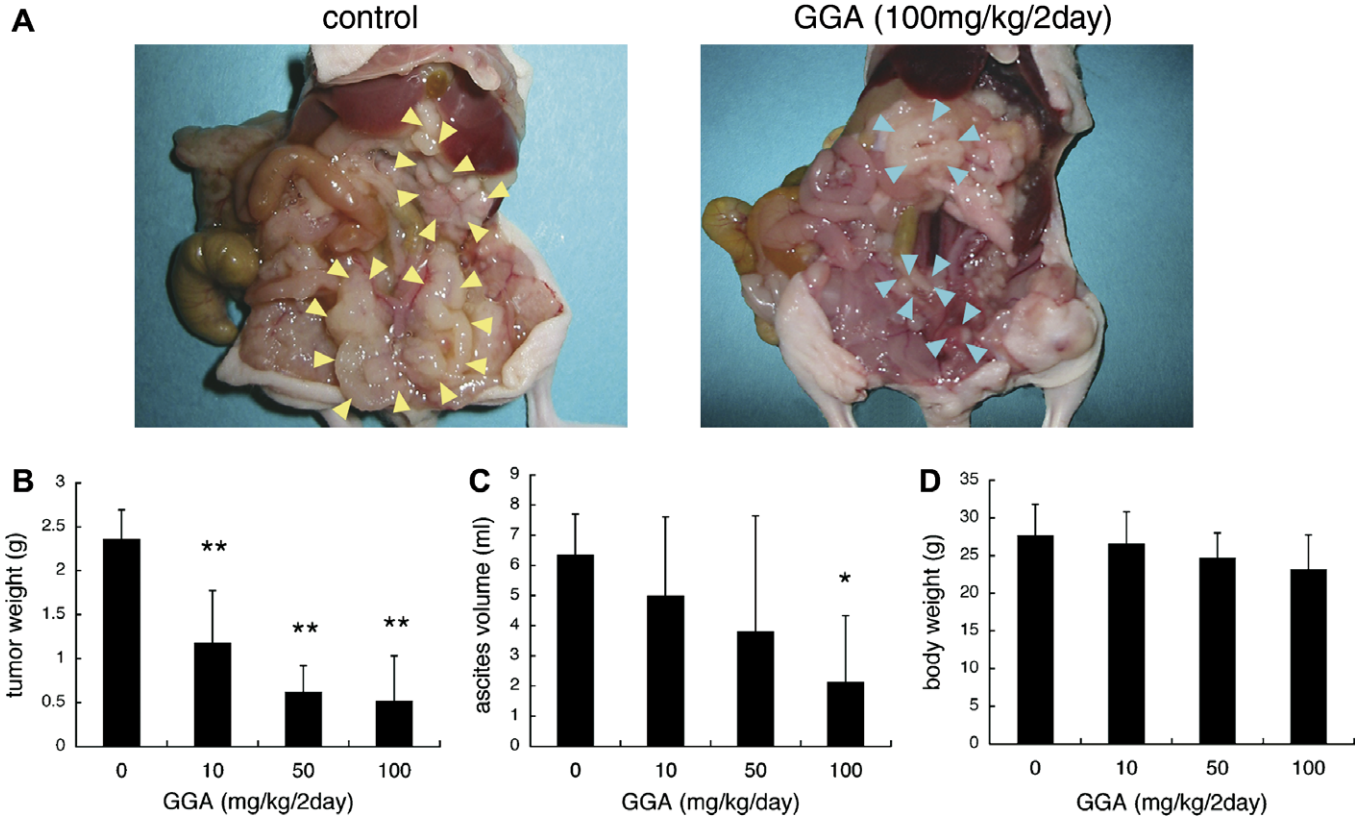

Fig. 4. The effect of GGA on i.p. tumor burden (B) and ascites formation (C) in athymic immunodeficient mice inoculated with Caov-3 cells. Athymic mice were inoculated with Caov-3 cells i.p., treated without or with oral GGA for 7 weeks. (A) Representative pictures of Caov-3 bearing mice without (left) or with oral GGA treatment (right). (B) Effects of GGA on tumor growth. (C) Effects of GGA on ascites formation. (D) Effects of GGA on body weight in Caov-3-inoculated mice. Data are expressed as the means; bars, ±SD. $^{*}p < 0.01$ and $^{**}p < 0.001$ relative to control.

Discussion

A distressingly high percentage of ovarian cancer patients with a complete response will eventually experience relapse [23,24]. There is universal interest in additional therapy after complete remission that might delay or prevent relapse. Because patients are clinically free of disease, preventive therapies ideally should also carry minimal toxicity and not adversely impact the patient's quality of life. The advantage of GGA is that it is already in clinical use and has a proven safety profile. Laboratory data revealed no detrimental events, even at the massive dosage of 500 mg/kg of body weight [26]. Therefore, we intend to examine the preventive effect of GGA after complete remission and applied GGA to the preventive protocol of *in vivo* ovarian cancer model [25]. We previously reported that GGA inhibited tumor cell invasion through modulating Rho/ROCK signaling *in vitro* [13]. In this study, GGA also inhibited the proliferation of cancer cells in a dose-dependent manner after longer treatment than in the experiments examining the anti-invasive effect. A few studies have examined the mechanism by which GGA inhibits the cancer cell proliferation and induces apoptosis [17,18]. In those reports, the anti-proliferative mechanism of GGA has not been clearly elucidated. We first revealed that GGA interfere Rho and Ras activation and prevents cancer cell invasion and proliferation.

Among the various signaling pathways triggered by tumor-progression factors is the Ras/Raf/MEK/Erk pathway. This pathway has been shown to play important roles in cell proliferation and the prevention of apoptosis [19], and aberrant activation of this pathway is commonly observed in malignantly transformed cells. In this study, LPA enhanced Ras activation in ovarian cancer cells and GGA reversed this activation to below the control level. Ras protein undergoes the following sequential posttranslational processing events. First, the cysteine (i.e., the C of the carboxyl-terminal CAAX sequence) is isoprenylated by protein farnesyltransferase or geranylgeranyltransferase type I [19]. Second, the last three amino acids of the protein (i.e., the-AAX) are cleaved off by Rce-1, an integral membrane protein of the ER [29]. Third, the newly exposed isoprenylcysteine is methylated by an ER membrane-bound methyltransferase, isoprenylcysteine carboxylmethyltransferase (Icmt) [30]. These modifications render the C terminus of RAS protein more hydrophobic, facilitating binding to membranes and inducing full function [31]. Neither GGOH nor FOH prevented the GGA-induced inhibition of Ras activation. Therefore, GGA might not interfere with the first step, isoprenylation of Ras. The structure of GGA suggests a possible interaction with Rce-1 or Icmt. Because both of these enzymes act on Ras and Rho it seems possible that GGA might interfere with the interaction between these enzymes and prenylated small GTPases.

Relatively long exposure and high concentrations of GGA inhibit cancer cell growth. The concentration range causing growth inhibition is more than 30 μM. On the other hand, motility inhibition occurs at more than 1 μM GGA. Furthermore, a pharmacokinetic study in humans reveled that the serum concentration of GGA after oral administration of the usual dosage is 1–10 μM [32]. We could not discriminate whether the anti-tumor effect *in vivo* is mediated through an anti-invasive effect, anti-proliferative effect or both. We further need to proceed to the pharmacodynamic/pharmacokinetic study of GGA.

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