Isoprenoid geranylgeranylacetone inhibits human colon cancer cells through induction of apoptosis and cell cycle arrest

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Geranylgeranylacetone (GGA), an isoprenoid compound, is a widely used antiulcer drug developed in Japan. GGA is structurally similar to plaunotol and geranylgeraniol, another isoprenoid reported to exert strong anticancer effects. In an earlier study, GGA was shown to inhibit ovarian cancer invasion by attenuating not only Rho activation, but also Ras–MAPK activation. In this study, we aimed to test whether GGA could have a therapeutic effect on colon cancer cells. As a result, we found that GGA induced a dose-dependent decrease in the proliferative activity through induction of cell apoptosis and cell cycle arrest in the G_1 phase. The induction of apoptosis was mediated by the activation of both caspase-8 and caspase-9 pathways. The induction of G_1 arrest was mediated by the increase of p21 and p27, and also the decrease of phosphorylated retinoblastoma protein levels. This study showed the potential anticancer activity of GGA. As this drug is already available in Japan for clinical use as an antiulcer/antigastritis agent, clinical trials will be designed to confirm its potential usefulness for cancer patients.

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Introduction

Geranylgeranylacetone (GGA), an isoprenoid compound developed in Japan, is a widely used oral antiulcer drug (Fig. 1a). GGA protects gastric mucosa from various stresses without affecting gastric acid secretion [1]. Moreover, the chemical structure of GGA is similar to that of geranylgeranylpyrophosphate, which is a component of the Rho metabolic pathway, as it is essential for geranylgeranylation of Rho [2]. In an earlier study, GGA was shown to inhibit Rho activity, cell motility, and invasion of cancer cells [3], in the same way as HMG-CoA reductase inhibitors [4–6] and bisphosphonates do [7,8]. GGA has also been shown to suppress cell growth, and induce differentiation or apoptosis by modulating small G protein activation in leukemia cell lines [9,10], and to inhibit ovarian cancer progression through inhibiting not only Rho, but also Ras–MAPK activity [11]. Some isoprenoids, such as docetaxel and paclitaxel [12,13], with strong anticancer effects [14,15], are already available in clinical settings. Recently, other isoprenyl compounds, such as farnesol, geranylgeraniol (GGOH), and geranylgeranoic acid have been shown to induce apoptotic cell death [16,17] and modulate cell motility [4,7]. GGOH is an intermediate product in the mevalonate pathway and functions as an essential regulator of cell proliferation [14,15,18], with known anticancer properties [16,19,20]. It was shown to induce caspase-dependent apoptosis of human hepatocellular carcinoma cells through inhibition of bcl-2 family proteins [21]. Recent studies have shown that GGOH has apoptosis-inducing activity in other cancer cell types as well, through activation of caspase-8 and suppression of bcl-xL [19,21,22]. Plaunotol is another isoprenoid structurally similar to GGOH, purified from the extract of a Thai medicinal plant named plau-noi, and clinically available in Japan as an antigastric ulcer agent. Its protective effect on gastric mucosa is mediated by several mechanisms, such as the increase of prostaglandin E_2 content in gastric tissue and the reduction of superoxide radicals generated by leukocytes [23–25]. Recently, we showed that plaunotol also exerts dual anticancer effects through inhibition of tumor angiogenesis [26] and direct inhibition of gastric and colon cancer cells [27,28]. Similar to other isoprenoids, the inhibitory effect of plaunotol was also dependent on caspase-mediated apoptosis [27,28]. At present, little is known of the anticancer properties of GGA. Similar to plaunotol, GGA is already available for clinical use in Japan as an antigastric ulcer agent, and if its anticancer property is confirmed, its use in clinical trials will be feasible. In

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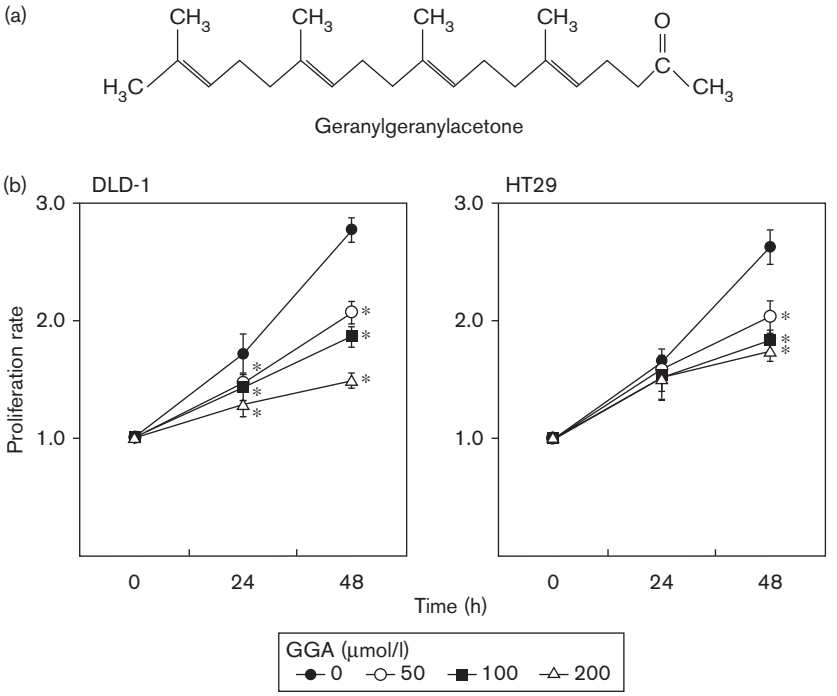
Fig. 1



(a) Chemical structure of geranylgeranylacetone (GGA). (b) Effect of GGA on the proliferation of colon cancer cells. The proliferative activity of DLD-1 and HT29, cultured without or with various concentrations of GGA for 24 or 48 h, was assessed by the MTS assay. The results are expressed as mean ± standard deviation of the results obtained in three independent experiments and an asterisk indicates statistical significance.

this study, therefore, we aimed to investigate the effect of GGA on colorectal cancer, which is one of the malignancies that has been markedly increasing over the last several decades in Japan, and is also among those with the highest incidences in the world. For this purpose, we used the in-vitro model of human colon cancer cell lines DLD-1 and HT29, and showed the significant inhibitory effect of GGA on cell proliferation, dependent partly on induction of apoptosis and partly on cell cycle arrest.

Materials and methods

Reagents

GGA was supplied by Eisai Co. (Tokyo, Japan). GGA was dissolved in dimethylsulfoxide (Sigma, St Louis, Missouri, USA) as a stock solution and then diluted with the culture medium for experiments. The final concentration of dimethylsulfoxide was maintained at 0.2% for all treatments, including control.

Cancer cell culture

Two human colon cancer cell lines, namely, DLD-1 and HT29, were obtained from the Japanese Cancer Research Resource Bank, and cultured in RPMI-1640 medium (Sigma-Aldrich, Tokyo, Japan) supplemented with 5% fetal calf serum and 1% antibiotic/antimycotic in an atmosphere of 5% CO_2 at 37°C.

Isolation of human umbilical vein endothelial cells and culture

Umbilical cords were obtained from normal pregnancies at delivery, after informed consent was obtained. Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cord blood vessels as described earlier [29]. Briefly, the umbilical vein was cannulated at both the edges, and 0.2% collagenase-I in Ca^{2+}–Mg^{2+}-free PBS (–) was added to the lumen of the vessel. The umbilical cord was incubated at 37°C for 20 min, and then the PBS (–) containing collagen was recovered and centrifuged to obtain the isolated cells. Cells were washed twice with PBS (–), suspended in complete medium consisting of human endothelial serum-free medium (Gibco, Life Technologies, Paisley, UK) supplemented with 5% fetal calf serum, 1% antibiotic/antimycotic, human vascular endothelial growth factor (5 ng/ml; PeproTech, Rocky Hill, New Jersey, USA), basic fibroblast growth factor (20 ng/ml; Gibco), and heparin–sodium salt (5 μg/ml; Sigma), and seeded in culture dishes previously coated with 0.1% gelatin. Cells were routinely cultured in complete medium in an atmosphere of 5% CO_2 at 37°C, passaged by trypsinization; up to 10 passages were used for the experiments.

In-vitro proliferation assay

Colon cancer cells were seeded at 2×10^3 cells/well in 96-well flat-bottomed plates in culture medium

containing different concentrations of GGA (0, 50, 100, or 200 μmol/l). HUVECs were seeded at 5×10^3 cells/well in 96-well flat-bottomed plates in culture medium and allowed to adhere for 24 h. The bottom of each well was earlier coated with 0.1% gelatin. Thereafter, medium containing differing concentrations of GGA was added to give the final concentrations of 0, 50, 100, 200 μmol/l. After 0, 24, or 48-h culture, the proliferative activity of the cells was determined by the MTS assay (CellTiter 96 nonradioactive cell proliferation assay; Promega, Madison, Wisconsin, USA), according to the manufacturer's recommendations. Proliferation rates were calculated as the ratios to values measured at day 0 that were considered 1.0.

Detection of apoptosis by flow cytometry

Colon cancer cells were treated with different concentrations of GGA (0, 50, 100, or 200 μmol/l) for 48 h. HUVECs were prepared and treated with GGA (0, 50, 100, or 200 μmol/l) for 24 or 48 h as described above. Cells were washed twice with PBS (−), incubated with fluorescein isothiocyanate (FITC)-conjugated Annexin V and propidium iodide (PI) (Biovision, Mountain View, California, USA) for 5 min at room temperature, and analyzed by flow cytometry (FACSCalibur; Becton-Dickinson, San Jose, California, USA). This method allows the distinction between early Annexin V–$FITC^+/PI^-$ and late Annexin V–$FITC^+/PI^+$ apoptosis cells. Data were collected in a FACSCalibur (Becton Dickinson) and analyzed using CellQuest software (Becton Dickinson).

Caspase activity

Caspase-3, 8, and 9 activities were evaluated using caspase-3, 8, and 9 detection kits (Oncogene, San Diego, California, USA). Cells treated with GGA (0, 50, 100, or 200 μmol/l) for 48 h were washed twice and suspended in PBS (−) containing FITC-conjugated specific caspase inhibitors DEVD-FMK (Asp-Glu-Val-Asp-fluoromethyl-ketone), IETD-FMK (Ile-Glu-Thr-Asp-fluoromethyl-ketone), or LEHD-FMK (Leu-Glu-His-Asp-fluoromethyl-ketone) for the detection of caspase-3, 8, or 9, respectively. Caspase inhibitors were allowed to permeate into the cells and bind irreversibly to the activated caspases for 1 h at 37°C. The fluorescence retained in the cells was analyzed by flow cytometry (FACSCalibur, Becton Dickinson). Data were analyzed using CellQuest software (Becton Dickinson).

Detection of cell cycle distribution by flow cytometry

Under subconfluent conditions of colon cancer cells, medium containing differing concentrations of GGA was added to yield the final concentrations of 0, 50, 100, and 200 μmol/l. After 48 h of culture, cells were harvested by trypsinization, washed twice in PBS (−). DNA contents of cells were measured using a DNA staining kit (CycleTEST PLUS DNA Reagent kit; BD Biosciences, Bedford, Massachusetts, USA). PI-stained nuclear fractions were obtained as described in the manufacturer's instructions. Data were analyzed using CellQuest Software (Becton Dickinson) with flow cytometry. Cell cycle distributions were calculated using ModFit LT 2.0 software (Verity Software House, Topsham, Maine, USA).

Western blotting

Cells were treated with different concentrations of GGA (0, 50, 100, and 200 μmol/l) for 48 h. Thereafter, cells were lysed with 200 μl of Tris–saline (50 mmol/l Tris–HCl, pH 7.6, and 150 mmol/l sodium chloride) containing various protease inhibitors (1 mmol/l EGTA, 0.1 mmol/l diisopropyl fluorophosphates, 0.5 mmol/l phenylmethylsulfonylfluoride, 1 mg/ml Na-P-tosyl-L-lysine chloromethyl ketone, 1 mg/ml antipain, 0.1 mg/ml pepstain, and 1 mg/ml leupeptin) and 1% Triton-X for 1 h (on ice) for protein extraction. SDS–polyacrylamide gel electrophoresis was performed as described earlier [30] using a Laemmli buffer system and 7.5 or 15% polyacrylamide gel (Ready Gel J; Bio-Rad, Hercules, California, USA). After the membrane strips had been electrophoretically transferred to polyvinylidine difluoride membranes (Immobilon P; Millipore, Bedford, Massachusetts, USA), they were incubated in the presence of either primary antibody. The primary antibodies used were mouse antihuman p21 and rabbit antihuman p27 and phosphorylated retinoblastoma protein (p-Rb), purchased from Cell Signaling Technology (Beverly, Massachusetts, USA). Mouse antihuman cyclin-dependent kinase 2 (CDK2), CDK4, cyclin D1, and cyclin E were purchased from MBL (Nagoya, Japan). Mouse antihuman β-actin was purchased from Sigma (Sigma-Aldrich), and used as a loading control. Thereafter, membrane strips were incubated with a 1 : 500 dilution of biotinylated anti-mouse immunoglobulin G (Vector Laboratories Inc., Burlingame, California, USA) as the secondary antibody, followed by treatment with the avidin–biotin complex (ABC Kit; Vector Laboratories Inc) solution for 30 min, and color development was carried out with a solution of diaminobenzidine (DABKit; Dako, Carpinteria, California, USA). Relative densitometric units were determined using the analysis software, Image J software (open source Image J Software available at *http://rsb.info.nih.gov/ij/*).

Statistical analysis

Statistical analysis was performed using PASW statistics software, Version 18 (SPSS Inc., Chicago, Illinois, USA). The unpaired Student's *t*-test was used to determine statistical significance. Differences at a P value less than 0.05 were considered statistically significant.

Results

Geranylgeranylacetone inhibited proliferation of colon cancer cells

The effect of GGA on colon cancer cells DLD-1 and HT29 was investigated *in vitro* using the MTS assay. As shown in Fig. 1b, in the presence of GGA, the proliferative activity of both colon cancer cell types was

dose-dependently inhibited. The number of viable cells 48 h after treatment with the highest dose (200 μmol/l) was 53.8% of untreated control in DLD-1 and 66.5% of untreated control in HT29, both decreases being statistically significant ($P < 0.05$).

Geranylgeranylacetone-induced apoptosis of colon cancer cells

To determine the mechanism of the inhibitory effect of GGA on the proliferative activity of colon cancer cells, we performed the apoptosis detection assay. Culture in the presence of GGA for 48 h resulted in a significant increase in the population of Annexin V^+/PI^- early apoptotic cells and Annexin V^+/PI^+ late apoptotic cells in a dose-dependent manner (Fig. 2b). Although both populations of Annexin V^+/PI^- and Annexin V^+/PI^+ cells were significantly increased, no increase in the population of Annexin V^-/PI^+ cells, which represent necrosis, was observed (Fig. 2a). These results suggested that the cell death induced by GGA was through induction of apoptosis, but not through necrosis.

Geranylgeranylacetone-induced apoptosis of colon cancer cells was caspase dependent

The activation of caspases 3, 8, and 9, which play the central roles in the apoptotic cascade, was investigated. As shown in Fig. 2c, all examined caspases were activated by GGA treatment for 48 h in a dose-dependent manner.

Geranylgeranylacetone-induced cell cycle arrest of colon cancer cells

To determine whether the antiproliferative effect of GGA on colon cancer cells could also be mediated through an alteration of the cell cycle, we analyzed the cell cycle distribution of GGA-treated colon cancer cells for 48 h by flow cytometry. As a result, treatment of colon cancer cells with GGA resulted in a dose-dependent inhibition of progression from the G_1 to the S phase, and in the consequent decrease of cells in the S phase, leading to an evident increase in the percentage of cells in the G_1 phase (Fig. 3b). Thus, the inhibitory effect of GGA on colon cancer cell proliferation was concluded to be a consequence of both apoptosis and cell cycle arrest.

Geranylgeranylacetone-induced cell cycle arrest was mediated by p-Rb, p21, and p27

Next, we examined the pattern of expression of the key cell cycle regulators, namely, p21, p27, cyclin D1, cyclin E, CDK2, CDK4, and p-Rb in GGA-treated colon cancer cells. We confirmed that each cell cycle regulatory protein was constitutively expressed in both colon cancer cell types. GGA treatment decreased p-Rb and increased p21 and p27 in a dose-dependent manner, whereas it did not show any significant effect on cyclin D1, cyclin E, CDK2, and CDK4 (Fig. 4).

The effect of geranylgeranylacetoneon normal human endothelial cells

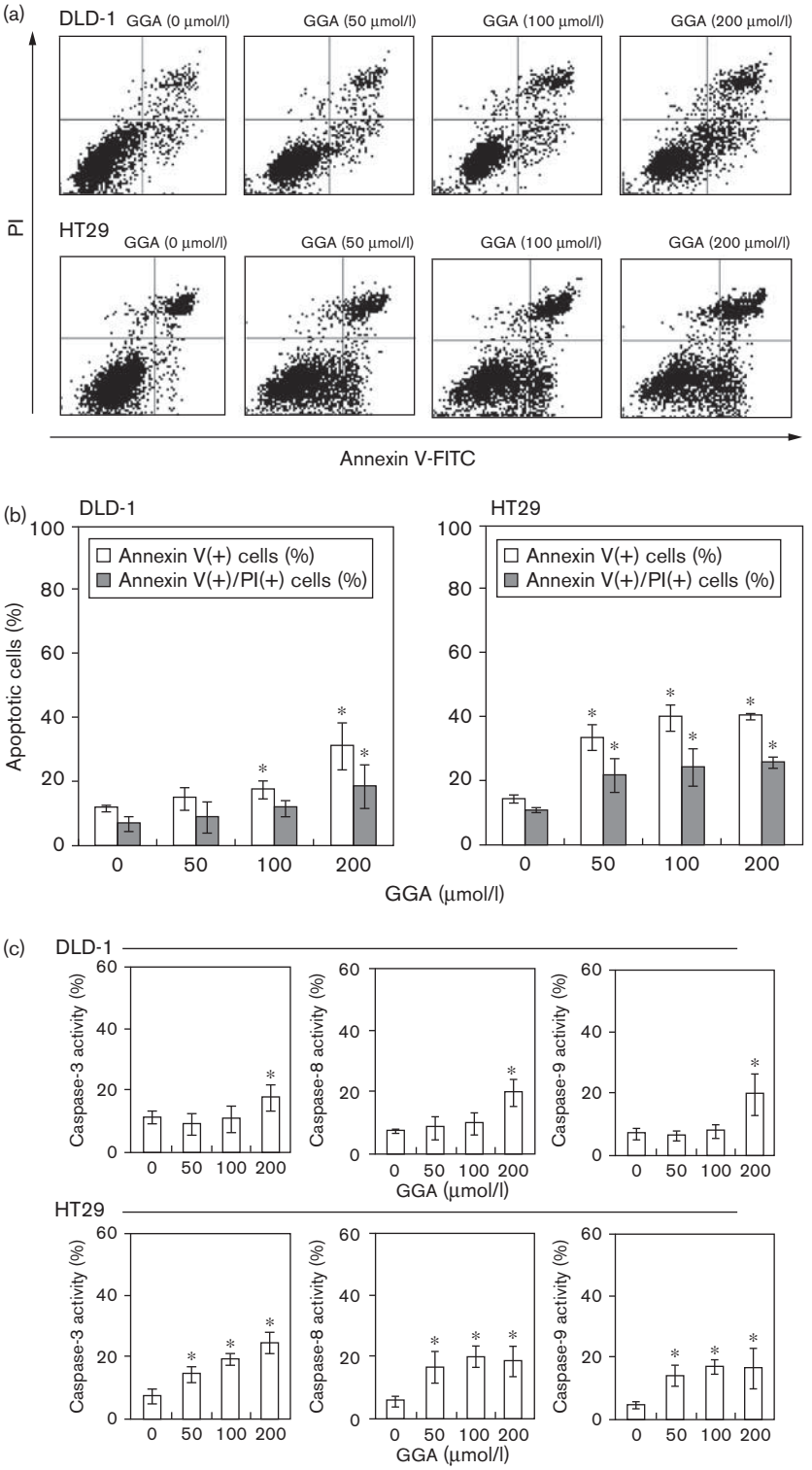
Human ECs were used as the in-vitro model of normal cells, and the effect of GGA on their proliferation and survival was tested. GGA had minimal inhibitory effect on the proliferation of ECs at doses of 50 and 100 μmol/l (Fig. 5a). A significant increase in the rate of early apoptotic cells was observed by treatment with GGA at the same doses, in a dose-dependent manner (Fig. 5b). At the highest dose of GGA tested (200 μM), almost all cells died, which was attributed to a cytotoxic effect of GGA (data not shown).

Discussion

GGA is known to have antiulcer effects through induction of heat-shock protein and nitric oxide synthase in gastric mucosa [31], and protective effects against myocardial ischemia/reperfusion injury [32] and antiviral effect against influenza virus infection [23]. However, only a few reports on its effects on cancer can be found in the literature [3,9,11]. We have earlier shown that plaunotol, another isoprenoid that is structurally similar to the GGA, is a promising anticancer agent, because of its direct inhibitory effect on gastric and colon cancer cells, and also because of an antiangiogenic effect. Plaunotol dose-dependently inhibited the proliferative activity of gastric and colon cancer cells, which was dependent on induction of cell apoptosis [27,28]. In addition, plaunotol inhibited the proliferative activity of ECs *in vitro*, and through the specific inhibition of integrin $\alpha_V\beta_3$, it inhibited the ability of ECs to adhere and spread on gelatin and vitronectin, and also to form tube-like structures on Matrigel; therefore, it was concluded that it inhibits important steps of angiogenesis [26].

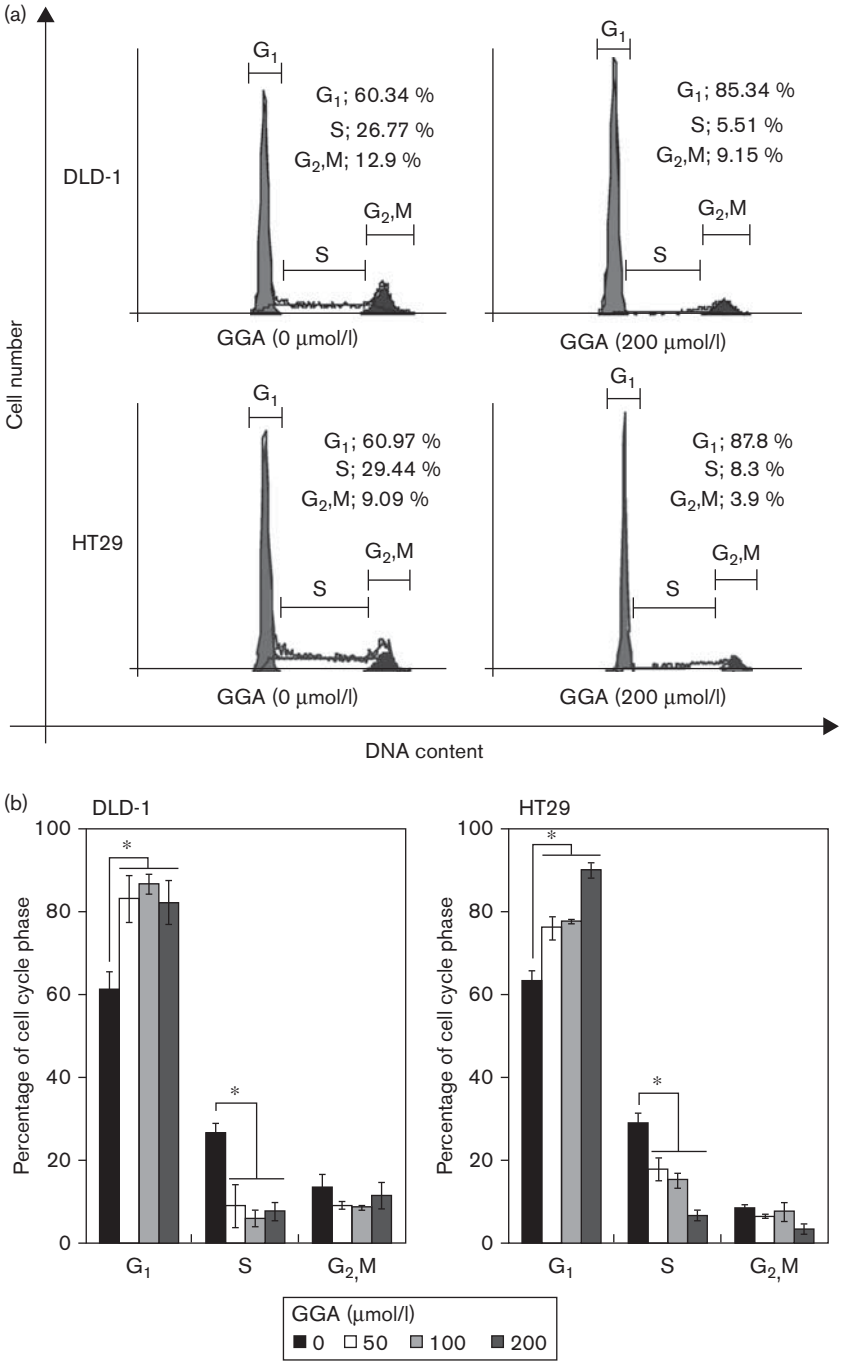
In this study, GGA had a strong inhibitory effect on both colon cancer cell types, DLD-1 and HT29, supporting the potential anticancer effects of isoprenoids on tumor progression. The inhibitory effects of GGA were confirmed to be dependent on the induction of apoptosis of colon cancer cells. A significant and dose-dependent increase of early and late apoptotic cells was observed 48 h after GGA treatment. It was associated with the activation of both major caspase pathways, the death receptor pathway, involving caspase-8, and the mitochondrial pathway, in which various signals can trigger the release of harmful proteins by mitochondria into the cytoplasm, leading to activation of caspase-9. Both pathways result in the downstream activation of caspase-3 [33]. Incubation with GGA invoked a dose-dependent activation of caspase-3. Cell cycle arrest induced by GGA in colon cancer cells was dependent on the increase of p21 and p27, and on a decrease in the expression of p-Rb. p21, also known as WAF1, Cip1, CAP20, and Sdil [34–38], and p27, also known as Kip1 [39,40], are the founding members of the Cip/Kip family of CDKIs (CDK

Fig. 2



Apoptotic effects induced by geranylgeranylacetone (GGA) on colon cancer cells. (a) Apoptosis was determined by staining the cells with Annexin V (*x*-axis) and propidium iodide (PI) (*y*-axis). Representative results are shown. (b) Data are expressed as relative numbers of Annexin V^+/PI^- early apoptotic cells and Annexin V^+/PI^+ late apoptotic cells after treatment with increasing concentrations of GGA for 48 h. (c) Activation of caspase-3, 8, and 9: DLD-1 and HT29 were incubated for 48 h without or with GGA, and the relative numbers of caspase-activated cells evaluated by flow cytometry are shown. The results represent mean ± standard deviation of three independent experiments and an asterisk indicates statistical significance.

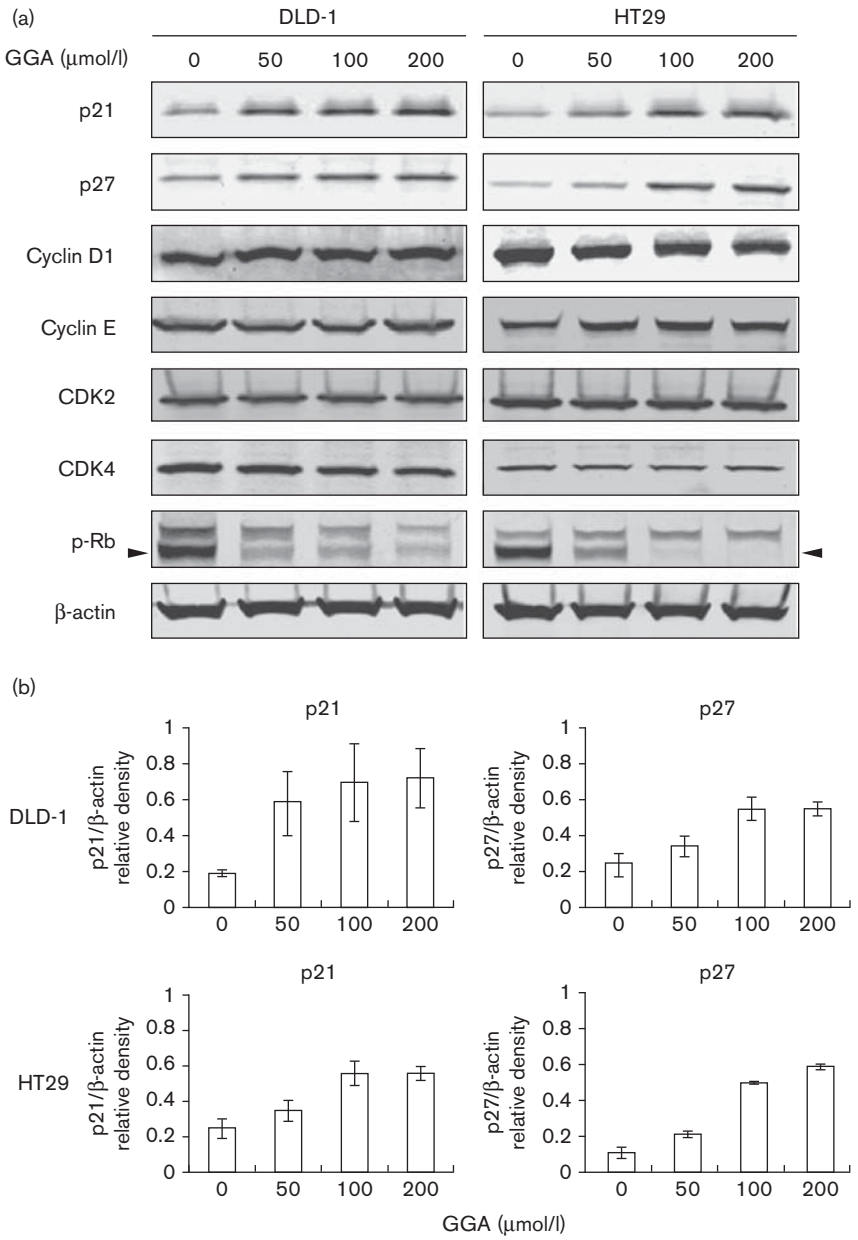
Fig. 3



(a) Cell cycle distribution of colon cancer cells treated without or with different doses of geranylgeranylacetone (GGA), analyzed by flow cytometry. Representative results are shown. (b) Treatment with GGA for 48 h resulted in a dose-dependent accumulation of cells in the G_1 phase, with a corresponding decrease in cells in the S phase. The results represent mean ± standard deviation of three independent experiments and an asterisk indicates statistical significance.

inhibitors). Overexpression of CDKI proteins suppresses cell cycle progression in the G_1 phase by inhibiting cellular cyclin/CDKs (CDKs) [41–43]. Tumor-suppressor proteins often cause G_1 arrest of cancer cells through upregulation of the expression levels and/or activities of CDKIs. Among these various CDKIs, p21 functions by decreasing cell cycle-regulated CDK activity, which in turn decreases the p-Rb family members and ultimately E2F transcriptional activity. p27, which binds to CDK2 and inhibits the CDK2/cyclin E complex, has drawn major

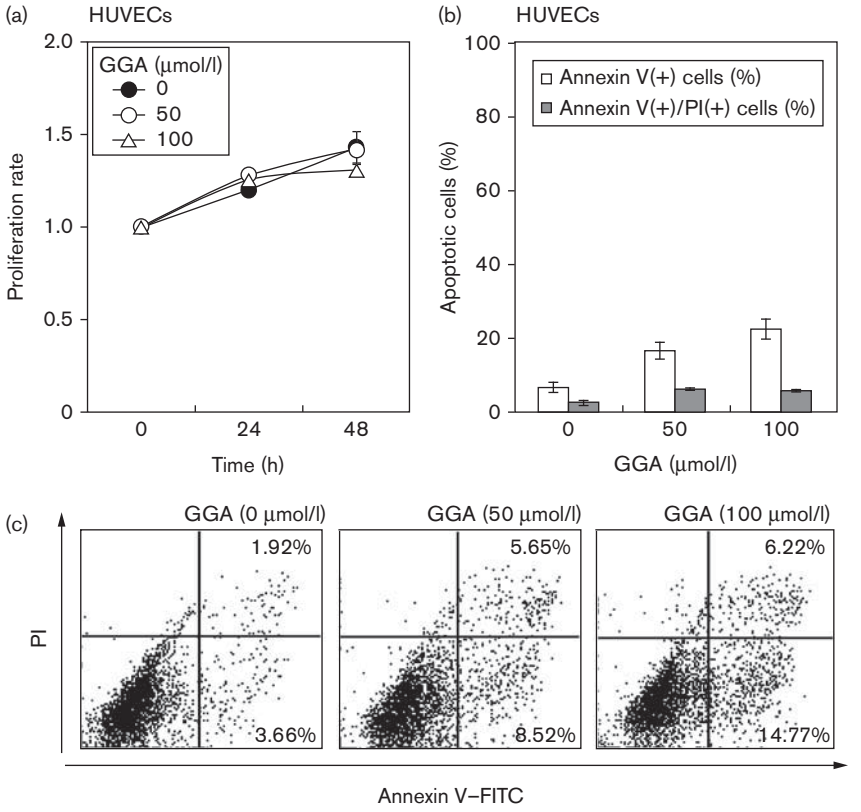
Fig. 4



The effect of geranylgeranylacetone (GGA) on the protein expression of cyclin-dependent kinase inhibitors (CDKIs), CDKs, cyclins, and phosphorylated retinoblastoma protein (p-Rb) in DLD-1 and HT29. (a) Cells were cultured for 48 h without or with GGA (50, 100, and 200 μmol/l), harvested, and the protein lysates subjected to 10% SDS–polyacrylamide gel electrophoresis for analysis of the main cell cycle regulators. The expression levels of CKIs, that is, p21 and p27, were increased in a dose-dependent manner by GGA treatment. pRb was reduced dose dependently (arrowheads, pRb). (b) p21 and p27 expression levels normalized to β-actin. The results represent mean ± standard deviation of two independent experiments.

attention in cancer because of its downregulation in a substantial number of human epithelial malignancies, such as breast, prostate, lung, colon, and head and neck. There is also clinical evidence of a reduced p27 level correlating with tumor aggressiveness and poor patient survival [44]. In this study, we showed that GGA suppressed the proliferation of colon cancer cells through p21 and p27 upregulation. pRb, which is necessary for the progression through G_1, is regulated primarily by the CDK4/cyclin D1 complex, whereas the CDK4/cyclin D1 complex regulated the passage of cells from the late G_1 to the S phase by p21. The GGA-induced cell cycle arrest, therefore, seems to be dependent on the downregulation of pRb in the late G_1 phase.

Fig. 5



The effect of geranylgeranylacetone (GGA) on endothelial cells (ECs). (a) Effect of GGA on the proliferation of ECs. GGA had minimal effect on the proliferative activity of ECs when tested at 50 and 100 μmol/l. (b) The percentages of Annexin V^+/propidium iodide (PI^-) early apoptotic cells and Annexin V^+/PI^+ late apoptotic cells after treatment with GGA for 48 h are shown. The percentage of early apoptotic cells increased dose-dependently of GGA. (c) Representative results of the apoptosis detection in GGA-treated cells after staining with Annexin V (*x*-axis) and PI (*y*-axis). In (a) and (b), results are expressed as mean ± standard deviation of the results obtained in three independent experiments. HUVECs, human umbilical vein endothelial cells.

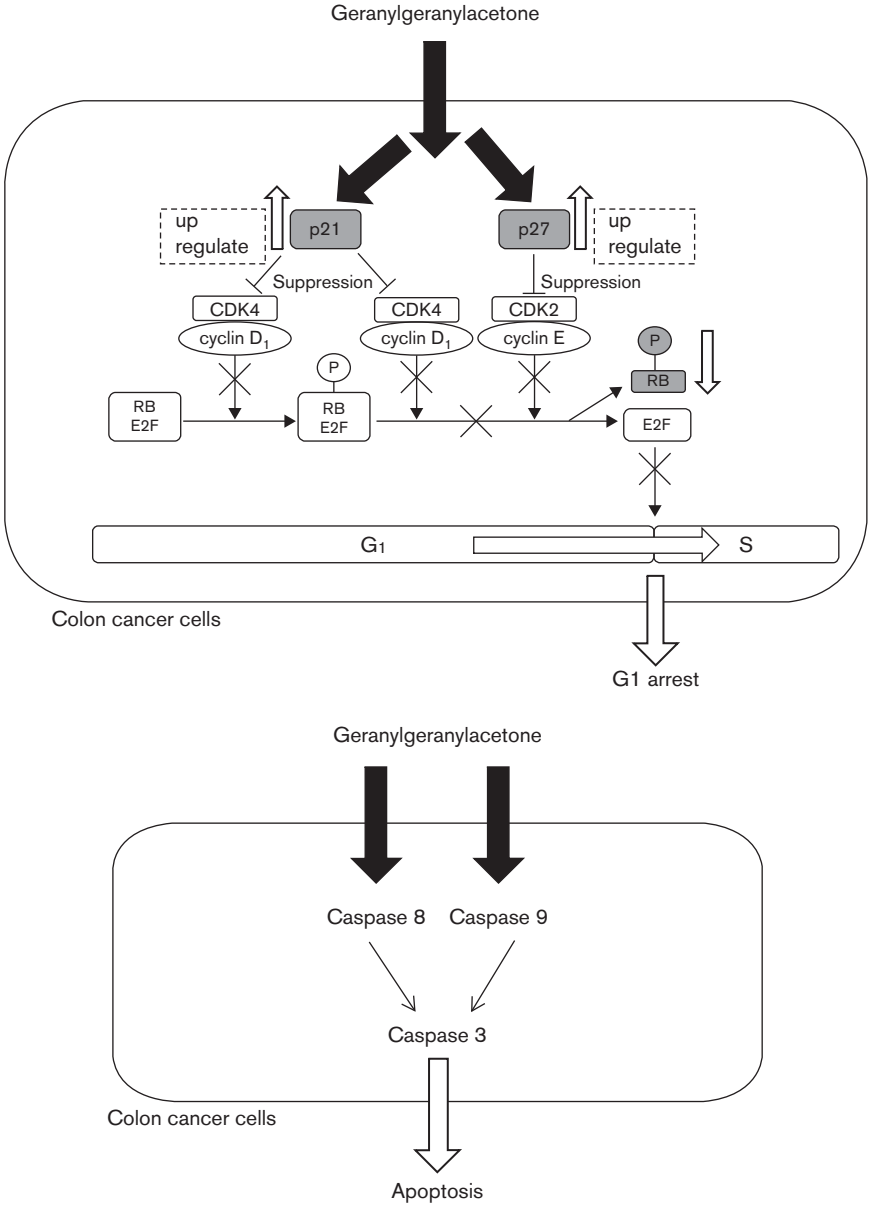
p53 mutation is found in around half of all human tumors [45], which disrupts p53-specific DNA binding, and transcriptional transactivation of target genes, such as p21, GADD45, Bax, Puma, and Noxa. In general, mutant p53 proteins are deficient for specific DNA binding, suggesting that DNA binding and transcriptional regulation of target genes are crucial for p53-mediated tumor suppression. Therefore, in addition to being an important clinical marker, p53 is also a novel therapeutic target. Recently, we analyzed the p53 gene sequences in 10 colon cancer cell lines, and found that, among them, eight (80%) had a p53 mutation of exons 5–8 (data not shown). In this study, GGA suppressed the proliferation of colon cancer cells through p21 and p27 upregulation. These results suggested that GGA should be more effective against colon cancer caused by mutation of the p53 gene.

In addition, to confirm the higher specificity of the inhibitory effect of GGA on cancer cells, the effect of GGA on human ECs, as an in-vitro model of normal cell, was investigated. Compared with colon cancer cells, the inhibitory effect of GGA (50 and 100 μmol/l) on the proliferation of ECs was smaller, and the apoptosis rate. But at the highest concentration (200 μmol/l) of GGA tested, a strong cytotoxic effect on ECs was observed (data not shown), although it was not as cytotoxic against colon cancer cells.

Taking these findings together, we concluded that, similar to other isoprenoids such as plaunotol and GGOH, GGA can exert strong anticancer activities, dependent on the induction of cell cycle arrest and apoptosis, and should be a promising new anticancer agent for the treatment of colon cancer. The hypothetic effects of geranylgeranylacetone on human colon cancer cells are summarized in Figure 6.

However, before being used as an anticancer agent under clinical conditions, it should be tested first in animal models. Therefore, these preclinical experiments are currently under way in our laboratory, and will be

Fig. 6



Summary of the hypothetic effects of geranylgeranylacetone on human colon cancer cells. CDK, cyclin-dependent kinase. P-RB, phosphorylated retinoblastoma protein.

included in our next contribution. In contrast, GGA is already commercially available in Japan, and therefore its clinical safety is confirmed. However, after oral administration of the usual dosage, the GGA serum concentration is 1–10 μmol/l [46], that is, 5–50 times smaller than was necessary to obtain the antiproliferative effects on colon cancer cells in this study. Therefore, there is a need to determine other routes of administration, such as intravenous or intratumoral, in an attempt to achieve doses higher than that achievable by oral administration. From our findings, it can be suggested that at doses lower than 100 μmol/l, the inhibitory effect of GGA is more pronounced against colon cancer cells than against normal ECs; however, there is a need to investigate the possible side effects on other normal tissues exposed to relatively high doses of GGA, and therefore this question will also be addressed in the animal experiments that are currently going on in our laboratory.

In conclusion, in this paper we provide evidence that GGA functions as a promising anticancer drug, by suppressing the proliferative activity of colon cancer cells

through induction of cell cycle arrest and apoptosis. It is still a preliminary trial, but as GGA is already available in Japan and clinically used as a unique antiulcer/antigastritis agent, after addressing some important questions in animal experiments, clinical trials will be conducted to confirm its anticancer effects observed in this study.

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