

Ganoderic acids suppress growth and invasive behavior of breast cancer cells by modulating AP-1 and NF- κ B signaling

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Abstract. Structurally related lanostane-type triterpenes, ganoderic acid A, F and H (GA-A, GA-F, GA-H), were identified in an oriental medicinal mushroom *Ganoderma lucidum*. In the present study we evaluated the effect of GA-A, GA-H and GA-F on highly invasive human breast cancer cells. We showed that GA-A and GA-H suppressed growth (cell proliferation and colony formation) and invasive behavior (adhesion, migration and invasion) of MDA-MB-231 cells. Our results suggest that GA-A and GA-H mediate their biological effects through the inhibition of transcription factors AP-1 and NF- κ B, resulting in the down-regulation of expression of Cdk4 and the suppression of secretion of uPA, respectively. Furthermore, the activity of ganoderic acids is linked to the hydroxylation in the position 7 and 15 (GA-A) and 3 (GA-H) in their triterpene lanostane structure. In conclusion, hydroxylated triterpenes from *G. lucidum* could be promising natural agents for the therapy of invasive breast cancers.

Introduction

Breast cancer is the most common malignancy in women and is the second leading cause of cancer death in the US. Approximately 178,480 new cases of breast cancer are expected to be diagnosed in 2007 (1). Breast cancer often progresses from the nonmetastatic and therapy-responsive phenotype to the highly invasive and metastatic phenotype, which is usually resistant to standard therapeutic procedures (2). The metastatic dissemination of tumors is responsible for treatment failure, morbidity and death in cancer patients (3). Nearly one third of women with breast cancer develops metastases and finally dies from this disease (4). Thus, the inhibition of metastasis is one of the potential therapeutic

strategies for the treatment of breast cancer patients. The invasion and metastasis of cancer comprise a complex multi-step process that includes tumor cell adhesion, extracellular matrix proteolysis, migration, proliferation at a distant site of the body and stimulation of angiogenesis (3,5). Urokinase-type plasminogen activator (uPA) is a serine protease that converts inactive plasminogen to active plasmin, cleaving several components of the extracellular matrix (3,5,6). uPA exerts its non-proteolytic activity through its interaction with uPA receptor (uPAR), which forms a complex with integrins and controls cell adhesion and migration (5). High levels of uPA and uPAR were reported in various human malignant cancers and are associated with advanced tumors and decreased survival time (5,6). Several transcription factors have been implicated in the regulation of expression of uPA and uPAR. For example, activator protein-1 (AP-1), nuclear factor- κ B (NF- κ B) and ETS family transcription factor (PEA3) control uPA expression, while AP-1, NF- κ B and Sp1 control uPAR expression (5,6). Besides regulating expression of uPA and uPAR, AP-1 and NF- κ B also regulate the expression of other genes involved in cell cycle progression (cyclin D1), cell survival (Bcl2, Bcl-x1), inflammatory tumor growth (cyclooxygenase-2, COX2), and angiogenesis (vascular endothelial growth factor, VEGF) among others (5,7-10). Constitutive activation of AP-1 and NF- κ B has been detected in highly invasive breast cancer cells (11,12). Therefore, the activation of AP-1 and NF- κ B is linked to the high proliferative potential, anti-apoptosis, and invasive and metastatic behavior of cancer cells (8,13,14). We recently demonstrated that constitutive activity of AP-1 and NF- κ B is associated with invasive behavior of breast cancer cells, and that inhibition of AP-1 and NF- κ B results in the suppression of invasiveness and growth of these cells (13-15).

The popular medicinal mushroom *Ganoderma lucidum* has been widely used in eastern Asia to promote health and longevity. The dried powder of *G. lucidum* has been used in traditional Chinese medicine (TCM) for more than 2000 years to prevent or treat different diseases, including cancer (16). The anticancer properties of *G. lucidum* have been attributed to either the isolated polysaccharides, which are responsible for the stimulation of the immune system, or triterpenes, which demonstrate cytotoxic activity against a variety of cancer cells including breast, prostate, lung, colon, sarcoma, hepatoma and leukemia cells (17,18). However, the identification and characterization of the most biologically active

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agents from *G. lucidum* proved to be difficult due to the complexity of *G. lucidum*. Recently, purified lanostanoid triterpenes isolated from *G. lucidum*, ganoderic acid X and ganoderic acid T induced apoptosis in human hepatoma and lung cancer cells, respectively (19,20), suggesting that the basic lanostane structure is necessary for the biological activity of purified triterpenes. Nevertheless, the relationship between the structure and function of *G. lucidum* triterpenes remains to be determined.

We previously demonstrated that *G. lucidum* inhibits invasiveness of breast cancer cells through the suppression of NF- κ B, resulting in the down-regulation of the expression of uPA and its receptor (uPAR) (13). We also found that *G. lucidum* inhibits the growth of breast cancer cells through cell cycle arrest at G₀/G₁, which was mediated by the suppression of NF- κ B signaling and the down-regulation of the expression of cyclin D1 (21). Our recent analysis by LC-MS demonstrated that extracts of *G. lucidum* contain ganoderic acid A, F and H (GA-A, GA-F, GA-H) (Fig. 1) (15). In the present study, we compared the anti-proliferative and anti-invasive activity of three purified triterpenes isolated from *G. lucidum* (GA-A, GA-F, GA-H), and demonstrated that the hydroxylation of the ganoderic acids affects their biological activity.

Materials and methods

Materials. Purified ganoderic acid A (79.12%), F (89.96%) and H (82.31%) were purchased from ChromaDex Inc. (Santa Ana, CA). Methanol was purchased from Sigma (St. Louis, MO). GA-A, GA-F and GA-H were dissolved in methanol at a concentration of 50 mM and stored at 4°C. Dulbecco's phosphate-buffered saline (DPBS) was purchased from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD).

Cell culture. The human breast cancer cell line (MDA-MB-231) was obtained from ATCC (Manassas, VA). MDA-MB-231 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing penicillin (50 U/ml), streptomycin (50 U/ml) and 10% fetal bovine serum (FBS). Media, supplements and FBS were from Invitrogen (Grand Island, NY).

Cell proliferation assay. Cell proliferation was determined by the tetrazolium salt method (MTT method), according to the manufacturer's instructions (Promega, Madison, WI). Briefly, MDA-MB-231 cells (2.5×10³/well) were cultured in a 96-well plate and treated with GA-A, GA-F and GA-H (0.10, 0.25, 0.50 mM) for 24, 48 and 72 h. At the end of the incubation period, the cells were harvested, and absorption was determined with an ELISA plate reader at 570 nm. Data points represent the mean ± SD in one experiment repeated at least twice.

Colony formation (anchorage-independent growth). MDA-MB-231 cells were harvested and seeded in 6-well plates coated with 1% agarose. Colony formation was assessed after incubation for 14 days in culture media with GA-A, GA-F and GA-H (0.10, 0.25, 0.50 mM), which was replaced every

4 days. Plates were stained with 0.005% Crystal Violet, and the colonies were counted manually under a microscope and photographed.

Cell adhesion assay. Cell adhesion of MDA-MB-231 cells was performed with Cytomatrix Adhesion Strips coated with human vitronectin (Chemicon International, Temecula, CA), according to the manufacturer's instructions. Briefly, MDA-MB-231 cells were treated with GA-A, GA-F and GA-H (0.10, 0.25, 0.50 mM) for 24 h, harvested, and counted. Cells (10⁵) were applied to the rehydrated vitronectin strips in a 96-well plate and incubated for 1.5 h at 37°C. The cells were stained with 0.2% Crystal Violet, washed, and the absorbance was determined at 570 nm with a microplate reader, as previously described (22). Data points represent the mean ± SD in one experiment repeated at least twice.

Cell migration assay. MDA-MB-231 cells were harvested and incubated with GA-A, GA-F and GA-H (0.10, 0.25, 0.50 mM) for 5 h. Chemokinesis was assessed in Transwell chambers (6.5 mm-diameter polycarbonate filters; 8 μm pore size) in the DMEM medium containing 10% FBS, as previously described (13,22). After fixing and staining, the number of migrating cells was determined microscopically by enumeration at 40x magnification from at least four random fields. Data points represent the mean ± SD of three individual filters within one representative experiment repeated at least twice.

Cell invasion assay. MDA-MB-231 cells were harvested and treated with GA-A, GA-F and GA-H (0.10, 0.25, 0.50 mM) for 24 h. Invasion was assessed in Transwell chambers (6.5 mm-diameter polycarbonate filters; 8 μm pore size) coated with 100 μl of Matrigel™ (BD Biosciences, Bedford, MA) diluted 1:3 with DMEM. After 24 h of incubation, the cells, which invaded through Matrigel were stained with hematoxylin, and their number was determined microscopically by enumeration at 40x magnification from at least four random fields. Data points represent the mean ± SD in one experiment repeated at least twice.

uPA secretion. DMEM media from MDA-MB-231 cells treated with GA-A, GA-F and GA-H (0.10, 0.25, 0.50 mM) for 24 h were collected and concentrated 10-fold by a Micon YM-10 filter (Amicon, Cambridge, MA). Secretion of uPA was detected by Western blot analysis of conditioned media with anti-uPA antibody (Oncogenes Research Products, Cambridge, MA), as described (13).

Immunocytochemical staining. MDA-MB-231 cells were cultured in multichamber slides (Nunc Inc., Naperville, IL) and treated with GA-A, GA-F and GA-H (0.10, 0.25, 0.50 mM) for 24 h. After 24 h, cells were fixed and then processed for immunocytochemical staining as described (23). Briefly, cells were incubated with prediluted blocking serum (DPBS containing 1.5% normal horse serum) for 1 h at room temperature, incubated with a rabbit anti-uPAR polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min at room temperature, and then incubated in the presence of a prediluted biotinylated anti-rabbit secondary antibody (Santa Cruz Biotechnology) for 30 min at room

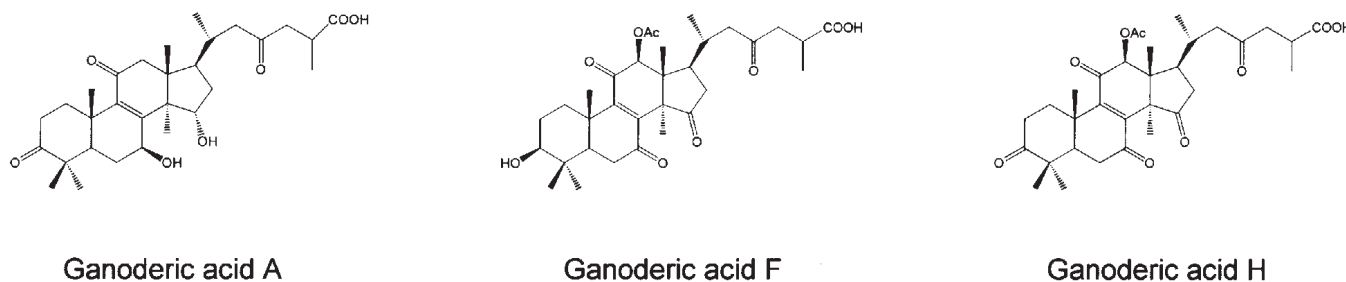


Figure 1. Structure of ganoderic acids.

temperature. Slides were incubated with AB enzyme reagent for 30 min and then incubated with peroxidase substrate solution (ABC staining system, Santa Cruz Biotechnology) until the desired stain intensity developed. Each step in the staining procedure was followed by three 5 min washes in PBS.

DNA transfection and luciferase assay. MDA-MB-231 cells (2.5×10^5 /well) were seeded into 6-well plates in DMEM with 10% FBS prior to transfection. Transient transfections were performed with the Effectene reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. Briefly, the cells were transfected with AP-1-luc or NF- κ B-luc (BD Biosciences Clontech, Palo Alto, CA) reporter constructs (1 μ g) and β -galactosidase expression vector pCH110 (1 μ g). Twenty hours after transfection, cells were washed once with DPBS, and the medium was replaced with DMEM with 10% FBS and incubated for 24 h. After a 24-h incubation, medium was replaced with DMEM containing no FBS, and cells were treated with GA-A, GA-F and GA-H (0.10, 0.25, 0.50 mM) for 24 h. β -galactosidase activity was measured in cell lysates, as previously described (24). Normalized amounts (equal numbers of β -galactosidase units) of cell extracts were used in the luciferase assay using Lmax luminometer (Molecular Devices, Sunnyvale, CA). Data points represent the mean \pm SD of three independent transfection experiments.

Preparation of nuclear and whole cell extract. MDA-MB-231 cells (1×10^7) were treated with GA-A, GA-F and GA-H (0.10, 0.25, 0.50 mM) for 24 h. Nuclear and whole cell extracts isolated from cells were prepared as previously described (24). The concentration of nuclear and total protein was determined according to the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA).

Western blot analysis. Equal amounts of proteins (20 μ g/lane) were separated on 4-12% SDS-PAGE (Invitrogen, Carlsbad, CA) and transferred to a PVDF membrane (Millipore, Bedford, MA). The membrane was incubated with the corresponding primary antibodies diluted 1:1000 in blocking solution, as follows: a rabbit anti-Cdk4 polyclonal antibody and a mouse anti- α -tubulin monoclonal antibody (Santa Cruz Biotechnology). Anti-mouse or anti-rabbit secondary antibodies (Amersham Biosciences, Buckinghamshire, UK) were used to detect and visualize by the ECL Western Blotting Detection system (Amersham Biosciences).

Electrophoretic mobility shift assay (EMSA). Oligonucleotide probes containing consensus sequences for AP-1 and NF- κ B binding sites were purchased from Promega, respectively. EMSA for AP-1 and NF- κ B was performed as previously described (14,24). Briefly, 1 μ l (40,000 cpm) of 32 P-labeled AP-1 or NF- κ B probe was added to the reaction mixture and incubated for another 20 min at room temperature. The reaction mixture was then separated on a 4.5% polyacrylamide gel running in 0.25X Tris/borate/EDTA (TBE) buffer. The specificity was confirmed with cold AP-1 or NF- κ B and nonspecific β -Gal oligonucleotides, and supershift analysis was performed with anti-NF- κ B p50, anti-NF- κ B p65, anti-c-Jun, and anti-c-Fos antibody or nonspecific antibody (IgG), respectively.

Statistical analysis. Data are expressed as the mean \pm SD of three experiments. Minitab statistical software for Windows (Minitab Inc., State College, PA) was used for statistical analysis. Statistical differences between means were evaluated using a one-way analysis of variance (ANOVA) followed by Tukey's pairwise comparisons. $p < 0.05$ was considered significant.

Results

Effect of GA-A, GA-F and GA-H on the proliferation of highly invasive breast cancer cells. Even with progress in the early diagnosis and therapy, some breast cancers demonstrate highly invasive characteristics that are usually refractory to medical therapy. Therefore, we evaluated structurally related ganoderic acids (GA-A, GA-F and GA-H; Fig. 1) on highly invasive breast cancer cells. MDA-MB-231 cells were treated with increasing concentrations of GA-A, GA-F and GA-H (0.10, 0.25 and 0.50 mM) for 24, 48 and 72 h, and proliferation was determined. As seen in Fig. 2, GA-A and GA-H significantly inhibited the growth of MDA-MB-231 cells. The growth of MDA-MB-231 cells treated with GA-A (0.50 mM) for 24, 48 and 72 h was inhibited by 41.7, 55.1 and 55.8%, respectively (Fig. 2A). Similarly, GA-H inhibited the growth of MDA-MB-231 cells by 38.3, 48.3 and 57.7%, respectively (Fig. 2C). However, the same concentrations of GA-F had no effect on the growth of MDA-MB-231 cells (Fig. 2B).

Effect of GA-A, GA-F and GA-H on the anchorage-independent growth of highly invasive breast cancer cells.

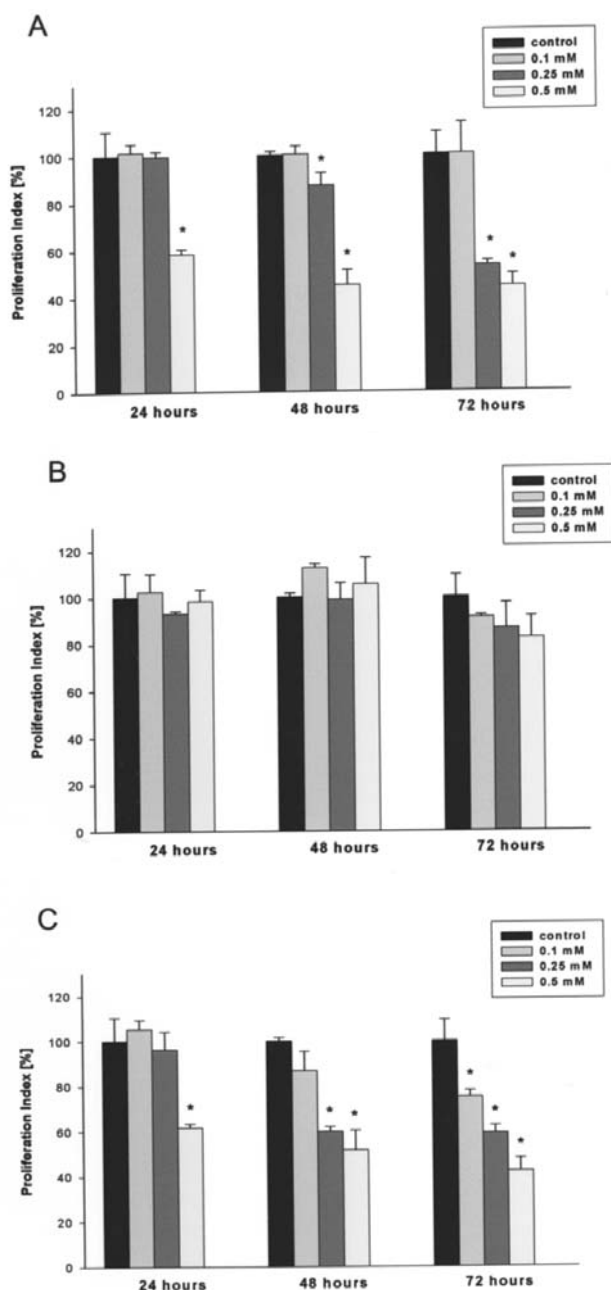


Figure 2. Effect of ganoderic acids on the proliferation of MDA-MB-231 cells. MDA-MB-231 cells were treated with (A) GA-A (0-0.5 mM), (B) GA-F (0-0.5 mM) and (C) GA-H (0-0.5 mM). Cell proliferation was determined by the tetrazolium salt method. Data are the means \pm SD of triplicate determinations. Similar results were obtained in at least two additional experiments; * $p < 0.05$.

The hallmark property of cancer cells, *in vitro*, is that they are capable of anchorage-independent growth. Cancer cells can survive growth under a nonadhesive or anchorage-independent condition (colony formation), and colony formation is correlated with the *in vivo* oncogenic potential of cancer cells. Therefore, colony formation is a key parameter for cells to acquire a metastatic potential (25). In order to evaluate the effects of ganoderic acids on colony formation of highly invasive breast cancer cells, MDA-MB-231 cells were plated on agarose and incubated for 14 days with culture media containing GA-A, GA-F and GA-H (0.10, 0.25, 0.50 mM). As seen in Fig. 3, GA-A and GA-H decreased the

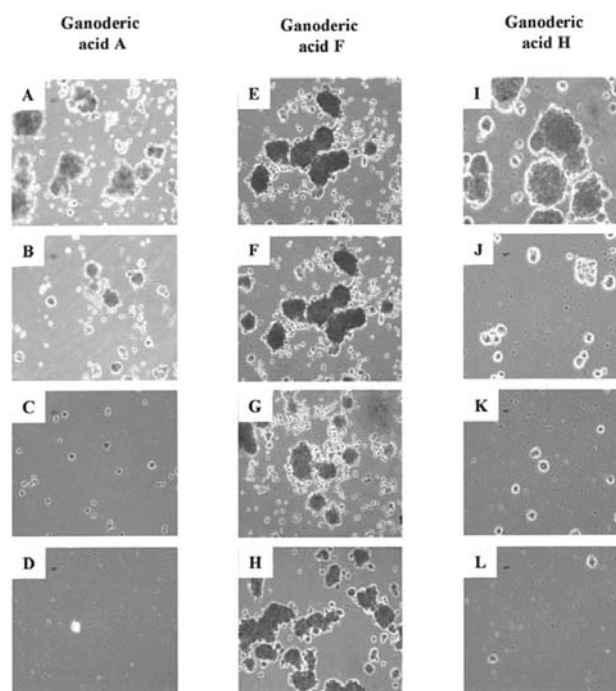


Figure 3. Effect of ganoderic acids on colony formation of MDA-MB-231 cells. Anchorage-independent growth (colony formation) of MDA-MB-231 cells was assessed on 1% agarose after incubation for 14 days with culture media containing GA-A: (A) control, (B) 0.1 mM, (C) 0.25 mM, (D) 0.5 mM; and containing GA-F: (E) control, (F) 0.1 mM, (G) 0.25 mM, (H) 0.5 mM; and containing GA-H: (I) control, (J) 0.1 mM, (K) 0.25 mM and (L) 0.5 mM.

number of colonies of MDA-MB-231 cells in a dose-dependent manner (Fig. 3A-D and I-L). However, GA-F had no effect on the amount of breast cancer cell colonies (Fig. 3E-H). These results suggest that GA-A and GA-H inhibited the anchorage-dependent (cell proliferation) as well as anchorage-independent (colony formation) growth of invasive breast cancer cells.

Effect of GA-A, GA-F and GA-H on invasive behavior of breast cancer cells. Adhesion of cancer cells to extracellular matrix or endothelial cells plays a key role in the process of metastasis. We previously demonstrated that an extract from *G. lucidum* inhibited the invasive behavior (adhesion, migration and invasion) of highly invasive human breast cancer cells (26). Therefore, we evaluated whether GA-A, GA-F and GA-H also inhibit the adhesion of MDA-MB-231 cells. As expected, GA-A and GA-H inhibited the adhesion of MDA-MB-231 cells, whereas GA-F did not show any effect (Fig. 4A). We found that GA-A reduced the adhesion of MDA-MB-231 cells to 71.1, 51.9 and 35.9% of the control at 0.10, 0.25 and 0.50 mM, respectively. Similarly, GA-H decreased the adhesion to 70.8, 57.9 and 43.9% of the control at 0.10, 0.25 and 0.50 mM, respectively. These results demonstrated that the inhibitory effects of GA-A and GA-H on the adhesion of MDA-MB-231 cells are dose-dependent.

The effect of GA-A, GA-F and GA-H on the motility of MDA-MB-231 cells was assessed by cell migration assay. Treatment of MDA-MB-231 cells with GA-A and GA-H demonstrated a significantly lower migration rate compared to the control (Fig. 4B). GA-A reduced the migration rate of

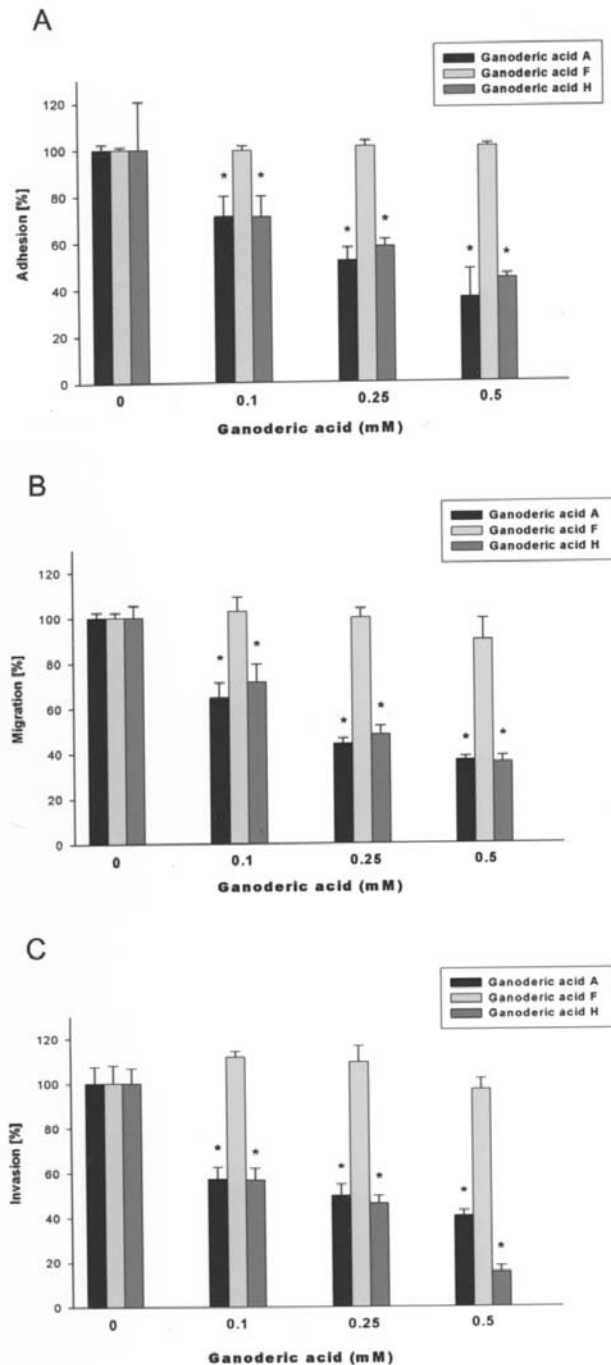


Figure 4. Effect of ganoderic acids on the invasive behavior of MDA-MB-231 cells. (A) Cell adhesion. MDA-MB-231 cells were treated with GA-A, GA-F or GA-H (0-0.5 mM) for 24 h, and cell adhesion to vitronectin was determined as described in Materials and methods. Each bar represents the mean \pm SD of three experiments; * $p < 0.05$. (B) Cell migration. Cell migration was determined after 5 h of incubation in the presence of GA-A, GA-F or GA-H (0-0.5 mM) in Boyden chambers as described in Materials and methods. Each bar represents the mean \pm SD of three experiments; * $p < 0.05$. (C) Cell invasion. Cell invasion was determined after 24 h of incubation in the presence of GA-A, GA-F or GA-H (0-0.5 mM) in Boyden chambers coated with Matrigel as described in Materials and methods. Each bar represents the mean \pm SD of three experiments; * $p < 0.05$.

MDA-MB-231 cells to 64.5, 44.0 and 36.6% of the control at 0.10, 0.25 and 0.50 mM, respectively. Similarly, GA-H decreased the migration rate to 71.3, 48.1 and 35.6% of the control at 0.10, 0.25 and 0.50 mM, respectively. In contrast,

GA-F at the same time point had no effect on the migration rate of MDA-MB-231 cells (Fig. 4B).

Next we examined whether GA-A, GA-F and GA-H also modulates the invasion of MDA-MB-231 cells, because invasion is another key factor for cancer expansion and metastasis. Consistent with anchorage-independent growth, adhesion and migration, GA-A and GA-H significantly inhibited the invasion of the MDA-MB-231 cells, whereas GA-F did not show any effect on cell invasion (Fig. 4C). GA-A reduced the invasion of MDA-MB-231 cells to 57.3, 49.9 and 40.4% of the control at 0.10, 0.25 and 0.50 mM, respectively. GA-H resulted in a similar reduction in the invasion rate to 56.9, 46.3 and 15.5% of the control at 0.10, 0.25 and 0.50 mM, respectively. These data indicate that the inhibitory effects of GA-A and GA-H on the invasion of MDA-MB-231 cells are dose-dependent.

Effect of GA-A, GA-F and GA-H on the constitutive activation of AP-1 and NF- κ B in MDA-MB-231 cells. AP-1 and NF- κ B are transcriptional activators implicated in promoting the invasive and metastatic potential of cancer cells. We previously demonstrated that *G. lucidum* decreased constitutive activation of AP-1 and NF- κ B (13), and inhibited adhesion, migration and invasion of MDA-MB-231 cells (13,26). The ability of GA-A and GA-H to decrease the invasive behavior of MDA-MB-231 cells suggests that ganoderic acids may affect AP-1 or NF- κ B signaling pathways responsible for the growth and metastatic behavior of breast cancer cells. In order to determine the effect of GA-A, GA-F and GA-H on the constitutive activation of AP-1 and NF- κ B, MDA-MB-231 cells were transfected with reporter gene constructs AP-1 and NF- κ B-luc, respectively, and then treated with GA-A, GA-F and GA-H. As expected, GA-A and GA-H significantly inhibited constitutive activation of AP-1 and NF- κ B, whereas GA-F had no effect on constitutively active AP-1 and NF- κ B in MDA-MB-231 cells (Fig. 5). Although our gel shift analysis demonstrated constitutive DNA-binding activity of AP-1 and NF- κ B, the DNA binding of AP-1 and NF- κ B was not affected by GA-A, GA-F and GA-H (data not shown). These data confirm that GA-A and GA-H suppress constitutive activation of AP-1 and NF- κ B at the trans-activation level, without affecting their DNA binding.

Effect of GA-A, GA-F and GA-H on the secretion of uPA and uPAR expression in MDA-MB-231 cells. The expression of uPA and uPAR are controlled by AP-1 and NF- κ B (14). Therefore, we evaluated whether the inhibition of AP-1 and NF- κ B by ganoderic acids results in the suppression of uPA secretion and uPAR expression. MDA-MB-231 cells were treated with GA-A, GA-F and GA-H for 24 h, and the expression of uPA was evaluated in cell-conditioned medium by Western blot analysis. The effects of GA-A, GA-F and GA-H on uPAR expression of MDA-MB-231 cells were examined by immunocytochemical staining. Our results showed that GA-A and GA-H markedly reduced the secretion of uPA from MDA-MB-231 cells. As expected, GA-F did not change the secretion of uPA in MDA-MB-231 cells (Fig. 6). Interestingly, GA-A, GA-F and GA-H did not cause any significant change in uPAR expression of MDA-MB-231 cells (Fig. 7). These results suggest that the inhibitory

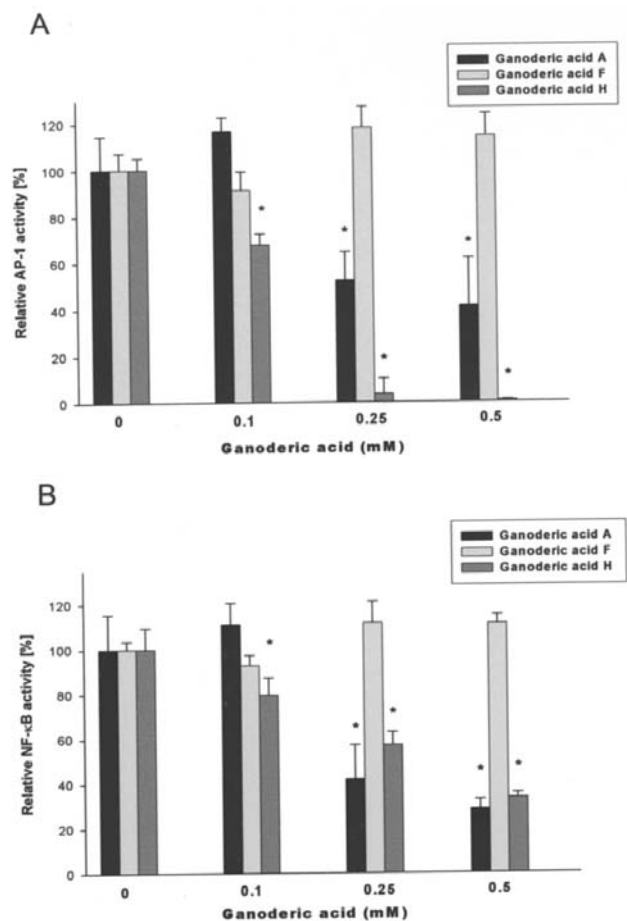


Figure 5. Effect of ganoderic acids on the activity of AP-1 and NF-κB in MDA-MB-231 cells. MDA-MB-231 cells were transfected with 1 μg AP-1-Luc (A), 1 μg NF-κB-Luc (B) reporter gene constructs and 1 μg β-galactosidase expression vector pCH110. Twenty-four hours after transfection, the cells were treated with GA-A (0-0.5 mM), GA-F (0-0.5 mM) and GA-H (0-0.5 mM) for 24 h. AP-1 and NF-κB activity was determined by luciferase assays as described in Materials and methods. The results are expressed as the percentage of relative AP-1 or NF-κB activity. Each bar represents the mean ± SD of three experiments; *p<0.05.

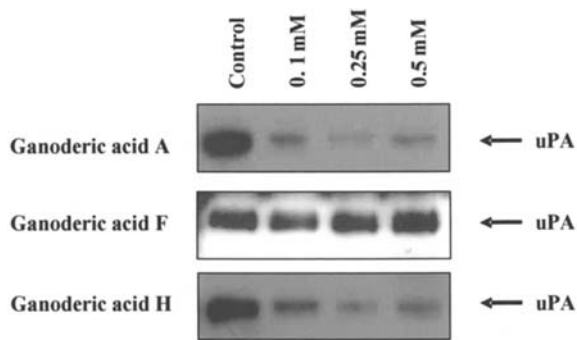


Figure 6. Effect of ganoderic acids on uPA secretion from MDA-MB-231 cells. MDA-MB-231 cells were treated with GA-A (0-0.5 mM), GA-F (0-0.5 mM) and GA-H (0-0.5 mM) for 24 h, and the expression of uPA was detected in conditioned media with anti-uPA antibody by Western blot analysis.

effects of GA-A and GA-H on the invasiveness of MDA-MB-231 cells can be associated with the down-regulation of uPA secretion, but not altered uPAR expression.

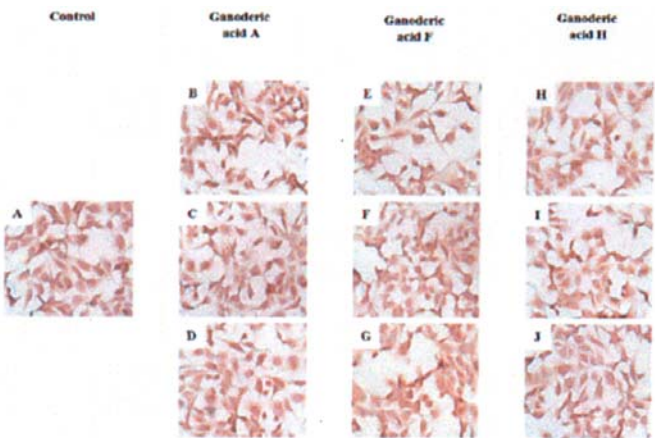


Figure 7. Effect of ganoderic acids on uPAR expression of MDA-MB-231 cells. MDA-MB-231 cells were cultured in multichamber slides and treated with GA-A (0-0.50 mM), GA-F (0-0.50 mM) and GA-H (0-0.50 mM) for 24 h. After 24 h, cells were fixed and then processed for uPAR expression by immunocytochemical staining as described in Materials and methods. (A) control; GA-A: (B) 0.1 mM, (C) 0.25 mM, (D) 0.5 mM; GA-F: (E) 0.1 mM, (F) 0.25 mM, (G) 0.5 mM; and GA-H: (H) 0.1 mM, (I) 0.25 mM, (J) 0.5 mM.

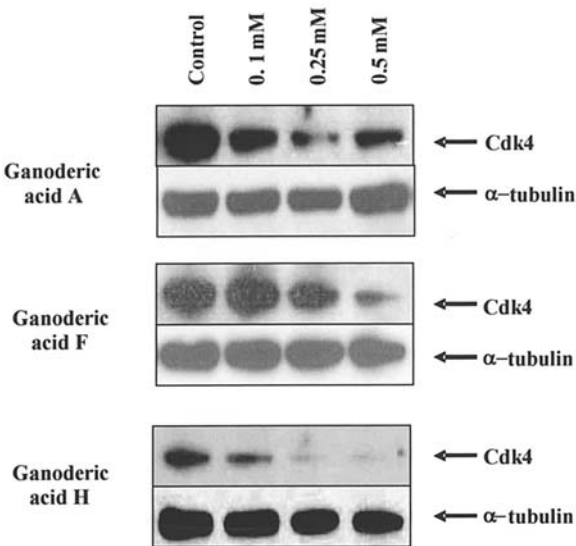


Figure 8. Effect of ganoderic acids on expression of Cdk4 protein in MDA-MB-231 cells. MDA-MB-231 cells were treated with GA-A (0-0.5 mM), GA-F (0-0.5 mM) and GA-H (0-0.5 mM) for 24 h, and whole cell extracts were subjected to Western blot analysis. The expression of Cdk4 and α-tubulin was evaluated by Western blot analysis with anti-Cdk4 and anti-α-tubulin antibodies. Equal protein loading was verified with anti-α-tubulin antibody as described in Materials and methods. The results are representative of three separate experiments.

Effect of GA-A, GA-F and GA-H on the expression of Cdk4 in MDA-MB-231 cells. The dysregulation of cell cycle control is another characteristic of cancer (27), and we previously showed that *G. lucidum* arrested the cell cycle in MDA-MB-231 cells at G₀/G₁ by the down-regulation of cyclin D1 and Cdk4 kinase (21). Therefore, we hypothesized that GA-A and GA-H would decrease the proliferation of MDA-MB-231 cells by a similar mechanism. The extracts from MDA-MB-231 cells treated with GA-A, GA-F and GA-H were subjected to

Western blot analysis with Cdk4. As shown in Fig. 8, GA-A and GA-H markedly decreased the expression of Cdk4 in MDA-MB-231 cells in a dose-response manner. As expected, GA-F did not reduce the expression of Cdk4 in these cells. Collectively, our results suggest that the inhibitory effects of GA-A and GA-H on the growth of MDA-MB-231 cells might be caused by the down-regulation of Cdk4 expression.

Discussion

We previously showed that extract from *G. lucidum* (containing polysaccharides and triterpenes) suppresses cell growth, cell adhesion, migration and invasion in invasive breast cancer cells (21,26). This extract contains GA-A, GA-F and GA-H (15). However, the effect of isolated GA-A, GA-F and GA-H on the growth and invasive behavior of breast cancer cells was not previously addressed. In the present study we showed that GA-A and GA-H inhibited growth and invasive behavior of breast cancer cells, whereas structurally related GA-F was ineffective. The anti-proliferate effect was mediated through the down-regulation of expression of Cdk4, leading to the inhibition of the cyclin D1/Cdk4 complex. This observation is in agreement with our previous study demonstrating cell cycle arrest at the G₁/G₀ phase of breast cancer cells and the down-regulation of Cdk4 by *G. lucidum* (21). Recently, Tang *et al* (20) showed that another triterpenoid purified from *G. lucidum* mycelia, ganoderic acid T, arrests lung cancer cells at the G₁/G₀ phase.

GA-A and GA-H also inhibited adhesion, migration and invasion with the down-regulation of uPA secretion in MDA-MB-231 cells. These data suggest that GA-A and GA-H inhibit the invasive behaviors of breast cancer cells by modulating the secretion of uPA. As shown above, GA-A and GA-H inhibit AP-1 and NF- κ B activity in highly invasive human breast cancer cells. Since AP-1 and NF- κ B binding sites exist at the promoter region of uPA (28), the suppression of secretion of uPA by GA-A and GA-H might be mediated by the inhibition of transactivation of AP-1 and NF- κ B. Nevertheless, it is possible that the inhibition of uPA secretion by GA-A and GA-H could be mediated by other mechanisms including posttranscriptional regulation of uPA mRNA (29). We previously showed that expression of uPAR is controlled by NF- κ B and AP-1, and the extract of *G. lucidum* down-regulates expression of uPAR in MDA-MB-231 as well as in PC-3 cells (13,14). However, in the current study we were not able to demonstrate the inhibitory effect of GA-A and GA-H on the expression of uPAR. Because the extract of *G. lucidum* used in our previous study contained both polysaccharides and triterpenes, it is possible that polysaccharide fractions, or their combination with triterpenes, were able to down-regulate uPAR expression.

As we clearly demonstrated, the triterpene structure of ganoderic acids plays a crucial role in their biological activity. Our data suggest that the activity of ganoderic acids could be especially linked to the hydroxylation of their lanostane triterpene structure. The active GA-H is hydroxylated at position 3 and GA-A at position 7 and 15; whereas inactive GA-F is not hydroxylated. In addition, other triterpenes with hydroxyl or acetoxy groups at the positions 3, 7 and/or 15

such as ganoderic acid C1, ganoderic acid C2 (30), ganoderic acid D (31), ganoderic acid T (20), ganoderic acid X (19), ganoderic acid Y (32), ganoderiol A (31), ganoderol B (32,33), lucidumol B and ganoderadiol (30,31) were shown to have inhibitory biological activity. Shiao (34) demonstrated that several Ganoderma triterpenes with hydroxyl or acetoxy groups in these positions were cytotoxic to hepatoma cells and inhibited histamine release, cholesterol synthesis, and farnesylation of Ras protein. Hajjaj *et al* (32) showed that ganoderic acid Y and ganoderol B inhibited cholesterol synthesis in human hepatic T9A4 cells. Li *et al* (19) reported that ganoderic acid X induced immediate inhibition of DNA synthesis and cell apoptosis in human hepatoma HuH-7 cells. Tang *et al* (20) demonstrated that ganoderic acid T induced apoptosis of metastatic lung tumor cells. Liu *et al* (31) also reported that ganoderic acid D, ganoderiol A, lucidumol B and ganoderadiol inhibited 5 α -reductase activity of rat microsomes. Akihisa *et al* (30) also showed that ganoderic acid C1 and ganoderic acid C2 have inhibitory effects on the induction of Epstein-Barr Virus early antigen. Liu *et al* (33) recently showed that ganoderol B has the ability to bind to the androgen receptor, inhibit androgen-induced growth of prostate cancer cells and suppress re-growth of the ventral prostate induced by testosterone in rats.

In conclusion, in this study we demonstrated a possible mechanism by which GA-A and GA-H isolated from *G. lucidum* inhibit the growth and invasive behaviors of human breast cancer cells. The anti-invasive effects of GA-A and GA-H on MDA-MB-231 cells may be mediated through the inhibition of AP-1/NF- κ B-dependent secretion of uPA, while the anti-proliferate effects of GA-A and GA-H on MDA-MB-231 cells may be mediated through the down-regulation of expression of Cdk4. Although our data suggest that the hydroxylation at positions 3, 7 and/or 15 in the lanostane triterpene structure is crucial for the biological activity of ganoderic acids, our study is limited only to the three triterpenes isolated from *G. lucidum*. Further studies are in progress to determine the relationship between the structure and biological activity of mushroom triterpenes for the development of novel agents for the treatment of breast cancer.

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