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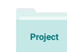

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Short Communication

Ganoderic Acid Me Inhibits Tumor Invasion Through Down-Regulating Matrix Metalloproteinases 2/9 Gene Expression

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Abstract. The effect of ganoderic acid Me (GA-Me), which was purified from the fermentation mycelia of the traditional Chinese medicinal mushroom Ganoderma lucidum as reported (Tang W, Gu TY, Zhong JJ. Biochem Eng J. 2006;32:205–210), on anti-invasion was investigated. Wound healing assay indicated that GA-Me inhibited cell migration of 95-D, a human highly metastatic lung tumor cell line, in dose- and time-dependent manners. Results of cell aggregation and adhesion assays showed that GA-Me promoted cell homotypic aggregation and inhibited cell adherence to extracellular matrix (ECM). In addition, GA-Me suppressed matrix metalloproteinases 2/9 (MMP2/9) gene expressions at both mRNA and protein levels in 95-D cells according to qRT-PCR and Western blotting, respectively. The results demonstrated that GA-Me effectively inhibited tumor invasion, and it might act as a new MMP2/9 inhibitor for anti-metastasis treatment of carcinoma cells.

Keywords: ganoderic acid Me (GA-Me), invasion, matrix metalloproteinases 2/9 (MMP2/9)

Ganoderma lucidum (Fr.) KARST (Polyporaceae), a traditional Chinese medicinal mushroom (1), has been used as a traditional medicine for the prevention and treatment of a variety of diseases, such as hepatitis, hepatopathy, hypertension, nephritis, bronchitis, HIV, platelet aggregation, immunological disorders, and cancers, in Asia for several thousand years (2). It is well documented that the extracts including polysaccharide or triterpene-enriched extract from *G. lucidum* inhibit cancer proliferation, induce cell cycle arrest, or apoptosis of human and mouse carcinoma cell lines (3, 4).

Interestingly, the anti-angiogenesis, anti-invasion, and anti-metastasis pharmacological functions of *G. lucidum* were recently found. The extracts of *G. lucidum* inhibited primary solid-tumor growth in the spleen, liver metastasis, and secondary metastatic tumor growth in the liver in intrasplenic Lewis lung carcinoma (LLC)-implanted mice, inhibited Martigel-induced angiogenesis (5), induced actin polymerization in bladder cancer cells in vitro (6), inhibited tumor cell adhesion (7), inhibited induced oxidative stress-invasiveness of cancer cells through suppression of interleukin (IL)-8 secretion (8), and suppressed motility and invasiveness of highly invasive breast and prostate cancer cells (9).

It is well recognized that the development of metastasis is a major cause of death in many human cancers. Use of natural products is emerging as an alternative to traditional medicines in treatment of cancer metastasis. For traditional herbal medicines, limited scientific evidence regarding their effectiveness and a lack of mechanistic understanding of their action have led to the fact that they are not being used in mainstream medicine in western countries. What is more, the treatment against metastasis is still far from satisfactory due to the lack of effective drugs. Obviously it is critical to find new effective drugs to fight against metastasis.

Until now, over one hundred ganoderma triterpenes have been identified, and several triterpenes were already purified from *G. lucidum* in relatively large quantitites (1), which makes bioassay experiments feasible. Ganoderiol F inhibited Martigel-induced angiogenesis (5). A pure lanostane triterpene, ganoderic acid

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Me (GA-Me), purified from *G. lucidum* by our group (1), could increase the immune function by enhancing the expression of T-helper type 1 (Th1) cytokines (IL-2 and IFN-γ), which led to the inhibition of tumor growth and lung metastasis (10). However, the direct anti-metastasis molecular mechanism of GA-Me was still unclear. Therefore, the present study was conducted to examine the anti-invasion/anti-metastasis mechanism of GA-Me, which is the same purified compound as reported by Tang et al. (1). Data are expressed as means ± S.E.M. One- or two-way ANOVA followed by Dunnett's multiple comparison tests were used for statistical analysis using OriginPro 8 (Origin Lab, Inc., Northampton, MD, USA). Our experimental results indicated that GA-Me suppressed invasion of human highly metastatic lung tumor 95-D cells through inhibiting MMP2/9 genes expression.

Tumor metastasis consists of numerous processes, including migration, homotypic and heterotypic cell-cell adhesion, cell-matrix interaction, invasion into surrounding tissues, release from the primary tumor, intravasation, adhesion to vascular walls, extravasation, and formation of new foci. Consequently, multiple factors are involved in the individual steps of metastasis. In normal tissues, cells are tightly associated with each other, so that they are generally not allowed to migrate freely. However, the malignant cells are more loosely associated and can freely detach from the primary tumor and migrate out. Our results indicated that GA-Me could facilitate the adhesion of cancer cells to each other, resulting in aggregate formation (Fig. 1A), which may contribute to prevention of the initial cell release. The cell aggregation assay was done as previously described (11). Briefly, a single-cell suspension was obtained through standard trypsinization procedures. A total of 2×10^5 95-D cells in 1 ml of RPMI medium 1640 (serum-free) with different concentrations of GA-Me was placed in polystyrene microtubes and shaken gently every 5 min for 1 h at 37°C. Figure 1A shows that GA-Me promoted spontaneous cell–cell aggregation in a dose-dependent manner. Following the treatment with increasing concentrations of GA-Me, the cell aggregates grew larger. The percentage of aggregated cells was about 48.4 ± 7.6% when cells were treated with 20 μm /ml of GA-Me for 1 h.

The wound healing assay was done as previously reported (11). For the wounding assays, confluent monolayers of 95-D cells were cultured in RPMI medium 1640 with 0.01% bovine serum albumin (BSA) in the absence or presence of different concentrations of GA-Me for 24 h after "wounding" of the cell layer with a pipette tip. The distance that the wounded edge of cells had moved was measured. By this assay, we observed that GA-Me effectively inhibited migration of 95-D cells. In Fig. 1B, the cellular motility of 95-D cells was obviously inhibited in a dose- and time-dependent manner by GA-Me. Compared to the control (untreated), GA-Me suppressed the migration capability of 95-D cells, and an increased suppression in cell migration was seen when 95-D cells were treated with a higher concentration of GA-Me (10 – 20 μg/ml, for 24 h). A clear dose–response effect was observed. What is more, at 6-h intervals, GA-Me (10 μg/ml) inhibited the cell motility effectively. This is consistent with data showing that *G. lucidum* extracts inhibited growth and induced actin polymerization in bladder cancer cells (6). In general, cell differentiation is associated with an increased F/G-actin ratio, whereas dedifferentiation and malignant transformation is associated with a decreased F/G-actin ratio (12). Our results imply that GA-Me may inhibit cell migration through inducing actin polymerization.

The key step of invasion is attachment of cancer cells to extracellular matrix (ECM) components. It is recognized that adhesive interactions of tumor cells with ECM components may play a critical role in the establishment of metastasis. To evaluate the anti-metastatic activity of GA-Me, we assessed the inhibitory effect of GA-Me on the adhesion and cell aggregation of tumor cells. We examined the effects of GA-Me on cancer cell adhesion to ECM proteins (Fig. 1C). The cell adhesion assay was done as previously reported (11). GA-Me inhibited Matrigel®-mediated attachment of 95-D cells. In the cells treated with 10 and 20 μg/ml GA-Me for 18 h, the adhesion ratio with respect to the control was 56.8 ± 3.2% and 20.5 ± 6.9%, respectively. The inhibitory effect on cell adhesion might contribute to the inhibition of invasion by GA-Me. The inhibition of tumor adhesion by the water extracts (polysaccharide) of *G. lucidum* was reported (7), but until now it has been unknown whether the triterpene had the similar effect. The results of this work provided new information about the effects of *G. lucidum* metabolites on tumor cell adhesion.

In addition, the cell viability was analyzed by both trypan blue dye exclusion assay and flow cytometry. The results showed that the cell viability of 95-D cells at 0, 5, 10, and 20 μg/ml of GA-Me was 98.3 ± 2.9%, 96.4 ± 0.6%, 93.1 ± 2.3%, and 92.4 ± 1.8%, respectively (data not shown). Apoptosis of tumor cells was only 2.3% at 20 μg/ml of GA-Me for 24 h. These results can be compared to the reported data on curcumin: Exposure of CL1–5 cells, which have high invasive capacities, to curcumin (0, 1, 5, 10, 20 μM) influenced the cell viability of CL1–5 cells in a concentration-dependent manner (92.3 ± 6.1% at 10 μM and 70.1 ± 7.3% at

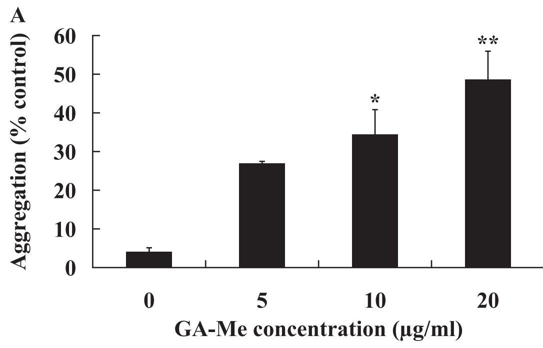
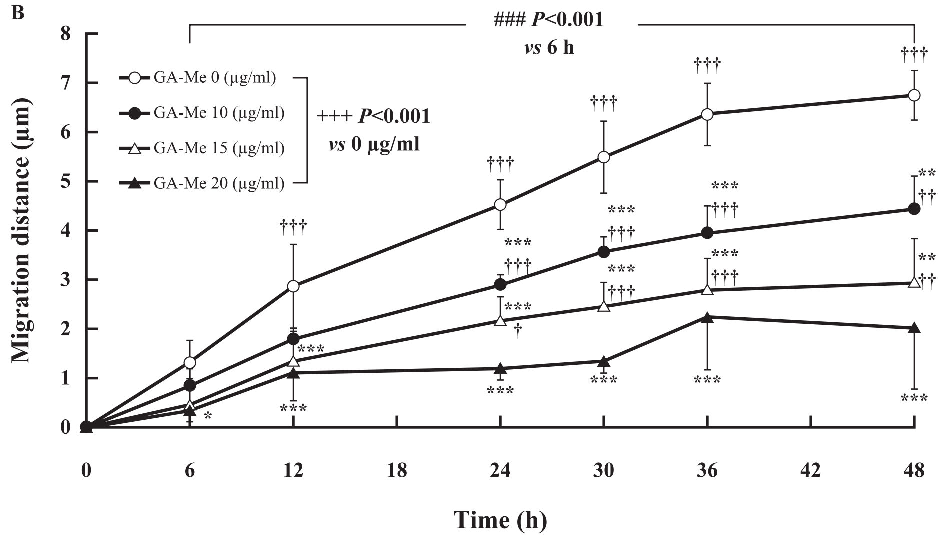
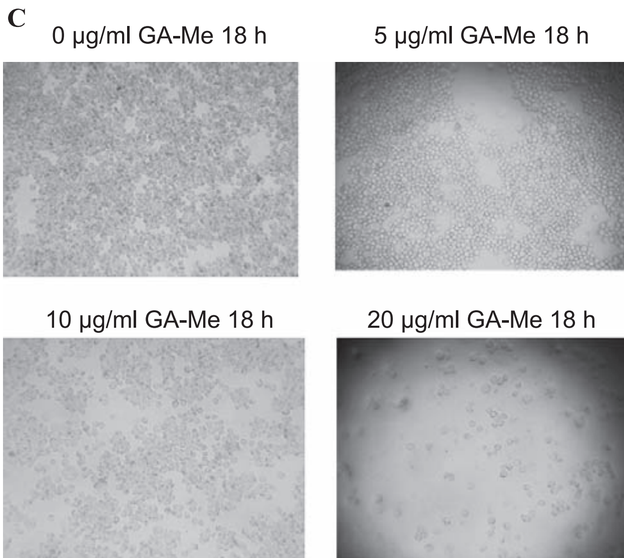
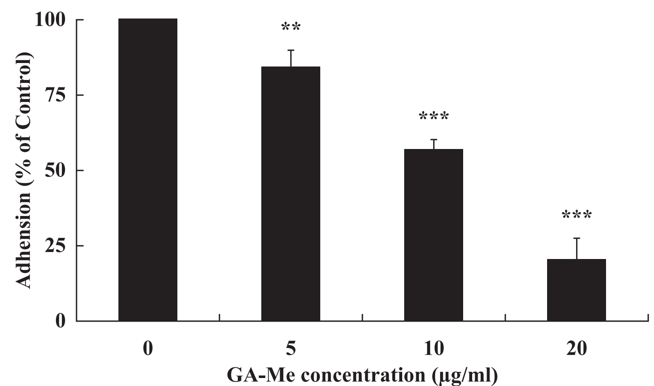


Fig. 1. Effect of ganoderic acid Me (GA-Me) on adhesion, aggregation, and migration of tumor cells. A: Effect of GA-Me on aggregation of 95-D cells. *P<0.05, **P<0.01 *vs* 0 μg/ml GA-Me (each group, n = 3). B: Effect of GA-Me on migration of 95-D cells. GA-Me: ^{+++}P<0.001 *vs* 0 μg/ml GA-Me, Time: $^{\#\#\#}P$<0.001 *vs* 6 h. The interaction of GA-Me concentration and time: *P<0.05, ***P<0.001 *vs* 0 μg/ml GA-Me at each time and $^{\dagger}P$<0.05, $^{\dagger\dagger\dagger}P$<0.001 *vs* 6 h at each concentration (each group, n = 4). C: Effect of GA-Me on cell adhesion on Matrigel®. **P<0.01, ***P<0.001 *vs* 0 μg/ml GA-Me (each group n = 3).







20 μM), and curcumin (1 – 20 μM) could significantly inhibit cell invasion of CL1–5 cells (56.0 ± 16.1% at 1.0 μM) (13).

After adhesion, another key step in the invasive progress is the degradation of a variety of ECM proteins by matrix metalloproteinases (MMPs), a well-known family of zinc-binding enzymes. MMPs play an important role in the process of cleaving ECM components. The expression levels of MMPs are correlated with tumor invasiveness. Therefore, inhibition of MMPs expression is regarded as a rational approach to metastatic disease therapy (14). The MMP2 and MMP9 are

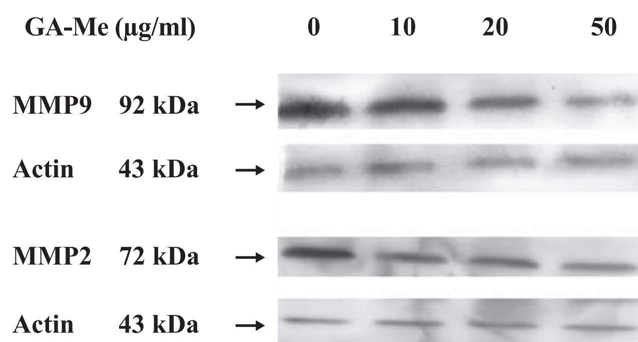


Fig. 2. Effect of GA-Me on expressions of MMP2 and MMP9 proteins. The 95-D cells were treated with different concentrations of GA-Me for 24 h. Equal amount of cellular total protein was separated on 10% SDS-PAGE with an actin as the load control and blotted with anti-MMP2 antibody or anti-MMP9 antibody and secondary antibody conjugated horseradish peroxidase. Then the blots were visualized with DAB substrate reagent (Pierce, Rockford, IL, USA). Total cellular protein was determined using the Bradford method. Experiments were repeated independently three times with similar results being obtained.

gelatinases and considered as the major proteolytic enzymes in the degradation of ECM during cancer cell progression and invasion. Interestingly, MMP2/9 expression levels are especially high in lung carcinoma and melanoma cells. In 95-D cells, invasive potential has been related to the activity and expression of MMP2/9 because they can degrade type IV collagen in the reconstituted basement membrane. To determine the effect of GA-Me on the MMP2/9 protein expression level of 95-D cells, western blot analysis was carried out. It was observed that treatment of cells with GA-Me dose-dependently resulted in down-regulation of MMP2/9 protein expression. This suggests that GA-Me inhibited the invasion of tumor cells by efficiently inhibiting the MMP2/9 protein expression (Fig. 2). Interestingly, our data were different from a previous report, where MMP2 was unchanged in the cells treated with either ethanol extracts or water extracts of *G. lucidum* (at 80 μg/ml for 24 h) and the control cells were just treated with water or ethanol (6). The difference may be the result that different extract mixtures have different effects on the anti-metastasis, and different compound(s) may have different even opposite effects. It is unclear what compound(s) in extract mixtures is responsible for the anti-metastasis, which also makes the study of structure-activity relationship difficult. Therefore, the use of a pure triterpene is required to reveal the mechanism of action of the compound(s) responsible for the anti-metastatic effects and to further screen and rationally design structurally similar lead compounds.

In order to investigate whether the suppression of MMP2/9 protein expression by GA-Me was due to reduced MMP2/9 mRNA expression, qRT-PCR analysis was employed on samples of total mRNAs extracted from 95-D cells after 24-h treatment. Briefly, 95-D cells were treated with different concentrations of GA-Me for 24 h. Total RNA was isolated by using 1 ml of Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's procedure. Residual genomic DNA was removed by incubating the RNA solution with 15 units of RNase-free DNase I (Takara Biotechnology Co., Ltd., Ohtsu) for 30 min at 37°C. The DNase reaction was stopped with a phenol/chloroform/isoamyl alcohol (25:24:1, by volume). A 2-μg sample of DNase-treated RNA was reverse-transcribed with a Reverse Transcription System (Promega Corporation, Madison, WI, USA). First-strand cDNA synthesis was carried out using 2 μg of DNase-treated total RNA in 25 μl of a solution containing first-strand buffer, 50 ng oligo$(dT)_{15}$ primers, 10 mM dNTP mixture, 25 U rRNasin Ribonuclease inhibitor, and 200 U reverse transcriptase at 42°C for 60 min. The cDNA mixtures were diluted five-fold in sterile distilled water, and 2-μl aliquots were subjected to real-time PCR using SYBR Green I dye. Real-time PCR was performed in 20 μl of a solution containing 2 × SYBR *premix Ex Tap*™ perfect real-time PCR version (Takara Biotechnology Co., Ltd.) and 10 μM of the following specific primers (sense and antisense):

GAPDH:
Sense primer: 5'-GCACCGTCAAGGCTGAGAAC-3',
Antisense primer: 5'-ATGGTGGTGAAGACGCCAGT-3',
MMP9:
Sense primer: 5'-TTGACAGCGACAAGAAGTGG-3',
Antisense primer: 5'-GCCATTCACGTCGTCCTTAT-3',
MMP2:
Sense primer: 5'-GTGTTCTTTGCAGGGAATGAAT-3',
Antisense primer: 5'-ACGACGGCATCCAGGTTATC-3'.

PCR was carried out in a thermal cycler (Rotor-Gene 3000; Corbett Research, Sydney, Australia), and the data were analyzed using Rotor-Gene 3000. The real-time PCR conditions were 95°C for 5 s and 60°C for 20 s for 40 cycles. The specificity of the PCR products was verified by adding melting curve analysis between 60°C and 95°C. The results of amplification of cDNA with primers specific for human MMP2/9 and GAPDH (as control) are shown in Fig. 3. The synthesis of MMP2/9 mRNA in 95-D cells pretreated with GA-Me for 24 h was also inhibited in a dose-dependent manner, as determined by qRT-PCR. Quantitative analysis indicated that 20 μg/ml GA-Me could reduce the mRNA level of MMP2 and MMP9 to 46.2% and 61.9%, respectively (Fig. 3). The dose-dependent responses suggest that GA-Me has the ability to inhibit the invasion of tumor

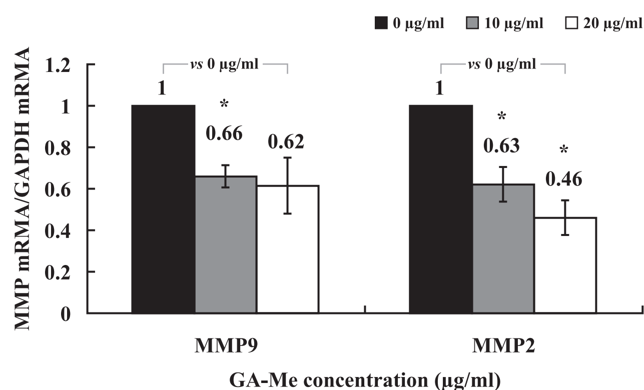


Fig. 3. Effect of GA-Me on mRNA level of MMP2 and MMP9 in 95-D cells. The qRT-PCR analysis was carried out after the cells were treated with different concentrations of GA-Me for 24 h. GAPDH was used as the control for normalization. Each real-time PCR was performed in triplicate, and the levels of mRNA expression were calculated and normalized to the level of GAPDH mRNA according to the $2^{-\Delta\Delta Ct}$ method at each different concentration. Experiments were repeated independently three times. *$P<0.05$ *vs* 0 μg/ml GA-Me (n = 3 for MMP9 and n = 3 for MMP2).

cells by diminishing the efficiency of gene transcription (Fig. 3). GA-Me suppressed MMP2/9 gene expression and hence decreased protein levels of MMP2 and MMP9 in 95-D cells.

It was demonstrated that GA-Me suppressed the expression of MMP2/9 and eventually inhibited cancer cell invasion. This conclusion is in agreement with those in previous studies that inhibitors of MMP2/9 significantly suppressed tumor metastasis in experimental animals (15). To our knowledge, our work is the first report showing that GA-Me effectively inhibited cancer cell invasion through inhibiting MMP2/9 gene expression. This is helpful for an in-depth understanding of the anti-invasion/anti-metastasis molecular mechanisms of ganoderic acids.

The anti-metastasis effects of GA-Me were associated with the promotion of cell aggregation and the inhibition of cell invasion, which was evidenced by inhibition of cell adhesion and motility as well as suppression of MMP2 and MMP9 genes expression. The results highlight the potential of GA-Me in the treatment of cancer metastasis as an MMP2/9 inhibitor. GA-Me could be a new promising candidate as an antimetastatic agent. The further elucidation of the molecular mechanisms responsible for the anti-invasive effects of GA-Me requires more studies in the future.

Acknowledgments

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