

Gambogic acid inhibits invasion of osteosarcoma via upregulation of TIMP-1

ZENG-FENG XIN, CHENG-CHUN SHEN, LI-JIANG TAO, SHI-GUI YAN and HAO-BO WU

Department of Orthopedic Surgery, The Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang 310009, P.R. China

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Abstract. Gambogic acid (GA), the natural product, has been demonstrated to be a promising chemotherapeutic drug for osteosarcoma (OS) due to its ability to induce apoptosis and cell cycle arrest. To date, no studies have examined the role of GA in metastatic bone disease. Matrix metalloproteinases (MMPs) play critical roles in invasion and metastasis, and the tissue inhibitors of metalloproteinase (TIMP) family regulates the activity of multifunctional metalloproteinases. In this study, we investigated the gene expression of matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) in OS cell lines treated by the GA. The expression of MMP-9 and TIMP-1 were studied by reverse transcription-polymerase chain reaction (RT-PCR) and western blotting. *In vitro* invasion of OS cell lines (Saos-2, MG-63) were investigated by the Matrigel invasion assay. Mean MMP-9 protein and mRNA expression was significantly suppressed; in addition, mean TIMP-1 protein mRNA expression were upregulated by increasing GA concentrations. GA reduced the invasiveness of OS cell lines dose-dependently. Furthermore, specific inhibition of TIMP-1 secretion with siRNA against TIMP-1 significantly reduced the effect of GA on OS cell lines. Overall, our findings suggest that GA reduces the invasive potential of OS cells via attenuation of MMP-9 and upregulation of TIMP-1. Moreover, TIMP-1 played an important role in the reduction of invasive potential of the OS cells which were treated by GA.

Introduction

Osteosarcoma (OS) is one of the most common malignant, aggressive tumors of bone in children and adolescents which requires a combined approach of surgery and systemic chemotherapy (1). The prognosis has improved markedly due to the introduction of aggressive chemotherapy into the multi-modal treatment regimen of OS. However, 30-50% of these patients with initially localized disease subsequently develop recurrence, which has an extremely poor clinical outcome, and 20% of all OS patients succumb to the disease due to tumor metastasis (2-5). As a consequence, new therapeutic strategies need to be actively explored to increase the survival rate of patients with OS.

The processes of tumor growth, invasion, and metastasis refer to tumor cell proliferation, cell migration and penetration through the extracellular matrix (ECM) (6). The balance in the activity between matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) is responsible for the proper maintenance of tissue. Disruption of this balance influences the metastasis and invasion of cancer and is associated with survival and prognosis (7,8).

MMPs are a family of zinc-dependent endopeptidases which have been shown to play a major role in ECM remodeling. MMPs comprise more than 25 members which are classified based on their substrate specificities and structural characteristics (9,10). In particular, the 92-kDa gelatinase B/Type IV (MMP-9) is one of the well-studied members of the MMP family, which is significantly involved in tumor invasion and angiogenesis. The ECM and basement membrane turnover is regulated by a delicate balance between the molecular factors that are involved in the production, activation, and inhibition MMPs and the production of natural TIMPs (11,12). Four members of the TIMP family have been identified (TIMP1-4) (13). TIMPs suppress MMP activity critical for ECM turnover associated with both physiological and pathological tissue remodeling. TIMP-1 is a multifunctional protein which specifically interacts with pro-MMP-9 and is subject to tight control at the level of transcription (14), whereas it has been shown that the elevated expression of TIMP-1 closely correlates with a more aggressive clinical behavior and poor prognosis in human cancer specimens (15-17). These findings suggest that TIMP-1 plays dual roles in cancer progression. Previous reports have indicated that MMP-9 and TIMP-1 are involved in the OS

Correspondence to: Dr Hao-Bo Wu, Department of Orthopedic Surgery, The Second Affiliated Hospital, School of Medicine, Zhejiang University, 88 Jie Fang Road, Hangzhou, Zhejiang 310009, P.R. China
E-mail: haobowu@hotmail.com

Abbreviations: GA, gambogic acid; MMPs, matrix metalloproteinases; OS, osteosarcoma; TIMPs, tissue inhibitors of matrix metalloproteinases; RT-PCR, reverse transcription-polymerase chain reaction

Key words: gambogic acid, osteosarcoma, invasion, tissue inhibitor of matrix metalloproteinase-1, matrix metalloproteinase-9

progression, and they have been shown to be associated with poor prognosis in human OS (18,19).

Gambogic acid (GA) (C₃₈H₄₄O₈), a natural compound extracted from gamboges, has recently been identified as a potent anticancer agent. Zhao *et al.* (20) reported that GA can induce apoptosis and cell cycle arrest of OS cell lines. In recent years, studies have shown that GA can equally inhibit the growth of a variety of tumor cells, including hepatoma, gastric and breast cancer (21-25). Moreover, it was also reported that GA exhibits low toxicity against normal tissues (26,27). The aim of our study was to examine the levels of MMP-9 and TIMP-1 in GA-treated OS cells in order to further investigate the anti-OS effect of GA.

Materials and methods

Reagents. GA was purchased from Sigma-Aldrich (St. Louis, MO, USA) with a purity of >95%. It was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and stored at -20°C at a concentration of 10 mmol/l; the final concentration of DMSO was <0.1%. The cell counting kit-8 (CCK-8) was purchased from Dojindo Laboratories (Japan). The primary antibodies against MMP-9, TIMP-1, GAPDH and secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Saos-2 and MG-63 cell culture. The established human OS cell lines MG-63 (CRL-1427TM, ATCC) and Saos-2 (HTB-85TM, ATCC) were obtained from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China), where they were tested and authenticated. MG-63 and Saos-2 cells were cultured in a humid atmosphere of 5% CO₂ and 95% air at 37°C in Dulbecco's modified Eagle's medium (DMEM) or McCoy's 5A Medium supplemented with 10% heat-inactivated fetal bovine serum (all were from Gibco-Life Sciences, USA). The medium was changed every 2-3 days, and cells were passaged twice a week. All experiments described were performed at least six times using cells at the exponential growth phase.

Effect of GA on cell proliferation by the cell counting kit-8 (CCK-8) assay. Saos-2 and MG-63 cells were seeded onto a 96-well culture plate at a density of 0.8-1x10⁴ cells/well. On the second day of culture, media were replaced with 100 µl of serum-free medium and GA at concentrations of 0-1 µM. On the third day, 10 µl of CCK-8 were added to each well and incubated for 1 h. The absorbance (A) was measured at 450 nm by a Microplate reader (Bio-Rad 550; Bio-Rad, USA). The percentage of cell viability was calculated as follows: cell viability (%) = A of experimental well/A of control well x100%. The IC₅₀ was the concentration of GA that caused 50% inhibition of cell viability.

Matrigel invasion assay. Invasion assay was performed using a transwell chamber (Neuro Probe, Inc., USA) and 8-µm pore size polycarbonate membranes (Costar, Cambridge, MA, USA) coated with Matrigel. Saos-2 and MG-63 cells were seeded at a density of 4x10⁴ in 350 µl of serum free-MEM in the upper compartment of the transwell. Complete MEM was placed in the lower chamber. Following overnight incuba-

tion at 37°C, the medium in the upper chamber was replaced with serum-free MEM and cells were treated with GA at 0, 0.5, 0.75 and 1 µM for 48 h of incubation at 37°C in a 5% CO₂ atmosphere. Non-invaded cells were wiped gently with the Matrigel. Finally, the invaded cells on the surface of the membranes were fixed and stained using Hemacolor® (Merck Millipore, Darmstadt, Germany). The number of invaded cells in six randomly selected microscopic fields (x200 magnifications) per membrane was counted.

Western blot analysis. Cells were treated with GA (0, 0.5, 0.75 and 1 µM) for 48 h, scraped into 1X cell lysis buffer (Cell Signaling Technology, USA), and incubated for 10 min on ice. Debris from the lysed cells was pelleted by centrifugation at 6,700 x g at 4°C for 5 min. The protein concentration of each sample was assayed using the bicinchoninic acid method (BCA kit) (Pierce, Rockford, IL, USA). Cell lysates, containing same amounts of protein, were mixed with equal volumes of 4X sample loading buffer, boiled for 5 min, cooled on ice for 5 min, and then analyzed by 10% Tris-HCl SDS polyacrylamide gel electrophoresis (SDS-PAGE). Protein was electrotransferred to a polyvinylidene difluoride membrane, and then blocked with 5% nonfat dry milk in 20 mM of TBS with 0.1% Tween-20 for 1 h at room temperature with shaking and incubated with the indicated primary antibodies followed by HRP-conjugated secondary antibody. After washing three times, bands were detected using ECL western blotting detection reagents (Santa Cruz Biotechnology, Inc.) and were then imaged with LAS-3000 (Life Science-Fujifilm Global).

Reverse transcription-polymerase chain reaction (RT-PCR). Cells were treated with GA (0, 0.5, 0.75 and 1 µM) for 48 h and washed twice with ice-cold 1X phosphate-buffered saline (PBS). Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. RNA (1 µg) was reverse-transcribed using the Superscript™ First-Strand Synthesis System for RT-PCR (Invitrogen) at 37°C. The following primers were used to determine target gene levels. β-actin, sense, 5'-CTGGAGCATGCC CGTATTTA-3' and antisense, 5'-TTTGGTCTTGCCACTTT TCC-3'; MMP-9, sense, 5'-AAGTGGCACCACCACAACAT-3' and antisense, 5'-TTTCCCATCAGCATTGCCGT-3'; and TIMP-1, sense, 5'-TTCCGACCTCGTCATCAGGG-3' and antisense, 5'-ATTCAGGCTATCTGGGACCGC-3'. All primers were checked against the GenBank Database to ensure no cross-reactivity with other known human DNA sequences. PCR cycles were performed using the following sequence: 94°C for 5 min, then 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C (for MMP-9) or 60°C (for TIMP-1) for 1 min, and polymerization at 72°C for 1 min, followed by 72°C for 7 min. RT-PCR products were visualized on 1.2% agarose gels electrophoresed in 0.5 TAE buffer containing 0.5 µg/ml ethidium bromide.

siRNA knockdown of TIMP-1. siRNA knockdown was used to inhibit mammalian TIMP-1. Cells were transfected with a pre-designed siRNA (100 nM) against TIMP-1 (sc-29505) using the siRNA Transfection Reagent (sc-29528). The transfection efficiency was >80% (transfection efficiency was assessed visually using control siRNA (fluorescein conjugates)

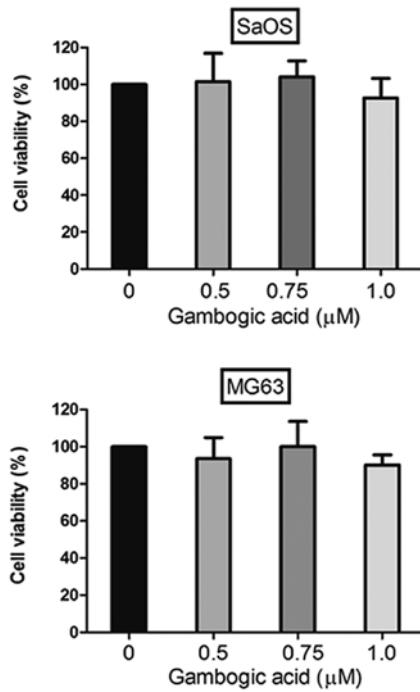


Figure 1. GA at concentrations up to 1.0 μM does not exert cytotoxicity on either Saos-2 or MG-63 cells. Both cell lines in serum-free MEM were left untreated or treated with the indicated concentrations of GA, incubated for 48 h and subjected to CCK-8 assay for cell growth quantification. The bar graph shows the absorbance (expressed as percentage of control) measured at 450 nm on an ELISA reader (n=6 independent experiments; mean ± standard deviation is shown).

(sc-36869) (all were from Santa Cruz Biotechnology, Inc.) and the extent of TIMP-1 knockdown was determined by western blot analysis of protein levels.

Statistical analysis. Intensity of bands was quantified using SPSS 17.0 software. Results are expressed as the means ± standard deviation. We performed Student's t-test statistical analysis. P<0.05 was considered to indicate statistically significant differences. Asterisks indicate the level of significance.

Results

Growth inhibition of OS cell lines by GA. We examined the role of GA on proliferation using a CCK-8 assay after treating both cell lines with GA. Saos-2 and MG-63 cells (0.8-1x10⁴ cell/well) were plated in each well of a 96-well plate. Control and 0.5, 0.75 and 1 μM GA-treated Saos-2 and MG-63 cells were incubated for 48 h. CCK-8 assay was performed 2 days after the treatment in order to quantify the proliferation of cells. GA treatment at 0-1 μM for 48 h did not significantly inhibit the growth of either cell line, therefore, there was no significant effect of GA on Saos-2 or MG-63 survival even at the concentration of 1 μM. We performed all subsequent experiments using GA concentrations ranging from 0 to 1 μM (Fig. 1).

GA suppresses the invasive ability of Saos-2 and MG-63 cells. We carried out Matrigel invasion assays to ascertain whether GA affects the invasive behavior of Saos-2 and MG-63 cells. We treated both cell lines with either control or GA (0.5, 0.75

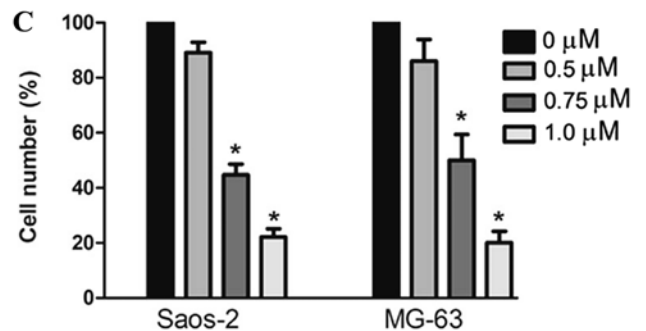
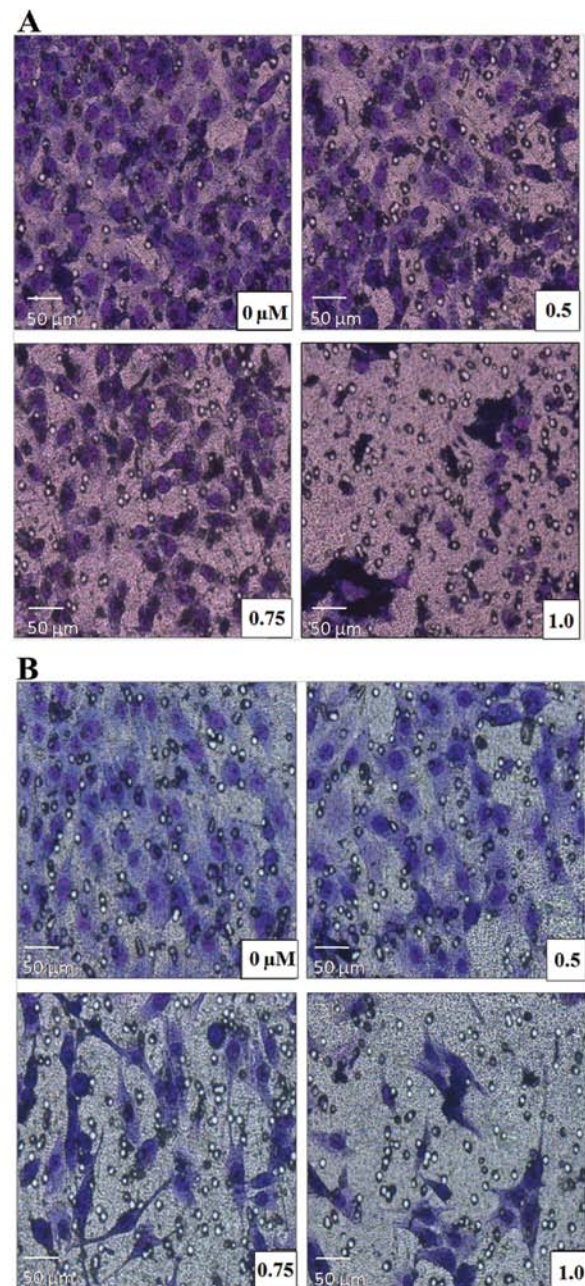


Figure 2. GA inhibits the invasiveness of (A) Saos-2, and (B) MG-63 cells. A 10-well chemotaxis chamber was used to measure the effect of GA on invasiveness upon treatment with the indicated concentrations of GA. A Matrigel-coated membrane inserted between the upper and lower chambers was stained with a Hemacolor rapid staining kit. The stained area represents cells which have migrated from the upper chamber. The number in panel denotes the concentration of GA added. (C) Bars represent cell number (expressed as percentage of control) of each image ± standard deviation. *P<0.05.

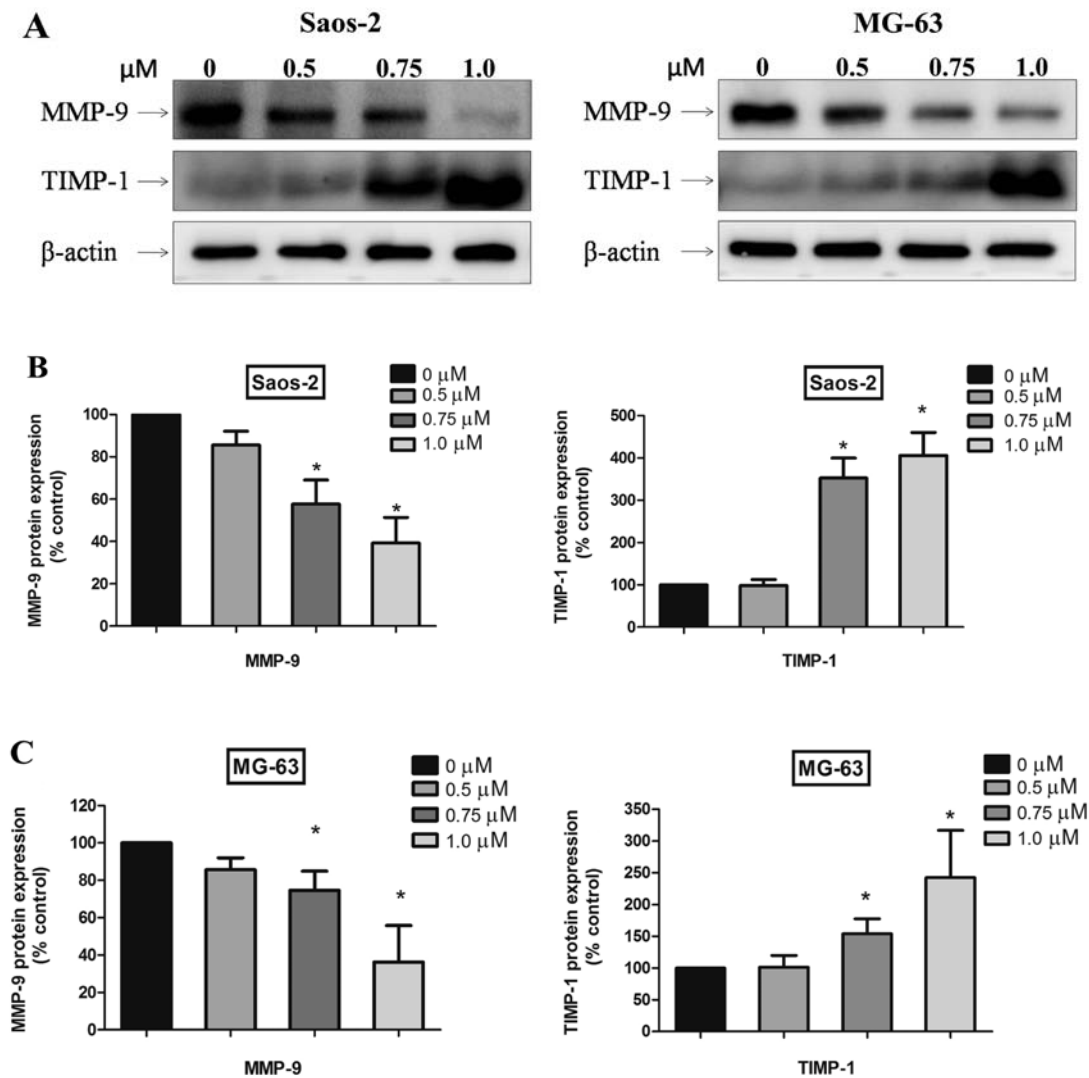


Figure 3. GA reduced the expression of MMP-9 and enhanced TIMP-1 proteins in Saos-2 and MG-63 cells. (A) Cells were treated with the indicated concentrations of GA for 48 h and were then processed for western blotting. β -actin was used as a loading control. (B and C) Protein levels of MMP-9 and TIMP-1 (expressed as percentage of control) ($n=6$). Numbers in the box represent the concentrations of GA in μM added to the cells. Bars denote average intensity (expressed as percentage of control) of each band \pm standard deviation. * $P<0.05$

and 1 μM). After 48 h of incubation, we removed transwells and stained invaded cells with Hemacolor[®] (Merck Millipore) and removed the non-invaded cells. The number of invaded Saos-2 and MG-63 cells treated by GA was significantly reduced, compared to the control, in a dose-dependent manner, indicating that GA inhibited the basal invasion capacity of Saos-2 and MG-63 cells ($P<0.05$) (Fig. 2).

GA suppresses MMP-9 and enhances TIMP-1 protein and mRNA levels in both cells. To elucidate the mechanism of invasive suppression of Saos-2 and MG-63 cells, we investigated the effect of GA on MMP-9 and TIMP-1 protein expression, which are key effectors for tissue invasion. The expression of TIMP was evaluated since the expression of TIMP suppresses the effect of MMP-9. Saos-2 and MG-63 cells were treated with GA and MMP-9 and TIMP-1 protein levels were observed by western blotting. Western blotting revealed that GA attenuated MMP-9 and increased TIMP-1 protein levels of both cell lines ($P<0.05$) (Fig. 3). RT-PCR was used to investigate the effects

of GA on MMP-9 and TIMP-1 at the transcriptional levels. GA was found to clearly attenuate MMP-9 mRNA levels and the mRNA transcripts of TIMP-1 in both Saos-2 and MG-63 cell lines ($P<0.05$) (Fig. 4).

Knockdown of TIMP-1 restrains the invasion ability of GA in both cell types. We carried out Matrigel invasion assays again to examine whether TIMP-1 affects the invasive behavior of Saos-2 and MG-63 cells. After transfection with siRNA TIMP-1 for 24 h, we treated both cell lines with either control or GA (1 μM). After 48 h of incubation, we removed transwells and stained invaded cells with Hemacolor[®] (Merck Millipore) and removed the non-invaded cells. The number of invaded Saos-2 and MG-63 cells treated by GA and siRNA TIMP-1 was significantly increased compared to the cells treated by siRNA TIMP-1 alone, demonstrating that TIMP-1 plays an important role in GA inhibiting the basal invasion capacity of Saos-2 and MG-63 cells ($P<0.05$) (Fig. 5).

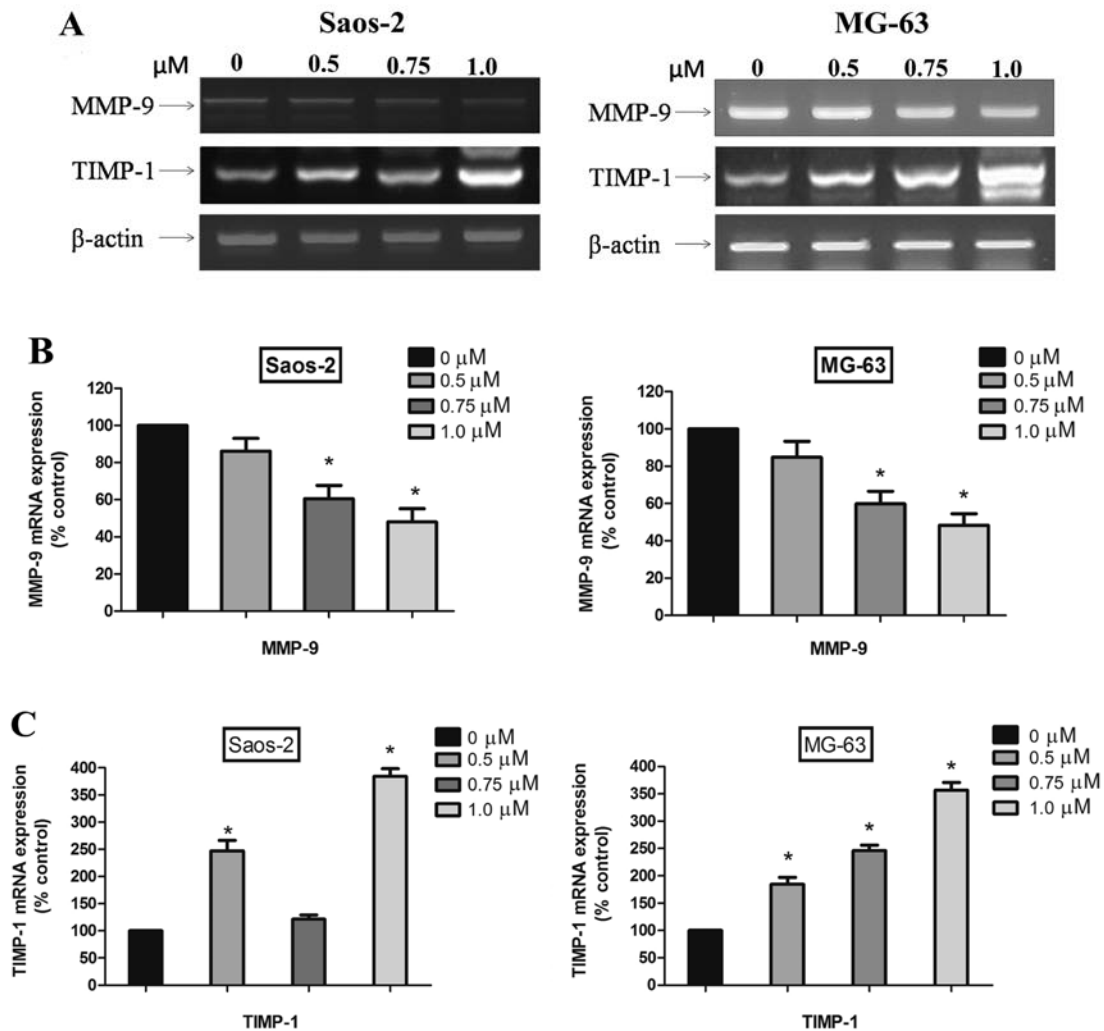


Figure 4. GA suppressed the expression of MMP-9 mRNA and enhanced TIMP-1 mRNA in Saos-2 and MG-63 cells. (A) Cells were treated with the indicated concentrations of GA for 48 h and were then processed for RT-PCR. (B and C) Quantitation of band intensity in (A) normalized to that of mRNA (expressed as percentage of control) (n=6). Numbers in the box represent the concentrations of GA in μM added to the cells. Bars represent average intensity (expressed as percentage of control) of each band \pm standard deviation. * $P < 0.05$

GA specifically enhances TIMP-1 protein and mRNA levels in both cells. To further elucidate the mechanism of invasive suppression of Saos-2 and MG-63 cells, we investigated the effect of GA on TIMP-1 protein expression after transfection with siRNA TIMP-1, which are key effectors for tissue invasion. Saos-2 and MG-63 cells were treated with GA and/or siRNA TIMP-1. TIMP-1 protein levels were observed by western blotting. Western blotting and RT-PCR revealed that GA increased only slightly the TIMP-1 protein and mRNA levels of both cell lines treated by siRNA TIMP-1 ($P > 0.05$) (Fig. 6). However, upregulation of TIMP-1 was significant in siRNA TIMP-1 and GA treated cell lines compared to siRNA TIMP-1 treated cell lines ($P > 0.05$).

Discussion

Tumor metastasis involves a series of mechanisms including vessel formation, cell attachment, invasion, migration and cell proliferation, whose regulation is complex. Numerous *in vitro* studies have demonstrated that GA exerts its antitumor effects by impeding tumor cell proliferation, inhibiting inva-

sion, inducing tumor cell apoptosis and suppressing MMPs (20,28). In addition, Matrigel is used for *in vitro* invasion assays. It contains a number of proteins such as laminin, collagen IV, heparan sulfate proteoglycan, entactin, nidogen and growth factors (e.g. TGF- β , fibroblast growth factor, tissue plasminogen activator). It has been used as a model in quantitative analysis. In the present study, we investigated the anti-metastatic effect of GA on the invasion and migration of human OS cell lines using Matrigel migration assay. We found that GA inhibited the *in vitro* migration and invasion ability of both cell lines (Fig. 2). Our results support the potential use of GA as a new strategy for anticancer therapy against migration and invasion of both cell lines.

Furthermore, it is well known that MMPs and TIMPs play key roles in tumor invasion and metastasis; as a result, the prognostic risk factors of recent studies in OS are focusing on the MMPs and TIMPs. Overall, studies on MMPs and TIMPs in cancer provide the rationale for developing anticancer drugs which target TIMP and MMP activities. TIMP-1 was described to specifically regulate proMMP-9 activation (15). Zhang *et al* (29) demonstrated that tumor

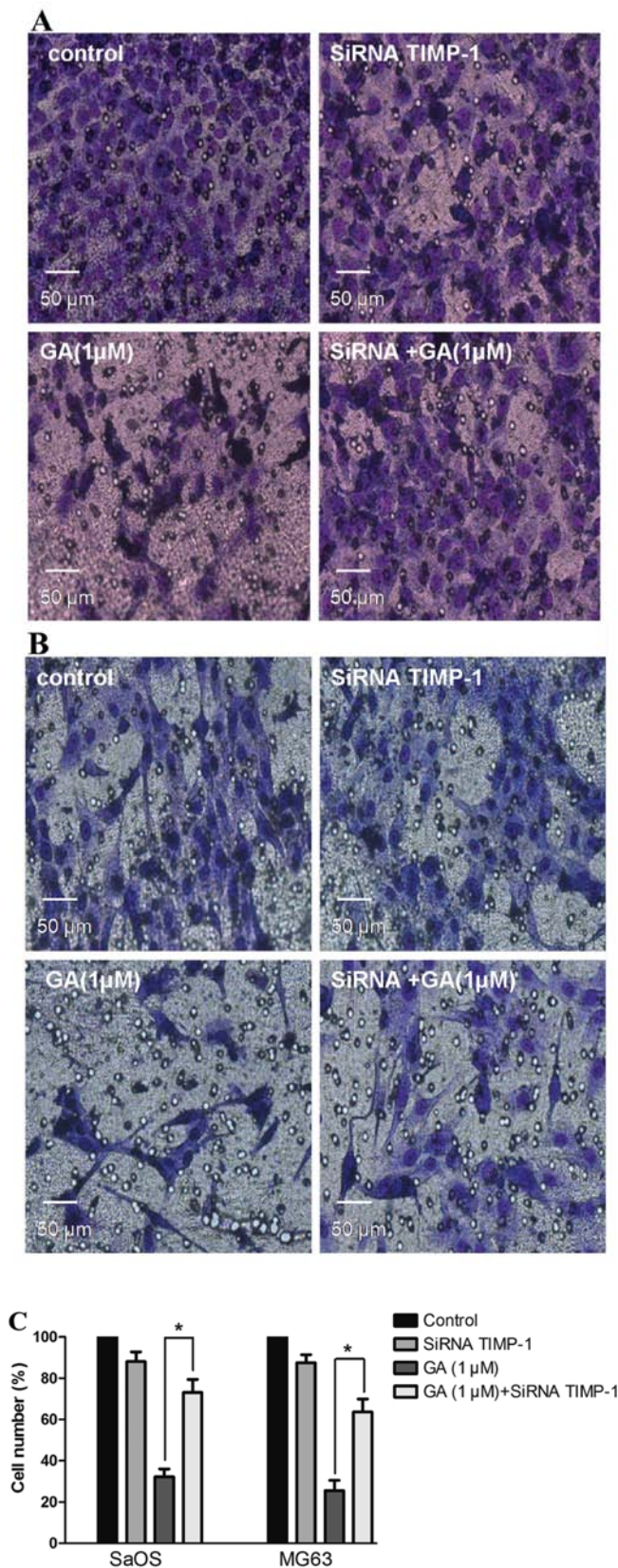


Figure 5. GA impedes the invasiveness of (A) Saos-2, and (B) MG-63 cells. However, the pharmaceutical effect of GA was inhibited when the expression of TIMP-1 was restrained by siRNA. A 10-well chemotaxis chamber was used to measure the effect of GA on invasiveness upon treatment with the indicated concentrations of GA. A Matrigel-coated membrane inserted between the upper and lower chambers was stained with a Hemacolor rapid staining kit. The stained area represents cells which have migrated from the upper chamber. (C) Bars represent cell number (expressed as percentage of control) of each image \pm standard deviation. * $P < 0.05$

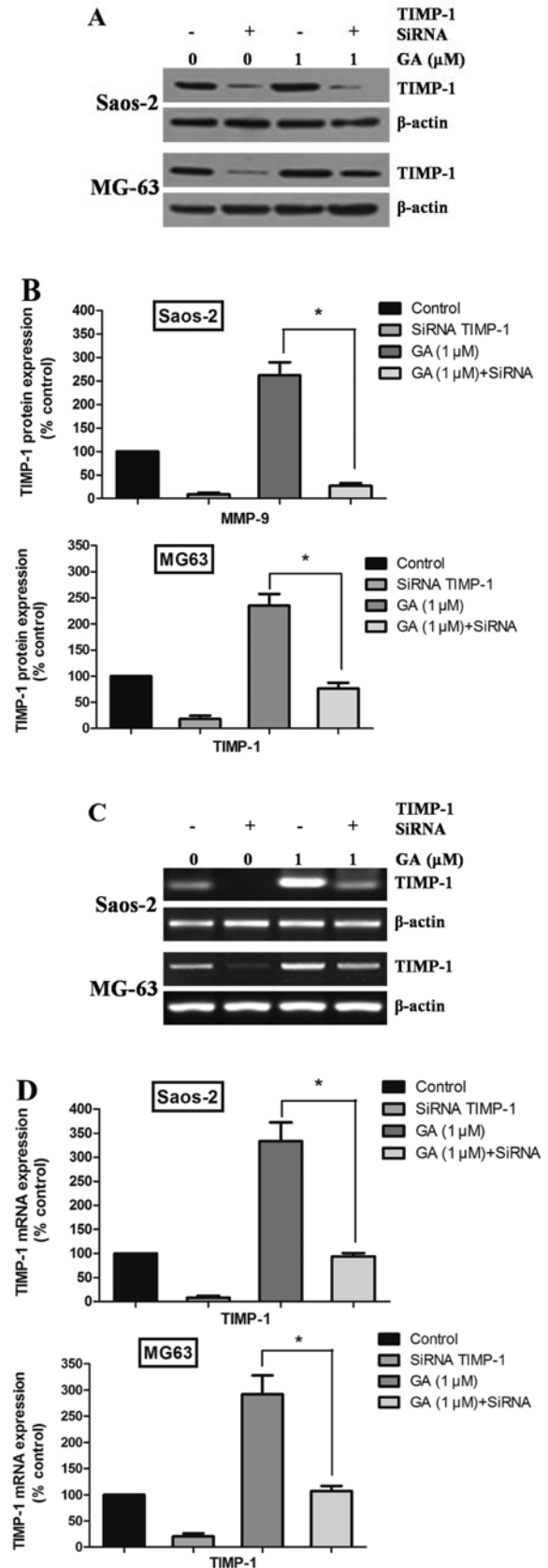


Figure 6. GA specifically enhanced TIMP-1 protein and mRNA levels in both cells. The expression of TIMP-1 proteins in Saos-2 and MG-63 cells were inhibited >80%. (A) Cells were treated with the siRNA and/or GA for 48 h and were then processed for western blotting. β -actin was used as a loading control. (B) Protein levels of TIMP-1 (expressed as percentage of control) ($n=6$). (C) Cells were treated with the indicated concentrations of GA for 48 h and were then processed for RT-PCR. (D) Quantitation of band intensity in (C) normalized to that of mRNA (expressed as percentage of control) ($n=6$). * $P < 0.05$

invasion and metastasis were more frequent in patients with tumors which secreted MMP-9 positively whereas they were significantly reduced if TIMP-1 was also expressed; thus, MMP-9 mainly functions to accelerate cancer invasion and metastasis, but TIMP-1 independently exerts an inhibitory function on cancer invasion and metastasis. However, Seo *et al* (30) suggested that the expression of TIMP-1, as well as the balance between the expression of MMP-9 and TIMP-1, indicates the progressed state of the tumor. It has recently been reported that MMP-2 and MMP-9 are present in human OS cells (31,32). In this study, GA inhibited MMP-9 expression and enhanced TIMP-1 at the protein levels; on the other hand, the mRNA expression of MMP-9 and TIMP-1 was also suppressed by GA.

As we described above, the results of the experiments of molecular biology were based on the assumption of survival rate of the cells greater than 80%. As a consequence, the effect of GA on OS cell invasion is probably not due to cell death. In terms of variation of MMP-9, TIMP-1 and previous theories, we concluded that GA promotes the expression of TIMP-1, probably further repressing the expression of MMP-9. Therefore, the balance between MMP-9 and TIMP-1 may be one of the mechanisms which lead to the inhibition of the OS cell invasion by GA. Of note, in this study, the pharmaceutical effect of GA was reduced when the expression of TIMP-1 was restrained by siRNA. This result revealed that TIMP-1 plays an important role in the reduction of the invasive potential of the OS cells which were treated by GA. The specific pathway of GA-induced TIMP-1 and MMP-9 expression requires further study to be elucidated.

In conclusion, our findings suggest that GA affects the expression and balance of MMP-9 and TIMP-1 in OS, and that this is responsible for its effect on OS cell invasiveness. This report provides the first evidence that GA interferes with the expression and balance of MMP-9 and TIMP-1 in OS cells *in vitro*. Furthermore, TIMP-1 plays an important role in the reduction of the invasive potential of the OS cells which were treated by GA. Although the precise molecular mechanism of cancer invasiveness and related targeted chemotherapy requires further clarification, these data indicate that GA may be a potential candidate for targeted drug therapy preventing recurrence and metastasis.

Acknowledgements

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