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Gallic acid inhibits migration and invasion in human osteosarcoma U-2 OS cells through suppressing the matrix metalloproteinase-2/-9, protein kinase B (PKB) and PKC signaling pathways

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ABSTRACT

Advanced cancer is a multifactorial disease which complicates treatment if the cancer cells have metastasized calling for the targeting of multiple cellular pathways. Gallic acid (GA) is known to possess multiple pharmacological activity including antitumor effects. This study investigated the mechanisms for the anticancer properties of GA on migration and invasion of human osteosarcoma U-2 OS cells. The migration and invasion in U-2 OS cells were determined by a Boyden chamber transwell assay. The expression levels and activities of MMP-2 and MMP-9 were measured by Western blotting, real-time PCR and gelatin zymography assays. All examined proteins levels from Western blotting indicated that GA decreased the protein levels of GRB2, PI3K, AKT/PKB, PKC, p38, ERK1/2, JNK, NF-κB p65 in U-2 OS cells. GA also inhibited the activities of AKT, IKK and PKC by *in vitro* kinase assay. GA suppressed the migration and invasive ability of U-2 OS cells, and it decreased MMP-2 and MMP-9 protein and mRNA levels and secreted enzyme activities *in vitro*. These results suggest that potential signaling pathways of GA-inhibited migration and invasion in U-2 OS cells may be due to down-regulation of PKC, inhibition of mitogen-activated protein kinase (MAPK) and PI3K/AKT, resulting in inhibition of MMP-2 and MMP-9 expressions.

1. Introduction

Bone cancer morbidity and mortality is a significant problem worldwide (Wermers et al., 2008). Osteosarcoma, a highly malignant bone tumor, characterized by formation of neoplastic bone tissue, is the most frequent malignant bone tumor in children and adolescents (Arndt and Crist, 1999; Thompson et al., 2002). Currently, treatment of bone cancer includes surgery, radiation, chemotherapy, or a combination of radiotherapy and chemotherapy, but such treatments are not overly successful. Numerous evidence has shown that natural products can be used to inhibit the development of cancer in multiple animal models (Hong and Sporn, 1997). These compounds can be used as antioxidants and cancer preventative agents or even as cancer therapy drugs

Abbreviations: AKT/PKB, RAC-alpha serine/threonine-protein kinase; ERK1/2, extracellular signal-regulated kinase 1/2; GRB2, growth factor receptor-bound protein 2; GA, gallic acid; JNK, c-Jun N-terminal kinase; IKK, IκB kinase; MAPK, mitogen-activated protein kinase; MMPs, matrix metalloproteinases; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C.

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(AbouEl Hassan et al., 2006). One of the major characteristics of cancer cell metastasis is altered adhesion ability between cells and the extracellular matrix (ECM) which is associated with invasion and migration of tumor cells. In the metastasis of tumor cells, matrix metalloproteinases (MMPs) and urokinase plasminogen activator (u-PA) are overexpressed (Farris et al., 2011), and MMPs have been shown to be involved in the invasion and metastasis of various tumor cells (Gullu et al., 2000; Mizutani et al., 2000). The inhibition of MMP expression or enzyme activity can be used as early targets for preventing cancer metastasis (Guruvayoorappan and Kuttan, 2008; Waas et al., 2003).

Gallic acid (3,4,5-trihydroxybenzoic acid; GA), a polyhydroxyphenolic compound, is abundant in natural plants such as gallnut, grapes, sumach, oak bark, green tea apple peels, grapes, strawberries, pineapples, bananas, lemons, and in red and white wine (Atkinson et al., 2004; Chakraborty et al., 2009; Kim et al., 2006; Ng et al., 2004; Shahrzad et al., 2001). GA has biological activities such as anti-bacterial (Kang et al., 2008), anti-viral (Kaur et al., 2009), anti-inflammatory (Kim et al., 2006), antioxidant (Inoue et al., 1994; Isuzugawa et al., 2001) and antitumor effects (Agarwal et al., 2006; Faried et al., 2007; Ji et al., 2009; Kawada et al., 2001; Veluri et al., 2006). Other beneficial actions of GA are anti-diabetic and anti-angiogenic effects nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activity through modulating the levels of MMP-2/9 and cytoskeletal reorganization signal pathway in gastric cancer cells (Ho et al., 2010). Although GA was found to inhibit the migration and invasion of osteosarcoma cells, the possible molecular mechanisms are unclear and not well investigated. Thus, the purpose of this study was to determine the anti-metastatic effects and signaling pathways of GA in the osteosarcoma cell line (U-2 OS) *in vitro*.

2. Materials and methods

2.1. Chemicals and reagents

Gallic acid (GA), dimethyl sulfoxide (DMSO), propidium iodide (PI) and trypan blue were purchased from Sigma–Aldrich Corp. (St. Louis, MO, USA). McCoy's 5A medium, L-glutamine, fetal bovine serum (FBS), penicillin–streptomycin, and trypsin–EDTA were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). Primary antibodies used for Western blotting analysis were obtained as follows: antibodies for growth factor receptor-bound protein 2 (GRB2), phosphoinositide kinase-3 (PI3K), AKT, PKC, p38, extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), NF-κB p65, MMP-2, MMP-9, p-p38, p-ERK1/2, p-JNK and α-tubulin, and second antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

2.2. Cell culture

The human osteosarcoma U-2 OS cell line was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). U-2 OS cells were plated onto 75 cm^2 tissue culture flasks with 90% McCoy's 5A medium. All cell media were supplemented with 2 mM L-glutamine, 10% FBS, 100 Units/ml penicillin and 100 μg/ml streptomycin and grown at 37 °C under a humidified 5% CO_2 atmosphere (Chen et al., 2011b; Huang et al., 2010).

2.3. Determination of cell viability and

Approximately 2×10^5 cells/well of U-2 OS cells were grown in 12-well plates and then were incubated with 5, 10, 20 and 40 μM GA or 0.5% DMSO (as a vehicle control) for 24 and 48 h. Cells were harvested by centrifugation. For viability determination, cells were stained with PI (5 μg/ml) and then analyzed using a PI exclusion method by flow cytometry (BD Biosciences, FACSCalibur, San Jose, CA, USA) as previously described (Chen et al., 2010; Lu et al., 2010).

2.4. Detection of cell number

Cells at a density of 2.5×10^5 were seeded in 12-well plates and then exposed to 5, 10, 20 and 40 μM GA or 0.5% DMSO (as a vehicle control) for 24 and 48 h. Cells were harvested and determined cell number using trypan blue stain by Countess Automated Cell Counter (Invitrogen/Life Technologist) (Chen et al., 2011a; Duan et al., 2011).

2.5. In vitro migration and invasion assays

The determinations of migration or invasion in U-2 OS cells was performed through 24-well Transwell inserts (8 μm pore filters; Millipore, Billerica, MA, USA) coated with 30 μg type I collagen (Millipore) (migration assay) or Matrigel (BD Biosciences, Bedford, MA, USA) (invasion assay), respectively (Chen et al., 2010; Liu et al., 2011). Initially, U-2 OS cells were cultured for 24 h in serum-free-McCoy's 5A medium and then cells were placed in the upper chamber of the transwell insert (2×10^4 cells/0.4 ml medium) and treated with 0.5% DMSO (as a control) or GA (20 or 40 μM) for 24 or 48 h. The medium containing 10% FBS was then placed in the lower chamber. A cotton swab was used to remove the non-migrated and invaded cells maintained in the upper chamber and the invasive cells were fixed with 4% formaldehyde and stained with 2% crystal violet. Finally, the cells in the lower surface of the filter which penetrated were counted and photographed under a phase-contrast microscope at 200× magnification. Each sample was analyzed duplicates of each treatment including the control and treated conditions were included and three independent experiments were performed as described elsewhere (Chen et al., 2010; Liu et al., 2011).

2.6. Gelatin zymography assay

Determination of the enzyme activities of MMP-2 and -9 in U-2 OS cells were measured after exposure to GA. U-2 OS cells (1×10^6 cells/well) were plated in 12-well tissue culture plates and then were incubated in serum-free McCoy's 5A medium in the presence of 0, 10, 20, 30 and 40 μM GA for 24 and 48 h. The conditioned medium was then collected and was separated by electrophoresis on 10% SDS–PAGE containing 0.2% gelatin (Sigma–Aldrich Corp.). At the end of electrophoresis, the gels were soaked in 2.5% Triton X-100 in dH_2O twice for a total of 60 min at 25 °C, then were incubated in substrate buffer (50 mM Tris HCl, 5 mM $CaCl_2$, 0.02% NaN_3 and 1% triton X-100, pH 8.0) at 37 °C for 18 h. Bands corresponding to activity of MMP-2 and -9 were visualized by negative staining using 0.2% Coomassie blue in 50% methanol and 10% acetic acid as described elsewhere (Lai et al., 2010; Liu et al., 2011). The NIH ImageJ software was applied to quantify these bands as previously described (Chiang et al., 2011; Wen et al., 2010).

2.7. Western blotting analysis

U-2 OS cells (1×10^6 cells/well) were placed in 6-well plates and incubated with GA (20 or 40 μM) for 24 h. Total protein amount was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) before being extracted with the PRO-PREP™ protein extraction solution (iNtRON Biotechnology, Seongnam, Gyeonggi-Do, Korea) according to the manufacturer's protocol. Protein

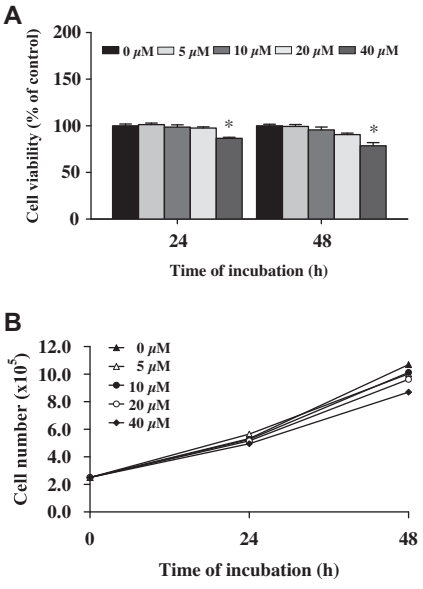

Fig. 1. GA affected on percentage of viable cells and cell number in U-2 OS cells *in vitro*. Cells were placed in 90% McCoy's 5A medium +10% FBS with 5, 10, 20 and 40 μM of GA for 24 and 48 h for percentages of viable cells. (A) The cells were collected and were analyzed for viability by a PI exclusion method and flow cytometry as described in Materials and Methods. (B) For cell number determination, cells were stained with trypan blue and determined Countess Automated Cell Counter (Invitrogen). Each point is mean ± S.D. in triplicate of at least two independent experiments. $^*p < 0.05$ was considered as statistically significant when compared with the untreated control.

abundance of GRB2, PI3K, AKT, PKC, p38, NF-κB p65, p38, ERK1/2, JNK, MMP-2, MMP-9, p-p38, p-ERK1/2 and p-JNK (Santa Cruz Biotechnology Inc.) were determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting as previously described (Lu et al., 2004; Yang et al., 2009). The relative abundance of each band was quantified using the NIH ImageJ (Chiang et al., 2011; Wen et al., 2010).

2.8. Real-time PCR of MMP-2 and -9 mRNA expressions

U-2 OS cells (1×10^6 cells/well) were placed in 6-well plates and incubated with GA (20 or 40 μM) for 24 h. Cells were collected and total RNA was extracted from each treatment as previously described (Chiang et al., 2011). RNA samples were reverse-transcribed at 42 °C with High Capacity cDNA Reverse Transcription Kit for 30 min according to the protocol of the supplier (Applied Biosystems, Foster City, CA, USA). The primers set as MMP-2F: CCCCAGACAGGTGATCTTGAC; MMP-2R: GCTTGCGAGGGAAGAAGTTG; MMP-7F: GGATGGTAGCAGTCTAGGGATTAACT; MMP-7R: AGGTTGGATACATCACTGCATTAGG; MMP-9F: CGCTGGGCTTAGATCATTCC; MMP-9R: AGGTTGGATACATCACTGCATTAGG; GAPDH-F: ACACCCACTCCTCCACCTTT; GAPDH-R: TAGCCAAATTCGTTGTCATACC. Applied Biosystems 7300 Real-Time PCR system was used for each assay in triplicate and expression fold-changes were derived using the comparative C_T (threshold cycle) method (Chen et al., 2010; Lin et al., 2009).

2.9. AKT, IκB kinase (IKK) and PKC using in vitro kinase assay

The peptide substrates, including KGSGS GRPRTSSFAEG for AKT1 and KKKKERLLDDRHDSGLDSMKDEE for IKKa/CHUK as well as the substrate Histone H1 and lipid activator for PKCα in base reaction buffer (20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 0.02% Brij35, 0.02 mg/ml BSA, 0.1 mM Na_3VO_4, 2 mM DTT and 1% DMSO) were prepared according procedures provided by the manufacturer (Reaction Biology Corp., Malvern, PA, USA). Any required cofactors (1.5 mM $CaCl_2$, 16 μg/ml Calmodulin, and 2 mM $MnCl_2$) were then delivered into the substrate solution above and gently mixed with indicated kinase and substrate solution. Various concentrations of GA (5, 10, 20, 40 and 80 μM in DMSO) were added and mixed into the kinase reaction mixture. The initiate the reaction was carried out after ^{33}P-ATP (specific activity 0.01 μCi/μl) into the reaction mixture and thereafter they were incubated for 120 min at room temperature. Reactions are spotted onto P81 ion exchange paper (Whatman #3698–915, Maidstone, England, United Kingdom) and filters were washed extensively in 0.1% phosphoric acid and counted as previously described (Coffer and Woodgett, 1991; Enomoto et al., 2005; Jones et al., 1991; Ma et al., 2011).

2.10. Statistical analysis

Statistical differences were determined using one-way analysis of variance (ANOVA) followed by Dunnett's post-test and considered significant at the $p < 0.05$ between experimental and control samples. All data are presented as means ± standard deviation (S.D.) in duplicate of at least three independent experiments.

3. Results

3.1. Effects of GA on percentage of viable and cell number U-2 OS cells in vitro

U-2 OS cells were treated with various concentrations (5, 10, 20 and 40 μM) of GA for 24 and 48 h. Cells were collected and determined viability by a PI exclusion and flow cytometry assays. For cell number determination, cells were stained with trypan blue and then measured using Countess Automated Cell Counter (Invitrogen). Fig. 1A shows that 40 μM GA treatment had a slight effect on reduction ($p < 0.05$) on viability (86% and 78% at 24 and 48-h

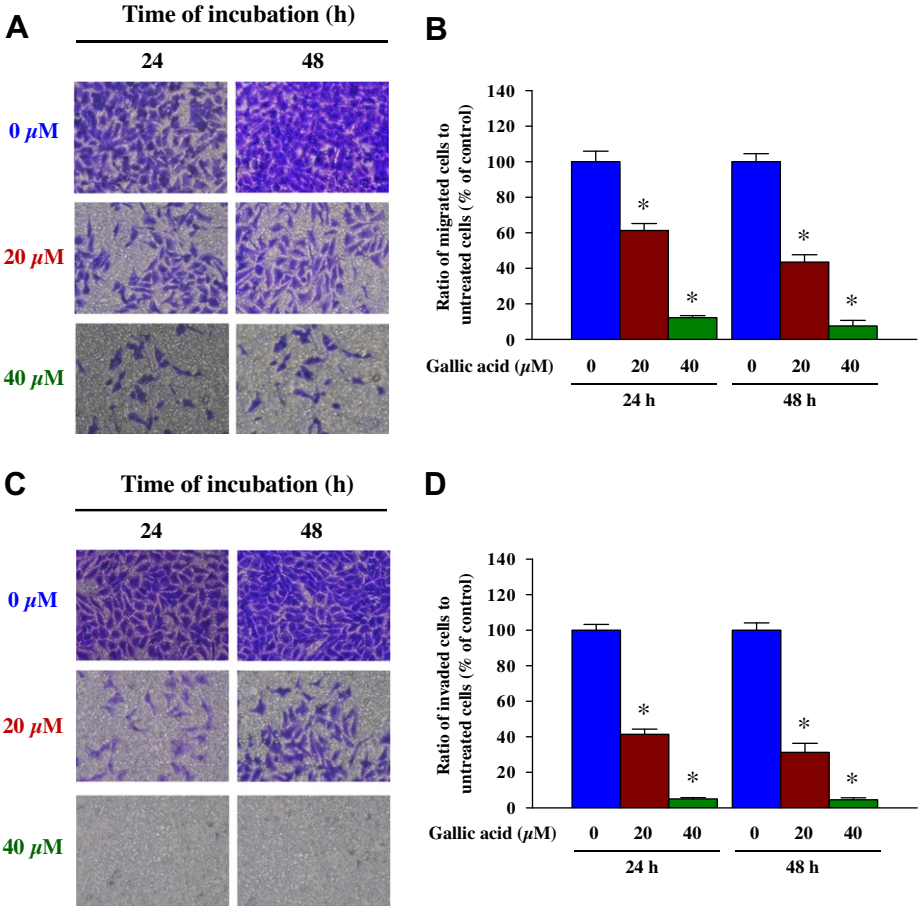

Fig. 2. GA suppressed cell migration and invasion of U-2 OS cells. Cells were treated with 0, 20 and 40 μM of GA for 24 and 48 h. (A) Cell migration was examined in a Boyden chamber and Matrigel-coated transwell with polycarbonate filters (pore size, 8 μm); (C) cell invasion was examined in a Boyden chamber (polycarbonate filters; pore size, 8 μm) were precoated with Matrigel. Migration (B) and invasion (D) abilities of U-2 OS cells were quantified by counting the number of cells that invaded the underside of the porous polycarbonate membrane under microscopy and represented the average of three independent experiments with duplicate samples. $^*p < 0.05$ was compared with the untreated control.

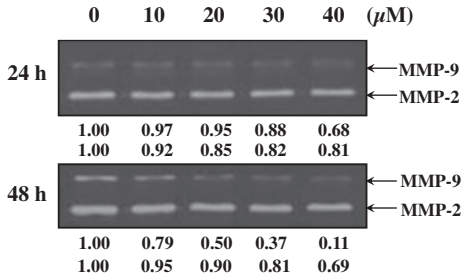

Fig. 3. GA decreased the MMP-2 and MMP-9 enzyme activities in U-2 OS cells. Cells (1×10^6 cells/well) were plated in 12-well were incubated with 0, 10, 20, 30 and 40 μM of GA for 24 and 48 h. The supernatant was harvested from examined cells and it was separated by gelatin zymography as described in Section 2. The images of MMP-2 and MMP-9 activities were quantitated by the NIH ImageJ software. Data are performed with representative at least three independent experiments with similar results.

incubation, respectively) when compared with the control group. GA at 40 μM treatment showed a decrease of cell number ($p < 0.05$) when compared with the 0-h treated group (Fig. 2B).

3.2. Effects of GA on migration and invasion of U-2 OS cells

We investigated the effects of GA on migration and invasion of U-2 OS cells and results are shown in Fig. 2A–D. The migration assay showed that GA had a significant inhibitory effect on cell migration at concentrations between 20 and 40 μM (Fig. 2A). Data in Fig. 2B indicated that the inhibition rate was at 40–84% and 60–92% when cells were incubated with GA for 24 and 48-h treatment, respectively. The invasion assay showed that U-2 OS cells moved from the upper chamber to the lower chamber in the absence of GA (control group) (Fig. 2C). However, the penetration of the Matrigel-coated filter by U-2 OS cells was inhibited in the presence of GA. Percent inhibition at 20–40 μM was at 60–90% and 66–94% (Fig. 2D) when cells were exposed to GA for 24 and 48 h exposure, respectively.

3.3. Effects of GA on the enzyme activity of MMP-2 and -9 in U-2 OS cells

The previous study has shown that MMP-2 and -9 play vital roles in tumor cell invasion (Zhang et al., 2004). We determined if GA could inhibit the secretion of MMP-2 and -9 in U-2 OS cells. Gelatin zymography was used to analyze the effects of 0, 10, 20, 30 and 40 μM GA-treated U-2 OS cells on MMP-2 and -9 activities for 24 and 48 h-incubations, respectively. Data in Fig. 3 indicated that GA at 20–40 μM dramatically reduced activity of both MMP-2 and -9 levels in U-2 OS cells.

3.4. Effects of GA on levels of proteins and genes associated with migration and invasion in U-2 OS cells

We further determined the effects of GA on the levels of proteins associated with migration and invasion in U-2 OS cells. Results showed in Fig. 4A and B and indicated that GA decreased

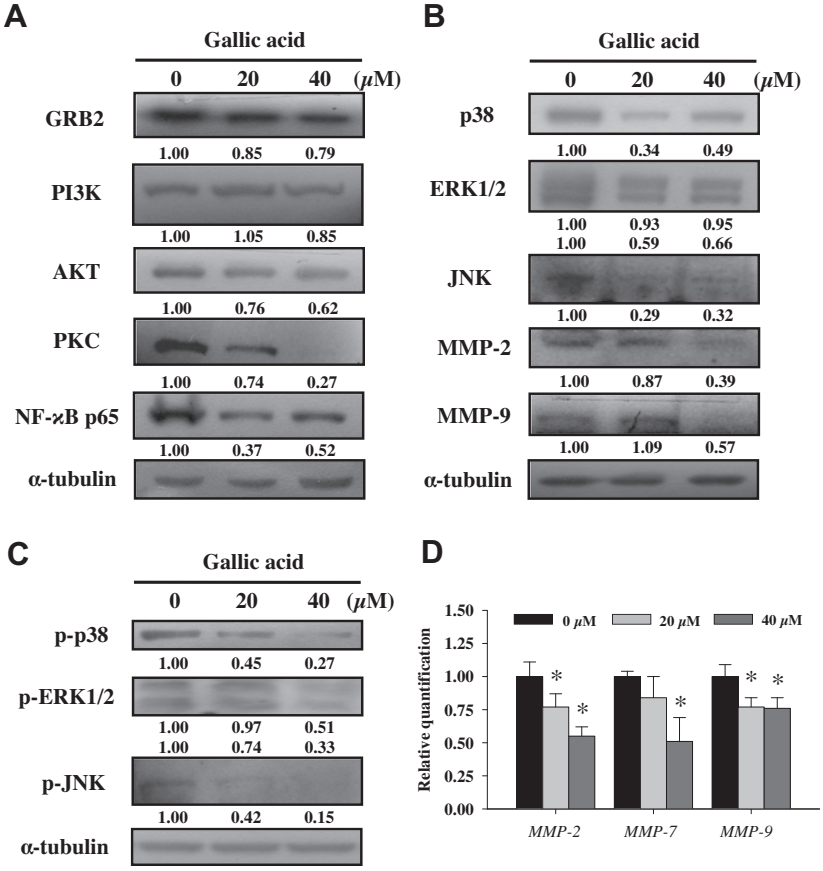

Fig. 4. GA altered the proteins and gene levels of associated with migration and invasion in U-2 OS cells. Cells were treated with 0, 20 and 40 μM of GA for 24 h. The total proteins were collected and the proteins levels were measured using Western blotting analysis. The levels of (A) GRB2, PI3K, AKT, PKC, NF-κB p65, (B) p38, ERK1/2, JNK, MMP-2, MMP-9, (C) p-p38, p-ERK1/2 and p-JNK were examined by SDS–PAGE and Western blotting. Results were shown with similar results of at least three independent experiments. (D) The total RNA was extracted from GA-treated U-2 OS cells, and then RNA samples were reverse-transcribed to cDNA for real-time PCR as described in Section 2. The ratios between MMP-2, -7, -9 and GAPDH mRNA are used and data represents mean ± S.D. in duplicate of at least three independent experiments. $^*p < 0.05$ was considered significantly.

protein levels of GRB2, PI3K, AKT, PKC, NF-κB p65 (Fig. 4A), p38, ERK1/2, JNK, MMP-2, MMP-9 (Fig. 4B), p-p38, p-ERK1/2 and p-JNK (Fig. 4C) in U-2 OS cells. The ERK signaling has been reported to up-regulate the expression of MMPs (Coffer and Woodgett, 1991; Enomoto et al., 2005; Jones et al., 1991). Results indicated that GA could inhibit the levels of ERK1/2 (Fig. 4B), p-ERK1/2 (Fig. 4C) and AKT/PKB (Fig. 4A), suggesting that GA may inhibit the ERK and PI3K/AKT signaling pathways in U-2 OS cells *in vitro*. It was reported that signaling pathways by p38, JNK, ERK1/2 and PI3K/AKT involve protein phosphorylation (Dalby et al., 1998; Mylonis et al., 2006). Our data indicated that GA suppressed the levels of p-p38, p-ERK1/2 and p-JNK in U-2 OS cells as shown in Fig. 4C. GA treatment in a dose-dependent manner for 24 h also reduced mRNA expression levels of MMP-2 and MMP-9 as seen in Fig. 4D. This is also confirmed by gelatin zymographic and Western blotting analyses in Figs. 3 and 4B, respectively, and the results indicated that GA inhibited the levels of protein and mRNA of MMP-2 and MMP-9 in U-2 OS cells.

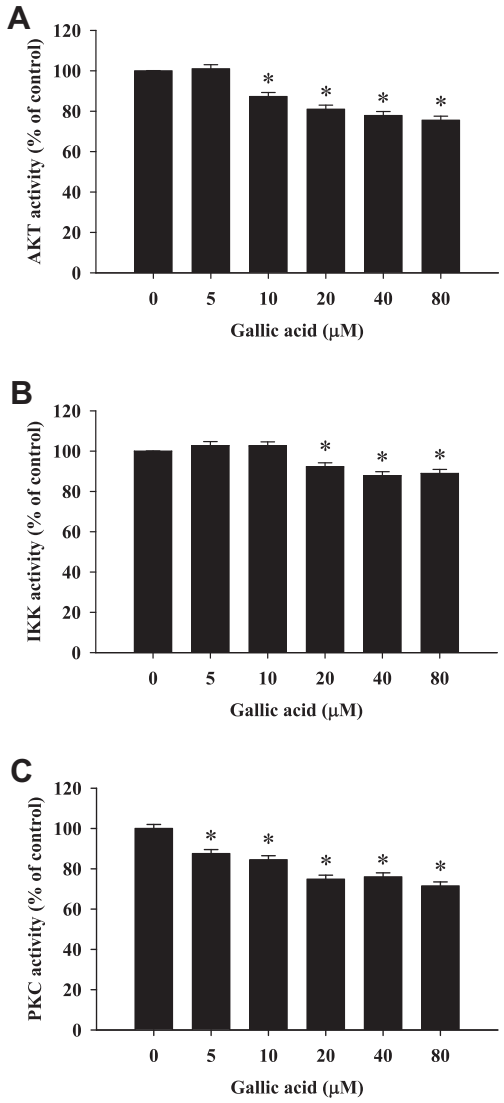

Fig. 5. GA reduced AKT/PKB, IKK and JNK activities *in vitro*. Different concentrations of GA (0, 5, 10, 20, 40 and 80 μM) were measured and the activities of AKT/PKB (A), IKK (B) and PKC (C) were done as described in Materials and Methods. Data represents mean ± S.D. of at least three independent experiments in duplicate. $^{*}p < 0.05$ was considered significantly.

3.5. Effects of GA on AKT/PKB, IKK and PKC activities by in vitro kinase assay

Data in Fig. 5A–C indicated that GA at 10, 20, 40 and 80 μM inhibited AKT/PKB activity (Fig. 5A). IKK activity was inhibited by GA at 20, 40 and 80 μM (Fig. 5B), and PKC activity was inhibited at 5, 10, 20, 40 and 80 μM (Fig. 5C).

4. Discussion

Tumor metastasis occurs through a series of steps including vessel formation, cell attachment, invasion, migration and cell proliferation, whose regulation is complex (Fidler, 2002). Numerous efforts have been taken to develop drugs that can inhibit metastasis, but the anti-metastatic agents have had mixed results (Fidler, 2002). In normal bone, matrix is constantly degraded and replaced by new matrixa a process involving a coordinated balance between bone degradation and synthesis (Chakraborti et al., 2003) and the proteolytic enzymes such as matrix metalloproteinases (MMPs) are required in this process (Duffy et al., 2008). Krupitza et al. demonstrated that GA was ineffective in lymphendothelial cells (LECs) gap formation and there was no influence on cell migration (Madlener et al., 2010). In our study, we investigated the anti-metastatic mechanisms of GA on the invasion and migration of human osteogenic sarcoma U-2 OS cells using a Boyden chamber assay to quantify the migratory potential of U-2 OS cells. We found that GA inhibited *in vitro* migration and invasion ability of U-2 OS cells (Fig. 2). Our results strengthen the potential use of GA as a new strategy for anticancer therapy against migration and invasion of U-2 OS cells.

MMPs play important roles in tumor angiogenesis, metastasis and stimulation of growth factor release from the ECM (Everts et al., 1992; Ortega et al., 2003). It is well-established that inhibitions of MMP gene expression or enzyme activity are early targets for preventing cancer metastasis (Zhang et al., 2004). It was reported that MMP-2 (72-kDa gelatinase A) and MMP-9 (92-kDa

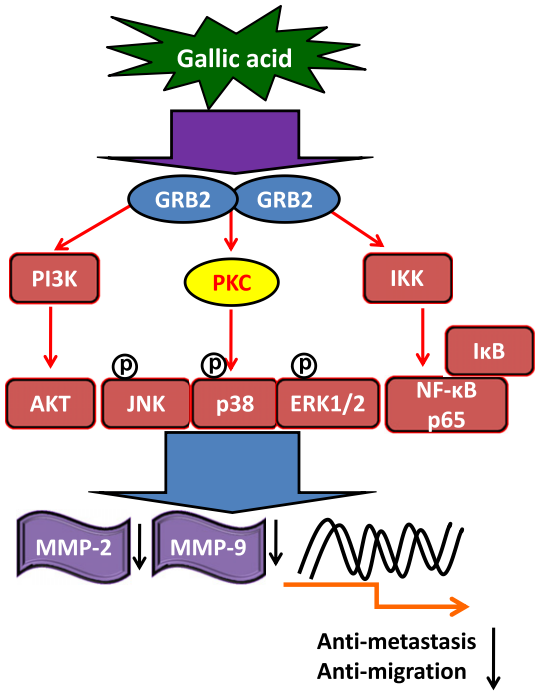

Fig. 6. The proposed a schematic presentation of the mechanisms of GA-inhibited migration and invasion of human osteosarcoma U-2 OS cells.

gelatinase B) are involved with the invasive metastatic potential of tumor cells. Recently, it was reported that MMP-2 and MMP-9 were present in human osteosarcoma cells (Guruvayoorappan and Kuttan, 2008; Waas et al., 2003). Herein, we clearly showed that GA inhibited the migration and invasion of U-2 OS cells *in vitro* and proposed that GA may account for its inhibitory effect on cancer metastasis. Firstly, GA could inhibit the protein activity of MMP-2 and MMP-9 (Fig 3) in U-2 OS cells, which are involved in degradation of extracellular matrix and play vital roles in cancer cell migration and invasion (Zhang et al., 2004). Secondly, GA inhibited the mRNA levels of MMP-2 and -9 (Fig. 4D). Thirdly, GA could inhibit the protein levels of MMP-2 and MMP-9 directly (Fig. 4B). Finally, GA inhibited levels of ERK1/2 which are key molecules of the ERK signaling pathway that have been shown to promote tumor invasion and metastasis. In the present study, GA also decreased the protein levels of AKT and JNK (Figs. 4A and B). It was reported that most integrins activate MAPK and PI3K, leading to activation of AKT/PKB (Bjornland et al., 2005; Chakraborti et al., 2003; Zhang et al., 2004). The JNK inhibitor (SP600125) was used to pre-treat the U-2 OS cells which then were exposed to GA, resulting in decrease the MMP-2 and MMP-9 protein levels (data not shown). We also found that GA inhibited the levels of AKT/PKB, IKK and PKC (Fig. 5) *in vitro* which was associated with inhibition of MMP-2 and MMP-9 mRNA levels (Fig. 4C) and protein levels (Fig. 4B). Such effects are consistent a decrease in migration of GA-treated U-2 OS cells.

To further explore the exact expression of GA-induced inhibition of invasion and migration, we performed a gelatin zymography assay to detect the activities of MMP-2 and MMP-9 in U-2 OS cells. The results showed that GA notably down-regulated activities of MMP-2 and MMP-9 in a dose-dependent manner. These results demonstrated that anti-metastatic effect of GA was associated with the inhibition of enzymatically degradative processes of metastasis in U-2 OS cells (Fig. 3). The present results suggest that GA might have efficacy if delivered to the bone site at concentrations between 20 and 40 μM, and thus preventing metastasis of bone cancer cells.

In the present study, results showed that GA reduced protein levels of PI3K, AKT/PKB, NF-κB, MMP-2 and MMP-9. PI3K activation stimulated the downstream target AKT/PKB which plays various and important roles including cell invasion (Duffy, 2004). GA also inhibited AKT/PKB activity (Fig. 5A) and it decreased the levels of PKC, p38 and JNK in U-2 OS cells (Fig. 4A and B). Several reports indicate that activation of PKC can cause translocation of the protein to membranes affecting the expression of MMP-9 *via* modulation of transcription factors such as NF-κB or Sp-1 through MAPK and PI3K signaling pathways (Eccles, 2004; Hollborn et al., 2007). GA inhibited the PKC activity (Fig. 5C). Therefore, the regulation of NF-κB, downstream of the PI3K/AKT and MAPK (ERK1/2, p38 and JNK) pathways, may be involved in GA-suppressed MMP-2 and -9 expression and invasion of U-2 OS cells. It was reported that PI3K activation stimulated the downstream target AKT/PKB, which plays various and important roles including cell invasion (Chung et al., 2004; Zhang et al., 2006). Other reports have shown that the activation of ERK and AKT/PKB is involved in the development of endometrial cancer (Hollborn et al., 2007). These findings suggest that GA has multiple anti-metastatic activities and it exhibits a potent cytotoxic effect that could be potentially utilized as an anti-metastatic agent for bone cancer in the future.

In conclusion, the present study determined the efficacy of GA on counteracting migration and invasion and identified molecular mechanisms of GA inhibition in human osteosarcoma U-2 OS cells. GA inhibited the activities of AKT/PKB, IKK and PKC in a dose-dependent manner. Potential signaling pathways depicted in Fig. 6 for GA-inhibited migration and invasion in U-2 OS cells which may be mediated through down-regulation of PKC, blocking MAPK, PI3K/AKT and NF-κB pathways, resulting in the inhibition of MMP-2 and MMP-9.

Conflict of Interest

The authors declare that there was no conflicts of interest.

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