RESEARCH ARTICLE

Ursolic acid, a naturally occurring triterpenoid, suppresses migration and invasion of human breast cancer cells by modulating c-Jun *N*-terminal kinase, Akt and mammalian target of rapamycin signaling

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Metastasis is one of the most important factors related to breast cancer therapeutic efficacy. Ursolic acid, a naturally occurring triterpenoid, has various anticancer activities. In this study, we first observed that ursolic acid exerted a dose- and time-dependent inhibitory effect on the migration and invasion of highly metastatic breast MDAMB231 cells at non-cytotoxic concentrations. This effect was associated with reduced activities of metalloproteinase-2 (MMP-2) and u-PA, which correlated with enhanced expression of tissue inhibitor of MMP-2 and plasminogen activator inhibitor-1, respectively. Ursolic acid suppressed the phosphorylation of Jun *N*-terminal kinase, Akt and mammalian target of rapamycin, but had no effect on the phosphorylation of ERK and p38. Ursolic acid also strongly reduced the levels of NFκB p65, c-Jun and c-Fos proteins in the nucleus of MDAMB231 cells. A time-dependent inhibition of the protein levels of Rho-like GTPases, growth factor receptor-bound protein 2, Ras and vascular endothelial growth factor in cytosol by ursolic acid treatment was also observed. In conclusion, we demonstrated that the anti-invasive effects of ursolic acid on MDAMB231 cells might be through the inhibition of Jun *N*-terminal kinase, Akt and mammalian target of rapamycin phosphorylation and a reduction of the level of NFκB protein in the nucleus, ultimately leading to downregulation of MMP-2 and u-PA expression. These results suggest that ursolic acid has potential as a chemopreventive agent for metastatic breast cancer.

Keywords:
Akt / Invasion / Jun *N*-terminal kinase / Mammalian target of rapamycin / Ursolic acid

1 Introduction

Breast cancer is one of the leading causes of malignancy-related death in the world. In 2008, an estimated 200 000 women will be diagnosed with this disease [1]. The principal mechanisms involved in breast cancer mortality are migration and invasion, where primary tumor cells disseminate and grow at a distant site, resulting in a secondary tumor. Unfortunately, the severe morbidity of metastatic breast cancer is not improved by surgery, radiotherapy or adjuvant chemotherapy, as metastatic breast cancer is highly resistant to chemotherapy and there is still no effective cure for patients with advanced stages of the disease [2]. Thus, it is imperative to search for novel targets and agents to prevent invasion and metastasis of breast cancer.

The phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway is involved in many cellular processes including tumorigenesis, cell cycle progression, cell motility, invasion and tumor angiogenesis [3]. Importantly, PI3K/Akt/mTOR signaling is often

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Abbreviations: Grb2, growth factor receptor-bound protein 2; **JNK,** c-Jun *N*-terminal kinase; **MMP-2,** metalloproteinase-2; **mTOR,** mammalian target of rapamycin; **PAI-1,** plasminogen activator inhibitor-1; **PI3K,** phosphatidylinositol 3-kinase; **RhoA,** Rho-like GTPases; **SRB,** sulforhodamine B; **TIMP-2,** tissue inhibitor of metalloproteinase-2; **VEGF,** vascular endothelial growth factor

activated in breast cancer due to genetic alterations of genes such as phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α isoform, phosphatase and tensin homolog deleted on chromosome 10 and tuberous sclerosis complex 1/2 that have frequently been found to be mutated or amplified in this pathway [4]. An *in vitro* experiment has demonstrated that the PI3K/Akt pathway is involved in breast cancer cell invasiveness through the enhancement of MMPs expression [5]. These observations suggest that the PI3K/Akt/mTOR pathway is aberrantly activated in the majority of invasive breast cancers. Therefore, blocking this pathway provides a promising target for novel therapeutic strategies.

Ursolic acid and oleanolic acid are naturally occurring triterpenoids that have been used in traditional medicine for centuries as an antibacterial, antifungal and anti-inflammatory agent [6]. The structures of these two compounds are shown in Fig. 1. Ursolic acid and oleanolic acid have also been used in the treatment of liver diseases [7] and hypertension [8] in addition to presenting immunomodulatory and hypoglycemic activities [9]. Several recent studies have indicated that ursolic acid and its derivatives inhibit the growth of cancer cells through cell cycle arrest and the stimulation of apoptosis [10, 11]. Because ursolic acid is relatively non-toxic to normal cells [12], an important implication of these finding is that this agent might play a useful role in the treatment of cancer. However, little is known regarding the anti-invasive activities of ursolic acid and oleanolic acid as well as their underlying mechanisms on breast carcinoma.

The purpose of this study was to examine the effect of ursolic acid and oleanolic acid on the invasion of MDAMB231 cells (a highly metastatic human breast cancer cell line). Additionally, in order to explore the underlying molecular mechanisms of the activity of ursolic acid in human breast cancer cell invasion and metastasis, the activity levels of MMPs, PI3K/Akt/mTOR signaling proteins and related transcription factors were also investigated.

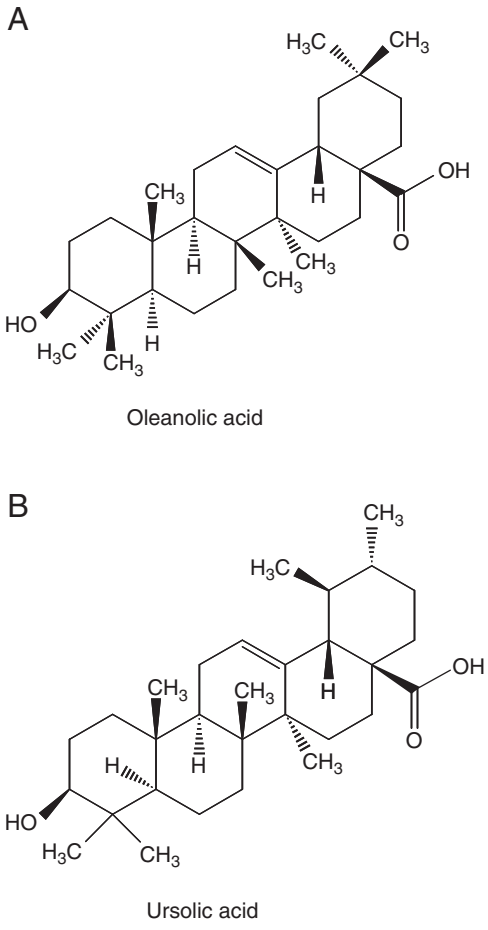

Figure 1. Structures of oleanolic acid (A) and ursolic acid (B).

2 Materials and methods

2.1 Chemicals

Triperpenoids (ursolic acid and oleanolic acid acid, 98% purity), rapamycin from *Streptomyces hygroscopicus* (≥95%, purity), IgG polyclonal antibody conjugated to peroxidase and Sulforhodamine B (SRB) dye were obtained from Sigma-Aldrich (St. Louis, MO, USA). The antibodies of total and phosphorylated MAPK/ERK1/2, p38 MAPK, SAPK/c-Jun *N*-terminal kinase (JNK), IκBα, c-Fos, c-Jun, IKK, NF-κB (p65), lamin B1 and β-actin were purchased from Cell Signaling Technology (Boston, MA, USA). Anti-Rho-like GTPases (RhoA), anti- growth factor receptor-bound protein 2 (Grb2), anti-Ras and anti-vascular endothelial growth factor (VEGF) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The inhibitors of JNK and Akt, SP600125 and LY294002 were obtained from Biosource (Camarillo, CA, USA); a TRIzol RNA isolation kit was obtained from Life Technologies (Rockville, MD, USA); and primers for RT-PCR, dNTP, reverse transcriptase and Taq polymerase were obtained from Gibco BRL (Cergy Pontoise, France). All other chemicals were of the highest pure grade available.

2.2 Cell lines and culture

The MDAMB231 human breast cancer cell lines were obtained from American Type Culture Collection (ATCC). MDAMB231 cell lines were maintained in RPMI 1640 medium supplemented with 10% v/v fetal bovine serum in a humidified atmosphere of 95% air and 5% CO_2. The media were changed three times *per* week. The cells were free of Mycoplasma contamination during the study period.

2.3 Analysis of cell viability (SRB assay)

Briefly, breast cancer cells were seeded into 96-well plates in growth medium at 3000 cells/well for 24 h. Then, the cells

were treated with ursolic acid and oleanoic acid at various concentrations (0, 5, 10, 25 and 50 μM) for various periods of time (24 and 48 h). After the exposure period, the cells were fixed with TCA for 1 h and stained with 100 μL of 0.4% w/v SRB for 10 min at room temperature. The bound dye was solubilized in 20 mM Tris base (100 μL/well) for 5 min on a shaker. Optical densities were read on a microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 562 nm.

2.4 Anchorage-independent growth

MDAMB231 cells were harvested and seeded in six-well plates coated with 1% agarose. Anchorage-independent growth was assessed after incubation for 10–14 days in culture media with or without ursolic acid (0, 10, 25 and 50 μM), 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.

2.5 Determination of metalloproteinase-2 and u-PA activities by zymography

The activity of metalloproteinase-2 (MMP-2) in the medium was measured using gelatin-zymography protease assays as previously described [13]. Briefly, collected media were prepared with SDS sample buffer without boiling or reduction, and subjected to 0.1% gelatin-8% SDS-PAGE electrophoresis. After electrophoresis, gels were washed with 2% Triton X-100 and then incubated in reaction buffer (40 mM Tris-HCl, pH 8.0, 10 mM $CaCl_2$, 0.02% NaN_3) for 12 h at 37°C to allow proteolysis of the gelatin substrate. The gel was then stained with Coomassie brilliant blue R-250 and molecular weights were estimated by reference to pre-stained SDS-PAGE markers. Visualization of u-PA activity was performed by casein-plasminogen zymography. Briefly, 2% w/v casein and 20 μg/mL plasminogen were added to 8% SDS-PAGE gels, and then electrophoresis and zymography were performed as described for the gelatin zymography.

2.6 *In vitro* scratch assay

For cell motility determination, MDAMB231 cells were seeded in six-well culture plates and grown to 80–90% confluence. After aspirating the medium, in the center of the cell, monolayers were scraped with a micropipette tip to create a denuded zone (gap) of constant width. Subsequently, cellular debris was washed with PBS, and MDAMB231 cells were exposed to various concentration of ursolic acid (0, 2.5, 5 and 10 μM). Photographs of the wound adjacent to reference lines scraped on the bottom of the plate were taken using a Nikon ECLIPSE TS100 microscope at various time points and the number of migrating cells was counted from these photographs.

2.7 Cell invasion and migration assay

The ability of MDAMB231 cells to pass through matrigel-coated filters was measured using the Boyden chamber invasion assay. Briefly, MDAMB231 cells were detached from the cell culture plates, washed with PBS buffer and resuspended in a serum-free RPMI1640 medium (5×10^4 cells/200 μL) in the presence or absence of ursolic acid. The cells were then seeded onto the upper chambers of matrigel-coated filter inserts. A serum-containing RPMI1640 medium (500 μL) was added to the lower chambers. After incubating for 24 h at 37°C, filter inserts were removed from the wells, the cells that invaded or migrated through the FluoroBlok membrane were stained with propidium iodide and fluorescence images were taken. The invasive cells were then counted using the Analytical Imaging Station software package (Imaging Research, ON, Canada).

To measure the ability of MDAMB231 cells on migration, cells were seeded into a Boyden chamber with 8 μm pore polycarbonate filters, which were not coated with matrigel. Migration of cells was treated with various concentration of ursolic acid. The migration assay was measured as described in the invasion assay.

2.8 RNA extraction and RT-PCR

Cellular RNA was extracted with a TRIzol RNA isolation kit as described in the manufacturer's manual. RNA concentration and purity were determined based on measurement of the absorbance at 260 and 280 nm. After the addition of RNase inhibitor (20 U), the total RNA was stored at −70°C. The sense and antisense primer sequences used were as follows: MMP-2 (473 bp): 5′-GGCCCTGTCACTCCTGA-GAT-3′, 5′-GGCATCCAGGTTATCGGGGA-3′; MT1-MMP (827 bp): 5′-CTCCTGCTCCCCCTGCTCACG-3′, 5′-CTC-ACCCCCAT AAAGTTGCTG-3′; u-PA (351 bp): 5′-TTGCGGCC ATCTACAGGAG-3′, 5′-ACTGGGGATCGT-TATACATC-3′; plasminogen activator inhibitor-1 (PAI-1) (254 bp): 5′-GGATCCAGCCACTGGAAA GGCAACATG-3′, 5′-GGATCCGTGCCGGACCACAAA GAGGAA-3′; tissue inhibitor of metalloproteinase-2 (TIMP-2) (496 bp): 5′-GGCGTTTTGCAATGCAGATGTAG-3′, 5′-CACAGGAG-CCGTCACTTCTCTTG-3′; and GADPH: 5′-CAAAA-GGGTCATCATCTCTGC-3′, 5′-GAGGGGCCA CACAGTC-TTC-3′, respectively. From each sample, 250 ng of RNA was reverse-transcribed, using 200 U of SuperScript II™ RNase-H reverse transcriptase, 20 U of RNase inhibitor, 0.6 mM dNTPs, and 0.5 mg/mL oligo (dT) 12–18. PCR was performed with Platinum Taq polymerase under the following conditions: 25 cycles of 94°C for 1 min, 55°C (u-PA and PAI-1) or 63°C (MMP-2, MT1-MMP, TIMP-2 and

GAPDH) for 1 min, 72°C for 2 min followed by 10 min at 72°C. A 10-μL aliquot from each PCR reaction was separated in a 1.8% agarose gel containing 0.2 mg/mL ethidium bromide.

2.9 Western blotting

The cell lysates and nuclear fractions were prepared using the Nuclear Extraction Kit (Panomics, Fremont, CA, USA). Samples (10 μg) of total cell lysates or nuclear fractions were size fractionated electrophoretically by a 10% polyacrylamide SDS-PAGE gel and transferred onto a polyvinylidene fluoride membrane using the BioRad Mini Protean electrotransfer system. The blots were subsequently incubated with 5% skim milk in PBST for 1 h to block nonspecific binding, and was probed overnight at 4°C with the antibodies against total and phosphorylated ERK, p38 MAPK, JNK, Akt and mTOR; IKK, IκBα, α-tubulin, NF-κB (p65), c-Jun, c-Fos, RhoA, Grb2, Ras, VEGF and lamin B. The membranes were sequentially detected with an appropriate peroxidase-conjugated secondary antibody incubated at room temperature for 1 h. Intensive PBS washing was performed after each incubation. After the final PBS washing, signals were developed using the ECL (enhanced chemiluminescence) detection system and Kodak X-OMAT Blue Autoradiography Film.

2.10 Statistical analysis

Each experiment was performed in triplicate and repeated three times. The results were expressed as means ± SD. Statistical comparisons were made by means of one-way analysis of variance, followed by a Duncan multiple-comparison test. Differences were considered significant when $p<0.05$.

3 Results

3.1 Ursolic acid, but not oleanolic acid, suppresses proliferation and colony formation of highly invasive breast cancer cells

The invasive behavior of cancer cells is directly linked to their metastatic potential, which leads to high cancer mortality. In order to investigate whether oleanolic acid and ursolic acid exhibit cytotoxic effects, the highly invasive MDAMB231 breast cancer cell line was treated with various concentrations of oleanolic acid and ursolic acid for 6, 12, 24 and 48 h, and the viability of cells was determined with SRB assays. The viability of MDAMB231 cells decreased in a dose- and time-dependent manner in the presence of ursolic acid, but not oleanolic acid (Fig. S1 of Supporting Information). High concentrations (25 or 50 μM) of ursolic acid, but not oleanolic acid, significantly suppressed cell growth. To further determine whether ursolic acid suppresses colony formation in highly invasive breast cancer cells, the anchorage-independent growth of MDA-MB-231 cells was evaluated. We observed that MDA-MB-231 cells formed colonies on agar after 14 days of incubation, and increasing concentrations of ursolic acid (0–50 μM) resulted in a significant reduction of the number of colonies in a dose-dependent manner (Fig. S2 of Supporting Information). The results showed that ursolic acid, but not oleanolic acid, inhibited anchorage-dependent and anchorage-independent growth of highly aggressive breast cancer cells. Moreover, these results also indicated that treatment with ursolic acid at different concentrations ranging from 0 to 10 μM exhibited no cytotoxic effects on the MDAMB231 cells. In the following invasiveness experiments, doses below 10 μM ursolic acid were applied to avoid the influence of cell growth on the observed parameters.

3.2 Ursolic acid inhibited MDAMB231 cell migration and invasion

In the wound-healing assay, cells were treated with various concentrations of ursolic acid for 24 h. The results showed that 10 μM ursolic acid exhibited the greatest inhibition of cell motility after 24 h of incubation (Fig. 2A). We further evaluated the inhibition of invasiveness by ursolic acid using a Transwell cell migration and invasion assay. Reductions in migration and invasion of MDAMB231 cells were observed when the ursolic acid concentration was above 2.5 μM. Ursolic acid induced a dose-dependent decrease in migration and invasion with increasing concentrations. In the 10 μM ursolic acid treated group, the migration and invasion of MDAMB231 cells showed 32 and 30% inhibition, respectively, compared with the non-ursolic acid treated group (Figs. 2B and 2C). The results demonstrated that ursolic acid significantly ($p<0.05$) inhibited the migration and invasion of the highly invasive MDAMB231 cells.

3.3 Ursolic acid inhibited MMP-2 and u-PA activities in MDAMB231 cells

The effects of ursolic acid on the MMP-2 and u-PA activities of MDAMB231 cells were analyzed by using gelatin and casein zymography. As shown in Fig. 3A, ursolic acid tremendously reduced MMP-2 activity in a dose-dependent manner, whereas the impacts of ursolic acid on MMP-7 and MMP-9 activities were inconclusive because an extremely low level of MMP-7 and MMP-9 was expressed in MDAMB231 cells, even in the absence of ursolic acid (data not shown). Moreover, u-PA activity was also inhibited by ursolic acid (Fig. 3B). The MMP-2 and u-PA activities were

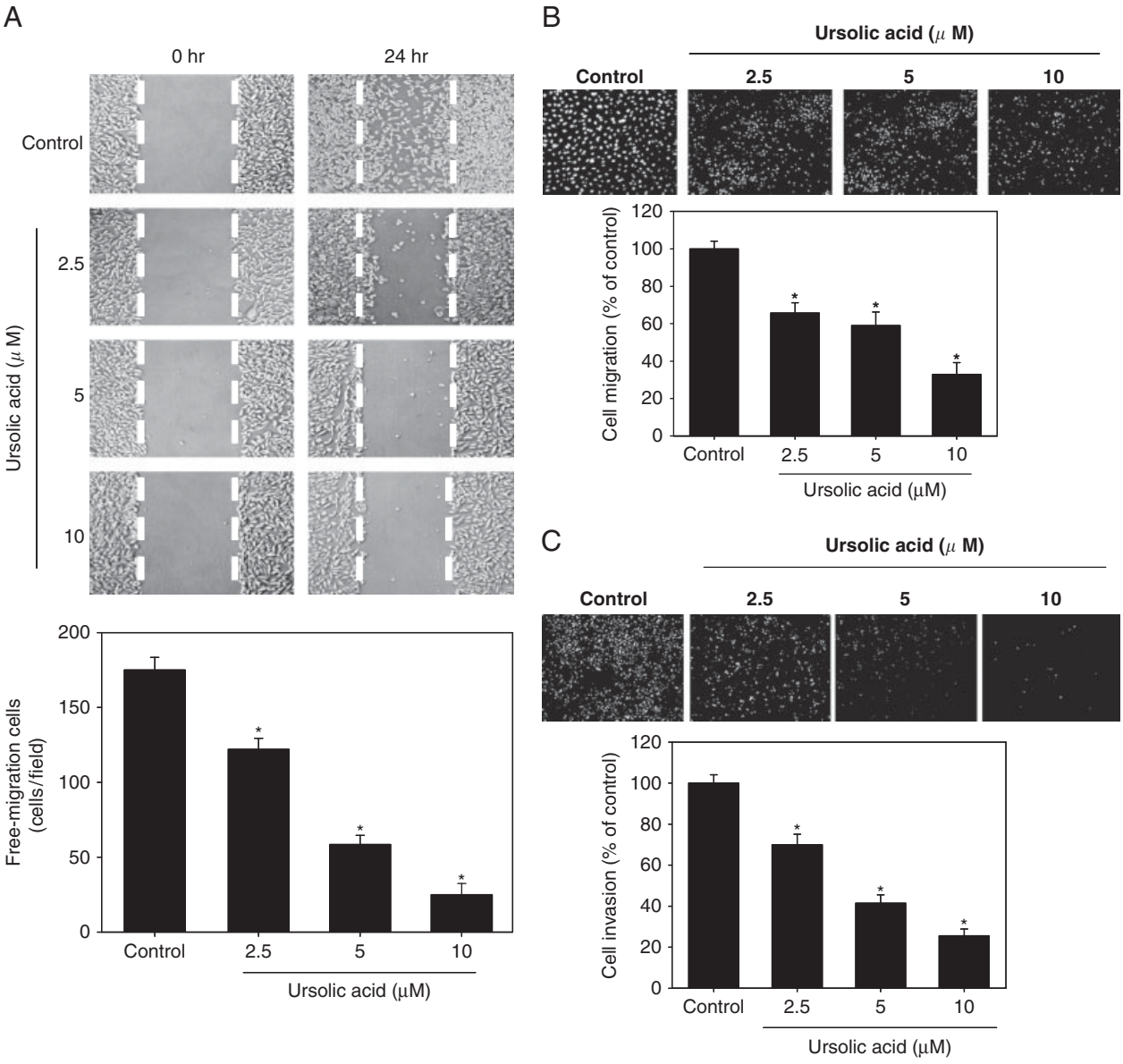

Figure 2. Effect of ursolic acid on migration and invasion of MDAMB231 cells. (A) The wound-healing assay for evaluating the inhibitory effect of ursolic acid on MDAMB231 cell migration. The cells migrating into the wound area were counted based on the dash line as time zero. Quantitative assessment of the mean number of cells in the denuded zone is expressed as mean ± SD ($n = 3$). $^*p < 0.05$, compared with the control. (B) Cell migration was measured using a Boyden chamber for 24 h with polycarbonate filters. (C) Cell invasion was measured using Transwell chambers for 24 h; the migration and invasion abilities of MDAMB231 cells were quantified by counting the number of cells that invaded to the underside of the porous polycarbonate as described in Section 2. $^*p < 0.05$, compared with the control.

significantly reduced by 35 and 42%, respectively, after treatment with 10 μM ursolic acid for 24 h. We further examined the effects of ursolic acid on MDAMB231 secretion of the TIMP-2 and PAI-1 protein levels by Western blot analysis. The immunoblotting showed that the TIMP-2 and PAI-1 protein levels were gradually increased with increasing concentrations of ursolic acid (Fig. 3C). Furthermore, after treatment of the cells with ursolic acid, the mRNA levels of MMP-2, u-PA, and MT1-MMP were significantly reduced to 25, 30, and 19% of the mRNA remaining, respectively, after a 10 μM ursolic acid treatment, while mRNA levels of TIMP-2 and PAI-1 were increased in a concentration-dependent manner, with levels as high as 250 and 230% detected after treatment by 10 μM ursolic acid (Fig. 3D). These results suggested that the anti-invasive effect of ursolic acid was related to the inhibition of degradation enzymes involved with tumor invasiveness. This study is the first to demonstrate the

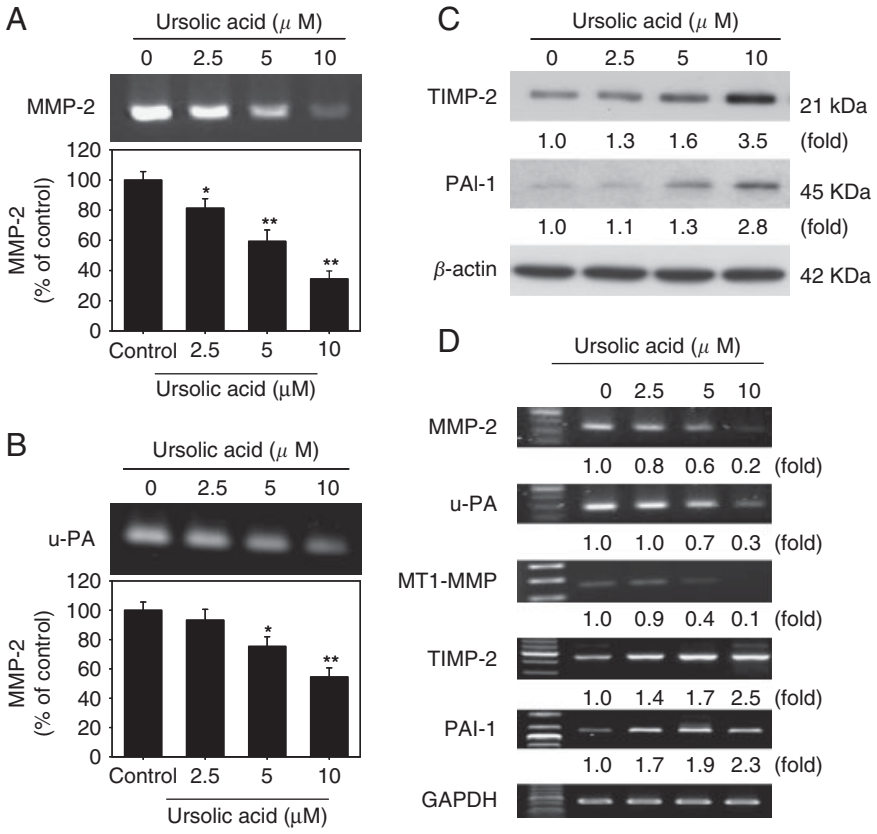

Figure 3. Effect of ursolic acid on the protein and mRNA levels of proteases and their endogenous inhibitors. MDAMB231 cells were treated with ursolic acid for 24 h and then subjected to gelatin zymography, casein zymography and Western blotting to analyze the activities of MMP-2 (A), u-PA (B), and TIMP-2 and PAI-1 (C), respectively, as described in Section 2. Determined activities of these proteins were subsequently quantified by densitometric analysis with the density of the control set to 100% as shown just below the gel data. (D) Total RNAs were extracted and subjected to semi-quantitative RT-PCR for MMP-2, u-PA, MT-1MMP, TIMP-2 and PAI-1, with GAPDH used as an internal control. Data are expressed as means ± SD ($n = 3$). $^*p < 0.05$, compared with the control.

molecular mechanisms by which ursolic acid reduces the invasiveness of a highly invasive human breast cancer cell line.

3.4 Ursolic acid inhibited the expression of MMP-2/u-PA in MDAMB231 cells by suppressing phosphorylation in the Akt, mTOR and JNK signaling pathway

Since we showed that treatment of MDAMB231 cells with ursolic acid inhibited cell invasion and the activities of MMP-2 and u-PA, the underlying molecular mechanisms were further investigated. As shown in Fig. 4A, ursolic acid inhibited the phosphorylation of Akt and its downstream target mTOR as well as JNK phosphorylation in a time-dependent manner in MDAMB231 cells. Treatment with 10 μM ursolic acid for 24 h resulted in decreases of 80, 70 and 70% in the phosphorylation of Akt, mTOR and JNK, respectively, as seen by comparing the densitometric analyses of blots from untreated cells, whereas it did not affect ERK and p38 phosphorylation. In further studies, we examined whether inhibition of MMP-2 and u-PA expression in MDAMB231 by ursolic acid was mediated through modulation of Akt, mTOR and JNK phosphorylation. As shown in Figs. 4B and 4C, treating the cells with the inhibitors of Akt (LY294002), mTOR (rapamycin) and JNK (SP600125) significantly reduced the expression of MMP-2 and u-PA. In particular, the combination treatment could reduce the expression of MMP-2 or u-PA by 46 or 38% (LY294002+ursolic acid), 52 or 40% (SP600125+ursolic acid), 49 or 50% (rapamycin+ursolic acid) and 89 or 86% (LY294002+SP600125+rapamycin+ursolic acid). These data revealed that ursolic acid inhibited the expression of MMP-2 and u-PA on MDAMB231 cells could partly occur through Akt, mTOR and JNK inactivation.

3.5 Ursolic acid inhibited cell invasion and migration in MDAMB231 cells through the inactivation of the Akt, mTOR and JNK signaling pathway

To further delineate whether the inhibition of cell invasion and migration by ursolic acid occurred mainly through the inhibition of Akt, mTOR and JNK signaling, MDAMB231 cells were pretreated with Akt (LY294002), mTOR (rapamycin) or JNK (SP600125) inhibitors and then incubated in the presence or absence of ursolic acid for 24 h. Compared with the control, the invasion and migration assays revealed that treatment with LY294002, SP600125, rapamycin or ursolic acid decreased cell migration and invasion by 19, 22, 21, and 61 and by 23, 22, 31, and 63%, respectively, and the

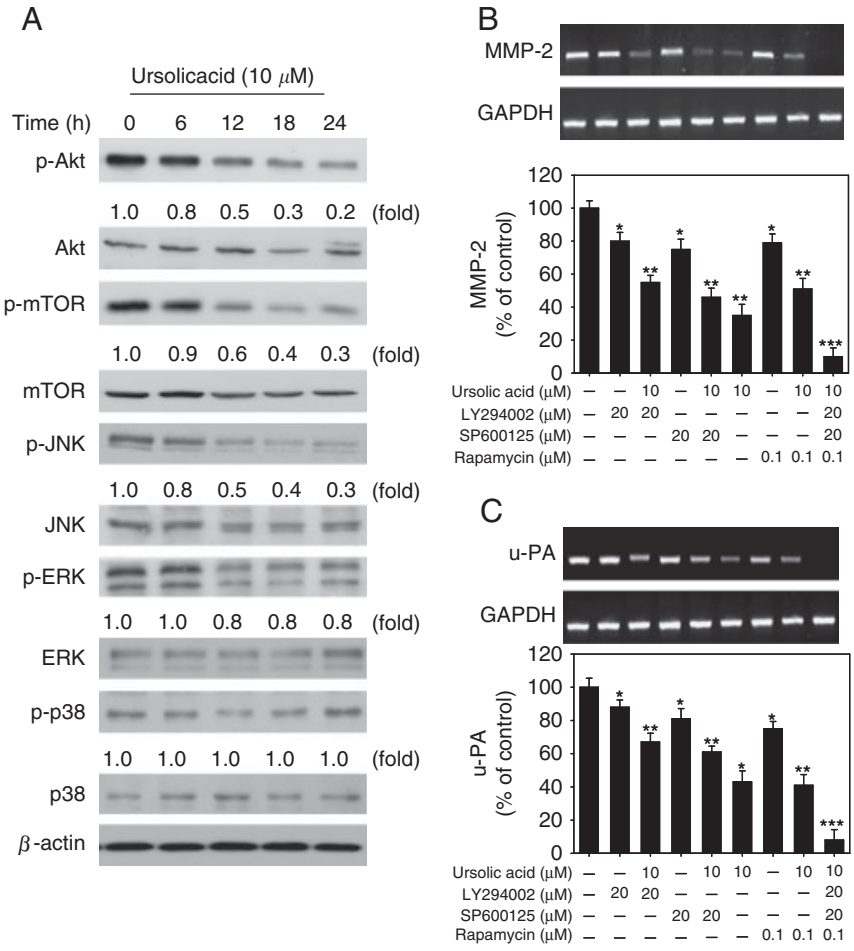

Figure 4. Effect of ursolic acid on the phosphorylation of Akt, mTOR and MAPK in MDAMB231 cells. (A) Immunoreactive bands of phosphorylated and non-phosphorylated Akt, mTOR, JNK, ERK, p38 MAPK and β-actin using specific antibody. (B) MDAMB231 cells were pretreated with PI3K inhibitor LY294002 (20 μM), JNK inhibitor SP600125 (20 μM) and mTOR inhibitor rapamycin (0.1 μM) for 30 min and then incubated in the presence or absence of ursolic acid (10 μM) for 24 h. Total RNAs were extracted and subjected to a semi-quantative RT-PCR for MMP-2 (B) and u-PA (C), with GAPDH used as an internal control. Data are expressed as means ± SD ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$, compared with the untreated control.

combination treatment (LY294002+SP600125+rapamycin +ursolic acid) further reduced cell migration and invasion by 88 and 92%, respectively (Fig. 5).

3.6 Ursolic acid reduced the nuclear levels of NF-κB, c-Jun, and c-Fos, as well as other proteins associated with invasion in MDAMB231 cells

The NF-κB and activated protein-1 families of transcription factors are known to translocate into the nucleus and regulate the expression of multiple genes involved in MMP-2 and u-PA expression. To measure the nuclear protein levels of NF-κB (p65), c-Jun and c-Fos, nuclear extracts were analyzed by Western blotting to assess the possible inhibitory effects of ursolic acid on NF-κB (p65), c-Jun and c-Fos. The levels of NF-κB (p65), c-Jun, and c-Fos proteins in the nucleus were decreased by the ursolic acid treatment in a time-dependent manner (Fig. 6A). Additionally, the results from Western blotting are also shown in Fig. 6B, which indicated that ursolic acid inhibited the upstream proteins of metastasis- and angiogenesis-related signaling cascades including RhoA, Grb2, Ras and VEGF.

4 Discussion

In recent years, attention has been focused on the anticancer properties of triterpenoids, for application in cancer prevention [14]. The antitumor effect of ursolic acid has been well documented in many different types of human cancers [15, 16]. In this study, we demonstrated for the first time that ursolic acid, but not oleaolic acid, at non-toxic concentration (2.5 to 10 μM) could effectively inhibit the invasion and migration of MDAMB231 cells (Fig. 2), and thus might represent a new strategy for anticancer therapy. However, the precise actions of ursolic acid on invasion and migration of MDAMB231 cells have not been reported. Our results suggested that inhibition was associated with a decreased in MMP-2 and u-PA at mRNA and activity levels, in which the JNK, Akt and mTOR signaling pathways was involved.

MMP-2 and u-PA, which are secreted by invasive cancer cells, play important roles in cancer cell invasion and metastasis because tumor cells must cross the type IV collagen-rich basement of vessel walls to spread to other sites during cancer metastasis [17]. Li *et al.* [18] revealed that MMP-2 activity is highly correlated with the risk for a

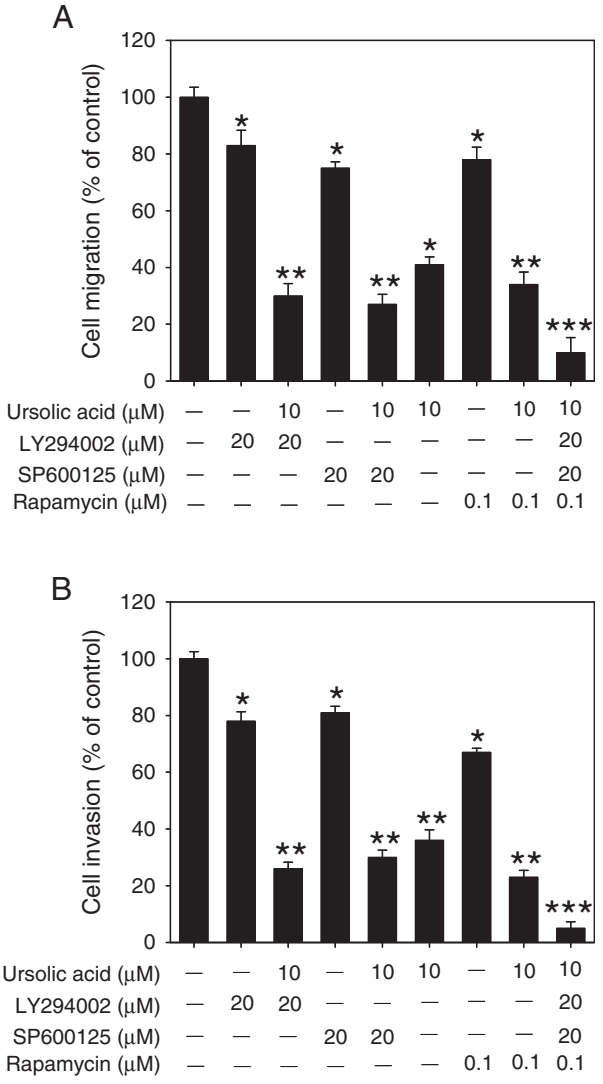

Figure 5. Effects of specific inhibitors and ursolic acid on cell invasion and migration. MDAMB231 cells were pretreated with PI3K inhibitor (LY294002, 20 μM), JNK inhibitor (SP600125, 20 μM), and mTOR inhibitor (rapamycin, 0.1 μM) for 30 min and then incubated in the presence or absence of ursolic acid (10 μM) for 24 h. Later, the cells were subjected to analyses for migration (A) and invasion (B) as described in Section 2. Data are expressed as means ± SD ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$, compared with the untreated control.

relapse in breast cancer patients. Furthermore, mice lacking MMP-2 gelatinase have a reduced tumor burden and decreased metastasis, as well as reduced tumor angiogenesis without developmental abnormalities [19]. Here, we showed that ursolic acid notably down-regulated the activities of MMP-2 and u-PA, while the levels of TIMP-2 and PAI-1 were enhanced (Fig. 3). To the best of our knowledge, this is the first scientific report relating the inhibitory effect of ursolic acid on breast cancer invasiveness to decreased production of tumor metastasis-related proteins such as MMP-2 and u-PA.

A major mechanism through which signals from extracellular stimuli are transmitted to the nucleus involves the activation of kinases. The c-JNK pathway represents one subgroup of MAP kinases activated by cytokines that promotes cell cycle progression and cell proliferation [20]. Akt is the serine/threonine kinase that promotes cell survival and blocks apoptosis, and consequently activates one of the downstream effectors mTOR by the phosphorylation of mTOR at Ser-2448. A large body of evidence suggests that the mTOR signaling pathway is dysregulated in premalignant or early malignant human tissues [21]. As shown in Fig. 4A, ursolic acid suppressed the phosphorylation of JNK, Akt and mTOR in a dose-dependent manner. In contrast, ursolic acid did not significantly affect the phosphor-ERK or p38 activities. The involvement of the MAPK pathway was further supported by the use of JNK, Akt and mTOR inhibitors in our experimental model. A pretreatment with specific inhibitors of JNK, Akt and mTOR could inhibit MMP-2 and u-PA expression (Figs. 4B and 4C).

The role of MAPKs in the regulation of MMP-2 and u-PA expression in carcinoma cells has been well-studied [22]. In carcinoma cancer cells, the PI3K/phosphatase and tensin homolog deleted on chromosome 10/Akt/mTOR pathway was shown to regulate invasiveness *via* MMPs [23]. Rapamycin and siRNA-mediated mTOR knockdown could inhibit cell proliferation, invasion and the secretion of MMP-2 and uPA [24]. Additionally, the PI3K and Akt signaling pathways play a crucial role in MMP gene regulation [25]. Our results suggest that the mechanism underlying the regulation of MMP-2 and u-PA and the reduction in cell invasion by ursolic acid could be through the inactivation of the JNK, Akt and mTOR signaling pathway (Fig. 5).

The transcription of MMPs and u-PA genes is regulated by upstream sequences, including motifs corresponding to NF-κB, activated protein-1, stimulatory protein-1 and polyoma virus enhancer activator-binding sites [26]. Therefore, it has been suggested that the suppression of NF-κB, c-Jun and c-Fos activities may inhibit tumor initiation, promotion and metastasis, and block the factors that bind to these regulatory elements, thus representing an appropriate approach to inhibit the synthesis of MMPs or u-PA. Previously, ursolic acid has been shown to induce apoptosis in melanoma cells through the down-regulation of c-Fos and NF-κB [27], and inhibition of NF-κB by ursolic acid has been demonstrated in leukemic cells stimulated by TNF-α [28]. Here, we have also found that the treatment of MDAMB231 cells with ursolic acid resulted in an inhibition of the nuclear translocation of the NF-κB p65, c-Jun, and c-Fos proteins (Fig. 6A).

Next, we examined the effect of ursolic acid on the levels of upstream proteins involved in metastatic signaling cascades. RhoA, a family member of the Rho-like GTPases, has been found to play important roles in distinct actin cytoskeleton changes that are required for cell adhesion, migration and invasion [29]. The Grb2 is an SH2 domain-containing docking module that participates in the

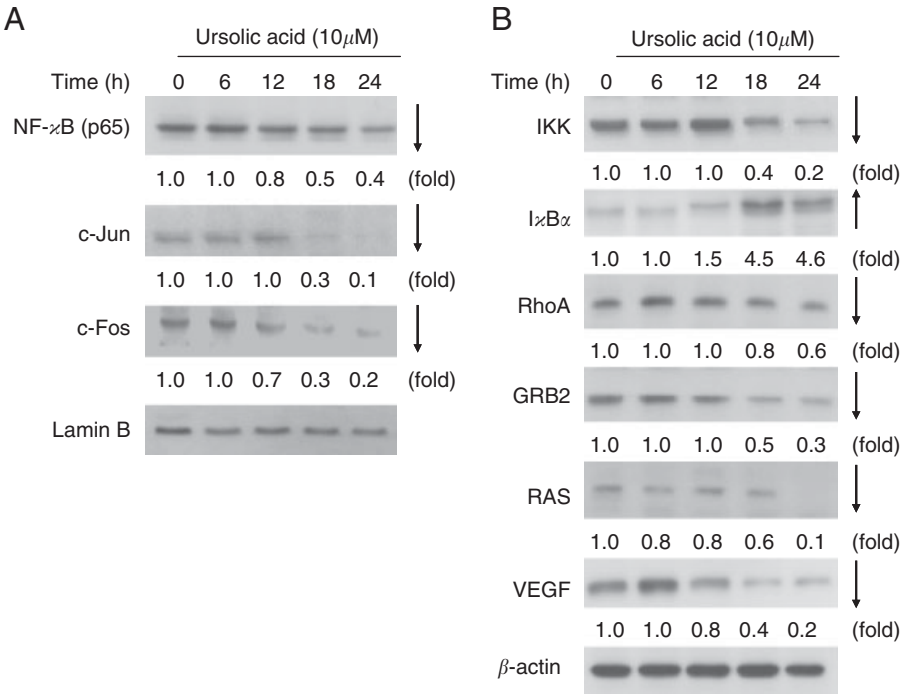

Figure 6. Western blotting to examine the effect of ursolic acid on the levels of proteins associated with invasion and migration in MDAMB231 cells. (A) Immunoreactive bands of nuclear p65, c-Jun, c-Fos, and Lamin B using specific antibody. (B) Immunoreactive bands of cytosolic IKK, IκBα, RhoA, Grb2, Ras, VEGF and β-actin using specific antibody. Levels of these proteins were quantified by densitometric analyses with the control set to 100% as shown below the gel data.

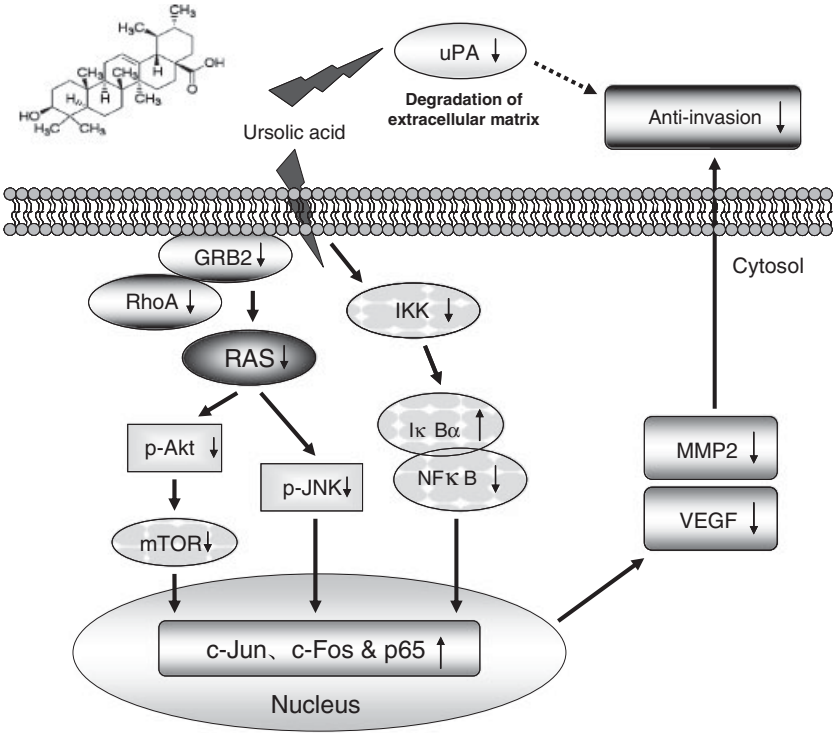

Figure 7. Proposed molecular mechanism for ursolic acid-inhibited invasion in human breast MDAMB231 cells.

oncogenic actions of certain protein-tyrosine kinase, including roles in erbB-2-dependent breast cancers and Met-dependent kidney cancers [30]. Additionally, Ras signaling may facilitate this migration *via* stimulation of angiogenesis by upregulation of VEGF [31]. An investigation by Zheng *et al.* [32] showed that VEGF promotes migration and invasion of cancer cells. Up-regulation of VEGF expression has been demonstrated to be strongly associated with tumor growth, angiogenesis and increased resistance to treatment of breast cancer [33]. Our results showed that ursolic acid

inhibited the expression of RhoA, Grb2, Ras and VEGF, which are the major factors causing the inhibition of migration and invasion of MDAMB231 cells (Fig. 6B).

However, the structure–activity relationship analysis regarding the relationship between anti-invasive potential and MMP-2 suppression by ursolic acid is still unclear. Ursolic acid and oleanolic acid have similar molecular structures but have different sites of methyl group on the ring E: if the methyl group at C_{19} of ursolic acid is moved to C_{20}, it changes to oleanolic acid [34]. In the present study, ursolic acid but not oleanolic acid has offered a remarkable anti-invasive activity against invasive human breast MDAMB231 cells. Since both the compounds are regio-isomers, the difference in their potency may be attributed to their structural arrangement of the substituent [35]. It is therefore speculated that methyl group at C_{19} of ursolic acid are crucial for MMP-2 inhibition. Further investigations to explore the structure–activity relationships for the action of ursolic acid and oleanolic acid on anti-invasive breast cancer cells may prove to be worthwhile.

In conclusion, our data indicated for the first time that ursolic acid could inhibit migration and invasion of human MDAMB231 breast cancer cells through Akt/mTOR and NF-κB signaling, resulting in the inhibition of MMP-2 and u-PA. Furthermore, ursolic acid reduced the levels of RhoA, Grb2, Ras, p-JNK, p-Akt and p-mTOR, leading to effects on NF-κB, resulting in inhibition of the expression of VEGF, MMP-2 and u-PA, which may have implications for the pathogenesis of breast cancer. It is therefore tempting to propose a simple mechanism for chemopreventive properties of ursolic acid (Fig. 7). Targeting the PI3K/Akt/mTOR pathway may be a potential adjuvant strategy to inhibit the migration and invasion of this highly metastatic cancer; however, more studies are needed to further justify ursolic acid as a novel agent with anti-metastatic capabilities in combination chemotherapy against breast cancer.

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