RESEARCH COMMUNICATION

Pristimerin Inhibits Breast Cancer Cell Migration by Up-regulating Regulator of G Protein Signaling 4 Expression

Xian-Min Mu[1,2&], Wei Shi[3], Li-Xin Sun[1], Han Li[1], Yu-Rong Wang[1], Zhen-Zhou Jiang[1&*], Lu-Yong Zhang[1,4&*]

Abstract

Background/Aim: Pristimerin isolated from *Celastrus* and *Maytenus* spp can inhibit proteasome activity. However, whether pristimerin can modulate cancer metastasis is unknown. Methods: The impacts of pristimerin on the purified and intracellular chymotrypsin proteasomal activity, the levels of regulator of G protein signaling 4 (RGS 4) expression and breast cancer cell lamellipodia formation, and the migration and invasion were determined by enzymatic, Western blot, immunofluorescent, and transwell assays, respectively. Results: We found that pristimerin inhibited human chymotrypsin proteasomal activity in MDA-MB-231 cells in a dose-dependent manner. Pristimerin also inhibited breast cancer cell lamellipodia formation, migration, and invasion *in vitro* by up-regulating RGS4 expression. Thus, knockdown of RGS4 attenuated pristimerin-mediated inhibition of breast cancer cell migration and invasion. Furthermore, pristimerin inhibited growth and invasion of implanted breast tumors in mice. Conclusion: Pristmerin inhibits proteasomal activity and increases the levels of RGS4, inhibiting the migration and invasion of breast cancer cells.

Keywords: Pristimerin - anti-cancer - proteasome inhibition - RGS4 - breast cancer

Asian Pacific J Cancer Prev, **13**, 1097-1104

Introduction

Breast cancer is the leading cause of cancer death among females in the world (Jemal et al., 2011). While primary breast cancer without metastasis usually can be surgically removed, breast cancer with distant metastasis is difficultly managed, leading to mortality (Chambers et al., 2002). Hence, it is critical to control breast cancer metastasis. Currently, there are a few therapeutic strategies for the control of breast cancer metastasis in the clinic. Therefore, the development and discovery of new medicines for the control of breast cancer metastasis will be of great significance in the management of patients with breast cancer in the clinic.

Metastasis is a complex process that is not fully understood. High levels of G-protein-linked receptor expression and the aberrant activation of G protein-coupled receptor (GPCR) in cancer cells have been thought to be implicated in the development of organ-specific metastasis of tumors. A variety of GPCRs was implicated in breast cancer metastasis, including PAR1, EP2, EP4, CXCR4, GPR30, and others (Dorsam and Gutkind, 2007). The redundant metastatic signals by different activated GPCRs simultaneously drive the migration of breast cancer cells, which makes the inhibition of breast cancer metastasis by a GPCR-specific inhibitor unfeasible. Ideally, simultaneous regulation of the majority of GPCR signaling may be a promising strategy for the control of breast cancer metastasis.

The regulator of G protein signaling 4 (RGS4) is an important regulator of GPCR signaling and can deactivate the Gi and Gq alpha subtypes through its GTPase-activating function, negatively regulating the GPCR-mediated pathway (Huang et al., 1997; Ross and Wilkie, 2000; Lappano and Maggiolini, 2011). Notably, a recent study indicates that RGS4 is a suppressor of breast cancer metastasis and that RGS4 inhibits the migration and invasion of breast cancer cells by down-regulating the metastasis-associated Gi-coupled receptors (PAR1 and CXCR4) signal transduction (Xie et al., 2009). RGS4 can inhibit the Rac1-dependent lamellipodia formation, a key process for the migration of cancer cells (Xie et al., 2009). Interestingly, RGS4 protein is usually degraded by proteasome, which promotes breast cancer cell migration and invasion (Lee et al., 2005; Bodenstein et al., 2007; Xie et al., 2009). Accordingly, we hypothesize that the down-regulation of proteasome activity to increase intra-plasmatic RGS4 levels may be effective in inhibiting and preventing breast cancer metastasis.

Pristimerin is a natural triterpenoid and is isolated from Celastrus and Maytenus spp. Previous studies have shown that pristimerin has anti-inflammatory activity and

*[1]Jiangsu Center of Drug Screening, [4]Key Laboratory of Drug Quality Control and Pharmacovigilance, Ministry of Education, China Pharmaceutical University, [2]The Secondary Affiliated Hospital of Nanjing Medical University, [3]Department of New Drug Screening, Jiangsu Chia-tai Tianqing Pharmaceutical Co., Ltd, Nanjing, China [&]Equal contributors *For correspondence: lyzhangchina@hotmail.com, jiangcpu@yahoo.com.cn*

can inhibit tumor cell proliferation by inhibiting the NF-κB pathway and cell cycling (Boire et al., 2005; Costa et al., 2008; Yang et al., 2008; Byun et al., 2009; Tiedemann et al., 2009). In addition, prestimerin has been reported to induce caspase-dependent apoptosis of breast cancer cells (Wu et al., 2005) and prostate cancer cells in vitro (Yang et al., 2008). Recently, pristimerin is reported to be a potent natural triterpenoid inhibitor of 20s proteasome chymotrypsin-like activity in prostate cancer cells (Tiedemann et al., 2009). However, whether pristimerin could inhibit proteasome activity and increase the levels of intra-plasmatic RGS4 in breast cancer cells , whether pristimerin could modulate breast cancer cell migration and invasiveness have not been systemically explored.

In the present study, we examined the effect of pristimerin on 20s proteasome chymotrypsin-like activity in breast cancer cells and on the migration and invasion in vitro and in vivo. Subsequently, we investigated the potential mechanisms by which pristimerin inhibited the migration and invasion in breast cancer cells. We found that pristimerin inhibited proteasome activity and up-regulated the levels of intra-plasmic RGS4, leading to the inhibition of migration and invasion in breast cancer cells. Our findings may provide new insights into the anti-tumor activity of pristimerin, and potentially, pristimerin may be valuable for the intervention of breast cancers.

Materials and Methods

Reagents

Pristimerin (purity ≥98%) was purchased from Enzo Life Sciences (Lausen, Switzerland). Other special materials used in the studies included MG132 (purity ≥ 98%, Calbiochem, Germany), Chloroquine (purity ≥ 98%, Sigma, Saint Louis, USA); cycloheximide (≥93.0%, sigmaUSA), Proteasome-Glo™ Chymotrypsin-Like Cell-Based Assay (Promega, Madison, USA), 20s-proteasome assay kit (Enzo Life Sciences, Farmingdale, USA), anti-RGS4 antibody (Abcam, USA), anti-β-actin antibody (Santa Cruz Biotech, Santa Cruz, USA), RGS4 siRNA (h)(Santa Cruz); Rhodamine phalloidin (cytoskeleton, Denver, USA), Matrigel (BD Biosciences, Bedford, USA), and All cell-culture reagents (Gibco-BRL, USA).

Cell culture

Human breast cancer cell line, MDA-MB-231, and mouse embryonic fibroblast cell line, NIH-3T3, were obtained from American Type Culture Collection (ATCC) and cultured in DMEM supplemented with 10% fetal bovine serum (FBS) at 37°C in an atmosphere of 5% CO_2, 95% air. The culture supernatants of NIH-3T3 cells were harvested as the conditioned medium (CM) and used as chemotactic reagents (Boire et al., 2005).

Chymotrypsin-like 20S proteasome assay

The impact of pristimerin on proteasome chymotrypsin-like activity was determined using the Chymotrypsin-like 20S proteasome assay kit, according to the manufacturers' instruction. Briefly, the purified human erythrocyte 20S proteasomes (0.1 μg/well) were mixed in triplicate with the supplied Suc-LLVY-AMC (75 μM), a fluorogenic substrate for chymotrypsin-like peptidase activity, in 50 μL of buffer in polystyrene microplates in the presence or absence of different concentrations (12.5-1000 nM) of pristimerin or MG132, a proteasome-specific inhibitor, and cultured at 37°C. The amounts of the cleaved substrate were measured every two minutes in a fluorescence microplate reader (at excitation of 360 nm and emission of 460 nm), and the proteasomal chymotrypsin-like activity was expressed as the velocity of AMC released (arbitrary fluorescence unit, AFU/min).

Cell-based proteasome activity assay

The impact of pristimerin on the proteasome chymotrypsin-like activity in MDA-MB-231 cells was measured using the Proteasome-Glo™ Chymotrypsin-Like Cell-Based Assay kit, according to the manufacturers' instruction. Briefly, MDA-MB-231 (8.0×10^3 cells/well) cells were cultured in 96-well plates overnight and the cells were treated in triplicate with 0.0625-1 μM pristimerin or 0.2-10 μM MG132 for two hours. The cells in medium alone were used as controls. Subsequently, the cells were exposed to the provided proteasome-Glo™ reagent containing a specific luminogenic proteasome substrate of Suc-LLVY-aminoluciferin and thermostable luciferase at 22°C for 15 minutes. Following proteasome cleavage of aminoluciferin, the generated luminescent signals by the luciferase reaction were measured using a luminometer (Thermo Fisher Scientific).

Transwell migration and invasion assays

The effects of pristimerin on the migration and invasion of MDA-MB-231 cells in vitro were determined by transwell migration assay, as described previously (Xie et al., 2009). Briefly, MDA-MB-231 cells (5×10^4/well) in the upper chambers were treated in triplicate with 0.25-1 μM pristimerin or 10 μM MG132. The bottom chambers were filled with 600 μL of CM from the cultured NIH3T3 cells (chemotactic reagents). The cells were treated in triplicate with individual compounds in the upper chambers that had been coated with 30 μg Matrigel for cell invasion assays. After incubation for 5 hours, the cells that had migrated through the membrane of the upper chambers were fixed with 4% paraformaldehyde and stained with 1% crystal violet, followed by imaging using an Olympus inverted microscope. The numbers of migrated and invaded cells in the bottom chambers were calculated.

Lamellipodium formation assay

The effect of pristimerin on the formation of lamellipodia in MDA-MB-231 cells was examined as described previously (Schlunck et al., 2004). Briefly, MDA-MB-231 cells were grown overnight on glass cover-slips. The cells were starved with 0.5% FBS DMEM for 24 hours and then treated in triplicate with 0.25-1 μM pristimerin or 10 μM MG132 in CM for 5 hours. The cells that had been cultured in CM alone were used as controls. Subsequently, the cells were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature, and permeabilized in 0.2% Triton X-100 in PBS for 15 min. The actin filaments (F-actin) in the cells were stained

with rhodamine-conjugated phalloidin in PBS. The coverslips were mounted on object slides using the Vectashield mounting medium containing DAPI and imaged using a Nikon TE2000-E inverted microscope. The lamellipodia were identified as smoothly protruding regions along the edge of the cell with perpendicular phalloidin-stained F-actin (Schlunck et al., 2004). A total of at least 1000 cells from more than six fields of each group of cells were counted for the percentages of lamellipodia$^+$ cells.

Western blot assay

To determine the effects of pristimerin on the levels of intra-plasmatic RGS4 protein, MDA-MB-231 cells were starved in serum-free DMEM for 12 hours and treated with, or without, 0.25-1 μM pristimerin or 10 μM MG132 for 5 hours. The cells were harvested and lyzed, following centrifuging. Individual protein lysates (60 μg/lane) were resolved by SDS-PAGE and transferred onto PVDF membranes. After blocked with 5% fat-free dry milk in TBST, the membranes were incubated with antibodies against RGS4 or β-actin at 4°C overnight, respectively. The bound antibodies were detected with HRP-conjugated secondary antibodies at 37°C for 1 h, and visualized using the enhanced chemiluminescence (ECL) reagents. The relative levels of RGS4 to β-actin expression were determined quantitatively by densitometric scanning using Imagej software.

Transfection for silencing the RGS4 expression

MDA-MB-231 cells at 7.5×10^5 cells/well were cultured in six-well plates overnight and transfected with 60 nmol RGS4-specific siRNAs or control-siRNAs (Santa Cruz Biotech, Santa Cruz, USA) using the transfection reagent, according to the manufacturer's instructions. Two days later, the efficacy of RGS4 silencing was determined by Western blot assays. Subsequently, the transfected cells were used for the transwell migration and invasion assays described above.

Breast cancer model in mice.

The experimental protocol was established. according to the guidelines of NIH Animal Research and Care, and was approved by the Animal Care and Use Committee of China Pharmaceutical University. Six week-old female BALB/c nude mice were obtained from the National Rodent Laboratory Animal Resources, Shanghai, China, and housed in a specific pathogen-free facility in a 12-h light-dark cycle in China Pharmaceutical University. The orthotopic mammary fat pad model of breast cancer was established, as previously described (Xie et al., 2009). Individual BALB/c nude mice were inoculated with 8×106 MDA-MB-231 cells in 100 μL Matrigel into a mammary fat pad. The growth of inoculated tumors was measured every other day using a digital caliper, and the tumor volumes were calculated using the ellipsoid formula. When the tumors grew to ~110 mm^3, the mice were randomized and treated subcutaneously with 1 mg/kg body weight of pristimrin or the same volume of vehicle every other day for six times (n=10 per group). Their primary tumors with surrounding fibroadipose tissues were dissected out, fixed in 10% formalin/PBS, and embedded in paraffin. The tumor tissue sections (4 μm) were stained with H & E, and at least five unconsecutive sections from individual tumor tissues were examined for evaluating the degrees of mammary fibroadipose tissue destruction caused by cancer invasiveness by two certified clinical pathologists in a blinded manner.

Statistical analysis

Data are expressed as mean ± standard error mean of individual groups from at least three separate experiments unless specifically stated. The difference among different groups was determined by ANOVA and post hoc Bonferroni correction, and the difference between two groups was analyzed by Student's t-test and X^2 test where applicable using the SPSS for Windows 17 software. A P value of <0.05 was considered statistically significant.

Results

Pristimerin inhibits proteasome chymotrypsin-like activity

Pristimerin has been found to inhibit proteasome function (Tiedemann et al., 2009). To verify the activity of pristimerin, we first measured the inhibition of pristimerin on the purified human 20S proteasome chymotrypsin-like activity in vitro. Following reaction of the purified 20S proteasomes with substrate in the presence or absence of different concentrations of pristimerin or the proteasome inhibitor of MG132, we measured the proteasome chymotrypin-like activity. As

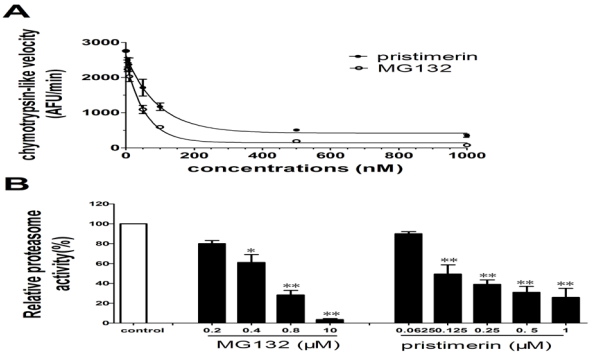

Figure 1. Pristimerin Inhibits Chymotrypsin-like Proteasome Activity in Vitro. The impacts of different concentrations of pristimerin on the activity of the purified human chymotrypsin-like 20S proteasomes and on the activity of chymotrypsin-like proteasome activity in MDA-MB-231 cells were determined by a substrate (suc-LLVY-AMC)-based enzymatic assay and a substrate-based luciferase-mediated luminescent assay, respectively. The proteasome-specific inhibitor of MG132 at different concentrations was used as positive controls. Data are expressed as mean ± SEM of the values of different groups from three separate experiments. A. Pristimerin inhibits human chymotrypsin-like 20S proteasome activity. The mean activity of the purified human chymotrypsin-like 20S proteasomes in the absence of inhibitor was 2758±215 AFU/min. B. Pristimerin inhibits chymotrypsin-like proteasome activity in MDA-MB-231 cells. The cells were treated with the indicated concentrations of pristimerin or MG132 for 2 hours, and the intracellular chymotryptic-like proteasome activities were determined. The activity of chymotrypsin-like proteasome in the vehicle-treated control MDA-MB-231 cells was 728649 ±66348 AFU and designated as 100%. $^*p<0.05$ or $^{**}p<0.01$ vs. the controls

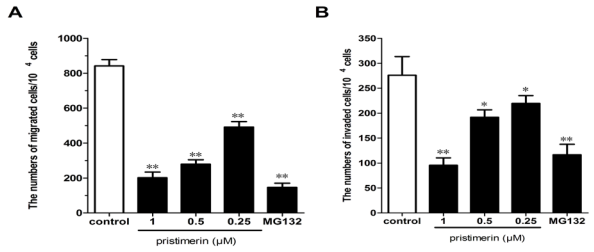

Figure 2. Pristimerin Inhibits the Migration and Invasion of MDA-MB-231 Cells in Vitro. MDA-MB-231 cells were treated with vehicle as the controls or with the indicated concentrations of pristimerin or 10 μM MG132 on the top chamber, and the impacts of pristimerin on the chemotactic reagent-induced breast cancer cell migration and invasion were determined using transwell assays without or with Matrigel, respectively. Data are expressed as mean ± SEM of the values of different groups from three separate experiments. $^{*}p<0.05$ or $^{**}p<0.01$ vs. the controls

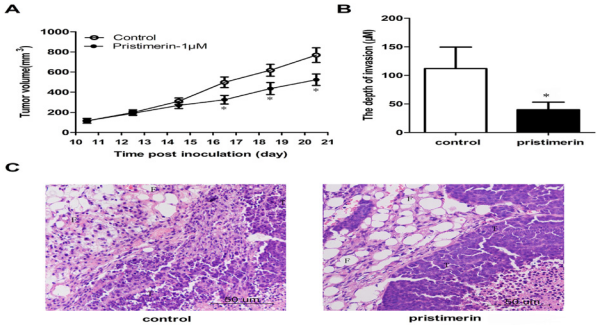

Figure 3. Treatment with Pristimerin Suppresses the Growth and Invasion of Implanted Tumors in Mice. BALB/c nude mice were inoculated with MDA-MB-231 cells into a mammary fat pad. When the tumors grew to ~110 mm^3, the mice were randomized and treated subcutaneously with 1 mg/kg of pristimrin or with the same volume of vehicle every other day for six times (n=10 per group). The growth of implanted tumors was monitored up to 20 days post inoculation. The breast tumors of individual mice were dissected and the tissue sections at 5 μM were stained by H&E, followed by imaging (magnification x200). The invasiveness of individual tumors was determined by quantitatively measuring the degrees of mammary fibroadipose tissue destruction caused by cancer invasiveness, and at least 15 high fields of three unconsecutive sections from individual tissue samples were examined. Data shown are representative images from each group and expressed as mean ± SEM of the values of individual groups from 2-5 independent experiments. A. The growth of implanted tumors. B. Micrographs of tissue samples. T: Tumor tissues. F: Fibroadipose tissue. C. Quantitative analysis of tumor invasion. $^{*}p<0.05$ or $^{**}p<0.01$ vs. the controls

shown in Figure 1A, pristimerin inhibited proteasome chymotrypin-like activity with an EC50 of less than 75 nM, and its inhibitory activities were dose-dependent, consistent with a previous report (Tiedemann et al., 2009). These data confirmed that pristimerin directly inhibited 20S proteasome chymotrypin-like activity in vitro.

Next, we examined whether pristimerin could inhibit proteasome chymotrypsin-like activity in breast cancer cells. MDA-MB-231 cells were treated with, or without, different concentrations of pristimerin or MG132 for two hours. The proteasome chymotrypsin-like activity in different groups of cells was determined by a proteasome activity-based luciferase assay. As shown in Figure 1B, the relative proteasome activity in the cells that had been treated with 0.125-1 μM pristimerin to that in the control cells cultured in medium alone was significantly reduced, and the inhibitory effects of pristimerin on proteasome activity in MDA-MB-231 cells appeared be dose-dependent. Similarly, treatment with MG132 also inhibited proteasome chymotrypsin-like activity in a dose-dependent manner. Collectively, our data clearly demonstrate that pristimerin directly targets proteasome and inhibits proteasome chymotrypsin-like activity in breast cancer cells in vitro.

Pristimerin inhibits the migration and invasion of breast cancer cells

A previous study has shown that inhibition of proteaosme activity is associated with the inhibition of MDA-MB-231cell migration and invasion (Xie et al., 2009). We tested whether treatment with pristimerin could modulate the migration and invasion of breast cancer cells by transwell assays in vitro. As shown in Figure 2A, treatment with 0.25-1μM pristimerin or 10μμM MG132 for 5 h significantly reduced the numbers of cells migrated through the membrane in response to the CM from the cultured NIH-3T3 cells, and its inhibitory effects were dose-dependent. Similarly, treatment with MG132 also inhibited the migration of breast cancer cells. A similar pattern of dose-dependent inhibition of pristimerin was observed in the migration of MDA-MB-436 cells (data not shown). Furthermore, we found that treatment with different doses of pristimerin inhibited the CM-mediated invasion of MDA-MB-231 cells and that treatment with 10 μM MG132 also inhibited the CM-mediated invasion of MDA-MB-231 cells (Figure 2B). Therefore, these data suggest that pristimerin inhibits proteasome activity and breast cancer cell migration and invasion in vitro.

Pristimerin inhibits the growth and invasion of implanted breast cancers in vivo

To investigate the effect of pristimerin on the growth and invasiveness of tumor cells in vivo, BALB/c nude mice were inoculated with MDA-MB-231 cells in their orthotopic mammary fat pads, and when the implanted tumors grew about 110 mm^3, the mice were randomized and treated subcutaneously with 1 mg/kg of pristimrin or the same volume of vehicle every other day for six times. We found that treatment with pristimerin inhibited the growth of implanted tumors in vivo. Evidentially, the average tumor volume in the control mice increased from 114.96 ± 22.85 to 767.92 ± 69.33 mm^3, whereas the average tumor volume in the pristimerin-treated group of mice increased from 115.76 ± 23.14 to 563.18 ± 50.50 mm^3 (Figure 3A). Characterization of the dissected tumor and surrounding tissue sections revealed that there was no clear separation between the tumor and surrounding mammary fibroadipose tissues and that many tumor cells had invaded into the surrounding mammary fibroadipose tissues in the vehicle-treated mice (Figure 3 B). In contrast, there was a clear border separating the tumor from surrounding fibroadipose tissues and the surrounding fibroadipose tissues remained normal structure. Quantitative analysis of the invasiveness of implanted tumors revealed that pristimerin treatment significantly inhibited the

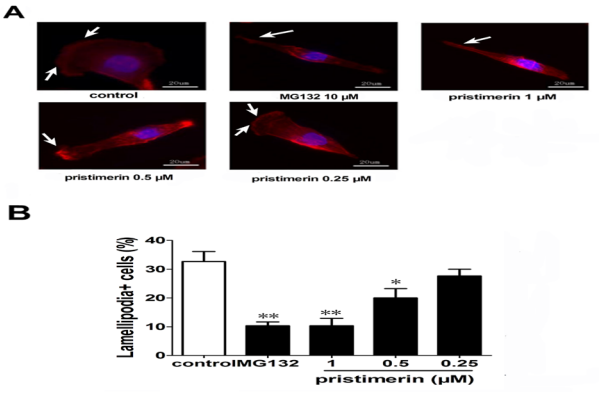

Figure 4. Pristimerin Inhibits the Formation of Lamellipodia in MDA-MB-231 Cells. MDA-MB-231 cells were starved for 24 hours in glass cover-slips and then treated with, or without, 0.25-1 μM pristimerin or 10 μM MG132 in CM for 5 hours. Subsequently, the cells were fixed, permeabilized, and stained with rhodamine-conjugated phalloidin, followed by mounting with medium containing DAPI and imaging under a fluorescent microscope. Data shown are representatives of photomicrographs (magnification x 400) and expressed as mean ± SEM of the percentage of cells with lamellipodia of different groups of cells from three independent experiments. A. The representative photomicrographs. Scale bar, 20 μm. Arrows: the lamellipodia at cell edges. B. Quantitative analysis. *p<0.05 or **p<0.01 vs. the controls

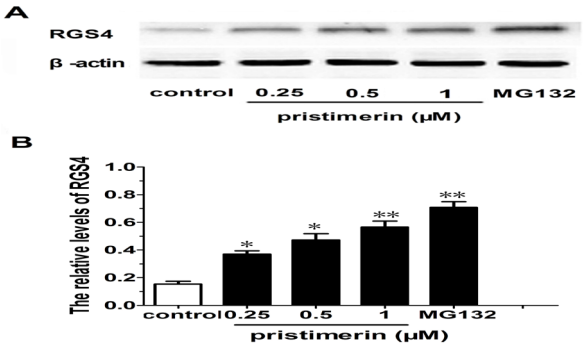

Figure 5. PristimerinIncreases the Levels of Intra-Plasmatic RGS4 in MDA-MB-231 Cells. MDA-MB-231 cells were treated with, or without, the indicated concentrations of primstimerin or10 μM MG132 for 4 h, and the relative levels of intracellular RGS4 to control β-actin were determined by Western blot assays, followed by quantitative analysis using densitometric scanning. Data shown are representative images and expressed as mean ± SEM of the values of individual groups from three separate experiments. A. The representative images. B. Quantitative analysis. *p<0.05 or **p<0.01 vs. the controls

invasiveness of implanted breast cancer in vivo (Figure 3C). Together, our novel data provide the first evidence that treatment with pristimerin inhibits the growth and invasion of implanted breast cancers in mice.

Pristimerin inhibits the lamellipodia formation in MDA-MB-231 cells

Activated GPCRs can induce actin cytoskeleton reorganization to form lamellipodia, which is the flattened F-actin-rich leading edge of migrating cells (Hobson et al., 2001). The formation of lamellipodia is a key process of cell migration and has been observed in MDA-MB-231 cells (Yamada et al., 2009). Given that pristimerin can directly inhibit proteasome activity and breast cancer cell migration and invasion, we reasonably tested the impact of pristimerin on the formation of lamellipodia in MDA-MB-231 cells in vitro. MDA-MB-231 cells were cultured in CM for five hours in the presence or absence of different concentrations of pristimerin or 10 μM MG132, and the cells were stained with rhodamine-conjugated phalloidin, followed by phontomicrography under a fluorescence microscope (Figure 4A). Control MDA-MB-231 cells that had been cultured in CM in the absence of pristimerin or MG132 displayed the typical lamellipodia at the edges of migrating cells. In contrast, a fine F-actin meshwork was observed over the cytoplasm of the cells that had been treated with MG132 and 1 μM pristimerin, accompanied by a fibrotic morphology, although atypical lamellipodia was observed in the cells treated with a lower dose of pristimerin. Quantitative analysis revealed that treatment with pristimerin or MG132 significantly reduced the percentages of lamellipodia-positive cells (p<0.05 or p<0.01, Figure 4B). Therefore, pristimerin significantly inhibits the CM-mediated lameppipodia formation in MDA-MB-231 cells in vitro, which may contribute to the inhibition of pristimerin on the migration and invasion of breast cancer cells.

Pristimerin increases the levels of intra-plasmatic RGS4 in breast cancer cells

Previous study has shown that RGS4 is a critical negative regulator of breast cancer cell migration and invasion (Xie et al., 2009). RGS4 is predominantly degraded by proteasome and the N-end rule pathway (Lee et al., 2005; Bodenstein et al., 2007) and pristimerin inhibits proteasome activity (Tiedemann et al., 2009). Accordingly, RGS4 may play an important role in the inhibition of pristimerin on breast cancer cell migration. To test this hypothesis, we treated MDA-MB-231 cells with different concentrations of pristimerin or 10 μM MG132 for four hours, and the levels of RGS4 in MDA-MB-231 cells were characterized by Western blot assays (Figure 5A). Clearly, treatment with pristimerin or MG132 increased the contents of RGS4 in MDA-MB-231 cells. Quantitative analysis revealed that treatment with pristimerin significantly increased the levels of RGS4, and its protective effects were dose-dependent (Figure 5B). However, treatment with a lysosome inhibitor of chloroquine (100 μM) failed to affect the levels of intra-plasmatic RGS4, further confirming that RGS4 is not degraded by lysosomes (data not shown). Given that RGS4 negatively regulates the migration and invasion of breast cancer cells, the increased levels of intra-plasmatic RGS4 by pristimerin may contribute to its inhibitory effect on the migration and invasion of MDA-MB-231 cells in vitro.

Knockdown of RGS4 expression attenuates the effect of pristimerin on the migration and invasion of breast cancer cells

To further determine the role of RGS4 in the pristimerin-mediated inhibition of MDA-MB-231 cell migration and invasion, MDA-MB-231 cells were transfected with the RGS4-specific siRNA or control siRNA for 48 h and the cells were treated with, or without, 1 μM pristimerin or 10 μM MG132 for five hours. Subsequently, the relative levels of RGS4 to β-actin in different groups of cells

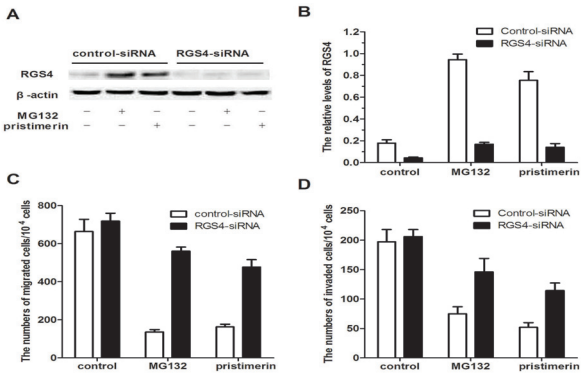

Figure 6. Knockdown of RGS4 Expression Attenuates Pristimerin-mediated Inhibition of Breast Cancer Cell Migration and Invasion in Vitro. MDA-MB-231 cells were transfected with control siRNA or the RGS4-psecific siRNAs for 48 h and treated with, or without, pristimerin or MG132 for 5 h, respectively. The relative levels of RGS4 to control β-actin expression in these cells were determined by Western blot assays. Subsequently, the ability of the transfected cells in response to chemotactic reagents was tested by transwell migration and invasion assays in the presence or absence of 1 μM pristimerin or 10 μM MG132, respectively. Data shown are representative images or expressed as mean ± SEM of the values of different groups from three separate experiments. A. Western blot analysis of RGS4 expression. B. Quantitative analysis of RGS4 expression. C. The effect of pristimerin on the migration of RGS4-silencing MDA-MB-231 cells. D. The effect of pristimerin on the invasion of RGS4-silencing MDA-MB-231 cells. $^{*}p<0.05$ or $^{**}p<0.01$ vs. the controls

were characterized by Western blot assays (Figure 6A). Transfection of the RGS4-specific siRNA dramatically reduced the levels of RGS4, indicating the silence of RGS4 expression. Furthermore, while treatment with pristimerin or MG132 increased the levels of RGS4 in the control siRNA-transfected cells by 4-6 fold, the same treatment only moderately elevated the levels of RGS4 in the RGS4-silencing MDA-MB-231 cells (Figure 6B).

We further examined the migration and invasion of RGS4-expressing and RGS4-silecning MDA-MB-231 cells. We found that knockdown of RGS4 expression enhanced breast cancer cell migration and invasion (Figure 6 C and D). Furthermore, while pristimerin or MG132 powerfully inhibited the migration and invasion of the control siRNA-transfected cells, the same treatment only moderately reduced the numbers of migrated and invaded breast cancer cells. Apparently, knockdown of RGS4 expression attenuated the pristimerin-mediated inhibition of breast cancer cell migration and invasion. These data indicate that increased levels of RGS4 by pristimerin are important for its inhibition of breast cancer cell migration and invasion in vitro.

Discussion

Pristimerin is a natural triterpenoid and can inhibit tumor cell proliferation and cell cycling as well as tumor growth in vivo (Costa et al., 2008; Tiedemann et al., 2009). In the present study, we found that treatment with pristimerin directly inhibited human 20S chymotrypsin-like proteasome activity and chymotrypsin-like activity in MDA-MB-231 breast cancer cells. Furthermore, we found that treatment with pristimerin inhibited the migration and invasion of MDA-MB-231 cells in vitro in a dose-dependent manner and the growth and invasion of implanted breast tumors in vivo. These novel data extend previous findings (Wu et al., 2005; Yang et al., 2008; Tiedemann et al., 2009) and suggest that pristimerin may have broad antitumor activity.

The proteasome plays a central role in the regulation of angiogenesis, metastasis, proliferation, cell cycling, and resistance to therapy (Glickman and Ciechanover, 2002; Adams et al., 2003; Dahlmann et al., 2007; Schwartz and Ciechanover, 2009; Valentiner et al., 2009). We found that pristmerin directly targeted the purified human 20S chymotrypsin-like proteasomes, consistent with a previous report (Tiedemann et al., 2009), and inhibited the chymotrypsin-like proteasomal activity in breast cancer cells. Given that inhibition of proteasome activity can regulate tumor growth and metastasis, our findings support the notion that pristimerin and similar proteasome inhibitors may inhibit the growth and metastasis of breast cancer through different mechanisms. We are interested in investigating whether pristimern can regulate angiogenesis and drug-resistance in breast cancers.

RGS4 is a G protein-specific GTPase-activating protein and has the GAP activity (Lee et al., 2005). RGS4 can selectively inhibit the Gi- and Gq-coupled GPCRs signaling (Huang et al., 1997; Xie GX et al., 2007), and modulation of RGS4 levels can regulate cancer cell metastasis (Xie et al., 2009). We found that treatment with pristimerin or the proteasome inhibitor of MG132 increased the levels of intra-plasmatic RGS4. However, treatment with a lysosome inhibitor failed to change the contents of intra-plasmatic RGS4 in breast cancer cells. These data are consistent with previous reports and further support the notion that RGS4 is predominantly degraded by the proteasome pathway (Varshavsky et al., 2000; Neves et al., 2002; Lee et al., 2005; Xie et al., 2009). Notably, high levels of RGS4 transcripts have been associated with increased viability, invasion, and motility of breast cancer cells (Yau et al., 2010). However, the levels of RGS4 mRNA transcripts are usually not correlated with levels of RGS4 protein in breast cancer cells and high levels of proteasome activity were detected in breast cancer cells (Chen and Madura, 2005; Yau et al., 2010). Apparently, RGS4 has a short half-life and is rapidly degraded in breast cancer cells. Indeed, a recent study has shown that there is an inverse correlation between the levels of RGS4 protein and metastatic capability of different human breast cancer cell lines (Xie et al., 2009). Furthermore, induction of RGS4 over-expression and blockade of RGS4 degradation inhibit cell migration and invasion (Albig AR et al., 2005; Xie et al., 2009). We found that pristimerin inhibited breast cancer cell migration and invasion in vitro and implanted breast tumor invasion in vivo. The inhibitory effect of pristimerin on the migration and invasion of breast cancer cells appeared to be associated with its ability to inhibit the chymotrypsin-like proteasome activity and to increase the levels of RGS4 in breast cancer cells. Evidentially, treatment with the proteasome inhibitor of MG132 also inhibited breast cancer cell migration and invasion. Furthermore, knockdown of RGS4 expression

attenuated the pristimerin-mediated inhibition of breast cancer cell migration and invasion. Notably, pristimerin has a more potent inhibitory effect than MG132 on the migration and invasion of RGS4-silencing breast cancer cells in vitro. This highly inhibitory effect suggests that pristimerin may also affect other molecules involved in regulating cancer metastasis. We are interested in further discovering the pharmacological mechanisms underlying the action of pristimerin in inhibiting tumor growth.

The GPCR activation triggers actin cytoskeleton reorganization to form lamellipodia, the flattened F-actin-rich leading edge of migrating cells, which is a critical process of cancer cell metastasis (Van Haastert and Devreotes, 2004). We found that treatment with pristimerin significantly reduced the chemotactic reagent-mediated lamellipodia formation in breast cancer cells in vitro. The inhibitory effect of pristimerin on the lamellipodia formation is likely mediated by its ability to increase the levels of intra-plasmatic RGS4 in breast cancer cells. Indeed, a previous study has shown that up-regulated RGS4 expression can disrupt the PAR1 or CXCR4 activation-mediated lamellipodai formation in tumor cells (Xie et al., 2009). There are other GPCRs involved in the regulation of cancer metastasis (Dorsam and Gutkind, 2007). It is possible that pristimern inhibits the proteasome activity and increases the levels of intra-plasmatic RGS4, which down-regulates the GPCR-mediated signaling and inhibits breast cancer cell migration and invasion. Therefore, our finding may provide new insights into pharmacological mechanisms underlying the action of pristimerin.

In summary, our data clearly demonstrated that pristimerin directly inhibited human chymotrypsin-like proteasome activity and increased the levels of RGS4 protein in MDA-MB-231 breast cancer cells. Furthermore, our data indicated that treatment with pristimerin inhibited the migration and invasion of breast cancer cells in a dose-dependent manner, which was attenuated by knockdown of the RGS4 expression. In addition, treatment with pristimerin inhibited the chemotactic reagent-mediated lamellipodia formation in MDA-MB-231 cells and suppressed the growth and invasion of implanted breast tumors in vivo. Therefore, our data indicate that pristimerin inhibits breast cancer migration and invasion by increases the levels of intra-plasmatic RGS4. Our findings may provide new insights into the pharmacological mechanisms underlying the action of pristimerin in regulating cancer metastasis.

Acknowledgements

This study was partially supported by grants from the Specific Fund for Public Interest Research of Traditional Chinese Medicine, Ministry of Finance (No. 200707008), and Mega-Projects of Science Research for the 11th Five-Year Plan: Standardized platform construction and scientific application in new technologies for new drug screening (No. 2009ZX09302-002), and the 111 Project (111-2-07) , and the International Scientific and Technological Cooperation Projects (No 2010DFB33710).

References

Adams J (2003). The proteasome: structure, function, and role in the cell. *Cancer Treat Rev*, **29 Suppl 1**, 3-9.

Albig AR SW (2005). Identification and characterization of regulator of G protein signaling 4 (RGS4) as a novel inhibitor of tubulogenesis. *Mol Biol Cell*, **16**, 609-25.

Bodenstein J, Sunahara RK, Neubig RR (2007). N-terminal residues control proteasomal degradation of RGS2, RGS4, and RGS5 in human embryonic kidney 293 cells. *Mol Pharmacol*, **71**, 1040-50.

Boire A, Covic L, Agarwal A, et al (2005). PAR1 is a matrix metalloprotease-1 receptor that promotes invasion and tumorigenesis of breast cancer cells. *Cell*, **120**, 303-13.

Byun JY, Kim MJ, Eum DY, et al (2009). Reactive oxygen species-dependent activation of Bax and poly(ADP-ribose) polymerase-1 is required for mitochondrial cell death induced by triterpenoid pristimerin in human cervical cancer cells. *Mol Pharmacol*, **76**, 734-44.

Chambers AF, Groom AC, MacDonald IC (2002). Dissemination and growth of cancer cells in metastatic sites. *Nat Rev Cancer*, **2**, 563-72.

Chen L, Madura K (2005). Increased proteasome activity, ubiquitin-conjugating enzymes, and eEF1A translation factor detected in breast cancer tissue. *Cancer Res*, **65**, 5599-606.

Costa PM, Ferreira PM, Bolzani Vda S, et al (2008). Antiproliferative activity of pristimerin isolated from Maytenus ilicifolia (Celastraceae) in human HL-60 cells. *Toxicol In Vitro*, **22**, 854-63.

Dahlmann B (2007). Role of proteasomes in disease. *BMC Biochem*, **8 Suppl 1**, S3.

Dorsam RT, Gutkind JS (2007). G-protein-coupled receptors and cancer. *Nat Rev Cancer*, **7**, 79-94.

Glickman MH, Ciechanover A (2002). The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol Rev*, **82**, 373-428.

Hobson JP, Rosenfeldt HM, Barak LS, et al (2001). Role of the sphingosine-1-phosphate receptor EDG-1 in PDGF-induced cell motility. *Science*, **291**, 1800-3.

Huang C, Hepler JR, Gilman AG, et al. (1997). Attenuation of Gi- and Gq-mediated signaling by expression of RGS4 or GAIP in mammalian cells. Proc Natl Acad Sci U S A, 94, 6159-63.

Jemal A, Bray F, Center MM, et al (2011). Global cancer statistics. *CA Cancer J Clin*, **61**, 69-90.

Lappano R, Maggiolini M (2011). G protein-coupled receptors: novel targets for drug discovery in cancer. *Nat Rev Drug Discov*, **10**, 47-60.

Lee MJ, Tasaki T, Moroi K, et al (2005). RGS4 and RGS5 are in vivo substrates of the N-end rule pathway. *Proc Natl Acad Sci U S A*, **102**, 15030-5.

Neves SR, Ram PT, Iyengar R (2002). G protein pathways. *Science*, **296**, 1636-9.

Ross EM, Wilkie TM (2000). GTPase-activating proteins for heterotrimeric G proteins: regulators of G protein signaling (RGS) and RGS-like proteins. *Annu Rev Biochem*, **69**, 795-827.

Schlunck G, Damke H, Kiosses WB, et al (2004). Modulation of Rac localization and function by dynamin. *Mol Biol Cell*, **15**, 256-67.

Schwartz AL, Ciechanover A (2009). Targeting proteins for destruction by the ubiquitin system: implications for human pathobiology. *Annu Rev Pharmacol Toxicol*, **49**, 73-96.

Tiedemann RE, Schmidt J, Keats JJ, et al (2009). Identification of a potent natural triterpenoid inhibitor of proteosome chymotrypsin-like activity and NF-kappaB with antimyeloma activity in vitro and in vivo. *Blood*, **113**, 4027-37.

Valentiner U, Haane C, Nehmann N, et al (2009). Effects of bortezomib on human neuroblastoma cells in vitro and in a metastatic xenograft model. *Anticancer Res*, **29**, 1219-25.
Van Haastert PJ, Devreotes PN (2004). Chemotaxis: signalling the way forward. *Nat Rev Mol Cell Biol*, **5**, 626-34.
Varshavsky IVDaA (2000). RGS4 is arginylated and degraded by the N-end rule pathway in vitro. *J Biol Chem*, **275**, 22931-41.
Wu CC, Chan ML, Chen WY, et al (2005). Pristimerin induces caspase-dependent apoptosis in MDA-MB-231 cells via direct effects on mitochondria. *Mol Cancer Ther*, **4**, 1277-85.
Xie GX PP (2007). How regulators of G protein signaling achieve selective regulation. *J Mol Biol*, **366**, 349-65.
Xie Y, Wolff DW, Wei T, et al (2009). Breast cancer migration and invasion depend on proteasome degradation of regulator of G-protein signaling 4. *Cancer Res*, **69**, 5743-51.
Yamada H, Abe T, Li SA, et al (2009). Dynasore, a dynamin inhibitor, suppresses lamellipodia formation and cancer cell invasion by destabilizing actin filaments. *Biochem Biophys Res Commun*, **390**, 1142-8.
Yang H, Landis-Piwowar KR, Lu D, et al (2008). Pristimerin induces apoptosis by targeting the proteasome in prostate cancer cells. *J Cell Biochem*, **103**, 234-44.
Yau C, Esserman L, Moore DH, et al (2010). A multigene predictor of metastatic outcome in early stage hormone receptor-negative and triple-negative breast cancer. *Breast Cancer Res*, **12**, R85.