Cyclopeptide RA-V inhibits cell adhesion and invasion in both estrogen receptor positive and negative breast cancer cells via PI3K/AKT and NF-κB signaling pathways

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Abstract: This is the first comprehensive report on the inhibitory activities on cell adhesion, migration, adhesion and ECM degradation in vitro, implying that RA-V is a potential anti-metastatic agent in breast cancer, and likely acts via PI3K/AKT and NF-κB signaling pathways in both ER-positive and ER-negative breast cancer cells.

1. Introduction

Breast cancer is the most frequent cancer diagnosed among women, accounting for 25% of all cancer cases. It is responsible for 12% of all cancer cases [1]. Approximately 20% of patients with breast cancer at early stage will develop metastasis [2]. Metastasis is responsible for more than 90% breast cancer death [3]. Anthracyclines, Taxanes® and 5-fluorouracil are commonly used in clinic for the treatment of metastatic breast cancer (MBC), but breast cancer recurrence and chemotherapy-resistant MBC are frequently observed. Therefore, MBC is still incurable as the strategy of developing an anti-metastatic drug lacks the full understanding of the entire metastatic process nowadays. Plant-derived compounds are rich sources of clinically used anti-cancer drugs, such as vinblastine, vincristine, irinotecan and paclitaxel [4]. RA-V (deoxybouvardin), shown in Fig. 1A, a natural cyclopeptide first isolated from the roots of Bouvardia ternifolia [5] with a molecular formula of C_{30}H_{48}N_{6}O_{9}. RA-V has also been isolated from the roots from other related species such as, Rubia cordifolia [6,7] and Rubia yunnanensis [8–10]. RA-V was shown to inhibit inflammation [10,11], tumor growth [6], angiogenesis [12] and induce apoptosis [13].

DNA microarray can efficiently screen differential gene expression by the gene expression profiling assay. Pathway analysis maps the genes from the microarray data to pathways according to Biocarta and Kegg databases. It ranks the pathways based on how relevant the pathways are to the genes in the microarray data, and gene ontology (GO) analysis covers 3 domains, cellular component, molecular function and biological process, so as to better describe gene product properties. These analyses can be carried out to study the microarray data, and
provide with a more comprehensive understanding of the biological activities of RA-V in cancer-related processes. In this study, microarray results revealed that RA-V was involved in suppressing cancer invasion and metastasis; and hence, the anti-metastatic and anti-invasive activities of RA-V and its underlying mechanisms in breast cancer cells were further examined.

Metastasis, an outgrowth of cancer cells in distant organs, is a complex cascade of events. Proteolytic enzyme degradation of extracellular matrix (ECM), cancer cell motility, proliferation and adhesion to ECM play critical roles in cancer invasion and metastasis. Matrix metalloproteinases (MMPs) also play roles in proteolytic enzyme degradation of ECM, and tissue inhibitor of metalloproteinases (TIMPs) and urokinase plasminogen activator system such as urokinase-type plasminogen activator (uPA) can tightly regulate the activity and level of MMPs. Many studies show that MMPs are not only responsible for degrading ECM, but also cell motility and adhesion[14] and metastasis[15].

**Pathway enrichment analysis showed top 5 signaling pathways in Hela cells in response to RA-V, according to Biocarta and Kegg databases.**

<table>
<thead>
<tr>
<th>Pathways</th>
<th>Hits</th>
<th>p-value</th>
<th>Examples of the genes involved</th>
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<tr>
<td>MAPK signaling</td>
<td>55</td>
<td>0.0001</td>
<td>↓ARRB1/2, ↑ATF4, ↓MAP2K6, ↓MAP3K8, ↓MAPK3, ↓FGFR3</td>
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<td>p33 signaling</td>
<td>21</td>
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<td>↓CASP9, ↓CD82, ↑IGFBP3, ↑PTEN, ↑TP53, ↓BDC3</td>
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<td>Wnt signaling</td>
<td>28</td>
<td>0.0167</td>
<td>↓FZD10, ↓TF711, ↓CXXC4, ↓PLCB1/3, ↓PORCN, ↓RAC3</td>
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<tr>
<td>MAPK kinase signaling</td>
<td>16</td>
<td>0.0392</td>
<td>↑CMYC, ↑MAPK3, ↑MAPK4, ↑MAPK11, ↑MAPK13, ↑MAPK2</td>
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<td>EGF signaling</td>
<td>7</td>
<td>0.0477</td>
<td>↓C-JUN, ↑C-KI, ↑EGF, ↑FGFR, ↑SRF, ↑STAT3</td>
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**Fig. 1.** (A) Chemical structure of cyclopeptide RA-V. (B–E) Data of microarray analysis. (B) The heatmap of differential expressed genes in RA-V treated and untreated control samples (n = 3). Hierarchical cluster analysis was carried out for all differential expressed genes (fold change ≥ 2 or ≤ 0.5; p value < 0.05) in RA-V-treated Hela cells, compared with the untreated control. Each column represented one sample. Red colored columns indicated up-regulated genes while green colored columns indicated down-regulated genes. (C) Hierarchical cluster analysis showed the differential expressions of tumor metastasis-related genes induced by RA-V. Each column represented one sample and each row represented one gene. The scale of color intensity was positively correlated to the fold change. (IGFBP6, insulin-like growth factor binding protein 6; ECM2, extracellular matrix protein 2; ICAM4, intercellular adhesion molecule 4; TIMP4, tissue inhibitor of metalloprotease 4; ITGA2B, integrin, alpha 2b; LAMA3, laminin, alpha 3; ALDH3A2, aldehyde dehydrogenase 3 family, member A2; TP53, tumor protein p53; LAMB3, laminin, beta 3; ALDHS4A1, aldehyde dehydrogenase 5 family, member A1; ALDH3B1, aldehyde dehydrogenase 3 family, member B1; IGF1R, IGF-like family receptor 1; COL1A1, collagen, type I, alpha 1; CFL2, coflin 2; MMP15, matrix metalloproteinase 15; ID1, inhibitor of DNA binding 1; IGFR, immunogluconulin superfamily, member 8; ID3, inhibitor of DNA binding 3; TIMP3, tissue inhibitor of metalloproteinase 3; ALDH1A1, aldehyde dehydrogenase 16 family, member A1; MMP28, matrix metalloproteinase 28; IGFR3, immunogluconulin superfamily, member 3; LAMB2, laminin, beta 2; COL4A5, collagen, type IV, alpha 5; COL4A4, collagen, type IV, alpha 4; IGFR2, IGF-like family member 2; TNC, tenascin C; IGF2BP1, insulin-like growth factor 2 mRNA binding protein 1; LAMA2, laminin, alpha 2; MMP7, matrix metalloproteinase 7; MMP19, matrix metalloproteinase 19; ITG8, integrin, beta 8; ITGAM, integrin, alpha M; LAMB1, laminin, beta 1; LAMB3, laminin, gamma 2). (D) RA-V induced cancer invasion and metastasis-associated GO categories. GO categories cover 3 domains, cellular component, molecular function and biological process. Genes with differential expressions were analyzed and subjected to GO analysis in accordance with the SBC Analysis System. GO categories (p value < 0.05; Hit ≥ 5) were considered to be significant. ‘Hits’ was considered as the number of the differential expressed genes according to each GO category. (E) Table showed the top 5 signaling pathways involved in Hela cells in response to RA-V. *The number of the differential expressed genes in a pathway according to Biocarta and Kegg databases. The enrichment p value of the pathway as determined by R-package Fisher’s Exact Test.
to the lung [16], MMP-1 and MMP-9 are associated with poor prognosis of patients suffering from breast cancer [17]. Overexpression of MMP-7 facilitates cancer invasion and also activation of MMP-2 and MMP-9 from their inactive forms in breast cancer cells [18]. TIMPs are capable of inhibiting MMP activities [19], uPA converts plasminogen into plasmin which directly degrades ECM proteins and indirectly activates pro-MMPs into active form of MMPs. Hence, increased expressions of MMPs, TIMPs and uPA are associated with increased cancer progression and reduced survival [20]. uPA can also induce cell migration. Constitutively active PI3K regulates cell migration by uPA secretion via NF-κB activation in breast cancer cells [21], and the regulation of cell migration in response to uPA requires FAK [22]. In addition, FAK is involved in the activation and secretion of MMP-2 and MMP-9 [23] as well as cell motility [24].

Once ECM has been degraded, it provides a path for cancer cells to migrate. Chemokine receptors can mediate cancer cell motility and are responsible for metastatic dissemination. Active chemokine receptors, CXCR4 and CCR7 are expressed in high levels in breast cancer cells and they are in response to their specific ligands CXCL12 and CXCL19 or CXCL21. Neutralizing anti-CXCR4 monoclonal antibody can inhibit such chemotaxis in vitro and inhibit metastasis in vivo [25]. Chemokine receptors expressed on breast cancer cells can direct these cells preferentially to particular sites where their ligands are over expressed during metastasis. Chemokine receptors also regulate cancer cell adhesion to ECM and metastatic dissemination through integrin [26]. In addition, coxin signaling pathway is involved in actin polymerization and mediates cell migration. Coxin induces actin polymerization and direct cancer cell migration, and total coxin is usually overexpressed in invasive cancer cells [27]. Coxin signaling pathway includes the involvement of small G-proteins, RhoA and cell division cycle 42 (Cdc42), coxin-regulating kinase (ROCK1), phosphorylated coxin and coxin.

Cancer cell adhesion to the ECM can also facilitate cancer invasion. Adhesion molecules such as vascular cellular adhesion molecule (VCAM), intracellular adhesion molecule (ICAM) are involved. Integrins are αβ heterodimers and are cell adhesion receptors that regulate cell-ECM adhesion and allow signal transduction across the membrane, as well as link the ECM to actin cytoskeleton. Migrating cancer cells at the leading edge adhere to the ECM and recruit actin cytoskeleton as well as promote membrane protrusion. On the other hand, cells at the rear edge detach from the ECM and disconnect to actin cytoskeleton. Various subunits of integrin are responsible for the adhesion to ECM components such as collagen, fibronectin and laminin [28].

Breast cancer progression usually begins with hormone-sensitive, non-metastatic cancer cells expressing estrogen receptor (ER) and subsequently progressing to hormone-insensitive, invasive and metastatic cancer cells with ER-negative phenotype. The response of the ER-negative cancer cells with increased metastatic potential to current chemotherapy or hormonal therapy is not compromising [29]. Therefore, the development of a novel anti-metastatic drug with high efficacy and low toxicity in both ER-positive and ER-negative breast cancers is urgently needed.

The present study aimed to investigate the anti-metastatic and anti-invasive effects of RA-V on two human breast cancer cells. ER-positive MCF-7 and ER-negative MDA-MB-231, representing slightly and highly invasive cancer cells, respectively, were used in this study. Here, our data demonstrated that RA-V inhibited breast cancer cell motility and migration, cell proliferation, cell-ECM adhesion and ECM-associated proteases including MMPs, TIMPs and uPA in both ER-positive MCF-7 and ER-negative MDA-MB-231 cells. RA-V was also demonstrated to exert its anti-invasive effect via PI3K/AKT and NF-κB signaling pathways.

2. Materials and methods

2.1. Preparation of RA-V

RA-V was extracted and isolated from the root of R. yunnanensis. The details of extraction and isolation procedures for RA-V have been reported previously [10,12]. RA-V powder was dissolved in dimethyl sulfoxide (DMSO) (Sigma, USA) at 10 mM as a stock solution. The stock was stored at −20 °C and reconstituted in a medium prior to use.

2.2. Cell culture

Human cervical cancer cells Hela and human breast cancer cells MDA-MB-231 and MCF-7 were purchased from American Type Culture Collection (ATCC, USA) during our study period (2008–2012). Hela cells were maintained in Dulbecco’s modified Eagle’s medium, while MDA-MB-231 and MCF-7 cells were maintained in RPMI medium 1640. The media were supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 100 units/mL of penicillin–streptomycin. All the culture media, PBS and supplements were obtained from Life Technologies (USA). The cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂. All the cells obtained from ATCC were immediately expanded and frozen down such that all cell lines could be restarted every 3–4 months from a frozen vial of the same batch of cells. Once resuscitated, cell lines were routinely authenticated through cell morphology monitoring and growth curve analysis.

2.3. Microarray and data analysis

Hela cells (6 × 10⁵/mL) were seeded in 6-well plates (Corning, USA) and incubated for 24 h. After another 24-h treatment with 100 nM of RA-V or DMSO (0.001%) as a control, the cells were washed twice by ice-cold PBS. Total RNA was extracted by using a TRIzol reagent (Life Technologies, USA) according to the manufacturer’s instructions. Its quantification and quality were assessed by spectrophotometry and with an Agilent 2100 Bioanalyzer (Agilent Technologies, USA) (Supplementary Fig. 1 and Table S1). Total RNA of the samples at 2 μg without degradation was amplified, labeled and hybridized. Gene expression profile was analyzed using the Agilent Whole Human Genome Oligo Microarray kit, 4 × 44K (Agilent Technologies, USA), consisting of approximately 41,000 genes and transcripts. Treated and untreated samples in triplicate were used in the microarray assay which was carried out by Shanghai Biochip Corporation (SBC) Limited (China). The experimental data were analyzed using the SBC Analysis System, including gene ontology (GO) and pathway enrichment analysis.

2.4. Cytotoxicity MTT assay and [methyl-3H]-thymidine incorporation assay

The cytotoxicity of RA-V on MDA-MB-231 and MCF-7 cells were assessed using MTT assay. Cells (5 × 10⁴/mL) were seeded in 96-well flat-bottom culture plate in 100 μL of medium and incubated overnight. RA-V at various concentrations in 100 μL of medium was added to give final concentrations of 3.125 to 200 nM. A medium containing vehicle solvent (0.5% (v/v) DMSO) was added to the wells as the untreated controls. Microplate was then incubated for 24 or 48 h. Thirty microliters of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (5 mg/mL) (Sigma, USA) was added and incubated for 3 h at 37 °C. Supernatants in each well were removed and 100 μL of DMSO was added. Once the violet crystal was dissolved in DMSO, the plate was read at 450 nm by a microplate reader (BioTek, USA). The change in optical density was represented as a fold of untreated control.

The effect of RA-V on breast cancer cell proliferation was assessed using [methyl-3H]-thymidine incorporation assay. Cells were seeded as described above. After incubation for 18 or 42 h, 50 μL of [methyl-3H]-thymidine solution (0.5 μCi) (PerkinElmer, USA) was added and incubated for another 6 h at 37 °C. Plates were then placed at −20 °C for at least 24 h. Once thawed, they were subjected to a scintillation counter (Packard, USA) measurement [12]. The change in count per minutes was represented as a fold of untreated control.
2.5. Scratch wound assay

Cell motility of MDA-MB-231 and MCF-7 cells was assessed using scratch wound assay. Cells (1 × 10^5/mL) were seeded in a 24-well plate and incubated overnight. The cells were starved with a medium in 1% (v/v) FBS for 24 h. The cells were scraped with a cross by a 200 μL pipette tip in the middle of well. Each well contained 2 crosses. The crosses of each well were photographed under an Olympus IX-71 microscope (Japan) at 0 h. A fresh medium with 3.125 to 25 nM of RA-V was added to the wells in triplicate and the plate was incubated for 24 h. The crosses of each well were photographed again at 24 h under a microscope. The percentages (%) of open wound area were measured and the change in open wound area (%) at 24 h from 0 h was calculated using the Tscratch software [12]. Motility was determined by the decrease of the open wound area.

2.6. Transwell migration assay

To assess the cell migration ability of MDA-MB-231 and MCF-7 cells, transwell migration assay was performed using a Boyden chamber. Cells (1.5 × 10^5/mL) were dissolved in 200 μL of medium with 1% (v/v) FBS containing RA-V at concentrations were added to the upper chamber of each transwell with 8 μm-pore size filter membrane (Corning, USA) to give a final concentration of RA-V at 6.25–25 nM in duplicate transwells. A culture medium (500 μL) with 10% (v/v) FBS, as a chemoattractant media, was added to the lower chamber. Transwells were incubated for 4 h at 37 °C. After incubation, cells were fixed with methanol for 3 min and stained with hematoxylin for 5 min. Cotton swab was used to scrap the cells on the top surface of the filter membrane of the upper chamber gently. Stained migrated cells were quantified by manual counting in a double-blinded manner. Migration was determined by the number of migrated cells.

2.7. Extracellular matrix (ECM) adhesion assay

To evaluate the effect of RA-V on MDA-MB-231 and MCF-7 cell-ECM adhesion, an Extracellular Matrix Cell Adhesion Array kit (Millipore, USA) was used. Each well of an eight-well strip was pre-coated with one of the seven different human ECM proteins (collagen I, collagen II, collagen IV, fibronectin, laminin, tenasin or vitronectin) or BSA, as a negative control. The assay was carried out according to the procedures recommended by the manufacturer. Briefly, cells were added to pre-coated wells treated with or without RA-V at 20 or 50 nM for 2 h. After washing, bound cells were stained and dissolved in an extraction buffer. The change in optical density was represented as a fold of untreated control.

2.8. Urokinase-type plasminogen activator (uPA) activity assay

The activity of uPA of MDA-MB-231 and MCF-7 cells was assessed by using uPA activity kit (Chemicon, Millipore, USA). Cells (1 × 10^5/mL) were seeded in a plastic 24-well plate overnight. They were then treated with RA-V (6.25–25 nM) in 0.5 mL culture medium with 1% (v/v) FBS and incubated for 16, 24 or 48 h. A culture medium was collected for the assay and centrifuged at 1000 × g for 10 min. Supernatants were subjected to gelatin zymography. The supernatants were resolved by centrifuged at 1000 × g for 10 min. Supernatants were subjected to gelatin zymography. The supernatants were resolved by 10% SDS polyacrylamide gel polymerized with 0.1% gelatin. After electrophoresis, the gels were washed with 2.5% Triton X-100 (Sigma, USA) for an hour and incubated in a development buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM CaCl_2) for 20 h at room temperature. The gels were then stained with 0.25% comassie blue R-250 (Sigma, USA) for 30 min and destained with 10% acetic acid in 5% ethanol until the clear zones were observed. The activities of MMP-2 and MMP-9 were observed as the clear zone, digested by MMP-2 and MMP-9 in the conditional medium in the gels, captured by a molecular imager, ChemiDoc XRS+ (Bio-Rad, USA). The clear zones were then quantified using ImageJ (NIH, USA).

2.9. Gelatin zymography

The activities of MMP-2 and MMP-9 (gelatinases) of MDA-MB-231 and MCF-7 cells were assessed by gelatin zymography. Cells (1 × 10^5/mL) were seeded in a type I collagen-pre-coated 24-well plate overnight. PBS was used to wash the wells twice to remove remaining FBS. The cells were then treated with RA-V (6.25–12.5 nM) in a culture medium replaced with 0.5 mL FBS-free culture medium and incubated for 72 h. A culture medium was collected and centrifuged at 1000 × g for 10 min. Supernatants were subjected to gelatin zymography. The supernatants were resolved by 10% SDS polyacrylamide gel polymerized with 0.1% gelatin. After electrophoresis, the gels were washed with 2.5% Triton X-100 (Sigma, USA) for an hour and incubated in a development buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM CaCl_2) for 20 h at room temperature. The gels were then stained with 0.25% comassie blue R-250 (Sigma, USA) for 30 min and destained with 10% acetic acid in 5% ethanol until the clear zones were observed. The activities of MMP-2 and MMP-9 were observed as the clear zone, digested by MMP-2 and MMP-9 in the conditional medium in the gels, captured by a molecular imager, ChemiDoc XRS+ (Bio-Rad, USA). The clear zones were then quantified using ImageJ (NIH, USA).

2.10. Western blot analysis

Protein expressions of MDA-MB-231 and MCF-7 cells were studied by Western blotting. Cells (1 × 10^6) in a 7 mL culture medium were seeded in a 100 mm culture dish and incubated overnight. Cells were incubated with RA-V (6.25, 12.5 or 25 nM) for various time-points (4, 8, 16, 24 or 48 h). After incubation, the attached and floating cells were harvested and washed twice with PBS. To obtain whole cell lysate, cell pellets were lysed with whole cell extraction buffer (% SDS, 10% glycerol, 625 mM Tris–HCl, pH 6.8) for 20 min with shaking on ice. To obtain cytoplasmic cell lysate, cell pellets were lysed with a cytoplasmic cell extraction buffer (Thermo Scientific, USA). The samples were then centrifuged at 14,000 × g for 15 min at 4 °C. The supernatants were collected and stored at −80 °C until used. Whole cell lysate and cytoplasmic cell lysate were subjected to Western blotting. The proteins were heated at 95 °C for 5 min and subsequently resolved by 10% SDS polyacrylamide gel. The proteins in the gel were then transferred to 0.45 mm PVDF membrane (Millipore, USA) at 90 V for 90 min. The blots were blocked with 5% non-fat dried milk in Tris-buffered saline Tween 20 (TBS) (20 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.1%, Tween-20). The blots were incubated with primary antibodies, beta-actin (Sigma, USA), MMP1, MMP2, MMP7, MMP9, uPA, TIMP-1, ER, ROCK1, pEGFR, EGFR (Abcam, USA), collin, p-collin, Cdc42, RhoA, TIMP-2, P3K, pP3K, AKT, pAKT, pNF-κB, pNF-κB, IκB and p-IκB (Cell Signalling, USA) overnight. The blots were then washed three times with TBS and were incubated with secondary horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Invitrogen, USA) for an hour. The signals on the blots were detected using an ECL solution (GE Healthcare Life Sciences, Sweden), and were captured by a molecular imager, ChemiDoc XRS+ (Bio-Rad, USA). The signals were quantified using ImageJ (NIH, USA).

2.11. Flow cytometry

Protein expressions of MDA-MB-231 and MCF-7 cells were studied by flow cytometry. Cells (1 × 10^6) in a 7 mL culture medium were seeded in a 100 mm culture dish and incubated overnight. Cells were treated with RA-V (6.25, 12.5 or 25 nM), LY294002 (20 μM, P3K inhibitor) and BAY11-7082 (5 μM, NF-κB inhibitor) (Sigma, USA) for 24 h. After incubation, the attached and floating cells were harvested and washed...
twice with PBS. Cells were stained with fluorochrome (FITC or PE)-conjugated monoclonal antibodies, CD47b (integrin α5), CD47e (integrin α5), CD47f (integrin α5), CD29 (integrin β1), CD184 (CXCR4), CD197 (CCR7) (Miltenyi Biotec, USA), VCAM-1, ICAM-1 (Abcam, USA) in accordance with the manufacturer’s specifications. Samples were mixed well and incubated at 4 °C for 30 min in the dark, and they were washed twice with PBS prior to analysis. Ten thousand events were accumulated for each analysis by the flow cytometer (Canto II, BD Biosciences, USA).

2.12. Estragen receptor (ER) binding assay

The binding of estrogen to ER in MCF-7 cells was assessed by using ER binding assay. Cells (1 × 10^6/mL) were seeded in a 100 mm culture dish overnight. They were then treated with RA-V (50 or 200 nM) or tamoxifen (1000 nM) for 6 and 24 h. The attached and floating cells were harvested and washed twice with PBS. To obtain cytoplasmic cell lysate, cell pellets were lysed with a cytoplasmic cell extraction buffer (Thermo Scientific, USA). The samples were then centrifuged at 14,000 × g for 15 min at 4 °C. Each sample (300 μg/mL) was mixed with 17β-estradiol (2 μg/mL) (PerkinElmer, USA) for 16 h at 4 °C. Charcoal solution (Sigma, USA) was added to the mixture with gentle shaking for 2 h at 4 °C. They were centrifuged at 10,000 × g for 10 min and supernatants were subjected to ER binding assay by a scintillation counter (Packard, USA).

2.13. Fibronectin adhesion assay

To determine whether the effect of RA-V on cell-ECM adhesion is via PI3K signaling or integrin signaling, fibronectin (Sigma, USA) at 20 μg/mL was coated on the wells of the 96-well plates (Corning, USA), followed by a 2-hour incubation at 37 °C. MCF-7 cells (1.5 × 10^5/mL) alone or in combination with LY294002 (20 μM) or integrin antagonist (Gly-Arg-Gly-Asp-Ser) (20 μg/mL) (Abcam, USA) for 2 h. After washing, bound cells were stained and dissolved in an extraction buffer (Millipore, USA). The change in optical density was represented as a fold of untreated control.

2.14. Statistical analysis

Data were expressed as mean ± SD. Statistical analyses and significance were calculated by one-way ANOVA, Dunnett test and analyzed using GraphPad PRISM software version 5.0 (GraphPad Software, USA). In all comparisons, p < 0.05 was considered as statistically significant.

3. Results

3.1. RA-V altered gene expressions involved in cancer cell adhesion, migration and invasion

To study the biological activities of RA-V in cancer, microarray analysis was carried out to investigate the change in gene expressions in Hela cells treated with RA-V. Using the Agilent Whole Human Genome Oligo Microarray, the gene expression profiling assay showed that 3449 genes were found to be differentially expressed when treated with RA-V at 100 nM (fold change ≥ 2 or ≤ 0.5; p < 0.05), compared with the untreated control. The heatmap of these genes was shown in Fig. 1B. Of them, 1639 genes were up-regulated and 1810 genes were down-regulated in RA-V-treated cells, compared with the untreated control. Some genes involved in cell adhesion and cancer invasion were affected significantly such as integrins, ECM-associated proteinases including MMPs, uPA and TIMPs, ALDHs and ID-1 (Fig. 1C). GO analysis was performed to understand the effects of RA-V in cancer cells in terms of cellular component, molecular function and biological process and revealed that RA-V strongly affected cell adhesion, cell motility, cell motion, ECM component binding and angiogenesis which were strongly associated with cancer invasion and metastasis (Fig. 1D). Pathway enrichment analysis was also carried out to further understand the effects of RA-V on gene expressions in signaling pathways according to KEGG and BioCarta databases. RA-V altered MAPK, p53, Wnt, MAPKK and EGF signaling pathways (Fig. 1E) which are involved in cancer progression and metastasis, according to KEGG and BioCarta databases. These microarray data strongly suggested that RA-V was involved in suppressing cancer invasion and metastasis.

3.2. RA-V induced cytotoxicity and inhibited cell proliferation in breast cancer cells

The cytotoxicity of RA-V on MDA-MB-231 and MCF-7 cells was determined by MTT assay (Fig. 2A). IC_{50} values of MDA-MB-231 were 106.6 nM for 24 h and 39.2 nM for 48 h, and of MCF-7 cells were 114.9 nM for 24 h and 78.6 nM for 48 h. Hence, RA-V at up to 25 nM was applied in the following culture assays as less than 70% cell viability when treated with concentrations of RA-V higher than 25 nM for 24 h. RA-V at 6.25 to 200 nM could significantly inhibit cell proliferation in MCF-7 cells when treated for 48 h (p < 0.001) (Fig. 2B). However, such anti-proliferative effect of RA-V was not found when treated for 24 h only (data not shown).

3.3. RA-V reduced cell motility and migration in breast cancer cells

In the scratch wound assay, the open wound area (%) of the cells treated with RA-V at 6.25 to 25 nM was significantly greater than that of the untreated (DMSO-treated) controls (Fig. 2C), indicating that RA-V significantly inhibited MDA-MB-231 and MCF-7 cell motility in a dose-dependent manner. Untreated controls have clearly migrated towards each other while the motility of both RA-V-treated breast cancer cell lines was inhibited. The migration of MDA-MB-231 and MCF-7 cells was also significantly inhibited in a dose-dependent manner when treated with RA-V at 12.5 and 25 nM using transwell migration assay (Fig. 2D). Cells have been migrated across the pore of the membrane from the upper chamber of the transwell to the lower chamber. The number of migrated cells was decreased when cells were treated with RA-V, compared to the untreated controls, suggesting that RA-V inhibited cancer cell migration.

3.4. RA-V reduced the expressions of chemokine receptors, CXCR4 and CCR7 in breast cancer cells

Chemokines can direct the migration and invasion of cancer cells, which express corresponding chemokine receptors, leading to metastasis to certain organs. In RA-V at 12.5 to 25 nM (Fig. 2E) they significantly and dose-dependently down-regulated the expressions of CCR7 and CXCR4 on MCF-7 cell surface and CXCR4 on MDA-MB-231 cell surface, determined by flow cytometry. RA-V at 25 nM significantly reduced CCR7 expression on MDA-MB-231 and MCF-7 cells.

3.5. RA-V inhibited the expressions of cofilin signaling molecules

Cofilin signaling is involved in actin polymerization and mediates cell migration. Results from Western blots revealed that RA-V at 6.25 to 25 nM significantly reduced cofilin expression in MDA-MB-231 and MCF-7 cells in a dose-dependent manner (Fig. 2F). The protein expressions of Rho family of GTPases (Cdc42 and RhoA) and cofilin-regulating kinase (ROCK1) which are involved in regulating cofilin activity were significantly inhibited by RA-V at 6.25 to 25 nM in a dose-dependent manner. RA-V at 12.5 to 25 nM also dose-dependently increased the expression of phosphorylated cofilin (inactive) in breast cancer cells.
3.6. RA-V reduced breast cancer cell adhesion to ECM

The ability of cancer cells to adhere to matrix proteins was assessed by ECM adhesion assay. RA-V inhibited breast cancer cell adhesion to extracellular matrix proteins (Fig. 3A). RA-V at 50 nM significantly decreased the adhesion of MDA-MB-231 cells to collagen I to 70% of control. RA-V at 50 nM significantly reduced MCF-7 cell adhesion to collagen I, collagen IV and fibronectin to 82%, 73%
and 81%, respectively, and could also slightly reduce its adhesion to laminin.

3.7. RA-V inhibited integrins, adhesion molecules, VCAM and ICAMs, and focal adhesion kinase (FAK) in breast cancer cells

Integrin and adhesion molecules, VCAM and ICAMs are involved in cell–cell or cell–matrix adhesion. Results from flow cytometry revealed that RA-V at 25 nM significantly reduced all integrins α5, α6, αβ3, and β1, and expression of MDA-MB-231 cells, while at 12.5 to 25 nM, RA-V significantly inhibited all these integrin expressions on MCF-7 cells in a dose-dependent manner (Fig. 3B and C). RA-V at 6.25 to 25 nM significantly inhibited VCAM-1 and ICAM-1 expressions on MDA-MB-231 and MCF-7 cells in a dose-dependent manner (Fig. 3D and E). This was consistent with the results from Western blotting (Fig. S2) which showed that RA-V could reduce VCAM-1 and ICAM-1 expressions in MDA-MB-231 and MCF-7 cells in a dose-dependent manner. FAK is a key protein tyrosine kinase involved in various signaling pathways triggered by integrin. FAK is responsible for cancer cell adhesion, migration and invasion [30]. Results from Western blot (Fig. 3F) showed that RA-V at 12.5 to 25 nM significantly reduced FAK expression in both MDA-MB-231 and MCF-7 cells treated for 24 h (Fig. 3G).

3.8. RA-V attenuated MMP-9 and uPA activities in breast cancer cells

MMPs and uPA (plasminogen activator) are ECM-associated proteases involved in matrix degradation. Gelatin zymography revealed the activities of MMP-9 and MMP-2 (gelatinases) secreted by MDA-MB-231 cells which were seeded on a collagen I-pre-coated well plate and treated with RA-V at 6.25 to 12.5 nM for 72 h (Fig. 4A). Collagen I was used as a substrate to stimulate the secretion of MMP-9 by breast cancer cells. The activity of MMP-9, but not MMP-2, secreted by MDA-MB-231 cells was significantly inhibited by RA-V at 6.25 to 12.5 nM in a dose-dependent manner (Fig. 4B). However, the activities of both MMP-2 and MMP-9 secreted by MCF-7 cells were negligible as no clear zones were observed in the stained gel (data not shown).

RA-V at 6.25 to 25 nM significantly inhibited the activity of uPA secreted by MDA-MB-231 cells incubated with RA-V for 24 h (Fig. 4C). The activity of uPA secreted by MCF-7 cells was also significantly inhibited when cancer cells treated with RA-V at 6.25 to 25 nM for 48 h (Fig. 4C), but not 24 h (data not shown).

3.9. RA-V down-regulated uPA, TIMP and MMP protein expressions

To determine the effects of RA-V on the expressions of the ECM-associated proteinases, MMPs, MMP-1, MMP-2, MMP-7 and MMP-9 protein expressions were studied in breast cancer cells using Western blot (Fig. 4D). RA-V at 25 nM significantly inhibited MMP-1, MMP-7 and MMP-9 expressions in MDA-MB-231 cells at 24 h (Fig. 4E). RA-V could reduce MMP-2 expressions in MDA-MB-231 although there was no significant difference. RA-V at 25 nM also significantly inhibited MMP-1, MMP-2, MMP-7 and MMP-9 expressions in MCF-7 cells at 48 h, but not at 24 h (data not shown). RA-V at 12–25 nM significantly inhibited MMP-1 expression in MCF-7 cells and inhibited MMP-9 and MMP-7 expressions in MDA-MB-231 cells in a dose-dependent manner.

Being a plasminogen activator and a MMP inhibitor respectively, uPA, TIMP-1 and TIMP-2 play a role in matrix degradation. Therefore, the inhibitory effects of RA-V on their expressions have been studied. RA-V at 6.25–25 nM inhibited TIMP-1 and TIMP-2 protein expressions in MDA-MB-231 for 24 h and MCF-7 cells for 48 h (Fig. 4E) in a dose-dependent manner. RA-V at 12.5–25 nM also significantly and dose-dependently inhibited uPA expressions in both breast cancer cell lines. Taken together with the results of the uPA activity assay, RA-V inhibited both the expressions as well as the activities of uPA proteins in MCF-7 and MDA-MB-231 cells.

3.10. RA-V inhibited the phosphorylation of EGFR in response to EGF in breast cancer cells

The results from pathway enrichment analysis (Fig. 1E) demonstrated that RA-V alters gene expressions in EGF signaling pathway in cancer cells. In addition, RA-V at 25 nM significantly inhibits the phosphorylation of cytoplasmic EGFR as well as reduces the expression of cytoplasmic EGFR at protein level in MDA-MB-231 and MCF-7 cells when treated for 8 h in the presence of EGF (Fig. 5A). The expression of cytoplasmic pEGFR is lower than that of cytoplasmic EGFR in RA-V-treated MDA-MB-231.

3.11. RA-V inhibited the protein expressions of AKT, PI3K and pPI3K in breast cancer cells

PI3K/AKT signaling is involved in cell survival and cell proliferation. PI3K is shown to associate with breast cancer cell migration, RA-V at 12.5 to 25 nM significantly reduced the pPI3K, PI3K and AKT levels in MDA-MB-231 cells at 8 h and in MCF-7 cells at 16 h in a dose-dependent manner (Fig. 5C and D). However, RA-V did not reduce the phosphorylation of AKT in both cancer cell lines.

3.12. RA-V reduced the phosphorylation of IκBα and NF-κB in breast cancer cells

To determine the effects of RA-V on NF-κB signaling transduction, the phosphorylation of both IκBα and NF-κB in the whole cell extracts of breast cancer cells were studied. RA-V at 25 nM significantly reduced the phosphorylation of NF-κB while the total levels of NF-κB were unchanged in both MDA-MB-231 and MCF-7 cells (Fig. 5E and F). RA-V at 25 nM also significantly inhibited the phosphorylation of IκBα in both cell lines.

3.13. RA-V exhibited its anti-invasive properties via PI3K and NF-κB signaling pathways in breast cancer cells

LY294002 (PI3K inhibitor) at 20 μM significantly reduced most of the expressions of integrins (Fig. 6A), specifically integrin α5 (p < 0.01) and

Fig. 2. Inhibitory effects of RA-V on breast cancer cell motility and migration. (A) Cytotoxic effects of RA-V on MDA-MB-231 and MCF-7 cells treated with RA-V at 3.125 to 200 nM for 24 or 48 h by MTT assay. Data were expressed as the mean fold of untreated control (mean ± SD of 3 independent experiments with 5 replicates each). (B) Anti-proliferative effects of RA-V on MDA-MB-231 and MCF-7 cells in a dose-dependent manner. Anti-proliferative assay was treated with or without RA-V at 6.25 to 200 nM for 48 h by MTT assay. Data were expressed as the mean fold of untreated control (mean ± SD of 3 independent experiments with 5 replicates each). (C) Anti-proliferative effects of RA-V on MDA-MB-231 and MCF-7 cells treated with RA-V at 6.25 to 200 nM for 48 h by [methyl-3H]-thymidine incorporation assay. (D) Anti-proliferative effects of RA-V on MDA-MB-231 and MCF-7 cells treated with RA-V at 6.25 to 25 nM for 24 h in the presence of EGF (Fig. 5A). The expression of cytoplasmic pEGFR is lower than that of cytoplasmic EGFR in RA-V-treated MDA-MB-231.

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LY294002 (PI3K inhibitor) at 20 μM significantly reduced most of the expressions of integrins (Fig. 6A), specifically integrin α5 (p < 0.01) and
integrin α6 (p < 0.001) in MDA-MB-231 and MCF-7 cells. However, BAY11-7082 (NF-κB inhibitor) at 5 μM could only significantly inhibit integrins α2 and α5 in MDA-MB-231 cells, and integrin β1 in MCF-7 cells (p < 0.05), suggesting that the effects of RA-V on reduced integrin expressions in breast cancer cells were mostly via PI3K/AKT signaling rather than NF-κB signaling. LY294002 also significantly inhibited the expression of chemokine receptors, CXCR4 and CCR7 in MDA-MB-231 and CCR7 in MCF-7 cells (Fig. 6B). On the other hand, reduced expression of CXCR4 but not CCR7 was found in both cell lines in the presence of BAY11-7082 (p < 0.05). Furthermore, as shown in Fig. 6C, a significant reduction in expressions of VCAM-1 and ICAM-1 were found in MCF-7 cells treated with LY294002 or BAY11-7082 (p < 0.01). Besides,
LY294002 significantly inhibited VCAM-1 (p < 0.05), while BAY11-7082 could significantly inhibit ICAM-1 in MDA-MB-231 cells (p < 0.001). LY294002 reduced ICAM-1 expression to 75% in MDA-MB-231 cells, although no significant difference was found. These findings suggested that RA-V inhibited cancer invasion via PI3K and NF-κB signaling.

In order to further determine whether RA-V exerts its anti-invasive effect via PI3K signaling, the effect of single and combined use of RA-V (25 nM) and LY294002 (PI3K inhibitor) (20 μM) on cell migration, adhesion and proliferation were further investigated. Both RA-V and LY294002 significantly reduced MCF-7 cell migration to 46% and 45%, respectively (Fig. 6D), and reduced MCF-7 cell-fibronectin adhesion to 72% and 82%, respectively (Fig. 6F). In addition, both RA-V and LY294002 attenuated 50% of the proliferation of MCF-7 cells (Fig. 6F). There was no significant difference between the RA-V treated group and the combined group of RA-V and LY294002 in MCF-7 cells according to Bonferroni’s multiple comparison test. These results demonstrate that the inhibitory effects of RA-V on MCF-7 cell migration, adhesion and proliferation are competitive with LY294002, and RA-V exerts its anti-invasive activities via PI3K signaling in hormone-responsiveness MCF-7 cells. For MDA-MB-231 cells, both RA-V and LY294002 significantly inhibited cell migration to 28% and 44%, respectively (Fig. 6D). There was no significant difference between the RA-V treated group and the combined group of RA-V and LY294002 in MCF-7 cells according to Bonferroni’s multiple comparison test. As the anti-adhesive effect of RA-V on MCF-7 cells is more compromised than MDA-MB-231 cells (Fig. 3A), only the underlying mechanism of RA-V on MCF-7 cell-ECM adhesion to fibronectin was investigated. In addition, RA-V and LY294002 reduced cell proliferation to 75% and 64%, respectively (Fig. 6F). The combined group was significantly lowered as compared to RA-V-treated group, but not LY294002-treated group. Therefore, such a significant decrease might be due to the inhibitory effect of LY294002, instead of their additive/synergistic effect. These results also revealed that the inhibitory effects of RA-V on MDA-MB-231 cell migration and proliferation were competitive with LY294002 (Fig. 6D–E), and RA-V exerted its anti-invasive activities via PI3K signaling in hormone-irresponsive MDA-MB-231 cells.

3.14. RA-V inhibit MCF-7 cell-fibronectin adhesion via integrin binding

Integrin antagonist (Gly-Arg-Gly-Asp-Ser) consists of the Arg-Gly-Asp sequence that is the attachment site for a variety of known integrins. Integrin antagonist (Gly-Arg-Gly-Asp-Ser) reduced MCF-7 cell adhesion to fibronectin, as integrins on MCF-7 cells can adhere to the integrin antagonist, instead of its ligands such as fibronectin. The fibronectin adhesion assay showed that RA-V, integrin antagonist or RA-V plus integrin antagonist significantly reduced MCF-7 cell-fibronectin adhesion to the same extent, which were about 75% (p < 0.001) (Fig. 6F). These results

![Fig. 4.](image-url)
revealed that RA-V could reduce MCF-7 cell adhesion to fibronectin via attenuating its integrin binding.

3.15. RA-V increased the ER expression of ER-positive breast cancer cells, MCF-7

In order to determine whether RA-V exerted its anti-cancer effects via ER, the binding of estrogen to ER and the expression of ER in ER-positive MCF-7 cells were investigated. Tamoxifen is an antagonist of estrogen receptor, and can inhibit estrogen-induced cell growth in an ER-dependent manner. Tamoxifen could also significantly increase ER expression (data not shown). RA-V at 12.5 to 25 nM also increased ER expression of MCF-7 in a dose-dependent manner (Fig. 7A). In addition, β-estradiol can trigger estrogen receptor endocytosis in ER-positive cells. Reduced cytoplasmic ER expression was found in MCF-7 cells in response to β-estradiol at 10 nM, suggesting that β-estradiol could induce receptor endocytosis in MCF-7 cells (Fig. 7B). The effect on estrogen receptor endocytosis was reversed in the presence of RA-V (p < 0.05) or tamoxifen. Increased ER expression in cytoplasm revealed that RA-V might inhibit receptor endocytosis induced by β-estradiol.

To further investigate whether increased ER expression is associated with ER binding for β-estradiol, ER binding assay and Western blotting were carried out. Both reduced ER binding and increased cytoplasmic ER expression of MCF-7 cells pre-treated with RA-V (50 or 200 nM) or tamoxifen (1000 nM) for 6 h or 24 h were found (Fig. 7C). However, when non-pre-treated cytoplasmic ER proteins mixed with β-estradiol and RA-V, no reduced ER binding to estrogen was found (data not shown). On the other hand, the mixture of non-pre-treated cytoplasmic ER proteins, β-estradiol and tamoxifen showed reduced ER binding to estrogen. This strongly suggests that RA-V does not compete with estrogen for the ER in a manner similar to tamoxifen. Instead, RA-V reduces the binding of β-estradiol to ER via affecting binding ability of ER in MCF-7 cells. Our results demonstrated that RA-V reduces the binding of β-estradiol to ER even with increased cytoplasmic ER expression, suggesting that RA-V triggers receptor endocytosis and inhibits estrogen-induced biological activity (Fig. 7D).

4. Discussion

Cancer cells need to acquire invasive abilities so as to metastasize. These processes require cancer cell proliferation, adhesion, migration and ECM proteolysis, and thus suppressing these steps may result in inhibiting tumor invasion. The cyclopeptide RA-V, isolated from the roots of R. yunnanensis, has been previously demonstrated as an anti-tumor and anti-angiogenic agent [6,7,10–13]. However, few studies have extensively studied the underlying mechanisms of the anti-migratory, anti-adhesive, anti-invasive and anti-proliferative effects of RA-V. Our microarray data strongly suggested that RA-V is a potential anti-metastatic agent as it could alter cancer cell adhesion, cell motility, cell motion, ECM component binding and angiogenesis which are involved in metastatic processes. Furthermore, GO analysis showed that RA-V could down-regulate some of the metastasis-related genes such as ICAM, MMPs and uPA, as well as interfere MAPK, p53, Wnt, MAPKK and EGF signaling pathways (p < 0.05) which play a critical role in cancer metastasis. In the present study, we demonstrated for the first time that RA-V inhibited breast cancer cell invasion by suppressing cancer...
cell proliferation, adhesion, migration and motility, likely via PI3K/AKT and NF-κB signaling pathways. RA-V was also shown to inhibit the protein expressions and activities of ECM-associated proteinases involved in ECM degradation.

RA-V has been shown previously to induce significant cytotoxicity on a variety of human cancer cells and endothelial cells [10,12,13]. Our study also showed that RA-V exerted significant cytotoxicity on MDA-MB-231 and MCF-7 cells (Fig. 2A). RA-V was previously demonstrated to sensitize breast cancer cells to mitochondria-mediated apoptosis through inhibiting PDK1-AKT with high RA-V concentrations i.e. 100–1000 nM [13]. However, in this study, lower concentrations of RA-V were applied in different cell culture assays because our approach was to inhibit cancer cell invasive ability at a low concentration to avoid inducing high cytotoxicity.

The migratory ability of cancer cells allows them to migrate to adjacent tissues and ultimately metastasize. Results from the scratch wound assay and transwell migration assay showed that RA-V significantly increased the change in the open wound area, uncovered by migrating cells, and reduced the number of migrated cells through the transwell membrane. The single and combined use of RA-V and LY294002 were also shown to inhibit breast cancer cell migration to a similar extent in transwell migration assay, revealing that RA-V reduced breast cancer cell migration via PI3K signaling. In addition, RA-V reduced the expressions of CXCR4 and CCR7, which are expressed in high levels in MDA-MB-231 and MCF-7 cells. RA-V potentially affected via PI3K and NF-κB signaling pathways. RA-V inhibited the expressions of coflin signaling molecules which are involved in actin polymerization as well as cell migration. Activation of chemokine receptors such as CXCR4 can also induce actin polymerization and mediate migration, adhesion as well as integrin activation [31]. These may contribute to the inhibitory effects of RA-V on cell migration.

ECM not only acts as a barrier to invasion, but also is involved in cell-ECM interactions during invasion. Our ECM adhesion assay data revealed that RA-V significantly inhibited cell adhesion of MDA-MB-231 to collagen I, and of MCF-7 to collagen I, collagen IV and fibronectin which are the major components of the ECM. RA-V reduced adhesion to collagen II and laminin in MCF-7 cells. Integrins on MCF-7 cells can adhere to the integrin antagonist instead of its ligands. Our fibronectin adhesion assay demonstrated that RA-V and RA-V plus integrin antagonist reduced MCF-7 cell-fibronectin adhesion to the same extent, and RA-V and RA-V plus LY294002 also attenuated such adhesion to the same extent (Fig. 6F). These results demonstrated the anti-adhesive effect of RA-V on MCF-7 cells underlying integrin binding and PI3K signaling. Furthermore, RA-V was shown to reduce FAK, integrin subunits, VCAM and ICAM expressions in both cell lines, in turn it might also contribute to the attenuation of cell adhesion to ECM proteins, and hence inhibited invasion.

Differential responses of RA-V on the adhesion of MDA-MB-231 and MCF-7 cells to ECM proteins may be due to their integrin expressions. MCF-7 and MDA-MB-231 cells have different levels of αv and β integrin subunits. Our results showed that much higher levels of α5, αv and αv and a slightly higher level of β1 were found in MDA-MB-231 cells as compared with MCF-7. Consistent with Morini’s group, they reported that MDA-MB-231 cells have high levels of αv and αv integrin subunits while MCF-7 cells have a moderate level of αv and low levels of αv. MCF-7 cells also express reduced levels of αv subunits compared with...
Fig. 7. (A) The effect of RA-V on estrogen receptor (ER) protein expression in MCF-7 cells by Western blot analyses. MCF-7 cells were treated with RA-V at 6.25 to 25 nM for 4 h. Whole cell lysate was subjected to Western blotting, and representative blots of MCF-7 cells were shown. (B) The effect of RA-V on ER protein expression in MCF-7 cells by Western blot analyses in response to β-estradiol. MCF-7 cells were treated with RA-V (25 nM) or tamoxifen (10 nM or 1000 nM) for 4 h in the presence of β-estradiol at 10 nM. Cytoplasmic cell lysate was subjected to Western blotting, and representative blots of MCF-7 cells were shown. (C–D) MCF-7 cells treated with (50 or 200 nM) or tamoxifen (1000 nM) for 6 or 24 h. Cytoplasmic cell lysates were subjected to (C) ER binding assay and (D) Western blotting. For ER binding assay, cytoplasmic cell lysates at 300 μg/mL were mixed with radioactive β-estradiol (2 μCi) for 16 h. For all Western blotting, the histogram showed the quantified results of the expression of proteins, which were normalized with corresponding β-actin protein expression and expressed as fold of control treated with estradiol only (mean ± SD of 3–4 independent experiments). Statistical differences were determined by one-way ANOVA, followed by Dunnett test, *p < 0.05, **p < 0.01, ***p < 0.001 against control treated with estradiol only. Statistical differences between untreated control and control treated with estradiol only by unpaired t-test, with ##p < 0.01. (E) Schematic diagram of the proposed underlying mechanisms of the anti-metastatic effect of RA-V.
MDA-MB-231 [32]. Different integrin subunits are responsible for cellu-
lar adhesion to different ECM proteins. For example, α5β1 and α6β1 are re-
sponsible for cellular adhesion to collagen and fibronectin, respective-
ly [28]. Therefore, the differential inhibitory effects of RA-V on cellular
adhesion to various ECM components may be due to the differential ex-
pression levels of α and β heterodimers between MDA-MB-231 and
MCF-7 cells. In addition, up-regulating β1 integrins of cancer cells
could lead to increased cell adhesion and invasion through collagen IV
[33] and fibronectin [34]. RA-V at 25 nM significantly reduced β1 inte-
рин expression on MCF-7 cells to a higher extent than that on
MDA-MB-231 cells (decreased to 46% in MCF-7; decreased to 78% in
MDA-MB-231) (Fig. 3C). This may explain why RA-V is slightly less ef-
fective to inhibit MDA-MB-231 cells to collagen IV and fibronectin.

Apart from inhibiting cancer cell adhesion and migration through the
ECM, attenuating the expression and activity of ECM-associated pro-
teinases including MMPs, uPA and TIMPs is also responsible for
inhibiting ECM degradation and cancer cell invasion. Results from
Western blot and gelatin zymography revealed that RA-V could reduce
MMP-1, MMP-2, MMP-7 and MMP-9 protein expressions as well as
MMP-9 activity in breast cancer cells. The activity of MMPs is tightly
controlled by a plasminogen activator, uPA and MMP inhibitors,
TIMPs. High level of uPA is associated with reduced relapse-free survival
and overall survival, breast cancer metastasis and a poor prognosis of
breast cancer [35,36]. RA-V reduced the protein expression and activity
of uPA involved in proteolytic enzyme degradation in breast cancer
cells. As TIMPs play a role in inhibiting MMP activity, it is expected
that low levels of TIMPs in cancer cells are in favor of cancer progression,
invasion and metastasis [37]. On the contrary, this potential anti-
metastatic agent, RA-V was shown to reduce the expressions of TIMP-
1 and TIMP-2 in breast cancer cells (Fig. 4D–E). Such controversy may
be due to the multifunction of TIMP-1 and TIMP-2. TIMPs can also stim-
ulate cell proliferation, inhibit apoptosis, and modulate angiogenesis
[38]. A preponderance of research has shown that high levels of TIMP-
1 [39] and TIMP-2 [40] proteins are significantly correlated and associ-
ated with poor outcome such as higher tumor stage and reduced overall
survival rate of patients suffering from breast cancer and breast cancer
metastasis. In short, breast metastasis can be facilitated by the imbalance
between MMPs and TIMPs rather than reduction of TIMP level
alone. Reduced activity and protein expressions of ECM-associated pro-
teinases, uPA and TIMPs of breast cancer cells by RA-V may therefore re-
sult in reduced cancer cell invasion and metastasis.

Our results from flow cytometry revealed that the expressions of
migration-related and adhesion-related molecules including chemo-
kine receptors, integrins and adhesion molecules (VCAM-1 and ICAM-
1) are the downstream molecules of PI3K and NF-κB signaling path-
ways. Constitutively active PI3K and NF-κB signaling pathways are usu-
ally found in highly invasive cancer cells such as MDA-MB-231 [41].
Blocking PI3K/AKT and NF-κB signaling pathways can result in de-
creased breast cancer cell migration [42] and the activity of ECM-
associated proteases including uPA and MMP-9 [21], RA-V was shown
to inhibit PI3K/AKT and NF-κB activations, so it might exert its anti-
metastatic effects through these signaling pathways. The RA-V treated
group and the combined group of RA-V and LY294002 exerted similar
anti-migratory, anti-adhesive and anti-proliferative effects on MDA-
MB-231 and MCF-7 cells, further confirming that RA-V exerts its anti-
migration, anti-adhesion and anti-proliferation via PI3K signaling
(Fig. 6D–F).

ER-positive breast cancer is responsive to anti-estrogen therapy and
usually has a better clinical outcome than an ER-negative one. However,
hormonal therapy is not applicable to ER-negative breast cancer, so it is
important to identify an anti-metastatic agent that is capable of inhibiting
ER-negative tumor growth in ER-independent manner. As RA-V can in-
duce anti-invasive effect on two breast cancer cell lines, ER-positive poor-
ly invasive MCF-7 and ER-negative highly invasive MDA-MB-231 cells, it
gave rise to a question that whether RA-V exerted its inhibitory effects
via ER-dependent and/or ER-independent pathways. RA-V could inhibit
cell migration, adhesion and invasion of ER-negative MDA-MB-231 cells,
suggesting that RA-V could exert its inhibitory effects via ER-

independent pathway. Beta-estradiol can trigger ER endocytosis in
MCF-7 cells, resulting in decreased cytoplasmic ER expressions. RA-V
could increase cytoplasmic ER expression in MCF-7 cells after a short
period of time following RA-V treatment, implying that it was able to
inhibit receptor endocytosis. RA-V reduced the binding of β-estradiol
to ER via affecting binding ability of ER instead of competing with
β-estradiol to ER. Our results strongly suggest that the inhibitory effect
of RA-V on cell migration, adhesion and matrix degradation is ER-

independent. The inhibitory effects of RA-V on cell migration, adhesion
and matrix degradation in ER-positive MCF-7 and ER-negative MDA-
MB-231 cells are in similar extent, implying that the inhibitory effect
of RA-V in MCF-7 cells on cell migration, adhesion and matrix degrada-
tion is ER-independent. A lower dose is required to induce ER-

dependent anti-cancer effects as compared with ER-independent one.
For example, tamoxifen, being an ER antagonist, can inhibit cancer cell
growth in ER-dependent manner at 10 nM in ER-sensitive cancer
cells, but at 10 μM in ER-independent manner in ER-negative MDA-
MB-231 cells [43]. However, RA-V inhibited both ER-positive and ER-
negative breast cancer cell migration and invasion at similar doses,
6.25–25 nM, suggesting that RA-V-induced ER-independent pathway
is more important in inhibiting breast cancer cell invasion.

In summary, RA-V can inhibit cell proliferation, cell adhesion, cell
migration and the activity and expression of ECM-associated proteo-
ases in breast cancer cells. RA-V inhibits the expressions of signaling mole-
cules involved in EGFR, PI3K/AKT and NF-κB signaling pathways in
both ER-negative and ER-positive breast cancer cells (Fig. 7E). RA-V is
also shown to induce anti-proliferative, anti-adhesive and anti-
migratory activities in both hormone-positive and hormone-negative
breast cancer cells via PI3K signaling. Although RA-V could reduce ER
binding, its inhibitory effect on cell migration, adhesion and matrix
degradation is ER-independent. These findings strongly support the
development of RA-V as an anti-metastatic agent and an alternative
therapeutic approach to combat hormone-independent breast cancers.

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Transparency document

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