Plant cyclopeptide RA-V kills human breast cancer cells by inducing mitochondria-mediated apoptosis through blocking PDK1–AKT interaction

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In the present paper, we examined the effects of a natural cyclopeptide RA-V on human breast cancer cells and the underlying mechanisms. RA-V significantly inhibited the growth of human breast cancer MCF-7, MDA-MB-231 cells and murine breast cancer 4T1 cells. In addition, RA-V triggered mitochondrial apoptotic pathway which was indicated by the loss of mitochondrial membrane potential, the release of cytochrome c, and the activation of caspase cascade. Further study showed that RA-V dramatically inhibited phosphorylation of AKT and 3-phosphoinositide dependent protein kinase 1 (PDK1) in MCF-7 cells. Moreover, RA-V disrupted the interaction between PDK1 and AKT in MCF-7 cells. Furthermore, RA-V-induced apoptosis could be enhanced by phosphatidylinositol 3-kinase inhibitor or attenuated by over-expression of AKT in all the three kinds of breast cancer cells. Taken together, this study shows that RA-V, which can induce mitochondria-mediated apoptosis, exerts strong anti-tumor activity against human breast cancer. The underlying anti-cancer mechanism of RA-V is related to the blockage of the interaction between PDK1 and AKT.

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Introduction

As the second most common cancer-related cause of death among females, breast cancer is considered as a major public health concern, accounting for nearly 1 in 3 cancers diagnosed among women in the United States (DeSantis et al., 2011). Therapeutic strategies employed to date have significantly improved the prognosis for patients with breast cancer (Findlay et al., 2008). Despite an improvement in overall survival, with the use of anthracyclines and taxanes, resistance to therapy and subsequent progression of disease are still observed in metastatic patients (Ribeiro et al., 2012). This emphasizes the need for investigating the molecular mechanisms responsible for breast cancer development and seeking effective and non-cytotoxic chemical agents for chemoprevention and treatment. In this respect, more and more researchers paid much attention to natural active compounds for cancer chemoprevention and treatment.

Plant-derived natural products occupy a very important position in the area of cancer chemotherapy (Gao et al., 2011; Li and Vederas, 2009). Molecules such as taxol, vincristine, vinblastine, camptothecin derivatives, and epipodophyllotoxin are invaluable contributions of nature to modern medicine. However, the quest to find out novel therapeutic compounds for cancer treatment and management is a never-ending venture. Recently, the anti-tumor activity of cyclic peptides has attracted much attention (Colombo et al., 2002; Tan and Zhou, 2006). RA-V (deoxybouvardin), an unique natural cyclopeptide which was derived from the medical plant Rubia yunnanensis, exhibits a variety of pharmacological activities including anti-tumor (Itokawa et al., 1984), anti-inflammatory (Fan et al., 2010), and anti-angiogenesis (Yue et al., 2011) activities. However, the exact anti-tumor mechanism of RA-V against human cancer remains vague.

Apoptosis is a major mechanism to eliminate cancer cells without elicitting damage to normal cells or surrounding tissues. The understanding of apoptosis has provided the basis for novel targeted therapies that induce cell death or sensitize cancer cells to established cytotoxic agents (Ghbrial et al., 2005). Two major pathways are involved in mammalian cells apoptosis — mitochondria-mediated intrinsic pathway and death receptor-mediated extrinsic pathway (Hengartner, 2000). Stress in the endoplasmic reticulum (ER) can also result in apoptosis (Hsin et al., 2012; Rasheva and Domingos, 2009). Mitochondria is involved in the so-called intrinsic pathway of apoptosis. Soluble proteins, including cytochrome c, were released from the intermembrane space of mitochondrial to the cytosol to initiate caspase activation (Martinou and Youle, 2011). The activation of caspase family proteases is the key step in cell apoptosis (Lavrik

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et al., 2005). Cytochrome c together with Apaf-1 is required for the activation of caspase-9 in the cytosol. Caspase-8 is the key initiative caspase in the death-receptor pathway. Upon ligand binding, caspase-8 is cleaved and activated (Wang et al., 2011). Caspase-4 appears to be cleaved and activated only by stimuli that activate ER stress (Hitomi et al., 2004). Thus, targeting apoptosis pathways in premalignant and malignant cells is an effective strategy for cancer prevention and treatment (Jo et al., 2012; Liu et al., 2011).

The phosphatidylinositol 3-kinase (PI3K)/AKT pathway is one of the most commonly activated signaling pathways in human cancer, which results in apoptosis resistance. PI3K catalyzes the phosphorylation of the 3-hydroxyl position of PIP2 (phosphatidylinositol 4,5-diphosphate) to PIP3 (phosphatidylinositol 3,4,5-triphosphate). PIP3 is responsible for facilitating the phosphorylation of AKT at Thr308 by 3-phosphoinositide dependent protein kinase 1 (PDK1) (Chakraborty et al., 2010). Physical association of PDK1 with AKT is sufficient for activation of this pathway (Calleja et al., 2009). Another phosphorylation event at Ser473 by the mammalian target of rapamycin (mTOR)-rictor complex is required for maximal AKT activity (Jacinto et al., 2006). Akt, which inhibits apoptosis, is the central effector of proliferation signaling pathways. Down-regulation of PI3K leads to downstream inactivation of AKT. In fact, AKT has been reported to be over-expressed in many kinds of human cancers including breast cancer (Di Cosimo and Baselga, 2008). Therefore, PI3K/AKT pathway is an attractive target for anticancer agents.

In this paper, we demonstrate that RA-V has a potent anti-cancer activity against human breast cancer. PI3K/AKT-dependent mitochondrial pathway is involved in the signaling of RA-V-induced apoptosis.

Materials and methods

Reagents. RA-V (Fig. 1A, purity > 99%) was isolated from R. yunnanensis as described before (Yue et al., 2011). RA-V was dissolved at the concentration of 20 mM in DMSO as a stock solution, stored at -20 °C, and diluted with medium for each experiment. DMEM, L-15, RPMI-1640, MEM, fetal bovine serum, JC-1 and lipofectamine 2000 were purchased from Life Technologies (Carlsbad, CA). Annexin V-FITC (fluorescein isothiocyanate)/PI kit and antibody to cytochrome c were purchased from BD Biosciences (San Jose, CA). MTT and PI3K inhibitor wortmannin were purchased from Sigma-Aldrich (St. Louis, MO). LDH assay kit was purchased from Promega (Madison, WI). Caspase-3, -8, -9 activity assay kits were purchased from Beyotime (Nantong, China). ProteoExtract Cytosol/Mitochondria Fractionation Kit was purchased from Merck Bioscience (Bad Soden, Germany). Plasmids pcDNA3-Myr-HA-AKT1, 2, and 3 were purchased from Addgene (Cambridge, MA). Antibodies to cleaved PARP, p-AKT (T308), p-AKT (S473), p-PDK1 (S241), COX IV and Caspase family were purchased from Cell Signaling Technology (Beverly, MA). Antibodies to PDK1, AKT, GAPDH, and α-Tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture. Human breast cancer MCF-7 cells were maintained in HG-Dulbecco’s modified Eagle’s medium; human breast cancer MDA-MB-231 cells were maintained in Leibovitz’s L-15 Medium; mouse breast cancer 4T1 cells were maintained in RPMI 1640 medium. All media were supplemented with 10% fetal bovine serum plus 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. All cells

![Fig. 1. RA-V killed various breast cancer cells in vitro. (A) Chemical structure of cyclopeptide RA-V. (B) MCF-7, MDA-MB-231 and 4T1 cells were seeded in 96-well plates and incubated with various concentrations of RA-V for 24 h, 48 h and 72 h respectively. The inhibitory rate of proliferation was determined by MTT assay. (C) MCF-7 and MDA-MB-231 cells were incubated with various concentrations of RA-V for 72 h, LDH released to the medium was determined by LDH assay. Data are mean±SEM of three independent experiments. *P<0.05, **P<0.01, ***P<0.001 vs. drug-untreated group.](image-url)
were purchased from Shanghai Institute of Cell Biology (Shanghai, China), and were cultured at 37 °C in a 5% (v/v) CO₂ atmosphere.

**Cytotoxicity assay.** The cytotoxic activity of RA-V was examined by MTT assay and LDH assay. Cells were treated with RA-V for indicated time in 96-well plates. For MTT assay, MTT solution (5 mg/ml in PBS) was added (10 μl/well) into each well and incubated with cells for 4 h at 37 °C. Then, the supernatant was discarded and the purple formazan crystals were dissolved in 100 μl of DMSO for 5 min. In the end, the plates were read on an automated microplate spectrophotometer (Sunrise, Tecan, Austria) at 570 nm. For LDH assay, the supernatant of one plate was harvested and the LDH released out of cells was detected. For another plate, cells were lysed in the well and the total cell LDH was detected. Cytotoxicity was determined by LDH released into the supernatant/total cell LDH. Assays were performed in triplicate on three independent experiments.

**Cell apoptosis assay.** Cells were measured by flow cytometry after addition of FITC-conjugated Annexin V and PI as previously described (Luo et al., 2011). Annexin V+ cells were considered as apoptotic cells, while Annexin V+/PI− cells were considered as apoptotic cells in the early phase.

**Western blot.** Western blot was done as described before (Sun et al., 2010). Briefly, cells were collected and lysed in lysis buffer containing protease inhibitor cocktail (Pierce, IL). Proteins were fractionated by SDS-PAGE and electrotransferred to PVDF membranes. Various antibodies were used for blotting. Detection was done by LumiGLO chemiluminescent system (KPL, Guildford, UK).

**Caspase activity analysis.** Cells were seeded in 6-well plate and cultured for 12 h. Then the cells were incubated with various concentrations of RA-V. Twenty-four hours later, cells were harvested. Caspase activity was determined following the instruction of commercial kit.

**Mitochondrial transmembrane potential assay.** Mitochondrial transmembrane potential assay was done as described before (Sun et al., 2009). Briefly, cells were incubated with JC-1 (5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetrathylbenzimidazol-carbocyanine iodide, 10 μg/ml) for 20 min at 37 °C. The staining buffer was removed and cells were washed with ice-cold PBS twice. Two milliliter DMEM was added to each well. Individual JC-1 loaded cells were observed with a fluorescence microscope and fluorescent images were captured by a digital camera. For flow cytometry, cells were harvested and incubated with JC-1, following by PBS washing. Then the cells were resuspended in PBS and detected.

**Subcellular fractionation.** Proteins in MCF-7 cells were separated into cytosolic and mitochondrial fractions using the ProteoExtract Cytosol/Mitochondria Fractionation Kit (Merck Bioscience, Bad Soden, Germany) according to the procedures provided by the manufacturer.

**Immunofluorescence analysis.** Cells on coverslips were washed twice with PBS and fixed in Fixation buffer (BD Cytofix™) at 4 °C for 15 min. Permeabilization of the cells was performed by incubating the cells with 0.1% saponin and 1% fetal bovine serum in PBS at 4 °C for 10 min. The cells were blocking with 3% BSA at room temperature for 1 h, and rinsed three times with PBS and then incubated with primary antibody against PDK1 (Santa Cruz) and AKT (Santa Cruz) at 4 °C overnight (0.1% saponin and 1% fetal bovine serum in PBS). For immunofluorescence detection of PDK1, cells were incubated with a secondary anti-mouse antibody (Alexa Fluor-594, Invitrogen) for 2 h. For AKT, cells were incubated with a secondary anti-rabbit antibody (Alexa Fluor-488, Invitrogen) for 2 h. Nucleus was stained with DAPI for 2 min. After staining, cells were rinsed four times with PBS and prepared for microscopic analysis. Images were acquired using a confocal immunofluorescence microscopy (Olympus) equipped with 60× oil-immersion objective.

**Transient transfection.** As mentioned before (Song et al., 2009), 2 μl lipofectamine 2000 and 2 μg plasmids were diluted in 50 μl MEM respectively, then mixed and incubated at room temperature for 20 min before addition of 800 μl MEM. The mixture was spread onto cells. Cells were switched to complete medium 6 h later. After 24 h of transfection, cells were incubated with various concentrations of RA-V for another 24 h.

**Statistical analysis.** Data are expressed as mean ± SEM. Student’s t test and one-way ANOVA test were used for statistical analyses of the data. All statistical analyses were conducted using SPSS 10.0 statistical software (SPSS, Chicago, IL). Cases in which P values of <0.05 were considered statistically significant.

**Results**

**RA-V inhibited the growth, adhesion and migration of human breast cancer cells**

The structure of cyclohexapeptide RA-V is presented in Fig. 1A. MCF-7 and MDA-MB-231 cells were widely used in studies on human breast cancer (Lacroix and Leclercq, 2004). In this study, these two cell lines and one mouse breast cancer 4T1 cells were used. RA-V suppressed the growth of all the three kinds of breast cancer cells in a dose- and time-dependent manner (Fig. 1B). After incubated with 1000 nM RA-V for 72 h, LDH released from MCF-7 and MDA-MB-231 cells were significantly increased up to 87.9% and 85.2%, respectively (Fig. 1C). In addition to breast cancer cells, the growths of several other human cancer cells were also inhibited by RA-V treatment in a dose-dependent manner. However, RA-V did not show obvious toxicity on normal cells including human stellate cells LX-2 and primary mouse lymphocytes (Supplementary Fig. S1).

MCF-7 cells were treated by various concentrations of RA-V for 24 h and then harvested for adhesion and migration assay. Results in Supplementary Fig. S2A showed that the adhesive abilities of MCF-7 to three kinds of extracellular matrixes (fibronectin, laminin and collagen) were significantly reduced by RA-V. The protein level of p-FAK (Y397) and p-Src (Y416) were also down-regulated by the treatment of RA-V (Supplementary Fig. S2B). The migratory ability of MCF-7 cells was also inhibited by RA-V in a dose-dependent manner (Supplementary Fig. S2C).

**RA-V induced mitochondria-mediated apoptosis of human breast cancer cells**

RA-V significantly induced the apoptosis of human breast cancer MCF-7 cells in a dose- and time-dependent manner (Fig. 2A). The similar results were observed in breast cancer MDA-MB-231 cells and 4T1 cells (Fig. 2B). To determine the potential mechanism of RA-V-induced apoptosis, the expressions of several apoptosis-related proteins were determined by Western blotting. After incubating with 100 nM RA-V for 0, 1.5, 3, 6, 12, 24 and 48 h, or with various concentrations of RA-V for 24 h, caspase-3, -9 and PARP in MCF-7 cells were cleaved in a time- and dose-dependent manner, while no obvious cleavages of caspase-4 and -8 were detectable (Supplementary Fig. S3). The similar results were seen in breast cancer MDA-MB-231 cells and 4T1 cells (Supplementary Fig. S4A). Consistently, the activities of caspase-3 and -9 of MCF-7 cells dose-dependently increased after RA-V treatment (Fig. 2C) while no change was found with caspase-4 and -8 activities (Figs. 2D, E). When MCF-7 cells were pretreated with the inhibitors of caspase-3 or caspase-9, the apoptosis induced by RA-V was remarkably inhibited (Fig. 2F). The similar results were also observed in MDA-MB-231 and 4T1 cells (data not shown).
The JC-1 assay showed that the fluorescence ratio (red+/red−) in MCF-7 cells was dose-dependently reduced by RA-V treatment (Figs. 3A, B), suggesting that RA-V disrupted the mitochondrial membrane potential of MCF-7 cells. To further examine the apoptosis pathway induced by RA-V, the mitochondrial protein and cytoplasmic protein were extracted from MCF-7 cells respectively. As shown in Fig. 3C, MCF-7 cells were incubated with RA-V for 24 h, cytochrome c was released from the mitochondria to the cytosol. COX IV was shown as a quality control for fractionations. These results indicate that RA-V triggers apoptosis through intrinsic mitochondria-mediated pathway, but not extrinsic death receptor pathway or endoplasmic reticulum stress-mediated pathway.

RA-V interrupted the interaction between PDK1 and AKT

PI3K/AKT pathway is one of the most important intracellular signaling which inhibits apoptosis (Franke et al., 2003). We further examined the PI3K/AKT signaling pathway to clarify the mechanism of RA-V-induced apoptosis. MCF-7 cells were incubated with RA-V for 0, 1.5, 3, 6, 12 and 24 h, the phosphorylation of AKT at Thr308 was measured by Western blotting. Image J analysis showed that p-AKT level decreased apparently at 24 h (Fig. 4A). The level of p-PDK1 and p-AKT were also examined at this time point. Under 24 hour treatment, RA-V inhibited the phosphorylations of PDK1 and AKT in a dose-dependent manner (Fig. 4B, Supplementary Fig. S4A). Moreover, the immunoprecipitation and immunofluorescence results showed that the interaction between PDK1 and AKT was remarkably blocked by RA-V (Figs. 4C, D).

PI3K inhibitor wortmannin synergistically enhanced RA-V-induced apoptosis through inhibition of AKT activation

Since the activation of AKT is regulated by PI3K, we examined the influence of wortmannin in AKT phosphorylation. The phosphorylation of AKT at Thr308 was inhibited by the treatment of PI3K inhibitor wortmannin (Fig. 5A). However, the instability of wortmannin led to a rebound in the phosphorylation level of AKT (Yuan et al., 2009). Base on this result, MCF-7 cells were pretreated by 1 μM wortmannin for 1.5 h before RA-V treatment. Not surprisingly, p-AKT (T308) in these cells was dramatically reduced under the pretreatment of wortmannin compared to treatment by RA-V alone (Fig. 5B). Such cells were also subjected to apoptosis assay. The flow cytometric results showed that wortmannin pretreatment further enhanced RA-V-induced apoptosis in MCF-7 cells (Fig. 5C). The similar results were seen in breast cancer MDA-MB-231 and 4T1 cells (Supplementary Fig. S4B-S4D).
Fig. 3. RA-V triggered mitochondria-mediated apoptosis in human breast cancer MCF-7 cells. MCF-7 cells were seeded in 6-well plate and incubated with 0, 10, 100 and 1000 nM RA-V for 24 h.  (A) Cells were stained with JC-1 and photographed by fluorescence microscope.  (B) Cells were harvested and stained with JC-1, the ratio of JC-1 red+/red− fluorescence was determined by flow cytometry. Data are mean ± SEM of three independent experiments. *P<0.05 vs. drug-untreated group. (C) The mitochondrial and cytoplasmic proteins were extracted from both vehicle- and RA-V-treated MCF-7 cells, respectively. Cytochrome c was determined by Western blotting. COX IV was shown as a quality control for fractionations. Data shown here is one of three different experiments.
AKT isoforms contributed to the resistance of MCF-7 cells to RA-V-induced apoptosis

To confirm the function of AKT in RA-V-induced apoptosis, AKT isoforms AKT1, AKT2 and AKT3 were over-expressed in MCF-7 cells respectively. After cells were transfection with 2 μg AKT1, AKT2 or AKT3 plasmids, the phosphorylation level of AKT at Ser473 remarkably increased (Fig. 6A). Over-expression of AKT in MCF-7 cells almost completely blocked the inhibitory effect of RA-V on p-AKT (S473) (Fig. 6B). In addition, RA-V-induced apoptosis significantly decreased after AKT over-expression (Figs. 6C, D). The similar results were observed in breast cancer MDA-MB-231 and 4T1 cells (Supplementary Fig. S4E, S4F). These results suggest that inhibition of PI3K/AKT pathway contributes to RA-V-induced apoptosis.

Discussion

In the present study, we reported that RA-V, a natural cyclopeptide, showed strong inhibitory activity on the growth, adhesion and migration of various breast cancer cells. This is the first
report on the chemotherapeutic effects of RA-V on human breast cancer cells, which may provide a potential option for the drug discovery and treatment of breast cancer. RA-V, which was previously found to exert antineoplastic effect, was clearly demonstrated to kill three kinds of breast cancer cells at very low concentrations (Fig. 1). It should be noted that RA-V, at the concentrations mentioned above, hardly affected the viability of the benign non-tumor human stellate LX-2 cells as well as primary murine lymphocytes, suggesting the selectivity of RA-V on tumor cells (Supplementary Fig. S1).

Cyclopeptide RA-V is isolated from *R. yunnanensis* (family Rubiaceae), which is widely distributed and used as anti-tumor herb in folk remedies in Yunnan province of China. In the examination for the mechanism of this cyclopeptide against breast cancer, RA-V was found to induce apoptosis in various breast cancer cells (Fig. 2). Apoptosis-resistant mechanism is known to play an important role in cancer pathogenesis, including tumor growth, metastasis and chemoresistance (Fuchs and Steller, 2011). Most cancer therapeutic approaches inhibit tumors by triggering cancer cell apoptosis (Liu et al., 2011). In the RA-V-induced apoptosis, caspase-3 and caspase-9 were cleaved, while no obvious cleavage of caspase-4 or caspase-8 was detected (Fig. 2), suggesting that extrinsic death receptor pathway and endoplasmic reticulum (ER)-stress-mediated apoptotic pathway were not involved. Enzyme activity assay of caspases also support this conclusion (Fig. 2D). For further confirmation, JC-1 staining was taken to detect the membrane potential of mitochondria. The membrane potential of mitochondria in MCF-7 cells was greatly reduced by RA-V, and cytochrome c was released from the mitochondria to cytoplasm (Fig. 3). All these results indicated that RA-V induced apoptosis in breast cancer cells through the mitochondrial pathway.

PI3K/AKT pathway is one of the most important intracellular signal transduction pathways. FAK is a critical mediator of the integrin signaling cascade, which modulates cell proliferation, apoptosis, adhesion, spreading and migration. Herein we showed that the phosphorylation of FAK (Y397) and Src (Y416) were remarkably reduced by RA-V treatment (Supplementary Fig. S2B). Previous studies have revealed that once activated by integrin and non-integrin stimuli, FAK
binds and activates several other molecules, such as Src (Sonoda et al.,
2000) and PI3K (Xie et al., 2011). Many critical cellular processes are
driven through PI3K/AKT pathway (Chang et al., 2003). There have been
several anti-tumor drugs targeting receptor tyrosine kinases (Ono et al.,
2004; Tuma, 2004). For instance, sunitinib (an inhibitor of vascular endo-
thelial growth factor receptor, platelet-derived growth factor receptor,
and other tyrosine kinase receptors) was reported to induce apoptosis
by inhibiting STAT3 and AKT signaling pathways (Yang et al., 2010).
RA-V has been reported to be able to inhibit the phosphorylation of
ERK1/2 in endothelial cells (Yue et al., 2011). In this study, we found
that RA-V treatment disrupted the interaction between PDK1 and AKT
and down-regulated p-PDK1 and p-AKT in breast cancer cells. AKT, an
important survival molecule in the signaling pathways involved in cell
growth, inhibits apoptosis by inactivating various apoptotic proteins
(Osaki et al., 2004). A recent study showed that the inhibition of PI3K/AKT
pathway is important for the apoptosis induced by HS-116 in HuH-7 cells
(Jung et al., 2012). Our investigation revealed that the PI3K inhib-
itor wortmannin showed a synergistic effect on the apoptosis induced
by RA-V via inhibiting the activation of AKT in breast cancer cells (Fig. 5).
Furthermore, our data also showed that over-expression of each of
the three AKT isoforms resulted in a blockade of apoptosis induced by RA-V
in breast cancer cells (Fig. 6). All these results indicated that the inhibition
on AKT activation contributed to RA-V-induced apoptosis.

Taken together, our results demonstrate that RA-V-induced apoptosis
in breast cancer cells is mediated by PI3K/AKT-dependent mitochondrial
apoptosis pathway. The relative low concentration of RA-V used in vitro
is attractive for its anti-cancer therapy in vivo. Our studies thus provide
a rational mechanism for the development of anti-cancer agents.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://

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Conflict of interest statement

The authors declare that there are no conflicts of interest.


