BCPT Basic & Clinical Pharmacology & Toxicology

Basic & Clinical Pharmacology & Toxicology, 2014, **115**, 507–511 Doi: 10.1111/bcpt.12270

Arctigenin Promotes Apoptosis in Ovarian Cancer Cells via the iNOS/NO/STAT3/Survivin Signalling

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(Received 26 March 2014; Accepted 6 May 2014)

Abstract: Arctigenin is a biologically active lignan extracted from the seeds of *Arctium lappa* and shows anticancer activity against a variety of human cancers. The aim of this study was to determine the effects of arctigenin on ovarian cancer cell proliferation and survival and associated molecular mechanisms. Human ovarian cancer OVCAR3 and SKOV3 cells were treated with arctigenin, and cell proliferation and apoptosis were assessed. Western blot analysis was used to examine signal transducer and activator of transcription-3 (STAT3) phosphorylation and survivin and inducible nitric oxide synthase (iNOS) expression. The involvement of STAT3/survivin/iNOS/NO signalling in arctigenin action was checked. Arctigenin treatment resulted in a significant and dose-dependent inhibition of cell proliferation. Arctigenin-treated cells showed a 4–6 times increase in the percentage of apoptosis, compared with control cells. Pre-treatment with Ac-DEVD-CHO, a specific inhibitor of caspase-3, counteracted the induction of apoptosis by arctigenin. Arctigenin treatment significantly inhibited STAT3 phosphorylation and survivin and iNOS expression. Arctigenin-induced apoptosis was impaired by pre-transfection with survivin-expressing plasmid or addition of chemical nitric oxide (NO) donors. Additionally, exogenous NO prevented the suppression of STAT3 phosphorylation and survivin expression by arctigenin. Arctigenin treatment inhibits the proliferation and induces caspase-3-dependent apoptosis of ovarian cancer cells. Suppression of iNOS/NO/STAT3/survivin signalling is causally linked to the anticancer activity of arctigenin. Therefore, arctigenin may be applicable to anticancer therapy for ovarian cancer.

Ovarian cancer is one of the most frequent gynaecological malignancies worldwide and the fifth leading cause of cancer mortality among women [1]. Due to the asymptomatic nature of the early disease, ovarian cancer is usually diagnosed at an advanced stage. The current standard therapy for ovarian cancer is surgical resection and adjuvant chemotherapy [2]. However, the therapeutic efficacy is hindered by tumour recurrence and chemoresistance [3]. Therefore, development of novel and effective therapeutic regimens is of importance for improving the prognosis of patients with ovarian cancer.

Constitutively activated signal transducer and activator of transcription-3 (STAT3) is causally linked to tumour development and progression in a variety of solid malignancies [4–6]. For instance, persistent activation of STAT3 signalling has been shown to promote breast cancer progression and resistance to chemotherapy by inducing the expression of the anti-apoptotic protein, survivin [6]. STAT3 activation also plays an important role in ovarian cancer growth and survival [7]. Han *et al.* [8] reported that silencing of STAT3 circumvents cisplatin resistance in ovarian cancer cells. Depletion of STAT3 causes efficient inhibition of intraperitoneal ovarian cancer growth in nude mice [9]. These findings suggest that STAT3 is a promising therapeutic target in ovarian cancer.

Arctigenin is a biologically active lignan abundantly found in the seeds of *Arctium lappa*. It has numerous pharmacological activities including antitumour, anti-inflammatory and anti-oxidant activities [10,11]. Zhao *et al.* [10] demonstrated that arctigenin suppresses lipopolysaccharide-stimulated nitric oxide (NO) production by reducing the expression of inducible nitric oxide synthase (iNOS). Constitutive intracellular production of iNOS and NO has been found to contribute to tumour growth and survival [12]. Activation of NO signalling confers resistance against carboplatin-/paclitaxel-induced apoptosis in ovarian cancer cells through up-regulation of survivin [13]. Most interestingly, it has been documented that arctigenin augments chemosensitivity of cancer cells to cisplatin through inhibition of STAT3 signalling [14]. These findings led us to suggest that arctigenin may exert anticancer effects against ovarian cancer via suppression of iNOS/NO and STAT3 signalling.

Therefore, in this study, we explored the cytotoxic effects of arctigenin against ovarian cancer cells and checked the involvement of iNOS/NO and STAT3 signalling.

Materials and Methods

Reagents and antibodies. Arctigenin with a purity of >97% was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Ac-DEVD-CHO and S-nitroso-N-acetylpenicillamine (SNAP) from Calbiochem (San Diego, CA, USA), and phenylmethylsulphonyl fluoride (PMSF), leupeptin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution from Sigma (St Louis, MO, USA). Lipofectamine 2000 reagents, foetal bovine serum (FBS) and RPMI 1640 medium were purchased from Invitrogen (Carlsbad, CA, USA). Annexin-V/propidium iodide (PI) apoptosis assay kit was purchased from Shenzhen Genmed Biological Company (Shenzhen, China) and enhanced chemiluminescence (ECL) system from Amersham (Piscataway, NJ, USA). Rabbit anticleaved caspase-3,

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anti-phospho-STAT3 (tyr705) and anti-STAT3 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Rabbit antibodies against iNOS, survivin and β-catenin were purchased from Santa Cruz Biotechnology. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was obtained from Beijing Zhong Shan Golden Bridge Biological Company (Beijing, China). Human survivin-expressing plasmid (pcDNA3.1-survivin) was kindly provided by Dr. Altieri (University of Massachusetts, Worcester, MA, USA).

Cell culture and treatment. The human epithelial ovarian cancer cell lines OVCAR3 and SKOV3 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS and antibiotics (100 units/mL of penicillin and 100 μg/mL streptomycin) in a humidified atmosphere of 95% air and 5% of CO_2 at 37°C. Cells were exposed to different concentrations (1–20 μM) of arctigenin for 48 hr, and cell viability was determined using the MTT assay as described below. For inhibition of caspase-3 activation, cells were pre-treated with Ac-DEVD-CHO (50 μM) for 2 hr before exposure to arctigenin. To check the involvement of iNOS/NO signalling in arctigenin action, cells were co-incubated with arctigenin and SNAP (100 μM), a chemical NO donor.

Plasmid transfection. Ovarian cancer cells were seeded at a density of 3×10^5 cells per well onto six-well plates and allowed to attach overnight. The cells were pre-transfected with empty vector or pcDNA3.1-survivin (100 ng/well) using Lipofectamine 2000 according to the manufacturer's instructions. To monitor the transfection efficiency, cells were transfected in parallel with green fluorescent protein-expressing plasmids (pGFP-N1; Clontech, Mountain View, CA, USA). The transfection efficiency was ~85%. After incubation for 24 hr, the transfected cells were treated with arctigenin and tested for apoptosis.

MTT assay. Briefly, cells (4×10^3 cells per well) were plated in 96-well plates and remained untreated or exposed to different concentrations of arctigenin. After incubation for 72 hr, the MTT solution (0.5 mg/ml) was added to the cell culture. After incubation for an additional 4 hr at 37°C, dimethyl sulphoxide was added to dissolve the formed blue formazan crystals. Absorbance at 570 nm was measured.

Apoptosis analysis. Cell apoptosis was detected using a commercially available apoptosis assay kit. This system comprises two individual strains: (i) annexin-V has a strong affinity for phosphatidylserine that translocates to the outer surface of the cell membrane during apoptosis and (ii) PI can intercalate into double-stranded nucleic acids and is only permeant to dead cells. Therefore, live, early apoptotic and late apoptotic/necrotic cells are designed as annexin-V-/PI−, annexin-V+/PI− and annexin-V+/PI+, respectively. Briefly, after treatment, cells were harvested through trypsinization and washed. The cell samples were incubated with 5 μl of fluorescein isothiocyanate (FITC)-conjugated annex in V and 5 μl of PI for 15 min. at room temperature in the dark. The 1× binding buffer (400 μl) was added to each sample tube and the samples were analysed on a FACSCalibur flow cytometer using Cell Quest Research Software (BD Biosciences, San Jose, CA, USA).

Protein isolation and Western blot analysis. Cells were lysed in a lysis buffer (20 nM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM PMSF and leupeptin). Proteins were separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis under denaturing conditions and were transferred to nitrocellulose membranes. The membranes were incubated with different primary antibodies overnight at 4°C, followed by HRP-conjugated secondary antibodies for 1 hr at room temperature. Immunoreactive complexes were detected with the ECL chemiluminescence detection system according to the manufacturer's protocol. The band density was measured using the Quantity One software (Bio-Rad, Hercules, CA, USA) and normalized against the density of β-actin.

Statistical analysis. All data were expressed as mean ± standard deviation (S.D.). The difference among the means of multiple groups was analysed by one-way analysis of variance (ANOVA) followed by the Tukey test. A p value of <0.05 was considered as statistically significant.

Results

Arctigenin treatment suppresses ovarian cancer cell proliferation.

To examine the effect of arctigenin on cell proliferation in ovarian cancer cells, OVCAR3 and SKOV3 cells were exposed to various concentrations of arctigenin for 48 hr. Cell proliferation was determined using the MTT assay. Arctigenin treatment significantly ($p < 0.05$) inhibited the cell proliferation in a dose-dependent manner (fig. 1). Arctigenin at 10 μM caused >50% reduction in the proliferation of both OVCAR3 and SKOV3 cells. If not stated otherwise, the concentration of 10 μM for arctigenin was used in the following experiments.

Arctigenin induces caspase-3-dependent apoptosis in ovarian cancer cells.

To check whether arctigenin-induced suppression of cell proliferation is attributed to apoptosis induction, the apoptosis of arctigenin-treated ovarian cancer cells was evaluated using the annexin-V-binding assay. As shown in fig. 2A, arctigenin treatment caused a four times increase in the percentage of annexin-V+ apoptotic OVCAR3 cells and six times for SKOV3 cells ($p < 0.05$ relative to untreated control). Western blot analysis further revealed a significant enhancement of caspase-3 cleavage in arctigenin-treated cells, when compared to untreated control (fig. 2B). To confirm the involvement of caspase-3 activation in arctigenin-induced apoptotic death, cells were pre-treated with Ac-DEVD-CHO for 2 hr before exposure to arctigenin. Notably, arctigenin-induced cell death was prevented by Ac-DEVD-CHO pre-treatment in both cell lines (fig. 2C).

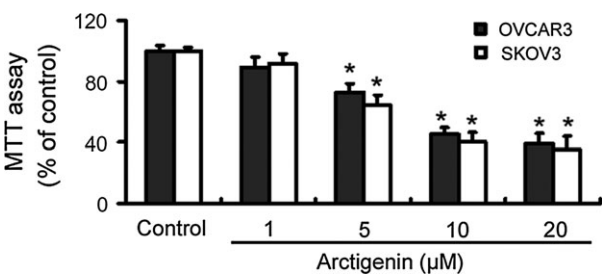

Fig. 1. Effect of arctigenin treatment on ovarian cancer cell proliferation. OVCAR3 and SKOV3 cells were exposed to different concentrations of arctigenin for 48 hr. Cell proliferation was determined using the MTT assay. The proliferation in untreated cells (control) was arbitrarily assigned 100%. *$p < 0.05$ *versus* control.

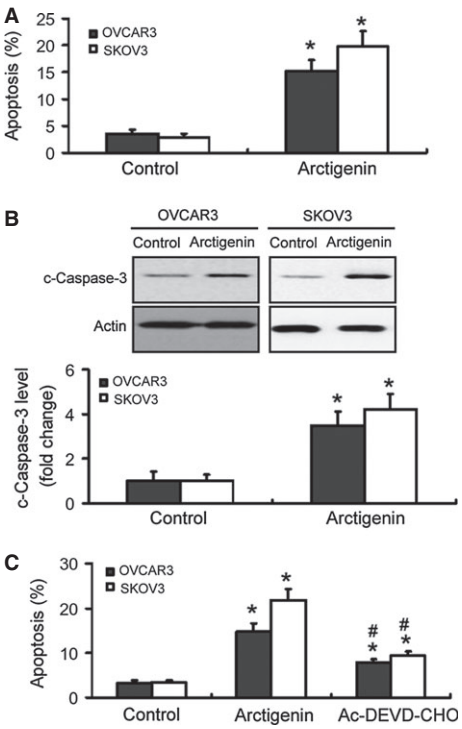

Fig. 2. Effect of arctigenin treatment on ovarian cancer cell apoptosis. Cells were exposed to arctigenin (10 μM) for 48 hr, and cell apoptosis was assessed. (A) The cells were stained with annexin-V and propidium iodide (PI), and the percentage of apoptotic cells was determined using flow cytometry. *$p < 0.05$ *versus* control. (B) Cells were tested for cleaved caspase-3 (c-caspase-3) levels by Western blot analysis. Upper panels: Representative blots of three independent experiments with similar results are shown. Lower panels: Densitometric analysis of cleaved caspase-3 levels. *$p < 0.05$ *versus* control. (C) Cells were pre-treated with Ac-DEVD-CHO for 2 hr before exposure to arctigenin (10 μM) for additional 48 hr. Cell apoptosis was determined using the annexin-V/PI staining assay. *$p < 0.05$ *versus* control; #$p < 0.05$ *versus* arctigenin treatment.

STAT3/survivin signalling mediates arctigenin-induced apoptosis.

To determine the effect of arctigenin on STAT3 activation, we measured the phosphorylation levels of STAT3 using Western blot analysis. The results showed that arctigenin inhibited the phosphorylation of STAT3 at residue Y705, compared with untreated control (fig. 3A). The protein level of survivin was consistently reduced by arctigenin treatment (fig. 3B). Next, we checked whether inactivation of STAT3/survivin signalling is responsible for arctigenin-induced apoptosis. As shown in fig. 3C, pre-transfection with survivin-expressing plasmid significantly ($p < 0.05$) blocked apoptotic death in arctigenin-treated OVCAR3 and SKOV3 cells.

Arctigenin-induced STAT3 inactivation is mediated via iNOS/NO signalling.

Western blot analysis demonstrated that both OVCAR3 and SKOV3 cells had high basal expression of iNOS (fig. 4A). Arctigenin treatment decreased the iNOS expression level by two to four times, compared with untreated control. Notably, co-incubation of cells with chemical NO donors reversed the suppression of STAT3 phosphorylation and survivin expression induced by arctigenin (fig. 4B). Moreover, the addition of exogenous NO significantly ($p < 0.05$) antagonized arctigenin-induced apoptosis in ovarian cancer cells (fig. 4C).

Discussion

Pre-clinical studies revealed that arctigenin has shown anticancer effects in many solid cancers such as colon cancer [15] and gastric cancer [16]. Hsieh *et al.* [17] showed that arctigenin induces apoptosis of oestrogen receptor-negative breast cancer cells. Our data revealed that arctigenin inhibited ovarian cancer cell proliferation in a dose-dependent manner. Such antiproliferative effect was associated with induction of apoptotic death. Caspase-3 is a well-established apoptosis mediator. We showed that arctigenin-induced apoptosis of ovarian cancer cells required enhanced cleavage of caspase-3, as blocking caspase-3 activity prevented apoptosis of arctigenin-treated cells. The activation of the caspase-3 apoptotic cascade by arctigenin was also described in non-small cell lung cancer [11].

Arctigenin has been shown to modulate multiple survival signalling pathways in different cellular contexts. Arctigenin can inhibit the unfolded protein response, which enhances cell survival and induces caspase-3-mediated apoptosis of cancer cells [15]. In non-small cell lung cancer, arctigenin enhances chemosensitivity to cisplatin through down-regulation of survivin expression [11]. Our data demonstrated that arctigenin treatment compromised the phosphorylation of STAT3 and suppressed the expression of survivin. Over-expression of survivin induced a reversal of arctigenin-induced apoptosis in ovarian cancer cells. The STAT3 pathway modulates the expression of several anti-apoptotic genes including survivin, and its inactivation may lead to reduced tumour cell survival. Zhang *et al.* [18] reported that oral administration of the STAT3 inhibitor BP-1-102 inhibits the growth of human breast and lung tumour xenografts. Another STAT3 inhibitor HO-3867 has been demonstrated to induce apoptosis of BRCA1-mutated ovarian cancer cells by down-regulating STAT3 downstream targets cyclin D1, Bcl-2 and survivin [19]. Arctigenin-mediated suppression of STAT3 in cancer cells has been previously reported [14]. Taken together, these findings indicate that the pro-apoptotic activity of arctigenin in ovarian cancer cells is mediated, at least in part, through inactivation of STAT3/survivin signalling.

Tight regulation of NO production is involved in cancer development. It has been documented that endogenous free radical NO can inhibit apoptosis and thereby promote colon cancer cell growth [20]. Induction of NO has been found to mediate the pro-survival effect of testosterone and prolactin in prostate cancer cells [21]. In ovarian cancer cells, low concentration of NO confers cytoprotectivity through up-regulation of survivin expression [13]. These studies suggest NO signalling as a potential anticancer therapeutic target. Arctigenin has shown potent inhibitory activity against iNOS pathway. Kou *et al.* [22] reported that arctigenin inhibits lipopolysaccharide (LPS)-induced iNOS expression in RAW264.7 cells. Yao

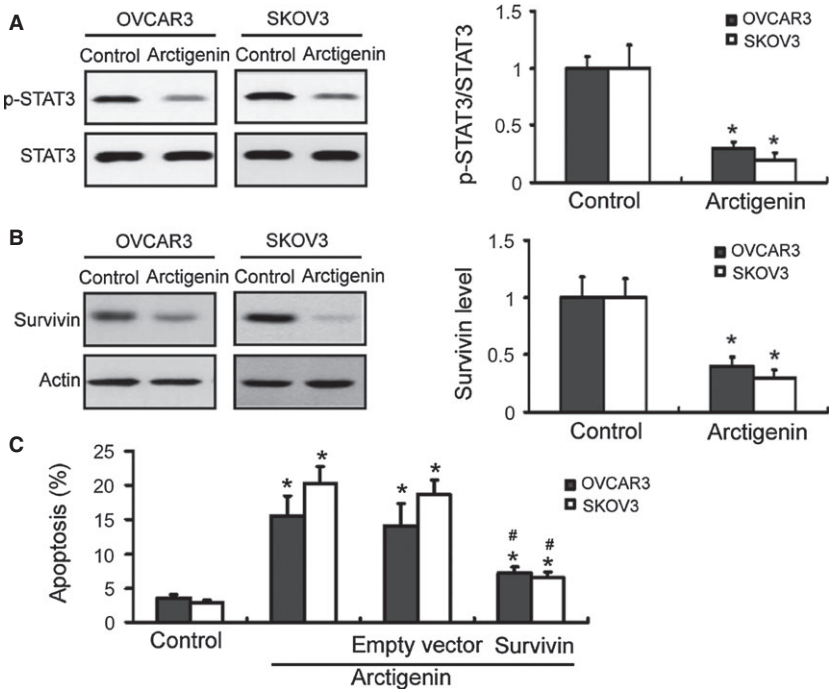

Fig. 3. STAT3/survivin signalling mediates arctigenin-induced apoptosis. Cells were exposed to arctigenin (10 μM) for 48 hr, and gene expression analysis was performed using Western blot analysis. (A) Effect of arctigenin on STAT3 phosphorylation. Left panels: Representative blots of three independent experiments with similar results are shown. Right panels: Bar graphs show the ratio between phosphorylated and total STAT3 levels determined by densitometry. $*p < 0.05$ *versus* control. (B) Western blot analysis of survivin protein levels. Left panels show representative blots of three independent experiments. Right panels show densitometric analysis of survivin levels. $*p < 0.05$ *versus* control. (C) Cells were pre-transfected with empty vector or survivin-expressing plasmid before exposure to arctigenin. After 48-hr incubation, cell apoptosis was measured. $*p < 0.05$ *versus* control; $^{\#}p < 0.05$ *versus* arctigenin treatment alone.

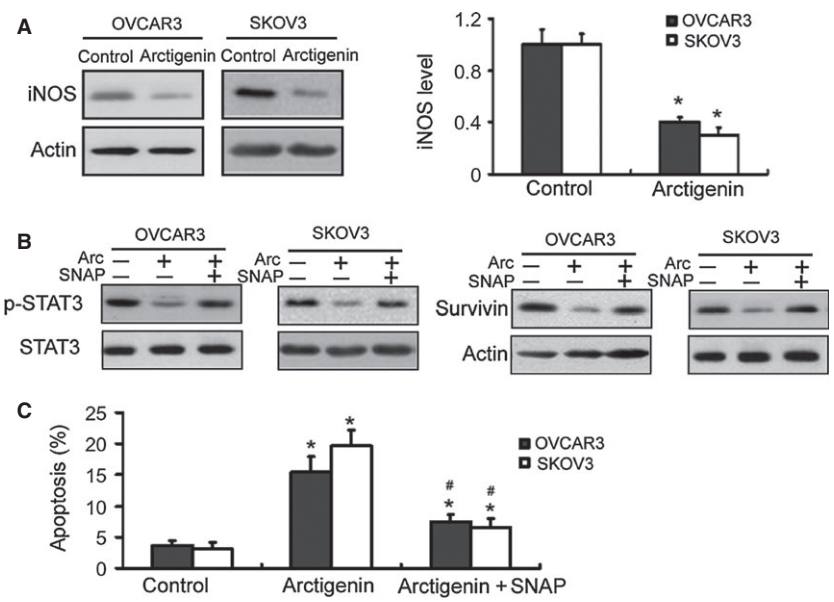

Fig. 4. Inducible nitric oxide synthase (iNOS)/NO signalling is involved in the anticancer activity of arctigenin. (A) Western blot analysis of iNOS expression levels in ovarian cancer cells treated with or without arctigenin (10 μM). Left panels show representative blots of three independent experiments. Right panels show densitometric analysis of iNOS levels. $*p < 0.05$ *versus* control. (B) Western blot analysis of total and phosphorylated STAT3 and survivin levels in ovarian cancer cells with or without pre-treatment with SNAP (100 μM) before exposure to arctigenin (Arc). Representative blots of three independent experiments with similar results are shown. (C) Cells were pre-treated with or without SNAP before exposure to arctigenin. After 48-hr incubation, cell apoptosis was measured. $*p < 0.05$ *versus* control; $^{\#}p < 0.05$ *versus* arctigenin treatment alone.

et al. [23] further revealed that such degradation of iNOS protein by arctigenin is due to CHIP-associated ubiquitination and proteasome dependency. Consistently, our data showed that arctigenin suppressed the iNOS expression in ovarian cancer cells. Most importantly, addition of exogenous NO prevented arctigenin-induced inactivation of STAT3/survivin signalling and apoptotic death in ovarian cancer cells. These findings indicate that arctigenin suppresses the pro-survival STAT3/survivin signalling in ovarian cancer largely through reduction in NO formation, which provides a novel molecular mechanism for the anticancer activity of arctigenin. However, there is a complex relationship between iNOS/NO expression and STAT3 activation. Arctigenin has been found to inactivate LPS-induced STAT signalling in RAW264.7 macrophages, consequently inhibiting the expression of iNOS [22]. A recent study has shown that iNOS activation and subsequent NO production leads to STAT3 S-nitrosylation/inactivation in microglia [24]. These studies suggest that iNOS can not only be stimulated by but also modulate the activation of STAT3 signalling. The interactions between iNOS/NO and STAT3 signalling pathways may be shaped by cellular context. Nevertheless, the detailed molecular mechanisms for the action of arctigenin in ovarian cancer need to be further clarified.

In conclusion, our data establish a link between iNOS/NO and STAT3/survivin signalling in the regulation of ovarian cancer cell survival. Arctigenin shows pro-apoptotic activity in ovarian cancer cells, which is associated largely with modulation of the iNOS/NO/STAT3 axis. Therefore, arctigenin may be a potential new therapeutic drug for ovarian cancer. Given the importance of STAT3 signalling in the apoptotic resistance of ovarian cancer cells, the utilization of arctigenin to improve chemotherapeutic efficacy warrants further investigation.

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