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Withaferin A Inhibits Experimental Epithelial-Mesenchymal Transition in MCF-10A Cells and Suppresses Vimentin Protein Level in Vivo in Breast Tumors

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Abstract

We have shown previously that withaferin A (WA), a bioactive component of the medicinal plant *Withania somnifera*, inhibits growth of cultured and xenografted human breast cancer cells and prevents breast cancer development and pulmonary metastasis incidence in a transgenic mouse model. The present study was undertaken to determine if the anticancer effect of WA involved inhibition of epithelial-mesenchymal transition (EMT). Experimental EMT induced by exposure of MCF-10A cells to tumor necrosis factor- α (TNF- α) and transforming growth factor- β 1 (TGF- β) was partially reversed by treatment with WA but not by its structural analogs withanone or withanolide A. Combined TNF- α and TGF- β treatments conferred partial protection against MCF-10A cell migration inhibition by WA. Inhibition of TNF- α and TGF- β -induced MCF-10A cell migration by WA exposure was modestly attenuated by knockdown of E-cadherin protein. MCF-7 and MDA-MB-231 cells exposed to WA exhibited sustained (MCF-7) or transient (MDA-MB-231) induction of E-cadherin protein. On the other hand, the level of vimentin protein was increased markedly after 24 h treatment of MDA-MB-231 cells with WA. WA-induced apoptosis was not affected by vimentin protein knockdown in MDA-MB-231 cells. Protein level of vimentin was significantly lower in the MDA-MB-231 xenografts as well as in MMTV-*neu* tumors from WA-treated mice compared with controls. The major conclusions of the present study are that (a) WA treatment inhibits experimental EMT in MCF-10A cells, and (b) mammary cancer growth inhibition by WA administration is associated with suppression of vimentin protein expression *in vivo*.

Keywords

Withaferin A; Vimentin; EMT; Breast cancer

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INTRODUCTION

Ingredients of traditional Indian medicine (commonly known as Ayurvedic medicine) continue to draw attention for identification of phytochemicals potentially useful for prevention and treatment of cancer [1]. *Withania somnifera* (also known as Ashwagandha or Indian winter cherry) is one such plant that is a key component of the Ayurvedic remedies used for the treatment of different disorders [1]. Anticancer effects of *W. somnifera* extract in experimental rodents have also been described [2,3]. For example, local injections of the leaf extract of *W. somnifera* every third day to the tumor resulted in growth retardation of human fibrosarcoma in athymic mice [2]. In a long-term chemically-induced cancer bioassay in mice, *W. somnifera* root extract administered in the diet resulted in prevention of benzo[a]pyrene-induced forestomach tumorigenesis [3].

Anticancer effect of *W. somnifera* is attributed to withanolides including withaferin A (WA). Anticancer effect of WA was first noted in the late sixties [4]. WA exhibited remarkable efficacy against oral carcinogenesis in hamsters induced by the chemical carcinogen 7,12-dimethylbenz[a]anthracene [5]. Several studies have demonstrated efficacy of WA against human tumor xenografts [6–8]. For instance, the growth of PC-3 human prostate cancer cells implanted in male nude mice was suppressed significantly by WA treatment [6]. We showed previously that the growth of MDA-MB-231 human breast cancer cells subcutaneously implanted in female athymic mice was significantly retarded by intraperitoneal injections of 4 mg WA/kg body weight (five times per week) without weight loss or any other signs of toxicity [8]. More recent studies from our laboratory have demonstrated significant inhibition of mammary tumor burden and pulmonary metastasis incidence in mouse mammary tumor virus-*neu* (MMTV-*neu*) mice after intraperitoneal administration of 0.1 mg WA, 3 times/week [9]. Mammary cancer prevention by WA in MMTV-*neu* mice was associated with increased apoptosis and inhibition of glycolysis [9].

The mechanisms underlying anticancer effect of WA are still not fully understood, but this phytochemical is known to exhibit a variety of effects in cultured cancer cells. For example, tumor proteasome was shown to be a target of WA in prostate cancer cells [7]. Exposure of human breast cancer cells to WA resulted in G₂/M phase cell cycle arrest [10] and apoptosis induction mediated by FOXO3a and Bim [8]. Apoptosis induction by WA was also shown in other cancer types including prostate, leukemia, and head and neck cancer cells to name a few [6,11,12]. Furthermore, WA has the ability to inhibit multiple oncogenic signaling pathways in cancer cells including nuclear factor- κ B [13], Notch-1 [14], signal transducer and activator of transcription 3 [15], and estrogen receptor- α [16]. On the other hand, WA treatment resulted in activation of Notch2 and Notch4 in human breast cancer cells [17]. Notch2 activation by WA impeded its inhibitory effect against breast cancer cell migration [17]. Anti-angiogenic and anti-metastatic effects of WA were also shown [18,19].

Intermediate filament protein vimentin is another target of WA [19–21]. WA treatment was shown to disrupt assembly and function of vimentin in breast cancer cells [19]. Bargagna-Mohan et al. [20] showed direct interaction of WA with vimentin through covalent modification of a cysteine residue present in the highly conserved α -helical coiled coil 2B domain. Another study showed caspase-dependent cleavage of vimentin upon treatment with

WA in soft tissue sarcoma cells [21]. Because induction of vimentin protein expression concomitant with loss of E-cadherin is a biochemical hallmark of epithelial-mesenchymal transition (EMT), we raised the question of whether WA inhibits EMT. EMT is a normal physiological process that is also implicated in cancer progression and metastasis [22]. In the present study, we have studied the effect of WA on EMT using human breast cancer cells (MCF-7 and MDA-MB-231), a non-tumorigenic human mammary epithelial cell line (MCF-10A), MDA-MB-231 xenografts from control and WA-treated mice, and mammary tumors from control and WA-treated MMTV-*neu* mice.

MATERIALS AND METHODS

Ethics Statement

Tumors from our previously published xenograft and chemoprevention studies [8,9] were used to determine the effect of WA administration on expression of E-cadherin and vimentin proteins *in vivo*. Use of mice and their care were in accordance with the University of Pittsburgh Institutional Animal Care and Use Committee guidelines.

Reagents

WA and its structural analogs withanone (WE) and withanolide A (WLA) were purchased from Enzo Life Sciences (Farmingdale, NY) or Chromadex (Irvine, CA). Purity of WA (Enzo Life Sciences) was 99%. Stock solutions of WA, WE, and WLA were prepared in dimethyl sulfoxide (DMSO; 100%) and diluted with medium prior to experimental procedures (final concentration of DMSO = 0.025%). WA was found to be stable for at least one year after storage of the DMSO stock at -20°C . WA concentrations used in the present study (1–5 μM) were selected based on the results of a pharmacokinetic study in mice [19]. This study showed that the mean C_{max} of WA in plasma was 1.8 μM following a single intraperitoneal injection of 4 mg/kg with a half life of about 1.3 h and AUC_{0-t} of 1.09 $\mu\text{M} \cdot \text{h}$ [19]. Clearance of WA from the plasma was rapid and the compound was not detectable in all mice at time points beyond 6 h [19]. 4',6-Diamidino-2-phenylindole (DAPI), tumor necrosis factor- α (TNF- α), and antibodies against actin, vimentin and α -tubulin were purchased from Sigma-Aldrich (St. Louis, MO). Transforming growth factor- β 1 (TGF- β) was purchased from Calbiochem-EMD Millipore (Billerica, MA). Cell culture media, antibiotic mixture, fetal bovine serum, and anti-occludin antibody were purchased from Invitrogen-Life Technologies (Grand Island, NY). An antibody against poly-(ADP-ribose)-polymerase (PARP) was purchased from Santa Cruz Biotechnology (Dallas, TX). Antibodies against E-cadherin, Caspase-3, cleaved Caspase-3, and cleaved PARP were purchased from Cell Signaling Technology (Danvers, MA) or BD Biosciences (San Jose, CA). Antibodies against fibronectin and vimentin (used for immunohistochemistry) were purchased from Santa Cruz Biotechnology. An antibody against Caspase-7 was from BD Pharmingen; anti-Caspase-9 antibody was from Stressgen-Enzo Life Sciences; anti-Caspase-8 antibody was from BioSource International-Life Technologies; and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was from GeneTex (Irvine, CA). Caspase inhibitors (zLEHD-fmk and zIETD-fmk) were purchased from MP Biomedicals (Solon, OH). A non-specific small interfering RNA (siRNA) was purchased from Qiagen (Valencia, CA). E-cadherin-targeted siRNA was purchased from Ambion-Life

Technologies. Vimentin-targeted small interfering RNA was obtained from Santa Cruz Biotechnology. The sequences of these siRNA are not disclosed by the suppliers.

Cell Lines and Cell Migration Assay

MDA-MB-231, MCF-7, and MCF-10A cells were obtained from American Type Culture Collection (Manassas, VA) and cultured as described previously [23]. Cell migration assay was performed using a Boyden chamber containing an 8 μ m filter as described previously [15]. Each experiment was performed in triplicate, and at least 3 non-overlapping areas on each filter were examined for quantitation of cell migration.

Western Blotting

Cell lysates and tumor supernatants were prepared as described previously [24,25]. The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto membrane, which was then treated with the desired primary antibody. After treatment with a secondary antibody, bands were visualized with the use of chemiluminescence reagent. For some experiments, the cells were pretreated for 2 h with 60 μ M of caspase inhibitors and then treated with WA in the absence or presence of caspase inhibitors for 36 h.

RNA Interference of E-Cadherin and Vimentin

For knockdown of E-cadherin, MCF-10A cells (1.25×10^5) were plated in 6-well plates. After overnight incubation to allow attachment, the cells were transfected with a control siRNA (100 nM) or E-cadherin-specific siRNA (100 nM) using OligoFECTAMINE (Invitrogen Life Technologies). After 24 h of transfection, the cells were treated with DMSO (control) or WA (2 μ M) in the absence or presence of TNF- α /TGF- β (10 ng/mL each) for 24 h. For knockdown of vimentin, MDA-MB-231 cells (1×10^5) were transfected with control siRNA (100 nM) or vimentin-specific siRNA (100 nM). After 48 h of transfection, the cells were treated with DMSO (control) or WA (2 or 4 μ M) for 24 h. Subsequently, the cells were collected and processed for western blotting, cell migration assay or apoptosis assays.

Immunofluorescence Microscopy

MCF-7 cells (1×10^5) were plated on coverslips and allowed to attach by overnight incubation. Cells were treated with DMSO (control) or 2 μ M WA for 24 h and fixed with 2% paraformaldehyde for 1 h at room temperature. Immunofluorescence microscopy for E-cadherin was performed as described previously [26]. Stained cells were examined under a Leica fluorescence microscope.

Apoptosis assay

Apoptosis induction was determined using Annexin V-FITC/Propidium Iodide Apoptosis Detection kit from BD Biosciences or histone-associated DNA fragment release into the cytosol ELISA kit from Roche Applied Science (Indianapolis, IN) according to the manufacturer's instructions. For quantitation of apoptosis by flow cytometry using Annexin V-FITC/Propidium Iodide kit, the cells were treated with DMSO or WA for 24 h, harvested and washed with phosphate buffered saline (PBS). Cells (1×10^5) were suspended in 100 μ L

of binding buffer, and stained with 4 μ L of Annexin V and 2 μ L of propidium iodide solution for 15 min at room temperature in the dark. Samples were then diluted with 200 μ L of the binding buffer. Stained cells were analyzed using a flow cytometer. For analysis of histone-associated DNA fragment release into the cytosol, MDA-MB-231 cells were transfected for 36 h with 200 nM of a control siRNA or 200 nM of a vimentin-targeted siRNA. The cells were treated with DMSO (control) or 4 μ M of WA for 48 h. In certain experiments, the cells were pretreated for 2 h with 60 μ M of caspase inhibitors and then treated with WA in the absence or presence of caspase inhibitors for 36 h.

Proliferation assay

Cell proliferation was determined by use of CellTiter 96[®] AQueous Non-Radioactive Cell Proliferation Assay kit from Promega (Madison, WI) according to the manufacturer's instructions. After transfection for 36 h with 200 nM of a control siRNA or 200 nM of a vimentin-targeted siRNA, MDA-MB-231 cells were plated in 96-well plate (2×10^3 cells per well) in quadruplicate. After overnight incubation, the cells were treated with DMSO (control) or WA (2 or 4 μ M) for 48 h.

Immunohistochemistry

Tumors were sectioned at 4–5 μ m, quenched with 3% H₂O₂ and then blocked with normal serum. Sections were then treated with anti-E-cadherin or anti-vimentin antibody. After washing with tris-buffered saline, the sections were treated with a secondary antibody. Brown color was developed by incubation with 3,3'-diaminobenzidine. The sections were counterstained with Hematoxylin and examined under a Leica microscope. Expression of E-cadherin or vimentin was quantified with the use of Aperio Image Scope software.

RESULTS

WA Inhibited TNF- α /TGF- β -induced EMT in MCF-10A Cells

Initially we used a well-established *in vitro* experimental EMT model involving TNF- α /TGF- β and a non-tumorigenic mammary epithelial cell line (MCF-10A) to determine the anti-EMT effect of WA, which is a C₅,C₆-epoxide steroidal lactone (structure of WA is shown in Figure 1A). As shown in Figure 1B, treatment of MCF-10A cells with 10 ng/mL each of TNF- α and TGF- β for 8 h resulted in 60% decrease in the level of E-cadherin protein and 5-fold increase in vimentin protein level compared to DMSO control. These results indicated EMT induction in MCF-10A cells after treatment with TNF- α /TGF- β . The WA treatment caused a nearly 14-fold increase in E-cadherin protein level compared with DMSO-treated control. The level of vimentin protein was decreased by about 50% upon exposure of MCF-10A cells to WA. The induction of E-cadherin resulting from WA exposure was partially attenuated in the presence of TNF- α /TGF- β (Figure 1B). On the other hand, the C₆,C₇-epoxide derivatives (WE and WLA) were ineffective in increasing E-cadherin protein level (Figure 1B). Induction of vimentin by treatment with TNF- α and TGF- β was sustained in the presence of WE or WLA (Figure 1B). In addition, unlike WA, both WE and WLA treatment caused upregulation of vimentin protein (Figure 1B). These results indicated inhibition of experimental EMT by WA but not by its C₆,C₇-epoxide derivatives.

WA Inhibited TNF- α /TGF- β -Induced MCF-10A Cell Migration

As shown in Figure 1C, the migratory potential of MCF-10A cells was significantly augmented in the presence of TNF- α /TGF- β (Figure 1C, c and h) when compared with DMSO control (Figure 1C, a and e). The MCF-10A cell migration was significantly decreased by treatment with 2 μ M WA (Figure 1C, b, and Figure 1D). Moreover, TNF- α /TGF- β stimulation conferred partial but significant protection against WA-mediated inhibition of MCF-10A cell migration (Figure 1C, d, and Figure 1D). WE (Figure 1C, f) and WLA (Figure 1C, g) were relatively less effective than WA for inhibition of MCF-10A cell migration (Figure 1D). The TNF- α /TGF- β also conferred protection against modest inhibition of MCF-10A cell migration observed in the presence of WE (Figure 1C, i) and WLA (Figure 1C, j).

We employed RNA interference technique to further test the role of E-cadherin in WA-mediated inhibition of EMT and cell migration. Level of E-cadherin protein was decreased by >99% after transient transfection of MCF-10A cells with an E-cadherin-targeted siRNA (Figure 2A). Migratory potential of control siRNA-transfected MCF-10A cells was increased markedly by treatment with TNF- α /TGF- β (Figure 2B,C). Knockdown of E-cadherin protein also caused a marked increase in migration capability of MCF-10A cells even in absence of TNF- α /TGF- β treatment (Figure 2B,C). WA treatment exhibited significant inhibitory effect against cell migration in both control siRNA transfected cells and E-cadherin siRNA transfected MCF-10A cells. However, TNF- α /TGF- β stimulation conferred modest but significant protection against WA-mediated inhibition of cell migration in both control siRNA transfected cells and E-cadherin siRNA transfected MCF-10A cells (Figure 2C). These results indicated impact of TNF- α /TGF- β stimulation as well as E-cadherin knockdown on MCF-10A cell migration inhibition by WA.

Effect of WA Treatment on EMT Markers in Human Breast Cancer Cells

EMT is characterized by downregulation of E-cadherin and occludin and induction of mesenchymal marker proteins such as vimentin and fibronectin [22]. Loss of E-cadherin expression has been observed in invasive breast cancers [27]. We used MCF-7 and MDA-MB-231 human breast cancer cells to further test anti-EMT effect of WA. The MCF-7 cell line is a well-accepted representative of epithelial type breast cancer cell line characterized by high expression of E-cadherin and occludin and tight cell-cell contacts [28]. Expression of mesenchymal marker protein vimentin is not detectable in the MCF-7 cells [28]. On the other hand, expression of E-cadherin is very low in the MDA-MB-231 cell line, which is a highly invasive cell line with mesenchymal characteristics (high expression of vimentin protein) [28]. The MCF-7 cell line exhibited visible cell-cell contact (Figure 3A). Immunofluorescence microscopy showed induction of E-cadherin protein expression upon treatment of MCF-7 cells with WA (Figure 3A). Consistent with these results, immunoblotting showed induction of E-cadherin and occludin and suppression of mesenchymal marker protein fibronectin in WA-treated MCF-7 cells in comparison with control (Figure 3B). As can be seen in Figure 3B, 8 h treatment of MDA-MB-231 cells with WA resulted in upregulation of E-cadherin protein expression and suppression of vimentin protein level, but these effects were transient. In addition, WA treatment for 24 h caused a marked increase in vimentin protein level in MDA-MB-231 cells (Figure 3B). However,

restoration of cell-cell contact could not be observed after treatment of MDA-MB-231 cells with WA either at 8 h time point or 24 time point (results not shown). These results indicated that while WA treatment affected the biochemical features of EMT in MCF-7 cells, a clear-cut reversal of this phenotype was not discernible in the MDA-MB-231 cell line after treatment with WA.

WA-Mediated Apoptosis Was Not Affected by Vimentin Protein Knockdown

We first determined the role of caspases in apoptosis induction by WA. As shown in Figure 4A and 4B, the levels of full-length caspases were decreased after treatment with WA in both MDA-MB-231 and MCF-7 cells albeit with different kinetics (caspase 3 is not expressed in MCF-7 cells). WA-mediated apoptosis, assessed by analysis of histone-associated DNA fragment release into the cytosol (Figure 4C), was partially but significantly attenuated in the presence of a specific inhibitor of caspase 9 (zLEHD-fmk). These results suggested involvement of intrinsic caspase pathway in apoptosis induction by WA.

Previous studies have suggested a role for vimentin in WA-induced apoptosis in soft tissue sarcoma cells [21]. Because WA treatment resulted in induction of vimentin protein in cultured MDA-MB-231 cells (Figure 3B), it was of interest to determine if proapoptotic effect of WA was altered by vimentin knockdown. Transient transfection of MDA-MB-231 cells with a vimentin-targeted siRNA resulted in 90% decrease in vimentin protein level (Figure 5A). Proapoptotic effect of WA in MDA-MB-231 cells transfected with a control siRNA and a vimentin-specific siRNA was examined by Annexin V-propidium iodide method, which measures both early and late apoptotic cells (Figure 5B). WA-mediated apoptosis was observed in both control siRNA transfected cells and vimentin-depleted cells (Figure 5C). However, depletion of vimentin protein was partially but significantly protective against WA-induced apoptosis at the 4 μ M dose (Figure 5C). This protective effect was not evident at the 2 μ M WA concentration (Figure 5C).

Because the results of Annexin V-propidium iodide method were not conclusive, additional experiments were performed to determine the effect of vimentin knockdown on cell proliferation inhibition and apoptosis induction by WA. Analysis of cell proliferation (Figure 5D), histone-associated DNA fragment release into the cytosol (Figure 5E), and cleavage of PARP and caspase-3 (Figure 5F) revealed that cell proliferation inhibition as well as apoptosis induction by WA was not affected by knockdown of vimentin protein. Based on these results, we conclude that vimentin induction has no meaningful impact on WA's ability to inhibit cell proliferation or induce apoptosis.

Effect of Vimentin Knockdown on WA-Mediated Inhibition of Cell Migration

Because vimentin protein is implicated in cell migration and invasion [22], we determined the effect of its knockdown on WA's ability to inhibit MDA-MB-231 cell migration. As shown in Figure 6A, vimentin knockdown itself reduced migratory potential of MDA-MB-231 cells. However, WA was more or less equally effective in inhibiting migration of MDA-MB-231 cells transfected with control siRNA and vimentin siRNA (Figure 6B). These results indicated that WA-mediated inhibition of MDA-MB-231 cell migration was not affected by vimentin protein level.

WA Decreased Vimentin Protein Expression in MDA-MB-231 Xenografts *In Vivo*

We have shown previously that WA administration significantly inhibits growth of MDA-MB-231 xenografts in female athymic mice [8]. We used tumors from this study to determine the *in vivo* effect of WA administration on vimentin and E-cadherin protein level. Initially, we performed western blotting using tumor lysates from control and WA-treated mice to examine the effect on E-cadherin and vimentin. As shown in Figure 7A, WA administration modestly increased E-cadherin expression, which was fairly low in MDA-MB-231 xenografts consistent with cellular data. However, the difference in E-cadherin expression between control and WA-treated tumor samples was not significant. Expression of vimentin protein seemed lower in the tumors from WA-treated mice compared with those of control mice but the difference was not significant (Figure 7A). Results of immunoblotting on vimentin expression were confirmed by immunohistochemistry (Figure 7B). Intensity of vimentin protein expression was significantly lower in the tumors from WA-treated mice (Figure 7B, c and d, and Figure 7C) compared with control (Figure 7B, a and b, and Figure 7C). These results showed *in vivo* suppression of vimentin protein expression by WA administration in MDA-MB-231 xenografts.

WA Treatment Decreased Vimentin Protein Expression in MMTV-*neu* Tumor *In Vivo*

Our recently published studies showed mammary cancer prevention by WA in MMTV-*neu* transgenic mice [9]. The tumor sections from MMTV-*neu* mice were also used to determine the *in vivo* effect of WA on vimentin and E-cadherin protein level. WA administration resulted in a modest increase in E-cadherin expression in the MMTV-*neu* mammary tumor (Figure 8A, c and d) compared with control (Figure 8A, a and b) but the difference did not reach statistical significance. On the other hand, intensity of vimentin protein expression was significantly lower in the mammary tumors from WA-treated MMTV-*neu* mice (Figure 8B, c and d) compared with those of control mice (Figure 8B, a and b).

DISCUSSION

The effect of WA treatment on blood chemistry is yet to be studied, but its effect on lung and liver histology has been determined in mice after intraperitoneal injection of 0.1–4 mg/kg [19]. There was no difference in the degree of necrosis or fibrosis in the lung parenchyma between control and the WA treatment groups [19]. In almost all cases, only minor necrosis (grade 1+; essentially normal) was observed in the lung. Lung fibrosis in WA treatment group was limited to grade 2+, which was similar to the vehicle-treated control mice [19]. Also, WA-treated mice exhibited only minor necrosis (grade 1+) in the liver, and several mice had grade 2+ and 3+ hepatic fibrosis in both the control group and the WA treatment group [19]. Based on these pre-clinical data and extensive usage of the WA-containing extract from *W. somnifera* plant in Ayurvedic medicine, it is reasonable to propose that WA has a favorable safety profile.

Previous studies from our laboratory have thoroughly characterized the mechanisms underlying WA-mediated apoptosis, including the role of anti-apoptotic and proapoptotic proteins, in breast cancer cells [8,29–31]. WA-mediated apoptosis is associated with induction of short and long forms of Bim in MCF-7 cells but upregulation of short and

extra-long forms of Bim in MDA-MB-231 cells [8]. Knockdown of Bim protein confers marked protection against WA-induced apoptosis in these cells [8]. The MCF-10A cells are significantly more resistant to apoptosis induction by WA compared with breast cancer cells [8]. Follow-up mechanistic studies revealed inhibition of complex III of the mitochondrial respiration leading to production of reactive oxygen species and activation of Bak in MCF-7 and MDA-MB-231 cells [29]. Consistent with the results in MCF-10A cells [8], WA-induced apoptosis, reactive oxygen species generation and Bak activation are not observed in a normal human mammary epithelial cell line HMEC [29] providing an explanation for the selectivity of the compound for cancer cells. In another study, we showed downregulation of inhibitor of apoptosis family proteins including survivin in WA-treated MDA-MB-231 and MCF-7 cells [30]. Furthermore, WA administration to MDA-MB-231 xenograft bearing athymic mice results in significant decrease in survivin protein expression *in vivo* [30]. We have also studied the role of mitogen-activated protein kinases (MAPK), including c-jun NH₂-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 MAPK, as well as the anti-apoptotic protein myeloid cell leukemia-1 (Mcl-1) in apoptotic cell death by WA [31]. The JNK, ERK, and p38 MAPK are hyperphosphorylated upon treatment with WA in MCF-7 cells as well as in a triple-negative breast cancer cell line (SUM159) [31]. Apoptosis by WA treatment in MCF-7 cells is significantly increased by inhibition of ERK and p38 MAPK [31]. On the other hand, WA-induced apoptosis in MCF-7 cells is partially but significantly blocked by a JNK inhibitor [31]. Inhibition of ERK or JNK is unable to alter WA-induced apoptosis in SUM159 cells [31]. Interestingly, exposure of MCF-7 and SUM159 cells to WA results in induction of long and short forms of Mcl-1 [8,31]. RNA interference of Mcl-1 triggers apoptosis consistent with its role as an anti-apoptotic protein [31]. In addition, apoptosis resulting from WA treatment is modestly but significantly augmented by knockdown of Mcl-1 protein [31].

The major objective of the present study was to test anti-EMT effect of WA. The EMT is a normal physiological process essential for embryonic development, tissue remodeling, and wound healing [32]. The epithelial phenotype is characterized by tight cell-cell junctions and polarity, whereas mesenchymal transition leads to disruption of the cell-cell contact with conversion to fibroblastic morphology and increased motility [32]. Accumulating evidence indicates a critical role for EMT in pathogenesis of aggressive cancers [32,33]. In human breast carcinoma, overexpression of EMT markers is associated with tumor aggressiveness, disease recurrence, unfavorable clinical and pathological variables, and reduced survival [27,34]. However, recent studies have questioned the validity of E-cadherin suppression in EMT at least in breast cancer cells [35]. Results presented herein indicate that WA partially reverses EMT induced by TNF- α and TGF- β in MCF-10A cells. Notably, this effect is evident at pharmacologically relevant concentrations of WA. Because EMT is implicated in cancer metastasis, it is not surprising that WA inhibits cancer metastasis [9,19].

Adherens junctions are critical for maintenance of the epithelial integrity [32,33]. The E-cadherin is believed to play an important role in safeguarding epithelial phenotype. This protein is frequently downregulated during cancer progression and correlates with poor prognosis [36,37]. The present study reveals that induction of E-cadherin protein contributes, at least in part, to WA-mediated inhibition of cell migration. Knockdown of E-

cadherin protein alone significantly increases migratory potential of the cells. Moreover, the WA-mediated inhibition of cell migration is partially but significantly attenuated by RNA interference of E-cadherin.

Vimentin, a mesenchymal filament protein, increases motility of cells in coordination with deetyrosinated microtubules to provide support to microtentacle extension of detached tumor cells [38]. Expression of vimentin in breast cancer cases of young women correlates with adverse pathological parameters [39]. The results of the present study indicate that exposure of TNF- α /TGF- β -stimulated MCF-10A cells to 2 μ M WA results in a marked decrease in vimentin protein level. Furthermore, WA-mediated inhibition of MDA-MB-231 xenograft growth is clearly associated with a significant decrease in expression of vimentin protein in the tumor *in vivo*. Thus, it is reasonable to speculate that suppression of vimentin by WA possibly contributes to its anti-metastatic effect [9,19].

In soft tissue sarcoma cells, WA treatment resulted in caspase-dependent degradation of vimentin, but this effect was much less pronounced in normal mesenchymal cells [21]. The proapoptotic effect of WA in soft-tissue sarcoma cells is abrogated after vimentin knockdown or inhibition of caspase-induced vimentin degradation [21]. The present study reveals that the apoptosis induction by WA is not altered to a meaningful extent upon vimentin knockdown.

In summary, the present study reveals that WA treatment reverses biochemical features of EMT induced by TNF- α and TGF- β in MCF-10A cells and suppresses expression of vimentin protein *in vivo* potentially contributing to its anticancer effects [8,9].

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Abbreviations

WA	withaferin A
WE	withanone
WLA	withanolide A
EMT	epithelial-mesenchymal transition
MMTV-<i>neu</i>	mouse mammary tumor virus- <i>neu</i>
DMSO	dimethyl sulfoxide
DAPI	4',6-Diamidino-2-phenylindole
TNF-α	tumor necrosis factor- α
TGF-β	transforming growth factor- β 1
PARP	poly-(ADP-ribose)-polymerase

siRNA	small interfering RNA
PBS	phosphate-buffered saline
MAPK	mitogen-activated protein kinase
JNK	c-jun NH ₂ -terminal kinase
ERK	extracellular signal-regulated kinase
Mcl-1	myeloid cell leukemia-1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase

References

1. Garodia P, Ichikawa H, Malani N, Sethi G, Aggarwal BB. From ancient medicine to modern medicine: Ayurvedic concepts of health and their role in inflammation and cancer. *J Soc Integr Oncol.* 2007; 5:25–37. [PubMed: 17309811]
2. Widodo N, Kaur K, Shrestha BG, et al. Selective killing of cancer cells by leaf extract of Ashwagandha: Identification of a tumor-inhibitory factor and the first molecular insights to its effect. *Clin Cancer Res.* 2007; 13:2298–2306. [PubMed: 17404115]
3. Padmavathi B, Rath PC, Rao AR, Singh RP. Roots of *Withania somnifera* inhibit forestomach and skin carcinogenesis in mice. *Evid Based Complement. Alternat Med.* 2005; 2:99–105.
4. Shohat B, Gitter S, Abraham A, Lavie D. Antitumor activity of withaferin A (NSC-101088). *Cancer Chemother Rep.* 1967; 51:271–276. [PubMed: 6062297]
5. Manoharan S, Panjamurthy K, Menon VP, Balakrishnan S, Alias LM. Protective effect of Withaferin-A on tumour formation in 7,12-dimethylbenz[a]anthracene induced oral carcinogenesis in hamsters. *Indian J Exp Biol.* 2009; 47:16–23. [PubMed: 19317347]
6. Srinivasan S, Ranga RS, Burikhanov R, Han SS, Chendil D. Par-4-dependent apoptosis by the dietary compound withaferin A in prostate cancer cells. *Cancer Res.* 2007; 67:246–253. [PubMed: 17185378]
7. Yang H, Shi G, Dou QP. The tumor proteasome is a primary target for the natural anticancer compound Withaferin A isolated from “Indian winter cherry”. *Mol Pharmacol.* 2007; 71:426–437. [PubMed: 17093135]
8. Stan SD, Hahm ER, Warin R, Singh SV. Withaferin A causes FOXO3a- and Bim-dependent apoptosis and inhibits growth of human breast cancer cells *in vivo*. *Cancer Res.* 2008; 68:7661–7669. [PubMed: 18794155]
9. Hahm ER, Lee J, Kim SH, et al. Metabolic alterations in mammary cancer prevention by withaferin A in a clinically relevant mouse model. *J Natl Cancer Inst.* 2013; 105:1111–1122. [PubMed: 23821767]
10. Stan SD, Zeng Y, Singh SV. Ayurvedic medicine constituent withaferin a causes G2 and M phase cell cycle arrest in human breast cancer cells. *Nutr Cancer.* 2008; 60:51–60. [PubMed: 19003581]
11. Malik F, Kumar A, Bhushan S, et al. Reactive oxygen species generation and mitochondrial dysfunction in the apoptotic cell death of human myeloid leukemia HL-60 cells by a dietary compound withaferin A with concomitant protection by *N*-acetyl cysteine. *Apoptosis.* 2007; 12:2115–2133. [PubMed: 17874299]
12. Samadi AK, Tong X, Mukerji R, Zhang H, Timmermann BN, Cohen MS. Withaferin A, a cytotoxic steroid from *Vassobia breviflora*, induces apoptosis in human head and neck squamous cell carcinoma. *J Natl Prod.* 2010; 73:1476–1481.
13. Ichikawa H, Takada Y, Shishodia S, Jayaprakasam B, Nair MG, Aggarwal BB. Withanolides potentiate apoptosis, inhibit invasion, and abolish osteoclastogenesis through suppression of nuclear factor- κ B (NF- κ B) activation and NF- κ B-regulated gene expression. *Mol Cancer Ther.* 2006; 5:1434–1445. [PubMed: 16818501]

14. Koduru S, Kumar R, Srinivasan S, Evers MB, Chendil D. Notch-1 inhibition by Withaferin-A: A therapeutic target against colon carcinogenesis. *Mol Cancer Ther.* 2010; 9:202–210. [PubMed: 20053782]
15. Lee J, Hahm ER, Singh SV. Withaferin A inhibits activation of signal transducer and activator of transcription 3 in human breast cancer cells. *Carcinogenesis.* 2010; 31:1991–1998. [PubMed: 20724373]
16. Hahm ER, Lee J, Huang Y, Singh SV. Withaferin A suppresses estrogen receptor- α expression in human breast cancer cells. *Mol Carcinog.* 2011; 50:614–624. [PubMed: 21432907]
17. Lee J, Sehrawat A, Singh SV. Withaferin A causes activation of Notch2 and Notch4 in human breast cancer cells. *Breast Cancer Res Treat.* 2012; 136:45–56. [PubMed: 22965833]
18. Mohan R, Hammers HJ, Bargagna-Mohan P, et al. Withaferin A is a potent inhibitor of angiogenesis. *Angiogenesis.* 2004; 7:115–122. [PubMed: 15516832]
19. Thaiparambil JT, Bender L, Ganesh T, et al. Withaferin A inhibits breast cancer invasion and metastasis at sub-cytotoxic doses by inducing vimentin disassembly and serine 56 phosphorylation. *Int J Cancer.* 2011; 129:2744–2755. [PubMed: 21538350]
20. Bargagna-Mohan P, Hamza A, Kim YE, et al. The tumor inhibitor and antiangiogenic agent withaferin A targets the intermediate filament protein vimentin. *Chem Biol.* 2007; 14:623–634. [PubMed: 17584610]
21. Lahat G, Zhu QS, Huang KL, et al. Vimentin is a novel anti-cancer therapeutic target; insights from *in vitro* and *in vivo* mice xenograft studies. *PLoS One.* 2010; 5:e10105. [PubMed: 20419128]
22. Tomaskovic-Crook E, Thompson EW, Thiery JP. Epithelial to mesenchymal transition and breast cancer. *Breast Cancer Res.* 2009; 11:213. [PubMed: 19909494]
23. Xiao D, Vogel V, Singh SV. Benzyl isothiocyanate-induced apoptosis in human breast cancer cells is initiated by reactive oxygen species and regulated by Bax and Bak. *Mol Cancer Ther.* 2006; 5:2931–2945. [PubMed: 17121941]
24. Xiao D, Srivastava SK, Lew KL, et al. Allyl isothiocyanate, a constituent of cruciferous vegetables, inhibits proliferation of human prostate cancer cells by causing G₂/M arrest and inducing apoptosis. *Carcinogenesis.* 2003; 24:891–897. [PubMed: 12771033]
25. Powolny AA, Bommarreddy A, Hahm ER, et al. Chemopreventative potential of the cruciferous vegetable constituent phenethyl isothiocyanate in a mouse model of prostate cancer. *J Natl Cancer Inst.* 2011; 103:571–584. [PubMed: 21330634]
26. Sehrawat A, Singh SV. Benzyl isothiocyanate inhibits epithelial-mesenchymal transition in cultured and xenografted human breast cancer cells. *Cancer Prev Res.* 2011; 4:1107–1117.
27. Gamallo C, Palacios J, Suarez A, et al. Correlation of E-cadherin expression with differentiation grade and histological type in breast carcinoma. *Am J Pathol.* 1993; 142:987–993. [PubMed: 7682767]
28. Blick T, Widodo E, Hugo H, et al. Epithelial mesenchymal transition traits in human breast cancer cell lines. *Clin Exp Metastasis.* 2008; 25:629–642. [PubMed: 18461285]
29. Hahm ER, Moura MB, Kelley EE, Van Houten B, Shiva S, Singh SV. Withaferin A-induced apoptosis in human breast cancer cells is mediated by reactive oxygen species. *PLoS One.* 2011; 6:e23354. [PubMed: 21853114]
30. Hahm ER, Singh SV. Withaferin A-induced apoptosis in human breast cancer cells is associated with suppression of inhibitor of apoptosis protein expression. *Cancer Lett.* 2013; 334:101–108. [PubMed: 22935676]
31. Hahm ER, Lee J, Singh SV. Role of mitogen-activated protein kinases and Mcl-1 in apoptosis induction by withaferin A in human breast cancer cells. *Mol Carcinog.* 2013 In Press.
32. Arias AM. Epithelial mesenchymal interactions in cancer and development. *Cell.* 2001; 105:425–431. [PubMed: 11371340]
33. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer.* 2002; 2:442–454. [PubMed: 12189386]
34. Creighton CJ, Chang JC, Rosen JM. Epithelial-mesenchymal transition (EMT) in tumor-initiating cells and its clinical implications in breast cancer. *J Mammary Gland Biol Neoplasia.* 2010; 15:253–260. [PubMed: 20354771]

35. Hollestelle A, Peeters JK, Smid M, et al. Loss of E-cadherin is not a necessity for epithelial to mesenchymal transition in human breast cancer. *Breast Cancer Res Treat.* 2013; 138:47–57. [PubMed: 23338761]
36. Mohammadizadeh F, Ghasemibasir H, Rajabi P, Naimi A, Eftekhari A, Mesbah A. Correlation of E-cadherin expression and routine immunohistochemistry panel in breast invasive ductal carcinoma. *Cancer Biomark.* 2009; 5:1–8. [PubMed: 19242056]
37. Kowalski PJ, Rubin MA, Kleer CG. E-cadherin expression in primary carcinomas of the breast and its distant metastases. *Breast Cancer Res.* 2003; 5:R217–R222. [PubMed: 14580257]
38. Whipple RA, Balzer EM, Cho EH, Matrone MA, Yoon JR, Martin SS. Vimentin filaments support extension of tubulin-based microtentacles in detached breast tumor cells. *Cancer Res.* 2008; 68:5678–5688. [PubMed: 18632620]
39. Chen MH, Yip GW, Tse GM, et al. Expression of basal keratins and vimentin in breast cancers of young women correlates with adverse pathologic parameters. *Mod Pathol.* 2008; 21:1183–1191. [PubMed: 18536655]

Figure 1.

WA inhibits TNF-α/TGF-β-induced EMT and cell migration in MCF-10A cells. (A) Chemical structures of withaferin A (WA), withanone (WE), and withanolide A (WLA). (B) Western blotting for E-cadherin and vimentin using cellular lysates from MCF-10A cells after 8 h treatment with DMSO (control), 2 μM of withanolides (WA, WE, and WLA) in the absence or presence of 10 ng/mL each of TNF-α and TGF-β. (C) Representative photomicrographs depicting MCF-10A cell migration after 24 h treatment with DMSO (panels a and e), WA (panel b), WE (panel f) and WLA (panel g), TNF-α/TGF-β alone (panels c and h), and combination of withanolides and TNF-α/TGF-β (panels d, i, and j) (×10 objective magnification). (D) Quantitation of MCF-10A cell migration from data shown in Figure 1C. Results shown are mean ± SD (n=3). Significantly different ($P < 0.05$)

compared with *DMSO control, and #between with and without TNF- α /TGF- β by one-way ANOVA followed by Bonferroni's multiple comparison test. Data shown are representative of at least two independent experiments.

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Figure 2.

E-cadherin knockdown promotes MCF-10A cell migration. (A) Western blot for E-cadherin using cellular lysates from MCF-10A cells transfected with a control siRNA (100 nM) or E-cadherin-targeted siRNA (100 nM) and treated for 24 h with DMSO (control) or WA (2 μM). (B) Representative photomicrographs depicting MCF-10A cell migration after transfection with 100 nM of a control siRNA or 100 nM of E-cadherin-targeted siRNA and treated for 24 h with DMSO (control), WA (2 μM) and/or TNF-α/TGF-β (10 ng/mL of each) (×10 objective magnification). (C) Quantitation of the results shown in Figure 2B. Results shown are mean ± SD (n=3). Significantly different ($P < 0.05$) compared with *corresponding DMSO-treated control, #between with and without TNF-α and TGF-β treatments, and \$between the indicated groups by one-way ANOVA followed with Tukey's adjustment. The results are representative of at least two independent experiments.

Figure 3.

Effect of WA treatment on EMT markers in breast cancer cells. (A) Immunofluorescence microscopy for E-cadherin protein expression in MCF-7 cells after 24 h treatment with DMSO or 2 μ M WA. (B) Western blots for E-cadherin, occludin, fibronectin, and/or vimentin using lysates from MCF-7 and MDA-MB-231 cells after 8 h or 24 h treatment with DMSO (control) or WA. Quantitation of protein expression change relative to corresponding DMSO control is shown above band. The blots were re-probed with anti-actin antibody or α -tubulin antibody for normalization. Each experiment was repeated at least twice and representative data from one such experiment are shown.

Figure 4.

The role of caspases in apoptosis induction by WA. Western blotting for full-length caspases using lysates from (A) MDA-MB-231 and (B) MCF-7 cells treated with WA for the indicated times. Quantitation of protein expression change relative to corresponding DMSO control is shown above band. The blots were re-probed with anti-actin antibody for normalization. (C) Quantitation of histone-associated DNA fragment release into the cytosol in MCF-7 cells. The cells were pretreated for 2 h with inhibitors of caspase 9 (zLEHD-fmk) or caspase 8 (zIETD-fmk) and then treated with DMSO (control) or WA in the absence or presence of the caspase inhibitors for 36 h. Results shown are mean $\pm$ SD (n=3).

*Significantly different ($P < 0.05$) between the indicated groups by one-way ANOVA followed by Bonferroni's multiple comparison test.

Figure 5.

Vimentin is dispensable for apoptosis induction by WA. (A) Western blot for vimentin protein using lysates from MDA-MB-231 cells transfected with 100 nM of a control siRNA (lane 1) or 100 nM of a vimentin-targeted siRNA (lane 2). (B) Representative flow histograms depicting apoptotic fraction in MDA-MB-231 cells transfected with a control siRNA (100 nM) and a vimentin siRNA (100 nM) and treated for 24 h with DMSO (control) or WA (2 or 4 μM). (C) Quantitation of apoptotic fraction (early+ late apoptotic cells) from results shown in Figure 5B. Results shown are enrichment of apoptotic fraction relative to corresponding DMSO-treated control [mean ± SD (n=3)]. *Significantly different ($P < 0.05$) between the indicated groups by one-way ANOVA followed by Bonferroni's multiple comparison test. (D) Proliferation of MDA-MB-231 cells transfected with 200 nM of a control siRNA or 200 nM of a vimentin-targeted siRNA and treated for 48 h with DMSO (control) or WA (2 or 4 μM). Results shown are mean ± SD (n=4). *Significantly different ($P < 0.05$) between the indicated groups by one-way ANOVA followed by Bonferroni's multiple comparison test. (E) Quantitation of histone-associated DNA fragment release into the cytosol in MDA-MB-231 cells transfected with 200 nM of a control siRNA or 200 nM of a vimentin-targeted siRNA and treated for 48 h with DMSO (control) or 4 μM of WA. Results shown are mean ± SD (n=3). *Significantly different ($P < 0.05$) between the indicated groups by one-way ANOVA followed by Bonferroni's multiple comparison test. (F) Western blot for vimentin, cleaved PARP, and cleaved caspase-3 using lysates from MDA-MB-231 cells transfected with 200 nM of a control siRNA or 200 nM of a vimentin-targeted siRNA and treated for 48 h with DMSO (control) or WA (2 or 4 μM). Similar results were observed in replicate experiments.

Figure 6.

Vimentin protein knockdown reduces MDA-MB-231 cell migration. (A) Representative photomicrographs depicting MDA-MB-231 cell migration after transfection with 100 nM of a control siRNA or 100 nM of a vimentin-targeted siRNA and treated for 24 h with DMSO (control) or WA (2 or 4 μM) (×10 objective magnification). (B) Quantitation of the results shown in Figure 6A. Cell migration relative to DMSO-treated control siRNA transfected cells (first bar from left) is shown. Results shown are mean ± SD (n=3). *Significantly different ($P < 0.05$) between the indicated groups by one-way ANOVA followed by Bonferroni's multiple comparison test. The results are representative of at least two independent experiments.

Figure 7.

WA administration decreases expression of vimentin protein *in vivo* in MDA-MB-231 xenografts. (A) Western blot for E-cadherin and vimentin using MDA-MB-231 xenograft supernatants from control and WA-treated mice. MDA-MB-231 xenograft supernatants from 4 different mice of each group were used. (B) Immunohistochemistry for vimentin protein expression in MDA-MB-231 xenograft sections from two mice each of the control group (a and b) and the WA-treatment group (c and d) (scale bar = 100 μ m). (C) Quantitation of vimentin protein expression from results shown in Figure 7B (n=7 for the control group and n=8 for the WA treatment group). Results shown are mean $\pm$ SD.

*Significantly different ($P < 0.05$) compared with control by Student's *t*-test.

Figure 8.

WA administration decreases expression of vimentin protein *in vivo* in mammary tumors from MMTV-*neu* mice. Immunohistochemistry for (A) E-cadherin and (B) vimentin protein expression in tumors from two representative mice each of the control group (a and b) and the WA treatment group (c and d). (scale bar = 20 μ m). Bar graphs show quantitation of E-cadherin and vimentin protein expression. Results shown are mean $\pm$ SD (n=7).

*Significantly different ($P < 0.05$) compared with control by Student's *t*-test.