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Peptidyl-prolyl *cis/trans* isomerase Pin1 regulates withaferin A-mediated cell cycle arrest in human breast cancer cells

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Abstract

We have reported previously that withaferin A (WA) prevents breast cancer development in mouse mammary tumor virus-*neu* (MMTV-*neu*) transgenic mice, but the mechanism is not fully understood. Unbiased proteomics of the mammary tumors from control- and WA-treated MMTV-*neu* mice revealed downregulation of peptidyl-prolyl *cis/trans* isomerase (Pin1) protein by WA administration. The present study extends these findings to elucidate the role of Pin1 in cancer chemopreventive mechanisms of WA. The mammary tumor level of Pin1 protein was lower by about 55% in WA-treated rats exposed to *N*-methyl-*N*-nitrosourea, compared to control. Exposure of MCF-7 and SK-BR-3 human breast cancer cells to WA resulted in downregulation of Pin1 protein. Ectopic expression of Pin1 attenuated G₂ and/or mitotic arrest resulting from WA treatment in both MCF-7 and SK-BR-3 cells. WA-induced apoptosis was increased by Pin1 overexpression in MCF-7 cells but not in the SK-BR-3 cell line. In addition, molecular docking followed by mass spectrometry indicated covalent interaction of WA with cysteine 113 of Pin1. Overexpression of Pin1^{C113A} mutant failed to attenuate WA-induced mitotic arrest or apoptosis in the MCF-7 cells. Furthermore, antibody array revealed upregulation of proapoptotic insulin-like growth factor binding proteins (IGFBPs), including IGFBP-3, IGFBP-4, IGFBP-5, and IGFBP-6, in Pin1 overexpressing MCF-7 cells following WA treatment when compared to empty vector transfected control cells. These data support a crucial role of the Pin1 for mitotic arrest and apoptosis signaling by WA at least in the MCF-7 cells.

Keywords

withaferin A; Pin1; mitotic arrest; chemoprevention

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CONFLICT OF INTEREST

The authors do not declare any conflict of interest.

1. INTRODUCTION

Impact from breast cancer, which is the most frequently diagnosed malignancy in women worldwide, is realized by nearly 40,000 deaths in the United States alone even after a comprehensive understanding of the risk factors and genomic landscape of the disease.^{1–3} Prevention is appealing to diminish the death and anguish from breast cancer as evidenced by clinical targeting of estrogen receptor positive breast cancers with selective estrogen receptor modulators as well as aromatase inhibitors.^{4–8} Ingredients of Ayurvedic medicine, which is practiced in India and neighboring countries, continue to garner research interest for prevention of breast and other cancers.^{9,10} Root of *Withania somnifera*, which is also known as Ashwagandha, Indian winter cherry or Indian ginseng belonging to the *Solanaceae* family of flowering plants, is used quite heavily in Ayurvedic medicine formulations.¹¹ Anticancer effect of this plant is attributable to steroidal lactones including withaferin A (WA), 12-deoxywithastramonolide, withanone, withanolide A, and so forth.^{11,12} Cellular studies have revealed a much greater sensitivity of breast cancer cells to growth inhibition by WA when compared to withanone or withanolide A.¹³

Cancer chemopreventive effect of WA has been demonstrated in preclinical rodent models of breast and other cancers.^{14–17} For example, the incidence of 7,12-dimethylbenz[*a*]anthracene-induced oral cancer in hamsters was completely inhibited by oral administration of 20 mg WA/kg body (three times/week) for 14 weeks.¹⁴ Oral administration of WA (3 and 5 mg/kg body weight) was also effective for prevention of prostate cancer development in a transgenic mouse model.¹⁵ Experimental evidence for chemopreventive efficacy of WA against breast cancer was provided by our research group.^{16,17} We were the first to demonstrate *in vivo* efficacy of WA for chemoprevention of human epidermal growth factor receptor 2-driven estrogen receptor-negative breast cancer using mouse mammary tumor virus-*neu* (MMTV-*neu*) transgenic mouse model.¹⁶ In this study, macroscopic mammary tumor size, microscopic mammary tumor area, and the incidence of pulmonary metastasis were significantly lower in MMTV-*neu* mice after 28 weeks of treatment with WA (~4 mg/kg body weight intraperitoneally, three times/week) compared with controls.¹⁶ In a follow-up study, we reported prevention of *N*-methyl-*N*-nitrosourea (MNU)-induced luminal-type (predominantly estrogen receptor-positive) breast cancer in rats by WA administration.¹⁷ Consistent with *in vitro* data, breast cancer chemoprevention by WA treatment in MMTV-*neu* mice as well as in the rat model was associated with reduced cell proliferation, increased apoptosis, and inhibition of breast cancer stem cell-like population.^{16–26}

We showed previously that breast cancer prevention by WA in MMTV-*neu* mice was associated with a significant decrease in protein levels of peptidyl-prolyl *cis/trans* isomerase (Pin1) through unbiased proteomics.¹⁶ The Pin1 protein, which plays an important role in mammary gland development as well as in various steps of breast cancer progression, catalyzes *cis/trans* isomerization of phospho-Ser/Thr-Pro motifs in many proteins.^{27–29} Because Pin1 controls isomerization of numerous cancer-relevant proteins, it is considered a valid therapeutic target.^{28,29} The present study was undertaken to determine the functional significance of Pin1 in chemopreventive mechanisms of WA in breast cancer.

2. MATERIALS AND METHODS

2.1 Reagents

Withaferin A (WA, purity > 95%) was purchased from ChromaDex (Irvine, CA) and dissolved in dimethyl sulfoxide (DMSO). Culture media were purchased from MediaTech (Manassas, VA). Fetal bovine serum and antibiotics were purchased from Invitrogen-Life Technologies (Carlsbad, CA). Annexin V/propidium iodide assay kit for apoptosis detection was purchased from BD Biosciences (San Jose, CA), whereas Cell Death Detection ELISA^{PLUS} kit was from Roche Diagnostics (Indianapolis, IN). Antibodies against Pin1 and Cdc25C were purchased from Cell Signaling Technology (Danvers, MA); anti-Cyclin B1 and anti-Cdc2 antibodies were from Santa Cruz Biotechnology (Dallas, TX); anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was from GeneTex (Irvine, CA); and anti-β-Actin and anti-β-Tubulin antibodies were from Sigma-Aldrich (St. Louis, MO). Recombinant human Pin1 protein was purchased from MyBioSource (San Diego, CA). Human Apoptosis Antibody Array was from Abcam (Cambridge, MA).

2.2 Cell lines and cell culture

Human breast cancer cell lines MCF-7 and SK-BR-3 were obtained from the American Type Culture Collection (Manassas, VA) and last authenticated by us in March of 2017. Monolayer cultures of MCF-7 and SK-BR-3 cells were maintained as suggested by the supplier. MCF-7 cells stably transfected with empty pcDNA3 vector or the same vector encoding for myc-tagged Pin1 were cultured as previously described.³⁰ SK-BR-3 cells were transiently transfected with empty pcDNA3 vector or the same vector encoding for myc-tagged Pin1. Mutant EGFP-N1-Pin1^{C113A} plasmid was provided by GENEWIZ (South Plainfield, NJ). MCF-7 cells were stably transfected with empty EGFP-N1 vector or mutant EGFP-N1-Pin1^{C113A} plasmid using FuGENE6, and stable clones were selected in the presence of 1 mg/mL of G418 for 2 months.

2.3 Immunohistochemistry

Immunohistochemistry for Pin1 protein in tumor sections of control- and WA-treated MMTV-*neu* mice was performed as described by us previously for other proteins.^{16,17} Immunohistochemical images were analyzed using the Aperio ImageScope software which provides quantitative assessment of immunohistochemical staining with specified algorithm-based scoring method. Quantitative results are expressed as H-score. The H-score is a widely accepted method for quantitation of immunohistochemical data. The H-score is based on intensity (0, 1+, 2+, and 3+) and % positivity (0–100%) and calculated using the formula: H-score = (% negative cells × 0) + (% 1+ cells × 1) + (% 2+ cells × 2) + (% 3+ cells × 3).¹⁶

2.4 Western blot analysis

Details of tumor supernatant preparation for immunoblotting have been described by us previously.¹⁶ Cells (5×10⁵ cells per dish) were seeded in 6-cm dishes, allowed to attach, and then exposed to DMSO or the indicated dose(s) of WA for specified time period. Details of cell lysate preparation and western blotting have been described by us previously.³¹ Membranes were stripped and re-probed with anti-GAPDH or anti-β-Actin antibody to

correct for difference in protein loading. Proteins of interest were quantitated by using UN-SCAN-IT gel automated digitizing system (Version 5.1, Silk Scientific, Orem, UT).

2.5 Confocal microscopy

Cells (1×10^5 cells per well) were plated on glass coverslips in 12-well plates in triplicate and allowed to attach by overnight incubation. After 24 h of treatment, cells were fixed in 2% paraformaldehyde followed by permeabilization with Triton X-100. After blocking with 3% bovine serum albumin in phosphate-buffered saline for 30 min at room temperature, cells were incubated with anti-Pin1 antibody for overnight at 4°C followed by incubation with Alexa Fluor 488-conjugated secondary antibody for 1 h. DRAQ5 was used to stain nuclear DNA. The coverslips were mounted and examined using a LEICA confocal microscope.

2.6 Analysis of cell cycle distribution

Quantification of the G₂ and mitotic population in DMSO- and WA-treated cells was accomplished by flow cytometry after staining the cells with propidium iodide and anti-phospho (S10)-Histone H3 antibody. Cells (5×10^5 cells per well) were seeded in 6-well plates in triplicate and allowed to attach by overnight incubation. Cells were then treated with DMSO or desired concentration of WA for 24 h. Subsequently, cells were trypsinized, washed with phosphate-buffered saline, and fixed in 70% ethanol at 4°C overnight. Cells were permeabilized with 0.25% Triton X-100 for 15 min at room temperature, incubated with Alexa Fluor 488-conjugated anti-phospho-(S10)-Histone-H3 antibody for 1 h, stained with propidium iodide for 30 min at room temperature, and analyzed using the BD Accuri C6 flow cytometer.

2.7 Apoptosis detection

Apoptosis was measured by the Annexin V/propidium iodide method. Cells (2×10^5 cells per well) were seeded in 6-well plates in triplicate, allowed to attach by overnight incubation, and then exposed to DMSO or desired concentration of WA for 24 h or 48 h. The floating and adherent cells were collected, washed with phosphate-buffered saline, and suspended in 100 µL of binding buffer supplied with the kit. Subsequently, 4 µL of Annexin V and 2 µL of propidium iodide was added to each sample. After 15 min of incubation in the dark, Annexin V and propidium iodide staining were analyzed using a flow cytometer. The Cell Death Detection ELISA^{PLUS} kit detects apoptotic death in cells by measuring histone-associated DNA fragment release into the cytosol. Cells (1×10^5 cells per well) were plated in triplicate in 12-well plates, allowed to attach, and then treated with DMSO or desired concentration of WA for 48 h. Cells were then processed for quantification of histone-associated DNA fragment release according to the manufacturer's instructions.

2.8 Molecular docking

Molecular docking was done using Hex8.0 software (<http://hex.loria.fr>). The structure used for docking was from Protein Data Base (PDB ID for human Pin1 3TBD) with resolution 2.27Å. Output of the docking was analyzed using Discovery Studio Client 3.5 software.

2.9 Mass spectrometric analysis of interaction of WA with Pin1

Recombinant human Pin1 protein was dialyzed in phosphate-buffered saline for 2 h to remove dithiothreitol. Subsequently, 2.5 µg of Pin1 protein was suspended in phosphate-buffered saline and incubated with 10 µM of WA or DMSO (control) for 4 h at 37°C. Protein sample was reduced/alkylated, precipitated, re-suspended in 100 mM triethyl ammonium bicarbonate buffer, and digested overnight with proteomic grade trypsin. The digested samples were loaded on an SCX column and eluted with 150 mM and 450 mM of ammonium acetate. The fractions were dried down in a speed-vac and suspended in 0.1% formic acid. Prior to liquid chromatography-tandem mass spectrometry (LC-MS/MS), peptides were desalted, dried in a speed-vac, and reconstituted in a solution containing 2% acetonitrile and 0.1% formic acid. For LC-MS/MS, each sample was loaded onto a C18 column (New Objective, Woburn, MA) using a Thermo Scientific Surveyor Auto-sampler operating in the no-waste injection mode. Peptides were eluted from the column using a linear acetonitrile gradient from 2 to 30% acetonitrile over 150 min followed by high and low organic washes for another 30 min using an LTQ XL mass spectrometer via a nanospray source with the spray voltage set to 1.8 kV and the ion transfer capillary set at 180°C. A full MS scan from m/z 350–1700 was performed by MS/MS scans on the three most abundant ions. Raw data files were searched against the most recent Uniprot database for human Pin1 protein. All searches were performed with Proteome Discoverer 1.4 (Thermo Scientific) and the SEQUEST HT algorithm using the MudPIT search protocol.

2.10 Antibody array

MCF-7 cells stably transfected with empty pcDNA3 vector or the same vector encoding for myc-tagged Pin1 were treated with DMSO or 1 µM WA for 24 h. Cells were then used for antibody array for proapoptotic [e.g., Bax, Bid, insulin-like growth factor binding protein (IGFBP)] and anti-apoptotic proteins (e.g., Bcl-2, inhibitor of apoptosis family proteins). Instructions from the supplier of the kit were followed for quantitation of protein expression changes.

2.11 Statistical analysis

Unpaired student's t-test was used for two sample comparisons. One-way analysis of variance (ANOVA) followed by Bonferroni's test was used for multiple comparison. Difference was considered significant at $P < 0.05$. Statistical analyses were performed using GraphPad Prism 7.02 (La Jolla, CA).

3. RESULTS

3.1 WA administration decreased Pin1 protein expression

Initially we used mammary tumor sections from control- and WA-treated (structure of WA is shown in Figure 1A) MMTV-*neu* mice to confirm the proteomics data showing downregulation of Pin1 by WA administration.¹⁶ Representative microscopic images depicting expression of Pin1 protein in mammary tumor sections of control- and WA-treated MMTV-*neu* mice are shown in Figure 1B. Protein level of Pin1 was modestly but statistically significantly lower in the mammary tumor sections of WA-treated mice

compared with control (Figure 1C). We also used tumor supernatants from control- and WA-treated rats¹⁷ to determine Pin1 expression by western blotting (Figure 1D). Level of Pin1 protein was lower by about 55% in breast tumors of WA-treated rats when compared to that of controls (Figure 1E) but the difference did not reach statistical significance due to large data scatter and small sample size (n=6). Nevertheless, these results indicated downregulation of Pin1 protein in breast tumors by WA administration in mice and rats.

3.2 Pin1 regulated cell cycle arrest by WA treatment

Next, we used two well-characterized human breast cancer cell lines (MCF-7 and SK-BR-3) to determine the functional significance of Pin1 downregulation in cancer chemopreventive mechanisms of WA. Arrest of cells in G₂/M phase of the cell cycle and apoptosis induction are two main effects of WA treatment in breast cancer cells.^{13,19,20} Pharmacokinetic studies in mice have shown a peak plasma concentration of 1.8 μ M following a single intraperitoneal injection of 4 mg WA/kg body weight.³² We therefore used WA concentrations of 2 μ M or lower in cellular experiments. Confocal microscopy indicated suppression of Pin1 protein level following WA treatment in both cell lines (Figure 1F), which was confirmed by western blotting (Figure 2A). Figure 2B shows overexpression of Pin1 protein in transfected MCF-7 (stable transfection) and SK-BR-3 cells (transient transfection) when compared to corresponding empty vector transfected control cells. Treatment of empty vector transfected control MCF-7 cells with 1 μ M WA for 24 h resulted in a >4-fold enrichment of mitotic fraction (Figure 2C, D). WA-mediated mitotic arrest was fully abolished by Pin1 overexpression in MCF-7 cells (Figure 2D). Likewise, the G₂/M phase cell cycle arrest resulting from WA exposure was significantly attenuated by stable overexpression of Pin1 in MCF-7 cells (Figure 2E). On the other hand, WA-mediated apoptosis determined by the Annexin V/propidium iodide method (Figure 2F) was significantly increased in Pin1 overexpressing MCF-7 cells when compared to empty vector transfected control cells at both 24 and 48 h time points. Similar to MCF-7 cells, mitotic arrest caused by WA treatment was significantly lower in Pin1 overexpressing SK-BR-3 cells when compared to empty vector transfected cells (Figure 2G). However, cell line difference was discernible with regards to apoptosis induction. Unlike MCF-7 cells (Figure 2F), Pin1 overexpression was protective against WA-mediated apoptosis in SK-BR-3 cells (Figure 2H). The reasons for cell line-specific differences in WA-induced apoptosis in MCF-7 *versus* SK-BR-3 is not yet clear but may be related to differences in expression levels of other proteins implicated in apoptotic cell death regulation.

3.3 Pin1 overexpression attenuated cyclin B1 stabilization by WA treatment in MCF-7 cells

We have shown previously that WA-mediated cell cycle arrest in MCF-7 cells is associated with stabilization of Cyclin B1 level but downregulation of Cdc25C, Cdc2, and β -Tubulin proteins.^{13,19} Downregulation of Cdc25C, Cdc2, or β -Tubulin proteins resulting from WA treatment was not affected by Pin1 overexpression in either MCF-7 or SK-BR-3 cells (Figure 3). However, effect of WA treatment was different on Cyclin B1 in MCF-7 *versus* SK-BR-3 cells. In the MCF-7 cell line, WA-mediated stabilization of Cyclin B1 was nearly fully abolished by Pin1 overexpression. To the contrary, Cyclin B1 protein level was decreased by WA treatment in both empty vector transfected and Pin1 overexpressing SK-

BR-3 cells (Figure 3). These results illustrate complexity and cell line-specific differences in regulation of cell cycle arrest by WA treatment.

3.4 Covalent interaction of WA with Cys¹¹³ of Pin1

WA is a Michael acceptor (electrophile) capable of reacting with sulfhydryl group of cysteine residues in proteins, including β -Tubulin and vimentin.^{13,33} Pin1 protein has two cysteine residues at positions 57 and 113. Molecular docking suggested interaction of WA with Cys¹¹³ of Pin1 (Figure 4A). The Cys¹¹³-WA adduct is shown as a sphere model with an atomic color scheme (carbon in *green*, oxygen in *red*, and hydrogen in *white*). Covalent interaction of WA with Cys¹¹³ was confirmed by mass spectrometry (Figure 4B–D). Figure 4B–D show Pin1 peptide sequence IKSGEEDFESLASQFSDCSSAK, and tandem mass spectrum of the peptide IKSGEEDFESLASQFSDCSSAK with sequence-specific product ions (b18 and y5) confirmed the presence of Cys¹¹³-WA adduct.

Given that Cys113-Pin1 is a crucial site for its catalytic activity²⁷ and evidence of WA adduct formation at this site in the present study, we generated Cys-113-Ala mutant of Pin1 (Pin1^{C113A}) for the functional study. We transfected MCF-7 cells with EGFP-N1 empty vector or the same vector encoding Pin1^{C113A}. Stable transfection was confirmed by microscopy for EGFP fluorescence (data not shown). Analyses of G₂/M phase cells (Figure 5A), mitotic fraction (Figure 5B), and apoptosis (Figure 5C) revealed that overexpression of Pin1^{C113A} mutant failed to attenuate WA-induced cell cycle arrest or apoptosis in the MCF-7 cells. Overexpression of Pin1^{C113A} mutant resulted in augmentation of WA-induced mitotic arrest, but not G₂/M population or apoptosis. These results indicated functional significance of WA interaction with Pin1 in mitotic arrest.

3.5 Pin1 overexpression resulted in induction of proapoptotic IGFBP family members following WA treatment in MCF-7 cells

Antibody array (composition of the array is shown in Figure 6A) was performed using empty vector transfected control and Pin1 overexpressing MCF-7 cells following 24 h treatment with DMSO or 1 μ M WA (Figure 6B). The most robust changes were evident for several members of the IGFBP family (IGFBP-3, IGFBP-4, IGFBP-5, and IGFBP-6) and death receptor pathway (DR6, sTNF-R1, sTNF-R2, and TRAIL-1) (Figure 6C). Figure 7A, B show antibody array data for other apoptosis regulating proteins. Interestingly, IGF-I, IGF-II, and IGF-1sR levels were also increased upon WA treatment in Pin1 overexpressing MCF-7 cells when compared to empty vector transfected control cells (Figure 7C). These results suggest that increased apoptosis following WA treatment in Pin1 overexpressing MCF-7 cells is likely mediated by induction of proapoptotic IGFBP family members and/or death receptor pathways.

4. DISCUSSION

The present study shows downregulation of Pin1 by WA treatment in breast cancer cells. Overexpression of Pin1 protein in breast cancer is associated with poor clinical outcome.^{29,34,35} Pin1 plays an important role in normal mammary development as well as in breast cancer progression by regulating stability or activity of numerous proteins.²⁹ Some of the

targets of Pin1 (e.g., estrogen receptor- α transcription, STAT3, NF- κ B, Notch-1) are also downregulated by WA treatment. For example, the Pin1 binding motif in STAT3 is Ser727, which promotes its transcriptional activity.³⁶ We have shown previously that WA treatment inhibits constitutive as well as interleukin-6-inducible activation of STAT3 in breast cancer cells.²¹ We also showed that WA-mediated inhibition of STAT3 activation was accompanied by suppression of Tyr705 and Ser727 phosphorylation and its dimerization.²¹ It is possible that WA treatment inhibits Ser727 phosphorylation of STAT3 in a Pin1-dependent manner. Likewise, we have shown previously that WA downregulates expression of estrogen receptor- α ,²³ which is another target of Pin1. Pin1 was shown to regulate N-terminal conformation and function of estrogen receptor- α .³⁷ Another study showed a role for Pin1 in regulation of estrogen receptor- α level through phosphorylation-dependent ubiquitination and degradation.³⁸ These examples illustrate that some effects of WA in breast cancer cells may be mediated by Pin1.

WA treatment causes arrest of breast cancer cells in G₂ and mitotic phases of the cell cycle, which is not a cell line-specific phenomenon.^{13,19} It was shown previously that Pin1 depletion in yeast and HeLa cells caused mitotic arrest but Pin1 overexpression in HeLa resulted in G₂ phase arrest.³⁹ Neither mitotic fraction nor G₂ population was affected by Pin1 overexpression in either MCF-7 or SK-BR-3 cells without WA treatment. While these results suggest cell line-dependent role for Pin1 in regulation of cell cycle progression, the mitotic arrest resulting from WA treatment was significantly attenuated by Pin1 overexpression in both MCF-7 and SK-BR-3 cells.

It is interesting to note that Pin1 overexpression accelerates WA-induced apoptosis in MCF-7 cells in association with induction of several proapoptotic members of the IGFBP family. For example, IGFBP-3 is the most abundant isoform in human serum and shown to inhibit cell proliferation and induce apoptosis in IGF-I-dependent (by binding to IGF-I and therefore preventing its interaction with its receptor) or -independent manner (e.g., inhibition of Akt in MCF-7 cells).⁴⁰ Likewise, IGFBP-5 overexpression resulted in G₂/M phase cell cycle arrest and apoptosis induction in human breast cancer cells.⁴¹ IGFBP-5-induced apoptosis was associated with a transcriptional increase in proapoptotic *bax* but downregulation of *bcl2* expression.⁴¹ We have shown previously that WA treatment causes an increase in *bax* and downregulation of *bcl2* in the MCF-7 cell line,²⁰ which may explain the proapoptotic effect of increased IGFBP-5 in this model. Overexpression of IGFBP-6 also inhibited rhabdomyosarcoma growth *in vivo*⁴² and its expression is also increased upon WA treatment in Pin1 overexpressing cells in comparison with empty vector transfected control MCF-7 cells (present study). It is important to point out that overexpression of Pin1 alone (without WA treatment) has no statistically significant effect on expression of IGFBP-3, IGFBP-4, IGFBP-5 or IGFBP-6. Increased expression of anti-apoptotic IGF-I and IGF-1sR by WA treatment in Pin1 overexpressing MCF-7 cells but not in the corresponding empty vector transfected control cells is also intriguing and suggests the complexity of the role of IGF and their binding proteins in regulation of WA-induced apoptosis in the context of Pin1 overexpression. SK-BR-3 cell line is insensitive to increased WA-mediated apoptosis upon Pin1 overexpression. Even though further work is needed to elucidate the mechanism for differential effect of Pin1 overexpression on WA-mediated apoptosis in MCF-7 *versus* SK-BR-3 cells, the difference may be related to p53 status. MCF-7 and SK-BR-3 cell lines

express wild-type and mutant p53, respectively. It has been shown previously that Pin1 activates the mitochondrial death program of p53.⁴³ We have shown previously that WA treatment increases protein level of p53 in MCF-7 cells.²³ In addition, WA-induced apoptosis in MCF-7 cell was partly but significantly attenuated by RNA interference of p53.²³

WA is an electrophile and hence can react with nucleophilic sites in proteins as illustrated by us previously for cysteine 303 of β -Tubulin.¹³ Because WA treatment causes production of reactive oxygen species in breast cancer cell lines (e.g., MCF-7), it has the ability to oxidize cysteine sulfhydryl group. Pin1 oxidation of Cys113 was shown to inhibit catalytic activity of the isomerase.^{44,45} The present study reveals covalent interaction of WA with Cys113 of Pin1. WA-induced apoptosis is not affected by overexpression of Pin1^{C113A} mutant. On the other hand, mitotic arrest resulting from WA treatment is augmented in MCF-7 cells with ectopic expression of the mutant Pin1. These results indicate functional significance of WA interaction with Pin1.

In conclusion, the present study reveals a role for Pin1 in regulation of cell cycle arrest and apoptosis by WA. Many of the mechanistic effects of WA (e.g., bax induction and bcl2 downregulation) may also be related to suppression of Pin1.

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Abbreviations

ANOVA	analysis of variance
DMSO	dimethyl sulfoxide
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
IGFBP	insulin-like growth factor binding protein
LC-MS/MS	liquid chromatography-tandem mass spectrometry
MNU	<i>N</i> -methyl- <i>N</i> -nitrosourea
Pin1	peptidyl-prolyl <i>cis/trans</i> isomerase
MMTV-<i>neu</i>	mouse mammary tumor virus- <i>neu</i>
WA	withaferin A

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FIGURE 1.

Withaferin A (WA) treatment downregulated protein level of Pin1 in breast cancer *in vivo* and *in vitro*. **(A)** Structure of WA. **(B)** Representative immunohistochemical images showing Pin1 expression in 3 different tumor sections of control- and WA-treated MMTV-*neu* mice (200× magnification; scale bar = 20 μm). **(C)** Quantitation of Pin1 expression in breast tumor sections of MMTV-*neu* mice. The bar graph shows mean Pin1 expression (H-score, positive pixel algorithm) with standard deviation (n = 7 for both control and WA treatment groups). *Statistically significant ($P < 0.05$) compared with control group by unpaired Student's t-test. **(D)** Western blot for Pin1 protein using breast tumor supernatants from control- and WA-treated rats. **(E)** Bar graph showing quantitation of Pin1 protein expression in rat tumors (mean ± SD; n = 6). **(F)** Representative confocal microscopic images (63× oil objective magnification; scale bar = 200 μm) showing Pin1 (green fluorescence) expression in MCF-7 and SK-BR-3 cells following 24 h treatment with DMSO or WA (2 μM). DRAQ5 (blue fluorescence) was used for nuclear staining. The results were consistent in replicate independent experiments.

FIGURE 2.

Pin1 overexpression in MCF-7 and SK-BR-3 cells attenuated WA-mediated mitotic arrest (A) Western blotting for Pin1 protein using lysates from MCF-7 and SK-BR-3 cells treated for 24 h with DMSO (control) or WA. (B) Western blotting for Pin1 protein using lysates from pcDNA3 empty vector (1) and myc-tagged Pin1 (2) transfected cells. Western blotting was performed at least twice. (C) Representative flow histograms depicting mitotic fraction (indicated by an arrow) in pcDNA3 empty vector transfected MCF-7 cells and those transfected with myc-tagged Pin1 and treated for 24 h with DMSO or 1 μ M of WA. (D) Quantitation of mitotic fraction in MCF-7 cells. Combined results from two experiments are shown as mean $\pm$ SD (n = 6). (E) Quantitation of G₂/M phase cells. Combined results from three experiments are shown as mean $\pm$ SD (n = 9). (F) Quantitation of total [early (Annexin V-positive) and late (Annexin V-positive and propidium iodide-positive)] apoptosis in

pcDNA3 empty vector transfected MCF-7 cells and those transfected with myc-tagged Pin1 and treated for 24 h or 48 h with DMSO or WA. Combined results from two experiments are shown as mean $\pm$ SD (n = 5~6). Effect of myc-Pin1 overexpression on WA-mediated (24 h treatment) **(G)** mitotic arrest and **(H)** total apoptosis in SK-BR-3 cells. Experiments were done at least twice with consistent results and representative data from one such experiment are shown as mean $\pm$ SD (n = 3). Statistically significant ($P < 0.05$) compared with the *corresponding DMSO-treated control or #between pcDNA3 and myc-Pin1 groups by one-way ANOVA followed by Bonferroni's multiple comparison test.

FIGURE 3.

Withaferin A (WA) treatment modulated expression of cell cycle regulatory proteins in MCF-7 and SK-BR-3 cells. Western blotting for Cdc25C, Cdc2, Cyclin B1, and β-Tubulin using lysates from pcDNA3 empty vector transfected and myc-tagged Pin1 overexpressing MCF-7 and SK-BR-3 cells treated for 24 h with DMSO or indicated doses of WA. Experiments were done at least twice and results were consistent.

FIGURE 4.

Withaferin A (WA) interacted covalently with Cys¹¹³ of human Pin1. **(A)** Model of the Cys¹¹³-WA adduct of human Pin1. *Right bottom*, comprehensive view of the model. The Cys¹¹³-WA adduct is shown as a sphere model with an atomic color scheme (carbon in *green*, oxygen in *red*, and hydrogen in *white*). *Left*, particulars of the WA-binding pocket. The human Pin1 model is shown as a ribbon diagram (helices as *spirals*, strands as *arrows*, and loops as *tubes*) with WA-interacting side chains as sticks (nitrogen in *blue*, carbon in *gray*, and oxygen in *red*), and the Cys¹¹³-WA adduct is shown as a stick model (nitrogen in *blue*, carbon in *green*, oxygen in *red*, and sulfur in *yellow*). **(B)** Human Pin1 peptide sequence IKSGEEDFESLASQFSDCSSAK. **(C)** Pin1 peptide sequence showing b-ions and y-ions. **(D)** Tandem mass spectrum of peptide IKSGEEDFESLASQFSDCSSAK with sequence-specific product ions (b18 and y5) confirming the presence of the Cys¹¹³-WA adduct.

FIGURE 5.

Overexpression of Pin1^{C113A} failed to attenuate withaferin A (WA)-induced mitotic arrest and apoptosis in MCF-7 cells. Quantitation of G₂/M phase cells (**A**), mitotic fraction (**B**), and release of histone-associated DNA fragments into the cytosol (**C**) in MCF-7 cells transfected with empty vector (EGFP-N1) and the same vector encoding Pin1^{C113A} mutant and treated with DMSO or the indicated dose(s) of WA for specified time periods. Combined results from multiple experiments are shown as mean $\pm$ SD (n = 9–12).

*Statistically significant ($P < 0.05$) compared to corresponding DMSO-treated control and

#between EGFP-N1 and Pin1^{C113A} groups by one-way ANOVA followed by Bonferroni's multiple comparison test.

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FIGURE 6.

Pin1 overexpression augmented WA-mediated increase in expression of proapoptotic IGFBP family proteins. **(A)** Human antibody array map with proapoptotic proteins numbered. **(B)** Antibody array showing expression of apoptosis regulating proteins. The antibody array was performed using lysates from pcDNA3 empty vector transfected MCF-7 cells or those transfected with myc-tagged Pin1 after 24 h treatment with DMSO or 1 μ M of WA. **(C)** Bar graph showing levels of proapoptotic proteins relative to DMSO-treated MCF-7 cells transfected with the empty vector (pcDNA3; mean $\pm$ SD; n = 2). Dotted lines indicate $\pm$ 2-fold changes in relative protein levels (i.e. 2 or 0.5).

Abbreviations: CD40, cluster of differentiation 40; CD40L, CD40 ligand; Cyto *c*, cytochrome *c*; DR6, death receptor 6; FasL, Fas ligand; IGFBP, insulin-like growth factor binding protein; SMAC, second mitochondria derived activator of caspase; sTNF-R, soluble

tumor necrosis factor receptor; TNF- β , tumor necrosis factor-beta; TRAILR, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor.

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FIGURE 7.

Pin1 overexpression and WA treatment altered the expression of anti-apoptotic proteins in MCF-7 cells. **(A)** Human antibody array map with anti-apoptotic proteins numbered. **(B)** Antibody array showing expression of anti-apoptotic proteins. Antibody array was performed using lysates from pcDNA3 empty vector transfected MCF-7 cells or those overexpressing myc-tagged Pin1 after 24 h treatment with DMSO or 1 μ M of WA. **(C)** Bar graph showing levels of anti-apoptotic proteins relative to DMSO-treated MCF-7 cells transfected with the empty vector (pcDNA3; mean $\pm$ SD; n = 2). Dotted lines indicate $\pm$ 2-fold changes in relative protein levels (i.e. 2 or 0.5).

Abbreviations: cIAP2, cellular inhibitor of apoptosis 2; HSP, heat shock protein; IGF, insulin-like growth factor; IGFBP, IGF binding protein; IGF-1sR, IGF-1 soluble receptor; TRAILR, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor; XIAP, X-linked inhibitor of apoptosis.