Par-4 Dependent Modulation of Cellular β-Catenin by Medicinal Plant Natural Product Derivative 3-azido Withaferin A

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Here, we provide evidences that natural product derivative 3-azido Withaferin A (3-AWA) abrogated EMT and invasion by modulating β-catenin localization and its transcriptional activity in the prostate as well as in breast cancer cells. This study, for the first time, reveals 3-AWA treatment consistently sequestered nuclear β-catenin and augmented its cytoplasmic pool as evidenced by reducing β-catenin transcriptional activity in these cells. Moreover, 3-AWA treatment triggered robust induction of pro-apoptotic intracellular Par-4, attenuated Akt activity and rescued Phospho-GSK3β (by Akt) to promote β-catenin destabilization. Further, our in vitro studies demonstrate that 3-AWA treatment amplified E-cadherin expression along with sharp downregulation of c-Myc and cyclin D1 proteins. Strikingly, endogenous Par-4 knock down by siRNA underscored 3-AWA mediated inhibition of nuclear β-catenin was Par-4 dependent and suppression of Par-4 activity, either by Bcl-2 or by Ras transfection, restored the nuclear β-catenin level suggesting Par-4 mediated β-catenin regulation was not promiscuous. In vivo results further demonstrated that 3-AWA was effective inhibitor of tumor growth and immunohistochemical studies indicated that increased expression of total β-catenin and decreased expression of phospho-β-catenin and Par-4 in breast cancer tissues as compared to normal breast tissue suggesting Par-4 and β-catenin proteins are mutually regulated and inversely co-related in normal as well as cancer condition. Thus, strategic regulation of intracellular Par-4 by 3-AWA in diverse cancers could be an effective tool to control cancer cell metastasis. Conclusively, this report puts forward a novel approach of controlling deregulated β-catenin signaling by 3-AWA induced Par-4 protein. © 2015 Wiley Periodicals, Inc.

Key words: Par-4; β-catenin; GSK3β; EMT; metastasis

INTRODUCTION

Beta-catenin (β-catenin) signaling is considered as the primary axis in cancer metastasis and drug resistance. Activating mutations of Wnt component are converged upon nuclear accumulation of β-catenin and are involved in tumor formation and development [1]. Deregulation of β-catenin signaling has been detected in a number of malignancies including prostate and breast cancer [2]. Progression of prostate cancer has been found to be intricately related to the loss of normal epithelial morphology, along with concomitant acquisition of invasive, metastatic, and ultimately fatal properties [3,4]. It is believed that cadherin–catenin complex plays a pivotal role in maintaining cellular homeostasis and disruption of this complex, primarily due to loss or reduced expression of E-cadherin and/or altered subcellular distribution of β-catenin, results in invasive behavior and poor clinical outcome in prostate cancer patients [5,6].

Inactivation of Glycogen synthase kinase 3 beta (GSK3β) by oncogenic processes depends on the phosphatidylinositol 3-kinase (PI3K) pathway leading to β-catenin dephosphorylation, its accumulation and translocation into the nucleus [7]. But the mechanisms underlying the regulation of GSK3β are poorly understood. As Akt in PI3K pathway serves a*

Abbreviations: DAPI, 4,6-diamidino-2-phenylindole; 3-AWA, 3-azido withaferin A; siRNA, small interfering RNA; GSK3β, Glycogen synthase kinase 3 beta; β-catenin, Beta-catenin; Par-4, Prostate apoptosis response 4; CaP, Prostate cancer.

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critical compensatory role by blocking the tumor suppressing property of GSK3β, removal of such a hindrance may improve this catastrophic condition. Unfortunately, inhibitors of this evolutionary conserved and biologically significant PI3K/Akt pathway have shown serious pitfalls while eliminating the disease [8]. Nevertheless, modulation of GSK3β activity may occur by other mechanisms for cancer cell survival and proliferation.

Prostate apoptosis response 4 (Par-4), a leucine zipper protein is intricately associated with tumor suppressing activity and its elevated expression is found in actively apoptosing cells [9]. On the other hand, Par-4 is down-regulated in many cancers [10–13], but in prostate carcinoma, the Par-4 inactivation occurs due to Akt1 mediated phosphorylation and abrogation of apoptotic potential of Par-4 [14]. Oncogenic transformation leads to activation of Akt that in turn phosphorylates number of downstream targets, majority of them are pro-apoptotic molecules including BAD, Caspase-9 and Par-4 [14,15]. Thus, stimulation of under expressed/inactivated Par-4 by strategic way could be a powerful tool for developing anti-cancer therapeutics.

Par-4, either intra-cellular or extra-cellular, could be stimulated by diverse therapeutic agents [16,17]. But induction due to dietary intake could have immense therapeutic windows and downgrades side effects including cytotoxicity/neurotoxicity. Withanolides are potential constituents of herbal medicinal plant, Withania somnifera and had long been prescribed as essential ayurvedic medication to treat vast range of ailments including inflammation, immunomodulatory and carcinogenesis [18–20].

Here, in continuation of our previous report, this study is designed to explore more detailed mechanism of 3-AWA induced intracellular Par-4 mediated effect on upstream effectors of MMP-2, the β-catenin localization and transcriptional regulation. Our investigation has revealed that Akt inactivation by 3-AWA treatment along with stimulation of endogenous Par-4 might be an essential tool to control deregulated β-catenin signaling in cancer progression.

MATERIALS AND METHODS

Cell Culture and Reagents

All cell lines were purchased from European Collection of Cell Culture (ECACC). Cells were cultured in RPMI 1640 and DMEM containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA). MEF cultured in RPMI 1640 and DMEM containing 10%

Supplemental Material
colonies were formed. Cells were then stimulated with VEGF (20 ng/ml) in the presence of various concentrations of the 3-AWA diluted in growth medium. After overnight incubation, colonies were fixed and stained with 0.2% crystal violet. Photographs were taken for individual colonies.

Matrigel Invasion Assay

The effect of 3-AWA treatment on cell invasion was determined using BD Biocoat Tumor Invasion Assay System (BD Bioscience, Bedford, MA) according to the instruction of the manufacturer. Briefly, DU 145 and MCF7 cells (1.2 × 10⁶) were cultured in the presence of different concentrations of 3-AWA or vehicle DMSO for 24 h in serum free media into the upper chambers/inserts, and the bottom wells were filled with chemoattractant (complete media with 10% FBS). Cells were allowed to migrate at 37 °C for 24 h the Matrigel-coated polycarbonate filters were floated and detached cells and photographed (time 0 h). Cells were successively treated with medium containing low serum (1.0%) in the presence of various concentrations of 3-AWA along with vehicle DMSO for 24 h. Wounded areas were progressively photographed with a Nikon D3100 inverted microscope camera (20× magnification). The percentage of wound closure was estimated by the following equation: % wound closure = [1−(wound area at t₁/ wound area at t₀)] × 100%, where t₁ is the time after wounding and t₀ is the time immediately after wounding.

Clonogenic Assay

The assay was performed according to the previously described method [17]. Briefly, plated cells were exposed to various concentrations of 3-AWA/vehicle DMSO for 5 days in the 37 °C incubator with 5% CO₂. Later on, the obtained colonies were fixed with 4% paraformaldehyde and were stained with 0.5% crystal violet solution. The colonies from the plates were counted and averaged from the observed fields randomly (n = 3) and photographed with a Nikon inverted microscope camera.

Immunoblotting

Immunoblotting experiments were performed according to the standard procedure. Briefly, after SDS-PAGE, proteins were blotted onto PVDF membrane (Millipore, Billerica, MA), which were then blocked for 1 h with 5.0% non-fat milk in PBS contains 0.1% Tween-20 and probed with indicated antibodies for 3 h at room temperature or overnight at 4 °C. Subsequently, the blots were washed and probed with species specific secondary antibodies (1:1000 dilution) coupled to horseradish peroxidase. After extensive washing with PBST, immunoreactive proteins were detected by enhanced chemiluminescence reagent (Millipore) exposure to Biomax light film (Eastman Kodak Co., Rochester, NY).

Preparation of Cytosolic and Nuclear Extract

Treated DU 145 and MCF7 cells were washed with ice-cold PBS and centrifuged. All steps of fractionation were carried out at 4 °C. Cell pellets were homogenized in 200 μl of ice cold hypotonic buffer (5 mM HEPES, pH 7.9, 10 mM KCl, 1 mM DTT, 0.5 mM PMSF, 1.5 mM MgCl₂, 1 mM NaVO₄, 10% protease cocktail inhibitor, 0.02 volumes of 10% NP-40) with mild vortexing by keeping on ice for 10 min. The cell suspension was accordingly centrifuged at 10000g for 1 min. The resulting cytosolic supernatants were stored at −80 °C. The pellets obtained were washed in 100 μl of hypotonic buffer and resuspended in 100 μl of hypertonic buffer (5 mM HEPES, pH 7.9, 0.25% NP-40, 25% glycerol, 500 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM PMSF, 1 mM NaVO₄, and 10% protease cocktail inhibitor), incubated in end to end rotor for 2 h at 4 °C following centrifugation at 10000g for 10 min at 4 °C. The supernatants obtained were stored at −80 °C for analysis of nuclear protein.

Immunocytochemistry

For immunostaining, PC-3, DU 145, MCF7, T47D, MEF Par-4+/+ and MEF Par-4−/− cells were plated in 8-well chamber slide at a seeding density of 3 × 10⁴ cells per well. Next day, cells were treated with mentioned concentrations of 3-AWA. After 24 h, cells were washed with PBS and fixed in 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.1% Triton X-100 in PBS for 5 min and was successively blocked with 1% BSA for 1 h. For detection of β-catenin localization, the cells were incubated with primary antibody (see legends) for 1 h and accordingly washed three times with PBS, then incubated with Alexa Fluor 488 conjugated secondary antibody for 1 h and washed, mounted with ultracruz mounting medium and analyzed by Meta confocal (Olympus fluoview FV 1000, Olympus, Singapore) microscope using fluoview FV-1000 software.
Gelatin Zymography

The gelatinase activity of MMP-2 was assessed by method standardized previously [17]. Accordingly, DU 145 cells in sub confluent culture (70–80% cell density of confluent culture) were transfected and/or treated with indicated conditions. The conditional media obtained from both treated and untreated samples were employed for protein estimation and equal amount of total proteins (20 μg) were mixed with sample buffer (2.0% SDS, 25% glycerol, 0.1% Bromophenol blue and 60 mM Tris-HCl, pH 6.8). Gelatin zymography of the samples were carried out using 7.5% SDS-polyacrylamide gels containing 0.1% gelatin at 100V for 3 h at 4°C. Gelatinase activity was detected by observing unstained bands on a blue background of Comassie stained gel.

Reporter Assay

A luciferase reporter assay system was used to measure β-catenin/TCF transcription activity. Briefly, DU 145 cells were plated overnight. TCF-firefly luciferase reporter construct containing either wild type TCF promoter binding sites (pTOP-FLASH) or mutant TCF promoter binding sites (pFOP-FLASH) was transfected and/or co-transfected with Renilla luciferase construct (pRL-TK) (E6911, Promega, Madison, WI) to normalize transfection efficiency. Four hours post-transfection, the cells were treated with either 3-AWA or vehicle DMSO for 36 h. The firefly luciferase activity and Renilla luciferase activity were analyzed by two step process using the Dual Glo reagents (E2940, Promega) according to the manufacturer's instruction.

siRNA Knockdown Assay

DU 145 cells were seeded in 6-well plates (for Western blotting) or 4-well chamber slide (for immunocytochemistry) and transient transfections were performed with DharmaFECT-1transfection reagent or Neon Transfection System (MPK5000, Invitrogen, Carlsbad, CA) according to manufacturer's protocol. The siRNA for Par-4 was: S-GAUG-CAAUUAACACACAGAdTdT-3.

Immunofluorescence Study

All animals used in this study were maintained in house (Central animal facility, Indian Institute of Integrative Medicine, Jammu, India). Animal studies were performed in accordance with experimental protocols that were approved by the Animal Ethics Committee of the Indian Institute of Integrative Medicine, Cancer Pharmacology Division, Council of Scientific and Industrial Research, Jammu, India. The experiment was performed as described previously [24]. Briefly, female Wistar rats (150–200 g body weight) with three regular cycles of 4 days were used in the experiments. Stages of the estrous cycle (pro-estrus and estrus) were confirmed by vaginal smears. Four rats were used for each time of the estrous cycle and sacrificed in a humane way. Uterus from each rat were removed carefully and kept in formalin solution. For immunofluorescence study, paraffin embedded uterine sections were prepared as described elsewhere [25]. Briefly, uterine tissue was isolated and fixed in 10% phosphate buffered formalin for at least 24 h. Then the paraffin sections were prepared and cut into 5 μm thick sections in a rotary microtome and mounted on the slide. Sections processed for immunofluorescence were incubated with total β-catenin antibody and Par-4 antibody at a dilution of 1:200, followed by incubation with Texas Red conjugated secondary antibody and mounting medium and viewed directly using confocal microscopy.

In Vivo Anti-Tumor Activity Against Sarcoma 180 Solid Tumor

To study the in vivo antitumor efficacy of 3-AWA, female Swiss albino mice (b.w. 18–23 g) were used. The experimental protocol was approved by the Institutional Animal Ethics Committee, CPCSEA, Indian Institute of Integrative Medicine, Jammu. Animals were fed with standard pelleted chow and maintained in a 22°C light dark cycle prior to and throughout the experiment. Initially, a dose response study was conducted taking various doses (1, 2.5, 5, 10, 15, 30, and 50 mg/kg) of 3-AWA and determined 10 mg/kg dose as maximum safe dose. A total of 18 animals were randomized and divided into three different groups. On day first, 10 x 10⁶ Sarcoma cells were injected intramuscularly into the right thigh of each animal. From the next day, the test group was treated with 10 mg/kg i.p. 3-AWA, the positive control group received 22 mg/kg i.p. 5-fluorouracil and the negative control group was administered with normal saline (0.2 ml, i.p.). The tumor sizes were measured with the help of a vernier caliper. Tumor weights of each animal was calculated using the following formula.

\[
\text{Tumor weight (mg)} = \frac{\text{Length (mm)} \times \text{width (mm)}^2}{2}
\]

The percentage tumor growth inhibition was calculated by comparing the average value of the treated groups with that of the control group. Tumor growth in saline-treated animals was taken as 100%.

Immunohistochemistry

For immunohistochemical detection of the Phospho-β-catenin, Total β-catenin and Par-4, paraffin embedded sections from normal and cancer breast tissues were prepared as described elsewhere [26]. Sections were incubated with primary antibody Phospho-β-catenin, Total β-catenin and Par-4.
(dilution 1:100) for overnight at 4°C, washed with PBS and further incubated with biotinylated secondary antibody for 60 min at room temperature. Sections were exposed to 3,3'-diaminobenzidine (DAB) substrate, counterstained with hematoxylin, dehydrated and mounted for confocal microscopy. The results were analyzed and confirmed by two individuals.

Statistical Analyses

Data were expressed as mean ± SEM of at least three independent experiments performed. Comparisons used Student’s t test. *P < 0.05 and **P < 0.01 values were assigned significance.

RESULTS

3-AWA Inhibits Gelatin Matrix Degradation, Cell Scattering, Invasion, and Migration of Cancer Cells

Withanolides are known to inhibit invasion capability of cancer cells [27], therefore, we were curious to investigate the mechanism based inhibitory property of 3-AWA on migration and invasion of DU 145 and MCF7 cells. The ability of prostate cancer cells to degrade the matrix was examined by culturing cells on a crosslinked fluorophore (FITC)-conjugated-gelatin matrix-coated coverslips for 24 h. Figure 1A clearly depicts that higher concentrations of 3-AWA inhibited the matrix gelatin degradation by aggressive DU
145 cells (indicated by arrows). Further, the threshold areas of degradation obtained from Image-J software highlights the degraded area convincingly support the gelatin matrix spots. On the other hand, MCF7 cells did not show gelatin degradation (data not shown). To further investigate the effect of 3-AWA on cell scattering, the predominant feature of a mesenchymal property of cancer cells, DU 145 cells were stimulated with VEGF in the presence of various concentrations of the 3-AWA diluted in growth medium. Scattered colonies were found only in DU 145 cells, not MCF7 (data not shown), by the typical change in morphology characterized by cell-cell dissociation and the acquisition of a migratory, fibroblast-like phenotype in vehicle treated cells and that eventually decelerated by treatment with different concentrations of 3-AWA (Figure 1B). We further envisaged whether 3-AWA treatment had any effect on fibroblast like phenotype observed by cells during EMT. In order to do this, MDA-MB-231 and PC-3 cells were treated with indicated concentrations of 3-AWA and accordingly cells were observed by bright field microscopy. Interestingly, it was found that 3-AWA treatment resulted in reversal of fibroblast like phenotype in both prostate and breast cancer cells in a dose dependent manner (Figure S1A).

The Boyden chamber invasion assay was carried out to determine the ability of DU 145 and MCF7 cells to invade through biological matrices in vitro. As shown in Figure 1C, treatment with 3-AWA of 3 and 5 μM inhibited invasion capability of DU 145 and MCF7 cells, respectively.

Additionally, to assess the effect of 3-AWA on cell motility, we found pronounced suppression of migration of DU 145 and MCF7 cells in the denuded zone in a dose-dependent manner (Figure 1D, S1B, and C). Further, colony formation ability of DU 145 and MCF7 cells were also significantly attenuated by 3-azidoWA treatment in a dose dependent manner as shown in Figure 1E, S1D, and E.

3-AWA Inhibits Expression of EMT Associated Markers and Upregulates Expression of E-cadherin, TIMP-1 in DU 145 and MCF7 Cells

Activation of an EMT program has been proposed as the critical tool for the acquisition of cancerous phenotypes by epithelial cancer cells. Thus, acquisition of invasiveness is primary step in EMT. Therefore, we sought to investigate the effects of 3-AWA on the expression of EMT specific markers viz. E-cadherin, Snail, MMP-2, TIMP-1 in DU 145, and MCF7 cells. Our immunoblot data demonstrated that 3-AWA reduced the expression of pro-EMT markers Snail, MMP-2 in a dose- and time-dependent manner (Figure 2A, B and D). In addition, 3-AWA upregulated the expressions of E-cadherin and TIMP-1, in an essentially similar fashion in both (DU 145 and MCF7) cell lines (Figure 2A and D). It is to be noted here that 3-AWA treatment strikingly enhanced the membrane localization of E-cadherin (Figure 2C). Since the activation of MMP-2 is crucial for ECM degradation, and is required for cell invasion, the effect of 3-AWA on the activation of MMP-2 was investigated. The results showed MMP-2 activity was markedly reduced by 3-AWA in a dose and time-dependent manner in DU 145 cells (Figure 2D). Moreover, expansion of vimentin cytoskeleton is deeply associated with formation of EMT/invasive phenotype to support mesenchymal features. Therefore, we investigated the effect of 3-AWA on vimentin expression in DU 145 cells by immunocytochemical and Western blot analysis. The data illustrated moderate inhibition of vimentin expression by 3-AWA treatment, suggesting the EMT inhibitory property of this novel derivative of Withaferin A (Figure 2D and E). As ZEB1 is a central regulator of EMT and overexpression of ZEB1 in cancer is associated with repression of E-cadherin and EMT, therefore, the effect of 3-AWA on expression of ZEB1 revealed that 3-AWA treatment attenuated the expression of pro-EMT marker ZEB1 in a dose- and time-dependent manner (Figure 2D).

3-AWA Upregulates Phosphorylation of β-Catenin, Modulates Downstream Effectors in β-Catenin Signaling and Negatively Regulates β-Catenin Transcriptional Activity

Emerging evidences suggest that the epigenetic control of E-cadherin and β-catenin/LEF activity is pivotal for establishing the metastatic potential of cancer cells [28]. Thus, activation of β-catenin is associated with its phosphorylation dependent nuclear accumulation, which led TCF/LEF mediated transcriptional stimulation of protooncogenes, viz. c-Myc, Cyclin D1 [29]. All of these factors negatively regulate E-cadherin expression and thus promotes tumorigenesis. Therefore, the effects of 3-AWA on the phosphorylation of β-catenin in prostate and breast cancer cells were investigated. The results showed that β-catenin phosphorylation (Ser 33/37/Thr 41) was augmented along with a decrease in nuclear localization of total β-catenin when prostate and breast cancer cells were treated with 3-AWA in both dose and time-dependent manner (Figure 3A, B, and S2). On the other hand 3-AWA treatment had hardly any effect on total β-catenin level in both prostate and breast cancer cell lines (Figure 3A, B, and S2).

Consistent with the increased phosphorylation of β-catenin, the expressions of c-Myc and Cyclin D1, downstream modulators of β-catenin signaling, were also downregulated following 3-AWA treatment (Figure 3C and D). Of note, the c-Myc level was reduced dramatically within 12 h of treatment regimen. Further, we confirmed the effect of 3-AWA on β-catenin transcriptional activity by TOP/FOP FLASH reporter assay. The results showed that 3-AWA treatment, not vehicle DMSO suppressed the TCF/LEF mediated transcriptional stimulation of β-catenin in a dose dependent manner (Figure 3E).
3-AWA Modulates β-catenin Localization in Prostate and Breast Cancer Cells

Cellular β-catenin localization profile has been implicated in cancer cell migration. It is reported in the literature that β-catenin translocation into the nucleus activates set of transcription factors to enhance EMT (Epithelial Mesenchymal Transition) and contrastly cytoplasmic pool of β-catenin facilitates catenin–cadherin complex formation at the cell membrane to suppress EMT [30]. Necessarily, we sought to determine the effect of 3-AWA on the sub-cellular localization of β-catenin and found that 3 and 5 μM of 3-AWA sharply attenuated nuclear β-catenin levels (Figure 4A and B). On the other hand, a dramatic accumulation of cytosolic/membrane β-catenin was achieved with these treatments. Further, immunocytochemical staining exhibited prominent cytosolic/membrane localization of β-catenin when the cells were treated with 3 μM of 3-AWA compared to vehicle DMSO treated cells (Figure 4C, S3). We have found that another cytotoxic candidate doxorubicin also similarly affected the β-catenin localization and sub-cellular distribution as efficient as 3-AWA (Figure S4A and B). Thus, all these results collectively suggest that 3-AWA potentially modulated β-catenin localization in prostate and breast cancer cells.

3-AWA Induces the Expression of Endogenous Par-4 and Modulates Upstream Effectors in β-Catenin Signaling

Par-4 is known to regulate many anti-apoptotic proteins to suppress tumorigenesis [14,16] and in our recent study, we have demonstrated that 3-AWA
mediated inhibition of MMP-2 was Par-4 dependent [17]. Moreover, a current study pointed out the role of Par-4 in metastatic cancer [31]. Rationally, we sought to examine whether there is any effect of 3-AWA on intracellular Par-4 induction and if so, how induction of Par-4 could modulate β-catenin signaling. Figure 5A and B shows a sharp elevation of intracellular Par-4 achieved within 12 h of drug exposure (3 μM of 3-AWA) without affecting nuclear integrity (less than 5% apoptotic population) as observed by Annexin V-FITC staining (data not shown) indicating the potential role of 3-AWA to stimulate endogenous Par-4 level in a dose and time dependent manner. Further, Akt-GSK3β axis is considered as a prime target for development of anticancer therapeutics against many cancers including prostate [32]. Our immunoblot results demonstrated the effect of 3-AWA on upstream effectors in β-catenin signaling. 3-AWA treatment remarkably reduced Akt phosphorylation (Ser 473) that further attenuated GSK3β phosphorylation at Ser 9. However, the total Akt and GSK3β level remained unaltered in dose and time dependent manner suggesting 3-AWA as a powerful anti-cancer agent to control the activation of upstream mediators of β-catenin pathway. (Figure 5C and D).

3-AWA Mediated Altered β-catenin Localization is Par-4 Dependent and Inhibition of Par-4 Activity Renders β-catenin Nuclear Accumulation

We observed that 3-AWA treatment augmented endogenous Par-4 level and modulated β-catenin's sub-cellular distribution. In the recent past, emerging evidences have suggested that Par-4 overexpression/induction could negatively regulate major cell survival proteins [33]. Therefore, we sought to investigate whether the knock down of endogenous Par-4 could restore β-catenin nuclear localization in the presence of 3-AWA. Surprisingly, we found that DU 145 cells transfected with siRNA Par-4 along with 3-AWA treatment failed to translocate β-catenin in the cytosolic compartment compared to the scrambled siRNA plus 3-AWA treated cells, where β-catenin was distributed in the cytosolic compartment compared to the scrambled siRNA plus 3-AWA treated cells, where β-catenin was distributed in the nucleus as well as cytosol (Figure 6A). Suppression of endogenous Par-4 by siRNA robustly elevated nuclear β-catenin level (threelfold increase compared to scrambled siRNA) and amplified c-Myc/Cyclin D1 level (data not shown) even in the presence of 3-AWA (Figure 6B). Further, siRNA mediated silencing of endogenous Par-4 plus 3-AWA treatment augmented nuclear β-catenin transcriptional activity compared with...
scrambled siRNA and 3-AWA group, as measured by Top-Fop reporter assay (Figure 6C). MMP-2 is shown as the downstream target of β-catenin [34,35] and one of our recent study revealed extracellular Par-4 mediated abrogation of invasion of prostate cancer cells through MMP-2 inhibition [17]. Necessarily, we investigated the effect of suppression of endogenous Par-4 on MMP-2 gelatinase activity and the results implied that siRNA Par-4 plus 3-AWA treatment failed to decrease MMP-2 levels as compared to scrambled siRNA and 3-AWA group (Figure 6D). Given that Par-4 and BCL-2 mutually regulate each other in maintaining cellular homeostasis [36], we suppressed endogenous Par-4 activity by transiently expressing GFP-BCL-2 and examined β-catenin compartmentalization in presence and absence of 3-AWA. GFP-BCL-2 alone facilitated nuclear accumulation of β-catenin that modestly attenuated in the presence of 3-AWA (Figure 6E) implying neutralization of GFP-BCL-2 activity by 3-AWA induced Par-4, restricted β-catenin’s nuclear entry. Previous studies implicated that Ras protein negatively regulates Par-4 in diverse cancer [37,38]. Keeping this in mind, we evaluated the localization of sub-cellular β-catenin in Ras transformed NIH 3T3 cells. We transiently transfected NIH 3T3 cells with EGFP/EFGP-HRas and followed the β-catenin’s sub-cellular distribution. Figure 6F shows EGFP-HRas transfected cells possessed high β-catenin.
levels in the nucleus and less cytosolic β-catenin as well as E-cadherin expression compared to EGFP transfected cells. All these results collectively suggest that Par-4 plays positive role in 3-AWA mediated sub-cellular distribution of β-catenin. Further, to examine the role of GFP-Par-4 overexpression on endogenous β-catenin, we transfected cells with GFP/GFP-Par-4 constructs and checked the sub-cellular β-catenin expression profile showing modest reduction in nuclear β-catenin level in GFP-Par-4 transfected wells compared to GFP transfected (data not shown). Further, our Western blot results suggested that GFP-Par-4 reduced phosphorylation of AKT Ser 473 (a primary phosphorylation site to impart Akt activation in prostate cancer) resulting in diminished GSK3β phosphorylation (~50%) at Ser 9 whereas total Akt and GSK3β levels were unchanged.

Although, E-cadherin expression profile is low in prostate cancer cells, [39] strikingly, a sharp amplification (2.5-3.0 fold increase) in E-cadherin expression was observed in GFP-Par-4 transfected cells compared to GFP transfected cells (Figure S5).

3-AWA Induced Endogenous Par-4 Indirectly Modulates Upstream Mediators of β-Catenin Signaling

GSK3β kinase is one of the major kinase regulating inner circuits of cellular metabolism and transformation. Inactivation of Glycogen synthase kinase 3 beta (GSK3β) by oncogenic processes depends on the phosphatidylinositol 3-kinase (PI3K) pathway leading to β-catenin dephosphorylation, its accumulation and translocation into the nucleus [7]. But the mechanisms underlying the regulation of GSK3β are poorly understood. As Akt in PI3K pathway serves a
critical compensatory role by blocking the tumor suppressing property of GSK3b, removal of this hindrance may improve such a catastrophic condition. Therefore, we examined if 3-AWA mediated upregulation of intracellular Par-4 had any positive/negative regulation on constitutively active GSK3b.

We assumed that transient transfection of cells with constitutively active GSK3b-S9A, which could not be phosphorylated by Akt (since Serine 9 is mutated to Alanine 9), might enhance phosphorylation of β-catenin protein in the presence of 3-AWA. Necessarily, we pre-treated DU 145 cells with or without Lithium Chloride, which is known to trigger phosphorylation (by Akt) of residual GSK3b (Ser 9), resulting GSK3b inhibition. Further, those cells were transfected with GSK3b-S9A following treatment with or without 3-AWA and analyzed by Western blot analysis for nuclear and cytosolic β-catenin levels. Histogram (lower right corner) represents the intensity of protein expression quantified by densitometric analysis. Data from three independent experiments were compared with untreated control and subjected to statistical analysis, *P < 0.05.

Molecular Carcinogenesis

Figure 6. 3-AWA mediated altered β-catenin localization is Par-4 dependent. (A) DU 145 cells were transfected with scrambled (control) siRNA/siRNA Par-4 as indicated following treatment with 3-AWA and subjected to immunocytochemistry analysis for β-catenin (green) localization. Scale bar, 50 μm. (B) The cell lysates from above were subjected to Western blot analysis for determining the expression of Par-4, nuclear, and cytosolic β-catenin levels. (C) Histogram represents the effect of Par-4 siRNA and/or 3-AWA along with or without DMSO on the β-catenin transcriptional activity in DU 145 cells. The β-catenin transcriptional activity was measured by transiently transfecting the cells with TCF luciferase reporter construct containing either TCF promoter binding sites (pTOP-FLASH) or mutant TCF promoter binding sites (pFOP-FLASH) with internal control plasmid containing Renilla luciferase gene along with or without indicated siRNAs. After 4h, the cells were treated with different concentrations of 3-AWA or DMSO for 36h. (D) DU 145 cells were transfected with siRNA as indicated following treatment with 3-AWA for indicated time periods. Conditioned media from above experiment were collected and subjected to gelatin zymography analysis for MMP-2 activity and coomassie blue staining for loading control. (E) DU 145 cells were transiently transfected with GFP or GFP-BCL-2 following treatment with or without 3-AWA and analyzed by Western blot analysis for nuclear and cytosolic β-catenin, Par-4 and BCL-2 expression. (F) NIH 3T3 cells were transiently transfected with expression constructs for EGFP or EGFP-H-Ras for 24h. Resulting cytoplasmic and nuclear extracts were immunoblotted to verify the levels of concerned proteins by relevant antibodies. Histogram (lower right corner) represents the intensity of protein expression quantified by densitometric analysis. Data from three independent experiments were compared with untreated control and subjected to statistical analysis, *P < 0.05.
in this two groups (with and without 3-AWA), suggesting that 3-AWA induced Par-4 had no advert effect on constitutively active GSK3β (Figure 7A, Lane 3, 4; top panel).

Since, Akt is a central player controlling β-catenin stability, we sought to correlate whether Akt inhibitor similarly abrogated β-catenin’s nuclear accumulation as 3-AWA exhibited and consequently modulated Par-4 activity. In order to do that, we treated the prostate and breast cancer cells with an AKT inhibitor AT7867 and performed immunoblot analysis and found that inhibition of AKT with AT7867 moderately elevated expression of Par-4 with concomitant de-phosphorylation of Akt and GSK3β (Figure 7B). Further, fractionation results also demonstrated that AKT inhibition with AT7867 altered nuclear β-catenin along with a rise in cytosolic β-catenin (Figure 7C) level compared to vehicle treatment, suggesting an identical role played by 3-AWA in regulating Akt-GSK3β.

One of the upstream mechanism that negatively regulates GSK3β include GSK3β phosphorylation on Ser 9 by Akt and pharmacological inhibition of GSK3β by Lithium Chloride and BIO treatments attenuate β-catenin phosphorylation at Ser 33/37/Thr 41 position [40-42]. As both Lithium Chloride or BIO significantly increases the phosphorylation (activation) of Akt [43], we were interested to investigate whether 3-AWA could rescue phosphorylation of β-catenin, that almost dephosphorylated due to Lithium Chloride or BIO pre-treatment. In order to do that, DU 145 cells were serum starved for 12 h following pre-treatment with indicated concentrations of LiCl or BIO for 4 h following treatment with or without 3-AWA for 24 h. The phosphorylation of p-β-catenin (Ser 33/37/Thr 41) was checked by Western blotting of the WCL. β-actin was used as a loading control. Histogram represents the level of protein expression quantified by densitometric analysis. The data given are representatives of three independent experiments performed, *P< 0.05, **P< 0.01.

Molecular Carcinogenesis
incubation with Lithium Chloride (20 mM) or BION (0.1 µM) for 4 h, then removed the media and kept for another 24 h in presence of 3-AWA. Western blotting results showed that 3-AWA upregulate phosphorylation of β-catenin in the lanes pre-treated with Lithium Chloride or BION (Figure 7D) indicating a prominent rescueing machinery involved to suppress/bypass the inhibitory role of Lithium Chloride/BION (on GSK3β) by 3-AWA targeting Akt. Further, to assess the effect of 3-AWA on localization of mutant β-catenin (S33Y), we performed transient transfection with pCMV β-catenin (S33Y) where Ser 33 is mutated to tyrosine. DU 145 cells, when transfected with β-catenin mutant (S33Y) alone or transfected with β-catenin mutant (S33Y) plus 3-AWA showed equal accumulation in nuclear β-catenin expression as compared to just 3-AWA treated cells, which exhibited a reduction of nuclear β-catenin (Figure 7E, Lanes 3, 4, and 5, top panel). This result suggests that 3-AWA mediated upregulation of Par-4 indirectly facilitated phosphorylation of β-catenin at Ser 33 position through activation of GSK3β and amplified β-catenin cytosolic accumulation compared to DMSO treated cells (Figure 7E, Lane 2 and 3, third panel from top). Notably, when β-catenin’s GSK3β phosphorylation site was mutated; 3-AWA induced Par-4 had no choice to rescue β-catenin from nucleus rendering lesser cytoplasmic accumulation of β-catenin (Figure 7E, Lanes 4 and 5, third panel from top). These data suggested that 3-AWA mediated GSK3β activation was not directly dependent on Par-4 rather than it relied on Par-4 mediated Akt regulation.

Par-4 and β-Catenin Expressions Are Inversely Related in Tumor Samples Obtained From 3-AWA Injected Mice as Well as Normal Versus Tumor Samples

In vivo antitumor activity of 3-AWA against Sarcoma-180 (solid) tumor was evaluated and as shown in Table 1, on the 13th day, the tumor growth inhibition was found to be 39.44% with 10 mg/kg i.p. dose of 3-AWA with an average tumor weight 861.84 ± 16.28 mg, whereas the tumor growth inhibition for the 5-FU (22 mg/kg i.p.) treated group was 54.35% with an average tumor size of 675.41 ± 22.26 mg, compared to the 1423.12 ± 24.34 mg for the control group. These results demonstrated that 3-AWA moderately inhibited the tumor growth in a safe and tolerable dose of 10 mg/kg b.w. (which was much less than the dose of 5-fluorouracil). Comparisons were made between the control and treated groups using the Student’s t-test (Table 1). The lysates of the tumor tissues obtained from 3-AWA injected as well as control mice were subjected to immunoblotting [45]. The result showed a distinct amplification of Par-4 and downregulation of β-catenin in tumor tissues obtained from 3-AWA injected mice, compared to the untreated control (Figure 8A).

Since, we observed an inverse relationship between the expression patterns of β-catenin and Par-4 due to 3-AWA treatment in an in vitro as well as in vivo set up, next, our aim was to find the level of these two evolutionarily conserved and physiologically important proteins, β-catenin and Par-4 in normal versus tumor samples. Immunohistochemistry studies of rat uterine tissues revealed a distinct inverse expression pattern of these two proteins. Of note, Par-4 expression is usually very high during proliferative phase (proestrus) but lower levels are reported during the secretory phase (estrus) [24]; therefore, we sought to correlate the pattern of Par-4 and β-catenin expressions in these two phases of rat menstruation cycle. Our immunofluorescence results showed lower levels of β-catenin during the proliferative phase (proestrus) and higher β-catenin expression was observed during the apoptotic/secretory phase (estrus), suggesting the inverse correlation of their expression patterns in normal cell (Figure 8B). Our result was also supported by the study of Yip et al, who showed that Wnt signaling was activated in estrus versus proestrus uterus [44]. Additionally, we explored whether the similar trend of inverse co-existence of Par-4 and β-catenin also existed in breast tumor tissue samples. Our immunohistochemistry results clearly identified an increased expression of total β-catenin and decreased expression of phospho-β-catenin and Par-4.

Table 1. In vivo Anti Cancer Activity of 3AWA

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Avg. body weights (g) of animals on days</th>
<th>13th day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st</td>
<td>5th</td>
</tr>
<tr>
<td>3AWA (10 mg/kg i.p.)</td>
<td>22.32</td>
<td>21.22</td>
</tr>
<tr>
<td>5-FU (22 mg/kg i.p.)</td>
<td>22.35</td>
<td>21.22</td>
</tr>
<tr>
<td>Normal saline (0.2 ml i.p.)</td>
<td>24.12</td>
<td>23.26</td>
</tr>
</tbody>
</table>

Values are mean ± S.E. (n = 6). *P < 0.05, **P < 0.01 versus normal saline control. 

Molecular Carcinogenesis
4 in breast cancer tissues as compared to normal breast tissue (Figure 8C).

DISCUSSION

Parent molecule Withaferin A is a druggable candidate with diverse pharmacological properties including anti-inflammatory, anti-angiogenic, cardioprotective and immunomodulatory activities [18,46]. Semi-synthetic modification of α,β-unsaturated functionality of ring A of Withaferin A [47], though, substantially magnified 3-AWA’s anti-cancer potentiality, it needs to be further elucidated. Here, in this report, we demonstrated that 3-AWA altered subcellular distribution pattern by modulating GSK3β via pro-apoptotic Par-4 mediated Akt inhibition.

Additionally, the outcome of this study strongly postulated that depletion of endogenous Par-4 restored nuclear β-catenin in presence of 3-AWA suggesting 3-AWA affected β-catenin sub-cellular distribution profile in a Par-4 dependent manner. Par-4, a leucine zipper domain protein is essential for apoptosis induction due to exogenous insults like UV or chemotherapy [48]. Moreover, Par-4 expression is downregulated in many cancers including renal cell carcinoma [10], lung cancer [49], breast cancer [50] and loss of Par-4 in mice leads to the spontaneous development of PIN (Prostate Intraepithelial Neoplasia) as well as endometrial tumors [51]. Although, previous studies have underscored major apoptotic role of Par-4, little is known about the anti-invasive/anti-metastatic role of Par-4 in various cancer. Our
earlier studies demonstrated anti-invasive potential of 3-AWA induced extracellular Par-4 to control cancer cell motility by inhibiting MMP-2 [17]. Here, we report a novel prospective mechanism of Par-4 which unveiled its greater therapeutic windows in prostate and breast cancer prevention. These findings implied that 3-AWA suppressed pro-metastatic ability of β-catenin by blocking its transcriptional activity through modestly augmenting the expression of E-cadherin and proved that 3-AWA could rescue GSK3β suppression (due to Lithium Chloride or BIO treatment) to induce β-catenin phosphorylation. Although, GSK3β is considered to play a major tumor suppressor role in prostate cancer cells, but hardly few reports are there in breast cancer. Additionally, as a proof of concept, the immunohistochemistry of breast tumor samples clearly revealed a downregulation of Par-4 levels with corresponding elevated β-catenin expression indicating a loss of function of the Par-4 protein in breast tumorigenesis. Further, we were interested to identify the effect of 3-AWA on β-catenin localization in Par-4 knock out model. Therefore, Par4+/- and Par-4-/- MEFs were treated with or without 3-AWA for 24 h following which immunocytochemistry was performed. Our immunocytochemistry study detected increased perinuclear/cytoplasmic accumulation of β-catenin in 3-AWA treated MEF Par-4+/- cells compared to Par-4-/- cells exhibiting dispersed β-catenin staining throughout the cells (Figure 8D). Interestingly, transient overexpression of Par-4 robustly augmented the cytoplasmic E-cadherin levels in prostate cancer cells, which is known to express low level of E-cadherin (Amin et al, unpublished data). Nevertheless, how Par-4 maintains (?) the cadherin-catenin integrity, yet to be investigated. Moreover, this study also sheds lights on the inverse co-relation of Par-4 and β-catenin expression in breast cancer tissues implicating a therapeutic perspective of Par-4. Although, several studies have demonstrated that the epigenetic control of E-cadherin and β-catenin/LEF activity is obligatory to designate the metastatic potential of cancer cells [28,29,52]. E-cadherin expression levels vary dramatically in different human tumors suggesting tumor prone microenvironment due to E-cadherin loss in EMT program [29]. Therefore, important natural products, which can retain the cellular E-cadherin level and suppress EMT of cancer cells, could be implemented for therapeutic perspective. In this report, we highlight novel activity of 3-AWA to suppress pro-EMT markers viz. Vimentin, Snail, and nuclear β-catenin to abrogate metastasis. Previous studies have demonstrated profound Akt activation due to Par-4 ablation in Par-4 KO as well as Par-4/PKCζ DKO cells suggesting Par-4 as a selective negative regulator of Akt [49]. Our result corroborates with earlier studies and vividly suggested that induction of endogenous Par-4 modulates Akt-GSK3β axis, which is engaged in a complex interplay of phosphoregulation in tumorigenesis. Though, recent constructive approaches [53,54], that have been undertaken to extraplate Par-4 in development of prostate cancer therapeutics, it has to be noted that only induction of Par-4 or transient overexpression of Par-4 is not adequate to suppress the hyperactive Akt in PTEN null prostate cancer cells, but a synergistic combination of 3-AWA with exogenous Par-4 only could consistently modulate Akt activation. The more detailed pharmacokinetic study will help in optimization of this synergistic approach in vivo prostate cancer model. It will be promising to interpret the circuit involved in engaging Par-4 for dual control of apoptosis and metastasis. Wnt/β-catenin signaling plays a pivotal role in breast as well as prostate cancer initiation, progression and metastasis [55]. To understand the role of Wnt/β-catenin signaling in two different cancers viz, prostate and breast, we carefully choose one aggressive (DU 145) and other non-aggressive (MCF7) cell lines. Immunoblots were obtained from 3-AWA injected mice as compared to 3-AWA treated MEF Par-4+/- cells compared to Par-4-/- cells exhibiting dispersed β-catenin staining throughout the cells (Figure 8D). More importantly, we observed the abrupt depletion of endogenous Par-4 expression in breast tumor samples with high level of β-catenin, which mimics the siRNA (Par-4) transfected endogenous Par-4 inhibition lead to higher nuclear β-catenin accumulation in DU 145 cells. Further, our in vivo results demonstrate 3-AWA as an effective tumor growth inhibitor and the tumor growth inhibition was 39.44% with only 10 mg/kg i.p. dose of 3-AWA. Interestingly, the immunoblots generated from tumor tissue lysates distinctly showed the inverse correlation of increased Par-4 expression with downregulation of β-catenin in tumor tissues obtained from 3-AWA injected mice as compared to control. Though previous studies suggest the loss of function/expression of Par-4 in many cancers, our report for the first time unveils Par-4 mediated β-catenin regulation by 3-AWA in prostate and breast cancer cells. Certain aspects of β-catenin regulation like β-catenin mediated TCF transcriptional regulation, β-catenin stability and its degradation leading to tumorigenecity remain to be entirely clarified. However, our finding supported the fact that 3-AWA induced Par-4 inhibited Akt activity (through PKCζ) [49] by ensuing that Akt was unable to phosphorylate GSK3β (Ser 9) as well as directly β-catenin at Ser 552 (major residue for β-catenin phosphorylation in tumorigenesis). Though, recent constructive approaches [53,54], that have been undertaken to extraplate Par-4 in development of prostate cancer therapeutics, it has to be noted that only induction of Par-4 or transient overexpression of Par-4 is not adequate to suppress the hyperactive Akt in PTEN null prostate cancer cells, but a synergistic combination of 3-AWA with exogenous Par-4 only could consistently modulate Akt activation. The more detailed pharmacokinetic study will help in optimization of this synergistic approach in vivo prostate cancer model. It will be promising to interpret the circuit involved in engaging Par-4 for dual control of apoptosis and metastasis.
Figure 9. Schematic diagram represents the effect of Par-4 induction by 3-AWA on β-catenin localization. Treatment of 3-AWA resulted in induction of Par-4 that in turn activates GSK3β by suppressing Akt. Eventually, active GSK3β phosphorylates β-catenin and thus prevents its nuclear accumulation.
regulation by Akt) and thus regulated its (β-catenin) transcriptional potential. Thus, due to 3-AWA treatment, the Par-4 mediated this dual control of β-catenin might be responsible for β-catenin cytosolic retention. It is tempting to speculate that despite its spontaneous mutation in β-catenin, only the threshold induction of Par-4 is necessary to secure the formation of cadherin–catenin complex for cell junction integrity. An appropriate example to support this is that the induced Par-4 effectively blocked MMP-2 activity [17] as well as augmented E-cadherin/TIMP-1 expression by sequestering nuclear β-catenin.

In conclusion, this study revealed a new dimension of Par-4 extrapolation for metastatic prostate cancer therapeutic development towards β-catenin driven oncogenesis. As a powerful anti-invasive agent, 3-AWA has shown to have a promising anti-cancer onco genesis. As a powerful anti-invasive agent, 3-AWA mediated induction of programmed cell death by Par-4 was not compromised by this novel β-catenin signal modulatory role, only future studies would unveil the mechanisms by which 3-AWA mediated Par-4 hinders Wnt signaling. This might facilitate the development of chemopreventive and therapeutic strategies for diverse cancers.

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REFERENCES


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