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Research Article

Par-4–Dependent Apoptosis by the Dietary Compound Withaferin A in Prostate Cancer Cells

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Abstract

Deletion or mutation of the androgen receptor (AR) renders prostate tumors refractory to apoptosis by androgen ablation, the mainstay of prostate cancer therapy. To identify novel therapeutics that can induce apoptosis regardless of the AR status of prostate cancer cells, we screened dietary herbal compounds using a reporter assay for the *prostate apoptosis response-4* (*Par-4*) gene, which induces p53- and PTEN-independent and cancer-selective apoptosis. One of the compounds, withaferin A (WA), a major constituent of the dietary compound *Withania somnifera*, induced Par-4–dependent apoptosis in androgen-refractory prostate cancer cells and regression of PC-3 xenografts in nude mice. Interestingly, restoration of wild-type AR in PC-3 (AR negative) cells abrogated both Par-4 induction and apoptosis by WA. Individually, WA and anti-androgens induced neither Par-4 nor apoptosis in androgen-responsive prostate cancer cells, yet in combination, WA and anti-androgen synergistically induced Par-4 and apoptosis in androgen-responsive prostate cancer cells. Thus, when judiciously combined with anti-androgens, WA inhibits survival of both androgen-responsive and androgen-refractory prostate cancer cells by a Par-4–dependent mechanism. As Par-4 up-regulation induces apoptosis in most tumor cells, our findings can be extended to high-throughput screens to identify synergistic combinations for both therapy-sensitive and therapy-resistant cancers. [Cancer Res 2007;67(1):246–53]

Introduction

Prostate cancer is the third leading cause of cancer-related deaths in men in the United States (1). About 234,460 new cases of prostate cancer are diagnosed annually in the United States alone, and the numbers are projected to increase as longevity expands the aging population (1). Both androgen and its cognate receptor [androgen receptor (AR)] are recognized risk factors in the development of prostate cancer (2–5). These observations are further corroborated by genetic evidence from transgenic mouse models, suggesting that increased AR signaling in the prostate is linked to an increase in precancerous lesions (6). Accordingly, the most effective treatment for early-stage prostate cancer includes suppression of AR function either by blocking androgen signaling with the anti-androgens bicalutamide (Casodex) or flutamide or by inhibiting the conversion of testosterone to the potent androgen dihydrotestosterone with finasteride. As prostate cancer cells are dependent on AR signaling for survival and growth, either the removal of androgen or blocking dihydrotestosterone synthesis leads to the induction of apoptosis in clinical prostate cancer as well as in cell culture and animal models of prostate cancer (7, 8). However, ~30% of these patients show relapse of the disease within 3 years as a result of the emergence of androgen-independent prostate cancer cells, which are either AR positive or AR negative (9, 10). The molecular mechanisms dictating the progression from androgen dependence to androgen independence are unclear due to the lack of suitable experimental models and molecular markers (11). In general, however, AR can be activated despite androgen blockade therapy in AR-positive prostate cancer (12). This is attributed to an increased sensitivity of AR to low concentrations of androgen due to mutations in AR, AR partner-protein interactions, or post-translational modifications; these underlying factors function to promote androgen depletion-independent signaling (13). Thus, alternative therapeutic approaches to inhibit AR function in a ligand-independent manner are needed to treat prostate cancer.

The proapoptotic protein prostate apoptosis response-4 (Par-4) was originally identified in prostate cancer cells undergoing apoptosis in response to ionomycin, which elevates intracellular Ca^{2+} levels (14). Par-4 is up-regulated in the rat ventral prostate after castration, and pretreatment of rats with the calcium channel blocker nifedipine prevents castration-inducible Par-4 up-regulation as well as apoptosis and involution of the ventral prostate (14). In a cell type– and inducer-specific manner, Par-4 expression is essential for apoptosis by cytokines, such as tumor necrosis factor (TNF) and TNF-related apoptosis-inducing ligand, which act via the death receptor signaling pathway, as well as by doxorubicin, etoposide, UV irradiation, growth factor deprivation, and ionizing radiation, which act via the intrinsic mitochondrial pathway (15, 16). The exact point at which Par-4 converges on these pathways is not yet delineated. Interestingly, ectopic Par-4 selectively induces apoptosis in cancer cells, but not in normal cells, and this cancer-selective function is mediated by its central core domain designated as the SAC domain (17). Both protein kinase A (PKA) phosphorylation, which results in the activation of Par-4, and nuclear entry are essential for apoptosis by either ectopic Par-4 or its SAC domain in cancer cells (18). Similar to the findings with ectopic Par-4, endogenous Par-4 confers apoptosis by a mechanism that requires PKA phosphorylation of its T155 residue as well as nuclear translocation for inhibition of NF-κB transcription activity (18). Par-4 action may be also mediated by interactions via its leucine zipper domain with partner proteins,

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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such as ζ protein kinase C (19) or Amida (20) in the cytoplasm and WT1 (21), THAP (22), or ZIPK/Daax (23) in the nucleus. Endogenous Par-4, however, is phosphorylated and inactivated by AKT and sequestered by 14-3-3 in the cytoplasm (24), thereby explaining why cancer cells survive in the presence of endogenous Par-4.

Our laboratory is interested in identifying natural agents with therapeutic potential against cancer and preferably minimal toxicity against normal cells. Because Par-4 induces cancer-selective apoptosis, we screened a panel of natural dietary compounds for Par-4 induction to identify compounds with apoptotic potential in prostate cancer cells. Our studies identified withaferin A (WA), a major constituent of the medicinal plant *Withania somnifera*, as the only compound in this panel that induced Par-4 expression in prostate cancer cells. The crystal structure of WA indicates that it is a highly oxygenated C-28 ergostane-type steroid with a 22,26-lactone and a 1-oxo-group (25). The ethnobotanical history of WA-containing herbal preparations in the treatment of cancer, inflammatory conditions (26, 27), and neurologic disorders (28), and the growth-inhibitory properties in tumor cell culture studies (29–31) prompted us to determine the mechanism of action of WA in prostate cancer cells. Our results suggest that WA induces apoptosis of prostate cancer cells and that this action is dependent on Par-4 function in both androgen-responsive and androgen-refractory prostate cancer cells.

Materials and Methods

Cell lines, plasmid constructs, and small interfering RNA duplexes. PC-3, LNCaP, and PzHPV-7 cells were purchased from American Type Culture Collection (Manassas, VA). The highly aggressive LNCaP-derivative LN3 cells were from Curtis Pettaway (The University of Texas M.D. Anderson Cancer Center, Dallas, Houston, TX). CWR22RV-1 cells were kindly provided by Dr. Michael Sramkoski (Case Western Reserve University, Cleveland, OH). PC-3 and CWR22Rv-1 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS); LNCaP and LN-3 cells were grown in DMEM supplemented with glutamine in 10% FBS and antibiotics; and PzHPV-7 cells were grown in keratinocyte serum-free medium from Sigma Chemical Co. (St. Louis, MO). In experiments that examined the role of dihydrotestosterone and AR in WA action, the androgen-responsive cells were grown in 10% charcoal stripped–serum containing medium lacking phenol red in the presence of dihydrotestosterone (Sigma Chemical). The small interfering RNA (siRNA) oligonucleotide duplexes for human Par-4, mouse Par-4, AR, or scrambled control were from Dharmacon, Inc. (Lafayette,CO). Human and mouse Par-4 show >85% similarity at the amino acid level. Importantly, all critical domains, especially those involved in the induction of apoptosis, are conserved in human and mouse Par-4. The human Par-4 siRNA sequence targets human Par-4 RNA at an area that shows maximal divergence from mouse Par-4; accordingly, only 11 of 19 nucleotides are similar in human and mouse Par-4 siRNA. The human Par-4 siRNA inhibits human Par-4, whereas the mouse Par-4 siRNA does not inhibit human Par-4, as confirmed by previous studies (24). Therefore, mouse Par-4 siRNA was used as a control in the studies done in PC-3 and CWR22Rv1 cells. The wild-type (WT) AR expression construct was from Gerhard Coetzee (University of Southern California, Los Angeles, CA). The Par-4 promoter-luciferase construct contained ~2 kb of human Par-4 promoter region upstream of the first ATG codon (Genbank accession no. AF541923) to drive luciferase expression in pGL3 (vector from Pierce Biotechnology, Rockford, IL).

Natural dietary compounds, caspase inhibitors, or AR inhibitors. Natural agents, such as curcumin (polyphenol from turmeric; ref. 32), epigallocatechin-3-gallate (polyphenol from green tea; ref. 33), lycopene (carotenoid from tomato; ref. 34), and resveratrol (phytoalexin from grape skin; ref. 35), were from Sigma Chemical; psoralidin (coumestan derivative found in the seed of *Psoralea corylifolia*; ref. 36) was isolated from Rasagenthi lehyam (37) and its purity and structure was confirmed by high-pressure liquid chromatography; and WA, genistein (isoflavone from soy beans; ref. 38), gingerol (vanilloid from ginger; ref. 39), plumbagin (naphthoquinone from the Plumbaginaceae and Drosereceae group of plants; ref. 40), rutin (bioflavanoid from the buckwheat plant *Fagopyrum esculentum*; ref. 41), and diosgenin (steroidal saponin from fenugreek; ref. 42) were from Chromadex (Santa Ana, CA). Caspase-3 inhibitor zDEVD-FMK was purchased from Calbiochem (San Diego, CA). Androgen antagonist and hydroxyl flutamide were purchased from Sigma Chemical. Based on initial optimization studies, we used 4 μmol/L amounts of WA, 1 μmol/L flutamide, or 2 μmol/L WA in combination with 1 μmol/L flutamide or 2 μmol/L Casodex.

Cell viability and apoptotic assays. Cells were treated with WA or vehicle (DMSO) for 12 or 24 h, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or apoptotic assays [Annexin V-FITC or terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL)] were done as described previously (32, 37, 43).

Western blot analysis. Cells were treated with WA (at the IC_{50} concentration for each cell line based on initial optimization studies) for various time intervals, and cell lysates were subjected to Western blot analysis using antibodies for Par-4, Bcl-2, and AR from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); cleaved (active) caspase-8 and cleaved (active) caspase-3 from Cell Signaling (Danvers, MA); or β-actin from Sigma Chemical as a loading control.

Reverse transcription-PCR analysis. Total RNA was isolated from cells after the indicated treatments using Trizol (Sigma Chemical, St. Louis, MO). The RNA (1 μg) was subjected to reverse transcription using the avian myeloblastosis virus reverse transcriptase kit from Roche (Indianapolis, IN). Double-stranded cDNA was synthesized by PCR using the forward primer (5′-GGCACACCTGGGAGCCGGATCC-3′) and the reverse primer (5′-CGCA-GCTTCCTCTTCTCGATCT-3′) for Par-4 and the GC-RICH PCR kit from Roche to amplify the products. PCR amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was similarly done as a control using 5′-AAGGTGAAGGTCGGAGTCAACG-3′ and 5′-CAGGGATGATGTT-CTGGAGAGC-3′ as forward and reverse primers, respectively. The optimized thermal cycling conditions were 95°C for 5 min followed by 28 cycles of amplification at 55°C, 68°C, and 95°C, each for 1 min. PCR products were resolved by electrophoresis on 1% agarose gels, stained with ethidium bromide, and documented by photography. Nucleotide sequencing of the PCR products was done at the University of California (Davis, CA).

Transient transfection and luciferase assays. Prostate cancer cells (at 80-90% confluency) were transiently transfected using LipofectAMINE Plus reagents (Life Technologies, Carlsbad, CA) with 4 μg of either the Par-4 promoter-luciferase construct or the NF-κB-luciferase construct (containing two tandem NF-κB-responsive sites) in the presence of β-galactosidase control or vector containing Renilla luciferase to normalize for transfection efficiency as described previously (17). Transfected cells were treated with WA or vehicle (DMSO) as indicated, and the cells were harvested after 24 h for luciferase or β-galactosidase activity assays.

Xenograft studies. We tested the effect of WA on tumors derived from PC-3 cells in 5 to 6 week male nude (*nu/nu*) mice (from Charles River Laboratories, Wilmington, MA), in accordance with the University of Kentucky Animal Care and Use Committee guidelines. About 5×10^6 cells were injected s.c. into 20 animals, and tumors were allowed to grow until they reached a volume of 50 mm^3. At this time, the animals were randomized into two groups, with 10 mice in each group, and WA (5 mg/kg body weight, dissolved in DMSO and diluted by PBS) or same amount of DMSO was dissolved in PBS given intratumorally 5 days a week for up to 4 weeks. WA dose was determined based on our initial optimization of experiments in mice. Tumor volume was measured daily over the observation period.

Immunocytochemical analysis. PC-3 cells, grown in chamber slides, were treated with WA and subjected to indirect immunofluorescence for active forms of caspase-8 and caspase-3 or for Par-4 using primary antibodies from Santa Cruz Biotechnology. A secondary antibody

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conjugated with the fluorescent dye Alexa Fluor 488 (green) or Alexa Fluor 594 (red) from Molecular Probes, Inc. (Eugene, OR) was used to detect the primary antibody. Immunocytochemical with normal rabbit antibody was used as a control. For protein localization, nuclei were stained with propidium iodide or 4′,6-diamidino-2-phenylindole hydrochloride (DAPI) for 20 min after cell fixation.

Fluorometry for caspase-3 activation. Caspase activation was analyzed using the ApoAlert caspase-3 fluorescent assay kit according to the manufacturer's instructions (Clontech, Mountain View, CA). Cell lysates were treated with caspase-3 inhibitor and reaction buffer, and after incubation for 30 min on ice, caspase-3 substrate (DEVD-AFC) was added and incubated for 1 h at 37°C. The samples were transferred to 96-well plates and read in a fluorometer with an excitation wavelength of 400 nm and emission wavelength of 505 nm. Nonconjugated AFC supplied with the kit was used to construct the calibration curve, and the caspase-3 activity was read against the calibration curve.

Statistical analysis. All experiments were done at least thrice to ascertain the reproducibility of the results. The data shown are representative of three experiments. The apoptosis data shown are a mean of four readings from each of the three experiments (total of 12 readings) ±SD error bars. The Student's *t* test was used to calculate statistical significance.

Results

Screening purified components of natural dietary products for Par-4 induction. As Par-4 is induced exclusively by apoptotic insults, and not during growth stimulation, growth arrest, or necrosis (14), and the elevation of Par-4 expression induces p53- and PTEN-independent apoptosis of cancer cells and tumor regression (44, 45), we developed a cell-based assay using Par-4 expression as a molecular indicator to screen for natural dietary compounds with apoptotic potential. The AR-negative (androgen refractory) prostate cancer cells, PC-3, which lack p53 and PTEN function and are sensitive to apoptosis by Par-4 (17), were transfected with either the Par-4 promoter-luciferase reporter construct or the control pGL3-luciferase construct lacking the Par-4 promoter to test a panel of natural dietary compounds for induction of Par-4. The transfectants were treated with either vehicle (DMSO) or various natural dietary compounds for 6 h, and induction of the Par-4 promoter was analyzed by luciferase assays. As seen in Fig. 1*A*, luciferase activity was induced by the Par-4 promoter in response to WA, not plumbagin (5 μmol/L), diosgenin (10 nmol/L), genistein (30 μmol/L), rutin (60 μmol/L), psoralidin (15 μmol/L), gingerol (40 μmol/L), curcumin (4 μmol/L), resveratrol (1 μmol/L), lycopene (1 μmol/L), or epigallocatechin-3-gallate (30 μmol/L). In contrast, WA did not induce luciferase activity in cells transfected with the pGL3 control vector (data not shown). To verify induction of endogenous Par-4 by WA, parental PC-3 cells were treated with the various compounds for 12 or 24 h, and whole-cell lysates were examined for Par-4 expression by Western blot analysis. As seen in Fig. 1*B*, Par-4 expression was induced by WA, but not by the other compounds. Thus, the Par-4 reporter assay was effective in identifying WA as a prospective natural product that may regulate apoptosis via Par-4.

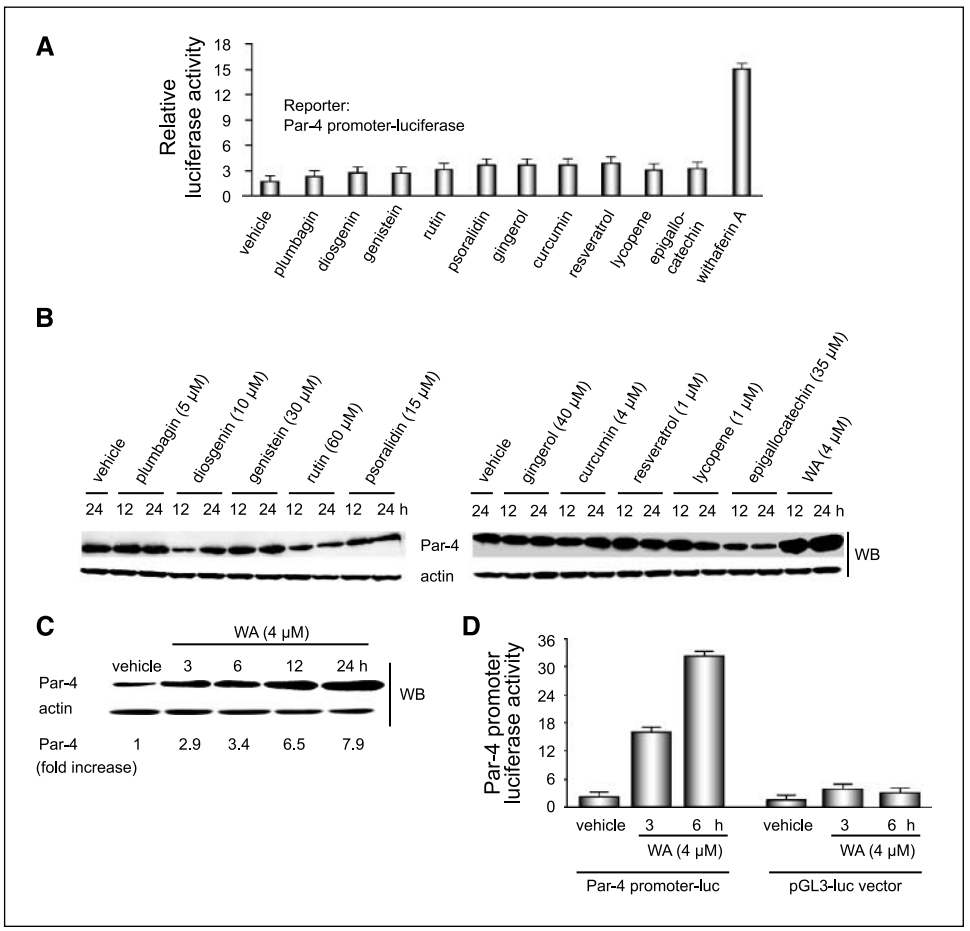

Figure 1. Par-4 expression is up-regulated by WA. *A,* PC-3 cells were transfected with the Par-4 promoter-luciferase reporter construct and the β-galactosidase construct and treated with various natural dietary compounds at their respective IC_{50} dose or vehicle for 6 h, and cell lysates were subjected to luciferase activity assays. The doses used were the following: 5 μmol/L plumbagin, 10 nmol/L diosgenin, 30 μmol/L genistein, 60 μmol/L rutin, 15 μmol/L psoralidin, 40 μmol/L gingerol, 4 μmol/L curcumin, 1 μmol/L resveratrol, 1 μmol/L lycopene, 35 μmol/L epigallocatechin-3-gallate, and 4 μmol/L WA. Relative luciferase activity normalized with respect to the corresponding β-galactosidase activity. *B,* PC-3 cells were treated with various natural dietary compounds or vehicle for 12 or 24 h, and whole-cell lysates were subjected to Western blot analysis for Par-4 or actin. *C,* PC-3 cells were treated with vehicle or WA (IC_{50} dose of 4 μmol/L) for the indicated time intervals, and whole-cell lysates were subjected to Western blot analysis for Par-4 or actin. Fold increase in Par-4 protein levels. *D,* PC-3 cells were transfected with either the Par-4 promoter-luciferase (*Par-4 promoter-luc*) reporter construct or pGL3-luciferase (*pGL3-luc*) control vector and β-galactosidase construct. The transfectants were then treated with WA or vehicle for 3 or 6 h, and cell lysates were subjected to luciferase activity assay. Relative luciferase activity normalized with respect to corresponding β-galactosidase activity.

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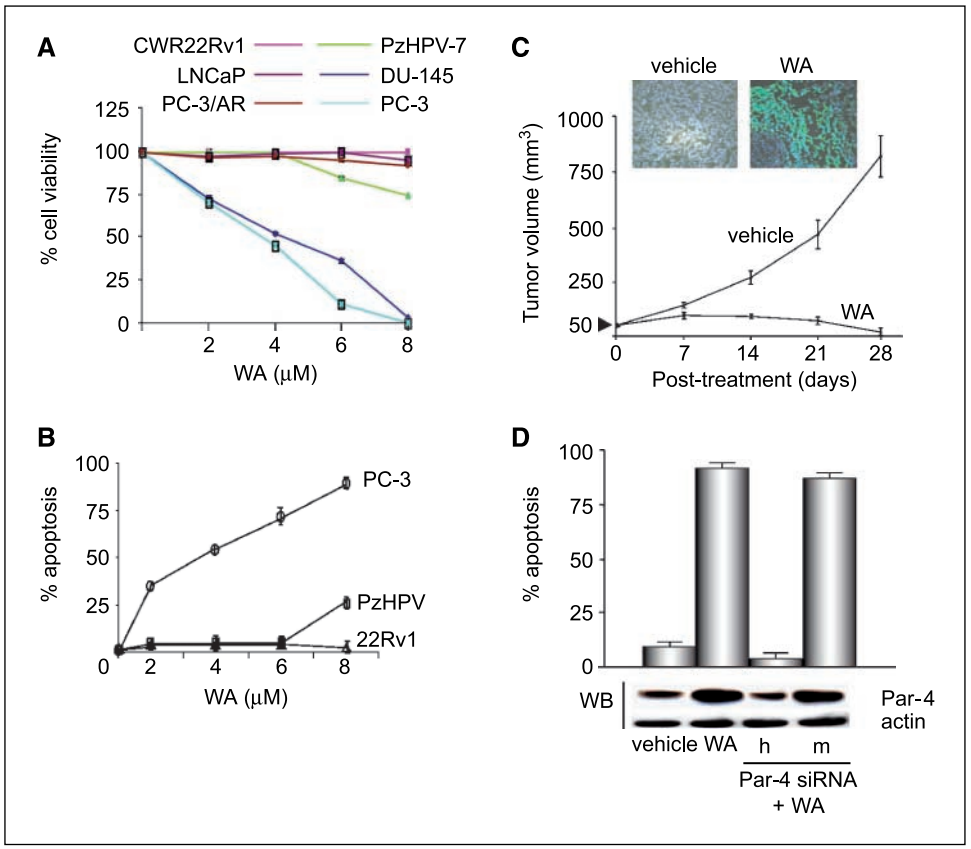

Figure 2. WA induces apoptosis by a Par-4–dependent mechanism. *A,* androgen-responsive (AR mutant) prostate cancer cells (LNCaP and CWR22Rv-1), androgen-refractory (AR negative) prostate cancer cells (PC-3 and DU-145), PC-3 cells transfected with WT AR (*PC-3/AR*), and a normal prostate epithelial cell line (PzHPV-7) were treated with various concentrations of WA for 24 h, and the cell viability was measured by MTT assay. *Points,* mean of 12 wells from three independent experiments; *bars,* SD. *B,* cells were treated with various concentrations of WA and apoptotic cells were scored after 24 h by Annexin V assays and confocal microscopy. *Points,* mean of 12 readings from three independent experiments; *bars,* SD. *C,* PC-3 cells were implanted s.c. in nude mice, and when the tumors reached a volume of 50 mm^3, they were injected intratumorally with vehicle or WA. Tumor growth was monitored over a 4-week period. Tumor volumes at 7-day intervals (*inset*). Tumors treated with vehicle or WA were sectioned at day 14 and subjected to TUNEL assay. *D,* PC-3 cells were transfected with human (*h*) or mouse (*m*) Par-4 siRNA and then treated with WA or vehicle for 24 h. Cells were either scored for apoptosis by confocal microscopy (*top*), or cell lysates were subjected to Western blot analysis (*WB*), to ascertain inhibition of Par-4 expression by siRNA (*bottom*).

To study the kinetics of Par-4 induction by WA, PC-3 cells were treated with WA (4 μmol/L) for various time intervals and Par-4 expression was examined by Western blot analysis. As seen in Fig. 1*C*, WA up-regulated Par-4 within 3 h of treatment and maximal up-regulation was seen at 24 h. Next, to ascertain that Par-4 is up-regulated by WA at the RNA and promoter level, PC-3 cells were treated with WA (4 μmol/L), and RNA from the cells was examined by reverse transcription-PCR (RT-PCR). Alternatively, cells were transiently transfected with either the Par-4 promoter-luciferase reporter or control pGL3-luciferase construct and treated with WA (4 μmol/L), and lysates were harvested for luciferase assays. RT-PCR analysis indicates that WA caused up-regulation of Par-4 RNA relative to the GAPDH control in PC-3 cells (Supplementary Fig. S1). Consistently, robust induction of Par-4 promoter activity by WA relative to vehicle-treated cells was noted (Fig. 1*D*). Together, these findings indicate that WA consistently up-regulates Par-4 expression in PC-3 cells.

WA induces apoptosis in prostate cancer cells. To determine whether WA inhibited prostate cancer cell survival, we did cell viability assays. Prostate cancer cells PC-3 and DU145 (both of which lack AR), PC-3 transfectants stably expressing ectopic WT AR (PC-3/AR), prostate cancer cells LNCaP and CWR22Rv-1 (both of which express AR mutant), and normal/immortalized prostate cancer cells PzHPV-7 were treated with various concentrations of WA for 24 h, and cell viability was quantified. WA caused significant inhibition of cell viability in the androgen-refractory PC-3 and DU145 cells relative to the AR-transfected PC-3/AR cells, LNCaP or CWR22Rv-1 (androgen responsive) prostate cancer cells, and immortalized PzHPV-7 cells (Fig. 2*A*). To resolve whether inhibition of cell viability by WA was a consequence of apoptosis, we treated PC-3, CWR22Rv-1, and PzHPV-7 cells with WA for 24 h and measured apoptosis. As seen in Fig. 2*B*, WA induced significant dose-dependent apoptosis in PC-3 cells relative to CWR22Rv-1 cells and PzHPV-7 cells. Moreover, WA induced time-dependent apoptosis over a 24-h period in PC-3 cells (Supplementary Fig. S2) and also translocated Par-4 to the nucleus (Supplementary Fig. S3). Together, these findings suggest that WA causes apoptosis in androgen-refractory prostate cancer cells.

To ascertain the effect of WA on prostate tumor growth, we tested the effect of WA on PC-3 xenografts in nude mice. The animals were injected intratumorally with WA or vehicle and tumor volumes were measured up to 4 weeks after the injections. As seen in Fig. 2*C*, tumors injected with vehicle continued to grow, whereas tumors injected with WA showed significant ($P < 0.001$) growth inhibition. Importantly, the WA-treated tumors failed to regrow during the 8-week observation period. When sections of the tumors were tested for Par-4 expression and apoptosis, a significant number of cells in the WA-treated tumors showed Par-4 up-regulation (Supplementary Fig. S4) and apoptosis (Fig. 2*C, inset*) relative to vehicle-treated tumors. Thus, WA caused induction of Par-4 expression, apoptosis, and regression of experimental tumors.

Role of Par-4 in the apoptotic action of WA. To determine whether Par-4 induction was causally involved in the apoptotic action of WA, we knocked down Par-4 expression in PC-3 cells by using human Par-4 siRNA and then treated the cells with vehicle or WA for 24 h. Cells transfected with mouse Par-4 siRNA or vector alone were used as control. The effect of Par-4

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siRNA was confirmed by Western blot analysis (Fig. 2*D, bottom*) and, after treatment with WA, Par-4 levels were inhibited in human Par-4 siRNA-treated cells (Fig. 2*D*). Importantly, we noted that WA-inducible apoptosis was inhibited in cells transfected with human Par-4 siRNA but not in cells transfected with control mouse Par-4 siRNA (Fig. 2*D, top*). Moreover, transfection with dominant-negative Par-4 abrogated WA-inducible apoptosis in PC-3 cells (Supplementary Fig. S5). These results indicate that Par-4 is essential for WA-inducible apoptosis.

WA induces caspase signaling and inhibits NF-κB activity by a Par-4–dependent pathway. In studies aimed at identifying the downstream signaling pathways involved in WA-inducible apoptosis of PC-3 cells, we noted that WA caused the activation of caspase-3, as judged by immunocytochemical fluorometry assays (Supplementary Fig. S6). Moreover, inhibition of caspase-3 activity by zDEVD-FMK led to inhibition of WA-inducible apoptosis (Fig. 3*A*), implying that caspase-3 activation was essential for WA-inducible apoptosis. Interestingly, Western blot analysis for Par-4 in cells treated with WA and the caspase-3 inhibitor indicated that WA induces Par-4 expression despite inhibition of caspase-3, implying that Par-4 induction occurs upstream of caspase-3 activation in the apoptotic pathway induced by WA. Further immunocytochemical studies indicated that WA induces the activation of caspase-8 and caspase-3 (Fig. 3*B*). To determine the role of Par-4 in caspase activation, we knocked down endogenous Par-4 in PC-3 cells using either human Par-4 siRNA or mouse Par-4 siRNA for control, treated the cells with WA, and did immunocytochemical for activated caspase-8 or caspase-3. As seen in Fig. 3*B*, siRNA for human Par-4, but not mouse Par-4, prevented the activation of caspase-8 and caspase-3 by WA. These results indicate that Par-4 induction is necessary for caspase-8 and caspase-3 activation by WA in PC-3 cells.

Because Par-4-inducible apoptosis involves inhibition of both NF-κB transcription activity (44) and downstream targets of NF-κB, including Bcl-2 (46), we examined whether WA regulated NF-κB activation and Bcl-2 expression. Interestingly, WA inhibited NF-κB-dependent luciferase activity (Supplementary Fig. S7) and caused down-regulation of Bcl-2 expression (Supplementary Fig. S8) in PC-3 cells. To determine whether Par-4 function was essential for inhibition of NF-κB signaling, we knocked down Par-4 expression in PC-3 cells by using Par-4 siRNA, transfected the cells with the NF-κB-luciferase construct, treated the cells with WA, and did luciferase assays for NF-κB activity and Western blot analysis for Bcl-2 expression. WA failed to inhibit NF-κB activity (Fig. 3*C*) and Bcl-2 expression (Fig. 3*D*) in cells where Par-4 expression was knocked down with human Par-4 siRNA but not when Par-4 levels were unaffected with mouse or control Par-4 siRNA. These findings imply that Par-4 function is necessary for

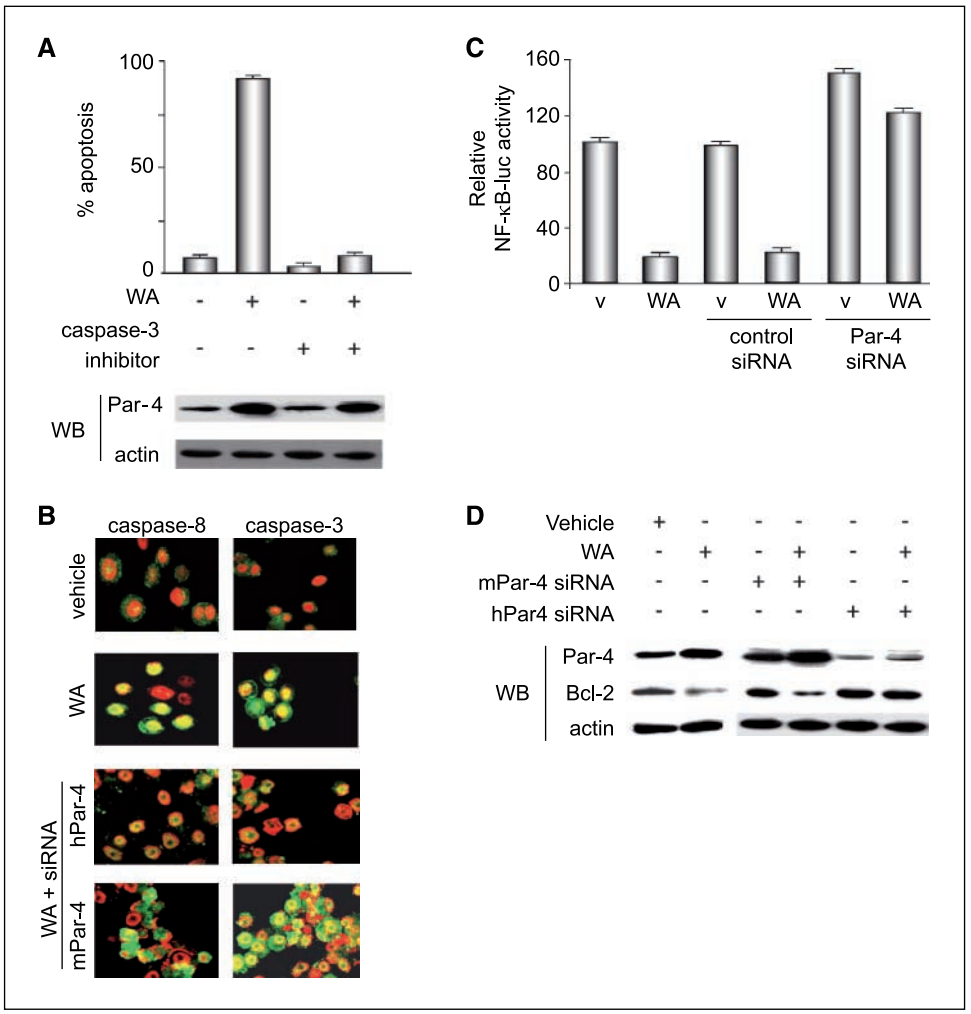

Figure 3. Caspase activation and inhibition of cell survival signaling by WA is Par-4 dependent. *A,* PC-3 cells were treated with caspase-3 inhibitor zDEVD-FMK and then exposed to WA or vehicle for 24 h. Cells were subjected to TUNEL assays to score for apoptosis (*top*), or cell lysates were examined for Par-4 expression by Western blot analysis (*bottom*). *B,* PC-3 cells were transfected with siRNA for either human Par-4 or mouse Par-4 and then treated with WA or vehicle for 24 h. The cells were then subjected to immunocytochemical for active forms of caspase-8 or caspase-3. FITC green fluorescence correlates with caspase activity. Nuclei were stained with DAPI (pseudo-colored red) and superimposed images are shown. *C,* PC-3 cells were transfected with siRNA for either human Par-4 or mouse Par-4 and then transfected with NF-κB reporter construct and β-galactosidase construct. The transfectants were treated with vehicle (*v*) or WA, and after 12 h, cell lysates were subjected to luciferase activity assays. Relative luciferase activity normalized with respect to corresponding β-galactosidase activity. *D,* PC-3 cells were transfected with siRNA for either human Par-4 or mouse Par-4 and treated with WA (4 μmol/L) or vehicle. The cells were then subjected to Western blot analysis for Par-4, Bcl-2, and actin.

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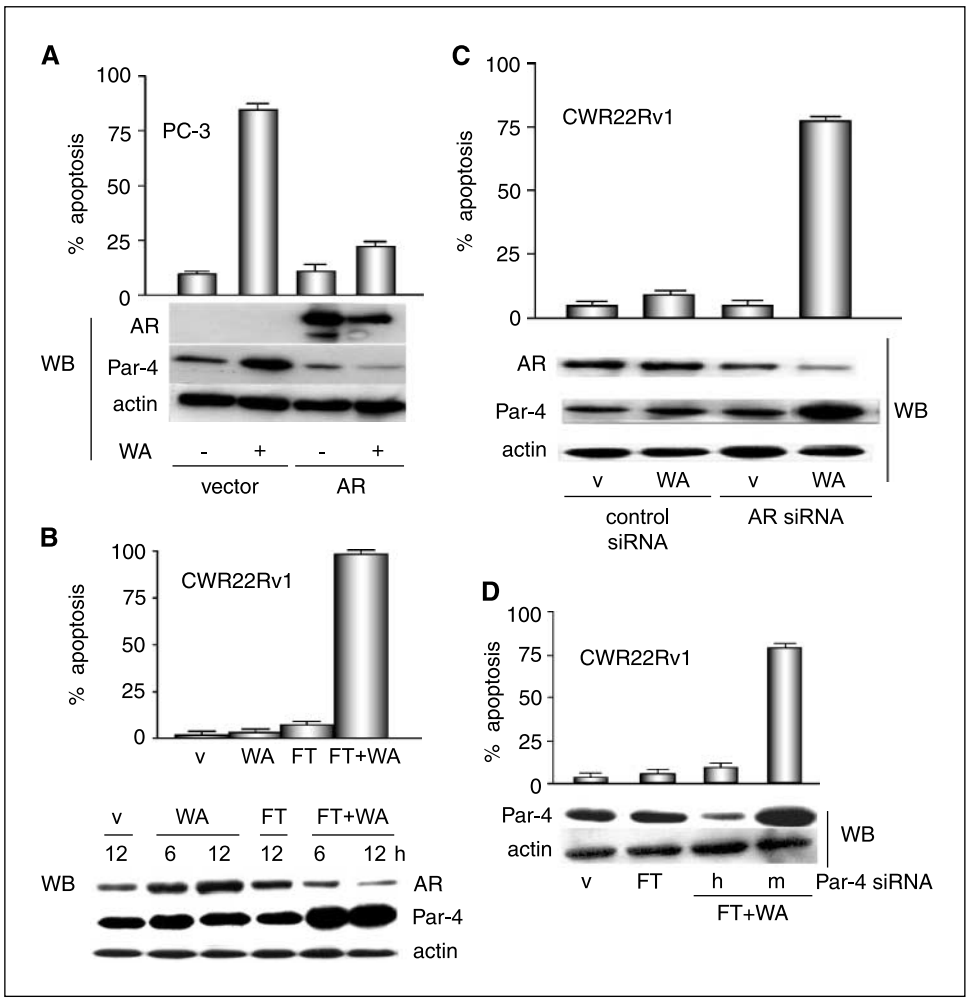

Figure 4. AR inhibits Par-4–dependent apoptosis in prostate cancer cells. *A,* PC-3 cells were transfected with either control vector or WT AR expression construct and then treated with WA (8μmol/L) or vehicle for 24 h. Whole-cell extracts were examined by Western blot analysis for AR and actin (*bottom*), or the cells were subjected to apoptosis assays (*top*). *B,* CWR22Rv-1 cells were treated with WA, flutamide (*FT*), vehicle, or flutamide plus WA for 6 or 12 h. Whole-cell extracts were examined by Western blot analysis for AR, Par-4, and actin (*bottom*), or the cells were subjected to apoptosis assays after 12 h (*top*). *C,* CWR22Rv-1 cells were transfected with siRNA for AR or scrambled siRNA duplexes for control, and the cells were then treated with WA or vehicle for 12 h. Whole-cell extracts were examined by Western blot analysis for AR, Par-4, and actin (*bottom*), or the cells were subjected to apoptosis assays (*top*). *D,* CWR22Rv-1 cells were transfected with siRNA for human Par-4 or mouse Par-4, and the cells were then treated with WA, flutamide, vehicle, or flutamide plus WA for 12 h. Whole-cell extracts were examined by Western blot analysis for Par-4 and actin (*bottom*), or the cells were subjected to apoptosis assays (*top*).

WA to down-regulate prosurvival NF-κB activity and Bcl-2 expression in PC-3 cells.

AR prevents Par-4 induction and apoptosis by WA. As WA induced apoptosis in androgen-refractory prostate cancer cells, but not in androgen-responsive prostate cancer cells, we sought to directly address whether AR modulates sensitivity to WA. PC-3 cells were transfected with an expression construct for either WT AR or control vector, and AR expression was verified by Western blot analysis (Fig. 4*A*). The transfectants were then treated with WA and scored for apoptosis. As seen in Fig. 4*A*, WA-inducible Par-4 expression and apoptosis were significantly inhibited ($P < 0.001$) in transfectants expressing WT AR relative to those expressing the control vector, suggesting that AR may confer resistance to apoptosis by WA.

Next, we determined the role of AR in resistance to WA-mediated apoptosis in prostate cancer cells that express endogenous AR (i.e., androgen-responsive prostate cancer cells CWR22Rv-1, LNCaP, and LN-3). First, we asked whether inhibition of AR signaling with anti-androgen could render CWR22Rv-1 cells susceptible to WA-inducible apoptosis. As seen in Fig. 4*B*, neither anti-androgen (flutamide) nor WA caused apoptosis in the androgen refractory prostate cancer cells. However, the combination of anti-androgen and WA caused significant induction of apoptosis in the cells (Fig. 4*B*). Moreover, Western blot analysis indicated that neither WA nor anti-androgen induced Par-4 expression (Fig. 4*B*); however, the combination of anti-androgen and WA down-regulated AR and up-regulated Par-4 expression in the AR-mutant cells.

We also knocked down AR expression in CWR22Rv-1 cells with AR siRNA to directly validate the role of AR in Par-4 induction and sensitivity to WA-mediated apoptosis. As seen in Fig. 4*C*, WA up-regulated Par-4 expression and induced apoptosis in CWR22Rv-1 cells transfected with AR siRNA but not in cells transfected with control siRNA. It is important to note that WA induces AR expression in CWR22Rv-1 cells, which in turn down-regulates Par-4 expression. This may be the reason for the resistance of the CWR22Rv-1 cells to WA. These results indicate that AR signaling prevents Par-4 induction and apoptosis by WA; therefore, inhibition of AR signaling restores Par-4 induction and sensitivity to WA.

Finally, we sought to determine whether Par-4 function was essential for induction of apoptosis by the combination of WA and flutamide in androgen-responsive cells. CWR22Rv-1 cells were transfected with either human Par-4 siRNA or mouse Par-4 siRNA and then treated with either the combination of flutamide and WA or vehicle. Inhibition of endogenous Par-4 with human Par-4 siRNA, but not with mouse siRNA, was confirmed by Western blot analysis (Fig. 4*D*). When the cells were scored for apoptosis, we noted that the combination of flutamide and WA induced apoptosis in cells transfected with mouse Par-4 siRNA but not in

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cells transfected with human Par-4 siRNA (Fig. 4*D*; Supplementary Fig. S9). Together, these findings suggest that inhibition of AR signaling facilitates Par-4–dependent apoptosis of androgen-responsive prostate cancer cells.

Discussion

Our findings suggest that WA preferentially induces apoptosis in androgen-refractory prostate cancer cells but not in androgen-responsive (WT AR or AR mutant) prostate cancer cells and normal/immortalized prostate epithelial cells. Interestingly, treatment of androgen-responsive prostate cancer cells with anti-androgens (flutamide and Casodex; data not shown), which on their own do not cause apoptosis, rendered the cells amenable to apoptosis by WA. Apoptosis by WA required the up-regulation of Par-4 expression for inhibition of NF-κB activity and for activation of the caspase cascade. Thus, this study identified, for the first time, the mechanism of apoptosis by WA. Moreover, our findings indicate that despite AR-mutation, prostate cancer cells are responsive to AR signaling, which sustains cell survival even in the presence of anti-androgens, and that a combination of WA and anti-androgens can synergistically induce Par-4 expression and apoptosis. Because androgen depletion independence is a critical determinant of aggressive prostate cancer that is refractory to conventional treatment, our findings provide new insight for the treatment of such tumors using a combinatorial approach with natural compounds, such as WA, which may work synergistically with anti-androgens to inhibit cell survival.

As Par-4 is induced exclusively by apoptotic agents (14), we used the Par-4 promoter construct to screen a panel of natural products for apoptotic activity in cancer cells. Moreover, mutations in the *Par-4* gene have not been reported in cancer cells, but the protein is rendered inactive by binding and phosphorylation by Akt activity, which is elevated in cancer cells (24). Par-4 function is essential for the induction of apoptosis by a broad range of agents, implying that endogenous Par-4 must be activated in response to these apoptotic agents (16). Two potential modes of Par-4 activation are the following: (*a*) up-regulation of Par-4 above the necessary thresholds to override inhibition by Akt, translocate Par-4 to the nucleus, and induce apoptosis (45) and (*b*) inhibition of Akt to release Par-4 for apoptosis (24). Our studies indicate that, from the compounds tested in this screen, only WA was able to induce Par-4 expression. Several other compounds from this panel have been reported in the literature to cause either growth-inhibitory or apoptotic effects in cancer cells, yet these compounds did not induce Par-4 expression. Apparently, these compounds induce apoptosis either by inhibiting Akt activity to release active Par-4 for apoptosis or by triggering a Par-4–independent mechanism. Our ongoing studies are poised to address the mechanism of prostate cancer cell apoptosis by the natural compounds that did not induce Par-4 expression. As Par-4 can induce apoptosis in the absence of p53 or PTEN function, both of which are often lost in cancer, our findings using the Par-4 reporter can be extended to high-throughput screens of natural compounds and small molecules for their anticancer/apoptotic potential. Most importantly, because ectopic Par-4 expression induces apoptosis exclusively in cancer cells and not in normal cells, the ideal compounds identified in the Par-4 reporter assays would preferentially induce Par-4–dependent apoptosis without activating other apoptotic pathways that would compromise cancer cell selectivity and consequently induce toxicity in normal cells. In the present study, WA failed to induce apoptosis in immortalized prostate epithelial cells, except at very high toxic doses. However, further studies using a broad panel of cell lines and *in vivo* models are essential to determine whether WA shows differential effects in normal and cancer cells.

Although several previous reports have described the growth-inhibitory potential of WA, the present study shows for the first time that WA inhibits cell viability by inducing apoptosis. Moreover, the mechanism of apoptosis involves induction of Par-4, activation of caspase-8 and caspase-3, and inhibition of the prosurvival activity of NF-κB. RNAi-mediated knockdown of Par-4 results in abrogation of WA-inducible caspase activation and inhibition of NF-κB activity, thereby positioning Par-4 induction by WA as an essential event upstream of caspase activation and NF-κB inhibition. Thus, WA, acting via induction of endogenous Par-4, activates a caspase-8- and caspase-3-dependent apoptotic pathway in prostate cancer cells.

AR function is integrally linked to prostate cancer progression. Initially, androgen independence was believed to be a consequence of AR loss, but it is now apparent that AR is expressed in hormone-refractory prostate cancer. There is a growing consensus, therefore, that the lack of responsiveness to anti-androgen therapy is a function of either enhanced sensitivity of AR to androgen levels (owing to mutations) or post-translational alterations in AR (13). Accordingly, both androgen-responsive or androgen-refractory prostate cancer cells have been identified in clinical specimens as well as in cell lines derived and established from advanced prostate cancer, as exemplified by the AR-negative PC-3 and DU145 cells and the LNCaP and CWR22Rv-1 (AR mutant) cells. Our findings suggest that, similar to AR-negative cells, AR-mutant cells, in which AR expression is knocked down by RNAi or AR signaling is ablated by anti-androgens, allow WA to induce Par-4. By contrast, AR-mutant cells with functionally active AR signaling do not allow WA to induce apoptosis. These findings on the suppression of Par-4 expression by AR signaling are consistent with our previous observation that castration, which depletes androgen and AR signaling, causes induction of Par-4 in the rat ventral prostate and that implantation of testosterone pellets prevents Par-4 induction following castration (47). Thus, AR signaling inhibits Par-4 induction by WA. Further studies should uncover the proximal events responsible for induction of the Par-4 promoter by WA and its suppression by AR signaling.

Most prostate tumors initially respond to androgen ablation therapy but later convert to androgen depletion-independent growth. At this advanced stage, AR inhibitors fail to function effectively, leaving little to no recourse for the patient. Because AR impedes the action of WA, but a combination of anti-androgens and WA induces apoptosis in androgen-independent prostate cancer cells, our studies underscore the significance of screening new compounds to identify synergistic combinations for prostate cancer therapy.

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