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Withaferin-A induces mitotic catastrophe and growth arrest in prostate cancer cells

Ram V Roy, **Suman Suman**, **Trinath P. Das**, **Joe Luevano**, and **Chendil Damodaran***
Department of Biomedical Sciences, Paul L Foster School of Medicine, Texas Tech University Health Sciences Center, El Paso, TX, USA

Abstract

Cell cycle deregulation is strongly associated with the pathogenesis of prostate cancer (CaP). Clinical trials of cell cycle regulators that target either the G_0/G_1 or G_2/M phase to inhibit the growth of cancers including CaP are increasing. In this study, we determined the cell-cycle regulatory potential of the herbal molecule Withaferin-A (WA) on CaP cells. WA induced irreversible G_2/M arrest in both CaP cell lines (PC3 and DU145) for 48 h. The G_2/M arrest was accompanied by upregulation of phosphorylated Wee1, phophorylated histone H3, p21 and Aurora-B. On the other hand, downregulation of cyclins (E2, A, and B1) and phorphorylated Cdc2 (Tyr15) was observed in WA-treated CaP cells. In addition, decreased levels of phosphorylated Chk1 (Ser345) and Chk2 (Thr68) were evident in WA-treated CaP cells. Our results suggest that activation of Cdc2 leads to accumulation in M-phase, with abnormal duplication, and initiation of mitotic catastrophe that results in cell death. In conclusion, these results clearly highlight the potential of WA as a regulator of the G2/M phase of the cell cycle and as a therapeutic agent for CaP.

1. Introduction

Prostate cancer (CaP) is the third most common cause of cancer-related deaths for men in Western countries [1]. In recent years, development of cutting-edge technologies for early detection of CaP and more effective therapeutic strategies have prevented CaP-related deaths. The pathogenesis of prostate tumors is initially androgen-dependent. Hence, hormone ablation therapy is the primary treatment for CaP [2]; however, many patients relapse to castration-resistant CaP [3].

In the mammalian cell cycle, the transition to G_2/M is tightly regulated by the kinase Cdc2/cyclin B [4]. Inhibition of Cdc2 activity occurs by specific phosphorylation at Tyr15 and Thr14 by two major regulators, Wee1 and myelin transcription factor 1 (Myt1) [5]. On the other hand, Cdc25C dephosphorylates Tyr15 on Cdc2 and converts this kinase to the active form, which is required for mitosis entry[6]. The activity of Cdc2, Cdc25C, and Wee1 proteins are stringently regulated at every phase of the cell cycle [7-9]. The cyclin B-Cdc2 complex localizes to the cytosol in the G2 phase and during prophase the complex is

*Chendil Damodaran, Center of Excellence in Cancer Research, Department of Biomedical Sciences, Paul L. Foster School of Medicine, Texas Tech University Health Sciences Center, El Paso, TX 79905, C.damodaran@ttuhsc.edu, Phone: 915-215-4228, Fax: 915-783-522.

activated and shuttles to the nucleus [10]. Cdc25 is regulated by checkpoint kinase (Chk1) and 14-3-3 proteins. Chk1 phosphorylates Cdc25C at Ser216, creating a consensus binding site for 14-3-3. The binding of 14-3-3 inhibits nuclear translocation from the cytoplasm, while unphosphorylated Cdc25C will migrate towards the nucleus [11, 12].

In recent years, natural compounds have drawn a great deal of attention because of their ability to suppress cancers as well as their potential to reduce the risk of cancer development [13]. One of the best studied natural bioactive compounds is withaferin-A (WA), a major constituent of the medicinal plant *Withania somnifer*. Extracts *of W. somnifera* have been historically used for the treatment of cancers, inflammation, and neurological disorders [14, 15]. Chemically, WA is a steroidal lactone and a highly oxygenated withanolide. The crystal structure of WA indicates a highly oxygenated C-28 ergostane-type steroid with 22, 26-lactone and 1-oxogroup.

WA exhibits an inhibitory effect against several different types of cancer cells. Briefly, in human leukemia cells, WA induced apoptosis in association with JNK and AKT signaling along with inhibition of NFκB activity [16]. WA also inhibited the growth of breast cancer cells in vitro and in vivo by molecularly inhibiting the levels of both transmembrane and cleaved Notch-1, inducing Notch-2 and -4 signaling cascades in MCF-7 and MDA-231 cells [17]. Previously, we reported that WA induces apoptosis in castration-resistant CaP cells but not in androgen-responsive and normal prostate epithelial cells by inducing the pro-apoptotic protein prostate apoptosis response-4 (Par-4) [18]. WA interferes with the ubiquitin–mediated proteasome pathway, leading to increased levels of poly-ubiquitinated proteins and subsequent inhibition of proliferation in human umbilical vein endothelial cells (HUVECs). This molecule also inhibits sprouting of HUVECs via inhibition of NFk-B activation [15].

In this study, we explored the cell cycle regulatory potential of WA on CaP cell lines. Our results suggest that activation of Cdc2 leads to the accumulation of cells in M phase, and results in abnormal duplication and mitotic catastrophe that initiates CaP cell death.

2. Materials and Methods

Chemicals and antibodies

WA, anti-CDK2, anti-CDK-4, anti-CDK-6, anti-cyclin D1, anti-cyclin D2, anti-cyclin D3, anti-cyclin E2, anti-H3, anti-$p21^{(Cip1)}$, anti-$p27^{(Kip1)}$, anti-β-actin, and horseradish peroxidase-conjugated anti-mouse, anti-goat, and anti-rabbit secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cyclin A, cyclin E, cyclin B1, p21, phospho-Wee1, phospho-Cdc2, Myt1, phospho-Chk1/2, 14-3-3, phopho-H3, H3 and Aurora B antibodies were obtained from Cell Signaling (Danvers, MA). Propidium iodide was purchased from Sigma (St. Louis, MO). Alexa Fluor 488, phalloidin, and Prolong gold antifade with DAPI mountant were purchased from Invitrogen (Grand Island, NY).

Cell culture

Human prostate cancer cell lines PC-3 and DU-145 were grown in Dulbecco's modified Eagle's medium and RPMI-1640, respectively, and supplemented with 10% heat-inactivated

fetal bovine serum and 1% antibiotics in multiwell plates at 37°C in a humidified atmosphere containing 5% CO_2. After overnight culture to allow cells to adhere, medium was replaced with or without WA at the indicated concentrations. WA was dissolved in dimethyl sulfoxide (DMSO), and the cells were treated with DMSO at a final concentration of 0.002%.

Cell viability

PC-3 and DU-145 cells were plated at a density of $3\times10_5$ cells/well in six-well plates in medium containing 10% fetal bovine serum. Cells were cultured for 24 h and treated with the addition of different concentrations of WA. Control cells were treated with the same volumes of DMSO, which never exceeded 0.002% of the total volume of the medium. After each treatment, cells were incubated at 37°C for 24 h in an atmosphere of 5% CO2. Viable cells were counted by trypan blue exclusion using a hemocytometer. Results were expressed as a percentage of the number of cells in DMSO-treated control cultures, and the half maximal inhibitory concentration (IC_{50}) values were calculated.

Western blot analysis

Cells were treated with WA at IC_{50} concentrations that had been optimized for each cell line based on preliminary studies for various time periods, and cell lysates were prepared using Pierce M-PER (Thermo Scientific) *lysis buffer containing* 1× *Halt protease inhibitor cocktail* (Pierce). Lysates were subjected to Western blotting. Proteins (25 ug) were subjected to Western blot analysis. Cdc2, Chk1, Chk2, cyclin A, cyclin E, cyclin B1, H3, phospho-H3, p21, Wee1, phospho-Wee1, phospho-Cdc2, Myt1, phospho-Chk1/2, 14-3-3, Aurora B proteins, and loading control β-actin were detected according to the method described [19].

Cell cycle analysis

Cells (PC-3 and DU-145) were plated at a density of 3×10^5 cells/well in six-well plates. After overnight attachment, cells were treated with 1 μM WA for the indicated times. The cells were stained with 0.5 g/L propidium iodide and analyzed using FACScan (Accuri Flow Cytometers Inc., MI, USA) as described previously [20].

Immunofluorescence staining

PC-3 cells were plated on glass coverslips and allowed to attach and grow to 60% confluence overnight. Following treatment with WA for 24 h, cells were washed three times with PBS, fixed in 4% paraformaldehyde for 20 min at room temperature, and permeabilized with 0.2% Triton X-100 for 20 min. Cells were incubated with antibodies to detect the localization and expression of pCdc-2 (Tyr15), α-tubulin, pH3 (Ser10) and Aurora-B. Cells were incubated with secondary antibodies conjugated to Alexa Fluor 488 (Green)/Alexa Fluor 567 (Red). Finally, cells were incubated with rhodamine-phalloidin and mounted using Antifade with DAPI mountant. Cells were analyzed using a Leica laser scanning confocal microscope.

Kinase assay

To determine the role of Chk1 and Chk2 levels in WA-treated CaP cells, CycLex Checkpoint Kinase Assay Kit-1 (CY-1162, MBL, International, MA) was used. Briefly, CaP cells were plated in 96-well plates that were pre-coated with a substrate corresponding to recombinant Cdc25C, which contains serine residues that can be phosphorylated by checkpoint kinases, including Chk1 and Chk2. The detector antibody specifically detects only the phosphorylated serine 216 residue on Cdc25C. To perform the test, the sample was diluted in kinase buffer, which was pipetted into the wells and allowed to phosphorylate the bound substrate after the addition of Mg_2^+ and ATP. The amount of phosphorylated substrate was measured via a binding reaction with a horseradish peroxidase-conjugated anti-phospho-Cdc25C Ser216 specific antibody, and this binding and catalyzes the conversion of the chromogenic substrate tetra-methylbenzidine from a colorless solution to a blue solution (or yellow after the addition of stopping reagent). A similar approach was used to detect Cdc2-cyclinB1 kinase activation (CY-1164, MBL International, MA) in WA-treated CaP cells.

Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using the RNeasy Micro Kit (QIAGEN, Valencia, CA), and cDNA was synthesized using the Applied Biosystems cDNA synthesis kit. Quantitative RT-PCR was performed using the $2^{-\Delta\Delta CT}$ method and SYBR Green supermix (QIAGEN, Hilden, Germany) on an Applied Biosystems multicolor real-time PCR detection system. Cycle threshold values were normalized to amplification measured for β-actin. Primers used for human p21 were (forward) 5′-CGATGCCAACCTCCTCAACGA-3′ and (reverse) 5′-TCGCAGACCTCCAGCATCCA-3′. Amplification was performed under the following conditions: 95°C for 5 min, followed by 45 cycles of 94°C for 30 s and 55°C for 40 s, with an extension at 72°C for 45 s.

3. Results and Discussion

WA inhibits cell viability and induces G_2/M cell cycle arrest in CaP cell lines

CaP cell lines (PC-3 and DU-145) were treated with different concentrations (0.5, 1, 2, 3, and 4 μM) of WA, and cell viability was measured with a trypan blue exclusion assay. A dose-dependent loss of cell viability was observed in both PC-3 and DU-145 cells after 24 h of WA treatment ($P<0.05$; Fig. 1A, 1B). To elucidate the mechanism underlying WA-mediated growth arrest in CaP cell lines, we treated PC-3 and DU145 cells with IC_{50} dose of WA for 12, 24, and 48 h time point. WA treatment significantly induces G_2/M arrest in both cell lines as early as 8 h (data was not shown). As seen in Fig. 1C, G_2/M phase accounted for 62.7% of the total population at 24 h and 60.7% at 48 h, whereas 28.3% and 25.3% of vehicle-treated cells were in G_2/M arrest at both time points respectively in PC-3 cells. Similarly, DU145 cells in G_2/M accounted for 36.75% of the total population at 24 h and 24.75% at 48 h, whereas 22% of vehicle-treated cells were in G_2/M arrest at both time points (Fig. 1D). These results suggest that WA arrests CaP cells in G_2/M phase.

Mechanism underlying WA-induced G2/M cell cycle arrest

Having ascertained that WA causes significant G_2/M cell cycle arrest in CaP cell lines, we probed the molecular mechanism by which WA causes this arrest. First, we investigated the expression patterns of cyclins by Western blot analysis. WA downregulated the expression of cyclin E, A, and B proteins respectively (Fig. 2A). As the G_2/M checkpoint for mitosis entry requires cyclin B and A expression [21, 22], the decreased levels of these proteins in our experiment suggest that CaP cells were arrested at the G2 phase of the cell cycle. Interestingly, we have also found increased expression of p21 at both the mRNA (Fig. 2C) and protein (Fig. 2B) levels following WA treatment. Our Western blot analysis revealed the appearance of two closely migrating bands suggesting p21 hyper-phosphorylated forms upon WA treatment (Fig.2B), which facilitates p21 binding to cyclin B1 to form a complex with Cdc2, thereby promoting kinase activation and G_2-M progression [23]. In general, p53 is the major activator of p21; however, PC-3 cells do not express p53. Hence, WA seemingly induces p53-independent p21 activation in PC-3 cells. Induction of p21 has been reported for both G1 and G2/M cell cycle arrest that eventually led to growth arrest [24]. These results clearly demonstrates that the WA-induced G_2/M cycle arrest in CaP cell lines requires decreased levels of cyclin A, E1, and B1 and increased activation of p21.

WA induces accumulation of active Wee1 in M phase in CaP cells

Transition to the G_2/M phase of the cell cycle is regulated by Cdc2 activity, and Wee-1 and Myt-1 negatively regulate Cdc2 function in mammalian cells [10, 12, 23, 25-27]. Hence, we examined the expression pattern of cell cycle these regulatory proteins in WA-treated CaP cells. An upregulation of pWee1 (Ser642) was seen at 12 and 24 h after WA treatment in PC-3 cells (Fig. 3A). On the other hand, downregulation of phosphorylation of Cdc2 at Tyr15 and of Cdc25c at Ser216 (data not shown) were observed following WA treatment. In addition, induction of Myt1 was downregulated in CaP cells (Fig. 3A), indicating that Myt-1 may not play a role in the regulation of the Cdc2-Cdc25c complex. Previous reports indicated that during M phase, the Cdc2-cyclin B complex is exported to the nucleus [9, 28]. In accordance, we found a punctate staining pattern in the membrane along with a decrease in cytoplasmic levels of pCdc2 (Tyr-15; Fig. 3B). These results demonstrate WA induces a delay in pWee1 degradation followed by activation of Cdc2 through Cdc25C, allowing cells to enter M phase but to arrest in pro-metaphase.

Published results suggest that Wee-1 is an important player that acts as a mitotic inhibitor; however, Wee-1 expression is controversial. For example, overexpression of Wee-1 has been reported for hepatocellular carcinoma [29], breast [30], and glioblastoma tumor tissues [25], while colon [31] and prostate tumor [32] tissues express very low levels of Wee-1. Thus, Wee-1 acts as a tumor suppressor, and therefore, activating Wee-1 could be a useful therapeutic target for CaP. Importantly, we demonstrated that the small molecule WA activates Wee-1 and induces growth arrest.

The role of Chk1 and Chk2 activation in WA-induced G2/M arrest

Next, we determined the role of Chk1 and Chk2 levels in WA-treated CaP cells, as a downregulation of phosphorylated Chk1 (Ser345) and Chk2 (Thr68) was evident (Fig. 4A). Interestingly, no significant changes in total Chk1 expression between WA-treated and

control cells were observed. Next, we determined that no significant changes occurred in kinase activity of Chk1 or the Cdc2-cyclin B complex in WA-treated CaP cells compared with control cells (Fig. 4B and 4C). These results suggest that WA caused G2/M arrest without altering the kinase activity of Chk1 or the Cdc2-cyclin B complex in CaP cells. Activation of Chk1 and Chk2 results in phosphorylation of Cdc25 during the G_1/S-phase transition and phosphorylation of Cdc25C during G_2/M [33]. In addition, activation of Chk1 induces Wee-1 and also arrests the cells in G2/M.

Another G2/M regulator is 14-3-3 [34], which is activated by p53. In our studies, no significant differences were noted in the expression of 14-3-3 in untreated and WA-treated CaP cells (Fig.4D).

G_2/M arrest involves accumulation of Aurora B and defects in α-tubulin depolymerization in WA-treated CaP Cells

During cell division, chromatin rearrangement is coordinated by a group of proteins called the chromosomal passage proteins, of which Aurora B is one.To understand the distribution of Aurora B during WA-induced G2/M arrest, we performed Western blot and confocal analyses. WA induced Aurora B expression at 12 and 24 h in CaP cells (Fig. 5A). Activation of Aurora B resulted in phosphorylation of histone H3 at serine 10, leading to initiation of chromosomal condensation [35]. Also, site-specific phosphorylation of H3 (Fig. 5A) after WA treatment suggests that Aurora B activation may initiate H3 activation in CaP cells. Activation of Aurora B causes spindle positioning, and when cells are arrested at G2/M, the expression of Aurora B reaches its maximum. When cells are preparing for division, Aurora B phosphorylates histone H3 (Ser10) and dissociates the heterochromatin from the chromosome. Therefore, we utilized confocal microscopy to analyze the localization of Aurora B in control as well as WA-treated CaP cells. Aurora B distributed uniformly at lower levels in the cytoplasm of non-dividing cells (Fig. 5B). In WA-treated CaP cells, Aurora B was strongly increased in the centromeric regions as determined by the punctate distribution. These findings suggest that the cells are arrested at the G2/M phase of the cell cycle (Fig. 5B). As more WA-treated cells became arrested in M phase, the proportion of cells with punctate staining was higher than in control cells. Similarly, we observed localization and accumulation of pH3 in the nucleus of WA- treated CaP cells compared to control cells (Fig. 5C). Thus, pH3 staining levels were higher in the treated cells and remained uniformly associated with the chromosomes inside the nucleus. These results demonstrate that more cells are in the process of active recruitment of condensation factors, and WA did not inhibit the early chromatin modeling factors.

One of the characteristic features of active cell division is redistribution of tubulin. PC-3 cells treated with WA were examined for distribution of α-tubulin. WA-treated cells exhibited bipolar spindles (Fig. 5D), suggesting defects in depolymerization of α-tubulin. As a result, cells failed to progress to the next phase. In control cells, microtubule bundles converged to well-defined single poles, and their corresponding chromosomes were situated in the equatorial plane, whereas in WA-treated cells, the spindles are highly distorted and consist of a curving microtubule array (Fig. 5D). In conclusion, our results demonstrate that WA induces cell cycle arrest at the G2/M phase and this arrest may inhibit the growth of

CaP cells. At the molecular level, cell cycle arrest is associated with inhibition of Wee1 expression, resulting in mitotic arrest that eventually leads to cell death. Future studies are required to further elucidate the potential therapeutic role of WA against CaP

References

1. Desantis C, Naishadham D, Jemal A. Cancer statistics for African Americans. CA Cancer J Clin. 2013 2013.
2. Harris WP, et al. Androgen deprivation therapy: progress in understanding mechanisms of resistance and optimizing androgen depletion. Nat Clin Pract Urol. 2009; 6(2):76–85. [PubMed: 19198621]
3. Nacusi LP, Tindall DJ. Targeting 5alpha-reductase for prostate cancer prevention and treatment. Nat Rev Urol. 2011; 8(7):378–84. [PubMed: 21629218]
4. Nurse P. Universal control mechanism regulating onset of M-phase. Nature. 1990; 344(6266):503–8. [PubMed: 2138713]
5. Liu F, et al. Overproduction of human Myt1 kinase induces a G2 cell cycle delay by interfering with the intracellular trafficking of Cdc2-cyclin B1 complexes. Mol Cell Biol. 1999; 19(7):5113–23. [PubMed: 10373560]
6. Perry JA, Kornbluth S. Cdc25 and Wee1: analogous opposites. Cell Div. 2007; 2:12. [PubMed: 17480229]
7. Coleman TR, Dunphy WG. Cdc2 regulatory factors. Curr Opin Cell Biol. 1994; 6(6):877–82. [PubMed: 7880537]
8. Heald R, McLoughlin M, McKeon F. Human wee1 maintains mitotic timing by protecting the nucleus from cytoplasmically activated Cdc2 kinase. Cell. 1993; 74(3):463–74. [PubMed: 8348613]
9. Masuda H, et al. Spatiotemporal regulations of Wee1 at the G2/M transition. Mol Biol Cell. 2011; 22(5):555–69. [PubMed: 21233285]
10. Hagting A, et al. MPF localization is controlled by nuclear export. EMBO J. 1998; 17(14):4127–38. [PubMed: 9670027]
11. Rezabkova L, et al. 14-3-3 protein interacts with and affects the structure of RGS domain of regulator of G protein signaling 3 (RGS3). J Struct Biol. 2010; 170(3):451–61. [PubMed: 20347994]
12. Lee J, Kumagai A, Dunphy WG. Positive regulation of Wee1 by Chk1 and 14-3-3 proteins. Mol Biol Cell. 2001; 12(3):551–63. [PubMed: 11251070]
13. Amin AR, et al. Perspectives for cancer prevention with natural compounds. J Clin Oncol. 2009; 27(16):2712–25. [PubMed: 19414669]
14. Bhattacharya SK, Satyan KS, Ghosal S. Antioxidant activity of glycowithanolides from Withania somnifera. Indian J Exp Biol. 1997; 35(3):236–9. [PubMed: 9332168]
15. Mohan R, et al. Withaferin A is a potent inhibitor of angiogenesis. Angiogenesis. 2004; 7(2):115–22. [PubMed: 15516832]
16. Oh JH, et al. Induction of apoptosis by withaferin A in human leukemia U937 cells through down-regulation of Akt phosphorylation. Apoptosis. 2008; 13(12):1494–504. [PubMed: 19002588]
17. Lee J, Sehrawat A, Singh SV. Withaferin A causes activation of Notch2 and Notch4 in human breast cancer cells. Breast Cancer Res Treat. 2012; 136(1):45–56. [PubMed: 22965833]
18. Srinivasan S, et al. Par-4-dependent apoptosis by the dietary compound withaferin A in prostate cancer cells. Cancer Res. 2007; 67(1):246–53. [PubMed: 17185378]
19. Das TP, Suman S, Damodaran C. Reactive oxygen species generation inhibits epithelial-mesenchymal transition and promotes growth arrest in prostate cancer cells. Mol Carcinog. 2013
20. Lim DY, et al. Inhibition of colon cancer cell proliferation by the dietary compound conjugated linoleic acid is mediated by the CDK inhibitor p21CIP1/WAF1. J Cell Physiol. 2005; 205(1):107–13. [PubMed: 15880444]
21. Spruck CH, Won KA, Reed SI. Deregulated cyclin E induces chromosome instability. Nature. 1999; 401(6750):297–300. [PubMed: 10499591]

22. Strohmaier H, et al. Human F-box protein hCdc4 targets cyclin E for proteolysis and is mutated in a breast cancer cell line. Nature. 2001; 413(6853):316–22. [PubMed: 11565034]
23. Dash BC, El-Deiry WS. Phosphorylation of p21 in G2/M promotes cyclin B-Cdc2 kinase activity. Mol Cell Biol. 2005; 25(8):3364–87. [PubMed: 15798220]
24. Adriaenssens E, et al. Growth signaling in breast cancer cells: outcomes and promises of proteomics. Biochem Pharmacol. 2002; 64(5-6):797–803. [PubMed: 12213572]
25. De Witt Hamer PC, et al. WEE1 kinase targeting combined with DNA-damaging cancer therapy catalyzes mitotic catastrophe. Clin Cancer Res. 2011; 17(13):4200–7. [PubMed: 21562035]
26. Honda R, Ohba Y, Yasuda H. 14-3-3 zeta protein binds to the carboxyl half of mouse wee1 kinase. Biochem Biophys Res Commun. 1997; 230(2):262–5. [PubMed: 9016762]
27. Decensi A, Costa A. Recent advances in cancer chemoprevention, with emphasis on breast and colorectal cancer. Eur J Cancer. 2000; 36(6):694–709. [PubMed: 10762741]
28. Lindqvist A, et al. Cdc25B cooperates with Cdc25A to induce mitosis but has a unique role in activating cyclin B1-Cdk1 at the centrosome. J Cell Biol. 2005; 171(1):35–45. [PubMed: 16216921]
29. Masaki T, et al. Cyclins and cyclin-dependent kinases: comparative study of hepatocellular carcinoma versus cirrhosis. Hepatology. 2003; 37(3):534–43. [PubMed: 12601350]
30. Iorns E, et al. Integrated functional, gene expression and genomic analysis for the identification of cancer targets. PLoS One. 2009; 4(4):e5120. [PubMed: 19357772]
31. Backert S, et al. Differential gene expression in colon carcinoma cells and tissues detected with a cDNA array. Int J Cancer. 1999; 82(6):868–74. [PubMed: 10446455]
32. Kiviharju-af Hallstrom TM, et al. Human prostate epithelium lacks Wee1A-mediated DNA damage-induced checkpoint enforcement. Proc Natl Acad Sci U S A. 2007; 104(17):7211–6. [PubMed: 17431037]
33. Warmerdam DO, Kanaar R. Dealing with DNA damage: relationships between checkpoint and repair pathways. Mutat Res. 2010; 704(1-3):2–11. [PubMed: 20006736]
34. Taylor WR, Stark GR. Regulation of the G2/M transition by p53. Oncogene. 2001; 20(15):1803–15. [PubMed: 11313928]
35. Hayashi-Takanaka Y, et al. Visualizing histone modifications in living cells: spatiotemporal dynamics of H3 phosphorylation during interphase. J Cell Biol. 2009; 187(6):781–90. [PubMed: 19995936]

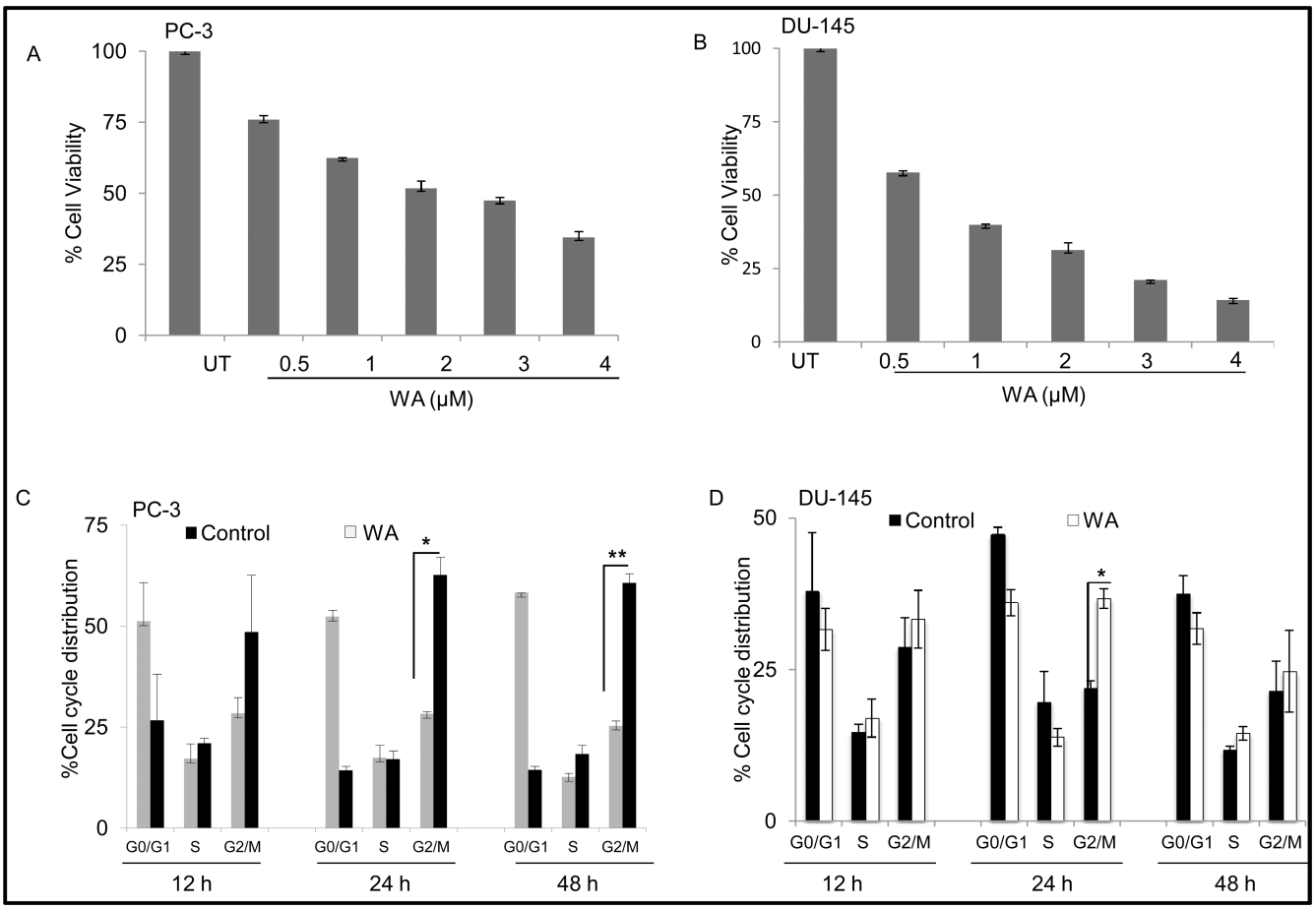

Figure 1. WA inhibits cell growth and induces G_2/M cell cycle arrest in CaP cell lines

(A) PC-3 and (B) DU145 cells were treated with either DMSO or the indicated dose of WA for 24 h, and the percentage of viable cells was determined using the trypan blue exclusion method. (C and D) PC3 and DU-145 cells were treated with WA (1 μM) for 12, 24, or 48 h and subjected to cell cycle analysis by flow cytometry. Values represent mean ± standard error of the mean of three independent experimental samples. **, $P<0.05$ versus vehicle.

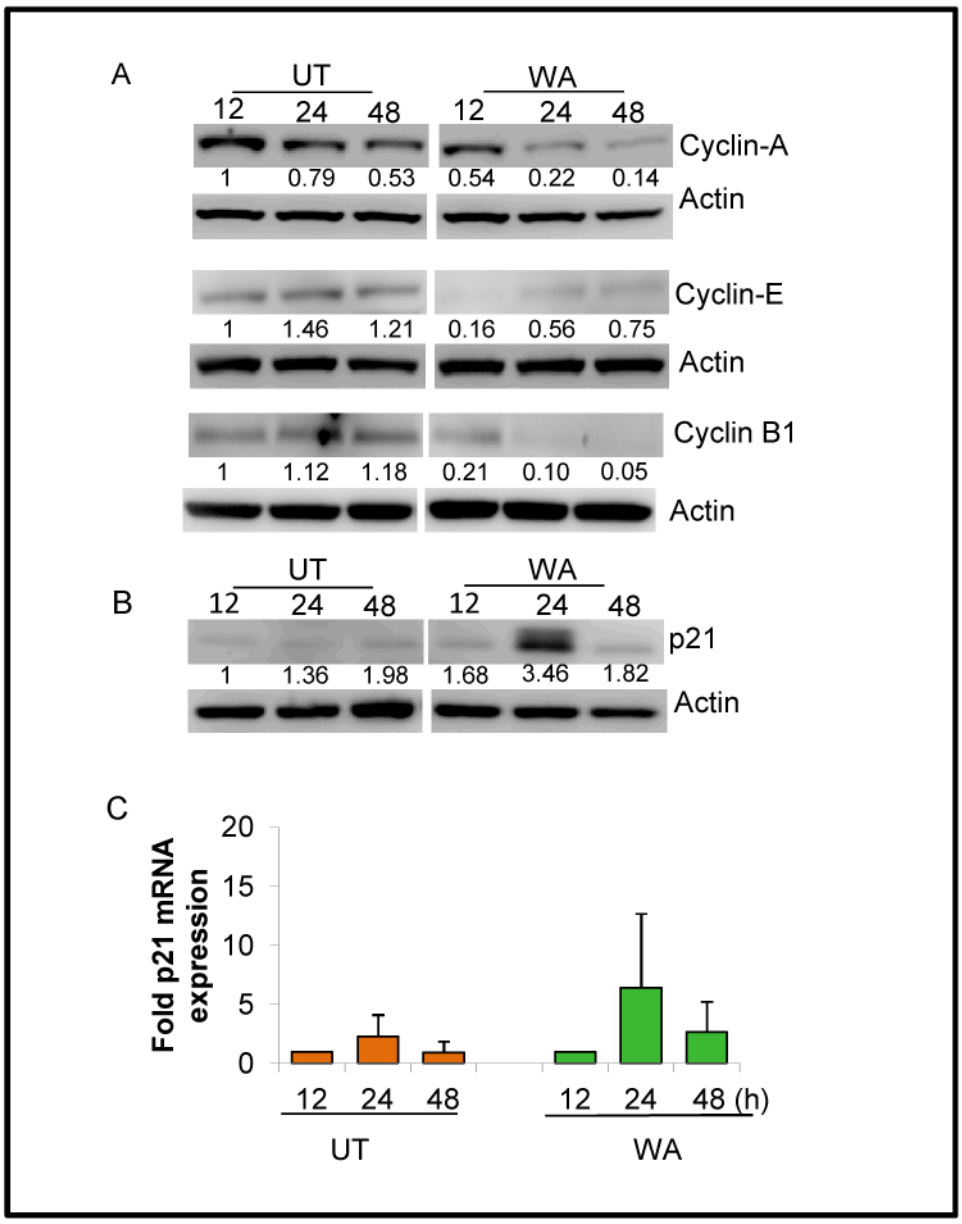

Figure 2. WA decreases cyclin-A, E2, and B1 protein levels in CaP cell lines

(A) PC3 and DU-145 cells were treated with either DMSO (untreated, UT) or WA, and cell lysates were subjected to Western blot analysis for cyclin-A, cyclin-E2, cyclin-B1expression.(B) PC3 and DU-145 cells were treated with either DMSO (untreated, UT) or WA, and cell lysates were subjected to Western blot analysis for p21 expression. (C) RT-PCR confirming the expression of p21 protein upon WA treatment in PC-3 cells.

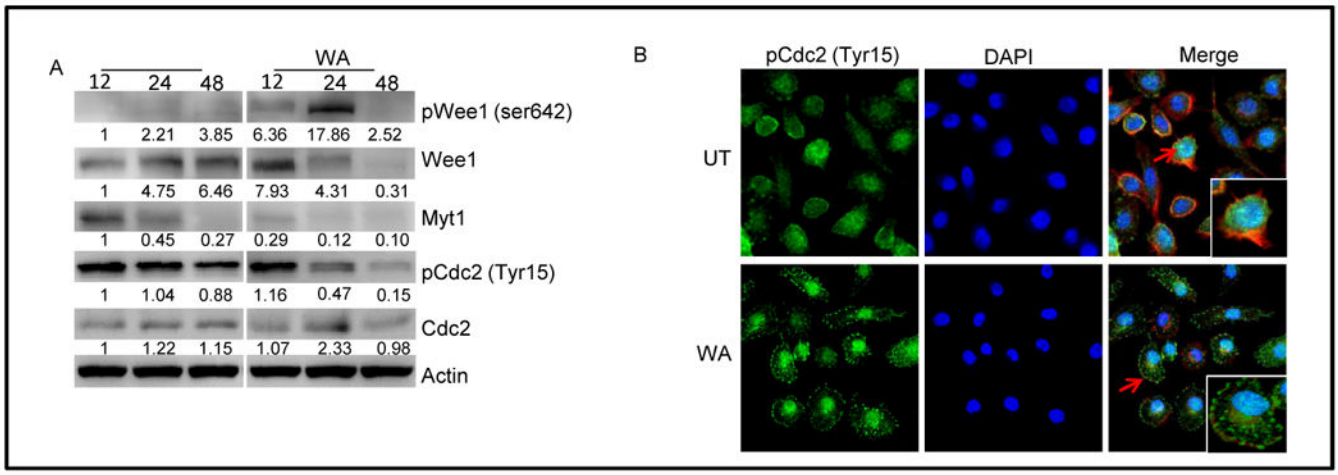

Figure 3. Regulatory effects of WA on Wee-1/Cdc2

PC3 cells were treated with WA (1 μM) for the indicated time periods, and cell lysates were subjected to Western blot analysis for (A) pWee1 and Wee1, pCdc2 (Tyr15) and Cdc25C (ser216), and total Myt-1 expression. Actin was used as a loading control. (B) CaP cells were treated with either WA (1 mM) or DMSO for 24 h and analyzed for Cdc2 (Tyr15) using immunofluorescence staining. Representative photographs were taken under a fluorescence microscope. A decrease in the punctate expression pattern were observed in the G_2/M-arrested cells (red arrow).

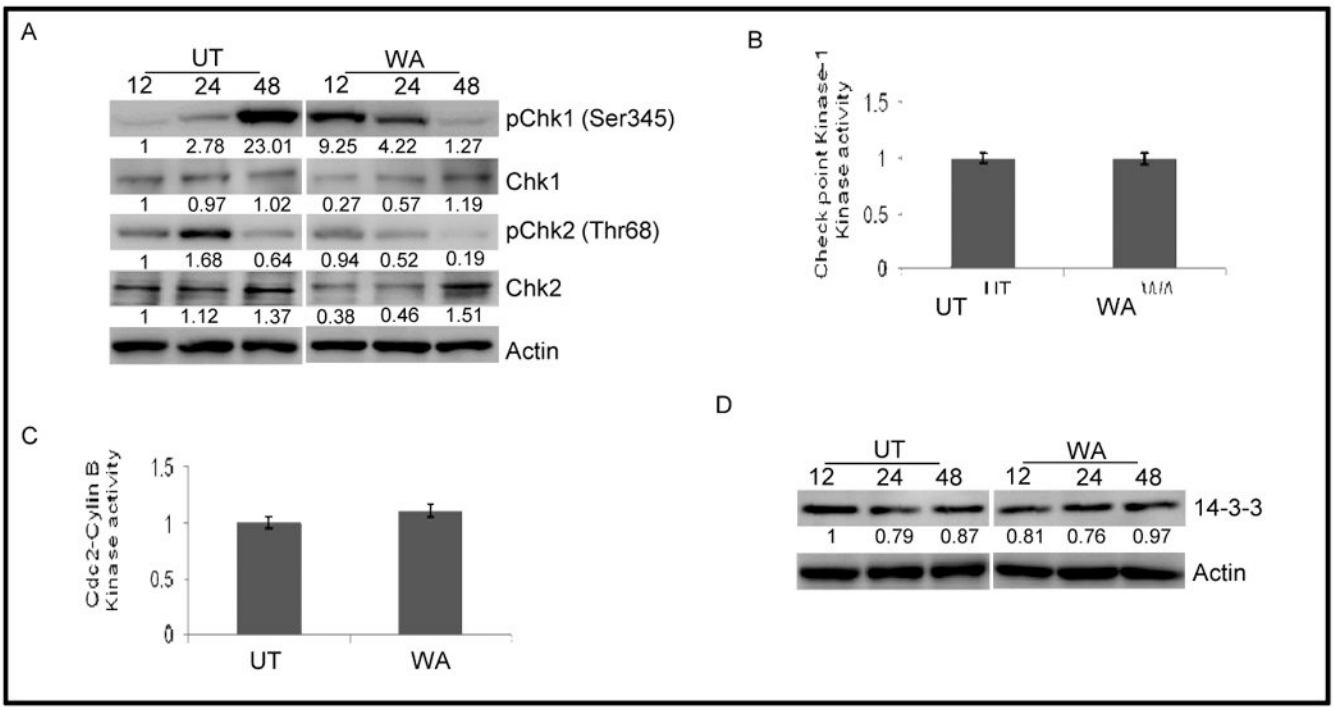

Figure 4. WA inhibits Chk1 and Chk2 expression

(A) CaP cells were treated with vehicle or WA, and cell lysates were subjected to Western blot analysis for pChk1 (Ser345), pChk2 (Thr68), Chk1.. Actin was used as a loading control. (B) Checkpoint kinase activation in WA-treated PC3 cells. No significant changes in Chk1 or Chk2 kinase activity were observed between vehicle and WA-treated PC-3 cells. (C) Cdc2-Cyclin B kinase activation in WA-treated PC3 cells. No significant change was observed in Cdc2-Cyclin B kinase activity between vehicle and WA-treated PC-3 cells. (D) CaP cells were treated with vehicle or WA, and cell lystates were subjected to Western blot analysis for total 14-3-3. Actin was used as a loading control.

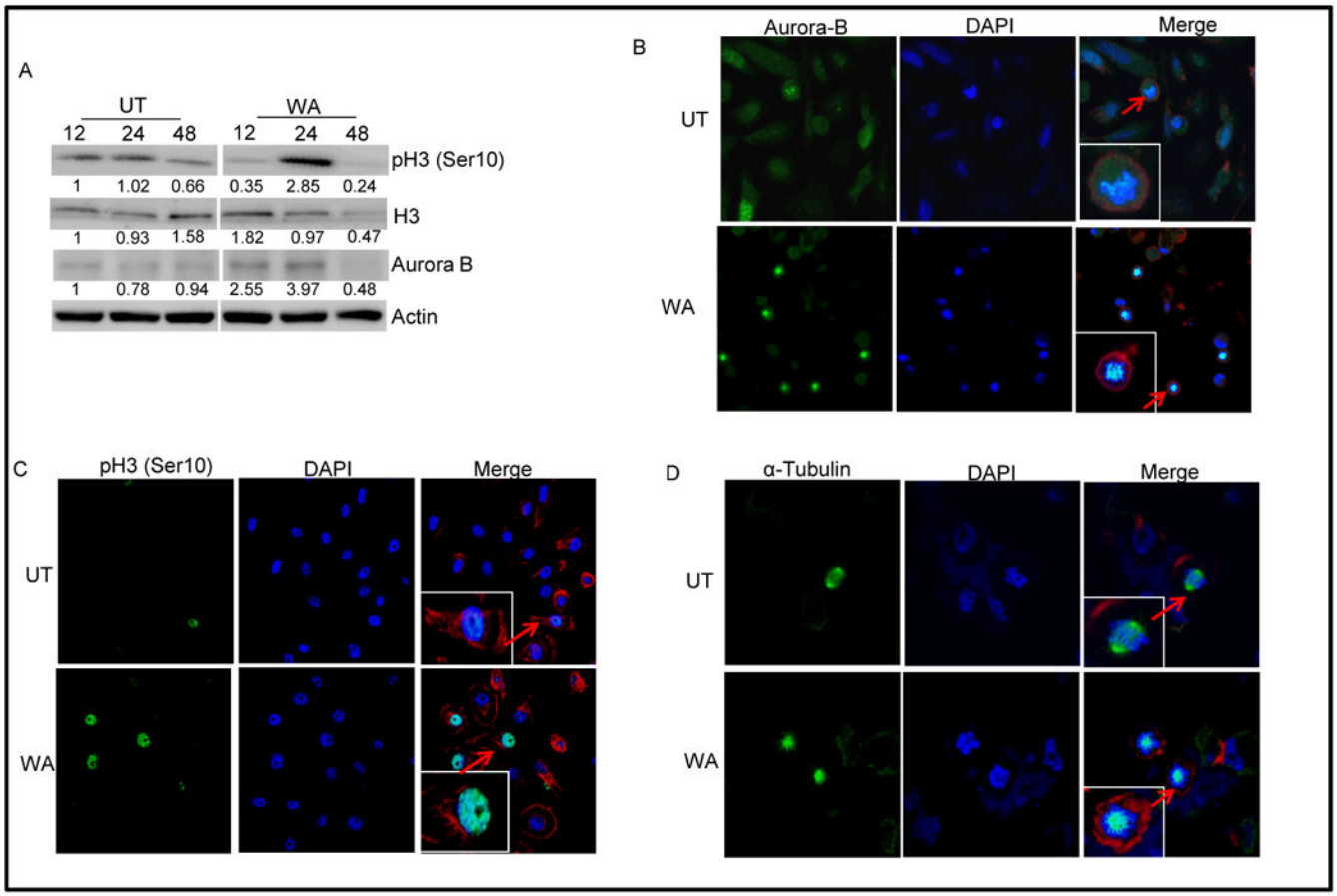

Figure 5. Mitotic arrest, alteration of the mitotic spindles, and accumulation of passage proteins altered by WA

(A) Western blot analysis of pH3 (Ser10), H3 and Aurora B expression in WA-treated CaP cells. Actin was used as a loading control. Cells were immunostained with antibodies to (B) Aurora B (C) phosphorylated histone H3 (Ser10), and (D) α-tubulin, and the nucleus was stained with DAPI. (C) WA induces G_2M markers.