Withaferin A induces p53-dependent apoptosis by repression of HPV oncoproteins and upregulation of tumor suppressor proteins in human cervical cancer cells

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Cervical cancer is caused by human papilloma virus (HPV) expressing E6 and E7 oncoproteins, which are known to inactivate tumor suppressor proteins p53 and pRb, respectively. Repression of HPV oncoproteins would therefore result in reactivation of tumor suppressor pathways and cause apoptosis in cancer cells. Withaferin A (WA), the active component of the medicinal plant Withania somnifera, has exhibited inhibitory effects against several different cancers. We examined the activity of WA on human cervical cancer cells in vitro and in vivo, WA potently inhibited proliferation of the cervical cancer cells, CaSkii (IC50 0.45 ± 0.05 μM). Mechanistically, WA was found to (i) downregulate expression of HPV E6 and E7 oncoproteins, (ii) induce accumulation of p53, (iii) increase levels of p21Waf1/Cip1 and its interaction with proliferating cell nuclear antigen (PCNA), (iv) cause G2/M cell cycle arrest, associated with modulation of cyclin B1, p34cdc2 and PCNA levels, (v) decrease the levels of STAT3 and its phosphorylation at Tyr705 and Ser237 and (vi) alter expression levels of p53-mediated apoptotic markers—Bcl2, Bax, caspase-3 and cleaved PARP. In vivo, WA resulted in reduction of nearly 70% of the tumor volume in athymic nude mice with essentially similar trend in the modulation of molecular markers as in vitro. This is the first demonstration indicating that WA significantly downregulates expression of HPV E6/E7 oncoproteins and restores the p53 pathway, resulting in apoptosis of cervical cancer cells. Together, our data suggest that WA can be exploited as a potent therapeutic agent for the treatment and prevention of cervical cancer without deleterious effects.

Introduction

Cervical cancer is the second leading cause of female cancer deaths worldwide. Infection with high-risk human papilloma virus (HPV)—such as HPV 16, 18, 31 and 33—plays a central role in the development of >99.5% cervical cancers (1). The two viral oncoproteins, E6 and E7, are known to induce cervical cancer by interacting with the tumor suppressor proteins, p53 and pRb, respectively, leading to their inactivation (2). These tumor suppressor proteins control signaling pathways that regulate the cell cycle and monitor and protect the integrity of the genome (3).

Unlike several cancers, where the p53 gene is mutated irreversibly, cervical carcinomas and cell lines uniquely harbor wild-type p53 and p105Rb genes. In this case, the growth-regulatory machinery of normal cells is masked by the expression of HPV E6 and E7 proteins (2). Therefore, the consequence of downregulation of HPV E6 and E7 proteins is shown to induce functional p53 (4). Activation of p53 can further induce downstream target genes involved in cell cycle arrest and apoptosis (5). The cytotoxic effect of p53 is mediated by transcriptional activation of the cyclin-dependent kinase (cdk) inhibitor, p21Waf1/Cip1 (6), whereas the apoptotic effect is mediated, in part, by activation of a proapoptotic gene product, Bax (7). The restoration of normal p53 function in cancer cells represents a viable option that might sensitize cells to cancer therapies or induce apoptosis directly (8). In fact, the possibility of reactivating the p53 pathway has been extensively studied in several cancers (9,10).

Despite advances in the treatment of cervical cancers, protocols for recurrent or persistent cancers and alternative treatment options with low toxicity are scanty. In recent years, natural products have been identified as promising sources of drugs for cancer prevention and treatment based on their ability to attack multiple molecular targets (11). Withaferin A (WA) (Figure 1A), is the principal active constituent of the Indian medicinal plant Withania somnifera, commonly referred to as 'ashwagandha’. Besides its use from ancient times against various ailments, this plant has shown antitumor, antiangiogenic and radiosensitizing activity (12,13). The anticancer activity of WA has been demonstrated in vitro and in vivo in prostate (14), breast (15,16) and pancreatic (17) cancers. The mechanisms behind it has been attributed to (i) inhibition of nuclear factor-kappa B activation (18), (ii) induction of Par-4 (14) and inhibition of HSP90 in prostate cancer cells (17), (iii) induction of G2/M arrest (15) and FOXO3a and Bim regulation in breast cancer (16) and (iv) inhibition of Noch-1 in colon cancer (19).

The antitumor potential of WA against cervical cancer has not yet been reported. Thus, the aim of this study was to determine the anti-proliferative activity of WA against cervical cancer cells and investigate the underlying molecular mechanisms of action. We here report repression of HPV E6 and E7 oncoproteins and restoration of p53 levels by WA, leading to sequential reactivation of p53-dependent tumor suppressor activity by downstream modulation of proteins involved in cell proliferation, cell cycle progression and apoptosis.

Materials and methods

Chemicals and reagents

WA was purchased from Chromadex (Irvine, CA). The following antibodies were used: Bax, Bcl2, caspase-3, p53, p21Waf1/Cip1, p34cdc2, E6, cyclin B1 (Santa Cruz Biotechnology, Santa Cruz, CA), poly (adenosine diphosphate-ribose) polymerase (PARP), proliferating cell nuclear antigen (PCNA), signal transducer and activator of transcription 3 (STAT3), pSTAT3(Tyr705), pSTAT3(Ser237), pCdk1, GFP (Cell Signaling Technologies, Danvers, MA), E7 (Abcam, Cambridge, MA) and β-actin (Sigma–Aldrich, St Louis, MO). All other chemicals used were of analytical grade.

Cell culture

The human cervical cancer cells CaSkii (HPV 16 + 18 positive), HeLa (HPV 18 positive), SiHa (HPV 16 positive), C33a (HPV negative) and primary human cervical cancer cells, SiHa, HeLa, C33a (ATCC, Manassas, VA) were cytogenetically tested and authenticated. Cervical cancer cells were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum. Primary HEK cells were cultured in basal cell dermal media supplemented with keratinocyte growth kit components and antibiotics. Cells were maintained in humidified atmosphere containing 5% CO2 at 37°C.

Cell proliferation assay

The effect of WA on cell viability was determined by the 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide (MTT) assay (20). Briefly, cells (5000 per well) were treated with WA (0–4 μM) for 6–72 h. The medium was changed and incubated with MTT (0.5 mg/ml) for 2 h, followed by solubilization in dimethyl sulfoxide and spectrophotometric measurement at 570 nm.

Cell morphology changes during apoptosis

Changes in cell morphology were studied using acridine orange and ethidium bromide (21). Briefly, CaSkii cells were treated with

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vehicle (dimethyl sulfoxide) or WA (0.5 μM) for 24 h. Cell suspension (0.5 × 10^6 cells/ml) was stained with acridine orange and ethidium bromide (10 μg/ml) and observed under fluorescence microscope (NIKON, Melville, NY) at ×100 magnification. In addition, cells were examined for nuclear morphology of apoptosis (chromatin condensation, DNA fragmentation) by labeling with 4′,6-diamidino-2-phenylindole (Sigma, St Louis, MO).

Annexin V/propidium iodide assay for apoptosis
For apoptosis assays, CaSki cells (1 × 10^6) were treated with WA (0–2 μM) for 24 and 48 h, stained with annexin V-FITC and propidium iodide (PI), according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA) and evaluated by BD FACScan flow cytometer (Becton Dickinson, San Jose, CA).

Cell cycle analysis by flow cytometry
CaSki cells (1 × 10^6) were synchronized by serum starvation for 72 h and later released by addition of complete media supplemented with 10% fetal bovine serum. Synchronized cells were treated with 0–2 μM WA for 12 and 24 h and cell cycle distribution was determined by PI staining using a BD FACScan flow cytometer as described previously (22). Data were analyzed using FlowJo 7.2.5 for Windows (Tree Star, Ashland, OR).

Protein extraction and western blot analysis
Whole cell lysates were prepared from CaSki cells (2 × 10^6) treated with WA or vehicle and tumor tissues from nude mice using RIPA buffer (Santa Cruz Biotechnology) and protease-inhibitor cocktail (Thermo Scientific, Waltham, MA). Nuclear and cytosolic fractions were isolated using NE-PER nuclear and cytoplasmic extraction kit (Thermo Scientific, Rockford, IL). The protein concentration of the lysate was determined using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Twenty to 40 μg of protein was used for western blot analysis as described previously (23). β-Actin was used to confirm equal loading in whole cell lysates and cytosolic fractions and TATA box-binding protein was used for nuclear fractions.

Confocal microscopy
CaSki cells were seeded in eight-well chamber slides and treated with WA (0.5 μM) or vehicle for 24 h. Cells were fixed in 4% paraformaldehyde for 15 min and permeabilized with 0.25% Triton X-100 for 10 min on ice. Cells were blocked with 1% bovine serum albumin for 30 min, washed with phosphate-buffered saline (PBS) and incubated with primary antibody diluted in 1% bovine serum albumin for 1 h at room temperature. Followed by 3× wash with PBS and incubated with secondary Alexa Fluor 594-conjugated antimouse antibody for HPV 16 E6 and E7 or Alexa Fluor 488-conjugated anti-mouse antibody for p53 was incubated for 1 h at room temperature. After 3× wash with PBS, cells were stained with Phalloidin 488 or 594 for actin filaments and 4′,6-diamidino-2-phenylindole for detecting nuclei. Cells were mounted and examined under Olympus Fluoview FV1000 confocal microscope. Images were captured at ×20 magnification and merged with FluoView software.

Coimmunoprecipitation
CaSki cells (2 × 10^6) were treated with WA (0.5 and 1 μM) or vehicle for 24 h and cell lysates were prepared as described previously (24). Primary antibodies were cross-linked with Dynabeads protein A (Invitrogen) and lysates were immunoprecipitated according to the manufacturer’s instructions and processed for western blot analysis.

Messenger RNA stability and quantitative real-time polymerase chain reaction
Effect of WA on messenger RNA (mRNA) stability of HPV and p53 genes was determined by using actinomycin D, a potent transcription inhibitor. CaSki cells were treated for 6 h with WA (0.5 μM) or vehicle and then blocked with 5 μg/ml actinomycin D. Cells were harvested at 0, 2, 4 and 8 h postactinomycin D treatment. Total cellular RNA was isolated using Trizol reagent (Invitrogen) as per manufacturer’s protocol and treated with DNase I. One-step quantitative polymerase chain reaction (qPCR) (Quanta Biosciences, Gaithersburg, MD) was performed using primers as described previously for p53 (25), HPV16 E6,
WA induces p53-dependent apoptosis in cervical cancer

Results

WA exhibits antiproliferative activity, induces morphological changes and apoptosis in CaSki cells

WA inhibited the growth of all the four cervical cancer cell lines, namely, CaSi, HeLa, SiHa and C33a in a dose-dependent manner (Figure 1B). The IC50 values in the ascending order were 0.2 ± 0.06, 0.45 ± 0.05, 1.0 ± 0.09 and 1.2 ± 0.1 μM for C33a, CaSi, HeLa and SiHa, respectively. In order to determine a possible mechanism of action of WA on cervical cancer cells, HPV oncogene E6, its target E7 and internal standard TATA-binding protein (26). After completion of the reverse transcription–polymerase chain reaction, Ct values were obtained from the ABI 7500 fast v2.0.1 software. The ΔΔCt method was used to represent mRNA fold change.

Protein stability

Translation inhibitor cyclohexamide (CHX) was used to determine the effect of WA on stability of HPV oncoproteins and p53 protein. CaSi cells (2 × 10^6) were treated with WA (0.5 μM) in presence or absence of CHX (100 μg/ml). Cells were harvested at different time points after CHX treatment, cell lysates were prepared for western blot analysis.

RNAi transfection

Silencer predesigned small-interfering RNA (siRNA) targeted to STAT3, p53 and fluorescent-conjugated non-targeting control siRNA (Cell Signaling Technologies) were used. Briefly, CaSi cells were seeded into six-well plates (2 × 10^5) in antibiotic-free media for at least 24 h before transfection. Briefly, cells were transiently transfected with STAT3 siRNA (100 nM), p53 siRNA (50 nM) or control siRNA (50 nM) using Lipofectamine 2000 according to the manufacturer’s protocol. Cells treated with control siRNA served as a negative reference and allowed to assess transfection efficiency under fluorescence microscopy. Cells were later treated with WA (0.5 μM) or vehicle for 24 h and lysates were prepared for western blot analysis.

GFP reporter plasmids and transfection

Plasmid pZM270 (kind gift from Prof Zheng, Zhi-Ming, NIH) contains the E6 and E7 coding regions, fused in frame to the C-terminus of GFP and GFP-p53 (Addgene, Cambridge, MA) were used for transient transfection in C33a (HPV-negative cervical cancer cell line) and H1299 (p53 null lung cancer cell line) cells, respectively. Cells were transfected with plasmid DNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Cells were treated with vehicle or WA (0.5 μM) for 24 h and lysates were prepared for western blot analysis and probed with GFP antibody.

Tumor xenograft study

CaSi cells (5 × 10^6) were mixed (1:1 volume) with Matrigel (BD Bioscience, Bedford, MA) and injected into the right flank of sixteen 5–6 week-old female athymic nu/nu mice (Harlan laboratories, Indianapolis, IN). On the following day, the mice were randomly divided into two groups of eight animals each. One group received intraperitoneal injection of WA (8 mg/kg WA in 100 μL PBS containing 10% dimethyl sulfoxide) on alternate days for 6 weeks. The other group received the vehicle alone in parallel. Once a week, the mice were weighed and tumors measured using digital Vernier calipers. Tumor volume was calculated using the formula: \[\text{Volume} = \text{length} \times \text{width} \times \text{height}\]. After euthanasia, the tumors were harvested, snap-frozen in liquid nitrogen and stored at −80°C until analysis.

Statistical analysis

The data points shown in the figures represent the mean ± SD. Differences between two groups were assessed using Student’s t-test. Comparisons among groups were made by analysis of variance. P value of <0.05 was considered statistically significant. In vitro assessments were performed in three independent experiments to confirm the results.

Fig. 2. WA induces morphological changes and apoptosis in CaSi cells. (A) Representative images of morphological changes in CaSi cell treated with 0.5 μM WA for 24 h. Upper row: ethidium bromide and acridine orange staining, dashed white arrow indicates membrane blebbing and solid white arrow indicates apoptotic cell. Lower row: 4′,6-diamidino-2-phenylindole staining for changes in nuclear morphology, dashed white arrow indicates chromatin condensation and solid white arrow indicates fragmented nuclei. (B) Representative FACS pictograms of cells treated with WA at indicated doses and times. Percent of annexin V-positive (early-apoptotic cells, lower right quadrant) and annexin V/PI-double-positive cells (late-apoptotic cells, upper right quadrant) are indicated (top). Percent of apoptosis at indicated dose of WA at 24 h (center) and 48 h (bottom). Results expressed as mean ± SD. One-way analysis of variance was performed to compare percent of early- and late-apoptotic cells between vehicle- and WA-treated cells. *P < 0.05; **P < 0.01; ***P < 0.001.
Fig. 3. WA modulates HPV oncogenes and restores p53. (A) CaSki cells were treated with vehicle or WA for 12 and 24 h. HPV16 E6 (left) and E7 (right) mRNA levels were measured by qPCR. Data represent mean ± SD of fold downregulation of mRNA levels relative to the control. **P < 0.01, ***P < 0.001. (B) CaSki cells were treated for 6 h with or without WA (0.5 μM) and RNA synthesis blocked with actinomycin D (5 μg/ml). Cells were harvested at indicated times and transcript levels of HPV16 E6 (left); E7 (center) and p53 (right) were determined by qPCR. Data represent mean ± SD of three experiments. (C), Transient transfection of a C33a (HPV negative) cells with pZMZ70 (GFP-HPV16 E6E7) (top row) and H1299 (p53 null) cells (bottom row) with GFP-p53 reporter is shown. Fluorescent microscope images show 60 and 90% transfection efficiency in C33a and H1299 cells, respectively. Cells were treated with vehicle or WA (0.5 μM) for 24 h and cell lysates were analyzed by western blotting using anti-GFP antibody. (D) Confocal photomicrographs (×20) of representative CaSki cells showing nuclear localization p53 protein. Cells were treated with vehicle (first row) and WA (0.5 μM) (second row) and isotype-matched negative control (third row) are shown. 4′,6-Diamidino-2-phenylindole was used to visualize DNA (first lane) and Phalloidin 488 (green) or 594 (red) used to visualize actin filaments (second lane). The scale represents a length of 40 μm. Western blot analysis of p53 protein from cytoplasmic (left) and nuclear (right) fractions of WA-treated CaSki cells at indicated doses. (E) Western blot analysis of indicated proteins after 12 (left) and 24 h (right) of WA treatment at indicated doses. (F) CaSki cells
p53 were analyzed by western blotting. Our results suggested that WA downregulated HPV E6 protein expression in all HPV-positive cell lines and increased p53 and p21 cip1/waf1 levels in a dose-dependent manner. These data suggest the involvement of p53-dependent apoptotic pathway in HPV-positive cervical cancer cells. However, in HPV-negative (C33a) cells expressing mutated p53, the levels remained largely unaltered whereas increased p21 cip1/waf1 levels were observed with WA treatment (Figure 1C). We chose CaSki cell line to perform detailed mechanistic studies behind the action of WA in this research work. The dose- and time-dependent effect of WA treatment on CaSki cells is shown in Figure 1D. It is noteworthy that the proliferation of primary epidermal keratinocytes was only slightly affected by WA (Figure 1E). The WA-treated CaSki cells elicited morphological changes and manifestations of early- and late-apoptosis such as membrane blebbing, chromatin condensation and DNA fragmentation (Figure 2A). To further confirm the apoptosis, annexin V binding was studied in the presence of WA. Representative blots are shown. (G) CaSki cells were transfected with si p53 (50 nM) for 24 h followed by treatment with either vehicle or WA at indicated doses for further 24 h and stained with annexin V/PI for apoptosis analysis by flow cytometry. Data represent mean ± SD of three independent experiments (*P < 0.05; **P < 0.01, analysis of variance).
V-FITC/PI staining was performed in WA- (0–2 μM) treated CaSki cells. We observed that WA resulted in a significant dose- and time-dependent induction of apoptosis. In comparison with vehicle treatment, we observed a significant increase in early-apoptotic cells at 24 h and a shift from early-apoptotic to late-apoptotic cell population at 48 h (Figure 2B). These results confirm the induction of apoptosis as a major mechanism behind antitumor activity of WA.

WA downregulates HPV oncoproteins and restores p53 levels

The effect of WA on HPV E6 and E7 oncoproteins in CaSki cells was investigated by qPCR. Our results indicate significant downregulation of both HPV16 E6 and E7 transcripts by WA (0.25–2 μM). Nearly 20-fold and 17-fold reduction in the mRNA expression levels of HPV16 E6 and E7, respectively, were observed with 2 μM WA after 24 h (Figure 3A). In addition, WA significantly decreased the mRNA stability of HPV16 E6 and E7 genes from 6.8 and 7.8 h to 1 and 1.8 h, respectively (Figure 3B). However, p53 mRNA stability was not significantly affected by WA. In order to determine upstream effect of WA on HPV and p53 gene transcription, GFP-reporter assay with pZMZ70 (GFP-HPV16 E6E7) in C33a (HPV negative) and GFP-p53 H1299 (p53 null) cells was performed. As evident by levels of the fusion proteins (Figure 3C), WA resulted in increased transcription of p53, whereas decreased transcription of HPV genes. In addition, confocal microscopic analysis revealed nuclear localization of p53 with WA treatment and this was further confirmed by western blot analysis of cytosolic and nuclear fractions from cells treated with WA (0–1 μM) (Figure 3D). Western blot analysis with whole cell lysate also confirmed decreased levels of E6 and E7 and increase in p53 and pRb by WA (Figure 3E). Decreased stability of HPV E6 and E7 protein was also observed in presence of WA, whereas stability of p53 protein largely remained unaltered (Figure 3F). These results confirmed that WA repressed both transcription and translation of E6 and E7 oncoproteins thereby leading to the restoration of the vital tumor suppressor pathways. We further demonstrated that abrogation of p53 by siRNA abolished the cell death induced by WA (Figure 3G) thus, substantiating the role of p53 in WA-induced cytotoxicity in cervical cancer cells.

WA induces G2/M phase arrest by modulating p53-dependent proteins involved in cell cycle progression

To determine the effect of WA on cell cycle perturbations, CaSki cell were synchronized at G0/G1 phase by 72 h serum starvation (Figure 4A). Synchronized cells treated with WA (0–2 μM) for 12 and 24 h showed a significant alteration in cell cycle progression (Figure 4B). In particular, the G2/M phase of cell cycle was arrested by WA in a dose- and time-dependent manner in contrast to vehicle-treated cells. Western blot analysis further revealed an increase in cyclin B1 levels, suggesting a possible late G2 or M phase arrest (Figure 4C). Since WA induced G2/M phase arrest we investigated its effect on p34^cdcl2, a key regulator of G2/M transition and its interaction with cyclin B1. Our data demonstrated substantial dose-dependent reduction in both p34^cdcl2 and phospho-Cdk1 levels (Figure 4C) as well as decreased interaction between p34^cdcl2 and cyclin B1 (Figure 4D), confirming G2/M arrest by WA. p21^cip1/waf1, a downstream target of p53, was upregulated, whereas PCNA levels decreased with WA treatment (Figure 4C). This latter result is important because p21^cip1/waf1 has been shown to bind directly with PCNA and inhibit cell cycle progression (27). Coimmunoprecipitation results confirmed increased interaction between p21^cip1/waf1,PCNA (Figure 4D). Thus, our results demonstrated G2/M arrest and modulation of p53-mediated cell cycle regulators by WA in CaSki cells.

WA inhibits STAT3 signaling proteins and modulates p53-dependent apoptotic markers

After finding significant effect of WA on cell cycle regulators, we sought to determine whether WA inhibited STAT3 activation and promoted p53-mediated apoptosis of tumor cells. We observed that WA decreased total STAT3 as well as the levels of pSTAT3 (Ser727) and pSTAT3 (Thr705) in a dose-dependent manner (Figure 5A). We also demonstrated that restoration of p53 levels with WA treatment was also, in part, due to STAT3 silencing, suggesting STAT3 inactivation as one of the mechanisms of action. Moreover, knockdown of p53 followed by treatment with WA resulted in lower STAT3 levels (Figure 5B), thus suggesting that WA-induced p53 to further cause decrease in STAT3 levels. Modulation of p53-dependent apoptotic markers such as decrease in Bcl2 and an increase in Bax levels were observed. Cytosolic cytochrome c levels were also increased with WA. p53 is also known to regulate the activation of caspase-3 to induce apoptosis. Thus, as expected, we observed reduced procaspase-3 levels with a dose-dependent increase of PARP cleavage (Figure 5A). These results suggest that WA regulated STAT3 expression and induced p53-dependent mitochondrially-mediated apoptosis.

WA inhibits growth of subcutaneous xenograft tumor in nude mice

Intraperitoneal injection of WA (8 mg/kg) on alternate days for 6 weeks caused significant inhibition of CaSki tumor xenograft growth in nude mice. WA resulted in nearly 70% reduction of tumor volume compared with control (270 ± 161 versus 81 ± 45 mm3; P ≤ 0.01) (Figure 6A and C). There were no signs of toxicity as reflected by unaltered body weights compared with the control group (Figure 6B). There was no mortality during the study period. Tumors were subcutaneously encapsulated with no visual signs of metastasis.

Fig. 5. WA modulates STAT3 and apoptosis-related proteins. (A) CaSki cells were treated with vehicle or WA at indicated doses for 12 h (left) and 24 h (right). Levels of indicated proteins were analyzed by western blot analysis. Cytosolic fractions were analyzed for cytochrome c levels. (B) CaSki cells were transfected with control siRNA, si STAT3 or si p53, followed by treatment with vehicle or WA (0.5 μM) for 24 h. Cell lysates were used for western blot analysis with STAT3, p53 and β-actin antibodies. Representative blots are shown.
Modulation of selected molecular markers in tumor tissues were essentially in agreement with that of the cell culture studies, suggesting p53-mediated apoptosis (Figure 6C).

Discussion

The main objective of the present study was to evaluate anticancer activity of WA and the associated mechanism of action in cervical cancer cells. In vitro treatment with WA markedly inhibited cell proliferation in all the four cervical cancer cell lines tested. The antiproliferative effect of WA was clearly associated with induction of apoptosis.

We investigated the mechanism behind the antiproliferative activity of WA by analyzing its effect on HPV16 E6 and E7 oncogenes. It is well documented that HPV E6 and E7 oncoproteins exert profound effects on tumor suppressor proteins p53 and pRb by accelerating their ubiquitin-mediated degradation. Most primary cervical cancers and cancer cell lines, including CaSki, HeLa and SiHa, are known to harbor wild-type p53 and p105Rb genes (2). Therefore, it is presumed that agents with a capability of repressing HPV oncoproteins would cause reactivation tumor suppressor pathways and have potential therapeutic implications in treating cervical cancers. To this end, we initially demonstrated that WA resulted in cytotoxicity in the three HPV-positive and one HPV-negative cervical cancer cell lines. The difference in sensitivity between the cell lines could be attributed to several factors such as growth rate, HPV type and copy number and basal p53 activity (28) among the cell lines. Our results suggested WA to repress HPV oncprotein E6 irrespective of HPV type and a favorable modulation in p53 and its effector molecule p21cip1/waf1 expression suggesting a possible p53-mediated apoptosis in these cells. In HPV-negative C33a cells bearing mutated p53, we did not observe significant change in p53 levels with WA; however, p21cip1/waf1 expression levels were increased in a dose-dependent manner. We selected CaSki cell line to deduce the mechanism of action in detail for following reasons: (i) infection with HPV is the etiological factor for 99.5% of cervical cancer, (ii) it harbors copies of both HPV 16 and HPV 18 and (iii) it had lower IC50 value compared with HeLa and SiHa.

Using CaSki cells, we demonstrated that WA not only decreased transcription of HPV oncogenes resulting in downregulation of mRNA expression but also reduced mRNA and protein stability of HPV16 E6 and E7 genes. We further questioned if repression of HPV oncogenes resulted in reactivation of dormant tumor suppressor pathways. Our results confirmed restoration of p53 levels by WA as indicated by...
increased transcription and nuclear localization of p53, which is vital to function as tumor suppressor. Although WA did not significantly influence the mRNA stability of p53, both protein levels and stability were improved.

Since induction of apoptosis is central to the tumor-suppressor activity of p53 (29), we investigated p53-dependent apoptotic pathways induced by WA. We demonstrated cell cycle arrest at the G2/M phase, with modulation of the major cell cycle regulators such as p34\(^{cd}c_2\), cyclin B1 and p21\(^{cip1/waf1}\). Cyclin B1 expressed during the late S to G2 phase immediately binds to p34\(^{cd}c_2\), leading G2 to M phase progression. Our results revealed that WA not only increased cyclin B1 and decreased p34\(^{cd}c_2\) and pCdk1 levels but also decreased cyclin B1-p34\(^{cd}c_2\) complex formation, causing cell cycle arrest at G2/M. A similar trend of cyclin B1 accumulation by WA was reported in breast cancer cells (15), p21\(^{cip1/waf1}\), a universal inhibitor of cdk, is known to inhibit p34\(^{cd}c_2\) (30). Therefore, the decrease in p34\(^{cd}c_2\) could also be due to increased p21\(^{cip1/waf1}\) caused by WA. Once induced, p21\(^{cip1/waf1}\) also has an ability to associate with PCNA and reinforce growth arrest by preventing DNA replication (31). WA-treated CaSki cells clearly demonstrated dose-dependent decrease in PCNA levels and an increased interaction between p21\(^{cip1/waf1}\) and PCNA. Hence, we presume that the proapoptotic effect of p21\(^{cip1/waf1}\), in part, could be due to its interaction with PCNA (32,33).

STAT3 activation has been implicated in tumor progression by promoting cell growth and angiogenesis and resistance to apoptosis induced by chemotherapeutic agents (34). Constitutive activation of STAT3 has been reported in several types of human cancer, including cervical cancer (23). Elevated levels of STAT3 phosphorylation associated with increased expression of Bcl-xL, survivin and mcl-1 has been reported in cervical cancer (35). Wild-type p53 expression has been shown to significantly diminish phosphorylation of STAT3, reduce STAT3 DNA-binding activity and inhibit STAT3-dependent transcriptional activity in cells expressing constitutively active STAT3 (36). In this research, we demonstrated that WA treatment not only decreased STAT3 phosphorylation at Tyr\(^{705}\) and Ser\(^{727}\) residues but also decreased total STAT3 levels. Our results are in agreement with a recent study that suggested inhibition of STAT3 activation by WA in human breast cancer cells (37). Moreover, WA-induced p53 expression could also, in part, be responsible for lowering STAT3 levels. Thus, our research suggested that WA inhibited STAT3 levels both directly as well as indirectly via the restored p53 expression in CaSki cells.

Several studies have demonstrated the direct activation of the proapoptotic protein Bax in p53-mediated apoptosis (7,38). We confirmed that WA-induced cell death in CaSki cells was accompanied by upregulation of Bax and downregulation of the antiapoptotic protein Bcl2. An increase in the Bax: Bcl2 ratio was found to increase the cells’ susceptibility to undergo apoptosis and stimulates the release of cytochrome c from mitochondria to cytosol (39,40). Subsequently, cleavage of procaspase-3 by cytochrome c resulted in the activated form of caspase-3 and PARP cleavage (41,42). This study clearly demonstrated that the restoration of p53 levels by WA, resulting in the modulation of the Bax/Bcl2 ratio and caspase-3 mediated apoptosis in CaSki cells.

Based on the high antiproliferative efficacy of WA against cervical cancer cells in vitro, we tested its efficacy against CaSki subcutaneous xenografts in athymic nude mice. Previous studies have used 4–30 mg/kg WA, with no apparent toxicity to the animals (12,16,43). In this study, the animals treated with WA (8 mg/kg) for 6 weeks exhibited a significant reduction in tumor volume (nearly 70%). Consistent with cell culture findings, we observed downregulation of HPV16 E6 and E7 oncoproteins and increase in p53 levels in the WA-treated animals. Modulation of the cell cycle regulators, STAT3 and other apoptotic markers were also in agreement with in vitro studies. Our data demonstrated that WA has a strong and significant in vivo antiproliferative activity against cervical cancer growth, in a p53-dependent manner, by molecular alterations leading to cell cycle arrest, inhibition of proliferation and induction of apoptotic cell death.

In summary, our results show that WA has profound in vitro and in vivo antiproliferative activity against cervical cancer. This is the first demonstration of the ability of WA to repress HPV E6 and E7 oncoproteins, resulting in sequential reactivation of p53-dependent tumor suppressor activity leading to growth inhibition of cervical cancer cells. Our findings provide a strong basis for the further exploration of WA as a therapeutic drug against cervical cancer, either alone or adjuvant to standard chemotherapeutic agents, to treat primary cancers or to prevent recurrence in cervical cancer patients.

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Conflict of Interest Statement: None declared.

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