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Herbal Extract of *Wedelia chinensis* Attenuates Androgen Receptor Activity and Orthotopic Growth of Prostate Cancer in Nude Mice

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Abstract

Purpose: *Wedelia chinensis* is a common ingredient of anti-inflammatory herbal medicines in Taiwan and southern China. Inflammation is involved in promoting tumor growth, invasion, and metastasis. This study aims to test the biological effects *in vivo* of *W. chinensis* extract on prostate cancer.

Experimental Design: The *in vivo* efficacy and mechanisms of action of oral administration of a standardized extract of *W. chinensis* were analyzed in animals bearing a subcutaneous or orthotopic prostate cancer xenograft.

Results: Exposure of prostate cancer cells to *W. chinensis* extract induced apoptosis selectively in androgen receptor (AR)–positive prostate cancer cells and shifted the proportion in each phase of cell cycle toward G_2-M phase in AR-negative prostate cancer cells. Oral herbal extract (4 or 40 mg/kg/d for 24-28 days) attenuated the growth of prostate tumors in nude mice implanted at both subcutaneous (31% and 44%, respectively) and orthotopic (49% and 49%, respectively) sites. The tumor suppression effects were associated with increased apoptosis and lower proliferation in tumor cells as well as reduced tumor angiogenesis. The antitumor effect of *W. chinensis* extract was correlated with accumulation of the principle active compounds wedelolactone, luteolin, and apigenin *in vivo*.

Conclusion: Anticancer action of *W. chinensis* extract was due to three active compounds that inhibit the AR signaling pathway. Oral administration of *W. chinensis* extract impeded prostate cancer tumorigenesis. Future studies of *W. chinensis* for chemoprevention or complementary medicine against prostate cancer in humans are thus warranted. (Clin Cancer Res 2009;15(17):5435–44)

Carcinoma of the prostate gland is the most common malignancy in males in the western world (1). Despite the low incidence of prostate cancer in oriental countries, statistics from Taiwan reveal prostate cancer deaths have continued to increase in the past two decades.[5] Androgen ablation therapy remains the most effective means of treating metastatic prostate cancer tumors (2, 3). This therapy often induces apoptosis in the majority of prostate cancer cells by blocking testosterone signaling at the androgen receptor (AR) and lowering the expression of AR-regulated genes including prostate-specific antigen (PSA), a serologic biomarker up-regulated by androgens (4, 5).

The CWR22 tumor model, based on implantation of a human prostate cancer in an athymic nude mouse, progresses in the same androgen-dependent manner as observed in clinical prostate cancer tumors. Further development from castration-relapsed CWR22 tumors created subline tumors and a cell line, 22Rv1, which retain expression of AR and show androgen stimulation of neoplasia (6, 7). Earlier, we devised the androgen-induced PSA-luciferase activity in prostate cancer 22Rv1 cells and a derived stable clone, 103E, as a cell-based assay to detect any AR modulator effect of herbal extract or compounds (8–10). To determine whether herbal remedies or compounds can inhibit prostate cancer growth in orthotopic sites, we constructed a tumor model by orthotopic implantation of luciferase-expressing 103E cells into nude mice to serially image prostate cancer growth. The present study used this bioluminescent tumor model to assess the preventive and therapeutic effects of a standardized herbal extract against prostate cancer.

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Translational Relevance

Herbal remedies are the most accepted complementary and alternative medicines used among patients with prostate cancer. Plant extracts contain various compounds that have been shown to inhibit many different process of inflammation. It is intriguing that *Wedelia chinensis*, an ingredient of anti-inflammatory herbal medicines, also contains a set of compounds that inhibit androgen receptor signaling and *in vitro* growth of prostate cancer cell lines. This study examines the use of a herbal extract to treat a clinically advanced prostate cancer in animal models with castration-resistant tumors. The herbal extract restrained the prostate cancer tumor growth and decreased angiogenesis without observable toxicity. This preclinical study thus provides strong evidence that *W. chinensis* is a good candidate for chemoprevention or complementary medicine.

We have shown previously that *Wedelia chinensis* (Asteraceae), an oriental herb, contains four compounds capable of suppressing androgen activity: indole-3-carboxylaldehyde, wedelolactone, luteolin, and apigenin (8). *In vitro* experiments showed that a combination of these bioactive compounds in the ratio present in the herbal extract synergistically inhibited the clonogenic growth of AR-positive prostate cancer cells. Luteolin and apigenin also had additive effects on AR-negative prostate cancer. In continued evaluation of the potential of *W. chinensis* extracts for treating prostate cancer, we prepared a standardized herbal extract of *W. chinensis* based on the potential anti–prostate cancer compounds, examined its action *in vivo* against prostate cancer models, and determined the *in vivo* bioavailability of the hypothesized active compounds. Evidence of the antitumor effect of the extract in cultured cell lines and the tumor specimens is also provided.

Materials and Methods

Plant material and extract preparation. The source of *W. chinensis* and preparation and extraction procedure was as described previously (8). The *W. chinensis* extract was dried *in vacuo* and reconstituted in DMSO as stock solutions. In animal experiments, mice were dosed by force feeding with 200 μL *W. chinensis* extract, freshly diluted from 0.5 and 5 mg/mL stocks with PBS into 10 volumes to attain dosage of 4 and 40 mg/kg.

Chemical reagents and antibodies. 5α-Dihydrotestosterone, puromycin, and propidium iodide (PI; Sigma-Aldrich), RNase (Amresco), and DMSO, ethanol, *n*-hexane, methanol, and acetonitrile (J.T. Baker) were of analytic reagent or high-performance liquid chromatography grade. Antibodies to AR (N-20), cyclin D1 (C-20), cyclin E (M-20), CDK2 (M-2), CDK4 (H-22), CDK6 (C-21), Rb (C-15), p27 (F-8), p21 (F-5), p53 (DO1), Bax, Bcl-xL, and β-actin were purchased from Santa Cruz Biotechnology. Caspase-3 and -7 antibodies were purchased from Neomarkers/Lab Vision and Oncogene Research Products, respectively. PSA antibodies were purchased from Chemicon and poly(ADP-ribose) polymerase and pRb (Ser^{780}) antibodies were purchased from Cell Signaling. Mouse monoclonal anti–Ki-67 (MIB-1) came from DAKOCytomation, the rat monoclonal anti-CD34 (MEC 14.7) came from Abcam, and the rabbit polyclonal anti-AR used in immunohistochemistry came from Santa Cruz Biotechnology.

Cell culture. LNCaP, PC-3, and 22Rv1 prostate cancer cell lines were obtained from the American Type Culture Collection. 103E was derived by stable transfection of p5.7kb-PSA-luciferase and pGK-puro and selected by puromycin resistance and androgen-dependent luciferase expression as described previously (8). LNCaP, 22Rv1, and 103E cells were cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone). PC-3 cells were cultured in DMEM (Hyclone) supplemented with 10% fetal bovine serum. Cultures were maintained in a humidified incubator at 37°C in 5% CO_2.

Luciferase, proliferation, and flow cytometry analysis of cell cycle and apoptosis. Luciferase assay and colony formation analyses were done as described previously (8). LNCaP, 22Rv1, and PC-3 cells were treated with indicated treatments for 48 h. The cell cycle of treated cells was examined by flow cytometry after cellular staining with PI and analyzed as described previously (11). For apoptosis analysis, treated cells were washed in PBS and resuspended in 500 μL staining solution containing FITC-Annexin V and PI in HEPES. After incubation at room temperature for 5 min, cells were analyzed by flow cytometry.

In vivo *studies.* Athymic (*nu/nu*) nude mice (6-7 weeks old) were obtained from the National Laboratory Animal Center and housed as described previously (9). All animal work was done in accordance with the protocol approved by the Institutional Animal Care and Use Committee, Academia Sinica, Taiwan. 22Rv1 cells (1×10^6) suspended in PBS were mixed 1:1 with Matrigel (BD Biosciences) and subcutaneously inoculated into the right flank of each mouse and allowed to reach a tumor diameter of 0.4 to 0.5 cm. For orthotopic implantation, a mouse prostate was exposed with a surgical incision and 103E cells in suspension (3×10^5 in 20 μL PBS) were injected into the left side of prostate. Two weeks after implantation, mice were randomly assigned to three groups ($n = 7$) that received vehicle control or *W. chinensis* extract at different dosages (4 or 40 mg/kg/d) by gavage in 200 μL PBS containing 10% DMSO. Subcutaneous tumors were measured twice per week using calipers and their volumes were calculated using a standard formula ($\text{width}^2 \times \text{length} \times 0.5$). Bioluminescence intensity of implanted tumors was monitored in living mice once a week. Body weight was measured weekly. Mice received 24 or 28 doses, and 24 h after the last dose, they were sacrificed to harvest plasma and tumors. A portion of each tumor was snap-frozen in liquid nitrogen and stored at -80°C until needed for analysis of attained compounds in the tumors, and the remainder was fixed in 10% formalin overnight.

Immunohistochemistry. The paraffin-embedded tumor sections (4 μm thickness) were heat immobilized, deparaffinized using xylene, and rehydrated in a graded series of ethanol with a final wash in distilled water. Antigen retrieval in Target Retrieval Solution (DAKOCytomation) in a Decloaking Chamber (Biocare Medical) was followed by quenching of endogenous peroxidase activity. Sections were then incubated with specific primary antibodies, including mouse monoclonal anti–Ki-67, rat monoclonal anti-CD34, and rabbit polyclonal anti-AR at 4°C overnight in a humid chamber. A Promark Polymer detection system (Biocare Medical) was used to detect the reaction products. *In situ* detection of apoptotic cells was carried out using terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay reaction as described previously (9). Tumor-associated angiogenesis was quantified by area microvessel density adapted from elsewhere (12). Microvessel area was defined as CD34-stained endothelial cell surface area, excluding vessel lumen. The regions with higher microvessel density were selected and images were captured with a Zeiss AxioCam HRc camera attached to a Zeiss AxioImager. Z1 microscope using a ×20 objective. Six tumor sections were used for each comparison, and five selected fields in each were examined and automatically quantified by Zeiss AxioVision Rel. 4.6 without interactive steps.

High-performance liquid chromatography analysis. Plasma (0.1 mL) and tumor samples (0.1 g) from each individual mouse were homogenized in a mixer ball mill (MM301; Retsch) for 5 min, deproteinized by adding 0.3 mL acetonitrile containing internal standard (genestein, 1 nmol for plasma and 100 nmol for tumor samples), and centrifuged

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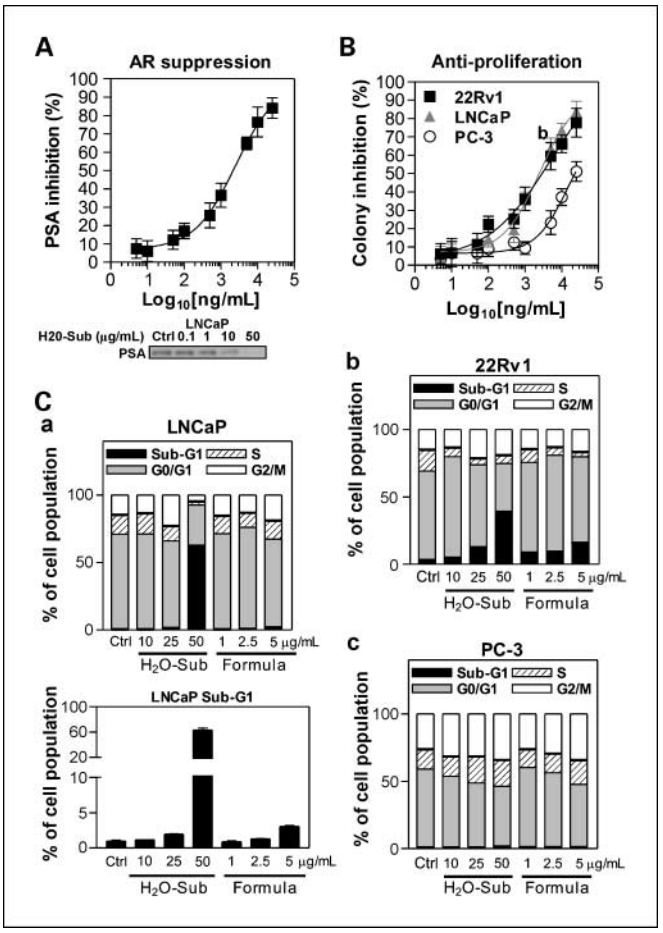

Fig. 1. Effects of *W. chinensis* extract on androgen activity and growth of prostate cancer cells. *A,* dose-response curves of *W. chinensis* extract in inhibiting 5α-dihydrotestosterone–induced PSA-luciferase reporter. 103E cells were grown and cotreated with 10 nmol/L 5α-dihydrotestosterone and indicated concentrations of *W. chinensis* extract for 24 h. PSA-luciferase activities of treated cells were measured to determine androgen activity. Mean ± SD of three independent experiments of three replicates. *Lower*, PSA expression in LNCaP cells co-treated with 10 nmol/L 5α-dihydrotestosterone and indicated amount of H_2O-Sub for 24 h. *B,* colony-forming growth was analyzed by growing LNCaP and 22Rv1 cells (AR-dependent prostate cancer cells) and PC-3 cells (AR-independent prostate cancer cells) in 24-well multidishes with indicated concentrations of *W. chinensis* extract for 12 d. Colony-forming growth was quantified, and dose-response curves are presented as percent inhibition with respect to control cells (vehicle treatment) of the same cell line. Mean ± SD of three independent experiments of three replicates. *C,* three different prostate cancer cell lines (*a-c*) were treated with extract (*H_2O-Sub*) or formula for 48 h. The cell cycle profiles of cancer cells after herbal treatment compared with control (ethanol treatment) were determined by flow cytometric analysis. *a,* results in LNCaP of different treatments (*top*) and magnified view of proportion of sub-G_1 cells (*bottom*).

at 16,800 × *g* for 10 min at 4°C. The supernatant was collected into the tube and evaporated to dryness by vacuum freeze drying. The residue was dissolved in 100 μL methanol and analyzed by analytic reverse-phase high-performance liquid chromatography using a 250 × 10 mm C_{18} Cosmosil column (Nacalai Tesque) on an Agilent 1100 system as described previously (8).

Data analysis. Dose-response curves and IC_{50} for PSA inhibition (Fig. 1A) and relative colony growth (Fig. 1B) were analyzed using a sigmoidal dose-response equation (variable slope) in Prism 3.02 (GraphPad) as described previously (8). Data are the mean ± SE for the indicated number of separate experiments. The statistical significance of differences between two groups of data (Figs. 3A and B, 4A and C, and 5B, C, D and 6C) was analyzed by paired *t* test and *P* values < 0.05 were considered significant.

Results

W. chinensis extract suppresses AR activity and clonogenic growth of prostate cancer cells. To investigate if W. chinensis may help prostate cancer treatment, we defined an extraction procedure that enriches the four hypothesized active compounds from crude extract of *W. chinensis*. The *W. chinensis* extract was standardized as 10% of its mass comprising the four potential active compounds (Supplementary Fig. S1). The effects of the whole extract of *W. chinensis* on AR suppression and clonogenic growth of prostate cancer cells were tested initially *in vitro*. Exposure to the herbal extract inhibited the androgen function in 103E and LNCaP prostate cancer cells in a dose-dependent manner (Fig. 1A). The same treatment also inhibited the clonogenic growth of prostate cancer cells (Fig. 1B). The IC_{50} for AR inhibition of 103E cells and antiproliferation of LNCaP, 22Rv1, and PC-3 cells were 2.5, 2.5, 4.9, and 14.6 μg/mL, respectively. Furthermore, the effect of *W. chinensis* extract varied according to the AR dependence of the target, with a much higher potency in AR-positive (LNCaP and 22Rv1) than AR-negative (PC-3) cell lines. These effects were in line with our previous data showing that a combination of the four potential bioactive compounds from *W. chinensis* differentially inhibited the clonogenic growth of prostate cancer cells, with higher sensitivity seen in AR-positive than AR-negative cells.

Differential effects of* W. chinensis *extract and bioactive compounds on cell cycle of prostate cancer cells according to AR expression. When the four bioactive compounds were applied together in the herbal extract or were reconstituted from individual components to the same proportion, both preparations induced sub-G_1 populations in AR-positive (LNCaP and 22Rv1) cells and shifted the cell cycle toward G_2-M phase in AR-negative (PC-3) prostate cancer cells (Fig. 1C). Because *W. chinensis* extract contains only 10% active compounds, its effects on cell cycle in prostate cancer cells were compared with a 1/10 dose of the formula. The proapoptosis effect was dose-dependent, more significant in 22Rv1 than LNCaP cell line, and more profound with the herbal extract than the formula. These results suggest that the possibility that other compounds in the extract may work together with the four candidate active compounds to boost the induction of apoptosis (13) and provide a rationale for the application of whole *W. chinensis* extract in prostate cancer treatment.

The ability of *W. chinensis* extract to induce cell apoptosis was further verified by examination of apoptotic cell numbers by flow cytometry (Fig. 2A), staining different cell lines with FITC-Annexin V, a stain of exposed surface phosphatidylserine in apoptotic cells, and PI, a marker of cell membrane permeability. Cells in early apoptosis are FITC-Annexin V positive and PI negative and late apoptosis both FITC-Annexin V and PI positive. When LNCaP and 22Rv1 cells were treated with *W. chinensis* extract (water subfraction) at doses of 10 or 50 μg/mL for 48 h, numbers of both early and late apoptotic cells increased dose-dependently (Fig. 2A, *a-f*). However, in Western blot analysis for other indicators of apoptosis, proteolytic cleavage of poly(ADP-ribose) polymerase, caspase-3, and caspase-7 and other changes were only seen at 50 μg/mL dose (Fig. 2B, *left* and *middle*). The herbal extract down-regulated the expression of the antiapoptotic regulator Bcl-xL without affecting the proapoptotic regulator BAX. In a separate experiment, using the same treatment in PC-3 cells, we found no significant

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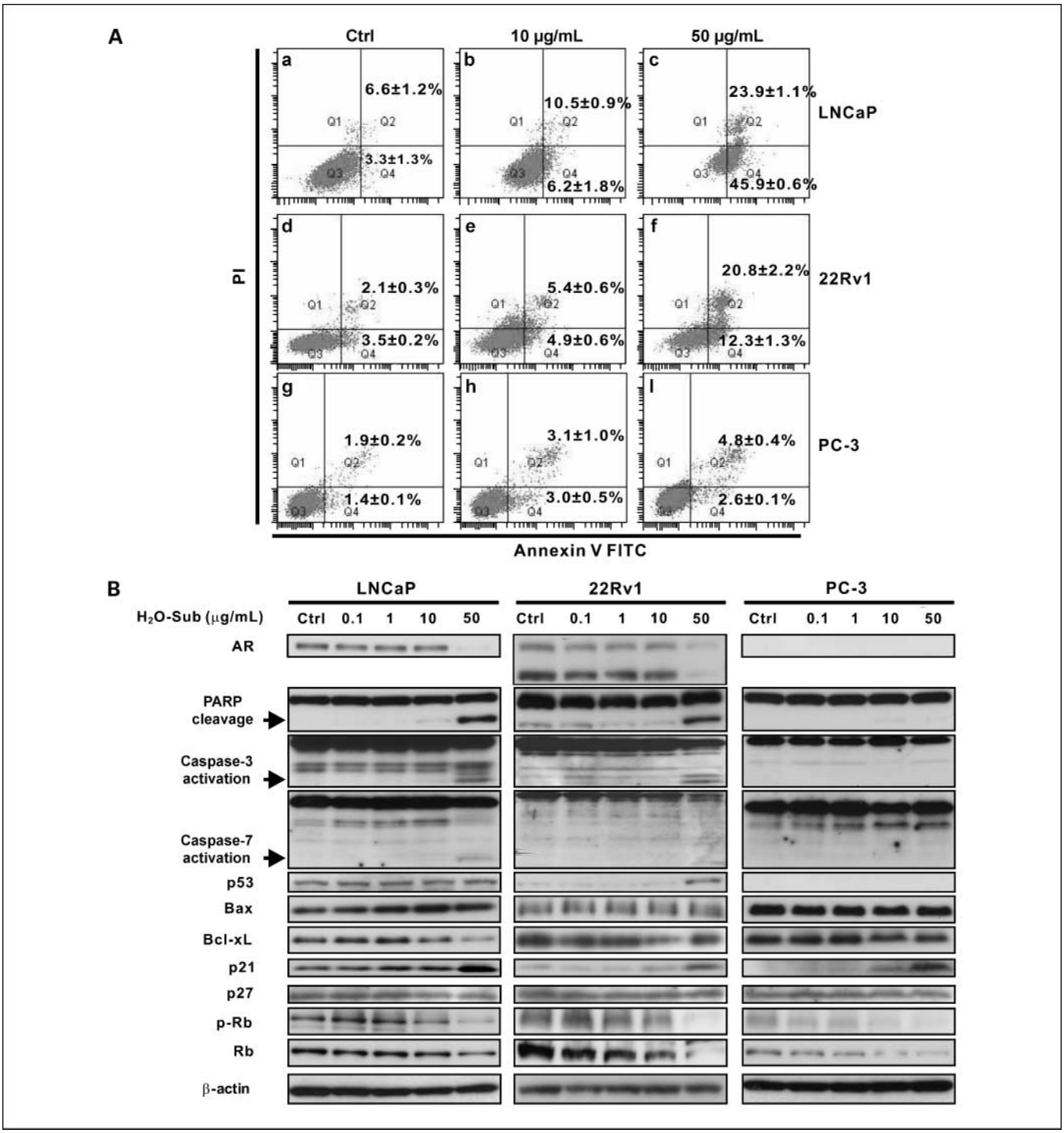

Fig. 2. *W. chinensis* extract induces apoptosis in AR-dependent prostate cancer cells. *A,* prostate cancer cell lines were treated with extract at 10 or 50 μg/mL for 48 h. After staining with FITC-Annexin V/PI, cells were analyzed by flow cytometry. Representative histograms for four set of experiments, quantitatively analyzed by BD FACSDiva software (BD Biosciences). *B,* effects of *W. chinensis* extract on the expression of molecular markers for apoptosis and cell cycle in prostate cancer cells. LNCaP and PC-3 cells were grown and treated with vehicle or indicated concentrations of *W. chinensis* extract for 48 h. Whole-cell extracts were then subjected to Western blotting using antibodies against poly(ADP-ribose) polymerase, caspase-3, caspase-7, Bax, anti–Bcl-xL pRb, Rb, p27, p21, and β-actin (used as loading control). Representative blots of three independent experiments.

changes in FITC-Annexin V/PI staining or these apoptotic markers (Fig. 2A, *g-i*, and B, *right*). Although the herbal extract did not cause apoptosis in PC-3 cells, it induced p21 expression and depleted the Rb pathway, the ability of which to arrest cell cycle at G_2-M phase has been shown earlier (14). Collectively, Western blot analysis of the *in vitro* effects of *W. chinensis* extract on apoptosis and cell cycle regulators corroborated the data obtained in flow cytometry.

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Oral* W. chinensis *extract attenuates growth of prostate cancer 22Rv1 xenograft in nude mice. 22Rv1 cells were derived from a human castration-relapsed tumor of prostate cancer origin and represent advanced tumors that are resistant to androgen ablation therapy. The effect of *W. chinensis* extract on prostate cancer growth *in vivo* was tested in athymic nude mice with a 22Rv1 tumor implanted subcutaneously. Treatment started after prostate cancer implant had established a palpable (0.4 cm diameter) tumor, and herbal extract was administrated by gavage once daily at doses of 4 or 40 mg/kg body weight. We monitored the prostate cancer growth by measuring tumor size and the tumor mass at the end of herbal treatment; both data revealed that oral intake of *W. chinensis* extract significantly retarded prostate cancer growth (Fig. 3A and B). Endpoint tumor mass showed dose-dependent growth suppression by *W. chinensis* extract, wherein the mean tumor masses of 4 and 40 mg/kg/d groups were 0.733 and 0.602 g compared with 1.057 g of the control group (4 versus 40 mg/kg; $P \leq 0.05$; Fig. 3B). During the 24-day treatment period, mice did not exhibit any symptoms of toxicity such as loss of appetite, decreased locomotion, or any other apparent signs of illness. As shown in Fig. 3C, body weight of herbal-treated mice (up to 40 mg/kg/d dosage) was not different from control-treated mice. Tumor-bearing mice representative of each group are shown in Fig. 3D.

To further mimic the tumor development in orthotopic site, we established a noninvasive imaging tumor model by implanting mouse prostate with 103E cells, a subline of 22Rv1 origin, which expresses luciferase under the regulation of PSA promoter. Two weeks after tumor cells were implanted, bioluminescence was clearly detectable. *W. chinensis* extract was administered by gavage once daily at doses of 4 or 40 mg/kg body weight for 4 weeks. The intensity of bioluminescence, which reflects the size of tumor, measured at weekly intervals was significantly inhibited in both groups treated with herbal extract (Fig. 4A). Tumors were dissected at necropsy, and macroscopic images of representative tumors from each group are shown in Fig. 4B. There was significant inhibition ($P < 0.01$, *t* test) of tumor growth, although the effect seemed to have already reached a maximum at 4 mg/kg/d (Fig. 4C). During the 28-day period of treatment, mice acted normally without any treatment-associated toxicity, and body weight of all mice was not influenced by administration of *W. chinensis* extract at either 4 or 40 mg/kg/d (Fig. 3D).

Oral* W. chinensis *extract induces apoptosis along with a decline in proliferation and angiogenesis in prostate cancer 103E xenografted nude mice. Our *in vitro* assays showed that *W. chinensis* extract inhibited cell proliferation and induced apoptosis in AR-dependent prostate cancer cells (Figs. 1 and 2); we therefore examined proliferation and apoptosis in the specimens of orthotopic tumors by immunohistochemistry. Staining for proliferative tumor cells with human-specific Ki-67 antibody and for apoptotic cells by the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling method revealed that oral *W. chinensis* extract (4 or 40 mg/kg/d for 28 days) decreased the number of proliferating prostate cancer cells (Fig. 5A, *a-c*) and increased the number of apoptotic tumor cells (Fig. 5A, *d-f*). The antiproliferation and proapoptosis effects were statistically significant (Fig. 5B and C). Despite treatment with the herbal extract, the tumors still expressed AR (Fig. 5A, *g-i*).The control group gave rise to tumors with a more bloody appearance than the herbal extract–treated groups (Fig. 4B), so we stained the tumors for CD34, a marker for endothelial cells and hematopoietic stem and progenitor cells. The antibody recognizes a neuraminidase-sensitive epitope on endothelium cells *in vivo*, particularly on small vessels, newly formed capillaries and developing vascular structures in embryonal structures. Indeed, infiltration of $CD34^+$ cells and the microvessel area in each field decreased in the herbal extract–treated groups, suggesting a dose-dependent antiangiogenesis effect (Fig. 5A, *j-l*, and D). All the antiproliferation, proapoptosis, and antiangiogenesis effects contribute to the reduced tumor growth caused by this herbal extract and infer the underlying mechanisms.

Oral* W. chinensis *extract achieved effective concentrations in plasma and in tumors in tested mice. To understand whether the *in vivo* efficacy may be attributable to those proposed bioactive compounds, we examined their levels in the plasma and tumors of tested mice 24 h after the last dose of herbal extract. None of the four compounds was detectable in the plasma of control groups, whereas concentrations of wedelolactone, luteolin, and apigenin in plasma were 0.05, 1.25, and 0.25 μmol/L, respectively (Fig. 6A and B, *left*). All three compounds were present at slightly but not significantly higher levels in the 40 mg/kg groups than in the 4 mg/kg groups. The ratio of wedelolactone, luteolin, and apigenin concentration in plasma was approximately the same as their ratio in *W. chinensis* extract, suggesting a similar bioavailability or coupled metabolism of wedelolactone, luteolin, and apigenin

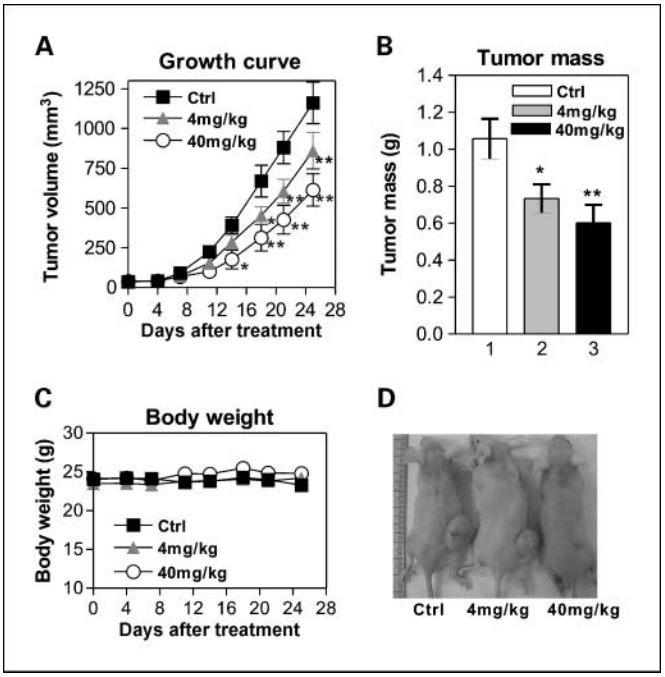

Fig. 3. *W. chinensis* extract attenuates tumor growth of CWR22Rv1 engrafted in the subcutis of nude mice. Subcutaneous CWR22Rv1 tumor xenografts were established in nude mice and mice were treated with *W. chinensis* extract via gavage once daily for 24 d. *A,* tumor volumes in each treatment group are presented as growth curves. *Points,* mean; *bars,* SE ($n = 6$). *B,* endpoint tumor mass of each treatment group. *Points,* mean; *bars,* SE ($n = 6$). *, $P \leq 0.05$; **, $P \leq 0.01$ (*t* test). *C,* mean body weight for each treatment group as a function of days of treatment. *D,* representative photograph of tumor-bearing mice in all tested groups.

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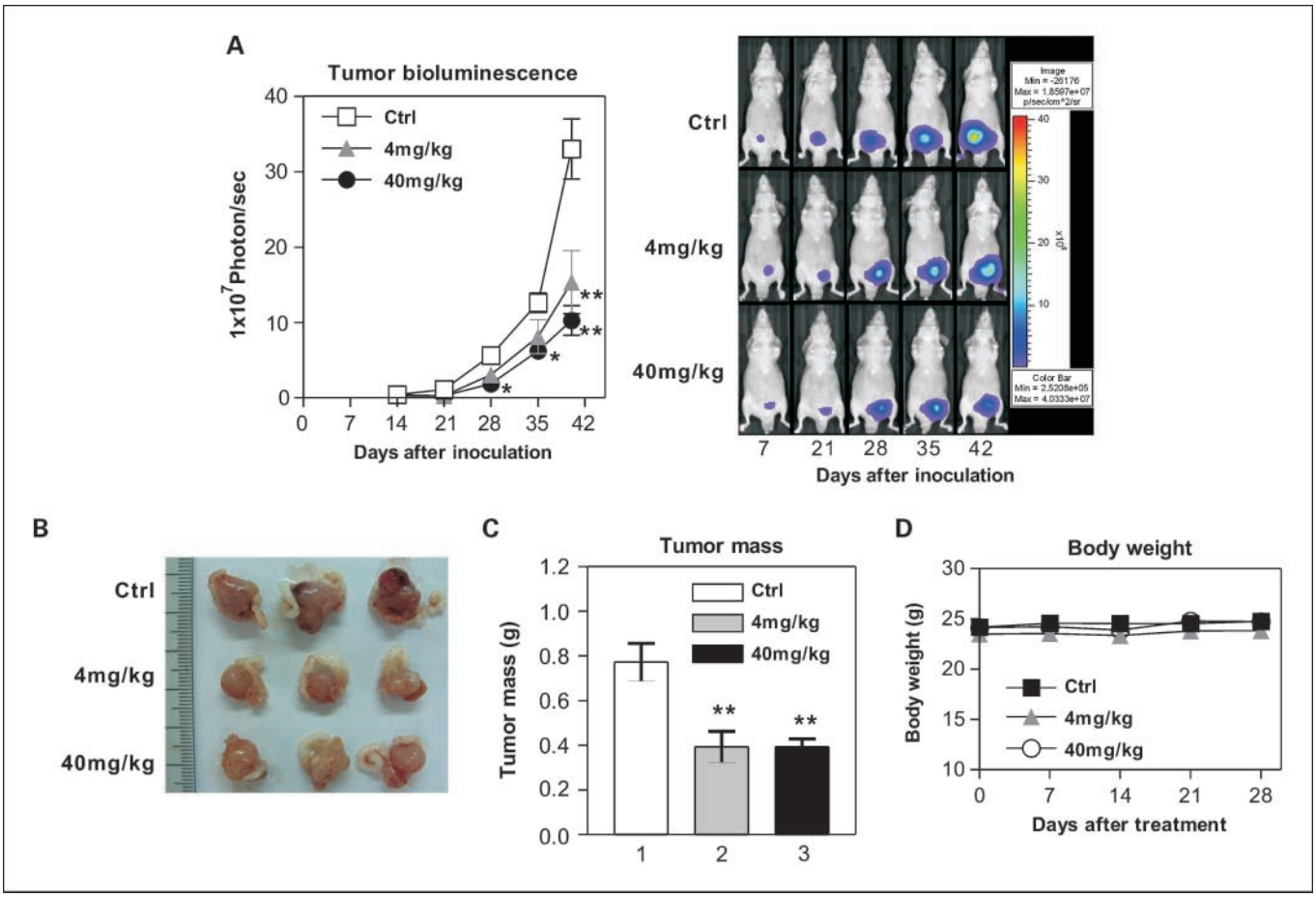

Fig. 4. *W. chinensis* extract attenuates tumor growth of 103E (a subline of CWR22Rv1) engrafted in the prostate of nude mice. Orthotopic 103E tumor xenografts were established in nude mice and mice were treated with *W. chinensis* extract via oral gavage once daily for 28 d. *A,* bioluminescence intensities in each treatment group were measured weekly and are presented as growth curves. *Points,* mean ($n = 7$); *bars,* SE. *B,* representative photograph of dissected tumors of all tested groups. *C,* endpoint mass of implanted tumors, including adherent seminal vesicle at the implantation site, were weighed and plotted and then analyzed for significant difference. *Columns,* mean ($n = 7$); *bars,* SE. Each treatment group was compared with control (*t* test). *, $P \leq 0.05$; **, $P \leq 0.01$. *D,* mean body weight for each treatment group as a function of day of treatment.

after ingestion of *W. chinensis* extract. Accumulation of wedelolactone, luteolin, and apigenin in the tumors of different treatment groups was found to parallel the plasma levels (Fig. 6A and B, *right*). These data endorse wedelolactone, luteolin, and apigenin as the active compounds of *W. chinensis* extract in attenuation of tumor development in prostate. However, it is not clear whether indole-3-carboxylaldehyde may contribute to the *in vivo* efficacy of the extract against prostate cancer.

***Oral wedelolactone, luteolin, and apigenin acted synergistically to suppress growth of prostate cancer 22Rv1 xenograft* in vivo.** To further analyze the combinational effect of wedelolactone, luteolin, and apigenin *in vivo*, we designed another set of animal experiments based on the median-effect principle of the mass-action law. Pure wedelolactone, luteolin, apigenin, or their formula (wedelolactone/luteolin/apigenin = 1:25:5) were orally administered once a day at equal dosage (2 mg/kg body weight) to nude mice bearing subcutaneous 22Rv1 xenografts. The tumor volume was followed twice a week during a 21-day treatment (Fig. 6C, *left*). Compared with control group, the resulting mean tumor mass in the wedelolactone, luteolin, apigenin, and formula groups decreased by 2.58%, 10.31%, 35.67%, and 39.54%, respectively (Fig. 6C, *right*). Statistical analysis suggests that neither wedelolactone nor luteolin singly were effective. The formula suppressed tumor growth to the same level as a five times larger amount of apigenin alone did. In this experiment, all the treated compounds were undetectable in the plasma in all groups even as short as 6 h after the last oral dose (data not shown), and apigenin was the only compound detectable in tumors of the apigenin and formula groups (Fig. 6D). The apigenin content of formula was 16.13% by mass, and apigenin level in formula-treated tumors was 22.82% of that in apigenin-treated tumors, which confirms that formula-treated tumors had a much lower systemic exposure to apigenin than the tumors treated with apigenin alone. In the same tumor model, oral administration of apigenin alone at doses of 20 and 50 μg/mouse/d (weighing 28 ± 3 g) suppressed tumor growth dose-dependently (15, 16). In concert with these data, one would anticipate a much lower level of tumor suppression in the formula group than the apigenin group. Single treatment with wedelolactone or luteolin resulted in undetectable bioavailability and biological effects, indicating their poor stability *in vivo*. However, the effect of the formula was higher than the level expected from its low apigenin content,

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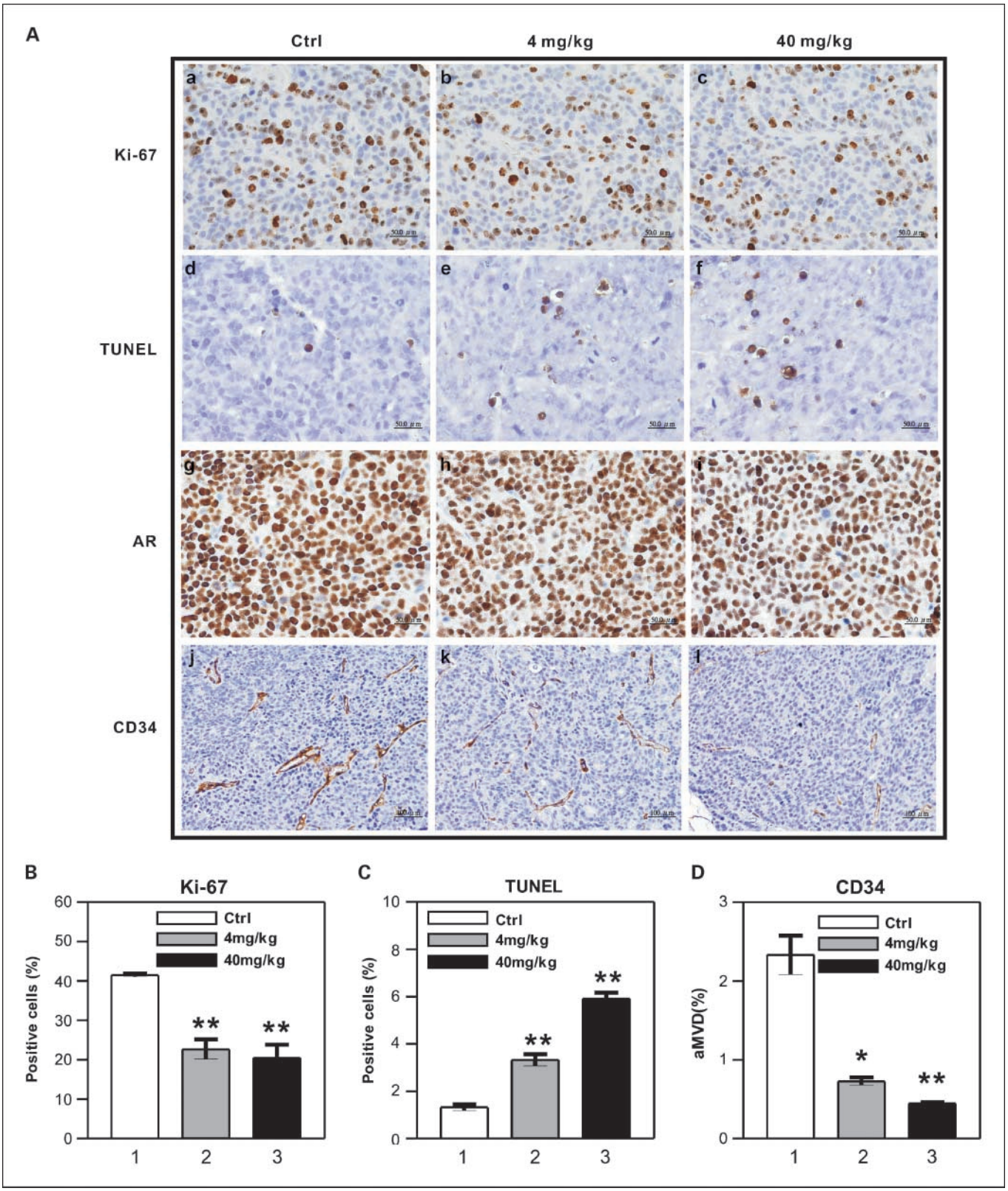

Fig. 5. Pathology analysis of *W. chinensis* functions *in vivo*. At the end of the herbal extract regimen, orthotopic 103E tumors were studied in detail. *A,* immunohistochemical staining of tumor proliferation with anti–Ki-67 (*a-c*), apoptosis analyzed by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay (*d-f*), AR expression (*g-i*), and CD34 to mark endothelial cells (*j-l*). Representative picture of each treatment group. Ki-67–positive (*B*) and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling–positive (*C*) fractions (%) were calculated as number of positive (reddish brown) cells × 100 / total number of cells counted. *D,* microvessel area (*aMVD*) was quantified to indicate angiogenesis. *Columns,* mean of five independent tumor samples from different mice, of which three random areas in each tumor were counted; *bars,* SE. Each treatment group was compared with the control (*t* test). *, $P \leq 0.05$; **, $P \leq 0.01$.

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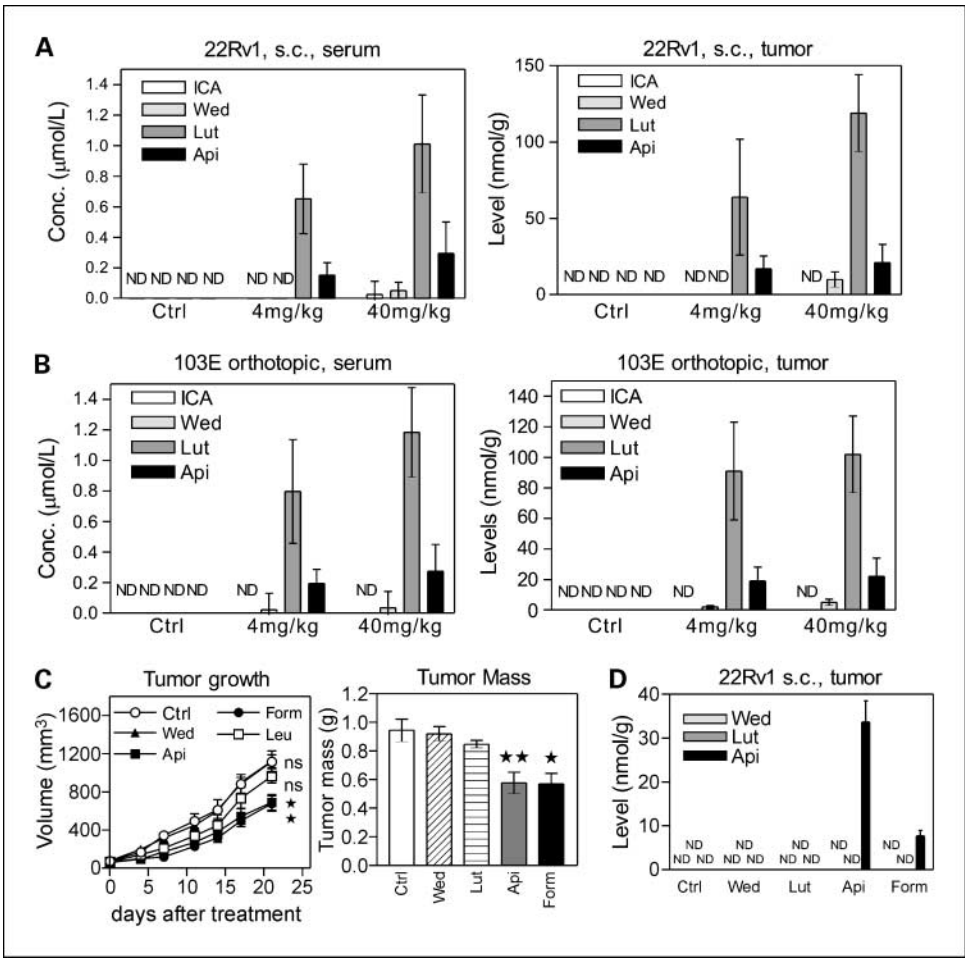

Fig. 6. Levels of bioactive compounds in plasma and tumors of tested mice after completion of the *W. chinensis* regimen. Concentrations of all four active compounds 24 h after the last dose were determined in plasma and tumor of mice with *A,* subcutaneous tumor. *B,* orthotopic tumor. *Columns,* mean; *bars,* SD ($n = 5$). *C,* groups of mice ($n = 6$) with subcutaneous prostate cancer 22Rv1 xenografts were treated with wedelolactone, luteolin, apigenin, or formula at 2 mg/kg/d dosage via gavage once daily for 21 d. *Left,* Tumor volumes in each treatment group are presented as growth curves. *Points,* mean; *bars,* SE ($n = 6$). *Right,* endpoint tumor mass of each treatment group. *Columns,* mean; *bars,* SE ($n = 6$). *, $P \leq 0.05$; **, $P \leq 0.01$ (*t* test). *D,* concentrations of active compounds in the tumors in *C* were determined 6 h after the last dose. *Columns,* mean; *bars,* SD ($n = 3$). *ND,* not detected.

which supports the synergistic hypothesis that cotreatment with wedelolactone and luteolin enhances the effect of apigenin *in vivo*. Alternatively, other potentially active compounds in the *W. chinensis* extract may also complement the total tumor-suppressive effect.

Discussion

The standard approach to new drug discovery has been to purify, synthesize, and administer the single compound thought responsible for a particular effect. However, carcinogenesis and cancer progression is a multistage process, in which multiple aberrations of signaling pathways may have arisen in patients. As single agents have not yet provided satisfactory results in prostate cancer treatment, the demand for complementary and alternative medicines has increased. Use of whole extracts of assorted plants has been an integral part of traditional medicine in different cultures, including for cancer treatment. A herbal extract is more convenient to prepare than isolation of single components and also can stabilize its active principles (17, 18). The various components in a herbal extract may have synergistic activities and buffer the toxicity of a single compound. However, inability to assure the quality and consistency of herbal extracts has been a major obstacle in the development of botanical preparations (19, 20). Our initial work on identifying the potential active principles of *W. chinensis* provides a sound basis to assure the quality and efficacy of the derived herbal extract. Concentrating on the active principles, we developed an analytic method for quality control and also operationally defined an extraction procedure to accomplish consistent *W. chinensis* preparations (Supplementary Fig. S1), which made it possible to carry out the current study.

Animal experiments are a relevant and necessary way to evaluate the efficacy of new drugs despite their cost in both expense and time. To avoid unnecessary animal experiments, bioassays using cell-based AR-mediated transcription and clonogenic growth of prostate cancer cells have proven their utility in the initial testing of herbal extracts and phytocompounds. When *in vivo* experimentation becomes necessary, the growth of tumor cells at an orthotopic site wherein cancer cells are fostered in a relevant tissue microenvironment is especially meaningful to reflect the natural history of clinical prostate cancer (21, 22). A tumor model with a characteristic that provides convenient and no-invasive assessment of tumor size has many advantages for the study of molecular mechanisms of disease progression and the evaluation of candidate therapeutics. We exploited the luciferase expression in prostate cancer cell line, 103E, to provide a longitudinal series of images of the growth of prostate cancer following orthotopic injection of 103E cells into nude mice. The current study showed that the bioluminescence of this tumor model is

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quantitative and predictive of the tumor mass on necropsy and validates this novel method.

W. chinensis contains four potential active compounds, the combination of which is synergistic *in vitro* and therefore can maximize efficacy against prostate cancer (8). Our cell cycle analyses here provide good evidence that both *W. chinensis* extract and a reconstituted formula induce apoptosis in AR-positive cells and shift the cell cycle toward the G_2-M phase in AR-negative prostate cancer cells (Figs. 1 and 2). These results also address the different mechanisms for the whole *W. chinensis* extract on AR-positive versus AR-negative prostate cancer cells (8). The ability of *W. chinensis* extract to arrest the cell cycle in AR-negative prostate cancer cells also suggests its potential to inhibit AR-negative prostate cancer tumors.

Because prostate cancer is typically diagnosed in men ages ≥50 years, even a slight delay in the onset and subsequent progression of prostate cancer could afford important health benefits (23, 24). Chemoprevention and chemotherapy concern the use of synthetic or natural products, singly or as a mixture, to block neoplastic inception or delay cancer progression (25). For various reasons, the most important of which may be public acceptance, natural products are preferred over synthetic agents for this role. In recent years, much progress has been made in identification of novel prostate cancer chemopreventive and chemotherapeutic agents from natural products (8, 9, 26–34). It should be noted that luteolin and apigenin, two major active compounds of *W. chinensis*, have been independently reported as having potential for prostate cancer treatment through different mechanisms. Luteolin and apigenin inhibit insulin-like growth factor-I receptor and Akt signaling in prostate cancer cells, induce cell cycle arrest and apoptosis, and as a result suppress subcutaneous growth of prostate cancer xenografts (15, 16, 35, 36). Luteolin can inhibit vascular endothelial growth factor–induced angiogenesis by targeting the phosphoinositide 3-kinase/Akt pathway for survival signals of endothelial cells (37). Apigenin also inhibits tumor-initiated angiogenesis by destabilizing hypoxia-inducible factor-1α and reducing vascular endothelial growth factor production in prostate cancer cells (38). Moreover, oral apigenin in TRAMP mice significantly decreased the prostate tumorigenesis and even abolished distant-site metastases through blockade of β-catenin signaling (39). These data agree with the antitumor effects and *in vivo* mechanisms seen following the use of *W. chinensis* extract, such as antiproliferation, proapoptosis, and antiangiogenesis, which are detailed in the current study. Previous data from our laboratory and others showed that luteolin and apigenin have similar potency toward both AR-positive and AR-negative prostate cancer (8, 15). However, the addition of wedelolactone and indole-3-carboxylaldehyde to luteolin and apigenin greatly enhanced their potency toward AR-positive prostate cancer, providing added benefit. Indeed, the dosage requirement in our trial (4 mg/kg/d × 10% purity of active compounds) is lower than the dosage used for luteolin and apigenin alone (16, 35).

The levels of three (wedelolactone, luteolin, and apigenin) of the four active compounds in the plasma (0.034, 1.184, and 0.274 μmol/L, respectively) and tumors (5, 102, and 22 nmol/g) of tested mice reached the beneficial levels (0.063, 1.402, and 0.275 μmol/L, respectively for 22Rv1) determined by our cell-based assays. The detection of wedelolactone, luteolin, and apigenin in plasma in the herbal extract–treated mice suggests an increase of their bioavailability when given in combination as the *W. chinensis* extract.

Although we administered two 10-fold different doses of herbal extract (40 versus 4 mg/kg) to the mice, plasma levels of wedelolactone, luteolin, and apigenin did not increase significantly in the higher group. The blood and tumor were sampled 24 h after ingestion, which is long enough for systemic clearance. It has been reported that apigenin is metabolized to luteolin *in vivo* and that their elimination half-lives in rat are only 1.75 ± 1.18 and 0.97 ± 1.25 h, respectively (40). However, a study of oral *Chrysanthemum morifolium* extract, which contains apigenin and luteolin, showed that both flavonoids have a quick absorption and a slow elimination phase, with apigenin absorbed more efficiently than luteolin in herbal extract, and suggested a possible accumulation of both flavonoids *in vivo* (41). We administered the single compounds and formula using the same vehicle as reported for apigenin (15). However, our experiment of single-compound or combinational treatment of wedelolactone, luteolin, and apigenin resulted in poor bioavailability, except for apigenin. Although combinational treatment did show an enhanced antitumor effect, the bioavailability of apigenin in our experiment was much lower than that reported by others (15, 16).

In our study, use of the whole extract provided several advantages: increased bioavailability of active compounds and favorable combinational effect. Together, our results show strong antitumor efficacy of the active principles in both subcutaneous and orthotopic xenograft models and suggest a wide safety margin for *W. chinensis* extract. These preclinical evaluations of *W. chinensis* are promising and indicate that this herbal intervention may provide a useful supplementary or alternative treatment to the current ablation therapy for prostate cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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References

1. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2008. CA Cancer J Clin 2008;58:71–96.
2. Crawford ED. Challenges in the management of prostate cancer. Br J Urol 1992;70 Suppl 1: 33–8.
3. Pienta KJ, Smith DC. Advances in prostate cancer chemotherapy: a new era begins. CA Cancer J Clin 2005;55:300–18, quiz 323–5.
4. Balk SP, Ko YJ, Bubley GJ. Biology of prostate-specific antigen. J Clin Oncol 2003;21:383–91.
5. Cleutjens KB, van der Korput HA, van Eekelen CC, van Rooij HC, Faber PW, Trapman J. An androgen response element in a far upstream enhancer region is essential for high, androgen-regulated activity of the prostate-specific antigen promoter. Mol Endocrinol 1997;11:148–61.
6. Attardi BJ, Burgenson J, Hild SA, Reel JR. Steroid hormonal regulation of growth, prostate specific antigen secretion, and transcription mediated by the mutated androgen receptor in CWR22Rv1 human prostate carcinoma cells. Mol Cell Endocrinol 2004;222:121–32.
7. Sramkoski RM, Pretlow TG II, Giaconia JM, et al. A new human prostate carcinoma cell

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line, 22Rv1. In Vitro Cell Dev Biol Anim 1999;35:403–9.

8. Lin FM, Chen LR, Lin EH, et al. Compounds from *Wedelia chinensis* synergistically suppress androgen activity and growth in prostate cancer cells. Carcinogenesis 2007;28:2521–9.
9. Lin FM, Tsai CH, Yang YC, et al. A novel diterpene suppresses CWR22Rv1 tumor growth *in vivo* through antiproliferation and proapoptosis. Cancer Res 2008;68:6634–42.
10. Tu WC, Wang SY, Chien SC, et al. Diterpenes from *Cryptomeria japonica* inhibit androgen receptor transcriptional activity in prostate cancer cells. Planta Med 2007;73:1407–9.
11. Cha TL, Chuang MJ, Wu ST, et al. Dual degradation of aurora A and B kinases by the histone deacetylase inhibitor LBH589 induces G_2-M arrest and apoptosis of renal cancer cells. Clin Cancer Res 2009;15:840–50.
12. Wester K, Ranefall P, Bengtsson E, Busch C, Malmstrom PU. Automatic quantification of microvessel density in urinary bladder carcinoma. Br J Cancer 1999;81:1363–70.
13. Chou TC. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev 2006;58:621–81.
14. Niculescu AB III, Chen X, Smeets M, Hengst L, Prives C, Reed SI. Effects of p21(Cip1/Waf1) at both the G_1-S and the G_2-M cell cycle transitions: pRb is a critical determinant in blocking DNA replication and in preventing endoreduplication. Mol Cell Biol 1998;18:629–43.
15. Shukla S, Gupta S. Molecular targets for apigenin-induced cell cycle arrest and apoptosis in prostate cancer cell xenograft. Mol Cancer Ther 2006;5:843–52.
16. Shukla S, Mishra A, Fu P, MacLennan GT, Resnick MI, Gupta S. Up-regulation of insulin-like growth factor binding protein-3 by apigenin leads to growth inhibition and apoptosis of 22Rv1 xenograft in athymic nude mice. FASEB J 2005;19:2042–4.
17. Eder M, Mehnert W. The importance of concomitant compounds in plant extracts. Die Pharmazie 1998;53:285–93.
18. Vickers A. Botanical medicines for the treatment of cancer: rationale, overview of current data, and methodological considerations for phase I and II trials. Cancer Invest 2002;20:1069–79.
19. Huie CW. A review of modern sample-preparation techniques for the extraction and analysis of medicinal plants. Anal Bioanal Chem 2002;373:23–30.
20. Khan IA. Issues related to botanicals. Life Sci 2006;78:2033–8.
21. Pettaway CA, Pathak S, Greene G, et al. Selection of highly metastatic variants of different human prostatic carcinomas using orthotopic implantation in nude mice. Clin Cancer Res 1996;2:1627–36.
22. Rembrink K, Romijn JC, van der Kwast TH, Rubben H, Schroder FH. Orthotopic implantation of human prostate cancer cell lines: a clinically relevant animal model for metastatic prostate cancer. Prostate 1997;31:168–74.
23. Klein EA, Thompson IM. Update on chemoprevention of prostate cancer. Curr Opin Urol 2004;14:143–9.
24. Neill MG, Fleshner NE. An update on chemoprevention strategies in prostate cancer for 2006. Curr Opin Urol 2006;16:132–7.
25. Bosland MC, McCormick DL, Melamed J, Walden PD, Zeleniuch-Jacquotte A, Lumey LH. Chemoprevention strategies for prostate cancer. Eur J Cancer Prev 2002;11 Suppl 2:S18–27.
26. Aziz MH, Dreckschmidt NE, Verma AK. Plumbagin, a medicinal plant-derived naphthoquinone, is a novel inhibitor of the growth and invasion of hormone-refractory prostate cancer. Cancer Res 2008;68:9024–32.
27. Cha TL, Qiu L, Chen CT, Wen Y, Hung MC. Emodin down-regulates androgen receptor and inhibits prostate cancer cell growth. Cancer Res 2005;65:2287–95.
28. Chinni SR, Li Y, Upadhyay S, Koppolu PK, Sarkar FH. Indole-3-carbinol (I3C) induced cell growth inhibition, G_1 cell cycle arrest and apoptosis in prostate cancer cells. Oncogene 2001;20:2927–36.
29. Guo J, Jiang C, Wang Z, et al. A novel class of pyranocoumarin anti-androgen receptor signaling compounds. Mol Cancer Ther 2007;6:907–17.
30. Hsu JC, Zhang J, Dev A, Wing A, Bjeldanes LF, Firestone GL. Indole-3-carbinol inhibition of androgen receptor expression and downregulation of androgen responsiveness in human prostate cancer cells. Carcinogenesis 2005;26:1896–904.
31. Jiang C, Lee HJ, Li GX, et al. Potent antiandrogen and androgen receptor activities of an *Angelica gigas*-containing herbal formulation: identification of decursin as a novel and active compound with implications for prevention and treatment of prostate cancer. Cancer Res 2006;66:453–63.
32. Yang H, Chen D, Cui QC, Yuan X, Dou QP. Celastrol, a triterpene extracted from the Chinese "Thunder of God Vine," is a potent proteasome inhibitor and suppresses human prostate cancer growth in nude mice. Cancer Res 2006;66:4758–65.
33. Yim D, Singh RP, Agarwal C, Lee S, Chi H, Agarwal R. A novel anticancer agent, decursin, induces G_1 arrest and apoptosis in human prostate carcinoma cells. Cancer Res 2005;65:1035–44.
34. Zi X, Zhang J, Agarwal R, Pollak M. Silibinin up-regulates insulin-like growth factor-binding protein 3 expression and inhibits proliferation of androgen-independent prostate cancer cells. Cancer Res 2000;60:5617–20.
35. Fang J, Zhou Q, Shi XL, Jiang BH. Luteolin inhibits insulin-like growth factor 1 receptor signaling in prostate cancer cells. Carcinogenesis 2007;28:713–23.
36. Shukla S, Gupta S. Molecular mechanisms for apigenin-induced cell-cycle arrest and apoptosis of hormone refractory human prostate carcinoma DU145 cells. Mol Carcinog 2004;39:114–26.
37. Bagli E, Stefaniotou M, Morbidelli L, et al. Luteolin inhibits vascular endothelial growth factor-induced angiogenesis; inhibition of endothelial cell survival and proliferation by targeting phosphatidylinositol 3′-kinase activity. Cancer Res 2004;64:7936–46.
38. Fang J, Zhou Q, Liu LZ, et al. Apigenin inhibits tumor angiogenesis through decreasing HIF-1α and VEGF expression. Carcinogenesis 2007;28:858–64.
39. Shukla S, MacLennan GT, Flask CA, et al. Blockade of β-catenin signaling by plant flavonoid apigenin suppresses prostate carcinogenesis in TRAMP mice. Cancer Res 2007;67:6925–35.
40. Wan L, Guo C, Yu Q, et al. Quantitative determination of apigenin and its metabolism in rat plasma after intravenous bolus administration by HPLC coupled with tandem mass spectrometry. J Chromatogr 2007;855:286–9.
41. Chen T, Li LP, Lu XY, Jiang HD, Zeng S. Absorption and excretion of luteolin and apigenin in rats after oral administration of *Chrysanthemum morifolium* extract. J Agric Food Chem 2007;55:273–7.

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