Compounds from *Wedelia chinensis* synergistically suppress androgen activity and growth in prostate cancer cells

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Chronic inflammation can augment tumor development in various types of cancers, including prostate cancer (PCa). Reduction of inflammation is therefore an important anticancer therapeutic opportunity. Here, we report four anti-proliferative phytocompounds in *Wedelia chinensis*, an oriental herbal medicine, identified through their ability to modulate the androgen receptor (AR) activation of transcription from prostate-specific antigen promoter in PCa cells. The 50% inhibition concentration values of indole-3-carboxyaldehyde, wedelolactone, luteolin and apigenin, were 34.9, 0.2, 2.4 and 9.8 μM, respectively. A formula that combined the phytocompounds in the same proportions as in the herbal extract decreased the dosage of each compound required to achieve maximal AR inhibition. In correlation with the AR suppression effect, these active compounds specifically inhibited the growth of AR-dependent PCa cells and as a combination formula they also synergistically suppressed growth in AR-dependent PCa cells. Our study has identified synergistic effects of active compounds in *W. chinensis* and demonstrated their potential in PCa prevention and therapy. The paradigm of multiple activities and synergism is a useful framework to investigate the therapeutic effects of whole extracts from assorted medicinal plant species.

Introduction

Prostate cancer (PCa) is a major cause of cancer-related deaths in males in Western countries (1). Androgen and androgen receptor (AR) functions play an essential role in the carcinogenesis and development of PCa (2,3). We thus targeted androgen signaling as a preventive or therapeutic approach for PCa. Prostate-specific antigen (PSA) level in the serum is widely used as a surrogate biomarker of PCa in both detection and therapeutic assessment (4). PSA expression is up-regulated by androgens and is tightly associated with growth of normal and cancerous prostatic cells. Therefore, it is rational to assess the AR activity in PCa by transcriptional activity of PSA promoter (5).

Chronic or recurrent inflammation and uncontrolled cell proliferation in the prostate have been strongly correlated with prostatic carcinogenesis and neoplastic conversion (6). An inflammatory signature in the prostate has also been studied for protection of the liver from toxicity (15,16). Epidemiological and experimental studies indicate that natural products show great promise of being used as a supplement by PCa patients who also received orthodox medication, suggesting a need among PCa patients for therapeutic improvement (12,13). Chinese medicinal plant documentation states that *Wedelia chinensis* (Osbeck) Merr., also called hwang-hua-mih-tsai in Taiwan, has medicinal anti-inflammatory properties (14). This Asteraceae plant is a perennial herb with bright yellow flowers and a light, camphor-like odor and is used to relieve fever and to reduce cough and phlegm. It has also been studied for protection of the liver from toxicity (15,16). Here, we examined the bioactive phytocompounds in *W. chinensis* for their potential in PCa chemoprevention or treatment.

To rationalize the usage of traditional herbal medicines, a systematic analysis of active compounds is necessary to provide rigorous scientific evidence for their efficacy and potency. Using the criterion of repression of AR activity in PCa cells, we identified four phytocompounds found in *W. chinensis* by bioactivity-guided fractionation. Our results show that all four phytocompounds suppressed the AR activity and growth of androgen-responsive PCa cells. The identification of several bioactive compounds led us to investigate their pharmacological integration in order for the crucial extraction to attain antitumor effects. A formula of a specific combination of the four active compounds based on their proportion in the original herbal extract synergized the suppressive effect on AR activity and growth of AR-dependent PCa cells. The potency of the combination formula was higher than any single constituent compound. Apigenin (Api) and luteolin (Lut) were also found to exert anti-proliferation effects on AR-independent PC-3 cells, thus the formula also additively suppressed growth of PC-3 cells. The potency of the formula which suppressed AR-expressing PCa cells was higher than that which suppressed AR-null PCa cells and is therefore specific. These results suggest that the use of *W. chinensis* extract to inhibit PCa cells is preferable to the use of purified single compounds.

Materials and methods

Plant materials, cells and reagents

Fresh whole plant of *W. chinensis* was purchased from a reputable Chinese medicinal herb store in Taiwan (July 2005). Authenticity of the plant species was validated by the specific morphological and anatomical features as reported by Jeng et al. (14). LNCaP, PC-3 and 22Rv1 cell lines were obtained from ATCC (American Type Culture Collection, Manassas, VA). Reporter plasmid (p5.7 kb-PSA-Luc) was constructed by inserting 5.7 kb PSA promoter into pcGL3 plasmid (Promega, Madison, WI). The formula (EtOAc), methanol and chloroform (CHCl₃) was identified by bioactivity-guided fractionation. The petrochemical compounds in *W. chinensis* and demonstrated their potential in PCa prevention and therapy. The paradigm of multiple activities and synergism is a useful framework to investigate the therapeutic effects of whole extracts from assorted medicinal plant species.

Plant extracts preparation

Whole air-dried *W. chinensis* plants (~100 g) were homogenized in EtOH with a blender and the crude herbal extract was further fractionated into different fractions as illustrated in Figure 1A. The EtOH extract was filtered through qualitative filter papers (diameter 12.5 cm, thickness 0.26 mm, pore size 5 μm, Adventec No. 2, Tokyo, Japan) by suction pump (Sibata, Tokyo, Japan). The filtrate was concentrated by evaporator (yield = ~5.1%, w/dry weight of plant material) and then washed with hot water. The water extract was further partitioned with EtOAc and divided into EtOAc and H₂O fractions through liquid-liquid partition. The EtOAc fraction (yield = ~1.8%, w/dry weight of plant material) was concentrated by evaporation and further partitioned with equal volume of CHCl₃ and H₂O. This H₂O subfraction (H₂O-Sub) was also concentrated by evaporation (yield = ~0.5%, w/dry weight of plant material) for further chromatographic purification.

Abbreviations: Api, apigenin; AR, androgen receptor; CHCl₃, chloroform; CL, combination index; DIM, diindolylmethane; EtOAc, ethyl acetate; EtOH, ethanol; H₂O-Sub, H₂O subfraction; HPLC, high-performance liquid chromatography; IC₅₀, 50% inhibition concentration; ICA, indole-3-carboxyaldehyde; I3C, indole-3-carbinol; Lut, luteolin; PCa, prostate cancer; PSA, prostate-specific antigen; PSA-Luc, prostate-specific antigen-luciferase; Wed, wedelolactone.
Compound isolation and identification

The H$_2$O-Sub was fractionated by silica gel column chromatography using a CHCl$_3$ and EtOAc developing system, resulting in the collection of another ethyl acetate-hexane subfractions 1-12 (EAH1-12). Compounds ICA (0.8 mg), Wed (1.5 mg), Lut (35.7 mg) and Api (6.8 mg) were purified from the EAH2–5 subfractions, using semi-preparative HPLC (Agilent 1100 series system, Palo Alto, CA) on a C$_{18}$ Cosmosil column (250 mm/C$_{18}$ 10 mm, Nacalai Tesque, Kyoto, Japan): mobile phase, methanol:water 53:2; flow rate, 1 ml/min at retention times of 14.7, 33.8, 34.6 and 38.1 min, respectively, detected by Ultraviolet absorption at 254 nm. The yields from whole air-dried plants of W. chinensis of compounds ICA, Wed, Lut and Api were 0.001, 0.002, 0.036 and 0.007% (w/w), respectively. The chemical structures of these four compounds were elucidated by spectroscopic analysis. Electrospray ionization mass spectrometry data were collected with LCQ Advantage mass spectrometer (Thermo Finnigan, San Jose, CA) and nuclear magnetic resonance spectra were recorded with Avance 500 and 300 MHz FT nuclear magnetic resonance spectrometers (Bruker, Bremen, Germany) at 500 MHz ($^1$H) and 75 MHz ($^{13}$C).

Luciferase assays

For luciferase activity, 2 × 10$^4$ cells of 22Rv1-derived 103E line were grown in 96-well multidishes with RPMI (Roswell Park Memorial Institute) 1640 medium containing 5% charcoal-coated dextran-stripped fetal bovine serum (HyClone, Logan, UT) for 1 day. The culture was refreshed with the same medium containing particular treatments as indicated in figures and grown for another 20 h. Cells were lysed by passive lysis buffer (Promega), then luciferase assay was performed using luciferase assay system (Promega) and VICTOR$^3$ multilabel counter (PerkinElmer, Turku, Finland) and normalized to lysate protein as measured by Coomassie (Bradford) Protein Assay Kit (Fermentas, Rockford, IL). Inhibition of AR by each treatment was calculated by relative luciferase activity induced by 10 nM 5α-dihydrotestosterone as 0% inhibition and vehicle as 100% inhibition.

Western blot analysis

An equal amount of cell lysate (40 μg per well, equivalent of 5 × 10$^5$ cells) was separated by 7.5–10% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins in the gel were electrotransferred onto polyvinylidene difluoride membrane (Millipore, Billerica, MA). After blocking in TBST (Tris-buffered saline Tween-20: 50 mmol/l Tris–HCl, 150 mmol/l NaCl containing 0.05% Tween-20) with 5% skim milk for 1 h, the membranes were incubated with primary antibodies overnight at 4°C (anti-PSA, Chemicon, Temecula, CA; anti-AR N-20 and anti-β-actin, Santa Cruz Biotech, Santa Cruz, CA) and then with horseradish peroxidase-conjugated secondary antibody for 1 h at 25°C. The membrane was washed three times with TBST and proteins were visualized using the enhanced chemiluminescence reagent (Pierce). The quantitative analysis of proteins was detected by Chemigenius$^2$ (Biolabo, Châtel-St-Denis, Switzerland).

Colony-forming assays

Colony-forming cell growth was performed by growing 2 × 10$^4$ 22Rv1, 103E, LNCaP or PC-3 cells in 24-well multidishes with indicated treatment for 12 days. The medium with the treatments indicated was refreshed every 3 days. Cell colonies were stained with 0.1% (w/v) crystal violet in phosphate buffer...
saline, dried and photographed. Cell-retained crystal violet was extracted with 20% (v/v) acetic acid in water and quantitatively measured by absorbance at 595 nm. Relative colony-forming growth of all treatments was analyzed and presented as a percentage of inhibition compared with control treatment of the same cell line.

**Statistical analysis and calculation of combination effects**

Data are represented as the means ± SDs for the indicated number of separate experiments. Statistical analysis was performed with the GraphPad Prism® 3.02 program (GraphPad Software, San Diego, CA). The statistical significance of differences between two groups of data (Figures 1B and 3B) were analyzed by paired t-test and P-values < 0.05 were considered significant. Statistically significant differences between the effect of the combination and the sum of individual compound effects in Figure 3A were analyzed using the non-parametric Wilcoxon matched pairs test. P-values < 0.05 were considered to be statistically significant. Dose–response curves for AR inhibition and relative colony-forming cell growth were plotted using a sigmoidal dose–response equation (variable slope) in GraphPadTM Prism® 3.02. Fifty percent inhibition concentration (IC50) and combination index (CI) were analyzed with the program CompuSyn (CompuSyn, Paramus, NJ) (17).

**Results**

**Establishment of the assay model system**

The androgen-responsive human PCa 22Rv1 cell line expresses AR and PSA and displays growth stimulation in response to androgen (18,19). We transfected a prostate-specific antigen-luciferase (PSA-Luc) reporter gene, driven by 5.7 kb PSA promoter, into the genome of 22Rv1 cells and derived a 103E clone. Stable transfection was demonstrated by the fact that PSA-Luc expression was induced in these cells by androgen at physiological concentration (10 nM 5α-dihydrotestosterone) and could be blocked by anti-androgen, nilutamide (Figure 1B, left). We could thus exploit the androgen-induced PSA-Luc activity in 103E cells as a cell-based assay for AR function in PCa cells to detect the bioactivity of plant extracts, fractions and phytocompounds.

**Bioactivity-guided fractionation of W. chinensis**

Our initial screening showed that *W. chinensis* suppressed AR activity, which is desirable for PCa prevention and therapy. Following the principle of reductionism, we partitioned the plant extracts of *W. chinensis* and further purified those phytocompounds that suppressed androgen induction of PSA-Luc in transfected 103E cells as described in Materials and Methods and Figure 1A. The H2O-Sub was then purified by column chromatography and HPLC for AR inhibitors and as a result four bioactive phytocompounds were identified (Figure 1A). The bioactivities of AR suppression in all extracts, solvent fractions and phytocompounds as determined by cell-based reporter gene expression assay are summarized in Table I.

The IC50 of the initial crude EtOH extract was 42.7 μg/ml. The bioactivity was enriched in the EtOAc partition and further enriched in H2O-Sub partition (Figure 1B, middle). As a result, the IC50 value decreased (20.5 μg/ml) and efficacy increased (~90% maximal inhibition, P < 0.01, Table I). The IC50 of the H2O-Sub dropped further to 5.6 μg/ml in contrast to the CHCl3 subfraction (30.3 μg/ml), which was much less potent and less efficacious (maximal inhibition of the H2O-Sub versus CHCl3 subfraction, P < 0.05, Table I). Thus, the major bioactivity of *W. chinensis* extract was further fractionated in the H2O-Sub.

**Chemical identification and functional characterization of AR inhibitory phytocompounds in W. chinensis**

Next, the H2O-Sub was further purified by column chromatography using normal phase silica gel and collected into 12 further subfractions (EAH1–12). As shown, EAH2–5 were highly effective at suppressing AR and thus were further purified by reverse phase HPLC (Figure 1B, right). Four bioactive phytocompounds were found contained in EAH2–5 in our HPLC analysis. As a representative, the HPLC chromatogram of EAH2 is shown in Figure 2A. Chemical structures of the bioactive compounds were then determined by anal-

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**Table I. Inhibitory activity on AR-mediated transcription of extracts, fraction and phytocompounds in *Wedelia chinensis***

<table>
<thead>
<tr>
<th>Treatments</th>
<th>IC50a (μg/ml)</th>
<th>Inhibitionmaxb (%)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracts or fractions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EtOH crude extract</td>
<td>42.7</td>
<td>61.3 ± 5.4</td>
<td></td>
</tr>
<tr>
<td>H2O fraction</td>
<td>59</td>
<td>48.5 ± 5.9</td>
<td></td>
</tr>
<tr>
<td>EtOAc fraction</td>
<td>20.5</td>
<td>90.2 ± 3.2**</td>
<td></td>
</tr>
<tr>
<td>CHCl3 subfraction</td>
<td>30.3</td>
<td>75.3 ± 6.1</td>
<td></td>
</tr>
<tr>
<td>H2O-Sub</td>
<td>5.6</td>
<td>85.7 ± 5.7h</td>
<td></td>
</tr>
<tr>
<td>Pure compounds</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICA</td>
<td>5.1</td>
<td>34.9</td>
<td>20</td>
</tr>
<tr>
<td>Wed</td>
<td>0.1</td>
<td>0.2</td>
<td>97.3 ± 3.9</td>
</tr>
<tr>
<td>Rut</td>
<td>0.7</td>
<td>0.2</td>
<td>95.5 ± 3.8</td>
</tr>
<tr>
<td>Api</td>
<td>2.6</td>
<td>9.8</td>
<td>94.0 ± 4.6</td>
</tr>
</tbody>
</table>

*a* (μg/ml or μM) represents the dose of each indicated treatment that gave 50% of the maximal response, which was calculated using a median-effect analysis in CompuSyn. ND, not determined. *b* Inhibitionmax (%) represents the maximal inhibition of each indicated treatment and data were mean ± SD from two independent experiments of four replicates. EtOAc versus H2O fractions and CHCl3 subfraction versus H2O-Sub were statistically compared. *P < 0.05; **P < 0.01.

**Combination of bioactive W. chinensis compounds synergistically increases AR suppression**

Having determined that the holistic crude extract of *W. chinensis* plant contains four AR-suppressive phytocompounds, we then investigated whether these four bioactive compounds applied together could be more potent or efficacious than when used in isolation, perhaps due to the simultaneous intervention of multiple oncogenic pathways. We therefore evaluated the combined effect of the four phytocompounds applied in combination and the respective contribution made by each. As analyzed quantitatively by HPLC and by purification yields, the molar ratio of the four principle active compounds in the H2O-Sub was ~1:1:25:5 for ICA:Wed:Lut:Api, respectively (see Materials and Methods). AR inhibition caused by three proportional doses of each compound according to the molar ratio above and the resulting bioactivity of combining the four compounds (CT, combination treatment) at three concentrations was investigated by PSA-Luc in 103E cells. As shown, combined treatment with the four compounds resulted in an additive or ‘greater than additive’ efficacy over individual compounds (Figure 3A).

The contribution of each bioactive compound to the combined effect was then evaluated by leaving one compound at a time out of the combination. Leaving out any one bioactive compound significantly decreased the efficacy of the combination (P < 0.05, Figure 3B). The reduction in percentage efficacy produced by excluding one bioactive compound from the combination was greater than the percentage efficacy of the compound used in isolation. For example, excluding 0.1 μM of ICA or 0.5 μM of Api, respectively, resulted in an ~20 and 22% reduction in the efficacy of the remaining combination (Figure 3B, columns 7 versus 3 and 7 versus 6) and significantly >4 or 7% efficiency of 0.1 μM of ICA or 0.5 μM of Api alone (Figure 3A, columns 6 and 9). These results suggest that each of the four bioactive compounds contributes to the enhancement of combined effect, thus emphasize the significance of the four bioactive compounds acting as a whole.
We then mixed the four compounds into a formula based on this original molar ratio, generated a dose–response curve and compared it with the dose–response curves of individual compounds. For AR suppression, the IC$_{50}$ of the formula was 0.3 μg/ml, which is more potent than ICA, Lut or Api alone and nearly as potent as Wed, the most potent among the four (Figure 3C and Table I). It is worth noting that 0.3 μg of the formula contains only 0.01 μg of Wed which means that use of the formula lowered the dosage requirement of all individual compounds to achieve the same efficacy. The combination effects of the formula was further quantitatively analyzed by currently the only method which is based on the median-effect principle of the mass action law (17). In this method, CI $< 1$ represents synergism, whereas CI $\geq 1$ represents additive effect or antagonism, respectively. As depicted by CI–effect plot, the CI of the formula for the AR inhibition ranges between 0.78 and 0.39, indicating that combining the four bioactive compounds caused a clear synergism (Figure 3E).

The AR suppression activity of the four bioactive compounds and their combined effect were also examined in LNCaP cells, another AR-dependent PCa cell line. Expression of endogenous PSA in treated cells was detected and inhibition of AR activity was analyzed. Data represent mean ± SD of two independent experiments of four replicates.

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A combination formula of the four bioactive compounds enhances anti-proliferation in AR-dependent PCa cells

Since AR is a critical growth determinant for PCa cells, the AR suppression effects of the *W. chinensis* compounds may also suppress the malignant growth of PCa cells. Colony-forming growth of AR-dependent PCa cells (22Rv1, 103E and LNCaP) and AR-independent PCa (PC-3) cells were tested for potential anti-proliferation effects of the four compounds either alone or combined in a specific formula.
that mimicked the proportions of the compounds found naturally in the plant. Growth suppression by each single compound and combined into the formula in four PCa cell lines is shown as dose–response curves (Figure 4). Surprisingly, data from 22Rv1, 103E and LNCaP cells show that the dose–response curves of the formula are located to the left of those of all individual compounds, suggesting a synergy or supra-additive effect among the four compounds (Figure 4A–C). Pharmacologically, dose-effect analyses reveal that the formula exerted higher anti-proliferation potency (lower IC\(_{50}\)) in AR-dependent PCa cells than all the individual compounds (Figure 4A–C). The efficacy (maximal inhibition) of each compound either alone or in combination was not significantly different when applied...
to AR-dependent PCa lines. The relative rankings of the slope of the dose–response curves (Wed > ICA > Api > Lut > CT), and the slopes themselves, are almost the same in all AR-dependent PCa cells (Figure 4A–C and Table II), although this relationship was not seen in AR-independent cells.

Though the four compounds were initially identified by AR suppression, Api and Lut also exerted anti-proliferation effects on androgen-independent PC-3 cells (Figure 4D and Table II). Wed and ICA showed higher anti-proliferation potency and efficacy in AR-dependent PCa cells than in AR-independent PCa cells (22Rv1 versus PC-3, P < 0.05) (Table II). More importantly, low dosages (submicromolar levels) of the formula suppressed growth of AR-dependent PCa cells more obviously than low dosages of all single compounds (Figure 4A–C). However, the formula was equally as efficacious and potent as either Lut or Api used alone in AR-independent PC-3 cells (Figure 4D and Table II), indicating that Lut and Api may affect the same set of targets in PC-3 cells. In agreement with the results described above, CI–effect plot for quantitative determining synergism in anti-proliferation demonstrated moderate synergism or synergism in three different AR-dependent PCa cell lines (CI = 0.78–0.26), whereas additive effect was found in AR-independent PC-3 cells (CI = 1.21–0.71, Figure 4E). In summary, use of the combination formula increased the potency of growth suppression in AR-dependent PCa cells and also extended its efficacy to AR-independent PCa cells.  

Discussion

Plant-derived compounds represent a major resource of chemopreventive and chemoprotective agents that provide a large number of candidate chemicals with a wide spectrum of chemical structures (23). To identify the bioactive compounds that exert a specific biological activity in medicinal herbs, bioactivity-guided fractionation that systematically purifies the activity-associated phytocompounds from herbal extracts is currently the most efficient approach. One such method is the use of high throughput biosassays to engineer relevant cells into a system that allows specific detection of critical signaling activity or pathways. We focused our work on identification of potential inhibitors using a cell-based assay system which detects AR activity in an androgen-responsive PCa cells, i.e. 22Rv1-derived 103E cells. Using AR activity in PCa cells as a surrogate, we purified four bioactive compounds from Asteraceae plant *W. chinensis* by bioactivity-guided fractionation and characterized the efficacy and potency of all four phytocompounds in suppressing AR (Figure 2B and Table I). Further analysis of colony-forming growth demonstrated their ability to suppress AR-dependent PCa cell growth (Figure 4A–C). Even though Api and Lut also suppressed the growth of AR-independent PCa cells, the result can be considered specific because the IC50 values for AR-independent PCa cells were markedly higher than AR-dependent PCa cells (Figure 4D and Table II). In androgen-independent PCa cells, Wed showed only partial efficacy and ICA was nearly ineffective (Figure 4D).

Our study of *W. chinensis* showed for the first time that flavonoid compounds Lut and Api inhibit AR function in PCa cells. Among the four principle AR inhibitors, *W. chinensis* extract contains higher amounts of Lut and Api than Wed and ICA. The IC50 values of Lut and Api for AR suppression were 2.4 and 9.8 μM, respectively (Figure 2B and Table I). These two flavonoids have previously been suggested to have anticancer properties. For example, their ability to inhibit fatty acid synthesis in cancer cells (LNCaP and MDA-MB-231) was linked to induction of apoptosis (24). Lut and Api have also been found to inhibit insulin-like growth factor-1 receptor signaling in PCa cells (25,26). Api inhibits IKK (IκB kinase) α kinase activity and NF-xb (nuclear factor-xb) signaling in PC-3 cells (27). More importantly, Api decreased the neoplasmic growth of androgen-dependent 22Rv1 and androgen-independent PC-3 cells implanted in athymic nude mice (28). The anti-proliferation effects of Api detailed in this study agree with these findings of Shukla *et al.* (25–27), lending validity to our approach of identifying bioactive extracts, fractions and compounds based on AR suppression in PCa cells. Future studies of the antitumor effect on PCa cells of the other bioactive compounds contained in *W. chinensis* herbal extract are merited.

This is also the first time that Wed has been found to inhibit AR activity with values as low as IC50 as 0.2 μM. Wed (>10 μM) has been reported to specifically inhibit kinase activities of both IKKα and IKKβ (29). IKK activity is constitutively activated in androgen-independent PCa cells and inhibition of IKK activity induces apoptosis in the PCa cells (30). We found here that Wed was a more potent anti-proliferative agent in AR-dependent PCa cells than AR-independent PCa cells, which contradicts the notion of differences in involvement of constitutive NF-xb activity between the two types of cells (PC-3 cells > LNCaP cells) (31). As a result, Wed suppression of PCa cell growth may involve IKKα or IKKβ-dependent but NF-xb-independent pathways (32).

In addition, ICA is a structural analog of indole-3-carbinol (I3C). Various anticancer effects have been reported for I3C and its metabolic dimer, diindolylmethane (DIM) (33). I3C forms DIM under acidic conditions; DIM is a more stable and more potent anticancer agent than I3C. DIM is suggested to have anticancer properties. For example, their ability to inhibit fatty acid synthesis in cancer cells (LNCaP and MDA-MB-231) was linked to induction of apoptosis (24). Lut and Api have also been found to inhibit insulin-like growth factor-1 receptor signaling in PCa cells (25,26). Api inhibits IKK (IκB kinase) α kinase activity and NF-xb (nuclear factor-xb) signaling in PC-3 cells (27). More importantly, Api decreased the neoplasmic growth of androgen-dependent 22Rv1 and androgen-independent PC-3 cells implanted in athymic nude mice (28). The anti-proliferation effects of Api detailed in this study agree with these findings of Shukla *et al.* (25–27), lending validity to our approach of identifying bioactive extracts, fractions and compounds based on AR suppression in PCa cells. Future studies of the antitumor effect on PCa cells of the other bioactive compounds contained in *W. chinensis* herbal extract are merited.

Table II. Dose-effect analyses of colony growth of active principles and formula in AR-dependent and AR-independent PCa cells

<table>
<thead>
<tr>
<th>Treatments</th>
<th>AR dependent</th>
<th></th>
<th>AR independent</th>
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<tbody>
<tr>
<td></td>
<td>22Rv1</td>
<td>103E</td>
<td>LNCaP</td>
</tr>
<tr>
<td><strong>IC50</strong> (μg/ml)</td>
<td><strong>Inhibitionmax (%)</strong></td>
<td><strong>Slope</strong></td>
<td><strong>IC50</strong> (μg/ml)</td>
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<tr>
<td>ICA</td>
<td>96.0</td>
<td>48.0 ± 4.8 (NS)</td>
<td>1.0</td>
</tr>
<tr>
<td>Wed</td>
<td>0.4</td>
<td>89.5 ± 2.1 (NS)</td>
<td>1.5</td>
</tr>
<tr>
<td>Lut</td>
<td>0.5</td>
<td>94.1 ± 4.4 (NS)</td>
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</tr>
<tr>
<td>Api</td>
<td>0.8</td>
<td>93.2 ± 3.0 (NS)</td>
<td>0.9</td>
</tr>
<tr>
<td>Formula</td>
<td>0.2</td>
<td>92.9 ± 2.9 (NS)</td>
<td>0.8</td>
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</table>

Potency was expressed as IC50 (μg/ml), which was calculated using a median-effect analysis in Compusyn.

Efficacy was expressed as maximal inhibition (%) of each indicated treatment and data represented mean ± SD from two independent experiments of four replicates. 103E versus 22Rv1, LNCaP versus 22Rv1 and PC-3 versus 22Rv1 were statistically compared. NS, not significant; "P < 0.01.

Slope of dose–response curves (Figure 4A–D) were analyzed using a sigmoidal dose–response equation (variable slope) in GraphPad® Prism® 3.02.

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Phytochemicals in medicinal herbs generally confer a lower potency or efficacy than optimized synthetic compounds; however, traditional medical practitioners often prescribe holistic treatment composed of several different natural products to patients. Crude extracts of medicinal plants that stand out in the practice of folk medicine often have a long history of empirical efficacy. Synergism and the interaction of multiple compounds contained in a holistic herbal extract are proposed mechanisms, but in many cases there is still a lack of rigorous scientific evidence. Our identification of four active compounds from the same plant and studies of the dose-effect analyses between single compounds and a reconstituted formula mimicking the ratios found in the crude extract may address the question of synergism. Measured by mass, which is interchangeable between different compounds and their combination formula, the formula turned out to be a potent anti-proliferation agent for AR-dependent PCa cells (Figure 4A–C). Therefore, maximal efficacy was attained with a reduced dosage of each of the individual compounds. The formula also showed a decrease in the slope of the dose–response curve, indicating a higher therapeutic index and less possibility of toxicity (Figure 4 and Table II). We speculate that multiple active principles may simultaneously interact with multiple molecular targets and therefore heighten pharmacological potency (decrease IC50 values), which may minimize the chance of side effects. It is not surprising that effects of all single compounds and their formulas on AR activity correlate with the effects on growth of multiple AR-positive PCa cell lines. Due to the activities of Lut and Api, the formula also suppressed growth of AR-independent PC-3 cells, albeit with a lower potency than on AR-dependent cells (Figure 4D) (28,42). PCa cells are often multifocal and heterogeneous, containing both AR-positive and AR-negative tumor cells. In this sense, the formula shows great promise of preventive and therapeutic actions for PCa.

Chemopreventive and therapeutic agents for PCa are mainly based on or in conjunction with down-regulation of androgen signaling (43,44). However, AR antagonism may cause adverse side effects and dose-limiting toxicities. For long-term chemoprevention, neutreuticals and dietary supplements from natural products are attractive to users. A holistic mixture or combination of phytochemicals from whole food consumption often exhibit stronger anti-proliferative or anticancer activities than purified single compounds (45). In this study, we provide evidence that the bioactive compounds in holistic herbal extracts may work synergistically to improve the therapeutic index and perhaps reduce the emergence of drug (anti-androgen) resistance. Ultimately, the pharmacokinetics and toxicology of each bioactive compound in the plant extract need to be further investigated in animal experiments.

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References

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