Characterization of Chemical Constituents in Scutellaria baicalensis with Antiandrogenic and Growth-Inhibitory Activities toward Prostate Carcinoma

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Abstract

Purpose: Botanical preparations are widely used by patients with prostate cancer. Scutellaria baicalensis, a botanical with a long history of medicinal use in China, was a constituent of the herbal mixture PC-SPES, a product that inhibited prostate cancer growth in both laboratory and clinical studies. Due to the difficulties encountered when evaluating the efficacy of complex natural products, we sought to identify active chemical constituents within Scutellaria and determine their mechanisms of action.

Experimental Design and Results: We used high-performance liquid chromatography to fractionate S. baicalensis and identified four compounds capable of inhibiting prostate cancer cell proliferation; baicalein, wogonin, neobaicalein, and skullcapflavone. Comparisons of the cellular effects induced by the entire extract versus the four-compound combination produced comparable cell cycle changes, levels of growth inhibition, and global gene expression profiles ($r^2 = 0.79$). Individual compounds exhibited antiandrogenic activities with reduced expression of the androgen receptor and androgen-regulated genes. In vivo, baicalein (20 mg/kg/d p.o.) reduced the growth of prostate cancer xenografts in nude mice by 55% at 2 weeks compared with placebo and delayed the average time for tumors to achieve a volume of $\sim$1,000 mm$^3$ from 16 to 47 days ($P < 0.001$).

Conclusions: Most of the anticancer activities of S. baicalensis can be recapitulated with four purified constituents that function in part through inhibition of the androgen receptor signaling pathway. We conclude that clinical studies evaluating the efficacy of these agents in the context of chemoprevention or the treatment of prostate cancer are warranted.

Prostate adenocarcinoma represents a major cause of cancer-related morbidity and mortality. One in six American men will develop prostate cancer and the disease claims $>30,000$ lives yearly (1). To reduce this tremendous health burden, new approaches have been directed toward extremes of the disease spectrum centering on strategies for prostate cancer prevention and for treating advanced androgen-independent cancers. In this context, environmental and lifestyle factors have been identified that influence prostate carcinogenesis. Nutritional studies indicate that diets rich in soy isoflavones, green tea, lycopene, vitamin E, and selenium are associated with reduced prostate cancer incidence (2–6). Phytoestrogens are thought to contribute to the lower frequency of prostate cancer found in countries with high soy consumption, such as China and Japan (7). In support of these observations, studies in rodent models of prostate cancer show that ingestion of soy isoflavones can delay prostate tumor growth, lower tumor incidence, and decrease the expression of the androgen receptor (AR; refs. 3, 4, 8).

Activation of the AR signaling pathway by androgenic ligands plays a permissive and potentially a promoting role in the development and progression of prostate cancer. Antiandrogen therapy, the initial treatment for advanced prostate cancer, is achieved through reductions in circulating androgen levels or inhibition of ligand binding to the AR. Although this approach is initially successful in slowing tumor growth, it is rarely curative due to the emergence of neoplastic cells capable of proliferating in a low-androgen environment (9). Importantly, the AR signaling pathway seems to be active in the vast majority of tumors described as “androgen independent” as shown by the expression of androgen-regulated genes (ARC), such as prostate-specific antigen (PSA). Several distinct mechanisms have been identified that seem capable of promoting AR signaling (10). These include overexpression of the AR by amplification, the selection of AR mutations conferring receptor activation through promiscuous ligand binding, and the cross-talk mediated by other growth factor signal transduction pathways. These observations affirm the critical importance of the AR pathway in sustaining prostate cancer.
cancer cell viability and support efforts designed to target the AR for therapeutic gain.

Numerous pharmacologic interventions have been developed in attempts to retard prostate tumor growth after the emergence of androgen-independent disease. Several cytotoxic chemotherapeutics have shown substantial palliative benefits but little improvement delaying disease progression or mortality (11). The inability of conventional approaches to reverse the progression of advanced disease coupled with a desire for therapies with fewer perceived toxicities has prompted patients and clinicians to consider unconventional or complementary alternatives. One such complementary therapy that garnered significant interest due to clinical studies reporting measurable responses in advanced prostate cancer consisted of a mixture of herbal extracts marketed under the name PC-SPES (12).

Importantly, laboratory assays indicated that one mechanism of growth-inhibitory activity was through modulation of the AR pathway (13). Although PC-SPES administration was generally well tolerated and early-phase clinical trials suggested therapeutic benefits, the difficulties associated with the analyses of poorly standardized and regulated compounds was highlighted through studies demonstrating variable quantities of synthetic drugs in lots of the dispensed PC-SPES capsules (14, 15). Although present in small quantities, several of the identified drugs could have contributed to both beneficial and adverse clinical effects seen with this therapy. However, the possibility that one or more natural constituents of the botanical extracts could exhibit anticancer activities has not been excluded. The objective of this study was to identify and characterize individual chemical compounds derived from specific botanical extracts reportedly used in the PC-SPES formulation that exhibit antiandrogenic and/or growth suppressive effects toward prostate carcinoma.

Materials and Methods

High-performance liquid chromatography fractionation of Scutellaria baicalensis. High-performance liquid chromatography (HPLC) analyses were done on HP1050 and HP1100 HPLC systems using a Discovery C18 (25 cm × 10 mm, 5 μm, Supelco, Bellefonte, PA) semipreparative column or a Supelcosil LC-18 (25 cm × 2.1 mm, 3 μm, Supelco) analytic column. Ethanol extract (1 mL) of S. baicalensis was injected in aliquots (100 μL each, 10 separate injections) onto the semipreparative column, which was eluted using gradient elution method A (Supplementary Material 1) and the absorbance at 270 nm was monitored. The eluate was collected in 30-second fractions and the solvent was removed from these fractions by evaporation. The dried fractions were resuspended in 1 mL DMSO and then were screened for growth inhibition of LNCaP cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. To assess the purity of the active fractions, aliquots (5 μL) were injected onto the analytic column, which was eluted using isocratic elution method B with monitoring at 270 nm. Methods for purifying larger amounts of compounds in the active fractions of Scutellaria are detailed in Supplementary Material 1.

General compound identification and purification methods. Low-resolution mass spectrometry (MS; electrospray ionization) was done on a Bruker Esquire ion trap mass spectrometer or a HP Series 1100 MSD. High-resolution MS was done using a Bruker APEX III 47e Fourier transform (ion cyclotron resonance) mass spectrometer. 1H nuclear magnetic resonance spectra were obtained on Tecmag or Bruker Avance 300 MHz spectrometer and 13C chemical shifts are reported in ppm (δ). Flash column chromatography was done using silica gel (grade 9385, 230-400 mesh, Merck, Whitehouse Station, NJ). Analytic TLC was done using silica gel Analtech GF plates (0.25 mm) and products were visualized using UV light. Unless otherwise noted, reagents were purchased from Sigma-Aldrich Corp. (St. Louis, MO) or Lancaster Synthesis (Windham, NH). Solvents were ACS reagent grade or better, and anhydrous solvents were used as received unless otherwise indicated. Bactaloin and baicalin were obtained from Sigma-Aldrich. Wogonin was obtained from Wako Pure Chemicals Industries Ltd. (Richmond, VA) Detailed methods for compound characterization using HPLC-MS are described in Supplementary Material 1.

Cell lines and tissue cultures. LNCAp and PC-3 cell lines were obtained from the American Type Culture Collection (Manassas, VA) and propagated according to the instructions of the supplier. Cells were grown in 10% fetal bovine serum for all experiments unless noted. Extracts were diluted in medium at indicated concentrations. Ethanol or DMSO treatments were used as a vehicle controls. Botanical products were obtained from Plum Flower Brand (Camden, NY). Extracts were derived by the addition of 3.2 g of individual herbs to 10 mL ethanol, incubation for 1 hour at 37°C followed by low-speed centrifugation, and filtration with a 0.45 μm filter. Drug treatments and suppliers olomoucine 50 μmol/L (Calbiochem, San Diego, CA), paclitaxel 10 μmol/L (Sigma-Aldrich), 2-methoxyestradiol 5 μmol/L (Sigma-Aldrich), and isoleucine-depleted RPMI (U.S. Biological, Swampscott, MA). The four-compound concentration stock consisted of 3.17 μmol/L baiacine, 1.08 mmol/L wogonin, 0.58 mmol/L neobaiacine, and 0.2 mmol/L skulcaplavone dissolved in DMSO.

Cell proliferation assay. The percentage of growth inhibition was determined by seeding 56-well microtiter plates with 5,000 cells per well and cells were allowed to adhere overnight followed by the addition of test compounds for 24 or 72 hours. Cell proliferation was measured by adding MTT (Sigma-Aldrich) at 1 mg/mL to the culture medium for 2 hours. Following the MTT addition, medium was removed and isopropanol was added to wells until the cells solubilized. MTT absorbance at 570 nm was measured with a MicroQuant spectrophotometer. Each data point represents the average of four separate experiments with each experiment containing eight wells.

Cell cycle analysis. Cells were treated with compounds for specific time periods and fixed in 70% cold ethanol. RNA was digested with 5 μL RNase (200 units/mL, Roche Applied Science, Indianapolis, IN) for 20 minutes at 37°C, after which 100 μL of 0.5 mg/mL propidium iodide (Roche) were added. Data acquisition was performed on a Becton Dickinson (Franklin Lakes, NJ) FASCScan cytometer; 10,000 gated events were counted for each sample. Data analysis was done using CellQuest and MPLiSS software. Each experiment was done in triplicate.

Northern blot analysis. Total RNA (10 μg) was fractionated on 1.2% agarose denaturing gels and transferred to nylon membranes by a capillary method (16). Blots were hybridized with DNA probes labeled with [α-32P]dCTP by random priming using the Rediprime II random primer labeling system (Amersham Biosciences, Piscataway, NJ) according to the manufacturer’s protocol. Filters were imaged and quantitated using a phosphor-capture screen and ImageQuant software (Amersham Biosciences, Piscataway, NJ). Equivalent loading and transfer of RNA samples was confirmed by staining membranes with 0.3% methylene blue.

Western blot analysis. Following treatment with the indicated compounds or vehicle controls, cells were lysed, proteins were isolated, and protein (20 μg) was loaded onto 4% to 12% bis-Tris gradient gels (Invitrogen, Carlsbad, CA). Gels were transferred to nitrocellulose membranes and blocked with 4% milk overnight at 4°C. Blots were probed with either anti-AR (AR N-20, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-actin (actin I-19, Santa Cruz Biotechnology), or anti-PSA [PSA 038(101) DAKO] antibodies. Either horseradish peroxidase–conjugated anti-rabbit, anti-goat, or anti-mouse were added (Pierce Biotechnology, Inc., Rockford, IL) followed by detection with a chemiluminescence kit (Pierce Biotechnology, Inc., Rockford, IL). Scanned autorad images were quantified using ImageQuant software (Molecular Dynamics).
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DNA microarray analysis. The protocol used for indirect labeling of cDNAs was a modification of a protocol described elsewhere (http://cmgm.stanford.edu/pbrown/protocols/aadUITPCouplingProcedure.htm; Supplementary Material 1).

Fluorescent array images were collected for both Cy3 and Cy5 using a GenePix 4000B fluorescent scanner (Axon Instruments, Foster City, CA). The image intensity data were extracted using GenePix PRO 4.1 software (Axon Instruments), and spots of poor quality determined by visual inspection were removed from further analysis. Each experiment was repeated with a switch in fluorescent labels to account for dye effects. For every experiment, each cDNA was represented twice on each slide, and the experiments were done in duplicate, producing four data points per cDNA clone per hybridization probe. Normalization of the Cy3 and Cy5 fluorescent signal on each array was done using GeneSpring software (Silicon Genetics, Redwood City, CA). Data were filtered to remove values from poorly hybridized cDNAs with average foreground minus background intensity levels of <300. Data from the four replicate cDNAs for each experiment were combined and the average ratios were used for comparative analyses. To compare the overall expression patterns of each cell line, log2 ratio measurements were statistically analyzed using the statistical analysis of microarrays procedure (ref. 17; http://www-stat.stanford.edu/~tibs/SAM/). A one-class t test was used to determine whether the mean gene expression for any group of samples differed from zero.

**Results**

Extracts of Scutellaria baicalensis inhibit androgen receptor expression and cell proliferation. We showed previously that treatment of the LNCaP prostate cancer cell line with an ethanolic extract of PC-SPES suppressed cell growth and inhibited the expression of the AR and ARGs (13). Extracts of eight different herbs were reportedly used in the formulation of PC-SPES: S. baicalensis, Glycyrrhiza glabra, Ganoderma lucidum, Isatis indigotica, Dendranthema morifolium tzvel, Panax notoginseng, Rubus fruticosus, and Serenoa repens (12). Using AR expression and cell proliferation as indicators of antiandrogenic activity, we individually evaluated ethanolic extracts of seven herbs (P. notoginseng was not available from a commercial vendor) to determine their ability to recapitulate these observations. We exposed LNCaP cells to extracts of each botanical and measured cell proliferation by MTT assay and AR transcript levels by Northern blot. S. repens, G. glabra, and S. baicalensis each inhibited LNCaP growth (Fig. 1A), but only S. baicalensis inhibited both cell proliferation and expression of the AR (Fig. 1B). The minimal concentration of S. baicalensis needed to decrease the AR message was determined to be between 2.5 and 5 µL/mL at 24 hours (Fig. 1C). We measured a 42% decrease in AR message after 8 hours of S. baicalensis treatment and the maximal inhibition occurred by 24 hours of treatment (Fig. 1D). Given these results, we chose to focus on identifying active compounds in extracts of S. baicalensis.

![Fig. 1. S. baicalensis inhibits LNCaP cell growth and decreases AR expression.](image-url)
Identification of individual chemical constituents in Scutellaria baicalensis with growth-inhibitory activity. To identify individual biologically active compounds within the chemically complex S. baicalensis extract, we separated the ethanol-soluble compounds by HPLC and screened each fraction for the ability to inhibit LNCaP cell growth by MTT assay. Two fractions (32 and 41) inhibited LNCaP growth (Fig. 2A). One major product was seen in fraction 32. MS was consistent with an elemental composition of C_{15}H_{10}O_{5}, which was identical to baicalein, a known constituent of S. baicalensis (18). Fraction 32 and commercially available baicalein also had identical nuclear magnetic resonance spectra and HPLC retention times, confirming the identification of this compound. Fraction 41 contained three major constituents, and the isolation of these compounds relative to other less abundant components was improved by extracting the herb with dichloromethane. Normal phase column chromatography and additional HPLC improved by extracting the herb with dichloromethane.

Comparative analysis of cell growth inhibition by active compounds in Scutellaria baicalensis. The growth-inhibitory activities of baicalein, wogonin, neobaicalein, and skullcapflavone were further examined by determining the IC_{50} toward LNCaP and PC-3 cells. Each cell line was treated with increasing concentrations of pure compounds for 72 hours. Cell growth was assessed by MTT assay. Each of the four compounds inhibited the growth of LNCaP cells >50% at concentrations below 50 μmol/L (Fig. 3A). The IC_{50} values for baicalein, wogonin, neobaicalein, and skullcapflavone were determined to be 13, 42, 22, and 11 μmol/L, respectively. PC-3 cells were less sensitive to all four compounds with the following IC_{50} values: baicalein 25 μmol/L, wogonin 50 μmol/L, and neobaicalein 35 μmol/L (Fig. 3B). Skullcapflavone did not inhibit PC-3 cell growth by >50% at concentrations below 100 μmol/L.

To begin an assessment of growth-inhibitory mechanisms, we determined if baicalein, wogonin, neobaicalein, and skullcapflavone could influence progression through specific phases of the cell cycle. Each compound was added to cells at their respective IC_{50} concentrations to ensure that a similar level of growth inhibition was present in the comparison of cell cycle distributions. After 24 hours of treatment, cells were stained with propidium iodide and analyzed for DNA content by flow cytometry. The percentage of cells in each cell cycle phase was compared with the vehicle control. In LNCaP cells, all four compounds led to an accumulation of cells in G1 phase was compared with the vehicle control. In LNCaP cells, all four compounds led to an accumulation of cells in G1 phase, with a diminished number of cells in G2-M phase (Fig. 3C). The percentage increase in G1 cell numbers ranged from 8% to 11%. With all compounds, the increase in G1-phase cells was accompanied by a decrease in S-phase cells, whereas the number of cells in G2-M was not substantially changed. The effects of the four compounds on PC-3 cells were quite different. In this AR null cell line, the treatment resulted in the accumulation of cells in the G2-M phase, with a diminished number of cells in G1 (Fig. 3D).

Comparative analyses of individual Scutellaria baicalensis constituents relative to the complete Scutellaria baicalensis extract. To determine if the combination of baicalein, wogonin, neobaicalein, and skullcapflavone could recapitulate the effects observed with the entire S. baicalensis preparation, the four compounds were combined at the concentrations we determined to be present in the complete botanical extract. Treatment of LNCaP cells with the complete S. baicalensis extract and the four-compound combination for 72 hours produced similar growth inhibition curves (Fig. 3E): 1 μL/mL extract inhibited cell growth 18% versus 24% for the four compounds, 3 μL/mL extract inhibited growth 54% versus 62% for the extract.

![Figure 2](https://example.com/figure2.png)

**Figure 2.** HPLC fractionation of S. baicalensis and identification of growth-inhibitory fractions. A. Absorbance at 270 nm from an ethanolic extract of S. baicalensis fractionated on a C18 reverse-phase column (solid line). Fold growth inhibition by 30-second HPLC fractions of Scutellaria on LNCaP cells (dashed line). B. Structures of growth-inhibitory flavonoids found in S. baicalensis.

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59% for the four compounds, and 5 μL/mL extract inhibited growth by 66% versus 69% for the four compounds. Based on the HPLC-MS calculations, the concentrations of the compounds at the extract’s IC₅₀ (2.7 μmol/L) were baicalein 8.6 μmol/L, wogonin 2.9 μmol/L, neobaicalein 1.6 μmol/L, and skullcapflavone 0.54 μmol/L. Cell cycle analysis also showed similar effects. Treatment of LNCaP cells for 24 hours with 3 μL/mL S. baicalensis extract or the four-compound combination increased the percentage of cells at G₁ by 12.1% and 13.5% relative to control, respectively (data not shown).

We next performed cDNA microarray analysis to compare and contrast cellular gene expression alterations resulting from treatment with complete extract (3 μL/mL) or the four-compound combination (3 μL/mL; Supplementary Material 4). To identify statistically significant changes in transcript abundance, we used the statistical analysis of microarrays algorithms (17). At a false discovery rate of 1.25%, S. baicalensis extract altered the expression of 1,645 transcripts and the four-compound combination of baicalein, neobaicalein, skullcapflavone, and wogonin altered the expression of 446 transcripts in LNCaP cells after 24 hours of treatment.
The antiandrogenic actions of baicalein, wogonin, neobaicalein, and skullcapflavone were confirmed by treating LNCaP cells with their respective IC50 doses for 24 and 72 hours and measuring AR and PSA protein levels by Western analysis (Fig. 4A). None of the four compounds produced measurable decreases in AR protein levels at these concentrations and time points analyzed. However, PSA protein levels were decreased by each compound at 24 hours. After 72 hours of exposure to baicalein, wogonin, neobaicalein, and skullcapflavone, PSA levels were reduced by 3.6-, 11.3-, 16.5-, and 2.0-fold relative to control, respectively. We did observe a decrease in AR protein levels at higher baicalein concentrations (20-30 μmol/L) than the 72-hour IC50 (13 μmol/L; Fig. 4B). We calculated that baicalein concentrations of 30 μmol/L would be present in S. baicalensis extract dilutions of 5 to 10 μL/mL. The comparative measures of compound activities and relative abundance indicate that baicalein is the chemical component within S. baicalensis that contributes the majority of antiandrogenic effects. For this reason, we undertook further studies to characterize in vivo and in vitro activities of baicalein.

**Influence of baicalein and cell cycle phase on androgen receptor and androgen-regulated gene expression.** AR expression has been shown previously to vary according to cell cycle phase, with loss of AR transcriptional activity observed at the G1-S transition (22). Thus, one mechanism explaining the reduction in AR expression and signaling following baicalein treatment could reflect the indirect result of cell cycle inhibition rather than a direct modulation of the AR. To address this experimentally, we measured the expression of AR and PSA following the propagation of LNCaP cells in conditions designed to arrest cells in specific cell cycle phases. Serum-free medium, charcoal-stripped medium, isoleucine-depleted medium, and the cyclin-dependent kinase inhibitor olomoucine induce G1 arrest (23), whereas paclitaxel and 2-methoxyestradiol arrest cells at G2-M (24, 25). After treating cells with 30 μmol/L baicalein for 24 hours, a 40% reduction in AR protein was measured by Western analysis (Fig. 4C). Growth in androgen-depleted medium (charcoal-stripped medium) and with 2-methoxyestradiol reduced AR protein by 33%. However, two treatments that cause G1 arrest independent of androgen signaling (isoleucine and olomoucine) did not change AR expression >7%, indicating that simply arresting cells in G1 is insufficient to modulate AR levels (Table 1). Despite causing a comparable level of growth inhibition at 24 hours, paclitaxel or olomoucine did not alter AR expression. In addition to lowering AR expression, baicalein treatment also reduced PSA protein by 84% relative to control (Table 1). The two treatments that deplete androgen from the medium caused PSA to decline by 80% (serum-free medium) and to undetectable levels (charcoal-stripped medium). In contrast, olomoucine, isoleucine, paclitaxel, and 2-methoxyestradiol caused only a 11% to 26% decline in PSA protein levels.

**Baicalein suppression of prostate tumor growth in vivo.** To determine if the growth-inhibitory activity observed with baicalein treatment in vitro could be recapitulated in vivo, we implanted the LuCaP 35 human prostate cancer xenograft (26) s.c. into athymic BALB/c mouse recipients and treated them with baicalein (20 mg/kg) or placebo five times weekly orally. The LuCaP 35 xenograft model closely resembles the biology of human prostate cancer in tumor response to androgen deprivation, reduction in PSA production, and ultimate progression to androgen-independent growth. Treatment was started after the tumors reached 200 mm3 in size and continued until the tumors reached 1,000 mm3 or compromised the animal. The average tumor volume in the...
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A. Western blot analysis depicting protein expression levels of AR, PSA, and actin after treatment of LNCaP cells for 24 hours with the four individual compounds (baicalein 13 μmol/L (B), wogonin 42 μmol/L (W), neobaicalein 25 μmol/L (N), or skullcapflavone 11 μmol/L (S)). B. AR, PSA, and actin protein expression following the addition of increasing concentrations of baicalein (BAC) for 24 hours. C. Western blot analysis of AR, PSA, and actin protein levels in LNCaP cells following treatment with DMSO 24 hours (CON), 30 μmol/L baicalein 24 hours (BAC), serum-free medium 72 hours (SFM), charcoal-stripped fetal bovine serum 72 hours (CS), 2-methoxyestradiol 24 hours (2ME), and 5 μmol/L 2-methoxyestradiol 24 hours (2ME).

Fig. 4. Antiandrogenic actions of compounds in S. baicalensis. A, Western blot analysis depicting protein expression levels of AR, PSA, and actin after treatment of LNCaP cells for 24 hours with the four individual compounds (baicalein 13 μmol/L (B), wogonin 42 μmol/L (W), neobaicalein 25 μmol/L (N), or skullcapflavone 11 μmol/L (S)). B, AR, PSA, and actin protein expression following the addition of increasing concentrations of baicalein (BAC) for 24 hours. C, Western blot analysis of AR, PSA, and actin protein levels in LNCaP cells following treatment with DMSO 24 hours (CON), 30 μmol/L baicalein 24 hours (BAC), serum-free medium 72 hours (SFM), charcoal-stripped fetal bovine serum 72 hours (CS), 2-methoxyestradiol 24 hours (2ME), and 5 μmol/L 2-methoxyestradiol 24 hours (2ME).

Discussion

The results of screening individual herbs that comprise PC-SPEs suggested that S. baicalensis was the botanical most likely to yield constituents with activities that cause both a decrease in AR expression and a reduction in prostate cancer cell growth. Scutellaria is reported to contain >35 flavonoid compounds and our HPLC fractionation of Scutellaria identified four flavonoids that inhibited cell proliferation: baicalein, neobaicalein, skullcapflavone, and wogonin. All four compounds share the same flavone backbone. The major differences between the chemical structures are the presence and placement of hydroxyl and methoxy groups. Previous studies involving baicalein and wogonin showed that these compounds influence multiple cellular processes (27–31). In contrast, little is known about the activities of skullcapflavone and neobaicalein.

The growth inhibition assays we carried out on LNCaP and PC-3 cells revealed differences in potency among the four compounds. In LNCaP cells, there was almost a 4-fold range in IC_{50} values after 72 hours of treatment (11-42 μmol/L). Overall, PC-3 cells were less sensitive to each of the compounds. Whether the difference between the two cell lines is related to the androgen sensitivity of LNCaP cells or the faster growth rate of PC-3 cells is unknown. Baicalein was shown previously to inhibit LNCaP and PC-3 cell growth with IC_{50}s of 29 and 25 μmol/L, respectively, although the PC-3 study was carried out in serum-free medium (32, 33). Wogonin-mediated inhibition of prostate cancer cell growth has not been described before, although it has been reported to reduce the proliferation of several nonprostate cell types (34–36). All four compounds induced a G_{1} arrest in LNCaP cells when added to the medium at equivalent growth-inhibitory concentrations. G_{1} arrest is a common finding among a diverse group of flavonoid compounds, such as epigallocatechin-3-gallate, silibinin, and genistein (37–39).

One potential mechanism leading to G_{1} cell cycle arrest in prostate epithelial cells involves inhibiting signal transduction pathways modulated by androgens and the AR. The androgen response pathway represents an important therapeutic target for the treatment of advanced prostate cancer as shown by a recent study showing that the singular common alteration in the emergence of androgen-independent cancers was up-regulation of AR expression; a molecular event that allowed for cell proliferation in a low-androgen environment (9). LNCaP cell growth is dependent on a functioning AR and these cells will undergo G_{1} arrest in androgen-depleted medium (22, 40). Of the flavonoids evaluated in the studies reported here, baicalein, wogonin, and neobaicalein each markedly inhibited the expression of the androgen-regulated PSA gene. DNA microarray analyses showed that many other genes known to be modulated by androgen signaling were down-regulated after exposure to these compounds. Several of these flavonoids, including baicalein, reduced transcripts encoding the AR, but at the IC_{50} doses originally studied none of the compounds seemed to reduce AR protein levels. However, treatment of LNCaP cells with 20 to 30 μmol/L baicalein treatment and grew to 1,000 mm³. The average time required to reach this size with baicalein was nearly thrice longer than placebo-treated animals (47–16 days; Fig. 5). Treatment-related toxicities were not observed in the mice receiving baicalein.
baicalein, a concentration above the IC_{50} value, did result in loss of AR protein within 24 hours. There is precedence for inhibition of PSA expression despite unchanged AR levels. The flavonoid silymarin has been shown to reduce PSA expression without changes in total cellular AR levels, although nuclear AR levels were diminished (41). Selenium and genistein inhibit AR binding to androgen response elements, preventing the formation of a transcriptional complex on the PSA promoter (42, 43). In addition, the c-Jun/c-Fos activator protein-1 protein complex has been shown to bind to AR. Once activator protein-1 is bound, the AR is prevented from activating transcription of target genes (44). Of interest, our microarray analyses showed that c-Jun transcripts were induced in LNCaP cells following exposure to the four *S. baicalensis* flavonoids. In a therapeutic context, interfering with AR function can be as effective as decreasing AR expression. In the case of the flavonoids studied here, the interference effect can be achieved with lower compound concentrations. The chemical structures of these compounds could serve as starting points for constructing synthetic derivatives with enhanced target activity and improved pharmacokinetics.

In addition to modulating the AR pathway, flavonoids identified in *S. baicalensis* have been shown to exhibit other effects that can influence cell growth. Baicalein inhibits 12-lipoxygenase at nanomolar concentrations (27). Elevated levels of 12-lipoxygenase and 12(S)hydroxyeicosatetraenoic acid are associated with advanced-stage, poorly differentiated, metastatic prostate tumor cells (45). When baicalein was given to the androgen-insensitive DU145 and PC-3 prostate cells, it caused cell cycle arrest as well as caspase-mediated apoptosis (33). In vivo experiments showed that baicalein doses of 250 mg/kg/d inhibited the growth of pancreatic tumor cells injected into mice (46) and reduced prostaglandin synthesis in rat glioma cells through inhibition of the mitogen-activated protein kinase pathway (47). *S. baicalensis* extracts were shown to reduce the growth of head and neck squamous cell carcinoma xenografts at a dose of 75 mg/kg five times weekly through a mechanism thought to involve inhibition of cyclooxygenase-2 activity (48). *In vitro* experiments showed that the *S. baicalensis* extract could inhibit cyclooxygenase-2 expression and prostaglandin E₂ synthesis, a finding not reproduced with baicalein alone. The inhibition of cyclooxygenase-2 gene expression has been attributed to wogonin (49), indicating that although the structures of the flavonoids in *S. baicalensis* are quite similar there are compound-specific effects that are relevant for modulating particular biochemical processes, such that the growth of certain neoplasms could be differentially affected.

An important question regarding the use of complex botanicals is whether their attributed biological activity can be reproduced with one or more purified chemical constituent(s) of the plant. The advantages of evaluating and administering individual pure compounds are many and

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Table 1. Effects of various treatments on LNCaP gene expression, cell cycle, and cell growth

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<th>Charcoal-stripped medium</th>
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<th>Isoleucine</th>
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</tr>
</tbody>
</table>

NOTE: G₁%, G₂%, and S%, percentage of cells in each phase of the cell cycle as determined by flow cytometry; MTT, assay for cell proliferation.
Mechanisms of Cell Growth Inhibition by S. baicalensis

The combination of these four compounds led to growth evaluated can be recapitulated with four purified isoflavones. *S. baicalensis* (50). The results reported here indicate that most of the may be attributed to undesirable chemicals within the plant and extraction procedures and reducing side effects that the complete extract could be attributed to this single compound. Clinical studies evaluating the efficacy of *S. baicalensis* toward prostate cancer could potentially substitute the four active flavonoids we have evaluated in this report. For some tumor types, particularly those not influenced by the cyclooxygenase-2-inhibitory activity provided by wogonin, baicalein alone would be sufficient. Clearly, there are caveats to this conclusion as we do not know the potential attributes of other *S. baicalensis* constituents in facilitating gastrointestinal absorption or other pharmacokinetic parameters. Further studies are warranted to determine if the cytotoxic effects of *S. baicalensis* toward other tumor types can be reproduced with combinations of these active compounds.

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References


Characterization of Chemical Constituents in *Scutellaria baicalensis* with Antiandrogenic and Growth-Inhibitory Activities toward Prostate Carcinoma

Michael Bonham, Jeff Posakony, Ilsa Coleman, et al.


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