

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 $\sim 1,000 \text{ mm}^3$ 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 (ARG), 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

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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 column (25 cm × 2.1 mm, 3 μm, Supelco) analytic column. Ethanolic 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 ¹H 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. Baicalin and baicalein 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 culture. 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 were olomoucine 50 μmol/L (Calbiochem, San Diego, CA), paclitaxel 10 nmol/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 mmol/L baicalein, 1.08 mmol/L wogonin, 0.58 mmol/L neobaicalein, and 0.2 mmol/L skullcapflavone dissolved in DMSO.

Cell proliferation assay. The percentage of growth inhibition was determined by seeding 96-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) FACScan cytometer; 10,000 gated events were counted for each sample. Data analysis was done using CellQuest and MPLUS 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).

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/aadUTPCouplingProcedure.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.

Animals and treatment protocol. Four- to 6-week-old male BALB/c athymic (*nu/nu*) mice were purchased from Charles River Laboratories (Wilmington, MA). All procedures were done in compliance with the University of Washington Institutional Animal Care and Use Committee and NIH guidelines. LuCaP 35 tumor bits (~25 mm³) were implanted s.c. Tumor growth was monitored by measuring tumor volume thrice weekly. Tumor volume was calculated as follows: length × width × width × 0.5236. Once tumors reached 250 mm³, treatment was initiated. Animals were then supplemented by gavage with control vehicle oil (corn oil/safflower oil/olive oil 8.5:2.7:28.4) or baicalin

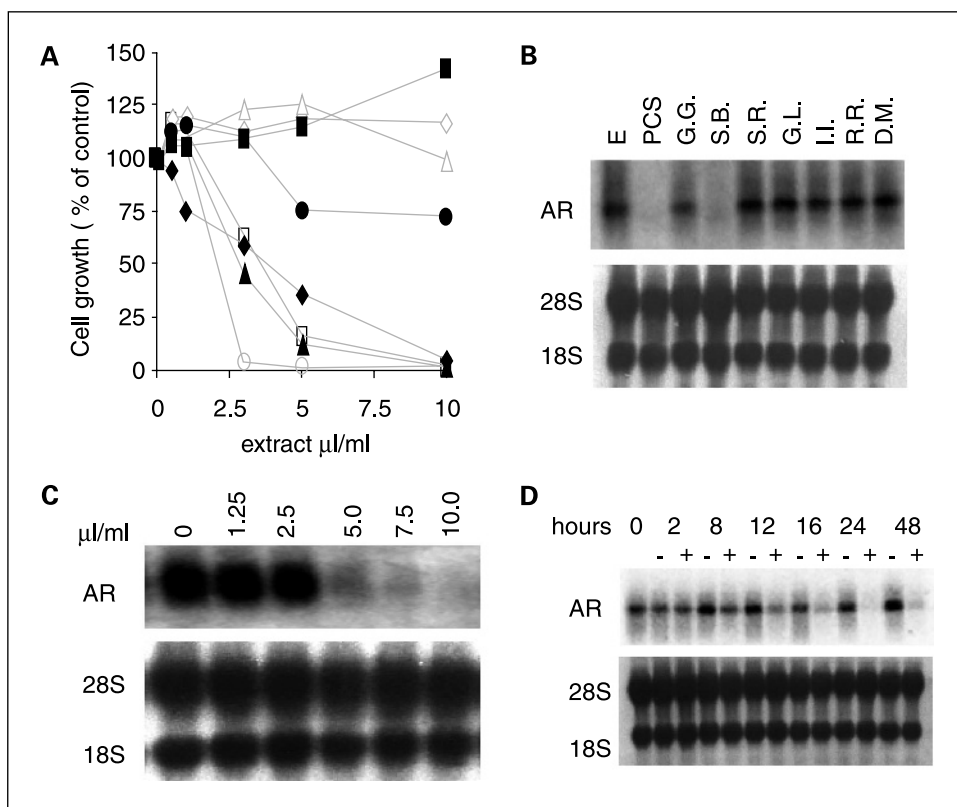
20 mg/kg/d in oil. Treatment was 5 of 7 days for 4 weeks. Animals were sacrificed when tumors reached ~1,000 to 1,400 mg or before the animals became compromised. The significance of differences in tumor growth rate was determined using Student's unpaired *t* test.

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*, *Rabdosia rubescens*, 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.

A, cell growth of LNCaP cells was measured by MTT assay after a 72-hour treatment with ethanolic extracts of the following botanicals: *S. baicalensis* (◆), *G. glabra* (▲), *G. lucidum* (△), *I. indigotica* (●), *D. morifolium tzvel* (◇), *R. rubescens* (■), and *S. repens* (○) or PC-SPES (□). **B**, LNCaP cells were treated with 5 μL/mL of the following extracts for 24 hours: ethanol control (E), PC-SPES (PCS), *G. glabra* (G.G.), *S. baicalensis* (S.B.), *S. repens* (S.R.), *G. lucidum* (G.L.), *I. indigotica* (I.I.), *R. rubescens* (R.R.), and *D. morifolium tzvel* (D.M.). AR expression was measured by Northern analysis. **C**, Northern blot analysis demonstrating AR message levels in LNCaP cells treated with increasing concentrations (μL/mL) of *S. baicalensis* extract for 24 hours. **D**, Northern analysis showing AR expression in LNCaP cells following treatment with 5 μL/mL ethanol (–) or *S. baicalensis* extract (+) at the indicated time points. Methylene blue staining of rRNA served as loading control.



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 components 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 was used to purify the active compounds from the dichloromethane extract. MS data of the three compounds were consistent with molecular formulas of $C_{16}H_{12}O_5$, $C_{17}H_{14}O_6$, and $C_{19}H_{18}O_8$, respectively. Based on these results and literature reports of compounds known to be in *S. baicalensis* (19, 20), it was suspected that the compounds were wogonin (molecular weight 284), skullcapflavone (molecular weight 314), and neobaicalein (molecular weight 374). Comparisons of HPLC retention times and nuclear magnetic resonance spectra between the purified compounds and standards from commercial sources (wogonin) or those we synthesized (skullcapflavone and neobaicalein synthesis methods are detailed as Supplementary Material 1) were carried out to

confirm the compound identities. The structures of the four compounds are shown in Fig. 2B. We next used HPLC-MS to determine concentrations of the identified compounds in the *S. baicalensis* extract. Based on the retention times of the standards and mass-specific ion detection, the peaks within the complex mixture were assigned to individual compounds (Supplementary Material 2). The concentrations of the compounds were calculated based on standard curves plotting peak area against the amount of compound standard injected (Supplementary Material 2). Baicalein was the most abundant of the four active compounds, present at 47.0 $\mu\text{g}/\text{mg}$ dried extract (Supplementary Material 3). Wogonin, neobaicalein, and skullcapflavone were present at 17.3, 12.0, and 3.4 $\mu\text{g}/\text{mg}$, respectively. The four active compounds combined made up 8.4% of the *S. baicalensis* extract's dry weight.

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 $\mu\text{mol}/\text{L}$ (Fig. 3A). The IC_{50} values for baicalein, wogonin, neobaicalein, and skullcapflavone were determined to be 13, 42, 22, and 11 $\mu\text{mol}/\text{L}$, respectively. PC-3 cells were less sensitive to all four compounds with the following IC_{50} values: baicalein 25 $\mu\text{mol}/\text{L}$, wogonin 50 $\mu\text{mol}/\text{L}$, and neobaicalein 35 $\mu\text{mol}/\text{L}$ (Fig. 3B). Skullcapflavone did not inhibit PC-3 cell growth by >50% at concentrations below 100 $\mu\text{mol}/\text{L}$.

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 G_1 phase at 24 hours (Fig. 3C). The percentage increase in G_1 cell numbers ranged from 8% to 11%. With all compounds, the increase in G_1 -phase cells was accompanied by a decrease in S-phase cells, whereas the number of cells in G_2 -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 G_2 -M phase, with a diminished number of cells in G_1 (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 $\mu\text{L}/\text{mL}$ extract inhibited cell growth 18% versus 24% for the four compounds, 3 $\mu\text{L}/\text{mL}$ extract inhibited growth 54% versus

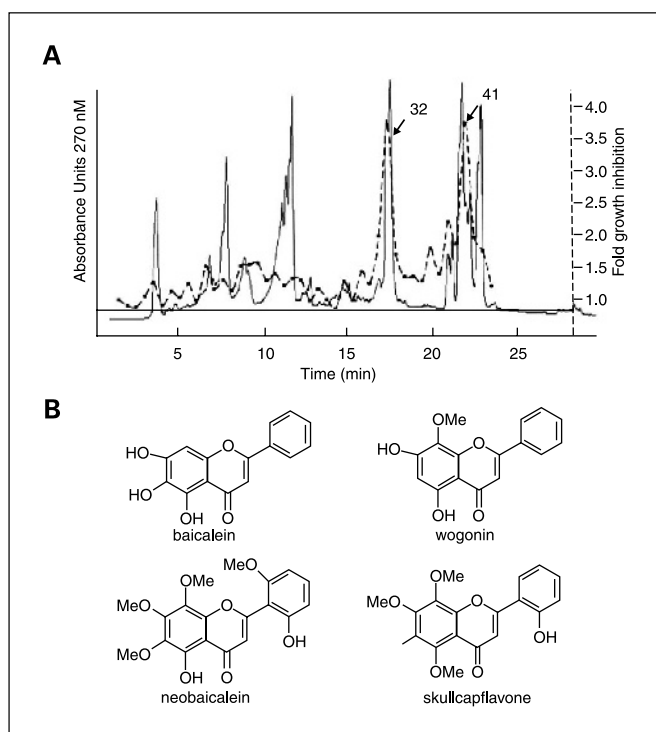


Fig. 2. HPLC fractionation of *S. baicalensis* and identification of growth-inhibitory fractions. *A*, absorbance at 270 nm from an ethanolic extract of *S. baicalensis* fractionated on a C18 reverse-phase column (solid line). Fold growth inhibition by 30-second HPLC fractions of *Scutellaria* on LNCaP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

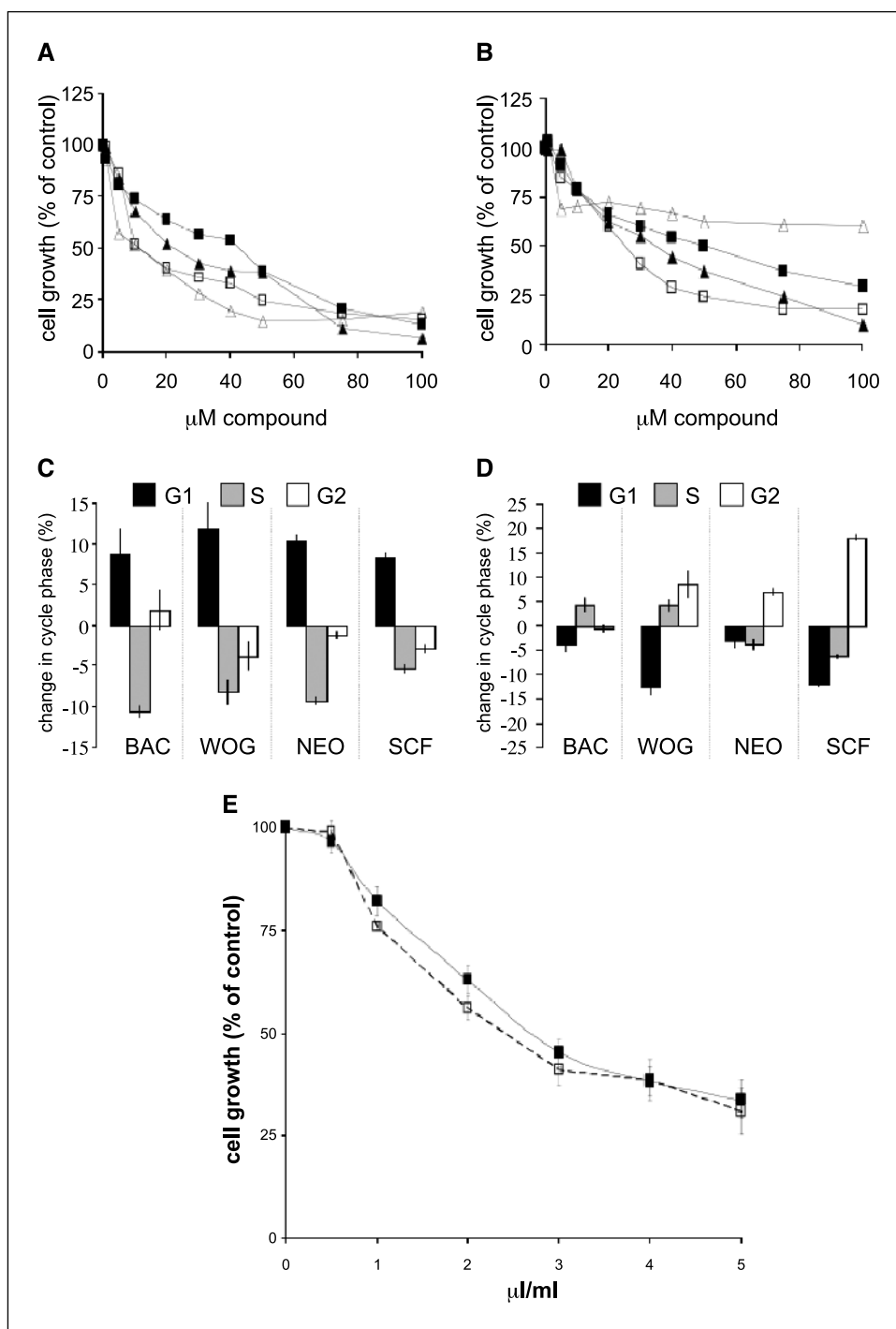


Fig. 3. Inhibition of prostate cancer cell growth with compounds isolated from *S. baicalensis*. LNCaP cells (A) or PC-3 cells (B) were treated with baicalein (\square), wogonin (\blacksquare), neobaicalein (\blacktriangle), or skullcapflavone (\triangle) for 72 hours. Cell growth was determined by MTT assay and values were expressed as the percentage of growth relative to the vehicle-treated control. LNCaP cells (C) or PC-3 cells (D) were treated with baicalein (BAC), wogonin (WOG), neobaicalein (NEO), or skullcapflavone (SCF) at their respective IC_{50} concentrations for 24 hours. The percentage change in each cell cycle phase relative to vehicle control for G₁ (dark gray), S (light gray), and G₂ (white) phases was determined by analyzing propidium iodide-stained cells by fluorescence-activated cell sorting. E, comparison of growth-inhibitory activities between the complete *S. baicalensis* extract and a combination of baicalein, neobaicalein, skullcapflavone, and wogonin. MTT assay following treatment with indicated dilutions of complete extract (solid line) or four-compound combination (dashed line).

59% for the four compounds, and 5 $\mu\text{L/mL}$ 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 IC_{50} (2.7 $\mu\text{L/mL}$) were baicalein 8.6 $\mu\text{mol/L}$, wogonin 2.9 $\mu\text{mol/L}$, neobaicalein 1.6 $\mu\text{mol/L}$, and skullcapflavone 0.54 $\mu\text{mol/L}$. Cell cycle analysis also showed similar effects. Treatment of LNCaP cells for 24 hours with 3 $\mu\text{L/mL}$ *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 $\mu\text{L/mL}$) or the four-compound combination (3 $\mu\text{L/mL}$; 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

(Supplementary Materials 5 and 6). Of the 446 transcripts significantly changed by the four-compound combination, 410 were also significantly altered by the *S. baicalensis* extract. All transcript ratios with a significant change in one treatment and an average signal intensity above background values in the other treatment were plotted, producing a r^2 value of 0.79 (Supplementary Material 4), indicating a high degree of similarity in cellular gene expression response. Although a larger number of transcript alterations were observed with the *S. baicalensis* extract, the magnitudes of most of these changes were quite small, although statistically significant. A two-class *t* test to directly identify genes expressed at significantly different levels between the two treatments determined that only 107 transcripts exhibited different expression alterations (false discovery rate, 1.25%; Supplementary Material 7). These transcripts corresponded to 85 unique genes, only 23 of which differed by >2-fold between the two treatments. As the treatment doses were selected to achieve equivalent growth inhibition, it is likely that the complete extract contains additional flavonoids or other compounds that contribute to additive or additional gene expression changes, but these genes did not seem to influence the proliferation or survival of LNCaP cells.

Comparison of cellular transcript and protein alterations resulting from treatment with baicalein, wogonin, neobaicalein, and skullcapflavone. We next sought to determine similarities and differences between the four active compounds identified in the *S. baicalensis* extract. Gene expression changes following treatment with baicalein, wogonin, neobaicalein, and skullcapflavone were determined by cDNA microarray analyses. LNCaP cells were treated for 8, 24, and 72 hours with compound doses based on the 72-hour IC_{50} to standardize cellular effects. These IC_{50} concentrations are higher than the amounts used in the previous experiments designed to assess the combination of individual compounds at the levels found in the complete *S. baicalensis* extract. At these IC_{50} doses, baicalein treatment (13 $\mu\text{mol/L}$) significantly altered the expression of 1,304 transcripts in at least one of the three time points relative to control. In comparison, neobaicalein (22 $\mu\text{mol/L}$), wogonin (42 $\mu\text{mol/L}$), and skullcapflavone (11 $\mu\text{mol/L}$) altered the expression of 2,076, 798, and 353 transcripts, respectively (Supplementary Materials 8-11). The expression data from the *S. baicalensis* extract, the four-compound combination, and the four individual compounds at 24 hours were simultaneously analyzed to compare effects on ARGs. Through literature reviews and our previous studies delineating the androgen response gene network (21), we identified 121 genes on the microarray that have been shown to be directly or indirectly regulated by androgenic hormones. Of these ARGs, 91 were significantly changed by at least one of the six treatments (Supplementary Material 4). The *S. baicalensis* extract significantly altered the expression of 53 ARGs by 1.5-fold compared with 33 by the four-compound combination. Individually, baicalein, wogonin, neobaicalein, and skullcapflavone significantly changed 31, 30, 41, and 3 ARGs by a factor of ≥ 1.5 -fold, respectively.

The antiandrogenic actions of baicalein, wogonin, neobaicalein, and skullcapflavone were confirmed by treating LNCaP cells with their respective IC_{50} 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 $\mu\text{mol/L}$) than the 72-hour IC_{50} (13 $\mu\text{mol/L}$; Fig. 4B). We calculated that baicalein concentrations of 30 $\mu\text{mol/L}$ would be present in *S. baicalensis* extract dilutions of 5 to 10 $\mu\text{L/mL}$. 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 G_1 -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 G_1 arrest (23), whereas paclitaxel and 2-methoxyestradiol arrest cells at G_2 -M (24, 25). After treating cells with 30 $\mu\text{mol/L}$ 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 G_1 arrest independent of androgen signaling (isoleucine and olomoucine) did not change AR expression >7%, indicating that simply arresting cells in G_1 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 xenografts (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 mm^3 in size and continued until the tumors reached 1,000 mm^3 or compromised the animal. The average tumor volume in the

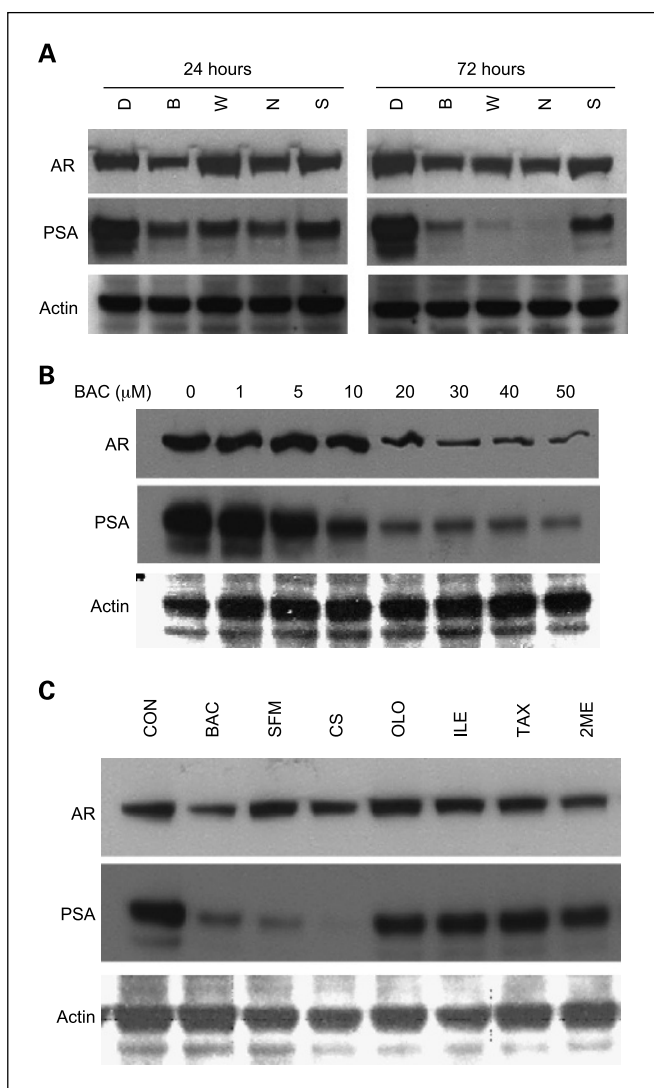


Fig. 4. Antiandrogenic actions of compounds in *S. baicalensis*. *A*, Western blot analysis depicting protein expression levels of AR, PSA, and actin after treatment of LNCaP cells for 24 hours with the four individual compounds [baicalein 13 μmol/L (*B*), wogonin 42 μmol/L (*W*), neobaicalein 25 μmol/L (*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), olomoucine 24 hours (OLO), isoleucine-depleted medium 72 hours (ILE), 10 nmol/L paclitaxel 24 hours (TAX), and 5 μmol/L 2-methoxyestradiol 24 hours (2ME).

control-treated mice doubled in size after 7.8 days of placebo treatment compared with 32 days for the baicalein-treated mice (Fig. 5). One week after initiation of placebo treatment, the average tumor volume increased 1.8-fold and by 2 weeks increased 3.5-fold. In contrast, the average tumor volume in mice treated with baicalein declined 1.8- and 2.2-fold at 1 and 2 weeks, respectively. In the first 2 weeks of treatment, the estimated trend for the placebo group was an increase of 41.9 mm³/d, whereas the estimated trend for the treatment group was a decrease of 7.5 mm³/d. The difference in trends was statistically significant ($P < 0.001$). The tumors in the baicalein-treated mice eventually developed resistance to

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.

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 IC₅₀ 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 IC₅₀s 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 G₁ arrest in LNCaP cells when added to the medium at equivalent growth-inhibitory concentrations. G₁ 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 G₁ 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 G₁ 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 IC₅₀ doses originally studied none of the compounds seemed to reduce AR protein levels. However, treatment of LNCaP cells with 20 to 30 μmol/L

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

	Control	Baicalein	Serum-free medium	Charcoal-stripped medium	Olomoucine	Isoleucine	Taxol	2-Methoxyestradiol
AR	100	58	100	68	103	93	95	67
PSA	100	16	20	0	78	82	89	74
G ₁ %	70	77	81	77	79	83	49	47
G ₂ %	12	14	12	16	13	12	34	37
S%	17	8	7	7	8	5	16	15
MTT	100	81	27	33	76	39	84	92

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.

baicalein, a concentration above the IC₅₀ 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

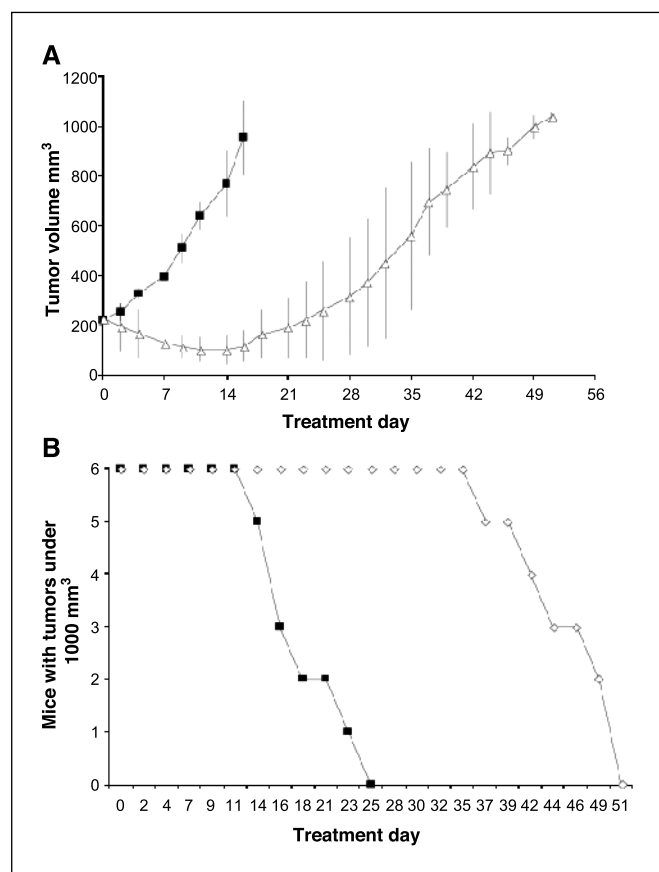


Fig. 5. Inhibition of LuCaP 35 prostate cancer xenograft growth by the oral administration of baicalein. LuCaP 35 xenograft tumor xenografts were implanted in nude mice, allowed to reach 250 mm³ in size, and treated with either baicalein (20 mg/kg) or placebo five times weekly orally. *A*, average tumor volume of placebo-treated mice (■; *n* = 6) or baicalein-treated mice (△; *n* = 6) plotted over time (days). For ethical reasons, mice were sacrificed at a tumor volume of ~1,000 mm³. *B*, number of mice remaining with tumor volumes ≤1,000 mm³ after treatment with placebo (■) or baicalein (△) for indicated days.

include eliminating inconsistencies involved in plant cultivation and extraction procedures and reducing side effects that may be attributed to undesirable chemicals within the plant (50). The results reported here indicate that most of the activities of *S. baicalensis* toward the prostate cell lines that we evaluated can be recapitulated with four purified isoflavones. The combination of these four compounds led to growth inhibition curves and cell cycle changes that were identical to those observed with the entire *S. baicalensis* extract. However, there were subtle differences in the transcript expression profiles between the entire extract and the four isoflavone combination that suggest the presence of additional active constituents within *S. baicalensis*. It is possible that these other activities could be mediators of antineoplastic activities in cell types that we did not study. The most abundant active compound in the *S. baicalensis* extract was determined to be baicalein, and most, although not all, of the activity seen with 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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