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

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\begin{abstract}
\textbf{Purpose:} Botanical preparations are widely used by patients with prostate cancer. \textit{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 \textit{Scutellaria} and determine their mechanisms of action.

\textbf{Experimental Design and Results:} We used high-performance liquid chromatography to fractionate \textit{S. baicalensis} and identified four compounds capable of inhibiting prostate cancer cell proliferation; baicaicen, wogonin, neobaicaicen, and skullcap flavone. 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. \textit{In vivo}, baicaicen (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 ($P < 0.001$).

\textbf{Conclusions:} Most of the anticancer activities of \textit{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.
\end{abstract}
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. Ethanolic extract (1 mL) of S. baicalensis was injected into 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 using gradient elution method A (Supplementary Material 1) and was visualized using UV light. Unless otherwise noted, reagents were purchased from Sigma-Aldrich (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 Chemical 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 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 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) FACS can cytometer; 10,000 gated events were counted for each sample. Data analysis was done using CellQuest and MPlLIS 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-1, 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 autoradiographs 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/adualITPCouplingProcedure.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 replicates 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 (>250 mm^3) were implanted s.c. Tumor growth was monitored by measuring tumor volume thrice weekly. Tumor volume was calculated as follows: length × height × width × 0.5236. Once tumors reached 250 mm^3, 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 baicalein 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. baicalensis* 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 μg/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 μg/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 (μg/mL) of *S. baicalensis* extract for 24 hours. D. Northern analysis showing AR expression in LNCaP cells following treatment with 5 μg/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_{13}H_{10}O_{6}, 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 neobacalein (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 neobacalein 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 μg/mg dried extract (Supplementary Material 3). Wogonin, neobacalein, and skullcapflavone were present at 17.3, 12.0, and 3.4 μg/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 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 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 µL/mL extract inhibited cell growth 18% versus 24% for the four compounds, 3 µL/mL extract inhibited growth 54% versus
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 IC_{50} (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.
(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 r² 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 IC₅₀ to standardize cellular effects. These IC₅₀ 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 IC₅₀ doses, baicalein treatment (13 μmol/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 μmol/L), wogonin (42 μmol/L), and skullcapflavone (11 μmol/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 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 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 mm³ in size and continued until the tumors reached 1,000 mm³ or compromised the animal. The average tumor volume in the
Mechanisms of Cell Growth Inhibition by 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), olomouecine 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).
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 E_{2} 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

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

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<th>Control</th>
<th>Baicalein</th>
<th>Serum-free medium</th>
<th>Charcoal-stripped medium</th>
<th>Olomoucine</th>
<th>Isoleucine</th>
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</table>

*NOTE: G_{s}% and S%, percentage of cells in each phase of the cell cycle as determined by flow cytometry; MTT, assay for cell proliferation.*

![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 mm^{3} 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 mm^{3}. B, number of mice remaining with tumor volumes ≤1,000 mm^{3} 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 inhibition growth curves and cell cycle changes that were identical to those observed with the entire *S. baicalensis* extract. However, there were subtle differences in the transient 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, baicalin 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 flavonoids.

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**References**


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