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Prevention and Management of Prostate Cancer Using PC-SPES: A Scientific Perspective[1,2]

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ABSTRACT Complementary and alternative therapies are increasingly used in the United States by individuals diagnosed with cancer. PC-SPES is a multiherb dietary supplement used by many patients with prostate cancer (CaP). The wide acceptance of PC-SPES for hormone-naive and end-stage CaP relates to clinical trials demonstrating significant efficacy and low toxicity. Although the clinical efficacy of PC-SPES is highly encouraging, its scientific basis has progressed more slowly. This article describes our understanding of the in vitro mechanisms of action of PC-SPES in androgen-dependent LNCaP cells. We first demonstrated significant suppression of cancer cell growth by restriction of cell cycle progression at G_1/S and drastic reductions in the expression of androgen receptor and prostate-specific antigen (PSA) by PC-SPES, providing a mechanistic rationale for its observed clinical effects. Further investigation of the anti-CaP properties of PC-SPES revealed that two of its multicomponent herbs, *Glycyrrhiza uralensis* and *Scutellaria baicalensis,* inhibited cell growth and down-regulated PSA in a manner comparable with PC-SPES. Exhaustive characterization of *S. baicalensis* resulted in the isolation of baicalein. Here we report that baicalein effectively suppressed growth and PSA expression and induced G_1/S arrest in LNCaP cells. Although baicalein cannot account for the entire activity of PC-SPES, it does display similar anti-CaP activities. These data suggest that a single herb or bioactive compound could suffice for CaP chemoprevention by effecting multiple changes in target cells to intervene in CaP progression. These studies provide the impetus for further evaluation of the composition herbs within PC-SPES and the precise characterization of their bioactive ingredients. J. Nutr. 132: 3513S–3517S, 2002.

KEY WORDS: • *PC-SPES* • *baicalein* • *chemoprevention* • *prostate cancer*

PC-SPES is a mixture of seven Chinese herbs *(Dendranthema morifolium, Ganoderma lucidium, Glycyrrhiza uraensis, Isatis indigotica, Panax pseudoginseng, Robdosia rubescens,* and *Scutellaria baicalensis)* and one American herb *(Serenoa repens)* (1–5). PC-SPES was formulated according to traditional Chinese medicine principles, encompassing ingredients that counteract, balance or circumvent potentially cytotoxic agents, minimizing toxicity; deliver a broad-based mechanistic platform with an array of overlapping and distinct antitumor activities that can be effectively directed to the various functional aberration sites characteristic of malignant cells; reduce the development of drug resistance in target cells; and overcome the heterogeneity and genetic variability intrinsically associated with localized and metastatic carcinomas.

PC-SPES is a popular herbal supplement consumed by individuals diagnosed with prostate cancer (CaP)[4] (6–10). Its efficacy as an antiprostatic dietary regimen has been supported by in vitro experiments, animal experiments and limited clinical trials (1–13). Reported benefits of PC-SPES in end-stage patients with CaP include rapid, marked reduction of serum prostate-specific antigen (PSA) and an overall improvement in morbidity and immune status (6–11,13,14). Because the time interval between the transition of CaP from the clinically treatable androgen-responsive to the incurable hormone-refractory states could be decades, this form of malignancy is amenable to chemopreventive intervention using agents such as PC-SPES. Some mechanistic insights into the positive clinical responses to PC-SPES have been provided by published studies from this laboratory and others (1–5,11). This laboratory first demonstrated the ability of PC-SPES, in in vitro studies using ethanol extracts (a method for isolating bioactive ingredients) of PC-SPES, to suppress cell growth accompanied by restriction of cell cycle traverse at the G_1/S

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[4] Abbreviations used: AR, androgen receptor; CaP, prostate cancer; PCNA, proliferating cell nuclear antigen; PSA, prostate-specific antigen.

and G_2/M checkpoints, induce apoptosis and significantly down-regulate the expression of androgen receptor (AR) and PSA.

Because PC-SPES is a complex herbal mixture, an important question regarding its use is whether its biological activity can be largely or exclusively ascribed to one or more individual herbs present in the mixture. This consideration led us to further test extracts from individual herbs comprising PC-SPES. The effects of each composition herb were determined by measuring effects on cell proliferation and AR and PSA expression using androgen-dependent LNCaP cells. These studies provided unequivocal evidence that *S. baicalensis* and *G. uralensis* displayed anti-CaP activities similar to those of PC-SPES (2). Particularly noteworthy was the observation that extracts of *S. baicalensis* had even greater potency in suppressing PSA than did PC-SPES, suggesting that this herb may contain bioactive ingredients uniquely targeted at the control of PSA expression.

Further study on *S. baicalensis* led to the discovery that baicalin and its metabolite baicalein, the principal anti-CaP bioflavonoids present in PC-SPES (15–17), are naturally occurring flavonoids with anti-inflammatory, antibacterial, antiproliferative and lipoxygenase-inhibitory activity (4,18–23). As part of our continuing effort to address various public health–related issues on PC-SPES and to further elucidate the chemopreventive potential of baicalein, we performed additional experiments on its anti-CaP properties. The scientific data described here support the notion that baicalein, as a single pure chemical entity, may suffice for the control of PSA expression in hormone-refractory patients with CaP. These findings are significant because PC-SPES was recently recalled because of the discovery that it contained prescribed medications, specifically, warfarin, diethylstilbestrol and indomethacin (24,25).

The issue of PC-SPES contamination is complicated, difficult to evaluate and not likely to be resolved immediately. The public health implications posed by its withdrawal from the market are of potential significance; therapeutic response failures, measured as increases in PSA levels, have been associated with the discontinuation of PC-SPES treatment (26).

Thus the precise characterization and titration of active components in PC-SPES, such as baicalein, may provide alternate and more specific treatment options and add to knowledge on their mechanisms of action. We suggest that further research on baicalein may lead to its strategic development as an alternative to PC-SPES in the treatment and management of CaP.

MATERIALS AND METHODS

Cell culture. Human CaP cell line LNCaP was purchased from American Type Culture Collection (Rockville, MD). LNCaP cells were maintained and cultured in RPMI-1640 medium containing L-glutamine, supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/mL), and streptomycin (172 μmol/L).

Ethanolic extracts from PC-SPES. PC-SPES (lot No. 5430171) was obtained from Botanic Lab. (Brea, CA). Standardized extracts of PC-SPES were prepared according to published procedures (1,14). Specifically, the contents of 1 capsule of PC-SPES containing 320 mg powdered extract were suspended in 1 mL of 70% ethanol and mixed by rotation on double platform rocker at 150 rpm (Midwest Scientific, Valley Park, MO) for 1 h at room temperature. Insoluble material was removed via centrifugation at 11,000 × *g* for 10 min. The ethanolic extract was filtered through a 0.22-μm filter (Millipore, Bedford, MA), divided into aliquots and maintained at 4°C. Before use, the stock solution was further diluted in RPMI-1640 medium to give the final indicated concentrations.

Baicalein. Baicalein was purchased from Sigma Chemical Co. (St. Louis, MO). Stock solutions (10 mmol/L) of baicalein were stored at −20°C. Before use, the stock solution was diluted in RPMI-1640 medium and added to cultures to provide the desired final concentrations.

Cell growth. LNCaP cells were seeded at 1×10^5 cells/mL in T75 flasks and allowed to attach overnight. Cells were incubated with 0, 19 and 93 μmol of baicalein/L. After a 3-d incubation, cells were harvested by trypsinization. Media from control and treated LNCaP cells were collected for determination of PSA by Western blot analysis. Cell number in control and treated cells was counted using an hemocytometer, and cell viability was determined by trypan blue dye exclusion (1,2,14).

Colony formation assay. LNCaP cells were cultured at 400 cells/mL of RPMI-1640 and 10% FBS in a 24-well plate. Cultures received 0, 4, 19 and 93 of baicalein μmol/L. After 14 d in culture, the cells were fixed and stained with 0.1% crystal violet to make colonies visible for counting (27). The experiments were performed in duplicate or triplicate.

Cell cycle analysis. Cell cycle phase distribution was analyzed by flow cytometry. Cultures were exposed to 0, 19 and 93 μmol of baicalein/L for 3 d and harvested. Cells were washed once with phosphate-buffered saline (PBS) and stained with 4′,6-diamidino-2-phenylindole at 2.9 μmol/L dissolved in buffer containing 100 mmol of NaCl and 2 mmol of $MgCl_2$ per L plus 0.1% Triton X-100 (Sigma Chemical) at pH 6.8 as previously described (5,15,27). The data from

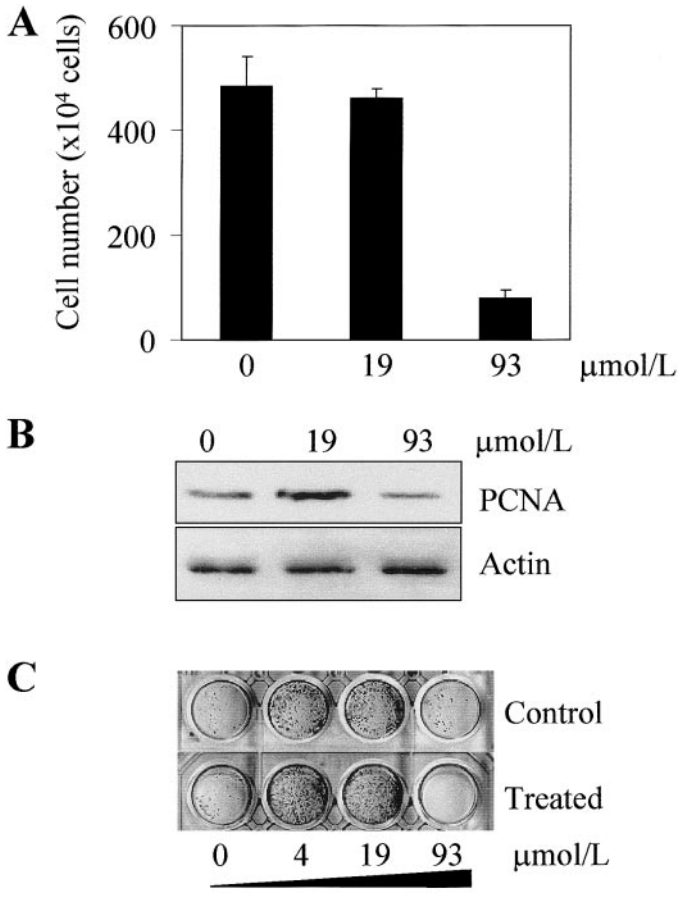

FIGURE 1 Effects of baicalein on growth of LNCaP cells. (*A*) Cells were treated for 3 d with the indicated concentration of baicalein. Growth was measured by counting the cell number using an hemacytometer. Results were averaged from three independent experiments and presented as means ± SD. (*B*) PCNA expression was evaluated by Western blot analysis using 3-d cultures treated with baicalein at 19 or 93 μmol/L. (*C*) Effect of baicalein at 0, 4, 19 and 93 μmol/L on clonogenicity of LNCaP cells.

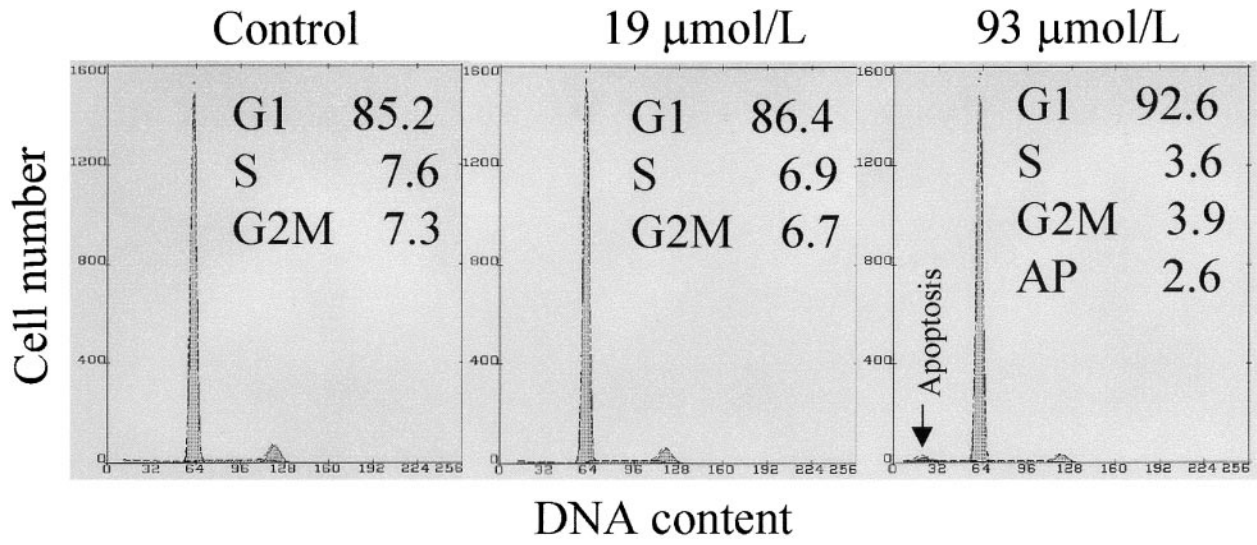

FIGURE 2 Effects of baicalein on cell cycle phase distribution in LNCaP cells. The cell cycle distribution of LNCaP cells treated with different concentrations of baicalein for 3 d was determined from DNA content frequency histograms. Quantitative assessment of distribution of cells (in percent) in each specific phase appeared in the insert.

each treatment were collected and analyzed by Multicycle software provided by Phoenix Flow Systems (San Diego, CA).

Protein extraction and Western blot analysis. Cells were suspended in buffer (50 μL/10^6 cells) containing 10 mmol of HEPES, pH 7.5, 90 mmol of KCl, 1.5 mmol of $Mg(OAc)_2$, 1 mmol of dithiothreitol, 0.5% Nonidet P-40, and 0.5 mmol of 5% glycerol supplemented with phenylmethylsulfonyl fluoride per L and 10 μg each of aprotinin, pepstatin and leupeptin per mL; cells were lysed by three freeze-thaw cycles (1,2,14,27). The extracts were centrifuged, and the clear supernatants were stored in aliquots at −70°C. Postmitochondrial extracts were prepared from control and treated cells using buffers supplemented with multiple protease inhibitors as previously described (1,2,14,27). Extracts from control and treated cells (10 μg) were separated on 10% sodium dodecyl sulfate–polyacrylamide gel, transferred onto nitrocellulose membranes and incubated with the respective primary [AR, PSA, proliferating cell nuclear antigen (PCNA) or actin] and secondary antibodies. Specific immunoreactive bands were made visible with the enhanced chemiluminescence system or by color reaction, as described in the manufacturer's protocol (Kirkegaard & Perry Laboratories, Gaithersburg, MD) (1,2,14,27). Reprobing of blots with different antibodies was done after stripping with a buffer containing 62.5 mmol of Tris-HCl/L, pH 6.7, 100 mmol of 2-mercaptoethanol/L, and 2% SDS at 50°C for 10–30 min. The membrane was also probed for actin to normalize for possible variations in transfer or loading. Intensity of the specific immunoreactive bands on the membrane was quantified by densitometry.

RNA extraction and reverse transcription–polymerase chain reaction (RT-PCR). Total cellular RNA was isolated from control and PC-SPES– or baicalein-treated LNCaP cells at d 3 by using TRIzol reagent (InVitrogen, Carlsbad, CA) according to protocols provided by the manufacturer. RNA purity was established by agarose gel electrophoresis, and the amount of RNA was quantified by measurement of the $A_{260/280}$ absorbance ratio. RT of RNA into cDNA was accomplished with Superscript RNase H^{--} reverse transcriptase (InVitrogen). The reverse-transcribed products were amplified by PCR using the following primer sets: AR, forward and backward primer sets, 5′-CTCTCTCAAGAGTTTGGATGGCT-3′ and 5′-CACTTGCACAGAGATGATCTCTGC-3′, expected size 342 bp; and PSA, forward and backward primer sets, 5′-CTCTCGTGGCAGGGCAGT-3′/5′-CCCCTGTCCAGCGTCCAG-3′, expected size 484 bp. The PCR products were separated in 1.2% agarose gels, and the relative intensities of fragments with the expected sizes were documented using an Innotech imaging system (San Leandro, CA) (27).

RESULTS

Effects of baicalein on LNCaP cell growth, PCNA expression and colony formation. Baicalin and its active metabolite baicalein were recently purified from PC-SPES, and its anti-CaP properties were demonstrated by this and other laboratories (15–17). In this study, we examined whether baicalein prevented cellular growth in LNCaP cells. When cells were incubated with 0, 19 and 93 μmol of baicalein/L, no significant growth suppression (~5%) was found at 19 μmol/L, whereas ~84% growth reduction was found at 93 μmol/L (**Fig. 1***A*). Because PCNA may be considered to be a marker for cell proliferation, we asked whether baicalein affected PCNA expression. Reduction in cell growth seen with baicalein treatment at 93 μmol/L was accompanied by down-regulation of

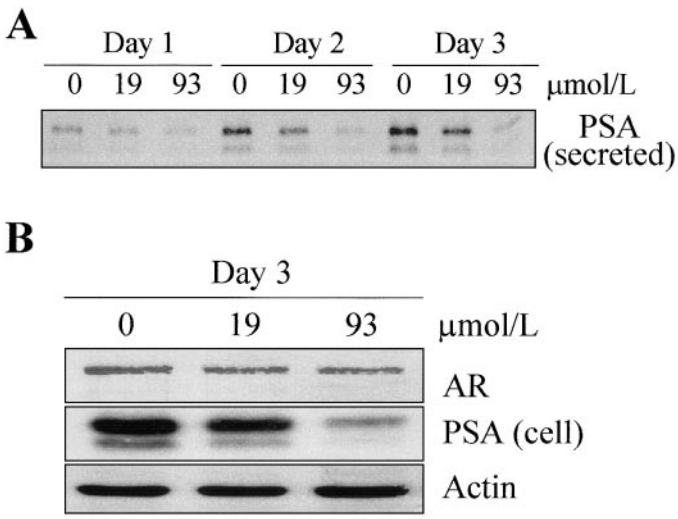

FIGURE 3 Control of AR and PSA expression by baicalein in LNCaP cells. (*A*) Immunoblot analysis of time-dependent changes in secreted PSA treated with baicalein at 19 or 93 μmol/L of for 1–3 d. (*B*) Western blot analyses of intracellular AR and PSA in control and 3-d baicalein-treated LNCaP cells.

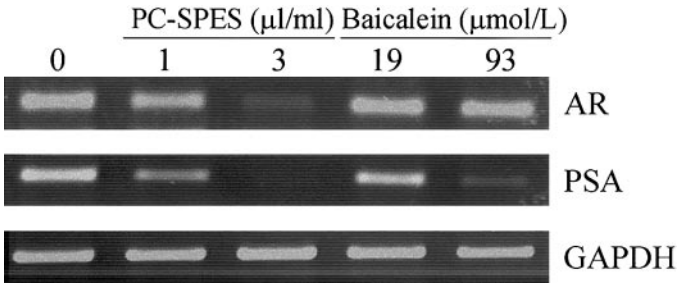

FIGURE 4 Control of AR and PSA expression by PC-SPES and baicalein in LNCaP cells. RT-PCR analysis of changes in control and 3-d PC-SPES–treated (1 or 3 μL/mL) or baicalein-treated (19 or 93 μmol/L) LNCaP cells. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to verify equivalent RNA loading.

PCNA (Fig. 1*B*). Further confirmation of the growth-suppressive properties of baicalein were obtained using the colony formation assay. Figure 1C shows a dose-dependent suppression of colony formation by increasing concentrations of 4, 19 and 93 μmol of baicalein/L. At the highest concentration of baicalein, the ability of LNCaP cells to form colonies was almost totally abolished (Fig. 1C).

Effects of baicalein on cell cycle progression. To further test the antitumorigenic property of baicalein, we monitored its effect on cell cycle distribution with flow cytometry. These studies show that at 93 μmol/L, baicalein elicited a G_1/S arrest in androgen-dependent LNCaP cells, with a 50% reduction in cell entry into the S phase (**Fig. 2**). In these experiments a very modest induction of apoptosis (2.6%) was observed after baicalein treatment at 93 μmol/L (Fig. 2).

Effects of baicalein on AR and PSA expression. Control of AR and PSA expression by baicalein in androgen-dependent LNCaP cells was also investigated. **Figure 3***A* shows time- and dose-dependent decreases in secreted PSA for baicalein treatment at 19 and 93 μmol/L at 1–3 d. We further tested whether PSA changes are accompanied by a commensurate change in the expression of its transcription factor, AR. Figure 3*B* shows that, by Western blot analysis, intracellular PSA levels were substantially lowered by baicalein treatment at 93 μmol/L, which was in direct contrast with the virtually unchanged intracellular AR protein expression. Similar analysis of AR and PSA expression at the RNA level by RT-PCR confirmed that although PSA RNA levels were suppressed in response to PC-SPES or baicalein, a parallel dose-dependent reduction of AR was seen only in PC-SPES–treated, not baicalein-treated, LNCaP cells (**Fig. 4**). These studies point to a clear distinction between the mechanism of action of PC-SPES and baicalein—namely, a decoupled regulation of AR and PSA levels in the case of baicalein and the apparent coordinated control of AR and PSA by PC-SPES.

On the basis of these findings, we conclude that androgen-dependent LNCaP cells respond to baicalein by suppression of cell proliferation, arrest of cell cycle progression at the G_1/S phase, a slight induction in apoptosis and a significant reduction in PSA unmatched by changes in the expression of AR. Overall, these phenotypic and genotypic changes (except AR) bear strong resemblance to those elicited by PC-SPES. A clear disparity in their mechanism of action, however, is revealed by RT-PCR analysis of AR and PSA expression.

We conclude that both PC-SPES and its bioactive component baicalein, at appropriate concentrations, arrest CaP cells in various checkpoints of the cell cycle, induce apoptosis and decrease PSA expression (1–5). Furthermore, in vitro studies showed that baicalein alone is unlikely to account for the total biological effects of PC-SPES especially with respect to AR expression (Fig. 4). However, baicalein does display anti-CaP activities similar to those of PC-SPES. Results of this study provide insights on how specific dietary flavonoids control proliferation and specific gene expression in CaP.

The isolation and characterization of individual herbs and their components from proprietary formulations have several broad implications. Unlike PC-SPES, which is and may remain commercially unavailable, baicalein remains available and potentially could be developed for the management and treatment of CaP. Once characterized, promising herbal agents such as baicalein may be incorporated into formulations with potentially synergistic activities and with the added benefit of avoiding confounding quality control issues of standardization and contamination.

ACKNOWLEDGMENT

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