

Effects of herbal preparation Equiguard™ on hormone-responsive and hormone-refractory prostate carcinoma cells: Mechanistic studies

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Abstract. The Equiguard™ is a dietary supplement comprised of standardized extracts from nine herbs, respectively, *Herba epimedii brevicornum Maxim* (stem and leaves), *Radix morindae officinalis* (root), *Fructus rosa laevigatae michx* (fruit), *Rubus chingii Hu* (fruit), *Schisandra chinensis (Turz.) Baill* (fruit), *Ligustrum lucidum Ait* (fruit), *Cuscuta chinensis Lam* (seed), *Psoralea corylifolia L.* (fruit), and *Astragalus membranaceus (Fisch.) Bge* (root). This proprietary product, formulated according to Chinese traditional medicinal concepts, is aimed at restoring harmony in the 'primordial (original) ying-yang' of the kidney, an organ which Chinese medicinal principles consider to be vital for invigorating as well as maintaining balance of the entire urological system. As the prostate is an integral component of the urological system, we performed *in vitro* studies to test the effects of ethanol extracts of Equiguard to modulate prostate growth and gene expression. These studies used prostate cancer cells mimicking the androgen-dependent (AD) and androgen-independent (AI) states of prostate carcinogenesis. Results show that Equiguard significantly reduced cancer cell growth, induced apoptosis, suppressed expression of the androgen receptor (AR) and lowered intracellular and secreted prostate specific antigen (PSA), and almost completely abolished colony forming abilities of prostate cancer cells. These data support the interpretation that this herbal formulation contains ingredients that collectively may be efficacious in preventing or treating AD and AI prostate carcinoma. The anti-prostatic activities of Equiguard may stem from its complex composition capable of targeting multiple signal transduction/metabolic pathways, to effectively correct, counteract or circumvent the impaired

or dysfunctional mechanisms accompanying different stages of prostate carcinogenesis.

Introduction

Prostate cancer (CaP) is the most commonly diagnosed neoplasm and produces the second-highest cancer mortality in U.S. males. Approximately 179,000 new cases were reported in 1999, with 37,000 deaths resulting from the disease (1,2). An upward trend in these statistics is expected due in part to a projected increase in life expectancy among adult males and wider implementation of PSA screening for CaP in its early stages (1,2). In individuals diagnosed with CaP, initially the cancer cells are slow growing and androgen-dependent (ADPc); ADPc patients may remain subclinical for an extended period of time. Ultimately the disease progresses to an androgen-independent state (AIPc), characterized by greater proliferation of cells, lack of responsiveness to androgen blockade, and high fatalities (2-5). The mainstay for localized CaP includes radical prostatectomy, radiation therapy, and hormonal modalities such as androgen deprivation using physical or chemical measures. These first-line treatments often result in positive responses in the majority of patients. However, relapse occurs with predictable frequency in a significant percentage of treated individuals; the recurrence is often accompanied by emergence of hormone-insensitive and -refractory clones. Expansion of these clones can result in establishment of hormone-insensitive states, which frequently will be rapidly followed by metastasis to sites beyond the confines of the gland and are not readily treatable (6). Thus, what is urgently needed are easily compliant preventive and treatment strategies. Research directed towards mechanistic understanding of such new strategies is also imperative.

Epidemiologic studies have consistently shown that age-adjusted incidence and mortality rates for clinical CaP display significant geographic variations and marked differences among various ethnic/racial groups. Genetic and epigenetic factors and their interplays contribute to the observed variable incidence (7,8). Diet and specific nutritional ingredients have been proposed to exert promoting as well as protecting roles in the progression and establishment of CaP clinically (9-13). Culture and regional customs, such as food and other lifestyle

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preferences, may also act to subsume potentially metastatic CaP in the latent, subclinical state (14-16).

The aforementioned considerations, coupled with the multifactorial, multi-stage, and heterogeneous nature of CaP, suggest that use of single agents for effective treatment of CaP will be challenging. An alternative and complementary approach is the development of combination and/or sequential therapies, which, in an atypical format, could include the use of herbs. Herbal therapies rely upon presentation of aggregate, ill-defined combinations of bioactive, inactive, and counter-active agents, with the aim that their collective manifestation results in reduced toxicity and appearance of new/novel activities. These features are considered important in cancer prevention/treatment since they may serve to counteract and circumvent overlapping molecular pathways, which typically characterize malignant states and which often impede success in cancer treatment. Herbal therapies are embodied in traditional Chinese medical practices, which approach disease treatment using a 'holistic/integrative' orientation quite distinct from the 'pharmaceutical' approaches of Western medicine. Typically, Chinese herbal prescriptions comprise of mixtures which, if properly prepared, deliver multiple bioactive agents to target cells/organs. Because this 'integrative' strategy emphasizes application of the total spectrum of bioactive ingredients present in a herbal mixture and evaluates success based on the 'well being/curing' of the patients as a whole, its concoction is not absolutely dependent upon precise knowledge of the specific defect/derangement in target cells. Moreover, the same beneficial clinical outcome may be achieved using formulations with no apparent identity.

Our laboratory has been investigating the potential efficacy of natural nutraceuticals and herbal products for treating prostate cancer. Previously, we have demonstrated the effects of ethanol extracts of PC-SPES in AD prostate cancer cells (14-16). PC-SPES is a multi-component herbal supplement clinically shown to be efficacious against locally advanced and metastatic prostate cancer. Most patients on PC-SPES show significant drop in serum testosterone and PSA levels. Pain relief and improved quality of life are also frequently observed (17-26). Our *in vitro* studies using LNCaP cells reveal that PC-SPES restricts cell growth, and reduces the expression of PSA concomitant with decreased expression of the androgen receptor, AR (14). Since use of PC-SPES has accompanying side effects reminiscent of individuals on diethylstilbestrol (19-24), it is of interest to seek other herbal formulations for the treatment of CaP. The Equiguard is a dietary supplement comprised of standardized extracts from nine Chinese herbs, respectively, *epimedium herb*, *morinda root*, *dodder seed*, *malaytea scurfpea fruit*, *glossy privet fruit*, *milkvetch root*, *palmleaf raspberry fruit*, *Cherokee rose fruit*, and *Chinese magnoliavine fruit*. This proprietary product was formulated to restore harmony in the kidney, which, according to Chinese traditional medicinal concepts is involved in regulating and maintaining balance of the entire urological system. Results reported herein, based on *in vitro* studies using prostate cancer cell lines mimicking the subclinical, hormone-responsive, and the advanced, hormone-refractory states of prostate carcinoma, show that ethanol extract of Equiguard profoundly reduced the expression of PSA and AR, effectively suppressed cell proliferation, and almost

completely abolished the colony forming properties of prostate cancer cells. These results suggest that Equiguard could be useful and applicable in the treatment of CaP.

Materials and methods

Cell culture. Human prostate cancer cells PC-3, DU 145, and LNCaP were obtained from ATCC, Rockville, MD. These cell lines were derived from various metastasized sites of prostate cancer patients, with prior exposure to hormone therapy (27-29). The JCA-1 cells were established in this institution from the primary prostate cancer site before any form of therapy was given to the subject (30). Androgen-responsive LNCaP cells are considered models of androgen-dependent forms of prostate cancer, whereas JCA-1, PC-3, and DU 145 cells may be more representative of prostate cancer in its advanced stages. Routinely, cells were cultured in RPMI 1640 media containing L-glutamine, supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml).

Preparation of ethanol extracts of Equiguard. Capsules of Equiguard (each capsule containing 340 mg powder) were provided by ICM Holdings, Inc. (Hong Kong) and stored in a refrigerator. The product was determined and certified to be free of heavy metal and bacterial contamination, by independent government approved service laboratories in Hong Kong. Assurance of quality control was provided by the company and was based on HPLC analysis, displaying a characteristic profile that was routinely checked and shown to be reproducible within a narrow range for different lots of Equiguard. The contents of each capsule were suspended in 70% ethanol (340 mg/ml) and stirred with intermittent mixing at 150 rpm for 60 min, room temperature. The insoluble material was removed by centrifugation and the soluble supernatant was sterilized by passing through a 0.22 µm filter. Before use, the stock was further diluted in tissue culture media to give the final indicated concentrations.

Effect of herbal extracts on prostate cancer cell proliferation and viability. Hormone-dependent LNCaP cells were seeded at 1×10^5 cells/ml in T-75 flasks and allowed to attach overnight. Other three cell types were seeded at 5×10^4 cells/ml. Cells were incubated with ethanol extracts (1 and 3 µl/ml) of Equiguard, prepared as described above. Following 72-h incubation, cells were harvested by trypsinization. Media from control and treated LNCaP cells were collected for analysis of PSA by immunoblotting or using the Tandem-E PSA kit purchased from Hybritech, Inc. Cell number in control and treated cells was counted using a hemocytometer and cell viability was determined by trypan blue dye exclusion (14-16).

Effects of Equiguard on colony formation. The procedure for clonal growth assay was performed as described (31). Prostate cancer cells were cultured at 200 cells/ml RPMI 1640 and 10% FBS in a 24-well dish. Treated cultures received ethanol extracts (1 and 3 µl/ml) of Equiguard. After 14 days in culture the cells were fixed and stained with 0.1% crystal violet to visualize colonies for counting. The experiments were performed in duplicates or triplicates.

Effects of Equiguard on cell cycle progression. Cell cycle phase distribution was analyzed by flow cytometry. Cultures were exposed to varying concentrations of the Equiguard extracts (1 and 3 $\mu\text{l/ml}$) for 3 days and harvested. Cells were washed once with PBS and stained with 1.0 $\mu\text{g/ml}$ DAPI containing 100 mM NaCl, 2 mM MgCl_2 and 0.1% Triton X-100 (Sigma) at pH 6.8 as previously described (32-34). The DNA-specific DAPI fluorescence was excited with UV-light and collected with appropriate filters in an ICP-22 (Ortho Diagnostic, Westwood, MA) flow cytometer. The data from each treatment were collected and analyzed by Multicycle™ software provided by Phoenix Flow Systems (San Diego, CA).

Measurement of intracellular and secreted PSA. The Tandem-E kit was used to measure intracellular and secreted PSA (16,18,32). The assay was based on the quantitative binding of PSA to alkaline phosphatase-conjugated anti-PSA monoclonal IgG and the cleavage of p-nitrophenyl phosphate by the IgG-conjugated alkaline phosphatase, yielding colored products that can be quantified by measuring absorbance at 405 and 450 nm (16,18,32).

Protein extraction and Western blot analysis. Cells were suspended in buffer (50 $\mu\text{l}/10^6$ cells) containing 10 mM HEPES, pH 7.5, 90 mM KCl, 1.5 mM $\text{Mg}(\text{OAc})_2$, 1 mM DTT, 0.5% NP40 and 5% glycerol supplemented with 0.5 mM PMSF, 10 $\mu\text{g/ml}$ each of aprotinin, pepstatin, leupeptin, and lysed by 3 freeze/thaw cycles (14,16,32-34). The extracts were centrifuged and the clear supernatants were stored in aliquots at -70°C . Ten μg postmitochondrial supernatant from control and treated cells were separated on 10% SDS-PAGE, followed by transfer onto nitrocellulose membranes. Following incubation with the respective primary (AR, PSA or actin) and secondary antibodies, specific immunoreactive bands were visualized using the enhanced chemiluminescence system (ECL) or by color reaction, as described by the manufacturer's protocol (Kirkegaard & Perry Laboratories) (16,32-34). Re-probing of blots with different antibodies was done after stripping with a buffer containing 62.5 mM Tris-HCl, pH 6.7, 100 mM 2-mercaptoethanol, 2% SDS, at 50°C for 10 min.

Isolation of RNA and analysis of PSA and AR expression by reverse transcription-polymerase chain reaction (RT-PCR). Total cellular RNA was isolated from control and treated LNCaP cells on day 3, using TRIzol reagent (Life Technologies, Inc.) according to protocols provided by the manufacturer. RNA purity and quantitation was determined by measuring the $A_{260/280}$ absorbance ratio. Reverse transcription of RNA into cDNA used Superscript™ Rnase H- reverse transcriptase (Gibco-BRL) at 42°C for 50 min. The RT products were amplified by PCR using AR, PSA, and β -actin specific forward/backward primer sets in a Hybaid thermal cycler. The AR primer had an expected size of 342 bp; the respective primers were 5'-CTCTCTCAAGAGTTTGGATGGCT-3' (primer 1) (location, 2896-2918) and 5'-CACTTGCACA GAGATGATCTCTGC-3' (primer 2) (location, 3214-3237). Primers for PSA were respectively 5'-CTCTCGTGGCAGG GCAGT-3' (location, exon 2) and 5'-CCCCTGTCCAGCG TCCAG-3' (location, exon 4), with 484 bp as the expected

size of the amplified product. Forward/backward primers for β -actin were 5'-GCAACTAAGTCATAGTCCGC-3' (location, 936-955) and 5'-CTGTCTGGCGGCACCACCAT-3' (location, 1170-1189) (expected size of amplified PCR product, 253 bp). The PCR products were separated in a 1.2% agarose gels and the relative intensity of the expected size fragments was documented using an Innotech imaging system.

Results

Studies with hormone-responsive LNCaP cells. Dissemination of tumor cells from the primary cancer site often involves their metastasis to lymph nodes. Accordingly, we first investigated the effects of 70% ethanol extracts of Equiguard on growth of LNCaP cells which are derived from an individual whose cancer metastasized to the lymph nodes (27), and which have been used as a model system for investigating parameters connected with androgen-responsiveness characteristic of prostate cancer cells (14,16,27,32).

Fig. 1A depicts that proliferation of cells were significantly inhibited by the addition of ethanol extracts of Equiguard. As little as 1 $\mu\text{l/ml}$ of extract was sufficient to cause a 30% reduction in cell growth after a 72-h incubation. Increase in Equiguard to 3 $\mu\text{l/ml}$ resulted in a proportional greater reduction in cell proliferation. To further confirm the growth-suppressive property of Equiguard, we utilized another growth characteristic of tumor cells in culture. Normal cells typically cease to proliferate as cell density increases; in contrast, tumor cells characteristically are unrestricted by contact inhibition and will continue to grow and form foci of clustered cell colonies. This assay, known as colony formation, is the basis for detecting the likelihood of neoplastic transformation. The assay can be performed by fixing and staining cells followed a defined period in culture and the number of colonies can be quantitated against the background (31). Fig. 1B shows that the clonogenicity of LNCaP cells is highly sensitive to ethanol extracts of Equiguard, with as little as 1 $\mu\text{l/ml}$ completely abolishing focus forming ability of LNCaP cells.

To further test the ability of ethanolic extracts of Equiguard to affect tumor cells in culture, we employed flow cytometry to measure cell cycle distribution. This is an automated technique that quantifies the relative number of cells in $G_0 + G_1$, S, or $G_2 + M$ phases of the cell cycle. Cultured cells are suspended as single cells, and stained with a fluorescent DNA dye (DAPI) (32-34). The sample of cells then passes a detector that records the relative DNA content based on the integrated intensity of fluorescence signal per cell. The data collected in this manner are transformed into a histogram. Fig. 1C shows the DNA histogram of LNCaP cells cultured with Equiguard extracts for 72 h: cells in G_2 and M phase of the cell cycle were unaffected, whereas cells in S phase decreased, concomitant with G_1 phase increase. In cells treated with the higher concentration of Equiguard, an additional peak (sub- G_1 cells), characteristic of cells undergoing apoptosis, became evident.

Effect of Equiguard on expression of PSA and AR. Another feature of the LNCaP cells is their ability to synthesize and secrete PSA, a 34-kDa tissue-specific glycoprotein with kallikrein-like serine protease activity (16,18,32). PSA is produced almost exclusively in epithelial cells lining the

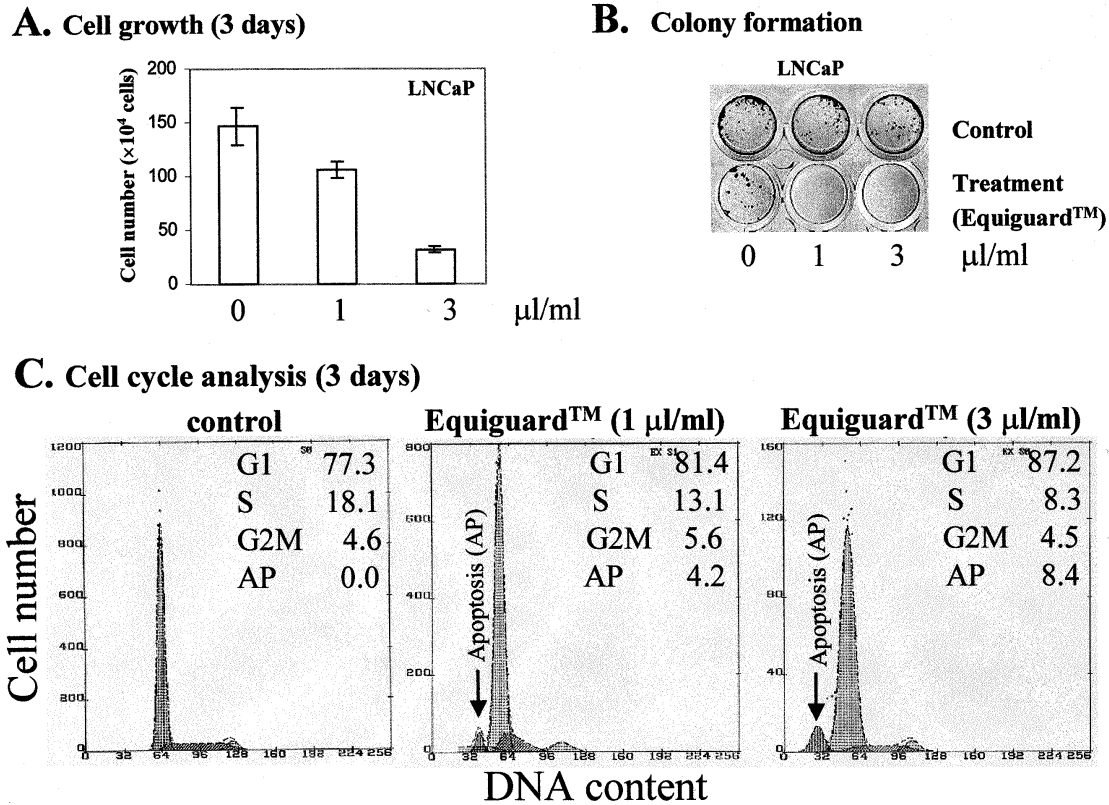


Figure 1. Effects of Equiguard on growth of LNCaP cells. (A), Cells were treated for 72 h with the indicated concentration of Equiguard, as described in Materials and methods. Growth was monitored by counting the cell number using a hemacytometer. (B), Effect of Equiguard on clonogenicity of LNCaP cells. (C), Effects of Equiguard on cell cycle phase distribution of LNCaP cells.

acini and ducts of the prostate, and is expressed in normal, benign prostate hyperplasia (BPH), and primary/metastatic prostate tissues. In normal prostate, serum PSA ranges from 0-4 ng/ml; elevated PSA (higher than 5 ng/ml) accompanies prostate carcinoma, benign prostate hyperplasia or prostatitis. PSA has been used as a serum marker to evaluate stages of prostate cancer, and for monitoring responses and progress of patients to different therapies (35-39).

Fig. 2A shows the decrease in PSA in the media of LNCaP cells after 1-3 days in culture with ethanol extracts of Equiguard. To test whether such decrease reflected a diminution of PSA expression, and to explore whether PSA changes is coordinated with regulation of its transcription factor AR, RNA was isolated from control and treated cells and the expression of PSA/AR was assessed by RT-PCR. Results in Fig. 2B show that both AR and PSA were significantly down-regulated in LNCaP cells by ethanol extracts of Equiguard.

Studies with hormone-independent DU 145 cells. In its advanced stages, prostate tumor often ends in distant target tissues such as the brain and bone. Accordingly, we investigated the effects of 70% ethanol extracts of Equiguard on growth of DU 145 cells, which were derived from brain metastasized prostate cancer cells. Both growth and clonogenicity were significantly inhibited by ethanol extracts of

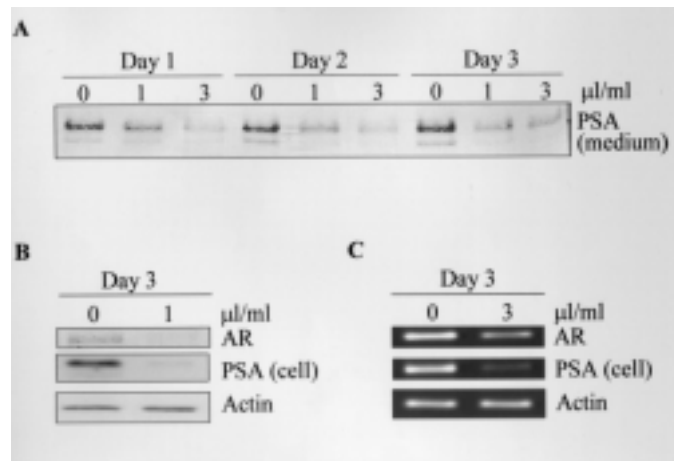


Figure 2. Control of AR and PSA expression by Equiguard in androgen-responsive LNCaP cells. (A), Immunoblot analysis of time-dependent changes in secreted PSA treated with two different concentrations of Equiguard. (B), Western blot analyses of intracellular AR and PSA in control and 72 h Equiguard-treated LNCaP cells. (C), RT-PCR analysis of changes in AR and PSA in control and 72 h Equiguard-treated LNCaP cells. In immunoblot analysis, postmitochondrial extracts were prepared from control and treated cells. Extracts were separated on 10% SDS-PAGE, transferred onto nitro-cellulose membranes, and incubated with the respective primary and secondary antibodies. Specific immunoreactive bands were visualized with enhanced chemiluminescence system (ECL) or by color reaction.

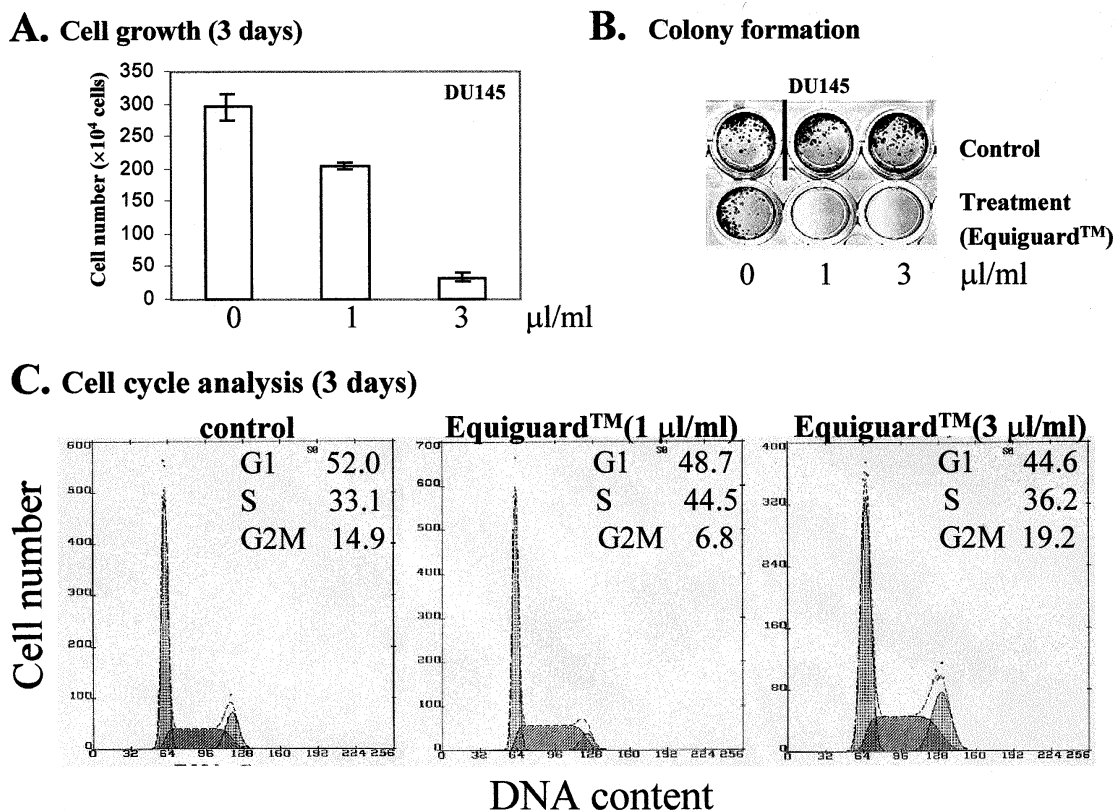


Figure 3. Inhibition of DU 145 cell growth by ethanol extract of Equiguard. (A), Proliferation of cells following a 72-h treatment with the indicated concentration of Equiguard. Growth was monitored by counting the cell number using a hemacytometer. (B), Effect of Equiguard on clonogenicity of DU 145 cells. (C), Effects of Equiguard on cell cycle phase distribution of DU 145 cells.

Equiguard. At the high concentration (3 µl/ml), proliferation decreased by 85% which was substantially more pronounced than that observed with LNCaP cells (Fig. 3A). Also, colony formation was markedly reduced by Equiguard (Fig. 3B). However, flow cytometric analysis did not reveal an arrest in G₁ nor an induction of apoptosis; rather, an increase in proportion of S phase cells and a decrease in G₂/M was seen at 1 µl/ml concentration (Fig. 3C). The differential cellular responses of LNCaP cells and DU 145 cells support the contention that multiple bioactive ingredients are present in Equiguard. Presence of a large array of diverse active ingredients in Equiguard attests to their potential for treating CaP, which is known to be heterogeneous (32).

Studies with hormone-independent JCA-1 cells. To obtain information on whether Equiguard exerts a comparable effect on non-metastasized, prostate tumors, its effect on growth of JCA-1 cells were studied. As illustrated in Fig. 4A, JCA-1 appeared to be more responsive to ethanol extracts of Equiguard, than LNCaP and DU 145 cells. Even at 1 µl/ml, a 45% suppression of cell growth was observed. This strong inhibition by Equiguard was validated using the colony formation assay (Fig. 4B). Flow cytometric analysis, however, demonstrated that the Equiguard primarily arrested cell cycle progression in the G₂/M phase. It should be noted that although JCA-1 cells were originally claimed to be established from a primary prostatic site prior to administration of hormonal therapy, suggesting that this cell line is an appropriate

representation of non-metastatic prostate cancer *in situ* (30), recent analysis using cytogenetics and DNA profiling methodologies, however, indicated that these cells are actually derived from T24 bladder carcinoma cells (40).

Studies with hormone-independent PC-3 cells. A major complication of prostate cancer is metastasis to bone. We investigated the growth response of PC-3 cells, derived from an individual whose cancer disseminated to the bone, to ethanol extracts of Equiguard. Even at the lowest concentration tested, a 70% reduction in cell growth was observed (Fig. 5A). Thus, compared to the other prostate cell lines, PC-3 cells were affected to the greatest degree by Equiguard. The basis of this growth disruption likely is attributed to a suppression of cell progression through S and G₂ + M phases, and additionally the induction of apoptosis.

Discussion

Mechanistic framework of anti-prostatic carcinogenic effects of Equiguard. The anti-prostatic properties of Equiguard, as a dietary supplement, have been validated in tissue culture studies described in this communication. Although the mechanism of action of Equiguard is not fully understood, its ability to regulate cell proliferation and PSA/AR gene expression may be readily comprehended using the scheme illustrated in Fig. 6. In line with traditional Chinese medicinal principles, efficacy of Equiguard relies upon combining

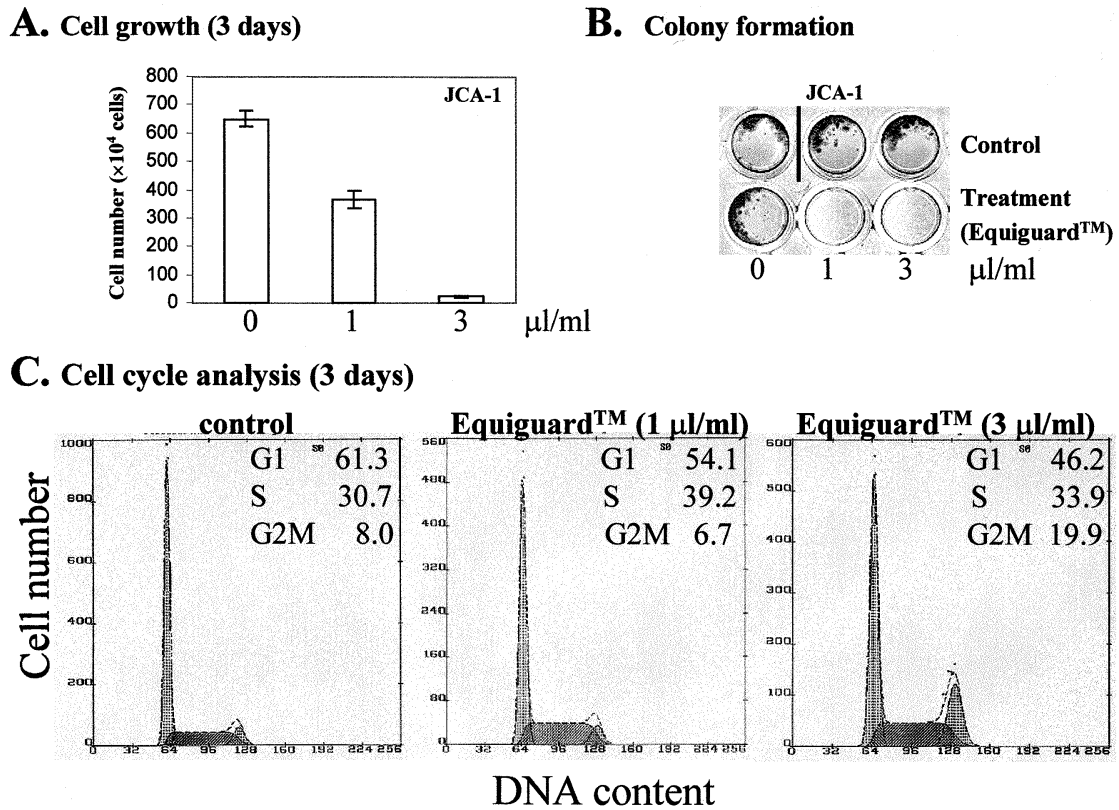


Figure 4. Responses of JCA-1 cells to Equiguard. (A), Cells were treated for 72 h with the indicated concentration of Equiguard, as described in Materials and methods. Growth was measured using a hemacytometer. (B), Effect of Equiguard on clonogenicity of JCA-1 cells. (C), Effects of Equiguard on cell cycle phase distribution of JCA-1 cells. As mentioned in the text, JCA-1 cells, originally believed to represent non-metastatic primary prostate carcinoma (30), have recently been shown to be a cross-contaminant of T24 bladder carcinoma cells, as evidenced by cytogenetic and DNA profiling analyses (40).

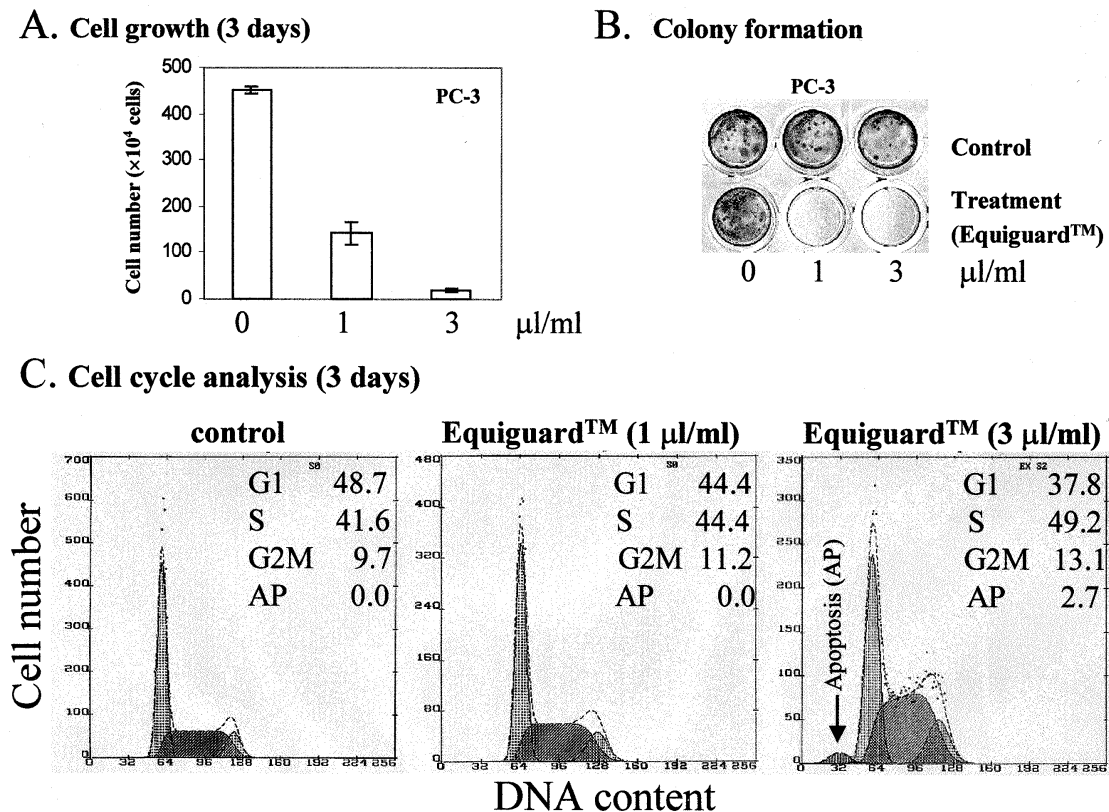


Figure 5. Interaction of Equiguard with PC-3 cells. (A), Analysis of cell proliferation after a 72-h treatment with the indicated concentration of Equiguard. Growth was assessed using a hemacytometer. (B), Inhibition of clonogenicity of PC-3 cells by Equiguard. (C), Changes in cell cycle phase distribution in Equiguard-treated PC-3 cells.

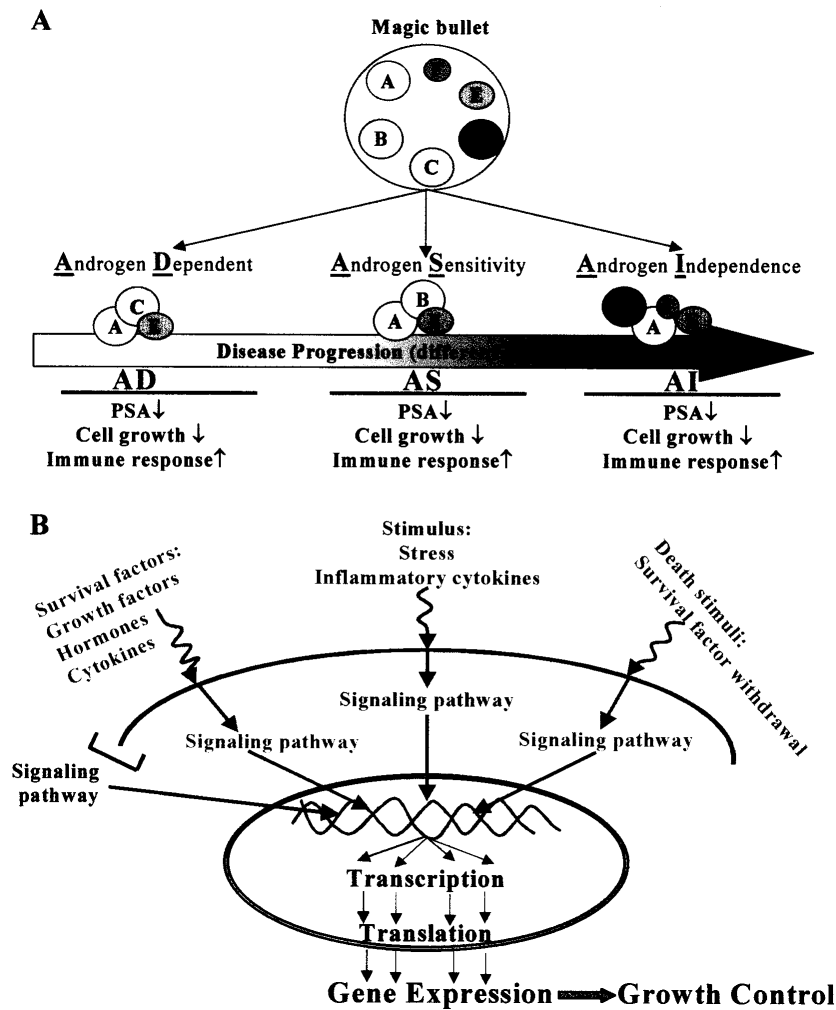


Figure 6. Proposed mechanistic model of Equiguard targeting various stages of prostate carcinogenesis. The scheme presented shows Equiguard is capable of acting at multiple targets which presumably underlies its ability to exert growth inhibitory and gene modulatory effects in prostate carcinoma cell lines mimicking the different stages of prostate cancer. Our hypothesis is that the herbal mixture probably is delivered to the target organ as a modular unit comprising of a cocktail of bioactive, inactive, and counter-active chemical ingredients manifesting a broad spectrum of biological activities, and hence are likely to be more effective compared to a single herb, with its much more limiting chemical profile. This hypothesis is in line with the basic concepts of Chinese traditional medicine, which espouses that functionality and efficacy of herbal formulations rely on strategic combination of different ingredients to potentially generate synergistic or novel activities.

multiple herbs to both enhance primary pharmaco-activity as well as to mitigate the toxicity of components present in the mixture. The success of Equiguard in treating both AD and AI prostate cancer cells may be due to unique combinatorial sets of active ingredients intrinsically present in Equiguard, capable of efficiently targeting multiple pathways, which functionally overlap to provide growth stimulatory advantage to prostate cells at different stages of carcinogenesis (Fig. 6).

Use of AD and AI prostate cancer cells provide scientific evidence for the anti-prostatic carcinogenic properties of Equiguard. On the basis that a single cancer cell injected into an appropriate host suffices to give rise to a tumor, many studies have been performed using cultured tumor cells, which have been considered invaluable as model systems for studying diseases such as cancer and for analyzing conditions and factors contributing to gene expression and regulation. In the present communication, four prostate cancer cell lines, three androgen-independent and one androgen-dependent, were used in preliminary studies to explore the mechanism of action

of Equiguard. Results of these experiments provide overwhelming evidence of the apparent effectiveness of ethanol extracts of Equiguard to inhibit proliferation, reduce PSA expression, and to elicit significant changes in cell cycle phase distribution. It would appear that PC-3 was most sensitive to the growth inhibitory effects of Equiguard; LNCaP and JCA-1 were affected to a comparable degree, and DU 145 cells were least inhibited. LNCaP and PC-3 cells were additionally induced to undergo apoptosis by this herbal supplement. Together, the combined weight of the evidence is consistent with an anti-tumor effect of Equiguard. Further studies with cells of non-urolithic lineages are needed to elucidate whether this effect is specific to prostate cancer or is of wider scope.

Prevention and treatment of CaP using natural products and herbal remedies. Considerable uncertainty and skepticism exist among scientists, healthcare professionals, and the public on the use of natural products and herbal remedies for treating various human diseases. Many outstanding issues remain: lack of state-of-the-art research, questions regarding identification

and characterization of active ingredients, product quality assurances, toxicity and possible interaction with medicines in use, absence of properly designed and hypothesis-driven clinical trials, missing details of mechanisms of action, etc. (14-16). These concerns notwithstanding, it is also becoming increasingly clear that there is a growing casual exposure to and at times significant reliance on this form of therapy, in conjunction with or exclusive of mainstream medicine (41-43).

Understanding and appreciation of herbal remedies, as an integral part of alternative/complementary/integrative medicine, can best be achieved by first accepting the paradigm of 'balanced/wholesome' therapeutics. This form of therapy differs from the conventional 'pharmaceutical' approach of identifying and isolating the most potent of the active principles, using *in vitro* or, more rarely, animal models and clinical studies. Instead, the 'balanced/wholesome' paradigm emphasizes the total spectrum of bioactive ingredients present and the 'well being/curing' of the patients as a whole. Applying this concept to Equiguard, it is likely that the efficacy of this herbal supplement is inextricably linked to 'group' administration of bioactive agents, resulting in effectual control of cellular proliferation and regulation of prostate specific gene expression, in target cells. This approach resembles the famed 'cocktail' approach, sometimes referred to as HAART (highly active antiretroviral therapy), for the treatment of HIV in infected individuals (44-46). Unlike HAART, however, whose success is predicated on detailed knowledge of the replication cycle of the HIV virus, herbal formulations such as Equiguard is not necessarily dependent upon precise knowledge of the specific molecular defect/derangement in target cells. Accordingly, it is possible to have identical clinical benefits using formulations that are totally different in its composition herbs.

In summary, we have shown that ethanol extract of Equiguard is effective in suppressing growth of AD and AI prostate cancer cells. Interestingly, the cell cycle progression was affected in different phases of the cycle, depending on the cell line. Thus, the progression of LNCaP cell was suppressed in G₁ phase, the remaining lines were affected more extensively in S phase. It is likely that diverse components of this complex herbal extract target different cell cycle regulatory elements in different cell lines. Further studies are required to examine in greater detail its mechanism of action. Also of interest and importance are further identification and elucidation of relevant chemicals present, in the context of efficacy against prostate carcinogenesis. A combination of physico-chemical principles, e.g., HPLC, together with gas chromatography and mass spectrometry, and mechanistically based biochemical and molecular assays, may be envisaged to achieve these objectives. Some of these initiatives are already underway in this laboratory. These studies in the future should broaden our understanding of the potential use of Equiguard as an herbal functional unit for the prevention and treatment of prostate cancer and other forms of malignant disorders.

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