Ethanolic extracts of herbal supplement Equiguard™ suppress growth and control gene expression in CWR22Rv1 cells representing the transition of prostate cancer from androgen dependence to hormone refractory status

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Received August 9, 2007; Accepted September 13, 2007

Abstract. Dietary supplements and botanical products are widely used by patients diagnosed with prostate cancer (CaP) as a primary or adjuvant form of treatment for their medical conditions in the United States. Many of the available products are complex mixtures composed of extracts from foreign plants, whose mechanism of action typically is not systematically and rigorously investigated. Laboratory studies employing precisely defined conditions and referenced methodologies are essential not only for standardization and characterization of the products, but are also important requisites for providing scientific evidence and molecular insights in regard to the clinical efficacies some of these products purportedly demonstrate. In previous studies from this laboratory, we serendipitously observed that Equiguard™, a dietary supplement formulated with extracts from nine Chinese herbs for preventing decline in renal functions associated with the aging process, contain 70% ethanol-extractable ingredients that displayed potent growth inhibitory activities in androgen-dependent (AD) LNCaP and androgen-independent (AI) DU-145 and PC-3 cells. Moreover, significant reduction in expression of the androgen receptor (AR) and prostate specific antigen (PSA) also occurred in Equiguard-treated LNCaP cells. Although these results offer the possibility that Equiguard confers chemoprevention for CaP, it remains undetermined whether Equiguard functions in CaP cell types that represent the transition of AD to the AI status. Further, details of its mechanism of action have not been fully elucidated. The studies described in this report focusing on CWR22Rv1 cells are intended to fill these gaps. These cells express AR and PSA, yet show weak responsiveness to androgens and largely proliferate in an AI-independent manner - features that mimic AD→AI in clinically advanced disease. Using the CWR22Rv1 cells, we showed that 70% ethanolic extracts of Equiguard effectively suppressed colony formation, inhibited cell proliferation, reduced expression of cell cycle regulatory proteins including cyclin D1, E2F, as well as lowered AR and PSA levels. Treatment of CWR22Rv1 cells with Equiguard also decreased cyclooxygenase 2 and led to increases in quinone reductase 1 and 2. These results provide further support that Equiguard possess multiple, chemopreventive attributes capable of disrupting the transition of AD→AI in clinically advanced CaP.

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Key words: Equiguard, CWR22Rv1 cells, cell cycle control, prostate specific antigen, androgen receptor, cyclooxygenase 2, quinone reductase

Introduction

According to recent published data, cancer-related mortalities in the United States when grouped by age have surpassed deaths attributed to coronary heart disease (1). In the case of prostate cancer (CaP), incidence rates from localized cases have increased steadily during most of the 1990s, and only in recent years has a slightly decrease in newly diagnosed cases been found among white men with a plateau seemingly reached among African Americans (2). Despite these encouraging trends, CaP remains the most commonly diagnosed non-cutaneous cancer in males and a leading cause of lethal malignancy worldwide (3-5).

Prostate cancer is a disease with significant medical, social and financial implications in many developed countries and increasingly, also developing countries. Localized and regionally confined disease responds readily and favorably to androgen ablation therapies, via surgical or medical castration. However, the improved clinical presentations of patients treated with such modalities are almost invariably followed by the ultimate appearance of hormone-hypersensitive and -independent clones and the recurrence of disease.

The incidence rate of latent CaP is quite similar across different geographical locations and races (6). In contrast, clinical CaP is more prevalent in the West, compared to countries in Asia (7,8). These sobering statistics have frequently been used to provide support for the notion that western diets promote the progression of latent CaP to its more aggressive, invasive form. However, an often overlooked possibility is that non-western diet and culture-specific lifestyles might induce the reversal of high-grade intra-

epithelial neoplasia (PIN) as well as render the maintenance of subclinical CaP, thereby confer protection by prolonging the time required for hormone-independent CaP status to be reached and established. No curative therapies currently exist for metastasized, hormone-independent CaP, underscoring the urgent need to develop cancer prevention strategies targeting the modulation of transition of androgen dependent (AD) to androgen-independent (AI) status. For these reasons, CaP patients have become increasingly interested in complementary and alternative medical options (CAM) on the premise that they might contain unique bioactive ingredients and distinct arrays of phytochemicals. First, CAM therapies have been the mainstay of treatment for the prevention and treatment of human diseases in many cultures. Second, herbal therapies are often prescribed as complex formulations containing multiple herbs, plants and botanical extracts, many of which are indigenous to countries other than the United States, and therefore might exhibit novel functions that amplify and harmonize the positive, clinically relevant effects while at the same time neutralize and minimize the toxic, adverse attributes of the constituents (9,10). It is also noteworthy that natural products and compounds derived from such sources historically have played a significant role in advancing the development of drugs and therapeutic agents (11-13).

Our laboratory has been interested in the study of dietary ingredients and plant-based supplements as modulators of AD→AI transition and as agents that may delay the establishment of hormone refractory prostate cancer (HRPC) (14-18). Our recent interests have been directed at a complex botanical formulation, denoted Equiguard (19-21), as a prototype of dietary supplement befitting the overarching rubric of plant-based cancer chemopreventive strategies. Equiguard contains mixtures of extracts derived from several plants indigenous to China, which was formulated to restore age-associated decline in renal functions. Through a laboratory screen of a number of cancer cell lines, we fortuitously observed that Equiguard displayed potent anti-CaP activities in cells mimicking the AD and AI states (21). We showed that ethanol extracts of Equiguard significantly suppressed cancer cell proliferation, restored apoptosis, inhibited expression of AR and PSA, and almost completely eliminated the colony forming capabilities of cancer cells (19-21). These results are consistent with the possibility that Equiguard may restrict, retard and obviate the transition of AD to AI, and facilitate establishment of clinically manageable instead of detrimental CaP.

It is of interest to query whether Equiguard functions in CaP cell types that represent the transition of AD to the AI status. Accordingly, in this report, we studied the effects of 70% ethanolic extracts of Equiguard using CWR22Rv1 cells. These cells were derived from a xenograft that was serially propagated in athymic male mice as a result of androgen withdrawal induced regression and relapse of the parent, androgen-dependent CWR22 xenograft (22). Notably, the CWR22Rv1 cells express AR and PSA, yet are marginally dependent on androgens and essentially grow in an AI manner (22). We found that ethanolic extracts of Equiguard effectively reduced colony formation, cell proliferation, and expression of several important cell cycle regulatory proteins in CWR22Rv1 cells including cyclin D1, E2F, and PCNA. Moreover, levels of prostate specific genes, AR and PSA, were also suppressed in CWR22Rv1 cells by Equiguard to a degree similar to those previously observed as in LNCaP cells (21). Treatment of CWR22Rv1 cells by Equiguard also increased phosphorylation at S15 of the tumor suppressor gene p53. We also observed decrease in cyclooxygenase 2 and increases in quinone reductase 1 and 2 expression in Equiguard-treated CWR22Rv1 cells.

Materials and methods

Cell culture. CWR22Rv1 cells (CRL-2505) were purchased from American Type Culture Collection (Rockville, MD) and maintained in RPMI-1640 media containing 2 mM L-glutamine, supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) (9,10,14-17,21,23,24). Cells were subcultured once every week by trypsinization and reseeding in new culture flasks.

Preparation of ethanol extracts of Equiguard. Equiguard, formulated from Chinese herbs according to a proprietary method and used in the present studies (lot 3BA03020528, expiration 28-5-05), was supplied by ICM Holdings, Ltd. (Hong Kong, P.R. China). It was certified to be free of heavy metal and bacteria contamination by government approved testing centers in Hong Kong. To prepare 70% ethanolic extract, the contents of each capsule was suspended in 6 ml of 70% ethanol, followed by intermittent mixing and vortexing for 60 min at room temperature and a centrifugation step to remove insoluble particulates. The clear supernatant was sterilized by filtration and stored in aliquots at 4˚C. Each aliquot was used only once and discarded. This method of preparation yielded a 6-fold diluted supernatant compared to those used in previous studies from our laboratory (19-21).

Effects of 70% ethanolic extracts of Equiguard on colony formation, cell proliferation, and cell cycle progression in CWR22Rv1 cells. The procedure for colony formation was as described (21,24,25). CWR22Rv1 cells maintained in RPMI-1640 supplemented with 10% FBS were seeded at 2000 cells/well in 6-well plates. Treated cultures received (0.5-7.5 μl/ml) 70% ethanolic extracts of Equiguard, prepared as described above. After 14 days in culture the cells were fixed and colonies were visualized by staining with 0.1% crystal violet. Effects of Equiguard on colony formation were determined by removing excess dye and documenting the formed colonies by photography. Next, each individual well in the plate was extracted with 0.1 ml 10% acetic acid to extract the cell-retained dye whose absorbance at 595 nm was measured to provide a quantitative determination of colony formation (26).

To assay the effects of Equiguard on cell growth, CWR22Rv1 cells were seeded at $5x10^4$ cells/ml in T-75 flasks and allowed to attach overnight. Cells were incubated with 1, 3, and 5 μl/ml ethanolic extracts of Equiguard and were harvested by trypsinization on days 1 and 3. Cell number was determined using a hemocytometer and viability was monitored by trypan blue dye exclusion, as described previously (14-16,19,20). Culture media were collected and saved for determination of secreted PSA.

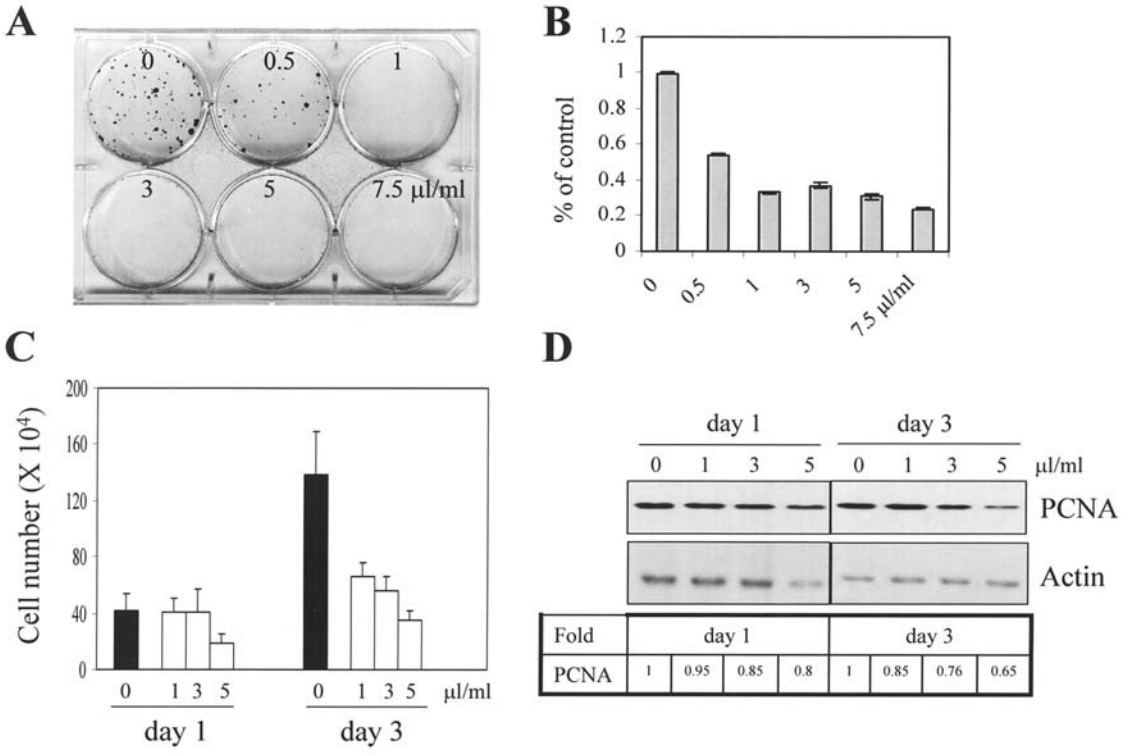


Figure 1. Effects of Equiguard on clonogenicity and proliferation of CWR22Rv1 cells. Results show the mean ± SD of at least 3 separate experiments. A, Clonogenicity following Equiguard treatment was measured as described in Materials and methods. The photographs showed colonies formed. B, Quantitative analysis of colony formation depicted in A as measured by absorbance of acetic acid extracted crystal violet dye retained by the cells at 595 nm. C, Effects of Equiguard on growth of CWR22Rv1 cells, as measured by trypan blue exclusion on days 1 and 3 after treatment. D, Effects of Equiguard on PCNA expression in control and Equiguard-treated cells. Relative changes in PCNA expression in control and days 1 and 3 treated cells were measured by Western blot analysis as described in Materials and methods. The intensities of the PCNA- and actin-immunoreactive bands were quantitated by densitometry and presented as relative expression levels, with the control values for each day of treatment showing as 1. A significant difference in growth was shown between control and Equiguard treated cells, $P<0.005$. Reduction in cell growth and PCNA expression by Equiguard was also significant.

Cell cycle phase distribution was analyzed by flow cytometry. Cultures were exposed to varying concentrations of the Equiguard extracts (1, 3 and 5 μl/ml) for 1 or 3 days and harvested. Cells were washed once with PBS and stained with 1.0 μg/ml DAPI containing 100 mM NaCl, 2 mM $MgCl_2$ and 0.1% Triton X-100 (Sigma) at pH 6.8 as previously described (21,23). 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 are collected and analyzed using the Multicycle™ software provided by Phoenix Flow Systems (San Diego, CA).

Measurement of intracellular and secreted PSA. Intracellular and secreted PSA levels were assayed as described (9,10,14-17, 21,23,24).

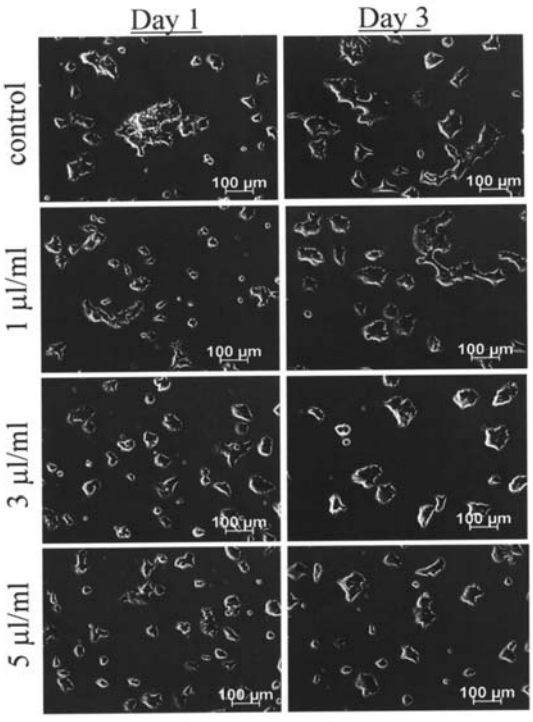
Protein extraction and Western blot analysis. Cell extracts were prepared by freeze/thaw cycles. Separation of proteins by 10% SDS-PAGE and immunoblot analysis, using the enhanced chemiluminescence system (ECL) or by color reaction, followed published procedures from this laboratory, as detailed (14-16,27).

Results

Effects of Equiguard on colony formation and cell proliferation in CWR22Rv1 cells. In previous studies from this laboratory, we have shown that Equiguard, a polyherbal dietary supplement, displayed anti-tumor activity against CaP cell lines mimicking the subclinical, hormone-responsive (LNCaP), and the advanced, hormone-refractory status (PC-3, DU-145 and JCA-1) (21). To determine whether Equiguard might show activity in individuals whose disease has progressed to a state of transition between androgen-dependence and independence, we tested its effects using CRW22Rv1 cells, an AR- and PSA-expressing cell line derived from androgen withdrawal induced regressed and subsequently relapsed progeny of the CWR22 xenograft (22).

First, the effects of 70% ethanolic extracts of Equiguard on colony formation were determined. This assay assesses the potential of tumor cells to repopulate based on retention of their ability to undergo sufficient rounds of cell division as to generate a visually defined and quantifiable 'foci/clones/colonies'. This is a simple, fast, inexpensive first-line method that gives a reasonably good predictive value (69%) with regard to patients' at-a-minimum response or resistance to a therapeutic regimen. Accordingly, CWR22Rv1 cells were treated with various concentrations of Equiguard, and the colonies formed were visually inspected after 14 days in culture by staining with crystal violet (Fig. 1A). Quantitative analysis of colony formation was based on spectrophotometric determination of cell-retained dye (Fig. 1B). A noticeable reduction in colony formation was already evident in cells treated with 0.5 μl/ml Equiguard; at ≥1 μl/ml, foci-forming ability of CWR22Rv1 cells was suppressed to the background

A



B

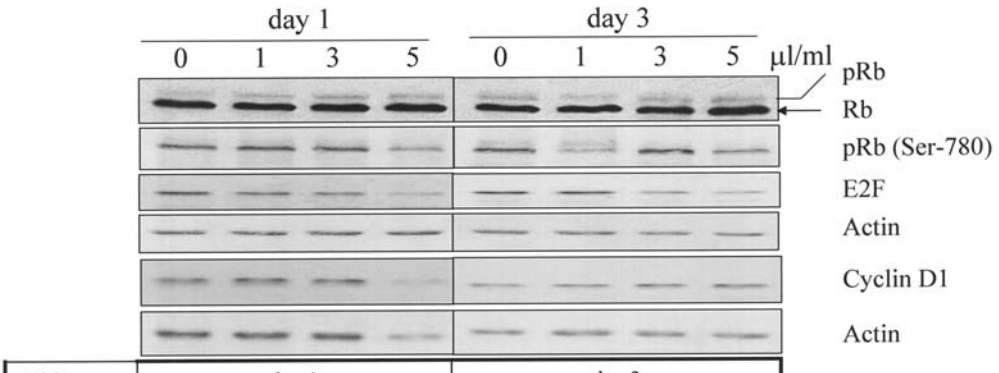
	Day 1				Day 3			
	control	1 µl/ml	3 µl/ml	5 µl/ml	control	1 µl/ml	3 µl/ml	5 µl/ml
G1	37.8	36.7	38.0	38.9	36.2	34.1	33.8	35.1
S	23.7	22.0	20.2	19.6	21.0	21.5	20.7	20.8
G2/M	38.5	41.2	41.8	41.5	42.8	44.4	45.5	44.1

Figure 2. Cell morphology and cell cycle phase distribution associated with treatment of CWR22Rv1 cells with ethanolic extracts of Equiguard. A, Morphological changes induced by Equiguard. The photographs (magnification x100) showed days 1 and 3 control and different dose treated cells. B, Cell cycle phase distribution changes of CWR22Rv1cells following 1-3-day treatment with different doses of Equiguard. Flow cytometric analysis was performed as described in Materials and methods.

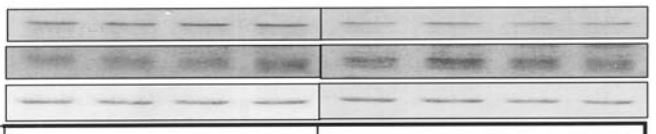
level. To confirm the growth modulatory effects of Equiguard, CWR22Rv1 cells were treated with various concentrations of Equiguard for 1-3 days, at the end of which cell number and viability was determined using the trypan blue exclusion assay. Dose-dependent suppression of growth was observed after 3 days of treatment, and even by day 1 at the highest concentrations used (Fig. 1C). The degree of suppression of cell growth was similar to that observed in the colony-forming assay, and correlated with reduction in PCNA expression, a commonly used index to validate proliferative activity, which was monitored by Western blot analysis and quantified by imaging analysis (Fig. 1D).

Effects of Equiguard on changes in cell morphology and cell cycle distribution in CWR22Rv1 cells. To further investigate the growth modulatory effects of Equiguard in CWR22Rv1 cells, we examined changes in cell morphology. CWR22Rv1 cells in culture grow as small patchy, cluster of cells, probably reflecting propensity for adherence to each other as cells proliferate. With addition of varying concentration of Equiguard, not only was a reduction in cell growth observed, cells also grew in a noticeably more disperse manner (Fig. 2A), suggesting that a significant alteration in cellular property resulted from exposure to extracts prepared from this polyherbal formulation. To obtain additional information on the nature of Equiguard-induced cellular changes, we performed flow cytometric analysis using control and treated cells. In these experiments, CWR22Rv1 cells were treated with the vehicle alone (0.1% DMSO) or with different concentrations of Equiguard. At 1 or 3 days post treatments, control and treated cells were collected, stained with DAPI, and the changes in cell cycle phase distribution was determined (Fig. 2B). Following a short-term treatment (day 1), a small change in cell cycle distribution was found, which was shown as a consistent increase in G_2/M phase cells, with a corresponding decrease in S phase cells, suggesting that ethanol extracts of Equiguard prolonged the transition of cells in the G_2/M phase of the cell cycle. By day 3 of treatment, however, this small change in cell cycle phase distribution was no longer evident. It should be noted that cell cycle analysis in the present studies used concentrations of Equiguard considerable lower than those of our earlier studies in AD and AI cells (21).

Effects of Equiguard on changes in the expression of cell cycle regulatory proteins and on the state of phosphorylation of the



Fold	day 1				day 3			
Rb (total)	1	1.1	1.2	1.25	1	0.87	1.1	1.15
pRb (ser780)	1	1.15	1.2	0.85	1	0.75	1.1	0.88
E2F	1	0.85	0.8	0.65	1	0.9	0.86	0.7
Cyclin D1	1	1.15	1.2	0.68	1	0.95	0.9	0.9



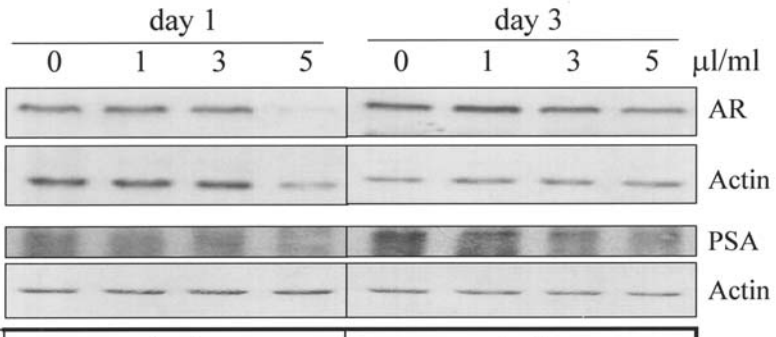
Fold	day 1				day 3			
P53	1	0.95	1	1.1	1	1.1	0.9	0.85
P53-p(ser15)	1	1.1	1.2	1.3	1	1.35	1.1	1.1

Figure 3. Changes in cell cycle regulatory proteins in control and 1-3-day Equiguard-treated CWR22Rv1 cells. Following a 1- or 3-day treatment with different doses (≤5 µl/ml) of Equiguard, cells were harvested and total protein extracts were prepared as described in Materials and methods. Aliquots of total extracts were separated by SDS-PAGE and analyzed for expression of Rb, pRb (S780), E2F, cyclin D1, the total and S15 phosphorylated p53. Immunoblots were quantified and shown as relative expression levels, with the control values for different days of treatment showing as 1.

tumor suppressor protein, p53. Since colony formation and cell proliferation was significantly suppressed in CWR22Rv1 cells treated with ethanolic extracts of Equiguard, we proceeded to determine cell cycle regulatory protein expression changes by Western blot analysis. Results are illustrated in Fig. 3. A transient inhibition of cyclin D1 expression was observed after 24-h treatment, whereas a more persistent decrease in E2F expression was also observed throughout the duration of the experiment. In contrast, little to no change in total or hyperphosphorylated form of Rb was found. Because status of the p53 is intimately linked both with cell cycle control and induction of apoptosis, we determined the effects of Equiguard on state of phosphorylation of S15 of the p53, which is known to coincide with the p53's ability to associate with different regulatory protein during cell cycle progression and in response to stress and DNA induced damage (28-30). Changes in expression and state of phosphorylation of the p53 were evaluated by taking control and treated samples at days 1 and 3. Fig. 4 shows that treatment with Equiguard resulted in modest but sustained increases in S15 without commensurate changes in expression of the p53.

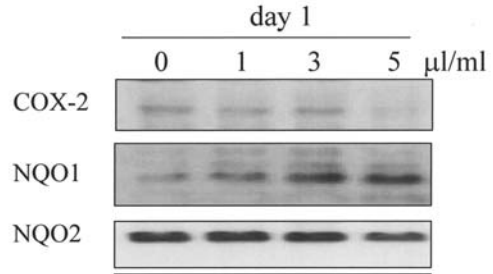
Changes in AR and PSA expression in Equiguard treated CWR22Rv1 cells. Changes in PSA is considered a key serum marker for CaP progression and for patients' response to treatment (31-34). Evidence to date suggests that the expression of PSA is usually coupled to changes in the AR (14,15,20,21). Therefore, we evaluated changes in the cellular content of these two prostate specific genes in control cultures and cells treated with Equiguard. CWR22Rv1 cells treated with different herbal extracts for 1 or 3 days were harvested and expression of proteins was analyzed by Western blot analysis using protein-specific antibodies. Significant suppression of AR and PSA expression occurred in day 1-treated cells (Fig. 4). The pronounced reduction in PSA persisted even at day 3 of treatment. These results show that control of PSA by Equiguard in general is linked to the status of AR in CWR22Rv1 cells. In addition, time- and dose-dependent measurement of secreted PSA in the culture media of control and treated cells showed that Equiguard also suppressed PSA secretion (data not shown).

Effects of Equiguard on expression of cycloxygenase 2 and quinone reductases. Evidence from epidemiological studies and clinical observations has provided support for the notion that persistent chronic inflammation plays an important yet not totally defined role in prostate carcinogenesis (35-37). In the early phase of inflammation, the enzyme cyclooxygenase 2 (Cox-2) facilitates the production of inflammatory prostaglandins. Cox-2 has been shown to be overexpressed in a variety of cancers (36-41). Further, there is compelling evidence from *in vitro* experimental studies that inhibition of COX-2 decreases cellular proliferation, increases apoptosis, and modulates genes involved in cell cycle regulation (36,37),



Fold	day 1				day 3			
AR	1	1.1	1.1	0.68	1	1.15	0.95	0.85
PSA	1	0.85	0.85	0.5	1	0.75	0.66	0.5

Figure 4. Effects of Equiguard on changes in intracellular AR/PSA in CWR22Rv1 cells. Following a 1- or 3-day treatment with Equiguard, cells were harvested and total protein extracts were prepared as described in Materials and methods. Aliquots of total extracts were separated by SDS-PAGE and analyzed for intracellular AR and PSA by immunoblot analysis. The results are presented as intensity of the Western blot analysis, and suggest that Equiguard affects intracellular AR and PSA in a somewhat coordinated manner.



Fold	day 1			
COX-2	1	0.85	0.9	0.7
NQO1	1	1.5	1.8	2.5
NQO2	1	0.95	1.08	1.5

Figure 5. Effects of Equiguard on changes in cyclooxygenase 2 (Cox-2) and quinone reductase (NQO1 and NQ02) expression. Following a 1-day treatment with varying amounts (≤5 μl/ml) of Equiguard, cells were harvested and total protein extracts were prepared as described in Materials and methods. Aliquots of total extracts were separated by SDS-PAGE and analyzed for the referenced protein expression by immunoblot analysis. The results shown were normalized against the expression of actin.

therefore we measured Cox-2 expression in control and treated cells. Results in Fig. 5 show that Cox-2 expression was significantly suppressed by treatment with ethanol extracts of Equiguard.

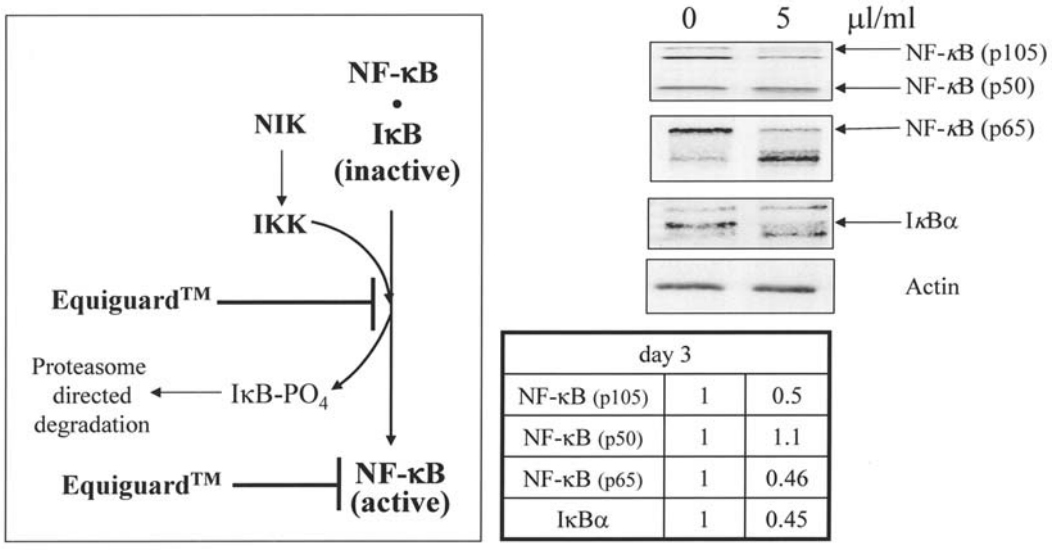
We also studied the genes encoding enzymes that are important for drug metabolism, specifically, detoxification by phase II enzymes, NQO1 and NQO2, which are genetically distinct but functionally homologous cytoplasmic flavoproteins that play a key role in the defense against chemical toxicants and carcinogenic agents (24,42,43). Results in Fig. 5 show that NQO1 was significantly upregulated by Equiguard. A more modest increase in NQO2 was similarly observed.

Effects of Equiguard on expression of NF-κB. To further explore the effects of Equiguard on regulation of Cox-2, we studied changes in nuclear factor NF-κB. Screen of NF-κB levels showed that this protein was expressed in a highly variable manner in different CaP cells. The LNCaP cells, which had a robust expression of NF-κB, were chosen for these experiments. Day 3 control and 5 μl/ml Equiguard-treated cells were lysed and total NF-κB was assayed using immunoblot analysis. A marked reduction in NF-κB expression (based on immunoreactivity to the p110 and p65 subunits of NF-κB) was clearly evident in Equiguard-treated cells (Fig. 6). The inhibition in NF-κB expression was accompanied by a comparable decrease of IκB expression, suggesting a possible causal relationship.

Discussion

Results of the present studies provide additional support for the notion that ethanolic extracts derived from the polyherbal supplement Equiguard effectively suppress proliferation, reduce clonogenicity, and exert differential gene regulatory activities in CWR22Rv1 cells, which were modeled after the transition of CaP from the androgen responsive to androgen refractory status (22). These new findings together with our earlier data using AD and AI CaP cells are consistent with the interpretation that Equiguard contains ingredients capable of acting on the entire spectrum of *in vitro* cell types representing the different stages of prostate carcinogenesis. Moreover, the bioactive agents contained in Equiguard must exert mechanisms whose effects not only target CaP cell division and proliferation, and induction of apoptosis, but also are capable of suppressing inflammation while stimulating the cellular detoxification capacity for chemicals such as quinones which are known to have potentially deleterious cellular consequences (44-49).

Comparison of the anticellular effects of Equiguard from experiments described in this report and from earlier studies shows that the CWR22Rv1 cells were most responsive to the 70% ethanolic extracts of this herbal formulation. To achieve the same degree of suppression of colony formation and cell growth in LNCaP and PC-3 cells, a 2-fold higher concentration



day 3		
NF-κB (p105)	1	0.5
NF-κB (p50)	1	1.1
NF-κB (p65)	1	0.46
IκBα	1	0.45

Figure 6. Effects of Equiguard on changes in NF-κB and in IκB untreated and Equiguard treated cells in LNCaP cells. Following a 3-day treatment with 5 μl/ml Equiguard, cells were harvested and total protein extracts were prepared as described in Materials and methods. Aliquots of total extracts were separated by SDS-PAGE and analyzed for expression of NF-κB and IκB by Western blot analysis. Relative expression of NF-κB and IκB was determined by densitometric scanning and presented as arbitrary units, after adjusting for expression of actin.

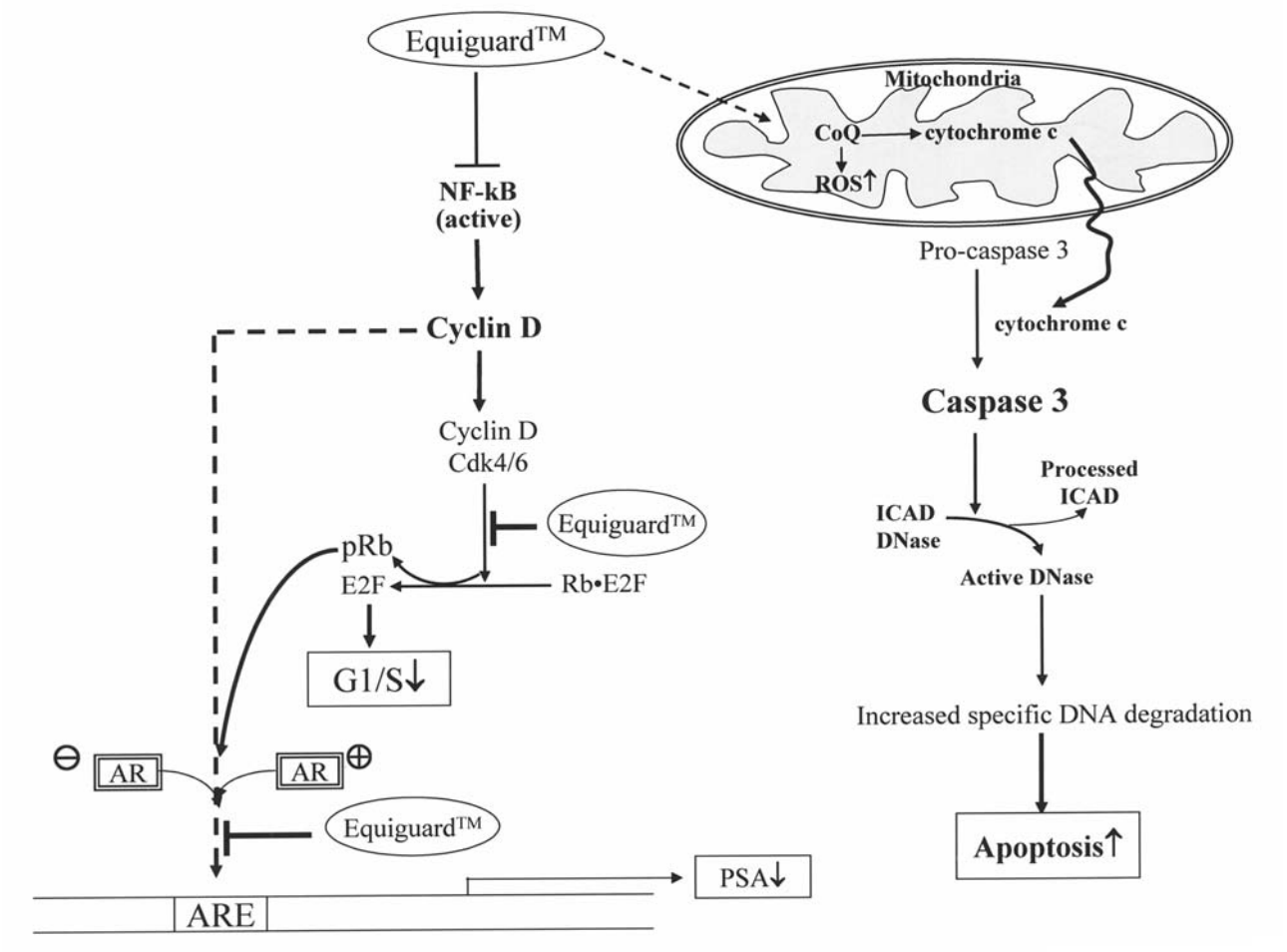


Figure 7. A proposed model for mechanism of inhibition of CaP by Equiguard. In this model, Equiguard is postulated as having diverse biological activities that impinge on prostate cancer cell proliferation and function. The ability of Equiguard to induce apoptosis is hypothesized to involve a disruption of mitochondria structure and function, leading to the release of cytochrome c, followed by subsequent activation of caspases and ensuring DNA damage and fragmentation. Equiguard also affects cell cycle progression and prostate specific gene expression, by suppressing the expression of nuclear factor NF-κB, which controls the transcriptional control of 'survival genes' including proteins having a pivotal role in G_1/S transition of the cell cycle. Inhibition of expression of cyclin D and pRb by Equiguard is surmised to affect the expression of PSA, a known biomarker for prostate carcinogenesis.

of the extract was required, whereas still higher doses of Equiguard must be added in culture for the same growth inhibitory effects to become evident in DU-145 and JCA-1 cells. Collectively, therefore, it would appear that this dietary supplement, originally formulated to re-invigorate age-associated deterioration in renal functions, indeed offer potential for preventing prostate-specific diseases including CaP. This may not be unexpected since the entire urological system including the prostate is considered an integral part of the kidney in Traditional Chinese Medicinal classification, both functionally and anatomically (9). Further, although direct extrapolation of laboratory findings to clinical outcomes would not be possible, our observations may also imply that AD patients and individuals with disease at the transition stage between AD and AI respond more readily and favorably to Equiguard than men whose cancer has progressed to the HRPC status. Definite proof for this possibility, however, must await appropriate animal model investigations and ultimately randomized clinical studies in humans. At a minimum, however, our *in vitro* studies have provided valuable mechanistic cues and disease-relevant end points that may be applicable not only to Equiguard but to other dietary supplements being considered for development in preventing and managing prostate cancer.

Mechanism of action of Equiguard and potential of polyherbal formulations and phytochemicals in the prevention of prostate cancer. Classically viewed as a single-cell disease in which a continuum of escalation in carcinogenicity among epithelial cells is believed to contribute to the range of clinical heterogeneity seen in patients, studies of tumor biology in recent decades have clearly demonstrated that cancer is a disease simple in its monoclonal inception yet complex in its progressive clinical presentations and diverse genetic and epigenetic dysfunctions. These advances have provided a solid scientific basis for why combined therapies targeting multiple steps and pivotal transition states in malignancies are preferred as hope and meaningful treatment/management options for cancer. This very same concept bears striking similarities to the empirical design and concept of using Chinese polyherbal formulations and phytochemicals as part of the armamentarium for cancer prevention including Equiguard. Moreover, because polyherbal formulations may have less clearly defined combinatorial characteristics, they actually might create a preferred setting to control the multifactorial nature of many malignancies and the complex, heterogeneous characteristics of clinically manifested cancer.

We present in Fig. 7 a model on how Equiguard harboring multi-targeted bioactive ingredients might function mechanistically to attenuate AD to AI transition. This model forms a framework for tackling HRPC since postponement or total aversion of the onset of AI, concomitant with the maintenance of AD, might result in a largely chronic medical condition rather than a terminal disease. In this model, Equiguard is postulated as having diverse biological activities that impinge on prostate cancer cell proliferation and function.

First, Equiguard affects cell cycle progression. Results of the present and previous studies show that Equiguard effectively targets cell cycle traverse - a molecular aberration presumably common to all malignant disorders. For example, suppression of cyclin D1 expression by Equiguard is of considerable significance since the D-type cyclins play a key role in the progression of cells through the G_1/S phase. Overexpression of cyclin D1 has been implicated in the etiology of a number of types of human cancer (50-53). A significant percentage of human prostate cancer specimens show cyclin D1 overexpression (4-25%) (50,54,55). Weinstein and coworkers reported an increase in cyclin D1 mRNA levels in CaP cells (56); D-type cyclins are critical for the androgen-dependent proliferation of prostate cells, by acting as transcriptional activators and repressors (57-60). Elevation of cyclin D1 not only increases cell growth and tumorigenicity in LNCaP cells, but also prevents castration-induced regression of tumors (61). Similarly, both in previous studies using LNCaP cells and in the present studies, we have shown that Equiguard increases the wild-type p53, specifically at S15. Equiguard also suppresses the expression of nuclear factor NF-κB, which controls the transcriptional control of 'survival genes' including proteins having a pivotal role in G_1/S transition of the cell cycle. NF-κB is a transcription factor with a ubiquitous distribution and capable of affecting the expression of many genes, involved in a multitude of important biological functions, notably, inflammation. Several members of the NF-κB family exist. These share in common a highly conserved Rel domain, which participates in DNA binding, dimerization, and interaction with its tethering protein IκB. The most abundant form of NF-κB is the p50/p65 heterodimer. Activation of NF-κB involves dislocation of the cytoplasmic NF-κB from its inhibitory protein IκB, followed by translocation into the nucleus. A key event preceding the cytoplasm-to-nucleus shuttling is the phosphorylation of IκB, followed by its degradation, by proteasome or other proteases (62). We have investigated changes in NF-κB expression and associated upstream signaling events. We showed that Equiguard down-regulated NF-κB as well as its upstream inhibitory protein IκB. Although we had surmised that Equiguard might exert its anti-inflammatory properties by increasing the expression of IκB, thereby forestalling the release of NF-κB from IκB in the cytoplasm and its subsequent translocation into the nucleus, our analysis actually showed that expression of both NF-κB and IκB to be reduced to a similar degree after a long-term exposure to Equiguard. We are currently re-investigating this phenomenon in greater detail.

Second, it is also noteworthy that treatment of CaP cells with Equiguard markedly induces apoptosis, at least in certain types of CaP cells (19). The apoptogenic abilities of Equiguard is hypothesized to involve a disruption of mitochondria structure and function, leading to the release of cytochrome c, followed by subsequent activation of caspases and ensuring DNA damage and fragmentation.

Third, Equiguard also affects prostate specific gene expression. In both LNCaP and now CWR22Rv1 cells, Equiguard has been shown to down-regulate the intracellular levels of AR and PSA, and in secreted PSA as well. This observation has considerable clinical relevance and implication as PSA levels are elevated in the sera of BPH and prostate cancer patients, and is widely considered as a serum marker for monitoring patients' response to standard and experimental therapies (63-66). As mentioned, cyclins D/E and Rb is

subject to control by Equiguard (19,20). Since they also act as co-modulators for the AR, we also hypothesize that control of their expression by Equiguard could concomitantly alter transcription of the AR-responsive gene, PSA. We suggest that the reduction in PSA by Equiguard is a result of its ability to reduce AR expression as well as to modulate AR co-regulators such as Rb and cyclins.

Fourth, as demonstrated in the present studies, Equiguard may also exert its anti-CaP effects by suppressing inflammation while enhancing cell detoxification activities and capacities. In support of this possibility, Equiguard was found to suppress Cox-2 expression while at the same time increase the levels of NQO1 and NQO2, a pair of oxidoreductases with an important role in the detoxification of procarcinogens.

How does Equiguard elicit this plethora of biological effects? This supplement has been formulated using the constituent herbs, respectively, *Epimedium brevicomum Maxim* (stem and leaves), *Morinda Officinalis How* (root), *Rosa laevigata Michx* (fruit), *Rubus chingii Hu* (fruit), *Schisandra chinensis (Turcz) Baill* (fruit), *Ligustrum lucidum Ait* (fruit), *Cuscuta chinensis Lam* (seed), *Psoralea corylifolia L.* (fruit), and *Astragalus membranaceus [Fisch] Bge* (root). The underlying theme of this integrative approach is that it will present responsive target cells with the total spectrum of bioactive, inactive, and counter-active ingredients present in a herbal mixture, the collective outcome of which likely will be: i) reduced toxicity, ii) appearance of new and novel activities, and iii) targeting those aspects of cancers pertinent to their propagation and/or establishment, while having minimal, subclinical overall toxicity in non-cancerous cells. Validity of this approach is supported by consideration of active ingredients present in individual herbs present in Equiguard. For example, icariin is a flavonoid found in *Epimedium brevicomum Maxim* that has been shown to protect against oxidative injuries in human umbilical vein endothelial cell line (67). Oral treatment with icariin in castrated rats reportedly improves erectile function, concomitant with an increase in expression of certain NOS in the corpus cavernosum (68). The root of *Morinda officinalis (Rubiaceae)* contain diterpenoids, notably, monotropein and deacetylasperulosidic acid, with anti-inflammatory activities (69) which presumably can apply to the inhibition of cox-2 and NF-κB observed in Figs. 5 and 6 (70). Dibenzocyclooctadiene lingnan activities capable of reversing P-glycoprotein (P-gp) mediated multidrug resistance (MDR) have been identified from *Schisandra chinensis (Turcz.) Baill* (71). *Ligustrum lucidum Ait* contains oleanolic acid and ursolic acid, novel triterpenoids capable of inducing the phase 2 response [e.g., elevation of NAD(P)H-quinone oxidoreductase and heme oxygenase 1], which is a major protector of cells against oxidative and electrophile stress (72-75). *Cuscuta chinensis Lam* contains ß-sitosterol with chemoprotective and chemopreventive effects (76,77). *Psoralea corylifolia* contains prenylflavones that inhibits nitric oxide synthase expression in activated microglial cells by inhibiting IκB-α degradation (78). *Astragalus membranaceus [Fisch]* Bge contains astragaloside IV whose anti-inflammatory activity of is mediated by inhibition of NF-κB activation and adhesion molecule expression (79). These biological activities associated with individual herbs present in Equiguard provide a reasonable explanation for the chemo-preventive attributes of Equiguard they might function synergistically to express novel bioactivities that circumvent overlapping molecular pathways which may hinder success in cancer treatment. Notably, this mechanistic framework is consistent with and in support of the general theme of TCM. It is also important to note that such approaches are not necessarily predicated on pre-existing knowledge of sequence of events leading to a particular form of malignancy.

While total appreciation and understanding of these and other aspects of TCM based on our current scientific knowledge is probably premature and debatable, acceptance of the practicality and validity of TCM, as illustrated by our studies of Equiguard in CaP cells, may draw analogy with advances made in 'structural' or 'systems' biology in recent years. The prevailing themes that are beginning to be widely accepted in these new sub-specialities of molecular biology revolve on molecular activities that often result from orderly and orchestrated assembly of macromolecules. Examples include formation of synthesome for DNA replication, enhanceosome for transcription, spliceosome in RNA processing, and COP9 signalosome for signal transduction. In these examples, the relevant activities have been demonstrated experimentally; yet, the fundamental principles and molecular details that result in the appearance of observed new activities are only beginning to be scientifically uncovered and appreciated. When these principles are applied to disease management, the implication is that small changes made in a complex herb formulation such as Equiguard may actually significantly affect or even alter its spectrum of biological and medical activities, the end result being either an expansion of its clinical applications or an altered scope of its measured clinical outcomes.

Acknowledgements

We are grateful for the support of an unrestricted grant from the Hong Kong Healthcare Center Ltd. We acknowledge with special thanks the vision and commitment of Ms. Vivien Chou and her family for mechanistic studies of Chinese herbals in the prevention and management of chronic diseases including prostate cancer.

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