

The protective effect of soybean phytochemicals on androgen responsive human prostate cancer cells LNCaP is likely mediated through modulation of hormone/cytokine-dependent pathways

Thomas T.Y. Wang¹, Stephen M. Boue², and Hari B. Krishnan³

¹USDA, ARS, BHNRC, Diet, Genomics, and Immunology Laboratory, Beltsville, MD 20854, USA

²USDA, Southern Regional Research Center, New Orleans, LA 70124, USA

³USDA, Plant Genetics Research Unit, ARS, Columbia, MO 65211, USA

*Corresponding author: Thomas Wang, PhD, ¹USDA, ARS, BHNRC, Diet, Genomics and Immunology Laboratory, 10300 Baltimore Avenue, Building 307C, Room 132, Beltsville, MD 20705, Telephone: 301-504-8459, Fax: 301-504-9456,

Key Words: androgen, estrogen, cancer prevention, cell cycle, gene expression

Submission date: October 14, 2011; Acceptance date: August 27, 2011; Publication date: August 30, 2011

Abstract

Background: Population studies suggested that consumption of a soy rich diet provides protective effects against several chronic diseases, including prostate cancer. However, the active components in soy as well as the mechanisms of action of soy's protective effects remain unclear. It would be important to elucidate these questions to support the use of soy in the prevention of chronic disease.

Methods: A cell culture model and molecular techniques were used as tools to identify a molecular signature induced by soy-derived phytochemicals.

Results: Soy phytochemicals inhibit growth of androgen responsive prostate cancer cells. Global gene expression analysis using DNA microarray and real time PCR analysis identified multiple pathways affected by the soy-derived phytochemicals genistein, daidzein, equol, and glyceollins in the androgen responsive human prostate cancer cell LNCaP. These pathways included androgen receptor-dependent pathways, insulin-like growth factors pathways, and cell cycle-related pathways. Soy-derived phytochemicals modulated these pathways in a concentration-dependent fashion.

Conclusion: Taking into consideration the physiological achievable concentration of diet-

derived soy phytochemicals, we propose the concentration-dependent cancer protective effect is likely mediated through modulation of hormone/cytokine-dependent pathways.

INTRODUCTION:

Prostate cancer is the most common non-cutaneous cancer among American men, and is ranked third as a cause of cancer deaths [1]. Since there is currently no effective cure for this disease, there is much interest in developing preventive strategies to reduce its impact [2]. Population and experimental studies have implicated that consumption of a diet that is rich in fruits, vegetables, and legumes is associated with a decreased risk for prostate and other forms of cancer [3,4]. Due to their expected safety and the fact that they are perceived as supportive of medical therapies, there is much interest in pursuing the development of food-derived products or compounds in prevention strategies [4]. However, the molecular targets, as well as the mechanisms of phytochemicals that contribute to their beneficial effects on cancer remain elusive. Further elucidation of the molecular targets and mechanisms would be important in defining their cancer preventive properties.

Phytoalexins constitute a chemically heterogeneous group of low molecular weight antimicrobial compounds that are synthesized *de novo* and accumulate in plants in response to stress [5, 6]. Soy and soy products contain several phytoalexins that have gained much interest, including genistein and daidzein (Figure 1) [7]. These constitutively produced isoflavones are considered as candidate diet-derived prostate cancer preventive compounds [8]. Daidzein can be metabolized to equol (Figure 1) by gut microbes and equol is purported to be the active component [9]. Initial interest in these compounds arose from studies that correlated consumption of soy products in Asian countries with a decreased incidence of hormone dependent cancers such as those of the mammary and prostate glands [10, 11]. Hence, a possible use for these compounds in mammary and prostate cancer prevention has been suggested [3, 7, and 10]. In addition to genistein and daidzein, the glyceollins (Figure 1) represent another group of phytoalexins whose biosynthesis is increased in response to plant stress signals [5, 6]. The glyceollin isomers I–III (Figure 1) have core structures similar to that of coumestrol and are derived from the precursor daidzein. The glyceollins (I–III) can be derived from the exposure of a soybean plant to the fungus *Aspergillus sojae*, a non-toxin-producing *Aspergillus* strain commonly used in the fermentation of soybeans to produce soy sauce and miso [12]. Compared with genistein and daidzein, purified glyceollins show greater inhibition of estradiol response (ER)-induced proliferation of breast cancer cells [13]. Glyceollins also have enhanced antagonism toward ER- α relative to ER- β , and lack the estrogen agonist activity of genistein and daidzein seen in low-estrogen conditions [13]. These findings suggest that soy protein enriched with glyceollins may have distinct estrogen-modulating properties compared with standard soy protein. However, the effects of the glyceollins on prostate cancer remain unclear.

In this report we use molecular techniques to evaluate prostate cancer cells in culture to illustrate the diverse pathways modulated by soy phytochemicals, the different activities between soy phytochemicals, and to show physiological concentrations of soy phytochemicals that induce cancer protective pathways.

METHODS:

Chemicals.

Dihydrotestosterone (DHT), dimethylsulfoxide (DMSO), genistein, daidzein and 17 β -estradiol were from Sigma Chemical Co. (St. Louis, MO). Equol was purchased from INDOFINE (Hillsborough, NJ). Cell culture media and reagents were purchased from Invitrogen (Carlsbad, CA). Acetonitrile (HPLC grade) and methanol were purchased from Aldrich Chemical Company. Water was obtained using a Millipore system and used during sample preparation procedures and HPLC analyses.

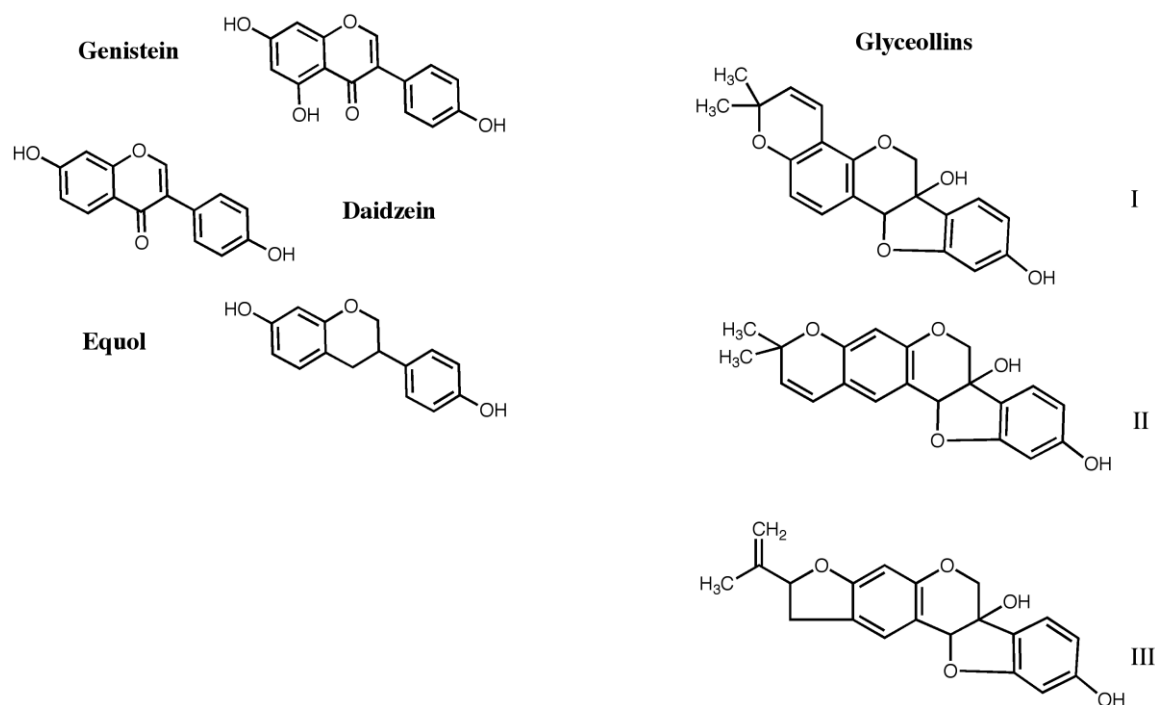


Figure 1. Soy phytochemicals structure.

Soybean treatment and harvesting.

Aspergillus sojae (SRRC 1125) cultures were grown at 25°C in the dark on potato dextrose agar. After 5 days, the inoculum was prepared by harvesting conidia (3.4×10^7 /ml) in 15 ml sterile, distilled H₂O. Seeds from commercial soybean variety Asgrow 5902 were surface-sterilized for 3 min in 70% ethanol followed by a quick deionized-H₂O rinse and two 2 min rinses in deionized-H₂O. Seeds were pre-soaked in sterile deionized-H₂O for 4-5 h, and then chopped for 2 min in a Cuisinart food processor. *Aspergillus sojae* spore suspension (300 ml) was applied to the cut surface of seeds on each tray. All trays were stored at 25°C in the dark for three days, rinsed with water to remove spores, and oven dried at 40° C for 24 h. Seeds were ground using a Waring blender before extraction.

Isolation of glyceollins (I-III).

The glyceollins I, II, and III were extracted from the 300g ground seeds with 1L methanol. The glyceollins were isolated using preparative scale HPLC using two Waters 25 mm 10 mm particle size mBondapak C18 radial compression column segments combined using an extension tube.

HPLC was performed on a Waters 600E System Controller combined with a Waters UV-VIS 996 detector. Elution was carried out at a flow rate of 8.0 ml/min with the following solvent system: A = acetonitrile, B = water; 5% A for 10 min, then 5% A to 90% A in 60 min followed by holding at 90% A for 20 min. The injection volume was 20 mL. The fraction containing the glyceollins was concentrated under vacuum and freeze-dried. The glyceollins were confirmed by UV-VIS spectrophotometry, mass spectrometry, and NMR as described [13]. A mixture of glyceollins I (68%), II (21%), and III (11%) were isolated (see Fig 1) and used in treatments. An average MW of 338 was used to calculate the concentration of glyceollins used in all cell culture experiments.

Cells and cell culture.

LNCaP human prostate cancer cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in Media A [RPMI 1640 medium with phenol red (Invitrogen, Carlsbad, CA), 2 mM L-glutamine (Sigma), 100 U/mL penicillin and 100 µg/mL streptomycin (BioSource International, Camarillo, CA) with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA)]. Cells were incubated in the presence of 5% CO₂ in air at 37°C.

Cell growth assay.

LNCaP cells (5×10^4 cells/well) were plated in 24-well plates (Costar) and treatments commenced 24 h later. Cells were treated with 0, 1, 5, 10 or 25 µM test compounds (DMSO as vehicle) for 0-96 h, and the medium containing test compounds was replaced every 24 h. Cell growth was analyzed using the sulforhodamine B (SRB) assay [14]. For experiments using the DHT or 17β-estradiol, cells were switched to Media B [RPMI 1640 medium without phenol red, 2 mM L-glutamine (Sigma), 100 U/mL penicillin and 100 µg/mL streptomycin with 10% charcoal dextran-treated FBS (CDS, Hyclone, Logan, UT)] 24 h after plating to minimize the effect of serum hormones. The cells were then incubated in Media B for an additional 24 h before the treatments were initiated.

Cell cycle analysis using flow cytometry. LNCaP cells (3×10^6 cells) were seeded into T-175 flask in Medium A. Twenty-four hours later the medium was changed to that containing vehicle (DMSO, 1µM, 5 µM or 25 µM test compounds). Cells were treated for 72 h and harvested for cell cycle analysis as previously described [15]

Determination of the effects on gene expression in LNCaP cells.

DNA microarray and RT-PCR were used to examine the effects of test compounds on gene expression. For microarray studies, LNCaP cells were seeded in 175 cm² tissue culture flasks (2×10^7 cells/flask) (Nunc, Roskilde, Denmark). Twenty-four h after plating, either test compound (at 1, 5 or 25 µM) or the vehicle DMSO was added to the media. The medium was changed every 24 h, and the test compound was replenished with each medium change. After 48 h of treatment, the cells were washed with phosphate-buffered saline (pH 7.4, Invitrogen) and total RNA extracted and microarray analysis was performed as previously described (16). Triplicate independent incubations were performed for each treatment.

For RT-PCR analysis, LNCaP cells were plated in 6-well plates (0.25×10^6 cells/well) in Media A. After twenty-four hours the medium was removed and replaced with fresh medium containing vehicle, 1, 5, or 25 μM test compounds. For experiments examining the effects of glyceollins on steroid hormone, LNCaP cells were plated in 6-well plates (0.25×10^6 cells/well) in Media A and switched to Media B containing 10% CDS 24 h after plating to minimize the effect of serum hormones. Twenty-four hours later, the medium was replaced with fresh medium containing 1 nM DHT or 17 β -estradiol with or without 0-25 μM test compounds. For all experiments fresh medium containing the test compounds was changed daily and cells were harvested for total RNA isolation using the Trizol method (Invitrogen) after 48 h [16]. Taqman real-time PCR was used to quantify expression of the mRNA [16]. Taqman real-time PCR Primer and probes for glyceraldehydes-3-phosphate dehydrogenase (G3PDH), PSA, cyclin-dependent kinase inhibitor (CDKN)1A and CDKN1B, NKX3.1(NK3 homeobox 1) and insulin like growth factor-1 receptor (IGF-1R) were purchased from Applied Biosystems (Foster City, CA).

Androgen receptor binding assays.

To assess the affinity of phytochemicals for androgen receptor (AR), the Androgen Receptor Competitor Assay Kit, Green (Invitrogen, Carlsbad, CA), a fluorescence polarization AR binding assay using a fluorescent androgen ligand FluormoneTM ALGreen, was performed according to manufacturer's protocol. DHT (0-1.5 μM) was used as a positive control. The concentration range of the test compounds used were: DIM and I3C, 0-50 μM ; genistein (GEN) and resveratrol (RES) 0-25 μM . Fluorescence polarization was detected using a TECAN ULTRA fluorescence plate reader (Tecan Systems Inc., San Jose, CA) set at 485 nm excitation and 535 nm emission wavelengths. Results were calculated as % Control = ((no compound control fluorescence units (FU) - with compound FU)/(no compound control FU - with 1.5 μM DHT FU)) x 100%. Triplicate assays were performed and results expressed as % Control +/- SD.

Statistics. All treatments were repeated at least 3 times, and representative experiments were presented. Experimental data was analyzed using the Prism 4 statistical software package (GraphPad software). Unpaired *t* tests were used for two group comparisons. For multiple group comparisons, ANOVA followed by post hoc analysis using Bonferroni's test were employed. Treatments effects with a *p* value of < 0.05 were considered significant.

RESULTS AND DISCUSSION:

Soy-derived compounds inhibit prostate cancer cell growth.

Soy isoflavones in general appeared to exert inhibitory effects on androgen responsive prostate cancer cells LNCaP. Figure 2A illustrates the effects of genistein and glyceollins on LNCaP cells. The effects appeared to be less in androgen non-responsive cells. Figure 2B shows an example of the effect of glyceollins on LNCaP cells and the androgen non-responsive human prostate cancer cells PC3. Daidzein appeared to be least effective based on concentration.

The growth inhibitory effects on prostate cancer cells appeared to be related to the modulation of the cell cycle, but there may be some differences in the mode of action. Treatment of LNCaP cells with the glyceollins for 72 h led to concentration-dependent effects on

G1/S arrest. Similarly, treatment of LNCaP cells with genistein (25 μM) for 72 h also leads to G1/G0 arrest. By contrast, genistein (25 μM) and not glyceollins (25 μM) treatments for 72 h lead to G2/M blockage in PC3 cells. Glyceollins treatment appeared to lead to S phase blockages in PC3 Cells. The cell cycle analysis did not reveal any significant effects on apoptotic events as indicated by lack of sub-2N PI staining of DNA.

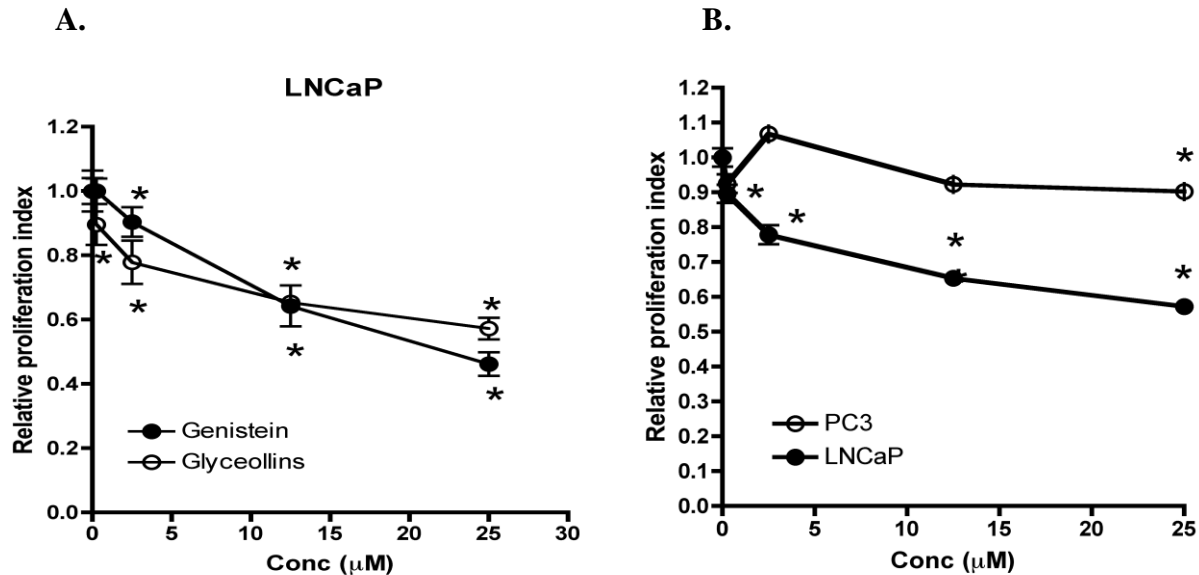


Figure 2. Effects of soy phytochemicals on prostate cancer cell growth. **A.** Effect of glyceollins and genistein on prostate cancer cell growth. LNCaP cells (0.25×10^6 cells/well) were plated on 6-well plates. Cell treatments with varied concentrations (0-25 μM) of glyceollins or genistein were started 24 h later for an additional 72 h and cell number determined as described in Materials and Methods. * represent significantly different from control at $p < 0.05$ ($n=6$). **B.** Effect of glyceollins on LNCaP and PC3 cell growth. LNCaP or PC3 cells (3×10^6 cell) were plated on 6-well plates. Cell treatments with varied concentrations (0-25 μM) of glyceollins were started 24 h later for an additional 72 h, and cell number determined as described in Materials and Methods. * represent significantly different from control at $p < 0.05$ ($n=6$).

Cell cycle effects of soy isoflavone correlate with up-regulation of cyclin inhibitors.

As shown in Figures 3 A, after 48 h treatment, glyceollins appeared to induce both CDKN1A and B mRNA levels. There were significant changes at 2.5 μM for both CDKN1A and B mRNA levels. Up regulation of these cyclin inhibitors were confirmed at the protein level. By contrast we only observed an induction of CDKN1A mRNA by genistein at 25 μM (Figure 3B), there were no changes in CDKN1B mRNA levels in LNCaP cells treated with genistein at all concentrations (0-25 μM) tested. Cyclin inhibitors are known to be induced by stress-dependent pathways such as DNA damage. CDKN1A responds to p53-dependent DNA damage pathway [17]. Hence, it is reasonable to conclude that genistein and glyceollins may cause DNA damage. Daidzein and equol at 25 μM did not show activation of cyclin inhibitors.

Microarray analysis identify regulation of multiple pathways affected by soy isoflavone.

The advent of DNA microarray technology [18] is an important tool to obtain global molecular signature of phytochemicals as well as making comparison between phytochemicals [16]. We used DNA microarray to examine the effects of isoflavones.

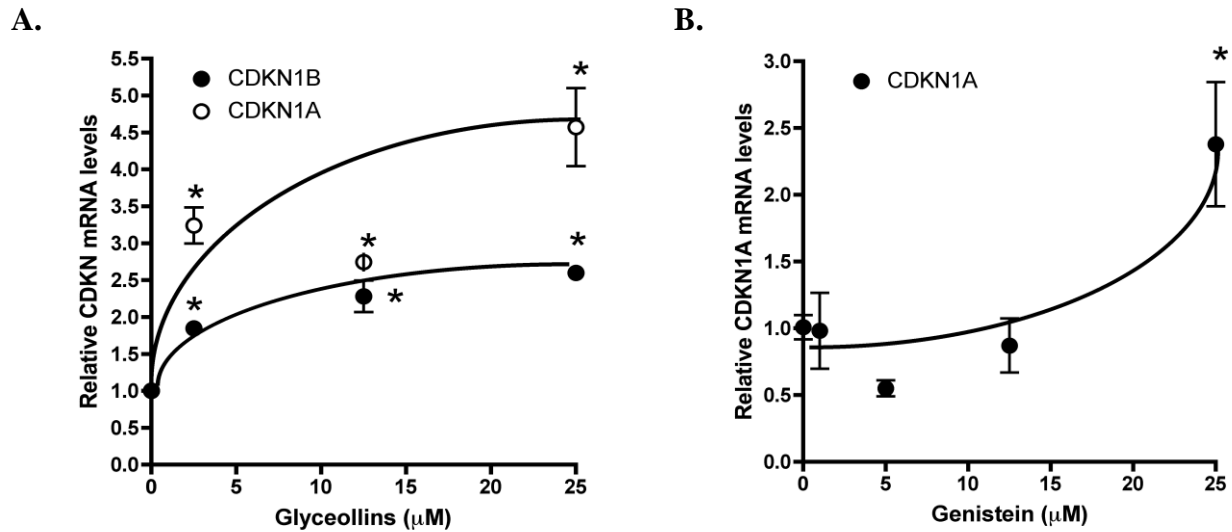


Figure 3. Effects of soy phytochemicals on cyclin inhibitors CDKN1A and B mRNA levels. **A.** Effect of glyceollins on CDKN1A and B mRNA levels. LNCaP cells cultured in 10% FBS were treated with 0, 2.5, 12.5, or 25 μM glyceollins for 48 h, total RNA isolated and mRNA levels of CDKN1A and B determined as described in Materials and Methods. Results are expressed as mean \pm SD (n=3). **B.** Effects of genistein on CDKN1A mRNA levels. LNCaP cells cultured in 10% FBS were treated with 0, 1, 5, 12.5 or 25 μM genistein for 48 h, total RNA isolated and mRNA levels of CDKN1A determined as described in Materials and Methods. Results are expressed as mean \pm SD (n=3). * represent significantly different from control at $p < 0.05$.

Equol, *daidzein*, and *genistein* exerted similar effects on androgen-responsive genes. Analyses of changes in gene expression seen upon exposure to the three compounds lead us to identify the androgen responsive genes (ARGs) [19] as a group of genes that are universally affected by these compounds (Figure 4A). Changes in the ARGs included both down- and up-regulated genes. In general, androgen up-regulated ARGs (such as KLK3/PSA) [20] that were down-regulated by treatments with the test compounds, and down-regulated ARGs (such as BCHE) that were up-regulated by treatment with the test compounds. The minimum threshold concentration that resulted in a change in gene expression appeared to be 1 μM . On the whole this group of genes was affected similarly by all the isoflavones tested, however, there were selected exceptions. For example, an androgen down-regulated gene, phosphoinositide-3-kinase regulatory subunit (PIK3R3) [21], was not affected by equol or daidzein at any tested concentration. PIK3R3 binds to activated protein tyrosine kinases (phosphorylated) through its SH2 domain, and regulates their kinase activity [21]. Interestingly, daidzein in particular appeared to regulate a greater number of ARGs at lower concentrations than did equol and genistein.

Expression changes for selected ARGs were all confirmed using real-time quantitative PCR. Treatment of LNCaP cells with genistein, daidzein or equol lead to concentration-dependent decrease in PSA/KLK3 mRNA levels. Similar inhibitory effects on other ARGs, such as SPAK [22] and NKX3.1 [23], mRNA levels were also confirmed using real-time PCR. This is similar to the effect of genistein on ARGs, as previously reported [16]. Interestingly, the effects of daidzein on ARGs appeared to plateau at or above 5 μ M (Figure 4B). This is different from the effects of equol (Figure 4A) and that of genistein (Figure 4B)

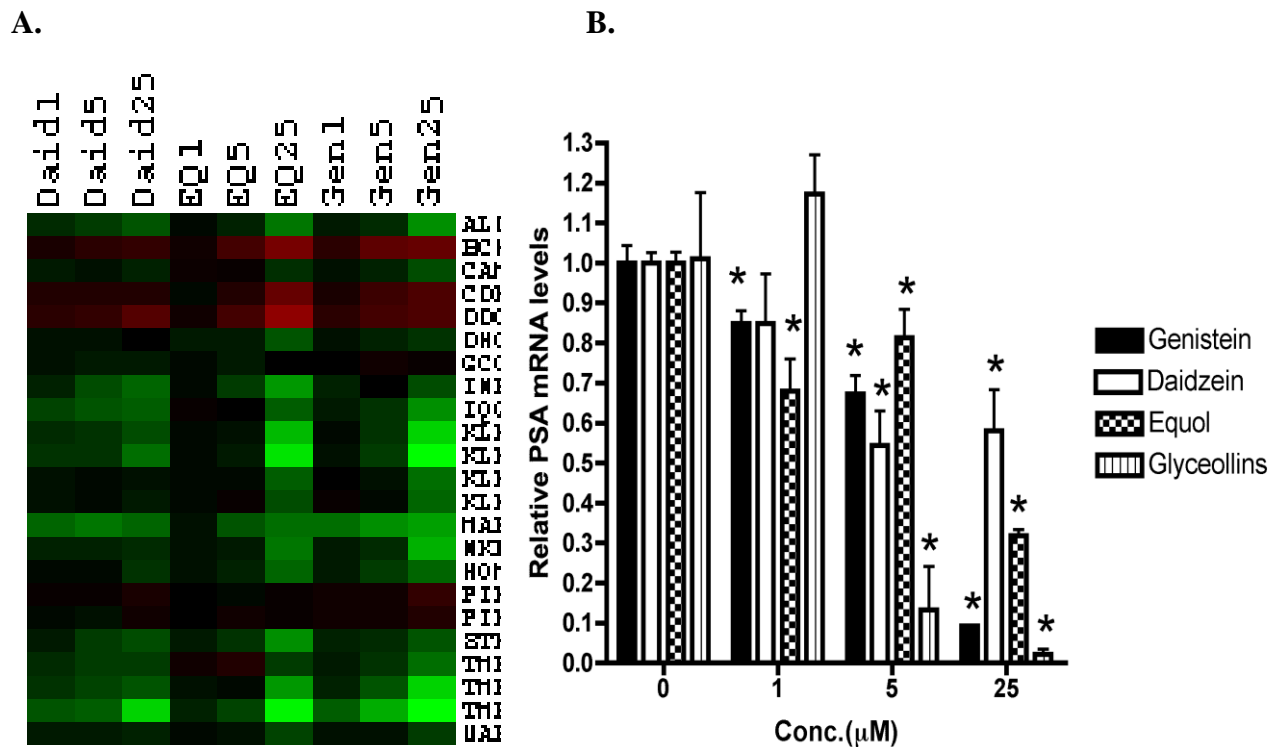


Figure 4. Global gene profiling analysis of phytochemical-treated LNCaP cells.

A. Microarray analysis. LNCaP cells were treated with 0, 1, 5 or 25 μ M equol, daidzein or genistein for 48 h. Subsequently, total RNA isolated and microarray analysis performed as described in Material and Methods. Cluster analysis was than performed on the data sets and heat map generated. Effects on androgen-responsive genes were identified. Red color represents up-regulated and green color represents down-regulated genes (as compared to vehicle-treated control), mean fold changes relative to control are listed next to each gene. **B.** RT-PCR confirmation of inhibitory effects of soy phytochemicals on PSA mRNA levels. LNCaP cells cultured in 10% FBS were treated with 0, 1, 5, 12.5 or 25 μ M test compounds for 48 h, total RNA isolated and mRNA levels of PSA determined as described in Materials and Methods. Results are expressed as mean \pm SD (n=3). * represent significantly different from control at p<0.05.

Equol, daidzein, and genistein exert similar effect on IGF-1R. Another pathway that was influenced similarly by the three isoflavones was the IGF-1 pathway, an important pathway related to prostate carcinogenesis as well as other cancers [24]. Microarray analysis demonstrated that all three compounds exerted an inhibitory effect on IGF-1R mRNA

expression, and real-time PCR analysis confirmed this observation (Figure 5). Treatments of LNCaP cells with daidzein, genistein, and equol all led to a concentration-dependent decrease in IGF-1R mRNA levels.

In addition, glyceollins appeared to act similarly on both androgen (Figure 4B, 6) and IGF-1R pathways (Figure 5) as confirmed by RT-PCR.

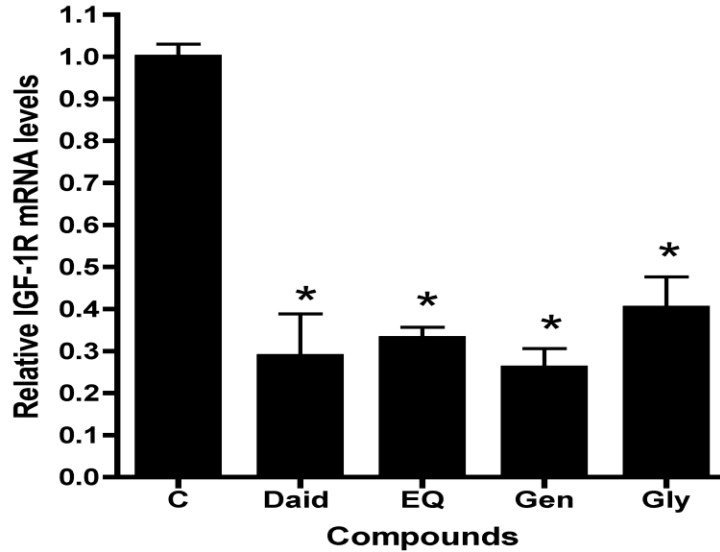


Figure 5. Effect of soy-derived phytochemicals on IGF-1R mRNA levels. LNCaP cells cultured in 10% FBS were treated with 25 μ M of daidzein (Daid), equol(EQ), Genistein (GEN) or Glyceollins(GLY) for 48 h, total RNA isolated and mRNA levels of IGF-1R determined as described in Materials and Methods. Results are expressed as mean \pm SD (n=3). * represent significantly different from control at $p < 0.05$.

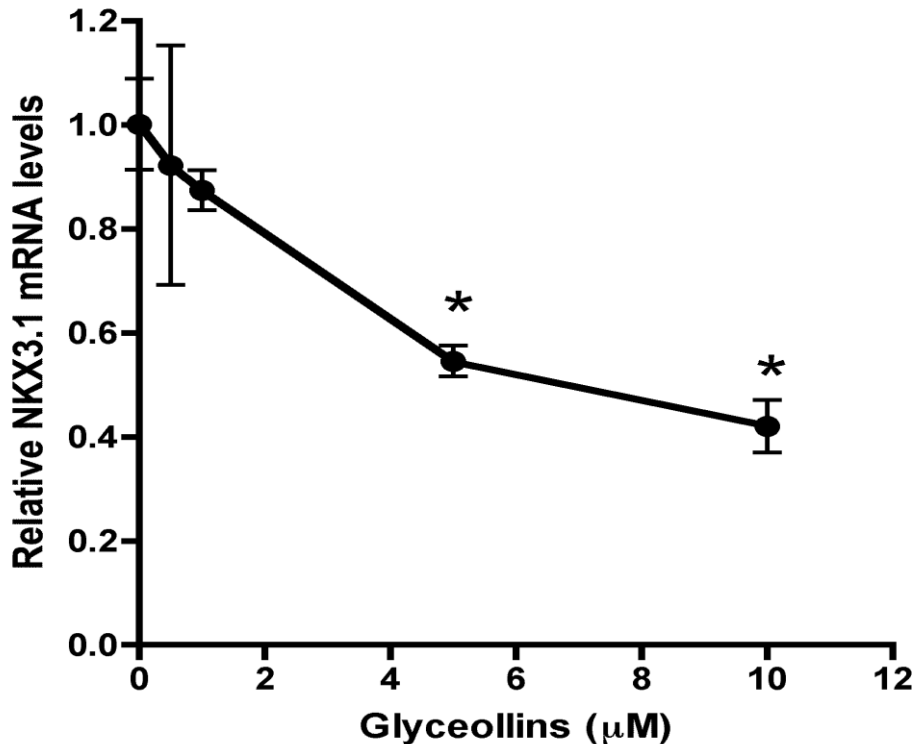


Figure 6. Effects of Glyceollins on the androgen responsive gene NKX3.1 mRNA levels. LNCaP cells cultured in 10% FBS were treated with 0, 0.5, 1, 5, or 10 μM test compounds for 48 h, total RNA isolated and mRNA levels of NKX3.1 determined as described in Materials and Methods. Results are expressed as mean \pm SD (n=3). * represent significantly different from control at $p < 0.05$.

Effects on xenobiotic metabolisms pathways.

Aryl hydrocarbon responsive pathways have long been associated with carcinogen metabolisms [25]. Several diet-derived compounds such as those derived from broccoli activate this pathway (26). Soy isoflavone appeared not to be effective in activating this pathway in LNCaP cells. We did not identify the regulation of these genes in our microarray analysis by genistein, daidzein or equol. This was confirmed in real time PCR, and there was little induction of the AHR-responsive gene CYP1A1 as compared to other phytochemicals such as diindolylmethane and sulforaphane (Figure 7).



Figure 7. Comparison of the effect of diet-derived phytochemicals on CYP1A1 mRNA levels. LNCaP cells cultured in 10% FBS were treated with the following concentration of the test compound. DIM (0-25 μM), I3C (0-100 μM), genistein (GEN, 0-25 μM), resveratrol (RES, 0-25 μM) or sulforaphane (SULFO, 0-10 μM) for 48 h, total RNA isolated and mRNA levels of CYP1A1 determined as described in Materials and Methods. Log 2 transformed mean expression values relative to vehicle control was analyzed using CLUSTER, a cluster analysis program, and visualized as HEAT map using TREEVIEW as previously described [55]. The intensity scale range is from -3 to 3. Red color indicates up regulation from vehicle control (0, Black) and green color indicates down regulation from vehicle control.

Effect of soy-derived phytochemicals on androgen responsive gene is mediated through modulation of estrogen-dependent pathway.

LNCaP cells express both androgen receptor and ER- β , and we have previously shown that exposure to estradiol can up regulate selected sets of androgen responsive genes [27]. The effect of isoflavones on androgen responsive genes in LNCaP cells appeared to be through modulation of estrogen-mediated pathways. Soy-derived phytochemicals genistein (Figure 8A) and glyceollins (Figure 8B) exert greater inhibitory effects on estrogen-induced increases in the androgen-responsive gene PSA. In addition, there appeared to be little interaction between isoflavones and the androgen receptor as shown by the lack of displacement of ligand from androgen receptor binding assay (Figure 9).

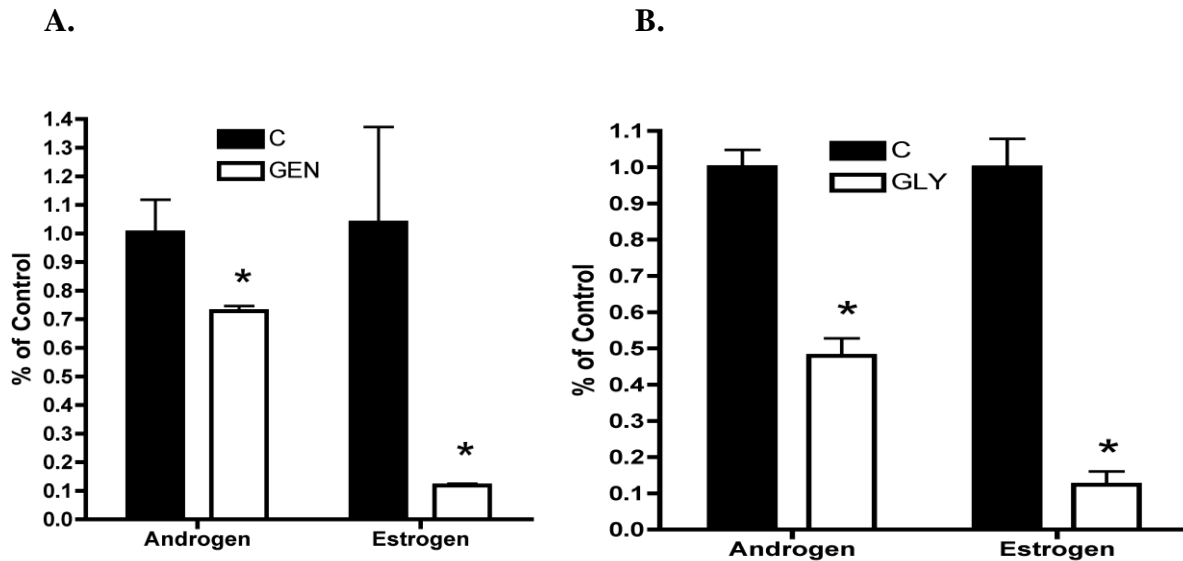


Figure 8. Effects of soy phytochemicals on androgen and estrogen induced increase of PSA in LNCaP cells. **A.** Effects of genistein on androgen or 17 β -estradiol induction of PSA mRNA level. LNCaP cells were plated in 6-well plates; 24 h after plating the medium was switched to Media B, which contains 10% CDS, for an additional 24 h. Cells were then treated with or without DHT (1 nM) or 17 β -estradiol (1 nM) in the presence or absence of genistein (25 μ M) for 48 h, total RNA was isolated and mRNA levels of PSA was determined as described in Materials and Methods. Results are expressed as % of DHT-treated control \pm SD (n=3). * represent significantly different from DHT or 17 β -estradiol-treated control at $p < 0.05$. **B.** Effects of glyceollins on androgen or 17 β -estradiol induction of PSA mRNA level. LNCaP cells were plated in 6-well plates; 24 h after plating the medium was switched to Media B, which contains 10% CDS, for an additional 24 h. Cells were then treated with or without DHT (1nM) or 17 β -estradiol (1 nM) in the presence or absence of glyceollins (25 μ M) for 48 h, total RNA was isolated and mRNA levels of PSA was determined as described in Materials and Methods. Results are expressed as % of DHT-treated control \pm SD (n=3). * represent significantly different from DHT or 17 β -estradiol-treated control at $p < 0.05$.

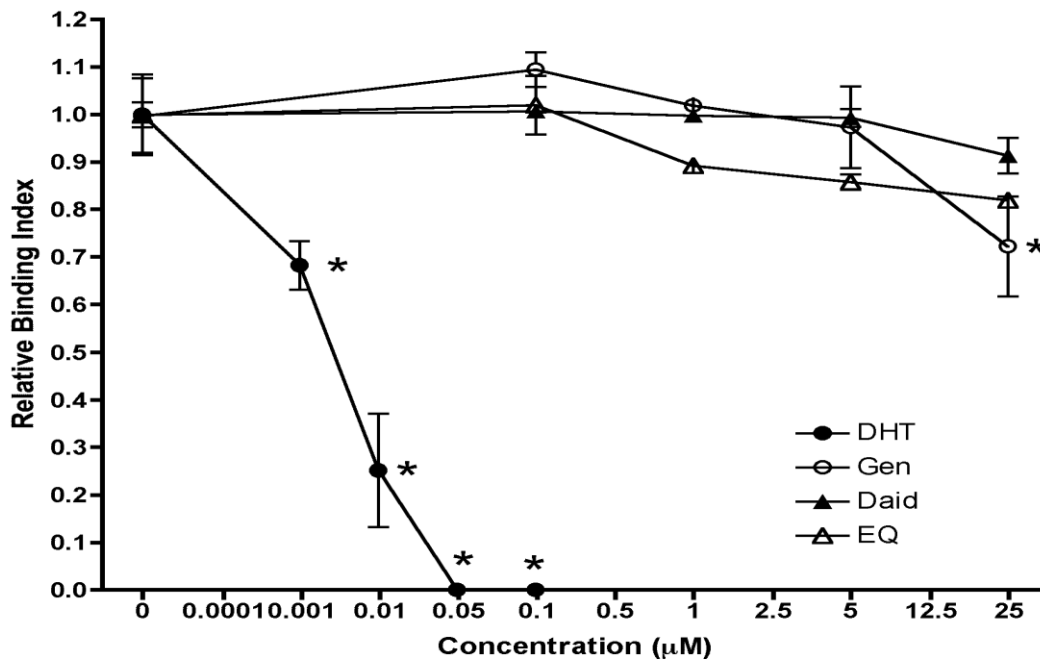


Figure 9. Interaction of soy phytochemicals and androgen receptor. Androgen receptor (AR) binding assays were performed to assess the affinity of daidzein (DAID), genistein (GEN), equol (EQ) for AR as described in Materials and Methods. Dihydrotestosterone (DHT) was used as positive control. Results are expressed as % Control \pm SD (n=3). Points with * are significantly different from vehicle control (0) at $p < 0.05$.

Consideration of physiological effect and potential targets pathways.

In a physiological setting such as consumption of isoflavone containing plant material, soy-derived phytochemicals can achieve a concentration around $\sim 10 \mu\text{M}$ in biological fluids. Taking this into account, we also analyzed the concentration threshold (lowest concentration required to observe an effect on gene expression analysis of pathways). Figure 10 illustrates a comparison of the difference in genistein concentration-dependent responses of PSA and CDKN1A. The isoflavone modulated androgen responsive genes at a low ($1 \mu\text{M}$) concentration. Similar results were also observed for IGF-1R. By contrast induction of cyclin inhibitor CDKN1A required $25 \mu\text{M}$ for genistein, but daidzein and equol were ineffective even at $25 \mu\text{M}$. Glyceollins appeared to be different from others since even at low concentrations both androgen and cyclin inhibitor pathways were activated.

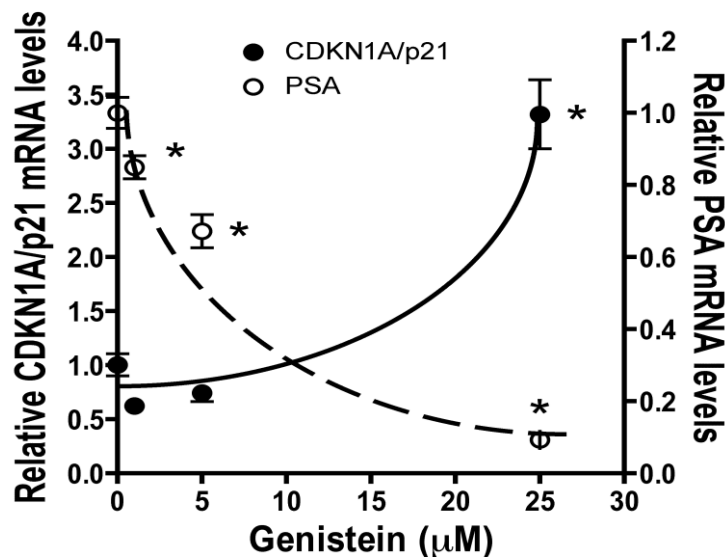


Figure 10. Concentration-dependent effects of genistein on PSA and CDKN1A mRNA levels. LNCaP cells cultured in 10% FBS were treated with 0, 1, 5, 12.5 or $25 \mu\text{M}$ genistein for 48 h, total RNA isolated and mRNA levels of PSA and CDKN1A determined as described in Materials and Methods. Results are expressed as mean \pm SD (n=3). * represent significantly different from control at $p < 0.05$.

CONCLUSION:

Our results illustrate several points: 1) Soy phytoalexins appeared to be active in inhibiting prostate cancer cells. 2) The compounds tested generally acted on hormone/cytokine-dependent pathways at physiologically achievable concentrations and, therefore, may represent a mechanism for their purported cancer protective effects. 3) The growth inhibitory effects of soy phytochemicals on prostate cancer cells may involve different pathways. At lower concentrations

they may exert their effect by inhibiting hormone/cytokines pathways. At higher pharmacological levels ($> 25 \mu\text{M}$) DNA damage pathways may also be activated by genistein, but not daidzein or equol. 4) Not all compounds are alike, for example, glyceollin especially induced cyclin inhibitors and inhibited androgen-dependent pathway at low concentrations.

Abbreviations: CDS, charcoal dextran-treated FBS; CDKN, cyclin-dependent kinase inhibitor; DHT, Dihydrotestosterone; DMSO, dimethylsulfoxide; ER, estrogen receptor; FBS, fetal bovine serum; G3PDH, glyceraldehydes-3-phosphate dehydrogenase; IGF-1R, insulin like growth factor-1 receptor; NK3 homeobox 1, NKX3.1; PI, propidium iodide; PSA, prostate specific antigen; SRB, sulforhodamine B.

Competing interests: The authors declare that they have no competing interests.

Authors' Contributions:

Thomas T.Y. Wang, Ph.D. is the principle investigator for this study providing oversight and contributed fundamental conceptualization for the research and writing of the manuscript.

Stephen M. Boue, Ph.D. contributed to the conceptualization of the research protocol and assisted in writing the manuscript.

Hari B. Krishnan, Ph.D. contributed to the conceptualization of the research protocol and assisted in writing the manuscript.

Acknowledgement and Funding supports: This work was supported by U.S appropriated funds to USDA project number 1235-51530-053-00 (TTYW), 6435-53000-001-00D (SMB), and 3622-21000-032-00D (HBK). Mention of trade names or commercial products is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. The USDA is an equal opportunity provider and employer

REFERENCES:

1. Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun M.J. Cancer statistics, CA Cancer J Clin 2007;57, 43-66.
2. Shirai T, Asamoto M, Takahashi S, Imaida K. Diet and prostate cancer. Toxicology 2002; 181-182:89-94.
3. Greenwald P, Clifford CK, Milner JA. Diet and cancer prevention. Eur J Cancer 2001; 37:948-965.
4. Greenwald P. Clinical trials of breast and prostate cancer prevention. J Nutr 2001; 131:176S-178S.
5. Darvill AG, Albersheim P. Phytoalexins and their elicitors: a defense against microbial infection in plants. Annu Rev Plant Physiol 1984; 35:243-275.

6. Paxton JD. Biosynthesis and accumulation of legume phytoalexins. In: Sharma RP, Salunkhe DK, editors. Mycotoxins and phytoalexins. Boca Raton (FL): C RCPress; 1991. p. 485-499.
7. Messina M, Nagata C, Wu AH. Estimated Asian adult soy protein and isoflavone intakes. *Nutr Cancer*. 2006; 55(1): 1-12.
8. Bemis DL, Katz AE, Buttyan R. Clinical trials of natural products as chemopreventive agents for prostate cancer. *Expert Opin Investig Drugs*. 2006; 15(10):1191-1200. Review.
9. Frankenfeld CL. Dairy consumption is a significant correlate of urinary equol concentration in a representative sample of US adults. *Am J Clin Nutr*. 2011 May;93(5):1109-16.
10. Messina MJ. Emerging evidence on the role of soy in reducing prostate cancer risk. *Nutr Rev*, 2003; 61:117-131.
11. Ziegler RG, Hoover RN, Pike MC, et al. Migration patterns and breast cancer risk in Asian-American women. *J Natl Cancer Inst* 1993; 85:1819-1827.
12. Chang TS, Ding HY, Tai SS, Wu CY. Metabolism of the soy isoflavones daidzein and genistein by fungi used in the preparation of various fermented soybean foods. *Biosci Biotechnol Biochem*. 2007; 71(5):1330-1333.
13. Burow ME, Boué SM, Collins-Burow BM, Melnik LI, Duong BN, Carter-Wientjes CH, Li S, Wiese TE, Cleveland TE, McLachlan JA. Phytochemical glyceollins, isolated from soy, mediate antihormonal effects through estrogen receptor α and β . *J. Clin. Endocrinol. Metab*. 2001; 86: 1750-1758.
14. Takahashi Y, Hursting SD, Perkins SN, Wang TC, Wang TT. Genistein affects androgen-responsive genes through both androgen- and estrogen-induced signaling pathways. *Mol Carcinog*. 2006; 45(1): 18-25.
15. Payton-Stewart F, Schoene NW, Kim YS, Burow ME, Cleveland TE, Boue SM, Wang TT. Molecular effects of soy phytoalexin glyceollins in human prostate cancer cells LNCaP. *Mol Carcinog*. 2009 Sep;48(9):862-71.
16. Takahashi Y, Lavigne JA, Hursting SD, Chandramouli GV, Perkins SN, Barrett JC, Wang TT. Using DNA microarray analyses to elucidate the effects of genistein in androgen-responsive prostate cancer cells: identification of novel targets. *Mol Carcinog*. 2004; 41(2):108-119.
17. Maddika S, Ande SR, Panigrahi S, Paranjothy T, Weglarczyk K, Zuse A, Eshraghi M, Manda KD, Wiechec E, Los M. Cell survival, cell death and cell cycle pathways are interconnected: implications for cancer therapy. *Drug Resist Updat* 2007;10(1-2):13-29. Review.
18. Mazumder A, Wang Y. Gene-expression signatures in oncology diagnostics. *Pharmacogenomics*. 2006 Dec;7(8):1167-73.
19. Nelson PS, Clegg N, Arnold H, et al. The program of androgen-responsive genes in neoplastic prostate epithelium. *Proc Natl Acad Sci USA* 2002;99:11890-11895.
20. Dixon SC, Knopf KB, Figg WD. The control of prostate-specific antigen expression and gene regulation by pharmacological agents. *Pharmacol Rev* 2001;53:73-91.
21. Pons S, Asano T, Glasheen E, et al. The structure and function of p55PIK reveal a new regulatory subunit for phosphatidylinositol 3-kinase. *Mol Cell Biol* 1995;15:4453-4465.

22. Johnston AM, Naselli G, Gonez LJ, Martin RM, Harrison LC, DeAizpurua HJ. SPAK, a STE20/SPS1-related kinase that activates the p38 pathway. *Oncogene* 2000;19:4290-4297.
23. Korkmaz KS, Korkmaz CG, Ragnhildstveit E, Kizildag S, Pretlow TG, Saatcioglu F. Full-length cDNA sequence and genomic organization of human NKX3A - alternative forms and regulation by both androgens and estrogens. *Gene* 2000;260:25-36.
24. Grimberg A, Cohen P. Role of insulin-like growth factors and their binding proteins in growth control and carcinogenesis. *J Cell Physiol* 2000;183:1-9.
25. Yu S, Kong AN. Targeting carcinogen metabolism by dietary cancer preventive compounds. *Curr Cancer Drug Targets*. 2007 Aug;7(5):416-24.
26. Wang TT, Schoene NW, Milner JA, Kim YS. Broccoli-derived phytochemicals indole-3-carbinol and 3,3'-diindolylmethane exerts concentration-dependent pleiotropic effects on prostate cancer cells: Comparison with other cancer preventive phytochemicals. *Mol Carcinog*. 2011 Apr 22. doi: 10.1002/mc.20774. [Epub ahead of print]
27. Takahashi Y, Perkins SN, Hursting SD, Wang TT. 17beta-Estradiol differentially regulates androgen-responsive genes through estrogen receptor-beta- and extracellular-signal regulated kinase-dependent pathways in LNCaP human prostate cancer cells. *Mol Carcinog*. 2007 Feb; 46 (2):117-29.