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Inhibition of Prostaglandin Synthesis and Actions by Genistein in Human Prostate Cancer Cells and by Soy Isoflavones in Prostate Cancer Patients

Srilatha Swami[1], **Aruna V. Krishnan**[1], **Jacqueline Moreno**[1], **Rumi S. Bhattacharyya**[1], **Christopher Gardner**[1], **James D. Brooks**[2], **Donna M. Peehl**[2], and **David Feldman**[1,†]

[1]Department of Medicine, Stanford University School of Medicine, Stanford, California 94305

[2]Department of Urology, Stanford University School of Medicine, Stanford, California 94305

Abstract

Soy and its constituent isoflavone genistein inhibit the development and progression of prostate cancer (PCa). Our study in both cultured cells and PCa patients reveals a novel pathway for the actions of genistein, namely the inhibition of the synthesis and biological actions of prostaglandins (PGs), known stimulators of PCa growth. In the cell culture experiments, genistein decreased *cyclooxygenase-2* (*COX-2*) mRNA and protein expression in both human PCa cell lines (LNCaP and PC-3) and primary prostate epithelial cells and increased *15-hydroxyprostaglandin dehydrogenase (15-PGDH)* mRNA levels in primary prostate cells. As a result genistein significantly reduced the secretion of PGE_2 by these cells. *EP4* and *FP* PG receptor mRNA were also reduced by genistein, providing an additional mechanism for the suppression of PG biological effects. Further, the growth stimulatory effects of both exogenous PGs as well as endogenous PGs derived from precursor arachidonic acid were attenuated by genistein. We also performed a pilot randomized double blind clinical study in which placebo or soy isoflavone supplements were given to PCa patients in the neo-adjuvant setting for 2 weeks prior to prostatectomy. Gene expression changes were measured in the prostatectomy specimens. In PCa patients ingesting isoflavones, we observed significant decreases in prostate *COX-2* mRNA) and increases in *p21* mRNA. There were significant correlations between *COX-2* mRNA suppression, *p21* mRNA stimulation and serum isoflavone levels. We propose that the inhibition of the PG pathway contributes to the beneficial effect of soy isoflavones in PCa chemoprevention and/or treatment.

Keywords

Genistein; soy; prostaglandins; *COX-2*; human prostate cells; prostate cancer patients

INTRODUCTION

Prostate cancer (PCa) is one of the most common forms of cancer affecting men in the US. Environmental factors, especially diet, have been shown to play a role in PCa risk.

[†]**Corresponding Author:** David Feldman, MD, Stanford University School of Medicine, 300 Pasteur Drive, Room S025, Stanford CA 94305-5103, Tel. 650-725-2910, Fax. 650-725-7085, dfeldman@stanford.edu.

Epidemiological studies as well as *in vitro* and animal data suggest that an increase in dietary isoflavones lowers the risk of several cancers including PCa[1]. Soy-derived isoflavones have a wide spectrum of biochemical activities including anti-cancer effects[1]. Asian men who consume large quantities of soy-based foods have reduced mortality and lower incidence of PCa than their Western counterparts[2, 3]. Furthermore, a rise in the incidence of PCa was reported among Asian immigrants to the US who adopted a Western diet, low in soy products[4–6]. Genistein, an isoflavone that structurally resembles estradiol, is found in higher concentrations in soy than any other food[4]. Genistein inhibits the growth of several types of cancer cells including PCa cells[7–9]. Data from animal studies also suggest that genistein may be useful in the prevention and treatment of PCa[10–12]. Several investigators have conducted pilot clinical trials examining the beneficial effect of the administration of soy isoflavones to PCa patients and some of these studies report decreases in the rate of rise in serum prostate specific antigen (PSA) levels due to soy isoflavones[13–15]. At the cellular level genistein exerts multiple actions to mediate its anti-cancer effects including: binding to estrogen receptors, inhibition of protein tyrosine kinases, inhibition of both NF-kappa B activation and Akt signaling, induction of apoptosis, anti-oxidant effects and suppression of metastasis and angiogenesis[16, 17]

Experimental evidence suggests that prostaglandins (PGs) are pro-inflammatory agents that play a key role in the carcinogenic process of many cancers including PCa[18] through the stimulation of cell proliferation, inhibition of differentiation and apoptosis as well as the augmentation of tumor cell invasiveness, metastasis and mutagenesis[19]. Genistein has been shown to decrease the synthesis of PGs in several normal and malignant cells[20–24]. In the current study we report that in established human PCa cell lines and in cultured primary human prostate epithelial cells genistein decreases the synthesis and biological actions of the PGs. Our results reveal that the major action of genistein on the PG pathway is the inhibition of the expression of *prostaglandin G/H synthase/cyclooxygenase-2* (*COX-2*), the enzyme that catalyzes the synthesis of PGs leading to a decrease in the synthesis and secretion of PGs by the prostate cells. Importantly our study shows that *in vivo* administration of moderate doses of soy isoflavones to PCa patients in the neo-adjuvant setting for 2–4 weeks prior to prostatectomy results in the suppression of *COX-2* expression in prostatectomy specimens. The inhibition of PG synthesis and actions provide an additional mechanism for the growth repressive effects of genistein in PCa. Our observations suggest that genistein or soy may play a therapeutic role in the chemoprevention and/or treatment of PCa.

MATERIALS AND METHODS

MATERIALS

PGE_2, $PGF_{2\alpha}$, and arachidonic acid were obtained from Cayman Chemical Co. (Ann Arbor, MI). Genistein, daidzein and phorbol 12-myristic 13-acetate (PMA) were from Sigma Aldrich (St.Louis, MO). Equol (a racemic mixture) was obtained from LC laboratories (Woburn, MA). Epidermal growth factor (EGF), insulin-like growth factor (IGF-1) and keratinocyte growth factor (KGF) were obtained from PeproTech (Rocky Hill, NJ). PD153035 and AG99 were from Calbiochem/EMD Biosciences (La Jolla, CA). Tissue culture media, supplements and fetal bovine serum (FBS) were obtained from GIBCO BRL

(Grand Island, NY). The *COX-2* promoter-luciferase plasmid containing a ~7 kb fragment of the human *COX-2* promoter (−7140 to +123) cloned into the luciferase reporter vector, pGL3-basic, was a kind gift from Dr. Stephen Prescott (University of Utah, Salt Lake City, UT).

METHODS

Cell culture—LNCaP and PC-3 cells were grown in RPMI 1640 medium supplemented with 5% FBS, 100 IU/ml of penicillin and 100 μg/ml streptomycin. Cells were maintained at 37°C with 5% CO_2 in a humidified incubator. Primary cells were derived from radical prostatectomy specimens from men undergoing surgery to treat PCa and propagated in culture as described previously[25]. The normal cell cultures (E-PZ-1 to-5) were derived from peripheral zone tissue with no histological evidence of cancer in adjacent sections. The cancer cell cultures used (E-CA-1 (Gleason grade 3/4), E-CA-2 (Gleason grade 4/3) and E-CA-3 (Gleason grade 3)) were derived from adenocarcinoma specimens. None of the patients had prior therapy or soy ingestion and were not the subjects in the pilot clinical trial for PCa.

Cell proliferation assays—LNCaP cells were seeded at an initial density of 1.5×10^5 cells/well in 6-well tissue culture plates and allowed to attach overnight in RPMI 1640 medium with 5% FBS. Cell cultures were shifted to medium containing 2% FBS and treated with either 0.1% ethanol vehicle or the indicated concentrations of drugs. Fresh media and drugs were replenished every other day. At the end of 6 days, cells were collected and processed for measurement of DNA content[26]. The proliferation of primary epithelial cells was determined in a high-density growth assay as described[27].

RNA isolation and Real-Time RT- PCR—Total RNA was isolated from vehicle- or drug-treated cells using Trizol reagent (Invitrogen, Lifetechnologies, Inc., Carlsbad, CA) as previously described[28]. Five μg of total RNA were used in reverse-transcription (RT) reactions using the SuperScript III first strand synthesis kit (Invitrogen) and gene expression was determined by real-time PCR using gene-specific primers[28]. The reactions were carried-out with the DyNamo SYBR green qPCR kit (Finnzymes, New England Biolabs, Ipswich, MA) as described previously[28] using an Opticon 2 DNA engine (Bio Rad, Hercules, CA). Melt curves were run with the PCR product to ascertain presence of a single peak. Relative changes in mRNA expression levels were assessed by the $2^{-\Delta\Delta C(T)}$ method[29]. Changes in mRNA expression of the different genes were normalized to either TATA binding-protein (TBP) gene or glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene as described previously[28].

Measurement of PGE_2 secretion—Subconfluent cell cultures were treated with vehicle or genistein for 72 h. Conditioned media were collected and secreted PGE_2 levels were quantitated using a PGE_2 monoclonal Enzyme Immunoassay kit (Cayman Chemical) according to the manufacturer's protocol.

Western blot analysis—Cell lysates were prepared from vehicle- or genistein-treated cells and subjected to Western blot analysis as described earlier[28]. The *COX-2* monoclonal

antibody (1:1000 dilution) was purchased from Cayman Chemicals. β-actin monoclonal antibody (dilution 1:500) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Immunoreactive bands were visualized using the enhanced chemiluminescence Western blot detection system (Amersham, Piscataway, N.J.) according to the manufacturer's instructions.

Transient transfections and luciferase assay—A ~7kb fragment of the human *COX-2* promoter fused to the luciferase reporter was transiently transfected into LNCaP cells using LipofectAmine reagent (Life Technologies). A renilla luciferase plasmid (pRLnull, Promega, Madison, WI) was co-transfected to control for transfection efficiency. Following transfections the cells were treated with 0.1% ethanol vehicle control (Con) or 10 μM genistein (G) for 6h either in the presence or absence of EGF (100 ng/ml), IGF-1 (100 ng/ml), KGF (100 ng/ml) or PMA (100 ng) added to the culture medium (RPMI + 5% FBS). Reporter and renilla luciferase activities were measured using the Dual luciferase assay kit (Promega) following the instructions of the manufacturer.

Soy isoflavone administration to PCa patients—Under a protocol approved by the Internal Review Board at Stanford, men diagnosed with PCa scheduled to undergo prostatectomy were enrolled in the study after providing informed consent. Eligibility criteria included: newly diagnosed adenocarcinoma by prostate needle biopsy, clinically localized disease, and no hormone therapy or radiation prior to surgery. The study was a double-blinded randomized placebo controlled trial in which participants were asked to take study tablets for a minimum of 2 weeks prior to surgery. Patients were randomly assigned to receive either a soy isoflavone supplement or a placebo. The isoflavone (Novasoy) tablets (27.2 mg isoflavone aglycones per tablet, 3 tablets/day) were provided and analyzed for isoflavone content by Archer Daniels Midland Co (Decatur, IL). The isoflavones were present at a ratio of 1.0:1.3 of genistein to daidzein. The placebo pills were manufactured to appear identical to the isoflavone tablets. The tablets were distributed at clinic visits and residual tablets were counted to assess compliance. The clinical characteristics of the patients in the placebo and Novasoy group are given in Table 1. Aliquots of the prostatectomy tissue (~50 mg) were snap frozen and stored at −80°C. The prostatectomy specimens contained both cancer and normal areas determined by histological analysis. RNA was isolated from the frozen tissue using the RecoverAll total nucleic acid isolation kit (Ambion, Austin, TX) and used for measurement of *COX-2*, *15-PGDH*, *p21*/waf1 and *GAPDH* mRNA levels by real-time RT-PCR. At the end of the study, serum isoflavone levels were measured by Dr. Adrian Franke (Cancer Research Center of Hawaii, Honolulu, HI) by liquid chromatography-mass spectrometry analysis[30].

Statistical Analysis—Statistical analyses were carried out using GraphPad Prism 5. Data were evaluated by ANOVA with Scheffé's F test as the post-hoc analysis. In the patient trial, unpaired samples were assessed using both Mann Whitney test (nonparametric variables) and Student t tests with Welch correction (parametric variables). Comparisons between paired samples were made using linear regression analysis and determination of the Pearson's correlation coefficient.

RESULTS

Regulation of COX-2 expression by genistein and other soy components

RT-PCR analysis revealed a significant inhibition of *COX-2* mRNA expression in both PCa cell lines and primary prostate epithelial cells (Figure 1 A–C). *COX-2* mRNA levels were suppressed in LNCaP cells (~50 % decrease) exposed to 10 μM genistein for 24 h while in PC-3 cells a more modest but statistically significant (~38%) decrease was seen (Figure 1A). We also examined the *COX-2* mRNA expression in 4 different primary epithelial cell cultures derived from normal human peripheral zone prostate tissue (E-PZ-1-4, Figure 1B) and three cell cultures derived from human PCa specimens (E-CA-1-3, Figure 1C). Genistein at 10 μM caused significant decreases in *COX-2* mRNA in 3 out of the 4 normal cell cultures tested (E-PZ-1, -2 and -4) and in 2 out the 3 cancer-derived cell cultures (E-CA-1 and -3) after 24 h of treatment. Panel D shows the results of a Western blot analysis of *COX-2* protein expression in the normal primary cell cultures E-PZ-3-5. The data reveal appreciable decreases in immunoreactive *COX-2* protein following 72 h of treatment with 10 μM genistein in E-PZ-4 and -5 cells. We also examined the effect of two other major soy components, daidzein and its metabolite equol on *COX-2* expression in LNCaP cells. When compared to vehicle-treated controls, 24-h treatment of cells with 10 μM daidzein or equol also resulted in a significant ($p<0.01$) down-regulation of *COX-2* mRNA levels (data not shown). *15-PGDH* mRNA levels did not appreciably change in LNCaP or PC-3 cells following 24h of genistein treatment. However, significant up-regulation of *15-PGDH* mRNA was seen in two of the normal (E-PZ-1 and -3) and two of the cancer-derived (E-CA-2 and -3) primary cell cultures following 24 h of genistein treatment (Figure 2, Panels B & C).

Regulation of EP and FP prostaglandin receptor mRNA expression by genistein

We examined the effects of genistein on the mRNA expression of the PG receptors *EP2*, *EP4* and *FP*. Genistein significantly decreased the *EP4* and *FP* mRNA levels by ~65 % and ~45 % respectively, in LNCaP cells at the end of 24 h of treatment (Figure 3A). The reduction in *EP2* mRNA was not statistically significant. Genistein did not significantly alter *EP* or *FP* receptor mRNA levels in PC-3 cells (Figure 3B). In the case of primary cells, both *EP2* and *EP4* receptor mRNA could be detected in normal as well as cancer-derived cultures while *FP* mRNA was undetectable. Significant decreases following genistein treatment were seen in *EP4* mRNA levels in 3 out of the 4 normal cell cultures, and 2 out the 3 cancer-derived cell cultures tested (Figure 3, Panels C & D). Genistein treatment did not change *EP2* mRNA expression significantly in the primary cells (data not shown).

Effect of genistein on prostaglandin levels

As a reflection of the effects of genistein on the expression levels of the PG synthesizing enzyme *COX-2* and the PG catabolizing enzyme *15-PGDH*, we examined PGE_2 production and secretion into the conditioned media from cell cultures exposed to 10 μM of genistein for 48 h. As shown in Figure 4A, genistein caused significant reductions in PGE_2 secretion in LNCaP cells (~60%), PC-3 cells (~75%) and in the primary epithelial cell cultures (~40–60%).

Effects of genistein on basal and prostaglandin-stimulated cell growth

The effects of genistein on the growth of the three normal primary prostate epithelial cell cultures E-PZ -1, -3 and -5 were determined. As shown in Figure 4B, addition of 10 μM genistein to the cultures caused highly significant inhibition of the growth of all the cell cultures tested (~70–85% inhibition as compared to control). We also analyzed the effect of genistein on the stimulation of LNCaP cell growth by exogenous PG addition as well as by endogenous PGs derived from the substrate arachidonic acid added to the culture medium. LNCaP cells were treated with arachidonic acid (3 μM), PGE_2 or $PGF_{2\alpha}$ (10 μM each) in the absence or presence of 10 μM genistein for 6 days and growth was assessed by measuring DNA. Our results revealed a moderate but statistically significant growth stimulation ($p<0.05$) due to the addition of arachidonic acid and a more pronounced growth stimulation ($p<0.01–0.001$) by the PGs (Figure 4C). Genistein (10 μM) had a marked growth inhibitory effect ($p<0.001$) on the basal (vehicle-treated) cell growth. In addition, genistein completely blocked the growth stimulation due to endogenous PGs derived from the added arachidonic acid as well as exogenous PG addition (Figure 4C).

Modulation of COX-2 promoter-luciferase activity by genistein

To determine whether genistein decreases *COX-2* gene expression by a direct transcriptional repression, we transiently transfected into LNCaP cells a plasmid containing a ~7 kb fragment (−7140 to +123) of the human *COX-2* promoter cloned into the pGL3-basic vector[31]. Luciferase activity was determined both under basal conditions and after treatment with known stimulators of *COX-2* expression with and without genistein co-treatment and the findings are shown in Figure 5A. Genistein had no effect on basal *COX-2*-luciferase activity. Addition of the growth factors EGF, KGF and IGF-1 or the phorbol ester PMA caused significant increases in *COX-2*-luciferase activity (~2-fold and ~3-fold over basal levels). Co-addition of genistein completely abolished EGF-, KGF- and IGF-1-stimulated *COX-2*-luciferase. However, the PMA-induced increase in *COX-2*-luciferase was not significantly affected by genistein co-treatment. As the receptors for EGF, KGF and IGF-1 exhibit tyrosine kinase activity and genistein is an inhibitor of protein tyrosine kinases, we examined the effect of known tyrosine protein kinase inhibitors on basal and EGF-stimulated *COX-2*-luciferase activity. As shown in Figure 5B, addition of EGF caused a ~2-fold increase in *COX-2*-luciferase activity over basal levels. The tyrosine protein kinase inhibitors AG99 and PD153035 abolished EGF stimulation of *COX-2*-luciferase. We also determined the effect of the PD153035 on *COX-2* mRNA expression in LNCaP cells in growth medium containing 5% FBS. Following treatment with 10 μM PD153035 for 24 h, a highly significant decrease in *COX-2* mRNA levels was seen in treated cells compared to cells exposed to the vehicle (Figure 5C).

Effect of soy isoflavone administration on gene expression in prostate surgical specimens from PCa patients

To determine whether the effects we demonstrated in cell culture were also relevant to human subjects, PCa patients scheduled for prostatectomy were given placebo or Novasoy tablets (27.2 mg isoflavone/tablet, 3 tablets/day) for 2 weeks prior to surgery. The assignment of patients to the placebo or isoflavone-treated groups was randomized and

double blinded. The characteristics of the patients and serum isoflavone levels are shown in Table 1. Serum total isoflavone levels in the group taking the Novasoy tablets were ~50-fold higher than the placebo group (Table 1). We measured the mRNA expression of *COX-2*, *15-PGDH* and the cell cycle inhibitor *p21/Waf1* in the prostatectomy specimens from the two groups. The expression of *GAPDH* mRNA was used as a control. A statistically significant decrease in *COX-2* mRNA levels ($p < 0.01$) was seen in the isoflavone-treated group (Figure 6A) when compared to the placebo group. In this group of patients, *COX-2* mRNA levels exhibited a significant ($p<0.05$) negative correlation with serum isoflavone levels achieved by Novasoy administration (Figure 6B). The expression of *15-PGDH* mRNA did not show a statistically significant change when placebo and soy isoflavone-treated samples were compared (data not shown). However, when compared to placebo-treated patients (Figure 6C), the soy isoflavone-treated patients showed a statistically significant increase in *p21* mRNA levels ($p < 0.01$). In the isoflavone-treated group, *p21* mRNA levels exhibited a significant ($p<0.05$) positive correlation with serum isoflavone levels (Figure 6D). Similar analyses examining the correlation of *COX-2* or *p21* mRNA expression in the prostate to serum isoflavone levels were not possible in the placebo group due to the very low levels of isoflavones in the serum samples in these patients. We also measured *COX-2* protein levels by Western blot in extracts from some of the prostatectomy specimens from the placebo- and soy isoflavone-treated groups. As shown in Figure 6E, *COX-2* protein expression in prostate tissue extracts from patients ingesting Novasoy (samples S1-S4 exhibiting *COX-2* mRNA levels of 0.04–0.5 arbitrary units) was appreciably lower than from patients on placebo (samples P1 & P2 exhibiting *COX-2* mRNA levels of 2 and 9 arbitrary units).

DISCUSSION

Genistein inhibits growth and induces apoptosis in several human PCa cells[7, 8]. Multiple molecular pathways appear to be involved in the anti-cancer effects of genistein[17]. These include the inhibition of cell cycle progression through the up-regulation of the *cyclin-dependent kinase inhibitor p21*[7, 9], down-regulation of *cyclin* expression[7], inactivation of *NF-κB*[16, 22] and modulation of the *androgen receptor*[32, 33]. Inhibition of tyrosine phosphorylation, particularly the expression and phosphorylation of EGF receptors in the prostate, may also play a role[34]. Our study shows that the regulation of the PG pathway is an additional mechanism by which genistein exerts its anti-proliferative effects on human prostate cells.

The main action of genistein on the PG pathway appears to be the suppression of *COX-2* expression as seen in the PCa cell lines (both LNCaP and PC-3) and in most of the primary cell cultures analyzed. We examined the effects of genistein in multiple isolates of primary prostate epithelial cells to account for the variations between the cell cultures as each culture is derived from an individual prostatectomy sample from a PCa patient. Since these cells are not immortalized, the cell cultures used were different in the various experiments depending on the availability at the time of analysis. In one of the normal primary cell cultures (E-PZ-1), we were able to carry out a complete assessment of the changes in all the key PG pathway genes, PGE_2 secretion and cell growth. However, it should be noted that the primary cell cultures used in the *in vitro* experiments were from untreated patients and were

not derived from the patients who participated in the pilot clinical study described in this report.

COX-2 basal expression is usually low to negligible in nearly all normal tissues, however, it can be induced by multiple factors including hormones, growth factors, cAMP, phorbol esters, inflammatory factors and cytokines[35]. In our studies we found appreciable *COX-2* expression in normal primary cell cultures presumably because the cultures were grown in defined media containing hormones, growth factors and cAMP inducers[36]. Genistein treatment caused significant decreases in both *COX-2* mRNA and protein levels in normal as well as cancer-derived primary cells and established PCa cell lines. The magnitude of COX-2 mRNA suppression was also higher in LNCaP cells, which represent a less aggressive and earlier stage of cancer when compared to the more aggressive and invasive PC-3 cells. There also seemed to be a greater degree of response among the primary cultures derived from normal prostate tissue than those derived from cancerous prostate tissue. Interestingly, significant increases in 15-*PGDH* mRNA was observed only in the primary cultures and not in the established PCa cell lines. The down-regulation of the PG receptor mRNA was seen in LNCaP cells and most of the primary cell cultures but was not seen in PC-3 cells. Taken together our data revealed that the primary cultures (both normal and cancer) and LNCaP cells (representative of a more differentiated or an earlier stage cancer) were more responsive to genistein treatment than the more aggressive and invasive PC-3 cells (representative of later stage cancer). The findings suggest that the use of genistein might be more beneficial earlier in the course of PCa.

The decrease in secreted PGE_2 levels seen in these cells mostly reflects the effect of genistein to inhibit PG synthesis by decreasing *COX-2* expression. The ability of genistein to inhibit arachidonic acid- stimulated LNCaP cell growth is due to its ability to inhibit endogenous PG synthesis from arachidonic acid by decreasing *COX-2* expression. Furthermore genistein abolished the growth stimulatory effects of added PGs indicating that the down-regulation of the PG receptors played a role in its action to inhibit the biological actions of PGs. Genistein has been shown to decrease PGE_2 synthesis through *COX-2* suppression in a variety of normal and malignant cells[20–24]. Our study is the first demonstration of the inhibition of *COX-2* expression by genistein in prostate cells. Decreases in COX-2 mRNA and protein were seen in all the cell models that we studied. Importantly we also showed significant decreases in *COX-2* expression in prostatectomy specimens following isoflavone administration to PCa patients.

COX-2 is regarded as a pro-inflammatory molecule that catalyzes the synthesis of PGs, which in many cancer cells exhibit growth stimulation, increased adhesion to extracellular matrix, resistance to apoptosis and stimulation of angiogenesis[19]. Several studies investigating *COX-2* expression in human PCa reported increases in *COX-2* expression in PCa and in high-grade prostate intraepithelial neoplasia (PIN)[37, 38]. Although two other studies did not find a consistent increase in *COX-2* expression in established PCa[39, 40], they did find *COX-2* over-expression in proliferative inflammatory atrophy (PIA) lesions, which are thought to be the precursors of PIN[40]. More recently, increased *COX-2* expression in prostate was reported in multiple studies of PCa patients[41–43]. We also found measurable levels of *COX-2* mRNA and protein in human PCa cells[28]. Most investigators agree that

local production of PGs by inflammatory cells increases the risk of prostate carcinogenesis and/or PCa progression[39, 40, 43, 44]. In our study of prostatectomy specimens we used represent tissue that was a heterogeneous mixture of both normal and tumor cells infiltrated with inflammatory cells. We demonstrate that in these prostatectomy specimens, *COX-2* is elevated in most samples and that genistein suppresses *COX-2* mRNA and protein expression. The data suggest that genistein, through its ability to down-regulate *COX-2* expression, may exhibit a beneficial chemopreventive and /or therapeutic effect in PCa regardless of whether the *COX-2* is being produced by the tumor cells or the infiltrating inflammatory cells.

Genistein is a phytoestrogen that can bind to the estrogen receptors (ER) α and β and regulate gene transcription[45]. Prostate tissue is known to express ERβ[45] and it has been shown that genistein has greater affinity for ERβ than ERα[45]. However, an investigation of the effects of genistein on ERβ is beyond the scope of this manuscript.

Our studies on the effect of genistein on *COX-2* promoter activity revealed that genistein abolished the activation of *COX-2* promoter by growth factors with known receptor tyrosine kinase activities. Further, tyrosine kinase inhibitors (AG99 and PD153035) mimicked the effects of genistein to suppress EGF-stimulated *COX-2* mRNA and promoter activity. These data suggest a contributing role for protein tyrosine kinase inhibition in the down-regulation of *COX-2* expression by genistein.

In vitro studies using genistein have shown that concentrations between 1 and 10 μM are required to inhibit growth of PCa cell lines[46–48]. In our study we found a dose dependent decrease in *COX-2* mRNA with 1 μM genistein showing a ~30% decrease (data not shown) and 10 μM showing a 50% decrease. The decrease with the lower concentration of genistein however was not statistically significant suggesting that concentrations >1 μM are needed to see a significant effect on *COX-2* gene expression. Based on studies of isoflavone administration to human subjects the highest serum genistein levels achievable by ingesting soy-rich diets appears to be 1–5 μM[49–51] However, studies in mice suggest that the concentration achieved within the prostate following genistein administration is about 10-fold higher than serum genistein levels[52]. In patients receiving oral isoflavone supplements the prostate tissue levels of the individual isoflavones were at least 2 to 3-fold higher than their levels in plasma[51]. In the current study we investigated the effects of administering moderate amounts of soy isoflavones (Novasoy) to PCa patients in a neo-adjuvant setting for ~2 weeks between diagnosis and prostatectomy. Soy administration for this short period of time resulted in serum total isoflavone levels of ~0.785 μM. Although these levels were lower than those reported in some human studies cited above[49–51] and the sample size was modest, our analysis revealed a statistically significant decrease in *COX-2* expression in the isoflavone-treated prostate samples ($p<0.01$), probably indicating achievement of higher concentrations within the prostate. In the current study soy administration to patients resulted in a ~6-fold increase in prostate tissue levels of total isoflavones and a ~4-fold increase in genistein levels when compared to their concentrations in plasma (Gardner et al 2008 – manuscript submitted). These observations suggest that although the achievable range for serum isoflavones is ~1–5 μM, the intraprostatic concentration is much higher approaching levels at which genistein exerts its biological effects on the PG pathway.

Additionally, it is also important to note that while our *in vitro* studies examined the effects of genistein as a single agent, the effects seen in prostatectomy samples might reflect the synergistic and/or cumulative effect of all the isoflavones whose levels were elevated in the patients following Novasoy administration. Importantly, we found a significant negative correlation between prostate *COX-2* mRNA and serum isoflavone levels in the isoflavone-treated group, suggesting that modest doses of soy supplements exhibit an anti-inflammatory effect in PCa patients.

As shown in Table 1, five patients in the placebo group and one patient in the isoflavone group exhibited disease recurrence at the end of 3 years of study. Further analyses of the patient characteristics revealed that although the assignment of patients to the placebo or treated groups was double-blinded and randomized, it was unfortunate that all the patients with Gleason scores >7 were in the placebo group offering a plausible explanation for the higher recurrence rate in the placebo-treated patients. Interestingly, however, prostate *COX-2* mRNA levels were very high in the 3 out of 4 patients in the placebo group who exhibited disease recurrence and 4 out of 4 with Gleason scores of 9. This observation is in agreement with the findings of Cohen et al.[41] who showed that elevated *COX-2* expression was associated with high Gleason score, poor prognosis and high rate of recurrence in PCa patients.

Isoflavone administration also resulted in significant increases in the mRNA expression of the cell cycle inhibitor *p21* compared to the placebo group. The gene expression levels however varied among the individual patients. The observed positive correlation between prostate *p21* mRNA and serum isoflavone levels is supportive of an antiproliferative effect of the administered isoflavones. In the placebo group, we generally found an inverse association between COX-2 mRNA and p21 mRNA levels. A similar trend was seen in the soy treated group, where all patients had a suppressed COX-2 and elevated p21 levels compared to the placebo group (See Figure 6). Interestingly, the patient who had the highest p21 mRNA had undetectable levels of COX-2 mRNA.

The variations in expression of genes such as *COX-2* and *p21* within the same experimental group (placebo or soy isoflavone group) may arise due to a number of factors such as the differences in the cellular composition of the tissue samples we studied (epithelial versus stromal cells and normal versus malignant epithelial cells) as well as the presence or absence of inflammatory cells. However, it is important to note that in spite of these and other possible variations, *COX-2* expression was consistently and significantly lower in the samples from patients ingesting soy isoflavones, suggesting that soy isoflavones are likely to suppress *COX-2* expression in more than one cell type within the prostate. The results indicate that ingesting modest amounts of soy can achieve adequate concentrations of genistein and other isoflavones in the prostate to mediate beneficial actions such as suppression of inflammation and proliferation by modulating gene expression within the prostate. Overall, the findings suggest that by decreasing the synthesis and actions of PGs, genistein/soy may exert a beneficial effect to inhibit the development and/or progression of PCa.

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Abbreviations

COX-2	cyclooxygenase 2
EP4	prostaglandin receptor subtype *EP4*
EGF	epidermal growth factor
FP	prostaglandin receptor subtype *FP*
FBS	fetal bovine serum
GAPDH	glyceraldehyde 3 phosphate dehydrogenase
IGF-1	insulin-like growth factor 1
KGF	keratinocyte growth factor
PG	prostaglandin
PGE	prostaglandin E
PGF	prostaglandin F
15-pgdh	15-prostaglandin dehydrogenase
PMA	phorbal 12-myristic 13-acetate

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Goals

1. To demonstrate important novel actions of isoflavones to inhibit the prostaglandin pathway in prostate cancer.
2. To confirm in patients with prostate cancer that ingestion of modest amounts of dietary isoflavones can achieve adequate serum levels to regulate prostate gene expression *in vivo* similarly to our *in vitro* findings.

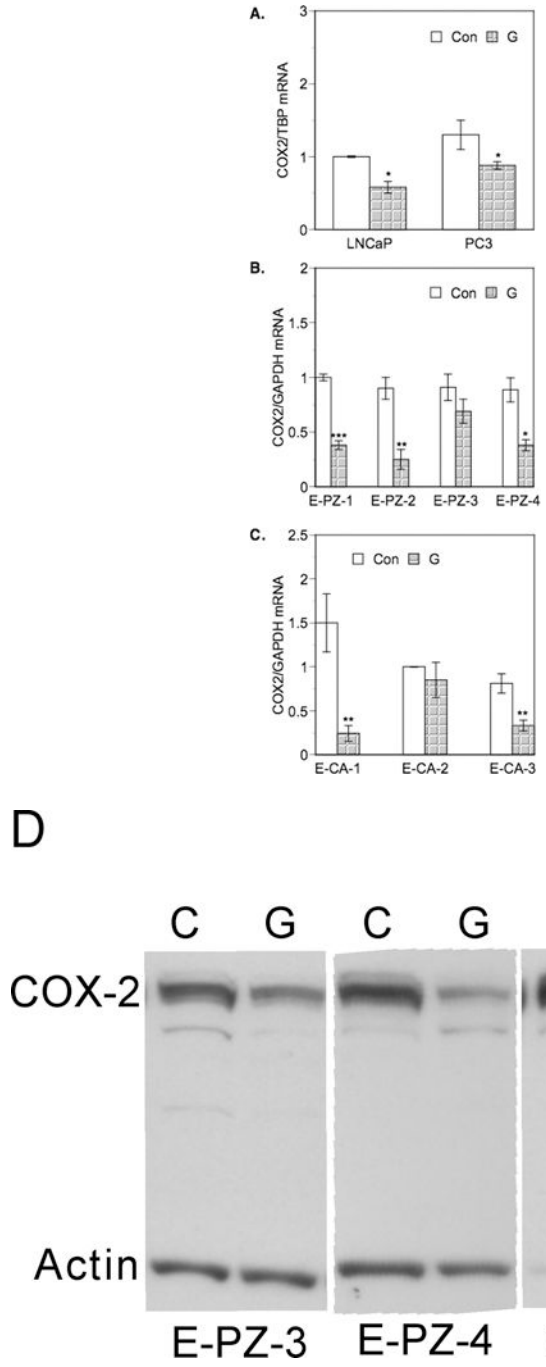

Figure 1. Effect of genistein on *COX-2* expression in PCa cell lines and primary prostatic epithelial cells

Sub-confluent cultures of LNCaP and PC-3 cells were treated with 0.1% ethanol vehicle (control, Con) or 10 M genistein (G) for 24 h. Total RNA was extracted and *COX-2* mRNA levels were determined by real-time RT-PCR analysis as described in *Materials and Methods. COX-2* mRNA were normalized to *TBP* mRNA levels and the ratios are given as fold change over control set at 1 (**Panel A**). Values represent mean ± SE of at least three individual experiments. * $p < 0.05$ as compared to control. Primary cultures of prostatic

epithelial cells obtained from the peripheral zone of normal prostate tissue (E-PZ) and adenocarcinoma (E-CA) were treated with either 0.01% ethanol vehicle (control, Con) or 10 μM genistein (G) for 24 h. Total RNA was extracted and the expression of *COX-2* mRNA was determined. *COX-2* mRNA levels were normalized to *GAPDH* mRNA levels and the ratios are given as fold change over control set at 1. Values represent mean ± SE for measurements conducted in triplicate. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ as compared to control. *COX-2* mRNA expression in normal prostatic epithelial cells is shown in **Panel B** and that of cancer-derived epithelial cells is shown in **Panel C. Panel D** is a representative Western blot showing COX-2 protein expression in primary normal prostatic epithelial cell cultures (E-PZ- 3-5) after 72 h of treatment with 0.01% ethanol vehicle (control, C) or genistein at 10 μM (G). The expression of β-actin was used as a control.

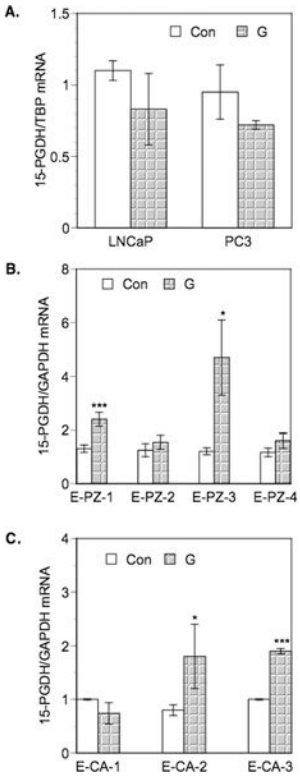

Figure 2. Effect of genistein on *15-PGDH* mRNA expression in PCa cell lines and primary prostatic epithelial cells

The various PCa cell lines and primary cell strains were treated with genistein and analyzed as described in Methods section. *15-PGDH* mRNA levels were normalized to *TBP* or *GAPDH* mRNA levels and the ratios are given as fold change over control set at 1. Values represent mean + SE for measurements conducted in triplicate. * $p< 0.05$, ** $p< 0.01$ and *** $p< 0.001$ as compared to control. *15-PGDH* mRNA levels in PCa cell lines are shown in **Panel A**, normal prostatic epithelial cells in **Panel B** and that of cancer-derived epithelial cells in **Panel C**.

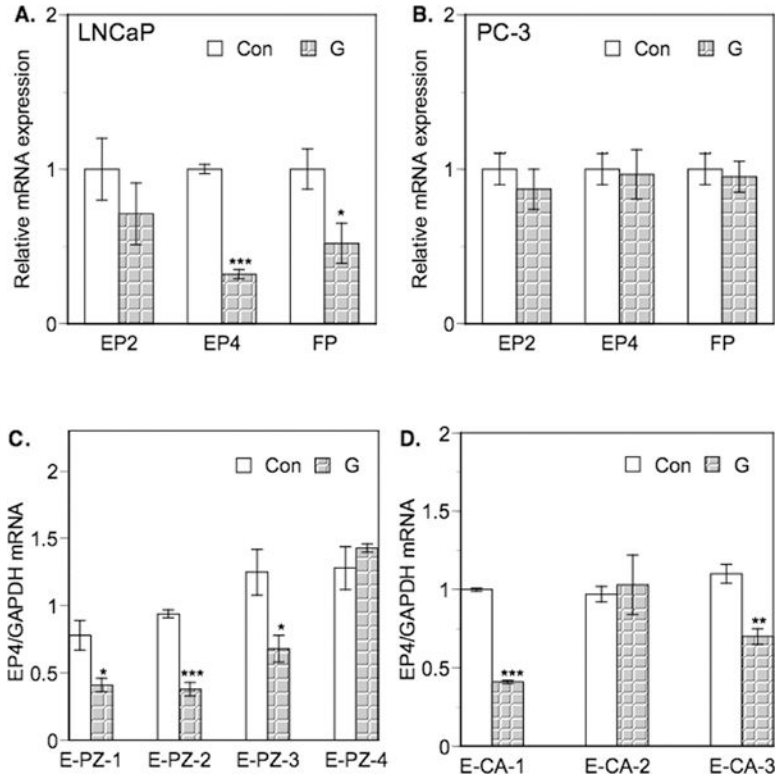

Figure 3. Regulation of PG receptor expression by genistein in LNCaP, PC-3 and primary prostatic epithelial cells

Sub-confluent cultures of LNCaP and PC-3 were treated with 0.1% ethanol (control, Con) or 10 μM genistein (G) for 24 h. Total RNA was extracted and *EP2*, *EP4* and *FP* mRNA expression was determined using real-time RT-PCR as described in *Materials and Methods*. Values were normalized to *TBP* mRNA levels and the ratios are given as fold change over control set at 1 (**Panel A**). Values represent mean ± SE of at least three individual experiments. * $p< 0.05$ and *** $p< 0.001$ as compared to control. The primary cell cultures were treated with genistein and analyzed for *EP4* receptor levels as described earlier. *EP4* PG receptor mRNA levels were normalized to *GAPDH* mRNA levels and the ratios are given as fold change over control set at 1. Values represent mean ± SE from three experiments. * $p< 0.05$, ** $p< 0.01$ and *** $p< 0.001$ as compared to vehicle. *EP4* mRNA expression in normal epithelial cells is shown in **Panel B** and that in cancer-derived epithelial cells is shown in **Panel C**.

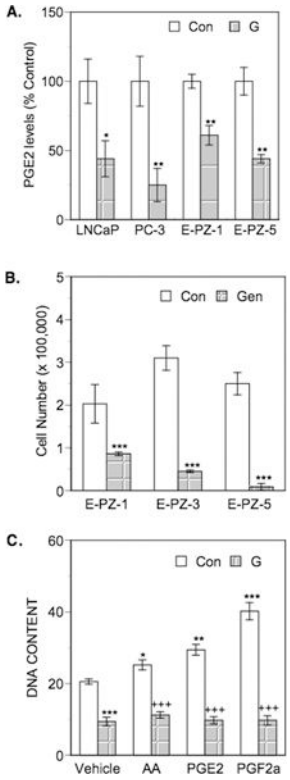

Figure 4. Effect of genistein on PGE_2 levels and cell growth

Panel A: Sub-confluent cultures of LNCaP, PC-3 and the primary normal prostatic epithelial cells E-PZ-1 and E-PZ-5 were treated with 0.01% ethanol vehicle (control, Con) or 10 μM genistein (G) for 72 h. Conditioned media from vehicle and genistein-treated cultures were collected and PGE_2 levels were determined using an EIA kit as described in *Materials and Methods*. Values are given as a percent of PGE_2 levels in vehicle-treated control set at 100%, which corresponded to 0.262 ± 0.04 pg/μg protein in LNCaP, 5.9 ± 1.2 pg/μg in PC-3, 175 ± 8.6 pg/μg in E-PZ-1and 0.029 ± 0.003 pg/μg in E-PZ-5 cells. Values represent mean ± SE from three experiments. *$p< 0.05$ and ** $p< 0.01$ as compared to control.

Panel B: Primary prostatic epithelial cells derived from normal human prostate tissue were seeded and cultured in defined growth media containing vehicle (0.01% ethanol) or 10 μM genistein. Cell growth at the end of 10 days was determined by a high-density growth assay as described in *Materials and Methods.* Values represented are mean ± SE from three experiments. *** $p< 0.001$ as compared to control.

Panel C: Semi-confluent LNCaP cells cultures in RPMI + 2% FBS were treated with 0.1% ethanol vehicle (control, Con), arachidonic acid (AA, 3 μM), PGE_2 (10 μM) or $PGF_{2\alpha}$ (10 μM) in the absence or presence of 10 μM genistein (G) added to the media. Fresh media and the various agents were replenished at the end of 3 days. Cell growth was determined at the end of 6 days by measuring DNA content as described in *Materials and Methods*. Values are expressed as μg of DNA/well and represent mean ± SE of at least three experiments. * $p< 0.05$, ** $p< 0.01$ and *** $p< 0.001$ when compared to vehicle. +++ $p< 0.001$ when compared to AA, PGE_2 or $PGF_{2\alpha}$ alone.

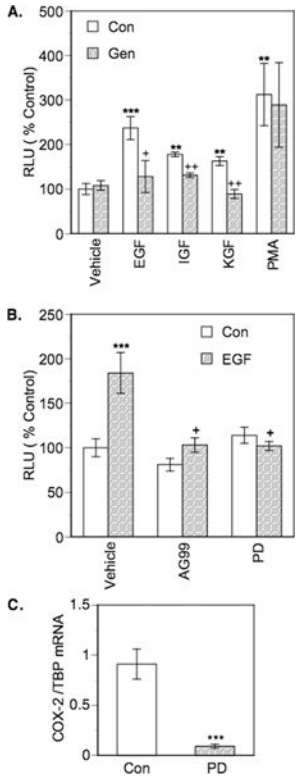

Figure 5.

A. Effect of genistein on *COX-2* promoter activity in LNCaP cells

A reporter construct containing a ~7kb fragment of the human *COX-2* promoter fused to the luciferase reporter was transiently transfected into semi-confluent cultures of LNCaP cells as described in *Materials and Methods*. A renilla luciferase plasmid was co-transfected to control for transaction efficiency. Following transfections the cells were treated with 0.1% ethanol vehicle (control, Con) or 10 μM genistein (G) either in the presence or absence of EGF, (100 ng/ml), PMA (1 μg/ml) for 6 hours and KGF (100 ng/ml) or IGF-1 (100 ng/ml) for 24 h. Luciferase activities were measured using the Dual luciferase assay kit. Results are expressed as relative lucifearase units (RLU), which represent the ratio of reporter luciferase to the renilla luciferase. Values are given as percent of control RLU set at 100%. * $p< 0.05$, ** $p< 0.01$ and *** $p< 0.001$ when compared to vehicle and + $p< 0.05$, ++ $p< 0.01$ as compared to EGF, IGF or KGF alone.

B. Effect of tyrosine kinase inhibitors on *COX-2* promoter activity

Following transfections (as described in Fig 4), cells were treated with 0.1% ethanol vehicle (control, Con) or 100 ng/ml EGF in the absence or presence of the tyrosine kinase inhibitors AG99 (10 μM) and PD153035 (10 μM) for 6 h. Values are given as percent of control RLU set at 100%. *** $p< 0.001$ when compared to vehicle and + $p< 0.05$ as compared to EGF treatment.

C. Effect of PD153035 on *COX-2* mRNA expression

LNCaP cells in culture media containing 5% FBS were treated with 10 μM PD153035 for 24 h. RNA was isolated and the expression of *COX-2* mRNA was determined as described in Figure 1. *COX-2* mRNA was normalized to TBP mRNA levels and the ratios are given as

fold change over vehicle treatment (control, Con) set at 1. *** $p < 0.001$ as compared to control.

All values represent mean ± SE of 3-6 experiments.

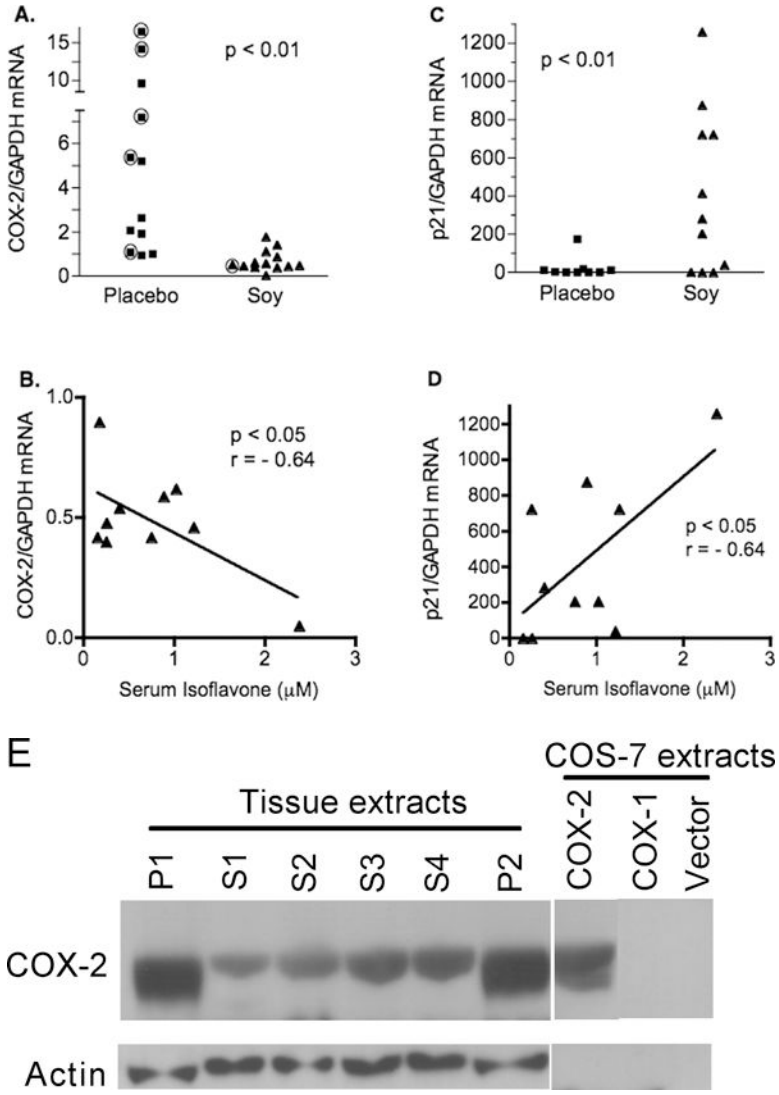

Figure 6. Analysis of gene expression in prostatectomy samples from PCa patients ingesting placebo or Nova soy Panel A

RNA was isolated from frozen tissues of prostatectomy specimens from PCa patients ingesting placebo (placebo, closed squares, n = 12) or Novasoy tablets (Soy, 30 mg/tablet, 3 tablets/day, closed triangles, n = 13) for 2–4 weeks prior to surgery. *COX-2* mRNA levels were determined by real-time RT-PCR, using specific primers as described in *Materials and Methods*. The expression of *GAPDH* gene is used as the control. Patients who had tumor recurrence at the end of three years are denoted with a circle around the symbol. **Panel B**. Correlation of prostate *COX-2* mRNA levels to serum isoflavone levels in patients ingesting Novasoy. **Panel C**. *p21* mRNA levels in the prostatectomy samples. **Panel D**. Correlation of prostate *p21* mRNA levels to serum isoflavone levels in patients ingesting Novasoy. **Panel E.** Western blot showing *COX-2* expression in extracts of tissue samples from patients ingesting placebo (P1 and P2) or soy (S1–S4). Positive and negative controls include extracts from COS-7 cells transfected with *COX-2* expression plasmid and COX-1

expression plasmid or vector plasmid respectively. The expression of β actin was used as a control.

TABLE 1

Patient Characteristics and Clinical Parameters

	PLACEBO	NOVASOY
Number of Patients	12	13
Age (yr)	58±2	57±2
PSA (ng/ml)	6.6±0.58	5.25±0.63
Clinical Tumor Stage	T1c (n=7) T2a (n=4) T2b (n=1)	T1c (n=9) T2a (n=4)
Gleason Score	6 (n=3) 7 (n=5) 9 (n=4)	6 (n=2) 7 (n=11)
Recurrences (at 3 years)	5	1
Serum Isoflavones (μM) Genistein Daidzein Equol	0.004±0.0022 0.011±0.0044 0.001±0.0005	0.424±0.110 0.164±0.036 0.093±0.065
Total Isoflavones	0.016±0.0033	0.785±0.214