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Combination of Calcitriol and Dietary Soy Exhibits Enhanced Anticancer Activity and Increased Hypercalcemic Toxicity in a Mouse Xenograft Model of Prostate Cancer

Jennifer Y. Wang, Srilatha Swami, Aruna V. Krishnan, and David Feldman[†]

Department of Medicine, Division of Endocrinology, Stanford University School of Medicine, Stanford, California 94305

Abstract

Background—The potential role of vitamin D and soy in prostate cancer (PCa) prevention/treatment has gained much attention in recent years. In this study, we evaluated the anticancer activity of calcitriol, the active form of vitamin D, dietary soy, and their combinations in a mouse model of PCa.

Methods—Athyimic male nude mice bearing PC-3 human PCa xenografts received diets containing 10 kcal% or 20 kcal% soy, calcitriol injections, or a combination of dietary soy and calcitriol. Changes in tumor growth, serum levels of 1,25(OH)₂D and calcium, and regulation of tumor gene expression were examined.

Results—The combination treatments resulted in substantially greater inhibition of tumor growth than either agent alone. Soy diets alone caused a modest elevation in serum 1,25(OH)₂D, whereas the calcitriol-soy combinations led to substantially elevated serum 1,25(OH)₂D, hypercalcemia, and in some cases lethal toxicity. The combinations enhanced calcitriol activity in regulating target gene expression, including greater up-regulation of anti-proliferative (p21, IGFBP-3) and pro-apoptotic (Bax) genes, increased inhibition of anti-apoptotic (Bcl-2) and cell cycle promoting (cyclin D1) genes, and suppression of prostaglandin (PG) synthesis and signaling (COX-2, 15-PGDH, PG receptors). Increases in serum calcium were accompanied by elevated expression of intestinal calcium absorption genes (TRPV6, calbindin-9k).

Conclusions—Soy increases the bioavailability of endogenous and administered calcitriol, thereby enhancing its anticancer effects and risk of hypercalcemia. Since both agents are easily available as dietary supplements, the increased potential for hypercalcemic toxicity becomes an important factor when considering the combined use of vitamin D and soy in PCa therapy.

Keywords

Vitamin D; calcitriol; soy; prostate cancer; CYP24A1; hypercalcemia

INTRODUCTION

Prostate cancer (PCa) is one of the most frequently diagnosed cancers among men in the United States and the second leading cause of cancer death in men after lung cancer [1]. While common treatment options include surgery, radiation, and androgen deprivation therapy, these methods are often insufficient for the treatment of advanced disease.

[†]Address correspondence and reprint requests to: David Feldman, MD, Stanford University School of Medicine, 300 Pasteur Drive, Room S025, Stanford CA 94305-5103, dfeldman@stanford.edu.

Extensive research has indicated a potential role for vitamin D and soy in the prevention and treatment of PCa [2–8].

Calcitriol (1,25-dihydroxyvitamin D₃) is the hormonally active form of vitamin D₃ (cholecalciferol). It regulates gene transcription by binding to the vitamin D receptor (VDR) and plays a major role in maintaining calcium homeostasis in the body. Additionally, calcitriol has been shown to exhibit anti-proliferative, pro-differentiating, pro-apoptotic, and anti-inflammatory actions on a variety of cancer cells, including PCa cells [5,8–12]. An important component of the anti-inflammatory activity of calcitriol is the inhibition of the synthesis and signaling of prostaglandins (PGs), which play an important role in prostate carcinogenesis [5,13–17]. Despite these beneficial anticancer activities, clinical applications of calcitriol have been limited by the increased potential for developing hypercalcemia, hypercalciuria, and renal stones at therapeutically effective doses [4]. One approach to limit the toxicity is to use lower concentrations of calcitriol in combination with other agents that enhance its anticancer activity.

Epidemiological and experimental evidence points to the potential benefits of soy isoflavones, in particular genistein, in PCa treatment and prevention [18–20]. Some of the anticancer effects of genistein in PCa cells include the induction of apoptosis, interference with cell signaling, reduction of androgen receptor expression, and inhibition of the PG pathway [19–22]. Genistein in combination with calcitriol causes enhanced inhibition of the proliferation of LNCaP PCa cells and primary human prostatic epithelial cells due to the synergistic up-regulation of VDR expression and vitamin D signaling [23,24]. Phytoestrogens have been shown to affect the sensitivity of breast cancer cells to vitamin D through regulation of the VDR promoter [25]. Previous studies from our lab have demonstrated that the combination of calcitriol and genistein inhibits the proliferation of the calcitriol-resistant human PCa cell line DU145 [26]. These observations suggest that combining genistein or soy with low doses of calcitriol might be a beneficial chemopreventive or therapeutic strategy in PCa [8,27,28].

We have shown that in addition to exerting anti-proliferative activity, genistein also acts as a noncompetitive inhibitor of the enzymatic activity of 25-hydroxyvitamin D-24-hydroxylase (CYP24A1), the P450 enzyme that catalyzes the initial conversion of calcitriol to less active metabolites [26]. By inhibiting CYP24A1 activity, genistein co-treatment effectively increased the half-life of calcitriol, up-regulated VDR expression, and thereby increased growth arrest and apoptosis of DU145 cells that were resistant when calcitriol was used alone [26]. However, these findings raise the possibility that the increased bioavailability of calcitriol due to co-administered genistein or soy would also be associated with increased toxic effects including hypercalcemia. Since vitamin D and soy are frequently used as dietary supplements, patients being treated with calcitriol or vitamin D supplements may also be supplementing with dietary soy, and the potential toxicity of combining these readily available agents needs to be evaluated.

Therefore, in the present study we evaluated the effects of combinations of calcitriol and soy in a mouse xenograft model of PCa. The data show that the combinations achieve significantly greater inhibition of tumor growth and enhanced anticancer activity compared to the individual agents. However, the combinations also increase the risk of hypercalcemia by prolonging serum calcitriol half-life and enhancing intestinal calcium absorption. These findings suggest that approaches aiming to reap the benefits of calcitriol and soy combinations in PCa treatment should proceed with caution.

MATERIALS AND METHODS

Materials

Calcitriol was kindly provided by Dr. Milan Uskokovic (BioXcell). Tissue culture media, supplements, and fetal bovine serum (FBS) were obtained from Gibco BRL (Grand Island, NY), Lonza (Walkersville, MD), and Mediatech Inc. (Herndon, VA), respectively. Research animal diets, including soy-supplemented diets, were obtained from Research Diets, Inc. (New Brunswick, NJ). The basal diet contains 1000 I.U. of vitamin D per kg, a moderately high level of vitamin D [29].

Methods

Cell Culture—PC-3 human PCa cells were routinely cultured in MEM medium containing 10% FBS, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Cells were maintained at 37°C and 5% CO₂ in a humidified incubator. Cell cultures were screened for the absence of murine viruses before inoculation into nude mice. Cultures grown to ~75% confluence in T-150 flasks were washed and trypsinized to obtain cell pellets under sterile conditions. Cell pellets were resuspended (~10×10⁷ cells/ml) in a small volume of sterile culture media and were mixed with an equal volume of Matrigel (BD Biosciences, Bedford, MA) prior to xenograft establishment in nude mice.

Xenograft establishment in nude mice—All animal procedures were performed in compliance with guidelines approved by the Stanford University Administrative Panels on Laboratory Animal Care (APLAC). Four- to six-week-old male athymic, nude (Foxn^{nu/nu}) mice purchased from Harlan Laboratories (Indianapolis, IN) were housed in a designated pathogen-free area in the Research Animal Facility, Stanford University School of Medicine, and fed irradiated mouse chow and autoclaved reverse osmosis-treated water. Xenografts were established in the flanks of mice by subcutaneous injections of PC-3 cells (~5 million) suspended in a small volume (~100 µl) of the culture medium–Matrigel mixture. The tumors were allowed to grow for three weeks and attained a size of 100 mm³ when treatment was initiated.

Treatments—Animals with established xenografts were randomly assigned to a control group and five treatment groups. Treatment groups received either injections of 0.1 µg calcitriol in 50 µl of 0.1% ethanol in sterile saline, soy-supplemented diet with 10 kcal% derived from soy protein (10%-soy), soy-supplemented diet with 20 kcal% from soy protein (20%-soy), or a combination of calcitriol injections and soy-supplemented diets. All diets were accessible *ad libitum*. Calcitriol was administered every other day by intraperitoneal (i.p.) injections. The mice in the control group received i.p. injections of 0.1% ethanol in sterile saline every other day and the basal diet. All diets contained 1000 I.U./kg of vitamin D [29]. All treatments were for a period of 4 weeks. Animal body weights were recorded weekly during the course of treatment and tumor volumes were calculated as described previously [30]. At the end of 4 weeks, the mice were subjected to CO₂ euthanasia and exsanguination by cardiac puncture 14 hours after the last injection of calcitriol. Sera were collected from the blood samples by centrifugation and stored at –80°C for later analysis. The tumors and intestinal mucosa were harvested and stored in Trizol reagent at –80° C until further use.

RNA isolation and real-time PCR—Tumor and intestinal mucosa samples were homogenized and total RNA was extracted using the Trizol reagent (Invitrogen, Life Technologies, Carlsbad, CA). Gene expression analysis by real-time RT-PCR was conducted as previously described [17]. Briefly, reverse transcription of the isolated RNA was performed with the SuperScript III First-Strand Synthesis kit (Invitrogen). Quantitative

real-time PCR was conducted with DyNamo SYBR green qPCR (Finnzymes, New England Biolabs, Ipswich, MA) on the Opticon 2 DNA engine (Bio Rad, Hercules, CA) using gene-specific primers, and mRNA expression levels, normalized to TATA-box binding protein (TBP) and GAPDH, were determined by the $2^{-\Delta\Delta C(t)}$ method as described previously [17].

Serum calcium and 1,25-dihydroxyvitamin D measurements—Serum calcium levels were measured using the QuantiChrom calcium assay kit (BioAssay Systems, Hayward, CA) according to manufacturer instructions. Levels of 1,25-dihydroxyvitamin D (1,25(OH)₂D), which includes both 1,25(OH)₂D₂ and 1,25(OH)₂D₃, were measured in pooled serum samples at Heartland Assays Inc. (Ames, IA). The assay involves a preliminary extraction and subsequent purification of vitamin D metabolites from serum using C₁₈-OH cartridges. Following extraction, the sample is then assayed using a competitive RIA procedure [31]. The RIA method employs a polyclonal antibody that is specific for both 1,25(OH)₂D₂ and 1,25(OH)₂D₃. The inter-assay and intra-assay coefficients of variation for this assay are 12.6% and 9.8%, respectively.

Statistical analysis—Statistical analyses were performed using the GraphPad Prism 5 (GraphPad Software, San Diego, CA). Data were evaluated by ANOVA and unpaired Student t tests with Welch correction.

RESULTS

Inhibition of Tumor Growth

PCa xenografts established in the flanks of male nude mice grew to ~100 mm³ in size by three weeks following tumor cell inoculation, at which time treatments were initiated. The tumors in the control group continued to grow over the next 4 weeks, reaching a mean volume of ~870 mm³ by week 7 (Figure 1). In calcitriol-treated mice, decreases in tumor volumes were evident by about 2 weeks after treatment was initiated. Continued tumor growth was evident in the calcitriol-treated group, but at a slower rate than controls. By the end of four weeks, calcitriol treatment resulted in a nearly 40% reduction ($p < 0.05$) in the mean tumor volumes compared to control (539 mm³ vs. 875 mm³). The magnitude of tumor inhibition seen in the mice receiving soy-supplemented diets was comparable to that due to calcitriol. Reductions in the tumor volumes were similar in the mice treated with the 10%-(564 mm³) and 20%-soy diets (574 mm³). Only the 20%-soy diet in combination with calcitriol was able to achieve a further reduction in final tumor volume compared to calcitriol alone, achieving a mean final tumor volume of 371 mm³ (~60% inhibition when compared to control and a 40% greater inhibition compared to either drug alone). In contrast, the 10%-soy diet combined with calcitriol resulted in a final tumor volume of 529 mm³, similar to the effect achieved by either agent alone.

Effects of calcitriol-soy combinations on body weight and serum levels of calcium and 1,25-dihydroxyvitamin D

Serum samples were obtained ~14 hours after the final calcitriol injection. No significant changes were observed in final body weight in the animals treated with calcitriol or the soy diets as single agents (Table I). However, serum calcium levels in animals treated with calcitriol registered a modest but statistically significant increase (10.0 mg/dl, $p < 0.05$). Signs of toxicity were most apparent in mice on the calcitriol-20%-soy combination treatment. Five of the sixteen mice in this group died within two weeks of treatment initiation. The body weights of the surviving mice receiving this combination treatment were significantly lower than the control mice at the end of 4 weeks, indicating toxicity (Table I). Serum calcium levels in the surviving mice treated with this combination showed a substantial and significant elevation demonstrating hypercalcemia (12.1 mg/dl, $p < 0.01$).

Elevations in serum calcium levels were also seen in the mice treated with the combination of calcitriol and 10%-soy, though to a lesser but still substantial degree (11.3 mg/dl, $p < 0.01$), and one mouse died in this group as well.

Serum 1,25(OH)₂D levels in the various experimental groups were also assayed (Table I). While serum 1,25(OH)₂D levels of animals receiving calcitriol injections were elevated as expected, 1,25(OH)₂D levels of the animals on the soy diets without calcitriol treatment were significantly increased as well, suggesting that the soy diets elevated endogenously synthesized 1,25(OH)₂D levels. Consistent with the significantly elevated serum 1,25(OH)₂D levels, both combination treatment groups showed significantly ($p < 0.001$) increased serum calcium levels. A dose-dependent effect was observed with the 20%-soy and calcitriol combination, resulting in much higher serum 1,25(OH)₂D and calcium levels than the 10%-soy and calcitriol combination. However, although elevated, no difference in serum 1,25(OH)₂D levels between 10%-soy alone and 20%-soy alone was observed at this time point. It should be emphasized that all blood samples were drawn 14 hours after the final calcitriol injection; the data therefore represent nadir values for serum 1,25(OH)₂D and calcium [32].

Changes in mRNA expression in PC-3 xenografts

We evaluated the ability of soy to modulate the regulatory effects of calcitriol on the expression of several target genes in the tumor. Because both the enhanced anticancer activity and increased toxicity were observed with the calcitriol-20%-soy combination, changes in gene expression when 20%-soy was administered alone or with calcitriol are presented here. Unless otherwise noted, treatments with the 10%-soy diet elicited similar responses as those seen with the 20%-soy diet, albeit to a somewhat lesser degree.

To understand the effects of soy supplementation on calcitriol-mediated transcriptional activity in the tumor, we first examined the gene expression of CYP24A1, whose transcription in target tissues is induced by calcitriol [8]. As expected, calcitriol increased CYP24A1 mRNA in the tumor 2.3-fold ($p < 0.05$) compared to untreated mice (Figure 2A). Soy supplementation alone did not appear to increase levels of CYP24A1 gene expression. The calcitriol-20% soy combination also significantly increased CYP24A1 mRNA 2.1-fold ($p < 0.05$), though this does not represent enhancement above the level achieved by calcitriol alone.

Next, we examined the expression of genes that are known to control proliferation (cyclin D1, p21 and IGFBP-3), apoptosis (Bcl-2 and Bax), and inflammation (COX-2, 15-PGDH, and EP receptors) in PCa. Significant increases in the mRNA levels of p21, an inhibitor of cyclin-dependent kinase (CDK) activity in promoting cell division, were seen in all treatment groups (Figure 2A). Consistent with the greatest degree of tumor retardation seen in this cohort, the 20%-soy and calcitriol combination increased p21 expression maximally (~2.5-fold over control mice, $p < 0.001$), which was significantly higher than calcitriol alone, indicating the potentiation of calcitriol effects by soy. Increases in the mRNA levels of IGFBP-3, a direct transcriptional target of calcitriol with anti-proliferative and pro-apoptotic effects [33], were also observed (Figure 2A). Calcitriol increased IGFBP-3 mRNA expression 1.8-fold over control, whereas soy did not produce a significant change. However, administration of soy in combination with calcitriol led to a further increase in IGFBP-3 mRNA level (~2.5-fold over control), indicating an enhanced effect. Soy co-administration greatly enhanced the down-regulation of the expression of the anti-apoptotic gene Bcl-2 and the up-regulation of the pro-apoptotic gene Bax by calcitriol (Figure 2B). Expression of mRNA for cyclin D1, an activator of CDK, was reduced approximately two-fold by calcitriol but was not enhanced by soy co-treatment (Figure 2B).

Significant up-regulation of the expression of the PG-degrading enzyme 15-PGDH was observed in all treatment groups (Figure 3A). Calcitriol and soy when used individually increased 15-PGDH mRNA levels approximately 2.5-fold over control. Combining the two agents significantly increased 15-PGDH mRNA levels over the increase seen with either agent alone, resulting in more than a five-fold increase over control. As expected, the expression of COX-2, the enzyme catalyzing PG synthesis, was significantly reduced by 50% by calcitriol (Figure 3B). Treatment with soy also decreased COX-2 expression levels to a similar degree. The combination of the two agents, however, produced no further decreases in COX-2 mRNA levels. Changes in the mRNA levels of three PG receptors were also analyzed. EP4 mRNA levels decreased significantly with both calcitriol and soy treatments, with the levels falling to 67% and 49% of control levels, respectively (Figure 3C). The combination was significantly more effective than calcitriol alone, reducing EP4 mRNA levels to 27% of control. Expression of the EP2 and FP receptors did not change significantly with the treatments (data not shown).

Changes in mRNA Expression in the Intestinal Mucosa

To further examine the mechanism of lethal hypercalcemia produced by the 20%-soy and calcitriol combination, the expression of genes involved in calcium transport, namely TRPV6 and calbindin-9k, was measured in the intestinal epithelia of the tumor-bearing mice (Figure 4). A significant induction of TRPV6 and calbindin mRNA expression was seen in the calcitriol-treated group. When used alone, the soy diets did not achieve any significant change in TRPV6 or calbindin-9k mRNA levels compared to control. In contrast, a striking enhancement of the increase in the expression of both calcium absorption genes was observed in the soy-calcitriol combination treatment groups. When calcitriol was combined with the 20%-soy diet, TRPV6 mRNA increased 55-fold, representing a substantial enhancement over the 10-fold increase seen with calcitriol alone. Calbindin-9k mRNA levels also increased more with the combination treatment than with calcitriol alone, resulting in a 20-fold increase in the group receiving the combination.

DISCUSSION

Pre-clinical research has demonstrated the benefit of calcitriol in the treatment of PCa, especially in combination with other cytotoxic agents [2,4,6,34], whereas large clinical trials thus far have been disappointing [35]. In this study we evaluated a potential treatment strategy, namely the combination of calcitriol and dietary soy. Evidence suggests that both these agents may have a beneficial role in the prevention and treatment of PCa [3,8–10,18–20,28,36,37]. Furthermore, earlier studies have demonstrated a synergistic enhancement of inhibition of prostate cell proliferation when these two agents are combined [24,26]. These observations provide the rationale for investigating the anticancer potential of calcitriol-soy combination *in vivo* using an animal model, wherein the potential for the development of hypercalcemic toxicity could also be evaluated.

Our data showed that calcitriol administration in combination with dietary soy protein resulted in more substantial inhibition of tumor growth than either agent alone, with the greatest inhibition being seen when the 20%-soy diet was combined with calcitriol. Consistent with enhanced inhibition of tumor growth was the enhancement by soy of calcitriol regulation of target gene expression, including greater up-regulation of anti-proliferative and pro-apoptotic genes and a more substantial decrease in the expression of anti-apoptotic and cell cycle regulatory genes. Enhanced inhibition of PG synthesis and signaling by calcitriol and soy was also observed in the tumor tissue, consistent with observations we have previously made in PCa cell cultures [16,17,27]. However, some calcitriol target genes (CYP24A1, COX-2, cyclin D1) examined in our study did not demonstrate an enhanced response to the combination treatment. This could be because

increases in circulating calcitriol levels following soy co-administration could have had a threshold effect on the expression of some genes, which are also direct targets of soy [22,23], or because the timing of our measurement of gene transcription missed the maximum changes in levels of expression of some genes.

As demonstrated by these findings *in vivo*, previous studies have established the ability of soy components such as genistein to potentiate calcitriol actions on gene transcription and prostate cell proliferation [19,21,24,26]. While the soy component genistein has independent actions on many of these genes, some of them, such as p21 and IGFBP-3, are also targeted by calcitriol [19,22,23]. Further vitamin D-independent actions of soy could also contribute to its anticancer activity in PCa cells [38,39]. However, we believe that the major mechanism underlying the synergistic activity of the calcitriol-soy combination is the ability of the soy component genistein to modulate the metabolism of vitamin D.

Metabolism of vitamin D in target tissues is mediated by two key enzymes: 1 α -hydroxylase (CYP27B1), which catalyzes the synthesis of calcitriol from 25(OH)D, and 24-hydroxylase (CYP24A1), which catalyzes the initial step in the degradation of 25(OH)D and calcitriol to inactive metabolites. One potential mechanism by which soy may influence the metabolism of calcitriol is by increasing its synthesis from dietary precursors. The standard AIN76 animal research diet contains 1000 I.U./kg of diet of vitamin D [29], as does the basal diet used in our study. Previous studies have shown increased expression of CYP27B1 mRNA in the colon of mice that received the AIN76 diet with genistein supplementation, but not in mice receiving AIN76 alone [40]. In our study, we found that serum 1,25(OH)₂D levels in mice treated with 10%- or 20%-soy were elevated in a dose-dependent manner to levels comparable to those in mice receiving only calcitriol injections, with maximal serum 1,25(OH)₂D levels achieved with the calcitriol-soy combinations. Thus, in order for circulating levels of 1,25(OH)₂D to increase in the absence of exogenous calcitriol administration, soy is likely enhancing the endogenous conversion of vitamin D from the diet to active calcitriol and/or inhibiting its degradation.

A second mechanism which we favor to underlie both the elevated 1,25(OH)₂D serum levels and the synergistic effect of the soy-calcitriol combination on tumor growth is the ability of genistein to inhibit the enzymatic activity of CYP24A1 [24,26], the main P450 enzyme in the calcitriol degradation pathway. Although genistein has also been shown to inhibit the transcription of CYP24A1 and CYP27B1 in DU-145 PCa cells [41,42], in this study we observed that CYP24A1 gene expression was not reduced below control levels in tumors from mice receiving soy-supplemented diets. Additionally, soy did not appear to attenuate CYP24A1 gene expression in the tumors when combined with calcitriol, allowing for comparable increases in CYP24A1 mRNA levels between the calcitriol-only and the combination treatment groups. In spite of this upregulation of CYP24A1 gene expression, serum 1,25(OH)₂D levels and calcitriol anti-tumor effects were both effectively enhanced by the combination treatment. Therefore, the ability of soy to prolong calcitriol serum half-life and enhance its activity must be primarily mediated through inhibition of the enzymatic activity of the 24-hydroxylase protein as we previously demonstrated [26] and not an effect on its mRNA expression. By inhibiting the degradation of calcitriol by CYP24A1, soy supplementation enabled higher levels of circulating calcitriol to exert a greater degree of anticancer activity, as our findings demonstrate. The role of CYP24A1 in antagonizing calcitriol activity against PCa has previously been explored using the P450 enzyme inhibitors liarozole and ketoconazole, whose combination with calcitriol resulted in greater tumor growth inhibition than achieved when either agent was used alone [43,44]. Accordingly, CYP24A1, now recognized as a candidate oncogene [45,46], has become the target of many potential therapeutic agents for PCa, including CYP24A1-resistant calcitriol analogs [47,48], tetralone derivatives [49], and other CYP24A1-inhibitory compounds.

Since CYP24A1 inhibition by soy increases calcitriol bioavailability [26], soy is expected to increase both calcitriol-mediated beneficial activity against PCa as well as its positive regulation of calcium absorption. Studies in CYP24A1-knock-out mice have demonstrated that the inability to degrade the active hormone greatly increases calcitriol activity, leading to decreased bone mineralization and high serum calcium levels [50]. Similarly, loss of function mutations in CYP24A1 have been shown to cause severe infantile hypercalcemia following vitamin D supplementation because of the inability to inactivate calcitriol [51]. The same mechanism would account for the findings in our study, where calcitriol-mediated up-regulation of the TRPV6 calcium channel and calbindin-9k [52–54] in intestinal epithelia was substantially enhanced by soy supplementation. The subsequent increase in calcium absorption led to hypercalcemia, which likely explains the increased mortality in the group treated with the calcitriol-20%-soy combination. In contrast, only modest elevations in serum calcium levels were seen in mice receiving calcitriol alone. Although we did not detect a significant difference in serum calcium levels between the two combination treatment groups, it is reasonable to assume that the deceased animals in the 20%-soy and calcitriol combination group had much higher serum calcium levels than the surviving mice in the immediate hours after calcitriol injections. This would suggest that actual peak values were higher than those determined when blood samples were drawn from surviving mice 14 hours after calcitriol injections, which may well represent nadir values [32]. The elevations in serum 1,25(OH)₂D levels and the degree of toxicity from soy-calcitriol co-administration also appeared to be dose-dependent. The 10%-soy diet clearly caused hypercalcemic toxicity, with one animal dying in this group. The higher 20%-soy concentration probably provided a greater degree of CYP24A1 inhibition and higher 1,25(OH)₂D levels, leading not only to more anticancer potency but also more severe hypercalcemia that proved fatal to several mice in this group. Thus, these results highlight the possible risk of increased hypercalcemia while using the calcitriol-soy combination in PCa therapy.

CONCLUSIONS

In conclusion, our study demonstrates the anticancer activities of calcitriol and soy *in vivo* in a mouse xenograft model of PCa. When combined, they exhibit enhanced anticancer activity but also increase the risk of toxicity from hypercalcemia. These findings, while further supporting the role of calcitriol and other dietary components in the treatment and prevention of PCa, also suggest the need for vigilance about the potential for toxicity if a combination of soy supplements and calcitriol administration or dietary vitamin D supplements are ingested. Dietary supplements of vitamin D were adequate to cause severe infantile hypercalcemia in children with CYP24A1 loss of function mutations because of their inability to inactivate 25(OH)D or 1,25(OH)₂D [51]. In our study, the modest levels of vitamin D in the basal diet also led to elevated serum calcium and 1,25(OH)₂D levels in the presence of soy supplements, likely due to soy inhibition of CYP24A1 activity. Since dietary vitamin D and soy are easily available agents, the potential risk of hypercalcemia becomes an important factor when considering the combined use of vitamin D and soy as a therapeutic approach in PCa.

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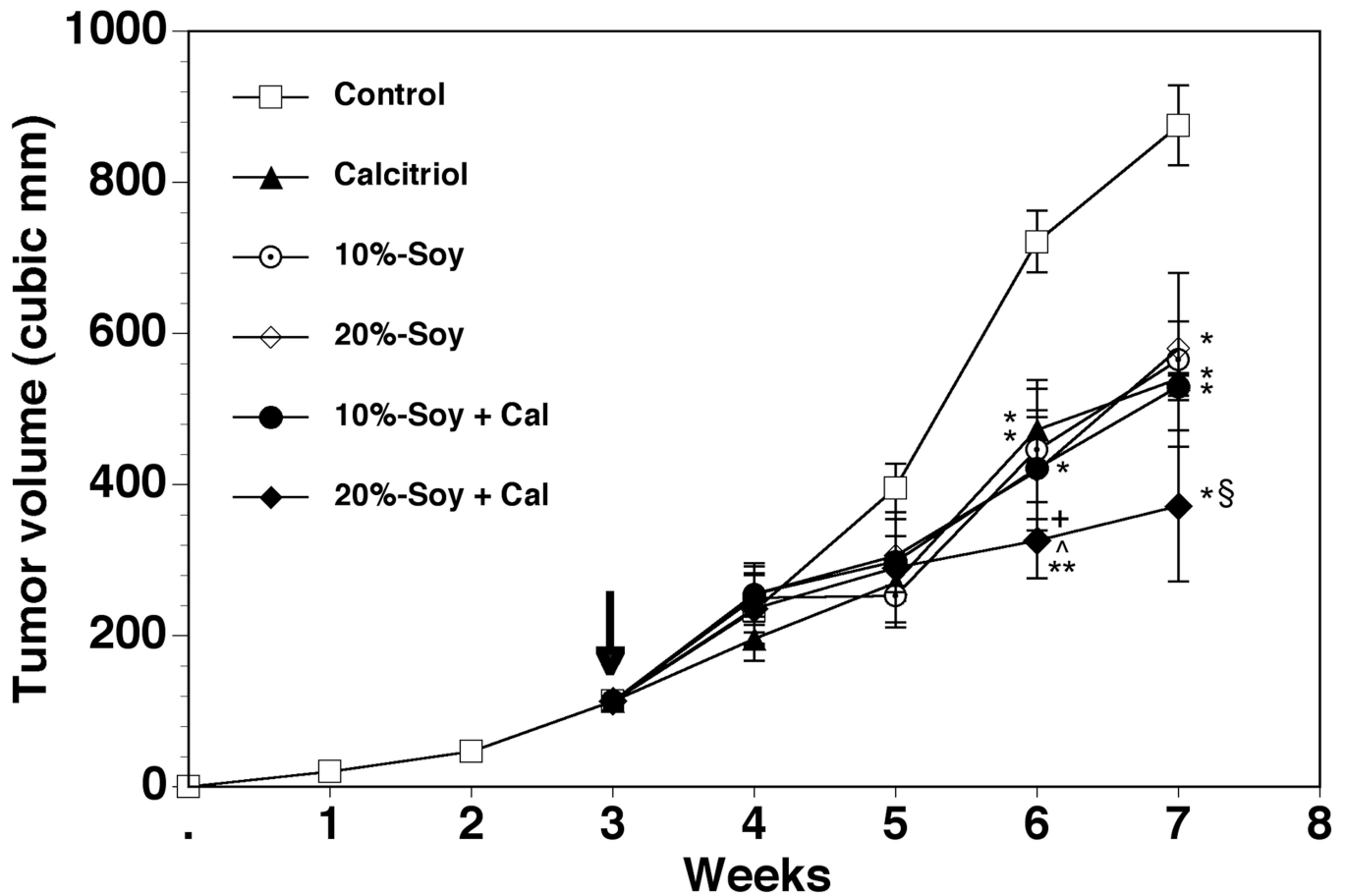


Figure 1. Effect of calcitriol, soy, and their combination on PC-3 xenograft growth

Human PC-3 cells were established in the flanks of male nude mice and allowed to grow for three weeks to approximately 100 mm³ before initiation of treatment (arrow). Treatment groups received either i.p. injections of 0.1 µg calcitriol in 50 µl of 0.1% ethanol in sterile saline every other day; soy-supplemented diet with 10 kcal% soy protein (10%-soy); soy supplemented diet with 20 kcal% soy protein (20%-soy); or a combination of calcitriol injections and soy-supplemented diets. Animals in the control group received i.p. injections of 0.1% ethanol in sterile saline every other day. Tumor volumes were measured weekly over the course of the four-week treatment period. Values represent mean ± SE. Control group n = 14; calcitriol group n = 16; 10%-soy group n = 13; 20%-soy group n = 14; 10%-soy+cal group n = 14; 20%-soy+cal group n = 12. * p < 0.05, ** p < 0.01 compared to control. + p < 0.05 compared to calcitriol. ^ p < 0.05 compared to 20%-soy. § Does not include animals that died before end of treatment course.

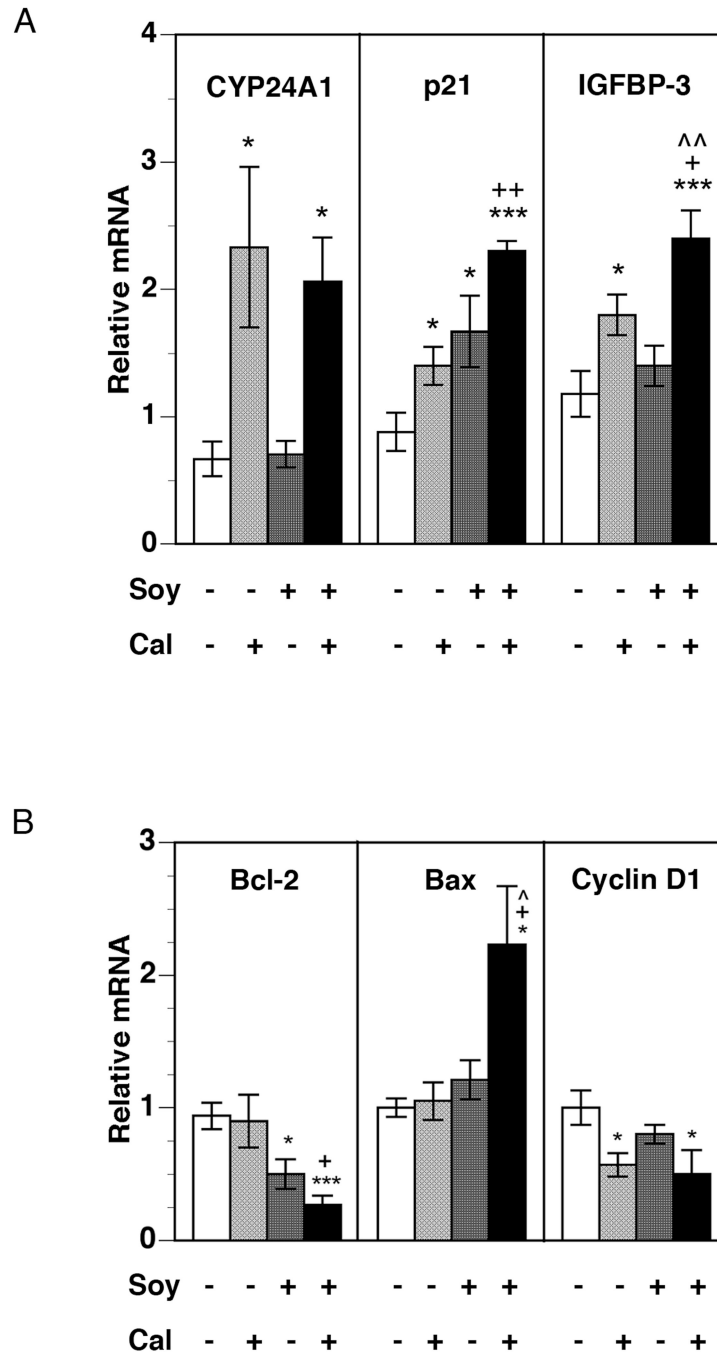


Figure 2. Changes in tumor mRNA expression of classical calcitriol target genes
 PC-3 xenografts were harvested from mice at the end of four weeks of treatment. Total RNA was isolated from harvested tumor tissue and mRNA levels of the calcitriol inactivating enzyme CYP24A1 (A), anti-proliferative genes p21 and IGFBP-3 (A), apoptosis-related genes Bcl-2 and Bax (B), and cell cycle promoter cyclin D1 (B) were analyzed by qRT-PCR, as described in Materials and Methods. Relative mRNA expression for each gene in tumors from control mice was set at 1. Values represent mean \pm SE of 6–11 measurements in each group. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to control. + $p < 0.05$, + + $p < 0.01$ compared to calcitriol. ^ $p < 0.05$, ^^ $p < 0.01$ compared to 20%-soy.

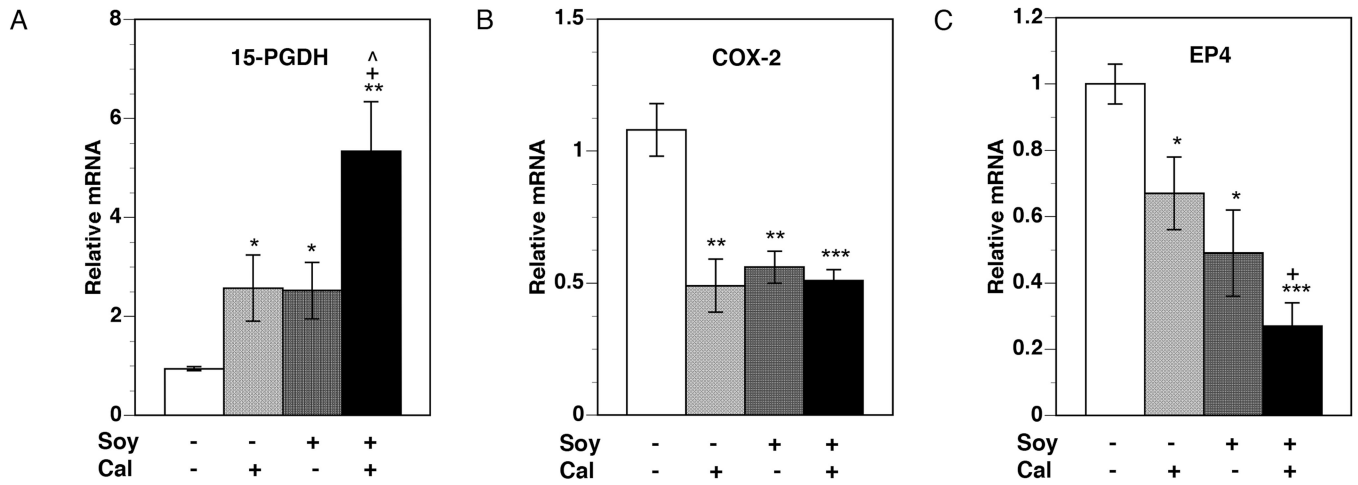


Figure 3. Changes in tumor mRNA expression of PG pathway genes

PC-3 xenografts were harvested from mice at the end of four weeks of treatment. Total RNA was isolated from harvested tumor tissue and mRNA levels of 15-PGDH (A), COX-2 (B), and PG receptor EP4 (C) were analyzed by qRT-PCR, as described in Materials and Methods. Relative mRNA expression for each gene in tumors from control mice was set at 1. Values represent mean \pm SE of 6–11 measurements in each group. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to control. + $p < 0.05$ compared to calcitriol. [^] $p < 0.05$ compared to 20%-soy.

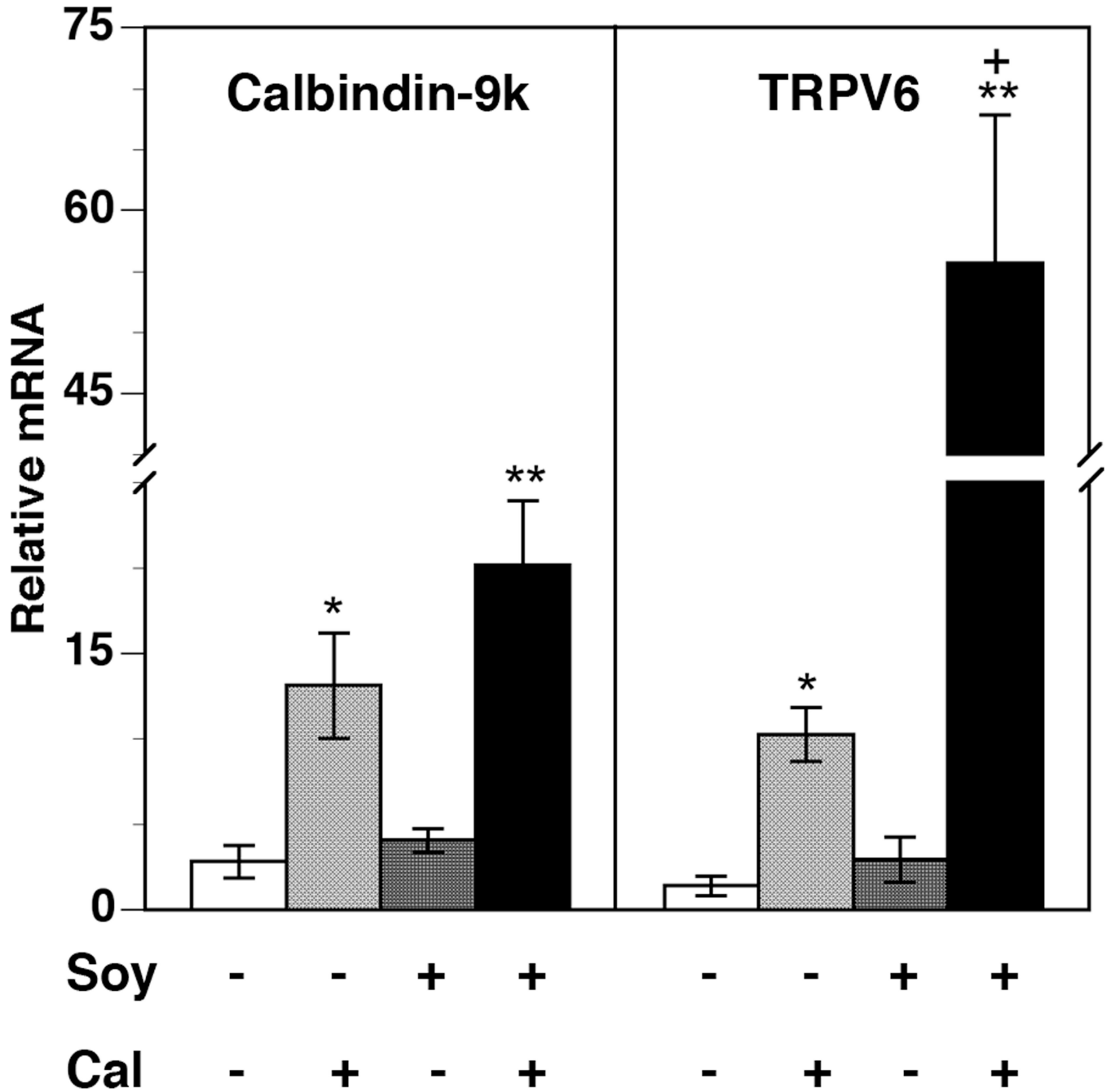


Figure 4. Changes in intestinal mucosa mRNA expression of calcium absorption genes
 Intestinal mucosa was collected from mice at the end of four weeks of treatment. Total RNA was isolated from the intestinal mucosa and mRNA levels of calbindin-9k and the TRPV6 calcium channel were analyzed by qRT-PCR. Relative mRNA expression for each gene in mucosa from control mice was set at 1. Values represent mean ± SE of 5–6 measurements in each group. * p < 0.05, ** p < 0.01 compared to control. + p < 0.05 compared to calcitriol.

TABLE I

Body weight, serum calcium, and serum 1,25(OH)₂D measurements

GROUPS	BODY WEIGHT (g) (n=14)	SERUM CALCIUM (mg/dl) (n=9)	SERUM 1,25(OH) ₂ D (pg/ml) (n=5)
Control	27.1±0.7	8.9±0.5	56.6±2
Calcitriol (0.1µg)	25.8±0.9	10.0±0.2 [*]	100.4±10 [*]
10%-Soy	28.1±0.7	9.1±0.6	133.8±16 ^{**}
20%-Soy	28.6±0.7	9.1±0.3	122.3±16 [*]
10%-Soy + Cal	25.1±0.6	11.3±0.4 ^{**}	170.2±19 ^{**+}
20%-Soy + Cal	24.6±0.5 [*]	12.1±0.8 ^{**}	266.7±20 ^{**+ ^^ §}

^{*} p < 0.05 when compared to control.

^{**} p < 0.01 when compared to control.

^{***} p < 0.001 when compared to control.

⁺ p < 0.05 when compared to calcitriol.

⁺⁺ p < 0.01 when compared to calcitriol.

^{^^} p < 0.01 when compared to 20%-soy.

[§] Does not include animals that died before end of treatment course.