

A Concentrated Aglycone Isoflavone Preparation (GCP) That Demonstrates Potent Anti-Prostate Cancer Activity *In vitro* and *In vivo*

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ABSTRACT

Purpose: Isoflavones have anticancer activities, but naturally occurring isoflavones are predominantly glycosylated and poorly absorbed. Genistein combined polysaccharide (GCP; Amino Up Chemical Co., Sapporo, Japan), is a fermentation product of soy extract and basidiomycetes myceliae that is enriched in biologically active aglycone isoflavones. This study analyzes GCP *in vitro* and *in vivo* for potential utility as a prostate cancer chemopreventive agent.

Experimental Design: Androgen-sensitive LNCaP and androgen-independent PC-3 cells were grown with various concentrations of GCP. *In vitro* cell growth was analyzed by the WST-1 assay, and apoptosis was assessed by fluorescence-activated cell sorting and detection of poly(ADP-ribose) polymerase cleavage using Western blot techniques. Effects of GCP on expression of cell cycle-regulatory proteins p53 (LNCaP only), p21, and p27 and the protein kinase Akt were considered using Western blot techniques. An *in vivo* LNCaP xenograft model was used to study the effects of a 2% GCP-supplemented diet on tumor growth in comparison with a control diet.

Results: GCP significantly suppressed LNCaP and PC-3 cell growth over 72 h (89% and 78% in LNCaP and PC-3, respectively, at 10 $\mu\text{g}/\text{ml}$; $P < 0.0001$). This reduction was associated with apoptosis in LNCaP cells, but not in PC-3 cells. GCP induced p27 and p53 (LNCaP only) protein expression within 6 h and suppressed phosphorylated Akt in both cell lines. The 2% GCP-supplemented diet significantly

slowed LNCaP tumor growth, increasing apoptosis ($P < 0.001$), and decreasing proliferation ($P < 0.001$) over 4 weeks.

Conclusions: GCP has potent growth-inhibitory effects against prostate cancer cell lines *in vitro* and *in vivo*. These data suggest GCP has potential as an effective chemopreventive agent against prostate cancer cell growth.

INTRODUCTION

Prostate cancer is the most common malignancy and the second leading cause of male cancer-related death in the United States (1). The incidence of prostate cancer increases rapidly with age, and a number of genetic and epigenetic factors have been implicated in the cause and progression of this disease. Despite extensive efforts to study the pathogenesis of prostate cancer, the factor(s) and conditions that promote progression of this malignancy are not well established. Additionally, suitably effective therapeutic approaches are still lacking, especially for the management of hormone-refractory prostate cancer (2). Many investigators and clinicians have recently begun to consider several forms of nutrition-based intervention to supplement the current available treatment options of surgery, androgen ablation, radiation, and chemotherapy (3–5). Most popular among such interventions for use in prostate cancer treatment include nutritional supplements, such as vitamin D₃, selenium, isoflavones, garlic, and green tea (6–10).

Epidemiological studies have suggested that the Asian diet, which is rich in soy phytoestrogens, including isoflavones, is one of the most significant contributing factors for the lower observed incidence and mortality of prostate cancers in Asia compared with Western countries (11, 12). Additionally, urinary excretion of isoflavones, as well as plasma and prostatic fluid isoflavone concentration, is elevated in Japanese men when compared with American men (13,14). Asian males who have moved to the United States were found to have increased mortality rates from prostate cancer equaling that of American-born men (15). Based on these and other similar data, many researchers have become significantly interested in studying soy isoflavones as potential chemopreventive agents for use against prostate cancer.

Isoflavones are nonsteroidal diphenolic compounds that are found in leguminous plants. These compounds are known to have estrogenic and antiestrogenic activities, in addition to growth-inhibitory activities that appear to be independent of the estrogen receptor (16). Genistein, a main isoflavone present in soy, has been reported to have significant protective effects against induced and spontaneous cancers that have been attributed to the induction of apoptosis and the inhibition of several receptor tyrosine kinase pathways, DNA topoisomerase II activity, proliferation, and angiogenesis (17–20). Genistein has also been demonstrated to induce cell cycle arrest at physiolog-

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ical concentrations ($\leq 20 \mu\text{M}$) and induce apoptosis in human prostate cancer cell lines (21, 22). Less data are available regarding the bioactivity of other soy isoflavones, such as daidzein and glycitein. However, a few studies have shown that both of these soy isoflavones also have anticancer activities in human prostate cancer cell lines, as well as in prostate cancer patients (16, 23).

Aglycone isoflavones are highly bioactive due to their unimpeded intestinal absorption, unlike their related glycosides, which are not absorbed across enterocytes (24). Glycosylated isoflavones are converted to aglycones via hydrolysis catalyzed by β -glycosidase, an intestinal enzyme that is also commonly found in microorganisms, plants, and some basidiomycete mushrooms (*i.e.*, *Ganoderma lucidum*). This study considers the bioactivity of a unique preparation of concentrated soybean isoflavones fermented with *G. lucidum* mycelia that is named Genistein Combined Polysaccharide (GCP; Amino Up Chemical Co., Sapporo, Japan) because of its high concentration of genistein. During the fermentation, a concentrated mixture of aglycone isoflavones is produced due to the hydrolytic cleavage of the sugar moiety from the isoflavone via *G. lucidum*-derived β -glycosidase. Compositional analysis of the final product demonstrated a significant conversion of isoflavone glycosides to their aglycone counterparts with a nearly 30-fold increase in genistein, a 16-fold increase in daidzein, and a 2-fold enrichment in glycitein (25). Additionally, β -glycosidase activity was observed to increase during the culture of the soybean extracts with *G. lucidum* from nearly undetectable levels up to 84.1 milliunits/ml over a 9-day culture period.

To examine the potential anti-prostate cancer activities of GCP, we have measured apoptosis and proliferation in both the androgen-sensitive LNCaP and the androgen-independent PC-3 prostate cancer cell lines. We have also considered the effects of GCP on the expression of important cell signaling proteins and cell cycle regulators such as phosphorylated Akt, p53, p21, and p27. Lastly, the *in vivo* effects of GCP were assessed on LNCaP xenografts in immunodeficient mice. We report here that GCP has potent antiproliferative and apoptotic-inducing activities both *in vitro* and *in vivo*, and given its availability as a simple dietary supplement, further clinical investigation for its use as a nutrition-based intervention in prostate cancer treatment is justified.

MATERIALS AND METHODS

Cell Culture. The androgen-sensitive human prostate cancer cell line LNCaP and the androgen-insensitive prostate cancer cell line PC-3 were obtained from American Type Culture Collection (Manassas, VA). LNCaP cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, glutamine, and antibiotics as described previously (26). PC-3 cells were grown in Ham's F12K medium with 2 mM L-glutamine adjusted to contain 1.5 g/liter sodium bicarbonate (90%) plus 10% fetal bovine serum, as recommended by American Type Culture Collection. The cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

Treatment with GCP. GCP, in a powder form, was supplied by Amino Up Chemical Co. This material was prepared by vortexing vigorously and dissolving in 50% ethanol

followed by centrifugation at 12,000 rpm at 4°C for 15 min to remove any insoluble debris. The brown supernatant containing the dissolved polysaccharide and genistein was used for additional experiments. For descriptive purposes, the active component(s) in this material is expressed as $\mu\text{g/ml}$ genistein, which dissolves completely under these conditions. Compositional analysis of the GCP extract revealed that 90 mg of genistein are present per gram of GCP extracted. Pure genistein was purchased from a commercial source (Sigma, St. Louis, MO) and dissolved in the same manner as GCP for use in cell proliferation studies.

Cell Proliferation Assays. LNCaP and PC-3 cells were seeded in 96-well plates at a density of 5000 cells/well in a final volume of 100 μl . Twenty-four h after seeding, the medium was removed and replaced with fresh medium containing the same concentration of ethanol (1%) as a vehicle control or with medium containing increasing concentrations of GCP (from 0.1 to 100 $\mu\text{g/ml}$, measured in terms of its genistein content) in a final volume of 100 μl . The culture was maintained in the CO₂ incubator for an additional period of 3 days. For each GCP concentration and time point, cultures were maintained in quadruplicates. At the end of the 24-, 48-, and 72-h time periods, 10 μl of WST-1 labeling solution (WST-1 cell proliferation assay kit; Roche Diagnostics, Indianapolis, IN) were added, and the cells were returned to the incubator for a period of 2 h. The absorbance of the formazan product formed was detected at 420 nm in a 96-well spectrophotometric plate reader, as described by the manufacturer.

Poly(ADP-ribose) Polymerase Immunoblotting. LNCaP and PC-3 cells were exposed to increasing concentrations of GCP as described earlier for a period of 24 h. The floating and adherent cells were pooled, washed three times with 1 \times PBS, and lysed in 1 \times RIPA buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS] containing a protease inhibitor mixture (Roche Diagnostics). Protein concentrations were measured spectrophotometrically using a modified Lowry assay protocol (DC Protein Assay; Bioroad, Hercules, CA). Normalized amounts of the cell lysate protein (25 μg) were electrophoresed through 7.5% denaturing polyacrylamide gels. The resolved proteins were transferred to nitrocellulose, blocked with 5% nonfat dry milk in 1 \times PBS buffer with 0.1% Tween 20, and probed with a monoclonal antibody against human poly(ADP-ribose) polymerase (Santa Cruz Biotechnology, Santa Cruz, CA). Protein bands were detected by the enhanced chemiluminescence procedure using luminol reagent (Santa Cruz Biotechnology) as described by the manufacturer.

Western Immunoblotting for Cell Cycle Regulators and Survival Signals. To determine the effects of GCP on the expression of cell cycle-regulatory proteins p53, p21, and p27 and the cell survival signal phosphorylated Akt, both LNCaP and PC-3 cells were treated with varying concentrations of GCP (0–40 $\mu\text{g/ml}$, genistein equivalence) for 24 h. Protein lysates were prepared and quantitated as described above. The lysates were electrophoresed through 7.5% denaturing polyacrylamide gels, transferred to nitrocellulose, and immunoblotted according to the standard methods described above. The p53 (DO-1), p21 (187), and p27 (F8) antibodies were purchased from Santa Cruz Biotechnology. The phosphorylated Akt (Ser⁴⁷³) monoclonal

antibody (clone 587F11) was purchased from Cell Signaling Technology (Beverly, MA), and the Akt (PKB-175) antibody was obtained from Sigma. Antibodies were diluted as recommended by the manufacturers for Western blotting.

Cell Cycle Analysis. Cultured LNCaP and PC-3 prostate cancer cells were exposed to 25 $\mu\text{g/ml}$ GCP for 24 h. Adherent cells were trypsinized and pooled with the cells in suspension, centrifuged, and washed three times with ice-cold PBS. A fraction of these washed cells were stained with trypan blue and counted to determine cell viability. The cells were adjusted to 1 million cells/ml and fixed in a 2:1 ratio (v/v) in chilled methanol overnight before staining with propidium iodide in the presence of RNase. Cell cycle distribution was analyzed on a Becton Dickinson (San Jose, CA) flow cytometer, and at least 10,000 cells were analyzed for each experimental condition. Data analysis was performed using CellQuest cell cycle analysis software.

Tumor Xenografts and GCP Treatment. Male nude mice (6–8 weeks old) were obtained from a commercial source (Harlan Inc., Indianapolis, IN) and housed in the nude mice facility at Columbia University. Thirty mice were randomized and split up into three groups and acclimatized for 1 week after arrival. All mice were injected with 1 million LNCaP cells on day 1 of the study in a final volume of 0.5 ml of a 50% suspension in Matrigel (Collaborative Biomedical Products, Becton Dickinson, Bedford, MA) using established procedures (27). The first group of 10 mice, the control group, was fed a regular diet for the entire course of the study (30 days after xenograft implantation). The second group of 11 mice, the prevention group, was switched to the experimental diet containing 2% GCP [prepared by Purina Test Diets (Richmond, IN)] 1 week before LNCaP xenograft implantation and continued on this diet for 30 days after implantation. The third group of 10 mice, the treatment group, were fed with the regular diet for exactly 1 week after the xenograft implantation and then switched over to the experimental diet for an additional 23 days. Animals in this third group were allowed to grow their tumors *in vivo* for 1 week before being switched to the experimental diet, thus reflecting the situation in the prostate cancer patient who already has an established tumor by the time he is examined by a clinician.

Three weeks after xenograft implantation, tumors were measured every 4–6 days for an additional 16 days, and tumor volumes were calculated using caliper measurements of height (h), length (L), width (W), and depth (volume = $\pi \times h (h^2 + 3a^2)/6$, $a = (L + W)/4$) (28). After the 30-day experimental period, the mice received i.p. injection with 0.5 ml of a 10 mM solution of bromodeoxyuridine [BrdUrd (BrdUrd Assay kit; Roche Diagnostics)] according to the instructions in the protocol exactly 1 h before being sacrificed. The animals were then humanely sacrificed, and their tumors were removed, fixed, and subjected to extensive analysis.

Immunohistochemical Detection of Cell Proliferation. Paraffin-embedded thin sections were dewaxed, rehydrated, and immunostained for BrdUrd using the *In Situ* Cell Proliferation Kit (POD Kit II; Roche Diagnostics) according to the protocol. Color development was achieved by using the fast red substrate for 1 h at room temperature. The slides were counterstained with hematoxylin, mounted with glycerol-gelatin, and covered with coverslips.

Immunohistochemical Detection of Apoptosis. Paraffin-embedded tumors were sectioned and immunostained using the *In Situ* Cell Death Detection Kit (POD TUNEL Assay; Roche Diagnostics) exactly according to the manufacturer's protocol. After the signal conversion by the POD reagent supplied, the slides were counterstained with Harris hematoxylin (Sigma) and mounted on coverslips with glycerol-gelatin (Sigma).

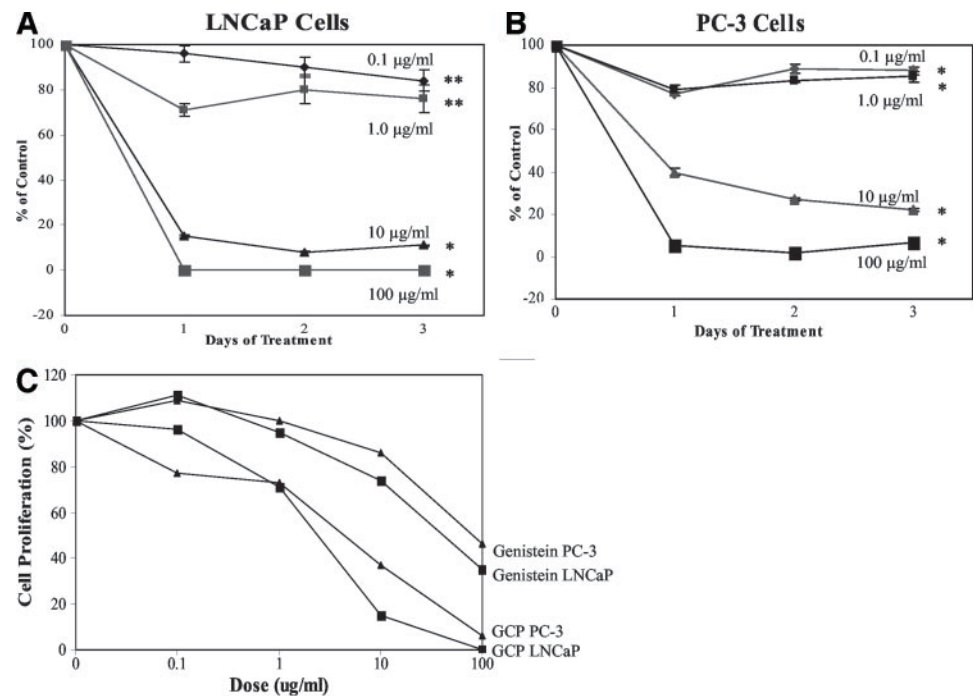
Histological Analysis and Scoring. Slides were examined by light microscopy at $\times 40$ magnification. Ten independent fields were counted for the tumor sections analyzed for both the terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) stain and the BrdUrd stain. Apoptotic cells were visualized by the brown-staining apoptotic nucleus after TUNEL labeling, and proliferating cells were identified by the characteristic red-staining nucleus after BrdUrd incorporation. Results were expressed as the mean of number of positive cells per high-power field.

Statistical Evaluation. All numerical data were expressed as the average of the values obtained for each group per time point. A skewed distribution of tumor volume was observed from a histogram analysis of the raw data. Therefore, data were log-transformed to help the distributions appear slightly more symmetric. A Kruskal-Wallis test was conducted to determine whether the mean volume was the same across all three groups, as well as to determine whether the rate of tumor growth was altered. Lastly, to more formally describe the relationship between tumor volume and treatment arm over time, a linear mixed effects model was used, regressing log-volume on treatment arm and week number with a random intercept corresponding to each mouse. This model accounts for correlation of observations within a mouse over time. Because of the skewed distribution of volume, we used log-volumes as the outcome because volume on the raw scale would violate the normality assumption required when fitting this model.

RESULTS

GCP Decreases the *in Vitro* Growth of Prostate Cancer Cell Lines by Affecting Proliferation and Apoptosis. This study analyzed the effect of GCP on the cell growth of both androgen-sensitive (LNCaP) and androgen-independent (PC-3) cells. The prostate cancer cell lines were exposed to increasing concentrations of the extract preparation (0.1–100 $\mu\text{g/ml}$ GCP) 1 day after seeding, and their viability was followed for 72 h using the WST-1 cell proliferation assay. Cell growth over the 72-h time period for both prostate cancer cell lines treated with GCP is shown in Fig. 1, A and B, respectively. The dose-response curves are presented as percentage of control growth on the corresponding day, and each value was measured in quadruplicates. The figures indicate that an exposure to GCP at a concentration between 1 and 10 $\mu\text{g/ml}$ and up to 100 $\mu\text{g/ml}$ strongly inhibited cell growth for both cell lines at all three time points. There was no growth inhibition evident in the vehicle (ethanol at a final concentration of 1%)-treated control cells. Additionally, GCP was found to be a more potent inhibitor of cell growth in both LNCaP and PC-3 cells when purified genistein was compared with equivalent concentrations of genistein in the GCP extract (Fig. 1C).

Fig. 1 Inhibition of proliferation of both (A) androgen-sensitive LNCaP and (B) androgen-independent PC-3 prostate cancer cell lines by GCP. Proliferation of both cell lines was assessed in the presence of various concentrations of GCP in a 96-well plate format. Cells were incubated for 72 h and pulsed for 3 h with WST-1. Absorbances were measured at 570 nm and expressed in relation to absorbances of control (untreated) cells. Values are expressed as means \pm SD ($n = 8$). *, $P \leq 0.0001$; **, $P \leq 0.005$ (compared with control cells in both LNCaP and PC-3 cells). C, comparison of purified genistein to amounts of GCP extract with equivalent concentrations of genistein on cell growth of both LNCaP and PC-3 cells over 24 h. Cell growth was analyzed by the WST-1 assay as described above.



GCP Induces Apoptosis in LNCaP Cells but not PC-3 Cells *in Vitro*. To determine whether the decrease in cell proliferation is brought about by induction of apoptosis, we looked for characteristic markers of apoptosis in the GCP-treated prostate cancer cells. Proteolysis of poly(ADP-ribose) polymerase from the native 113 kDa protein to a characteristic immunoreactive 89-kDa fragment is an indicator of the activation of the poly(ADP-ribose) polymerase protease (CPP32/prICE), an event that occurs during the initiation of the apoptotic process. Western blot analysis revealed poly(ADP-ribose) polymerase proteolysis occurred in LNCaP cells after treatment with 10–40 $\mu\text{g/ml}$ GCP for 24 h, as seen in Fig. 2A. However, no poly(ADP-ribose) polymerase cleavage fragment was observed in the PC-3 cells after a 24-h treatment with GCP at the same concentrations used with the LNCaP cells.

Next, flow cytometric analysis was used to examine the cell cycle distribution of both the parental LNCaP and PC-3 cells in the absence and presence of 25 $\mu\text{g/ml}$ GCP for 24 h. As can be seen from Fig. 2, B and C, a broad peak of cells equaling 39% of the total cell population appeared in the sub- G_0 population of the GCP-treated LNCaP cells, as compared with only 4% of the control LNCaP cells localizing in the sub- G_0 region. This elevated population of cells localizing in the sub- G_0 peak after GCP treatment is suggestive of an ongoing extensive apoptotic process or cytotoxic response. Interestingly, no significant increase in the sub- G_0 population was observed in the GCP-treated PC-3 cells in comparison with the control PC-3 cells. However, GCP appeared to cause an accumulation of PC-3 cells in the G_2 -M phase, as seen by the 38% increase in the number of cells that partitioned into this population compared with the control (Fig. 2, B and C). These results, in combination with the growth inhibition induced by GCP in both LNCaP and

PC-3 cells (Fig. 1), strongly suggest that GCP has selective actions on both cell types. It appears to initiate the process of programmed cell death in LNCaP cells, whereas it acts only as a cytostatic agent in PC-3 cells.

GCP Increases Expression of Cell Cycle Regulators p53, p21, and p27 and Decreases Expression of Phosphorylated Akt. To investigate potential molecular effectors of GCP action on these prostate cancer cell lines, we conducted a focused analysis to determine how GCP treatment might influence expression of some critical molecules involved in cell cycle progression and apoptosis. A Western blot analysis of the effects of increasing concentrations of GCP on Akt expression and phosphorylation showed that Akt expression was progressively diminished in both LNCaP and PC-3 cells as the dose of GCP was increased and the phosphorylated form (Ser⁴⁷³) was coordinately decreased (Fig. 3A). Akt in its active form (phosphorylated Akt) is known to signal resistance to apoptosis and to suppress the expression of the critical cell cycle regulator p27 through suppression of forkhead transcription factor activity (29). Indeed, when we evaluated p27 expression using the Western blot approach, we observed that GCP treatment increased expression of p27 protein in LNCaP and PC-3 in a dose-dependent manner (Fig. 3B). We also evaluated the effects of GCP on the expression of another apoptosis/cell cycle-regulatory protein, p53, and found that it is highly up-regulated in the LNCaP cells that are wild-type for p53 (Fig. 3C) and that this up-regulation was also associated with increased expression of p21, a critical downstream target of p53. The PC-3 cells are null for the p53 gene, and our analysis of p21 expression in these cells under the same experimental conditions did not identify any effect of GCP on the expression of this molecule (data not shown) in this cell line.

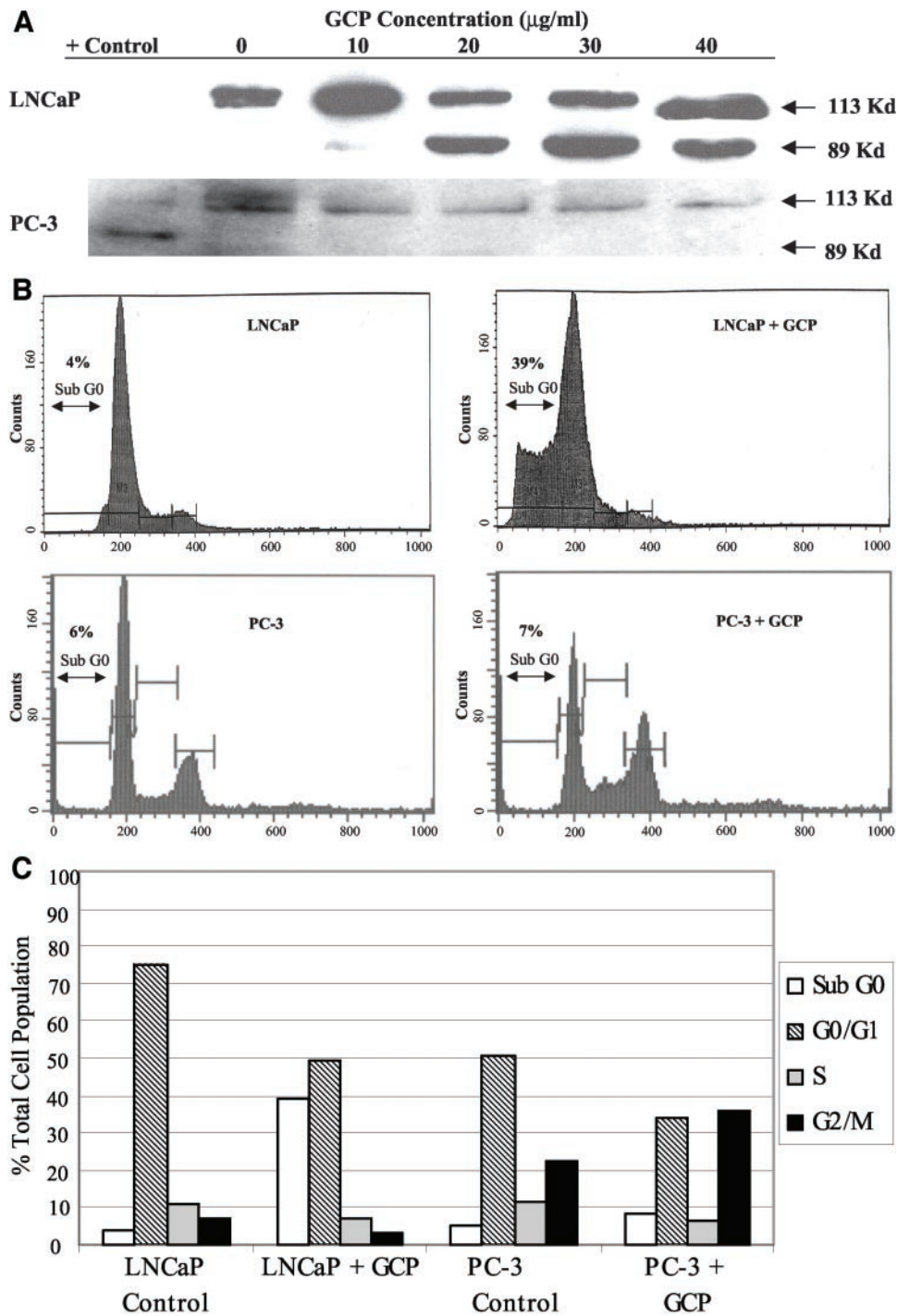


Fig. 2 GCP induces apoptosis in both LNCaP and PC-3 cells. *A*, cells were treated with various concentrations of GCP or vehicle alone (1% ethanol in distilled H₂O) for 24 h. At the end of the treatment, both floating and attached cells were pooled, and cell lysates were prepared in 1× radioimmunoprecipitation assay buffer followed by SDS-PAGE and Western analysis, as described in “Materials and Methods.” Membranes were probed with anti-poly(ADP-ribose) polymerase antibody, and the presence of the immunoreactive poly(ADP-ribose) polymerase cleavage product at 89 kDa indicated that cells were undergoing apoptosis. Blots are representative of two to three individual experiments. *B*, cells were exposed to 25 $\mu\text{g/ml}$ GCP for 24 h. Adherent and floating cells were pooled, washed three times in PBS, and fixed in a 2:1 ratio (v/v) in chilled methanol overnight before staining with propidium iodide in the presence of RNase. Cell cycle distribution was analyzed on at least 10,000 cells for each experimental condition. Data analysis was performed using CellQuest cell cycle analysis software. *C*, quantitative data from cell cycle analysis above were graphed to illustrate the percentage of cells in each phase of the cell cycle.

GCP Shows Significant Antitumor Effect *in Vivo*. The effect of treatment of athymic nude mice with an experimental diet containing 2% GCP over the 4-week experimental period after xenograft implantation is shown in Fig. 4. By day 3 after s.c. injection of the LNCaP cells, more than 80% of the mice had palpable flank tumors. During the course of this study, no mice suffered any untoward toxicity from the GCP, and none died. As

can be seen in Table 1, a comparison of the median volume across the arms at each week illustrates that the mice in the control arm have consistently higher median tumor volumes than those in either the prevention or treatment arms. A Kruskal-Wallis test was performed at each of the four time points to assess whether the mean volume was the same across the three treatment groups. Significant differences ($P < 0.05$) were ob-

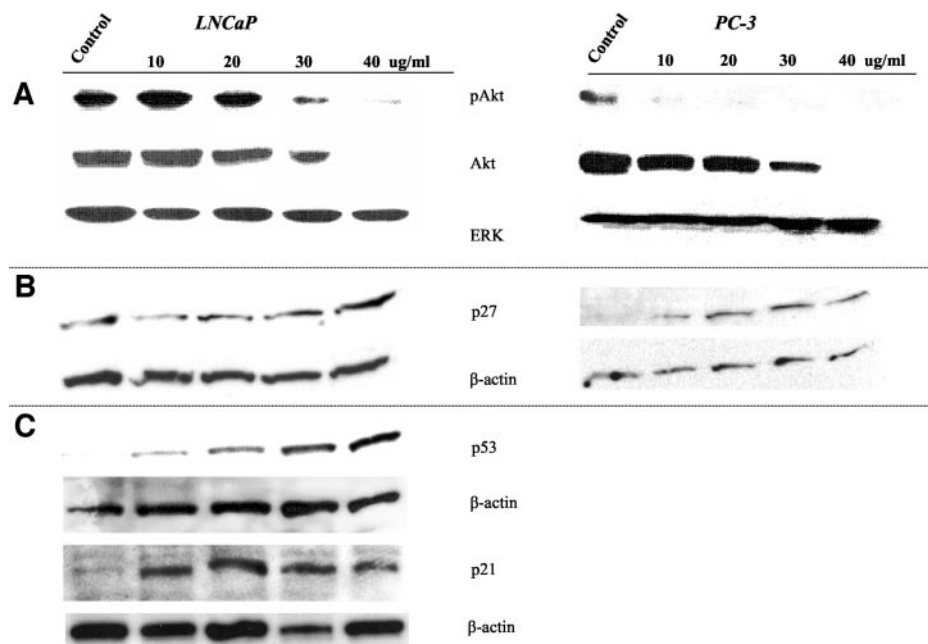


Fig. 3 Effects of GCP on cell cycle-regulatory proteins and survival signals. **A**, LNCaP and PC-3 cells were treated with various concentrations of GCP for 24 h. Cell lysates were prepared as described in "Materials and Methods," followed by SDS-PAGE and Western analysis. Membranes were probed with phosphorylated Akt (Ser⁴⁷³), Akt, and extracellular signal-regulated kinase (as a lane loading control) antibodies. **B**, LNCaP and PC-3 cells were treated with various concentrations of GCP for 6 h. Cell lysates were prepared as described in **A** and analyzed by SDS-PAGE/Western blotting techniques. Membranes were probed with anti-p27 and anti- β -actin (lane loading control). **C**, the same procedure was performed as described in **B**, except membranes were probed with anti-p53 and anti-p21 antibodies. PC-3 cells do not express p53, and p21 expression was not induced. All blots are representative of two to three individual experiments.

served in tumor volumes at time points 1, 3, and 4 ($P = 0.0158$, 0.3182, 0.025, and 0.049 for weeks 1, 2, 3, and 4, respectively). Further pairwise comparisons at weeks 1, 3, and 4 were not

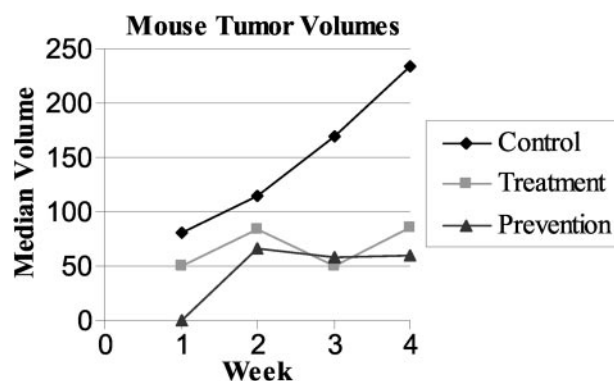


Fig. 4 Effect of GCP on growth of LNCaP tumors in nude mice. One million actively growing LNCaP cells mixed with an equal volume of Matrigel (Collaborative Biomedical Products) were injected s.c. into the right flank of nude mice. The control group ($n = 10$) was fed a regular diet for the entire course of the study (30 days after xenograft implantation). The prevention group ($n = 11$) was fed the experimental diet containing 2% GCP 1 week before LNCaP xenograft implantation and continued on this diet for 30 days after implantation. The treatment group ($n = 10$) was fed the regular diet for exactly 1 week after xenograft implantation and then switched over to the experimental GCP diet for an additional 23 days. Median tumor volume of each group is plotted against date. Significance of the observed trends is analyzed in Tables 1 and 2.

conducted due to the small number of mice in each group. The data do demonstrate, however, that the treatment and prevention groups had statistically significant lower tumor volumes than the control group at weeks 1, 3, and 4. Also, the treatment and prevention groups do not appear to be markedly different from one another throughout the experiment.

Furthermore, a linear regression was fit to the volume data over the four time points to obtain an estimate of the rate of volume increase among the three experimental groups (data not shown). A Kruskal-Wallis test determined that the median of the slopes was not significantly different ($P = 0.19$), demonstrating no difference in the rate of tumor growth among the three treatment groups.

To more formally describe the relationship between tumor volume and treatment over time, the data were fit with a linear mixed effects model regressing log-volume on treatment arm and week number with a random intercept corresponding to each mouse (Table 2). This model accounts for the correlation of observations within a mouse over time. The results indicate that there is a significant treatment effect where the P is 0.005. Point estimates that correspond to the prevention and treatment groups are in comparison with the control group. These data state that mice in the prevention group at a particular time have a reduced log-tumor volume of 1.58 on average compared with mice in the control group; similarly, mice in the treatment group have a 1.50 decrease in log-tumor volume compared with mice in the control group. Additionally, for every unit increase in time point, an average increase in log-volume of 0.52 is expected. The con-

Table 1 Descriptive statistics for volume by time and treatment group

Week	Prevention			Treatment			Control		
	Med	1st q	3rd q	Med	1st q	3rd q	Med	1st q	3rd q
1	0.00	0.00	56.20	50.45	0.00	73.90	80.15	59.73	165.20
2	66.50	50.80	102.60	83.60	69.45	100.50	115.20	67.45	197.20
3	57.70	32.65	108.90	50.30	31.25	69.38	169.90	68.65	362.20
4	59.10	49.40	176.40	86.00	50.20	113.00	233.70	114.40	487.50

Abbreviations: Med, median volume; 1st q, first quartile; 3rd q, third quartile.

stant increase is a constraint we imposed when fitting this particular model because we assumed a linear relationship between log-volume and week. A more complicated regression model was fit including an interaction term between week number and treatment group. Because this interaction term was not found to contribute significantly to an explanation of the variability of log-volume, results from this model were not included.

GCP Increased Apoptosis and Decreased Proliferation in Tumor Xenografts from Treatment Groups. Histological analysis of the tumor sections stained with H&E illustrated that the control animals had LNCaP tumors typical of an epithelial cell tumor with increased nuclear to cytoplasm ratio, prominent nucleoli, and increased mitotic figures compared with the GCP-treated animals (data not shown). LNCaP tumors excised from GCP-treated mice showed increasing areas of fibrosis, some necrotic regions, and areas that were characteristically acellular, which most likely represented the extracellular matrix bed (Matrigel) injected with the prostate cancer cells. The tumor mass in the GCP-treated tumor sections was uniformly <30%, as compared with the nearly 100% tumor mass representation in the control sections. Pathological features of the GCP-treated animals strongly suggest that these LNCaP tumors *in vivo* are undergoing apoptosis, contributing to a marked decrease in the cell number, as shown *in vitro*.

To consider the possibility that GCP induced apoptosis in the LNCaP cells, paraffin-embedded thin sections of the tumors were analyzed for TUNEL-positive nuclei, as seen in Fig. 5. Both the prevention and treatment groups demonstrated statistically significant increases of >2-fold in the number of TUNEL-positive nuclei ($P < 0.001$), suggesting an increase in apoptosis of the tumor cells after GCP intake. No significant difference was noted between the treatment and prevention groups, however.

The *in vivo* effects of the GCP diet on tumor cell proliferation in comparison with the control diet were examined using immunohistochemical detection of incorporated BrdUrd. As can be seen in Fig. 5D, at least a 2-fold decrease in the number of BrdUrd-positive nuclei was observed in both the prevention and treatment groups compared with the control group ($P < 0.05$). The magnitude of the decrease in LNCaP tumor cell proliferation after treatment with the GCP diet is the same as that observed for the increase in tumor cell apoptosis occurring in the prevention and treatment groups, suggesting that the decrease in proliferation observed could likely be due to the increase in apoptosis. These data are similar to data observed *in vitro* for LNCaP cells, but not PC-3 cells, in which GCP appeared to stop growth without inducing apoptosis.

DISCUSSION

In this study, we demonstrate that GCP, a soy isoflavone extract fermented with *G. lucidum* and rich in aglycone isoflavones, has significant anti-prostate cancer activities in both *in vitro* and *in vivo* systems. GCP inhibited cell proliferation in both the androgen-sensitive LNCaP and androgen-independent PC-3 prostate cancer cell lines. Interestingly, poly(ADP-ribose) polymerase cleavage data and cell cycle analysis indicated that GCP induced apoptosis in the LNCaP cells, whereas it only had a cytostatic effect in the PC-3 cells. Furthermore, the preparation elicited an increase in expression of p27 in both cell lines and p53 and p21 in LNCaPs, whereas expression of both Akt and phosphorylated Akt was decreased. *In vivo* studies conducted on immunodeficient mice with LNCaP xenografts demonstrated that a diet containing 2% GCP significantly reduced tumor volume and increased apoptosis within the tumors. This study further supports other laboratory and epidemiological data suggesting that increased consumption of soy-based products has chemopreventive properties and, perhaps, decreases the potential for prostate cancer progression.

The mechanism of action by which isoflavones inhibit proliferation and induce apoptosis in a variety of cancer cells has yet to be clearly determined. Due to the weak estrogenic activity of dietary isoflavones, much research has focused on their effects against hormone-responsive cancers and used breast and prostate cancer cell lines as model systems (30, 31). However, the mechanisms of action of genistein and other isoflavones do not appear to be limited to estrogen receptor activation. Recent data have suggested that genistein can also induce a G₁ block in the LNCaP prostate cancer cell line, as well as inactivate nuclear factor- κ B in both LNCaP and PC-3 cells (20, 32). Genistein has also been demonstrated to be a potent inhibitor of the epidermal growth factor receptor tyrosine kinase and specialized tyrosine kinase-mediated signals involved in the genesis of new blood vessels in the tumor (16, 19).

Individual isoflavones have been reported to have various effects on cell cycle progression in different cells, including

Table 2 Mixed effects linear regression model where log-volume is the response

Variable	Estimate	SE	T-statistic	P
Intercept	3.62	0.47	7.64	<0.001
Treatment arm				
Prevention	-1.58	0.54		
Treatment	-1.50	0.55		
Week	0.52	0.11	4.72	<0.0001

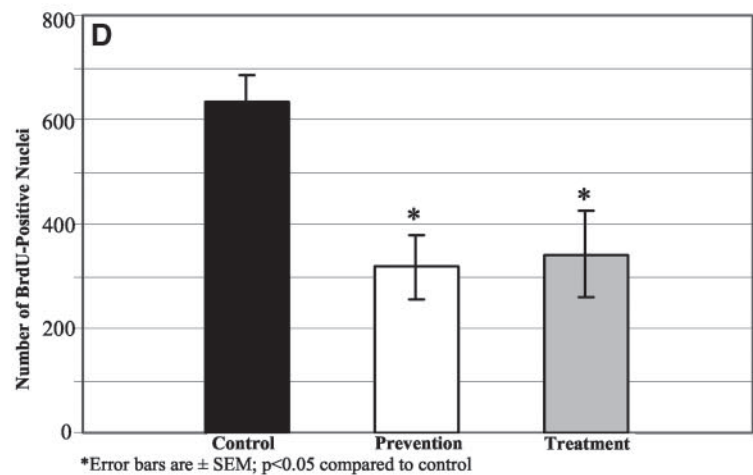
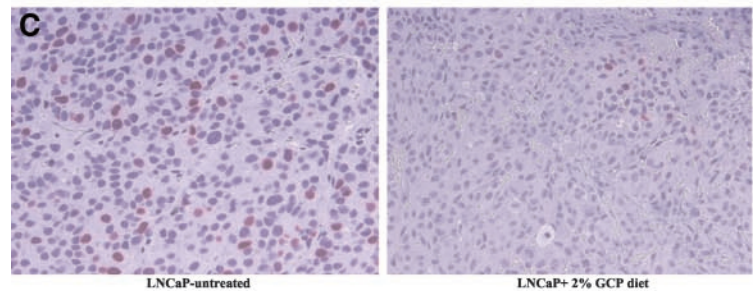
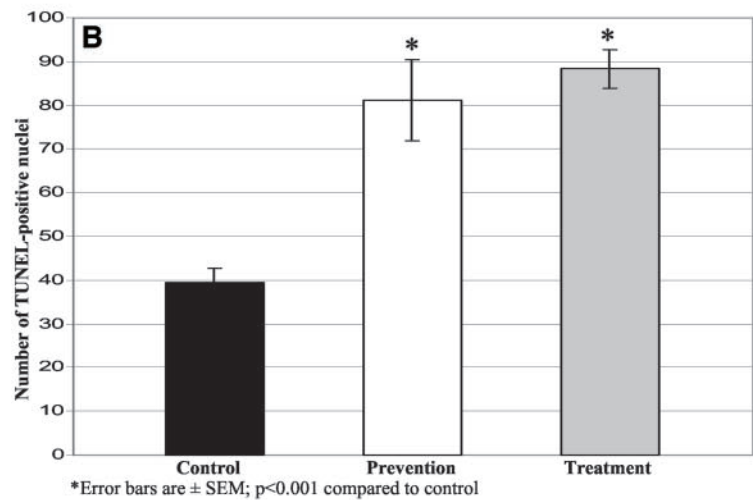
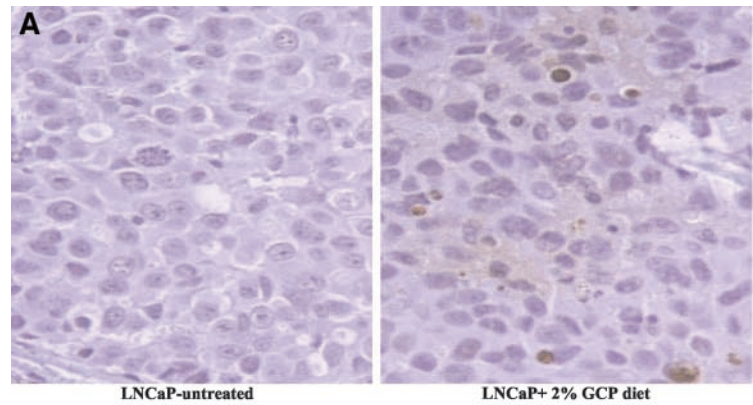


Fig. 5 Effect of 2% GCP-supplemented diet on apoptosis and LNCaP tumor cell growth as measured by TUNEL staining and BrdUrd incorporation of the LNCaP tumors *in vivo*. **A**, *in situ* analysis for apoptosis by the TUNEL assay in tissue sections from LNCaP tumors from both control and GCP-fed mice. The primary fluorescein-dUTP signal was converted into the peroxidase signal by using an anti-fluorescein antibody suitable for analysis by light microscopy. Slides were counterstained with hematoxylin. The representative field in control tissue demonstrates negligible peroxidase staining, indicating very little apoptosis. The representative field in LNCaP tumor tissue from GCP-fed mice shows a marked increase in apoptosis. Magnification, $\times 20$. **B**, graphical representation of number of TUNEL-positive nuclei per treatment group. Error bars indicate SE. *, $P < 0.001$. **C**, BrdUrd incorporation in LNCaP tumors from both control and GCP-fed mice showing red spots of cell nuclei that are actively undergoing DNA synthesis. After BrdUrd immunostaining, the slides were counterstained with hematoxylin. Magnification, $\times 20$. **D**, graphical representation of number of BrdUrd-positive nuclei per treatment group. Error bars indicate SE. *, $P < 0.05$.

blocking at various phases of the cell cycle or having no effect on cell cycle distribution at all (33–35). Whereas Shen *et al.* (21) observed a genistein-induced G₁ block in LNCaP cells, we saw no significant effect on cell cycle progression through G₁ in LNCaP cells after treatment with GCP, which contains a mixture of isoflavones. Rather, we observed a marked increase in the apoptotic/necrotic cell population to nearly 40% of the total cells without affecting progression through S, G₂ + M, or G₁ phases in the LNCaP cells. The dramatic rise in the sub-G₀ peak suggests that GCP-treated cells initiate the process of apoptosis irrespective of their cell cycle status and independent of cell cycle arrest. This could indicate that GCP, which contains a mixture of isoflavones and other potentially bioactive compounds, is capable of inducing this process in both the nonproliferating and proliferating compartments of the cancer cells, unlike many conventional chemotherapeutic agents that act solely on proliferating cells. For example, inhibition of cell proliferation and apoptosis induced by 5-fluorouracil is linked with cell cycle arrest primarily in the G₁ phase of the proliferative compartment of the tumor cells, leaving the nonproliferating cancer cells essentially untouched (36). Incidentally, the cell cycle stage-independent induction of apoptosis by GCP is reminiscent of a similar process that occurs after treatment of colon adenocarcinoma cells with nonsteroidal anti-inflammatory drugs such as sulindac and its metabolites (37).

Although GCP inhibited cell growth in both PC-3 and LNCaP cell lines, the preparation was unable to induce an apoptotic response in the PC-3 cells. Genistein has been reported in the literature to induce apoptosis in PC3-M cells, a metastatic variant of PC-3 cells; however, others have found that bioflavonoids were unable to induce apoptosis in PC-3 cells (34, 35). Importantly, GCP did induce a significant inhibition of proliferation and appeared to block the cell cycle at the G₂-M phase in PC-3 cells. This observed inhibition of cell proliferation is consistent with data reported by others in the literature regarding the effects of isoflavones on PC-3 cells (34). Inhibition of cell cycle progression in the aggressive, androgen-independent PC-3 prostate cancer cell line suggests that GCP could have potential utility as a nutrition-based intervention for advanced prostate cancer treatment, in addition to chemoprevention.

The exact mechanisms of action by which isoflavones exert cell growth-inhibitory effects have yet to be elucidated. One proposed mechanism suggests that genistein inhibits 5 α -reductase, which would result in decreased androgen receptor activation (38). Our data, however, demonstrates that the soy isoflavone enriched preparation (GCP) containing genistein, daidzein, and glycitein inhibits cell growth in both androgen-sensitive and androgen-independent prostate cancer cell lines. This observation indicates that the mechanism(s) by which GCP acts is not dependent on the inhibition of signaling via the androgen receptor axis alone because this response is observed in the androgen receptor-negative prostate cancer cell line PC-3.

In attempt to better understand how GCP is inhibiting cell growth, we analyzed the expression of a few important cell cycle-regulatory molecules. In both PC-3 and LNCaP cell lines, dose-dependent increases in protein expression levels were observed for the cyclin-dependent kinase inhibitor p27. This action, in and of itself, has the potential to suppress cell cycle

progression. In LNCaP cells, which have a wild-type p53 phenotype, we also observed a strong up-regulation of p53 and its downstream target p21 in a dose-dependent manner after treatment with GCP. In contrast, the PC-3 cells, which are p53 null, do not up-regulate p21 when treated with GCP. These observations may be pertinent to the differential phenotypic effects of GCP on these two cell lines, wherein both of these cell lines experience inhibition of cell cycle progression, but only LNCaP cells undergo apoptosis in response to GCP treatment. It is important to note that we also observed a dramatic decrease in expression of phosphorylated Akt, a cell survival signal, in both prostate cancer cell lines. This action, as well, has the potential to influence the differential apoptotic response in these two cell lines because LNCaP cells are more sensitive to inhibition of Akt action than PC-3 cells (39). Due to the complex composition of GCP, it is possible that a strong survival signal is being transduced through another cell survival/mitogenic pathway such as the mitogen-activated protein kinase pathway, which is enabling these cells to avoid apoptosis. An additional possibility could be attributed to the different genetic background of the two cell lines. More work is required to determine the differential responses to GCP between the LNCaP and PC-3 cells regarding apoptosis, and this is the focus of ongoing studies.

We found that the antiproliferative and apoptosis-inducing effects of GCP on the LNCaP cells *in vitro* were effectively extrapolated to the *in vivo* situation. Our studies indicate that this biologically active mixture can markedly decrease the overall tumor volume in nude mice under both treatment and prevention conditions when ingested as a regular diet supplemented with 2% GCP. Immunohistochemical analyses of the tumor xenografts indicated significant inhibition of tumor cell growth and an induction of apoptosis. Our data suggest that GCP does have a significant growth-inhibitory effect on LNCaP tumors xenografted into nude mice. Whether or not these data will translate to humans is under consideration at this time. However, a recently published observational study (23) on a small cohort of Australian men showed that dietary isoflavones do increase apoptosis in low- to moderate-grade prostate carcinoma, giving further support to the potential role of dietary isoflavones in prostate cancer treatment protocols. Initial clinical experience with GCP supplementation in prostate cancer therapy is already being tested, and anecdotal evidence identifies the potential for benefit (40).

The contributions of other components present in GCP, such as the polysaccharide fraction of the *G. lucidum* mycelia, need to be further explored. This study was not designed to analyze the purported immune-stimulating or other activities of these polysaccharides and the product (GCP) as a whole, and this is the focus of future work. However, research done by other investigators has suggested that the mycelia of basidiomycetes such as *Leninus edodes* and *G. lucidum* express many biologically active compounds (41, 42). Basidiomycetes polysaccharides have been reported to express antihypertensive, immune-modulating, antibacterial, antiviral, and antitumor activities (43–46). *G. lucidum* polysaccharides, specifically, have also been commonly used as antitumor agents in folk medicine (47). Moreover, there is the distinct possibility that novel natural products are produced during the fermentation process involved

in the production of GCP. Further work is needed to elucidate such compounds and analyze their potential bioactivity.

Given the difficulties in treatment of hormone-refractory prostate cancer, clinicians, researchers, and patients are avidly looking toward other means that could enhance the current cure rates achievable by conventional therapies such as radiation and chemotherapy. The significance of nutrition in disease onset and progression is quickly gaining recognition, and nutrition-based intervention is becoming an important treatment modality in all specialties of medicine, including urology. Although more research is needed, dietary isoflavones are emerging as a potentially beneficial nutritional therapy for the prevention and treatment of prostate cancer.

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