Combined Inhibitory Effects of Green Tea Polyphenols and Selective Cyclooxygenase-2 Inhibitors on the Growth of Human Prostate Cancer Cells Both *In vitro* and *In vivo*

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Abstract

Purpose: Cyclooxygenase-2 (COX-2) inhibitors hold promise for cancer chemoprevention; however, recent toxicity concerns suggest that new strategies are needed. One approach to overcome this limitation is to use lower doses of COX-2 inhibitors in combination with other established agents with complementary mechanisms. In this study, the effect of (−)epigallocatechin-3-gallate (EGCG), a promising chemopreventive agent from green tea, was tested alone and in combination with specific COX-2 inhibitors on the growth of human prostate cancer cells both *in vitro* and *in vivo*.

Experimental Design: Human prostate cancer cells LNCaP, PC-3, and CWR22Rν1 were treated with EGCG and NS398 alone and in combination, and their effect on growth and apoptosis was evaluated. *In vivo*, athymic nude mice implanted with androgen-sensitive CWR22Rν1 cells were given green tea polyphenols (0.1% in drinking water) and celecoxib (5 mg/kg, i.p., daily, 5 days per week), alone and in combination, and their effect on tumor growth was evaluated.

Results: Combination of EGCG (10-40 μmol/L) and NS-398 (10 μmol/L) resulted in enhanced (*a*) cell growth inhibition; (*b*) apoptosis induction; (*c*) expression of Bax, pro-caspase-6, and pro-caspase-9, and poly(ADP)ribose polymerase cleavage; (*d*) inhibition of peroxisome proliferator activated receptor γ; and (*e*) inhibition of nuclear factor-κB compared with the additive effects of the two agents alone, suggesting a possible synergism. *In vivo*, combination treatment with green tea polyphenols and celecoxib resulted in enhanced (*a*) tumor growth inhibition, (*b*) lowering of prostate-specific antigen levels, (*c*) lowering of insulin-like growth factor-I levels, and (*d*) circulating levels of serum insulin-like growth factor binding protein-3 compared with results of single-agent treatment.

Conclusions: These data suggest synergistic and/or additive effects of combinatorial chemopreventive agents and underscore the need for rational design of human clinical trials.

Limited options for the management of prostate cancer and its increasing incidence necessitate search for novel preventive approaches for this disease. One such approach is through chemoprevention, a means of cancer management by which the occurrence of the disease can be entirely prevented, slowed, or reversed by the administration of one or more naturally occurring and/or synthetic compounds (1, 2). Indeed, prostate cancer represents an ideal candidate disease for chemoprevention because it is typically diagnosed in elderly men; therefore, even a modest delay in the neoplastic development achieved through pharmacologic or nutritional intervention could result in substantial reduction in the incidence of the clinically detectable disease (3). It is proposed that prevention of cancer is more feasible and holds better promise with the use of multiple agents that can hit multiple targets in the process of carcinogenesis (4–7). There are many advantages of a combinatorial chemopreventive approach, the most important considerations of which are (*a*) an attempt to hit more than one critical molecular target, (*b*) lower dose requirement lessening the concern of associated toxicities, and (*c*) likeliness of human acceptability. Consistent with this view, there is currently an increasing interest in the use of a combination of two or more agents, at low doses with differing modes of action, for chemoprevention (6–13).

Aberrant or increased expression of cyclooxygenase-2 (COX-2), the rate-limiting enzyme involved in the conversion of arachidonic acid to prostaglandins, has been implicated in the pathogenesis of many cancer types (14). We observed overexpression of COX-2 in human prostate adenocarcinoma (15). Taking advantage of this finding, we showed suppression of prostate carcinogenesis by dietary supplementation of a selective COX-2 inhibitor celecoxib in transgenic adenocarcinoma

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mouse prostate, a model in which prostate cancer development occurs in a manner similar to human disease (16). Narayanan et al. (6, 8) reported similar effects of celecoxib and other nonsteroidal anti-inflammatory drugs in the transgenic adenocarcinoma mouse prostate model. Oral administration of celecoxib to mice with xenograft prostate tumors increased tumor latency, decreased tumor growth, and enhanced antitumor activity in combination with other cytotoxic drugs, which was associated with caspase-3 and caspase-9 activation (17). COX-2 antisense-expressing PC-3ML tumors show growth delay compared with vector controls, and this growth inhibition was further slowed in combination with other chemotherapeutic drugs (18). We have also shown that oral infusion of a decaffeinated polyphenolic fraction isolated from green tea [green tea polyphenol (GTP)] at a human achievable dose significantly inhibits prostate cancer development, progression, and metastasis, and enhances tumor-free and overall survival in transgenic adenocarcinoma mouse prostate (19). This finding has also subsequently been verified from other laboratories (20, 21). Continuing our efforts to define molecular targets for prevention of prostate cancer by green tea (22), we recently observed that (−)epigallocatechin-3-gallate (EGCG), a major polyphenolic constituent of GTP, also inhibits COX-2 without affecting COX-1 expression (23).

Epidemiologic and case-control studies suggest that both COX-2 inhibitors (24, 25) as well as oral consumption of green tea (26, 27) hold promise in chemoprevention of prostate carcinogenesis. A concern relevant to the use of COX-2 inhibitors is the associated adverse side effects that recently resulted in the withdrawal of VIOXX and concerns about CELEBREX that were being investigated as potential cancer chemopreventive agents (7, 28). The major reasons for the side effects were related to high doses of these agents, and it was generally concluded that COX-2 inhibitors induce cardiovascular problems when prescribed at high doses over long durations (29). Although COX-2 inhibitors offer a rational choice for prevention of cancer, we hypothesized that lower doses of COX-2 inhibitors may prove to be more beneficial in prevention of cancer if given in combination with other agents with complementary mechanisms. This study was therefore designed to investigate prostate cancer chemoprevention through a mechanism-based approach using COX-2 inhibitors at low doses in combination with GTP. It is important to mention here that for our *in vitro* assays, we decided to use NS-398, a specific COX-2 inhibitor, and EGCG, the major polyphenol in green tea. This choice was based on our previous experience of working with EGCG and the fact that pure agents rather than complex mixtures offer a rational choice for deciphering mechanism of action. For our *in vivo* experiments, we decided to use celecoxib, a nonsteroidal anti-inflammatory drug approved for cancer chemopreventive studies, and GTP. This choice was based on real-life human situation, keeping in mind that humans are more likely to drink green tea rather than a purified compound from green tea and could be persuaded to take celecoxib.

Materials and Methods

Materials. The human reactive monoclonal and polyclonal antibodies against poly(ADP)ribose polymerase (PARP), Bcl-2, Bax, caspase-6, caspase-9, vascular endothelial growth factor, and proliferating cell nuclear antigen were purchased from Upstate Biotechnology (Lake Placid, NY). Anti–peroxisome proliferator activated receptor γ (PPAR-γ) antibody was obtained from Upstate Biotechnology; anti–phosphorylated (phospho-) nuclear factor-κB (NF-κB) p65 and anti–COX-2 were obtained from Cell Signaling (Beverly, MA). Anti-mouse or anti-rabbit horseradish peroxidase (HRP) conjugate secondary antibodies were obtained from Amersham Life Science, Inc. (Arlington Height, IL). ELISA kits for insulin-like growth factor-I (IGF-I) and IGF binding protein 3 (IGFBP-3) assays were purchased from Diagnostic Systems Laboratories (Webster, TX). The BCA Protein Assay Kit was purchased from Pierce (Rockford, IL). Novex precast Tris-glycine gels were obtained from Invitrogen (Carlsbad, CA). GTP (>95% pure) and EGCG (>98% pure) were obtained from Tokyo Food Techno Co. Ltd. (Tokyo, Japan). NS-398 was purchased from Sigma Chemical Co. (St. Louis, MO), and celecoxib (200 mg capsules) was purchased from a local pharmacy.

Treatment of cells. Human prostate carcinoma cells CWR22Rv1, LNCaP, and PC-3 were obtained from American Type Culture Collection (Rockville, MD). Cells were cultured in RPMI 1640 (Life Technologies, Rockville, MD) medium with 2 mmol/L L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mmol/L HEPES, and 1.0 mmol/L sodium pyruvate, and supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. The cells were maintained under standard cell culture conditions at 37°C and 5% CO_2 in a humid environment. The cells (70-80% confluent) were treated with EGCG (10, 20, and 40 μmol/L) in PBS and NS-398 (10 μmol/L) in ethanol alone and in combination for 24 h in complete cell culture medium. Cells that were used as controls were incubated with the maximum used amount of ethanol only.

Cell growth and viability. The effect of combination of EGCG and NS-398 on the viability of cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were plated at 1×10^4 per well in 200 μL complete culture medium. The next day, cells were treated with EGCG (10-40 μmol/L) and NS-398 (10 μmol/L) alone and in combination for 48 h. Each concentration was repeated in 10 wells. After incubation for a specified time at 37°C in a humidified incubator, cell viability was determined. MTT (5 mg/mL in PBS) was added to each well and incubated for 2 h, after which the plate was centrifuged at 500 × *g* for 5 min at 4°C. The MTT solution was removed from the wells by aspiration. After careful removal of the medium, 0.1 mL buffered DMSO was added to each well, and plates were shaken. The absorbance was recorded on a microplate reader at the wavelength of 540 nm. The effect of each compound alone and in combination on growth inhibition was assessed as percentage of cell viability in which vehicle-treated cells were taken as 100% viable.

Apoptosis detection by fluorescence microscopy. The Annexin V-FLUOS Staining Kit (Roche Applied Biosciences, Mannheim, Germany) was used for the detection of apoptotic cells. The cells were grown to ~50% confluency on cell culture slides and then treated with EGCG (10, 20, and 40 μmol/L) and NS-398 (10 μmol/L) alone and in combination for 48 h. The cells were then incubated with Annexin V-FLUOS labeling reagent for 10 min and then analyzed using fluorescence microscopy (excitation at 450-500 nm and detection at 515-565 nm). Cells with green fluorescence were scored as apoptotic. For quantitation, apoptotic cells were counted in 10 randomly selected fields and represented as mean apoptotic cells.

Protein extraction and Western blotting. The cells (60-70% confluent) were treated with EGCG (10-40 μmol/L) and NS-398 (10 μmol/L) alone and in combination in complete medium. Twenty-four hours later, the medium was aspirated and the cells were washed with cold PBS (10 mmol/L, pH 7.4) followed by incubation in ice-cold lysis buffer [50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L EDTA, 20 mmol/L NaF, 100 mmol/L Na_3VO_4, 0.5% NP40, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride (pH 7.4)] with freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III, Calbiochem, La Jolla, CA) over ice for 30 min. The cells

were scraped and the suspension was collected in a microfuge tube and passed through a 21.5-gauge needle to break up the cell aggregates. The lysate was cleared by centrifugation at 14,000 × *g* for 15 min at 4°C, and the supernatant (total cell lysate) was stored at −80°C for further analysis. For Western blotting, 25 to 40 μg of protein were resolved over 8% to 12% polyacrylamide gels and transferred to a nitrocellulose membrane. The blot was blocked in blocking buffer [5% nonfat dry milk/1% Tween 20; in 20 mmol/L TBS (pH 7.6)] for 1 h at room temperature, incubated with appropriate monoclonal or polyclonal primary antibody in blocking buffer for 2 h at room temperature or overnight at 4°C, followed by incubation with appropriate secondary antibody HRP conjugate. The blots were exposed to enhanced chemiluminescence (Amersham Life Science) and subjected to autoradiography using XAR-5 film obtained from Kodak (Rochester, NY). Densitometric measurements of the bands in Western blot analysis were done using digitalized scientific software program, UN-SCAN-IT, purchased from Silk Scientific Corporation (Orem, UT).

ELISA for prostate-specific antigen, IGF-I, and IGFBP-3. Quantitative sandwich enzyme immunoassay techniques were used to quantitate prostate-specific antigen (PSA; Anogen, Mississauga, Ontario, Canada) IGF-I and IGFBP-3 (Diagnostic Systems Laboratories) levels in the serum of nude mice by following the manufacturer's protocol. Briefly, standard, control, and serum samples were added to the plates precoated with monoclonal antibodies. After a washing step, a standardized preparation of HRP-conjugated polyclonal antibody specific for each protein was added to the wells followed by thorough washing of the wells to remove all unbound components. Next, a 3,3′,5,5′-tetramethyl-benzidine substrate solution was added to each well. The enzyme (HRP) and substrate were allowed to react over a short incubation period. The enzyme-substrate reaction was terminated by the addition of 2 N solution of sulfuric acid, and the color change was measured spectrophotometrically at the wavelength of 450 ± 2 nm. The concentration of each protein in the sample was determined by comparing the absorbance of the samples to the standard curve.

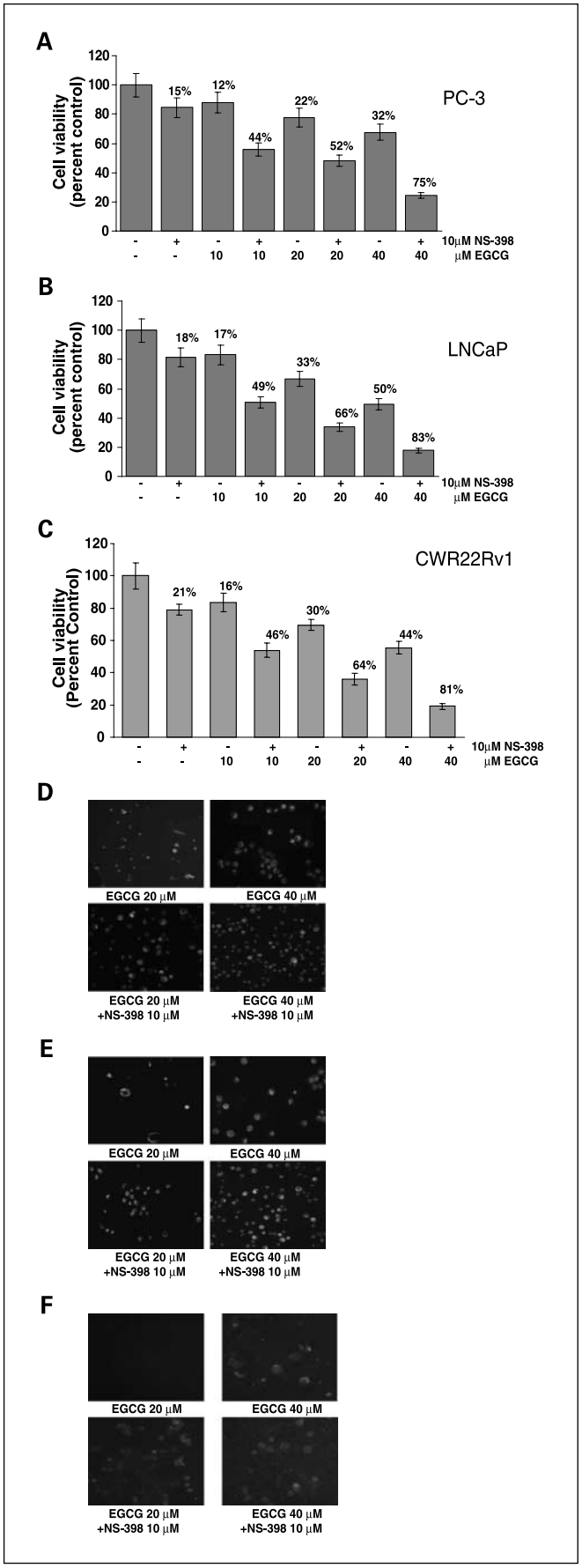
In vivo *tumor xenograft studies.* Forty 6 to 8-week-old male athymic nu/nu mice were obtained from NxGen Biosciences (San Diego, CA) and housed in the animal care facility of University of Wisconsin, School of Medicine, at standard conditions (in laminar airflow cabinets under pathogen-free conditions with 12 h light/12 h dark schedule) and fed *ad libitum* with autoclaved Harlan Teklad Sterilizable Rodent Diet (Harlan Teklad, Madison, WI). CWR22R*v*1 cells were detached from the culture dishes by trypsinization and then collected, washed, and resuspended in RPMI 1640. To establish CWR22R*v*1 tumor xenografts, mice were injected s.c. with 1×10^6 CWR22R*v*1 cells mixed with 50 μL RPMI + 50 μL Matrigel (Collaborative Biomedical Products, Bedford, MA) in the right and left flanks of each mouse. The mice were then randomly divided into four groups each consisting of 10 animals. Animals in group I (control) received drinking water; group II received 0.1% GTP (w/v, *ad libitum*) in drinking water; group III received celecoxib 5 mg/kg, i.p., daily, 5 days per week; and group IV received 0.1% GTP (w/v, *ad libitum*) in drinking water and celecoxib 5 mg/kg, i.p., daily, 5 days per week. GTP and celecoxib were chosen for *in vivo* studies to simulate a real-life human situation with the thinking that humans are more likely to consume up to four cups each day of brewed green tea and could be persuaded to take celecoxib. Body weight, diet, and water consumption were recorded twice weekly throughout the study. Once xenografts started growing, their sizes were measured twice weekly. The tumor volume was calculated by the formula $0.5238 L_1 L_2 H$, where L_1 is the long diameter, L_2 is the short diameter, and H is the height of the tumor. This formula is derived from an equation for calculating the volume of a hemi-ellipsoid, the geometric figure most nearly approximating the shape of tumors (30). Five animals from each group were removed from the study when tumors reached a volume of 1,300 mm^3 in the control group. These mice were killed, and their tumors were excised, weighed, and stored at −80°C until additional biochemical analysis. The remaining five animals in each group were allowed to remain in the study and monitored until tumors reached a volume of 1,300 mm^3. All animals were euthanized once tumors reached a volume of 1,300 mm^3 according to the guidelines laid down for the use and care of laboratory animals. Protocols for animal handling and experimentation were approved by the Research Animal Resource Center, of the University of Wisconsin-Madison. Every week, blood samples were collected either by the "mandibular bleed" or by the "retro-orbital bleed." The sera were separated by allowing the blood to clot at room temperature and centrifuging for 5 min at 4°C and then stored at −20°C for further biochemical analysis.

Densitometry and statistical analysis. All *in vitro* assays were repeated in three independent experiments, and only representative blots were presented. Immunoblots were scanned by HP Precisionscan Pro 3.13 (Hewlett-Packard, Palo Alto, CA). Densitometry measurements of the scanned bands were done using digitalized scientific software program UN-SCAN-IT (Silk Scientific Corporation). Data were normalized to β-actin or suitable loading controls and expressed as mean ± SE followed by appropriate statistical analysis. A quantitative measure of the degree of drug interaction in terms of additive effect, synergism, or antagonism was evaluated by the median-effect equation (31). This was achieved with the help of a software program Calcusyn (Biosoft, Cambridge, United Kingdom). A combination index (CI) value of <1 was considered a synergistic effect, and a CI value of 1 was considered as additive. Intergroup comparisons were made using one-way ANOVA followed by Tukey's multiple comparison tests, and values of $P < 0.05$ were considered statistically significant.

Results

Effect of EGCG and NS-398, alone and in combination, on cell growth and apoptosis of human prostate cancer cells. To evaluate the effect of combination on the growth of human prostate cancer cells, we selected androgen-sensitive LNCaP and CWR22R*v*1 and an androgen-independent PC-3 human prostate cancer cell line. This choice was based on the premise that prostate cancer at the time of diagnosis represents a mixture of androgen-dependent and androgen-independent cancer cells, and a treatment protocol aimed at eliminating both types of cells would be ideal. Treatment of LNCaP, CWR22R*v*1, and PC-3 cells with NS-398 (10 μmol/L) and increasing doses of EGCG (10-40 μmol/L) alone for 48 h resulted in a dose-dependent inhibition of cell growth as analyzed by MTT assay (Fig. 1A-C). However, cell growth inhibition was enhanced by combination resulting in 15% to 28% more inhibition compared with the additive effects of the agents, suggesting a possible synergism (CI < 1) between the two agents (Fig. 1A-C). Because COX-2 inhibitors and GTPs have been shown to induce apoptosis, we next determined if the growth-inhibitory effects of the NS-398 and EGCG in combination correlated with enhanced induction of apoptosis. Using an Annexin-labeled fluorescent kit, we observed enhanced apoptosis of LNCaP, CWR22R*v*1, and PC-3 (Fig. 1D-F) cells treated with NS-398 and EGCG in combination for 48 h compared with individual effects of each agent alone.

Effect of EGCG and NS-398 treatment, alone and in combination, on the protein expression of Bax, Bcl-2, caspases, and PARP in human prostate cancer PC-3 cells. To understand the molecular basis of the effects of combination, we studied several molecules involved during the initiation and execution of apoptosis. Levels of proapoptotic Bax were increased by EGCG and NS-398, and this increase was augmented by combination of the two compounds (Fig. 2A). At the same



time, levels of antiapoptotic Bcl-2 were decreased synergistically by a combination of EGCG and NS-398 (Fig. 2B; CI <1). Modulations in the expression of Bax and Bcl-2 by these agents resulted in change in the ratio of these molecules in a way that favored apoptosis (Fig. 2C). Using immunoblot analysis, we also assessed the effect of combination of EGCG and NS-398 on the protein expressions of the caspases that are activated during apoptosis. We observed increased activation of the initiator caspase-9 and effector caspase-6 by EGCG and NS-398, which was synergistically enhanced by their combination (Fig. 3A and B). Finally, expression of cleaved PARP, a marker of cell apoptosis, was synergistically increased by a combination of EGCG and NS-398 (Fig. 2D).

Effect of EGCG and NS-398 treatment, alone and in combination, on the protein expression of phospho–NF-κB-p65 and PPAR-γ in human prostate cancer PC-3 cells. It has been shown that even in the absence of any COX inhibitory activity, nonsteroidal anti-inflammatory drugs can modify cancer cell kinetics in a way that is consistent with an anticancer effect, and there has been a growing appreciation of the validity of this idea (32, 33). In line with this concept, we observed inhibition of NF-κB and PPAR-γ by EGCG and NS-398 (Fig. 3C and D). Densitometric analysis of the blots suggested significantly greater inhibition by combination compared with the individual effects of the two agents (Fig. 3C and D).

Effect of oral consumption of GTPs and i.p. administration of celecoxib, alone and in combination, on the growth of CWR22Rv1 tumors and on serum PSA, IGF-I, and IGFBP-3 levels in athymic nude mice. To determine the *in vivo* relevance of our *in vitro* findings, we selected athymic nude mice and injected them with androgen-sensitive CWR22Rv1 cells. The choice was based on the fact that these cells form rapid and reproducible tumors and secrete PSA in the bloodstream of the host. The effect of GTP (0.1% in drinking water) and celecoxib (10 mg/kg, i.p., daily, 5 days per week), alone and in combination, was tested on the growth of tumors in this xenograft mouse model. It took 48 days for the tumor volume to reach 1,300 mm^3 in mice that received both celecoxib and GTP compared with GTP alone (36 days) and celecoxib alone (40 days; Fig. 4). In the control animals, this tumor volume was reached in only 28 days (Fig. 4). At 28 days, there was 81% inhibition in tumor growth with combination of GTP and celecoxib compared with 42% with GTP alone and 57% with celecoxib alone (Fig. 4A-C). In parallel to tumor growth inhibition, we also observed significant decrease in the PSA levels in celecoxib and GTP-treated mice. The mice that received

Fig. 1. Effect of EGCG and NS-398 treatment, alone and in combination, on cell growth and apoptosis of human prostate cancer PC-3, LNCaP cells, and CWR22Rv1. Cell growth analysis: PC-3 (*A*), LNCaP (*B*), and CWR22Rv1 (*C*) cells were treated with EGCG (10-40 μmol/L) and NS-398 (10 μmol/L) alone and in combination for 48 h, and cell growth was determined by MTT assay in a 96-well plate as detailed in Materials and Methods. Columns, mean of three separate experiments in which each treatment was repeated in 10 wells; bars, SE. Values above each column reflect percentage change compared with control. Apoptosis detection: PC-3 (*D*), LNCaP (*E*), and CWR22Rv1 (*F*) cells were grown on cell culture slides and treated with EGCG (20 and 40 μmol/L) and NS-398 (10 μmol/L), alone and in combination, for 48 h. Apoptosis was determined by a commercially available kit as detailed in Materials and Methods. Representative photomicrographs from each treatment group showing induction of apoptosis (*green fluorescence*). Data were from one experiment repeated thrice with similar results.

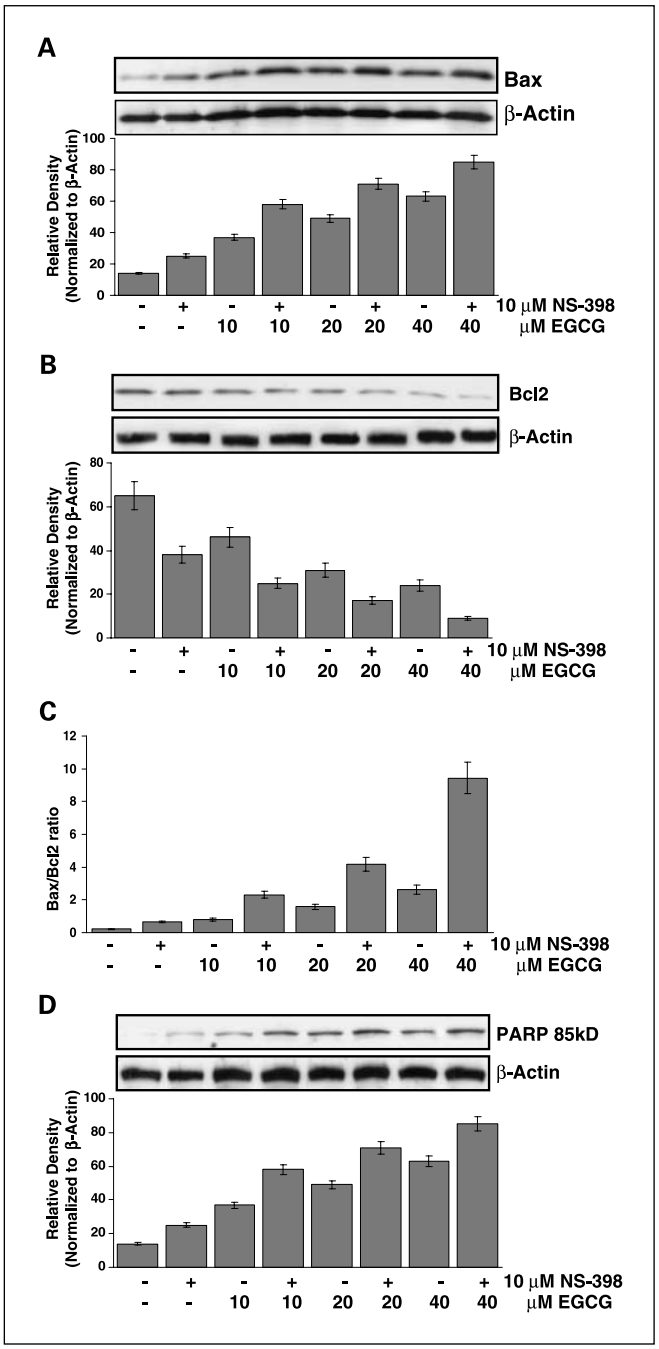


Fig. 2. Effect of EGCG and NS-398 treatment, alone and in combination, on the protein expression of Bax (*A*), Bcl-2 (*B*), 85 kDa PARP (*D*), and on the Bax to Bcl-2 ratio (*C*) in human prostate cancer PC-3 cells. The cells were treated with EGCG (10-40 μmol/L) and NS-398 (10 μmol/L) alone and in combination and harvested 24 h following the treatments. Total cell lysates were prepared, and 50 μg of protein were subjected to SDS-PAGE followed by Western blot analysis using specific antibodies and secondary HRP conjugates. The protein was detected by chemiluminescence. The details are described in Materials and Methods. Equal loading was confirmed by stripping the membrane and reprobing it with β-actin. Histograms, relative densities of the bands normalized to β-actin. Data were from a typical experiment repeated thrice with similar results.

a combination of GTP and celecoxib registered a greater decrease ($P < 0.001$) in PSA levels compared with mice that were treated with celecoxib alone or GTP alone (Fig. 4D). High circulating levels of PSA result in increased levels of IGF-I due to an increased proteolysis if IGFBP-3 in plasma (34). Higher circulating levels of IGF-I have been associated with increased risk of both prostate cancer and possibly benign prostatic hyperplasia (35). Greater rates of cell proliferation induced by

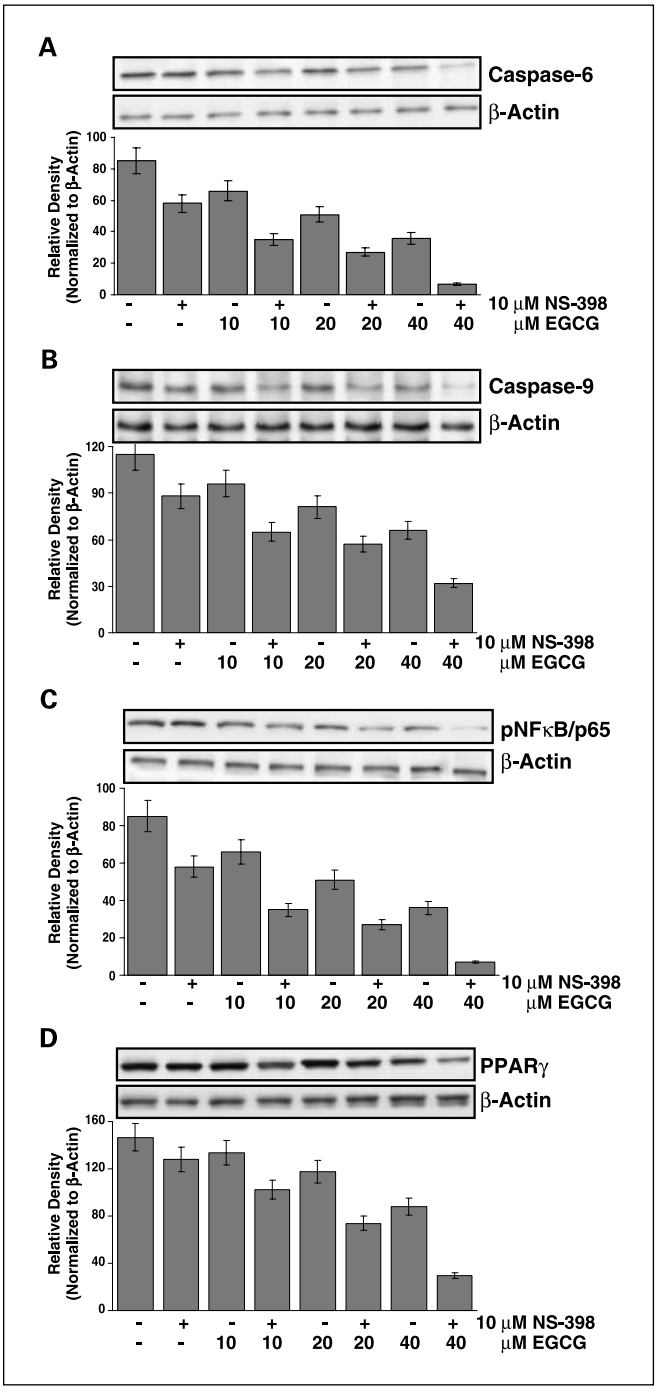


Fig. 3. Effect of EGCG and NS-398 treatment, alone and in combination, on the protein expression of caspase-6 (*A*), caspase-9 (*B*), phospho – NF-κB/p65 (*C*), and PPAR-γ (*D*) in human prostate cancer PC-3 cells. The cells were treated with EGCG (10-40 μmol/L) and NS-398 (10 μmol/L) alone and in combination and harvested 24 h after the treatments. Total cell lysates were prepared and 50 μg of protein were subjected to SDS-PAGE followed by Western blot analysis using specific antibodies and secondary HRP conjugates. The protein was detected by chemiluminescence. The details are described in Materials and Methods. Equal loading was confirmed by stripping the membrane and reprobing it with β-actin. Histograms, relative densities of the bands normalized to β-actin. Data were from a typical experiment repeated thrice with similar results.

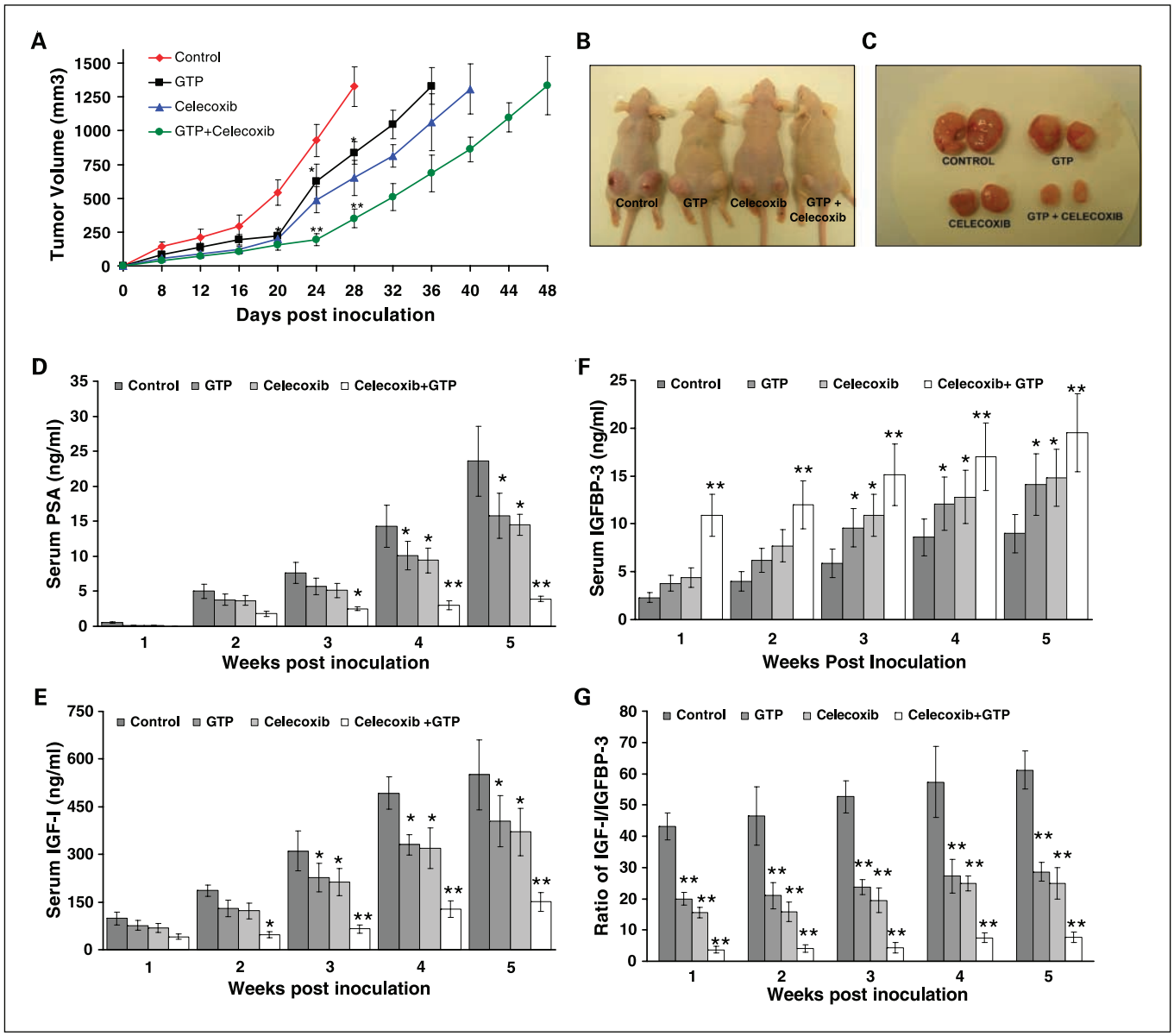


Fig. 4. Effect of oral consumption of GTPs and i.p. administration of celecoxib, alone and in combination, on the growth of CWR22Rν1 tumors and on serum PSA, IGF-I, and IGFBP-3 levels in athymic nude mice. *A,* athymic nude mice were injected s.c. with 1×10^6 CWR22Rν1 cells mixed with 50 μL RPMI + 50 μL Matrigel on the right and left flanks. The mice were then randomly divided into four groups of 10 animals each: group I received drinking water; group II received 0.1% GTP (w/v) in drinking water; group III received celecoxib 5 mg/kg, i.p., daily, 5 d/wk; group IV received 0.1% GTP (w/v) in drinking water and celecoxib 5 mg/kg, i.p., daily, 5 d/wk. Once tumors started growing, their sizes were measured twice weekly and tumor volume was calculated. Points, mean of eight animals; bars, SD. *, $P < 0.01$; **, $P < 0.001$, compared with control. *B,* photographs of representative mice with tumors from each group. *C,* photographs of excised tumors from each group. *D* to *F,* blood was withdrawn every week postinoculation, and serum PSA, IGF-I, and IGFBP-3 levels were analyzed by ELISA as described in Materials and Methods. *G,* IGF-I to IGFBP-3 ratios were obtained from the values in (*E*) and (*F*). Columns, mean of eight animals; bars, SD. *, $P < 0.01$; **, $P < 0.001$, compared with control (water-fed mice).

IGF-I may be a key biological pathway underlying these disorders (36, 37). We observed a significant ($P < 0.001$) decrease in the levels of serum IGF-I in mice that received a combination of GTP and celecoxib compared with mice that received celecoxib or GTP alone (Fig. 4E). This change was evident as early as 2 weeks posttreatment. Inversely, circulating levels of IGFBP-3 significantly increased in mice that received GTP and celecoxib in combination and was evident as early as 1 week posttreatment (Fig. 4F). An examination of the IGF-I to IGFBP-3 ratios suggested low values throughout the treatment protocol, indicating low availability of IGF-I in the circulating serum (Fig. 4G) in animals receiving GTP and celecoxib in combination. High IGF-I to IGFBP-3 molar ratio is associated with an increased risk of prostate cancer and is considered an important risk factor for prostate cancer (38, 39).

Effect of oral consumption of GTPs and i.p. administration of celecoxib, alone and in combination, on the protein expression of Bax, Bcl-2, and 85 kDa PARP, and on the Bax to Bcl-2 ratio in CWR22Rν1 established tumors in athymic nude mice. Tumor tissues from control and treated mice were examined for biochemical variables at 28 days posttreatment when control

tumors had reached a volume of 1,300 mm^3. At this time, tumor volume in the GTP-alone group was 835 mm^3, in celecoxib alone group was 650 mm^3, and in mice that received a combination of GTP and celecoxib was 350 mm^3. Similar to the effects observed *in vitro*, we observed increase in Bax levels and decrease in Bcl-2 levels by a combination of EGCG and NS-398 (Fig. 5A). Modulations in the expression of Bax and Bcl-2 resulted in change in the ratio of these molecules in a way that favored apoptosis, leading to the caspase proteolytic cascade and cleavage of PARP, and ultimately resulting to cell death (Fig. 5A-D).

Effect of oral consumption of GTPs and i.p. administration of celecoxib, alone and in combination, on protein expression of proliferating cell nuclear antigen, PPAR-γ,vascular endothelial growth factor, and phospho–NF-κB/p65 in CWR22Rν1 established tumors in athymic nude mice. Because our results suggested that the combination of GTPs and celecoxib induced tumor growth inhibition, we examined several molecules that have relevance to tumor growth. Our observations suggested decreased expression of proliferating cell nuclear antigen, a marker for cell proliferation, in tumors of mice that had received a combination of GTP and celecoxib (Fig. 6A). Levels of vascular endothelial growth factor, a marker for tissue angiogenesis, were also low in tumors of mice that received GTP and celecoxib in combination compared with mice that received either GTP or celecoxib alone (Fig. 6C). Further, protein expression of PPAR-γ and NF-κB were also significantly decreased in tumors of mice that received a combination of GTP and celecoxib, compared with tumors of mice that received GTP or celecoxib alone (Fig. 6B and D).

Discussion

All cancers arise as a result of multiple defects in signaling pathways and continue to accumulate new genetic mutations as they progress. The probability of a single-agent therapy against cancer, therefore, diminishes with time; hence, a multipronged therapy has been advocated both for chemoprevention as well as long-term control of cancer (4–7). Although single agents initially have remarkable activity, tumors in the regressing cancers usually acquire resistant features resulting in recurrence of the disease. This is especially true for prostate cancer in which androgen ablation initially helps to regress tumors; however, soon, tumors become hormone refractory and fail to respond to primary treatment.

The concept of combination of drugs for treatment of cancers is not new; however, the idea of combination with two distinct agents for prevention of cancers is exciting but is still in its infancy (10, 40, 41). The recent concern associated with the use of COX-2 inhibitors has thrown doubts on the very concept of prevention by COX-2 inhibitors (29, 42, 43). One approach to overcome the limitations associated with COX-2 inhibitors is the use of a combinatorial approach by the use of agents at low

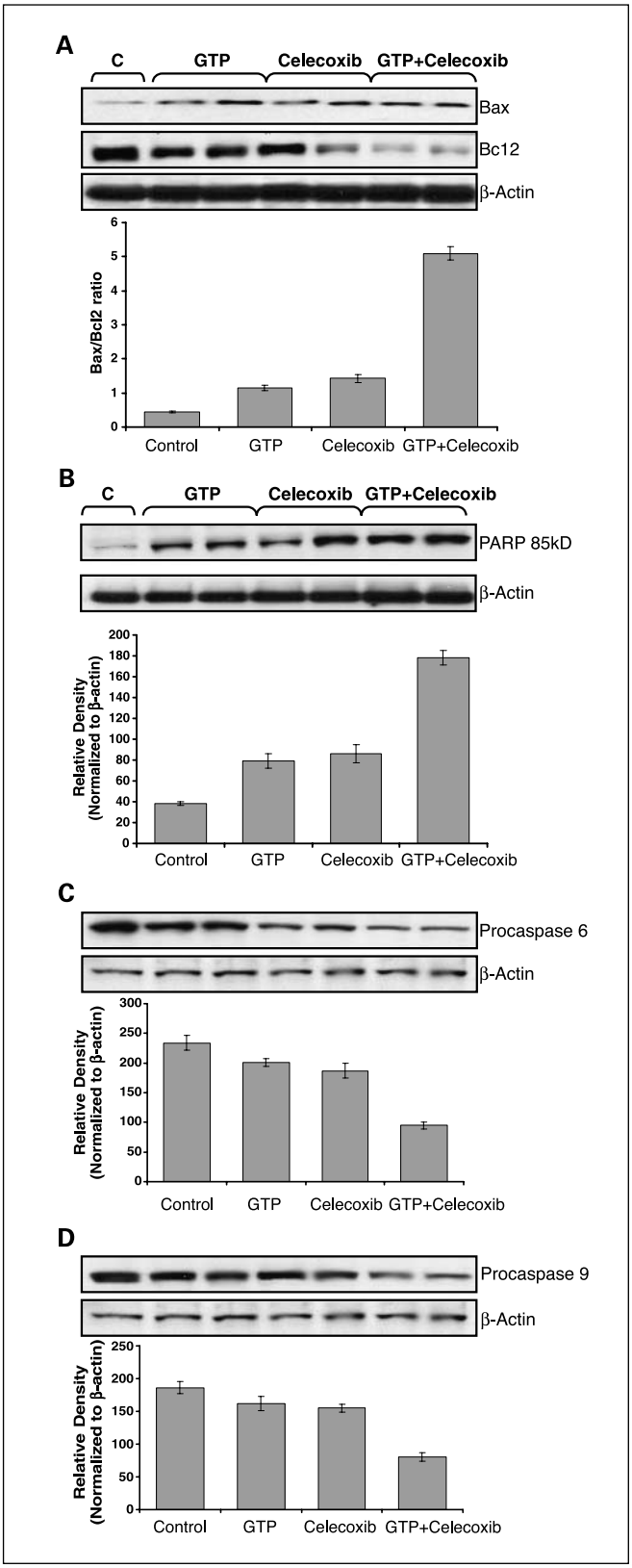


Fig. 5. Effect of oral consumption of GTPs and i.p. administration of celecoxib, alone and in combination, on the protein expression of Bax (*A*), Bcl-2 (*B*), 85 kDa PARP (*D*), and on the Bax to Bcl-2 ratio (*C*) in CWR22Rν1 established tumors in athymic nude mice. Approximately 1 million CWR22Rν1 cells were s.c. injected into the left and right flanks of each mouse to initiate tumor growth as described in Materials and Methods. The animals were treated with GTP and celecoxib alone and in combination throughout the experiment. *C,* control. Tumor tissues were excised from each group for protein expression analysis by Western blot analysis. Equal loading was confirmed by stripping the membrane and reprobing it with β-actin. Columns, mean of eight animals; bars, SD.

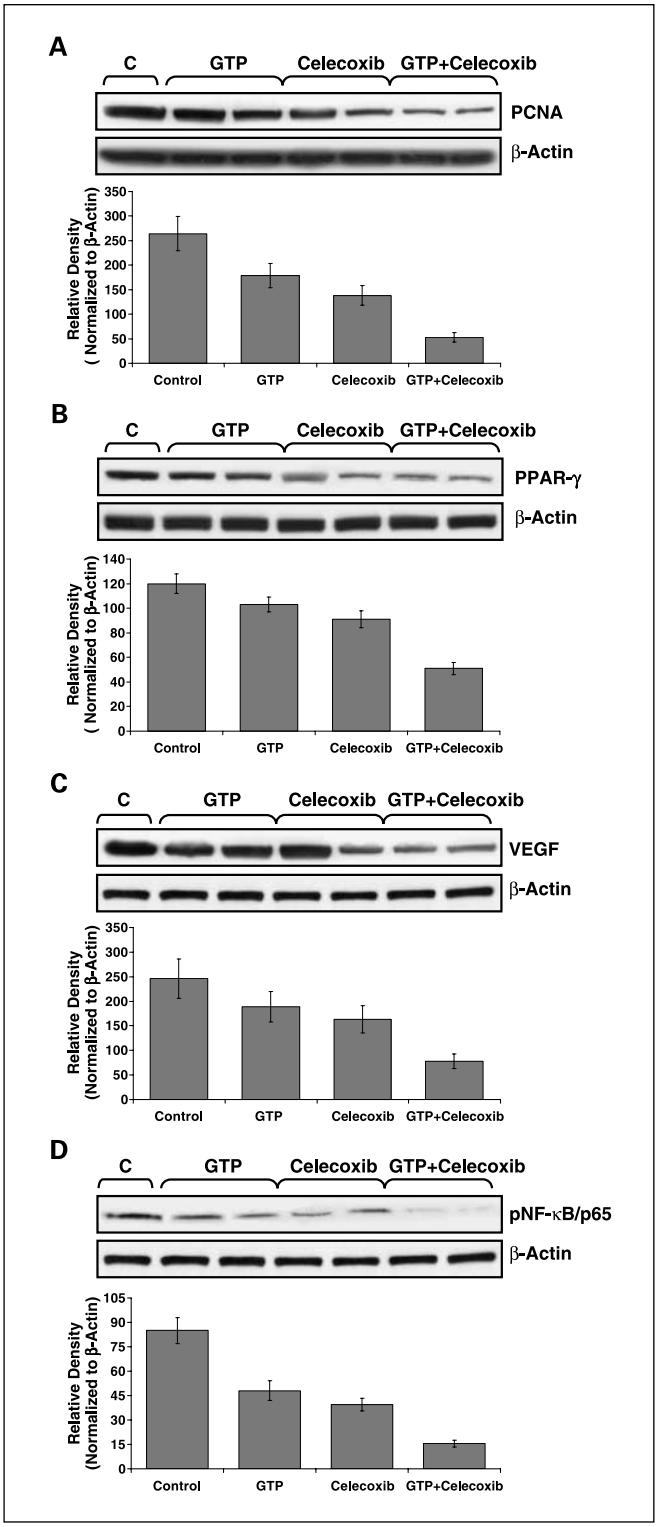


Fig. 6. Effect of oral consumption of GTPs and i.p. administration of celecoxib, alone and in combination, on protein expression of proliferating cell nuclear antigen (*PCNA*; *A*), PPAR-γ (*B*), vascular endothelial growth factor (*VEGF*; *C*), and phospho – NF-κB/p65 (*D*) in CWR22Rν1 established tumors in athymic nude mice. Approximately 1 million CWR22Rν1 cells were s.c. injected into the left and right flanks of each mouse to initiate tumor growth as described in Materials and Methods. The animals were treated with GTP and celecoxib alone and in combination throughout the experiment. Tumor tissues were excised from each group for protein expression analysis by Western blot analysis. Equal loading was confirmed by stripping the membrane and reprobing it with β-actin. Columns, mean of eight animals; bars, SD.

doses and with complementary mechanisms (7, 44, 45). We hypothesized that combination of a specific COX-2 inhibitor in low doses with GTP, which, in addition to inhibiting COX-2, also inhibits various signaling molecules, will afford an additive and/or synergistic inhibitory effect on the development of prostate cancer.

Although growth inhibition and apoptosis of prostate cancer cells has been shown with EGCG and COX-2 inhibitors, we here report for the first time a synergism between these agents against prostate cancer cells. The importance of both positive and negative regulators of cell growth is well documented in neoplasia (44). Bcl-2 has proved to be unique among proto-oncogenes in blocking programmed cell death and Bax, a conserved homologue that heterodimerizes *in vivo* with Bcl-2 and promotes cell death. A preset Bax to Bcl-2 ratio seems to determine the survival or death of cells following an apoptotic stimulus (46). Changes in the Bax to Bcl-2 ratio lead to the destabilization of the mitochondrial membrane and release of apoptotic factors. These factors induce the caspase proteolytic cascade and cleavage of PARP, ultimately leading to cell death. At least two pathways, the death receptor and the mitochondrial pathways, are known to activate the effector caspases via the initiator caspases (47). Our observations suggest that the mitochondrial pathway triggers the activation of initiator caspase-9, which activates the effector caspases-6, followed by proteolytic cleavage of PARP (48). It has been shown that nonsteroidal anti-inflammatory drugs affect cellular homeostasis by targeting molecules through COX-2–independent mechanisms. The idea that nonsteroidal anti-inflammatory drugs actually have a variety of molecular targets not only provides a much-needed explanation of apparently disparate observations but also highlights the importance of COX-2–independent targets for cancer prevention and possibly treatment (49).

In the present study, we report an increased efficacy of selective COX-2 inhibitors in combination with polyphenols from green tea for inhibition of growth of human prostate cancer cells both *in vitro* and *in vivo*. This effect was mainly observed owing to increased apoptosis after increased activation of caspase-6 and caspase-9. Further, we identified mechanisms that account for increased cytotoxic effects by a combination of GTPs and selective COX-2 inhibitors. Thus, COX-2 selective inhibitors (e.g., celecoxib and NS-398) can suppress growth of cancer cells both by modulating multiple targets. Our data also suggest that COX-2 inhibitors can also produce synergic effects when used in combination with other nontoxic, natural anticancer agents. Our results assume significance in the wake of recent observations that suggest increased cardiovascular side effects associated with long-term use of high doses of COX-2 inhibitors. COX-2 inhibitors are being evaluated as cancer chemopreventive agents in subjects at high risk for developing cancers. There are at present more than 20 clinical trials investigating the possible use of COX-2 inhibitors as cancer-preventive/therapeutic agents in combination with other chemotherapeutic agents. However, to our knowledge, none of these trials has taken into consideration the possibility of combination with a dietary agent with proven cancer-preventive activity. Thus, we conclude that COX-2 selective inhibitors at low doses in combination with polyphenols from green tea may be a promising approach for prevention of prostate cancer.

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