Exisulind in Combination with Celecoxib Modulates Epidermal Growth Factor Receptor, Cyclooxygenase-2, and Cyclin D1 against Prostate Carcinogenesis: *In vivo* Evidence

Bhagavathi A. Narayanan,[1] Bandaru S. Reddy,[2] Maarten C. Bosland,[3] Dominick Nargi,[1] Lori Horton,[1] Carla Randolph,[1] and Narayanan K. Narayanan[1]

Abstract

Purpose: Nonsteroidal anti-inflammatory drugs mediate anticancer effects by modulating cyclooxygenase-2 (COX-2)-dependent and/or COX-2–independent mechanism(s); however, the toxicity issue is a concern with single agents at higher doses. In this study, we determined the combined effect of celecoxib, a COX-2 inhibitor, along with exisulind (sulindac sulfone/Aptosyn) at low doses in prostate cancer.

Experimental Design: We used a sequential regimen of *N*-methyl-*N*-nitrosourea + testosterone to induce prostate cancer in Wistar-Unilever rats. Following carcinogen treatment, celecoxib and exisulind individually and their combination at low doses were given in NIH-07 diet for 52 weeks. We determined the incidence of prostatic intraepithelial neoplasia, adenocarcinomas, rate of tumor cell proliferation, and apoptosis. Immunohistochemical and Western blot analysis were done to determine COX-2, epidermal growth factor receptor (EGFR), Akt, androgen receptor, and cyclin D1 expression. Serum prostaglandin E_2 and tumor necrosis factor-α levels were determined using enzyme immunoassay/ELISA assays.

Results: The rats that received celecoxib in combination with exisulind at low doses showed a significant decrease in prostatic intraepithelial neoplasia and adenocarcinomas as well as an enhanced rate of apoptosis. An overall decrease in COX-2, EGFR, Akt, androgen receptor, and cyclin D1 expression was found associated with tumor growth inhibition. Reduced serum levels of COX-2 protein, prostaglandin E_2, and tumor necrosis factor-α indicated anti-inflammatory effects. A strong inhibition of total and phosphorylated form of EGFR (Tyr^{992} and Tyr^{845}) and Akt (Ser^{473}) was significant in rats given with these agents in combination.

Conclusions: In this study, we show for the first time that the combination of celecoxib with exisulind at low doses could prevent prostate carcinogenesis by altering key molecular events.

Prostate cancer is one of the most common malignancies in men in the United States and in other Western countries (1). Based on key etiologic factors linked to human prostate cancer, researchers estimate that inflammation contributes to the development of a higher number of human cancers, including cancer of the colon, liver, and prostate (2, 3). Molecular changes that occur during stepwise growth of prostate cancer have been characterized by a shift in the expression of genes and proteins mediating inflammation (4–6). Although there are inconsistencies about the role of cyclooxygenase-2 (COX-2) in prostate cancer development, a growing number of studies provide evidence on the overexpression of COX-2 and increased prostaglandin biosynthesis [prostaglandin E_2 (PGE_2)] in benign and malignant human prostate (5–15). Furthermore, COX-2 and epidermal growth factor receptor (EGFR) represent the more promising pharmacologic targets in cancer progression, as they exhibit cross-talk in cancer cells (16). Subsequent reports on racial disparity of EGFR overexpression and novel mutations in prostate cancer among African-Americans (17) provide compelling evidence on the possible interaction between COX-2 and EGFR in human prostate carcinogenesis. Yet another downstream factor connecting COX-2 is the oncogenic protein cyclin D1, which is also implicated in EGFR signaling and androgen receptor (AR) regulation in prostate cancer (18, 19). As chemoprevention is one of the best strategies to prevent prostate cancer, and nonsteroidal anti-inflammatory drugs are considered to be potential chemopreventive agents, it is essential to understand their mode of action. Despite a growing number of clinical reports on the lower risk of prostate cancer among nonsteroidal anti-inflammatory drug users (20–23), concerns about the side effects among the users of selective

Authors' Affiliations: [1]Department of Environmental Medicine, New York University School of Medicine, Tuxedo, New York; [2]Department of Chemical Biology, Rutgers, The State University of New Jersey, Piscataway, New Jersey; and [3]Department of Pathology, College of Medicine, University of Illinois at Chicago, Chicago, Illinois

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Requests for reprints: Bhagavathi A. Narayanan, Department of Environmental Medicine, New York University School of Medicine, Tuxedo, NY 10987. Phone: 845-731-3624; Fax: 845-351-4510; E-mail: bhagavathi@env.med.nyu.edu.

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COX-2 inhibitors (e.g., a caution from Food and Drug Administration; refs. 24, 25) further ignite the need to investigate their specific mode of action and key targets. In our earlier studies, we have used various doses of COX-2 inhibitors with other agents in cell culture and in animal models to understand their mode of action (5, 6, 26). Although the blockade of EGFR activation by anti-EGFR agents has been proposed as one of the potential mechanisms to prevent human cancer (27), their interactions with COX-2 and EGFR signaling pathways in prostate cancer are not clear (28, 29). Although cyclin D1 is a multifaceted regulator of cell cycle and AR (30), the potential use of nonsteroidal anti-inflammatory drugs as chemopreventive agents and their mode of action targeting COX-2, EGFR, AR, and cyclin D1 in prostate cancer have not been determined. However, combination of potential agents at low doses is considered to be very efficacious in minimizing toxicity compared with the use of individual agents at higher dose levels (9, 31–35).

Based on our earlier reports on the potential use of COX-2 inhibitors as chemopreventive agents for prostate cancer (5, 6, 26), this study was focused to determine the anticancer effects of COX-2 inhibitors with other agents in combination at low doses. Overall, our goal was (*a*) to investigate the mode of action of a COX-2 inhibitor, celecoxib, in combination with a cyclic GMP phosphodiesterase inhibitor, exisulind (sulindac sulfone/Aptosyn), in prostate cancer and (*b*) to examine the combined effect of exisulind and celecoxib in abrogating the complex interaction between COX-2, EGFR, Akt, cyclin D1, and AR in prostate cancer. We used a Wistar-Unilever rat model in which prostate cancer was induced by a sequential regimen of *N*-methyl-*N*-nitrosourea (MNU) + testosterone (36, 37). Using this model, we have shown for the first time that the combination of a COX-2 inhibitor, celecoxib, with a cyclic GMP phosphodiesterase inhibitor, exisulind (sulindac sulfone/Aptosyn), is more effective in preventing prostate cancer growth. To our knowledge, this is the first report on the use of a low-dose combination of celecoxib with exisulind in preventing carcinogen-induced prostate cancer in a preclinical model.

Materials and Methods

Animals and diets. Male Wistar-Unilever (HsdCpb:WU) rats (6-7 weeks of age) were purchased from Harlan Sprague Dawley and maintained in quarantine for 2 weeks before they were transferred to a holding room in the Department of Environmental Medicine Satellite Animal Facility at New York University School of Medicine (Tuxedo, NY). The rats were housed in cages with wood chip bedding and maintained under controlled conditions (21°C and 50% relative humidity) in a 12-h light/dark cycle. The diet NIH-07 (Harlan Teklad) was stored at 4°C before it was mixed with the experimental diets. During the study, rats were permitted free access to basal diet and/or experimental diet and water. All rats were inspected at least once daily to monitor their general health status and weighed weekly.

Agents and dose selection. For the present study, we used celecoxib purchased from Focal Vision International. Exisulind (sulindac sulfone/Aptosyn-OSI-461) was provided by OSI Pharmaceuticals. Experimental diets were prepared weekly by mixing celecoxib (500 ppm) or exisulind (1,000 ppm) with the basal diet. For combination studies, a dose of 250 ppm of celecoxib with 500 ppm of exisulind was mixed with NIH-07 diet. Selection of the dose for the agents used in this study was based on our earlier studies, where the maximum tolerated dose was determined to be 1,500 ppm for celecoxib and 800 ppm for exisulind (5, 38, 39). All control and experimental diets containing celecoxib and/or exisulind were stored in a cold room.

Prostate tumor induction. Prostate cancer induction in Wistar-Unilever rats was carried out following the protocols outlined by Bosland (40, 41) and Bosland et al. (42). After quarantine, all rats in the experimental groups (28 per group) received daily oral (gavage) dose of flutamide purchased from Sigma for 21 days (days 1-21) at a dosage of 20 mg/kg body weight for 21 consecutive days to inhibit androgen synthesis. One day after the final dose of flutamide, rats received one s.c injection of 10 mg/kg body weight of testosterone propionate (Sigma Chemical Co.) in corn oil at a concentration of 50 mg/mL. The sequence of antiandrogen administration followed by androgen alone results in maximal stimulation of prostatic epithelial proliferation at 60 h after the first dose of testosterone. Sixty hours after the first dose of testosterone propionate, rats in designated experimental groups received a single i.v injection of 30 mg/mL of MNU (Ash Stevens) per kilogram body weight via tail vein under anesthesia (fentanyl-droperidol). Two weeks after MNU administration, all MNU-treated rats received continuous exposure to testosterone via two s.c. implants of silastic tubes (3-cm tube; Dow Corning), containing 40 mg of crystalline testosterone (Sigma Chemical), under light anesthesia (fentanyl-droperidol).

Administration of chemopreventive agents. Dietary administration of 500 ppm of celecoxib and 1,000 ppm of exisulind individually and 250 ppm of celecoxib + 500 ppm of exisulind in combination was started at day 21 after the tumor induction procedure by carcinogen treatment. All the dietary regimens for the experimental rats (n = 28) were continued until termination of the study at the end of 52 weeks. Control rats (n = 28) received NIH-07 diet only. Body weights were recorded every week. At the end of the study, the whole prostate was examined grossly for tumors. Blood, plasma and prostate tissues, seminal vesicle, and testes were collected for further histologic analysis. Part of dorsolateral prostate tissues isolated from the control and experimental group of rats was immediately processed for RNA and protein extraction for biochemical analysis. Chemopreventive effect of celecoxib in combination with exisulind was determined by comparing their individual effects on overall health, body weight, tumor burden, rate of proliferation, and apoptosis.

Histologic evaluation of tumor burden. Paraffin-embedded dorsolateral prostate tissue sections (5 μm thick) were used for the histologic determination of tumor incidence. Tissue sections from each group of rats stained with H&E were examined for neoplastic and cellular changes. Epithelial stratification and related changes indicative of prostatic intraepithelial neoplasia (PIN) and adenocarcinomas of the dorsolateral prostate were recorded as described earlier (5, 6, 42–44). In this study, tumor growth inhibition was determined by measuring the total number of PIN lesions, adenocarcinomas, level of Ki-67 staining indicating tumor proliferation, and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay revealing the rate of apoptosis.

Immunohistochemical staining for Ki-67. To determine the chemopreventive effect of exisulind in combination with celecoxib on prostate cancer growth, we did immunostaining for Ki-67. Mouse monoclonal antibody for Ki-67 (Santa Cruz Biotechnology) was incubated (1:100 dilution) with deparaffinized tissue sections (5 μm thick) from the dorsolateral prostate of control and experimental rats. Immunostaining procedures were followed as described in our earlier studies (6). The reactive proteins were detected using avidin-biotin-horseradish peroxidase complex and 3,3′-diaminobenzidine tetrahydrochloride as the chromogen. Negative controls were treated with serum instead of primary antibody. Positive staining for Ki-67 was quantified from five slides per rat.

Detection of apoptosis by TUNEL assay. Apoptosis induced by exisulind and celecoxib individually and in combination at low doses in the rat dorsolateral prostate was determined by TUNEL assay using ApopTag *In situ* Apoptosis Detection kit (InterGen). Briefly, 5-μm-thick formalin-fixed tissue sections of the dorsolateral prostate from

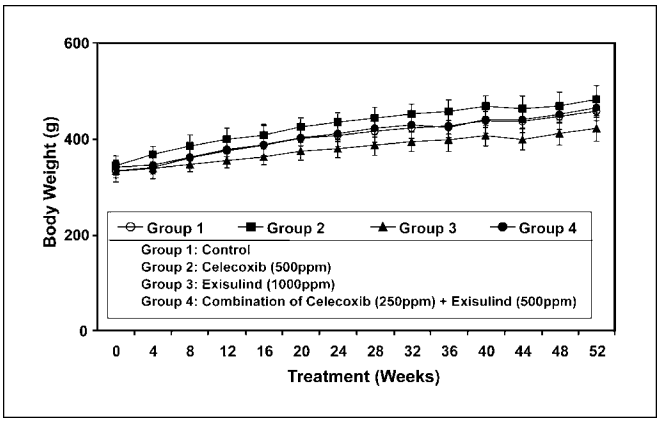

Fig. 1. Effect on body weight: MNU/testosterone-treated Wistar-Unilever rats were given celecoxib and exisulind individually and in combination as described in Materials and Methods. A pairwise and timeline comparisons of the animal body weights using repeated measures of ANOVA revealed an overall weight gain in control and treatment groups ($P < 0.001$).

treatments and control group of rats were deparaffinized and washed with PBS and permeabilized with 20 mg/mL of proteinase K. The samples were then equilibrated with buffer, and the DNA strand breaks were labeled with anti-digoxigenin-peroxidase using reagents from ApopTag Plus Peroxidase kit. The reactions were terminated using a stop buffer provided by the manufacturer and washed twice with PBS. Counterstaining was done with 0.5% methyl green (w/v) and sodium acetate (0.1 mol/L). An AX70 microscope (Olympus) was used to detect TUNEL-positive apoptotic cells and quantified with Image-Pro Plus software (Media Cybernetics). Positive staining of 100 cells per field from three independent tissue sections of same treatment groups was processed in a similar way to determine the mean percentage of apoptotic cells.

Immunohistochemical detection of COX-2, EGFR, cyclin D1, and AR. To determine the effect of exisulind in combination with celecoxib on the molecular targets of prostate cancer in the dorsolateral prostate tissues, we did immunohistochemical analysis to detect the tissue level expression of COX-2, EGFR, cyclin D1, and AR. Mouse monoclonal antibodies for COX-2 (Cayman), EGFR (Cell Signaling Technology, Inc.), cyclin D1, and AR (Santa Cruz Biotechnology) were incubated (1:100 dilution) with deparaffinized tissue sections (5 μm) of the dorsolateral prostate removed from control and experimental rats. The proteins were detected using avidin-biotin-horseradish peroxidase complex and 3,3′-diaminobenzidine tetrahydrochloride as the chromogen. Negative controls were treated with serum instead of primary antibody. We used tissue sections of MNU-induced rat mammary tumor staining with the respective antibody (except for AR) for positive control.

Measuring PGE_2 and tumor necrosis factor-α levels. We used Correlate-enzyme immunoassay/ELISA-PGE_2 (Assay Designs) to measure the level of PGE_2 in the serum collected from the control and experimental rats. At the time of sacrifice, serum from control and experimental groups of rats was frozen and stored at -80°C. To do enzyme immunoassay/ELISA-PGE_2 assays, first, a standard PGE_2 stock of 50,000 pg/mL was used for subsequent lower dilutions. The assay involved the use of a monoclonal antibody to PGE_2, which binds in a competitive manner with the PGE_2 in the sample, or an alkaline phosphatase molecule that is covalently attached to PGE_2. After a short incubation time, the enzyme reaction was stopped and the yellow color generated was read on the microplate reader at 405 nm. A similar but modified protocol using enzyme immunoassay/ELISA-tumor necrosis factor-α (TNF-α) assay kit (Assay Designs) was used specifically to measure the level of TNF-α in the serum using the standards provided by the manufacturer. The results presented in this study are based on four sets of data from independent assays.

Western blot analysis. Total protein extracted from rat dorsolateral prostate tissues of the control and experimental groups was fractionated on a SDS-PAGE as described earlier (26). Briefly, 100 mg of dorsolateral prostate tissue from each group were used to isolate protein with an extraction buffer containing 150 mmol/L NaCl, 10 mmol/L Tris (pH 7.2), 5 mmol/L EDTA, 0.1% Triton X-100, 5% glycerol, and 2% SDS in addition to a mixture of protease inhibitors (Boehringer Mannheim). Aliquots of protein (50 μg/lane) were fractionated on 10% SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes. The Western blot procedure was carried out to detect phosphorylated EGFR (Tyr^{992} and Tyr^{845}; Cell Signaling Technology), Akt (Ser^{473}), and total Akt using specific monoclonal antibody (Santa Cruz Biotechnology). Specific antibody was used to detect COX-2 (Cayman) and COX-1 (Santa Cruz Biotechnology) proteins. The level of β-actin expression was used for equal loading.

Statistical analysis. Measures of tumor growth inhibition in terms of regression in the number of PIN lesions and adenocarcinoma of the dorsolateral prostate, rate of apoptosis induced by the agents, and Ki-67 indicating the rate of proliferation determined via immunohistochemical analysis were compared among the experimental and control groups using one-way ANOVA followed by Tukey's multiple comparisons procedure (45).

Results

General health of the animals. The health and the physical activity of the rats in each group were remarkably good until the day of sacrifice after 52 weeks of treatment with celecoxib and

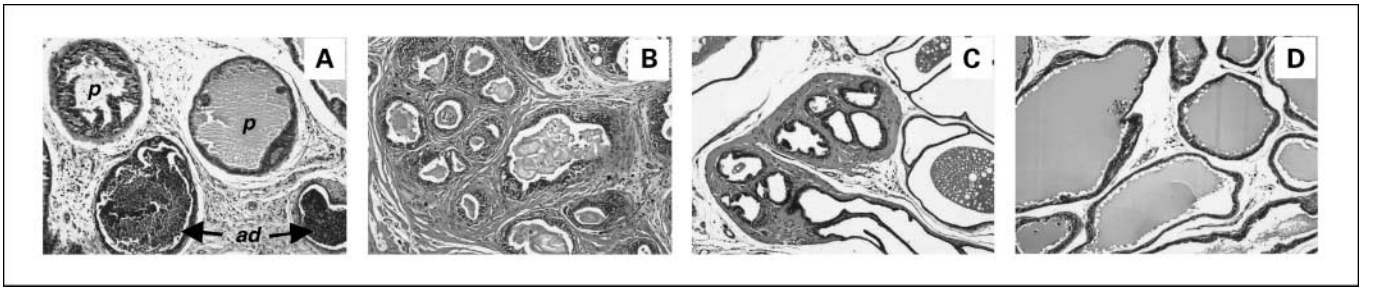

Fig. 2. Histologic determination of tumor growth inhibition: histologic changes observed in the H&E-stained dorsolateral prostate sections were evaluated to determine the effects of dietary celecoxib and sulindac sulfone given individually and in combination on tumor growth inhibition in MNU/testosterone-induced dorsolateral prostate in Wistar-Unilever rats. *A,* control dorsolateral prostate with PIN lesions (*p*) showing papillary projections of proliferating epithelial cells characteristic of PIN and highly invasive and proliferating adenocarcinomas (*ad*). Arrows, significant cellular changes after treatment. *B,* dorsolateral prostate tissue sections from rats treated with celecoxib (500 ppm) showing reduced epithelial projections and cell proliferation. *C,* dorsolateral prostate tissue section from rats treated with exisulind (1,000 ppm) showing reduced tumor cell proliferation. *D,* dorsolateral prostate tissue section from rats treated with combination of celecoxib (250 ppm) + exisulind (500 ppm) showing remarkable decrease in the epithelial cell layer proliferation, indicating significant growth inhibition compared with individual treatments.

Table 1. Inhibitory effects of dietary celecoxib and exisulind individually or in combination on rat prostate tumor incidence

Group	Treatment	No. rats*	Survival rate (%)	Tumor incidence (%) †	
				PIN	Adenocarcinoma
1	Control ‡	21	75.0	71.33 ± 4.04	61.67 ± 5.51
2	Celecoxib (500 ppm)	24	85.7	43.67 ± 3.21§	34.00 ± 3.46§
3	Exisulind (1,000 ppm)	22	78.6	15.33 ± 3.33§	12.67 ± 3.06§
4	Celecoxib (250 ppm) and exisulind (500 ppm)	23	82.1	7.17 ± 1.65‖¶**	6.08 ± 1.52‖¶**

NOTE: Five independent sections of the dorsolateral prostate sections from rats in each group were stained and examined to determine tumor incidence (%) in terms of number of PIN lesions and adenocarcinomas. The total number of lesions counted in 10 high-power fields is presented for control versus celecoxib or exisulind or their combination.
*Number of rats (*n*) sacrificed at the end of bioassay out of a total of 28 rats assigned per group.
† Mean ± SD.
‡ Control: carcinogen (MNU/testosterone) treatment.
§Group 1 versus groups 2 and 3: significant difference, $P < 0.001$.
‖Group 1 versus group 4: significant difference, $P < 0.0001$.
¶Group 2 versus group 4: significant difference, $P < 0.001$.
**Group 3 versus group 4: significant difference, $P < 0.05$.

exisulind individually and in combination. Based on the evaluation of the internal organs and general health, we determined that there is no toxicity either in the control (MNU/testosterone) or in the experimental rats. An overall weight gain was observed in the group of rats that received the control (NIH-07) as well as the experimental diet ($P < 0.001$; Fig. 1). Although an insignificant but a small decrease in the body weight was observed among the rats in the exisulind-treated group, there was no sign of toxicity due to treatments. The steady-state plasma level of celecoxib and exisulind in the rats was compared with that from earlier studies (38, 39) of Dr. Bandaru Reddy (coauthor of this article and also a veterinarian). The level of celecoxib in the plasma was estimated to be 2.00 μg/mL for 250 ppm and 2.5 μg/mL for

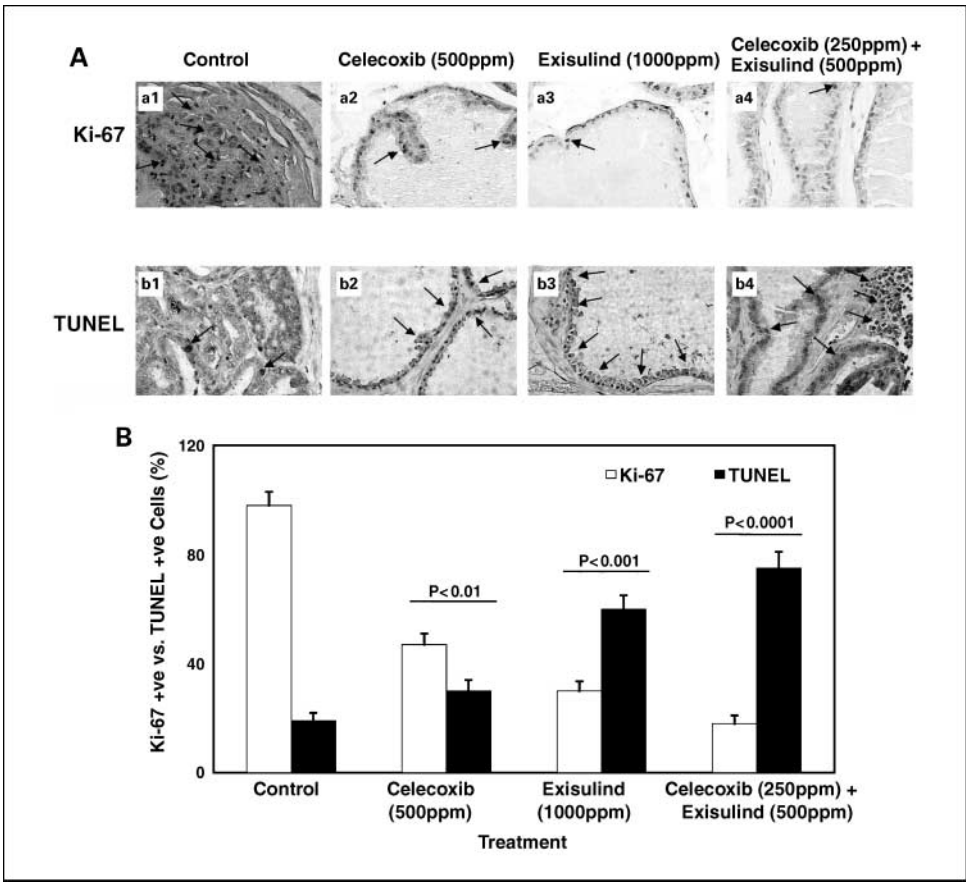

Fig. 3. Immunohistochemical detection of Ki-67–positive and TUNEL-positive apoptotic cells: effects of dietary celecoxib and exisulind given individually and in combination in MNU/testosterone-induced dorsolateral prostate of Wistar-Unilever rats. *A,* effect on the proliferation marker Ki-67 (*arrows*, Ki-67–positive cells; *a1-a4*) and apoptosis (*arrows*, TUNEL-positive cells; *b1-b4*). *B,* quantification of the tumor cell proliferation rate (Ki-67) and apoptosis measured by TUNEL-positive cells. The results were compared among the experimental and control groups and are presented as a bar graph.

500 ppm. The serum level of exisulind (sulindac sulfone) in our study was found to be closer to the level reported by Kapetanovic et al. (46) in which dosing of sulindac sulfone (200 ppm) via diet resulted in measurable and steadier plasma concentrations of 36.5 (2.5), the areas under the concentration-time curve [$AUC_{24\ h}$ sulindac sulfone (μg/h/mL)] in rats. The rat serum levels of celecoxib and exisulind, reported in this study, were further confirmed (independent confirmation) with the earlier reports of Reddy et al. (38, 39).

Chemopreventive effect. To determine chemopreventive efficacy, we first examined the individual and combined effect of celecoxib with exisulind on prostate cancer incidence. We examined the neoplastic changes induced in these rats by the regimen of MNU + testosterone, which is highly specific for the prostate as shown by earlier studies (36, 37, 47, 48). A detailed histologic examination of the dorsolateral prostate revealed the presence of a higher number of PIN and adenocarcinomas in the rats receiving flutamide, testosterone propionate, and MNU followed by chronic exposure to testosterone. Tumor growth inhibition was determined histologically based on the cellular changes associated with PIN and adenocarcinomas (Fig. 2). Research specialists who have no knowledge on the treatment regimens determined the total number of PIN and adenocarcinomas per rat. As per the dietary regimen described, the MNU +

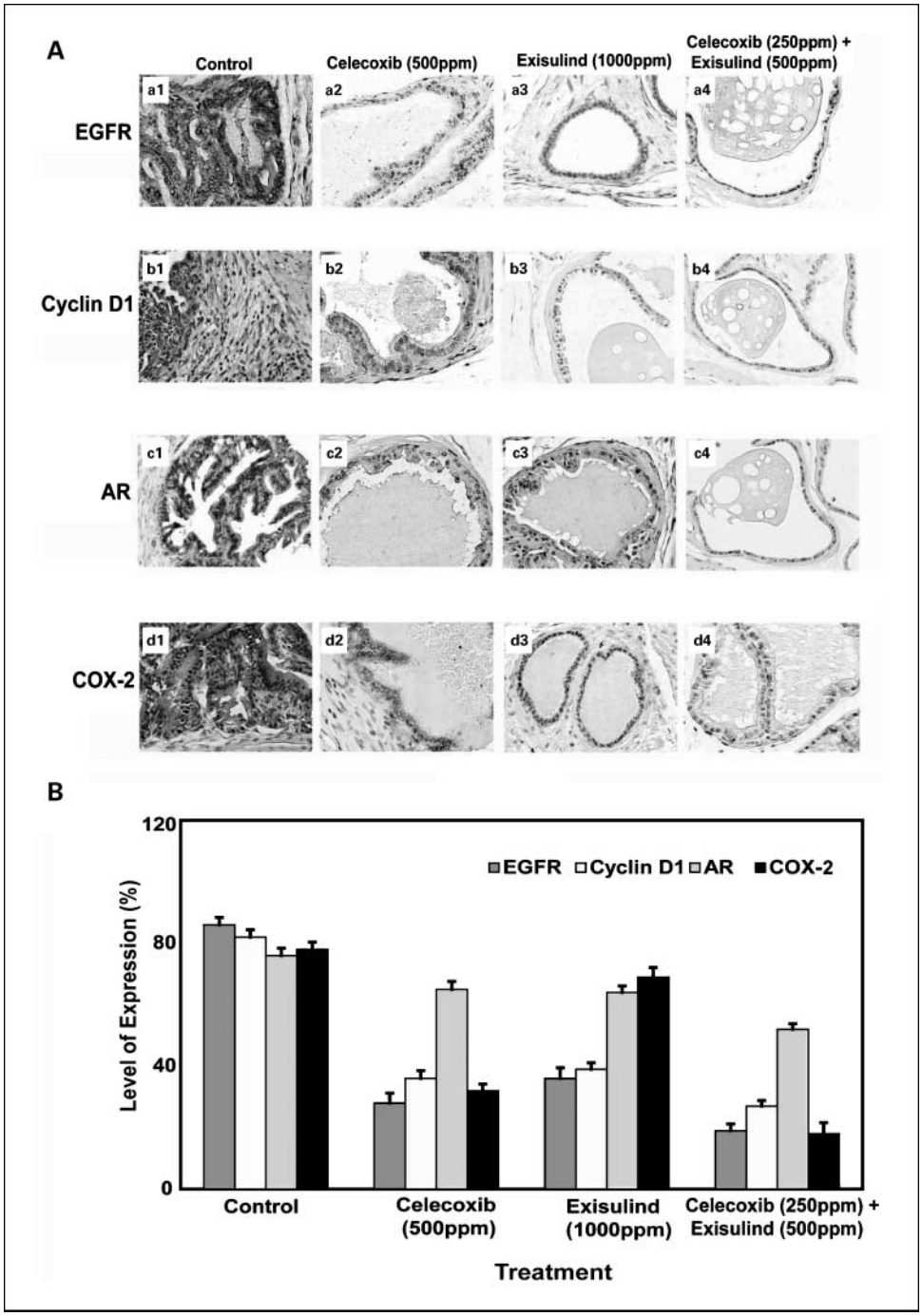

Fig. 4. Immunohistochemical detection of specific targets: the inhibitory effects of dietary celecoxib and exisulind individually and in combination on the expression of potential molecular targets in MNU/testosterone-induced dorsolateral prostate in Wistar-Unilever rats were determined at the tissue level by doing immunohistochemical analysis using specific monoclonal antibody for each target as described in Materials and Methods. *A,* total EGFR (*a1-a4*), cyclin D1 (*b1-b4*), AR (*c1-c4*), and COX-2 (*d1-d4*). *B,* semiquantification of the differences in the expression of various markers was compared among the experimental and control groups and is presented as a bar graph.

testosterone–treated rats fed with diets containing celecoxib (500 ppm) or exisulind (1,000 ppm) alone showed an overall tumor growth inhibition in terms of total number of PIN and adenocarcinomas of the dorsolateral prostate. However, the rats fed with diets containing combination of celecoxib (250 ppm) with exisulind (500 ppm) showed a remarkable decrease in the tumor incidence compared with the control, suggesting that low-dose combination of celecoxib with exisulind is more effective against prostate cancer when compared with the solitary effects of celecoxib or exisulind (Table 1). Interestingly, this finding on tumor growth inhibition by celecoxib in combination with exisulind was associated with reduced immunostaining for Ki-67, indicating a decrease in the rate of tumor proliferation (Fig. 3A, *a1-a4*). The rate of tumor growth inhibition is presented as a bar graph (Fig. 3B).

Effect on apoptosis. The rate of apoptosis determined by TUNEL assay was instrumental in determining the chemopreventive effect of celecoxib in combination with exisulind. As shown in Fig. 3A (*b1-b4*), an increase in the rate of apoptosis was evident in the dorsolateral prostate of rats receiving combination treatment compared with the individual effects as shown in the bar graph (Fig. 3B).

Effect on potential molecular targets. Elevated levels of COX-2, EGFR, cyclin D1, and AR independently represent as promising targets in prostate cancer development. We measured their expression at the tissue level before and after treatment with celecoxib and exisulind individually at higher doses and in their combination at low doses for 52 weeks. As shown in Fig. 4A, based on the immunohistochemical detection, our findings clearly indicate the inhibition of EGFR

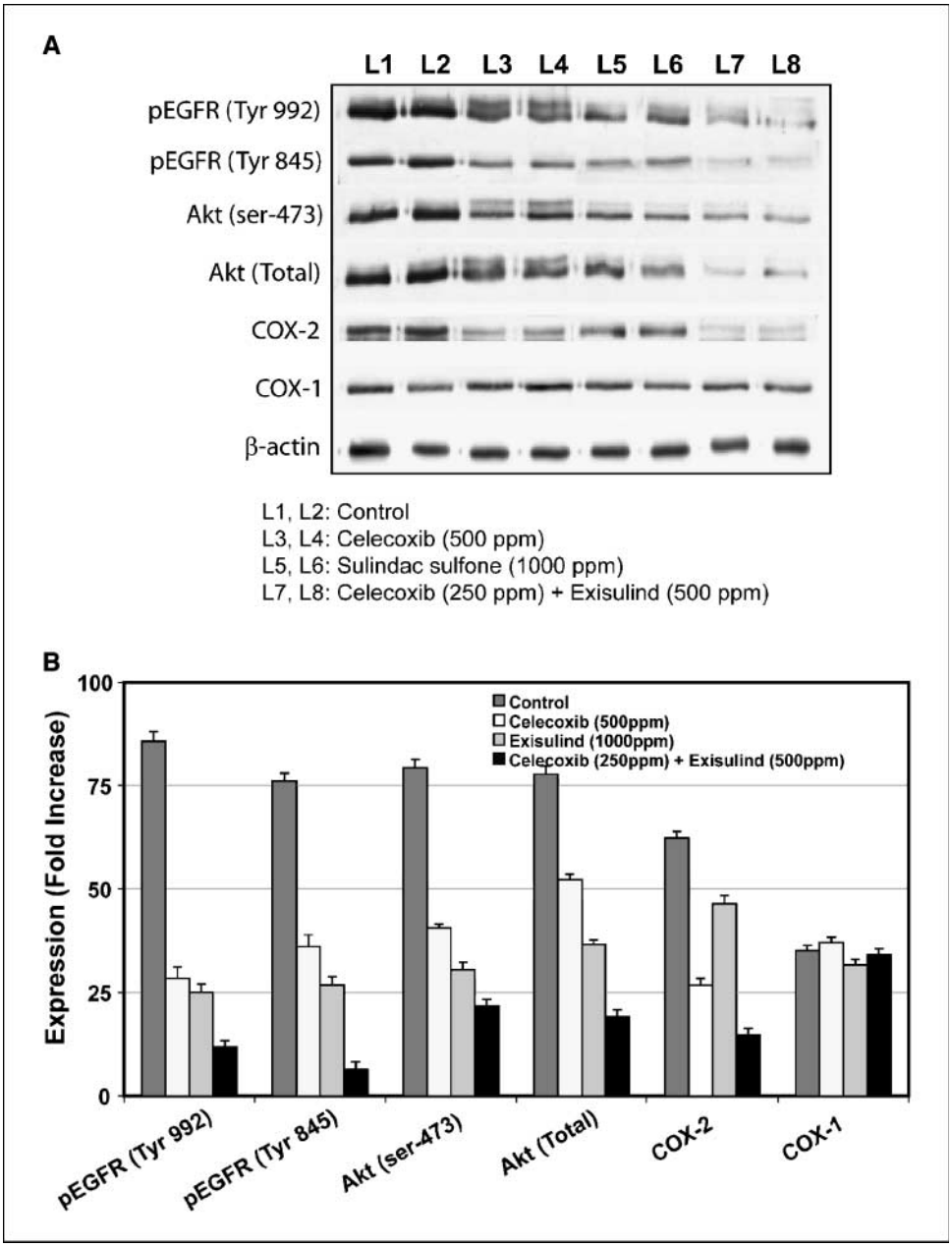

Fig. 5. Western blot analysis: total protein extracted from the dorsolateral prostate of the experimental and control group was used for Western blot analysis as described in Materials and Methods. *A,* effects of dietary celecoxib and exisulind individually and in combination on the expression of COX-1, COX-2, Akt (total), Akt (Ser^{473}), and phosphorylated EGFR (*pEGFR*; Tyr^{845} and Tyr^{992}) proteins were determined using specific antibody. Note: total protein samples from two rats for each group representing control and experiment were used for Western blot. Lanes L1 and L2, control; lanes L3 and L4, celecoxib; lanes L5 and L6, exisulind; lanes L7 and L8, combination of celecoxib and exisulind. *B,* quantification of the difference in the expression levels (average of two samples) was compared between control and experimental groups and is presented as a bar graph.

(*a1-a4*), cyclin D1 (*b1-b4*), AR (*c1-c4*), and COX-2 (*d1-d4*) expression in the dorsolateral prostate of rats receiving diet supplemented with celecoxib in combination with exisulind more effectively than the agents alone. Further, in-depth analysis indicated a parallel decrease in the expression level of EGFR and cyclin D1 in response to similar treatments. A semiquantification of the expression levels of these molecular targets is presented in Fig. 4B.

Specific effect on EGFR and Akt phosphorylation. To address the question on whether the combination of low doses of celecoxib and exisulind modulates the expression level of EGFR and Akt, beyond the primary effect on COX-2, we examined the phosphorylation status of EGFR and Akt. As shown in Fig. 5A, a strong inhibition of total and phosphorylated form of EGFR (Tyr^{992} and Tyr^{845}) was evident in rats that received the combination of celecoxib with exisulind compared with the effect by individual agents (Fig. 5B). Although the inhibitory effects exerted by individual agents vary, a strong inhibition of total and phosphorylated Akt (Ser^{473}) suggests that Akt is down-regulated among the rats that received the combination of celecoxib with exisulind. Our findings suggest that the low-dose combination regimens of celecoxib with exisulind are more effective against prostate cancer development than the agents alone.

Inhibition of COX-2, PGE_2, and TNF-α. To determine the effect of celecoxib in combination with exisulind on selected mediators of inflammation, first, Western blot analysis was done to detect COX-1 and COX-2 protein levels. Protein extracted from dorsolateral prostate tissue of the experimental rats did not show a significant effect on COX-1 expression. However, dietary intake of celecoxib for 52 weeks reduced the expression of COX-2 in the dorsolateral prostate, but a weak inhibitory effect was observed in rats with exisulind treatment. Most importantly, a significant decrease in COX-2 expression was evident in the prostate of the rats receiving the combination of celecoxib with exisulind when compared with exisulind alone (Fig. 5A). The bar graph represents the level of COX-2 expression as determined by the densitometric analysis (Fig. 5B).

Findings from enzyme immunoassay/ELISA assay for serum PGE_2 level showed a significant decrease in rats that received combination of celecoxib with exisulind ($P < 0.05$) as shown in Fig. 6A compared with the individual effects. Similarly, serum analysis for TNF-α revealed a remarkable decrease in the group of rats receiving both individual and combination ($P < 0.05$) treatments with celecoxib (Fig. 6B). However, a weak inhibitory effect was observed in rats that received exisulind alone.

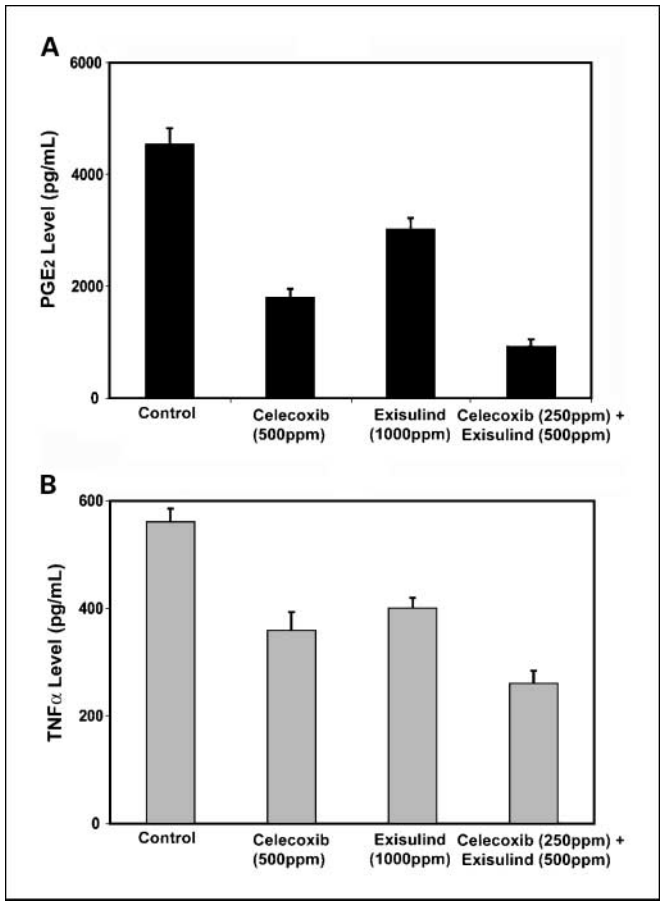

Fig. 6. Effects on PGE_2 and TNF-α levels: effect of dietary celecoxib and exisulind individually and in combination in the serum levels of PGE_2 and TNF-α of MNU/testosterone-induced dorsolateral prostate in Wistar-Unilever rats was measured using enzyme immunoassay-enzyme assay kit as described in Materials and Methods. Levels of PGE_2 and TNF-α were measured in pg/mL. The results from the control group were compared with treatment groups. *A,* levels of PGE_2. *B,* levels of TNF-α.

Discussion

The results presented in this study clearly show a novel but diverse mode of action by celecoxib in combination with exisulind against MNU/testosterone-induced prostate cancer. Essentially, the overall tumor growth inhibition detected by apoptosis in rats given with celecoxib in combination with exisulind is orchestrated by a novel interaction between four independent mechanisms primarily targeting inflammation, cell cycle, AR regulation, and EGFR signal transduction pathways. Findings from our study with MNU/testosterone-induced rat prostate cancer model recall earlier studies of Pollard and Luckert (49–51), where a similar rat model was used to show the efficacy of nonsteroidal anti-inflammatory drugs, such as peroxicum and indomethacine, against intestinal and prostate tumors. Consistently, in our earlier studies using cell lines derived from the MNU/testosterone model, we showed the individual effects of celecoxib in modulating COX-2–dependent and COX-2–independent mechanisms particularly affecting the expression of COX-2 and cyclin D1 (26). Overall, our findings suggest that combination of celecoxib and exisulind not only enhances apoptosis but also exerts anti-inflammatory effect as illustrated by the reduced levels of COX-2, PGE_2, and TNF-α.

Several earlier studies have shown that exisulind is an inhibitor of cyclic GMP phosphodiesterase and is also an effective apoptosis inducer (5, 6, 52–55). A significant observation in this study was that exisulind alone was very effective in reducing tumor growth, which exceeded the effect of celecoxib alone, albeit at a higher dose, and thus supports a COX-2–independent mechanism. However, this is the first report on the use of exisulind in combination with celecoxib showing anti-inflammatory effects in addition to enhancing the effect on apoptosis against prostate cancer.

Interestingly, our findings on exisulind in combination with celecoxib indicated a remarkable decrease in the expression level of EGFR (total and phosphorylated forms), suggesting an inhibitory effect on tyrosine kinase pathways. Although tyrosine kinase receptors are required to increase catalytic activity in tumor cells by mediating phosphorylation of Tyr^{845} (56, 57) or Tyr^{992}, findings from this study showed how phosphorylation of Tyr^{845} or Tyr^{992} could be altered by celecoxib in combination with exisulind against prostate cancer development. However, the biological significance of the specific effect on Tyr^{845} or Tyr^{992} has not yet been determined.

Another important observation in this study is on the inhibitory effect of celecoxib in combination with exisulind on total Akt and phosphorylated Akt (Ser^{453}) against prostate cancer development and agrees with our earlier reports (5). It is evident from a recent study that PGE_2-mediated G protein receptor EP4 is involved with phosphatidylinositol 3-kinase–activated Akt. Activated Akt is significantly increased in UVB-induced mouse skin cancer (58), suggesting a potential role of celecoxib and exisulind in combination in exerting an anti-Akt effect and thus sheds light on G protein receptor signal transduction pathways involving E-prostanoid receptors. Further, our findings suggest a significant decrease in the expression level of cyclin D1 and AR protein in the dorsolateral prostate tissue of rats given with celecoxib in combination with exisulind. This critical finding reveals an insight into the coexistence of cyclin D1 and AR at the tissue level, which could be abrogated by celecoxib and exisulind in combination. Our findings are also consistent with the earlier reports on the inhibition of AR by the exisulind alone (59). The association and dependency of AR with D-type cyclins in prostate cancer have been reported earlier (30). In this study, we provide evidence that celecoxib in combination with exisulind prevented the close association between COX-2, EGFR, AR, and cyclin D1 by more than a single mode of action and resulted in tumor growth inhibition. Findings from this study conclude that the combination of potential COX-2 inhibitors with apoptosis-enhancing agents at lower doses could improve the chemopreventive efficacy certainly in carcinogen/testosterone-induced prostate cancer.

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