Regression of Mouse Prostatic Intraepithelial Neoplasia by Nonsteroidal Anti-inflammatory Drugs in the Transgenic Adenocarcinoma Mouse Prostate Model

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ABSTRACT

Purpose: Epidemiologic studies have revealed a decreased risk of colon cancer among people who have regularly taken cyclooxygenase (COX)-2 inhibitors such as aspirin or other nonsteroidal anti-inflammatory drugs (NSAIDs). Whereas the selective COX-2 inhibitor celecoxib and exisulind, a metabolic product of sulindac, have gained increasing attention as efficacious chemopreventive agents against colon and prostate cancer, not much is known about the underlying molecular targets and mechanisms. Moreover, the side effects of NSAIDs are a major obstacle for large-scale application to the prevention of cancer in humans; for example, in the United States in 1998, there were 16,550 deaths from NSAID-induced gastrointestinal complications. The toxicity associated with these compounds is raising concerns, and more needs to be known about their mode of action and molecular targets.

Experimental Design: We used the transgenic mouse prostate (TRAMP) model, which exhibits similarities with human prostate cancer, including epithelial origin, progression from the PIN stage to adenocarcinoma, and metastasis by a transgene that is hormonally regulated by androgens. In addition to histologically analyzing the PIN lesions of the dorsolateral prostate from TRAMP mice, we delineated the molecular targets and mechanisms of celecoxib and exisulind against mouse PIN lesions. We performed Western blot analysis of the total protein lysate from the tissues of mouse PIN lesions to measure the level of expression of androgen receptor, vascular endothelial growth factor, nuclear factor-κB p65, Bcl-2, AKT (total and phosphorylated Ser473), p53, cyclin-dependent kinase inhibitor p21WAF1/CIP1, p27, BAX, and caspase-3 to demonstrate the COX-2–dependent mechanism involved in the inhibition of PIN lesions of the dorsolateral prostate by both celecoxib and exisulind.

Results: We found for the first time that (a) both celecoxib and exisulind as dietary supplements induce strong inhibitory effects against prostate cancer at doses of 800 and 500 ppm, respectively, after 16 weeks; (b) the histologic analysis of the dorsolateral prostate after 2 weeks of treatment indicated a reduction of PIN lesions from 75% to 19% with celecoxib and to 16% with exisulind; (c) more importantly, those few PINs and adenocarcinomas in the groups treated with celecoxib or exisulind showed more apoptotic cells, lower levels of proliferating cell nuclear antigen, and a lower number of mitotic cells. To understand the molecular mechanisms involved in the inhibition of PIN lesions, first, we examined the expression of molecular targets involved in angiogenesis and inflammatory processes. It was clearly evident from Western blot analysis of the total protein lysate derived from the dorsolateral prostate tissues with PIN lesions that expression of androgen receptor, vascular endothelial growth factor, nuclear factor-κB p65, and Bcl-2 is down-regulated more effectively by celecoxib. Down-regulation of AKT protein (total and phosphorylated at Ser473) and Bax signaling by celecoxib clearly indicates an inhibition of the survival gene and the pathological process that could otherwise lead to adenocarcinoma.

Conclusions: Overall, the findings from this study clearly show the effectiveness of celecoxib and exisulind in reducing the PIN lesions by modulating a cascade of molecular targets involved in COX-2–dependent and –independent mechanisms. Whereas these agents are already in clinical trial or in use as chemopreventive agents, findings from this study demonstrate the difference in their mode of action, thus helping us to understand the side effects.

INTRODUCTION

The incidence of prostate cancer, one of the most common malignancies in men in the United States and in other Western countries, is gradually showing an upward trend (1). Recent epidemiologic reports indicate an increase in the number of men being diagnosed with prostate cancer worldwide (2–6). Prostate cancer growth in humans has been viewed as a multistage process with an early onset of minor histologic changes progressing slowly to metastatic lesions of higher grade (7). A population-based case control study (8), as well as several related reports (9, 10) on nonsteroidal anti-inflammatory drugs (NSAIDs), revealed a trend toward reduced prostate cancer risk associated with regular intake of NSAIDs. Moreover, increased insight into the biology of prostate cancer and the emergence of new chemopreventive agents offer significant means to test new
prevention strategies (11–17). In this connection, it is interesting to note that findings from human studies and preclinical model assays have suggested a potential role for selective cyclooxygenase (COX)-2 inhibitors in the prevention of colon cancer (18–27), but less information exists regarding the role of COX-2 inhibitors against prostate cancer. Whereas the selective COX-2 inhibitor celecoxib induced a dose-dependent cytotoxic effect in colon and breast cancer cells (20, 28), exisulind (sulindac sulfone), a weak inhibitor of COX-2, induced apoptosis in mammary cancer models and thus also inhibited cancer growth (29, 30). Although COX-2 inhibitors play an important role in tumor growth inhibition, the mechanisms underlying selective COX-2 inhibitors are not limited to the COX-2 target but involve COX-2–independent pathways that modulate AKT expression levels as seen in hepatocarcinoma cells (31–33). The effectiveness of these two agents against prostatic intraepithelial neoplasia (PIN) that expresses a low level of COX-2 has not been sufficiently investigated. To address this issue, and also to evaluate the efficacy of celecoxib and exisulind individually against prostate cancer, we used the transgenic mouse prostate (TRAMP) model, which exhibits many similarities to human prostate cancer, including epithelial origin, progression from the PIN stage to adenocarcinoma, and metastasis by a transgene that is hormonally regulated by androgens (34–37), so that it serves as a suitable model. The advantage of the TRAMP model is that well-differentiated neoplasia is generally observed in 100% of these mice between 8 and 12 weeks of age; the metastases to distant sites develop between 18 and 24 weeks of age, and all of the mice display primary tumors and metastases to distant sites (38, 39). This model has been used extensively to evaluate the chemopreventive and therapeutic efficacy of several agents against prostate cancer, including difluoromethylornithine and R-flurbiprofen (40, 41). In this report, for the first time, we present results on the efficacy of celecoxib and exisulind against PIN lesions of prostate cancer, whichwas assessed by measuring the total number of PIN lesions, the level of proliferating cell nuclear antigen (PCNA), and the rate of apoptosis. More importantly, we have elucidated the effects of celecoxib and exisulind on several molecular targets (including AKT signaling) that could play an essential role in PIN development via COX-2–independent mechanisms. The overall findings from this study clearly indicate that celecoxib and exisulind play a significant role in reducing the number of PIN lesions by down-regulating the expression of key molecular targets involved in angiogenesis and inflammatory processes. Whereas these agents are already in clinical trial or in use, findings from this study clearly demonstrate their mode of action at the molecular level, which is vital to understand the side effects. More importantly, our findings indicate early molecular targets of PIN lesions to be examined in the human clinical samples and thus will enable us to design more effective prevention strategies.

### MATERIALS AND METHODS

#### Animals and Diets

Hemizygous 6-week-old male TRAMP [C57BL/6-TgN(TRAMP)8247Ng] and control (C57BL/6) mice were purchased from JAX Mice and Services (Bar Harbor, ME). The mice were maintained in quarantine for 2 weeks in a holding room at the Institute for Cancer Prevention (Valhalla, NY). They were housed in cages with wood chip bedding in a temperature-controlled room (68°F–72°F) with a 12-h light/dark cycle, at a relative humidity of 45% to 55%. All ingredients of the semi-purified AIN-76A diet were purchased from Diets Inc. (Bethlehem, PA). The modified AIN-76A diet consisted of 20% casein, 0.3% L-methionine, 52% corn starch, 13% dextrose, 5% corn oil, 5% alphacel, 3.5% AIN mineral mixture, 1% AIN vitamin mixture, and 0.2% choline bitartrate. The ingredients were stored at 4°C before preparation of the experimental diets.

#### Chemopreventive Agents and Dose Selection

We used celecoxib (SC-58635), supplied by Searle Research and Development (Pharmacia, St. Louis, MO), and sulindac-sulfone (exisulind-CP461) provided by Cell Pathways, Inc. (Horsham, PA). Experimental diets were prepared weekly by mixing celecoxib (800 ppm) or exisulind (500 ppm) with modified AIN-76A diet. The diets were stored in a cold room. In this study, celecoxib at 800 ppm and exisulind at 500 ppm were evaluated for their chemopreventive effect. The above-mentioned doses of celecoxib and exisulind were selected on the basis of a maximum tolerated dose (80% maximum tolerated dose) assay.

#### Study Design and Experimental Procedure

After the quarantine, 6-week-old TRAMP mice were randomly distributed by weight into experimental and control groups (n = 10), as shown in Table 1, and fed the experimental diet for a period of 16 weeks. A cohort of TRAMP mice was used as control group. Nontransgenic mice (C57BL/6) were also used as an overall control group. The animals in both control groups were fed AIN-76A diet only. Body weights were recorded weekly, and the animals were monitored daily for their general health. The mice were sacrificed by CO2 euthanasia at 8 or 24 weeks of age.

#### Histopathology

Before sacrifice, each mouse was anesthetized and weighed. The genitourinary tract (GUT) consisting of the bladder, urethra, seminal vesicles, ampuller gland, and prostate was excised, weighed, and dissected under a micro-

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**Table 1** Efficacy study design using the TRAMP (C57BL/6-TgN 8247Ng) mouse model

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of animals</th>
<th>Period of treatment (wks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Fed with AIN-76A diet only</td>
<td>10</td>
<td>16</td>
</tr>
<tr>
<td>Celecoxib treatment</td>
<td>Fed with 800 ppm of celecoxib in AIN-76A diet</td>
<td>10</td>
<td>16</td>
</tr>
<tr>
<td>Exisulind treatment</td>
<td>Fed with 500 ppm of exisulind in AIN-76A diet</td>
<td>10</td>
<td>16</td>
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scope. The dorsal prostate, lateral prostate, ventral prostate, and anterior prostate lobes of the prostate were microdissected into individual lobes whenever possible and fixed overnight in 10% formalin and then transferred into 70% EtOH. Paraffin-embedded tissue sections of 5-μm thickness were used for histology. Hematoxylin and eosin (H&E)-stained sections were examined for neoplastic changes. Histologic evaluations for the presence of epithelial stratification and related changes indicative of PIN and adenocarcinoma of the dorsolateral prostate were conducted by assessing the changes that are characteristic for TRAMP prostate histology as described previously (36, 44). All tissues from our study were evaluated and reviewed in collaboration with the help of a designated pathologist. For the histologic, immunohistochemical, and Western blot analyses, we used dorsolateral prostate tissue samples from treatment and control groups sacrificed after 2 weeks (for PIN lesions) or 16 weeks (for adenocarcinoma).

**Scoring of Apoptotic Cells.** Apoptosis was assessed by the identification of cells showing morphologic changes associated with condensation of nuclear material by examination of H&E-stained sections under a light microscope at high-power fields. The apoptotic index was estimated by the percentage of apoptotic cells scored under ×40 magnification. The percentage was calculated by dividing the total number of apoptotic cells by the total number of cells per section in 10 different fields. The results presented are for the dorsolateral prostate tissue derived from each mouse in the experimental and control groups.

**Immunohistochemical Detection of Proliferating Cell Nuclear Antigen.** We used paraffin-embedded dorsolateral prostate tissue sections of 5-μm thickness for all immunohistochemical analyses. After rehydration, antigens were retrieved by a process involving microwaving with antigen-unmasking fluid (Vector Laboratories, Burlingame, CA) twice for 5 minutes, with a 3-minute interval. After 15 minutes at room temperature, the sections were washed and blocked with 10% normal horse serum. The sections were then incubated for 1 hour with primary mouse anti-PCNA antibody (clone PC10; diluted 1:200 in serum; Lab Vision, Fremont, CA) at room temperature. Overall expression levels of PCNA were detected using a universal labeling kit (horseradish peroxidase/3,3′-diaminobenzidine) from Ventana Medical Systems (Tucson, AZ). The Image Pro software program (Media Cybernetics, Silver Spring, MD) was used to quantitate the total number of PCNA-positive cells in a minimum of 10 fields at ×40 magnification.

**Western Blots for Molecular Targets.** Total protein from control TRAMP dorsolateral prostate and tumor tissues from the group of mice fed experimental and control diets for 2 weeks and 16 weeks, respectively, were isolated. Briefly, 100 mg of tissue was extracted with 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, Mannheim, Germany). Aliquots of protein (20 μg/lane) were fractionated on 10% SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes. The Western blot protocol was carried out as described previously (28). The level of β-actin expression was used as the internal control for equal loading. The antibodies for COX-1 and COX-2 were purchased from (Cayman, Ann Arbor, MI). Reactive protein bands were developed with chemiluminescence detection reagents [enhanced chemiluminescence (ECL); Amersham Biosciences, Piscataway, NJ].

Western blot analysis was extended to measure the level of expression of androgen receptor (AR), nuclear factor (NF)-κB p65, vascular endothelial growth factor (VEGF), AKT (total and phosphorylated Ser473), BclII, p53, cyclin-dependent kinase inhibitor p21WAF1/CIP1, p27, BAX, and caspase-3 using specific antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) to demonstrate the COX-2–independent mechanism involved in the inhibition of PIN lesions of the dorsolateral prostate by both celecoxib and exisulind. Densitometric analysis of the protein bands was performed with Gel-Pro Analyzer software (Media Cybernetics) as described previously (28).

**Measuring Prostaglandin E2 Levels.** We used the Asay Designs (Ann Arbor, MI) Correlate-EIA-prostaglandin (PG) E2 enzyme immunoassay to measure the PGE2 level in the serum and dorsolateral prostate tissue from TRAMP mice. At the time of sacrifice, serum and dorsolateral prostate tissue with PIN from TRAMP control and experimental groups of celecoxib- and exisulind-treated mice was snap-frozen and stored at −80°C. Frozen tissues were ground in liquid nitrogen using a mortar and pestle, weighed, and extracted for PGE2 as per the manufacturer’s recommendation. PGE2 standard with a stock of 50,000 pg/mL PGE2 was used for subsequent lower dilutions. The assay involves the use of a monoclonal antibody to PGE2 to bind in a competitive manner the PGE2 in the sample or an alkaline phosphatase molecule, which has PGE2 covalently attached. After a short incubation time, the enzyme reaction was stopped, and the yellow color generated was read on a microplate reader at 405 nm. The intensity of bound yellow color is inversely proportional to the concentration of PGE2 in the sample. The results presented here are based on data from three independent assays.

**Statistical Analysis.** Measures of COX-2, PGE2, PCNA, GUT weight, the number of PIN lesions, and so forth were compared among the groups using one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons procedure (45). Differences in body weight measured over time were evaluated through graphic summaries and repeated-measure ANOVA models. All tests were considered statistically significant at P < 0.01.

**RESULTS**

**General Observations.** An overall weight gain was observed in those TRAMP mice that received the control diet AIN-76A and experimental diets containing celecoxib and exisulind. The experimental mice that consumed the diet with either celecoxib (800 ppm) or exisulind (500 ppm) for 16 weeks showed no signs of toxicity but had a small decrease in total weight gain as compared with the control group (Fig. 1). At the time of sacrifice, the lower GUT (including testes, bladder, prostate, and seminal vesicles) was removed, and the total wet weight was recorded in grams and is presented in Fig. 2. A difference in the total body weight and GUT weight appeared to be attributable to the inhibition of cell proliferation and tumor growth in the experimental mice compared with the weight gain in the TRAMP control animals.
Primary and Secondary Targets of NSAIDs

Histopathological Evaluation of the Dorsolateral Prostate. Histologic evaluation of the H&E-stained dorsolateral prostate tumor tissues revealed that the tissue sections from the control TRAMP mice examined between 8 and 10 weeks of age had developed PIN with characteristic changes as described by Shappell et al. (44), in addition to information from other studies in identifying the presence of papillary projections of epithelial cells in 75% of the total acini counted (Fig. 3A and B). All of the TRAMP mice in each group (100%) had developed prostate cancer after 24 weeks of age. Adenocarcinoma with multiple layers of stromal fibroblasts of the dorsolateral prostate was seen in all TRAMP mice (Fig. 3C and D).

Regression of Murine PIN Lesions by Celecoxib or Exisulind Treatment. Feeding supplements of celecoxib or exisulind to TRAMP mice for 2 weeks at 800 and 500 ppm, respectively, reduced the number of histologically identifiable PIN lesions. Both celecoxib and exisulind effectively reduced the total number of PIN lesions in comparison with controls. The total number of PIN lesions determined in the treatment groups versus the control group of mice is shown in Fig. 4; the reduction of lesions was from 75% to 19% (SE, ±4.26) with celecoxib (P < 0.0001) and from 75% to 16% with exisulind (SE, ±6.63; P < 0.0001).

Celecoxib- and/or Exisulind-Induced Apoptosis. Numerous cells identified in the H&E-stained sections showed condensed nuclei associated with higher nuclear to cytoplasmic ratio (confirmed with light microscope), reflecting apoptotic cell death in the tumors of the dorsolateral prostate observed after feeding of celecoxib or exisulind feeding for 16 weeks. The well-defined and characteristic apoptotic cells in the PIN and adenocarcinoma of the TRAMP are shown in Fig. 5A–D. Quantification of the rate of apoptosis induced by these agents is presented in Fig. 5E.

Effect of Celecoxib and Exisulind on Proliferating Cell Nuclear Antigen. Proliferating cell nuclear antigen is an auxiliary protein for DNA polymerase. It reaches maximal expression during the S phase of the cell cycle. Hence, PCNA has been widely used as an index of the proliferative activity of a tumor. In this study, we measured PCNA labeling only in the adenocarcinoma. The level of PCNA-positive cells was higher in TRAMP control adenocarcinoma than was the level of expression in adenocarcinoma of the mice treated with celecoxib or exisulind after 16 weeks. Lower PCNA labeling indicates less proliferation and hence the regression of prostate cancer (Fig. 6A–C). Quantitative aspects of the PCNA expression in different groups are presented in Fig. 6D.

Effect of Celecoxib or Exisulind on COX-1, COX-2, and PGE2 Expression. Protein extracted from prostate tissue sample at the early stage of PIN lesions, i.e., after 2 weeks of treatment, did not show either COX-1 or COX-2 expression. However, dietary intake of celecoxib for 16 weeks reduced the expression of COX-2 in the dorsolateral prostate adenocarcinoma tissue. Western blot analysis of the total protein from adenocarcinoma after 16 weeks of treatment indicated a marked inhibition of COX-2 by celecoxib by comparison with that in the exisulind-treated or control TRAMP mice, as shown in Fig. 7A. However, we also observed a minor impact on the COX-1 protein in the celecoxib-treated as well as the exisulind-treated group of mice. The bar graph represents the level of COX-2 expression as determined by densitometric analysis (Fig. 7B). Although the level of PGE2 is higher in the prostate tissue than in the serum, both agents inhibited PGE2 very effectively, with a small difference between celecoxib and exisulind (P < 0.005) as shown in Fig. 7C.

Effects of Celecoxib or Exisulind on AR, NF-κB p65, VEGF, BclIII, and AKT Expression Levels. To examine the molecular targets that are involved in NSAID-mediated regression of PIN lesions, we investigated the level of expression of...
AR, NF-κB p65, VEGF, BclII, and AKT. These molecular targets are linked to the androgenic, angiogenic, and inflammatory processes of tumor progression. Western blot analysis of the total protein of prostate tissue with PIN lesions indicated reduced expression of AR, NF-κB p65, VEGF, total and phosphorylated AKT (Ser473), and BclII in the celecoxib-treated mice compared with that seen in the exisulind-treated or control TRAMP mice, as shown in Fig. 8A. The bar graph represents the level of expression as determined by the densitometric analysis (Fig. 8B). Down-regulation of AKT protein (total and phosphorylated at Ser473) signaling by celecoxib clearly indicates an inhibition of the survival gene and the associated pathological process that could otherwise lead to adenocarcinoma. Although exisulind also inhibited AKT phosphorylated at Ser473, a small apparent increase in the total AKT level was unexpected, and this could be related to several other factors, which need to be examined further in the TRAMP model when tumor growth is neuroendocrine related. Our findings emphasize that the level of AKT and NF-κB p65 expression in the exisulind-treated TRAMP mice is independent of its total and significant effect in inducing apoptosis and thus reducing the PIN lesions.

Celecoxib- or Exisulind-Mediated COX-2–Independent Molecular Targets. Histologic evaluation of both celecoxib- and exisulind-treated mice showed reduced PIN lesions of the dorsolateral prostate and was associated with a higher number of apoptotic cells. Therefore, we examined the molecular targets that enhanced the mechanism of apoptosis. Interestingly elevated expression levels of tumor suppressor gene p53; cyclin-dependent kinase inhibitors such as p21WAF1/CIP1, p27, and proapoptotic BAX; and caspase-3 were evident from Western blot analysis; these are shown in Fig. 9A. Higher expression levels of p53 and p21WAF1/CIP1 were very clearly evident in the celecoxib- and exisulind-treated groups of mice, suggesting a significant role of a p53-dependent apoptosis mechanism. The bar graph represents the level of expression of the above-mentioned markers as determined by densitometric analysis (Fig. 9B).

![Fig. 3](image-url) Histologic evaluation of PIN and adenocarcinoma. A, H&E staining of the TRAMP dorsolateral prostate showing histologic changes associated with mouse PIN lesions after 8 weeks of age. B, higher magnification (×40) of the section showing the lesions with papillary projections of proliferating epithelial cells characteristic of PIN. C, a section through the adenocarcinoma of the prostate after 24 weeks of age. D, adenocarcinoma at higher magnification (×40) showing penetration of highly proliferating cells through the basement membrane of PIN-involved glands (invasive carcinoma).

![Fig. 4](image-url) Effect of celecoxib and exisulind against PIN lesions. Dorsolateral prostate from the TRAMP mice sacrificed after 2 weeks of treatment with celecoxib (800 ppm) or exisulind (500 ppm) as described in Materials and Methods was examined for PIN lesions. The bar graph represents the total percentage of PIN lesions identified in tissue sections from a total of five mice in each group. The total number of lesions counted in 10 high-power fields are presented for control versus celecoxib or exisulind, where P < 0.0001.
Fig. 5  Apoptosis induced by celecoxib and exisulind. Histologic demonstration of the presence of apoptotic cells in the TRAMP prostate after administration of either celecoxib (800 ppm) or exisulind (500 ppm), along with the modified AIN-76A diet for 16 weeks. TRAMP control mice were fed only the modified AIN-76A diet. H&E-stained sections showing the condensed and fragmented nuclear material characteristic of apoptotic cells are shown: A, control; B, celecoxib treatment; C, exisulind treatment; and D, higher magnification of apoptotic cells (arrow indicates apoptotic cells).

Fig. 6  Effect of celecoxib and exisulind on the proliferation marker PCNA. Immunohistochemical determination of PCNA levels in PIN lesions of the dorsolateral prostate. A, PCNA expression in control TRAMP. B, PCNA expression in celecoxib (800 ppm)-treated TRAMP. C, PCNA expression in exisulind (500 ppm)-treated TRAMP. The difference in the PCNA expression in the control versus experimental groups is presented in D as a bar graph. Celecoxib, *P* < 0.001; exisulind, *P* < 0.0001.
DISCUSSION

The present study is part of an ongoing investigation of the chemopreventive efficacy of NSAIDs against prostate cancer in preclinical assays. Recent reports on NSAIDs against prostate cancer strongly support the need to understand the molecular mechanisms behind their action in a dose- and time-dependent manner (46, 47). First of all, we confirm the suitability of the TRAMP model for prostate cancer chemoprevention studies because it enables examination of the effects of the agents on PIN and subsequent stages, including adenocarcinoma of the dorsolateral prostate. Whereas the TRAMP model assays are widely used to foster the understanding of several molecular events related to prostate cancer (34–36, 48, 49), thus far only a few investigators have reported using this assay for studies on the chemopreventive effects against prostate cancer. These include Gupta et al. (40), who tested the efficacy of difluoromethylornithine, and Wechter et al. (41), who examined the efficacy of R-flurbiprofen against prostate cancer. However, these studies provide insufficient information regarding the effect of chemopreventive agents on the early lesions in the dorsolateral prostate that are precursors for prostate cancer. Transgenic mice spontaneously develop prostate cancer through a series of well-defined stages, including PIN lesions that already show the characteristic alteration leading to markers of cell proliferation (34, 44). Therefore, we used the TRAMP assay to investigate the chemopreventive effects of celecoxib and exisulind at specific doses based on maximum tolerated dose assays.

Celecoxib is a cancer-preventive agent that has been highly effective as an inhibitor of colon cancer in preclinical models (20, 24, 25, 50). The data presented here clearly demonstrate that administration of either celecoxib or exisulind at 800 or 500 ppm, respectively, in the diet for 16 weeks is nontoxic. An important observation of this study is that mice sacrificed after only 2 weeks of dietary administration of celecoxib or exisulind showed a lower number of PIN lesions compared with the control, suggesting that these doses are very effective for preventing the occurrence of early lesions of the dorsolateral prostate. More importantly, these few PIN lesions and, later, the adenocarcinoma of the dorsolateral prostate showed more apoptotic and fewer PCNA-positive cells compared with the control, reflecting that celecoxib and exisulind inhibit cell proliferation and induce apoptosis. Our earlier studies with celecoxib

![Image](cancerres.aacrjournals.org/cone/7733/fig5_e.jpg)

**Fig. 5 Continued.** E, apoptotic cells counted in 10 high-power fields were quantified and presented (bar graph) as a percentage of apoptotic cells.

![Image](cancerres.aacrjournals.org/cone/7733/fig7_a.jpg)

**Fig. 7** Effect of celecoxib and exisulind on COX-1, COX-2, and PGE₂ expression in TRAMP dorsolateral prostate. A, Western blot analysis to detect changes in the expression of COX-1 and COX-2 in dorsolateral prostate tissue samples from control and experimental groups after administration of either celecoxib (800 ppm) or exisulind (500 ppm) for 16 weeks. Reactive protein bands for COX-1 and COX-2 were analyzed using ECL detection with specific antibodies as described in Materials and Methods. B, Expressed proteins were quantified as the ratio to control β-actin. Each bar represents the approximate amount of protein corresponding to the banding pattern that was measured by densitometric analysis performed with Gel-Pro Analyzer software (Media Cybernetics). C, Serum and dorsolateral prostate tissue from control TRAMP, celecoxib (800 ppm)-treated, and exisulind (500 ppm)-treated mice were analyzed for PGE₂ using correlate-EIA-PGE₂ enzyme immunoassay. Levels of PGE₂ (measured in pg/mL) in the control were compared for treatment with celecoxib and or exisulind.
and exisulind (28), along with reports from the literature (30), clearly support the effectiveness of these two agents in inducing apoptosis and inhibiting prostate cancer cell growth at very low doses. The histologic analysis of apoptotic cells and PCNA levels clearly correlate with the chemopreventive efficacy of these agents.

In recent years, there has been great interest in the question of whether NSAIDs, including COX-2 inhibitors such as celecoxib, affect carcinogenesis solely through COX-2 inhibition or also via COX-2–independent mechanism(s). Although the precise mechanisms by which celecoxib inhibits prostate carcinogenesis are not fully known, we propose, on the basis of our earlier findings with in vitro models and the observations of the present study, that COX-2 inhibitors operate by inducing apoptosis and by inhibiting cell proliferation irrespective of their effects on COX-2 function. However, it is also very clear from this study that a decrease in COX-2 by celecoxib is associated with a more significant decline in PGE2 levels in the prostate tissue as compared with the serum (as shown in Fig. 7C). PGE2 is a major downstream mediator of COX-2 that promotes cellular proliferation and angiogenesis, makes cells resistant to apoptosis, enhances invasiveness, and modulates immunosuppression. COX-2 and COX-2–derived PGE2 may be involved in

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**Fig. 8** Expression of AR, NF-κB p65, VEGF, AKT, and Bcl/II in the dorsolateral prostate with PIN. A. The results are presented for the tissue samples analyzed in the experimental groups after administration of either celecoxib (800 ppm) or exisulind (500 ppm) for 2 weeks. Reactive protein bands were analyzed using ECL detection with specific antibodies as described in Materials and Methods. B. Expressed proteins were quantified as the ratio to control β-actin. Each bar represents the approximate amount of protein corresponding to the banding pattern that was measured by densitometric analysis performed with Gel-Pro Analyzer software (Media Cybernetics). The significant difference between treatment and control, determined from pairwise comparisons, resulted in a P of <0.001.

**Fig. 9** Effect of celecoxib and exisulind on p53 expression and Bax activation in TRAMP dorsolateral prostate. A. The results are presented for the tissue samples analyzed in the experimental groups after administration of either celecoxib (800 ppm) or exisulind (500 ppm) for 16 weeks. Reactive protein bands were analyzed using ECL detection with specific antibodies as described in Materials and Methods. Expressed proteins were quantified as the ratio to control β-actin. B. Each bar represents the approximate amount of protein corresponding to the banding pattern that was measured by densitometric analysis performed with Gel-Pro Analyzer software (Media Cybernetics). The significant difference between treatment and control was determined from pairwise comparisons resulting in a P of <0.001.
Colon and mammary carcinogenesis (51). Therefore, COX-2–selective inhibitors may have a role in prostate cancer prevention, in which expression of COX-2 and PGE$_2$ appears to be a late event in prostate carcinogenesis. It is noteworthy that we have shown COX-2 inhibition through celecoxib treatment but less of an effect on COX-2 by exisulind, yet both agents reduced PIN lesions and caused more apoptotic cells in the adenocarcinoma of the dorsolateral prostate than are present in spontaneous tumors in the control mice in a COX-2–independent manner. These observations suggest that inhibition of prostate cancer by celecoxib and exisulind activated the downstream targets of p53 and apoptosis activators such as BAX and caspase-3, indicating a convergence in the mechanism of the two agents as they inhibit PIN lesions. The NSAID-induced sequence of alterations of key molecular targets involved in the development of PIN lesions to adenocarcinoma is clearly demonstrated in this study.

![Diagram](image-url)

Fig. 10  Molecular targets and mechanisms underlying the development of mouse PIN lesions and inhibition by NSAIDs. On the basis of our findings from this study using the TRAMP model assay, we derived a possible mechanism underlying the cascade of molecular events that resulted in the regression of PIN lesions and adenocarcinoma in mice fed with celecoxib and/or exisulind for a period of 2 and 16 weeks, respectively. As depicted in this figure, we conclude that the mechanisms involved in the regression of PIN lesions by celecoxib and/or exisulind are the result of several molecular changes in the PIN lesions of the dorsolateral prostate, mediated by an antiandrogenic effect resulting in inhibition of the expression of AR and nuclear transcription factor NF-$\kappa$B p65. The cascade of events initiated at this point reduced the expression of VEGF, causing an antiangiogenic effect, followed by a reduced inflammatory response that was evident from the lower expression of COX-2 in the dorsolateral prostate adenocarcinoma. The observation that the expression of survival genes AKT (total and phosphorylated at Ser$^{473}$) and BclII was down-regulated effectively by celecoxib suggests a close link between the reduced number of PIN lesions and lower expression of survival genes or proteins. Interestingly, both celecoxib and exisulind activated the downstream targets of p53 and apoptosis activators such as BAX and caspase-3, indicating a convergence in the mechanism of the two agents as they inhibit PIN lesions. The NSAID-induced sequence of alterations of key molecular targets involved in the development of PIN lesions to adenocarcinoma is clearly demonstrated in this study.

Colon and mammary carcinogenesis (51). Therefore, COX-2–selective inhibitors may have a role in prostate cancer prevention, in which expression of COX-2 and PGE$_2$ appears to be a late event in prostate carcinogenesis. It is noteworthy that we have shown COX-2 inhibition through celecoxib treatment but less of an effect on COX-2 by exisulind, yet both agents reduced PIN lesions and caused more apoptotic cells in the adenocarcinoma of the dorsolateral prostate than are present in spontaneous tumors in the control mice in a COX-2–independent manner. These observations suggest that inhibition of prostate cancer by celecoxib and exisulind is mediated through both COX-2–dependent and –independent mechanisms, strengthening the concept of several investigators that more than one molecular mechanism is involved in tumor inhibition by these chemopreventive agents (20, 21, 52–54).

Phosphatidylinositol 3'-kinase/AKT is an important survival signal pathway that has been shown to be crucial in the regulation of balance between the proapoptotic and survival signal.

Our results showed a significant decrease in total and phosphorylated AKT at Ser$^{473}$ with celecoxib compared with the control. There is no significant correlation among the number of PIN lesions, the rate of apoptosis, and the total AKT level in the exisulind-treated TRAMP mice. Our current ongoing studies are focused more on AKT regulation in exisulind-treated prostate cancer cells. However, studies also indicate constitutive activation of AKT in insulin-like growth factor II-overexpressing rhabdomyosarcoma cells (55). Exploring the role of AKT in the activation of NF-$\kappa$B p65 in prostate cancer cells is very important to understand its role in the initiation of PIN. Although AKT expression is an early event in colon carcinogenesis (56), the exact transition point of AKT activation in the prostate that leads to tumor growth is not yet clear. This suggests that a better understanding of AKT signaling, specifically by exisulind against prostate cancer, is needed.

We conclude that the mechanisms involved in the regression of PIN lesions by celecoxib and/or exisulind are the result of several molecular changes in the PIN lesions of the dorsolateral prostate, mediated by an antiandrogenic effect resulting in inhibition of the expression of AR and nuclear transcription factor NF-$\kappa$B p65 as illustrated in Fig. 10. The cascade of events initiated at this point reduced the expression of VEGF, causing an antiangiogenic effect, followed by a reduced inflammatory response that was evident from the lower expression of COX-2 in the dorsolateral prostate adenocarcinoma. The observation that the expression of survival genes AKT (total and phospho-
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