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Inhibition of Rat Mammary Gland Carcinogenesis by Simultaneous Targeting of Cyclooxygenase-2 and Peroxisome Proliferator-activated Receptor γ

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ABSTRACT

We examined the effect of celecoxib, a cyclooxygenase-2 (COX-2) inhibitor, and *N*-(9-fluorenyl-methyloxycarbonyl)-L-leucine (F-L-Leu), a peroxisome proliferator-activated receptor γ (PPARγ) agonist, separately and combined, on the development of methylnitrosourea (MNU)-induced rat mammary gland carcinogenesis. Celecoxib and F-L-Leu significantly reduced tumor incidence and multiplicity ($P < 0.05$). Combining both agents exerted higher (synergistic) cancer inhibition than separate treatments ($P < 0.05$). The effects of the test drugs on COX-2 and PPARγ expression and on the synthesis of prostaglandin E_2 (PGE_2) and 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) were examined in rat mammary normal (MNU-untreated), uninvolved, and tumor (MNU-treated) tissues. Celecoxib and F-L-Leu, separately, inhibited COX-2 and up-regulated PPARγ expression. These effects were paralleled by inhibition of PGE_2 synthesis and up-regulation of 15d-PGJ_2. Combined treatment resulted in higher alterations in COX-2 and PPARγ transcripts and PG synthesis compared with separate administrations. The effect of the test agents on Bcl_2, BAX, and protein kinase Cα expression levels were examined in the rat mammary gland and the pro-(BAX:Bcl_2) and anti-[PKCα*(Bcl_2/BAX)] apoptotic ratios were evaluated. Each drug increased the proapoptotic ratio by 2- to 7-fold and reduced the antiapoptotic ratio by 2- to >8-fold in all tissues. Combined treatment, however, resulted in >9- to 14-fold up-regulation in the proapoptotic processes and 15- to >30-fold down-regulation in the antiapoptotic ones. Analyses were also carried out on the drug-induced modulation of cell cycle regulators and proliferation markers (cyclin-dependent kinase 1 and proliferating cell nuclear antigen). F-L-Leu and celecoxib each reduced the cyclin-dependent kinase 1 and proliferating cell nuclear antigen expression in the tumor. Higher down-regulation was attained in all tissues by combined treatment where cyclin-dependent kinase 1 and proliferating cell nuclear antigen almost retained the expression levels observed in the normal glands. In conclusion, simultaneous targeting of COX-2 and PPARγ may inhibit mammary cancer development more effectively than targeting each molecule alone. COX-2 inhibitors and PPARγ agonists coordinately mediate their anticancer effect via both COX-dependent (inhibition of COX-2, activation of PPARγ, and modulation PG synthesis) and COX-independent (induction of proapoptotic factors and inhibition of cell proliferation) pathways.

INTRODUCTION

The high prevalence of breast cancer (1) and the limited options for treatment provide a strong rationale for identifying new, selective molecular targets for pharmacological modulation and chemopreventive intervention. Cyclooxygenase-2 (COX-2) and peroxisome proliferator-activated receptor-γ (PPARγ) are among the regulatory molecules that have emerged as promising candidates for breast cancer prevention (2). COX-2 and its products, *e.g.*, prostaglandin E_2 (PGE_2), induce inflammation and mediate a number of apoptotic and cell growth-signaling pathways and may play a role in carcinogenesis (3). Similarly, inactivation of PPARγ influences the transcription of genes involved in cell proliferation and apoptosis (4, 5). Several lines of evidence suggest a possible coordinated action and cross-talk between the two molecules (6, see below). In human breast cancer, independent studies demonstrate that COX-2 and PPARγ, respectively, exhibit induction (7–10) and inactivation (10, 11). Experimental evidence suggests that inhibition of COX-2 (12–14), *e.g.*, by celecoxib, and activation of PPARγ (15–17), *e.g.*, by glitazones or 15-deoxy-$\Delta^{12, 14}$-PGJ_2 (15d-PGJ_2), prevent carcinogen-induced mammary cancer in animals. Recently, combinational targeting of more than one molecule, *e.g.*, estrogen receptors and PPARγ (15) or retinoid X receptors and PPARγ (16), has emerged as an approach that provides more effective prevention with fewer side effects than targeting separate molecules. We thought, therefore, that simultaneous targeting of COX-2 and PPARγ might act additively, if not synergistically, to inhibit the development of mammary cancer.

Although several mechanisms are proposed to explain the antitumorigenic action of COX-2 inhibitors and PPARγ ligands in the mammary gland, their effect on apoptotic signaling and cell proliferation has received particular attention (6). Available information suggests that tissue growth depends upon the balance between cell growth and death. The two rates are normally balanced in the adult resting normal mammary gland so that overgrowth does not occur (18). Apoptosis plays a critical role in the homeostasis of the mammary glands, *e.g.*, in tissue remodeling during postlactation involution (19). A major pathway implicated in apoptosis in the normal (and cancerous) mammary gland is the intrinsic or mitochondrial signaling triggered and mediated by Bcl_2 family members, *e.g.*, BAX and Bcl_2 (19, 20). The antiapoptotic Bcl_2 is detected in 80% of breast cancer cases and is related to metastasis, chemoresistance, and poor prognosis (19). The proapoptotic BAX, on the other hand, is expressed in high levels in the normal breast tissue but only weakly or not at all in tumor (21). Loss of BAX correlates with shorter survival time and faster tumor growth (22, 23). The interaction between BAX and Bcl_2 determines the net apoptotic gains and can subsequently play a critical role in cancer development and prevention (19, 24, 25). Many cancer therapeutic (26–28) and preventive (24, 29) agents mediate their action by altering the activity and expression of Bcl_2-family genes/proteins. Similarly, COX-2 inhibitors and PPARγ ligands induce proapoptotic signaling in human breast cancer cells by up-regulating BAX and down-regulating Bcl_2 (30–32) transcription. Several antiapoptotic factors, *e.g.*, protein kinase C (PKC)α, influence BAX-Bcl_2 interaction and, thereby, mediate cell survival (33).

Another mechanism by which COX-2 inhibitors and PPARγ ligands may inhibit carcinogenesis is by attenuating cell growth and inducing cell cycle arrest (6, 32). This action may occur because of the ability of these agents to reduce the expression of markers of cell proliferation, *e.g.*, proliferating cell nuclear antigen (PCNA; Refs. 34, 35) and Ki-67 (36, 37), and deregulate the activity of factors involved in cell cycle progression, *e.g.*, cyclin-dependent kinases (cdks; Ref. 38). Accordingly, during mammary carcinogenesis, the extent to which COX-2 inhibitors and PPARγ ligands influence the dynamic interaction between apoptosis and cell proliferation may play a role in their cancer preventive efficacy.

In this report, we examine the efficacy of celecoxib, a COX-2

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inhibitor (Fig. 1*A*), and *N*-(9-fluorenyl-methyloxycarbonyl)-L-leucine (F-L-Leu), a PPARγ agonist (Fig. 1*A*), separately and in combination, on the inhibition of *N*-methyl-*N*-nitrosourea (MNU)-induced rat mammary gland carcinogenesis, a well-established model for breast cancer (39). Moreover, to identify some of the molecular pathways involved in the anticancer potentials of these agents, we evaluated their effect on an array of markers of apoptosis and cell proliferation known to be involved in mammary gland development as well as susceptibility to cancer.

MATERIALS AND METHODS

Animals, Treatments, and Tumor Induction

Female Sprague Dawley rats (n = 100), purchased from Taconic Farms (Germantown, NY), were housed at 22 ± 2°C, 50% humidity with a 12-h light-dark cycle. Tap water was provided *ad libitum* throughout the experiment. Rats were acclimatized for 1 week on an AIN-93G diet (Dytes Co., Bethlehem, PA). The composition of the experimental diet has been described previously (40). As shown in Fig. 1*B*, animals were given a single i.p. injection of 50 mg/kg MNU (Sigma Chemical Co., St. Louis, MO) at 21 days of age (39). MNU was dissolved in 0.05% acetic acid in normal saline and used within 30 min of preparation (13). One week later, rats were randomized into four equal experimental groups that received AIN-93G diet alone or containing the maximum tolerated dose of F-L-Leu (250 ppm; Sigma; Ref. 41) and celecoxib (1500 ppm; SC-58635, kindly supplied by Pharmacia, Skokie, IL; Ref. 14) separately or in combination. To circumvent the effect of MNU treatment and to examine the response of normal tissues to the test drugs, 40 age-matched rats were randomized similarly to the above four groups but not administered MNU (Fig. 1*B*). All animals were weighed twice weekly and at autopsy. MNU-treated groups were palpated for mammary lesions every other day. At 90 days of age (*i.e.*, 8 weeks after MNU), all animals were killed by CO_2 inhalation. Mammary tumors and uninvolved (preneoplastic) tissues were collected from MNU-treated rats, whereas normal mammary tissues were obtained from the MNU-untreated groups. Tissues were stored at −80°C for analysis. Liver, kidney, stomach, upper intestinal tract, and abdominal adipose tissue were also collected, weighed, and examined for abnormalities. This study had institutional approval and followed all guidelines for appropriate animal treatment.

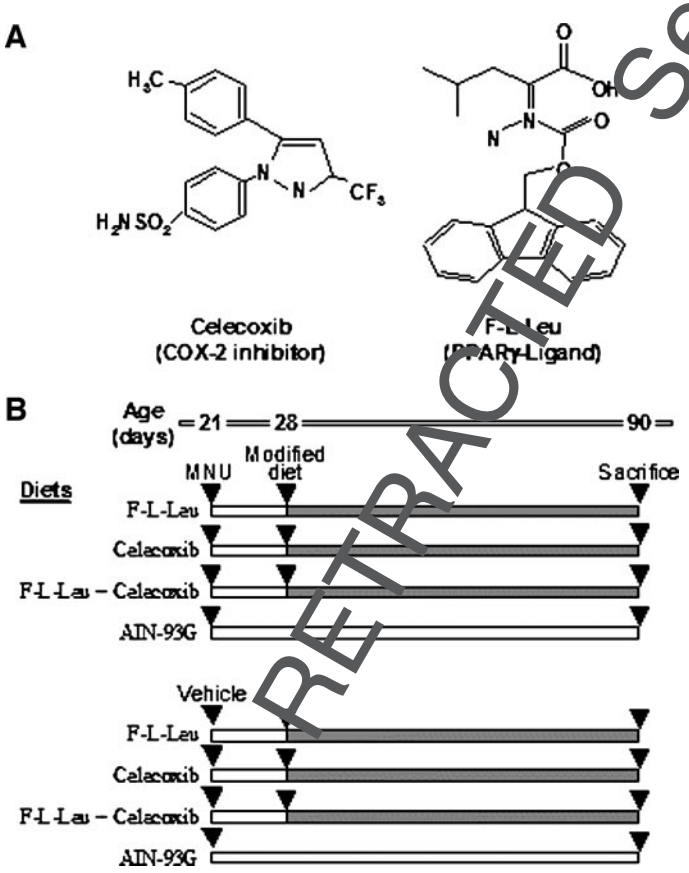

Fig. 1. Chemical structures of the test agents (*A*) and the experimental design (*B*). *Shaded areas* in the experimental design (*B*) represent durations of drug treatment.

Analysis of mRNA and Protein Expression

Reverse Transcription-PCR. Total RNA was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA) from mammary epithelial cells that were isolated from the whole mammary tissue as described previously (42). RNA was subjected to reverse transcription-PCR reaction using Titanium One-Step Reverse Transcription-PCR (Clontech, Palo Alto, CA) protocol (10). Reverse transcription-PCR reactions were carried out in a total volume of 50 μl buffer containing 0.5–1 μg of RNA, Moloney murine leukemia virus reverse transcriptase, 20 μM oligo(dT) primer, 50 mM Tricine, 20 mM KCl, 3 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate, 1× Taq enzyme mix provided by the manufacturer, 20 units of recombinant RNase inhibitor, and 45 μM PCR primers that have already been described for COX-1, COX-2, PPARγ, BAX, Bcl_2, PKCα, and β-actin (Table 1). Using a hot-lid Gene-Amp 9700 thermocycler (Perkin-Elmer, Norwalk, CT), RNA was reverse transcribed at 50°C for 60 min, followed by 5 min at 94°C. The PCR cycling conditions used in analyzing the apoptotic markers are shown in Table 1. Under each cycling condition, the yield of the amplified products was linear with respect to the input RNA and cycle number. The assay resolved a 2-fold difference in the amount of input RNA. Furthermore, the yields were linear when the PCR reactions were carried out for 20, 30, or 35 cycles. PCR products were separated in 1.5–2% agarose gels and visualized by ethidium bromide staining. Appropriate negative controls were carried out as described previously (41, 42) to rule out contamination of RNA with genomic DNA and to ensure that no cDNA synthesis would occur before reverse transcriptase inactivation. Digitized images of the stained cDNA products were captured as 8-bit digital TIFF files using a DC290 Digital Camera (Eastman Kodak Co., Rochester, NY). The intensity of each band was measured using Kodak Digital 1D-Image Analysis 3.6 software. Transcripts were normalized to the corresponding β-actin band and expressed as arbitrary density units.

Western Blot Analysis. Protein isolation was carried out by lysing mammary epithelial cells in 1.5 ml lysis buffer (1% SDS, 1.0 mM sodium orthovanadate, and 10 mM Tris, pH 7.4) and centrifugation at 16,000 × g for 10 min at 4°C. The protein content of the supernatant was quantified by Bio-Rad reagent (Bio-Rad Laboratories, Inc., Hercules, CA), using the method of Bradford (48). Diluted supernatants were incubated for 5 min at 100°C with Laemmli sample buffer (Bio-Rad), and 10–20 μg of protein were loaded per well in 7.5–12% SDS-acrylamide gels, separated by electrophoresis, and electrotransferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked with 5–10% nonfat dry milk (in TBS buffer containing 0.05% Tween 20) and probed for 1–2 h with COX-1, COX-2, PPARγ, BAX, Bcl_2, PKCα, PCNA, cdk1, and β-actin polyclonal antibodies. COX-1 and COX-2 antibodies were purchased from Cayman Chemicals (Ann Arbor, MI), whereas other primary and all secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz Biotechnology, CA). Antibody dilutions (1:100–1:1000) were carried out in 5% nonfat dry milk, and membranes were incubated with peroxidase-conjugated antirabbit or antigoat antibodies at 1:500 to 1:1000 dilution. Membranes were reprobed at least two to three times with markers of the same pathway (*i.e.*, COX and PPARγ or Bcl_2 family members), and β-actin signal was assessed for each membrane. Signals were detected by enhanced chemiluminescence (Perkin-Elmer Life Science, Boston, MA), integrated and normalized to the corresponding β-actin band and expressed as arbitrary density units.

Analysis of Prostaglandin Synthesis

Levels of PGE_2 and 15d-PGJ_2 were examined in the whole tissue homogenate by enzyme immunoassay (EIA) as described earlier (10, 40). EIA for PGE_2 and 15d-PGJ_2 was carried out using Correlate-EIA kits (Assay Designs Inc., Ann Arbor, MI) according to the manufacturer's instructions. The cross-reactivities of the EIA for a number of eicosanoids were determined by the vendor. The competitive PGE_2 EIA analysis exhibits cross-reactivity with PGE_1 (70%), PGE_3 (16.3%), and $PGF_1\alpha$ (1.4%). 15d-PGJ_2 analysis cross-

Table 1 *Primers and PCR thermocycling parameters*

Primer	Sequence	PCR conditions	Product (bp)	Ref.
COX-1	5′-CCTTCCGTGTGCCAGATTAC 3′-TCACCCGCCAGATAGTCAAC	25 cycles: 94°C, 30 s; 65°C, 30 s; 68°C, 1 min Finale extension: 68°C, 2 min	645	43
COX-2	5′-CTGTATCCCGCCCTGCTGGTG 3′-ACTTGCGTTGATGGTGGCTGTCTT	25 cycles: 94°C, 30 s; 65°C, 30 s; 68°C, 1 min Final extension: 68°C, 2 min	279	40
PPARγ	5′-TGTGTGACAGGAAACAGCTATGACCATG 3′-ATGCAGGTTCGTAAAACGACGGCCAGT	30 cycles: 94°C, 30 s; 65°C, 30 s; 72°C, 1 min Final extension: 72°C, 2 min	384	44
BAX	5′-ACAAAGATGGTCACGGTCTGCC 3′-GGTTCATCCAGGATCGAGACGG	25 cycles: 94°C, 30 s; 65°C, 30 s; 68°C, 1 min Final extension: 68°C, 2 min	429	45
Bcl_2	5′-CTCAGTCATCCACAGGGCGA 3′-AGAGGGGCTACGAGTGGGAT	25 cycles: 94°C, 30 s; 65°C, 30 s; 68°C, 1 min Final extension: 68°C, 2 min	450	45
PKCα	5′-TGAACCCTCAGTGGAATGAGT 3′-GGCTGCTTCCTGTCTTCTGAA	30 cycles: 93°C, 30 s; 60°C, 45 s; 74°C, 45 s Final extension: 74°C, 10 min	325	46
β-actin	5′-GTGGGCCGCTCTAGGCACCAA 3′-CTCTTTGATGTCACGCACGATTTC	25 cycles: 94°C, 30 s; 65°C, 30 s; 68°C, 1 min Final extension: 68°C, 2 min	540	47

reacts with PGJ_2 (49%), Δ^{12}-PGJ_2 (5.9%), and PGD_2 (4.9%). Color intensity was measured at 405 nm using a SpectraMax-250 microplate reader (Molecular Devices, Sunnyvale, CA). Levels of PGE_2 and 15d-PGJ_2 were expressed as ng/g wet tissue. Standard curves and reaction negative and positive controls were generated for PGs and assayed simultaneously with the samples.

Statistical Analysis

Differences between the control and experimental groups in the final body weight and liver:body weight ratios were determined by Mann-Whitney's test. ANOVA test and Dunnett's test, adjusted for multiple comparison, were used to evaluate differences among groups. Analysis of differences in tumor incidence (% palpable tumors) was examined using χ^2 test with *P*s subjected to Bonferroni criterion. Student's *t* test with Welch's correction (assuming no equal variance between groups) and the Mann-Whitney test were used to compare tumor multiplicity (tumors/rat) and average tumor weight between controls and test groups. Inhibition of tumor incidence was evaluated using the following equation: $I = [(x - y)/(x)] \times 100$ where *I* is the percentage of tumor inhibition, *x* is tumor incidence in controls, and *y* is tumor incidence in the experimental groups (14). Tumor inhibition was compared between control and test group(s) using χ^2 test with *P*s adjusted for Bonferroni criterion. ANOVA test using the Kruskal-Wallis test (*i.e.*, assuming no equal variance between groups) and Dunnett's test, adjusted for multiple comparisons, were used to evaluate the differences in tumorigenesis among treatments.

Differences in apoptotic and proliferation markers attributable to the test drugs or between normal, uninvolved, and tumor tissues were evaluated by two-way ANOVA test, whereas differences between individual groups (or tissue types) were carried out using Student's *t* test. Because BAX and Bcl_2 play opposing roles in determining the net apoptotic signal (49), a ratio of BAX:Bcl_2 was created. This variable has proapoptotic numerator (BAX) and antiapoptotic denominator (Bcl_2), and therefore, higher ratio values implicate higher proapoptotic gains or lower antiapoptotic rates. Additionally, because more PKCα promotes protection against apoptosis via involvement in the activation/inactivation of members of Bcl_2 family (33), the interactive variable PKCα*Bcl_2/BAX was generated to consider this interaction. In this variable, because of the antiapoptotic factors in the numerator and the proapoptotic BAX in the denominator, higher ratio values implicate higher levels of antiapoptotic signaling or lower proapoptotic processes. The proapoptotic ratio BAX:Bcl_2 and the antiapoptotic variable PKCα*Bcl_2/BAX were evaluated and compared between groups and tissues by two-way ANOVA. Correlational analyses between protein and mRNA expression levels for individual markers were carried out across tissues and treatments by determining the coefficients of correlation (r^2) using a polynomial linear regression model. All tests were performed using the SAS System 8.0 (SAS Institute, Cary, NC).

RESULTS

General Observations. In response to F-L-Leu and/or celecoxib treatments, no gross or histological changes in liver, kidney, stomach, or upper intestinal tract were observed in the experimental groups. MNU treatment did not induce tumors at sites other than the mammary gland. Body weights were evaluated twice weekly over the course of the experiment and were not different among groups (data not shown). No differences were noted between treatments in the final body weight or the liver:body weight ratio (Table 2), and we did not observe differences in the abdominal adipose tissue weight or food consumption (data not shown).

Table 2 *Effect of F-L-Leu and celecoxib on the inhibition of rat mammary gland cancinoma*[a]

Group	Final body weight (g)		Liver:body weight ratio		Tumor latency (days)	Tumor incidence (%)[b]	Tumor multiplicity (tumor/rat)[c]	ATW[d] (g)[c]	Tumor inhibition (%)[b]
	Normal	MNU-treated	Normal	MNU-treated					
(A) Control	210 ± 11	212 ± 7	4.5 ± 0.31	4.3 ± 0.14	31	80	1.40 ± 0.29	1.54 ± 0.56	
(B) F-L-Leu (250 ppm)	207 ± 8	208 ± 6	4.2 ± 0.26	4.2 ± 0.18	40	53 (0.05)[e]	0.73 ± 0.21 (0.05, 0.06)	0.42 ± 0.23 (0.07, 0.02)	33 (0.05)
(C) Celecoxib (1500 ppm)	208 ± 9	209 ± 4	4.4 ± 0.47	4.7 ± 0.11	38	47 (0.03)	0.60 ± 0.19 (0.02, 0.03)	0.41 ± 0.14 (0.07, 0.03)	41 (0.03)
(D) F-L-Leu + Celecoxib	210 ± 7	203 ± 3	4.6 ± 0.32	4.5 ± 0.22	53	14 (0.002) (0.01 *vs.* B or C)[f]	0.20 ± 0.14 (0.001, 0.001)	0.08 ± 0.07 (0.021, 0.001)	82 (0.002) (0.01 *vs.* B or C)[f]
p[g]	NS	NS	NS	NS	0.001	0.001	0.003	0.001	0.001
p[i]	NS	NS	NS	NS	<0.05 (A *vs.* D)	<0.05 (A *vs.* D)	<0.01 (A *vs.* D)	<0.01 (A *vs.* D)	<0.05 (A *vs.* D)

[a] Values represent mean ± SE.
[b] *P*s from χ^2 test, adjusted using Bonferroni criterion.
[c] *P*s from Student's *t* test with Welch's correction (*i.e.*, assuming no equal variance between groups) and followed by *P*s from the Mann-Whitney test.
[d] ATW, average tumor weight in all animals per group.
[e] All *P*s are *versus* group A, except as noted.
[f] *P*s from the Mann-Whitney test.
[g] Differences among treatments were evaluated by ANOVA test using the Kruskal-Wallis test (*i.e.*, assuming no equal variance between groups).
[h] NS, represents a nonsignificant difference between groups.
[i] Results were adjusted for multiple comparison using Dunnett's test and differences between treatments were emerged only between controls and the combined treatment group.

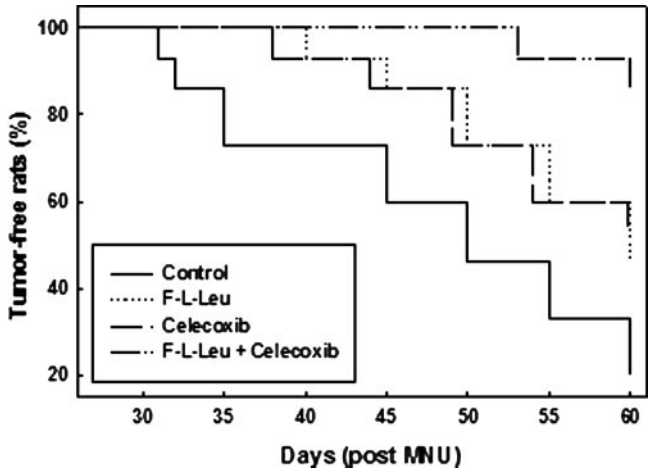

Fig. 2. The Kaplan-Meier cumulative risk of rat mammary gland carcinomas in response to treatments with F-L-Leu and celecoxib. Overall, $P = 0.0015$ (log-rank test). For the combined treatment group *versus* control, $P = 0.0002$, when adjusted for multiple comparisons by using Dunnett's test.

Inhibition of Rat Mammary Tumorigenesis. The effect of F-L-Leu and/or celecoxib on mammary gland tumorigenesis is shown in Table 2. Compared with drug-untreated controls, each agent significantly reduced the final tumor incidence, multiplicity, and average tumor weight and extended the tumor latency. Inhibition of mammary cancer by F-L-Leu and celecoxib reached 33 and 41%, respectively. The effect of the test drugs on tumor incidence and latency was reflected by their ability to improve the Kaplan-Meier cumulative risk of mammary cancer (Fig. 2). We used comparable doses of F-L-Leu and celecoxib (*i.e.*, maximum tolerated doses), and hence, as expected, no differences were noted between the drugs in cancer inhibition. Combined treatment with F-L-Leu and celecoxib elicited higher inhibition in average tumor weight, multiplicity, and incidence (>80%), longer latency, and further improvement in the Kaplan-Meier cumulative cancer risk compared with separate treatments or controls (Table 2 and Fig. 2). When the effects of the test drugs on tumorigenesis were adjusted for multiple comparisons (Dunnett's test), cancer was inhibited significantly only by the combined treatment, implicating a possible synergistic interaction between the test agents on mammary carcinogenesis.

Regulation of COX-2 and PPARγ Expression and PG Synthesis. The effects of F-L-Leu and/or celecoxib were examined on COX-1 and COX-2 and PPARγ expression in the rat mammary normal, uninvolved, and tumor tissues (Fig. 3) and on the tissue levels of PGE_2 and 15d-PGJ_2 (Fig. 4). COX-1 expression was not affected by the test drugs in the three tissue types and was not changed significantly by MNU administration. As expected, COX-2 was undetectable in the normal tissue but was induced in response to MNU. Tumors exhibited 2-fold higher COX-2 expression compared with the uninvolved tissue ($P > 0.05$). F-L-Leu and celecoxib down-regulated COX-2 expression, respectively, by 2- and 3-fold in the tumor tissue, whereas combined treatment resulted in >6-fold decrease compared with the corresponding controls. In contrast to COX-2, PPARγ mRNA and protein signals were detected in all tissues but with varying intensities (Fig. 3). Tumors exhibited the lowest PPARγ expression compared with uninvolved (2-fold) or normal (4-fold) tissues. F-L-Leu and celecoxib, separately or in combination, exerted a modest up-regulation on PPARγ expression (~2- to 3-fold).

Analysis of PG synthesis indicated that levels of PGE_2 in tumors from drug-untreated rats were, respectively, 2- and 5-fold higher than uninvolved and normal tissues. Significant inhibition in PGE_2 synthesis was attained in all tissues by F-L-Leu (~1.5-fold), celecoxib (~2-fold), and their combination (ranged from 4-fold in the MNU-treated tissues to 12-fold in the normal gland). The increases in PGE_2 levels after MNU treatment was paralleled by inhibition in the synthesis of 15d-PGJ_2 (which reached concentrations below the detection limit of 0.1 ng/100 mg tissue in the tumor). Both normal and uninvolved tissues exhibited increases in the 15d-PGJ_2 in response to F-L-Leu (~2-fold), celecoxib (~2- to 3-fold), and their combination (>5-fold). Normal tissues had the highest responses to the drug-induced alterations in PG synthesis. Although levels of PGE_2 (or 15d-PGJ_2) were different ($P < 0.001$) among tissues and treatments, adjusting the data for multiple comparisons (Dunnett's test) indicated that treatment-related differences exist mainly between controls and the groups that received combined treatment ($P < 0.05$).

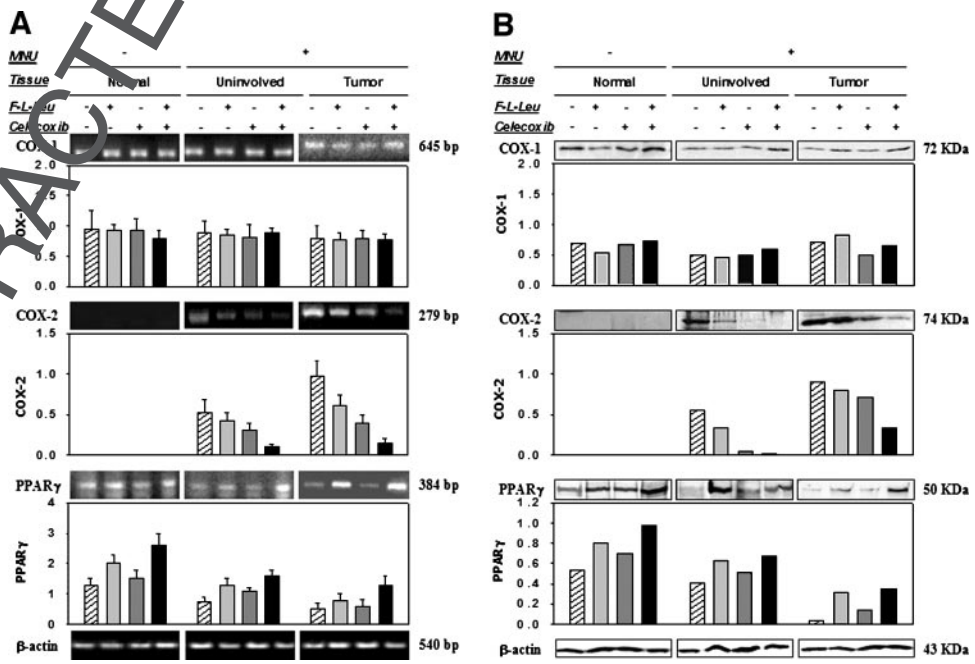

Fig. 3. COX-1 and -2 and PPARγ mRNA (*A*) and protein (*B*) expression in the rat mammary glands in response to F-L-Leu and celecoxib treatments. Band intensities were adjusted for the corresponding β-actin signals, and values are expressed as arbitrary density units. Expression levels represent means of three to four animals (*A*) or six rats (*B*) per group; *bars*, SD. Protein and mRNA expression levels were correlated across tissues and treatments (r^2 varied from 0.67 for PPARγ to 0.7 for COX-2, $P < 0.05$). Statistical differences between groups were evaluated using the two-way ANOVA test. *P*s showed significant differences in COX-2 and PPARγ levels (but not COX-1) among treatments and tissues ($P < 0.0$). When results were adjusted for multiple comparisons (Dunnett's test), differences among the experimental groups were between the controls and the combined treatments in both MNU-treated or untreated rats ($P < 0.05$).

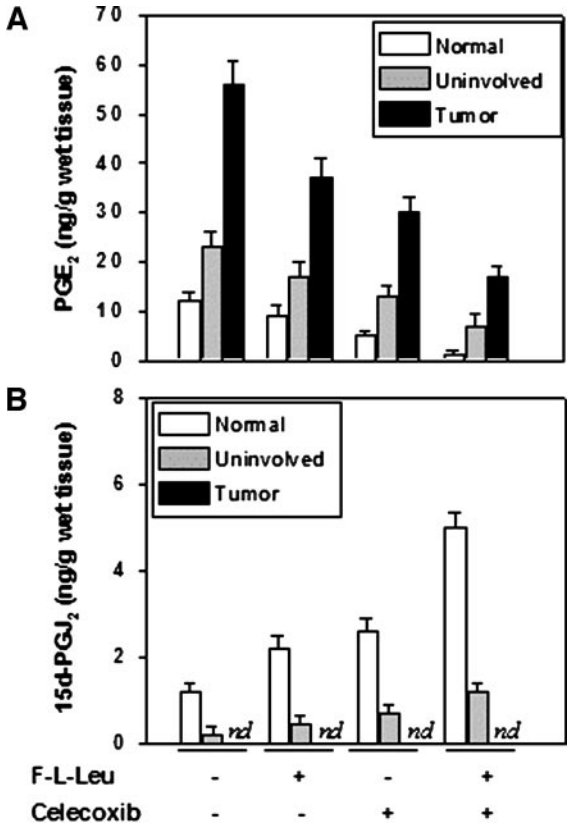

Fig. 4. Changes in the tissue levels of PGE_2 (*A*) and 15d-PGJ_2 (*B*) in the rat mammary gland in response to F-L-Leu and celecoxib treatments. Data represent means (four to six animals/group); *bars*, SD. 15d-PGJ_2 was not detected (*nd*) in the tumor tissues, *i.e.*, below the limit of detection (0.1 ng/100 mg wet tissue). Statistical differences between experimental groups were evaluated using two-way ANOVA test. *P*s showed significant differences in the levels of PGE_2 and 15d-PGJ_2 among treatments and tissues ($P < 0.001$). When results were adjusted for multiple comparison (Dunnett's test), differences among the experimental groups were mainly between the controls and the combined treatments in both MNU-treated and untreated rats ($P < 0.05$).

Effect on Apoptotic Markers. The effect of F-L-Leu and celecoxib on the expressions of the BAX, Bcl_2, and PKCα were examined in the rat mammary normal, uninvolved, and tumor tissues (Fig. 5). Normal tissues from drug-untreated animals contained higher BAX (4- to 5-fold) and lower Bcl_2 (2- to 3-fold) and PKCα (2- to 3-fold) than the corresponding uninvolved or tumor tissues. BAX expression was higher than Bcl_2 in the normal glands (BAX:Bcl_2 ratio >1), whereas Bcl_2 was higher in the uninvolved and tumor tissues (BAX:Bcl_2 ratio <1). This may be attributable to the significant ($P < 0.05$) induction of Bcl_2 (and down-regulation of BAX) in response to MNU treatment (Fig. 5). F-L-Leu and celecoxib resulted in ~3-fold change in Bcl_2 (decreases) and BAX (increases) expression when administered separately and in 3- to 5-fold altered expression when combined. Because BAX and Bcl_2 play opposing roles in determining the net apoptosis, a ratio of BAX:Bcl_2 was created as an indicator of the drug-induced proapoptotic effects (Fig. 6*A*). F-L-Leu and celecoxib, respectively, increased the proapoptotic ratio by 2- and 4-fold in the normal glands, 3- and 4-fold in the uninvolved tissues, and 4- to 7-fold in the tumors. Combined treatment, however, exerted higher increases in the BAX:Bcl_2 ratio ($P < 0.05$) than separate treatments (>9-fold in the normal glands and >14-fold in the uninvolved or tumor tissues).

Separate administrations of F-L-Leu and celecoxib elicited >1.5-fold down-regulation in the PKCα expression in all tissues, whereas combining both agents induced higher decreases ($P < 0.05$) than in controls (2.5- to 3-fold) or separate treatments (2-fold). Because more PKCα promotes protection against apoptosis via influencing the activities of Bcl_2 family members, the interactive factor PKCα*Bcl_2/BAX was generated to consider this relationship and as an indicator of the drug-induced antiapoptotic effects (Fig. 6*B*). As expected, normal glands exhibited the lowest PKCα*Bcl_2/BAX compared with other tissues (4-fold lower than uninvolved and 16-fold lower than tumors). F-L-Leu and celecoxib, respectively, reduced the antiapoptotic signaling by >2- and 5-fold in the normal glands, 3- and 6-fold in the uninvolved tissue, and >5- and >8-fold in the tumors. Compared with the corresponding controls, combined treatment elicited >14-fold lowering in the PKCα*Bcl_2/BAX values in the normal tissue, 20-fold in the uninvolved tissue, and ~30-fold in the tumor tissue.

Effect on Markers of Cell Cycle and Proliferation. The effect of F-L-Leu and/or celecoxib was also examined on markers of cell cycle regulation (cdk1) and cell proliferation (PCNA) in the rat mammary epithelial cells from normal, uninvolved, and tumor tissue (Fig. 7). Apparently, cdk1 protein was barely detectable in the resting normal mammary tissues, whereas PCNA was undetectable. Both cdk1 and PCNA were up-regulated as the tumor emerged. In the uninvolved

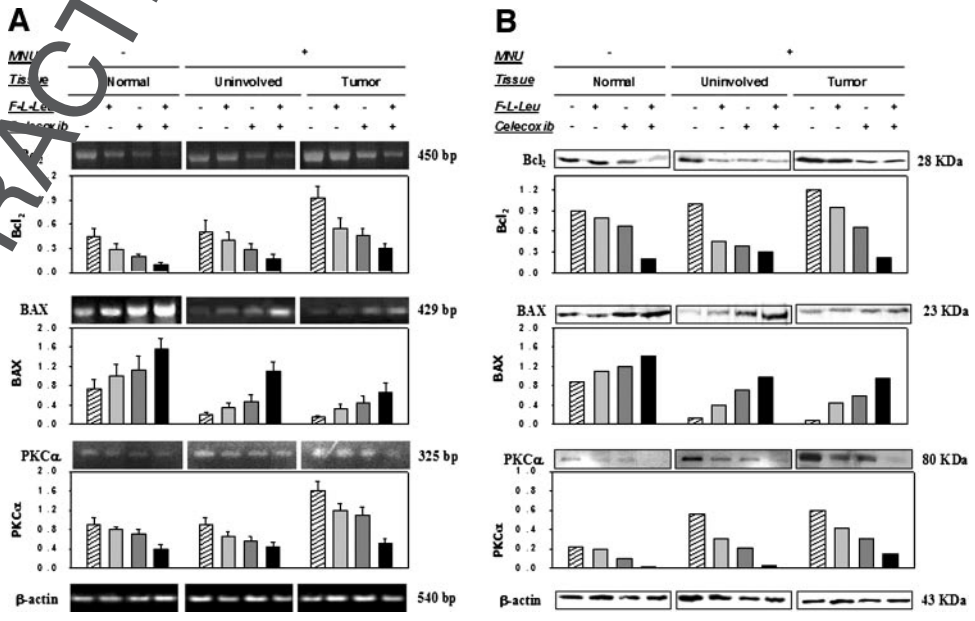

Fig. 5. Bcl_2, BAX and PKCα mRNA (*A*) and protein (*B*) expression in the rat mammary glands in response to F-L-Leu and celecoxib treatments. For experimental details and statistical differences, see Fig. 3. Protein and mRNA expression levels were correlated across tissue types and treatments (r^2 varied from 0.62 for Bcl_2 to 0.8 for BAX, $P < 0.05$).

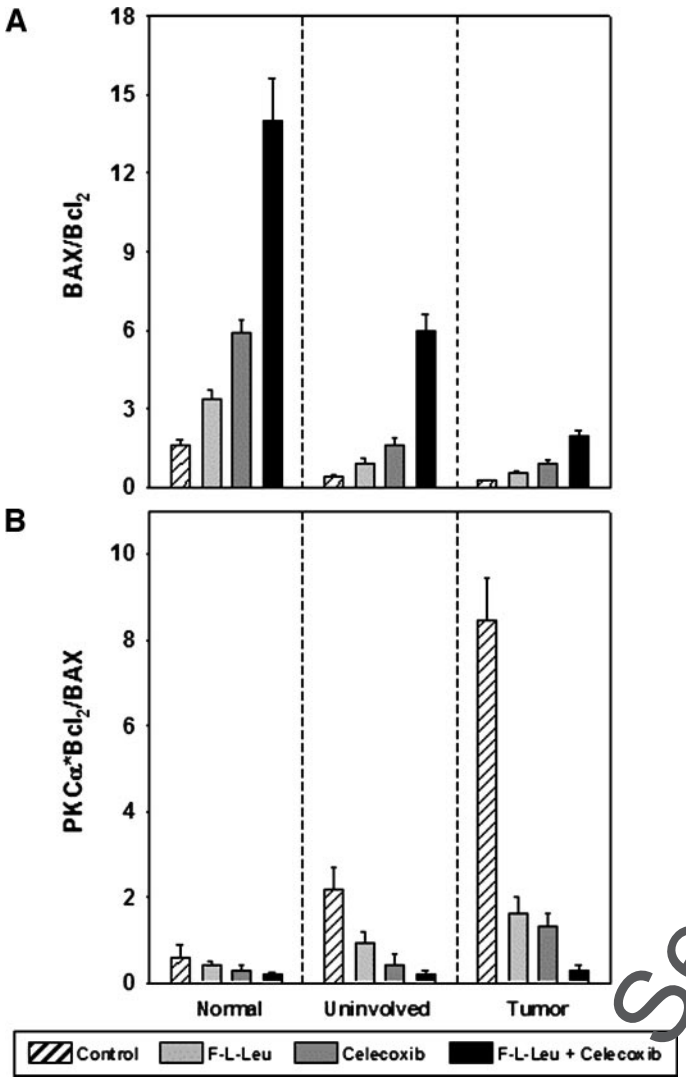

Fig. 6. Changes in the proapoptotic BAX:Bcl_2 ratio (*A*) and the antiapoptotic variable PKCα*Bcl_2/BAX (*B*) in the rat mammary gland in response to F-L-Leu and celecoxib treatments. Pro- (*A*) and anti- (*B*) apoptotic variables were created from the mRNA levels of Bcl_2, BAX and PKCα (Fig. 5) in the normal, uninvolved, and tumor tissues. Data (means of three to four animals/group; *bars*, SD) represent the ratio of arbitrary density units for BAX:Bcl_2 or for PKCα*Bcl_2/BAX adjusted for the corresponding β-actin mRNA signals. Statistical differences between experimental groups were evaluated using the two-way ANOVA test. *P*s showed significant differences in the pro- and antiapoptotic values among treatments and tissues ($P < 0.01$). When results were adjusted for multiple comparisons (Dunnett's test), differences in the tumor tissue for BAX:Bcl_2 ratio and in the normal tissue for PKCα*Bcl_2/BAX among the experimental groups were mainly between the controls and the combined treatments in both MNU-treated and untreated rats ($P < 0.05$). Differences were significant among all treatments in other tissues ($P < 0.05$).

tissues, cdk1 was detectable and PCNA was slightly induced. Over 10-fold induction in both markers occurred in the tumors compared with the uninvolved tissue. Both F-L-Leu and celecoxib reduced the expression of cdk1 and PCNA, particularly in the tumor tissue (~6-fold). Combined treatment exerted a higher down-regulation of cdk1 (~10-fold) and retained PCNA at the undetectable levels observed in the normal glands.

DISCUSSION

COX-2 and PPARγ have emerged as promising candidate molecules for the prevention of breast cancer (2). Evidence suggests that induction of COX-2 and inactivation and/or transcriptional down-regulation of PPARγ can be key components in the genesis of breast cancer (6, 10). In the present study, we examined the effect of a COX-2 inhibitor (celecoxib) and a PPARγ agonist (F-L-Leu), separately and in combination, on the development of rat mammary cancer. The toxicity profile of the test agents (Table 2) is in general agreement with reports demonstrating no toxic reactions or weight changes in animals administered celecoxib (14) or F-L-Leu (41) at doses equivalent to those examined here. As noted previously for various COX-2 inhibitors (12–14) and PPARγ ligands (15–17), celecoxib and F-L-Leu, separately, can inhibit rat mammary gland carcinoma (Table 2; Fig. 2). The present study, however, is the first demonstration that combined treatment with both classes of agents can exert an additive, if not synergistic, inhibition in mammary cancer (Table 2). Similar observations were reported for other combinational targeting protocols, *e.g.*, PPARγ and estrogen receptor (15) or PPARγ and retinoid X receptor (16).

Although COX-2 and PPARγ mediate different pathways, they modulate common molecular targets and may, therefore, synergistically inhibit cancer development (6). To identify some of the molecular pathways involved in the anticancer potentials of COX-2 inhibitors and PPARγ ligands, we examined three different, but related, pathways for their response to drug treatment. These are: (*a*) direct drug-related pathway (COX-2, PPARγ and PG synthesis); (*b*) intrinsic apoptotic signaling pathway (BAX and Bcl_2 and their modulator PKCα); and (*c*) cell cycle regulation (cdk1) and proliferation (PCNA) pathways.

COX-2 inhibitors and PPARγ agonists down-regulated the expression of COX-2 and modestly up-regulated that of PPARγ (Fig. 3) with a concurrent inhibition in PGE_2 synthesis and increases in 15d-PGJ_2 levels (Fig. 4). Several lines of evidence implicate an interrelationship between COX-2 and PPARγ pathways (6). For example, COX-2 and PPARγ are inversely correlated in both rat (this study) and human (10) models. Moreover, activation of PPARγ inhibits COX-2 (50), whereas inhibition of COX-2 activates PPARγ (51). COX-2 inhibitors act as partial PPARγ agonists (52), whereas PPARγ agonists partially inhibit COX-2 and PGE_2 synthesis (5, 53). These observations may explain the moderate up-regulation of PPARγ expression by celecoxib and the inhibition of COX-2 by F-L-Leu in the mammary gland. Combinational treatment with COX-2 inhibitors and PPARγ ligands may, therefore, lead to a synergistic inhibition of COX-2 and activation or transcriptional up-regulation of PPARγ compared with separate treatments as observed in the present study.

COX-2 inhibitors and PPARγ agonists were thought to mediate

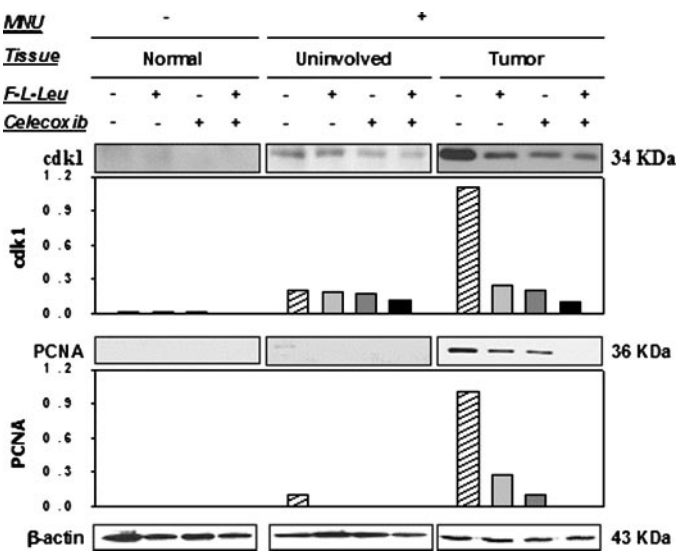

Fig. 7. Protein expression of markers of cell cycle (*cdk1*) and cell proliferation (*PCNA*) in the rat mammary gland in response to F-L-Leu and/or celecoxib treatments.

their antitumorigenic effect coordinately via COX-2- and PPARγ-dependent mechanisms of action (reviewed in Ref. 6). In normal cells, COX-1 and PGD_2 synthase trigger PGD_2 and its PGJ metabolites to predominate and help maintain a quiescent state and active PPARγ status. In response to carcinogenic agents and during tumorigenesis, COX-2 is induced and switches cells over from PGD_2 synthase-dominated PGs (*e.g.*, 15d-PGJ_2) to PGE_2 synthesis. COX-2 inhibitors and PPARγ ligands inhibit PGE_2 synthesis and switch the cell PG synthesis back to a PGD_2 and PGJ series-dominated state. This interaction may explain the higher modulation in PG synthesis elicited by the simultaneous targeting of COX-2 and PPARγ compared with separate treatments.

In general, the up-regulation in PGJ_2 synthesis (and activation PPARγ) and inhibition in COX-2 (and PGE_2 synthesis) induced by the test drugs may influence cancer prevention by various mechanisms, including rendering cells more susceptible to apoptosis or attenuating cell growth and proliferation (6, 23). COX-2 induction and/or PPARγ inactivation increase(s) the resistance of mammary cells to apoptosis by up-regulating Bcl_2 (31, 32) and may, thereby, affect cancer development. The intrinsic apoptotic signaling mediated by Bcl_2 family members (*e.g.*, BAX and Bcl_2) is an important factor in mammary normal development (18) as well as in breast carcinogenesis (see below). The interaction between BAX and Bcl_2 dictates susceptibility to apoptosis and is implicated in breast cancer (19), prognosis (25), and chemoprevention (25). Higher BAX:Bcl_2 values indicate enhanced proapoptotic signaling or lower antiapoptotic processes (49, 54). In breast cancer, BAX:Bcl_2 ratios are usually low, reflecting low pro-apoptotic rates (19). In the present study we noted that BAX expression was higher than Bcl_2 in the normal glands, whereas Bcl_2 was higher in the cancerous tissues (Fig. 5). Celecoxib and/or F-L-Leu down-regulated Bcl_2 expression and up-regulated BAX levels in all tissues. Consequently, the BAX:Bcl_2 ratio was increased by 2- to 9-fold in the normal tissue and 4- to 14-fold in cancerous tissues. These alterations may, therefore, be involved in the proapoptotic effects of COX-2 inhibitors and PPARγ ligands and can mediate their antitumorigenic action.

In addition to the interaction between BAX and Bcl_2 (29), the activity of Bcl_2 family members can be affected by their posttranslational modification with phosphorylation (33, 55), *e.g.*, by PKCα. Forced expression of PKCα in human cells influenced the activity of Bcl_2 (56) by increasing its phosphorylation, which led to a >10-fold increased resistance to drug-induced cell death (33). The overexpression of PKCα in human (57) and animal (this study) tumors may, therefore, reduce apoptotic rates via Bcl_2 family members and, subsequently, hinder the pro-apoptotic effects of COX-2 inhibitors and PPARγ ligands. The multiplicative factor PKCα*Bcl_2/BAX was considered previously (58, 59) to predict the drug-induced effects on the prosurvival processes attributed to the PKC-Bcl_2 signaling. Values of PKCα*Bcl_2/BAX were 16-fold lower in the normal glands than in tumors. In agreement with previous findings for other COX-2 inhibitors (60) and PPARγ ligands (61), in the present study, celecoxib, and F-L-Leu down-regulated PKCα expression and the PKCα*Bcl_2/BAX factor in all tissues. The involvement of PKCα in the transcriptional regulation of COX-2 and PPARγ (62, 63) implies that PKCα inhibition may avert further COX-2 induction and/or PPARγ inactivation during tumorigenesis. The relationship between the apoptotic potential of the test drugs and their anticancer activity suggests that COX-2 inhibitors and PPARγ ligands can be considered in apoptosis-inducing prevention or therapy. In fact, many cancer therapeutic (26–28) and preventive (24, 29) agents exert their effect in target tissues primarily by inducing apoptosis.

Another possible mechanism by which COX-2 inhibitors and PPARγ ligands mediate their anti-cancer activity is via modulating the expression of factors involved in cell cycle regulation and proliferation (6). For example, PPARγ ligands (36) and nonsteroidal anti-inflammatory agents (64) markedly reduce the levels of Ki-67. Combining both classes of agents synergistically inhibits human breast cancer cell cycle progression (32). PCNA, another marker of cell proliferation (65), is abundant in the rapidly growing cells (preneoplastic and malignant) and can be significantly up-regulated (>10-fold) in mammary tumors of both human (66) and animal (67, this study) models. The marked decrease in the PCNA levels after F-L-Leu or celecoxib treatment in the rat mammary tumors (Fig. 7) was shown previously to be dose dependent for other PPARγ ligands (34) and COX-2 inhibitors (35), which may explain the higher effect elicited by combinational treatment on PCNA compared with separate administrations (Fig. 7).

The increase in PCNA during tumorigenesis is usually accompanied by induction in genes/proteins involved in cell cycle (68). An important player in cell cycle regulation is cdk1. It is required twice during the cell cycle, first in the G_1 phase for onset of S-phase and again in G_2 for onset of mitosis (69). Increased activity and expression of cdk1 occur in several types of human cancer (70–72) and in rat mammary tumors (Fig. 7). F-L-Leu and celecoxib, separately and in combination, down-regulated cdk1 expression (Fig. 7) and increased its phosphorylation, which resulted in ~2-fold inhibition in the activity (data not shown). Similar to other anticancer agents, *e.g.*, genistein (73), COX-2 inhibitors and PPARγ ligands seem to mediate their action by interacting with cdk1 to attenuate the transition of G_1 to S-phase and/or G_2 to M phase. In support, we reported earlier that the test drugs accumulate human breast cancer cells in the G_0-G_1 phase (32) and therefore, may exert their effects during early cell cycle (*i.e.*, G_1 to S-phase). The drug-induced effects on the cell cycle are usually paralleled by increases in the apoptotic rates (32, 74, 75). When these effects occur concurrently with inhibition of COX-2, modulation of PG synthesis and activation of PPARγ, some of the mechanisms by which COX-2 inhibitors and PPARγ ligands exert their anticancer effects may be elucidated.

In conclusion, simultaneous targeting of COX-2 and PPARγ inhibited mammary cancer development more effectively (and possibly synergistically) than targeting each molecule alone. COX-2 inhibitors and PPARγ agonists appear to mediate their action on mammary tumorigenesis by both COX-dependent and -independent mechanisms. COX-independent pathways implicated in the cancer preventive activity of these agents include concurrent inhibition of cell growth and induction in apoptotic rates. Further studies are warranted to examine the simultaneous targeting of COX-2 and PPARγ in apoptosis-inducing therapy and in the prevention of human breast cancer.

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