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Sulindac Derivatives Inhibit Growth and Induce Apoptosis in Human Prostate Cancer Cell Lines

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ABSTRACT. We examined the activity of two metabolites of sulindac (a nonsteroidal anti-inflammatory drug), sulindac sulfide and sulindac sulfone (exisulind, Prevatec™), and a novel highly potent analog of exisulind (CP248) on a series of human prostate epithelial cell lines. Marked growth inhibition was seen with the BPH-1, LNCaP, and PC3 cell lines with IC_{50} values of about 66 μM, 137 μM, and 64 nM for sulindac sulfide, exisulind, and CP248, respectively. DNA flow cytometry and 4′,6′-diamido-2-phenylindole (DAPI) staining indicated that these three compounds also induced apoptosis in all of these cell lines. Similar growth inhibition also was seen with the PrEC normal human prostate epithelial cell line, but these cells were resistant to induction of apoptosis at concentrations up to 300 μM, 1 mM, and 750 nM of sulindac sulfide, exisulind, and CP248, respectively. Derivatives of LNCaP cells that stably overexpress bcl-2 remained sensitive to growth inhibition and induction of apoptosis by these compounds. *In vitro* enzyme assays indicated that despite its high potency in inhibiting growth and inducing apoptosis, CP248, like exisulind, lacked cyclooxygenase (COX-1 and COX-2) inhibitory activity even at concentrations up to 10 mM. Moreover, despite variations of COX-1 and COX-2 expression, the three benign and malignant prostate cell lines showed similar sensitivity to growth inhibition and induction of apoptosis by these three compounds. Therefore, sulindac derivatives can cause growth inhibition and induce apoptosis in human prostate cancer cells by a COX-1 and -2 independent mechanism, and this occurs irrespective of androgen sensitivity or increased expression of bcl-2. These compounds may be useful in the prevention and treatment of human prostate cancer. BIOCHEM PHARMACOL **58**;7:1097–1107, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. apoptosis; bcl-2; cyclooxygenase; nonsteroidal anti-inflammatory drug; prostate cancer; sulindac

In the United States, prostate cancer is currently the most frequently diagnosed cancer and the second leading cause of cancer death in men. It is estimated that in 1997 approximately 317,100 new cases were diagnosed and 41,400 men died of prostate cancer [1]. Treatment for advanced prostate cancer often involves either surgical gonadectomy or drugs that inhibit androgen production and action, thereby inducing apoptosis in androgen-dependent prostate cancer cells [2]. Unfortunately, prostate cancers often metastasize to lymph nodes, bone, and other distant sites, and the metastases tend to become androgen-independent subsequent to hormonal therapies. Since the advanced stages of prostate cancer are often refractory to the currently available forms of chemotherapy, these patients have a poor prognosis [3]. Prostate tumors that display increased expression of the anti-apoptosis protein bcl-2 are associated with a poor prognosis. This has been attributed to the finding that increased bcl-2 expression apparently enhances the resistance of prostate cancers to hormonal and other forms of therapy [4, 5].

Sulindac is a NSAID‖ that has been used primarily for the treatment of chronic inflammatory diseases such as rheumatoid arthritis. In recent years, this drug has become of interest for the treatment of patients with FAP [6, 7]. In a double-blind, randomized, placebo-controlled study of 22 FAP patients, treatment with sulindac showed a statistically significant decrease in the number and size of polyps, although the benefits of sulindac were not sustained once the treatment was terminated [8]. The sulfone metabolite of sulindac (exisulind, (Z)-5-fluoro-2-methyl-1-[[4-(methylsulfonylbenzyl)phenyl]methylene]-idene-3-ylacetic acid, Fig. 1) has been shown to also induce

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‖ *Abbreviations*: NSAID, nonsteroidal anti-inflammatory drug; FBS, fetal bovine serum; TUNEL, Terminal transferase-mediated dUTP-digoxigenin Nick End Labeling; DAPI, 4′,6′-diamido-2-phenylindole; FAP, familial adenomatous polyposis; COX, cyclooxygenase; SRB, sulforhodamine B; and PMSF, phenylmethylsulfonyl fluoride.

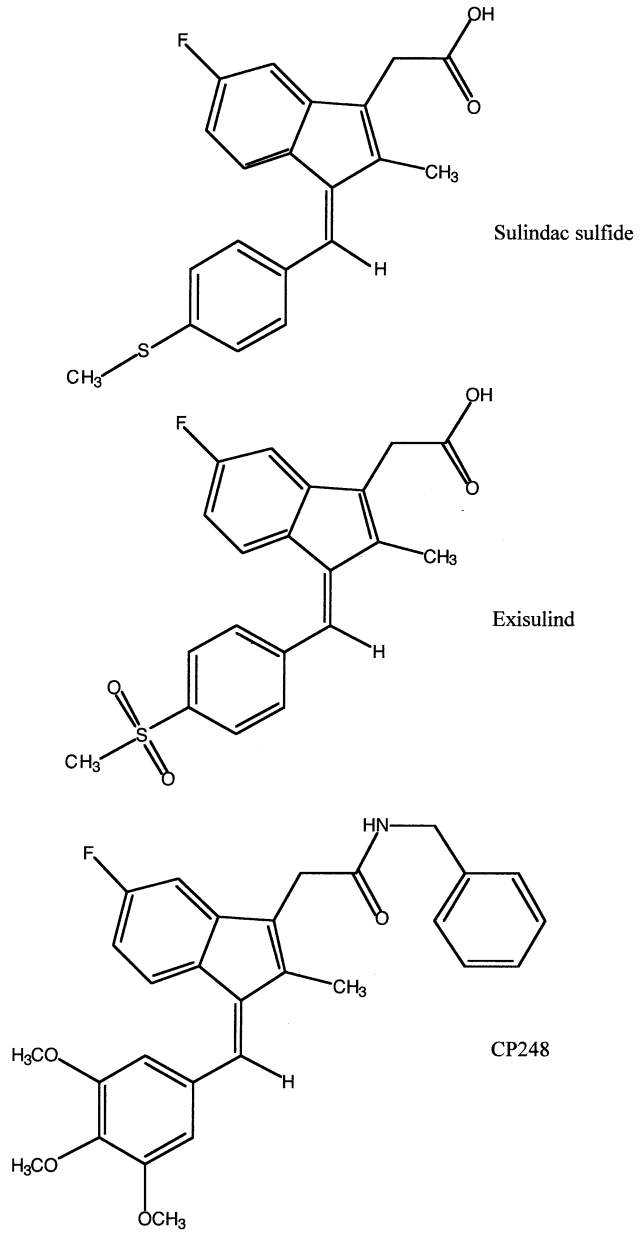

FIG. 1. Chemical structures of sulindac sulfide, exisulind, and CP248.

polyp regression and prevent polyp recurrence in FAP patients (unpublished studies). These clinical studies, as well as experimental animal studies [9, 10], clearly indicate that sulindac and exisulind have an inhibitory effect on neoplastic lesions of the colon. Mechanistic studies indicate that sulindac or its derivatives suppress proliferation [11] and induce apoptosis [12] in colonic tissue of patients with FAP. These compounds also inhibit growth and induce apoptosis in human colon cancer cell lines [12–16], and we have described similar effects in an immortalized normal rat intestinal epithelial cell line [17]. The antineoplastic effects are not limited to colon cancer, since sulindac and exisulind can inhibit experimental mammary carcinogenesis in rats [18, 19], and they also inhibit growth and induce apoptosis in human breast carcinoma cell lines [19, 20].

Despite these promising findings related to colon and breast cancer, to our knowledge the NSAIDs have not been studied previously with respect to prostate cancer. As an initial approach to this question, we examined the effects of three sulindac derivatives on the growth of human prostate cancer cell lines. The compounds examined were sulindac sulfide [(Z)-5-fluoro-2-methyl-1-(*p*-methylthiobenzylidene)-3-indenylacetic acid], a reduced metabolite of sulindac [21]; sulindac sulfone (exisulind), an oxidized natural metabolite [21]; and CP248 [(Z)-5-fluoro-2-methyl-(3,4,5-trimethyoxybenzylidene)-3-(*N*-benzyl)-indenylacetamide], an exisulind analog (Fig. 1). Two prostate cancer cell lines were used, LNCaP, which is androgen-sensitive [22, 23], and PC3, which is androgen-insensitive [23]. We also used two derivatives of LNCaP cells (B6 and B5) that were engineered to stably overexpress an exogenous bcl-2 protein [2], to determine if bcl-2 had a protective effect. We included the BPH-1 cell line that was established from human benign prostatic hyperplasia tissue [24], to determine the effect of the drugs on benign prostatic hyperplasia, and the PrEC normal epithelial prostatic cell line. Our studies provide evidence that all three of these compounds can inhibit growth and induce apoptosis in human prostate cancer cells, despite the fact that they are androgen-sensitive or express increased levels of bcl-2.

MATERIALS AND METHODS

Drugs and Antibodies

Sulindac metabolites and CP248 were obtained from Cell Pathways, Inc. The drugs were dissolved in 100% DMSO and added to the cell culture medium at a final concentration of 0.1% DMSO. The bcl-2 primary antibody was obtained from Santa Cruz Biotechnology, Inc. The β-actin primary antibody was obtained from Sigma Biosciences. COX-1 and COX-2 primary antibodies were obtained from Oxford Biomedical Research, Inc.

Cell Culture

The human prostate cancer cell lines LNCaP and PC3 were purchased from the American Type Culture Collection. Derivatives of the LNCaP cell line that stably overexpress bcl-2 were described previously [2]. The benign prostatic hyperplasia cell line BPH-1 was provided by Dr. Simon W. Hayward (University of California). The PrEC normal epithelial prostate cell line was purchased from Clonetics. The BPH-1, LNCaP, and PC3 cell lines were grown in RPMI-1640 medium supplemented with 10% FBS, 100 units/mL of penicillin G, and 100 mg/mL of streptomycin (Life Technologies Inc.), and maintained at 37° in a humidified atmosphere containing 5% CO_2. The PrEC normal prostate cells were grown in PrEGM medium according to the manufacturer's instructions.

Assays for Apoptosis

Changes in cell morphology characteristic of apoptosis were examined by fluorescence microscopy of DAPI-stained cells. The indicated cell lines were first plated at 5×10^5 cells per 60-mm diameter culture plate. Twenty-four hours later, appropriate dilutions of the sulindac compounds were added to the medium. Seventy-two hours later both the adherent and floating cells were collected, and 5×10^3 of these cells were plated onto Colorfrost/*Plus* glass slides (Fisher Scientific). The cells were dried and fixed with 10% neutral buffered formalin for 10 min, and then with graded ethanol for 30 min. The slides were washed twice for 3 min with PBS. Next, the slides were stained with 0.15% DAPI in PBS for 30 min at room temperature and in the dark, followed by three 30-min washes with PBS, also at room temperature and in the dark. The slides were gel-mounted and examined by fluorescence microscopy. Triplicate random fields were chosen, each with a minimum of 300–600 cells. Both the total number of cells and the number of cells displaying evidence of apoptosis, i.e. fragmented nuclei, were counted, and the results were expressed as "percent apoptosis."

Apoptosis also was assayed using the Cell Death Detection $ELISA_{plus}$ assay, according to the manufacturer's protocol (Boehringer-Mannheim). In brief, 10^4 cells/well were treated with the indicated concentrations of each of the three drugs for 48 hr. The cells were washed and then lysed in 200 μL of lysis buffer per well, at room temperature for 45 min. The plates were centrifuged, and 20 μL of supernatant was transferred to appropriate wells on an ELISA plate. Eighty microliters of the immunoreagent was added per well, and the plates were incubated for 2 hr on a Vortemp plate shaker (500 rpm at room temperature). The plates were washed three times with incubation buffer and dried. One hundred microliters of the photometric substrate was added per well, and absorbance (405–490 nm) was read 5 min later. The amounts of histone-DNA fragments were computed as a ratio to the vehicle control. Experiments were done in triplicate.

Western Blots

The levels of the bcl-2 protein were evaluated by western blot analysis of protein extracts obtained from the various cell lines. Cell pellets containing approximately 10^7 cells were suspended in 200 mL of ice-cold Cell Lysis Buffer (50 mM HEPES, 150 mM NaCl, 2.5 mM EGTA, 1.0 mM EDTA, 1.0 mM DTT, 0.1% Tween-20, 10% glycerol, 10 mM β- glycerophosphate, 1.0 mM NaF, 0.1 mM Na_3VO_4, 10 mg/mL of leupeptin, 10 μg/mL of aprotinin, and 1.0 mM PMSF, at pH 7.5), and then sonicated. Insoluble debris was removed by centrifugation at 10,000 *g* for 10 min. Protein concentrations were determined using the Bio-Rad protein assay system (Bio-Rad Laboratories). Lysates containing 50 μg of protein were mixed with an equal volume of 2x sample buffer (125 mM Tris–HCl, pH 6.8, 20% glycerol, 4% SDS, 0.02% bromophenol blue, 10% 2-mercaptoethanol) and boiled for 5 min. The samples were cooled on ice for 5 min, briefly centrifuged, and subjected to 10% SDS–PAGE. Proteins were transferred to a nitrocellulose membrane for 2 hr at 70 V in 25 mM Tris–HCl, pH 8.0, 195 mM glycine, and 10% methanol. The membrane was blocked with 3% BSA in 1x TBS buffer (20 mM Tris–HCl, pH 8.0, 0.136 M NaCl, 0.5% Tween 20) for 1 hr, and the bcl-2 antibody was added for another hour. After five washes in 1x TBS buffer, the membrane was incubated with a sheep anti-mouse IgG-horseradish peroxidase complex (Amersham Life Science) for 1 hr, followed by five more washes in 1x TBS buffer. Chemiluminescence was visualized using the ECL kit (Amersham). The blot was exposed to Kodak XAR-5 X-ray film for 10 min and then developed. A similar procedure was used with the β-actin, COX-1, and COX-2 primary antibodies. The intensities of the bands were quantitated on a Molecular Dynamics computing densitometer (Molecular Dynamics) using ImageQuant software version 3.2.2.

Growth Curves and IC_{50} Values

Cells were plated, in triplicate, at a density of 1×10^5 cells/well in 60-mm diameter plates. Twenty-four hours later, various concentrations of sulindac sulfide, exisulind, CP248, or DMSO (0.1% final) were added to the culture media. Seventy-two hours after adding the test compounds, the number of attached viable cells was counted using a Coulter Counter, model Z_F, (Coulter Electronics Inc.). The percent of growth, using the DMSO controls as the 100% value, and the IC_{50} values, with their respective standard deviations, were determined using Microsoft Excel 5.0 software. DMSO at 0.1% had no significant effects on the growth of these cell lines.

For the SRB assays, cells were plated in 96-well plates, and the indicated concentrations of the drugs were added (see Fig. 3). Six days later, 50 μL of 50% trichloroacetic acid was used to fix the cells in each well for 1 hr at 4°. Then each plate was washed five times with distilled water and allowed to dry at room temperature for 1–2 hr. Next, the cells were stained with 4% SRB in 1% acetic acid by adding 100 μL to each well for 10 min at room temperature. Each plate was washed four times with 1% acetic acid and dried at room temperature for 1–2 hr. The adherent dye was solubilized by rocking in 100 μL/well of 10 mM Tris at room temperature for 10 min. Finally, optical densities were measured at 540 nm. Growth inhibition was calculated as the percent inhibition with respect to the vehicle control. Experiments were done in duplicate.

Flow Cytometry

Cells (10^6) were treated with high concentrations of sulindac sulfide, exisulind, and CP248 and collected after 24, 48, and 72 hr. The cells were resuspended in 1 mL of PBS, and 5 mL of 70% ethanol in distilled H_2O was added

slowly dropwise while vortexing the tube. After 30 min at 4° the cells were centrifuged at 2400 *g* for 5 min and resuspended in 200 μL of PI dye (0.1 mg/mL of propidium iodide and 0.6% NP-40 in distilled H_2O) and 200 μL of 2 mg/mL of RNAse A. The tubes were incubated at room temperature and in the dark for 30 min. The cells were filtered through 41-μm spectra/mesh filters (Spectrum). The DNA content of 10^4 cells stained with propidium iodide was measured with a FACScan instrument equipped with FACStation running Cell Quest software (Becton Dickinson). Cell cycle analysis of DNA histograms was performed with the Multicycle program (Phoenix Flow Systems). The percent of the total DNA that was present as a subdiploid peak was determined, as additional evidence for apoptosis [25–27].

COX Assays

The COX-1 inhibitory activity of the sulindac derivatives was determined essentially as described previously [28]. In brief, prostaglandin H synthetase 1 (Cayman Chemical) was incubated with 100 μM arachidonic acid (Sigma) and cofactors (0.5 mM glutathione, 0.5 mM hydroquinone, 0.625 μM hemoglobin, and 1.25 mM $CaCl_2$ in 100 mM Tris–HCl, pH 7.4) at 37° for 20 min, in the presence of the indicated sulindac compounds or only the solvent (1% DMSO, final concentration). The reaction was terminated by adding the colorimetric reagent, thiobarbituric acid, to form the product, malonaldehyde, and quantitated by a spectrophotometer at 530 nm. The COX-2 inhibitory activity was determined similarly using prostaglandin H synthetase 2 (Cayman Chemical).

Statistical Analysis

Growth curve and apoptosis data were analyzed using a two-sample *t*-test of the difference between two populations of means (Data Desk Software 5.0.1; Data Description, Inc.) Differences were considered significant at $P < 0.05$.

RESULTS

Growth Inhibition

The chemical structures of sulindac sulfide, exisulind, and CP248 are shown in Fig. 1. To determine their effects on growth, increasing concentrations of these three compounds were added to exponentially growing cultures of the various cell lines. Seventy-two hours later the numbers of cells were counted, and this value was expressed as a percent of control cultures, i.e. parallel cultures treated only with the vehicle DMSO (0.1%). The effects obtained with the androgen-sensitive LNCaP cell line, its two bcl-2-overexpressing derivatives B6 and B5, the androgen-insensitive PC3 cell line, and the BPH-1 cell line are shown in Fig. 2A. Figure 2B is a western blot that confirms previous evidence [2] that the B6 and B5 derivatives express increased levels of bcl-2, when compared to the parental LNCaP cell line. Densitometric analysis in which the results were normalized by using β-actin as an internal control indicated a 1.6- and 2.3-fold increase in the bcl-2 protein for the B6 and B5 cell lines, respectively, when compared with the parental LNCaP cells. Concentrations of sulindac sulfide and exisulind in the range of 50–150 μM, and of CP248 in the range of 0.1 μM, caused marked growth inhibition of all of the five cell lines (Fig. 2A). The growth rates of the untreated cultures of the LNCaP, B6, and B5 cell lines were similar (data not shown) [see also Ref. 2]. The respective IC_{50} values with the parental LNCaP cell line were 48, 114, and 0.06 μM (Table 1). Similar values were obtained with the two bcl-2 overexpression derivatives. The IC_{50} values were somewhat higher for the B5 derivative, by about 28% for exisulind and about 16% for CP248, suggesting that a high increase in bcl-2 expression might offer slight protection against the growth inhibitory effects of these compounds. However, statistical analysis comparing the difference in means of the three LNCaP cell lines, at various concentrations with each of the three sulindac derivatives, showed no statistical significance.

The IC_{50} values obtained with all five cell lines are summarized in Table 1. Within a factor of less than two these values were similar to those obtained with the LNCaP cells, for all three compounds. With all of the cell lines sulindac sulfide was about twice as potent as exisulind as an inhibitor of growth, whereas CP248 was about 1000 times more potent than sulindac sulfide (Table 1). The extremely high potency of CP248, with an IC_{50} value in the range of 50–100 nM, is a remarkable feature of this compound. Similar IC_{50} values for CP248 were observed with the HL-60, HT-29, NCI-H82, and MDA-MB-231 cell lines (unpublished studies).

A 6-day growth inhibition study was done comparing LNCaP and PC3 cells with the normal prostate epithelial cell line PrEC at increasing concentrations of the three compounds using the SRB binding assay (Fig. 3). All three compounds were equally effective in inhibiting growth of both the LNCaP and the PrEC prostate cell lines. Again, CP248 was about 1000 times more potent than sulindac sulfide. By comparison, exisulind was approximately two times less potent than sulindac sulfide in inhibiting all three cell lines.

Apoptosis Assays

Since sulindac sulfide and exisulind can induce apoptosis in colonic tissue *in vivo* [10, 29] and since sulindac and its derivatives can also induce apoptosis in human colon [13, 14] and breast cancer cell lines [19, 20], we also examined the effects of these compounds on apoptosis in this set of prostate cancer cell lines.

We compared the effects of the three sulindac derivatives on apoptosis in the derivatives of LNCaP cells that overexpressed bcl-2 with the effects in the parental LNCaP cells. Initially, we examined apoptosis in these LNCaP cells after treating them with 50, 100, and 150 μM sulindac

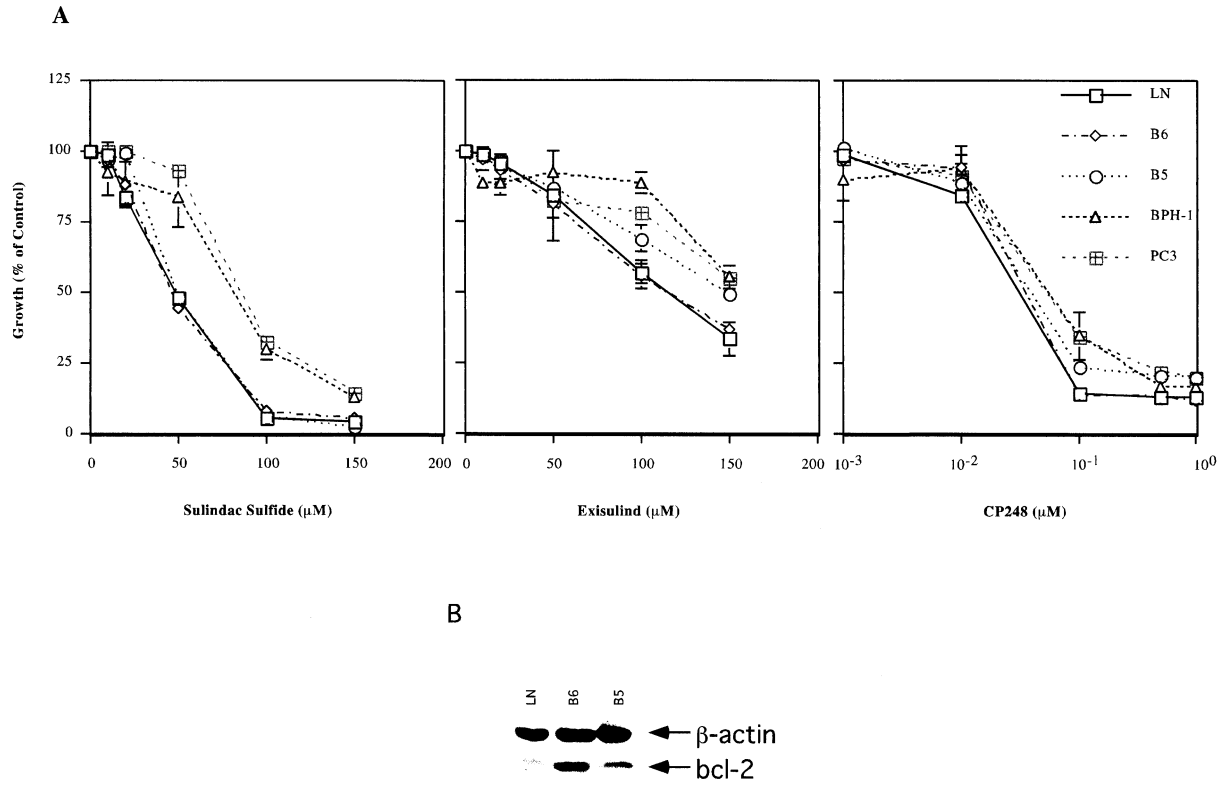

FIG. 2. (A) Growth inhibition of BPH-1, PC3, LNCaP (LN), and two derivatives of LNCaP, B6 and B5, that express increased levels of bcl-2. One hundred thousand exponentially dividing cells were exposed to the indicated concentrations of sulindac sulfide, exisulind, or CP248 for 72 hr, and the percent of growth was calculated, with 100% representing control cultures treated only with the solvent (0.1% DMSO). The values represent the means and standard deviations of triplicate assays. (B) Western blot analysis for levels of expression of bcl-2 in the three cell lines. Also shown is a blot for β-actin as a control for protein loading.

sulfide or exisulind and 0.01, 0.1, and 1 μM CP248. No significant differences were seen among the three cell lines (data not shown), but the extent of apoptosis in all cases was not very high (less than 5%). Therefore, the three cell lines then were treated with high concentrations of these compounds for 72 hr, to maximize the apoptotic effects. We determined the percent of cells (both floating and attached) that displayed morphologic evidence of apoptosis, i.e. nuclear fragmentation, after staining the treated cells with DAPI. We used this method to detect apoptosis since it gave more reproducible results than the TUNEL assay (data not shown). The results are summarized in Table 2. In the 0.1% DMSO-treated parental LNCaP cultures, and in the B6 and B5 derivatives, the number of apoptotic cells was about 2% (or less) of the total cell population. Treatment of the parental LNCaP cells with 500 μM sulindac sulfide, 600 μM exisulind, or 5 μM CP248 gave values of about 23, 16, and 28% apoptosis, respectively. Similar results were obtained with the B6 and B5 derivatives, although in some cases it appeared that increased expression of bcl-2 partially protected these cells (Table 2). However, these differences were not statistically significant. It is apparent that the compound CP248 is much more potent than both sulindac sulfide and exisulind in inducing apoptosis (Table 2) and growth inhibition (Fig. 2) in the LNCaP cell line.

An additional method for assessing apoptosis is to use

TABLE 1. IC_{50} Values for sulindac sulfide, exisulind and CP248 for various prostate cell lines*

Cell line	Compound name (I.D.)	IC_{50} (μM)
LNCaP-LN	Sulindac sulfide	48.0
	Exisulind	114.2
	CP248	0.06
LNCaP-B6	Sulindac sulfide	45.9
	Exisulind	114.3
	CP248	0.06
LNCaP-B5	Sulindac sulfide	48.6
	Exisulind	147.6
	CP248	0.07
PC3	Sulindac sulfide	85.3
	Exisulind	160.3
	CP248	0.08
BPH-1	Sulindac sulfide	81.2
	Exisulind	157.5
	CP248	0.08

* Assays were performed as described in the legend of Fig. 2, and the IC_{50} values were calculated as described in Materials and Methods. There was no significant difference between the IC_{50} values of the LNCaP (LN) parental cells and the B6 and B5 derivatives of this cell line. The standard deviations are indicated in Fig. 2A.

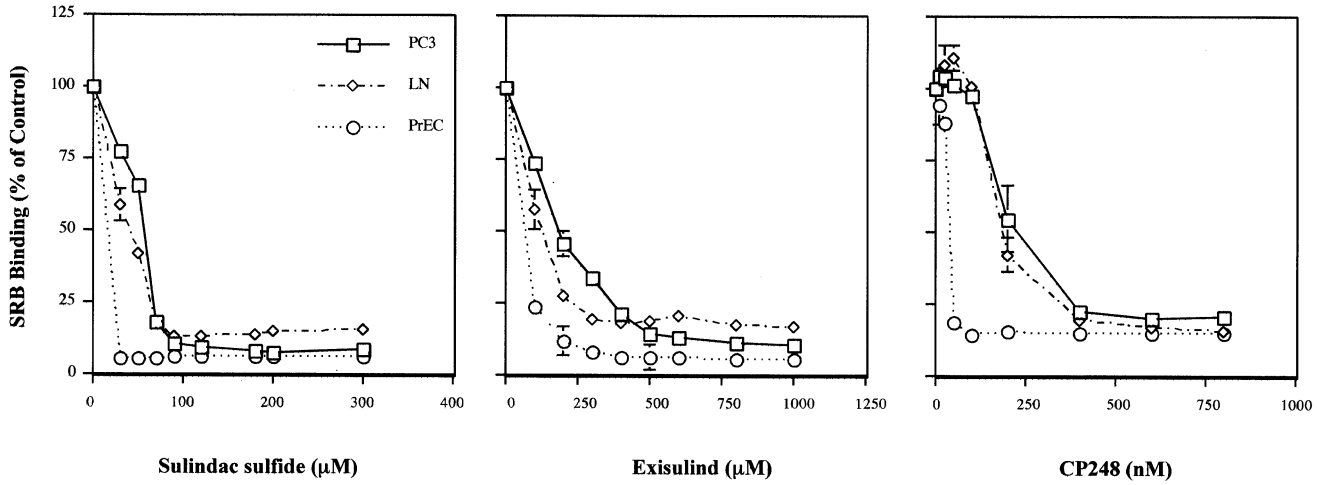

FIG. 3. Effects of increasing sulindac sulfide, exisulind, and CP248 on inhibition of the growth of PrEC normal human epithelial prostate cells, in comparison to LNCaP and PC3 cells. Ten thousand exponentially growing cells were exposed to either the solvent alone (0.1% DMSO) or the indicated concentrations of the three drugs, for 6 days, and assayed for SRB binding at 550 nm. One hundred percent value represents the SRB binding of the vehicle alone (0.1%). (DMSO-alone control O.D. values for the various experiments are as follows: sulindac sulfide: PC3, 1.148; LNCaP, 0.683; PrEC, 1.796. Exisulind: PC3, 1.08; LNCaP, 0.617; PrEC, 1.786. CP248: PC3, 1.14; LNCaP, 0.677; PrEC, 1.893.) The values represent the means and the bars represent the ranges of duplicate assays.

FACS analysis to determine the percent of cells that have a sub-G_1 DNA content [25–27]. Therefore, we also employed this method to determine the extent of apoptosis induced by the three sulindac compounds in our series of prostate cancer cell lines. Again, each cell line was treated with the vehicle alone (0.1% DMSO) or relatively high concentrations of the sulindac compounds, i.e. 500 μM sulindac sulfide, 600 μM exisulind, or 5 μM CP248, for 72 hr. Then the cells were collected, stained with propidium iodide, and the percent of cells with a sub-G_1 DNA content was determined by FACS analysis (Table 3). When the parental LNCaP cell line was treated with sulindac sulfide, exisulind, or CP248, 43, 34, and 74% of the cell population displayed sub-G_1 DNA content. Qualitatively similar results were obtained with the LNCaP-B6, LNCaP-B5, and PC3 cell lines (Table 3). Therefore, this method of assessing apoptosis also demonstrated that these three sulindac compounds induced apoptosis in both the androgen-sensi-

TABLE 2. Comparison of the effects of sulindac compounds on the induction of apoptosis in the parental LNCaP cell line and the B6 and B5 derivatives that express increased levels of bcl-2*

Cell line	Compound name (I.D.)	Concentration (μM)	DAPI (% apoptosis)
LNCaP-LN	0.1% DMSO		1.7 ± 0.6
	Sulindac sulfide	500	23.3 ± 1.3
	Exisulind	600	16.4 ± 3.1
	CP248	5	27.5 ± 1.6
LNCaP-B6	0.1% DMSO		2.1 ± 0.3
	Sulindac sulfide	500	20.3 ± 2.9
	Exisulind	600	12.9 ± 3.5
	CP248	5	28.8 ± 1.7
LNCaP-B5	0.1% DMSO		0.2 ± 0.1
	Sulindac sulfide	500	20.6 ± 1.9
	Exisulind	600	9.1 ± 1.2
	CP248	5	22.2 ± 3.0

* Cells were treated with the indicated concentrations of the drugs for 72 hr and then examined for morphologic changes in DAPI-stained cells. The values indicate the means ± SD of triplicate assays for the percent of cells that displayed evidence of apoptosis.

TABLE 3. Effects of sulindac compounds on the induction of apoptosis in prostate cell lines, as measured by their sub-G_1 DNA content*

Cell line	Compound	% of Cells with sub-G_1 DNA content
LNCaP-LN	0.1% DMSO	0
	500 μM Sulindac sulfide	43
	600 μM Exisulind	34
	5 μM CP248	74
LNCaP-B6	0.1% DMSO	0
	500 μM Sulindac sulfide	38
	600 μM Exisulind	30
	5 μM CP248	64
LNCaP-B5	0.1% DMSO	0
	500 μM Sulindac sulfide	32
	600 μM Exisulind	26
	5 μM CO248	43
PC3	0.1% DMSO	0
	500 μM Sulindac sulfide	26
	600 μM Exisulind	35
	5 μM CP248	43

* Cells were treated with the indicated concentration of the drugs for 72 hr. The cells were then stained with propidium iodide, and 10^4 cells were analyzed with a FACScan instrument equipped with a FACStation. The values indicate the percent of the total cell population that had a sub-G_1 DNA content in a single assay.

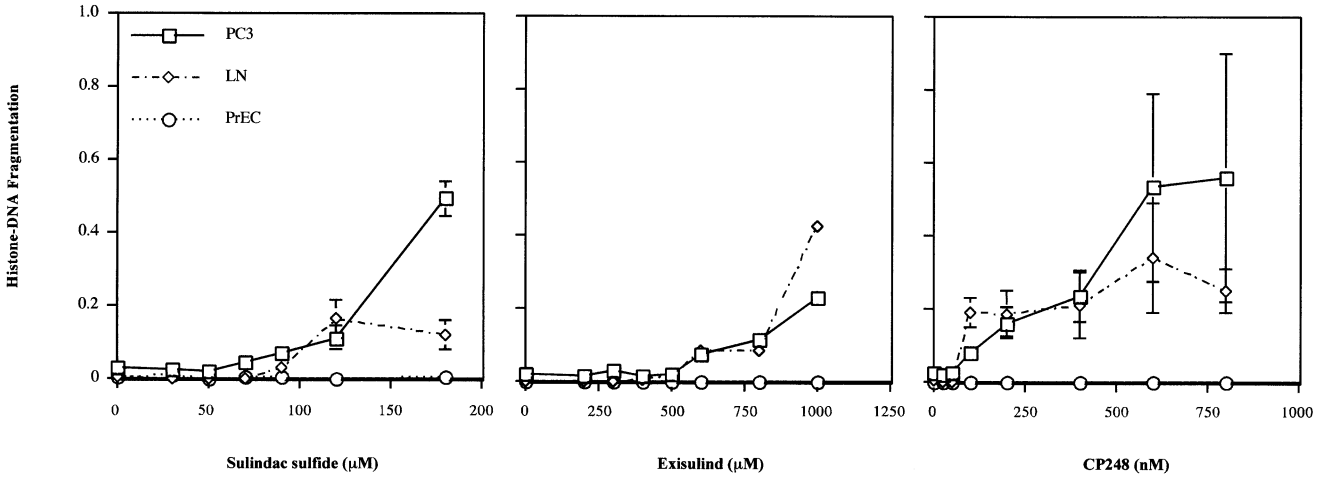

FIG. 4. Effects of sulindac sulfide, exisulind, and CP248 on apoptosis in PrEC normal epithelial prostate cells, in comparison with effects of LNCaP and PC3 cells. Ten thousand exponentially growing cells were exposed to either the solvent alone (0.1% DMSO) or the indicated concentrations of the three drugs for 48 hr, and assayed for histone-DNA fragmentation at 440 nm. The values represent the means and standard deviations of triplicate assays.

tive LNCaP and androgen-insensitive PC3 cell lines, as well as in the derivatives of the LNCaP cell line that express increased levels of bcl-2. This method also appeared to be more sensitive than the DAPI staining method (compare Tables 2 and 3). The same flow cytometry data showed no consistent effects of sulindac sulfide or exisulind on the percent of cells in the G_1, S, or G_2/M phases of the cell cycle, although treatment of the LNCaP and PC3 cells with CP248 increased the fraction of cells in the G_2/M phase (data not shown). The significance of the latter finding is currently being studied.

We also compared the sensitivity of the PrEC normal prostate epithelial cells to induction of apoptosis by these compounds with that obtained with the LNCaP and PC3 prostate cancer cell lines, using the Cell Death Detection $ELISA_{plus}$ assay. After 48 hr, approximately 50% of PC3 cells underwent apoptosis at 200 μM sulindac sulfide, 25% apoptosis at 1 mM exisulind, and 50% apoptosis at 800 nM CP248 (Fig. 4). LNCaP cells underwent 18% apoptosis with 200 μM sulindac sulfide, 40% apoptosis with 1 mM exisulind, and 20% apoptosis with 800 nM CP248. In stark contrast, the PrEC cells showed no indication of apoptosis at the same concentrations over the same time period. Furthermore, at similar concentrations, PrEC cells remained resistant to apoptosis up to 100 hr of drug treatment (unpublished studies).

COX Assays

To determine if reduction of prostaglandin synthesis is necessary for the growth inhibitory and apoptosis-inducing effects of the sulindac derivatives used in the present study, we examined the abilities of these three compounds to inhibit the *in vitro* activities of COX-1 and -2. Sulindac sulfide was a potent inhibitor of COX-1 (sulindac sulfide COX-1 value was statistically different from exisulind and CP248 starting at 1 μM), as previously reported [21], and also COX-2 (sulindac sulfide COX-2 value was statistically different from exisulind and CP248 starting at 1000 μM), with IC_{50} values of approximately 1 and 100 μM, respectively (Fig. 5). In contrast, exisulind was ineffective in inhibiting either COX-1 or -2, even at concentrations up to 10 mM (Fig. 5). Despite the high potency that CP248 displayed for inhibiting cell growth and inducing apoptosis (Fig. 2, Tables 1–3), this compound also lacked inhibitory activity for COX-1 or COX-2 at concentrations up to 10 mM (Fig. 5).

COX-1 and -2 western blots were done to evaluate the level of expression of these two proteins in whole cell extracts. Only trace levels of the constitutive COX-1 protein were detected in the PrEC, LNCaP, and PC3 prostate cell lines, whereas the BPH-1 cell line expressed moderate amounts of the COX-1 protein (Fig. 6). The COX-2 protein was expressed at negligible levels in the LNCaP and PC3 cells, while there were relatively high levels of COX-2 expression in the PrEC and BPH-1 cell lines.

DISCUSSION

When compared to results previously obtained with other cell lines [12, 20], the present studies indicate that human prostate cancer cells are similar to human colon and breast cancer cells in their sensitivity to growth inhibition by sulindac sulfide and exisulind. This is the first published study with the CP248 compound in any cell line, and demonstrates that it is a highly potent analog of exisulind. Indeed, CP248 is about 100 to 1000 times more potent than exisulind or sulindac sulfide with respect to both growth inhibition and induction of apoptosis (Fig. 2, Tables 1–3). It is also highly potent in its effects on other human cancer

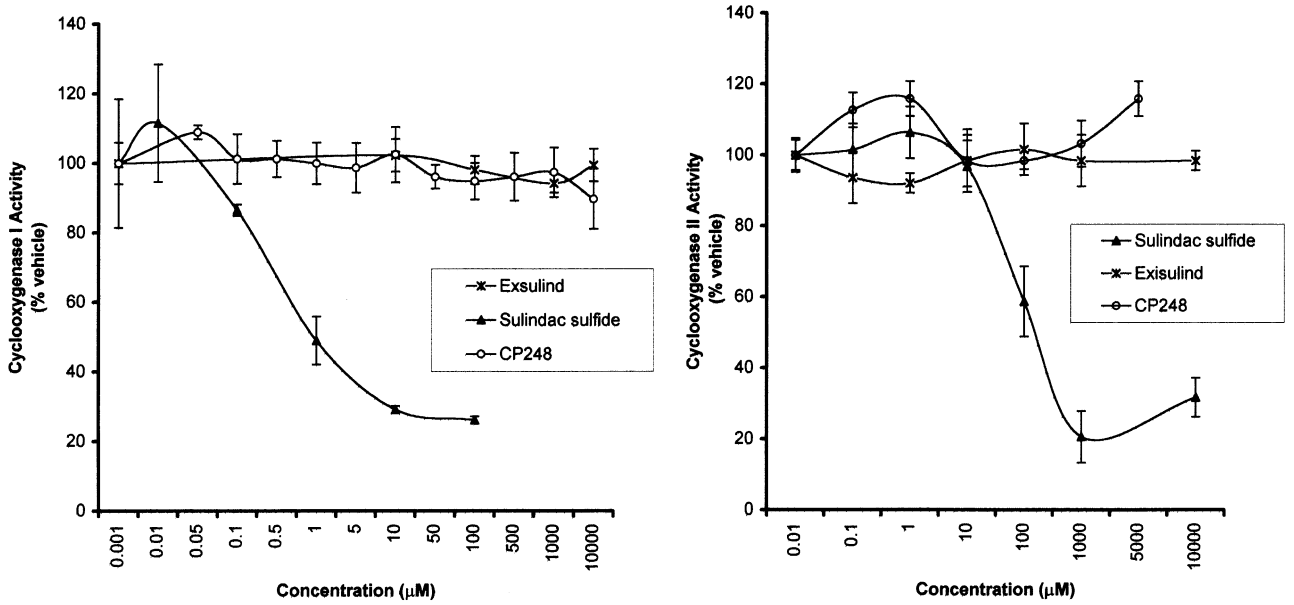

FIG. 5. Comparison of the effects of sulindac sulfide, exisulind, and CP248 on *in vitro* COX-1 and COX-2 enzyme activity. Cyclooxygenase activity was determined as described previously [21]. One hundred percent values (COX-1, O.D. = 0.026; COX-2, O.D. = 0.021) represent the activity of the enzymes in the vehicle alone (1% DMSO). The values represent means and standard deviations of triplicate assays.

cell lines derived from breast, lung, and colon tumors (unpublished results).

Bcl-2 is a 25–26 kDa protein that inhibits the action of numerous inducers or mediators of apoptosis including UV radiation [29, 30], γ-radiation [31], heat shock [32], neurotropin withdrawal [33], tumour necrosis factor [34], reactive oxygen species [35], calcium [36, 37], interleukin-1β-converting enzyme [38], c-Myc [39], and p53 [29]. Bcl-2 is specifically relevant to the present study since increased expression of this protein in prostate cancer is associated with increased resistance to hormonal and other forms of treatment [2, 33, 40]. Therefore, the results of the present study, indicating that derivatives of LNCaP cells that express increased levels of bcl-2 remain sensitive to growth inhibition and the induction of apoptosis by sulindac-related compounds (Fig. 1 and Tables 1–3), are of considerable importance. Although in some cases the LNCaP derivatives appeared to be less sensitive to exisulind or CP248, these differences were not statistically significant. The level of protein expression of the pro-apoptotic protein and bcl-2 counterpart bax was the same in these derivatives as in the parental LNCaP cells (data not shown). Assays for DNA fragmentation using the DNA laddering method also showed that the sulindac-related compounds induced apoptosis in both the parental and bcl-2 overexpressing LNCaP cells (data not shown). These results are in contrast to those obtained by Raffo *et al.* [2], who found that increased expression of bcl-2 markedly protected LNCaP cells from loss of viability and induction of apoptosis caused by serum starvation, treatment with the phorbol ester TPA (phorbol 12-myristate 13-acetate), or treatment with a Fas antibody. Therefore, the sulindac class of compounds may be effective in the treatment of prostate cancers, even in the subset of tumors that overexpress bcl-2. Furthermore, we found that even though the DU145 prostate cancer cell line does not express bcl-2 [41, 42], its sensitivity to growth inhibition and induction of apoptosis by the sulindac compounds was similar to that of the LNCaP cells (data not shown). In recent unpublished studies, done in collaboration with Dr. Cy Stein, we found that derivatives of LNCaP cells that express a 6-fold increase in the expression of the anti-apoptotic protein bcl-x_L also remain sensitive to the effects of these sulindac compounds. Taken together, these results suggest that sulindac derivatives induce growth inhibition and apoptosis via a pathway(s) that is independent of bcl-2 and bcl-x_L.

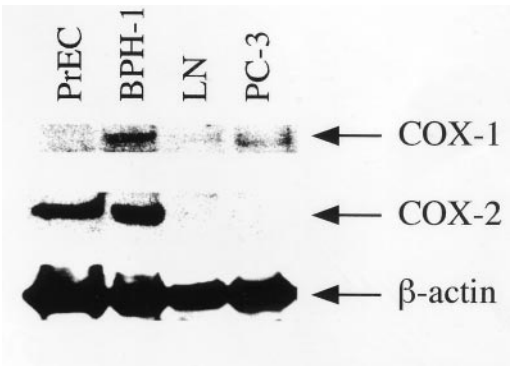

FIG. 6. Western blot analysis for COX-1 and -2 protein expression in prostate cell lines. β-Actin was used as a loading control.

At the present time the precise mechanisms by which sulindac compounds exert their growth inhibitory, apoptotic, and tumor inhibitory effects are not known. Sulindac sulfide and related NSAIDs inhibit COX-1 and -2. This

blocks the synthesis of prostaglandin E_2 and also could divert arachidonic acid into the lipoxygenase pathway, thus increasing the synthesis of leukotrienes [43, 44]. Alternatively, the increased level of arachidonic acid could result in activation of sphingomyelinase, which would cause increased levels of ceramide, a compound that also can induce apoptosis [45]. Our *in vitro* enzyme assays (Fig. 5) confirm previous studies indicating that sulindac sulfide inhibits COX-1 [21], and we found that it also inhibited COX-2. However, exisulind was ineffective in inhibiting either COX-1 or -2, even when tested at concentrations 100x greater than those effective for inhibiting cell growth (Fig. 5). The latter result is consistent with previous studies [18, 19, 46]. We found that CP248 also failed to inhibit COX-1 or -2, even at 10 mM, despite the fact that it inhibited cell growth and induced apoptosis at nanomolar concentrations. Therefore, the growth inhibitory and apoptotic effects of exisulind and CP248 are not due to inhibition of COX-1 or -2. It is still possible that the COX inhibitory activity of sulindac sulfide contributes to its growth inhibitory and apoptosis-inducing activity. However, this is also not certain, since sulindac sulfide can inhibit growth and induce apoptosis in the HCT-15 human colon cancer cell line, even though this cell line lacks detectable COX-1 or -2 activity [15, 16]. Furthermore, our LNCaP and PC3 cells showed trace amounts of COX-1 and -2 expression via both western blotting (Fig. 6) and RT–PCR (unpublished studies), whereas the BPH-1 cell line showed relatively high levels of COX-1 and COX-2 protein (Fig. 6). Yet, these prostate cancer cells still underwent growth inhibition and apoptosis comparable to that of the BPH-1 cells. These results lead us to conclude that a non-cyclooxygenase cellular pathway(s) remains to be identified to explain the antineoplastic mechanisms of action of NSAIDs and related compounds.

Furthermore, it seems likely that in prostate cancer cells, the growth inhibitory and apoptotic effects of these compounds may be mediated, at least in part, by separate mechanisms. Concentrations that caused complete growth inhibition in prostate cancer cells induced apoptosis in less than 30% of the total cell population (compare Figs. 1 and 2 and Tables 1 and 2). Therefore, the *in vitro* growth inhibitory effects of these compounds on prostate cancer cells do not appear to be due simply to the induction of apoptosis. The PC3 and DU145 cell lines carry inactivating mutations in the *p53* gene [47, 48], and yet the sulindac compounds also caused growth inhibition and induced apoptosis in these cells. Therefore, similar to the situation with human colon cancer cells [14, 49], the effects of these compounds in prostate cancer cells appear to be mediated by a p53-independent pathway.

In summary, this paper provides the first evidence that sulindac sulfide and exisulind are effective inhibitors of the growth of human prostate cancer cells and that they also induce apoptosis in these cells. This paper also describes for the first time a novel sulindac derivative, CP248. We have shown that this compound is approximately 1000-fold more potent than sulindac sulfide in inhibiting cell growth and approximately 100-fold more potent than sulindac sulfide in inducing apoptosis, even though CP248 does not inhibit COX-1 or COX-2. We also provide evidence that the growth inhibitory and apoptotic effects of sulindac derivatives are seen in both the androgen-sensitive LNCaP cells and the androgen-insensitive PC3 cells, as well as in derivatives of LNCaP cells that stably express increased levels of the anti-apoptotic protein bcl-2. Separate studies indicate that when administered in the diet at non-toxic doses, exisulind also inhibited the growth of LNCaP cells in a xenograft model in nude mice [50]. However, induction of apoptosis was not observed in the PrEC normal epithelial prostate cells at comparable concentrations up to 100 hr of treatment. These data are consistent with previous results indicating that exisulind increased apoptotic rates in regressing adenomas but not in normal mucosa from APC patients treated with exisulind (unpublished studies). Taken together, these findings provide evidence that this class of compounds, when used alone or in combination with other agents, may be therapeutically effective against prostate cancer and/or benign hyperplasia of the prostate. As a result of these novel findings, exisulind is currently undergoing a phase III clinical trial for prevention of recurrence of disease in patients with human prostate cancer.

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