

Sulindac Sulfide, an Aspirin-like Compound, Inhibits Proliferation, Causes Cell Cycle Quiescence, and Induces Apoptosis in HT-29 Colon Adenocarcinoma Cells

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Abstract

Nonsteroidal antiinflammatory drugs (NSAIDs), have cancer preventive and tumor regressive effects in the human colon. They lower the incidence of and mortality from colorectal cancer and sulindac reduces the number and size of polyps in patients with familial adenomatous polyposis.

We studied the effect of sulindac, and its metabolite sulindac sulfide, on the proliferation of HT-29 colon adenocarcinoma cells. Both compounds reduced the proliferation rate of these cells, changed their morphology, and caused them to accumulate in the G₀/G₁ phase of the cell cycle. These responses were time- and concentration-dependent and reversible. In addition, these compounds reduced the level and activity of several cyclin-dependent kinases (cdks), which regulate cell cycle progression. Sulindac and sulindac sulfide also induced apoptosis in these cells at concentrations that affected their proliferation, morphology, and cell cycle phase distribution. Sulindac sulfide was approximately six-fold more potent than sulindac in inducing these cellular responses.

Our results indicate that inhibition of cell cycle progression and induction of apoptotic cell death contribute to the anti-proliferative effects of sulindac and sulindac sulfide in HT-29 cells. These findings may be relevant to the cancer preventive and tumor regressive effects of these compounds in humans. (*J. Clin. Invest.* 1995. 96:491–503.) **Key words:** sulindac • apoptosis • cyclin-dependent kinases • colon cancer • proliferation

Introduction

Nonsteroidal antiinflammatory drugs (NSAIDs)¹ are among the most commonly used medications. Their use dates back to 1875 when sodium salicylate was given to patients with rheumatic fever as an anti-pyretic (1). They are currently utilized in a

variety of clinical situations and are often administered for long periods of time, in certain cases for many years. Indications requiring such long-term use include the prevention of myocardial infarction and cerebrovascular disease and the treatment of chronic inflammatory diseases such as rheumatoid arthritis.

Several epidemiological studies have demonstrated an association between the long-term consumption of NSAIDs and a reduced incidence of and mortality from colon cancer (e.g., most recently, [2, 3]). Sulindac (Clinoril®) has been shown to reduce the number and size of colonic and rectal adenomatous polyps in patients with familial adenomatous polyposis (FAP) (4, 5), although this effect may not be permanent (6). In addition, animal studies have shown that NSAIDs have anti-neoplastic effects in the colon. Aspirin (7), sulindac (8), piroxicam (9), and indomethacin (10) all reduce the number and size of tumors arising in the colon of rodents administered colonic carcinogens such as dimethyl-hydrazine or azoxymethane.

Because sulindac has such a marked anti-tumor effect, regressing established adenomatous polyps in patients with FAP, it is important to understand the mechanisms mediating this phenomenon. These theoretically could include the regulation of the dynamics of cell proliferation in the colonic mucosa. This hypothesis is supported by the work of Bayer et al. (11, 12), who demonstrated that indomethacin reversibly inhibits the proliferation of human fibroblasts and rat hepatoma cells and arrests them in the G₁ phase of the cell cycle.

Over the past several years, some of the molecular events governing progression through the various phases of the eukaryotic cell division cycle have been elucidated. Two families of proteins have been shown to play a central role in regulating the major transition points of the cell cycle in all eukaryotic organisms: the cyclins and the cyclin dependent kinases (cdks), (see references 13 and 14 for recent reviews). The cyclins comprise a group of regulatory proteins that share stretches of amino acid sequence homology. The cdks are a family of serine/threonine protein kinase subunits that associate with cyclins before their activation in discrete phases of the cell cycle (13, 14). All cdks share amino acid sequence homology with p34^{cdc2}, the prototypical molecule of this family. Recently, another family of proteins that play a key role in cell cycle regulation, the cyclin-dependent kinase inhibitors, have been described which interact with cdks or cyclin-cdk complexes to inhibit cdk catalytic activity (15).

The cdks that participate in regulating cell cycle progression include: (a) p34^{cdc2} which, in concert with the A- and B-type cyclins, controls the G₂/M transition (16–19); (b) p33^{cdk2}, which regulates the G₁/S transition (16, 18, 20) in association with cyclin E (21–24); and (c) p34^{cdk4} (25, 26) and p40^{cdk6} (27) which, in conjunction with the D-type cyclins (D1, D2, or D3), control progression through G₁ (28). In most proliferating cells, the steady state levels of the cdks do not change apprecia-

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1. *Abbreviations used in this paper:* cdks, cyclin-dependent kinases; FAP, familial adenomatous polyposis; NSAIDs, nonsteroidal antiinflammatory drugs.

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bly throughout the cell cycle (29, 30). Thus cdk activity, and therefore advancement through the cell cycle, is regulated in part by the distinctive periodic expression patterns of the different cyclins.

Since indomethacin has been shown to reduce the proliferation and alter the cell cycle of noncolonic cells (11, 12, 31), it is conceivable that NSAIDs affect colonic epithelial cells in a similar fashion. Therefore, we studied the effect of sulindac and sulindac sulfide, clinically important compounds in the chemoprevention of colon cancer, on the proliferation and the cell cycle of HT-29 colon adenocarcinoma cells.

As sulindac sulfide is considered the active metabolite of sulindac, we elected to use concentrations of sulindac sulfide that could conceivably be achieved in the colon *in vivo*. A large fraction of sulindac is converted to sulindac sulfide in the colon by colonic bacteria (32). Therefore, high luminal concentrations of sulindac sulfide are achieved in the colon (33). In addition, animal studies have shown that sulindac sulfide is concentrated in the mucosa of the colon at levels several-fold higher than in the serum (34). Colonic epithelial cells can be exposed to concentrations up to 20-fold higher than those in serum (33); serum levels of sulindac sulfide are about 10–15 μM when sulindac is given orally at doses which regress polyps in FAP patients (35, 36). Sulindac, was used at concentrations several times higher than sulindac sulfide to see if the parent compound could mimic its effects.

It is also possible that the mechanisms responsible for the anti-proliferative effects of the NSAIDs on cultured cells is multifactorial. Apoptotic cell death is another mechanism that could contribute to reduced cell growth. Apoptosis has been the focus of intense scrutiny and has become better understood at the molecular genetic level (for recent reviews see references 37–39). It is a genetically determined and evolutionarily conserved process that occurs in a variety of physiological and pathophysiological contexts including: (a) the elimination of self-reactive immune cells; (b) embryonic tissue development; and (c) tumorigenesis. Therefore, we also examined whether these compounds caused apoptosis in HT-29 colon cancer cells.

Methods

Cell lines. The human colon adenocarcinoma cell line HT-29 (ATCC HTB 38) and the promyelocytic leukemia cell line HL-60 (ATCC CCL 240) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Culture medium consisted of McCoy's 5A (Cellgro[®], Mediatech, Inc., Herndon, VA) supplemented with 10% FBS (Gemini Bioproducts, Inc., Calabasas, CA) for HT-29 cells, and RPMI 1640 (Cellgro[®]) supplemented with 10% heat-inactivated FBS for HL-60 cells. In addition, both types of culture media were supplemented with nonessential amino acids, penicillin (50 U/ml), and streptomycin (50 $\mu\text{g}/\text{ml}$) (all from GIBCO BRL, Gaithersburg, MD). HT-29 cells were grown as monolayers in 100- or 150-cm² plates and HL-60 cells were grown in suspension in 100-cm² plates. Both were incubated at 37°C, in 5% CO₂ and 90% relative humidity. Cell morphology was recorded daily. To document morphologic alterations, cells grown for 72 h with or without supplemental sulindac or sulindac sulfide were photographed in the culture dishes using an inverted microscope (Diaphot-TMD; Nikon Inc., Melville, NY). Only areas representative of the morphology of the cells throughout the entire dish were photographed.

Reagents. Sulindac (Sigma Chemical Co., St. Louis, MO) was dissolved in 1 M Tris-HCl, pH 7.5; the final pH was adjusted to 7.2 with 10 N NaOH. Sulindac sulfide (desoxy-sulindac, generously provided by Merck and Co., Rahway, NJ), was dissolved in DMSO (Fisher Scientific, Fair Lawn, NJ). All compounds were added to the culture

medium and cells prior to plating them. The DMSO concentration was adjusted to 0.6% in all sulindac sulfide-supplemented media. Different batches of sulindac sulfide required slightly different final concentrations (within a narrow range) to induce similar effects on the HT-29 cells.

Immunological reagents. The p34^{cdk2} antibody was raised by immunizing rabbits with a peptide (CDNQIKKM) from the COOH terminus of p34^{cdk2} (GIBCO BRL). The p33^{cdk2} antiserum (Pharmingen, San Diego, CA) used in immunoprecipitations was produced by immunizing rabbits with a COOH-terminal peptide (CDVTKPVPHLRL). The p33^{cdk2} and p34^{cdk4} antibodies used for Western blots (Santa Cruz Biotechnologies, Santa Cruz, CA) were also raised in rabbits to a COOH-terminal peptide of p33^{cdk2} or p34^{cdk4}, respectively.

Cell proliferation and cell cycle analysis. Single cell suspensions were obtained from monolayers of HT-29 cells as follows. First, cells were washed with PBS, pH 7.2, supplemented with 1% BSA (PBS/BSA), and then incubated with Hank's based Enzyme-free Cell Dissociation Solution (Specialty Media, Inc., Lavallette, NJ), as per the manufacturer's instructions. Cells were then gently scraped from the dishes in the presence of PBS/BSA supplemented with 0.05% Tween-20. Cells floating in the culture medium were harvested by low-speed centrifugation, and then pooled with the adherent cell fraction. The cells were washed and resuspended in 1 ml of PBS/BSA. Aliquots of cells were counted using a hemacytometer and tested for viability by the trypan-blue dye exclusion method. Trypan-blue (Sigma) was added to the cell suspension at a final concentration of 0.08%. Cells that stained blue were considered "nonviable." Proliferation is reported as the number of cell counted at any given time point divided by the number of cells that were initially plated.

For cell cycle analysis, cells were fixed with ice-cold 70% ethanol, incubated at -20°C for a minimum of 30 min, then treated with 0.1% NP-40 for 5 min on ice, and then washed twice with PBS/BSA. Cells were then resuspended in 40 $\mu\text{g}/\text{ml}$ propidium iodide (Sigma) and 200 $\mu\text{g}/\text{ml}$ RNase type IIA (Sigma) in PBS/BSA and incubated at room temperature for 30 min before measuring their DNA content using a FACS-scan (Becton Dickinson Labware, Franklin Lakes, NJ). Raw data of the distribution of DNA content retrieved from the FACS scan was expressed as a percentage of the G₀/G₁ through G₂/M populations, inclusive. The MultiPlus software program (Phoenix Flow Systems, San Diego, CA) was used to generate DNA content frequency histograms and to assist in data analysis.

Thin-layer chromatography. The possibility that sulindac was converted to sulindac sulfide in the culture medium was evaluated by TLC. Culture medium was aspirated and mixed 1:1 (vol/vol) with CHCl₃/CH₃OH (1:1, vol/vol). A 10- μl aliquot from this mixture was spotted onto silica gel GF TLC plates (Analtech Inc., Newark, DE); 3 mM solutions of authentic sulindac and sulindac sulfide were also spotted in parallel. The plates were developed with ethylacetate/acetic acid (97/3, vol/vol) and the compounds were visualized by short-wave UV illumination.

Western blots. Cells were plated onto 150-cm² dishes at a density of 3×10^6 cells per dish and grown for 72 h, with and without sulindac or sulindac sulfide, and were harvested as described. Their cell cycle distribution was determined by measuring the DNA content in an aliquot of these cells. The remainder of the cells were washed with PBS, lysed with 2–4 vol of 2 \times Laemmli's buffer without supplemental bromophenol blue (0.12 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol) (40), heated to 95°C for 10 min, and then briefly sonicated. The protein content of these extracts was measured spectrophotometrically with bicinchoninic acid (41) (BCA protein assay reagent; Pierce Chem. Co., Rockford, IL). The accuracy of this method was verified by resolving a small aliquot by SDS-PAGE electrophoresis followed by Coomassie blue staining. 50 μg of total protein from control and drug-treated samples were resolved on 10% SDS-PAGE gels along with prestained protein molecular weight standards (Sigma). Gels were then Western blotted onto PVDF membranes (Polyscreen[®], Dupont-NEN, Boston, MA) (as described in Harlow and Lane [42]). Upon completion of the transfer, the membranes were blocked with 5% milk (Carnation nonfat dry milk; Carnation Co., Los Angeles, CA) in TBS/0.05% Tween-20 (TBS-

T) for a minimum of 2 h at room temperature. Membranes were washed with two changes of TBS-T, then reacted with the primary antibodies at a 1:1,000 dilution in 5% milk/TBS-T for 1 h at room temperature. Following primary antibody incubation, the blots were washed four times with TBS-T, and then incubated with the secondary antibody, a peroxidase-conjugated goat anti-rabbit antibody (GIBCO BRL) diluted 1:5,000 in 5% milk/TBS-T for a minimum of 1 h at room temperature. Then, after additional washing with TBS-T, the cdk proteins were detected with a chemiluminescence assay system (Renaissance[®]; Dupont-NEN). After the specific proteins were visualized, the blots were stained with 0.1% India ink in TBS with 0.3% Tween-20 to verify that equal amounts of total protein were present in all of the lanes (42).

Immunoprecipitation and histone H1 kinase assays. Control and drug-treated HT-29 cells were harvested and 2×10^6 viable cells were lysed in the following buffer: 0.1% NP-40, 50 mM Hepes, pH 7.5, 250 mM NaCl, 100 μ g/ml PMSF, 2 μ g/ml aprotinin, 2 μ g/ml leupeptin, 0.1 μ g/ml sodium orthovanadate, 10 mM NaF, 5 mM DTT, and 5 mM EDTA. The lysates were incubated with 50 μ l of adult rabbit serum (Hazelton, Denver, PA) for 30 min and then mixed for 1 h with the pellet from 1 ml of a 10% suspension of lyophilized heat killed *Staphylococcus aureus*, Cowan I strain (Zysorbin; Zymed Laboratories, Inc., South San Francisco, CA) followed by centrifugation at 12,500 g for 10 min as a preclearing step. For p34^{cdc2} immunoprecipitations (IPs), one half of the precleared lysate was incubated with 5 μ l of p34^{cdc2} antibody, and the other half with 5 μ l of normal adult rabbit serum as a negative control. Similarly for p33^{cdc2} IPs, precleared lysates were divided and one half was incubated with 5 μ l of a p33^{cdc2} antiserum and the other with 5 μ l of the negative control normal adult rabbit serum. After a 1-h incubation on ice, the immune complexes were precipitated by incubation with protein A coupled to sepharose beads (Pharmacia Biotech, Inc., Piscataway, NJ). The beads bearing the immunoprecipitates were thoroughly washed with lysis buffer, and then with cdc2 (50 mM Tris-HCl, pH 8, 10 mM MgCl₂, 1 mM DTT) or cdk2 (50 mM Hepes, pH 7, 10 mM MgCl₂, 1 mM DTT) kinase wash buffers. The beads were then pelleted and aspirated dry. Histone H1 kinase assays were performed by the addition of cdc2 kinase (50 mM Tris-HCl, pH 8, 10 mM MgCl₂, 1 mM DTT, 25 μ M ATP, 5 μ Ci ³²P γ -ATP [Dupont-NEN]) or cdk2 kinase (50 mM Hepes, pH 7, 10 mM MgCl₂, 1 mM DTT, 12.5 μ M ATP, 20 μ Ci ³²P γ -ATP) buffers supplemented with 100 μ g/ml histone H1 (Boehringer-Mannheim Corp., Indianapolis, IN). The kinase assays, performed at room temperature for 30 min, were terminated by the addition of 2 \times Laemmli's buffer containing 200 mM DTT. The products of these reactions were resolved on 10% SDS-PAGE gels. Upon completion of the electrophoresis, gels were immersed in 0.25% Coomassie blue, followed by destaining with 0.25% MeOH and 0.07% acetic acid, then by autoradiography. Following visualization of the ³²P-labeled histone H1 in this manner, the Coomassie-stained histone H1 bands were excised from the gels. The degree of incorporation of ³²P into the histone H1 present in the individual gel slices, expressed as cpm emitted from the samples, was determined in a scintillation counter (Beckman Instruments, Inc., Fullerton, CA) by the Cerenkov method.

Apoptosis. The cells were evaluated for evidence of apoptosis by three different assays: (a) measurement of the DNA content of cells by propidium iodide staining and FACS analysis (43, 44), to detect a subdiploid peak of DNA, indicative of the DNA fragmentation occurring with apoptosis; (b) acridine orange staining to identify cellular morphologic changes characteristic of apoptosis (45); and (c) agarose gel electrophoresis of genomic DNA to detect the DNA degradation associated with apoptosis (46).

Acridine orange staining. Single cell suspensions of control and drug-treated HT-29 cells in PBS/BSA were fixed with 70% ethanol and frozen at -20°C for at least 30 min. They were washed and resuspended in PBS and treated with ice-cold 0.1% Triton X-100, 0.08 N HCl, and 0.15 M NaCl. The cells were then stained with 20 μ M acridine orange (Sigma) in a phosphate-citric acid buffer (pH 6) containing 1 mM EDTA and 0.15 M NaCl. The suspension of stained cells was centrifuged at 750 g and cells were then resuspended in PBS. One drop of this suspension was placed on a microscope slide and the cells were evalu-

ated by UV fluorescence microscopy. The morphologic criteria used to quantify the fraction of apoptotic cells on these slides included the presence of: (a) cytoplasmic and nuclear shrinkage; (b) chromatin condensation; and (c) cytoplasmic blebbing with maintenance of the integrity of the cell membrane (zeiosis) (37). The percentage of apoptotic cells among all of the cells present on the slide was determined by counting 10 randomly selected high-power fields.

For fluorescence microscopy, 50,000 freshly harvested cells from control or drug-treated dishes were applied to glass slides by centrifugation at 1,000 rpm in a cytospin well (Cytospin 2; Shandon Inc., Pittsburgh, PA) for 5 min. The slides were fixed by immersion in acetic acid/ethanol (1:9, vol/vol) for at least 20 min. Upon drying, the slides were treated with HBSS, and stained with acridine orange as above. Following several washes with HBSS, the cells were examined with a UV fluorescence microscope and photographed (Labophot, Nikon Inc.).

DNA degradation. HT-29 cells were plated at a density of 3×10^6 cells/dish and treated with control or drug-supplemented medium for 72 h. HL-60 cells, plated at a density of 0.4×10^6 cells/ml, were treated with control or drug-supplemented medium for 4 h. Genomic DNA was obtained from 1×10^6 HT-29 cells (both viable and nonviable) and $2-5 \times 10^6$ HL-60 cells by lysis in 0.04 M Tris-Acetate, 1 mM EDTA, 0.25% NP-40. These extracts were digested with 100 μ g/ml boiled RNase IIA (Sigma) for 1 h at 37°C followed by treatment with proteinase K (Boehringer-Mannheim) at a concentration of 1 mg/ml at 37°C for 18 h. Aliquots of DNA from the equivalent of $1-2 \times 10^5$ cells, were mixed with sample buffer (final concentration: 0.025% bromophenol blue/ \pm 0.025% xylene cyanole/3.0% glycerol) and resolved in 1.8% agarose gels impregnated with ethidium bromide (0.1 μ g/ml). To analyze greater amounts of DNA, isolated genomic DNA was extracted with phenol:chloroform (1:1, vol/vol) then chloroform, followed by ethanol precipitation. The resulting DNA was dissolved in H₂O and its amount present in solution was quantified by measuring its absorbance at 260 nm. 10 μ g of this DNA, mixed with sample buffer, was resolved on 1.8% agarose gels. DNA was visualized by UV transillumination and photographed using Polaroid 667 Film.

Results

Sulindac and its metabolite, sulindac sulfide, reduce the proliferation and alter the morphology of HT-29 adenocarcinoma cells

Sulindac and sulindac sulfide reduce the proliferation of HT-29 cells. To evaluate the effect of sulindac and sulindac sulfide on the proliferation rates of colon cancer cells in vitro, HT-29 cells were plated at a density of $0.75-1.0 \times 10^6$ cells per 100-cm² tissue culture dish or at a density of 3.0×10^6 cells per 150-cm² dish, with or without sulindac or sulindac sulfide supplemented in the culture medium. Cells were harvested 24, 48, or 72 h after plating. The number of viable cells present in the culture dishes, following incubation with either sulindac-supplemented (100, 200, 400, 600, and 1,200 μ M) or control medium, is shown in Fig. 1. This figure demonstrates that control HT-29 colon cancer cells entered log growth phase \sim 24 h after plating. Cells treated with sulindac exhibit a profound concentration-dependent reduction in their proliferation rate over the 72-h test period. At sulindac 1,200 μ M, the proliferation curve was flat, i.e., there was no net increase in the number of viable cells following treatment with this concentration over the entire 72 h.

Sulindac sulfide, the reduced molecular species of sulindac, also inhibited the accumulation of HT-29 cells. It was more potent than sulindac in inhibiting HT-29 cell proliferation. As illustrated in Fig. 2, compared to the control cells (grown in McCoy's medium supplemented with 0.6% DMSO), sulindac sulfide caused a concentration-dependent reduction in HT-29

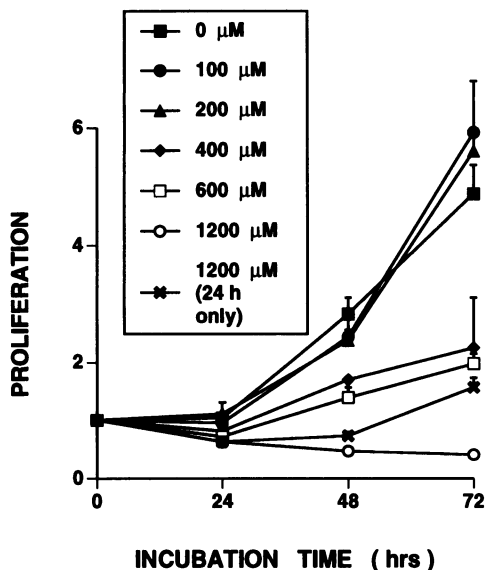


Figure 1. Growth of HT-29 cells treated with sulindac. Cells were grown in the presence of various concentrations of sulindac for 72 h and counted, as described in Methods. —x— line represents the reversibility study: cells were treated with sulindac 1,200 μM for only 24 h, when the sulindac-supplemented medium was replaced by control medium for an additional 24 or 48 h. The values for proliferation are the ratio between the number of cells counted at each time point divided by the number initially plated. Values are mean \pm SEM (n ranges between 6 and 20).

cell proliferation. At 175 and 200 μM , sulindac sulfide strongly inhibited the proliferation of these cells, with a sustained diminution of the number of viable cells throughout the 72-h treatment period.

It should be emphasized that sulindac sulfide was added to the culture dishes at concentrations that we estimated are achievable *in vivo* in colon tissue (see below).

Sulindac and sulindac sulfide alter the morphology of HT-29 cells. Sulindac and sulindac sulfide treatment induced distinctive morphological changes in HT-29 cells. Normally, 72 h after being plated at a density of 1×10^6 cells per 100-cm² dish with control medium supplemented with 0.6% DMSO, HT-29 cells grow in aggregates with individual cells assuming polygonal or rectangular shapes (a representative area is shown in Fig. 3 a). Cells treated with 150 μM sulindac sulfide (Fig. 3 b) for 72 h also grew in groups, but developed elongated cellular processes and assumed unusual shapes. Treatment with 200 μM sulindac sulfide for 72 h induced striking morphological changes in these cells. As demonstrated in Fig. 3 c, these cells grew as small, single cells, often separated from neighboring cells, and displayed prominent long filamentous processes. These processes were similar to, but more prominent than, those seen following treatment with lower concentrations of sulindac sulfide (Fig. 3 b). In addition, cells treated with 200 μM sulindac sulfide developed small, round, and highly refractile intracellular bodies distributed throughout their cytoplasm (Fig. 3 c). These changes were evident as early as 12 to 24 h after incubation of the HT-29 cells with sulindac sulfide (data not shown).

Sulindac treatment resulted in similar, if not identical, morphological changes in these cells, but at higher concentrations

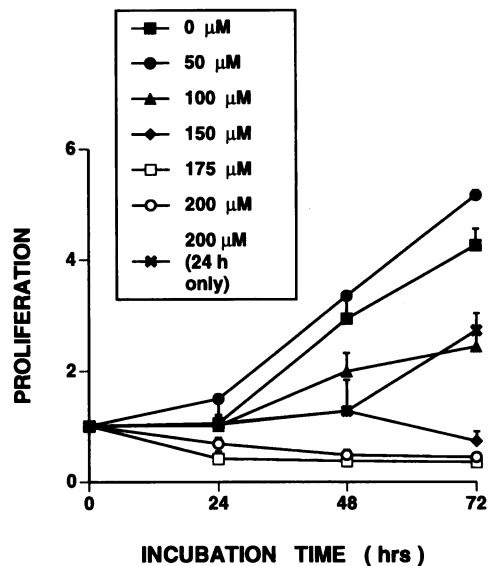


Figure 2. Growth of HT-29 cells treated with sulindac sulfide. Cells were grown in the presence of various concentrations of sulindac sulfide for 72 h and counted, as described in Methods. —x— line represents the reversibility study: cells were treated with sulindac sulfide 200 μM for only 24 h, when the sulindac sulfide-supplemented medium was replaced by control medium for an additional 24 or 48 h. The values for proliferation are the ratio between the number of cells counted at each time point divided by the number initially plated. Values are mean \pm SEM (n ranges between 5 and 30).

than were required to see an effect with sulindac sulfide. Sulindac 1,200 μM (Fig. 3 g) induced the alterations in cell morphology similar to those seen with 200 μM sulindac sulfide (Fig. 3 c). In addition, sulindac 600 μM (Fig. 3 f) induced changes similar to those seen at the 150 μM concentration of sulindac sulfide (Fig. 3 b). These morphological changes were also evident as early as 12 to 24 h after incubation with sulindac (data not shown). In addition, there was virtually no difference in the morphology of cells incubated in medium with (Fig. 3 a) or without (Fig. 3 e) supplemental DMSO at a concentration of 0.6%.

The effect of sulindac and sulindac sulfide on the proliferation and morphology of HT-29 cells is reversible. We assessed whether the effects of sulindac and sulindac sulfide on HT-29 cell proliferation and morphology were reversible. Cells were treated with sulindac (1,200 μM) or sulindac sulfide (200 μM) for 24 h, then were washed and refed with control medium (McCoy's or McCoy's supplemented with 0.6% DMSO) and allowed to grow for an additional 24 or 48 h. As shown in Fig. 1, the proliferation rate of cells initially treated with high concentrations (1,200 μM) of sulindac approached that of the control cells 48 h after replacement of drug-supplemented medium with control medium. At this time, these cells assumed the morphological characteristics of control cells (Fig. 3 h).

Sulindac sulfide also had a reversible effect on HT-29 cell proliferation and morphology. As shown in Fig. 2, cells treated with sulindac sulfide (200 μM) for the initial 24 h achieved proliferation rates approaching those of the control cells 48 h after replacement of drug-supplemented medium with control medium. Likewise, these cells then assumed normal shapes and sizes (Fig. 3 d). The morphologic alterations were rapidly re-

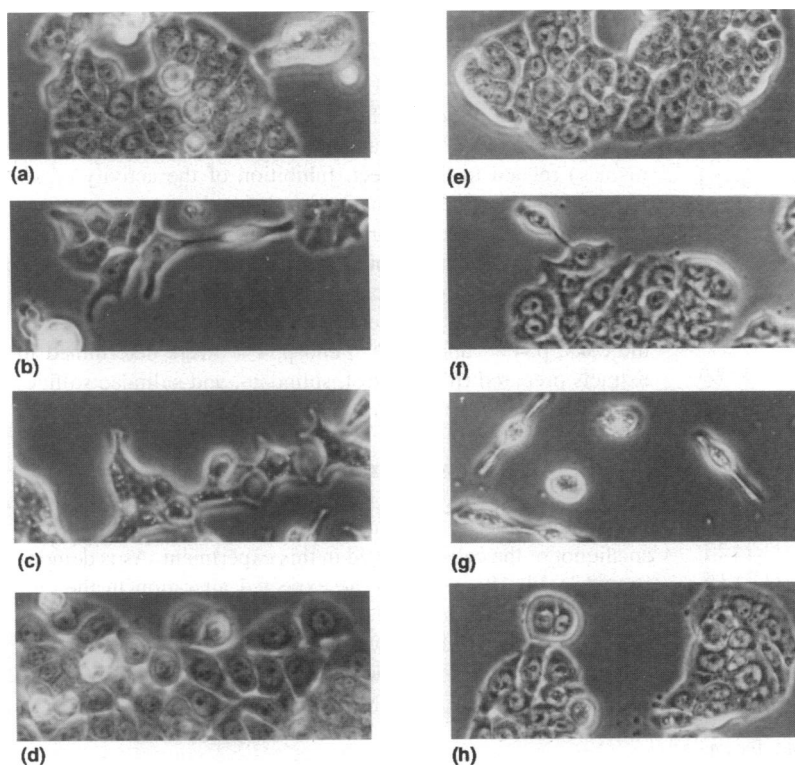


Figure 3. Photomicrographs of HT-29 cells treated with sulindac sulfide or sulindac. Cells were treated with various concentrations of sulindac sulfide (a, 0 μM ; b, 150 μM ; c, 200 μM) for 72 h; or with 200 μM for only 24 h (d), when the sulindac sulfide-supplemented medium was replaced with control medium for an additional 48 h. Similarly, cells were treated with sulindac (e, 0 μM ; f, 600 μM ; g, 1,200 μM) for 72 h, or with 1,200 μM for only 24 h (h), when the sulindac-supplemented medium was replaced by control medium for an additional 48 h. Only areas portraying the representative morphologic changes of the cells throughout the entire dish were photographed and presented in this figure. $\times 40$.

versed (data not shown), often 24 h after removal of sulindac sulfide.

The effect of sulindac is not mediated exclusively by conversion to sulindac sulfide in the culture medium

Since sulindac sulfide reduced HT-29 cell proliferation and altered their morphology at concentrations approximately six-times lower than its parent compound, we evaluated whether sulindac was converted to sulindac sulfide in the culture medium bathing the cells to a degree that would suggest sulindac sulfide was the mediator of the effect of sulindac on these cells. To address this, HT-29 cells were incubated with either control medium or with sulindac- or sulindac sulfide-supplemented medium. 72 h after plating, the medium was aspirated from the dishes and an aliquot was resolved by thin layer chromatography. A chromatogram of control and drug-supplemented culture

media is shown in Fig. 4. At concentrations previously demonstrated to inhibit HT-29 cell proliferation, we did not detect appreciable conversion of sulindac to sulindac sulfide, or vice versa, in the medium bathing the cells after 72 h of incubation. This figure shows that sulindac 1,200 μM was not converted to sulindac sulfide, at least to levels approaching 150 μM , which produced a comparable effect on proliferation and morphology.

Sulindac and sulindac sulfide alter the distribution of DNA content of HT-29 cells

To explore the mechanism responsible for the anti-proliferative effect of sulindac and sulindac sulfide treatment on HT-29 colon adenocarcinoma cells, we assessed the effect of these compounds on their cell division cycle. Cells treated with the test compounds were harvested 24, 48, or 72 h after plating, and their DNA content was assessed. The cell cycle phase distribution of HT-29 cells incubated for 72 h in control or sulindac-supplemented medium (between 100 and 1,200 μM) is shown in Table I (top). At 48 h, the change in the cell cycle phase distribution in response to sulindac was concentration-dependent between 400 and 1,200 μM . At 72 h, sulindac caused a nonlinear concentration-dependent alteration in the cell cycle phase distribution of these cells; with a significant effect noted only at its highest concentration. At the 1,200 μM concentration, sulindac increased the proportion of cells in the G_0/G_1 phase and decreased the proportion in the S and G_2/M phases of the cell cycle. Fig. 5 also shows the cell cycle phase distribution of control and sulindac-treated cells harvested at 24, 48, and 72 h after plating. As is evident in this figure, the effect of sulindac 1,200 μM on the redistribution of cells in the cell cycle was recognized as early as 24 h after the cells were plated and persisted throughout the entire 72-h treatment period.

Like sulindac, sulindac sulfide treatment produced a concen-

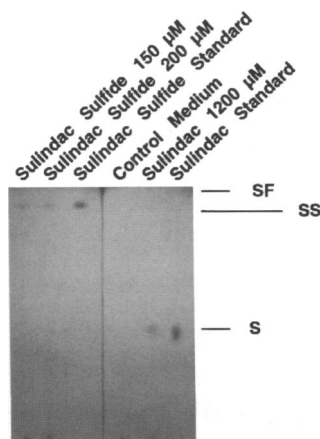


Figure 4. Thin layer chromatogram of sulindac and sulindac sulfide. Medium from HT-29 cells treated with sulindac 1,200 μM or sulindac sulfide 150 and 200 μM was applied to TLC plates, along with authentic compounds (3 mM) (S, sulindac, SS, sulindac sulfide) and resolved, as described in Methods. SF, solvent front.

Table I. Cell Cycle Phase Distribution of HT-29 Cells Treated with Sulindac or Sulindac Sulfide for 72 h

Concentration (μM)	% G_0/G_1	% S	% G_2/M
Sulindac			
0	52.4 \pm 2.0	21.6 \pm 1.3	24.9 \pm 2.1
100	56.4 \pm 3.2	25.5 \pm 5.7	17.8 \pm 3.3
200	54.5 \pm 4.1	24.8 \pm 5.6	20.1 \pm 8.8
400	50.4 \pm 1.8	14.6 \pm 0.6	33.4 \pm 2.1
200	51.6 \pm 2.0	18.3 \pm 1.1	29.3 \pm 1.8
1200	77.5 \pm 3.5	8.4 \pm 2.3	14.1 \pm 2.4
1200 (24 h only)	50.2 \pm 0.4	30.0 \pm 2.4	20.4 \pm 2.0
Sulindac sulfide			
0	51.4 \pm 1.7	20.1 \pm 1.2	26.6 \pm 1.9
50	54.8 \pm 0.9	19.1 \pm 0.6	26.3 \pm 0.3
100	52.2 \pm 1.9	23.1 \pm 1.4	23.1 \pm 1.4
150	69.3 \pm 2.7	11.5 \pm 1.2	17.2 \pm 1.8
175	80.5 \pm 2.6	8.4 \pm 0.7	11.2 \pm 2.1
200	79.3 \pm 1.8	7.3 \pm 0.9	11.7 \pm 1.7
200 (24 h only)	15.6 \pm 6.3	26.0 \pm 1.1	57.8 \pm 5.2

In each panel, the last row of results represents a reversibility study; cells were treated with sulindac 1,200 μM or sulindac sulfide 200 μM for 24 h, when the medium was replaced with control medium for an additional 48 h before harvest and FACS analysis. Values are mean \pm SEM (n ranges between 6 and 25).

tration-dependent effect on the cell cycle phase distribution of HT-29 cells, but again at a lower concentration range. Also like sulindac, the concentration dependence was not linear. Compared to controls, at 72 h sulindac sulfide markedly increased the proportion of cells in G_0/G_1 and markedly decreased the proportion of cells in the S and G_2/M phases (Table I, *bottom*). This effect was evident as early as 24 h after plating at ≥ 150 μM and persisted for the entire 72-h experimental period (Fig. 6). Fig. 7 shows DNA content frequency histograms from control and sulindac sulfide-treated cells after 72 h of incubation. The changes in the cell cycle phase distribution in response to sulindac sulfide are also clearly visible in this figure which also demonstrates the appearance of a sub-diploid peak in the sulindac sulfide-treated cells (Fig. 7 *middle* and see below). Sulindac-treated cells did not manifest such a sub-diploid peak after 72 h of incubation (data not shown).

The effect of both sulindac and sulindac sulfide on the cell cycle phase distribution of HT-29 cells was reversible. Figs. 5 and 6 and Table I show the cell cycle phase distribution of HT-29 cells 24 or 48 h after a 24-h period of treatment with sulindac (1,200 μM) or sulindac sulfide (200 μM). Fig. 5 *b* shows that 24 h after the removal of 1,200 μM sulindac, a lower proportion of the cells were in G_0/G_1 and a greater percentage in S phase compared to control and sulindac-treated cells. 48 h after removal of sulindac, the cell cycle phase distribution was no different from control cells (Fig. 5 *c* and Table I, *top*).

After the removal of 200 μM sulindac sulfide from the culture medium 24 or 48 h after an initial period of drug treatment, a lower proportion of the cells were in G_0/G_1 and a higher percentage in the S and G_2/M phases compared to either control cells or those continuously treated with sulindac sulfide (Figs. 6, *b* and *c* and 7, *bottom*, and Table I, *bottom*).

Sulindac and sulindac sulfide reduce the level of p34^{cdc2}, p33^{cdk2}, and p34^{cdk4} in HT-29 colon cancer cells

Since high concentrations of sulindac and sulindac sulfide had such a marked effect on the cell cycle distribution of HT-29 colon cancer cells, we evaluated potential molecular mechanism(s) related to this effect. Inhibition of the activity of selected cyclin-dependent kinases, has been shown in many systems to induce cell cycle arrest (18). We wished to determine whether drug treatment could alter the levels and activity of certain cdks that are critical for the regulation of cell cycle progression in eukaryotic cells. Therefore, the relative levels of the cdks, p34^{cdc2} and p33^{cdk2}, and p34^{cdk4} were determined in extracts prepared from control, sulindac-, and sulindac sulfide-treated cells by Western blot. As illustrated in Fig. 8 *a*, sulindac and sulindac sulfide led to a reduction in the level of p34^{cdc2} protein. This effect was maximal at 1,200 μM sulindac and 175 μM sulindac sulfide, where little p34^{cdc2} protein was detectable. The cell cycle phase distribution was measured in parallel from an aliquot of the cells collected in this experiment. As is demonstrated in Fig. 8 (*bottom*), the expected alteration in the cell cycle phase distribution (high G_0/G_1 and low S and G_2/M phases) was also observed with sulindac 1,200 μM and sulindac sulfide 175 μM . As is shown in Fig. 8 *b*, sulindac 1,200 μM and sulindac sulfide 175 μM profoundly reduced the level of

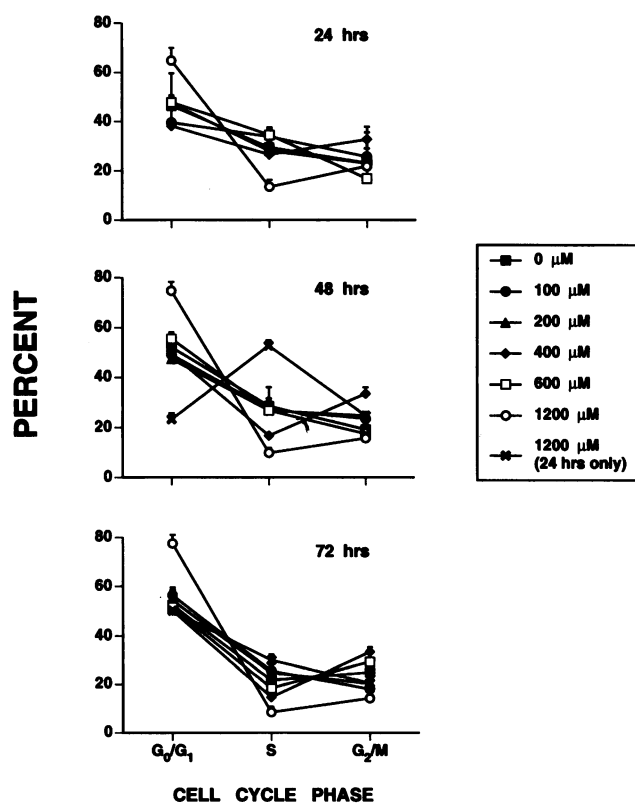


Figure 5. Cell cycle phase distribution of HT-29 cells treated with sulindac. HT-29 cells were treated with various concentrations of sulindac and harvested at 24 (*a*), 48 (*b*), or 72 (*c*) h. Their cell cycle phase distribution was determined by FACS analysis, as described in Methods. —x— in *b* and *c* represents the reversibility study: cells were treated with sulindac 1,200 μM for only 24 h, when the sulindac-supplemented medium was replaced with control medium for an additional 24 (*b*) or 48 (*c*) h. Values are mean \pm SEM (n ranges between 6 and 20).

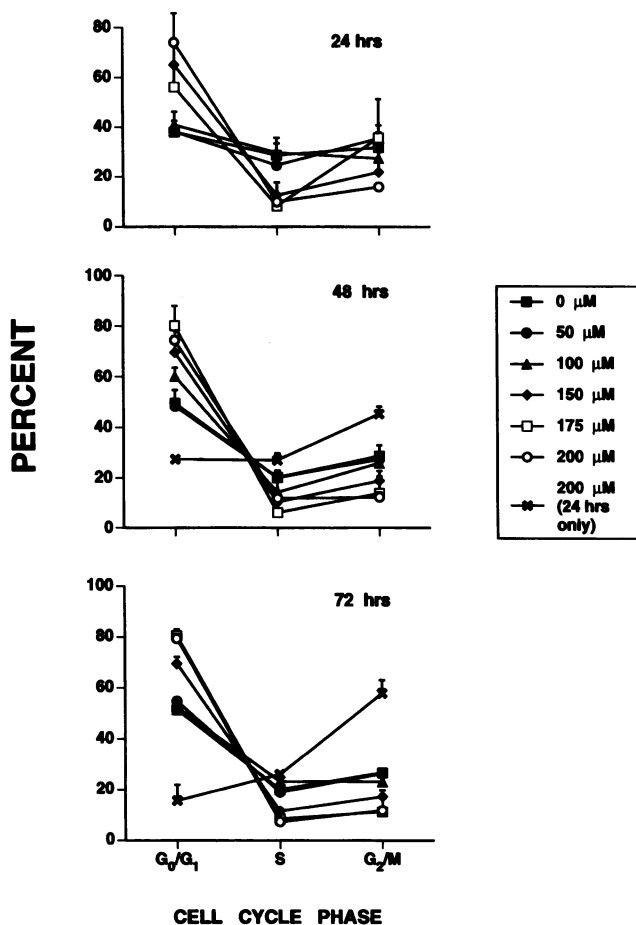


Figure 6. Cell cycle phase distribution of HT-29 cells treated with sulindac sulfide. HT-29 cells were treated with various concentrations of sulindac sulfide and harvested at 24 (a), 48 (b), or 72 (c) h. Their cell cycle phase distribution was determined by FACS analysis, as described in Methods. —x— in b and c represents the reversibility study: cells were treated with sulindac sulfide 200 μ M for only 24 h, when the sulindac sulfide-supplemented medium was replaced by control medium for an additional 24 (b) or 48 (c) h. Values are mean \pm SEM (*n* ranges between 5 and 30).

p33^{cdk2} protein, which regulates the G₁ to S transition. Finally, Fig. 8 c shows that high concentrations of sulindac and sulindac sulfide also reduced the level of p34^{cdk4}, though to a lesser extent than the other cdk. Lower concentrations of sulindac (600 μ M) and sulindac sulfide (100 μ M) resulted in minimal, if any, reduction in levels of p34^{cdk2}, p33^{cdk2}, or p34^{cdk4} (Fig. 8, a–c). In addition, as expected, the lower concentrations of these compounds did not affect the cell cycle phase distribution of these cells (Fig. 8, bottom, Figs. 5 and 6). Therefore, the changes in the abundance of the cdk proteins were noted only when sulindac and sulindac sulfide induced an effect on the cell cycle in these cells.

To confirm that equal amounts of total protein were loaded in each of the lanes on these blots, after they were probed with the appropriate antibodies, they were treated with India ink to visualize the total protein content in all of the lanes. There were approximately equal amounts of total protein in all of the lanes of these membranes, including the lanes where the cdk were profoundly reduced (data not shown).

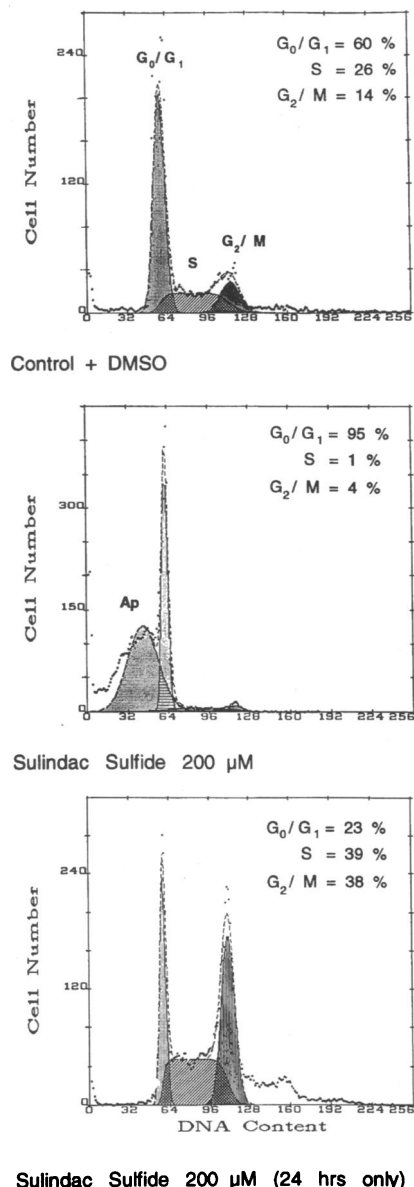


Figure 7. DNA content frequency histograms of HT-29 cells treated with sulindac sulfide. Cells were treated with 200 μ M sulindac sulfide for 72 h and their DNA content was determined by FACS, as described in Methods. (Top) Control cells. (Middle) sulindac sulfide-treated cells. (Bottom) Reversibility study: cells were treated with sulindac sulfide for 24 h, when the sulindac sulfide-supplemented medium was replaced by control medium for an additional 48 h. The cell cycle phase distributions for each treatment are indicated within each panel. Sulindac sulfide-treated cells developed a novel peak below the G₀/G₁ position on the histograms (Ap), which corresponds to apoptotic HT-29 cells.

Longer exposures of these Western blots revealed very low levels of cdk proteins in the lanes containing proteins extracted from cells treated with sulindac 1,200 μ M and sulindac sulfide 175 μ M (data not shown). Upon determining that the level of these cdk were severely reduced by sulindac and sulindac sulfide treatment, we then examined whether the reduction in cdk levels was accompanied by a change in their enzymatic activity. Therefore, immunoprecipitation-histone H1 kinase assays, using lysates from control and sulindac- and sulindac sulfide-

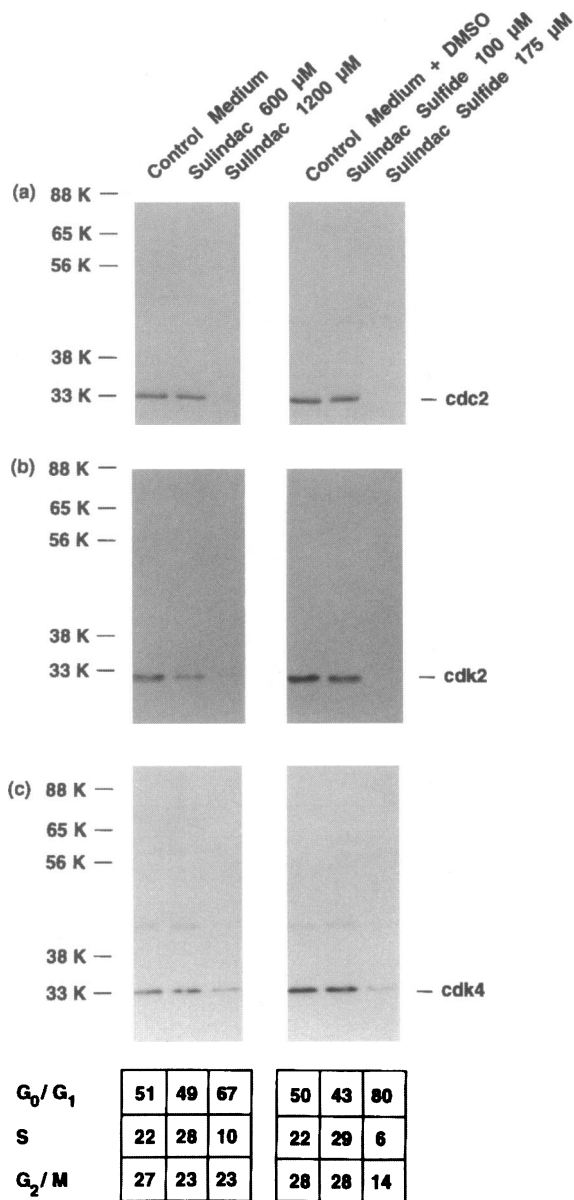


Figure 8. Western blots of cdk proteins from HT-29 cells treated with sulindac or sulindac sulfide. Cells were treated with either sulindac (600 or 1,200 μ M) or sulindac sulfide (100 or 175 μ M). Proteins (a, p34^{cdc2}; b, p33^{cdk2}; c, p34^{cdk4}) were extracted, fractionated on 10% SDS-PAGE gels, and Western blots performed, as described in Methods. The cell cycle phase distribution of these cells (bottom) was determined in an aliquot of these cells by FACS analysis, as described in Methods. The positions of molecular weight standards are depicted to the left of each panel. The levels of all three cdk were decreased in response to sulindac 1,200 μ M and sulindac sulfide 175 μ M; there was little or no change in response to lower concentrations of these compounds. The faint band in the lower panels (c) has not been identified. This protocol was repeated two additional times and similar results were obtained each time.

treated cells, were performed to measure the in vitro catalytic activity of p34^{cdc2} and p33^{cdk2} (47) in these cells. We did not measure the in vitro activity of p34^{cdk4} in this study. As shown in Fig. 9, a and b, there was a severe reduction in the histone H1 kinase activity of p34^{cdc2} and p33^{cdk2}, measured by the intensity of the substrate histone H1 bands on the gels and by their

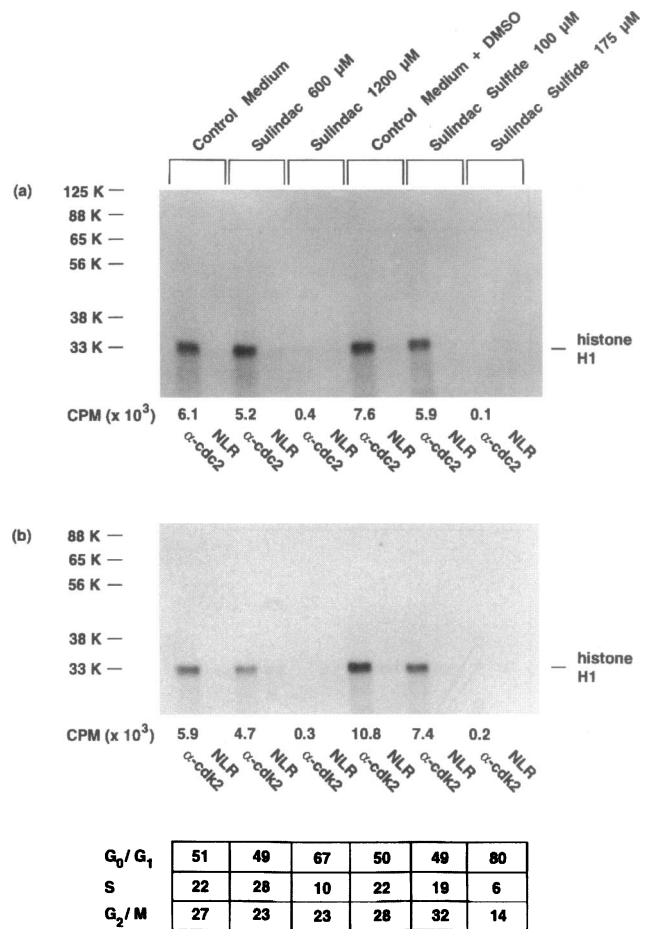


Figure 9. Histone H1 kinase assays of HT-29 cells treated with sulindac or sulindac sulfide. p34^{cdc2} and p33^{cdk2} were immunopurified in native form from a lysate of HT-29 cells treated with either sulindac (600 or 1,200 μ M) or sulindac sulfide (100 or 175 μ M) as described in Methods. Their ability to phosphorylate exogenously added histone H1 in vitro was determined using γ -³²P-ATP as the donor. Control reactions involved immunoprecipitation of the lysates using a nonspecific normal adult rabbit serum (NLR). The specific reaction is indicated as α -cdc2 (a) or α -cdk2 (b). The products of the reaction were fractionated on 10% SDS-PAGE gels and visualized by autoradiography. Each band was cut out of dried gels and quantitated, as described in Methods, and the counts are indicated below each lane; all control reactions had counts $< 0.4 \times 10^3$ cpm. The cell cycle phase distribution of these cells (bottom) was determined in an aliquot of these cells by FACS analysis, as described in Methods. The positions of molecular weight standards are depicted to the left of each panel.

relative incorporation of label, after 72 h of treatment with sulindac 1,200 μ M and sulindac sulfide 175 μ M. Compared to control, the histone H1 kinase activity of p34^{cdc2} and p33^{cdk2} was reduced 93.4 and 95%, respectively, by sulindac 1,200 μ M and 98.7 and 98.1%, respectively, by sulindac sulfide 175 μ M. These concentrations also reduced the proliferation and altered the morphology and cell cycle phase distribution of these cells (Fig. 9, bottom). There was no effect on the associated histone H1 kinase activity of p34^{cdc2} (Fig. 9 a) or p33^{cdk2} (Fig. 9 b) upon treatment with sulindac or sulindac sulfide at lower concentrations that had minimal, if any, effect on cdk levels on Western blot (Fig. 8) or on the cell cycle phase distribution of these cells (Fig. 9, bottom). To exclude the possibility that

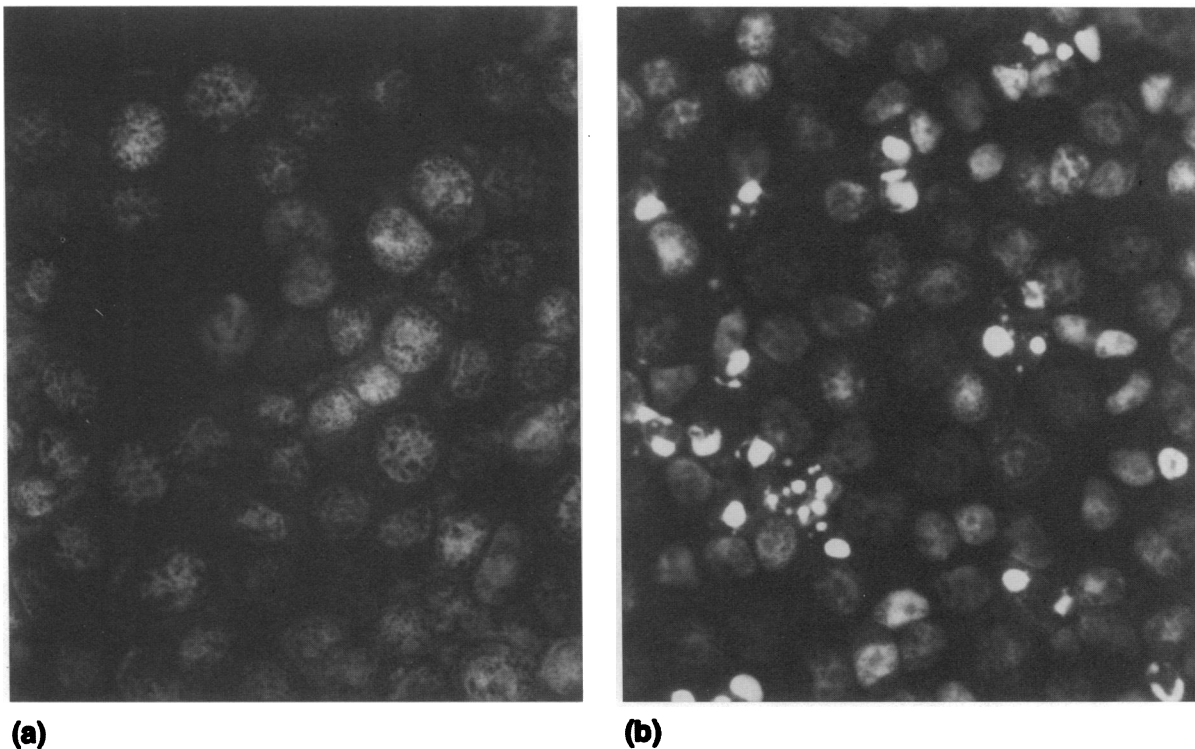


Figure 10. Fluorescence photomicrograph of HT-29 cells treated with sulindac sulfide. Control cells (a) and those treated with sulindac sulfide (175 μM) for 72 h (b) were stained with acridine orange and evaluated by UV fluorescent microscopy, as described in Methods. Changes in the cells typical of apoptosis: the presence of cytoplasmic and nuclear shrinkage; chromatin condensation; and cytoplasmic blebbing with maintenance of the integrity of the cell membrane (*zeiosis*) was seen in (b). $\times 40$.

these compounds directly inhibit the catalytic activity of p34^{cdc2} and p33^{cdk2}, sulindac (1,200 μM) and sulindac sulfide (175 μM) were added to the p34^{cdc2} and p33^{cdk2} IP-kinase assays from lysates of HT-29 cells incubated in control medium for 72 h. There was no difference in the associated histone H1 kinase activity in these reactions compared to those without the compounds added to the kinase buffer (data not shown).

Hence, in a concentration-dependent manner, though nonlinear and restricted to the higher concentrations tested, sulindac and sulindac sulfide: (a) induced a significant shift in the cell cycle phase distribution towards G₀/G₁; and (b) reduced the levels of three critical cell cycle regulators, p34^{cdc2}, p33^{cdk2}, and p34^{cdk4} in HT-29 colon adenocarcinoma cells. Furthermore, the reduction in the levels of p34^{cdc2} and p33^{cdk2} was also accompanied by a reduction in their catalytic activity. On a molar basis, sulindac sulfide was six times more potent than sulindac in bringing about these effects. These data indicate that critical cell cycle transition points, G₁ \rightarrow S, G₂ \rightarrow M and possibly the progression through G₁ were profoundly inhibited by treatment with these compounds. Given the known significance of inhibiting cdk function on cell cycle progression (17, 18), these results suggest that high concentrations of sulindac and sulindac sulfide increase the fraction of cells with G₀/G₁ DNA content by arresting them in the G₁ or G₀ phase of the cell cycle through an effect on the molecular components that regulate cell cycle transitions.

Sulindac sulfide induces apoptosis in HT-29 colon cancer cells and HL-60 promyelocytic leukemia cells

Treatment of HT-29 colon cancer cells with sulindac and sulindac sulfide reduced the net cell accumulation over time. Both

compounds also arrested a substantial fraction of these cells in the G₀ or G₁ phase of the cell cycle. This phenomenon could explain, in part, the anti-proliferative effect of high concentrations of these compounds on cultured HT-29 cells. However, we suspected that the anti-proliferative effect of higher concentrations of sulindac sulfide, in particular, might be multifactorial because a large percentage of HT-29 cells treated with high concentrations of sulindac sulfide developed less than a diploid amount of DNA on FACS analysis (Fig. 7 b). This sub-diploid peak in DNA content correlates with the morphologic and biochemical hallmarks of apoptosis (43, 44). Therefore, we sought to determine whether sulindac sulfide treatment induces apoptosis in HT-29 colon cancer cells.

HT-29 cells were treated with either control medium or sulindac sulfide- (100 μM , 150 μM , or 200 μM) supplemented medium for 72 h after which their morphology was evaluated by acridine orange staining. Sulindac sulfide, at concentrations that altered the cell cycle distribution of these cells, induced morphological hallmarks of apoptosis. As is evident in Fig. 10, a high fraction of 175 μM sulindac sulfide-treated cells exhibit nuclei with condensed and fragmented chromatin. In addition, the plasma membrane remained intact around these aberrant nuclei and, in some cells, the condensed DNA appeared to bud off from the remainder of the cell. Table II shows that after 72 h of sulindac sulfide ($\geq 150 \mu\text{M}$) treatment, 15–26% of the cells developed apoptotic changes. Table II also demonstrates that this effect was concentration dependent. Sulindac also induced apoptosis in a concentration-dependent manner, but to a far lesser degree and at higher concentrations than sulindac sulfide (Table II). When treated with control medium, sulindac 600 μM , or sulindac sulfide 100 μM , HT-29 cells did not exhibit

Table II. Apoptosis in HT-29 Cells Treated with Sulindac or Sulindac Sulfide

Drug	Percent apoptosis
μM	
Sulindac	
0	0
600	0.6 ± 0.2
1200	7.0 ± 1.6
Sulindac sulfide	
0	0.4 ± 0.2
100	0.1 ± 0.1
150	15.2 ± 2.4
175	15.5 ± 0.5
200	26.1 ± 3.2

The percentage of apoptosis, determined as described in Methods, after treatment with various concentrations of these compounds, is represented as the mean \pm SEM of at least three duplicate experiments.

appreciable levels of apoptosis. Thus apoptosis was prominent in sulindac- and sulindac sulfide-treated cells only at the higher concentration ranges which induced an effect on the cell cycle distribution of these cells.

The degradation of genomic DNA, another marker of apoptosis, was also analyzed to measure the apoptotic response of HT-29 cells to sulindac sulfide treatment. Fig. 11 *a* shows that treatment with 200 μM sulindac sulfide resulted in degradation of the genomic DNA of these cells, leading to a conspicuous smear below the predominant genomic DNA band. The DNA smear pattern in HT-29 cells was correlated with greater amounts of apoptosis seen after acridine orange staining (Fig. 10) and with the height of the sub-diploid peak in DNA content noted on FACS analysis (Fig. 7 and data not shown). Fig. 11 *b* also shows genomic DNA prepared in the same fashion from HL-60 cells treated for 4 h with either control medium or sulindac sulfide- (50–200 μM) supplemented medium. The HL-60

cells developed a nucleosomal ladder pattern of DNA degradation, considered distinctive of apoptosis, on treatment with higher concentrations of sulindac sulfide. A characteristic ladder pattern was also observed in sulindac sulfide-treated HT-29 cells when greater amounts of DNA were resolved on agarose gels (Fig. 11 *c*).

Thus, sulindac sulfide induced apoptosis in HT-29 colon adenocarcinoma cells in three assays commonly used to demonstrate this phenomenon.

Discussion

Our findings demonstrate that the NSAIDs sulindac and sulindac sulfide have profound effects on HT-29 colon adenocarcinoma cells in vitro. These compounds reduce the proliferation rate of these cells, cause changes in their morphology, induce them to accumulate in the G_0/G_1 phase of the cell cycle, and induce cell death by apoptosis. These responses are time-dependent, reversible, and are prominent at the higher concentrations of sulindac and sulindac sulfide tested. The response of these cells to sulindac is not due to extensive conversion to sulindac sulfide in the culture medium during incubation with the HT-29 cells.

Our findings that sulindac and sulindac sulfide have profound anti-proliferative effects on HT-29 colon adenocarcinoma cells are in agreement with previous studies demonstrating that many of the NSAIDs inhibit the proliferation of nonintestinal cultured cell lines (11, 12, 31, 48–51). For example, sulindac sulfide, one of the compounds we studied, inhibited the accumulation of lapine chondrocytes (51). Sulindac sulfide and, to a lesser degree, sulindac inhibit cyclooxygenase (prostaglandin synthetase) activity (52). However, whether inhibition of eicosanoid synthesis plays a role in the effect of sulindac or sulindac sulfide on cell proliferation remains unresolved (12, 53).

We also report that sulindac and sulindac sulfide cause a reversible change in the morphology of HT-29 cells. These morphological changes, long cytoplasmic extensions and intracytoplasmic vesicles, were similar to those reported by Neupert and Müller who treated rat embryo fibroblasts with indomethacin

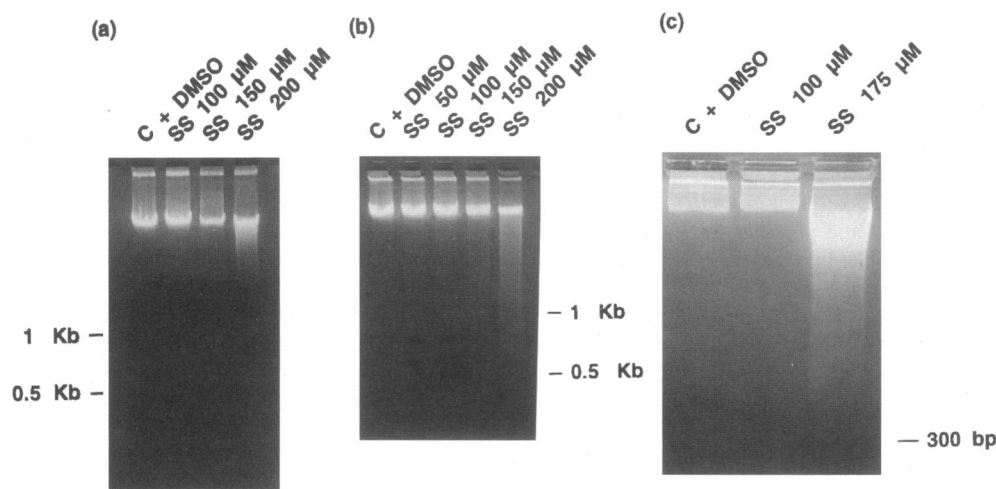


Figure 11. Electrophoresis of genomic DNA from HL-60 and HT-29 cells treated with sulindac sulfide. Genomic DNA was isolated from HT-29 colon adenocarcinoma cells (*a*) or HL-60 promyelocytic leukemia cells (*b*), and fractionated on 1.8% agarose gels, as described in Methods. HT-29 cells treated with sulindac sulfide 200 μM show a smear pattern which was not seen in control cells or those treated with sulindac sulfide 100 μM ; cells treated with sulindac sulfide 150 μM show a weak smear pattern (*a*). The typical ladder pattern often associated with apoptosis was seen when genomic DNA was isolated by the

same method from HL-60 cells treated with increasing concentrations of sulindac sulfide (*b*). A ladder pattern of DNA degradation was seen in DNA extracted from HT-29 cells treated with sulindac sulfide 175 μM when 10 μg of DNA was resolved on the agarose gels (*c*, lane 3). The position of DNA molecular weight standards are shown to the side of each panel. The DNA in each lane of *a* corresponds to 1×10^5 cells, whereas in *b* it corresponds to 2×10^5 cells, in *c* it corresponds to 10 μg . C, control; SS, sulindac sulfide.

cin (48). It is noteworthy that, in our experiments, the morphological changes caused by sulindac and sulindac sulfide occurred over the concentration ranges which also reduced the proliferation of the HT-29 cells.

Our study demonstrates that, in addition to reducing the proliferation and altering morphology, sulindac and sulindac sulfide alter the cell cycle distribution of HT-29 cells. Sulindac and sulindac sulfide caused an increase in the proportion of cells in the G₀/G₁ phase and a decrease in the percentage of cells in the S and G₂/M phases. This effect was reversible and occurred at the higher end of the concentration range that altered cell morphology and inhibited cell proliferation. Previous studies on nonintestinal cells have described a similar cell cycle effect in response to indomethacin treatment (11, 12, 31). In our work, the combination of cell type (colon) and class of NSAID (sulindac and sulindac sulfide) is important because of the striking human data that show the colon to be a key target for the action of these drugs (3–5).

The changes in the cell cycle phase distribution of HT-29 cells in response to sulindac and sulindac sulfide treatment, were accompanied by profound reductions in the level and activity of key proteins that regulate progression through the cell cycle. Sulindac and sulindac sulfide reduced the level of cdks (p34^{cdk2}, p33^{cdk2}, p34^{cdk4}), and also the *in vitro* kinase activity of p34^{cdk2} and p33^{cdk2}; we did not measure the *in vitro* kinase activity of p34^{cdk4} in this study. The relative deficiency in the activity of these key proteins may contribute to the accumulation of these cells in the G₀/G₁ phase following treatment with sulindac and sulindac sulfide. The reduced levels of these proteins may explain their reduced activity. However, the possibility that cdk inhibitors also play a role in this phenomenon has not yet been ruled out (15). The mechanisms through which these compounds influence the levels of these cdk proteins remains unknown. It is conceivable that exogenous sulindac and sulindac sulfide produce this effect by influencing signaling pathways in these cells that control the levels of these proteins. Regardless of the exact molecular mechanism involved, the reduction in the level and activity of certain cdks may be important for the anti-proliferative effect of these compounds.

Sulindac and, more potently, sulindac sulfide induce apoptosis in HT-29 colon adenocarcinoma cells. To our knowledge, this is the first report to document that a class of NSAIDs can induce cell death by apoptosis in colonic epithelial cells. Apoptosis in response to sulindac sulfide treatment was documented by showing the appearance of: (a) morphological changes characteristic of apoptosis; (b) a sub-diploid peak of DNA content on FACS analysis; and (c) genomic DNA degradation. Evidence that apoptosis was induced by sulindac was based on the detection of morphological changes only; no sub-diploid peak was noted on DNA content frequency histograms. The apoptosis induced by these compounds likely also contributes to their effect on the growth rate of HT-29 cells. Furthermore, additional studies we have performed, which will be reported elsewhere, have shown that sulindac sulfide induces apoptosis in other colon cancer cell lines such as HCT-15 (54).

Under the influence of higher concentrations of sulindac and sulindac sulfide, the cell cycle distribution, the activity of cell cycle kinases and the rate of apoptosis all changed in parallel. These responses occurred at concentrations of sulindac above 600 μ M and of sulindac sulfide above 100 μ M. However, the correlation between the effect of these compounds on cell proliferation and on these responses was not as strict at lower

concentrations. For example, sulindac sulfide 100 μ M did not produce a robust effect on the cell cycle phase distribution, the level and activity of the cdks, or on apoptosis at 72 h. Nevertheless, at this concentration, cell proliferation was inhibited by 42%. Similar observations were made with sulindac. Proliferation, as is measured in our model system, is likely the result of a complex interplay between many factors such as apoptosis, cell cycle progression, and other responses. Our data cannot ascertain, with precision, the individual contribution of these factors to the final outcome, i.e., proliferation. The effect of these two compounds on HT-29 cell proliferation at lower concentrations remains unexplained. However, the clear-cut responses of these cells to higher concentrations at 72 h likely affect cell proliferation. Clearly, further investigation is required to fully explain the anti-proliferative effect at lower concentrations of sulindac and sulindac sulfide.

The ability of sulindac sulfide to induce apoptosis in tumor cells is not restricted to those derived from the colon. Sulindac sulfide also produced apoptosis in HL-60 promyelocytic leukemia cells, at concentrations similar to those that induced apoptosis in HT-29 cells. Unlike colon cells that showed a smear pattern when DNA was size fractionated on agarose gels, apoptosis in HL-60 cells was accompanied by a typical DNA ladder pattern. The reason for this difference is unclear. Since apoptosis is a genetically programmed process, our findings suggest that the cellular pathways responsible for sulindac sulfide-induced apoptosis are likely to be present in non-intestinal cells as well. Wild-type p53 protein is thought to be an important mediator of apoptosis, especially in response to DNA damage (55, 56). Since HL-60 cells lack p53 protein (57) and HT-29 cells do not contain wild-type p53 protein (58), the apoptosis induced by sulindac sulfide is likely independent of wild-type p53 protein.

It remains to be seen whether the results presented here, obtained from an *in vitro* tissue culture study, can be extrapolated to humans. In fact, such an extrapolation cannot be made based on our data. However, it is possible that the concentrations of sulindac sulfide used here can be reached in the colon *in vivo*. For example, humans given sulindac sulfide, at doses that regress polyps in FAP patients, achieve plasma concentrations of sulindac sulfide of \sim 10–15 μ M (35, 36). Pharmacological studies have demonstrated that colon tissue levels of sulindac sulfide far exceed those in plasma; tissue to plasma ratios in the colon are in the range of 10–20 (33, 34). In addition, since approximately half of the sulindac sulfide in plasma originates from sulindac via bacterial metabolism in the colon (32), high concentrations of this compound reaches colon cells through the lumen. As a result of this analysis through luminal exposure and tissue accumulation, it is conceivable that high concentrations of sulindac sulfide, in the range that produced the cellular effects we observed *in vitro*, can be achieved in the colon of humans. Finally, since both compounds are highly protein bound (1), the effective concentration (or concentration of free molecules) in the culture dishes is really unknown.

Sulindac, in humans, reaches plasma levels of \sim 10 μ M but, in contrast to sulindac sulfide, it does not accumulate in colonic tissue (34). Thus, it is less likely that sulindac achieves tissue levels comparable to those that were effective in this study (1,200 μ M). However, the possibility that high luminal levels are reached in the microenvironment of the colonic crypts cannot be excluded. Overall, by virtue of its reported tissue accumulation and *in vitro* potency, sulindac sulfide appears the

more likely of the two compounds responsible for the biological changes that have been observed with sulindac in vivo.

It is also conceivable that apoptosis might occur in the human colon in response to sulindac. There is limited histologic evidence that treatment with mefenamic acid and diclofenac can induce apoptosis in the human colon. Lee has shown induction of apoptosis in the crypts of colorectal biopsy specimens from patients who developed gastrointestinal symptoms in response to these NSAIDs (59). The apoptosis noticed in his study, uncharacteristically however, was associated with an inflammatory cell infiltration in the lamina propria and the crypts.

Apoptosis in the gut mucosa may be relevant to the pathogenesis of gastrointestinal malignancies. Qualitative differences in apoptosis in the small intestine, as compared with that in the colon and rectum, may help to explain the disparate incidence rates of adenocarcinoma between these organs (60). Also of interest, sodium butyrate, a short-chain fatty acid generated in high concentrations in the colon by bacterial fermentation of dietary fiber (61) induced apoptosis in HT-29 cells (62). The inverse relationship between fruit and vegetable fiber intake in the diet and colorectal cancer is generally accepted (63). Whether apoptosis plays a role in this process is an interesting hypothesis, but at this time, there is no data to support it.

In summary, we conclude that the NSAIDs sulindac and sulindac sulfide have anti-proliferative effects on cultured HT-29 colon cancer cells in vitro. Relatively high concentrations of sulindac and sulindac sulfide cause a reversible accumulation of cells in the G₀ or G₁ phase of the cell cycle that is accompanied by reduced levels and activity of certain cdks. In addition, similar concentrations of these compounds induce apoptosis in HT-29 cells. Sulindac sulfide is approximately six times more potent than sulindac in inducing these responses. The induction of apoptosis and cell cycle quiescence are both anti-proliferative responses that likely contribute to the inhibition of HT-29 cell proliferation observed when they are treated with high concentrations of these compounds. We speculate that these responses may also contribute to the cancer preventive and tumor regressive properties of sulindac in vivo.

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