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Apoptosis Primarily Accounts for the Growth-inhibitory Properties of Sulindac Metabolites and Involves a Mechanism That Is Independent of Cyclooxygenase Inhibition, Cell Cycle Arrest, and p53 Induction[1]

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ABSTRACT

Sulindac causes regression of and prevents recurrence of colonic adenomas in patients with familial adenomatous polyposis. Although cell cycle arrest and apoptosis have been proposed, the mechanism of action is poorly understood. In this study, we characterized the growth-inhibitory effects of active metabolites of sulindac in cultured colon adenocarcinoma cells by determining the contribution of apoptosis and cell cycle arrest and the requirement for cyclooxygenase (COX) inhibition and p53 involvement and compared the effects of sulindac metabolites with the chemotherapeutic drug, 5-fluorouracil (5-FU). Time course and dose-response experiments demonstrated that increased apoptosis paralleled the growth-inhibitory effects of the sulfide and sulfone. A relationship among a series of nonsteroidal anti-inflammatory drugs was observed between potency for growth inhibition and ability to induce apoptosis but not potency to inhibit COX. For example, the sulfone was at least 5000-fold less potent than the sulfide for inhibiting COX but only 6.5-fold less potent for inducing apoptosis. Moreover, the prostaglandin analogue, dimethyl-prostaglandin E_2, failed to reverse the apoptosis-inducing effects of the sulfide. Sulindac metabolites caused G_1 cell cycle arrest in proliferating cells but were comparably effective in nonproliferating cells. In contrast, 5-FU treatment was less effective in nonproliferating cells. Combined treatment with sulindac metabolites and 5-FU did not result in an additive apoptotic response. Treatment of cells with 5-FU increased p53 protein levels, whereas sulindac metabolites did not induce expression. Saos-2 cells, which lack p53, responded to sulindac metabolites but not 5-FU. These results show that apoptosis primarily contributes to growth inhibition by sulindac metabolites. The biochemical pathway does not require COX inhibition or p53 induction and appears to be fundamentally different from the apoptotic response to 5-FU.

INTRODUCTION

Over the past 20 years, it has become apparent from experimental models of carcinogenesis that NSAIDs[3] have cancer chemopreventive properties, although their application to human cancer and the extent of their benefits in the clinic is presently a matter of intense investigation. Early evidence that NSAIDs have chemopreventive efficacy came from rodent models of carcinogenesis that demonstrated that certain NSAIDs inhibit the growth of transplanted tumors (1, 2) or chemical- and radiation-induced carcinogenesis (3–12). Separately, Waddell and Loughry (13) in 1983 and Waddell *et al.* (14) later in 1989 demonstrated provocative evidence that one NSAID, sulindac, caused regression of and prevented recurrence of adenomatous colorectal polyps in patients with FAP. Subsequently, several controlled clinical trials confirmed Waddell's observations and demonstrated the utility of sulindac for treating precancerous lesions in FAP patients (15–22).

An explanation for the antineoplastic properties of NSAIDs was first suggested by Adolphie *et al.* (22) in 1972, who reported that certain NSAIDs were capable of inhibiting the proliferation of cultured HeLa cells by causing cell cycle arrest. On the basis of the observation that indomethacin and aspirin inhibited the growth of transplanted tumor cells, several additional reports were published that showed that NSAIDs inhibit the growth of tumor cells in culture and that the cellular mechanism may involve an arrest of the cell cycle in G_1 (23–25). There have, however, been conflicting reports from *in vivo* studies regarding the possibility that inhibition of colonic epithelial cell proliferation accounts for the ability of sulindac to either cause regression of adenomas and/or prevent their recurrence in FAP patients (19, 20, 26). Recently, several groups have shown that certain NSAIDs induce apoptosis of various cultured tumor cell lines (27–30). Although there has been no direct *in situ* evidence to indicate that, for example, regressing adenomas display increased rates of apoptosis, Pasricha *et al.* (26) demonstrated that colonocyte cell suspensions prepared from mucosa biopsies of FAP patients treated with sulindac displayed higher levels of apoptosis relative to mucosa biopsies obtained from the patients prior to treatment. Studies showing that apoptosis is altered during the progression of colorectal cancer (31) provide further support for the possibility that apoptosis contributes to the antineoplastic properties of NSAIDs. Because both cell cycle arrest and increased apoptosis have been reported to occur under similar conditions in cell culture models (27, 28), it is conceivable that both a reduction of cell proliferation and an increase in cell death occur in response to sulindac treatment *in vivo*. However, from a mechanistic point of view, it is not clear if these two processes are linked whereby apoptosis occurs in response to cell cycle arrest.

The anti-inflammatory properties of NSAIDs are known to be mediated by COX inhibition (32), and many have attributed their antineoplastic properties to reduction of prostaglandin levels in the target tissue (33). On the basis of studies involving sulindac metabolites, some investigators have recently questioned the involvement of COX inhibition in mediating the colon cancer chemopreventive properties of NSAIDs (34). Sulindac is a prodrug that is metabolized after p.o. administration to either a sulfide or sulfone derivative. The sulfide is known to be a potent inhibitory agent of COX and is exclusively responsible for the anti-inflammatory properties of sulindac (35). The sulfone, on the other hand, does not inhibit COX, types I or II (36), and does not have anti-inflammatory properties (35). In studies involving rodent models of chemically induced mammary (36) and colon (37)[4] carcinogenesis, direct administration of the sulfone in the diet was shown to result in a chemoprotective benefit similar to

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[3] The abbreviations used are: NSAID, nonsteroidal anti-inflammatory drug; FAP, familial adenomatous polyposis; COX, cyclooxygenase; 5-FU, 5-fluorouracil; IC_{50}, 50% inhibitory concentration; EC_{50}, 50% effective concentration; PGE_2, prostaglandin E_2; SRB, sulforhodamine B.

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sulindac. The possibility that COX inhibition does not mediate the antineoplastic properties of NSAIDs is of considerable clinical significance because reduction of prostaglandin levels is known to be responsible for the gastrointestinal and renal toxicity that accompanies chronic NSAID administration (38, 39). If COX inhibition is not necessary or sufficient for the antineoplastic properties of NSAIDs, then it should be feasible to develop less toxic NSAID-like drugs for treating patients with FAP.

Recent advancements in the understanding of cell cycle and apoptosis regulation suggest a myriad of potential targets that could be responsible for the antineoplastic properties of NSAIDs. The tumor suppressor gene product, p53, is a key component in regulating cell cycle progression, and many apoptotic stimuli are known to involve a p53-dependent pathway (40). For example, the ability of cancer chemotherapeutic drugs and other DNA-damaging agents (*i.e.*, ionizing radiation) to cause cell cycle arrest and induce apoptosis has previously been shown to occur by a mechanism involving overexpression of p53 (41). However, p53-independent pathways of apoptosis have also been described (40). Because sulindac metabolites cause cell cycle arrest in G_1 similar, for example, to the chemotherapeutic drug, 5-FU, it is possible that these two classes of drugs share a common intracellular pathway for activating cell death processes. The present study was undertaken using cultured human colon adenocarcinoma cells as a model to investigate the mechanism responsible for tumor cell growth inhibition by sulindac metabolites and to compare the effects of sulindac metabolites with 5-FU.

MATERIALS AND METHODS

Drug Treatments and Preparation. The sulfide and sulfone metabolites of sulindac were synthesized as described previously (27, 42). Aspirin, salicylic acid, tolmetin, sulindac sulfoxide, diclofenac, dimethyl-PGE_2, and 5-FU were purchased from Sigma Chemical Co. (St. Louis, MO). Naproxen, ibuprofen, piroxicam, and indomethacin were purchased from Biomol (Plymouth Meeting, PA). Stock solutions of sulindac metabolites, NSAIDs, and chemotherapeutic drugs were made at 1000× concentrations in 100% DMSO (Sigma) and then diluted with RPMI media for cell culture testing. The final concentration of DMSO for all treatments was maintained at 0.1%. All drug solutions were prepared fresh on the day of testing.

Cell Culture and Dosing Schedules. The cell lines, HT-29, SW-480, and Saos-2, were obtained from American Type Culture Collection (Rockville, MD) and grown in RPMI media (Celox, Hopkins, MN) supplemented with 5% FCS (Gemini Bio-Products, Inc., Calabases, CA) and 2 mM glutamine, 100 units/ml penicillin, 100 units/ml streptomycin, and 0.25 μg/ml amphotericin obtained from Life Technologies, Inc. (Grand Island, NY). Cultures were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. The cultures were passaged at preconfluent densities using a solution of 0.05% trypsin and 0.53 mM EDTA (Celox). All experiments involved cells that were passaged no more than 10 times. Cells were plated at the following densities to obtain cultures used for the experiments: 500 or 10,000 cells/well for 96-well flat-bottomed microtiter plates as indicated, 0.5×10^6 cells per 12.5 cm^2 flask, 1×10^6 cells per 25 cm^2 flask, or 4×10^6 cells per 75 cm^2 flask. Assays using preconfluent conditions (SRB and DNA fragmentation assays) involved treating cells by adding the appropriate amount of drug stock solution directly to the medium the day following plating at the above densities. Assays requiring confluent conditions were plated at the densities listed above and allowed to grow 10 days to confluence before dosing. Cells were dosed and replenished with fresh medium on day 10. For some experiments, treatment effects were compared between proliferating and nonproliferating cultures. Treatment of proliferating cultures was performed as described above for confluent cultures. Treatment of nonproliferating cultures was performed under the same conditions, except the cultures were dosed 48 h after medium was replenished (day 12), at which time the majority of the cell population was in G_1.

Growth Inhibition. The growth-inhibitory effect of sulindac metabolites, NSAIDs, and chemotherapeutic drugs was determined by either a reduction of viable cell number after trypan blue staining or by the SRB protein-binding assay. Trypan blue stain was determined from either 25- or 75-cm^2 flasks, and both attached and floating cells were evaluated. After treatment, the medium was withdrawn, and the attached cells were trypsinized for 5 min at 37°C, centrifuged for 15 min ($300 \times g$), and resuspended in the original medium containing floating cells. An aliquot was combined with an equal volume of trypan blue dye (Sigma) and counted using a hemacytometer. Trypan blue-excluding cells were counted, and results were expressed as viable cell number. The SRB assay was performed on cells cultured and treated in 96-well microtiter plates as described previously (27, 43).

Cell Cycle Distribution. The proportion of cells in G_1, S, and G_2-M phases of the cell cycle was determined by flow cytometric analysis of DNA content. Cell cycle distribution was measured after 24 h of treatment with either sulindac metabolites or 5-FU. DNA content was then determined after labeling cells with propidium iodide as described previously (44). In brief, cell suspensions from confluent HT-29 cell cultures were prepared by trypsinization and washing two times with PBS, followed by centrifugation at $300 \times g$. Cells (1×10^6) were labeled by resuspension in a 1-ml solution containing 4 mM sodium citrate, 50 μg/ml propidium iodide, 0.02% NP40, and 20 μg/ml RNase (Sigma). The suspension was incubated overnight at 4°C to allow maximum labeling of the DNA. Total DNA content per cell was quantified by fluorescence at 585 nm using a Coulter's Epic 752 model flow cytometer. The resulting histogram was analyzed using ModFit software (Verity House Software, Topsham, ME).

Morphological Measurement of Apoptosis. Confluent cultures were assayed for apoptosis and necrosis by fluorescence microscopy following labeling with acridine orange and ethidium bromide, as described by Duke and Cohen (45). Floating and attached cells were collected as described above and washed three times in PBS. One-ml aliquots of 1×10^6 cells were centrifuged ($300 \times g$), the pellet was resuspended in 25 μl of media, and 1 μl of a dye mixture containing 100 μg/ml acridine orange and 100 μg/ml ethidium bromide prepared in PBS, and mixed gently. Ten μl of mixture were placed on a microscope slide and covered with a 22-mm^2 coverslip and examined under ×40 dry objectives using epillumination and filter combination. An observer blinded to the identity of treatments scored at least 200 cells/sample. Live cells were determined by the exclusion of ethidium bromide stain. Live and dead apoptotic cells were identified by nuclear condensation of chromatin stained by the acridine orange or ethidium bromide, respectively. Necrotic cells were identified by uniform labeling of the cell with ethidium bromide.

Measurement of Apoptosis by DNA Fragmentation. In some experiments, apoptosis was measured by the level of fragmented DNA contained in cell lysates following treatment with sulindac metabolites or other NSAIDs. The method for measuring fragmented DNA used a commercially available photometric enzyme-immunoassay kit (Cell Death Detection ELISA ^{Plus}; Boehringer-Mannheim, Mannheim, Germany). The immunoassay involved mouse monoclonal antibodies directed against DNA and histones, respectively, which allowed for the determination of mononucleosomes and oligonucleosomes in the soluble fraction of cell lysates. Cells were plated at a density of 10,000 cells/well in 180 μl of medium to 96-well microtiter plates and incubated for 24 h. Cells were then treated with 20-μl aliquots of appropriately diluted compounds. After 48 h of treatment, the microtiter plate was centrifuged (15 min; $300 \times g$) to collect both floating and attached cells. The cell pellets were then lysed and assayed for fragmented DNA using the protocol specified by the manufacturer. Absorbance was determined by absorbance (405–490 nm) using a SpectraMax 340 microplate reader (Molecular Devices, Sunnyvale, CA). EC_{50}s were determined by data analysis software (Softmax Pro; Molecular Devices, Inc.). Fold induction of apoptosis was calculated by dividing the maximal absorbance from the test compound with the absorbance from vehicle treatment.

COX Assay. COX inhibitory activity of a panel of NSAIDs was determined by a protocol essentially as described previously (46). In brief, prostaglandin H synthetase 1 (Cayman Chemical, Ann Arbor, MI) 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°C for 20 min in the presence of various NSAIDs or their solvent (1% DMSO, final concentration). The reaction was terminated by the addition of trichloroacetic acid. Enzyme activity was measured by the thiobarbituric acid color reaction of malonaldehyde formed in the reaction and

determined by a spectrophotometer at 530 nm. IC_{50}s were calculated from a five-point dose-response curve.

p53 Western Blot. For measuring p53 levels, whole-cell lysates were obtained from HT-29 or Saos-2 cell cultures that had been treated for 6 days as described above. Proteins (20 μg) from SDS-treated samples were separated by SDS-PAGE on a 12% resolving gel, electroeluted to nitrocellulose membranes, and probed for p53 protein using an antibody that cross-reacts with both wild and mutant p53 (Oncogene Science, Inc., Uniondale, NY). The complex was bound to peroxidase-coupled sheep antimouse antibody (Amersham Corp., Arlington Heights, IL), and the reaction product was visualized by the Enhanced Chemiluminescence detection kit for Western blotting (Amersham).

RESULTS

Growth Inhibition and Apoptosis. Time course experiments were performed, and simultaneous measurements of viable cell number and apoptosis were made to determine the kinetics of sulindac sulfide-induced apoptosis in relation to growth inhibition (*i.e.*, reduction of viable cell number). Fig. 1*a* shows that total viable cell number in vehicle-treated HT-29 cell cultures increased by approximately 3-fold within 4 days. Relative to control cultures, cultures treated with sulindac sulfide (120 μM) displayed a 6-fold reduction of viable cell number after 4 days of treatment. To directly compare apoptosis with growth inhibition, apoptosis measurements were made from the same cultures used to measure viable cell number. As shown in Fig. 1*b*, sulindac sulfide treatment markedly increased the percentage of cells displaying morphological characteristics of apoptosis, and this effect accompanied in time the reduction of viable cell number. For example, after 24 h of treatment with sulindac sulfide, at least 50% of the total cell population was apoptotic, and there was an approximate 40% reduction in viable cell number. After 48 h of treatment, the percentage of apoptotic cells increased to approximately 80% of the total cell population, at which time a maximal reduction of viable cell number was observed. Treatment with sulindac sulfide at 120 μM did not increase the percentage of cells displaying necrotic characteristics (*i.e.*, labeled by ethidium bromide) within the duration of the experiment (data not shown).

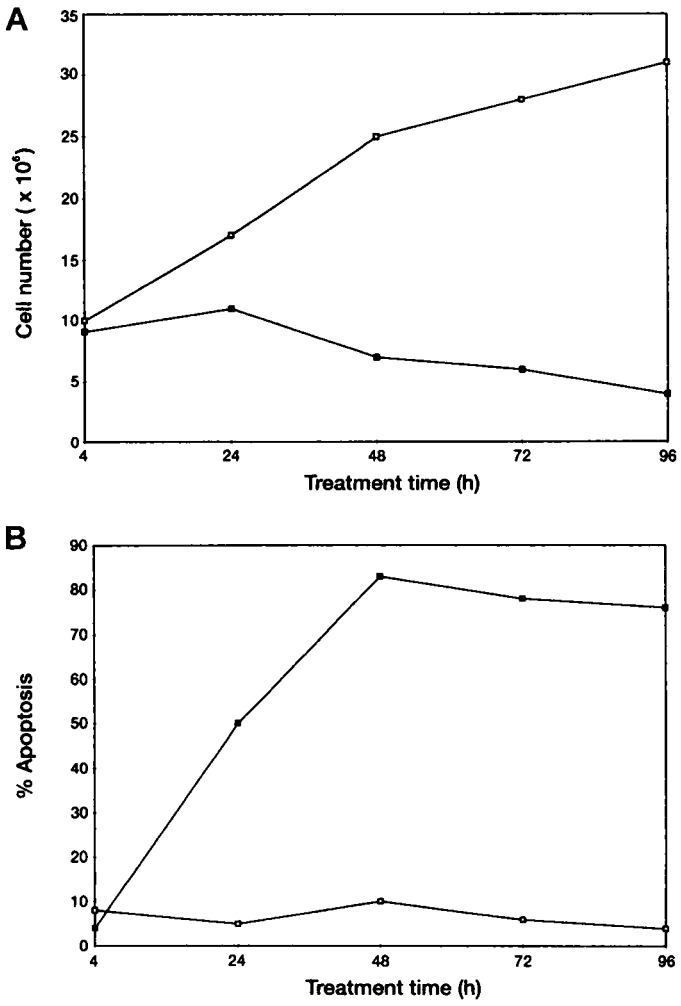

Fig. 1. Time course for growth inhibition (*A*) and apoptosis (*B*) of HT-29 cells after treatment with sulindac sulfide. HT-29 cells (4 × 10^6 cells) were established in 75-cm^2 flasks and allowed to growth for 3 days prior to treatment. Treatment was initiated at time 0 with either sulindac sulfide (final concentration, 120 μM; ■) or vehicle (0.1% DMSO; □), which was added with fresh medium. After the indicated time, the number of viable and apoptotic cells that were attached or floating was determined from the same flask as described in "Materials and Methods." The data shown are from one of three representative experiments. All data shown were collected from the same experiment.

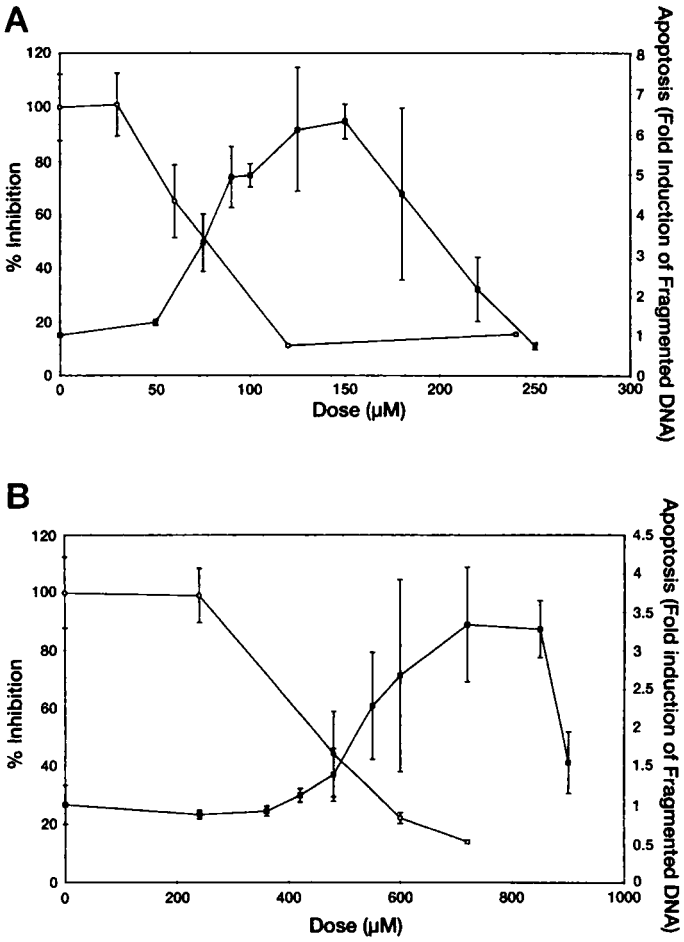

Fig. 2. Sulindac sulfide (*A*) and sulfone (*B*) growth inhibition and DNA fragmentation in Sw-480 cells. SW-480 cells were plated at a density of 10,000 cells/well in 96-well microtiter plates and allowed to grow 1 day prior to initiating treatment. Growth inhibition (□; *left axis*) was measured by the SRB assay after 6 days of treatment, and apoptosis was measured by DNA fragmentation (■; *right axis*) after 48 h of treatment as described under "Materials and Methods." Growth inhibition and DNA fragmentation was determined within the same experiment. *Bars*, SD.

In addition to morphological evaluation, apoptosis induction by sulindac metabolites was independently confirmed using an assay developed to measure DNA fragmentation in monolayer cultures; DNA fragmentation is a known biochemical indicator of programmed cell death (47). A dose-response experiment as in Fig. 2 shows that treatment of SW-480 cells with either sulindac sulfide or sulfone for 48 h significantly increased levels of fragmented DNA by approximately 6- and 3-fold, respectively. The EC_{50} values calculated from

Table 1 *Growth-inhibitory, apoptosis-inducing, and COX inhibitory activity of a series of NSAIDs*

Treatment	Growth inhibition[a] (IC_{50}, μM)	Apoptosis[b] (EC_{50}, μM)	COX inhibition[c] (IC_{50}, μM)
Aspirin	>3,000	>2,500	235
Salicylic acid	>3,000	>2,000	>10,000
Naproxen	>1,000	>750	103
Ibuprofen	600	>650	474
Tolmetin	550	>1,000	55
Sulindac sulfoxide	380	>550	>10,000
Sulindac sulfone	250	475	>10,000
Diclofenac	90	100	0.4
Indomethacin	75	75	1.4
Sulindac sulfide	50	65	1.8

[a] IC_{50}s of drugs for inhibiting HT-29 cell growth were determined from a 6-point dose-response curve (duplicate samples) by the SRB assay as described under "Materials and Methods."

[b] EC_{50}s for drugs to induce apoptosis of HT-29 cells were determined from a 6-point dose-response curve (duplicate samples) by the DNA fragmentation assays as described under "Materials and Methods."

[c] IC_{50}s for inhibiting COX (type 1) were determined from a 5-point dose-response curve (duplicate samples) as described under "Materials and Methods."

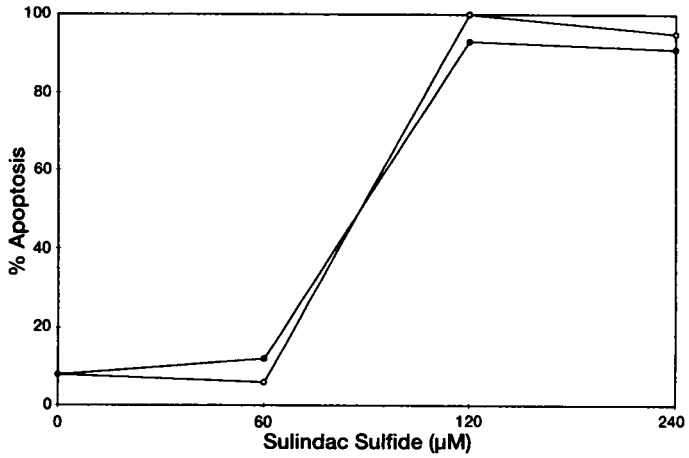

Fig. 3. Effect of dimethyl-PGE_2 on sulindac sulfide induction of apoptosis. HT-29 cells (1×10^6 cells) were plated in 25-cm^2 flasks, allowed to grow for 10 days, and treated with the indicated dose of sulindac sulfide in the presence (■) or absence (□) of dimethyl-PGE_2 (5 μM) for 6 days. Apoptosis was measured by morphology as described under "Materials and Methods."

four different experiments were highly reproducible, with values of 65 μM for the sulfide and 425 μM for the sulfone. Dose escalation with both sulindac sulfide (*i.e.*, >200 μM) and sulfone (*i.e.*, >800 μM) caused the DNA fragmentation response to become diminished. This was likely the result of necrotic cell death because these doses increased the percentage of cells uniformly labeled with ethidium bromide, as determined by morphology assays of apoptosis (data not shown).

To confirm that the dose of sulindac metabolites effective for increasing DNA fragmentation corresponded to the active dose range for inhibiting cell growth, cell number was measured by the SRB assay in corresponding cultures used for measuring DNA fragmentation as described above. As shown in Fig. 2, treatment for 6 days with either sulindac sulfide or sulfone reduced SRB staining, and this effect occurred within the same dose range that was effective for increasing DNA fragmentation.

To further determine whether apoptosis accounts for NSAID inhibition of cell growth, a series of NSAIDs with different potencies for growth inhibition were evaluated for their ability to induce apoptosis. As summarized in Table 1, compounds such as aspirin, salicylic acid, and naproxen, which failed to inhibit HT-29 cell growth, also failed to induce apoptosis within the dose range evaluated. Other compounds such as tolmetin, ibuprofen, and sulindac sulfoxide inhibited HT-29 cell growth but with low potency. These compounds also failed to induce apoptosis, even when tested at concentrations appreciably higher than their IC_{50} for growth inhibition. By comparison, compounds such as sulindac sulfide and sulfone, diclofenac, and indomethacin inhibited cell growth with higher potency and induced apoptosis with an EC_{50} value comparable to their IC_{50} value for growth inhibition. None of the compounds caused necrotic cell death at doses that were growth inhibitory (data not shown).

Requirement for COX Inhibition. Using the same series of NSAIDs evaluated above, we determined if potency for COX inhibition correlated with their potency to inhibit cell growth and induce apoptosis. As summarized in Table 1, there was no apparent relationship among these compounds between their potency to inhibit COX and their potency to inhibit cell growth or induce apoptosis. The majority of compounds that were capable of inhibiting COX required appreciably higher concentrations to inhibit cell growth and induce apoptosis. Most striking were the effects of compounds such as sulindac sulfone and sulfoxide that essentially lacked COX-inhibitory activity, yet were capable of inhibiting cell growth at doses comparable to other drugs showing high potency for COX inhibition.

Add-back experiments were also conducted to determine whether the stable prostaglandin analogue, dimethyl-PGE_2, could reverse or limit the apoptosis-inducing activity of an active NSAID, such as sulindac sulfide. As shown in Fig. 3, the apoptosis-inducing activity of sulindac sulfide in HT-29 cells was not affected by exogenous dimethyl-PGE_2. Dimethyl-PGE_2 treatment alone did not affect apoptosis. PGE_2 and the prostaglandin precursor, arachidonic acid, also did not reverse the growth-inhibitory effects of sulindac sulfide (data not shown).

Table 2 *Effect of sulindac metabolites on cell cycle distribution, cell growth, and apoptosis in resting and proliferating HT-29 cells*

Treatment	Cell cycle distribution[a]			Cell growth[b]		Apoptosis[c]	
	%G_1	%S	%G_2-M	Cell no.	% reduction	% apoptotic cells	Fold induction
Proliferating cells[d]							
Vehicle	42.5	35.4	22.1	5.98×10^6		8	
Sulfide (120 μM)	95.7	4.4	2.1	1.50×10^5	97.5	71	8.9
Sulfone (480 μM)	85.8	11.9	2.3	8.0×10^5	84.6	71	8.9
Nonproliferating cells[e]							
Vehicle	91.1	4.2	4.7	3.81×10^6		11	
Sulfide (120 μM)	89.7	4.4	5.8	8.01×10^5	79.0	90	8.2
Sulfone (480 μM)	85.4	4.4	10.1	1.27×10^6	66.7	58	5.3

[a] Determined from duplicate flasks after 24 h of treatment.

[b] Determined from duplicate flasks after 6 days of treatment.

[c] Determined from duplicate flasks after 6 days of treatment using the same flasks used to measure viable cell number.

[d] HT-29 cells (12.5-cm^2 flasks) were grown until day 10, and medium was replenished. Treatment was initiated on day 10.

[e] Same as proliferating cultures except treatment was initiated on day 12.

Table 3 *Effect of 5-FU on cell cycle distribution, cell growth, and apoptosis in resting and proliferating HT-29 cells*

Treatment	Cell cycle distribution[a]			Cell growth[b]		Apoptosis[c]	
	%G_1	%S	%G_2-M	Cell no.	% reduction	% apoptotic cells	Fold induction
Proliferating cells[d]							
Vehicle	41.9	44.6	13.5	4.45×10^6		12	
5-FU (50 μM)	92.8	6.5	0.1	5.90×10^5	86.8	75	6.3
Nonproliferating cells[e]							
Vehicle	81.7	12.6	9.3	2.65×10^6		15	
5-FU (50 μM)	76.8	17.8	5.4	1.55×10^6	41.5	42	2.8

[a] Determined from duplicate flasks after 24 h of treatment.
[b] Determined from duplicate flasks after 6 days of treatment.
[c] Determined from duplicate flasks after 6 days of treatment using the same flasks used to measure viable cell number.
[d] HT-29 cells (12.5-cm^2 flasks) were grown until day 10, and medium was replenished. Treatment was initiated on day 10.
[e] Same as proliferating cultures except treatment was initiated on day 12.

Requirement for Cell Cycle Arrest and Comparison with Chemotherapeutic Drugs. To determine the relative contribution of cell cycle arrest and apoptosis to growth inhibition by sulindac metabolites, simultaneous measurements of cell cycle distribution, apoptosis, and viable cell number were performed under conditions involving either rapidly proliferating or nonproliferating HT-29 cell cultures. Established cultures of HT-29 cells contain greater than 90% of cells in G_1 as they reach confluence and/or exhaust nutrients in the medium (27). Replenishment of such cultures with fresh medium simulates a synchronized progression into S phase, with approximately 60% of the cell population in S and G_2-M phase 24 h after medium replenishment. Greater than 90% of the cells return back to G_1 within 48 h after medium replenishment and remain in G_1 phase for up to 7 days in culture. Table 2 shows the comparative effect of treatment with sulindac sulfide or sulfone if the drugs were added at the same time as medium replenishment (*i.e.*, proliferating cultures) or 48 h after medium replenishment (*i.e.*, nonproliferating cultures). In proliferating cultures, sulindac metabolites effectively blocked cell cycle progression as determined by measuring cell cycle distribution after 24 h of treatment. Under these conditions and after 6 days of treatment, sulindac sulfide and sulfone reduced viable cell number by 97.5 and 84.6%, respectively, and induced apoptosis to a comparable level (8.9-fold). As expected, treatment of nonproliferating cultures with sulindac metabolites did not alter cell cycle progression. However, under these resting conditions, sulindac sulfide and sulfone inhibited cell growth by 79 and 66.7%, respectively, and induced apoptosis by 8.2- and 5.3-fold, respectively. These results demonstrate that sulindac metabolites are capable of causing appreciable growth inhibition and apoptosis under conditions where cell cycle arrest does not occur. The effectiveness of 5-FU for inhibiting cell growth and inducing apoptosis in proliferating and nonproliferating cultures was next determined. As summarized in Table 3, 5-FU treatment of proliferating cultures caused G_1 arrest, reduction of viable cell number (86.8%), and induction of apoptosis (6.3-fold) to a level comparable to sulindac sulfide. In contrast to treatment with the sulfide or sulfone, which caused comparable effects on proliferating and nonproliferating cultures, 5-FU treatment was appreciably less effective in nonproliferating cultures (42% reduction in viable cell number; 2.8-fold induction of apoptosis) relative to proliferating cultures.

To test the possibility that 5-FU and sulindac metabolites share common pathways for the induction of apoptosis, the effects of combined treatment on apoptosis were evaluated. As shown in Fig. 4, sulindac sulfone or sulfide induced apoptosis in approximately 40–50% of the cell population in the absence of 5-FU. Combination treatment with 5-FU did not result in a greater apoptotic response compared with 5-FU treatment alone.

p53 Induction. To determine whether p53 is induced during the apoptotic response to sulindac metabolites and 5-FU, the expression of p53 protein was measured by Western blotting using whole-cell extracts prepared from treated HT-29 cell cultures. Levels of p53 were measured in the same cultures where the percentage of apoptotic cells had been predetermined to directly compare expression levels with the degree of apoptosis. As shown in Fig. 5, treatment of HT-29 cells with sulindac sulfide (120 μM) and 5-FU (50 μM) markedly induced apoptosis to comparable levels. Sulindac sulfone (480 μM) also induced apoptosis, but the effect was less pronounced. Densitometric scanning of multiple gels demonstrated that sulfone treatment did not alter p53 expression, whereas 5-FU treatment significantly increased expression by greater than 3.5-fold. Sulfide treatment, on the other hand, decreased p53 levels by approximately 50%.

An osteosarcoma cell line, Saos-2, which lacks functional p53 (48), was used to assess the potential involvement of p53 in the apoptotic response to sulindac metabolites and 5-FU. Saos-2 cells where grown under the same conditions as HT-29 cells and treated with similar doses of drugs to directly compare the response of the two cell lines to drug treatment. As shown in Fig. 6, sulindac metabolites induced apoptosis of Saos-2 cells to a level comparable to HT-29 cells (Fig. 5). By contrast, 5-FU treatment caused an approximate 6-fold induction of apoptosis in HT-29 cells but caused less than a 2-fold induction in Saos-2 cells at the same dose. To confirm that Saos-2 cells lacked p53 protein, extracts from HT-29 and Saos-2 cells were probed for p53 by Western blotting. Although p53 could readily be detected in extracts from vehicle-treated HT-29 cells, no p53 could be detected in either vehicle- or drug-treated Saos-2 cell extracts (data not shown).

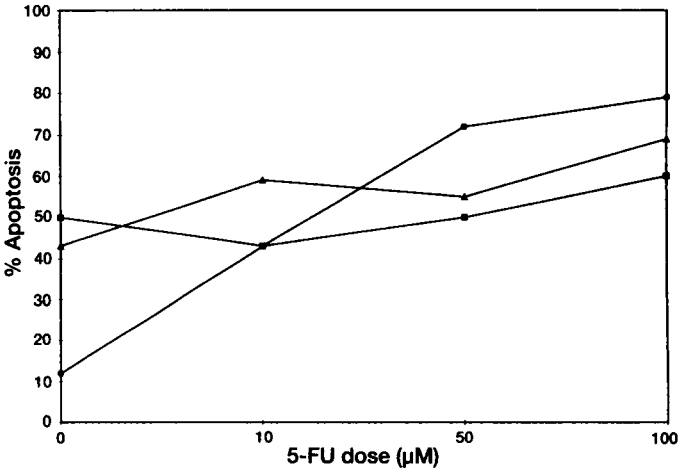

Fig. 4. Effects of sulindac sulfide or sulfone on 5-FU-induced apoptosis of HT-29 cells. HT-29 cells (1×10^6 cells) were plated in 25-cm^2 flasks, allowed to grow for 10 days, and treated with the indicated dose of 5-FU in the absence (●) or presence of 60 μM sulindac sulfide (□) or 240 μM sulfone (▲) for 6 days. Apoptosis was determined by morphology as described under "Materials and Methods."

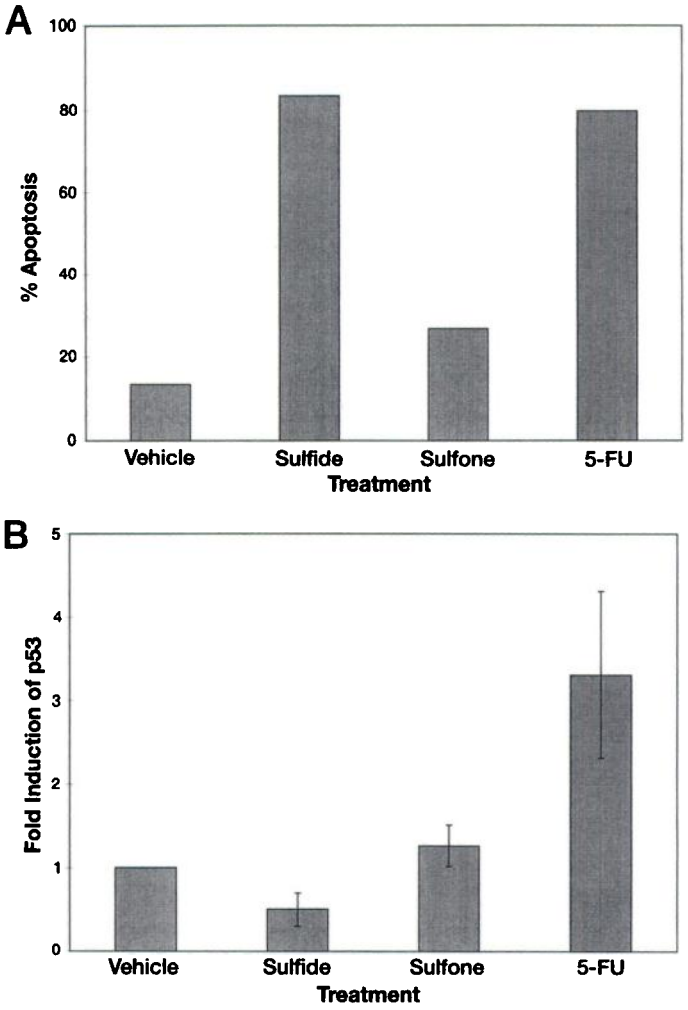

Fig. 5. Effects of sulindac metabolites and 5-FU on apoptosis (*A*) and p53 expression (*B*) in HT-29 cells. HT-29 cells (4×10^6 cells) were plated in 75-cm^2 flasks, allowed to grow 10 days, and treated with sulindac sulfide (120 μM), sulfone (480 μM), or 5-FU (50 μM) for 6 days. Apoptosis was determined by morphology as described under "Materials and Methods" and represents the average of two separate experiments. p53 protein levels were determined from the same cultures as used for apoptosis measurements and detected by Western blotting as described under "Materials and Methods." Fold induction of p53 was quantified by densitometric scanning of the area of the p53 protein band from four different gels (two different cell preparations). *Bars*, SD.

DISCUSSION

Using cultured human colon adenocarcinoma cells, the observations described in this study demonstrate that apoptosis primarily accounts for the growth-inhibitory activity of sulindac metabolites. Experiments involving simultaneous measurement of apoptosis and viable cell number showed that increased apoptosis accompanies growth inhibition in time and that doses effective for both processes are comparable. Moreover, a correlation was observed among a series of NSAIDs between their potency to inhibit cell growth and ability to induce apoptosis. Although sulindac metabolites are capable of causing cell cycle arrest under conditions involving mitogenic stimulation, we observed appreciable growth inhibition and apoptosis under conditions where cells were maintained in G_1 throughout the course of treatment.

COX inhibition does not appear to be necessary or sufficient for the growth-inhibitory or apoptosis-inducing properties of NSAIDs. Sulindac sulfone, for example, which lacks COX-inhibitory activity at concentrations up to 10 mM, inhibited cell growth and induced apoptosis similar to the sulfide that inhibits COX at an IC_{50} of 1.8 μM, a potency difference of at least 5000-fold. Although the sulfide showed a 6.5-fold lower EC_{50} relative to the sulfone for inducing apoptosis, this difference may be attributed to factors other than COX inhibition. For example, the sulfide is significantly more lipophilic than either the sulfone or sulfoxide (log P difference of approximately 2), and this could enhance membrane penetration, thereby lowering the effective dose range independent of an effect on COX. With regard to other NSAIDs, we found that doses effective for inhibiting cell growth or inducing apoptosis were appreciably higher than those effective for COX inhibition. In addition, add-back experiments demonstrated that the stable prostaglandin analogue, dimethyl-PGE_2, did not reverse or limit the apoptosis inducing effects of sulindac sulfide. This observation is consistent with results described by other investigators testing other prostaglandins (29, 49). Although COX inhibition is a characteristic property of NSAIDs, we speculate that this effect is ancillary for their antineoplastic benefits. Together with evidence demonstrating that sulindac sulfone has chemopreventive properties in rodent models of experimental carcinogenesis (36, 37),[4] these observations suggest that intracellular targets other than COX are responsible for apoptosis-inducing properties of NSAIDs.

Chemotherapeutic drugs act by numerous biochemical mechanisms that result in the disruption of DNA synthesis or replication. Cell cycle arrest and inhibition of rapidly proliferating cell populations are, in fact, hallmarks of efficacy of most chemotherapeutic drugs, as well as their toxicity to tissues that have rapid rates of cell turnover. Although the primary target of NSAIDs has not yet been defined and is undoubtedly different from chemotherapeutic drug targets, we have shown that apoptosis induced by sulindac metabolites is fundamentally distinct from that induced by 5-FU at both the cellular and biochemical levels. Sulindac metabolites were equally active in both proliferating and nonproliferating cells, whereas 5-FU was markedly less effective in nonproliferating cells relative to proliferating cells. The expression of p53 was significantly elevated in apoptotic cells by 5-FU treatment, whereas treatment with sulindac metabolites did not induce its expression. In fact, sulindac sulfide reduced p53 expression, an effect reported previously by other investigators (50). Lastly, from experiments involving combination treatment, we found no evidence that sulindac metabolites and 5-FU caused an additive or synergistic apoptotic response. Therefore, in contrast to sulindac metabolites, apoptosis induced by 5-FU appears to be linked with cell cycle arrest. *In vivo* studies support this possibility. For example, acute treatment

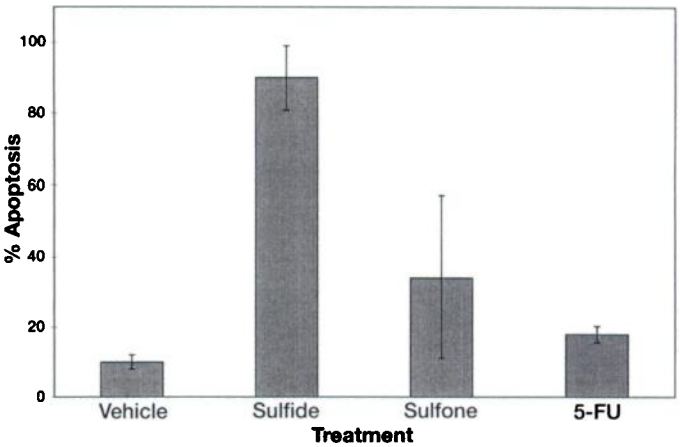

Fig. 6. Effects of sulindac metabolites and 5-FU on the induction of apoptosis in p53-negative Saos-2 cells. Saos-2 cells (1×10^6 cells) were plated in 75-cm^2 flasks, allowed to grow for 10 days, and treated with sulindac sulfide (120 μM), sulfone (480 μM), or 5-FU (50 μM) for 6 days. Apoptosis was determined by morphology as described under "Materials and Methods." Saos-2 cells were confirmed to lack p53 protein by Western blotting extracts prepared from the same cultures as used for apoptosis measurements and detected as described under "Materials and Methods."

of rats with 5-FU causes a pronounced increase in apoptosis in normal colonic mucosa as determined by terminal deoxynucleotidyl transferase-mediated nick end labeling of formalin-fixed tissue (51). Because apoptotic cells were exclusively present in the proliferative compartment of the crypt, increased apoptosis was likely the result of cell cycle arrest of rapidly proliferating colonocytes.

On the basis of observations described in this report, we propose that increased apoptosis is a key mechanism responsible for the ability of sulindac to cause regression of and prevent recurrence of polyps in FAP patients. This hypothesis and the possibility that COX inhibition is not necessary for adenoma regression is presently being investigated in a Phase I-II clinical trial involving treatment of FAP patients with sulindac sulfone (FGN-1).[5] Preliminary evidence from *in situ* measurements of apoptosis in polyp biopsies from these patients revealed higher apoptosis labeling indices as a result of both the dose and duration of FGN-1 treatment. Moreover, polyps that showed evidence of regression (*i.e.*, flattening and size diminution) had significantly higher rates of apoptosis relative to exophytic polyps present before or after treatment. Biopsies from normal colonic mucosa showed that FGN-1 treatment did not alter apoptosis rates in normal tissue. The biochemical mechanism responsible for the selectivity by which sulindac sulfone induces apoptosis of neoplastic cells is presently under investigation.

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