Antiproliferative Effect of Nonsteroidal Antiinflammatory Drugs against Human Colon Cancer Cells

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Abstract

Several lines of evidence suggest that nonsteroidal antiinflammatory drugs may be effective in preventing colorectal cancer. These include animal experiments, case-control studies, and clinical experience with sulindac in promoting the regression of adenomatous polyps in adenomatous polyposis coli. We determined the antiproliferative activity of various nonsteroidal antiinflammatory drugs, including two sulindac derivatives, against human colon cancer cells in vitro. Ht-29, SW480, and DLD-1 cells were continuously incubated with serial drug dilutions for 6 days prior to fixation. Cell number was determined using the sulforhodamine B assay, and drug concentrations which inhibited cell growth by 50% were estimated for each agent by interpolation. All drugs exhibited antiproliferative activity against Ht-29 and DLD-1 cells, and most inhibited SW480 cells. For Ht-29 cells, the 50% inhibitory concentration varied from 55 μM for diclofenac to 2100 μM for 5-aminosalicylic acid, with three drug groups of high, intermediate, and low potency evident. Inhibition of cell growth by sulindac sulfide was reversible following drug removal. Nonsteroidal antiinflammatory drugs exert an antiproliferative effect against human colon cancer cells with a wide range of potencies. A cytostatic response was demonstrated with sulindac sulfide. These data further support the potential role of these agents for chemoprevention of colorectal neoplasia.

Introduction

NSAIDs have considerable potential as chemopreventive agents for colorectal cancer. Two recent case-control drug surveillance studies and one large cohort study found that patients with regular aspirin use had a reduced incidence or decreased death rate from colorectal cancer (1-3). Several different NSAIDs have been shown to reduce the formation of both colon adenomatous polyps (the precursor lesion of colon cancer) and cancers in experimental animals given known carcinogens (4-10). NSAIDs have also been demonstrated in animal models to inhibit the growth and clinical expression of transplanted tumors and metastatic spread (11-15), and to potentiate the antitumor effects of immunotherapy, radiotherapy, and cytotoxic drug therapy (16-21). Perhaps most intriguing are the reports that the NSAID sulindac promotes regression and inhibits recurrence of adenomatous colon polyps in patients with adenomatous polyposis coli (22-26).

NSAIDs inhibit cell growth and [3H]thymidine incorporation into cellular DNA in various cell culture models (e.g., rat hepatoma and colon carcinoma and human fibroblast) and in human rectal mucosa explants (27, 28). Indomethacin exerts a cytostatic effect by reversibly freezing cells in the G1 phase in vitro (29). We now extend these earlier observations in assessing the relative inhibitory effects of a series of 16 NSAIDs against 3 well-characterized human colon carcinoma cell lines. This assay is used as a primary screening tool by the National Cancer Institute’s Division of Cancer Treatment (Developmental Therapeutics Program) to identify candidate chemopreventive drugs (30).

Materials and Methods

Cell Culture Technique. The human colon carcinoma cell lines Ht-29, DLD-1 and SW480 were obtained from ATCC, Bethesda, MD and have been previously characterized (31). Cells were maintained in monolayer culture in RPMI 1640 supplemented with 5% fetal bovine serum (Gemini Bio-products, Inc., Calabases, CA) and 2 mM glutamine in a humidified atmosphere of 95% air and 5% CO2 at 37°C. Cell lines were monitored for the absence of Mycoplasma using the Gen-Probe Mycoplasma Detection System (Gen-Probe, Inc., San Diego, CA). Subculturing was done at subconfluent densities. The cells were dispersed with a solution of 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid.

Measurement of Cell Proliferation. Cell number was determined using the SRB colorimetric protein stain assay (30). Cells (500 cells/well) were plated in nonperimeter wells in 96-well microtiter plates and incubated for 24 h at 37°C prior to addition of drugs. All NSAIDs tested except sulindac sulfide and sulindac sulfone were purchased from Sigma Chemical Co. (St. Louis, MO). Sulindac sulfide and sulindac sulfone were synthesized as outlined below. The sulindac compounds were solubilized in 100% ethanol and all other agents in 100% DMSO. Concentrations were diluted with media so that final concentrations within wells

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3 The abbreviations used are: NSAID, nonsteroidal antiinflammatory drug; SRB, sulforhodamine B; DMSO, dimethyl sulfoxide; IC50, 50% inhibitory concentration.
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contained a maximum of 0.5% DMSO or 1% ethanol. For initial screening, each agent was tested at 5–6 10-fold dilutions, generally starting with a maximum concentration of 100–300 μM as solubility permitted. Smaller serial dilutions were tested subsequently with selected agents to further clarify a drug concentration which achieved an IC₅₀. For each plate, 12 wells were designated as media controls, 6 wells were designated as DMSO or ethanol controls, and the remainder of the wells were designated for drug dilutions with 6 wells per drug concentration. After 6 days of culture, cells were fixed by the addition of cold trichloroacetic acid to a final concentration of 10%. Plates were incubated at 4°C for 60 min; then the supernatant was aspirated and the plates were washed with deionized water. SRB (Aldrich Chemical Co., Milwaukee, WI) solution was formulated to 0.4% w/v in 1% acetic acid; 10 μl was added to each well and the plates were incubated for 10 min at room temperature. Unbound SRB was removed by washing with 1% acetic acid followed by air drying. Bound stain was solubilized with 50 mM unbuffered Tris and absorbance was read by an automated spectrophotometer at a single wavelength of 540 nm.

To assess whether inhibition of Ht-29 cell growth is reversible, microtiter plates were prepared as described, and after 24 h, drug was added at a single concentration to designated wells. Cultures were incubated with drug or control media continuously until assayed (after 1–7 days of drug exposure) or drug was removed after 3 days and replaced with control media after a single wash for the remainder of the experiment (from 1 to 4 days after drug removal).

**Synthesis of Sulindac Sulfide and Sulindac Sulfone.** (Z)-5-Fluoro-2-methyl-1-(p-methylthiobenzylidene)-3-indenylacetic acid (sulfide) and (Z)-5-fluoro-2-methyl-1-(p-methylsulfonylbenzylidene)-3-indenylacetic acid (sulfoxide) were prepared according to the procedures described by Shuman et al. (32) and were found to be 99.7% pure. (Z)-5-Fluoro-2-methyl-1-(p-methylsulfonylbenzylidene)-3-indenylacetic acid (sulfone) was prepared as follows; to a cooled, stirred mixture of sulfoxide (100 g, 0.281 mol) in MeOH (250 ml) and CH₄CN (500 ml), CH₄ONa (4.4 m in MeOH, 68.5 ml, 0.30 mol) was added dropwise. After the mixture clears, NaHCO₃ (47 g, 0.56 mol) is added, followed by the dropwise addition of H₂O₂ (30% in H₂O, 3.63 ml, 0.56 mol) keeping the reaction temperature below 5°C. After sitting at −10°C for 18 h, NaHCO₃ is filtered off and washed with MeOH. The cooled (0°C) and stirred filtrate is neutralized to pH 7 by the dropwise addition of HCl (1 M, 350 ml). The crystalline mass is stirred at 0°C for 1 h, filtered off, and washed with MeOH to give 93 g (89% yield); melting point, thin layer chromatography, infrared spectroscopy, ¹H nuclear magnetic resonance, ¹³C nuclear magnetic resonance, and elemental analysis confirm purity to be above 99.7%.

**Analysis of Data.** For each drug, a minimum of three experiments measuring the growth inhibition of Ht-29 cells was conducted. One or two experiments with each drug were conducted with DLD-1 and SW480 cells. For these experiments, the mean ± SD of absorbance data from at least 6 replicated wells were calculated for each drug concentration tested. From these means, the inhibitory effect of the drug at each concentration was expressed as:

\[
\text{% inhibition} = \frac{\text{absorbance of treated cells}}{\text{absorbance of control cells}} \times 100
\]

The IC₅₀, the drug concentration causing a 50% reduction in the absorbance relative to the control wells, was then estimated by interpolation from the inhibitory effects measured at each concentration tested. The IC₅₀ values from each experiment were then compared in an analysis of variance to test for differences among the drugs. The statistical significance between all pairs of drugs was assessed using Tukey’s multiple comparisons test (33).

**Results**

The mean estimated IC₅₀ values of 18 different NSAIDs (including 3 sulindac compounds) for Ht-29 cells are listed in Table 1. The pairwise comparisons of the antiproliferative potency of these agents in Ht-29 cells is demonstrated in Fig. 1. Grouping of the drugs into high, intermediate, and low potency categories is apparent. No statistically significant differences were noted among the estimated IC₅₀ values for salicylic acid, acetylsalicylic acid, and 5-aminosalicylic acid. However, the estimated IC₅₀ values of these three low potency drugs were judged to be significantly higher than for all other drugs tested. On the other end of the potency spectrum, the IC₅₀ values for diclofenac, mefenamic acid, sulindac sulfide, and indomethacin could not be statistically distinguished from each other using this data, nor could they be statistically distinguished from oxypHENbutazone or sulindac sulfone on this basis. The 4 high potency drugs were, however, judged to have significantly lower IC₅₀ values than the other 12 drugs. Fenoprofen, phenylbutazone, flurbiprofen, sulindac sulfoxide, piroxicam, ibuprofen, ketoprofen, naproxen, and tolmetin, therefore, appear to constitute a class of intermediate potency drugs.

Representative drug concentration time curves for three NSAIDs of various potencies in Ht-29 cells are dem-
onstrated in Fig. 2. Each curve represents the mean of at least 3 experiments with the estimated IC\textsubscript{50} for diclofenac, fenoprofen, and salicylic acid equivalent to 55, 240, and 1735 \(\mu\)M, respectively. Concentration time curves for the three sulindac compounds are shown in Fig. 3. Estimated IC\textsubscript{50} values for these agents are sulindac sulfide (40 \(\mu\)M), sulindac sulfone (120 \(\mu\)M), and sulindac sulfoxide (280 \(\mu\)M). Inhibition of tumor cell growth by sulindac sulfide was reversible after the removal of drug, suggesting a cytostatic effect (Fig. 4). Following a 3-day drug exposure, cells resumed growth at a rate similar to control cultures after a delay of 24–48 h. Cell growth remained markedly suppressed as long as cultures were exposed to drug (up to 7 days). In contrast, a cytotoxic response was evident with 5-fluorouracil in which cell growth did not recover despite drug removal (Fig. 4).

The antiproliferative activity of most of the NSAIDs was also assessed in two additional human colon cancer cell

Fig. 1. Relative antiproliferative activity of NSAIDs against Ht-29 cells as compared using Tukey's multiple comparisons test. Vertical lines, groups of drugs which cannot be statistically distinguished on the basis of their IC\textsubscript{50} values from the drug marked by the open circle.

Fig. 2. Drug concentration time curves for diclofenac (---), fenoprofen (---), and salicylic acid (---) in Ht-29 cells. The estimated IC\textsubscript{50} values are diclofenac (55 \(\mu\)M), fenoprofen (240 \(\mu\)M), and salicylic acid (1733 \(\mu\)M). Each curve represents the mean ± SEM from 3 experiments.

Fig. 3. Drug concentration time curves for sulindac sulfide (--), sulindac sulfone (- -) and sulindac sulfoxide (+ +) in Ht-29 cells. The IC\textsubscript{50} values are sulindac sulfide (40 \(\mu\)M), sulindac sulfone (120 \(\mu\)M), and sulindac sulfoxide (280 \(\mu\)M). Each curve represents the mean ± SEM from 3 experiments.

Fig. 4. A and B, reversibility of the antiproliferative effect of sulindac sulfide and 5-fluorouracil on Ht-29 colon cancer cell line growth. Drug sulindac sulfide (100 \(\mu\)M) or 5-fluorouracil (10 \(\mu\)M) or control media was added to cell cultures at 24 h. Control wells (a) were assayed at timed intervals out to 8 days; wells with drug were exposed between days 1–4, then washed free of drug and incubated in control media until assayed (b), or continually exposed to drug (c). A cytostatic response to sulindac sulfide is demonstrated in which inhibition of cell growth is reversible (values for b at days 7 and 8 are significantly higher than corresponding values for c). The response to 5-fluorouracil is cytotoxic in that there was no reversal of 5-fluorouracil growth inhibitory effects after its removal. Horizontal solid bars, drug exposure duration.
lines, DLD-1 and SW480 (Table 1). Although fewer replicative experiments were performed as compared to the data collected with the Ht-29 cell line, the IC50 values observed with each of the tested NSAIDs against the DLD-1 and SW480 cells tend to sort these agents into similar categories of high, intermediate, and low potency. For example, tolfenamic acid tends to fall into the low potency group and sulindac sulfide and oxyphenbutazone fall into the high potency group of NSAIDs with respect to the IC50 values determined for each of the drugs in the three different cell lines.

Discussion

This study demonstrates that a wide variety of NSAIDs exert antiproliferative activity against human colon cancer cells in vitro. As reported previously with indomethacin (27, 29), we found that sulindac sulfide exerts a cytostatic rather than cytotoxic effect in that a normal rate of cell growth resumes after removal of drug. This response in vitro concurs with reported clinical experience that sulindac prevents regrowth of macroscopic colorectal polyps in patients with adenomatous polyposis coli only with continuous drug dosing (22–23).

The best characterized pharmacological effect of the NSAIDs is to diminish prostaglandin synthesis by inhibiting cyclooxygenase, which catalyzes the formation of prostaglandin precursors from arachidonic acid. Prostaglandins are potent mediators of numerous biological responses and probably play a role in maintaining cellular viability and in modulating both normal and neoplastic cell proliferation (34–37).

The mechanism by which the NSAIDs exert their cytostatic effects against Ht-29 colon cancer cells is unclear, but inhibition of prostaglandin synthesis would appear to be a plausible hypothesis because a variety of experimental animal and human tumors contain and/or synthesize high levels of prostaglandins (7, 34, 38, 39). In addition, prostaglandin synthesis has been associated with tumors promoting increased metastatic potential (34, 40–42), and it predicts which tumors will demonstrate slowing of growth in response to indomethacin (43).

Other lines of evidence are contrary to the concept that inhibition of prostaglandin synthesis is central to the NSAID antiproliferative effect. Relatively high levels of prostaglandins have been reported to inhibit tumor cell growth both in vivo and in vitro, and to inhibit differentiation in some tumor cell lines (34, 36, 44–49). Exogenous prostaglandins have also been demonstrated to inhibit basal mucosal DNA synthesis in colon explants from animals (50, 51). DeMello et al. (52) reported that NSAID concentrations which inhibit cell growth in rat hepatoma and human fibroblast cell lines in vitro correlate poorly with concentrations reported to inhibit cyclooxygenase in other studies. In addition, prostaglandin synthetic activity was not detected in the rat hepatoma cell line, and exogenous prostaglandins did not reverse the antiproliferative effects of indomethacin.

Our results concur with those of DeMello et al. (52) in that the antiproliferative effect of NSAIDs does not appear to correlate with cyclooxygenase inhibitory activity. Piroxicam, the most potent cyclooxygenase inhibitor assessed in this study, demonstrated intermediate activity against the human colon cancer Ht-29 cell line, whereas sulindac sulfide and sulindac sulfone which are reported to have minimal or no prostaglandin inhibitory activity (53, 54) are in the high potency range. Hypothetically, sulindac sulfone might have to some extent been reduced intracellularly to sulindac sulfide, a known potent inhibitor of cyclooxygenase; however, sulindac sulfone is a metabolite resulting from irreversible oxidation of sulindac sulfoxide (53).

Mechanisms postulated to explain the antiproliferative/antitumor effects of NSAIDs other than through prostaglandin modulation include: (a) NSAID interference with a spectrum of membrane-associated processes including G protein signal transduction, transmembrane calcium flux, and cell-to-cell binding (55–57); (b) in addition to cyclooxygenase, NSAID inhibition of the activity of other enzymes including phosphodiesterase, cyclic AMP-dependent protein kinase, and some folate-dependent enzymes (58–60); (c) NSAID inhibition of cyclooxygenase cooxidation of nonlipid substrates to carcinogens during prostaglandin synthesis (61–63); and (d) NSAID enhancement of a multitude of immunological responses which may have an important role in restoring host defenses.

In conclusion, all of the tested NSAIDs demonstrate antiproliferative activity in two human colon cancer cell lines (Ht-29, DLD-1) and most agents inhibit proliferation in a third line (SW480). In addition, studies, we have observed similar results in human lung and breast carcinoma and melanoma cell lines (A-427, MCF7/S, UACC375) (data not shown). Although the effects of NSAIDs on colon cancer cells may not be generalizable to all predysplastic or dysplastic cells, the ability of sulindac to promote regression and prevent recurrence of adenomatous polyps in the setting of adenomatous polyposis coli suggests that these drugs have a potential role for the chemoprevention of colorectal neoplasia. Which, if any, of the NSAIDs might prevent the development/growth of sporadic adenomatous colonic polyps in humans is currently unknown. Because most toxicity associated with chronic NSAID therapy is related to antiprostaglandin activity, low-dose therapy and/or utilization of agents with less potent prostaglandin inhibition conceptually would be more attractive as a potential chronic chemoprevention treatment.

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