

Phospho-Sulindac (OXT-328), a Novel Sulindac Derivative, Is Safe and Effective in Colon Cancer Prevention in Mice

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BACKGROUND & AIMS: Nonsteroidal anti-inflammatory drugs (NSAIDs) are effective cancer chemopreventive agents. However, chronic administration of NSAIDs is associated with significant side effects, mainly of the gastrointestinal tract. Given these limitations, we synthesized phospho-sulindac (P-S; OXT-328), a novel sulindac derivative. **METHODS:** Here, we evaluated the safety and efficacy of P-S in preclinical models, including its mechanism of action with human colon cancer cell (HCCC) lines and animal tumor models. **RESULTS:** (1) Compared with sulindac, P-S is much more potent in inhibiting the growth of cultured HCCCs and more efficacious in preventing the growth of HT-29 xenografts in nude mice. P-S also prevents the growth of intestinal tumors in Apc/Min mice. (2) In combination with difluoromethylornithine (DFMO), P-S reduced tumor multiplicity in Apc/Min mice by 90%. (3) P-S is much safer than sulindac as evidenced by its in vitro toxicologic evaluation and animal toxicity studies. Mechanistically, P-S increases the intracellular levels of reactive oxygen and nitrogen species, which are key early mediators of its chemopreventive effect. Moreover, P-S induces spermidine/spermine N¹-acetyltransferase enzymatic activity, and together with DFMO it reduces polyamine levels in vitro and in vivo. **CONCLUSIONS: P-S displays considerable safety and efficacy, 2 pharmacologic properties that are essential for a potential cancer chemopreventive agent, and thus merits further evaluation.**

Keywords: Colon Cancer; Phospho-Sulindac; Sulindac; Polyamines; Reactive Oxygen Species.

Colon cancer, one of the most frequent human malignancies in the Western world, is a preventable disease. In 2009, the National Cancer Institute estimated that in the United States there were approximately 150,000 new cases and 50,000 deaths from colorectal cancer. Thus, the development of safe and effective chemopreventive agents represents a pressing need.

The nonsteroidal anti-inflammatory drug (NSAID) sulindac, alone or in combination with difluoromethylornithine (DFMO), has been shown to be effective in the prevention of colon cancer.¹⁻³ A recent study by Meykens et al,³ reaffirmed the feasibility of this approach. In

humans, compared with controls, sulindac plus DFMO reduced the recurrence of ≥ 1 adenomas by 70% and of more advanced adenomas by 92%. However, a limiting factor in the long-term use of sulindac is its toxicity, primarily gastrointestinal and renal toxicities, that can affect $\leq 20\%$ of patients.⁴ These considerations prompted us to synthesize phospho-sulindac (P-S; OXT-328; Figure 1A), a novel derivative of sulindac, and to evaluate its safety and efficacy against colon cancer. We anticipated enhanced safety of P-S, because it is modified at its -COOH moiety, which is considered critical for its toxicity.⁵

Here, we report the enhanced potency of P-S over sulindac in inhibiting the growth of human colon cancer cell lines, its efficacy in animal tumor models, its apparent safety on the basis of on toxicologic evaluation and animal testing, and its ability to provide a mechanism for its chemopreventive effect.

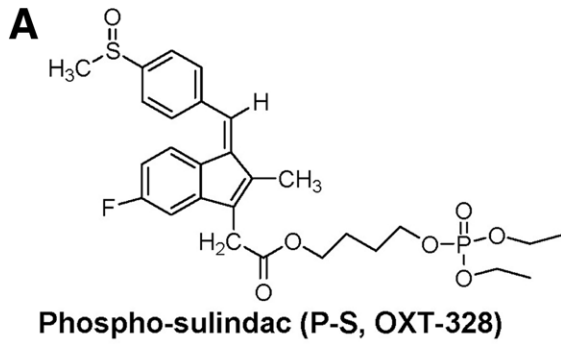
Materials and Methods

Reagents and Cell Culture

P-S was synthesized as reported.⁶ Sulindac, A23187, and N-acetyl-L-cysteine (NAC) were from Sigma. Human colon cancer cell lines and the normal human colon cell line NCM460 were grown as monolayer as suggested by American Type Culture Collection (Manassas, VA). Cell growth was determined with the MTT assay (Promega, Madison, WI) following the manufacturer's instructions.⁷ Cells were treated with P-S with or without DFMO for ≤ 48 hours. Cell proliferation was assayed by 5-bromo-2'-deoxyuridine incorporation; apoptosis and necrosis were assayed by staining with Annexin V-fluorescein isothiocyanate and propidium iodide (PI) and the

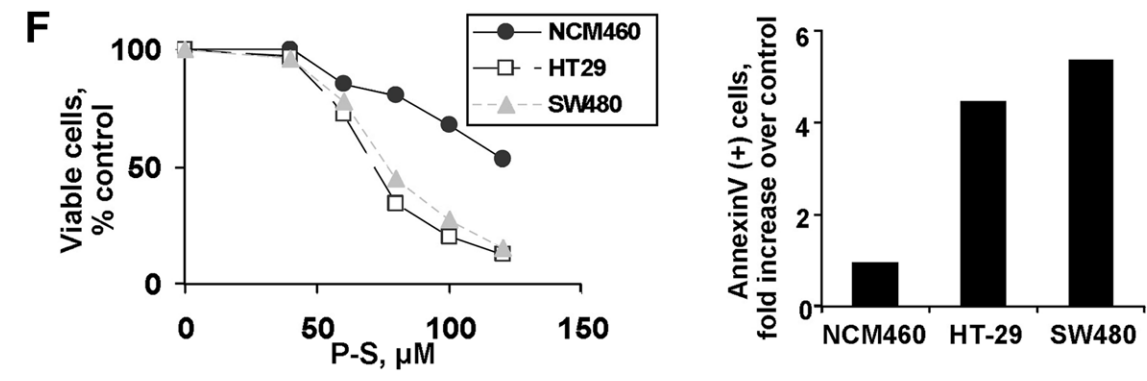
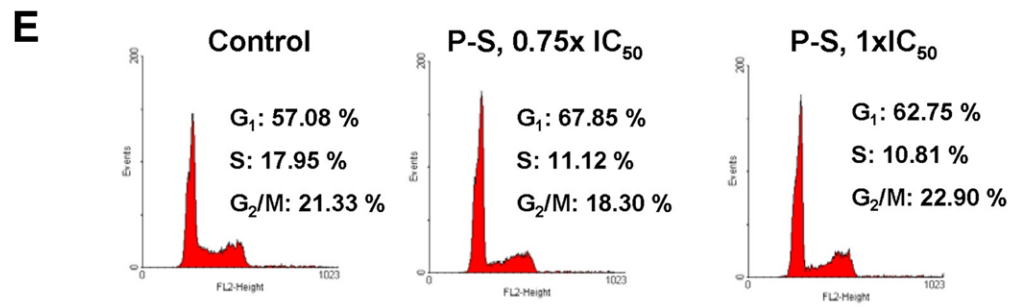
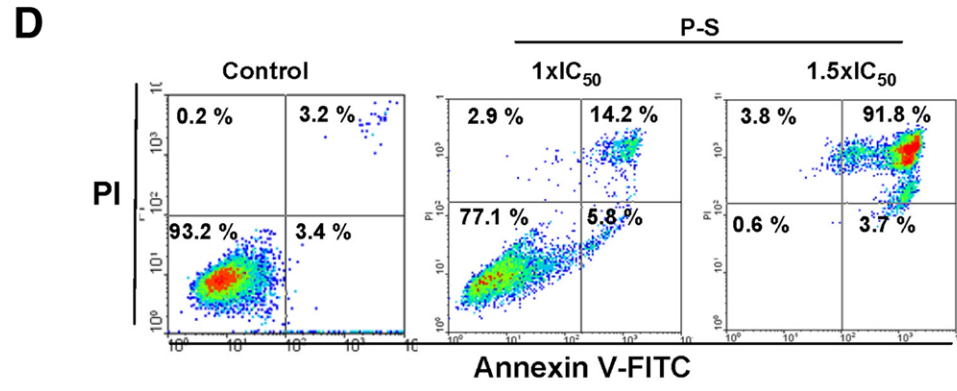
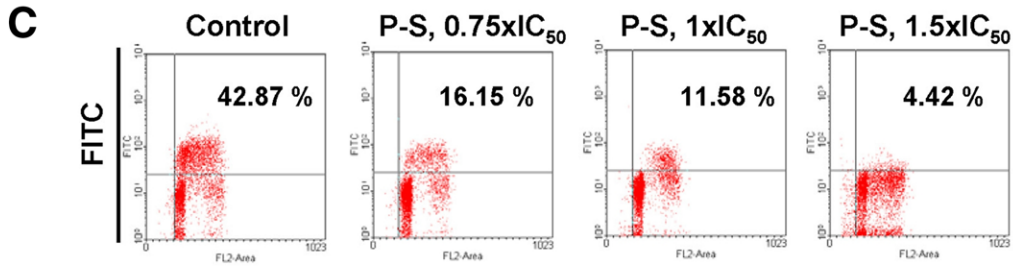
Abbreviations used in this paper: BSO, buthionine sulfoximine; COX, cyclooxygenase; DCFDA, dichlorodihydrofluorescein diacetate; DFMO, difluoromethylornithine; ERK, extracellular-signal regulate kinase; GSH, glutathione; IC₅₀, concentration that inhibits 50%; JNK, Jun kinase; MAPK, Mitogen-activated protein kinase; NAC, N-acetyl-L-cysteine; NF- κ B, nuclear factor- κ B; NSAID, nonsteroidal anti-inflammatory drug; P-S, phospho-sulindac; RONS, reactive oxygen and nitrogen species; SAT1, spermidine/spermine-N¹-acetyltransferase; siRNA, short interfering RNA; Trx-1, thioredoxin-1.

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Cell line	Phospho-sulindac	Sulindac	Potency enhancement
HT-29	71.7 ± 5.5	> 1000	> 13.9
SW480	76.7 ± 6.2	> 1000	> 13.0
HCT116	75.0 ± 4.1	> 1000	> 13.3
HCT15	70.0 ± 7.7	> 1000	> 14.2
LoVo	71.0 ± 5.4	> 1000	> 14.0
Caco2	79.3 ± 4.9	> 1000	> 12.6



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fluorescence intensities were analyzed by FACScaliber (BD Bioscience, San Jose, CA); and cell cycle was assayed by flow cytometry, all as described.⁷

Redox Assays

Reactive oxygen and nitrogen species (RONS) levels were determined with the use of the general RONS probe dichlorodihydrofluorescein diacetate (DCFDA).⁶ HT-29 cells treated with the test compound(s) for 2 hours were loaded with DCFDA, and fluorescence intensity was analyzed by FACScaliber (BD Bioscience).⁷ Mitochondrial superoxide was determined by fluorescence microscopy. Cells seeded overnight in glass-bottomed culture dishes (MatTek, Ashland, MA) were treated with the test compound(s) for 0–60 minutes and assayed by fluorescence microscopy.⁶ Glutathione (oxidized and reduced) was determined by the glutathione (GSH) reductase-coupled 5,5'-dithiobis(2-nitrobenzoic acid) assay.⁸ The thioredoxin redox status assay was performed as described.⁶

Electrophoretic Mobility Shift Assay

Nuclear fractions were isolated from 2×10^6 cells (treated and controls) and subjected to electrophoretic mobility shift assay.⁷ Oligonucleotides containing the consensus sequence for nuclear factor- κ B (NF- κ B) or organic cation transport 1 (OCT-1; control) were end-labeled with [γ -³²P]-adenosine triphosphate with the use of T4 polynucleotide kinase.

Cyclooxygenase Activity and PGE₂ Levels

Cyclooxygenase (COX) activity and prostaglandin E₂ (PGE₂) levels were determined by immunoassays, following the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI).

Polyamines and Spermidine/Spermine-N1-Acetyltransferase Activity

Cells treated with test compound(s) for 24 hours were assayed for polyamine levels by high-performance liquid chromatography (internal standard: 1,7-diaminoheptane).⁹ For the spermidine/spermine-N1-acetyltransferase (SAT1) activity, cells (3×10^6) treated with test compound(s) were harvested by scraping, disrupted by sonication in buffer [10 mmol/L Tris-HCl (pH 7.5), 2.5 mmol/L DTT, 1 mmol/L EDTA], and pelleted. SAT1

activity was based on the amount of labeled N¹-acetyl-spermidine synthesized from [¹⁴C]acetyl-CoA and unlabeled spermidine.¹⁰

Short Interfering RNA Gene Knock-Down

Cells were transfected with 200 ng of control or *sat1* short interfering RNA (siRNA) in lipofectamine 2000 (Invitrogen, Carlsbad, CA) and 48 hours later were replated and treated with test compound(s).

Animal Studies

All studies were approved by our Institutional Animal Care and Use Committee.

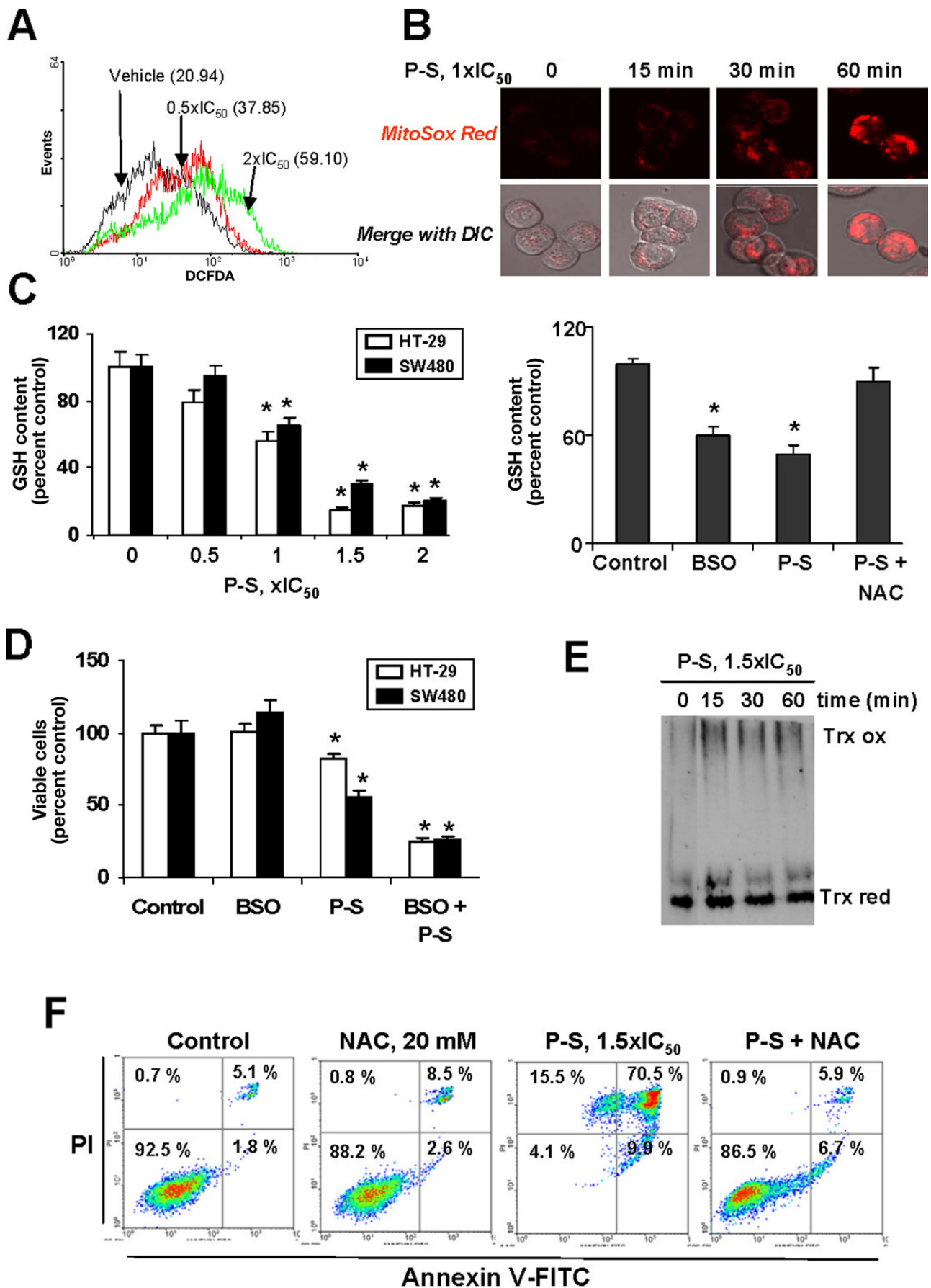
Acute toxicity. Six-week-old female C57BL/6J^{+/+} mice (n = 8/group) were treated for 5 days by oral gavage with equimolar amounts of P-S (317 mg/kg/d) or sulindac (200 mg/kg/d) or vehicle. Body weight was recorded every other day. All mice were necropsied.

Survival study. Six-week-old female nude mice (n = 6/group) were treated for 21 days by oral gavage with vehicle, P-S (100 mg/kg/d), or sulindac 66 mg/kg/d (equimolar) or 100 mg/kg/d (equi-dose). Body weight was recorded every other day. On day 21, surviving animals were killed, serum was collected, and their liver, kidneys, and pancreas were removed, preserved in formalin, and evaluated histologically.

Gastrointestinal toxicity. The gastrointestinal toxicity of P-S was determined in rats following a standard protocol.¹¹ Six-week-old Sprague Dawley rats (n = 3/group) were treated for 4 days by gavage with vehicle, indomethacin 4.75 mg/kg/d (positive control), or equimolar amounts of P-S (317 mg/kg/d) or sulindac (200 mg/kg/d). On day 5, animals were injected with 1% Evans blue solution and killed, and the number and size of small intestinal ulcerations were recorded. The toxicity scoring ranges between 0 (intact small intestine with no ulcerations or mucosal damage) and 5 (animal dies before study's end).¹¹

Efficacy in nude mouse xenografts. Six-week-old female NCr nude mice (Taconic Farms, Germantown, NY; n = 6/group) were treated by gavage with sulindac (33 mg/kg/d), P-S (50 or 100 mg/kg/d), or vehicle (corn oil). Five days later, mice were inoculated subcutaneously with 2×10^6 HT-29 colon cancer cells on each flank.

Figure 1. Phospho-sulindac (P-S; OXT-328) inhibits cell proliferation and induces cell cycle arrest and cell death by apoptosis in colon cancer cells. (A) Chemical structure of P-S. (B) IC₅₀ values for colon cancer cells treated with P-S or sulindac for 24 hours (mean \pm SEM). (C) Histograms of 5-bromo-2'-deoxyuridine incorporation in HT-29 cells treated for 24 hours without (control) or with 0.75 \times , 1 \times , or 1.5 \times IC₅₀ P-S. FITC, fluorescein isothiocyanate. (D) The percentage of SW480 apoptotic cells, determined by flow cytometry with the use of dual staining (Annexin V and propidium iodide), are indicated in each quadrant. (E) Cell cycle progression in HT-29 cells. Representative profiles of the distribution of cells in G₁, G₂/M, and S phases are shown. (F) Differential cytotoxic effect of P-S in colon cancer cells compared with the normal human colon cell line NCM460. (Left) NCM460, HT-29, and SW480 cells were incubated without or with variable concentrations (0–120 μ mol/L) of P-S for 24 hours. Cell growth was expressed as percentage of control. (Right) Cell death by apoptosis was determined by flow cytometry in NCM460, HT-29, and SW480 cells incubated without or with 110 μ mol/L P-S for 24 hours. Results are expressed as fold-increase compared with the percentages of apoptotic cells in the NCM460 cells.



Tumor size was determined with a digital microcaliper (tumor volume = [length \times width \times (length + width/2) \times 0.56]). Eighteen days after cell inoculation, animals were killed, and tumors were removed and weighed.

Efficacy in APC^{Min/+} mice. (1) For the Treatment protocol study, 11-week-old male C57BL/6J APC^{Min/+} (The Jackson Laboratories, Bar Harbor, ME; n = 10/group) were treated by gavage with P-S 100 mg/kg/d or vehicle (corn oil) for 28 days. At time of killing, their small intestine and colon segments were removed and opened longitudinally and tumors were counted under a magnifying lens. (2) For the prevention protocol study, 6-week-old C57BL/6J APC^{Min/+} mice (n = 9/group) received daily the following: group 1, vehicle (corn oil) by gavage; group 2, P-S 100 mg/kg by gavage; group 3, DFMO 2% in drinking water; and group 4, P-S and DFMO as above. Seven weeks later, all animals were killed; their small intestine and colon were removed and processed as above. Tissue samples were preserved in formalin and studied by immunohistochemistry, following standard protocols⁸ or snap-frozen for biochemical analyses.

Statistical Analysis

Results, from at least 3 independent experiments and expressed as mean \pm SEM were analyzed by 1-factor analysis of variance followed by Tukey test for multiple comparisons. $P < .05$ was statistically significant.

Results

P-S Inhibits the Growth of Cultured Colon Cancer Cells Through a Strong Cytokinetic Effect

To study the effect of P-S on cell growth, we determined the 24-hour concentration that inhibits 50% (IC₅₀) values of P-S and sulindac in various human colon cancer cells (Figure 1B). The IC₅₀ values of P-S varied little among these cell lines (70–79.3 μ mol/L), whereas those of sulindac were consistently >1000 μ mol/L, indicating a potency enhancement of >14.2 -fold.

We then elucidated the underlying cytokinetic effect of P-S in HT-29 and SW480 cells. P-S markedly reduced concentration-dependent cell proliferation (Figure 1C). For instance, at its IC₅₀ P-S reduced HT-29 cell proliferation from 43% in controls to 12% and at $1.5 \times$ IC₅₀ to 4%,

accounting for 73% and 91% reduction, respectively. P-S induced concentration-dependent apoptosis in HT-29 and SW480 cells. Both early apoptosis and late apoptosis were present, but the latter was predominated. At 24 hours, in SW480 cells, the annexin V⁺ cells increased from 5.6% in control to 20% at P-S $1 \times$ IC₅₀ and to 95.5% at $1.5 \times$ IC₅₀ (Figure 1D). Finally, P-S blocked the G₁-to-S transition. The percentage of cells in G₁ phase was significantly higher in cells treated with $0.75 \times$ IC₅₀ P-S than in controls (HT-29 cell, 80% vs 57%; SW480 cells, 68% vs 57%; Figure 1E).

We next compared the effect of P-S on colon cancer cells against that on the normal human colon epithelial cell line NCM460. After 24-hour treatment with 100 μ mol/L P-S, only 20.4% and 27.7% of HT-29 and SW480 cells, respectively, remained viable. However, under the same experimental conditions, 67.7% of NCM460 cells were viable (Figure 1F). In addition, incubation of HT-29 and SW480 cells with 110 μ mol/L P-S generated a 4.5- and 5-fold increase in annexin V⁺ cells. In contrast, only a 1-fold increase in annexin V⁺ cells was observed for NCM460 cells (Figure 1F). This indicates that P-S decreases cell growth and induces apoptosis preferentially in colon cancer cells compared with a normal epithelial colon cell line.

Cell Signaling Effects of P-S

We examined the effect of P-S on the redox status of colon cancer cell line, an effect potentially important for its mechanism of action,^{6,8} its effect on polyamines, known to be critical to the anticancer effect of sulindac,² and a series of redox-sensitive signaling pathways.

P-S induces oxidative stress in colon cancer cells. To explore the effect of P-S on cellular RONS, we loaded SW480 cells treated with $0.5 \times$ or $2 \times$ IC₅₀ P-S for 1 hour with DCFDA, a general RONS probe. The level of RONS in response to P-S at $0.5 \times$ and $1 \times$ IC₅₀ for cell growth increased 1.8- and 2.8-fold over control (Figure 2A), whereas P-S at $1 \times$ and $1.5 \times$ IC₅₀ gave intermediate results (not shown). In HT-29 cells, P-S $1 \times$ IC₅₀ increased mitochondria superoxide levels in a time-dependent manner (Figure 2B). We then evaluated GSH, a major antioxidant system in mammalian cells, levels in response to P-S. Treatment of HT-29 cells with P-S $1 \times$ IC₅₀ for 4 hours decreased GSH levels by 50% (Figure 2C). The GSH

Figure 2. P-S induces RONS in colon cancer cells: effect on cell growth and apoptosis. (A) DCFDA fluorescence was measured by flow cytometry in SW480 cells treated with $0.5 \times$ IC₅₀ or $2 \times$ IC₅₀ P-S for 1 hour. (B) P-S induces a time-dependent increase in mitochondrial superoxide levels. HT-29 cells incubated with $1 \times$ IC₅₀ P-S for different time periods. After adding the selective MitoSOX probe, cells were subjected to confocal microscopy ($\times 40$). (C) P-S reduces GSH content. (Left) GSH levels were determined in HT-29 cells incubated with various concentrations of P-S (left) or with $1 \times$ IC₅₀ P-S, BSO, or with NAC and P-S for 4 hours (right). Values are shown as means \pm SEM of 3 independent experiments; * $P < .05$ vs control. (D) HT-29 and SW480 cells were treated with 100 μ mol/L BSO followed by treatment with P-S for 24 hours. Cell growth was expressed as percentage of control. (E) P-S affects the thioredoxin system. The levels of oxidized (Trx ox) and reduced (Trx red) Trx-1 were detected by native immunoblot in whole-cell lysates from SW480 cells treated with P-S $1.5 \times$ IC₅₀ for different time periods. (F) NAC prevents cell death induced by P-S. HT-29 cells were either untreated or pretreated with NAC for 1 hour followed by P-S for 24 hours. Cell death by apoptosis was determined by flow cytometry. FITC, fluorescein isothiocyanate.

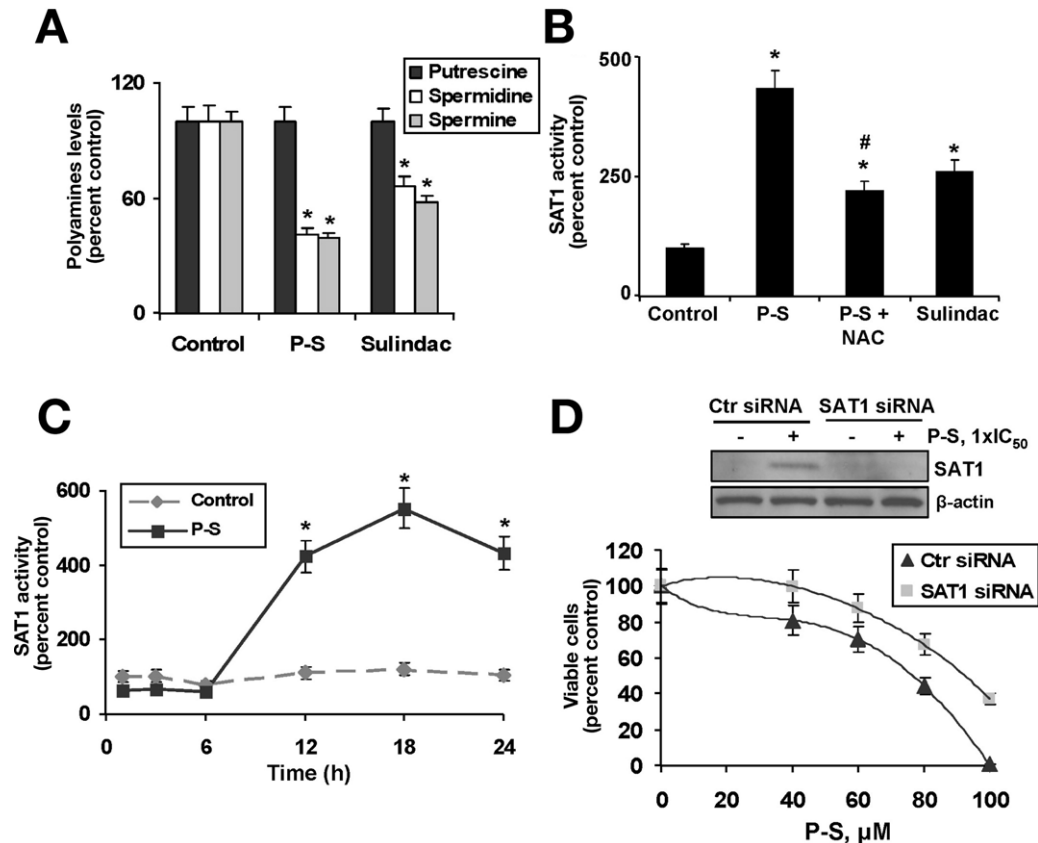


Figure 3. P-S reduces polyamines levels by inducing SAT1 in colon cancer cells. (A) Polyamine levels in SW480 cells treated with P-S 80 μ mol/L or sulindac 800 μ mol/L for 24 hours. * $P < .05$ vs control. (B) P-S induces SAT1 enzymatic activity. SW480 cells were incubated with P-S or sulindac for 24 hours. * $P < .05$ vs control; # $P < .05$ vs P-S. (C) Time dependency on the effect of P-S on SAT1 activity. * $P < .05$ vs control. (D) Silencing SAT1 attenuates P-S-induced cell growth reduction. SW480 cells were transfected with either control or SAT1 siRNA. After transfection, cells were treated with various concentrations of P-S for 24 hours, and cell growth was evaluated; * $P < .05$ vs control (bottom). After transfection a subset of cells were treated with $1 \times IC_{50}$ P-S for 24 hours. Immunoblots to verify SAT1 silencing were performed on whole cell extracts obtained from these cells (top).

synthase inhibitor buthionine sulfoximine (BSO) had a similar effect (41% reduction), whereas pretreatment for 1 hour with NAC largely abrogated the effect of P-S on GSH. Furthermore, P-S induced the oxidized form of thioredoxin-1 (Trx-1), a key antioxidant enzyme, most notably between 15 and 60 minutes (Figure 2E).

We next examined whether P-S may stimulate apoptosis by inducing a state of oxidative stress. Pretreatment with NAC for 1 hour, before P-S, reduced SW480 cell death by 84% (Figure 2F). Moreover, GSH depletion induced by BSO enhanced the cell growth-induced inhibition by P-S (Figure 2D). Although P-S inhibited the growth of HT-29 and SW480 cells ($IC_{50} > 80 \mu$ mol/L under this experimental protocol), pretreatment with 100 μ mol/L BSO for 24 hours reduced the IC_{50} to 25 and 26 μ mol/L for HT-29 and SW480 cells, respectively (Figure 2D). Overall, these findings indicate that RONS decide the fate of colon cancer cells in response to P-S.

P-S reduces the level of polyamines by inducing SAT1 activity in colon cancer cells. Sulindac is known to reduce polyamine levels in colon cancer cells. Thus, we evaluated whether P-S could have a similar effect. Treat-

ment of HT-29 or SW480 cells with $1 \times IC_{50}$ P-S for 24 hours markedly diminished the levels of spermidine and spermine, without affecting putrescine levels (Figure 3A).

We examined the effect of P-S on SAT1 activity, an enzyme known to be induced by sulindac.¹² Although incubation for 24 hours with 85 μ mol/L P-S increased SAT1 activity by 3- and 4.4-fold ($P < .05$ vs control), 800 μ mol/L sulindac increased it by 1.5- and 2.5-fold ($P < .05$ vs control) in HT-29 and SW480 cells, respectively (Figure 3B). Because RONS can modulate SAT1 activity,¹⁰ we tested whether NAC could prevent the induction of SAT1 activity by P-S. Indeed, NAC 20 mmol/L prevented, in part, the effect of P-S on SAT1, suggesting a redox-dependent effect (Figure 3B). Of note, the effect of P-S on SAT1 was time dependent. Although no difference in SAT1 activity was observed during the first 6 hours of treatment with P-S, after 12 hours, activity was increased 4-fold compared with controls and continued to a maximum of 5.5-fold increase after 18 hours (Figure 3C).

To evaluate the role of SAT1 in P-S-induced cell growth inhibition, we silenced SAT1. Compared with

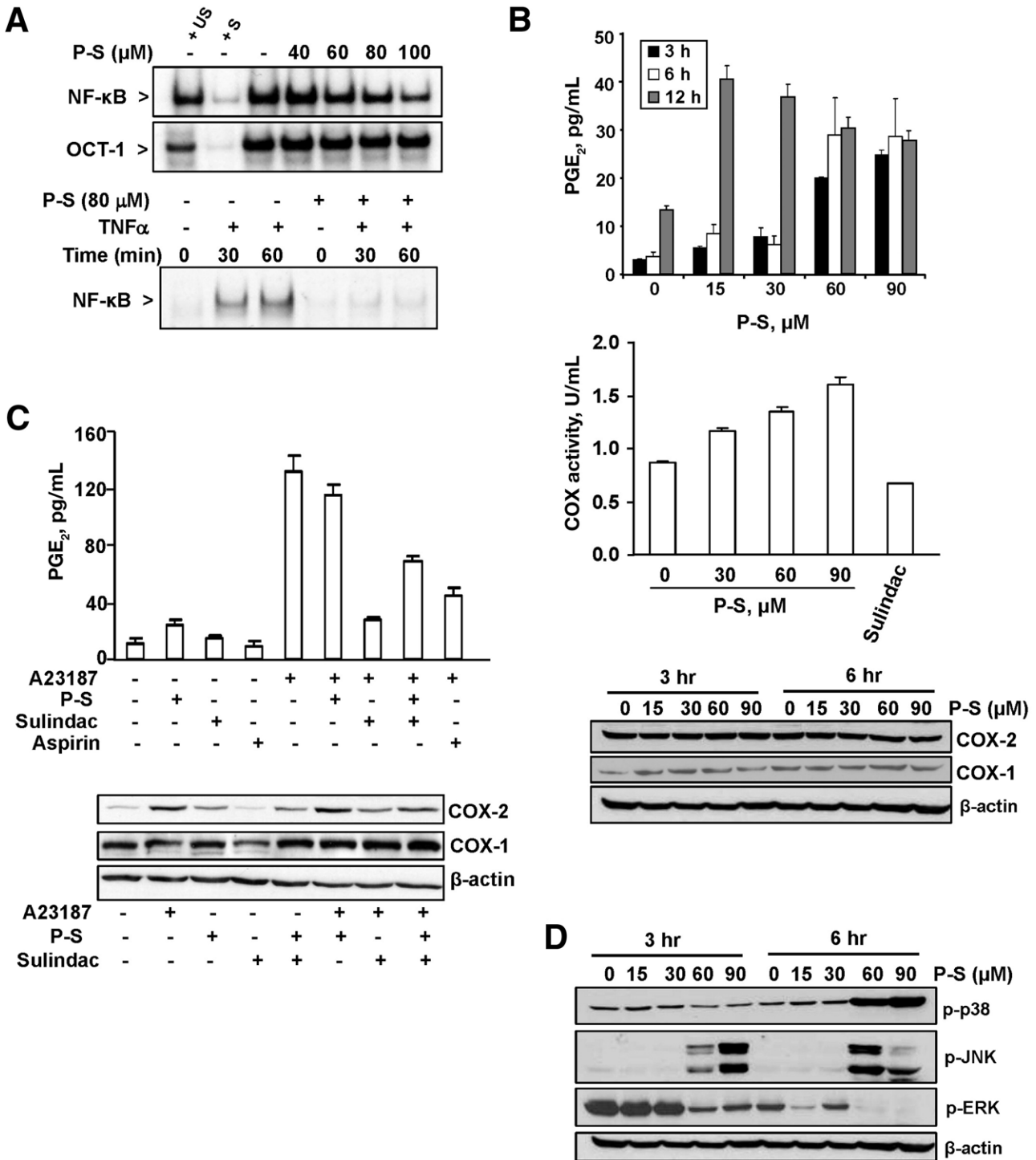
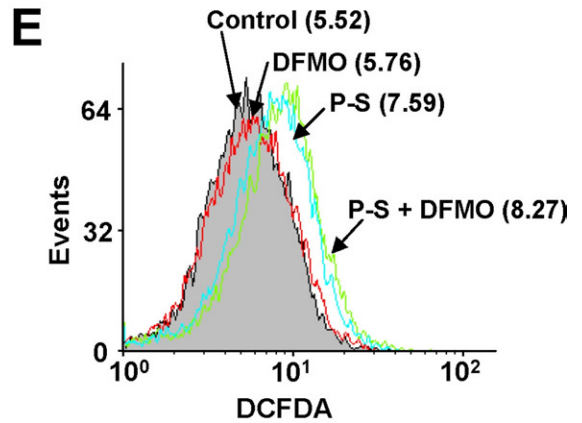
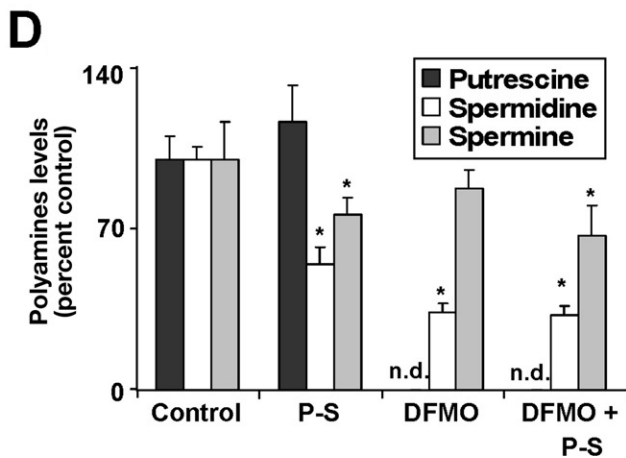
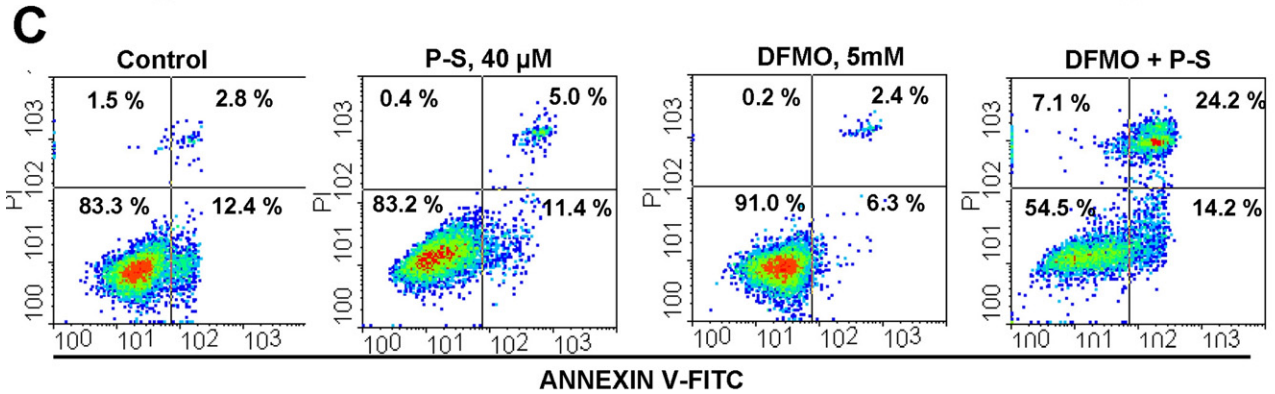
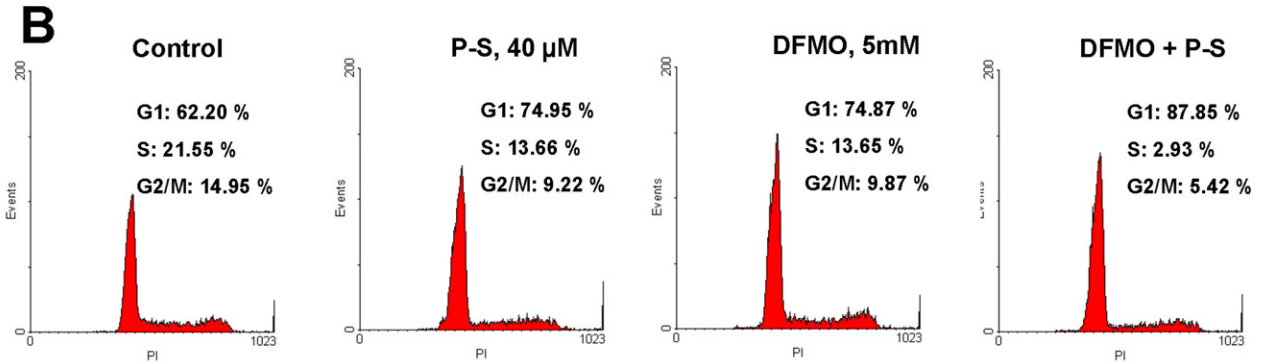
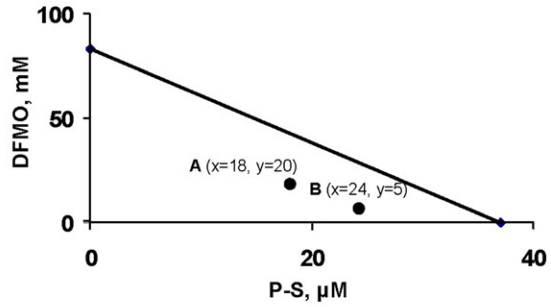
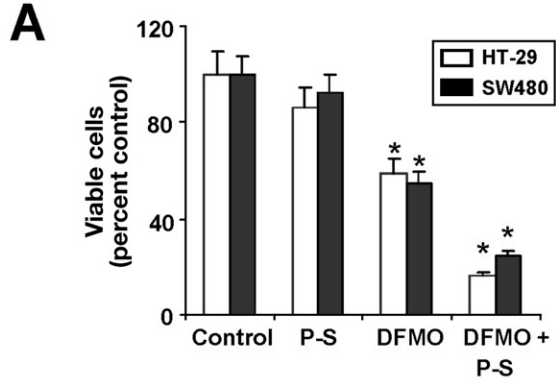


Figure 4. P-S modulates redox-sensitive signaling pathways in colon cancer cells. (A) P-S inhibits constitutive and tumor necrosis factor α (TNF- α)-induced NF- κB activation. (Top) Electrophoretic mobility shift assay for NF- κB and OCT-1 of nuclear fractions isolated from HT-29 cells after 4 hours of treatment without or with 40–100 $\mu\text{mol/L}$ P-S. To determine the specificity of each transcription factor–DNA complex, the control nuclear fraction (–) was incubated in the presence of 100-fold molar excess of unlabeled oligonucleotide containing the consensus sequence for either the specific (+ S) or an unspecific (+ US) transcription factor. (Bottom) EMSA for NF- κB of nuclear fractions isolated, after 4 hours of preincubation without or with P-S and further 0, 30, or 60 minutes of incubation without (–) or with 10 ng/mL TNF α . n.s.: not specific. (B) P-S increased PGE $_2$ levels, measured by enzyme-linked immunoabsorbent assay (ELISA), in a time- and concentration-dependent manner; and up-regulated COX-2 activity, measured by ELISA after a 6-hour incubation with P-S or 1.2 mmol/L sulindac. COX-2 and COX-1 expression, by immunoblot in total fractions isolated from HT-29 cells treated with P-S, were unaffected. (C) P-S did not prevent the increase in PGE $_2$ levels induced by A23187. HT-29 cells were pretreated with P-S $1 \times \text{IC}_{50}$, sulindac 1.2 mmol/L, or aspirin 0.4 mmol/L for 30 minutes followed by A23187 5 $\mu\text{mol/L}$ for 3 hours. PGE $_2$ levels, measured by ELISA, and COX-2 and COX-1 expression, measured by immunoblots, are shown. (D) P-S modulates MAPKs. Levels of phosphorylated p38, JNK, and ERK (p-p38, p-JNK, and p-ERK, respectively) were measured by immunoblot in total fractions isolated from HT-29 cells treated with P-S. β -Actin levels are shown as loading controls.



controls, knocking-down *sat1* rendered SW480 cells resistant to the growth inhibitory effect of P-S. Although cells transfected with nonspecific siRNA required 75 $\mu\text{mol/L}$ P-S to decrease their number of viable cells by half, cells transfected with siRNA against *sat1* required 93 $\mu\text{mol/L}$ P-S (24% more) for the same effect (Figure 3D).

P-S modulates redox-sensitive signaling molecules. *NF- κ B*. A redox-sensitive dimer, NF- κ B modulates cell growth and inflammation, especially in cancer.¹³ Treatment of HT-29 cells with P-S suppressed NF- κ B activation in a concentration-dependent manner (Figure 4A). Moreover, although TNF- α rapidly activated NF- κ B, a 4-hour preincubation with P-S abrogated this effect.

Eicosanoids. COX, considered important in cancer, is inhibited by sulindac. We evaluated the effect of P-S on the COX/PGE₂ cascade. In HT-29 cells, P-S left unaltered the expression of COX-2 and COX-1, but it increased COX activity, leading to significantly increased PGE₂ production (Figure 4B). The COX-2 inhibitors sulindac and aspirin abrogated the increase in PGE₂ levels by the calcium ionophore A23187, but P-S failed to prevent it (Figure 4C), suggesting that it lacks an anti-COX effect. P-S plus sulindac suppressed COX-2 expression and PGE₂ levels, although less than sulindac alone.

Mitogen-activated protein kinases. Mitogen-activated protein kinases (MAPKs) are important mediators of intracellular signaling, whereas p38 and Jun kinase (JNK), 2 of the major MAPKs, are redox dependent.¹⁴ In HT-29 cells, P-S up-regulated phosphorylated p38 (p-p38) and JNK (p-JNK). P-S (15 or 30 $\mu\text{mol/L}$) failed to induce formation of p-JNK, but at 60 and 90 $\mu\text{mol/L}$ it greatly increased p-JNK formation at 3 and 6 hours (Figure 4D). In the case of p38, 60 and 90 $\mu\text{mol/L}$ P-S markedly stimulated its phosphorylation, which became evident at 6 hours. Finally, P-S clearly decreased extracellular signal-regulated kinase 1 (ERK1)/ERK2 phosphorylation, which is important in cell survival.¹⁵

DFMO Synergizes With P-S to Inhibit Colon Cancer Cell Growth

By analogy with sulindac, which synergizes with DFMO to prevent colon cancer,³ we expected synergy between DFMO and P-S. At 48 hours, DFMO 5 mmol/L and P-S 40 $\mu\text{mol/L}$ each alone modestly inhibited cell growth, but their combination was more effective than the sum of the 2. The reductions in cell number were as

follows: (1) in HT-29 cells: DFMO, 14%; P-S, 41%; both, 84%; and (2) in SW480 cells: DFMO, 8%; P-S, 45%; both, 75%. The isobologram confirms as clear cut the pharmacologic synergy between the 2 agents (Figure 5A).

DFMO and P-S also synergize to inhibit cell cycle phase transitions. For example, the proportion of HT-29 cells in S phase was 13.6% for either alone and was reduced to 2.9% for both (Figure 5B). A similar synergistic effect was observed in the induction of apoptosis. After 48 hours of incubation with DFMO and P-S, the percentage of apoptotic cells was 38.4%, compared with 8.7% and 16.5% for DFMO and P-S alone, respectively (Figure 5C). Of note, the concentrations of both compounds were below their IC₅₀s for cell growth.

To elucidate the mechanism of the combination, we measured polyamines levels. As expected, P-S decreased spermidine and spermine, whereas DFMO completely abrogated putrescine levels. Combination of P-S and DFMO decreased the levels of the 3 polyamines measured (Figure 5D). We also evaluated if DFMO could enhance the increase in RONS levels induced by P-S. Although P-S increased RONS levels after 1 hour of incubation, cotreatment with DFMO did not additionally increase RONS levels in SW480 cells (Figure 5E).

P-S Shows No Genotoxicity and No Gastrotoxicity in Rats and Mice

We evaluated the safety of P-S by examining its genotoxicity by the Ames test and its gastrointestinal and other toxicity in mice and rats and compared it with sulindac.

Genotoxicity. The mutagenic potential of P-S was evaluated by measuring its ability to induce reverse mutations at selected loci of 2 strains of *Salmonella typhimurium* in the presence and absence of S9 activation (performed by BioReliance, Rockville, MD). All these studies were negative for genotoxicity.

Acute toxicity. Mice were treated for 5 days by oral gavage with equimolar amounts of P-S (317 mg/kg/d) or sulindac (200 mg/kg/d) or vehicle. Mice surviving to the end of the study were killed. P-S- and vehicle-treated mice (1) maintained their weight (P-S: 16.3 \pm 1.2 to 15.7 \pm 1.2; vehicle: 16.1 \pm 1.0 g to 15.7 \pm 1.2; mean \pm SD) and (2) showed no evidence of gastrointestinal or other toxicity; (3) all were alive and healthy at the conclusion of the study; and (4) inspection of the heart,

Figure 5. DFMO enhances P-S-induced inhibition of colon cancer cell growth. (A *left*) Cell viability was determined in HT-29 and SW-480 cells after 48 hours of incubation with 5 mmol/L DFMO, 40 $\mu\text{mol/L}$ P-S, or both. Results are expressed as percentage of control; * P < .02 vs control. (A *right*) In this isobologram the additivity line connects the IC₅₀ value of each compound used alone. A and B represent 2 different dose pairs of each compound (their respective concentrations are shown in parentheses). The location of both A and B below the additivity line signifies synergy. (B) Cell cycle progression in HT-29 cells incubated for 48 hours as indicated. Representative profiles of the distribution of cells in G₁, G₂/M, and S phases are shown. (C) The percentages of apoptotic cells determined by flow cytometry with the use of dual staining (Annexin V and propidium iodide) are indicated in each quadrant. Representative images are shown. (D) Polyamines levels in SW480 cells treated with P-S 1 \times IC₅₀, DFMO 5 mmol/L, or a combination of both for 24 hours; * P < .05 vs control; n.d.: not detected. (E) DCFDA fluorescence was measured by flow cytometry in SW480 cells treated with P-S 1 \times IC₅₀, DFMO 5mmol/L, or both for 1 hour.

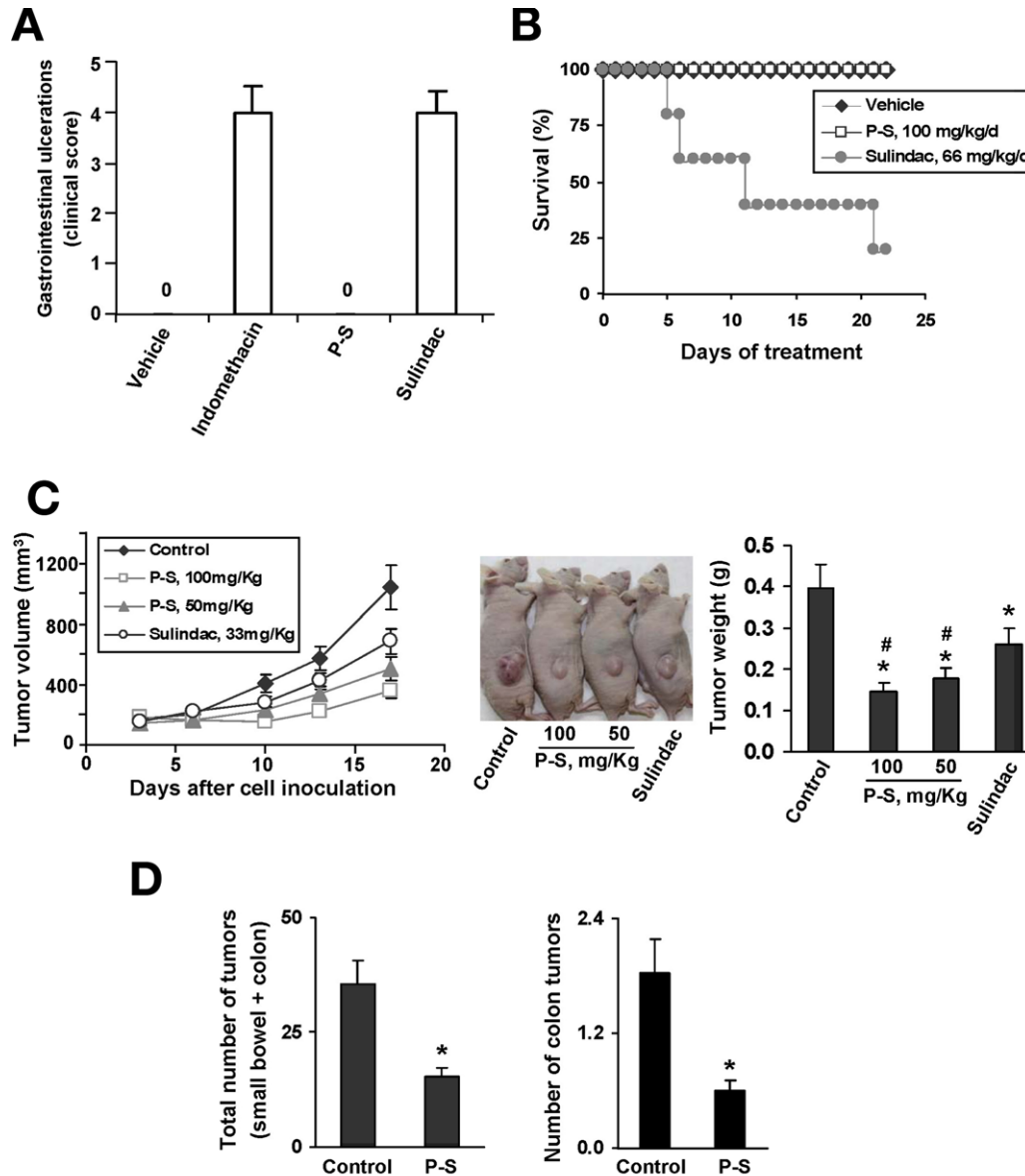
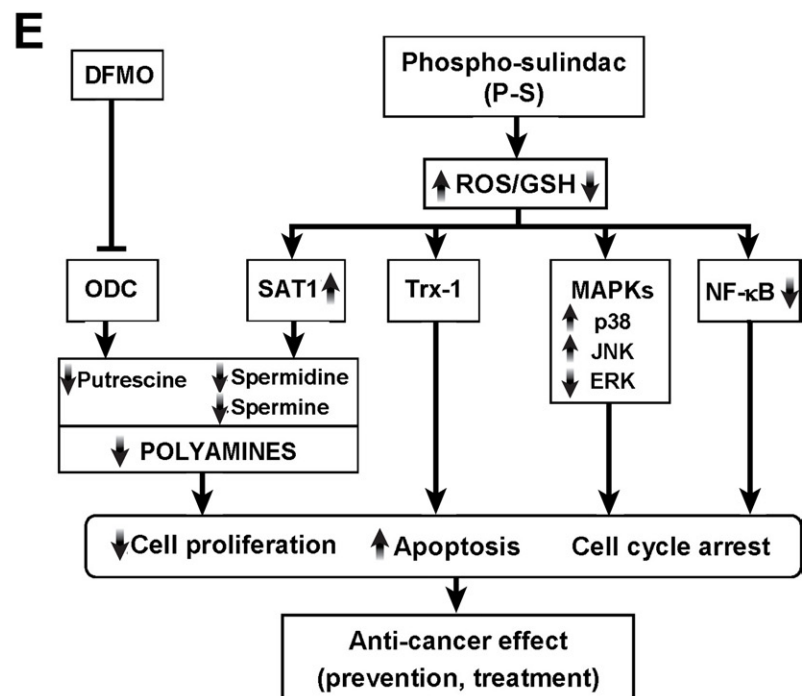
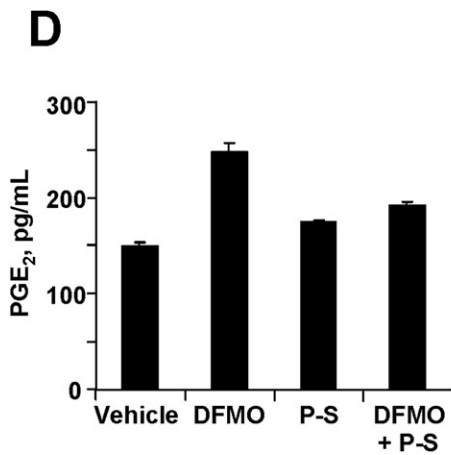
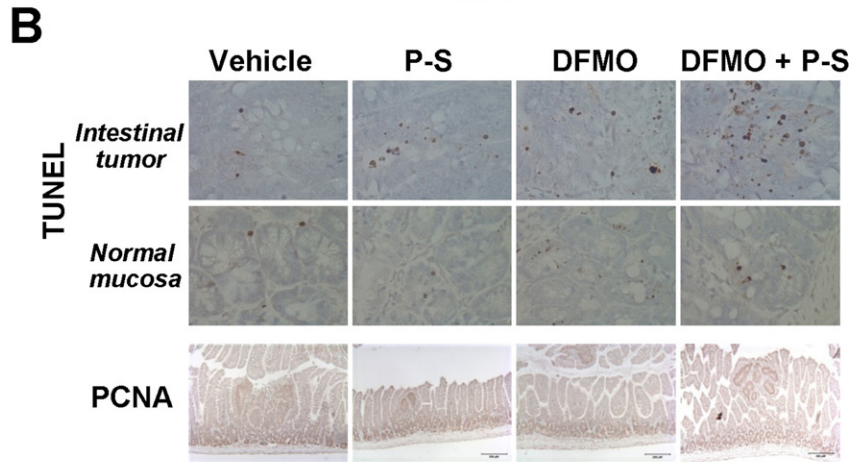
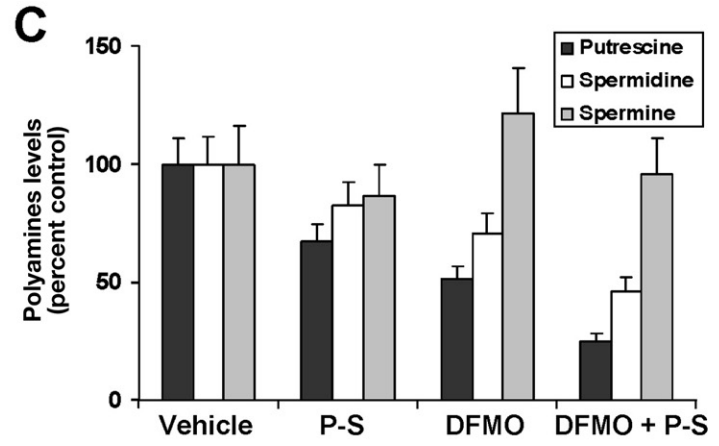
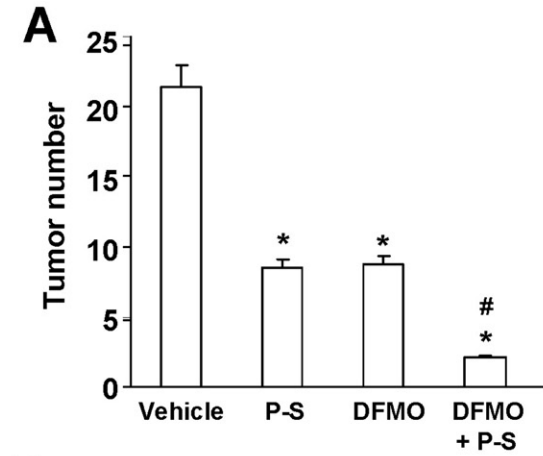


Figure 6. P-S is a safe and effective antitumor agent against colon cancer in vivo. (A) Acute gastrointestinal toxicity of sulindac and P-S. Rats were treated with P-S or sulindac or indomethacin or vehicle, as described in “Materials and Methods.” At day 5, the number and sizes of small intestine ulcerations were counted and scored according to the protocol.¹¹ P-S-treated rats showed no gastrointestinal toxicity. (B) Survival curve for mice treated daily for 3 weeks with equimolar doses of P-S or sulindac. (C) Effect of P-S and sulindac on colon cancer xenografts in nude mice. (Left) Tumor volume growth over time for mice treated with control, P-S 50 mg/kg/d, P-S 100 mg/kg/d, and sulindac 33 mg/kg/d. (Center) The difference in tumor size among the groups is apparent. (Right) Mass of the dissected tumors. All values are mean \pm SEM; * P < .05 vs vehicle-treated mice; # P < .05 vs sulindac-treated mice. (D) Effect of P-S on tumor multiplicity in *Apc^{Min/+}* mice. The total number of tumors per animal in both small and large bowel (left) as well as those only in colon (right) was greatly reduced after 4 weeks of treatment with P-S; * P < .02 vs control mice.

Figure 7. P-S in combination with DFMO inhibits tumor multiplicity in *Apc^{Min/+}* mice. (A) The number of tumors per animal was greatly reduced after 9 weeks of treatment with P-S, DFMO, or both compared with controls, * P < .01 vs controls; # P < .01 vs P-S- or DFMO-treated groups. (B) Combination between DFMO and P-S inhibited proliferation and selectively increased apoptosis in intestinal tumors. Intestinal tissue sections from *Apc^{Min/+}* mice treated without (vehicle) or with P-S, DFMO, or both were stained for proliferating cell nuclear antigen expression as a proliferation marker ($\times 10$) or by the terminal deoxynucleotidyl-transferase-mediated 2'-deoxyuridine 5'-triphosphate-biotin nick-end labeling (TUNEL) method as an apoptosis marker ($40\times$). (Right) A total of 10 fields from tumor and normal mucosa for each group were examined and counted ($n = 5/\text{group}$). Results are expressed as the Apoptotic index (TUNEL+ cells \pm SEM per $40\times$ field); * P < .01 vs control. (C) Polyamine content in small intestine of *Apc^{Min/+}* mice treated with P-S, DFMO, or both. (D) Intestinal PGE₂ levels of *Apc^{Min/+}* mice treated with P-S, DFMO, or both. (E) Scheme showing the target pathways by which P-S alone, or in combination with DFMO, leads to colon cancer prevention.



lungs, spleen, kidneys, and liver showed no abnormalities. In contrast, sulindac-treated mice (1) lost 20% of their weight (16.3 ± 1.2 g to 13.0 ± 0.5 g; mean \pm SD) and (2) showed significant mortality: 75% versus 0% for P-S and vehicle (5 of the 8 mice died: 1 on day 2; 2 on day 3; 2 on day 4; and 1 on day 5); (3) necropsies showed upper gastrointestinal toxicity with macroscopically evident gastric ulcers in 3 mice, gastric bleeding in 1 mouse, and perforation in 1 mouse. The stomachs of sulindac-treated animals were larger than those of the other 2 groups, and in some the liver appeared hyperemic.

Gastrointestinal toxicity. Rats were treated for 4 days by oral gavage with equimolar amounts of P-S (317 mg/kg/d) or sulindac (200 mg/kg/d), indomethacin (4.75 mg/kg/d), or vehicle. As expected,¹¹ treatment with indomethacin produced predominantly medium and large ulcerations (>4 mm) in the small intestine, generating to a clinical score of 3.8. Vehicle- and P-S-treated rats showed no gastrointestinal toxicity, with no signs of ulcerations or mucosal damage (Figure 6A). In contrast to P-S and similar to indomethacin, medium and large ulcerations in the small intestine were observed in the sulindac-treated rats.

Survival of mice and organ toxicity. Mice were treated for 3 weeks by oral gavage with vehicle, P-S (100 mg/kg/d), or sulindac 66 mg/kg/d (equimolar) or 100 mg/kg/d (equi-dose). All vehicle- and P-S-treated mice were alive and healthy by the end of the study. However, 80% of the mice treated with sulindac (66 mg/kg/d) died by 3 weeks (Figure 6B), and all mice treated with sulindac (100 mg/kg/d) died after 7 days of treatment (Supplementary Figure 1). Liver function tests (aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, γ -glutamyl transpeptidase, and total bilirubin) were similar between P-S- and vehicle-treated mice. Finally, histologically the liver, pancreas, and kidneys of mice treated with P-S showed no signs of toxicity (Supplementary Figure 2).

P-S Alone and in Combination With DFMO Inhibits Colon Carcinogenesis In Vivo

We evaluated the chemotherapeutic potential of P-S and sulindac in a subcutaneous xenograft model of HT-29 cells. At killing, although P-S (50 or 100 mg/kg/d by gavage) reduced tumor growth by 51% and 65%, respectively, compared with controls, sulindac (33 mg/kg/d, equimolar to P-S 50 mg/kg/d) reduced tumor growth by 34%. At both doses P-S reduced tumor growth significantly more than sulindac ($P < .05$; Figure 6C). In *Apc^{Min/+}* mice (predisposed to develop tumors in the small intestine and colon), P-S 100 mg/kg/d by gavage for 4 weeks decreased the number of tumors in the small intestine by 57.2% compared with controls ($P < .002$); in the colon such reduction was 61.8% ($P < .02$; Figure 6D). In both studies P-S was well tolerated with no weight loss during treatment.

In another study, P-S (100 mg/kg by gavage) and DFMO (2% in drinking water) were administered alone and combined to *Apc^{Min/+}* mice between 6 and 13 weeks

of age. DFMO and P-S alone reduced the number of all intestinal tumors by 50.8% and 52.1%, respectively, but their combination reduced it by 90.2% ($P < .0001$; Figure 7A). The combination of P-S plus DFMO achieved this effect by inhibiting cell proliferation and inducing apoptosis (Figure 7B). Interestingly, compared with the vehicle-treated group, P-S plus DFMO selectively induced apoptosis in the intestinal tumors but not in the normal mucosa (Figure 7B). Mechanistically, this combination reduced total intestinal polyamine levels (Figure 7C) but did not alter PGE₂ tissue levels among the various groups (Figure 7D).

Discussion

The successful pharmacologic agent against cancer must be effective and must lack significant side effects. Here, we demonstrate that P-S seems to meet both these requirements, markedly inhibiting intestinal tumors by itself and to a greater extent in combination with DFMO and appearing much safer than sulindac in preclinical studies.

P-S is a potent inhibitor of colon cancer cell lines (>14-fold more potent than sulindac) and exerts a profound inhibitory effect in preclinical models of colon cancer. Although sulindac reduced the volume of colon xenografts by 34%, P-S at an equimolar dose reduced it by 51%, and by 65% at a higher dose. Furthermore, P-S reduced the number of all intestinal tumors in *Apc^{Min/+}* mice by 57.2%. Importantly, in contrast to sulindac, which stimulates tumor formation in the colon of *Apc^{Min/+}* mice,¹⁶ P-S reduced the number of colon tumors by 61.8%. This inhibitory effect of P-S appears to be caused by a triple cell kinetic effect: inhibition of proliferation, induction of apoptosis and necrosis, and blockage at the G₁/S cell cycle transition; the proapoptotic effect being the dominant. Of note, a normal human colon epithelial cell line was resistant to P-S, indicating selectivity between normal and transformed colon cells. DFMO synergized with P-S in vivo, reducing the number of tumors in *Apc^{Min/+}* mice by 90.2%; their cytotoxic effect was synergistic, and apoptosis was induced selectively in the intestinal tumors and not in the normal mucosa. These results indicate that P-S with and without DFMO is effective and acts selectively against intestinal tumors.

Our work unraveled key parts of the mechanism of action of P-S (Figure 7E). A major event is the induction of oxidative stress, followed by the activation of signaling cascades, involving polyamines, the thioredoxin system, MAPKs, and NF- κ B. The centrality of the oxidative stress (elevated RONS, suppressed GSH, and increased oxidized Trx-1 levels) was underscored by manipulating the system: the antioxidant NAC greatly attenuated the apoptotic effect of P-S and decreased the activity of SAT1, the enzyme that acetylates and exports polyamines from the cells; BSO, which depletes intracellular GSH, enhanced its growth inhibitory effect; and as previously shown, knocking-down the expression of *trx-1* rescued cells from the proapoptotic effect of P-S.⁶ These effects, combined

with the inhibition of ornithine decarboxylase by DFMO, culminates in the profound cytokinetic effect manifested as dramatic colon cancer prevention.

The inhibition of NF- κ B activation by P-S and changes in 3 major branches of MAPKs (activation of p38 and JNK and suppression of ERK1/2) are consistent with the growth inhibitory effect of P-S and probably mediate part of it. On the one hand, the expression of COX-2 and COX-1 were not affected by P-S; on the other hand, COX activity and PGE₂ levels increased in vitro and remained unchanged in vivo. In contrast to sulindac, P-S did not inhibit COX-2 expression or PGE₂ levels in response to the calcium ionophore A23287. Although sulindac inhibits COX, it is apparent that modifying it at its carboxylic group to generate P-S abrogates its ability to inhibit COX. This is in agreement with a recent study that confirms the crucial role of sulindac's carboxylic moiety for COX-2 binding.⁵ Indeed, the complex mode of action of sulindac includes COX-dependent and -independent effects.

Increased polyamine levels have long been associated with colon carcinogenesis and are considered targets of both DFMO and NSAIDs, including sulindac.¹² Sulindac stimulates polyamine acetylation and export by increasing SAT1 activity.¹² Similar to sulindac, P-S per se reduced the levels of spermidine and spermine by inducing SAT1 activity. Indeed, SAT1 is responsible, in part, for the cell growth inhibitory effect of P-S; knocking-down *sat1* expression rescued cell growth inhibition by P-S. The synergy of P-S and DFMO in suppressing polyamine levels is apparent both in vitro and in vivo.

The safety of an agent is of extreme importance. Although NSAIDs, including sulindac, are established as prototypical colon chemopreventive agents,¹⁷ their extended use in chemoprevention is limited because of significant toxicity, mostly of the gastrointestinal tract.⁴ Unlike sulindac, the novel P-S is a safer chemopreventive agent on the basis of the lack of genotoxicity and the absence of gastrointestinal toxicity in rats and mice.

In summary, our data indicate that the novel drug P-S is a safe and effective drug in preclinical models, 2 essential pharmacologic properties for any candidate chemopreventive agent. Therefore, P-S merits further evaluation as a potentially chemopreventive agent against colon cancer.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2010.06.044.

References

1. Beazer-Barclay Yet al WF, Hamilton SR, Vogelstein B, Kinzler KW. Sulindac suppresses tumorigenesis in the Min mouse. *Carcinogenesis* 1996;17:1757–1760.
2. Gerner EW, Meyskens FL Jr. Polyamines and cancer: old molecules, new understanding. *Nat Rev Cancer* 2004;4:781–792.

3. Meyskens FL Jr, McLaren CE, Pelot D, et al. Difluoromethylornithine plus sulindac for the prevention of sporadic colorectal adenomas: a randomized placebo-controlled, double-blind trial. *Cancer Prev Res (Phila Pa)* 2008;1:32–38.
4. Roberts J 2d, Morrow J. Analgesic-antipyretic and antiinflammatory agents and drugs employed in the treatment gout. In: Hardman JG, Limbird LE, eds. *Goodman and Gilman's The Pharmacological Basis of Therapeutics*. 10th ed. New York: McGraw-Hill, 2001:687–731.
5. Piazza GA, Keeton AB, Tinsley HN, et al. A novel sulindac derivative that does not inhibit cyclooxygenases but potently inhibits colon tumor cell growth and induces apoptosis with antitumor activity. *Cancer Prev Res (Phila Pa)* 2009;2:572–580.
6. Sun Y, Rigas B. The thioredoxin system mediates redox-induced cell death in human colon cancer cells: implications for the mechanism of action of anticancer agents. *Cancer Res* 2008;68:8269–8277.
7. Zhao W, Mackenzie GG, Murray OT, Zhang Z, Rigas B. Phosphoaspirin (MDC-43), a novel benzyl ester of aspirin, inhibits the growth of human cancer cell lines more potently than aspirin: a redox-dependent effect. *Carcinogenesis* 2009;30:512–519.
8. Gao J, Liu X, Rigas B. Nitric oxide-donating aspirin induces apoptosis in human colon cancer cells through induction of oxidative stress. *Proc Natl Acad Sci U S A* 2005;102:17207–17212.
9. Seiler N, Knodgen B. High-performance liquid chromatographic procedure for the simultaneous determination of the natural polyamines and their monoacetyl derivatives. *J Chromatogr* 1980;221:227–235.
10. Chopra S, Wallace HM. Induction of spermidine/spermine N1-acetyltransferase in human cancer cells in response to increased production of reactive oxygen species. *Biochem Pharmacol* 1998;55:1119–1123.
11. Whiteley PE, Dalrymple SA. Models of inflammation: measuring gastrointestinal ulcerations in the rat. In: Enna SJ, ed. *Current Protocols in Pharmacology*. Volume 3. New York: John Wiley & Sons Inc, 1998.
12. Babbar N, Ignatenko NA, Casero RA Jr, Gerner EW. Cyclooxygenase-independent induction of apoptosis by sulindac sulfone is mediated by polyamines in colon cancer. *J Biol Chem* 2003;278:47762–47775.
13. Karin M. Nuclear factor-kappaB in cancer development and progression. *Nature* 2006;441:431–436.
14. Benhar M, Engelberg D, Levitzki A. ROS, stress-activated kinases and stress signaling in cancer. *EMBO Rep* 2002;3:420–425.
15. Balmanno K, Cook SJ. Tumour cell survival signalling by the ERK1/2 pathway. *Cell Death Differ* 2009;16:368–377.
16. Yang K, Fan K, Kurihara N, et al. Regional response leading to tumorigenesis after sulindac in small and large intestine of mice with *Apc* mutations. *Carcinogenesis* 2003;24:605–611.
17. Baron JA. What now for aspirin and cancer prevention? *J Natl Cancer Inst* 2004;96:4–5.

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Conflicts of interest

The authors disclose no conflicts, except for B.R., who has an equity position in Medicon, Inc.

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