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# 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 *N*$^1$-acetyltransferase enzymatic activity, and together with DFMO it reduces polyamine levels in vitro and in vivo. **CONCLUSIONS: P-S displays considerable safety and efficacy, two 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 Meyskens 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 ≤20% of patients.[4] These considerations prompted us to synthesize phospho-sulindac (P-S; OXT-328; Figure 1*A*), 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 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–fluo-

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*Abbreviations used in this paper:* BSO, buthionine sulfoximine; COX, cyclooxygenase; DCFDA, dichlorodihydrofluorescein diacetate; DFMO, difluoromethylornithine; ERK, extracellular-signal regulate kinase; GSH, glutathione; $IC_{50}$, concentration that inhibits 50%; JNK, Jun kinase; MAPK, Mitogen-activated protein kinase; NAC, *N*-acetyl-L-cysteine; NF-κB, nuclear factor-κB; 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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rescein isothiocyanate and propidium iodide (PI) and the 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 $2 \times 10^6$ cells (treated and controls) and subjected to electrophoretic mobility shift assay.[7] Oligonucleotides containing the consensus sequence for nuclear factor-$\kappa$B (NF-$\kappa$B) or Octamer transcription factor 1 (OCT-1; control) were end-labeled with [$\gamma$-$^{32}$P]–adenosine triphosphate with the use of T4 polynucleotide kinase.

### *Cyclooxygenase Activity and PGE$_2$ Levels*

Cyclooxygenase (COX) activity and prostaglandin E$_2$ (PGE$_2$) levels were determined by immunoassays, following the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI).

### *Polyamines and Spermidine/Spermine-N$^1$-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-N$^1$-acetyltransferase (SAT1) activity, cells ($3 \times 10^6$) 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 $N^1$-acetylspermidine synthesized from [$^{14}$C]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.

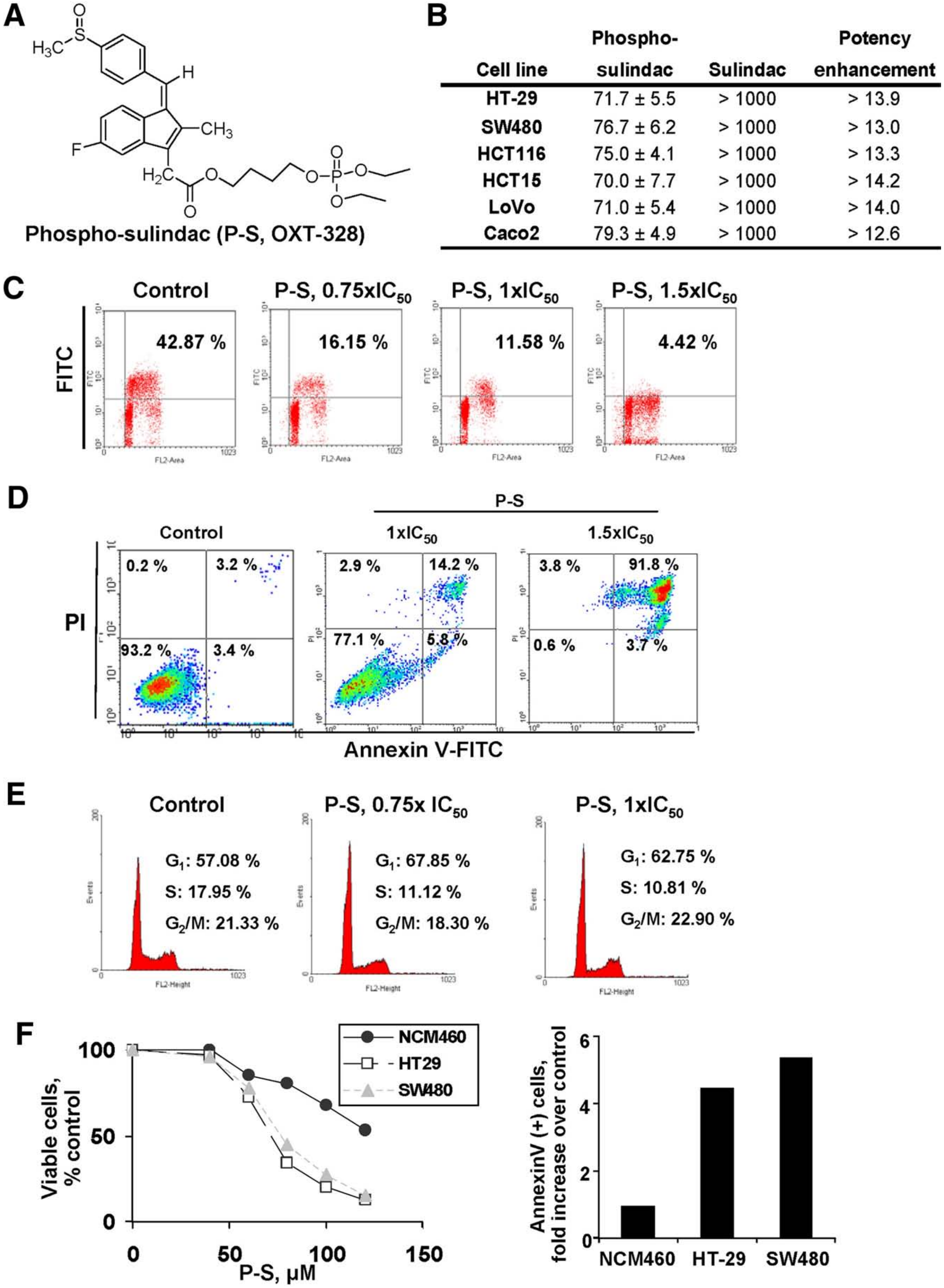
**Acute toxicity.** Six-week-old female C57BL/6J$^{+/+}$ mice (n = 8/group) were treated for 5 days by oral gavage with equimolar amounts of P-S (317 mg/kg/d) or sulindac (200 mg/kg/d) or vehicle. Body weight was recorded every other day. All mice were necropsied.

**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 $2 \times 10^6$ HT-29 colon cancer cells on each flank. Tumor size was determined with a digital microcalipter (tumor volume = [length × width × (length + width/2) × 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 protocols[8] or snap-frozen for biochemical analyses.



| 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 |

### *Statistical Analysis*

Results, from at least 3 independent experiments and expressed as mean ± 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 and sulindac on cell growth, we determined in various human colon cancer cells the 24-hour concentration values that inhibit their growth by 50% (IC$_{50}$; Figure 1*B*). The IC$_{50}$ values of P-S varied little among these cell lines (70–79.3 μmol/L), whereas those of sulindac were consistently >1000 μmol/L, indicating a potency enhancement of >14.2-fold.

We then elucidated the underlying cytokinetic effect of P-S on HT-29 and SW480 cells. P-S markedly reduced concentration-dependent cell proliferation (Figure 1*C*). For instance, at its IC$_{50}$ P-S reduced HT-29 cell proliferation from 43% in controls to 12% and at 1.5× IC$_{50}$ 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× IC$_{50}$ and to 95.5% at 1.5× IC$_{50}$ (Figure 1*D*). Finally, P-S blocked the G$_1$-to-S transition. The percentage of cells in G$_1$ phase was significantly higher in cells treated with 0.75× IC$_{50}$ P-S than in controls (HT-29 cell, 80% vs 57%; SW480 cells, 68% vs 57%; Figure 1*E*).

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 μmol/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 1*F*). In addition, incubation of HT-29 and SW480 cells with 110 μmol/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 1*F*). 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× or 2× IC$_{50}$ P-S for 1 hour with DCFDA, a general RONS probe. The level of RONS in response to P-S at 0.5× and 1× IC$_{50}$ for cell growth increased 1.8- and 2.8-fold over control (Figure 2*A*), whereas P-S at 1× and 1.5× IC$_{50}$ gave intermediate results (not shown). In HT-29 cells, P-S 1× IC$_{50}$ increased mitochondria superoxide levels in a time-dependent manner (Figure 2*B*). 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× IC$_{50}$ for 4 hours deceased GSH levels by 50% (Figure 2*C*). The GSH 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 2*E*).

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 2*F*). Moreover, GSH depletion induced by BSO enhanced the cell growth-induced inhibition by P-S (Figure 2*D*). Although P-S inhibited the growth of HT-29 and SW480 cells (IC$_{50}$ > 80 μmol/L under this experimental protocol), pretreatment with 100 μmol/L BSO for 24 hours reduced the IC$_{50}$ to 25 and 26 μmol/L for HT-29 and SW480 cells, respectively (Figure 2*D*). 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-

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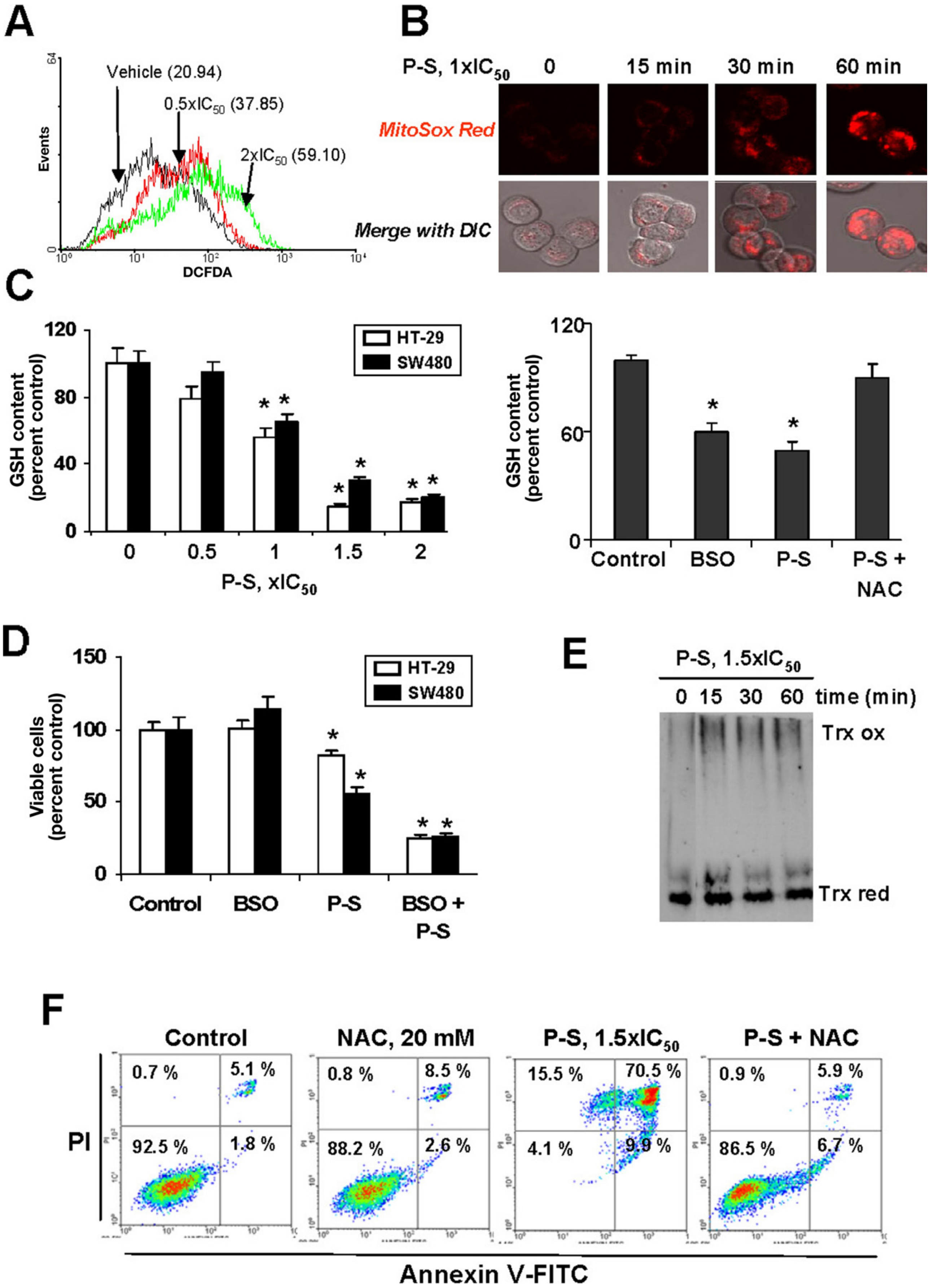
**Figure 1.** Phospho-sulindac (P-S; OXT-328) inhibits cell proliferation and induces cell cycle arrest and cell death by apoptosis in colon cancer cells. (*A*) Chemical structure of P-S. (*B*) IC$_{50}$ values (μmol/L) for colon cancer cells treated with P-S or sulindac for 24 hours (mean ± SEM). (*C*) Histograms of 5-bromo-2′-deoxyuridine incorporation in HT-29 cells treated for 24 hours without (control) or with 0.75×, 1×, or 1.5× IC$_{50}$ P-S. FITC, fluorescein isothiocyanate. (*D*) The percentages 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 G$_1$, G$_2$/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 various concentrations (0–120 μmol/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 μmol/L P-S for 24 hours. Results are expressed as fold-increase compared with the percentages of apoptotic cells in the NCM460 cells.


A
Vehicle (20.94)
0.5xIC50 (37.85)
2xIC50 (59.10)
Events
64
0
10⁰
10¹
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DCFDA
B
P-S, 1xIC50
0
15 min
30 min
60 min
MitoSox Red
Merge with DIC
C
GSH content (percent control)
120
80
40
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0
0.5
1
1.5
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P-S, xIC50
HT-29
SW480
*
GSH content (percent control)
120
60
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Control
BSO
P-S
P-S + NAC
D
Viable cells (percent control)
150
100
50
0
Control
BSO
P-S
BSO + P-S
HT-29
SW480
E
P-S, 1.5xIC50
0
15
30
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time (min)
Trx ox
Trx red
F
Control
NAC, 20 mM
P-S, 1.5xIC50
P-S + NAC
PI
0.7 %
5.1 %
92.5 %
1.8 %
0.8 %
8.5 %
88.2 %
2.6 %
15.5 %
70.5 %
4.1 %
9.9 %
0.9 %
5.9 %
86.5 %
6.7 %
Annexin V-FITC

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

We examined the effect of P-S on SAT1 activity, an enzyme known to be induced by sulindac.[12] Incubation for 24 hours with 85 $\mu$mol/L P-S increased SAT1 activity by 3- and 4.4-fold ($P < .05$ vs control), but 800 $\mu$mol/L sulindac increased it by 1.5- and 2.5-fold ($P < .05$ vs control) in HT-29 and SW480 cells, respectively (Figure 3*B*). 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 3*B*). 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 5.5-fold increase after 18 hours (Figure 3*C*).

To evaluate the role of SAT1 in P-S–induced cell growth inhibition, we silenced *sat1*. Compared with controls, knocking-down *sat1* rendered SW480 cells resistant to the growth inhibitory effect of P-S. Although cells transfected with nonspecific siRNA required 75 $\mu$mol/L P-S to decrease their number of viable cells by half, cells transfected with siRNA against *sat1* required 93 $\mu$mol/L P-S (24% more) for the same effect (Figure 3*D*).

**P-S modulates redox-sensitive signaling molecules.** *NF-κB.* A redox-sensitive dimer, NF-κB modulates cell growth and inflammation, especially in cancer.[13] Treatment of HT-29 cells with P-S suppressed NF-κB activation in a concentration-dependent manner (Figure 4*A*). Moreover, although TNF-α rapidly activated NF-κB, 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/$PGE_2$ 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 $PGE_2$ production (Figure 4*B*). The COX-2 inhibitors sulindac and aspirin abrogated the increase in $PGE_2$ levels by the calcium ionophore A23187, but P-S failed to prevent it (Figure 4*C*), suggesting that it lacks an anti-COX effect. P-S plus sulindac suppressed COX-2 expression and $PGE_2$ 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 $\mu$mol/L) failed to induce formation of p-JNK, but at 60 and 90 $\mu$mol/L it greatly increased p-JNK formation at 3 and 6 hours (Figure 4*D*). In the case of p38, 60 and 90 $\mu$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 $\mu$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 5*A*).

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 5*B*). 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 5*C*). Of note, the concentrations of both compounds were below their $IC_{50}$s 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 5*D*). 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, co-

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**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× $IC_{50}$ or 2× $IC_{50}$ P-S for 1 hour. (*B*) P-S induces a time-dependent increase in mitochondrial superoxide levels. HT-29 cells incubated with 1× $IC_{50}$ P-S for different time periods. After adding the selective MitoSOX probe, cells were subjected to confocal microscopy (×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× $IC_{50}$ P-S, BSO, or with NAC and P-S for 4 hours (*right*). Values are shown as means ± SEM of 3 independent experiments; *$P < .05$ vs control. (*D*) HT-29 and SW480 cells were treated with 100 $\mu$mol/L BSO for 24 hours followed by treatment with P-S 1× $IC_{50}$ 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× $IC_{50}$ 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.

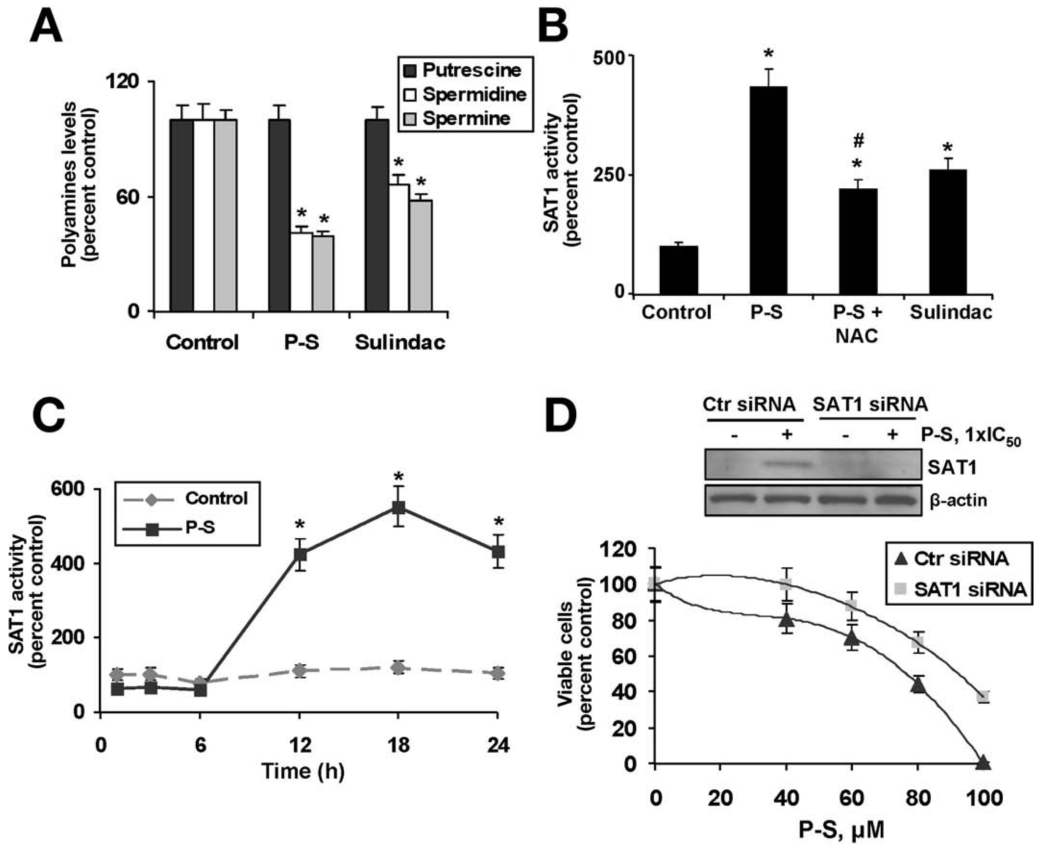


**Figure 3.** P-S reduces polyamines levels by inducing SAT1 in colon cancer cells. (*A*) Polyamine levels in SW480 cells treated with P-S 80 $\mu$mol/L or sulindac 800 $\mu$mol/L for 24 hours. *$P < .05$ vs control. (*B*) P-S induces SAT1 enzymatic activity. SW480 cells were incubated with P-S or NAC and 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× $IC_{50}$ P-S for 24 hours. Immunoblots to verify SAT1 silencing were performed on whole cell extracts obtained from these cells (*top*).

treatment with DFMO did not additionally increase RONS levels in SW480 cells (Figure 5*E*).

### *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 ± 1.2 to 15.7 ± 1.2; vehicle: 16.1 ± 1.0 g to 15.7 ± 1.2; mean ± 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, 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 ± 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 6*A*). In contrast to P-S and similar

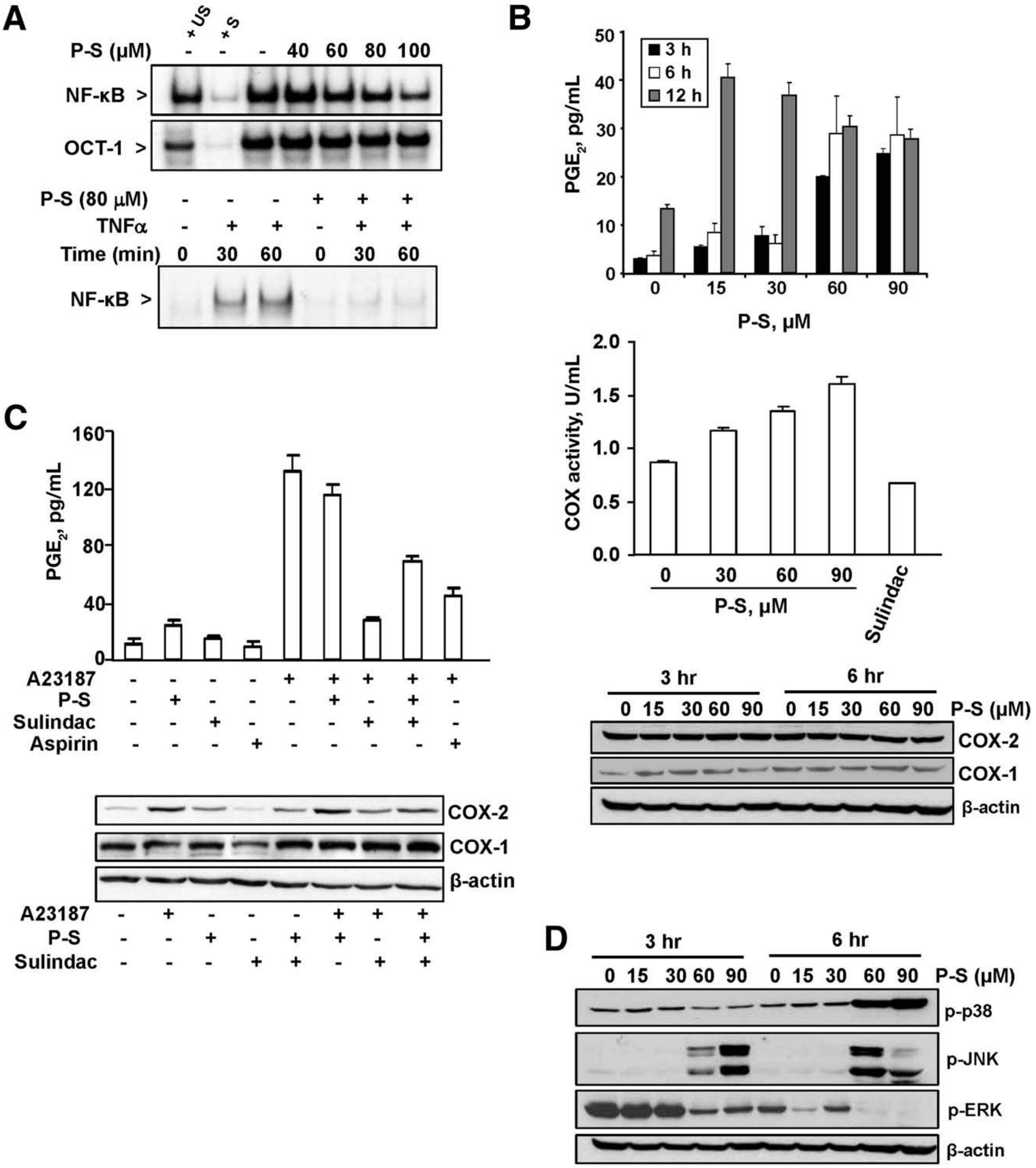
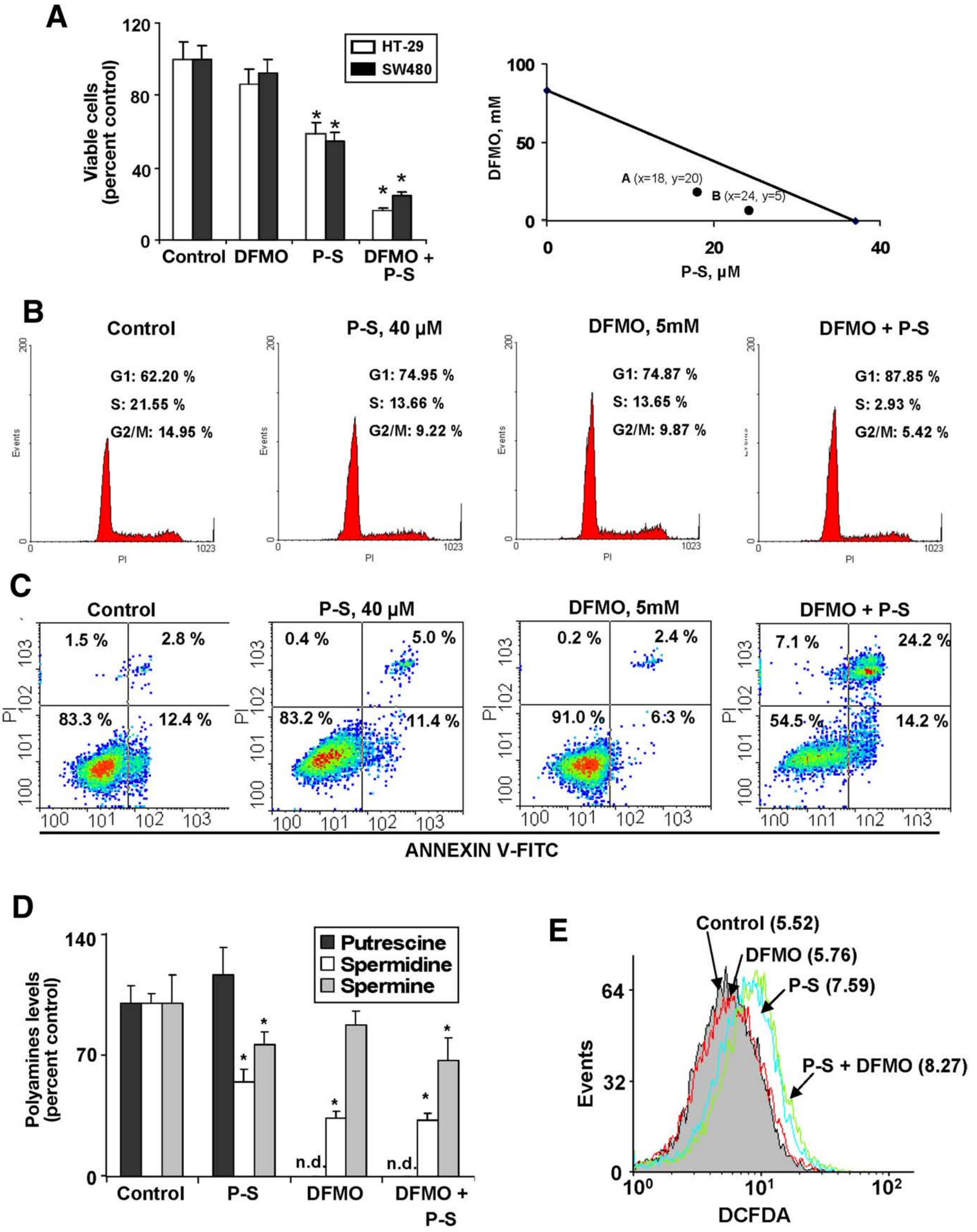


**Figure 4.** P-S modulates redox-sensitive signaling pathways in colon cancer cells. (*A*) P-S inhibits constitutive and tumor necrosis factor $\alpha$ (TNF-$\alpha$)–induced NF-$\kappa$B activation. (*Top*) Electrophoretic mobility shift assay for NF-$\kappa$B and OCT-1 of nuclear fractions isolated from HT-29 cells after 4 hours of treatment without or with 40–100 $\mu$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-$\kappa$B 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$\alpha$. n.s.: not specific. (*B*) P-S increased PGE$_2$ levels (*top*), measured by enzyme-linked immunoabsorbent assay (ELISA), in a time- and concentration-dependent manner; and up-regulated COX-2 (*center*) activity, measured by ELISA after a 6-hour incubation with P-S or 1.2 mmol/L sulindac. (*Bottom*) 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× IC$_{50}$, sulindac 1.2 mmol/L, or aspirin 0.4 mmol/L for 30 minutes followed by A23187 5 $\mu$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. $\beta$-Actin levels are shown as loading controls.

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 6*B*), 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, $\gamma$-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 6*C*). 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 6*D*). 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 7*A*). The combination of P-S plus DFMO achieved this effect by inhibiting cell proliferation and inducing apoptosis (Figure 7*B*). 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 7*B*). Mechanistically, this combination reduced total intestinal polyamine levels (Figure 7*C*) but did not alter PGE$_2$ tissue levels among the various groups (Figure 7*D*).

## 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 G$_1$/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 cytokinetic 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 7*E*). A major event is the induction of oxidative stress, followed by the activation of signaling cascades, involving polyamines, the thioredoxin system, MAPKs, and NF-$\kappa$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-$\kappa$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

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**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 $\mu$mol/L P-S, or both. Results are expressed as percentage of control; *$P < .02$ vs control. (*Right*) In this isobologram the additivity line connects the IC$_{50}$ 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 G$_1$, G$_2$/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× IC$_{50}$, 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× IC$_{50}$, DFMO 5mmol/L, or both for 1 hour.

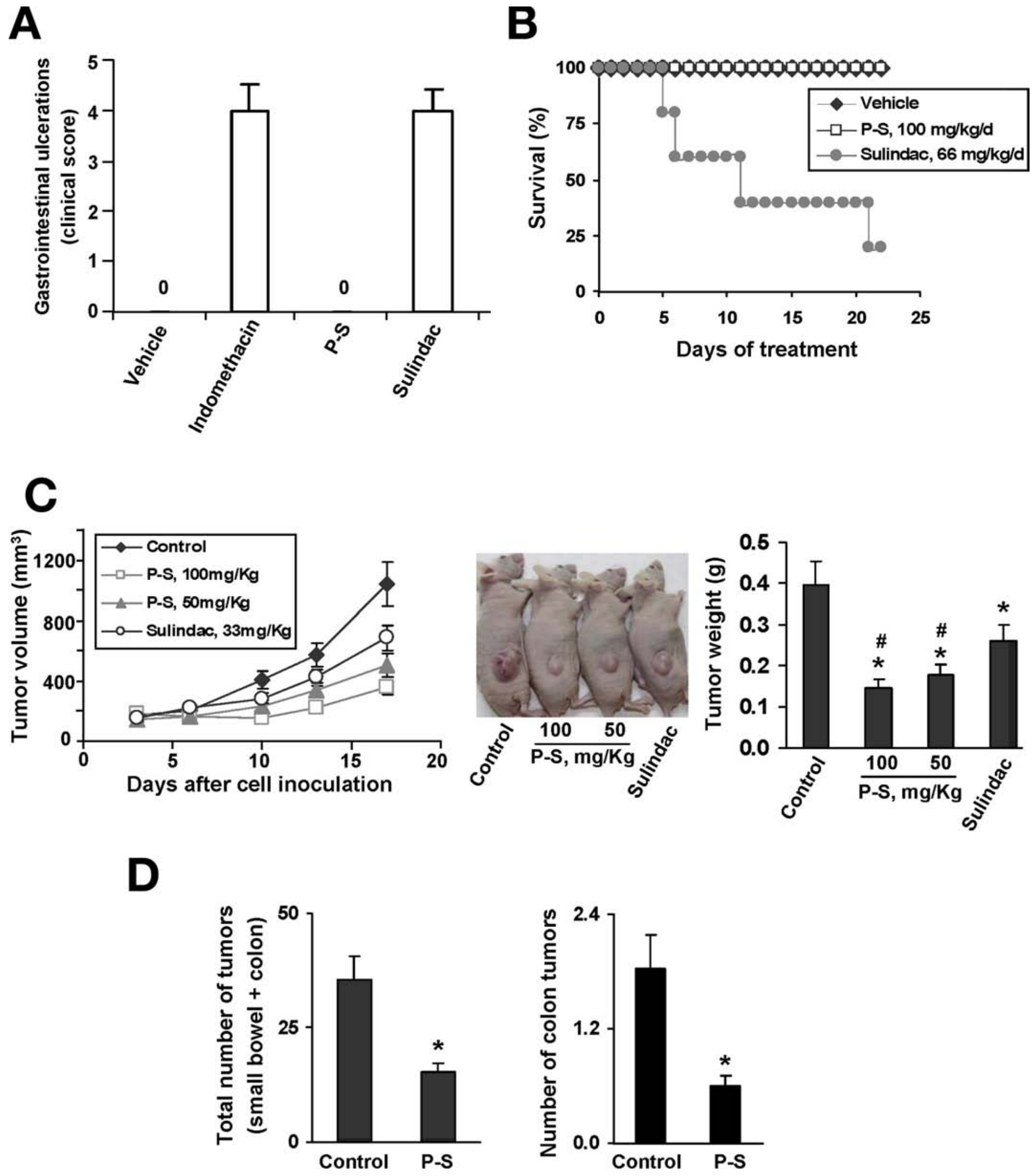
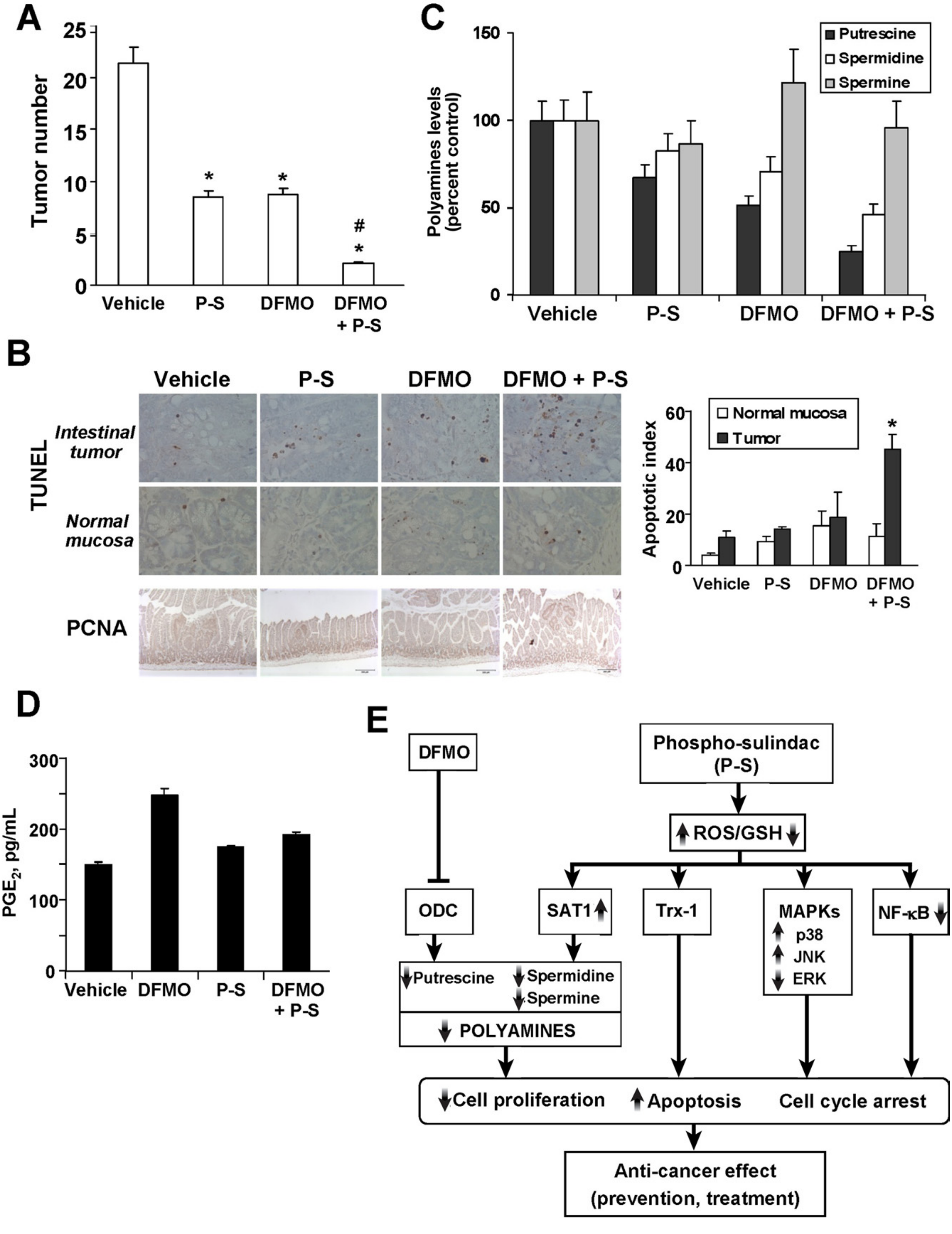


**Figure 6.** P-S is a safe and effective antitumor agent against colon cancer in vivo. (*A*) Acute gastrointestinal toxicity of sulindac and P-S. Rats were treated with P-S or sulindac or indomethacin or vehicle, as described in "Materials and Methods." At day 5, the number and sizes of small intestine 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 ± 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 (×10) or by the terminal deoxynucleotidyl-transferase–mediated 2′-deoxyuridine 5′-triphosphate–biotin nick-end labeling (TUNEL) method as an apoptosis marker (40×). (*Right*) A total of 10 fields from tumor and normal mucosa for each group were examined and counted (n = 5/group). Results are expressed as the Apoptotic index (TUNEL+ cells ± SEM per 40× field); $^{*}P < .01$ vs control. (*C*) Polyamine content in small intestine of $Apc^{Min/+}$ mice treated with P-S, DFMO, or both. (*D*) Intestinal $PGE_2$ levels of $Apc^{Min/+}$ mice treated with P-S, DFMO, or both, measured by enzyme-linked immunoabsorbent assay, remain unchanged among the various groups. (*E*) Scheme showing the target pathways by which P-S alone, or in combination with DFMO, leads to colon cancer prevention.


A
Tumor number
25
20
15
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5
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*
*
#
*
Vehicle
P-S
DFMO
DFMO + P-S
C
Polyamines levels (percent control)
150
100
50
0
■ Putrescine
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Vehicle
P-S
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Vehicle
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Phospho-sulindac (P-S)
↑ROS/GSH↓
ODC
SAT1↑
Trx-1
MAPKs
↑ p38
↑ JNK
↓ ERK
NF-κB↓
↓Putrescine
↓Spermidine
↓Spermine
↓ POLYAMINES
↓Cell proliferation
↑Apoptosis
Cell cycle arrest
Anti-cancer effect (prevention, treatment)

inhibitory effect of P-S and probably mediate part of it. On the one hand, the expression of COX-2 and COX-1 was not affected by P-S; on the other hand, COX activity and $PGE_2$ levels increased in vitro and remained unchanged in vivo. In contrast to sulindac, P-S did not inhibit COX-2 expression or $PGE_2$ levels in response to the calcium ionophore A23187. 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.

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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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