

**Phospho-sulindac (OXT-922) inhibits the growth of human colon cancer cell lines:
A redox/polyamine dependent effect**

Liqun Huang¹, Caihua Zhu¹, Yu Sun¹, Gang Xie¹, Gerardo G. Mackenzie¹, George Qiao¹, Despina Komninou², Basil Rigas^{1*}

¹*Division of Cancer Prevention, Department of Medicine, Stony Brook University, Stony Brook, NY,*

²*Medicon, Inc, Stony Brook, NY*

† These authors contributed equally to this work.

*To whom correspondence should be addressed.

Tel: +1 631-444-9538; Fax: +1 631-444-9553; Email: basil.rigas@stonybrook.edu

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Abbreviations: COX, cyclooxygenase; ROS, reactive oxygen species; PI, propidium iodide; DAF-FM, 4-amino-5-methylamino-2,7-difluorofluorescein; DCFDA, 2,7-dichlorodihydrofluorescein diacetate; DHE, dihydroethidium; JC-1, 5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolylcarbocyanine iodide; MAPK, mitogen-activated protein kinase; NAC, N-acetyl-L-cysteine; NF- κ B, nuclear factor- κ B; SSAT, spermidine/spermine N¹-acetyltransferase; DFMO, difluoromethylornithine; Trx-1, Thioredoxin-1

ABSTRACT

Non-steroidal anti-inflammatory drugs (NSAIDs) such as sulindac are promising chemoprevention agents against colon cancer, but their weak potency and side effects limit their use for both chemoprevention and chemotherapy. Here, we evaluated the effect of a new sulindac derivative, phospho-sulindac or OXT-922, on the growth of human cancer cell lines and its mechanism of action. OXT-922 inhibited the growth of human cancer cell lines originating from colon, pancreas and breast about 11- to 30-fold more potently than sulindac. This effect was mediated by a strong cytotoxic effect. Compared to control, OXT-922 inhibited cell proliferation by up to 67%, induced apoptosis 4.1 fold over control and blocked the G₁ to S cell cycle phase transition. OXT-922 suppressed the levels of cell cycle regulating proteins including cyclins D₁ and D₃ and CDKs 4 and 6. The levels of intracellular reactive oxygen species (ROS), especially those of mitochondrial O₂⁻, were markedly elevated (5.5 fold) in response to OXT-922. ROS collapsed the mitochondrial membrane potential and triggered apoptosis, which was largely abrogated by antioxidants. OXT-922 suppressed nuclear factor κ B (NF- κ B) activation and downregulated thioredoxin-1 (Trx-1) expression. It also suppressed the production of prostaglandin E₂ (PGE₂) and decreased cyclooxygenase-1 (COX-1) expression. Similar to sulindac, OXT-922 enhanced spermidine/spermine N¹-acetyltransferase activity, reduced the cellular polyamine content, and synergized with difluoromethylornithine (DFMO) to inhibit cancer cell proliferation and induce apoptosis. Our results suggest that OXT-922 possesses promising anti-cancer properties and deserves further evaluation.

INTRODUCTION

Substantive epidemiological, *in vitro* and animal studies indicate that non-steroidal anti-inflammatory drugs such as aspirin, sulindac, and piroxicam have anti-tumorigenic activities against colorectal cancer. NSAIDs inhibit cell cycle progression and cell proliferation and induce apoptosis in various cancer cell lines; several underlying molecular mechanisms have been suggested [1]. Inhibition of cyclooxygenase COX activity by NSAIDs, which leads to reduced prostaglandin synthesis, is considered important for their anti-tumorigenic activity [2]. On the other hand, an extensive array of non-COX targets of NSAIDs relevant to their anticancer properties has been identified. For example, NSAIDs also inhibit the growth of cancer cells expressing no COX-1/COX-2, and this effect might depend on NF- κ B signaling inhibition, oxidative stress or reduced polyamine synthesis [3-5].

Among the nearly 50 approved NSAIDs, aspirin and sulindac are probably the best studied. Interventional clinical trials have demonstrated the cancer preventive effect of both, but neither had an effect strong enough to be clinically useful [6-9]. As for any agent, besides efficacy, the safety of NSAIDs is another relevant consideration. NSAIDs, even though widely used, do carry significant toxicity, primarily gastrointestinal and renal [10-11]. Such toxicity is expected to be more pronounced if they are to be used on a long-term basis for cancer prevention. Several attempts have been made to overcome the limitations of conventional NSAIDs, mainly those concerning their efficacy and safety. Some of these efforts include the chemical modification of the NSAID molecule in the hopes of generating pharmacologically superior derivatives. Two examples are the nitric oxide donating NSAIDs [12] and a modified sulindac [13]. These and other studies suggest that modifying the NSAIDs molecule may lead to useful new compounds.

In the present study, we evaluated the growth inhibitory effect of OXT-922, the novel sulindac derivative shown in Fig. 1. We found that the growth inhibitory effect of OXT-922 in cancer cells was largely mediated by a) elevated levels of reactive oxygen species (ROS), which, in turn, activated relevant cell signaling, leading to apoptosis, and b) enhanced spermidine/spermine *N*¹-acetyltransferase (SSAT) activity, which reduced polyamine levels and inhibited cell proliferation (cell renewal). Our work elucidates the anti-cancer mechanism of action of OXT-922 and highlights the potential of this compound as an anti-cancer drug.

MATERIALS AND METHODS

Reagents and cell culture

OXT-922 was provided by Medicon Inc (Stony Brook, NY). Annexin V-FITC, dihydroethidium (DHE), 2,7-dichlorodihydrofluorescein diacetate (DCFDA), 4-amino-5-methylamino-2,7-difluoro fluorescein (DAF-FM) and MitoSOX Red were purchased from Invitrogen (Carlsbad, CA). N-acetyl-L-cysteine (NAC) was purchased from EMD4BioSciences (Brookfield, WI). McCoy's 5a medium (modified), MEM (Eagle), RPMI 1640, and antibiotics were from Mediatech (Manassas, VA). FCS was from Thermo Scientific (Waltham, MA). Trolox and Tempol were from Sigma (St Louis, MO). Melittin was from Enzo Life Sciences (Plymouth Meeting, PA). Human colon (HT-29 and SW480), pancreatic (BxPC-3 and MIA PaCa-2) and breast (MDA-MB-231 and MCF-7) cancer cell lines (American Type Culture Collection, Manassas, VA) were grown in media recommended by American Type Culture Collection.

Cell kinetic assays

Cells were seeded at a density of 5×10^4 cells/cm² and allowed to attach for 24 h, when various treatments were applied. Cell viability was measured by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay following the protocol of the manufacturer (Roche Diagnostics). For cell proliferation assay, HT-29 cells, treated with OXT-922, were pulse-labeled with 10 μ M bromodeoxyuridine (BD Bioscience, San Jose, CA) 30 min prior to harvesting and analyzed by flow cytometry. To measure cell death, cells were treated with OXT-922 for 24 h, harvested by trypsinization, stained with fluorescein isothiocyanate-conjugated Annexin V and propidium iodide (PI) according to the manufacturer's protocol and analysed by flow cytometry. For cell cycle analysis, cells were fixed by cold 70% ethanol and stained with PI following standard protocols prior to flow cytometric analysis.

Determination of ROS

We determined ROS levels with confocal microscopy and flow cytometry. To determine mitochondrial O₂^{•-} production, cells were seeded in 35-mm glass bottom culture dishes. After each treatment, cells were stained with 5 μ M MitoSOX Red for 10 min or with 5 μ M dihydrorhodamine for 15 min. Live cells were kept in a 5% CO₂ chamber and examined under a Zeiss LSM510 confocal microscope. For flow cytometry, after treatment with the OXT-922 in 6-well plates, cells were trypsinized and stained with 10 μ M DCFDA for 30 min at 37 °C and their fluorescence intensity was analyzed by FACS Caliber (BD Bioscience, San Jose, CA).

Determination of mitochondrial membrane potential

The mitochondrial membrane potential was determined by flow cytometry using the 5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) cationic dye (Invitrogen). Briefly, SW480 cells were incubated with OXT-922 at 1 IC₅₀ for 3 h, when cells were trypsinized and washed once with phosphate-buffered saline. The supernatant was discarded and cells were incubated with 5 μM JC-1 for 30 min at 37°C, protected from light and analyzed by flow cytometry.

Western blotting

After each treatment, cells were lysed on ice with 1% Triton X-100 lysis buffer with 2.5 mM 4-nitrophenylphosphate, 1% SDS and 0.25% sodium deoxycholate for 30 min. For each sample, 30 μg of cell lysate were loaded onto SDS-electrophoresis gel and transferred onto a nitrocellulose membrane. The membrane was then immunoblotted with primary antibodies followed by secondary antibodies conjugated with horseradish peroxidase (HRP) from Santa Cruz Biotechnology. Enhanced chemiluminescence (ECL) was used to visualize the bands on X-ray film.

Electrophoretic mobility shift assay (EMSA)

After the indicated treatment, cell nuclear fractions were isolated from 3x10⁶ cells using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific). The NF-κB activity was detected by using LightShift chemiluminescent EMSA kit (Thermo Scientific) according to the manufacturer's instructions. Briefly, the nuclear extracts were incubated with biotin-labeled DNA probes at 37 °C for 20 min, then loaded onto the polyacrylamide gel and transferred to a nylon membrane. The membrane was exposed to UV-light for 10 min for cross-linking of the transferred DNA, then incubated with stabilized streptavidin-horseradish peroxide conjugate in blocking buffer for 15 min and covered with substrate working solution, followed by exposure to X-ray film.

PGE₂ assay

PGE₂ levels in culture media were determined using the kit from Cayman Chemical according to the manufacturer's instruction.

Spermidine/spermine N¹-acetyltransferase (SSAT) assay

Cells (3×10^6) were seeded in plates overnight and then treated with OXT-922 for 24 h. Cells were washed twice with cold homogenization buffer (10 mM Tris-HCl, pH 7.5; 2.5 mM DTT; 1 mM EDTA), scraped, disrupted by sonification and then centrifuged at 15,000x *g* at 4 °C for 10 min. 25 μ L of supernatant were incubated with 3 mM spermidine and 10 μ M [14 C] acetyl-CoA in a final volume of 50 μ L for 10 min at 37 °C. The reaction was stopped by the addition of 20 μ L 1M NH₂OH-HCl and heating in boiling water for 3 min. The resulting samples were centrifuged, 30 μ L of supernatant were spotted onto P-81 phosphocellulose discs and scintillation was counted. Results were expressed as a percentage of control.

RESULTS

OXT-922 inhibits the growth of various human cancer cell lines: A strong cytokinetic effect

The growth inhibitory effect of OXT-922 was evaluated by the MTT assay in human cancer cell lines. As shown in Table 1, the 24-hour IC₅₀ of cell lines originating from the colon, pancreas and breast ranged from 18 μ M (MDA-MB-231) to 92 μ M (MIA-PaCa-2). The breast cancer cell lines were more sensitive to OXT-922 than others. We also tested the growth inhibitory effect of sulindac. In agreement with previous reports [14], its effect was weak and the IC₅₀ values varied from 489 μ M (BxPC-3) to 1,173 μ M (HT-29). Compared to sulindac, OXT-922 was more potent in all 6 cancer cell lines tested; the potency enhancement ranged between 11 and 30 fold. However, the normal human colon mucosal epithelial cell line NCM460 is resistant to OXT-922, with an IC₅₀ of 181 μ M (2.5-fold higher than the average IC₅₀ of the two colon cancer cell lines); the potency enhancement was only 3 fold compared to sulindac.

We explored the cytokinetic effect of OXT-922 to assess the mechanism of its growth inhibitory effect. OXT-922 induced apoptosis in HT-29 cells after 24-h treatment with OXT-922 at 2 IC₅₀ concentration (Fig. 2A). The proportion of apoptotic cells [Annexin V(+)/propidium iodide (PI)(-) and Annexin V(+)/PI(+)] increased about 4.1 fold, from 12.9% to 52.9%. Late apoptotic cells [Annexin V(+)/PI(+)] increased even more (about 8.5 fold over control). The antioxidant NAC effectively blocked the apoptosis induced by OXT-922. After treatment with OXT-922 2 IC₅₀ and NAC 2 mM, 76.1% cells survived, while only 41.1% cells survived in the absence of NAC. Cell proliferation was evaluated by the bromodeoxyuridine (BrdU) incorporation method. As shown in Fig. 2B, OXT-922 reduced BrdU incorporation in HT-29 cells in a concentration-dependent manner. At 1 IC₅₀ concentration, MDA-922 decreased BrdU positive cells from 37.0% to 16.7%, and at 1.5 IC₅₀ concentration it decreased them further to 12.2%. After 24 h of incubation with 1 IC₅₀ OXT-922, a significant G₁ to S arrest was observed, with the proportion of cells in G₁ phase increasing from 59.8% to 85.1% (Fig. 2C). In both colon cancer cell lines tested, OXT-922 suppressed the expression of proteins regulating this part of the cell cycle. For example, the levels of cyclin D₁, cyclin D₃, CDK4 and CDK6 were suppressed in a concentration-dependent manner, more prominently in HT-29 cells. CDK4 and CDK6, each complexes with cyclin D, an interaction that is critical for mitotic cells to overcome the G₁ restriction point [15]. Thus, our findings suggest that OXT-922 blocks the transition through the G₁ restriction point. OXT-922 also upregulated the CDK inhibitor p21 in HT-29 (but not in SW480) cells and left unaffected the protein levels of p15, p27 and

p53 (Fig. 2D). Taken together, these changes provide at least a partial explanation of the cell cycle effect of OXT-922.

OXT-922 enhances ROS levels in colon cancer cells

ROS play a critical role in the mechanism of action of some anticancer compounds, including paclitaxel, arsenic trioxide and NO-aspirin [16-19]. Therefore, we determined the ROS levels in HT-29 cells after their treatment with OXT-922. As shown in Fig. 3A, OXT-922 increased the fluorescence of the general ROS probe, DCFDA, which reacts with nearly 10 individual ROS [20-21]. Compared to control, OXT-922 1 IC₅₀ enhanced ROS levels in SW480 cells by about 135% in 1 h. Pretreatment of the cells with 2 mM NAC for 2 h suppressed the upregulated ROS levels by 65%; the same result was obtained with HT-29 cells (Fig. S1A).

As shown in Fig. 3, we determined the levels of individual ROS using specific probes (indicated in parentheses): a) NO (DAF-FM); b) intracellular superoxide anion, O₂⁻, (DHE); c) mitochondrial O₂⁻, (MitoSOX Red); and d) peroxynitrite, ONOO⁻, (DHR). Compared to control, OXT-922 treatment increased the intracellular levels of NO by 69% and of O₂⁻ by 141%. Of note, mitochondrial levels of O₂⁻ were markedly elevated in response to OXT-922, increasing 5.5-fold (the geometric mean of probe fluorescence went from 12.0 to 66.3). The elevated mitochondrial O₂⁻ levels were confirmed using fluorescence confocal microscopy (Fig. 3E, right panel). The levels of ONOO⁻ were also elevated 128% in SW480 cells (Fig. 3E, left panel). Elevation of all these ROS levels was also observed in HT-29 cells in response to OXT-922 (Fig. S1).

OXT-922 induces redox-dependent apoptosis

ROS accumulation is critical for the induction of apoptosis by many anticancer agents [16,22]. As the O₂⁻ levels in mitochondria were markedly enhanced by OXT-922, we first tested for the loss of mitochondrial membrane potential, which is indicative of apoptosis [23-24]. To this end, we used the unique fluorescent cationic dye, JC-1, which exhibits potential-dependent accumulation in mitochondria and forms red fluorescent J-aggregates [25]. As shown in Fig. 4A, treatment with OXT-922 1 IC₅₀ for 3 h decreased red fluorescence signal intensity by 59.4%, indicating the collapse of mitochondrial membrane potential. We then examined the status of apoptosis-related proteins in HT-29 cells treated with OXT-922. The caspase cascade was probed in a concentration-dependent manner after treatment with various concentrations of OXT-922 for 24 h (Fig. 4B). Initiator caspases such as caspase-8, and -9 and the effector caspase-2, -3, and -7 [26] were cleaved and activated in HT-29 cells following OXT-922 treatment. OXT-922 also

suppressed the expression levels of phosphorylated and total Akt, which is critical for cell proliferation and survival [27].

Oxidative stress can modulate cell survival through multiple mechanisms [28]. However, cell death can also lead to ROS enrichment [29]. To distinguish the causality between OXT-922-induced ROS production and apoptosis, we evaluated the effect of antioxidants on OXT-922-induced apoptosis. The MTT assay showed that OXT-922 reduced cell viability in a concentration-dependent manner, and that this effect was effectively blocked by the antioxidants NAC, Tempol and Trolox [30-31] (Fig. 4C). NAC pretreatment also greatly blocked OXT-922-induced caspase-3 cleavage (Fig. 4D), and protected cells from OXT-922 induced apoptosis (Fig. 2A), indicating that oxidative stress is a major player in the induction of apoptosis by OXT-922.

The effect of OXT-922 on MAPK, Trx, NF- κ B and COX signaling pathways in colon cancer cells

Prior work has demonstrated the extensive effects of NSAIDs on cell signaling pathways including MAPKs, NF- κ B and Trx [32-34]. Thus, we explored whether OXT-922 affected these signaling pathways. The levels of phosphorylated p38, JNK and ERK were not significantly affected by OXT-922 (data not shown). In contrast, the activation of NF- κ B was largely inhibited by OXT-922, as revealed by the electrophoretic mobility shift assay (EMSA) shown in Figure 4E. Certain anticancer agents modulate NF- κ B signaling through thioredoxin oxidation and activation of apoptosis signal-regulating kinase-1 (ASK1) [17,35]. However, OXT-922 did not alter the oxidation level of Trx-1 neither did it reduce the physical association between ASK1 and Trx-1 (Fig. S2). Instead, it directly downregulated the Trx-1 protein level (Fig. 4F and Fig. S3). Finally, OXT-922 suppressed the expression of COX-1, notably at its IC₅₀ concentration and above, but had no effect on COX-2 expression over the same range of concentrations (Fig. 4G). OXT-922, however, suppressed both the baseline and stimulated (by melittin) production of PGE₂ by HT-29 cells; the latter effect was pronounced reaching 86% (Fig. 4H).

The effect of OXT-922 on polyamines; synergy with DFMO

It is reported that the growth inhibitory effect of sulindac on colon cancer cells is due, in part, to its ability to change polyamine metabolism [5]. The polyamines (putrescine, spermidine, and spermine) are abundant polycations, which are often elevated in neoplastic cells and affect numerous processes in carcinogenesis. Polyamine levels are tightly regulated by the biosynthetic enzyme ornithine decarboxylase [36] and the catabolic enzyme SSAT [37]. Sulindac enhances SSAT activity by increasing its transcription leading to suppressed polyamine content of cells [5]. We, therefore, evaluated whether

OXT-922 affects polyamine levels. As shown in Fig. 5, compared to control, OXT-922 significantly reduced the levels of polyamines in SW480 cells, while its combination with difluoromethylornithine (DFMO) further decreased putrescine levels. In addition, treatment of HT-29 cells with OXT-922 1 IC_{50} for 24 h enhanced SSAT activity by >150%. Similar results were obtained in SW480 cells, explaining in part the effect of OXT-922 on polyamines. However, OXT-922 enhanced ODC activity in SW480 cells, which may reflect feedback by suppressed polyamine levels; this enhancement was completely inhibited by DFMO (Fig. S4).

Given the clinically important effect of the combination of sulindac with DFMO, the most widely studied inhibitor of polyamine synthesis, on the prevention of colon cancer [38], we evaluate whether OXT-922 synergizes with DFMO to inhibit colon cancer cell growth. The isobolograms depicted in Fig. 5, indeed, establish the synergy between OXT-922 and DFMO, in inhibiting cell growth evaluated both at 72 h. Each of the two concentration pairs of OXT-922 and DFMO are well below the additivity line. This synergistic effect on cell growth is brought about by synergistic effects on cell proliferation (cell renewal) and cell death. Treatment with 5 mM DFMO or 30 μ M OXT-922 alone had a very modest anti-proliferative effect on SW480 cells but when applied together, they suppressed the proportion of BrdU positive cells from 51.6% to 34.4%. Similarly, 48-h treatment with 5 mM DFMO or 15 μ M OXT-922 alone showed a weak proapoptotic effect, but when these agents were used together, they greatly induced apoptosis (50.3% apoptotic cells as opposed to the expected 28%).

DISCUSSION

Our findings establish that OXT-922 displays a far greater growth inhibitory effect on human cancer cell lines than conventional sulindac. This effect, studied in detail in colon cancer cell lines, is brought about through two distinct effects, one on ROS-dependent cell signaling and the other on the polyamine pathway. Both of these effects culminate in strong cytokinetic changes, consisting of diminished cell proliferation, enhanced apoptosis, and a block in cell cycle. The net effect is marked inhibition of cell growth. These effects are depicted in Figure 6. Of potential clinical importance was the finding that OXT-922 synergized with DFMO, the clinically applicable inhibitor of ODC.

Our results reveal the uniform enhancement of OXT-922 potency compared to conventional sulindac. In the tested 6 cancer cell lines originating from different human tissues, the average enhancement is 19.2 fold. The six IC_{50} s are within the fairly narrow range of 18-92 μ M. Taken together, these findings suggest that OXT-922 may possess a broad spectrum of anticancer activity. In colon cancer cells, which were studied in greater detail, the growth inhibitory effect of OXT-922 is associated with G_1 to S cell cycle arrest, inhibition of proliferation and induction of apoptosis. Low concentration of OXT-922 (1 IC_{50}) significantly reduced the proportion of cells in S phase and blocked DNA synthesis inhibiting cell renewal, while high concentration of OXT-922 (2 IC_{50}) remarkably induced cell death in over 50% of the cells (1 IC_{50} caused 14.8% more apoptotic cells than control group, data not shown). The relative contribution of these two effects to the overall growth inhibition may vary according to the concentration of OXT-922.

A key finding of our study is that OXT-922 is able to significantly induce ROS production in colon cancer cells and that this effect is primarily responsible for the induction of apoptosis. Prior work has revealed that sulindac induces ROS production in cancer cells [39-41]. However, this effect is relatively weak, requiring prolonged exposure to high concentrations of sulindac. In contrast, OXT-922 at low concentration and within 1 h significantly enhanced ROS levels (detected with the general ROS probe DCFDA). When we explored the effect of OXT-922 on individual ROS species, it was clear that the most pronounced effect concerned the induction of mitochondrial $O_2^{\cdot-}$, which was elevated 5.5 fold compared to control. The remaining three species, NO, ONOO $^-$ and whole cell $O_2^{\cdot-}$, were modestly induced (about twice of control). Based on these findings, it appears that the mitochondria are the main target of OXT-922 for ROS induction.

ROS have a dual function in living cells: they can serve either as essential signaling molecules, mainly at low concentrations, or they can harm many cell constituents, especially at high concentrations

[42]. Maintaining ROS homeostasis is crucial for normal cell growth and survival. In HT-29 cells, treatment with OXT-922 (1 IC_{50}) for 3 h collapsed the mitochondrial membrane potential, an early indication of the initiation of cellular apoptosis. Longer exposure to OXT-922 caused cleavage of caspases and evident apoptosis. The ROS scavenger NAC was able to block caspase-3 cleavage and abrogate the apoptosis induced by OXT-922. Other antioxidants like Trolox and Tempol also abrogated the cytotoxicity of OXT-922. These results confirm the essential role of ROS in OXT-922-induced apoptosis.

Prior work by us has demonstrated that derivatives of NSAIDs modulate signaling pathways including MAPKs, COX, the Trx system and NF- κ B [17,33,43]. Our current results show that OXT-922 exerts no effect on MAPK signaling, and suppresses NF- κ B activity without Trx-1 oxidation. Interestingly, OXT-922 directly downregulates the expression of Trx-1. Considering the importance of Trx-1 in cancer development [44-45], the downregulation of Trx-1 by OXT-922 may contribute to its anti-cancer activity, a potential mechanism that deserves further study. OXT-922 had a rather unusual effect on the COX isozymes, in that it suppressed the expression of COX-1 while not affecting that of COX-2. The production of PGE₂, the dominant PG produced by HT-29 cells, was markedly suppressed by OXT-922, especially following stimulation by melittin [3]. Of note the combination of OXT-922 with conventional sulindac had no additive effect, indicating perhaps maximal inhibition by either compound.

OXT-922 has a significant effect on the polyamine pathway, suppressing the levels of all three members (putrescine, spermidine, and spermine) that were assayed. This effect is partly due to its ability to stimulate the activity of SSAT, the enzyme that acetylates spermine and spermidine and exports them from the cell. An important finding was the synergy between OXT-922 and DFMO, which was due mainly to a marked enhancement of apoptotic cell death and to a lesser extent to inhibition of proliferation.

Taken together, our findings indicate that OXT-922, a derivative of sulindac belonging to the broader pharmacological category of modified NSAIDs, displays properties that are relevant to the control of cancer. Of particular interest are: a) its cell signaling mechanism involving two pathways that bear heavily on the fate of the cancer cell, and b) its ability to synergize with DFMO. OXT-922 is a promising novel agent worthy of further probing for its anticancer properties.

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CONFLICT OF INTEREST STATEMENT

The authors have nothing to declare except for Basil Rigas who has an equity position in Medicon Inc.

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Table I . The growth inhibitory effect of OXT-922 on human cells

	Cell lines	IC ₅₀ : ?M		Enhancement (fold)
		Sulindac	OXT-922	
Colon	HT-29	1,173	70	17
	SW480	900	73	12
	NCM 460	528	181	3
Breast	MCF-7	1,128	38	30
	MDA-MB-231	530	18	30
Pancreatic	BxPC-3	489	32	15
	MIA-PaCa-2	1,036	92	11

FIGURE LEGENDS

Fig. 1. The chemical structure of sulindac and OXT-922.

Fig. 2. The cytokinetic effect of OXT-922. (A) HT-29 cells treated with OXT-922 for 24 h were stained with PI and Annexin V and analyzed by flow cytometry. The numbers inside each box represent the percentage of cells in each category. *Lower panel:* cells were pretreated with 5 mM NAC for 2h. (B) BrdU incorporation assay in HT-29 cells following OXT-922 treatment for 24 h. The number in the right upper box indicates the percentage of cells in S phase. (C) Cell cycle profiles generated from fluorescence-activated cell sorting of propidium iodide-stained cells 24 h after OXT-922 treatment. (D) Cells were exposed to the indicated concentrations of OXT-922 for 18 h and analyzed by western blotting to determine protein levels. These results are representative of three independent experiments, which generated similar results.

Fig. 3. OXT-922 enhances ROS levels in SW480 cells. (A) Fluorescence intensity histograms of SW480 cells loaded with DCFDA for 30 min and subjected to flow cytometry. Cells were treated with OXT-922 for 1 h and pretreated, as indicated, with 2 mM NAC for 2 h. (B-D) Similar analyses were performed using the molecular probes DAF-FM (for NO), DHE (for intracellular $O_2^{\cdot-}$) and MitoSOX Red (for mitochondrial $O_2^{\cdot-}$). The numbers in parentheses (A-D) are the corresponding geometric means of fluorescence intensity. (E) Peroxyntirte (*left*) and mitochondrial $O_2^{\cdot-}$ (*right*) were detected by confocal microscopy as in *Materials and Methods* in SW480 cells treated with or without OXT-922 (1 IC_{50}) for 1 h, and then loaded with 5 μ M MitoSOX Red or 5 μ M dihydrorhodamine for 15 min.

Fig. 4. OXT-922 induces redox-dependent apoptosis and modulates NF- κ B and COX signaling. (A) HT-29 cells were treated with OXT-922 (1 IC_{50}) for 3 h, then stained with 2 μ M JC-1 for 30 min and fluorescence intensity was detected by flow cytometry. The numbers in parentheses are the corresponding geometric means of fluorescence intensity. (B) Immunoblots for HT-29 cells treated with the indicated concentrations of OXT-922 for 24 h. Cytochrome C was determined in the cytosolic fraction. (C) HT-29 cells were plated in 96-well plates (1×10^4 cells/well). After pretreatment with or without 2 mM NAC or 200 μ M Trolox or 250 μ M Tempol for 2 h, cells were treated with OXT-922 for another 36 h, and cell viability was tested by MTT assay. (D) HT-29 cells were treated with or without 2 mM NAC for 2 h, and then cells were incubated with OXT-922 for an additional 24 h. Protein levels of cleaved caspase-3

were tested by western blot. (E) After a 6-h treatment with OXT-922, NF- κ B activation was determined by non-radioactive EMSA using a biotinylated NF- κ B DNA probe as in *Materials and Methods*. (F) Cells were treated with the indicated concentrations of OXT-922 for 3 h and the Trx-1 levels were determined by immunoblot. Loading control: actin. (G) HT-29 cells were treated with the indicated concentrations of OXT-922 for 18 h and COX-1/2 levels were determined by immunoblot. (H) HT-29 cells were treated with the indicated compounds (1 IC₅₀) for 0.5 h, then treated with or without melittin (10 μ M) for another 2.5 h. PGE₂ levels were determined in the culture media.

Fig. 5. Synergy between DFMO and OXT-922. (A) SW480 cells were incubated with 1 IC₅₀ OXT-922 for 24 h and polyamine levels were determined by HPLC. Polyamine levels of control group are: putrescine, 0.64 nmol/mg protein; spermidine, 4.17 nmol/mg protein and spermine, 4.19 nmol/mg protein. (B) Cells were treated with 1 IC₅₀ OXT-922 for 24 h and SSAT activity was determined as in *Materials and Methods*. Values in A and B are *mean*±*SEM*. All differences from the corresponding controls are significant (*P* values <0.01). SSAT activity of control groups are 9.6–2.4 (SW480) and 7.0–0.4 (HT-29) pmol/mg protein/min. (C) The isobologram, based on cell viability, establishes the synergy between OXT-922 and DFMO. The additivity line connects the IC₅₀ value of each compound when used alone. A and B, representing two different concentration pairs of each compound (*x*=abscissa; *y*=ordinate), are well below the additivity line. (D) The BrdU incorporation assay was used to detect the de novo DNA synthesis of cells following treatment with OXT-922 and DMFO as indicated. The number in the right upper box indicates the percentage of cells in S phase. (E) Annexin V and PI staining, as in *Materials and Methods*, was used to detect apoptosis in HT-29 cells after 48-h treatment with OXT-922 and DMFO. The Annexin V(+)/propidium iodide (PI)(-) and Annexin V(+)/PI(+) (two right quadrants) are apoptotic cells. The numbers inside each box represent the percentage of cells in each category.

Fig. 6. Proposed mechanism of the cancer growth inhibitory effect of OXT-922. OXT-922 induces oxidative stress and enhances SSAT activity. Oxidative stress induces apoptosis by inducing mitochondrial membrane potential collapse, downregulating Trx-1 and suppressing NF- κ B activity, and blocks G₁-S transition through downregulating the expression of cyclin D₁/D₃ and CDK-4/6. Enhanced SSAT activity decreases the levels of polyamines (synergy with DFMO) and inhibits cell proliferation. *Arrows*, positive effect; *T-shaped arrows*, negative effect.

Figure S1

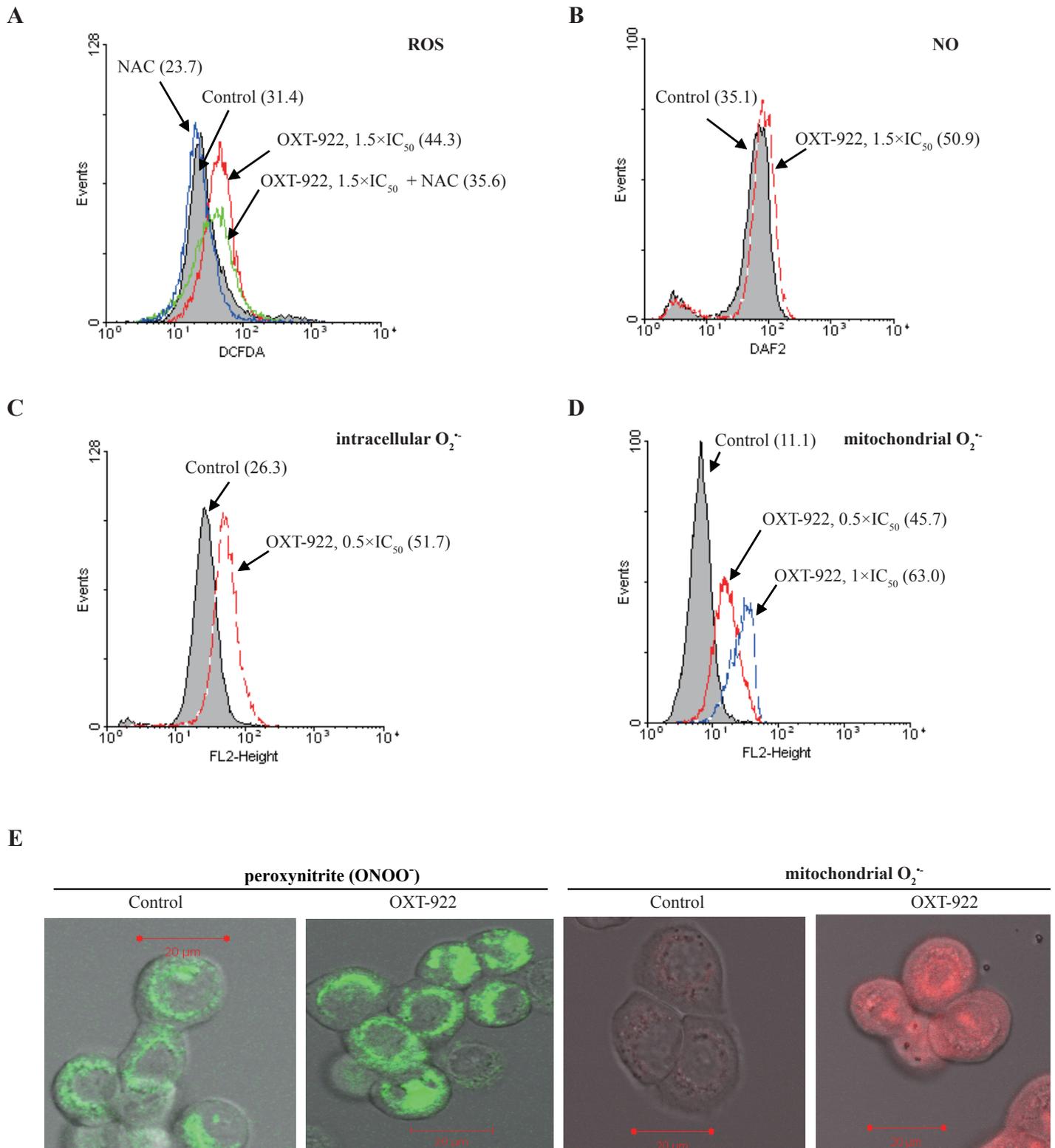


Figure S2

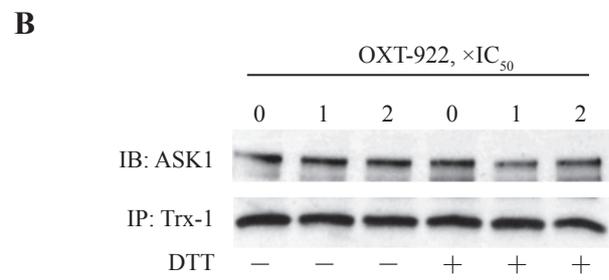
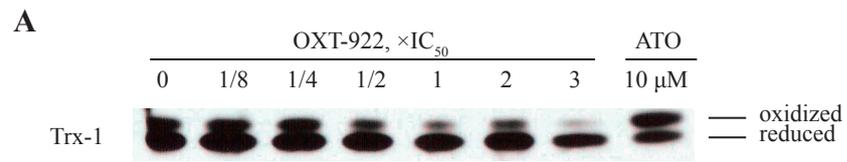


Figure S3

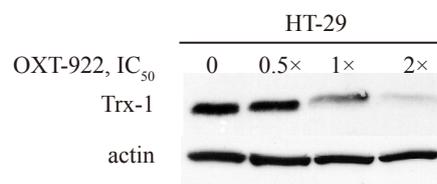


Figure S4

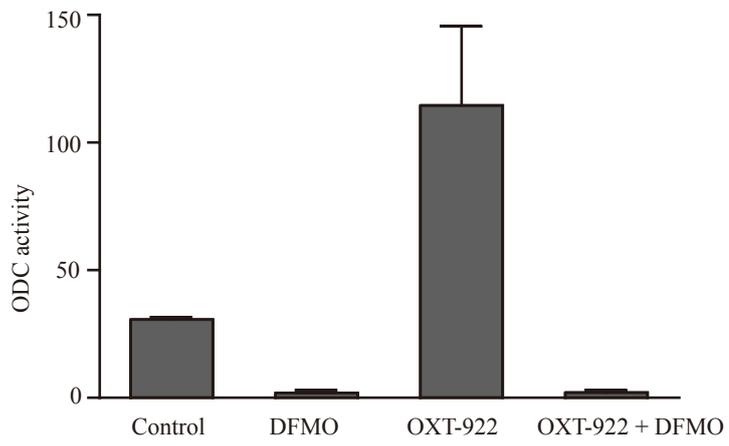


Figure 1

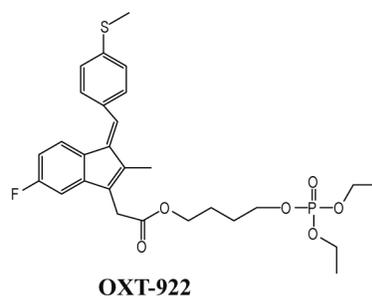


Figure 2

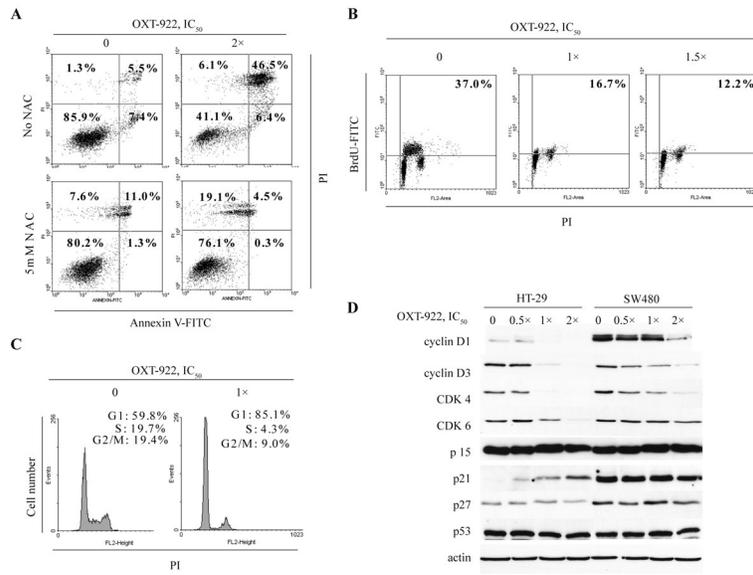


Figure 3

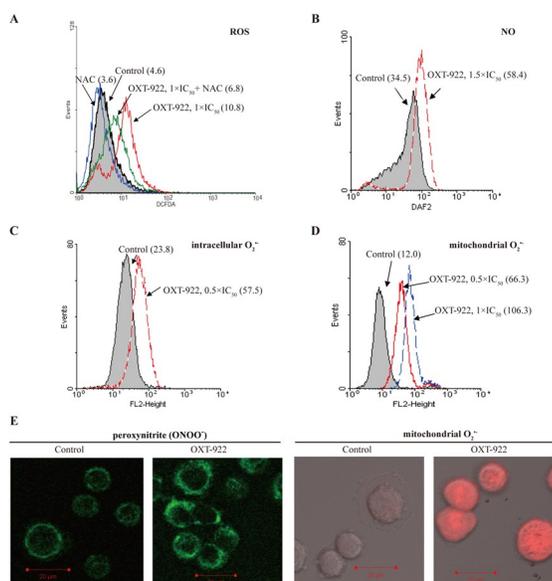


Figure 4

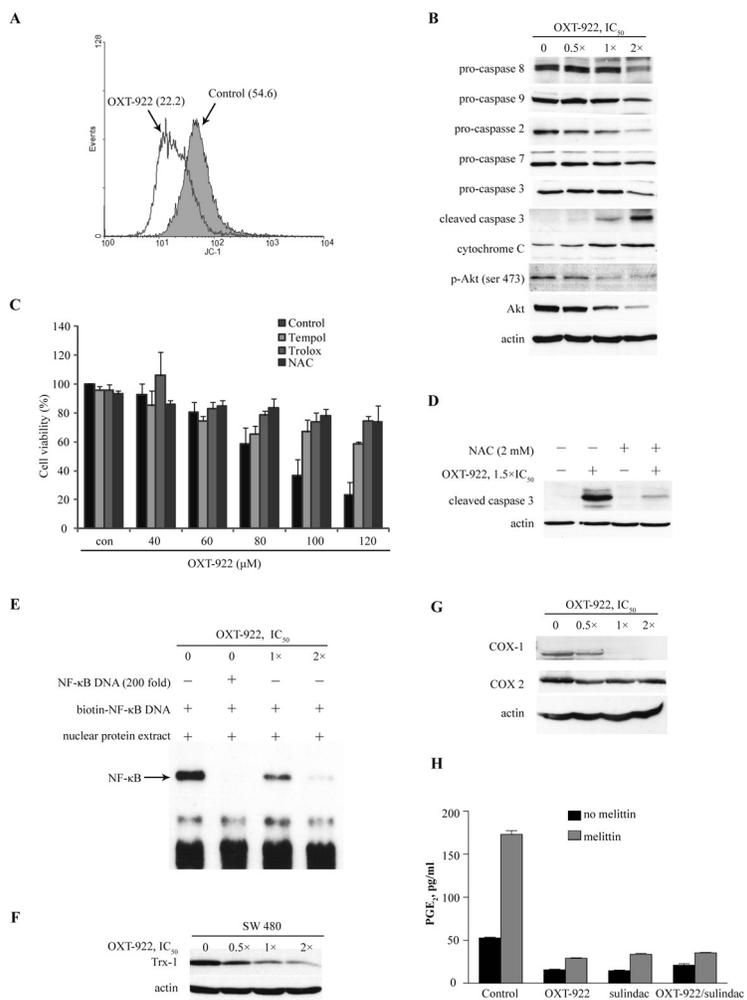


Figure 5

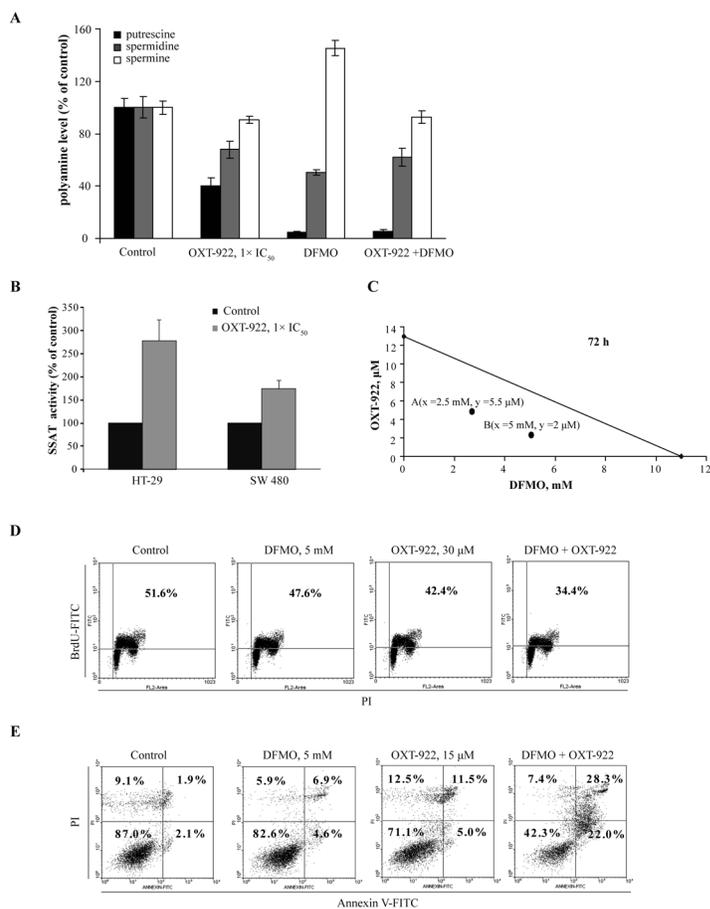


Figure 6

