# The Thioredoxin System Mediates Redox-Induced Cell Death in Human Colon Cancer Cells: Implications for the Mechanism of Action of Anticancer Agents

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## Abstract

**Anticancer agents act, at least in part, by inducing reactive oxygen and nitrogen species (RONS). We examined the redox effect on SW480 and HT-29 colon cancer cells of four anticancer compounds, arsenic trioxide, phosphoaspirin, phosphosulindac, and nitric oxide–donating aspirin (NO-ASA). All compounds inhibited the growth of both cell lines ($IC_{50}$, 10–90 μmol/L) and induced RONS detected by a general RONS molecular probe. NO-ASA, which induced at least four individual RONS (NO, $H_2O_2$, superoxide anion, and peroxynitirte), induced apoptotic and necrotic cell death that was RONS-mediated (cell death paralleled RONS levels and was abrogated by *N*-acetyl cysteine but not by diphenylene iodonium, which displayed prooxidant activity and enhanced cell death). Nuclear factor-κB and mitogen-activated protein kinases were modulated by RONS. Thioredoxin-1 (Trx-1), an oxidoreductase involved in redox regulation, was heavily oxidized in response to RONS and mediated the growth inhibitory effect of the anticancer agents; knocking-down *trx-1* expression by small interfering RNA abrogated cell death induced by them. These compounds also inhibited the activity of Trx reductase that reduces oxidized Trx-1, whereas the Trx reductase inhibitor aurothiomalate synergized with NO-ASA in the induction of cell death. Our findings indicate that the Trx system mediates to a large extent redox-induced cell death in response to anticancer agents. This mechanism of action may be shared by more anticancer agents and deserves further assessment as a candidate mechanism for the pharmacologic control of cancer.** [Cancer Res 2008;68(20):8269–77]

## Introduction

Thioredoxin (Trx) is an a oxidoreductase involved in redox regulation and cell signaling (1–4). Trx is a member of the Trx system that also includes Trx reductase (TrxR) and NADPH. The ubiquitously expressed Trx system and glutathione (GSH) are the two main antioxidant systems that reduce thiol (-SH) groups. Three distinct forms of Trx have been identified: the 12-kDa cytosolic Trx-1; Trx-2, the mitochondrial isoform; and SpTrx, which is highly expressed in spermatozoa. Trx-1 acts as an intracellular reductase using two vicinal cysteine residues (Cys32 and Cys35) at its conserved active site (-Cys-Gly-Pro-Cys-). The redox-active center of reduced Trx-1 interacts with the oxidized protein that it is about to reduce, which has a disulfide bond (S-S; ref. 1). Trx-1 reduces the target protein by converting its disulfide bond to two-SH groups, whereas, in the process, Trx-1 itself becomes oxidized, with its two -SH groups forming a disulfide. TrxR and NADPH reduce Trx-1 back to its original state. Trx-1 has three additional cysteines, Cys62, Cys69, and Cys73. Further oxidation of Trx-1 first leads to the formation of a disulfide bond between Cys62 and Cys69 and, next, to a disulfide bond between Cys73 of two different Trx-1 molecules, which ultimately leads to the formation of a dimer.

Numerous proteins are redox-regulated by the Trx system (5). Examples of such regulation include the reduction by Trx-1 of a cysteinyl residue of the p50 subunit of nuclear factor-κB (NF-κB), which is required for its DNA binding, and the activation through similar reduction reactions of many transcription factors. Trxs, implicated in a number of diseases (2, 3), seem to play a vital role in cancer biology and in cancer response to chemotherapeutic agents. Trx, overexpressed in pancreas, colon, lung, and other cancers, suppresses apoptosis by activating the Akt pathway or through the apoptosis signal-regulating kinase-1 (ASK1; ref. 6).

In recent years, it has become clear that anticancer agents act, at least in part, by inducing reactive oxygen and nitrogen species (RONS). At low concentrations, RONS seem to protect the cell, whereas at higher concentrations, they can damage many biological molecules, such as DNA, proteins, and lipids, and may initiate cell death (7). Among the anticancer compounds that generate RONS is nitric oxide–donating aspirin (NO-ASA), a promising chemopreventive agent (8). NO-ASA generates a state of oxidative stress through which it affects redox-sensitive signaling pathways, leading ultimately to the elimination of the neoplastic cell via apoptosis or necrosis (9).

Given the apparent importance of RONS in the mechanism of action of these compounds, we studied this phenomenon in human colon cancer cell lines. Whereas we have focused on NO-ASA, we have also included in our study phosphoaspirin, a structurally similar anticancer derivative of aspirin, which is devoid of the NO-releasing moiety (10), phosphosulindac, a derivative of sulindac, that is also chemopreventive against colon cancer,[1] and arsenic trioxide ($As_2O_3$), an agent that is highly effective in acute promyelocytic leukemia (11). Here, we present results demonstrating that these anticancer agents induce oxidative stress leading to apoptosis and necrosis and that the Trx system plays a key role in the induction of cell death by them.

**Note:** Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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[1] Our unpublished observations.

## Materials and Methods

### Reagents and Culture Media

McCoy's 5a medium (modified), MEM (Eagle), RPMI 1640, and antibiotics were from Fisher-Mediatech. FCS was from Hyclone. NO-ASA [2-(acetyloxy) benzoic acid 4-(nitrooxymethyl)-phenyl ester], phosphoaspirin [2-(acetyloxy) benzoic acid 4-(diethylphospho)-phenyl ester], and phosphosulindac were a gift from Chemi-Master International (East Setauket, NY; ref. 12). Dihydroethidium and 2′,7′-dichlorofluorescine diacetate (DCFDA) were from Calbiochem. Dihydrorhodamine, MitoSOX Red, Annexin V, and propidium iodide (PI) were from Invitrogen. Anti–mitogen-activated protein kinases (MAPK) antibodies were from Cell Signaling, anti-ASK1 was from Santa Cruz Biotechnology, and antibodies to Trx-1 and TrxR were from Abcam. Arsenic trioxide (ATO), aurothiomalate (ATM), and all other reagents were from Sigma Chemical.

### Cell Culture and Cell Viability Assays

The HT-29 and SW480 human colon adenocarcinoma cell lines were from American Type Culture Collection (ATCC). Cells, grown as recommended by ATCC, were seeded at $5 \times 10^4$ cells/cm$^2$ and allowed to attach for 24 h, followed by various treatments, as indicated. Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay following the protocol of the manufacturer (Roche Diagnostics).

### Cell Death Assays

**PI incorporation assay.** Cells were seeded in 12-well plates and treated as indicated. After each treatment, cells were detached using a cell scraper, fixed in 70% ethanol for 1 h, stained with PI (50 μg/mL) and RNase A (4 units/mL) for 30 min, and subjected to flow cytometry analysis.

**Lactate dehydrogenase assay.** After cells were treated with the test drug for 24 h, lactate dehydrogenase (LDH) concentration was determined in the culture medium using the Cytotoxicity Detection kit$^{PLUS}$ (Roche Diagnostics).

**Annexin V and PI staining.** After cells were treated with the test drugs in 12-well plates, they were trypsinized and stained with Annexin V-FITC (100× dilution) and PI (0.5 μg/mL) for 15 min. Annexin V-FITC and PI fluorescence intensities were analyzed by FACSCaliber (BD Bioscience). Annexin V (+)/PI (−) cells are apoptotic cells, Annexin V (+)/PI (+) cells have undergone secondary necrosis, and Annexin V (−)/PI (+) cells are necrotic cells.

### Determination of RONS

**Determination of RONS levels by confocal microscopy.** To determine mitochondrial superoxide anion production, cells were seeded in 35-mm glass bottom culture dishes (MatTek). After each treatment, cells were stained with 5 μmol/L MitoSOX Red for 10 min or with 5 μmol/L dihydrorhodamine for 15 min. Live cells were kept in a 5% $CO_2$ chamber and examined under a Zeiss LSM510 metaconfocal microscope.

**Determination of RONS levels by SpectraMax M5.** Cells seeded in 96-well plates were loaded with either 5 μmol/L DCFDA for 30 min or 5 μmol/L DAF-2 for 30 min or 5 μmol/L Amplex Red for 15 min in plain RPMI medium and then treated with the test drug as indicated. The emission for each dye was read by SpectraMax M5 (Molecular Devices). In experiments using *N*-acetyl-cysteine (NAC), cells were pretreated with 20 mmol/L NAC for 4 h in complete medium, followed by DCFDA for 30 min in medium without phenol red and serum, and then were treated with the test drug as indicated.

**Determination of RONS by FACSCaliber.** After treatment with the test drug in 12-well plates, cells were trypsinized and stained with 10 μmol/L DCFDA for 30 min at 37°C and the fluorescence intensity was analyzed by FACSCaliber (BD Bioscience).

### Western Blotting

After each treatment, cells were lysed on ice with 1% Triton X-100 lysis buffer with 2.5 mmol/L 4-nitrophenylphosphate, 1% SDS, and 0.25% sodium deoxycholate for 30 min. The cell lysate (30 μg) was loaded onto SDS-electrophoresis gel and transferred onto a nitrocellulose membrane. The membrane was then immunoblotted with anti–phosphorylated p38, anti–phosphorylated extracellular signal-regulated kinase (ERK), or anti–phosphorylated c-Jun-$NH_2$ kinase (JNK) 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

After treatment with the test drug, cells were gently scraped into the medium and electrophoretic mobility shift assays (EMSA) were performed on nuclear extracts, as previously described (13). To assay NF-κB binding, we used a commercially available Gel Shift Assay System (Promega Corporation).

### NF-κB (Human p50) Transcription Factor Assay by ELISA

After treatment with the test drug, cells were gently scraped into the medium and nuclear extracts were isolated, as previously described (13). The NF-κB activity was analyzed using an ELISA kit from Panomics.

### Assay for ASK1–Trx-1 Complex Formation

Immunoprecipitation of ASK1–Trx-1 complexes was performed using Protein A/G PLUS-Agarose beads as per the instructions of the manufacturer (Santa Cruz Biotechnology). Briefly, cells were lysed in immunoprecipitation assay buffer, and half of each cell lysate was incubated with 1 mmol/L DTT for 30 min. The lysates were then precleared by adding 40 μL Protein A/G PLUS-Agarose beads and incubating for 30 min at 4°C. Cell lysates were then incubated with 6 μg anti–Trx-1 and 40 μL Protein A/G PLUS-Agarose beads for 4 h at 4°C. The immunoprecipitates were boiled in 1× electrophoresis sample buffer, and samples were subjected to SDS-PAGE analysis.

### Trx Redox Status Assay

$10^6$cells were lysed in 6 mol/L guanidinium chloride, 50 mmol/L Tris/HCl (pH 8.3), 3 mmol/L EDTA, and 0.5% Triton-X-100 containing 50 mmol/L iodoacetic acid (14). After 30 min at 37°C, the excess iodoacetic acid was removed using Microspin G-25 columns (GE Healthcare Life Sciences). Oxidized and reduced Trx-1 were separated by native PAGE. The gel was electroblotted onto a nitrocellulose membrane and probed with a Trx-1 antibody, followed by HRP-conjugated secondary antibody. Bands corresponding to Trx-1 were visualized by ECL.

### Trx Gene Silencing by Transfecting Specific Small Interfering RNA

$0.8 \times 10^5$ SW480 cells seeded in 12-well plates were transfected with 100 nmol/L Trx small interfering RNA (siRNA) or nonspecific control siRNA (Dharmacon) for 72 h using Lipofectamine 2000 (Invitrogen).

### Trx Reductase Activity Assay

After treatment with the test drug, cells were lysed and TrxR activity was determined in the protein lysate using a commercially available kit, as per the instructions of the manufacturer (Cayman Chemical). In this assay, TrxR uses NADPH to reduce 5,5′-dithiobis-(2-nitrobenzoic acid) to 5-thio-2-nitrobenzoic acid (TNB).

## Results

**Anticancer agents induce multiple RONS in colon cancer cell lines.** As expected, all four of the anticancer compounds studied (structures in Supplementary Fig. S1) inhibited the growth of SW480 and HT-29 human colon cancer cells. Under our experimental protocol, the $IC_{50}$s of growth inhibition after 24 hours of treatment were as follows: (*a*) NO-ASA: HT-29 cells = 25 ± 2.3 μmol/L (mean ± SE, for this and all subsequent values), SW480 cells = 38 ± 4.0 μmol/L; (*b*) phosphoaspirin: HT-29 cells = 39.3 ± 2.9 μmol/L, SW480 cells = 90.3 ± 2.8 μmol/L; (*c*) phosphosulindac: HT-29 cells = 65 ± 2.3 μmol/L, SW480 cells = 98 ± 4.0 μmol/L; and (*d*) arsenic trioxide: SW480 cells = 10 ± 1.3 μmol/L.

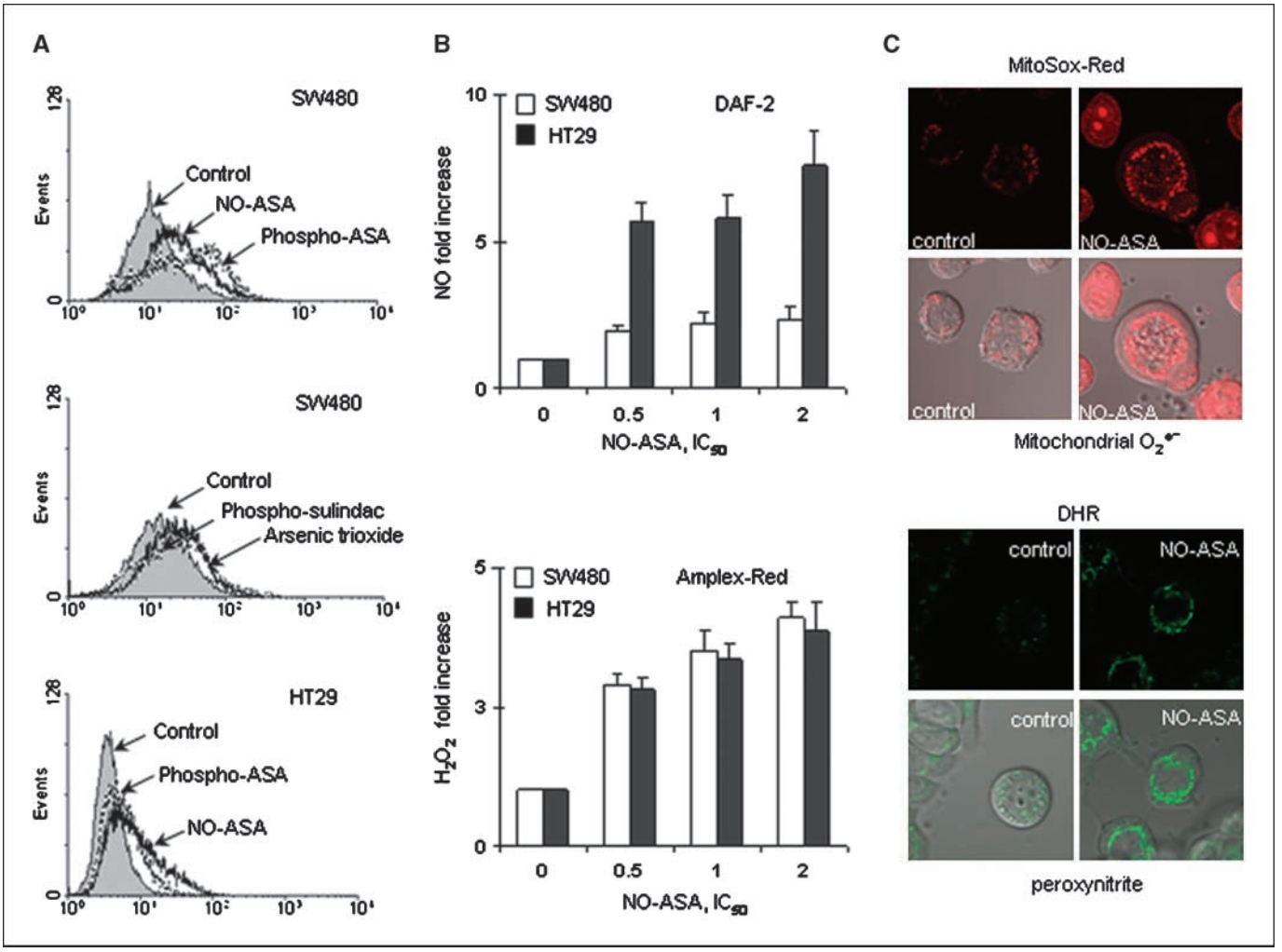


**Figure 1.** Anticancer agents induce multiple RONS in colon cancer cell lines. *A,* HT-29 and SW480 cells were treated with various compounds for 3 h, as indicated, followed by DCFDA staining for 30 min. The fluorescent intensity of each sample was read by flow cytometry. *B, top,* HT-29 and SW480 cells were preincubated with the NO probe, DAF-2, for 30 min, followed by treatment with the test agent for 3 h, when intracellular NO levels were determined by flow cytometry, as in Materials and Methods. Results are shown as fold increase compared with control cells. *Bottom,* HT-29 and SW480 cells were preincubated for 15 min with the $H_2O_2$ probe Amplex Red, followed by treatment with each of the test drugs for 3 h, when intracellular $H_2O_2$ levels were determined based on their fluorescence as in Materials and Methods. Results are expressed as fold increase compared with untreated controls. *C,* HT-29 cells were treated with 50 μmol/L NO-ASA ($2 \times IC_{50}$) for 30 min, followed by 5 μmol/L MitoSox ($O_2^{\bullet-}$ probe) for 10 min (*top*) or 5 μmol/L dihydrorhodamine (peroxynitrite probe) for 15 min (*bottom*). The production of $O_2^{\bullet-}$ (*top*) and peroxynitrite (*bottom*) was observed using a Zeiss LSM510-meta confocal microscope. In each panel, the top row represents fluorescent images and the bottom row represents them merged with the corresponding differential interference contrast image. Values are mean ± SE.

As shown in Fig. 1, all four compounds induced RONS, as detected by the general molecular probe DCFDA, which responds to at least 10 individual reactive species (15, 16). Of note, at identical multiples of their respective $IC_{50}$s, sulindac produced more RONS than phosphosulindac; ASA (5 mmol/L) failed to induce any RONS, whereas NO-ASA and phosphoaspirin did (Supplementary Fig. S2). We then investigated the effect of NO-ASA on the levels of four individual RONS, NO, $H_2O_2$, $ONOO^-$, , and $O_2^{\bullet-}$. After 3 hours of treatment, NO-ASA increased, in both cell lines, the levels of NO and $H_2O_2$ in a concentration-dependent manner. NO levels were increased 2-fold to 7-fold over control and $H_2O_2$ levels 3.5-fold to 4.5-fold over control. Imaging by confocal microscopy showed that NO-ASA promoted $O_2^{\bullet-}$ and $ONOO^-$formation in mitochondria as early as 30 minutes after they were exposed to NO-ASA.

**NO-ASA induces RONS-dependent cell death.** The two cell lines, SW480 and HT-29, responded to NO-ASA in a similar manner. As shown in Fig. 2*A*, NO-ASA induced colon cancer cell death, both by apoptosis and necrosis. The induction of apoptosis by NO-ASA was greatest at the $1 \times IC_{50}$ concentration, when 20% of SW480 cells and 16% of HT-29 cells were apoptotic, diminishing progressively as the concentrations of NO-ASA increased further. The induction of necrosis showed a steady increase in response to increasing concentrations of NO-ASA; at $4 \times IC_{50}$, the highest NO-ASA concentration studied, 74% and 53% of SW480 and HT-29 cells, respectively, were necrotic.

RONS are known to induce cell death in various cancer cell lines (7, 17). On the other hand, RONS can be produced during cell death (18). To distinguish whether the cell death induced by NO-ASA was RONS-dependent or whether RONS production was the result of cell death, we used two antioxidants, NAC and diphenylene iodonium (DPI; refs. 19–22). However, the results obtained with these two compounds were different. As seen in Fig. 2*B*, after treatment with NO-ASA for 16 hours, 20 mmol/L NAC blocked cell

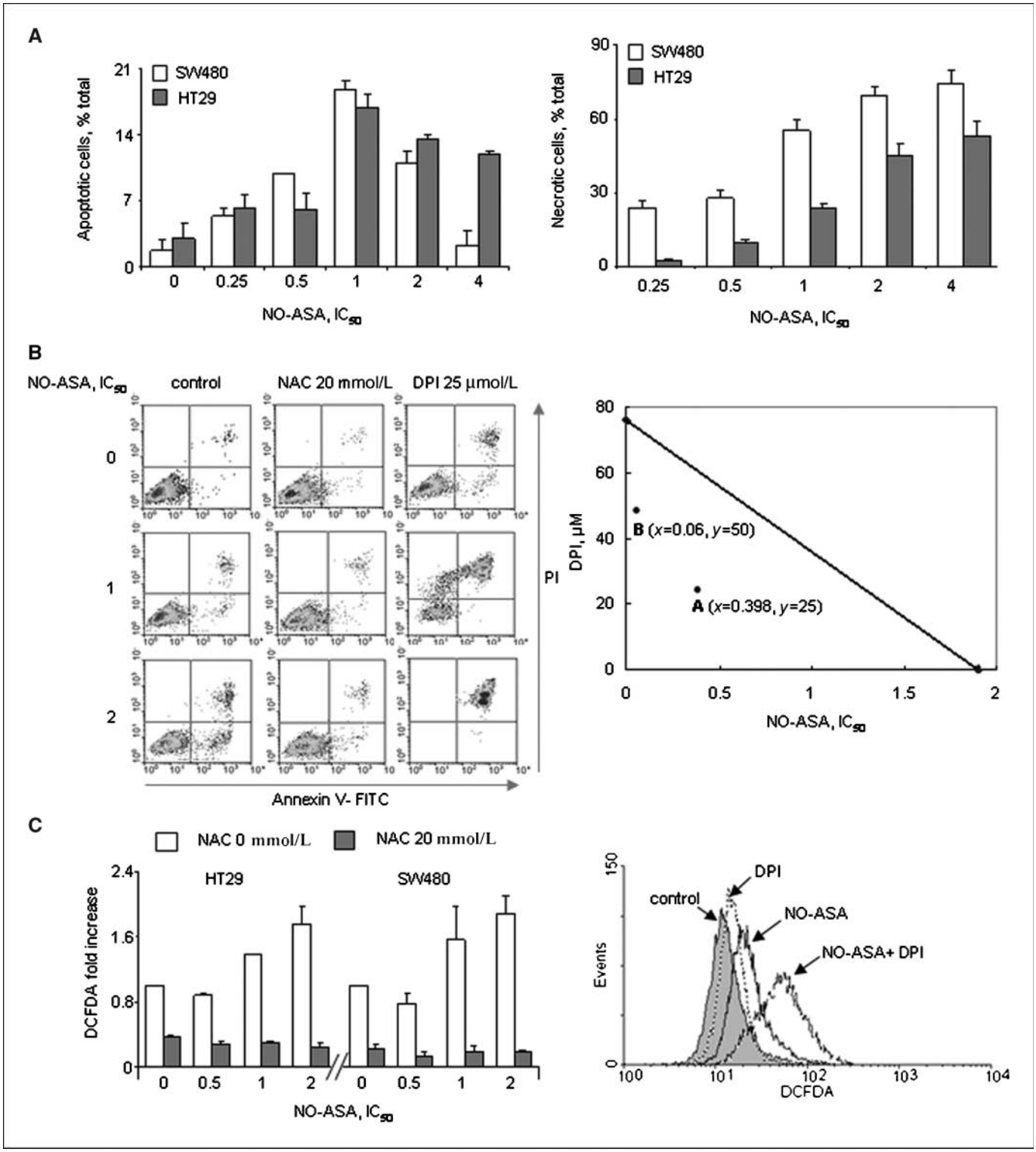


**Figure 2.** NO-ASA induces oxidative stress-dependent cell death. *A,* SW480 and HT-29 cells were treated with various concentrations of NO-ASA for 24 h, and cells were collected for apoptotic or necrotic cell death analysis. PI incorporation, determined by flow cytometry, was used to detect apoptotic cell death (sub-$G_1$ population in DNA histograms; *left*). LDH assay from cultured supernatants was used to determine necrosis (*right*). Please note the difference in scale between the two graphs. *B,* SW480 cells were pretreated with either NAC for 4 h or DPI for 2 h, followed by treatment with NO-ASA for 18 h as indicated. Annexin V and PI staining was used to detect necrosis and apoptosis (*left*). The isobologram (*right*) is based on the results of cell death by Annexin V and PI staining and was used to analyze potential synergy on cell death between NO-ASA and DPI. The additivity line connects the $IC_{50}$ value of each compound used alone. *A* and *B* represent two different dose pairs of each compound (their respective concentrations are shown for each point). Both *A* and *B* seem well below the additivity line signifying synergy. *C,* RONS production was detected by staining with DCFDA (general RONS probe). *Left,* both HT-29 and SW480 cells were pretreated with NAC, followed by NO-ASA treatment for 3 h; the DCFDA fluorescence intensity was measured by SpectraMax. The induction of RONS in treated samples is shown as fold increase compared with control cells. *Right,* SW480 cells were pretreated with DPI for 2 h, followed by NO-ASA treatment for 3 h; the DCFDA fluorescent intensity was measured by flow cytometry. Values are mean ± SE.

death significantly. For example, at $2\times IC_{50}$, NO-ASA cell death (Annexin V–positive cells) was reduced by 50% by NAC (30.4% in control, 14.2% in NAC). In contrast, 25 μmol/L DPI potentiated NO-ASA–induced cell death, increasing the Annexin V–positive cells from 30.4% to nearly 100% at NO-ASA $2\times IC_{50}$. Examined by isobolograms (23), the combined effects of DPI and NO-ASA on cell death represent a clear-cut case of pharmacologic synergy.

We also examined RONS levels in response to NAC and DPI. As shown in Fig. 2*C*, 20 mmol/L NAC prevented the induction of RONS by NO-ASA, keeping their levels at baseline. In the DPI study, NO-ASA at $1\times IC_{50}$ increased RONS levels of 83% over control. DPI, in contrast to NAC, increased both basal RONS levels (17% over control) and NO-ASA–induced RONS levels (204% increase over cells treated solely with NO-ASA and nearly 3-fold over control).

In these cells, the level of RONS paralleled the degree of cell death either by apoptosis or necrosis. As Fig. 2*A* and *C* shows, such a correlation existed also for the extent of cell death after treatment with NAC or DPI (Fig. 2*B* and *C*). The type of cell death (apoptosis versus necrosis) seems to be dependent on the levels of RONS. For example, lower NO-ASA concentrations (e.g., $1\times IC_{50}$) induced lower RONS levels, which activated the apoptotic signaling pathway. Higher NO-ASA concentrations (e.g., $>2\times IC_{50}$) led to higher RONS levels, in which case cell death was due predominantly to necrosis (Figs. 1*A* and *B* and 2*A*). In DPI-pretreated SW480 cells, the massive RONS production by NO-ASA (3-fold over control) caused necrosis in nearly all cells.

**Anticancer drug–induced RONS modulate NF-κB and MAPK signaling in colon cancers cells.** NF-κB and MAPK are major determinants of cell renewal and cell death. In general, NF-κB, whose activation and binding to DNA can be modified by RONS (24), modulates cell kinetics, but in each case, the specific response depends upon the biological context (24). MAPKs are serine/threonine-specific protein kinases that respond to extracellular stimuli and regulate various cellular activities, such as gene expression, mitosis, differentiation, cell survival, and cell death (25). The three major MAPKs, p38, ERK, and JNK, are at least partial mediators of RONS-induced cellular response.

In our system, we studied the effect of the altered RONS levels on NF-κB and MAPK activation. NO-ASA inhibited NF-κB activation after 3 h of treatment (Fig. 3*A*). Pretreatment with 20 mmol/L NAC for 4 h restored NF-κB activity to control levels. Pretreatment with 25 μmol/L DPI, however, could not restore NF-κB activity; in fact, it inhibited NF-κB activity slightly more than NO-ASA alone. Similar results were obtained with the other three compounds that we studied. In all cases, the activity of NF-κB (assayed either by an ELISA method or by EMSA; Fig. 3*A*) was inhibited by each one of them, and this inhibition was abrogated by pretreatment with 20 mmol/L NAC for 16 h.

As indicated by their increased phosphorylation, NO-ASA activated p38, ERK, and JNK as early as 1 hour after initiation of treatment in a concentration-dependent manner (Fig. 3*B*). The activation of these MAPKs could be blocked by pretreatment with 20 mmol/L NAC. DPI had no effect on the activation of MAPKs induced by NO-ASA.

**NO-ASA dissociates the ASK1–Trx-1 complex.** ASK1, also known as MAPK kinase kinase 5 (abbreviated as MAP3K5), is part of the MAPK cascade (26). ASK1 is inactive when bound to reduced Trx. When Trx is oxidized by RONS, Trx and ASK1

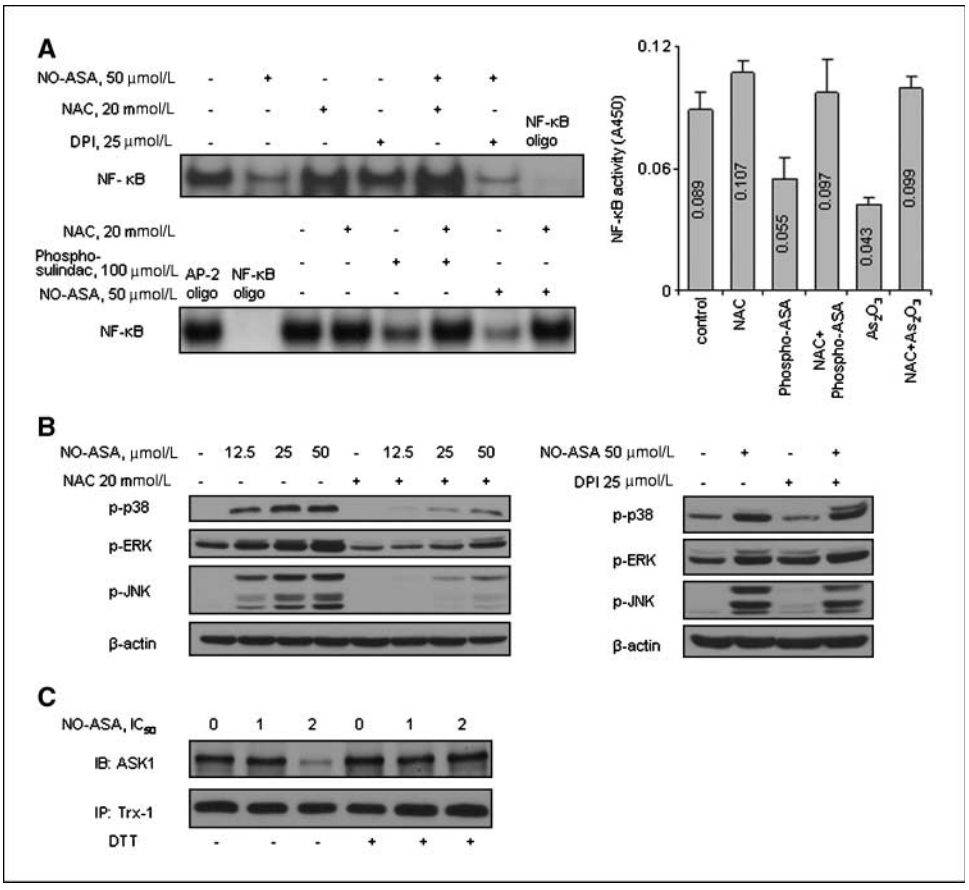


**Figure 3.** NO-ASA activates RONS-dependent signaling in colon cancer cells. *A, top left,* SW480 cells were preincubated with 20 mmol/L NAC for 4 h or 10 μmol/L DPI for 2 h, followed by NO-ASA treatment for 3 h. NF-κB activity was determined by EMSA in nuclear extracts. *Top right,* NF-κB activity (mean ± SD) in SW480 cells treated with phosphoaspirin or $As_2O_3$, each at $2\times IC_{50}$ for 4 h; pretreatment with NAC 20 mmol/L was for 16 h. Numbers in columns indicate the ODA450 values. *Bottom left,* HT-29 cells were treated with phosphosulindac or NO-ASA for 4 h and pretreated with NAC 20 mmol/L for 16 h, as indicated. Nuclear proteins were subjected to EMSA. AP-2 oligonucleotide was used as a nonspecific competitor. *B,* MAPK phosphorylation was determined by immunoblotting of total cell lysates. Cells were pretreated with either NAC (*left*) or DPI (*right*). Loading control, β-actin. *C,* HT-29 cells were treated with NO-ASA for 1 h. Cell protein lysates were treated with or without DTT 1 mmol/L for 30 min and then subjected to immunoprecipitation (*IP*) using an anti-Trx-1 antibody and the precipitates were immunoblotted (*IB*) for ASK1.

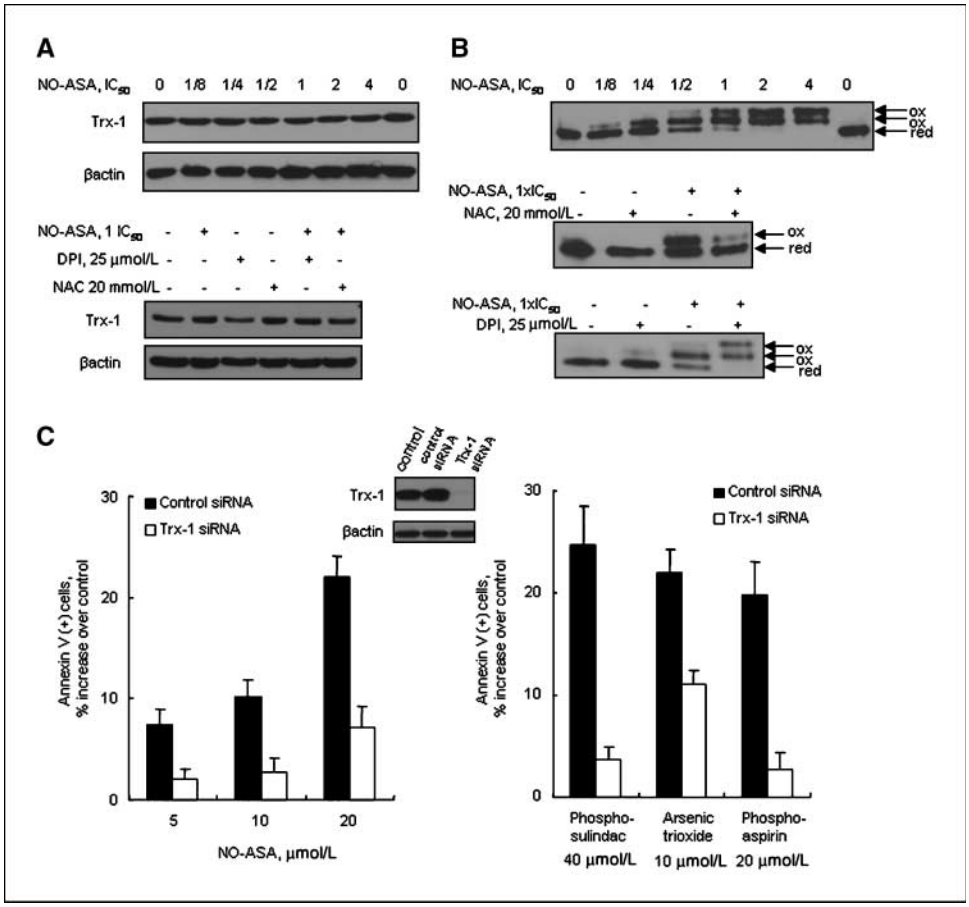


**Figure 4.** NO-ASA oxidizes Trx-1, which mediates oxidative stress-induced cell death. *A,* the level of Trx-1 was detected by Western blot in whole-cell lysates from SW480 cells treated with NO-ASA for 1 h with or without pretreatment with NAC or DPI as indicated. *B,* after the same treatment as in *A*, cell lysates were processed, as in Materials and Methods, to identify Trx-1 redox forms indicated as follows: *red,* reduced form; *ox,* oxidized form; the topmost ox band is the one most oxidized. Cell lysates from HT-29 cells produced similar results. *C,* SW480 cells were transfected with *trx-1* siRNA or nonspecific siRNA for 72 h, and total cell lysates were analyzed by Western blotting (*top small panel*). After transfection with siRNA, SW480 cells were treated with NO-ASA (*left bottom*), phosphosulindac, arsenic trioxide, or phosphoaspirin, as indicated, and cell death was evaluated by Annexin V staining. All drugs increased Annexin V (+) cells, a response severely suppressed in the absence of Trx-1. Values are mean ± SE.

dissociate and ASK1 autophosphorylates, becoming an active MAPKKK. Phosphorylation of ASK1 protein can lead to apoptosis or other cellular responses, depending on cell type. We explored the status of the ASK1–Trx-1 complex in HT-29 cells in response to NO-ASA.

As shown in Fig. 3*C*, treatment of HT-29 cells for 1 hour with NO-ASA at $2\times IC_{50}$ decreased dramatically the amount of ASK1 associated with Trx-1. This decrease resulted from the dissociation of the ASK1–Trx-1 complex due to NO-ASA–induced oxidation of Trx-1 (Fig. 4). This conclusion is supported by the finding that incubation of the cell lysate with the reducing agent DTT restored the integrity of the ASK1–Trx-1 complex.

**Oxidized Trx participates in oxidative stress-induced cell death.** Trx-1 is one of the most important molecules linking RONS to NF-κB and MAPK (5). Trx-1 modulates the activity of NF-κB by interacting directly with it and of MAPK through the ASK1-Trx signaling complex. In our system, NO-ASA dramatically influenced RONS-dependent NF-κB and MAPK activities, as well as cell death. Consequently, we determined whether NO-ASA together with NAC or DPI could influence the cellular levels and the redox status of Trx-1.

As shown in Fig. 4*A*, NO-ASA did not change the total protein level of Trx-1, and pretreatment with NAC or DPI had no effect on it. However, we found significant changes in the oxidation status of Trx-1 in response to treatment with NO-ASA. As described in Materials and Methods, to detect the oxidized and reduced forms of Trx-1, protection of the oxidation status of its thiols during protein extraction was essential. When fully reduced, the five cysteines of Trx-1 generate its reduced form, whereas when oxidized, they generate its oxidized forms (1). After 1 hour of treatment of HT-29 cells with NO-ASA, the redox status of Trx-1 changed in a concentration-dependent manner (Fig. 4*B*). At low NO-ASA concentrations (1/8, 1/4, 1/2, and $1\times IC_{50}$), we observed both reduced and oxidized forms of Trx-1, whereas at high concentrations (2 and $4\times IC_{50}$), the reduced band was undetectable and only two oxidized forms were present. SW480 cells gave similar results (data not shown).

In both HT-29 and SW480 cells, the changes in the redox state of Trx-1 in response to NO-ASA corresponded closely to the changes in RONS levels. Furthermore, 20 mmol/L NAC, which completely suppressed RONS, reduced the oxidized form of Trx-1 that was generated by NO-ASA. However, 25 μmol/L DPI, which greatly enhanced RONS levels in the presence of NO-ASA (Fig. 2*C*), also enhanced further the oxidation of Trx-1. Combined with NO-ASA, DPI generated two oxidized forms compared with one oxidized and one reduced form in cells treated with NO-ASA alone (Fig. 4*B*). These findings suggest that RONS generated by NO-ASA (and DPI when it was used) modulated the redox status of Trx-1.

We then silenced the expression of *trx-1* in SW480 cells using siRNA and determined whether the absence of Trx-1 interfered with the cell death effect of NO-ASA. As shown in Fig. 4*C*, 72 hours after transfection, the protein level of Trx-1 decreased dramatically, with only a faint band being detected in the Western blot. Compared with cells treated with control siRNA, NO-ASA induced much less death in cells transfected with *trx-1* siRNA. Tested at three different concentrations of NO-ASA, the absence of Trx-1

reduced the extent of apoptosis-related cell death by 65% to 70%. Annexin V (A) positive cells represent either apoptosis [A(+)/PI(−)] or secondary necrosis [A(+)/PI(+)]. Trx-1 was also required for the ability of the other three compounds to induce cell death. When SW480 cells, with knocked down expression of Trx-1, were treated in a similar manner with each of the other three compounds, apoptosis-related cell death was reduced significantly in each case (85% by phosphosulindac, 50% by arsenic trioxide, and 86% by phosphoaspirin). The latter finding indicates a generalized property of Trx-1 rather than one restricted to the effect of NO-ASA.

**Anticancer agents inhibit Trx reductase.** Of the four compounds that we studied, only arsenic trioxide has been previously evaluated for its effect on Trx-1 (27). This proapoptotic agent is known to oxidize Trx-1 by inducing RONS production and by inhibiting the activity of TrxR, the enzyme which normally reduces oxidized Trx-1. We studied, therefore, the effect of all four compounds on both the expression and activity of TrxR in SW480 and HT-29 cells.

After treatment with NO-ASA for 1 h, TrxR activity was decreased concentration-dependently in SW480 and HT-29 cells (Fig. 5*A*). The other three compounds also reduced the activity of TrxR. However, the protein level of TrxR was not changed by NO-ASA, even when the incubation period was prolonged to 6 hours or when high concentrations of NO-ASA (up to $4\times IC_{50}$) were used. Thus, changes in TrxR levels are an unlikely explanation for its reduced activity.

To further evaluate the effect of oxidized Trx-1 on oxidative stress-induced cell death, we used the TrxR inhibitor aurothiomalate (ATM), which prevents the conversion of oxidized Trx-1 to its reduced form (28). Cells pretreated with 50 or 100 μmol/L ATM for 24 hours were exposed to NO-ASA at its $0.5\times IC_{50}$ concentration for 16 hours. ATM increased cell death from 12% (NO-ASA alone) to 34% or 41% at 50 or 100 μmol/L ATM, respectively. At a higher NO-ASA concentration ($1\times IC_{50}$), ATM increased cell death from 29% (NO-ASA alone) to 52% or 62% at 50 or 100 μmol/L ATM, respectively. Examined by isobolograms (23), the effects of ATM on cell death represent pharmacologic synergy (Fig. 5*C*).

Combined with the siRNA results, these findings strongly suggest that the oxidized form of Trx-1 provides a critical contribution to cell death in response to anticancer agents that share the ability to induce RONS.

## Discussion

Our data show that four anticancer agents generate in human colon cancer cell lines a state of oxidative stress, which involves at least four individual RONS. This effect is accompanied by changes in two critical cell signaling cascades, NF-κB and MAPK,

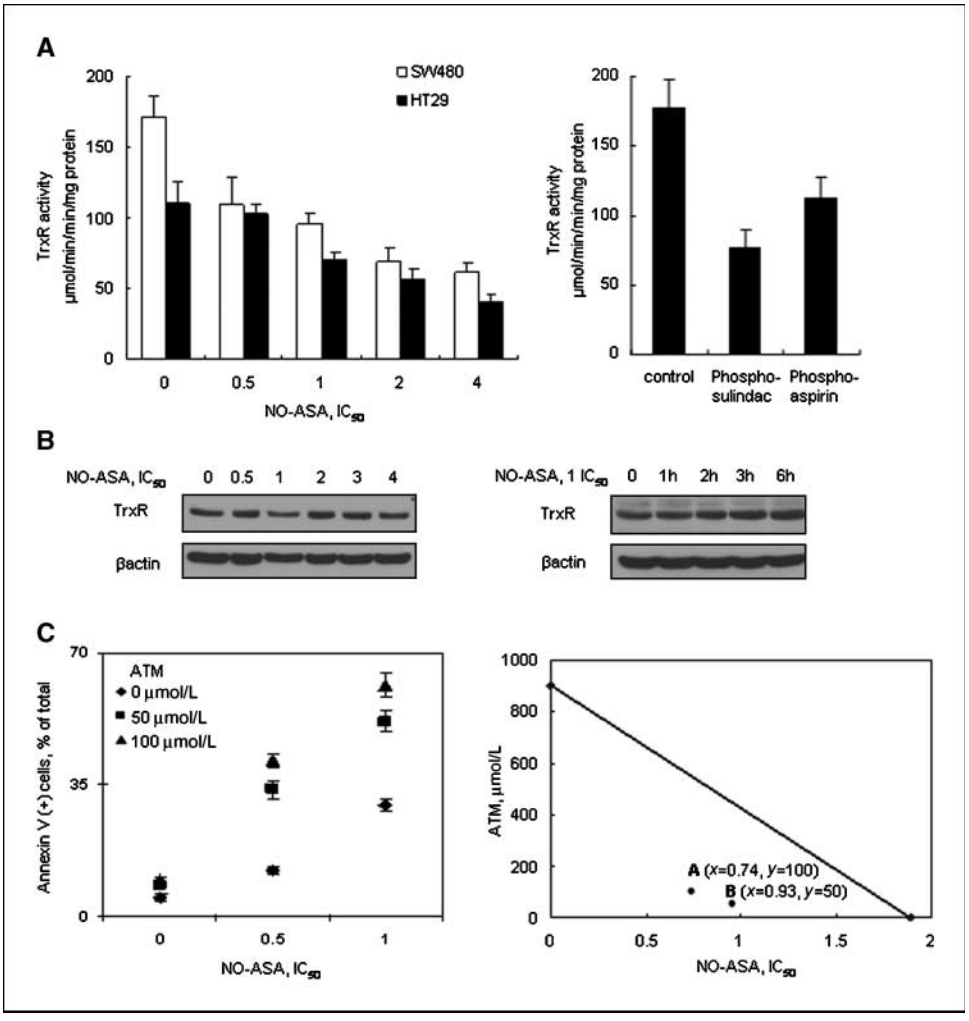


**Figure 5.** Anticancer agents decrease TrxR activity and a TrxR inhibitor potentiates cell death. *A,* SW480 and HT-29 cells were treated with NO-ASA for 1 h, as indicated, harvested, and their TrxR activity was measured, as in Materials and Methods (*left*). HT-29 cells were treated for 1 h with the other three anticancer agents, each used alone at its respective $IC_{50}$. *B,* the expression of TrxR was determined in SW480 cells treated with various concentrations of NO-ASA for 1 h (*left*) or with $1\times IC_{50}$ for up to 6 h (*right*). *C,* SW480 cells were treated with various concentrations of NO-ASA for 16 h, after 24 h pretreatment with or without the TrxR inhibitor ATM. The percentage of Annexin V (+) cells is shown. ATM enhanced greatly the NO-ASA–induced cell death. The isobologram on the right, based on the results of cell death by Annexin V staining, evaluated potential synergy between NO-ASA and ATM. The additivity line connects the $IC_{50}$ value of each compound when used alone. *A* and *B* represent two different dose pairs of each compound (the respective concentrations of each are shown next to each point). Values are mean ± SE.

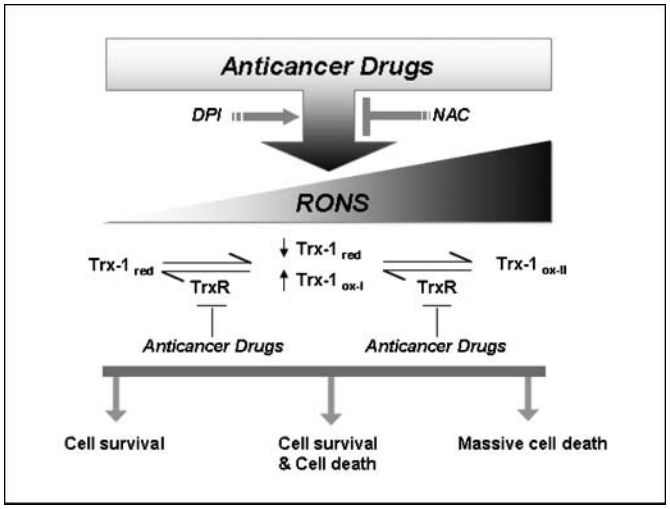


**Figure 6.** The redox status of Trx-1 modulates cell survival. Dependence on intracellular RONS levels. This diagram depicts the relationship between Trx-1, TrxR, and RONS. As RONS levels increase, Trx-1 changes from its completely reduced form (*Trx-1$_{red}$*) to partially oxidized (*Trx-1$_{ox-I}$*) to its fully oxidized form (*Trx-1$_{ox-II}$*). TrxR, which reverses the oxidation of Trx-1, was also inhibited by the compounds we studied. At low RONS concentrations, when Trx-1 is in its reduced form, cell death is minimal. As RONS concentrations increase, cell death is evident, becoming massive at the highest RONS concentrations. The predominance of apoptosis at intermediate RONS levels is replaced by necrosis at higher RONS levels.

both responsive to RONS, and culminates in apoptotic or necrotic cell death, with the specific type of cell death depending on RONS levels. The Trx system plays a pivotal role in the induction of oxidative stress, as Trx-1 and TrxR are altered in a way that prevents them from countering the prooxidant effect of RONS.

The four anticancer agents that we used are structurally diverse. Three of them have shown anticancer properties in preclinical models, whereas the fourth, arsenic trioxide, is the drug of choice for the treatment of promyelocytic leukemia. Their $IC_{50}$s for cell growth are similar, ranging between 10 and 90 μmol/L. Our data establish that their common property is the induction of RONS in two colon cancer cell lines, documented by the response of the general RONS probe DCFDA. In the case of NO-ASA, which was studied in greater detail, the induction of RONS involved at least four individual species, NO, $H_2O_2$, $ONOO^-$, and $O_2^{\bullet-}$. These compounds encompass a wide range of reactivities and biological behavior. The mitochondria showed the most notable production of $O_2^{\bullet-}$. The reaction of $O_2^{\bullet-}$ with NO may be the sole source of the very reactive (and damaging) $ONOO^-$. The ultimate result of the generation of RONS was cell death; the etiologic relationship between the two was underscored by the abrogation of cell death by NAC, which blocked the generation of RONS by NO-ASA. A remarkable finding was the clear relationship between RONS levels and the type of cell death: apoptosis at lower RONS concentrations and necrosis at higher levels.

Contrary to expectations, DPI potentiated the generation of RONS and, consequently, the degree of cell death. In fact, DPI synergized with NO-ASA, as was formally shown by our data analysis. DPI, considered predominantly as an antioxidant, is also known to have prooxidant properties, and this may indeed be the case here. It is unclear what determined the type of response to DPI.

RONS are increasingly appreciated as significant signaling molecules, a role distinct from their potentially catastrophic effects, which occur at high concentrations and represent the situation conventionally described as oxidative stress (29). NO-ASA activated MAPKs, a signaling pathway also known to be redox responsive (25). NAC prevented, to a large extent, the activation of these pathways, establishing a potential cause-and-effect relationship with RONS. In addition, all four compounds inhibited NF-κB activation. In each case, RONS seem to have been the responsible signaling molecules, as NAC abrogated their effect on NF-κB and DPI, which unexpectedly increased RONS levels, inhibited further the activity of NF-κB (which was already inhibited by NO-ASA). An important proximal signaling molecule in this cascade is ASK1, which, as mentioned, is physically associated with reduced Trx-1. Our data confirmed this association and also showed that NO-ASA, which oxidized Trx-1, released ASK1, whereas reduction of Trx-1 by the reducing agent DTT favored formation of the Trx-1–ASK1 complex.

The redox status of the cell regulates NF-κB activity, but the specific response (NF-κB inhibition or activation) seems to be dependent on cell type and perhaps on the individual RONS involved (24, 30). Direct interactions of RONS with the NF-κB subunits or with proteins in the NF-κB regulatory cascade have been described. Cysteine residue(s) of NF-κB subunits are involved in its recognition of and binding to DNA, such as the redox-sensitive Cys62 residue of p50, whereas the redox control mechanism mediated by Trx may regulate NF-κB–dependent gene expression (31, 32). The most reasonable explanation of our data is that the four anticancer agents induced RONS, which interacted either directly or indirectly with NF-κB, inhibiting its ability to bind to its cognate DNA recognition sequence (inhibition of NF-κB activation). The effects of NAC and DPI strongly support this notion.

The Trx system, along with GSH, is currently thought to be at the heart of the antioxidant response of a mammalian cell. In the face of high RONS levels in response to NO-ASA, it is easy to anticipate that Trx-1, with its wide substrate specificity, was extensively oxidized as it engaged in the reduction of its many oxidized client proteins. Here again, the degree of Trx-1 oxidation paralleled the amount of RONS, and such oxidation was prevented by pretreatment with NAC and exacerbated by DPI. At the highest NO-ASA concentrations, the oxidation of Trx-1 seemed complete. Trx-1 seemed to play a central role in the growth inhibitory effect of NO-ASA. In particular, when its expression was knocked down by siRNA, the proapoptotic effect of NO-ASA and other three compounds was abrogated; this effect was nearly complete (except for the 50% reduction by arsenic trioxide).

TrxR, the enzyme that reduces oxidized Trx-1, was significantly inhibited by these compounds, and this explains, at least partially, the persistence of the oxidized form of Trx-1. That the interaction between Trx-1 and its reductase is important in these cells was further substantiated by the finding that a TrxR inhibitor actually synergized with NO-ASA to inhibit cell death. It is, therefore, likely that the Trx system suffered a dual assault consisting of (*a*) inhibition of TrxR by these agents and (*b*) oxidation of Trx-1, as it had to reduce its client proteins that were extensively oxidized by RONS. The end result was the oxidation of Trx-1, which in its extreme form became complete and culminated in massive cell death (Fig. 6).

The exact mechanism by which oxidized Trx-1 participates in the induction of cell death by these compounds is not

fully delineated. It is, however, clear that NF-κB and MAPKs are modulated in our experimental system. Both pathways (*a*) have been previously documented to be critical for the growth inhibitory effect of NO-ASA, (13, 33) and (*b*) depend on Trx for their oxidation status that determines their biological activity. Based on the known role of Trx-1 in the regulation of transcription factors, it is likely that the oxidation of Trx-1 is proximal to NF-κB and MAPKs. Indeed, in the case of MAPK activation, the release of ASK1 from its complex with Trx-1 represents a convincing mechanistic link between the two.

In conclusion, our data show the significant regulatory role of the Trx system in the induction of cell death by four diverse anticancer compounds. It is reasonable to consider that the mechanism presented here may be shared by more compounds in addition to those studied by us, and thus, it deserves further assessment as a candidate mechanism for the pharmacologic control of cancer.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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