

# Anti-Inflammatory, Antiproliferative, and Cytoprotective Activity of NO Chimera Nitrates of Use in Cancer Chemoprevention<sup>S</sup>

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## ABSTRACT

Nonsteroidal anti-inflammatory drugs (NSAIDs) have shown promise in colorectal cancer (CRC), but they are compromised by gastrototoxicity. NO-NSAIDs are hybrid nitrates conjugated to an NSAID designed to exploit the gastroprotective properties of NO bioactivity. The NO chimera ethyl 2-((2,3-bis(nitrooxy)propyl)disulfanyl)benzoate (GT-094), a novel nitrate containing an NSAID and disulfide pharmacophores, is effective in vivo in rat models of CRC and is a lead compound for design of agents of use in CRC. Preferred chemopreventive agents possess 1) antiproliferative and 2) anti-inflammatory actions and 3) the ability to induce cytoprotective phase 2 enzymes. To determine the contribution of each pharmacophore to the biological activity of GT-094, these three biological activities were studied in

vitro in compounds that deconstructed the structural elements of the lead GT-094. The anti-inflammatory and antiproliferative actions of GT-094 in vivo were recapitulated in vitro, and GT-094 was seen to induce phase 2 enzymes via the antioxidant responsive element. In the variety of colon, macrophage-like, and liver cell lines studied, the evidence from structure-activity relationships was that the disulfide structural element of GT-094 is the dominant contributor in vitro to the anti-inflammatory activity, antiproliferation, and enzyme induction. The results provide a direction for lead compound refinement. The evidence for a contribution from the NO mimetic activity of nitrates in vitro was equivocal, and combinations of nitrates with acetylsalicylic acid were inactive.

Colorectal cancer (CRC) is a leading cause of death, providing impetus for discovery of new chemopreventive agents. Chemoprevention strategies for CRC have targeted antiproliferative and anti-inflammatory actions on colonocytes containing populations subject to carcinogen-induced DNA damage. Although, the exact mechanisms of colon carcinogenesis remain to be revealed, there are clear biomarkers that cor-

relate with drug intervention and tumorigenesis, in particular biomarkers of inflammation and of proliferation.

Aberrant crypt foci (ACF) are seen as an early precursor stage to adenomas and cancer, which facilitate early diagnosis. The ACF is a monoclonal structure that arises from mutations within a single crypt stem cell, thought to result from a single mutational event within an isolated colon crypt. ACF are strongly linked to colon cancer risk, and in animal models, such as the murine azoxymethane (AOM) carcinogen model, a good correlation between ACF number and tumorigenesis has been reported for a variety of chemopreventive agents (Corpet and Taché, 2002). ACF are a reliable biomarker for colon cancer in preclinical models and are of use in

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**ABBREVIATIONS:** CRC, colorectal cancer; ACF, aberrant crypt foci; AOM, azoxymethane; iNOS, inducible nitric-oxide synthase; NSAID, nonsteroidal anti-inflammatory drug; ASA, aspirin; GI, gastrointestinal; NO-NSAID, NO-donating nonsteroidal anti-inflammatory drug; BECS, 1,2-bis-(2-ethoxycarbonylphenyl)sulfane; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; ARE, antioxidant responsive element; LPS, lipopolysaccharide; L-NAME, *N*<sup>ω</sup>-nitro-L-arginine methyl ester; CM, conditioned media; PBS, phosphate buffered saline; ERK, extracellular signal-regulated kinase; ERK, extracellular signal-regulated kinase; NQO1, NAD(P)H-dependent quinone oxidoreductase; CI, chemopreventive index; RT-PCR, reverse transcription-polymerase chain reaction; TNF, tumor necrosis factor; IL, interleukin; COX, cyclooxygenase; PG, prostaglandin; SPE/NO, spermine diazeniumdiolate salt; DETA/NO, diethyltriamine diazeniumdiolate; BF, 4'-bromoflavone; ISDN, isosorbide dinitrate; PTIO, 2-phenyl-4,4,5,5-tetramethylimidazoline-*l*-oxyl-3-oxide; CD, concentration required to double the specific activity of NAD(P)H-dependent quinone oxidoreductase; ODQ, 1H-[1,2,4]oxadiazolo[3,4-*a*]quinoxalin-1-one; MAPK, mitogen-activated protein kinase; PD 98059, 2'-amino-3'-methoxyflavone; GTN, nitroglycerin; DADS, dipropyl disulfide and diallyl disulfide; Keap1, protein Kelch-like ECH-associated protein 1; NCX 4016, 2-acetoxybenzoate-2-(1-nitroxymethyl)phenyl ester; GT-947, ethyl 2-(1,3-bis(nitrooxy)propan-2-ylthio)benzoate; GT-794, ethyl 2-(2,3-bis(nitrooxy)propylthio)benzoate; GT-103, 2-((2,3-bis(nitrooxy)propylthio)carbonyl)phenyl acetate; GT-094, ethyl 2-((2,3-bis(nitrooxy)propyl)disulfanyl)benzoate; GT-015, 3,3'-disulfanediybis(propane-3,2,1-triyl) tetranitrate.

drug discovery. Furthermore, in humans, ACF demonstrate increased expression of markers of proliferation, and inflammation [e.g., inducible NO synthase (iNOS)] (Hao et al., 2001).

Nonsteroidal anti-inflammatory drugs (NSAIDs), such as acetylsalicylic acid (ASA; aspirin), have shown promise in CRC clinical trials (Rosenberg et al., 1998). However, chronic NSAID use has fatal side effects from severe gastrointestinal (GI) damage, and efficacy at low doses in CRC is problematic. Exploration of the use of nitric oxide donor NSAIDs (NO-NSAIDs) in CRC represented a logical progression, because NO-NSAIDs were originally designed to deliver the biological activity of NO to mollify the GI damage caused by NSAIDs. The most widely studied NO-NSAID, NCX 4016, is an NO-ASA that entered National Cancer Institute-sponsored clinical trials for CRC prevention having shown promise in pre-clinical studies (Bak et al., 1998; Rao et al., 2006).

NO-NSAIDs are hybrid nitrate drugs; aliphatic nitrates conjugated via a labile ester linkage to an NSAID (Bolla et al., 2005). Nitrates, themselves, have been in clinical use for more than 130 years in cardiovascular therapy (Thatcher et al., 2004; Thatcher, 2007). There is a substantial body of work on NO-ASA related to CRC; however, key gaps in our knowledge have been highlighted by Rigas and coworkers (Kaza et al., 2002); and importantly, structural optimization of NO-NSAIDs specifically for CRC chemoprevention has not been explored. The NCX 4016 phase 2 CRC chemoprevention clinical trial was recently halted because of potential drug toxicity, amplifying the need for a deeper understanding of structure-activity relationships. It is possible that drug optimization of a nitrate-NSAID hybrid for CRC would circumvent the toxicity reported for NCX 4016.

The novel nitrate GT-094, in simile with NO-NSAIDs, contains an NSAID and a nitrate, but it also has a disulfide pharmacophore, known to be effective in chemoprevention. GT-094 was validated as a lead compound for chemoprevention by studies in the rat/AOM model of human CRC, which demonstrated significant reductions in ACF number and several biomarkers linked to CRC (Hagos et al., 2007). In this article, GT-094 is studied in vitro and the pharmacophore elements are structurally deconstructed to gain insight into the relationship of structure to anti-inflammatory, antiproliferative, and cytoprotective activity. The results affect future development strategies for optimization of nitrate-NSAID hybrid drugs for CRC.

## Materials and Methods

### Materials

Full synthesis and characterization data for novel compounds is presented in Supplemental Data. 1,2-Bis-(2-ethoxycarbonylphenyl)-sulfane (BECS), NCX 4016, GT-015, and GT-094, all described previously, were synthesized by minor modification of literature procedures, purified, and fully characterized (Nicolescu et al., 2002; Hagos et al., 2007). Buffered formalin, xylene, and alcohols (histology grade) were from Thermo Fisher Scientific (Waltham, MA). All other biochemical reagents were from Sigma-Aldrich (St. Louis, MO).

### Biology

Caco-2 human colonic adenocarcinoma cells obtained from American Type Culture Collection (Manassas, VA) were grown in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 supplemented with 1% penicillin-streptomycin, 20% fetal bovine serum (FBS), non-

essential amino acids (1 mM), sodium pyruvate (0.1 mM), and 1.5 g/l sodium bicarbonate, and they were incubated in 5% CO<sub>2</sub> at 37°C. HT-29 human adenocarcinoma cells were supplied by Dr. Geneveva Murillo (Illinois Institute of Technology Research Institute, Chicago, IL). Cells were maintained in RPMI 1640 medium, supplemented with 1% antibiotic-antimycotic, 1% L-glutamine (200 mM), and 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA), and they were incubated in 5% CO<sub>2</sub> at 37°C. RAW 264.7 mouse macrophage-like cells, provided by Dr. J. Cook (University of Illinois at Chicago, Chicago, IL), were maintained in DMEM, supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum, and they were incubated in 5% CO<sub>2</sub> at 37°C. The mouse alveolar macrophage MH-S cell line was a generous gift from Dr. J. Cook (University of Illinois at Chicago). MH-S cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum and 1% penicillin-streptomycin-amphotericin. Hepa 1c1c7 murine hepatoma cells were supplied by Dr. J. P. Whitlock, Jr. (Stanford University, Stanford, CA), and they were cultured in  $\alpha$ -minimal essential medium with 1% penicillin-streptomycin and 10% fetal bovine serum (Atlanta Biologicals). HepG2 cells stably transfected with antioxidant/electrophile responsive element (ARE)-luciferase reporter gene were kindly provided by Dr. A. N. Kong (Rutgers University, Piscataway, NJ) and cultured in Ham's F-12 medium with 10% fetal bovine serum, 1% penicillin-streptomycin, 1% nonessential amino acids, and 0.2 mg/ml insulin (Chen et al., 2000). MCF-7 WS8 human breast cancer cells (a kind gift from Dr. V. C. Jordan, Fox Chase Cancer Center, Philadelphia, PA) were maintained in RPMI 1640 medium containing 10% FBS (Atlanta Biologicals), 2 mM L-glutamine, 0.1 mM nonessential amino acids, and 6  $\mu$ g/ml bovine insulin (Sigma-Aldrich). Estrogen-free media were prepared by supplementing 3 $\times$  dextran-coated charcoal-treated FBS to phenol-red free RPMI 1640 medium, whereas other components remained the same. Assays were performed in at least two separate cell passages.

**Proliferation Assay.** Cells were seeded in 96-well plates at a density of  $2 \times 10^4$  cells/ml in 190  $\mu$ l of media. After 24-h incubation, test samples were added to each well, and the cells were incubated for an additional 24, 48, or 96 h. The cells were fixed by adding ice-cold 20% trichloroacetic acid, and they were incubated for 30 min at 4°C. The plates were then washed with water and air-dried. The trichloroacetic acid-fixed cells were stained with 100  $\mu$ l of 0.4% sulforhodamine B and incubated for 30 min at room temperature. The free dye was washed with 1% aqueous acetic acid, and the plates were air-dried. The bound dye was solubilized by adding Tris base (10 mM; 200  $\mu$ l). The plates were placed on an orbital shaker (100 rpm) for 10 min at room temperature. The absorption was measured at 515 nm, using a plate reader and normalized to control cells.

**Griess Assay.** Macrophage cells were plated at a concentration of  $12 \times 10^4$  cells/ml (or as otherwise stated in text and figure legends) in a 96-well plate. Cells were plated in DMEM and incubated at 37°C for 24 h. The medium was changed, allowing cell induction by addition of lipopolysaccharide from *Escherichia coli* 055:B5 (LPS; Sigma-Aldrich) in the medium. Test compounds were added, and at the indicated time points, 100  $\mu$ l of the supernatant was removed and incubated with the Griess reagent [100  $\mu$ l; 0.5% sulfanilamide, 0.05% (*N*-1-naphthyl)ethylenediamine dihydrochloride, 2.5% H<sub>3</sub>PO<sub>4</sub>, and 97% H<sub>2</sub>O by weight] for 30 min at room temperature in the dark. The absorbance was measured at 530 nm on a Dynex MRX II microplate spectrophotometer and calibrated using a standard curve constructed with sodium nitrite to yield NO<sub>2</sub><sup>-</sup> concentration. Where indicated in the text, subtraction of NO<sub>2</sub><sup>-</sup> formed in cell incubations of nitrates in the absence of LPS was used to determine NO<sub>2</sub><sup>-</sup> production from endogenous NOS-mediated production of NO.

In preliminary experiments with the NOS inhibitor in RAW 264.7 cells, cells were plated at a concentration  $25 \times 10^4$  cells/well in a 24-well plate for 24 h; L-NAME (10  $\mu$ M) was added; after a further 40 min, cells were induced with LPS (1  $\mu$ g/ml); after 24 h, 50  $\mu$ l of the supernatant was withdrawn and analyzed in the Griess assay using calibration curves obtained under identical conditions. For assay

using conditioned media (CM) from noninduced and LPS-induced RAW 264.7 cell cultures, cells were cultured as described above and incubated for 12 h in the absence and presence of LPS and L-NAME. Aliquots of supernatant were removed to a 96-well plate and further incubated for 12 h with or without GT-094 (100  $\mu$ M) before Griess assay.

In experiments to study the effect of cell density and of arginine depletion, RAW 264.7 cells were plated at either 5, 10, or 20  $\times 10^4$  cells/well for 24 h; media were replaced with FBS-free media containing GT-094 (100  $\mu$ M); after a further 30 min LPS (1  $\mu$ g/ml) was added, and cells were incubated for 20 h before collection of supernatant aliquots for Griess assay. For study of arginine depletion, cells were plated at 20  $\times 10^4$  cells/well; after 24 h, the media were replaced with FBS-free media (AthenaES, Baltimore MD) with or without L-arginine. GT-094 (100  $\mu$ M) was immediately added, followed after 30 min by LPS. The Griess assay was performed after 20-h incubation.

**Immunoblot Analysis.** Cells were plated 400 to 500  $\times 10^4$  cells/dish in 100-mm dishes. After 24 h, cells were treated with the test compounds at the desired concentrations. After the desired time period, cells were lysed as follows: media were removed and cells were washed twice with ice-cold PBS followed by addition 250  $\mu$ l of ice-cold 1 $\times$  lysis buffer (Cell Signaling Technology Inc., Danvers, MA). Cells were scraped with the lysis buffer and transferred into an Eppendorf tube and left on ice for 30 min while vortexing occasionally. The tubes were centrifuged at 12,000g for 15 min, and the supernatant was transferred into a fresh tube and stored at  $-80^\circ\text{C}$ . Protein concentrations were determined using a standard protein assay kit (Bio-Rad Laboratories, Hercules, CA). Proteins were separated by 8% SDS gel, and then they were transferred to polyvinylidene difluoride membrane. The membrane was incubated with the desired primary antibody (p53, phospho-p53, phosphorylated ERK, and ERK from Cell Signaling Technology Inc.; iNOS from Santa Cruz Biotechnology, Inc., Santa Cruz, CA; actin from Sigma-Aldrich) in 3% (w/v) nonfat dry milk in buffer, and then they were incubated with the secondary antibody (anti-rabbit IgG or anti-mouse IgG) conjugated with horseradish peroxidase (Cell Signaling Technology Inc.) in 3% (w/v) nonfat dry milk in buffer. Immunoreactive proteins were detected by chemiluminescence (reagents from Santa Cruz Biotechnology Inc.) and visualized after exposure to BioMax film (Eastman Kodak, Rochester, NY).

**NQO1 Activity Assay.** Induction of NAD(P)H-dependent quinone oxidoreductase (NQO1) activity was assessed in Hepa 1c1c7 murine hepatoma cells as described previously, with minor modifications (Prochaska and Santamaria, 1988). In brief, Hepa 1c1c7 cells were seeded in 96-well plates at a density of  $1.25 \times 10^4$  cells/ml in 190  $\mu$ l of media. After 24-h incubation, test samples were added to each well, and the cells were incubated further (0–24 h). The medium was decanted, and the cells were incubated at  $37^\circ\text{C}$  for 10 min with 50  $\mu$ l of 0.8% digitonin and 2 mM EDTA solution, pH 7.8. The plates were then agitated on an orbital shaker (100 rpm) for 10 min at room temperature, and 200  $\mu$ l of reaction mixture [0.7 mg/ml bovine serum albumin, 0.3 mg/ml 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide, 25 mM Tris-HCl, 0.01% Tween 20, 5  $\mu$ M FAD, 1 mM glucose 6-phosphate, 30  $\mu$ M NADP, 2 U/ml glucose-6-phosphate dehydrogenase, and 50  $\mu$ M menadione] was added to each well. After agitation on a shaker for 5 min, the plates were scanned at 595 nm. The specific activity of NQO1 was determined by measuring NADPH-dependent menadiol-mediated reduction of 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide to blue formazan. Induction of NQO1 activity was calculated by comparing the NQO1 specific activity of sample-treated cells with that of solvent-treated cells. CD values represent the concentration required to double NQO1 induction. The chemopreventive index (CI =  $\text{IC}_{50}/\text{CD}$ ) is calculated from the  $\text{IC}_{50}$  value for inhibition of cell growth (Song et al., 1999), which was measured for Hepa 1c1c7 cells by decanting medium from 96-well plates, followed by addition of 200  $\mu$ l of 0.2% crystal violet solution in 2% ethanol. After 10 min the plates were

rinsed for 2 min with water and dried. The bound dye was solubilized by incubation at  $37^\circ\text{C}$  for 1 h with 200  $\mu$ l of 0.5% SDS in 50% ethanol. The absorption of crystal violet was measured at 595 nm.

**RT-PCR Analysis.** RAW 264.7 cells were plated at a concentration of  $400 \times 10^4$  cells/ml in a 100-mm dish. After 24 h, cells were treated with GT-094 and 1  $\mu$ g/ml LPS. After 4 h, media were removed, and cells were washed with PBS and lysed by adding TRIzol (Invitrogen, Carlsbad, CA). Cells were scraped and transferred into Eppendorf tubes; chloroform (200  $\mu$ l) was added, and the cells were vortexed at room temperature. The tubes were centrifuged at 12000 rpm for 10 min at room temperature, and the supernatant ( $\sim 600$   $\mu$ l) was transferred to a new tube, and 1 $\times$  volume ( $\sim 600$   $\mu$ l) of isopropanol was added. The tubes were left at  $-20^\circ\text{C}$  for 30 min and centrifuged at 14,000 rpm at  $4^\circ\text{C}$  for 15 min. The supernatant was discarded, and the pellets were washed with 70% ethanol (1 ml), centrifuged at 14,000 rpm for 10 min, and ethanol was removed. The pellets left for 1 to 2 min to dry. The pellets were dissolved in diethyl pyrocarbonate-treated water (Ambion, Austin, TX). Optical density was measured at 260/280 nm to determine the concentration of RNA. Total RNA (5  $\mu$ g) was reverse transcribed by using SuperScript II RT, dNTP and oligo(dT)<sub>12-18</sub> as described in the manufacturer's protocol (Invitrogen). cDNA (2  $\mu$ l) was amplified by PCR. PCR reactions were carried out in a volume of 50  $\mu$ l containing Taq DNA polymerase (5 U/ml), 0.2 mM dNTP, 1 $\times$  reaction buffer, and 200 pmol of 5' and 3' primers (Operon Biotechnologies, Huntsville, AL; see Supplemental Data for details). After one cycle of denaturation, amplification cycles were performed for TNF- $\alpha$ , IL-1 $\beta$ , IL-6, iNOS, and COX-2: 1 min of  $94^\circ\text{C}$  denaturation, 1 min of  $60^\circ\text{C}$  annealing, and 1 min  $72^\circ\text{C}$  extension. After amplification, electrophoresis was performed on 1% agarose gel and visualized by ethidium bromide staining and UV light.

**Prostaglandin Analysis.** After plating for 24 h, RAW 264.7 cells were treated with GT-094 at different concentrations, and 1  $\mu$ g/ml LPS was added after 30 min. After a further 24 h, media were collected and analyzed for prostaglandins. Then, 1 N citric acid (40  $\mu$ l) and 10% butylated hydroxytoluene (5  $\mu$ l) were added immediately to inhibit oxidation. Before extraction, 20  $\mu$ l of  $\text{PG}_{\text{E}_2\text{-D}_4}$  (100 ng/ml) was added to each sample as an internal standard. Prostaglandins were extracted from cell suspensions by adding 2 ml of hexane/ethyl acetate [1:1 (v/v)] and vortex mixing for 2 min. Samples were centrifuged (1800g for 10 min at  $4^\circ\text{C}$ ), and the upper organic layer was then transferred to a glass test tube on ice. The extraction step was repeated twice, and the organic phases were evaporated to dryness under a stream of nitrogen at room temperature. All extraction procedures were performed under low light and low temperature conditions to minimize potential photo-oxidation or thermal degradation of eicosanoid metabolites. Samples were reconstituted in 200  $\mu$ l of methanol before liquid chromatography/tandem mass spectrometry analysis (Yang et al., 2002). Liquid chromatography/tandem mass spectrometry was performed using an API 3000 mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a high-performance liquid chromatograph (Shimadzu, Kyoto, Japan). Prostaglandins were chromatographically resolved using a Luna 3- $\mu$ m, phenylhexyl, 2  $\times$  150-mm analytical column (Phenomenex, Torrance, CA) using a methanol/ammonium acetate (10 mM; pH 8.5) gradient at a flow rate of 400  $\mu$ l/min. Individual analytes were detected and quantified according to the literature (Yang et al., 2002).

## Results

**Chemistry.** Seven synthetic compounds were selected, containing various structural elements, for comparison with the parent GT-094 and both ASA and the classical nitrate ISDN (Fig. 1). Efficient synthesis of dinitrates requires oxidative nitration procedures, which inherently are not compatible with oxidizable functional groups such as sulfides;

therefore, a new synthesis was developed to allow access to dinitrates GT-794 and GT-974.

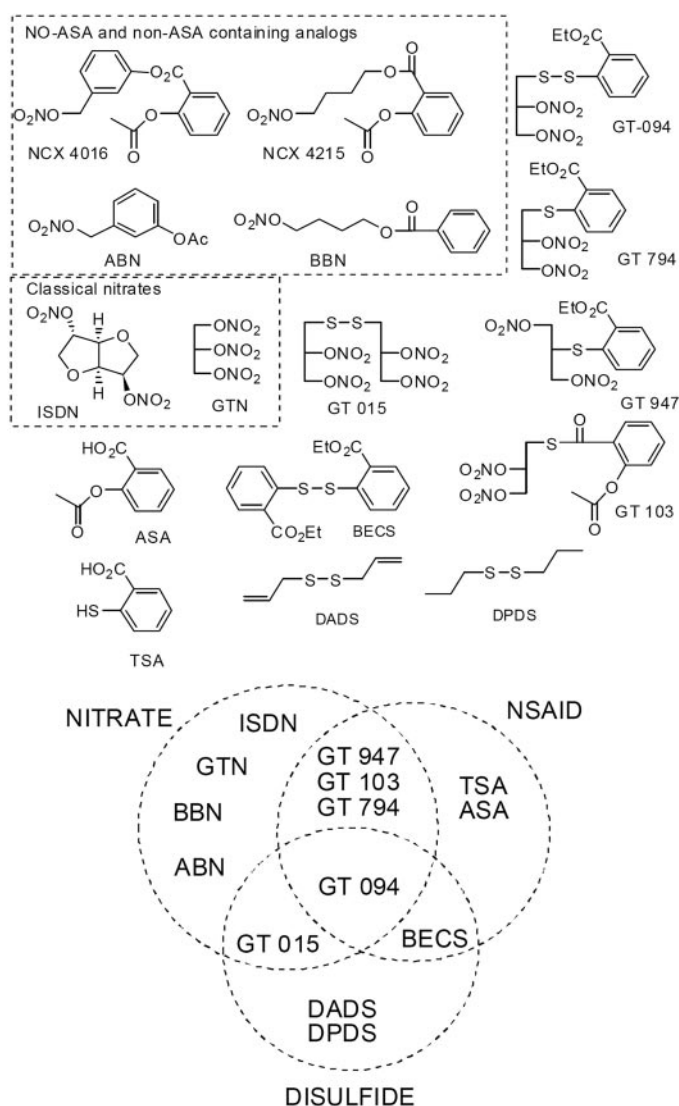
**Antiproliferative Activity.** The *in vivo* antiproliferative activity of GT-094 in the colon in the rat/AOM model of CRC was reported previously (Hagos et al., 2007). In two human colon cancer cell lines studied herein, Caco-2 and HT-29, GT-094 was not cytotoxic at the concentrations studied ( $\leq 100 \mu\text{M}$ ), but it was antiproliferative, with a measured  $\text{IC}_{50}$  value of  $41 \mu\text{M}$  (Fig. 2A) and  $59 \mu\text{M}$ , respectively. Likewise, the disulfanyl tetranitrate GT-015 and the disulfanyl bis-thiosalicylate BECS significantly inhibited growth of Caco-2 and HT-29 cells (Fig. 2, B and C). GT-094 showed similar antiproliferative potency in RAW 264.7 cell culture in the absence of added LPS ( $\text{IC}_{50} = 51 \mu\text{M}$ ; Fig. 2C). BECS inhibited cell growth more potently than GT-094, and both GT-749 and GT-947, analogs of GT-094 missing only the disulfide pharmacophore, decreased cell growth by 50% at  $100 \mu\text{M}$  (Fig. 2D), suggesting that the sulfanyl nitrate ele-

ment possesses antiproliferative properties, but that they are weaker than those of the disulfide element.

**Anti-Inflammatory Activity: iNOS Induction and NO Release.** The murine macrophage-like RAW 264.7 cell line is routinely used to examine the ability of agents to block cellular inflammatory response, and it represents a model for anti-inflammatory action toward activated macrophages. Treatment with the bacterial endotoxin LPS reliably induces various cytokines and also triggers expression of iNOS, the activity of which is readily assessed by measurement of inorganic nitrite ( $\text{NO}_2^-$ ). Although not a direct measure of NO production,  $\text{NO}_2^-$  is a major product of NO oxidative metabolism that can readily be quantified using the Griess assay. However, the majority of nitrates undergo denitration to  $\text{NO}_2^-$  as the major product (Thatcher et al., 2004). Therefore, to quantify endogenous  $\text{NO}_2^-$  production due to iNOS activity,  $\text{NO}_2^-$  production from nitrate test compounds must be accounted for.

In noninduced, untreated cells, no significant basal  $\text{NO}_2^-$  was detected, whereas ISDN ( $100 \mu\text{M}$ )-treated cells accumulated  $2.7 \pm 0.5 \mu\text{M}$   $\text{NO}_2^-$ , and cells treated with GT-094 (10, 50,  $100 \mu\text{M}$ ) generated  $3.4 \pm 0.2$ ,  $16.2 \pm 0.3$ , and  $38.7 \pm 0.7 \mu\text{M}$   $\text{NO}_2^-$ , respectively (Fig. 3B). ASA had no influence on  $\text{NO}_2^-$  release from ISDN, and no increase in  $\text{NO}_2^-$  production from ISDN or GT-094 was seen from 12 to 24 h. A more complete time course of  $\text{NO}_2^-$  release was measured for GT-015 compared with the true NO donor SPE/NO (Fig. 3A). In noninduced cells, SPE/NO ( $100 \mu\text{M}$ ) gave  $\text{NO}_2^-$  ( $97 \mu\text{M}$ ) within 5 h. This represents 50% of the maximum theoretical yield of  $\text{NO}_2^-$  from SPE/NO (Maragos et al., 1991), given the theoretical 1:1 conversion of NO to  $\text{NO}_2^-$  from autoxidation (Ford et al., 1993). This yield of  $\text{NO}_2^-$  compares with 30 to 40% of the maximum theoretical yield of  $\text{NO}_2^-$  produced from denitration of GT-094 in incubations with noninduced cells (Fig. 3B). Because these are not sealed systems, loss of NO by effusion is expected. It was further observed that denitration of GT-094 occurred to a substantial extent in cell culture supernatant drawn from both induced and noninduced RAW 264.7 cells (Fig. 4).

The proposed method to quantify endogenous  $\text{NO}_2^-$  production by iNOS measures  $\text{NO}_2^-$  release in induced cells and subtracts the  $\text{NO}_2^-$  levels observed in noninduced cells. In theory, if denitration of GT-094 was modified in LPS-induced cells, this methodology would be problematic. Therefore, the denitration of GT-094 was further studied in RAW 264.7 cells, in which endogenous  $\text{NO}_2^-$  production was attenuated, using the NOS inhibitor L-NAME or media absent the NOS substrate L-arginine. The NOS inhibitor L-NAME ( $10 \mu\text{M}$ ) inhibited  $\text{NO}_2^-$  production by  $46.1 \pm 3.5\%$  at 24 h in LPS-induced RAW 264.7 cells. The denitration of GT-094 was measured in supernatants from RAW 264.7 cells incubated with or without LPS and L-NAME (Fig. 4). The results demonstrated that LPS induction did not have a significant effect upon the amount of  $\text{NO}_2^-$  produced by GT-094, that GT-094 was stable to denitration in culture media, and that denitration was efficient in the presence of factors expressed by both induced and noninduced cells. These observations suggested that the reactivity of GT-094 would be dependent upon cell density. This was confirmed by measurement of  $\text{NO}_2^-$  production from GT-094 ( $100 \mu\text{M}$ ) as a function of cell density (Fig. 5A). In media depleted of L-arginine, LPS induction of iNOS does not result in NO or  $\text{NO}_2^-$  production (Fig. 5B). This



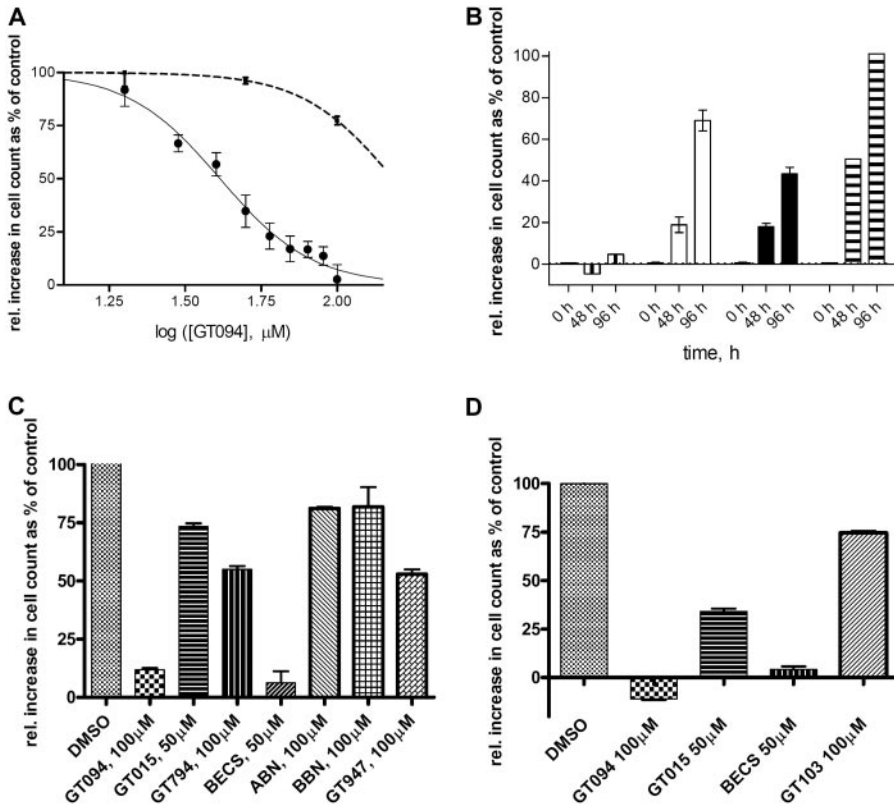
**Fig. 1.** Top, structures of compounds used in this study compared with NO-ASA (NCX 4016 and NCX 4215) and classical nitrates (ISDN and GTN). Bottom, Venn diagram showing how the study compounds dissect the component structural elements of the NO chimera GT-094 (nitrate, disulfide, and NSAID).

experiment shows categorically that factors induced by LPS in RAW 264.7 cells do not influence GT-094 denitration. The methodology proposed is therefore sound, with the caveat that both GT-094 reactivity and iNOS induction are dependent on cell density.

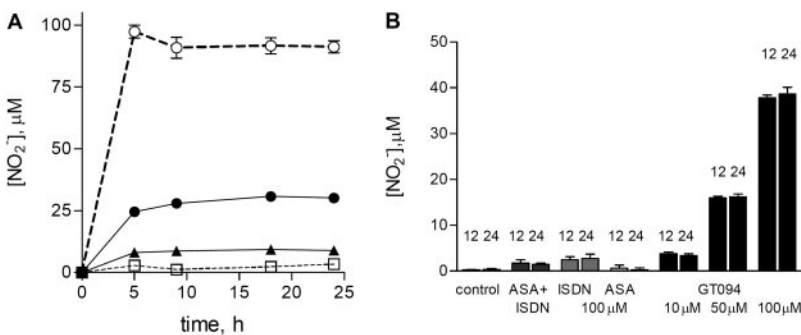
The time course for generation of NO<sub>2</sub><sup>-</sup> from induced RAW 264.7 cells (Fig. 6A) was thus corrected using our methodology to yield endogenous NO<sub>2</sub><sup>-</sup> produced by iNOS (Fig. 6B). The observed time course for endogenous production of NO<sub>2</sub><sup>-</sup> from RAW 264.7 cells in response to LPS stimulation is entirely compatible with literature reports describing the time course of iNOS induction (Connelly et al., 2001). By measuring NO<sub>2</sub><sup>-</sup> at 24 h in cell culture with and without LPS, and correcting for background release, the anti-inflammatory activity toward iNOS induction was compared for the variety of compounds selected to provide structural elements of GT-094 (Fig. 6C; and see Fig. 1). The classical nitrates ISDN and nitroglycerin (GTN) had negligible effects on LPS-induced elevation of NO<sub>2</sub><sup>-</sup>. The disulfides BECS, GT-015, and GT-094, in a concentration-dependent manner, substantially inhibited NO<sub>2</sub><sup>-</sup> production. However, the sulfanyl nitrate GT-794

was without effect; GT-794 differs from GT-094 in only one S atom; it is identical in every structural element, except for the disulfide. ASA had no effect on NO<sub>2</sub><sup>-</sup> production; and NO<sub>2</sub><sup>-</sup> itself, which is clearly a major breakdown product of GT-015 and GT-094, did not inhibit endogenous NO<sub>2</sub><sup>-</sup> production. The ISDN drug combinations (at the same concentrations delivered singly) had little additive activity of note on NO<sub>2</sub><sup>-</sup> production.

The observations in RAW 264.7 cells with the true NO donor SPE/NO demonstrated that NO at low concentrations stimulated iNOS expression and NO<sub>2</sub><sup>-</sup> production, but at high concentrations it modestly inhibited NO<sub>2</sub><sup>-</sup> formation. The first observation is compatible with a proposed role for NO from endothelial NOS early in macrophage activation (Connelly et al., 2003); the second observation is compatible with proposed negative feedback, or autoinhibition of iNOS induction by higher levels of iNOS-derived NO (Hinz et al., 2000). To confirm the generality of the observations on RAW 264.7 cells, the alternative MH-S macrophage cell line was studied with GT-015 and SPE/NO, measuring NO<sub>2</sub><sup>-</sup> production; confirming the observations on RAW 264.7 cells (Fig. 6C).

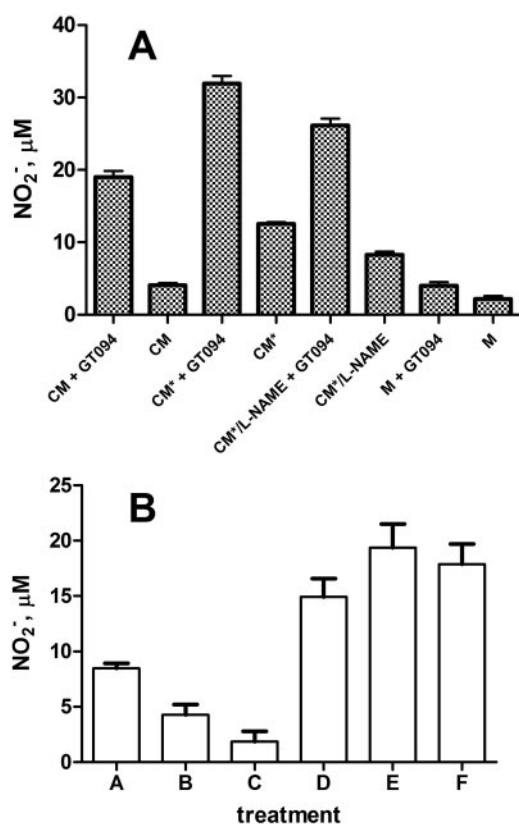


**Fig. 2.** Effects of GT-094 and structural components on cell proliferation. Cell count as a function of time on incubation with test compounds normalized to 0% at time 0 and 100% as dimethyl sulfoxide vehicle control, using sulforhodamine B cell staining quantified at 515 nm to estimate cell population. A, Caco-2 cells incubated with GT-094 (solid line) or DETA/NO (dashed line) for 48 h. IC<sub>50</sub> (GT-094) = 41 ± 2 μM. B, Caco-2 cells incubated with test compounds, control (horizontal line at 48 or 96 h), GT-015 (50 μM; open bars at 48 or 96 h), BECS (50 μM; closed bars at 48 or 96 h), GT-094 (100 μM; vertical line at 48 or 96 h). C, HT-29 cells treated with test compounds for 48 h. D, RAW 264.7 cells treated with test compounds for 24 h. Data show mean and S.E.M.



**Fig. 3.** Production of NO<sub>2</sub><sup>-</sup> from test compounds in RAW 264.7 cell incubations in the absence of LPS. A, time course for NO<sub>2</sub><sup>-</sup> production by an NO donor (SPE/NO 100 μM, open circles; SPE/NO 1 μM, open squares) and by nitrates (GT-015 30 μM, closed circles; GT-015 10 μM, closed triangles). B, comparison of NO<sub>2</sub><sup>-</sup> production in RAW 264.7 cell incubations of nitrates: ISDN (with or without ASA) and GT-094 at 12 h (left bar of pair) and 24 h (right bar of pair). Data determined by Griess assay show mean and S.D. For measurement of [NO<sub>2</sub><sup>-</sup>] ≥ 4 μM, average error was <4% from at least triplicate measures.

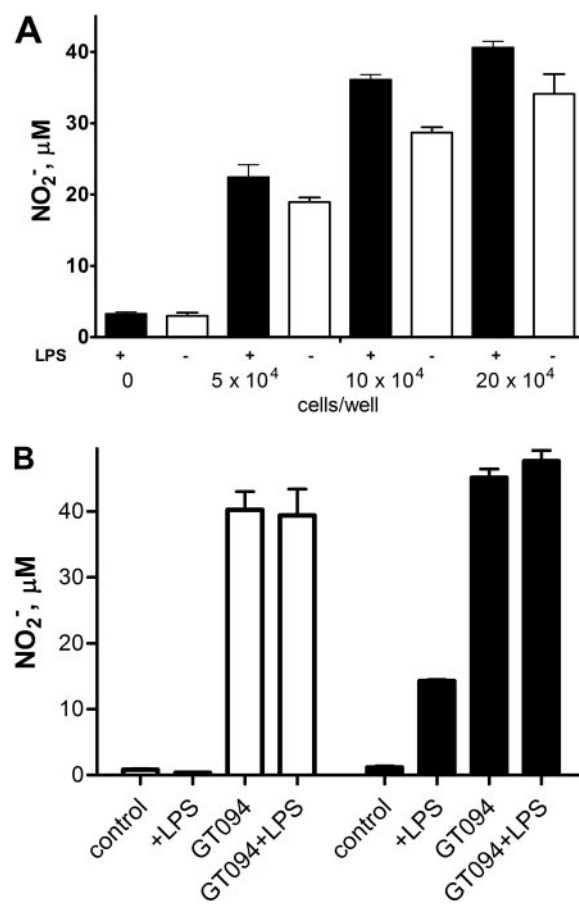
To further explore the GT-094 reactivity, the capacity of denitration to yield NO was studied using protein phosphorylation in MCF-7 cells as a sensitive cellular NO sensor. Thomas et al. (2004) exhaustively explored this cell model system using diazeniumdiolate salts to generate various fluxes of NO: phosphorylation of p53 and of ERK was shown to be a sensor for intracellular NO, responding to true NO donors in a time- and concentration-dependent manner: p53 phosphorylation required higher concentrations of NO, whereas ERK was phosphorylated at lower concentrations. Incubation of cells with GT-094 (100  $\mu$ M) did not increase levels of phospho-p53 with time, in contrast to the true NO donor SPE/NO (100  $\mu$ M), whereas both GT-094 (100  $\mu$ M) and SPE/NO (10  $\mu$ M) gave ERK phosphorylation (Fig. 7). The interpretation of these data is that GT-094 may function as a cellular NO donor, but the fluxes of NO produced are not high.



**Fig. 4.** Production of NO<sub>2</sub><sup>-</sup> from incubation of GT-094 in RAW 264.7 cell culture supernatants to test the effect of LPS induction on denitration of GT-094. A, primary data on nitrite release from GT-094 incubated for 12 h in supernatants from cell cultures ± LPS and ± L-NAME (10  $\mu$ M) as NOS inhibitor (M, media without cells; CM, supernatant; CM\*, supernatant from LPS-induced cells). B, secondary data analysis. Column A, endogenous NO<sub>2</sub><sup>-</sup> production by NOS after LPS induction. Column B, endogenous NO<sub>2</sub><sup>-</sup> production after LPS induction in the presence of L-NAME. Column C, NO<sub>2</sub><sup>-</sup> production from GT-094 in media alone. Column D, NO<sub>2</sub><sup>-</sup> production from GT-094 with noninduced cell supernatant. Column E, NO<sub>2</sub><sup>-</sup> production from GT-094 after LPS induction. Column F, NO<sub>2</sub><sup>-</sup> production from GT-094 after LPS induction from induced cells treated with L-NAME. Cells were plated for 24 h before addition of LPS or LPS + L-NAME. Supernatant was removed after a further 12-h incubation to provide reaction solutions. GT-094 (100  $\mu$ M) was incubated for 12 h in these solutions before Griess assay. Data show mean and S.D. Column key: A, CM\*-CM; B, CM\*/L-NAME-CM\*; C, M + GT094 - M; D, CM + GT094 - CM; E, CM\* + GT094 - CM\*; and F, (CM\*/L-NAME + GT094) - (CM\*/L-NAME).

**Anti-Inflammatory Activity and PG Release.** Western blots of lysates from RAW 264.7 cells confirmed that GT-094 blocked iNOS protein expression rather than activity. In the present study, the data on GT-094 was extended to measure cytokines and inflammatory proteins at the mRNA level using RT-PCR (Fig. 8). LPS stimulation of RAW 264.7 cells was shown to produce a typical macrophage-like activation, leading to induction of cytokines (TNF- $\alpha$ , IL-6, and IL-1 $\beta$ ) and enzymes mediating the inflammatory response (COX-2 and iNOS), as shown by elevation of mRNA levels relative to noninduced control cells. GT-094 was observed to inhibit induction of all cytokines and iNOS in a concentration-dependent manner, but to have no effect on expression of COX-2. The lack of an inhibitory effect on COX-2 expression is compatible with reports on NO-ASA (Williams et al., 2003). The observation that COX-2 mRNA levels are elevated by LPS induction in the presence and absence of GT-094 demonstrates that the anti-inflammatory actions of this drug are not simply the result of inhibition of cell growth and function.

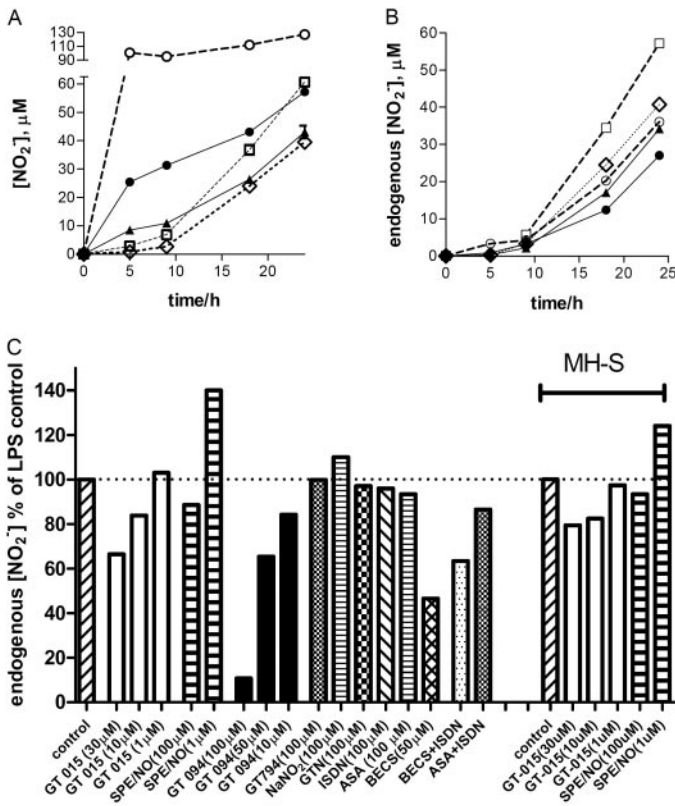
NSAID-containing hybrid drugs are anticipated to inhibit COX-2, inhibiting production of PGs. In RAW 264.7 cells,



**Fig. 5.** Effect of cell density (A) and arginine depletion (B) on production of NO<sub>2</sub><sup>-</sup> from GT-094 in RAW 264.7 cell culture incubation. A, cells were plated at densities of zero, 5, 10, and 20 × 10<sup>4</sup> cells/well for 24 h, supernatant was replaced with FBS-free media containing GT-094 (100  $\mu$ M), and LPS (1  $\mu$ g/ml) was added after 30 min; NO<sub>2</sub><sup>-</sup> was measured by Griess assay after a further 20-h incubation. B, cells were plated at 20 × 10<sup>4</sup> cells/well for 24 h, supernatant was replaced with FBS-free media, containing GT-094 (100  $\mu$ M), and LPS (1  $\mu$ g/ml) was added after 30 min; NO<sub>2</sub><sup>-</sup> was measured by Griess assay after a further 20-h incubation. Open bars, media with L-arginine; closed bars, media without L-arginine. Data show mean and S.E.M.

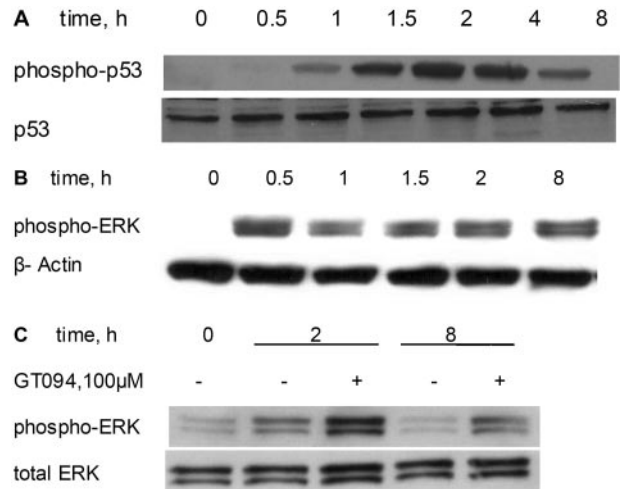
GT-094 was observed to inhibit production of PG<sub>D2</sub> and PG<sub>E2</sub> induced by LPS (Fig. 9). The measured IC<sub>50</sub> value of 4 μM demonstrates that the thiosalicylate pharmacophore of GT-094 delivers the anticipated COX-2 inhibitory activity, which is expected to contribute to the chemopreventive profile of GT-094 in CRC (Wang and Dubois, 2006). For comparison, the concentration dependence of the anti-inflammatory activity of GT-094 toward iNOS induction is also shown (Fig. 9B).

**Induction of NQO1.** To investigate structure-activity relationships for induction of detoxifying phase 2 enzymes by GT-094 and its structural components, compounds were administered to Hepa 1c1c7 cells in different concentrations, and NQO1 activity was measured. The Hepa 1c1c7 cell system is commonly used to screen chemopreventive agents and potential anticarcinogens, because basal NQO1 enzyme activity is relatively low and is readily induced to measurable levels that allow comparison of efficacy and potency (Kang and Pezzuto, 2004). A standard procedure measures induction of NQO1 after incubation of agents for 48 h using 4-bromoflavone (BF) as a positive control. (Song et al., 1999) However, comparison of the time courses for BF and GT-094 showed that GT-094 activity was maximal at 24 h (data not shown); thus, a 24 h incubation was used for assay of NQO1 activity.

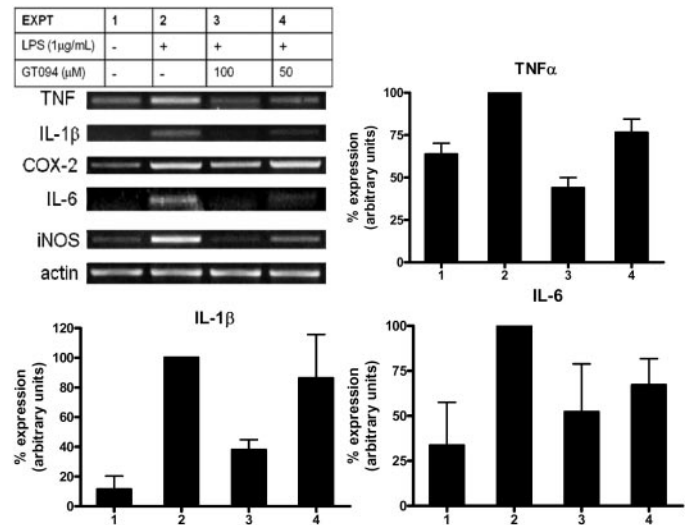


**Fig. 6.** Production of NO<sub>2</sub><sup>-</sup> in cells subject to iNOS induction by LPS (1 μg/ml): RAW 264.7 cells (A and B) and RAW 264.7 cells or MH-S cells (C). A, time course for NO<sub>2</sub><sup>-</sup> production in the absence (Δ) or presence of an NO donor (SPE/NO 100 μM, ○; SPE/NO 1 μM, □) or nitrate (GT-015 30 μM, ●; GT-015 10 μM, ▲). B, time course of endogenous NO<sub>2</sub><sup>-</sup> production calculated by subtraction of measurements in the absence from measurements in the presence of LPS (symbols as in A). C, endogenous production of NO<sub>2</sub><sup>-</sup> from incubations of test compounds with RAW 264.7 cells or MH-S cells at 24 h. Data determined by Griess assay show mean and S.D. For measurement of [NO<sub>2</sub><sup>-</sup>] ≥ 4 μM, average error was <4% from at least triplicate measures.

Concentration-dependent induction of NQO1 activity was measured for GT-094, GT-015, BECS, and for GT-103 in Hepa 1c1c7 cells (Fig. 10A). The three disulfides were equipotent, whereas GT-103, incorporating S-nitrate and NSAID moieties but no disulfide group, induced NQO1 activity, but it was less efficacious. Induction of NQO1 by ISDN was not significant, and only 1.5-fold induction of NQO1 was seen in the presence of the slow NO donor DETA/NO (Fig. 10B). The nitron radical scavenger PTIO, often referred to as a “specific trap for NO,” has been used to provide evidence for the role of NO in biochemical mechanisms. For this reason, the influence of PTIO on the induction of NQO1 by GT-094 and DETA/NO was examined (Fig. 10B). PTIO (50 μM) was observed to lower NQO1 activity induced by GT-094 and DETA/



**Fig. 7.** Immunoblots of proteins from MCF-7 cells used to sense NO release. A, cells treated with the NO donor SPE/NO (100 μM) and probed with antibodies for phospho-Ser15-p53 or p53 showing time-dependent phosphorylation; GT-094 (100 μM) gave no p53 phosphorylation in identical experiments with GT-094. B, cells treated with SPE/NO (1 μM) and probed with antibodies for phospho-ERK and β-actin showing ERK phosphorylation. C, cells treated with GT-094 (100 μM) and probed with antibodies for phospho-ERK and total ERK showing ERK phosphorylation induced by GT-094. Representative blots from triplicate experiments are shown.

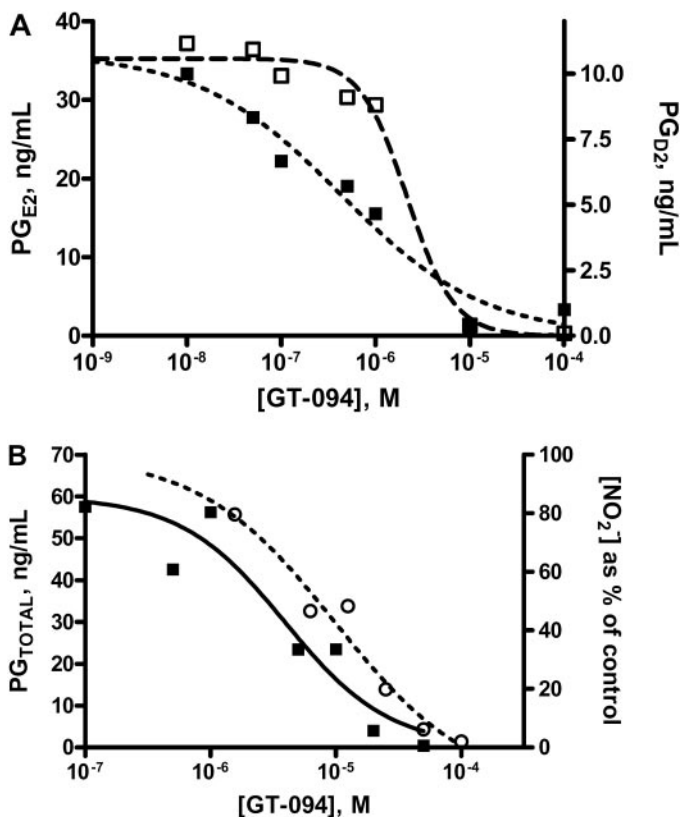


**Fig. 8.** Effects of GT-094 on LPS induced expression of inflammatory markers (TNF-α, IL-1β, IL-6, COX-2, and iNOS) in RAW264.7 cells measured by RT-PCR after 4-h incubation. Data quantitation and representative gel images are shown from two to three separate determinations.

NO, but it did not reach significance (one-way analysis of variance with Tukey's post test). DETA/NO at 1.5 mM has been reported to induce NQO1, but this high concentration is not relevant to understanding the activity of GT-094 at  $\leq 0.1$   $\mu$ M.

Measurement of  $IC_{50}$  values for inhibition of Hepa 1c1c7 cell growth by GT-094, GT-015, and BECS (105, 99, and 104  $\mu$ M, respectively) allowed estimation of CI. The CD and CI values for GT-094, GT-015, and BECS were very similar: CD = 58, 67, and 57  $\mu$ M and CI = 1.8, 1.5, and 1.8, respectively.

**Activation of ARE-Luciferase.** The mechanism of NQO1 induction by GT-094 and related compounds was studied in HepG2-ARE-C8 cells by measuring the induction of a luciferase reporter by activation of the ARE (Kang and Pezzuto, 2004). HepG2-ARE-C8 cells are human hepatocarcinoma cell lines that have been stably transfected with the pARE-T1-luciferase construct. Luciferase induction in this cell line reflects the ability to activate the endogenous ARE that regulates expression of many phase 2 enzymes. A good correlation between ARE-luciferase induction and NQO1 induction is usually anticipated; concentration-dependent induction of ARE-luciferase by GT-094 was indeed observed. Moreover, the time course of ARE-luciferase induction by GT-094 was compared with the benchmark chemopreventive agent BF (Fig. 10D). Both agents were observed strongly to activate ARE, giving 10- to 15-fold luciferase induction relative to control, with differences in time dependence at the 24-h time point.



**Fig. 9.** Inhibition of LPS-induced inflammatory response in RAW 264.7 cells by GT-094. Determination of PG release by liquid chromatography/mass spectrometry. A, concentration response for PG<sub>D2</sub> (right axis; ■) and PG<sub>E2</sub> (left axis; □). B, concentration response for inhibition of total PG production (PG<sub>D2</sub> + PG<sub>E2</sub>; ■, solid line;  $IC_{50}$  = 3.9  $\mu$ M) compared with inhibition of endogenous nitrite production (○, dashed line;  $IC_{50}$  = 10  $\mu$ M; measured by Griess assay).

In further experiments, the influence of PTIO was again studied; definitively, PTIO did not decrease ARE-luciferase induction by GT-094 (Fig. 10C). The biological activity of PTIO is in reality not specific for NO scavenging (Goldstein et al., 2003; Nicolescu et al., 2006); therefore, the influence of the soluble guanylyl cyclase inhibitor ODQ was examined; surprisingly, ODQ significantly increased ARE-luciferase induction by GT-094, although ODQ alone had no effect (one-way analysis of variance with Tukey's post test). The mitogen-activated protein kinase kinase [mitogen-activated protein kinase (MAPK)/ERK kinase] inhibitor PD 98059 was also studied, in part because of the known capability of NO to activate the MAPK/ERK pathway (Thomas et al., 2004). The mitogen-activated protein kinase kinase inhibitor was observed to give significant induction of ARE-luciferase in its own right and this effect was additive with that of GT-094 in induction of ARE-luciferase (Fig. 10C). It is noteworthy that a similar effect of PD 98059 was reported on ARE-luciferase activity in HepG2 cells by Kong and coworkers; and furthermore, was reported to be additive with the inductive effect of the garlic-derived diallyl trisulfide (Chen et al., 2004).

## Discussion

A recent review concluded that NSAIDs and COX inhibitors reduce the incidence of colonic adenomas but that the adverse events, including cardiovascular events, and gastrotoxicity, give a risk/benefit ratio that does not support their use in CRC chemoprevention in average-risk individuals (Rostom et al., 2007). The cytoprotective effects of NO in the GI tract involve actions similar to those of PGs, for example, stimulation of mucus secretion and mucosal blood flow. Nitrates are NO mimetic drugs (Thatcher et al., 2004); thus, GTN is able to substitute for PG action in the GI tract to counterbalance pharmacological COX inhibition (MacNaughton et al., 1989). The examination of hybrid nitrates that are NSAID prodrugs (NO-NSAIDs) was therefore a rational new approach to CRC chemoprevention (Bak et al., 1998; Bolla et al., 2005). This approach led to the NO-ASA derivative NCX 4016, originally designed for use in pain, entering clinical trials for CRC chemoprevention (Rao et al., 2006). The cessation of these trials, reportedly because of *in vitro* genotoxicity, emphasizes the need for thorough study of molecular and structural pharmacology as a basis for optimizing nitrate drugs.

GT-094, coined an NO chimera drug because it incorporates two ancillary pharmacophores in addition to the NO mimetic nitrate group, bears comparison with NO-ASA and is a good lead compound for discovery of nitrates chemopreventive in CRC, containing ancillary pharmacophores known to have chemopreventive properties in humans: an NSAID (a thiosalicylate ester; Brannigan et al., 1976; Halaschek-Wiener et al., 2000; Kapadia et al., 2000) and a disulfide. Proof-of-principle for GT-094 was obtained in the standard animal model for CRC, the formation of ACF in the colon of rats treated with AOM carcinogen: delivered after carcinogen administration in this AOM/rat model, GT-094 reduced ACF number by 45%, reduced colon crypt proliferation by 30 to 69%, reduced iNOS levels by 33 to 67%, and elevated levels of the anti-oncogene p27 in the distal colon (Hagos et al., 2007).

In the current work, antiproliferative activity, anti-inflammatory activity (previously reported for GT-094 *in vivo*), and

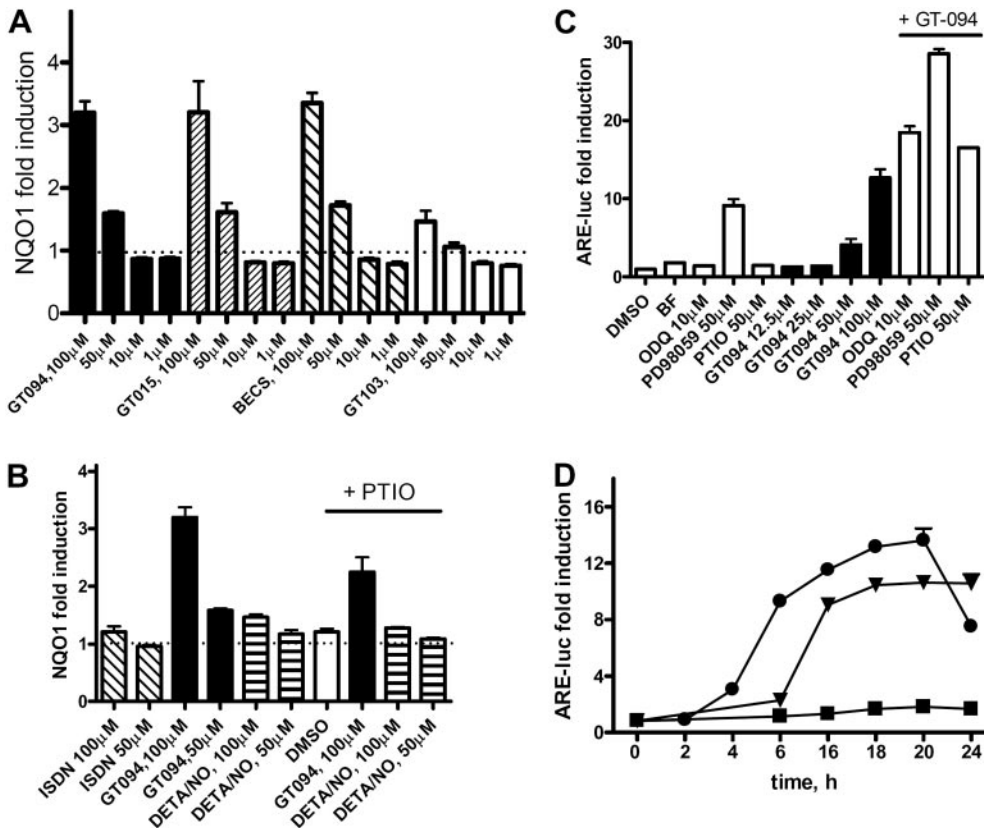


induction of cytoprotective phase 2 gene products are studied in vitro for compounds that deconstruct the three-component structural elements of GT-094, nitrate, NSAID, and disulfide, with the intent of understanding the contribution of each structural element to activity. The classical nitrates GTN and ISDN were selected for study, in addition to ABN and BBN, which are organic nitrate elements of NO-ASA, but without the NSAID moiety. The combination of ISDN + ASA was used, because this combination provides both nitrate and NSAID components. BECS contains both the disulfide and the thiosalicylate NSAID elements of GT-094, whereas GT-015 contains the disulfide and nitrate elements. Likewise, GT-947, GT-794, and GT-103 contain elements of GT-094 (Fig. 1). Finally, diazeniumdiolate salts were used as true NO-donor control compounds (DETA/NO is a slow NO donor,  $t_{1/2} \sim 20$  h; SPE/NO is a moderate donor,  $t_{1/2} \sim 40$  min).

GT-094 was antiproliferative in the Caco-2 human colon cancer cell line, inducing a transient G<sub>2</sub>/M phase cell cycle block (Hagos et al., 2007). Cell count measurements were used to estimate antiproliferative activity in Caco-2, HT-29, and RAW 264.7 cells and to provide estimates of CI in Hepa 1c1c7 cells. Of the component compounds tested, only the disulfides, BECS, GT-094, and GT-015 at concentrations below 100  $\mu$ M significantly inhibited cell growth (Fig. 2). Disulfides such as dipropyl disulfide and diallyl disulfide (DADS) have been reported to reduce ACF formation in the rat/AOM model and contribute to the chemopreventive properties of allium family botanicals (Sumiyoshi and Wargovich, 1990; Wargovich et al., 1996). It is suggested that the disulfide group of DADS contributes to the antiproliferative activity (Knowles and Milner, 2003; Druesne et al., 2004); although the allyl group seems strongly to influence NQO1

induction (Munday and Munday, 2004). DADS was reported to induce a G<sub>2</sub>/M phase cell cycle block in colon cancer cells, including Caco-2 cells (Wargovich et al., 1996; Knowles and Milner, 2003; Druesne et al., 2004), possibly by inhibition of histone deacetylase activity (Suzuki et al., 2006). NCX 4016 was reported to be weakly antiproliferative in colon cancer cell lines ( $165 \mu\text{M} < \text{IC}_{50} < 250 \mu\text{M}$ ), inducing a G<sub>2</sub>/M phase block at 250  $\mu$ M, whereas ASA was devoid of activity (Tesei et al., 2003).

LPS induces an inflammatory response in RAW 264.7 cells that includes expression of cytokines and of enzymes, including COX-2 and iNOS. GT-094 was shown to retain NSAID activity, inhibiting LPS-induced prostaglandin synthesis, but not inhibiting COX-2 transcription (Figs. 8 and 9). The LPS-induced elevated COX-2 transcription was unaffected by GT-094, but GT-094 inhibited elevated iNOS and cytokine transcription. Inhibition of iNOS expression was compatible with the observed concentration-dependent reduction in measured iNOS activity (Figs. 6 and 8). The relationship of anti-inflammatory activity to structure was studied by measuring NO<sub>2</sub><sup>-</sup> as a reflection of endogenous NOS activity. GT-094, BECS, and GT-015 inhibited iNOS activity, whereas component compounds and their combination had little or no effect, demonstrating that a disulfide structural element is required for anti-inflammatory activity (Fig. 6). The NO donor SPE/NO demonstrated the anticipated effects in RAW 264.7 cells, including a very modest inhibition of iNOS activity at higher concentration, compatible with proposed roles for NO in signaling iNOS up- and down-regulation (Connelly et al., 2001, 2003). The observed concentration dependence of the activity of the true NO donor, SPE/NO, was dissimilar to that



**Fig. 10.** Induction of NQO1 or ARE-luciferase activity in liver cells as -fold induction relative to vehicle control. A, induction of NQO1 by test compounds in Hepa 1c1c7 cells after 48-h incubation. B, induction of NQO1 in Hepa 1c1c7 cells by GT-094, ISDN, and the NO donor DETA/NO, with and without PTIO, after 48 h. C, activation of ARE and induction of luciferase reporter activity in HepG2 cells by test compounds with or without pathway inhibitors measured at 20 h. D, time course for induction of ARE-luciferase in HepG2 cells by GT-094 (100  $\mu$ M; ●) and BF (1  $\mu$ M; ▼) versus control (■), relative to vehicle control at time 0. Data show mean and S.E.M.

of GT-015 and GT-094, casting doubt on NO being the mediator of the effects of the nitrates observed in these cells.

A further recognized contributor to chemoprevention is induction of cytoprotective phase 2 enzymes, such as NQO1, in particular via activation of the ARE, which has been reported for a number of chemopreventive agents (Prester and Talalay, 1995). The activation of the ARE and the induction of NQO1 and luciferase activity in human liver cancer cells is anticipated for compounds, including disulfides (Song et al., 1999), that are electrophiles or oxidants toward thiol sensor proteins such as the cytosolic, thiol-rich regulatory protein Kelch-like ECH-associated protein 1 (Keap1) (Zhang and Hannink, 2003). Nitrates are also able to act as oxidizing agents, especially toward thiols; indeed, oxidation of protein thiols is likely to accompany reductive bioactivation of nitrates. There are also reports that NO itself is also able to oxidize and nitrosate Keap1, leading to ARE activation (Gao et al., 2006; Buckley and Whorton, 2008). However, neither ISDN nor the NO donor DETA/NO was observed to induce NQO1 at a level comparable with that of the disulfides studied herein, despite the high concentrations of NO donors used.

In theory, all disulfides have the potential to be electrophilic toward thiols via thiol-disulfide exchange reactions, leading to GSH depletion, oxidation of the thiol groups of redox sensor proteins, and ARE activation, but significant differences in reactivity are expected. For example, DADS was reported to induce ARE-luciferase in HepG2 cells 2-fold, whereas dipropyl disulfide had no effect (both 250  $\mu\text{M}$ ) (Chen et al., 2004). Chemopreventive disulfides, such as DADS, induce detoxifying phase 2 enzymes via multiple pathways converging on ARE activation, and involving, in part, the transcription factor nuclear factor-erythroid 2-related factor 2 and Keap1. The disulfides that were seen to have antiproliferative and anti-inflammatory activity in the present study, GT-094, GT-015, and BECS, were assayed for NQO1 induction in Hepa 1c1c7 cells and ARE-luciferase induction in HepG2 cells, demonstrating concentration-dependent activity at levels equal or greater than those reported for DADS (Fig. 10). The simplest rationale is that the disulfide structural element is dominant, reflecting properties similar to DADS. Addition of PTIO, commonly used as a selective NO scavenger, reduced NQO1 induction by GT-094 and DETA/NO, but not significantly. The effects of adjuvant PTIO were further examined on ARE-luciferase induction, confirming that this "NO trap" did not reduce ARE activation by GT-094. Addition of NO/cGMP and MAPK/ERK pathway inhibitors provided no evidence for involvement of NO in ARE activation, whereas the activity profile was fully compatible with that reported for DADS and a related trisulfide (Chen et al., 2004).

There is an extensive literature on the in vitro activity of NO-NSAID hybrid nitrates: studies have reported inhibition of Wnt signaling, disruption of nuclear factor- $\kappa\text{B}$  function, and reduction of cyclin D<sub>1</sub> levels by NO-ASA (Kashfi et al., 2002; Kaza et al., 2002; Williams et al., 2003). True NO donors, such as DETA/NO, have been observed to be antiproliferative in Caco-2 cells via inhibition of ornithine decarboxylase (Buga et al., 1998); however, in HT-29 cells, high concentrations of NO donor, or NO donor plus ASA, were needed to inhibit cell growth: DETA/NO, IC<sub>50</sub> ~ 750  $\mu\text{M}$  and DETA/NO + ASA, IC<sub>50</sub> at 700 and 375  $\mu\text{M}$  (Kashfi et al.,

2005). In vitro studies on NCX 4016 have ascribed activity to sustained release of low levels of NO (Gao et al., 2005; Bratasz et al., 2006), although other studies have demonstrated NO-independent biological activity for NO-ASA (Dunlap et al., 2007, 2008; Hulsman et al., 2007). The release of intracellular NO can be addressed through application of the NO sensor paradigm pioneered by Thomas et al. (2004), who studied MCF-7 cells incubated with NO donors. In the present study, protein phosphorylation was caused by GT-094 (100  $\mu\text{M}$ ) in a pattern similar to that produced by SPE/NO (10  $\mu\text{M}$ ), but not SPE/NO (100  $\mu\text{M}$ ), ruling out production of higher fluxes of NO from GT-094. However, it must be noted that the disulfide DADS (100  $\mu\text{M}$ ) also has been reported to elevate intracellular levels of phosphorylated ERK (Knowles and Milner, 2003).

Hybrid and chimeric drugs, by design, incorporate structural elements that provide additive or synergistic pharmacophores. GT-094 is a chimeric drug containing NSAID, NO mimetic, and disulfide pharmacophores, which like NO-NSAID drugs, has demonstrated chemopreventive properties in animal models of CRC. In theory, each pharmacophore element of GT-094 could provide anti-inflammation, antiproliferation, and induction of phase 2 enzymes; however, in the variety cell lines studied, the evidence is that the disulfide structural element is the dominant contributor to all observed activity. There is no unequivocal evidence in these vitro systems that the NO mimetic activity of GT-094 contributes. The combination of classical nitrates with ASA demonstrated no activity in vitro. Thus, the inclusion of the disulfide structural element in this NO chimera is expected to contribute strongly to the chemopreventive profile. GT-094 manifests the required NSAID activity toward prostaglandin synthesis, and it is anticipated that in vivo the intended and invaluable gastroprotective effects of the NO mimetic nitrate will come into play.

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