Colon cancer chemoprevention by a novel NO chimera that shows anti-inflammatory and antiproliferative activity *in vitro* and *in vivo*

Ghenet K. Hagos,[1] Robert E. Carroll,[2] Tatiana Kouznetsova,[2] Qian Li,[1] Violeta Toader,[1] Patricia A. Fernandez,[1] Steven M. Swanson,[1] and Gregory R.J. Thatcher[1]

[1]Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago and [2]Department of Medicine, University of Illinois at Chicago and Chicago Veterans Administration Medical Center (West Side Division), Chicago, Illinois

Abstract

Chemopreventive agents in colorectal cancer possess either antiproliferative or anti-inflammatory actions. Nonsteroidal anti-inflammatory drugs (NSAID) and cyclooxygenase-2 inhibitors have shown promise, but are compromised by side effects. Nitric oxide donor NSAIDs are organic nitrates conjugated via a labile linker to an NSAID, originally designed for use in pain relief, that have shown efficacy in colorectal cancer chemoprevention. The NO chimera, GT-094, is a novel nitrate containing an NSAID and disulfide pharmacophores, a lead compound for the design of agents specifically for colorectal cancer. GT-094 is the first nitrate reported to reduce aberrant crypt foci (by 45%) when administered after carcinogen in the standard azoxymethane rat model of colorectal cancer. Analysis of proximal and distal colon tissue from 8- and 28-week rat/azoxymethane studies showed that GT-094 treatment reduced colon crypt proliferation by 30% to 69%, reduced inducible NO synthase (iNOS) levels by 33% to 67%, reduced poly(ADP-ribose)polymerase-1 expression and cleavage 2- to 4-fold, and elevated levels of p27 in the distal colon 3-fold. Studies in cancer cell cultures recapitulated actions of GT-094: antiproliferative activity and transient G_2-M phase cell cycle block were measured in Caco-2 cells; apoptotic activity was examined but not observed; anti-inflammatory activity was seen in the inhibition of up-regulation of iNOS and endogenous NO production in lipopolysaccharide (LPS)-induced RAW 264.7 cells. In summary, antiproliferative, anti-inflammatory, and cytoprotective activity observed *in vivo* and *in vitro* support GT-094 as a lead compound for the design of NO chimeras for colorectal cancer chemoprevention. [Mol Cancer Ther 2007;6(8):2230–9]

Introduction

The initiation of colon cancer is thought to begin with a single mutational event within an isolated colon crypt. The aberrant crypt foci (ACF), or a specific dysplastic subset of these lesions, are seen as an early precursor stage to adenomas and cancer. The ACF itself is a monoclonal structure that arises from mutations within a single crypt stem cell. That these ACF structures directly develop into adenomas is still a matter of debate, but ACF are strongly linked to colon cancer risk, and in animal models, such as the murine azoxymethane carcinogen model, a good correlation between ACF number and tumorigenesis has been reported for a wide variety of chemopreventive agents (1). The ACF number is a reliable biomarker of colon cancer in preclinical models, and furthermore, in humans, ACF show increased expression of markers of proliferation [proliferating cell nuclear antigen (PCNA)], and inflammation [inducible NO synthase (iNOS); ref. 2].

Colorectal cancer is a leading cause of death, and chemoprevention of colorectal cancer represents an important therapeutic target and an unmet need. Chemopreventive strategies for colorectal cancer have targeted antiproliferative and anti-inflammatory actions on colonocytes containing populations subject to carcinogen-induced DNA damage. In many cases, these chemopreventive agents act to modulate the levels of abnormally expressed proteins and to inhibit proliferation. In most cases, the exact mechanism of action is uncertain, although again, there are clear biomarkers that correlate with drug intervention in the development of ACF and tumorigenesis, in particular biomarkers of inflammation and of proliferation.

Nonsteroidal anti-inflammatory drugs (NSAID), such as acetylsalicylic acid (ASA), have shown promise in colorectal cancer clinical trials (3), but carry the burden of severe gastrointestinal side effects and loss of efficacy at low doses. Selective cyclooxygenase-2 (COX-2) inhibitors, which possess attenuated gastrointestinal side effects, have recently been confirmed by the U.S. Food and Drug Administration to manifest serious cardiovascular side effects. A further class of anti-inflammatory agents, nitric oxide donor NSAIDs (NO-NSAIDs), was originally designed to use the biological activity of NO to mollify the gastrointestinal damage caused by NSAIDs. NCX 4016

Received 1/30/07; revised 5/12/07; accepted 6/15/07.

Grant support: University of Illinois at Chicago Cancer Center, NIH grants CA-102590 and CA-80360, and VA Merit Review to R.E. Carroll.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Gregory R. Thatcher, Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, 833 S Wood Street, Chicago, IL 60612. Phone: 312-355-5282. E-mail: thatcher@uic.edu

doi:10.1158/1535-7163.MCT-07-0069

is an NO-ASA that is currently in National Cancer Institute (NCI)–sponsored clinical trials for colorectal cancer prevention, having shown promise in preclinical studies (Fig. 1; ref. 4). NO-NSAIDs are aliphatic nitrates conjugated via a labile ester linkage to an NSAID (5) and represent a new direction for nitrate therapeutic agents, which have been in clinical use for more than 130 years in cardiovascular therapy.

Rigas et al. (6) have positively assessed the potential for NO-ASA in cancer therapy and chemoprevention, but pointed out key gaps in our knowledge: the mechanism of action is not clearly defined; it is not known how classic nitrates compare with NO-NSAIDs; and drug optimization of improved NO-NSAIDs has been superficially explored. The novel nitrate, GT-094, is a prototype NO chimera, containing pharmacophores known to be effective in chemoprevention, including an NSAID (Fig. 1). To validate GT-094 as a lead compound for optimization of a nitrate specifically for colorectal cancer chemoprevention, GT-094 was studied in the traditional rat/azoxymethane colorectal cancer model. An extended rat/azoxymethane study provided tissues to examine drug effects on protein regulation and proliferation. Studies in cell culture showed that anti-inflammatory, antiproliferative, and cytoprotective effects seen *in vivo* were recapitulated *in vitro*. The NO chimera, GT-094, is reported for the first time as a lead for drug discovery and a probe compound for exploration of mechanisms of colon cancer chemoprevention.

Materials and Methods

Materials

Azoxymethane was obtained from the NCI Chemical Carcinogen Reference Standard Repository, MidWest Research Institute. Buffered formalin, xylene, and alcohols (histology grade) were from Fisher Scientific. Vincristine and all other biochemical reagents were from Sigma. The iNOS and poly(ADP-ribose)polymerase (PARP) antibodies were from Santa Cruz Biotechnology. Male Fisher 344 rats were obtained from Harlan Sprague-Dawley Labs and studied from 8 weeks of age (200–250 g).

Animal Care and Maintenance

Rats were allowed free access to water and Harlan Teklad 8640 mash diet. The rats were housed in micro-isolator cages with GreenTru laboratory bedding (Green Products) and exposed to a controlled light cycle of 14:10 h (light/dark) at 25°C. This study was approved by the University of Illinois at Chicago IACUC.

Carcinogen Administration, Drug Dosing, and Observation Protocol

Rats received 15 mg/kg of azoxymethane s.c. twice, 7 days apart (total dose of 30 mg/kg). Drug administration was initiated 2 days after the last dose of azoxymethane. In the 8-week treatment protocol, the drug was given in feed for 8 weeks after initiation of drug treatment to animals in four arms ($n = 8$). Treatment diets were supplemented with arm 1: ASA (117 ppm); arm 2: GT-094 (233 ppm); or arm 3: ASA (117 ppm) + nitrates [ISDN 93 ppm; mannitol dinitrate (MDN) 80 ppm]. The control group (arm 4) received standard diet. The amount of ASA in feed was calculated to deliver ~10 mg/kg/day, the same dosage used in the study of Bak et al. (4). The amounts of GT-094 and of ASA/nitrates in feed were calculated to be roughly equimolar with the ASA dosage. In arm 2 only, GT-094 was supplemented with twice-weekly administration of GT-094 by gavage [3.5-mg dose, i.e., 1 mL of a 5% DMSO solution (70 mg/mL) made up in 1% carboxymethylcellulose]. In the 28-week treatment protocol, a separate longer term study ($n = 11$) was conducted in which drugs were given 2 days after the last dose of azoxymethane for 8 weeks, using identical dosing to arms 2 and 4 in the 8-week treatment protocol. Immediately after the 8-week drug administration (i.e., in week 11 of the study), the administration of GT-094 by gavage was terminated, and the GT-094 treatment arm received GT-094 in feed alone for a further 20 weeks. Immediately after this 20-week period, animals were sacrificed. For reasons not associated with drug treatment, one animal was lost in the control group. In both protocols, rats were weighed weekly to examine drug perturbations of feeding and body weight (which were not observed) and were euthanized by CO_2 asphyxiation either 8 or 28 weeks after drug administration was initiated. Two hours before sacrifice, rats received vincristine 1 mg/kg by i.p. injection, thereby allowing the proliferative effects of azoxymethane on the colon to be assessed. In colon tissue from the 28-week study, proliferation was measured by vincristine metaphase arrest; inflammation was quantitated by measuring iNOS protein in Western blots and by immunohistochemistry; DNA damage was estimated by

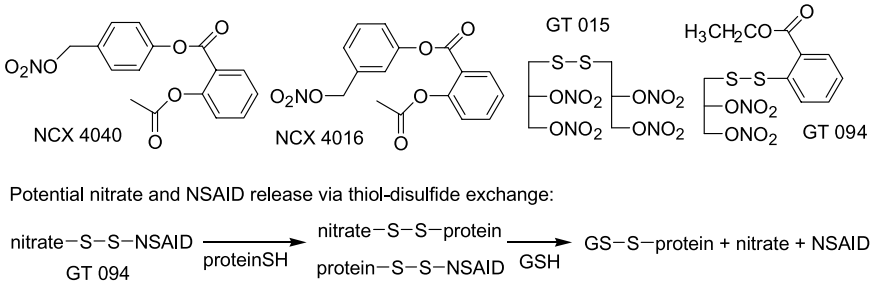

Figure 1. Structures of aliphatic nitrates including NO-ASA (NCX 4016, NCX 4040) and NO chimera, GT-094. GT-094 is expected to undergo rapid thiol/disulfide exchange with protein sulfhydryl groups leading to NSAID (thiosalicylate) release.

immunoassay of PARP protein; and p27 levels were estimated by Western blots.

ACF Analysis

Immediately postsacrifice, colons were excised, rinsed with ice-cold PBS, and opened longitudinally under direct vision using a Nikon SMZ-U stereomicroscope. Overall colon length as well as polyp and tumor location was determined by mounting the specimen on a glass slide marked in 1-cm increments. The colon was then stained in ice-cold 0.3% (v/v) methylene blue in PBS for 40 to 60 min. After staining, the surface of the colon was again examined but now at 40× to identify ACF. ACF were identified using the criteria of Bird and McLellan (7), and their position was recorded. To be considered an ACF, each structure had to have four of the five following criteria: crypts that were two to three times larger than normal; a thickened layer of epithelial cells; an increased pericryptal area; slit-shaped lumina; and be microscopically elevated above the plane of normal crypts in the preparation.

Tissue Pathology

Gross examination of the liver, lungs, and peritoneum for secondary tumors was done in all animals. In the dissected rat colon, tumor number, size, weight, and location were recorded. Most colonic tumors were adenocarcinomas, and thus, we used only those for the analysis of variables. Each tumor was fixed individually, with the remainder of the colon processed as single specimen. All tissues were formalin fixed and paraffin embedded according to standard Armed Forces Institute of Pathology (AFIP) protocol. Blocks were sectioned (5 μm) using a Spencer Model 820 microtome (American Optical), heat fixed at 70°C for 20 min, and stained with H&E according to standard AFIP protocol.

Quantitative Metaphase Crypt Analysis

The number of proliferating colonic crypt cells was determined by counting vincristine-arrested cellular metaphases from freshly dissected crypts according to the method of Goodlad (8). Briefly, after ACF analysis (described above), the proximal and distal colon were separately fixed in Carnoy's solution for 3 h and then stored in 70% alcohol. Individual crypts were prepared by hydrating in graded alcohols followed by immersion in 1 mol/L HCl for 10 min at 60°C. Colon tissue was counterstained with Schiff's reagent for 45 min until a deep magenta color developed. Crypts were isolated under direct stereomicroscopic vision after adding two drops of 45% (v/v) acetic acid and spreading under a large (24 × 40 mm) coverslip. Metaphase nuclei from 20 separate crypts were identified and counted at 400× from both the distal and proximal colon of all animals.

Western Blot for iNOS and PARP

Protein lysates were prepared from excised rat colon in cold NaCl (150 mmol/L), EDTA (1 mmol/L), EGTA (1 mmol/L), sodium PPi (2. 5 mmol/L), β-glycerophosphate (1 mmol/L), Na_3VO_4 (1 mmol/L), 1% (v/v) Triton X-100, 1 μg/mL leupeptin, phenylmethylsulfonyl fluoride (1 mmol/L), Tris-HCl (20 mmol/L; pH, 7), before electrophoretic resolution via SDS-PAGE and transfer to nitrocellulose for immunoblotting. The blots were developed on Kodak Biomax film using a Luminol enhanced chemiluminescence kit. Quantitation of band intensity at 130 kDa is done using Adobe Photoshop image analysis software (Adobe Systems Inc.).

Immunohistochemistry

Immunohistochemistry was done using a two-stage modification of an immunoperoxidase technique that we have previously described (9). Briefly, after quenching peroxidase activity with 3% hydrogen peroxide and blocking nonspecific binding with goat serum, a 1:50 dilution of primary iNOS antibody was applied to 5-μm tissue sections and incubated at 4°C overnight. The sections were washed in PBS, followed by incubation with a horseradish peroxidase–labeled anti-rabbit immunoglobulin G for 30 min using the DAKO EnVision+ System. After a final wash in PBS, the polymer-bound antibody is detected with liquid 3,3′-diaminobenzidine substrate chromogen system for 3 to 5 min. The chromogen-stained tissue is counterstained for 30 s in Gill's modified hematoxylin and iNOS expression measured by Q-IHC as we have previously described (9).

Cell Culture Studies

Caco-2 human colonic adenocarcinoma cells obtained from the American Type Culture Collection were grown in DMEM/F-12 supplemented with 1% penicillin-streptomycin, 20% fetal bovine serum, nonessential amino acids, sodium pyruvate, and 1.5 g/L sodium bicarbonate and incubated in 5% CO_2 at 37°C. At confluency, cells were seeded in 96-well plates at a density of 2×10^4 cells/mL in 190 μL media. After 24 h incubation, test samples were added to each well, and the cells were incubated for an additional 48 or 96 h. The cells were fixed with ice-cold 20% trichloroacetic acid, and cell count was done using sulforhodamine B (SRB) staining. The absorption was measured at 515 nm using a plate reader. Assays were done in three separate cell cultures. Cell cycle fluorescence-activated cell sorting (FACS) analysis was done by the propidium iodide–trypsin method on two separate cell cultures. After 24 h incubation with or without drug, cells were lysed with trypsin/EDTA and centrifuged (600 × *g*), and the pellet was resuspended in RPMI/PBS/10% FCS. Aliquots of 1×10^6 cells were transferred to FACS tubes and centrifuged (600 × *g*), and the pellet was resuspended in citrate buffer (100 μL) before tryptic digest, RNase treatment, and staining with ice-cold propidium iodide (416 μL/mL) and spermine tetrahydrochloride solution (1.16 mg/mL) before FACS analysis.

The annexin V apoptosis assay was carried out on an alternative human colon adenocarcinoma cell line, HT-29, supplied by Dr. Murillo (Illinois Institute of Technology, Chicago, IL). Cells were maintained in RPMI 1640, supplemented with 1% antibiotic-antimycotic, 1% L-glutamine (200 mmol/L), and 10% fetal bovine serum (Atlanta Biologicals), and incubated in 5% CO_2 at 37°C. Incubations of HT-29 cells with GT-094 (50 or 100 μmol/L) were done according to standard methods (annexin V–FITC apoptosis detection kit; Sigma). After the indicated time points, the

media were harvested. After workup, both floating and trypsinized cells were treated with annexin V–FITC and propidium iodide and analyzed by flow cytometry (Beckman Coulter Elite ESP) to quantitatively assess cells undergoing apoptosis.

The mouse RAW 264.7 cells, provided by Dr. J. Cook (University of Illinois at Chicago, Chicago, IL), were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 50 μg/mL penicillin, and 50 μg/mL streptomycin in bacteriologic plates at 37°C in humid 5% CO_2. Cells were cultured for no more than four passages. Cells were plated in DMEM and incubated at 37°C for 12 h. The medium was changed, allowing cell induction by addition of lipopolysaccharide (LPS) in the medium. Cell lysate supernatant from drug-treated and control incubations was taken for assay at 5, 12, and 24 h. Supernatant was assayed for NO_2^- using the Griess assay. Cell lysates were assayed for cell number using the SRB method or for iNOS content using Western blot immunoassay.

Results

GT-094 Significantly Reduced Azoxymethane-Induced ACF

In the 8-week study, mean ACF number was significantly reduced in the GT-094–treated group (24 ± 4) of rats compared with azoxymethane-treated control rats (44 ± 9; $P < 0.05$; Fig. 2) by one-way ANOVA using Dunnett's post-test. Furthermore, the effects of ASA (44 ± 4) or ASA plus nitrates (42 ± 7) failed to reach significance for the reduction of ACF formation. In the 28-week study, the incidence of tumors in rats treated with azoxymethane alone was 50% (5:10), whereas tumors developed in 36% (4:11) of GT-094–supplemented animals; similarly, tumor multiplicity was reduced 1.8 versus 1.5 tumors per animal, as well as mean tumor weight 169 ± 81 mg versus 96 ± 36 mg in the GT-094–supplemented animals, but these differences did not reach statistical significance by unpaired t test.

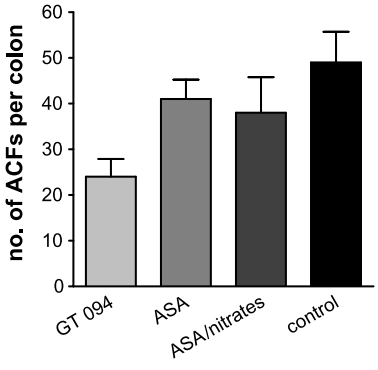

Figure 2. Colon ACF quantified in Fischer rats (n = 8) treated with azoxymethane, followed by administration of drugs for 8 wks: ASA (117 mg/kg); GT-094 (233 mg/kg); nitrates (ISMN 93 mg/kg; MDN 80 mg/kg), representing approximately equimolar doses. *Bars*, SE. One-way ANOVA with Tukey's post-test, $P < 0.05$ for GT-094 versus control.

GT-094 Reduced Proliferation and Increased p27 Expression in Azoxymethane-Treated Colons

In response to azoxymethane, normal crypts residing in the proximal and distal colon of the rat undergo an increase both in the number of proliferating crypt cells and an apical migration of metaphase-arrested cells up the native crypt (10). We evaluated proliferation 28 weeks after azoxymethane administration by counting metaphase-arrested cells in whole crypt preparations from five treated and untreated animals. In the proximal colon, GT-094 reduced proliferation by 69% [13.9 ± 7.6 versus 4.4 ± 2.6 metaphases per crypt (mean ± SD); $P < 0.05$]. Crypt proliferation rates in the distal colon were more modestly reduced [13.9 ± 2.9 versus 9.1 ± 2.9 metaphases per crypt (mean ± SD); $P < 0.05$], but this 30% reduction remained significant (Fig. 3).

Expression of the cell cycle inhibitor p27 was not significantly altered within the proximal and distal colon of untreated animals after 28 weeks of azoxymethane administration. However, GT-094–supplemented animals displayed marginally reduced p27 expression in the proximal colon (despite decreased levels of proliferation) compared with untreated animals, but also showed a marked (~3-fold) increase in expression in the distal colon where DNA damage and ACF formation occur ($P < 0.05$; Fig. 3).

GT-094–Altered Expression of Markers of Inflammation (iNOS) and DNA Damage (PARP)

The iNOS expression identified in the 10-week study by immunohistochemistry was predominantly within and adjacent to colon crypt as previously shown in other azoxymethane models (11). ASA alone reduced iNOS expression by 27%, whereas GT-094 further reduced iNOS production by 67%, a ~2.5-fold reduction compared with azoxymethane alone. The extent of this iNOS inhibition in GT-094–treated animals did not persist through 30 weeks of carcinogen exposure, declining to 33% of the levels expressed in untreated animals, although this reduction remained significant (Fig. 3; $P < 0.05$).

PARP-1 expression and cleavage were evaluated as a secondary marker of inflammation and DNA damage (12). Again, GT-094–treated animals showed significantly reduced amounts of cleaved and uncleaved PARP-1 expression in the distal colon compared with that of azoxymethane-treated animals. Uncleaved PARP was ~4-fold higher in the azoxymethane-alone group, whereas the cleaved protein, representing cellular damage, was 2-fold higher in the animals not protected by GT-094 (Fig. 3).

GT-094 Was Antiproliferative in Colon Cancer Cells but not Proapoptotic

Data collected *in vivo* showed antiproliferative and anti-inflammatory actions of GT-094 in rat colon; therefore, studies were conducted to determine if these effects were recapitulated *in vitro*. A concentration-response curve was obtained for GT-094 in the colon cancer, Caco-2, cell line, using SRB dye staining to obtain cell number at 48 h incubation, giving IC_{50} ~40 μmol/L (Fig. 4). At 100 μmol/L, GT-094 was cytostatic. The antiproliferative

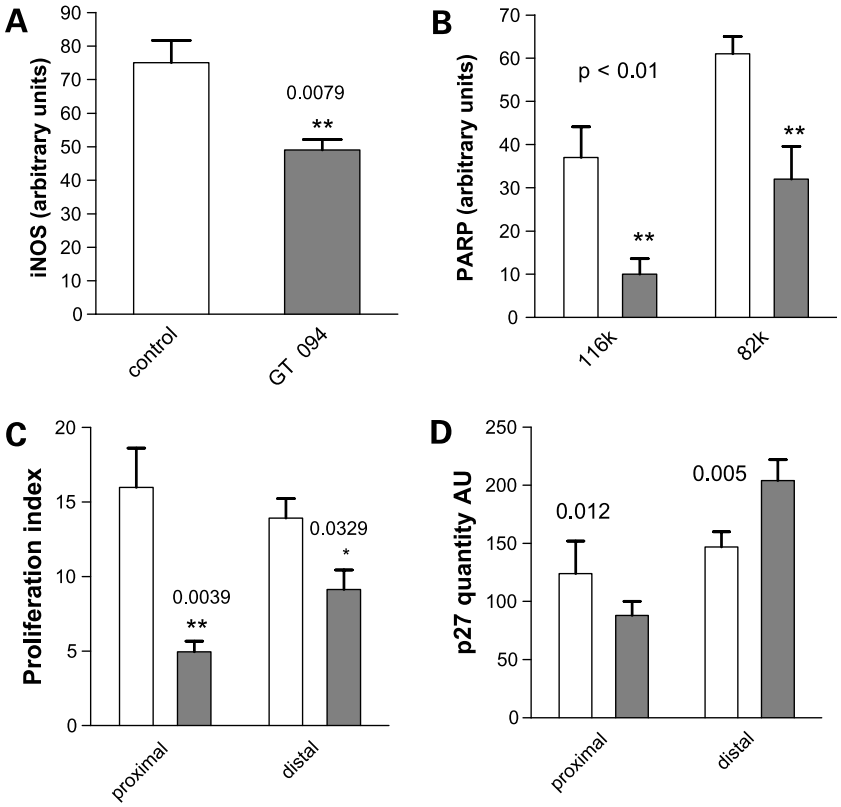

Figure 3. Quantitative analysis of (**A**) iNOS; (**B**) PARP (intact and cleaved), from Western blot analysis of colon tissue lysates; (**C**) proliferation measured by vincristine-arrested cellular metaphases; (**D**) p27 protein from Western blot analysis of colon tissue lysates. Colon tissue dissected from rats treated over 2 wks with azoxymethane carcinogen and for a further 28 wks with GT-094. Data ($n = 5$) show mean and SE and P values analyzed by unpaired t test at 95% confidence.

effects of GT-094 were further studied in Caco-2 cells by cell cycle FACS analysis, demonstrating a transient G_2-M phase block that was apparent at 6 h, but which was diminished at 24 h (Fig. 5). The Caco-2 cell cycle analysis gave no qualitative evidence for apoptosis induced by GT-094, but this aspect was studied in more detail by FACS analysis using annexin and propidium iodide staining in the HT-29 colon cancer cell line. The data analysis showed that GT-094 does not induce apoptosis in HT-29 colon cancer cell culture (Fig. 5).

GT-094 Inhibited Induction of iNOS and NO Production in RAW 264.7 Cells

The murine alveolar macrophage-like RAW 264.7 cell line has frequently been used to examine interference of agents with cellular inflammatory response. LPS treatment induces iNOS, which can be quantified by Western blot or by measurement of NO_2^- production because NO_2^- is the ultimate product of NO oxidative metabolism. Using the Griess assay, ~30 μmol/L NO_2^- was measured in LPS-induced RAW 264.7 cells after 24 h incubation. The majority of nitrates, including ISDN, undergo denitration to NO_2^- as the major product (13). The supernatant from RAW 264.7 cells was assayed for NO_2^- in the absence of LPS induction: no significant amount of NO_2^- was detected in untreated cells; whereas ISDN-treated cells accumulated 2.7 ± 0.5 μmol/L NO_2^-. Cells treated with GT-094 (10, 50, and 100 μmol/L) contained 3.4 ± 0.2, 16.2 ± 0.3, and 38.7 ± 0.7 μmol/L NO_2^-, respectively, corresponding to ~33% yield from GT-094. No significant differences were seen in [NO_2^-] produced from nitrates at 9, 12, and 20 h. Simple subtraction of NO_2^- in the absence and presence of LPS induction yielded values for endogenous NOS-mediated NO_2^- production (Fig. 6). GT-094 substantially inhibited NO_2^- production in a concentration-dependent manner, which was not seen for ISDN at high concentration (100 μmol/L) with or without added ASA (100 μmol/L). Western blots of lysates from treated and untreated RAW 264.7 cells, with and without LPS induction, confirmed that GT-094 inhibited iNOS protein expression in response to LPS induction.

Discussion

NO-NSAIDs are nitrates conjugated to an NSAID drug moiety via a labile linker. Collectively, NO-NSAIDs containing an ASA moiety are termed NO-ASA; one isomer, NCX 4016, has entered clinical trials for colorectal cancer chemoprevention. NCX 4016, together with its isomer, NCX 4040, have been the subject of elegant studies by Rigas et al. (6). NO-ASA molecules were originally designed to exploit the NO bioactivity of the nitrate group to attenuate the serious gastrointestinal toxicity caused by the action of NSAIDs; for example, NCX 4016 first entered clinical trials in 1996 for use in pain and inflammation. Despite NO-ASA not having been chemically optimized for chemoprevention, the published results are impressive (14). Given this promise, a prototype nitrate was selected, GT-094, to provide a lead compound for further structure-activity

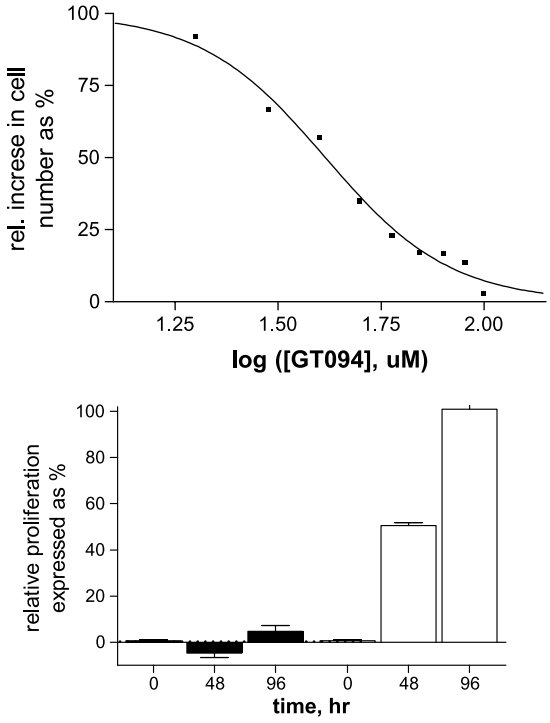

Figure 4. Caco-2 cell count as a function of time on incubation with GT-094. Cells were fixed with trichloroacetic acid and stained with 0.4% sulforhodamine B; protein-bound dye was extracted and quantified at 515 nm to estimate cell population. **A**, concentration-response data assayed at 48 h showing the relative increase in cell number as a percentage of the untreated control, fitted to a curve with $IC_{50} = 41 \pm 2$ μmol/L. **B**, time course of cell proliferation relative to the untreated control group at 96 h (mean and SE): *filled columns*, GT094 (100 μmol/L); *open columns*, control.

studies toward optimization for colorectal cancer chemoprevention (Fig. 1). Herein, we report *in vivo* and *in vitro* studies on GT-094, showing attributes considered beneficial for a chemopreventive agent.

Nitrate Therapeutics and NO Chimeras

The classic nitrate nitrovasodilator, nitroglycerin (GTN), has been in clinical use in therapy of angina for more than 130 years. The classic nitrate, ISDN, is important because it is now being used as a cardioprotective agent, expanding the clinical paradigm of nitrates to chronic, prophylactic therapy (15). Organic nitrates contain the nitrooxy group ($-ONO_2$) that provides bioactivity that mimics that of NO (13). Nitrates are also readily able to act as oxidizing agents toward thiols. Bioactivation of nitrates provides NO bioactivity, with no evidence for production of the higher levels of NO that can be observed from NO donors such as NONOates (13). Novel nitrates containing a disulfide linkage (e.g., GT-094) have been shown to differ from classic nitrates, manifesting NO bioactivity including antioxidant capacity and cytoprotection (16–19). GT-094 is coined an NO chimera because it incorporates ancillary pharmacophores, a disulfide and an NSAID (thiosalicylate), both shown to be effective in colorectal cancer chemoprevention (Fig. 1). Thiosalicylates have been reported as NSAIDs (20, 21), and a thiosalicylate derivative has been explored in colon cancer therapy (22). Disulfides, notably diallyl disulfide (DADS), have been studied as garlic-derived chemopreventive agents that reduce ACF formation in the rat/azoxymethane model (23, 24). Reports suggest that the disulfide group of DADS is responsible for the antiproliferative activity (16). DADS was reported to induce a G_2-M phase cell cycle block in HCT-15 cells (25) and to be anti-antiproliferative in Caco-2 cells (24, 26). The antiproliferative colorectal cancer chemopreventive agent, butyrate, was also reported to induce a G_2-M phase block in Caco-2 cells (at 5 mmol/L; refs. 24, 27).

NSAIDs, NO-ASA, and Colorectal Cancer

Epidemiologic data suggest an inverse relationship between colorectal cancer risk and regular use of NSAIDs. Clinical trials with NSAIDs also showed that NSAID treatment caused regression of pre-existing colon adenomas in patients with familial adenomatous polyposis (FAP). However, even the use of low-dose ASA can damage the gastric mucosa. NSAID gastrotoxicity seems to be closely related to the inhibition of COX and PG biosynthesis. NO has cytoprotective properties in the stomach and in other organs, exhibiting actions in the gastrointestinal tract similar to those of PGs, such as the stimulation of mucus secretion and maintenance of mucosal blood flow. NO solutions and GTN were reported to be able to substitute for PG action in the gastrointestinal tract to counterbalance pharmacologic COX inhibition (28), providing the impetus for development of NO-NSAIDs; hybrid nitrates that conjugate a nitrate group to an NSAID via a labile acyl linker (4–6, 29, 30).

There is an extensive literature on the *in vitro* activity of NO-NSAIDs and NO-ASA. Importantly, in cell culture, NO-NSAIDs are reported to be potent COX-independent, antiproliferative agents in contrast to the parent NSAIDs (6, 31, 32). Moreover, the combination of ASA with very high concentrations of true NO donors (0.7 mmol/L) was only weakly antiproliferative (33), supporting the argument that antiproliferative activity is a property of the benzyl nitrate pharmacophore. Interestingly, two recent studies reported that the activity of NCX 4016 resulted at least in part from the oxidant properties of the compound (34, 35).

NOS, NO, and Colorectal Cancer

The role of inflammation in colorectal cancer is well accepted: iNOS is invoked as a component of inflammatory carcinogenesis. The use in colorectal cancer of drugs that are traditionally regarded as NO donors seems counterintuitive, but the correlation between NO, NOS, and colorectal cancer is not so straightforward. Levels of iNOS and eNOS are reported to be elevated in carcinoma tissue in response to azoxymethane, and similar observations on iNOS in human colorectal cancer have been made (36), but increased eNOS has also been correlated with improved survival in colorectal cancer (37). Inhibitors of iNOS have been observed to reduce ACF levels in the rat/azoxymethane model (38), but in one report in this model, a nonspecific NOS inhibitor increased ACF levels (39). In the

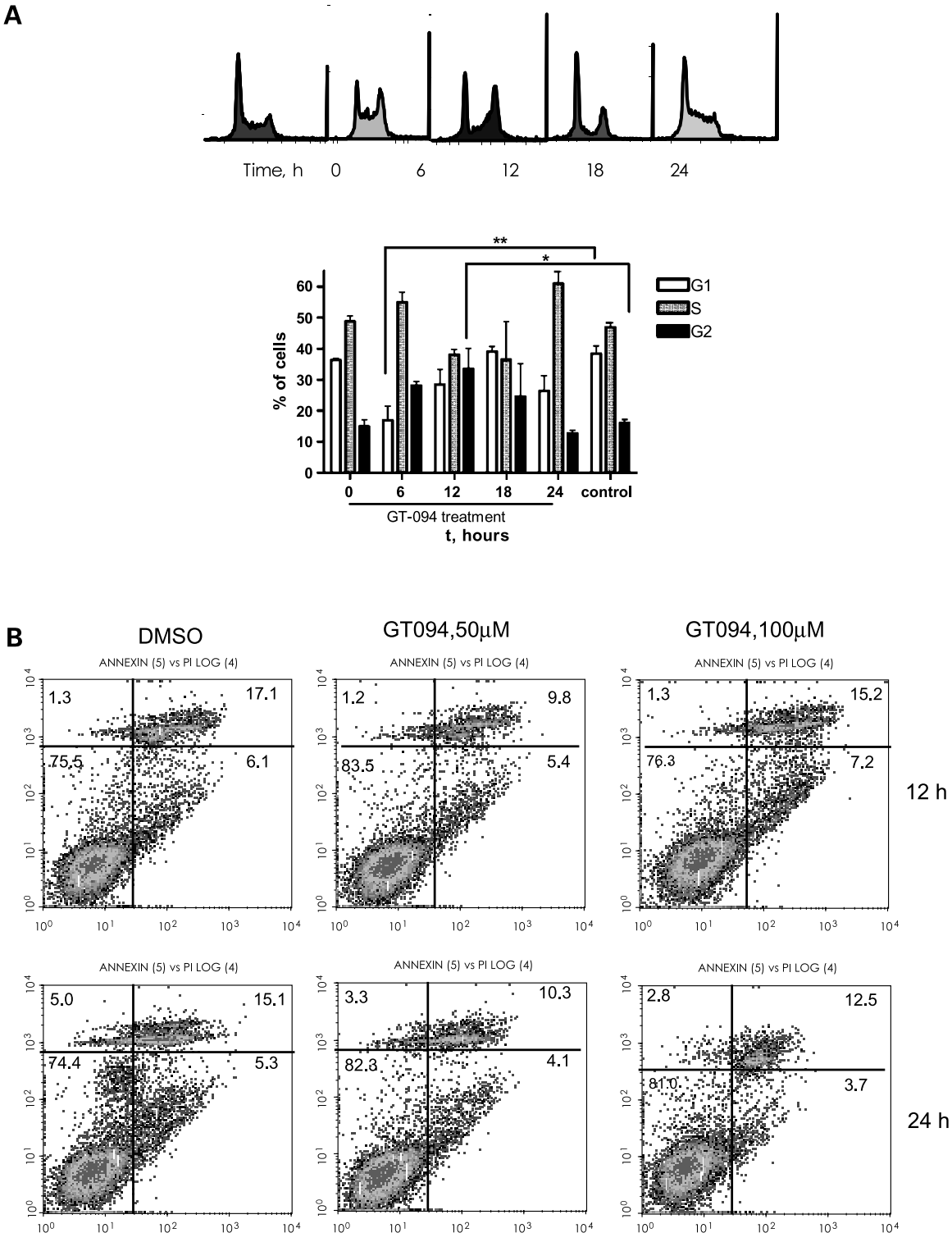

Figure 5. **A,** cell cycle FACS analysis of propidium bromide – treated Caco-2 cells incubated with GT-094 (100 μmol/L). Detailed cell cycle analysis of one Caco-2 culture representative of the two different passages assayed. Chart compilation of FACS analyses of percentage cells in G_1, S, and G_2-M phase as a function of time clearly showing transient accumulation of cells in G_2-M phase in the drug-treated group. Untreated cells (*control*) showed no change in distribution with time. Data show mean and SD analyzed by one-way ANOVA with Newman-Keul's post-test. **, $P < 0.01$; *, $P < 0.05$. **B,** the effect of GT-094 on HT-29 colon cancer cell apoptosis assayed by staining with annexin and propidium iodide and subjected to flow-cytometric analysis as described in Materials and Methods. Numbers represent percentage of cells in each subcategory. Data shown are from a single experiment that was repeated in two subsequent cell passages, yielding data within 10% of the data shown.

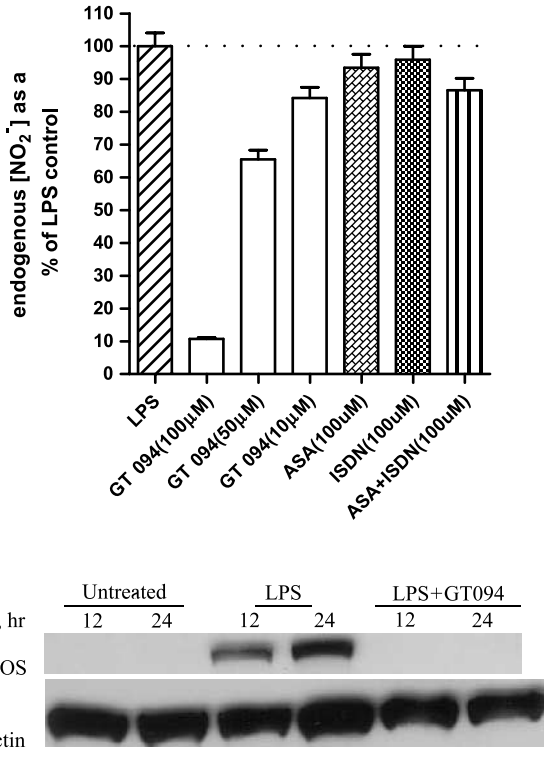

Figure 6. Measurements of endogenous NO_2^- production in RAW 264.7 cells induced by LPS at 24 h, with Western blots shown for iNOS protein in untreated, LPS-treated, and LPS-treated cells administered with GT-094 (100 μmol/L). Triplicate cultures in quadruplicate ±SE.

Apc(min/+) FAP model, $iNOS^{-/-}$ knock-outs had more intestinal adenomas (40). Ornithine decarboxylase activity is raised in colorectal cancer and is reduced by NO, providing a possible rationale for some of these observations (39–41). The evidence for a causative role for NO from NOS in colorectal cancer is problematic, but the evidence supports iNOS as a biomarker for colorectal cancer. Furthermore, agents that lower iNOS in RAW 264.7 cells have been shown to reduce ACFs in the rat/azoxymethane model (1), supporting the cellular iNOS level as a biomarker in drug discovery.

Nitrate Therapeutics in Colorectal Cancer Chemoprevention

The progression of NCX 4016 into clinical trials for colorectal cancer is supported by rodent models. The first study on NCX 4016 in a modified rat/azoxymethane model used as an intrarectal dose of trinitrobenzene-sulfonic acid normally used in models of inflammatory colitis, and the drug was given concurrently with carcinogen (4). Drug treatment (p.o. ASA, 10 mg/kg/day, or NCX 4016, 18.5 mg/kg/day) yielded a 65% reduction of ACFs by ASA alone and an 85% reduction by NCX 4016. In a second animal study, NCX 4016 and NCX 4040, a regioisomer of NCX 4016 with more potent cytotoxic activity were studied in the Apc(min/+) mouse model of FAP, in which tumors develop spontaneously in the small intestine (42): delivered intrarectally at high dose (100 mg/kg/day; 21 days), a reduction of 60% in tumor incidence was observed for NCX 4040, but the effect of NCX 4016 was not significant. Tissues from these NO-ASA animal studies showed no evidence for inhibition of COX or PG synthesis and no evidence for an antiproliferative effect. A very recent rat/azoxymethane study reported significant reductions in tumor incidence and multiplicity after 46 weeks of treatment with high-dose NCX 4016 (14).

The actions of GT-094 *in vitro* and *in vivo* are not identical to those reported for NO-ASA. The G_2-M phase cell cycle block observed for GT-094 has not been reported for NO-ASA and may result from the disulfide pharmacophore of GT-094 because DADS was reported to show a similar antiproliferative profile. The antiproliferative activity of DADS in Caco-2 and HCT-29 cells has been ascribed to the modulation of histone acetylation and $p21^{waf1/cip1}$ expression (26). NO-ASAs are antiproliferative but, in addition and in contrast to GT-094, have been reported to induce apoptosis and PARP cleavage in colon cancer cells (6, 31, 33, 34). However, the differences in the properties of the NO-ASA isomers should be noted: NCX 4040 and NCX 4060, in contrast to NCX 4016, were significantly cytotoxic: in HT-29 cells, NCX 4016 gave an antiproliferative IC_{50} of 200 μmol/L and only 5% apoptosis, whereas under similar conditions, the isomers were 100- to 200-fold more potent and induced 100% apoptotic and atypical cell morphology (33, 34). GT-094 was seen to be antiproliferative in rat and in human colon cancer cells, but showed evidence neither for inducing apoptosis in HT-29 cells nor for PARP cleavage *in vivo*. The reported differences between GT-094, NCX 4016, and NCX 4040 emphasize that the nitrate structure influences activity, one rationale being the well-known capacity of NO to exert both pro- and antiapoptotic actions dependent on NO flux, cell type, and other factors (43). Induction of apoptosis in neoplastic cells *in vivo* is seen as a contributor to chemoprevention, although not a definitive requirement. Further studies are needed to compare the activity of chemopreventive nitrates toward apoptosis and differentiation *in vivo*.

In the present study, GT-094 is shown to act as an antiproliferative agent that reduces ACF in the rat/azoxymethane model and reduces iNOS expression *in vitro* and *in vivo*. The strongest evidence for NO-ASA actions on iNOS was reported in the recent rat/azoxymethane study delivering high-dose NCX 4016 in feed, where both antiproliferation and iNOS inhibition were observed (14), in contrast to two previous NO-ASA animal studies that did not show such effects. There is a known sensitivity to dosage in rat/azoxymethane studies, and in this respect, the observation in the present study that a single-dose combination of ASA with ISDN did not significantly reduce ACF cannot be viewed as definitive; however, it is noted that *in vitro*, the combination therapy was also not efficacious.

In the newly reported rat/azoxymethane work on NCX 4016, drug was delivered in feed at 3,000 ppm (14). The initial azoxymethane/ACF study on NCX 4016 used a lower dose, and in our work, an approximately equimolar

dosage of GT-094 in feed was chosen (233 ppm). GT-094 gave a significant reduction in ACF, but at this dose after 28 weeks, data on tumor reduction did not reach significance relative to azoxymethane-treated control ($n = 10$). At the 10-fold higher dose in the NCX 4016 study, a significant reduction in both the incidence and multiplicity of noninvasive colon adenocarcinomas was reported after 46 weeks ($n = 36$). NCX 4016 was also antiproliferative, reducing colonocyte PCNA expression by 14%, and was anti-inflammatory, reducing iNOS activity by 47%, but not having any significant effect on iNOS expression. In comparison and in contrast, GT-094 reduced azoxymethane-induced colon crypt proliferation by 30% to 69% and iNOS expression in the rat colon by 33% to 67%. NCX 4016 reduced azoxymethane-induced COX activity in the colon, which was not measured for GT-094; and GT-094 reduced azoxymethane-induced PARP cleavage and increased p27 expression in the distal colon, which was not reported for NCX 4016. The nitrates, NO-ASA and GT-094, thus do not show identical activity, but do manifest a variety of beneficial actions, both *in vitro* and more importantly *in vivo*, that support the further exploration and optimization of nitrates for colorectal cancer chemoprevention.

Acknowledgments

Daniel D. Lantvit is thanked for technical assistance. This project was done in part using compound(s) provided by the National Cancer Institute's Chemical Carcinogen Reference Standards Repository operated under contract by Midwest Research Institute, no. N02-CB-07008.

References

1. Corpet DE, Tache S. Most effective colon cancer chemopreventive agents in rats: a systematic review of aberrant crypt foci and tumor data, ranked by potency. Nutr Cancer 2002;43:1 – 21.

2. Hao XP, Pretlow TG, Rao JS, Pretlow TP. Inducible nitric oxide synthase (iNOS) is expressed similarly in multiple aberrant crypt foci and colorectal tumors from the same patients. Cancer Res 2001;61:419 – 22.

3. Rosenberg L, Louik C, Shapiro S. Nonsteroidal antiinflammatory drug use and reduced risk of large bowel carcinoma. Cancer 1998;82: 2326 – 33.

4. Bak AW, McKnight W, Li P, et al. Cyclooxygenase-independent chemoprevention with an aspirin derivative in a rat model of colonic adenocarcinoma. Life Sci 1998;62:367 – 73.

5. Bolla M, Almirante N, Benedini F. Therapeutic potential of nitrate esters of commonly used drugs. Curr Top Med Chem 2005;5:707 – 20.

6. Kaza CS, Kashfi K, Rigas B. Colon cancer prevention with NO-releasing NSAIDs. Prostaglandins Other Lipid Mediat 2002;67:107 – 20.

7. Bird RP, McLellan EA, Bruce WR. Aberrant crypts, putative precancerous lesions, in the study of the role of diet in the aetiology of colon cancer. Cancer Surv 1989;8:189 – 200.

8. Goodlad RA, Lee CY, Wright NA. Cell proliferation in the small intestine and colon of intravenously fed rats: effects of urogastrone-epidermal growth factor. Cell Prolif 1992;25:393 – 404.

9. Carroll RE, Matkowskyj K, Saunthararajah Y, Sekosan M, Battey JF, Benya RV. Contribution of gastrin-releasing peptide and its receptor to villus development in the murine and human gastrointestinal tract. Mech Dev 2002;113:121 – 30.

10. Hirose Y, Tanaka T, Makita H, et al. Suppressing effects of 6-(2,5-dichlorophenyl)-2,4-diamino-1,3,5-triazine and related synthetic compounds on azoxymethane-induced aberrant crypt foci in rat colon. Jpn J Cancer Res 1996;87:549 – 54.

11. Kohno H, Suzuki R, Sugie S, Tanaka T. Suppression of colitis-related mouse colon carcinogenesis by a COX-2 inhibitor and PPAR ligands. BMC Cancer 2005;5:46.

12. Hagberg H, Wilson MA, Matsushita H, et al. PARP-1 gene disruption in mice preferentially protects males from perinatal brain injury. J Neurochem 2004;90:1068 – 75.

13. Thatcher GR, Nicolescu AC, Bennett BM, Toader V. Nitrates and no release: contemporary aspects in biological and medicinal chemistry. Free Radic Biol Med 2004;37:1122 – 43.

14. Rao CV, Reddy BS, Steele VE, et al. Nitric oxide-releasing aspirin and indomethacin are potent inhibitors against colon cancer in azoxymethane-treated rats: effects on molecular targets. Mol Cancer Ther 2006;5: 1530 – 8.

15. Taylor AL, Ziesche S, Yancy C, et al. Combination of isosorbide dinitrate and hydralazine in blacks with heart failure. N Engl J Med 2004; 351:2049 – 57.

16. Nicolescu AC, Zavorin SI, Turro NJ, Reynolds JN, Thatcher GRJ. Inhibition of lipid peroxidation in synaptosomes and liposomes by nitrates and nitrites. Chem Res Toxicol 2002;15:985 – 98.

17. Smith S, Dringenberg HC, Bennett BM, Thatcher GRJ, Reynolds JN. A novel nitrate ester reverses the cognitive impairment caused by scopolamine in the Morris water maze. Neuroreport 2000;11:3883 – 6.

18. Thatcher GRJ, Bennett BM, Dringenberg HC, Reynolds JN. Novel nitrates as NO mimetics directed at Alzheimer's disease. J Alzheimers Dis 2004;6:S75 – 84.

19. Zavorin SI, Artz JD, Dumitrascu A, et al. Nitrate esters as nitric oxide donors: SS-nitrates. Org Lett 2001;3:1113 – 6.

20. Kapadia GJ, Azuine MA, Takayasu J, et al. Inhibition of Epstein-Barr virus early antigen activation promoted by 12-*O*-tetradecanoylphorbol-13-acetate by the nonsteroidal anti-inflammatory drugs. Cancer Lett 2000; 161:221 – 9.

21. Brannigan LH, Hodge RB, Field L. Biologically oriented organic sulfur chemistry: 14. Antiinflammatory properties of some aryl sulfides, sulfoxides, and sulfones. J Med Chem 1976;19:798 – 802.

22. Halaschek-Wiener J, Wacheck V, Schlagbauer-Wadl H, Wolff K, Kloog Y, Jansen B. A novel Ras antagonist regulates both oncogenic Ras and the tumor suppressor p53 in colon cancer cells. Mol Med 2000;6: 693 – 704.

23. Sumiyoshi H, Wargovich MJ. Chemoprevention of 1,2-dimethylhydrazine – induced colon cancer in mice by naturally occurring organosulfur compounds. Cancer Res 1990;50:5084 – 7.

24. Wargovich MJ, Chen CD, Jimenez A, et al. Aberrant crypts as a biomarker for colon cancer: evaluation of potential chemopreventive agents in the rat. Cancer Epidemiol Biomarkers Prev 1996;5:355 – 60.

25. Knowles LM, Milner JA. Diallyl disulfide induces ERK phosphorylation and alters gene expression profiles in human colon tumor cells. J Nutr 2003;133:2901 – 6.

26. Druesne N, Pagniez A, Mayeur C, et al. Diallyl disulfide (DADS) increases histone acetylation and p21waf1/cip1 expression in human colon tumor cell lines. Carcinogenesis 2004;25:1227 – 36.

27. Harrison LE, Wang QM, Studzinski GP. Butyrate-induced G_2-M block in Caco-2 colon cancer cells is associated with decreased p34cdc2 activity. Proc Soc Exp Biol Med 1999;222:150 – 6.

28. MacNaughton WK, Cirino G, Wallace JL. Endothelium-derived relaxing factor (nitric oxide) has protective actions in the stomach. Life Sci 1989;45:1869 – 76.

29. Wallace JL, McKnight W, Del Soldato P, Baydoun AR, Cirino G. Antithrombotic effects of a nitric oxide-releasing, gastric-sparing aspirin derivative. J Clin Invest 1995;96:2711 – 8.

30. Wallace JL, Reuter B, Cicala C, McKnight W, Grisham MB, Cirino G. Novel nonsteroidal anti-inflammatory drug derivatives with markedly reduced ulcerogenic properties in the rat. Gastroenterology 1994;107: 173 – 9.

31. Kashfi K, Ryan Y, Qiao LL, et al. Nitric oxide-donating nonsteroidal anti-inflammatory drugs inhibit the growth of various cultured human cancer cells: evidence of a tissue type-independent effect. J Pharmacol Exp Ther 2002;303:1273 – 82.

32. Williams JL, Nath N, Chen J, et al. Growth inhibition of human colon cancer cells by nitric oxide (NO)-donating aspirin is associated with cyclooxygenase-2 induction and β-catenin/T-cell factor signaling, nuclear factor-κB, and NO synthase 2 inhibition: implications for chemoprevention. Cancer Res 2003;63:7613 – 8.

33. Kashfi K, Borgo S, Williams JL, et al. Positional isomerism markedly affects the growth inhibition of colon cancer cells by nitric oxide-donating aspirin *in vitro* and *in vivo*. J Pharmacol Exp Ther 2005;312:978 – 88.

34. Gao J, Liu X, Rigas B. Nitric oxide-donating aspirin induces apoptosis in human colon cancer cells through induction of oxidative stress. Proc Natl Acad Sci U S A 2005;102:17207 – 12.

35. Bratasz A, Weir NM, Parinandi NL, et al. Reversal to cisplatin sensitivity in recurrent human ovarian cancer cells by NCX-4016, a nitro derivative of aspirin. Proc Natl Acad Sci U S A 2006;103: 3914 – 9.

36. Takahashi M, Wakabayashi K. Gene mutations and altered gene expression in azoxymethane-induced colon carcinogenesis in rodents. Cancer Sci 2004;95:475 – 80.

37. Mortensen K, Christensen IJ, Nielsen HJ, Hansen U, Larsson LI. High expression of endothelial cell nitric oxide synthase in peritumoral microvessels predicts increased disease-free survival in colorectal cancer. Cancer Lett 2004;216:109 – 14.

38. Rao CV, Indranie C, Simi B, Manning PT, Connor JR, Reddy BS. Chemopreventive properties of a selective inducible nitric oxide synthase inhibitor in colon carcinogenesis, administered alone or in combination with celecoxib, a selective cyclooxygenase-2 inhibitor. Cancer Res 2002; 62:165 – 70.

39. Schleiffer R, Duranton B, Gosse F, Bergmann C, Raul F. Nitric oxide synthase inhibition promotes carcinogen-induced preneoplastic changes in the colon of rats. Nitric Oxide 2000;4:583 – 9.

40. Scott DJ, Hull MA, Cartwright EJ, et al. Lack of inducible nitric oxide synthase promotes intestinal tumorigenesis in the Apc(Min/+) mouse. Gastroenterology 2001;121:889 – 99.

41. Buga GM, Wei LH, Bauer PM, Fukuto JM, Ignarro LJ. NG-Hydroxy-L-arginine and nitric oxide inhibit Caco-2 tumor cell proliferation by distinct mechanisms. Am J Physiol 1998;275:R1256 – 64.

42. Williams JL, Kashfi K, Ouyang N, del Soldato P, Kopelovich L, Rigas B. NO-donating aspirin inhibits intestinal carcinogenesis in Min (APC(Min/+)) mice. Biochem Biophys Res Commun 2004;313:784 – 8.

43. Chung HT, Pae HO, Choi BM, Billiar TR, Kim YM. Nitric oxide as a bioregulator of apoptosis. Biochem Biophys Res Commun 2001;282: 1075 – 9.