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Nitrates and NO-NSAIDs in Cancer Chemoprevention & Therapy: In Vitro Evidence Querying the NO Donor Functionality

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Abstract

Properties of the NO-ASA family of NO-donating NSAIDs (NO-NSAIDs), notably NCX 4016 (**mNO-ASA**) and NCX 4040 (**pNO-ASA**), reported in more than a hundred publications, have included positive preclinical data in cancer chemoprevention and therapy. Evidence is presented that the antiproliferative, the chemopreventive (antioxidant/electrophile response element (ARE) activation), and the anti-inflammatory activity of NO-ASA in cell cultures is replicated by X-ASA derivatives that are incapable of acting as NO donors. **pBr-ASA** and **mBr-ASA** are conisogenic with NO-ASA, but are not NO donors. The biological activity of **pNO-ASA** is replicated by **pBr-ASA**; and both **pNO-ASA** and **pBr-ASA** are bioactivated to the same quinone methide electrophile. The biological activity of **mNO-ASA** is replicated by **mBr-ASA**; **mNO-ASA** and **mBr-ASA** are bioactivated to different benzyl electrophiles. The observed activity is likely initiated by trapping of thiol biomolecules by the quinone and benzyl electrophiles, leading to depletion of GSH and modification of Cys-containing sensor proteins. Whereas all NO-NSAIDs containing the same structural “linker” as NCX 4040 and NCX 4016 are anticipated to possess activity resulting from bioactivation to electrophilic metabolites, this expectation does not extend to other linker structures. Nitrates require metabolic bioactivation to liberate NO bioactivity, which is often poorly replicated in vitro, and NO bioactivity provided by NO-NSAIDs in vivo provides proven therapeutic benefits in mitigation of NSAID gastrotoxicity. The in vivo properties of X-ASA drugs await discovery.

Keywords

Chemoprevention; quinone oxidoreductase; hybrid drugs; antioxidant response element; quinone methide; nitrates; inflammation; NO-ASA; NO-NSAID

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¹Abbreviations: ARE, antioxidant responsive element; ASA, acetylsalicylic acid; BF, 4'-bromoflavone; CI, chemopreventive index; DCP, 2,6-dichlorophenolindophenol; EDTA, ethylenediaminetetraacetic acid; FAD, flavin adenine dinucleotide; GST, glutathione-S-transferase; Keap1, Kelch-like ECH-associated protein 1; MTT, thiazolyl blue tetrazolium bromide; NADP, β-nicotinamide adenine dinucleotide phosphate; NQO1, NAD(P)H-dependent quinone oxidoreductase; Nrf2, nuclear factor-erythroid 2 related factor 2; NSAID, non-steroidal anti-inflammatory drugs; NO-NSAIDs, NO-donating NSAIDs; SSA, 5-sulfosalicylic acid.

Introduction

Chemopreventive agents are natural product and synthetic molecules that act in primary cancer prevention and secondary prevention of cancer recurrence. These agents delivered chronically over periods of years act on a number of cellular targets in at-risk populations and must possess excellent safety characteristics. Chemoprevention contrasts with chemotherapy, since chemotherapeutic agents are designed to be cytotoxic in order to kill cancer cells, and often cause serious adverse events including death of normal host cells. Curiously, the activity profile of chemopreventive agents may have less in common with chemotherapeutics than with weak carcinogens, since compounds which possess weak carcinogenic activity have the potential to protect against carcinogenesis [1]. The excellent safety profile required for a chemopreventive agent provided the initial impetus for the study of NO-donating non-steroidal anti-inflammatory drugs (NO-NSAIDs) in colorectal cancer (CRC) chemoprevention, since these drugs were designed to supply NO to attenuate NSAID gastrotoxicity.

NO-NSAIDs are hybrid nitrates, designed to conjugate a “NO-donating moiety”, in the form of an organic nitrate functional group with existing, well-known NSAIDs, leading to new pharmacologically active molecules of potential use in various therapeutic areas [2] By design the optimum molecular fragment carrying the nitrate group should be able to preserve or enhance the pharmacokinetic and pharmacodynamic properties of the selected NSAID. The conjugation (or hybridization) of the NSAID uses enzymatically cleavable ester bonds in order to have the NSAID and the NO-releasing moiety released in the biological milieu after absorption and distribution of the hybrid drug. The use of various chemical fragments bearing the nitrate ester group has been reported. These “spacer” or “linker” fragments, possessing varied stability have been assumed to be inert, therefore the new pharmacological properties of NO-NSAIDs, when superior to the parent NSAID, have been ascribed to NO [3;4;5]. It is important to note that the clear identification of the metabolic steps by which NO-NSAIDs produce NO has not been established [6].

A parallel approach to developing novel nitrate therapeutics has been to incorporate one or more pharmacophores into a nitrate ester, termed an NO chimera.[7;8] **GT-094** is an NO chimera that contains a thiosalicylate NSAID moiety and a disulfide group, both pharmacophores having shown promise in vitro and in vivo in colon cancer chemoprevention. **GT-094** bears comparison to NO-ASA, since both contain salicylate NSAID fragments and organic nitrate groups. In the case of **GT-094**, these fragments are linked by a disulfide that is intended to deliver biological activity, whereas in the case of most NO-ASA derivatives, the linker is intended to be biologically inert.

Consideration of the metabolic chemistry of a subset of NO-ASA derivatives, including NCX 4040 (**pNO-ASA**) and NCX 4016 (**mNO-ASA**), suggested that the linker should be biologically active. This prediction has been confirmed in vitro: the cytotoxic and apoptotic activity of **pNO-ASA** has been shown to be a property of the “inert” linker [9]; and, the antiproliferative activity of both **mNO-ASA** and **pNO-ASA** has been demonstrated to result from the metabolic chemistry of the linker fragments [10]. No unequivocal evidence was reported to suggest that this activity resulted from any NO-donor properties of these NO-ASA derivatives. Recently, a phase 2 clinical trial of **mNO-ASA** in CRC chemoprevention was halted because of concerns over genotoxicity of its benzyl nitrate metabolite NCX 4015, emphasizing the importance of a fuller understanding of the relationship of chemical structure to biological activity.

In this paper, in vitro data are presented on **GT-094**, NO-ASA, and control compounds conisogenic with NO-ASA, which do not contain a NO-donating or nitrate group. In addition to antiproliferative activity, the observed anti-inflammatory activity and the induction of

cytoprotective phase 2 enzymes are shown to be NO independent in cell culture. It is also discussed that these properties are not universal for all NO-NSAIDS, but should be expected for an important subset of NO-ASA and NO-NSAID derivatives that contain a specific linker fragment.

EXPERIMENTAL PROCEDURES

Reagents

All chemicals and reagents were purchased from Sigma (St. Louis, MO) or Aldrich (Milwaukee, WI) unless stated otherwise. **pNO-ASA** and **mNO-ASA** were purchased from Cayman Chemicals (Ann Arbor, MI). X-ASA derivatives, **GT-094**, and metabolites were synthesized as described in the literature [7;10]. The Comet Assay Kit was purchased from Trevigen Inc. (Gaithersburg, MD).

Cell cultures

HT-29 human adenocarcinoma cells were supplied by Dr. Genoveva Murillo (IIT Research Institute, Chicago, IL). Cells were maintained in RPMI 1640 medium, supplemented with 1% antibiotic-antimycotic, 1% L-glutamine (200mM), 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA), and incubated in 5% CO₂ at 37 °C. RAW 264.7 mouse macrophage-like cells, provided by Dr. J. Cook (UIC, Chicago, IL), were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% penicillin-streptomycin, 10% fetal bovine serum and incubated in 5% CO₂ at 37°C. The mouse alveolar macrophage MH-S cell line was a generous gift from Dr J. Cook (UIC, 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 were cultured in α -MEM with 1% penicillin-streptomycin and 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA). HepG2 cells stably transfected with ARE-luciferase reporter gene were kindly provided by Dr. A.N. Kong (Rutgers University, Piscataway, NJ) and cultured in F-12 medium with 10% fetal bovine serum, 1% penicillin-streptomycin, 1% non-essential amino acids and 0.2 mg/mL insulin [11].

Proliferation assay—Cells were seeded in 96-well plates at a density of 2×10^4 cells/mL in 190 μ L media. After 24 h incubation, test samples were added to each well and the cells incubated for an additional 24, 48, or 96h. The cells were fixed by adding ice cold 20% trichloroacetic acid and 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 μ L of 0.4% sulforhodamine B (SRB) 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 μ 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. For Hepa 1c1c7 cells, cells were plated and treated as described for the NQO1 assay. After the cells were treated with test samples for 48 h, the medium was decanted, and 200 μ L of 0.2 % crystal violet (CV) solution in 2 % ethanol was added. After incubation at 37 °C for 10 mins, the plates were rinsed for 2 min with water and dried. The bound dye was then solubilized by incubation at 37 °C for 10–20 mins with 200 μ L of 0.5% SDS in 50% ethanol and agitation for 20 min. The absorption of CV was measured at 595 nm, and the IC₅₀ values were determined using GraphPad Prism 4.00.

NQO1 activity assay—For cultured hep 1c1c7 liver cells, the induction of NQO1 activity was assayed as described previously with minor modifications [12]. Briefly, cells were seeded in 96-well plates with proper density in 190 μ L of media. After 24 h of incubation, 10 μ L test

samples, DMSO or 4-bromoflavone (BF) as positive control were added to each well and the cells were incubated for an additional 48 h. The medium was decanted, and the cells were incubated at 37 °C for 10 min with 50 μ 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 μ L of reaction mixture [0.7 mg/mL bovine serum albumin (BSA), 0.3mg/mL (3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide) (MTT), 25 mM Tris-HCl, 0.01% Tween 20, 5 μ M FAD, 1 mM glucose-6-phosphate, 30 μ M NADP, 2 U/mL glucose-6-phosphate dehydrogenase, and 50 μ 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 MTT 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. Chemopreventive Index (CI=IC₅₀/CD) is calculated from IC₅₀ for inhibition of cell growth [13].

ARE-luciferase reporter assay—HepG2-ARE-Luc cells were plated in a 6-well plate for 48 h [14]. Cells were treated with DMSO, test samples, or positive control (BF) and incubated for an additional 8 h. The luciferase activity was determined according to the protocol provided by the manufacturer (Promega, Madison, WI). Briefly, cells were washed with cold PBS twice and lysed with 500 μ L reporter lysis buffer. After centrifugation, 20 μ L of the supernatant was used for determination of the luciferase activity by a luminometer (FLUOstar OPTIMA, BMG Labtechnologies, Germany). The luciferase activity was normalized to protein concentration using the BCA assay. The experiment was repeated on three separate cell passages (representative data is shown) and expressed as foldinduction over control (treated cells/DMSO treated cells $\pm$ SD).

NO_x assay—A solution containing substrate and porcine liver esterase (0.15 mg of enzyme/0.4 mL) in 50 mM phosphate buffer (pH 7.0, 0.4 mL total volume) was incubated for 10 min at 37 °C. Reaction was quenched by addition of acetonitrile (0.6 mL) and centrifugation for 10 min. The solution was filtered and analyzed by chemiluminescence. A chemiluminescence detector (Sievers Research Inc, Model 280i) attached to a computer was used to measure NO production by injections of reaction headspace gas at 5 and 10 min of reaction. The detector was calibrated using DEA/NO for which the rate of NO release at pH 7.4 is known [15]. Production of nitrite was assayed using the Griess reagent (0.5% sulfanilamide, 0.05% (N-1-naphthyl) ethylenediamine dihydrochloride) in 2.5% w/w H₃PO₄. Griess reagent (150 μ L) and reaction mixtures (50 μ L) were mixed and incubated in a 96-well plate; absorbance was measured at 530 nm in a microplate reader and compared to a NaNO₂ standard curve. Nitrate production was measured by extension of the Griess method, using a fresh solution of VCl₃ (80 mg in 1N HCl 10 mL) to reduce inorganic nitrate to nitrite. Construction of calibration curves in the presence and absence of GSH and esterase for both measurement of NO₂⁻ and NO₃⁻ showed that the thiol diminished the response of the assay to NO₃⁻, possibly by trapping of an intermediate in V-mediated reduction of NO₃⁻ (supplemental data). The difference between the absorbance values in the Griess/VCl₃ assay and the Griess assay indicates the absorbance due to reduced nitrate, when a KNO₃ standard calibration curve is used.

GSH Levels—The total cellular glutathione (GSH + GSSG) levels were measured by an enzymatic recycling procedure with some modifications. Briefly, HepG2 cells were seeded in 6-well plates. The cells were treated with DMSO and sample compounds. The cells were washed twice with PBS (50 mM, pH 7.4) and homogenized with 200 μ L ice cold 5% SSA (5-sulfosalicylic acid). Lysates were centrifuged (8,000 rpm) and supernatant was assayed for total glutathione levels by adding the reaction mixture [NADPH (0.3 mM), 5,5'-dithiobis-(2-nitrobenzoic acid) (0.6 mM) and GSH reductase (0.5 units/ml) in PBS (125 mM) with EDTA

(6.3 mM pH 7.5)]. The absorbance was measured at 412 nm at 25°C. The mean GSH concentration in DMSO vehicle treated cells measured at 10 min was 145 μ M. GSH concentrations in the samples were calculated based on a standard curve.

DNA damage in Hepa 1c1c7 cells using Single-Cell Gel Electrophoresis Assay (Comet assay)—This assay was performed according to the manufacturer's recommendations (Trevigen, Inc. Gaithersburg, MD). Briefly, Hepa 1c1c7 cells (3×10^5 cells/mL) were incubated with DMSO, **1a**, or **1b** (10 μ M and 25 μ M) 1h. After incubation, the cells were collected and centrifuged (1,500 rpm 5 min.). The supernatant was discarded and the cells were suspended in PBS (2 mL, Ca^{2+} and Mg^{2+} free). The cell suspension (30 μ l) was added to 300 μ l of 1% LMAgarose (37°C) and 50 μ l of the resulting solution was added to the CometAssay slide. Slides were placed at 4°C in the dark for 10 min and thereafter placed in lysis solution (2.5 M sodium chloride, 100 mM EDTA pH 10, 10 mM tris base, 1% sodium lauryl sarcosinate, and 1% Triton X-100) for 30 min. The slides were removed from the lysis solution; the slides were transferred in the dark to alkali solution (pH >13, 300 mM NaOH, 1 mM EDTA) for 40 min. After electrophoresis, slides were stained with SYBR Green (50 μ l), viewed with a fluorescence microscope, and analyzed using CometScore Freeware (<http://www.autocomet.com>) and the data presented as the percent DNA in the tail. Each sample contained at least 80 cells.

Griess assay—Macrophage cells were plated at a concentration of 12×10^4 cell/mL 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 LPS (lipopolysaccharide from E. coli 055:B5; Sigma, St Louis, MO) in the medium. Test compounds were added, and at the indicated time points, 100 μ L of the supernatant was removed and incubated with the Griess reagent (100 μ L; 0.5% sulfanilamide, 0.05% (N-1-naphthyl)ethylenediamine dihydrochloride, 2.5% H_3PO_4 and 97% H_2O 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_2^- concentration. Subtraction of $[\text{NO}_2^-]$ formed in cell incubations of nitrates in the absence of LPS, was used to determine NO_2^- production from endogenous NOS mediated production of NO.

Results

The structures of the compounds used in this study and their ability to be bioactivated to quinone methide or benzyl electrophiles are detailed in figure 1 and figure 2. Release of NOx species was measured in incubations with esterase to give a simulated metabolic profile. **mNO-ASA**, **oNO-ASA**, and **pNO-ASA** are chemically susceptible to nucleophilic substitution at carbon, giving denitration to NO_3^- as a product; in the case of **pNO-ASA**, inorganic nitrate was the sole product of denitration observed in vitro (Table 1; [10]). In incubations of high concentrations of **mNO-ASA** with esterase, all three NOx species were detectable (Table 1).

Both **pBr-ASA** and **pNO-ASA** were reported to activate ARE in HepG2 liver cells with high efficacy and similar potency [10]. To further explore the similarities and differences between the mechanism of action of **pBr-ASA** and **pNO-ASA**, the time course for ARE activation was measured using an ARE-luciferase reporter construct (Fig. 3). The similar time course for activation of ARE was observed for both NO-ASA and Br-ASA.

Dunlap et al. reported data for depletion of GSH, cell viability (measuring antiproliferative and cytotoxic actions), activation of ARE, and induction of NQO1 in liver cells for all NO-ASA and X-ASA derivatives [10]. It is informative to draw correlations between these actions of these compounds and their ability to be bioactivated to a quinone methide (Fig. 4). The quinone forming drugs rapidly deplete cellular GSH and are potent inhibitors of cell growth (with the

exception of **pMs-ASA**); and a reasonable correlation is seen between potency for inhibition of cell growth and for induction of NQO1. Evidence for the reactivity of the benzyl electrophile formed in bioactivation of **mBr-ASA** was obtained in the Comet assay measuring DNA single strand breaks in hepa 1c1c7 cells (Fig. 5). A similar concentration dependence was observed for **pNO-ASA** and **pBr-ASA**, which form the same quinone methide, whereas **mBr-ASA** and **mNO-ASA**, which form different benzyl electrophiles, differed; **mNO-ASA** showed no significant genotoxicity at the concentration studied (Fig. 5).

The antiproliferative activity of NO-NSAID compounds has been remarked on in a variety of cell lines, since the parent NSAIDs are not antiproliferative [16]. The NO chimera, **GT-094**, bears comparison with NO-ASA; **GT-094** contains an NSAID pharmacophore, and possesses similar actions in inhibiting Caco-2 colon cancer cell proliferation, reducing iNOS activity, and being effective in an animal model of CRC [7]. **GT-094** was also observed to be antiproliferative in a variety of cell lines (Fig. 6).

Activation of NQO1 in liver cells was previously studied in response to the range of X-ASA derivatives and compared to **mNO-ASA** and **pNO-ASA** [10]. These data provide one aspect of the correlation shown in figure 4, in which it can be seen that NQO1 induction is not a special feature of NO-ASA derivatives. The role of NO is often investigated with the use of PTIO, which among other actions can act as an NO scavenger. The effect of PTIO on induction of NQO1 in hepa 1c1c7 cells by **pNO-ASA** and **mNO-ASA**, revealed no significant inhibition by PTIO (Fig. 7).

Treatment of RAW 264.7 cells with the bacterial endotoxin LPS reliably induces expression of iNOS, the activity of which is assessed by measurement of inorganic nitrite (NO_2^-), a major product of NO oxidative metabolism, which can readily be quantified using the Griess assay. **GT-094** has been reported to inhibit iNOS induction in these cells [7]. All four compounds studied, **pNO-ASA**, **pBr-ASA**, **mNO-ASA** and **mBr-ASA**, were observed significantly to reduce iNOS activity (Fig. 8); in the case of the quinone donors and **mBr-ASA**, iNOS activity was reduced almost to basal levels.

Discussion

NO-NSAID structural classification

Figure 1 shows the structures of representative NO-NSAIDs. As prodrugs, NO-NSAID are designed to liberate an NSAID and an NO-donating nitrate through enzymic cleavage of a labile linker in biological milieu. There are two general classes of NO-NSAIDs: those designed to provide a second moiety with potential biological activity; and those without this feature. The NO-ASA, NCX 2219, is an example of the former, which is designed to liberate the ferrulic acid antioxidant moiety, however, both classes have in common the liberation of a parent nitrate; for example, both NCX 2219 and NCX 4215 are designed to liberate the parent nitrate, 4-nitrooxybutanol (NCX 3000). Based upon chemical principles and published literature, NCX 3000 is predicted to possess simple metabolic properties akin to isosorbide mononitrate; in contrast, NO-NSAIDs that liberate parent nitrates containing a hydroxybenzyl group (NCX 4015, pHBN, oHBN) are anticipated to have more complex metabolic properties. In the 1980's, comparable hydroxybenzyl derivatives were reported by Widlanski and co-workers to liberate an electrophilic quinone methide at an enzyme active site, leading to enzyme inhibition [17; 18]. In simile, both **pNO-ASA** and **o-NO-ASA** would be predicted to act as caged quinone methides, leading to covalent modification of biomolecules after bioactivation in a biological milieu.

Why are NCX 4017 and NCX 4042 unsatisfactory control compounds?—Several papers studying the biological activity of **mNO-ASA** and **pNO-ASA** have used as control

compounds, NCX 4017 and NCX 4042, respectively; these are denitrated derivatives of the NO-ASA. These compounds and similar “R-ASA” derivatives lack one important feature required for an appropriate control compound, a good leaving group (Fig. 2). From the 1950’s, benzyl nitrates were subject to numerous elegant studies as mechanistic probes, directed in part at the S_N1 - S_N2 continuum [19;20;21;22;23;24]. Benzyl nitrates were selected simply because nitrate (the conjugate base of nitric acid) is a good leaving group towards nucleophilic substitution, and the benzyl carbon readily rehybridizes to an sp^2 carbocation. Thus, the X-ASA derivatives, conisogenic with NO-ASA, represent appropriate control compounds for unequivocal definition of the role of NO from NO-ASA; in X-ASA, X is a good leaving group (chloride, bromide, methanesulfonate) (Fig. 1). As depicted in figure 2, X-ASA and NO-ASA are bioactivated to electrophiles by esterase action, whereas R-ASA, reported as control compounds in the literature, are not bioactivated. R-ASA lack both NO-donor capability and the ability to be bioactivated to electrophiles in the biological milieu. Differences in biological activity between NCX 4042 and NCX 4040 reported in the literature [25; 26], are due to NCX 4040 being a quinone methide donor, and not associated with donation of NO.

NO-ASA as quinone methide donors

Basic chemical principles and literature reports from the 1980’s predict that **pNO-ASA** and **oNO-ASA** will undergo ester hydrolysis leading to rapid loss of NO_3^- and formation of a quinone methide. A quinone methide is a highly reactive electrophile and Michael acceptor, the generation and reactivity of which has been rigorously studied using laser flash photolysis [27;28]. The half-life for uncatalyzed addition of water to the quinone methide formed from **pNO-ASA** is 200 ms; as a short-lived reactive intermediate, a quinone methide is trapped by GSH, other biomolecules, and even weak nucleophiles such as water (Fig. 2). It is predicted that the biological activity of **pNO-ASA** and **oNO-ASA** will be replicated by X-ASA conisogeners that are not able to release NO or NOx.

Defining the role of NO using X-ASA

The exact mechanisms of biotransformation of NO-ASA hybrid nitrates to NO are undefined [6]. The majority of nitrates readily undergo denitration mediated by glutathione *S*-transferase (GST), cytochrome P450 oxidoreductase (CYP), NADPH cytochrome P450 reductase (CPR), and aldehyde dehydrogenase type-2 (ALDH2), to yield NO_2^- [29;30]. Nitrates susceptible to nucleophilic substitution at C, such as **mNO-ASA**, **oNO-ASA**, and **pNO-ASA**, will be denitrated to release various amounts of NO_3^- ; in the case of **pNO-ASA**, inorganic nitrate is the sole product of denitration in vitro (Table 1; [10]). In incubations of high concentrations of **mNO-ASA** with esterase, all three NOx species were detectable [Table 1], but after administration of **mNO-ASA** in extensive metabolic studies in rats [31;32;33;34;35], plasma NO_2^- levels were reported to be unchanged ($\sim 1 \mu M$) over 6 h, and plasma NO_3^- was elevated from $\sim 20 \mu M$ to $\sim 130 \mu M$. Denitration of nitrates directly generates NO_3^- and NO_2^- as products; since these are also products of NO oxidation, unequivocal evidence for NO release is easily obtained. Many contemporary pharmacokinetic studies measure NOx ($NO + NO_3^- + NO_2^-$), thereby losing important metabolic and mechanistic information and obfuscating the meaning of NO donor; there is no doubt that NO-ASA (in all its forms) is an NOx donor! To simplify the question of the role of NO, we postulate that if the biological activity of NO-ASA is recapitulated by the conisogenic X-ASA derivative, then this activity is not associated with any NO donor properties of NO-ASA.

Quinone forming NO-ASA and X-ASA

Hulsman et al. put forward a postulate similar to that above, but limited the biological activity studied to cytotoxicity and limited the NO-ASA versus X-ASA comparison to **pNO-ASA** versus **pCI-ASA** [9]. These workers examined cytotoxicity in colon cancer cells and assayed

DNA damage and PARP cleavage as a measure of apoptosis: the activity of both the X-ASA and NO-ASA derivatives was cytotoxic and apoptotic, with **pCl-ASA** more than twofold higher in potency. It was concluded that both X-ASA and NO-ASA formed the same cytotoxic quinone with no involvement of NO or of ASA.

Two different quinone methide forming X-ASA derivatives, **pBr-ASA** and **pMs-ASA** were compared to **pNO-ASA** in liver cells in an independent study [10]. This research was designed to test whether a further attribute of NO-ASA previously attributed to NO [36], could be explained instead by quinone methide formation; induction of phase 2 (detoxifying) enzymes via activation of antioxidant/electrophile response element (ARE). Quinone electrophiles are well-known to be able to activate ARE and induce phase 2 enzymes, such as NAD(P)H dependent quinone oxidoreductase (NQO1) [37;38]. Covalent modification of the cysteine residues of the redox sensor protein Keap1 by chemopreventive agents such as Michael acceptors has been shown to lead to activation of ARE via Nrf2 [39]. Although activation of pathways via Nrf2 and Keap1/Nrf2 may not be obligatory for phase 2 enzyme induction, Nrf2 gene knockout experiments show substantial reductions in the ability of chemopreventive agents to induce NQO1 [40;41]. Nevertheless, the quinone methide metabolites, anticipated to be released from X-ASA and NO-ASA derivatives, would be expected to activate ARE via electrophilic mechanisms including Keap1 modification.

In Hepa 1c1c7 and HepG2 liver cells, **pBr-ASA** and **pNO-ASA** were observed to be equally cytotoxic (inhibition of Hepa 1c1c7 cell growth: $IC_{50}(\text{pBr-ASA}) = 13 \mu\text{M}$; $IC_{50}(\text{pNO-ASA}) = 12 \mu\text{M}$), and to induce NQO1 (concentration needed for 1.5-fold induction: $C_{1.5}(\text{pBr-ASA}) = 3 \mu\text{M}$; $C_{1.5}(\text{pNO-ASA}) = 7 \mu\text{M}$). In addition, **pBr-ASA** and **pNO-ASA** were observed to be equally reactive towards GSH both (a) in test tube incubations with esterase and (b) in HepG2 cells where stoichiometric depletion of GSH was measured after only 10 min incubation [10]. Both **pBr-ASA** and **pNO-ASA** were reported to activate ARE in HepG2 cells with high efficacy and similar potency. To further demonstrate the likely identical mechanism of action of **pBr-ASA** and **pNO-ASA**, the time course for ARE activation was measured using an ARE-luciferase reporter construct (Fig. 3). These results with **pBr-ASA** confirm the quinone methide postulate for **pNO-ASA** as it relates to cell death, and extend the quinone methide postulate to activation of ARE and induction of NQO1.

Unexpectedly, **pMs-ASA**, was reported to have lower reactivity towards cellular GSH than **pBr-ASA** and **pNO-ASA**, and consequently was observed to be less potent in inhibition of cell growth ($IC_{50} = 50 \mu\text{M}$) and induction of NQO1 ($C_{1.5} = 40 \mu\text{M}$). Furthermore, **mBr-ASA** and **mNO-ASA** were both observed to inhibit cell growth and induce NQO1, although with significantly lower potency than **pBr-ASA** and **pNO-ASA** [10]. Since **mBr-ASA**, and **mNO-ASA** cannot form a quinone methide, an alternative mechanism must come into play.

NO-ASA and X-ASA as electrophile donors

The *o*- and *p*-substituted derivatives of X-ASA and NO-ASA are bioactivated to a quinone methide, but bioactivation of the *m*-substituted derivatives of X-ASA and NO-ASA leads instead to a benzyl electrophile as shown in figure 2. The observation of NQO1 induction and antiproliferative activity for **mBr-ASA** and **mNO-ASA** indicates that this less reactive electrophile is able trigger similar pathways to the quinone. Figure 4 shows a secondary analysis of the data reported in Dunlap et al. for all NO-ASA and X-ASA derivatives [10]: the quinone forming drugs rapidly deplete cellular GSH and are potent inhibitors of cell growth (with the exception of **pMs-ASA**); and a reasonable correlation is seen between potency for inhibition of cell growth and for induction of NQO1. These correlations suggest that the highly reactive quinone methide engenders high potency; but also that the benzyl electrophile ($\text{BrCH}_2\text{C}_6\text{H}_4\text{OH}$) produced from **mBr-ASA** is more reactive than that produced from **mNO-ASA** ($\text{O}_2\text{NOCH}_2\text{C}_6\text{H}_4\text{OH}$). Further evidence for the reactivity of the benzyl electrophile from

mBr-ASA was obtained in the Comet assay measuring DNA single strand breaks in hepa 1c1c7 cells (Fig. 5). The similar concentration dependence seen for **pNO-ASA** and **pBr-ASA** is compatible with the fact that they generate the identical quinone electrophile and appear to undergo bioactivation at similar rates. In contrast, **mBr-ASA** and **mNO-ASA**, undergo bioactivation to different benzyl electrophiles, consequently, **mBr-ASA** was significantly genotoxic, but **mNO-ASA** at the same concentration was not.

NO chimeras and NO-NSAIDs

NO chimeras have been defined as drugs that incorporate an NO mimetic nitrate group with at least one additional pharmacophore [7;8]. In contrast to NO-NSAIDs that, by design, contain a labile linker and release an intact FDA approved drug, a labile linker is not a requirement in an NO chimera, and the ancillary pharmacophore is not required to be an intact clinical drug. **GT-094**, under development as a lead compound for discovery of nitrates chemopreventive in CRC, in addition to the NO mimetic nitrate group, contains two ancillary pharmacophores known to have chemopreventive properties in man: an NSAID (a thiosalicylate ester [42;43; 44]); and a disulfide (garlic contains chemopreventive disulfides [45;46]). Proof-of-principle for **GT-094** was obtained in the standard animal model for CRC, the formation of aberrant crypt foci (ACF) in the colon of rats treated with azoxymethane (AOM) carcinogen: delivered after carcinogen administration in this AOM/rat model, **GT-094** reduced ACF number by 45%, reduced colon crypt proliferation by 30–69%; reduced iNOS levels by 33–67%; and elevated levels of the anti-oncogene p27 in the distal colon [7]. **GT-094** was reported to act as an antiproliferative agent that reduced ACF in the rat/AOM model and as shown in figure **GT-094** is antiproliferative in a variety of cell types. **GT-094** was also observed to reduce iNOS expression both in vitro and in vivo [7]. The strongest evidence for NO-ASA actions on iNOS was reported in the recent rat/AOM study delivering high dose **mNO-ASA** in feed, where both antiproliferation and iNOS inhibition were observed [47]; in contrast, two previous NO-ASA animal studies did not show such effects. In the recent rat/AOM work on **mNO-ASA**, at high dose, a significant reduction in both the incidence and multiplicity of noninvasive colon adenocarcinomas was reported after 46 weeks; **mNO-ASA** was antiproliferative; and was anti-inflammatory reducing iNOS activity, but did not inhibit iNOS expression. In contrast, **GT-094** inhibited AOM-induced iNOS expression in the rat colon and LPS-induced expression in vitro, and reduced AOM-induced PARP cleavage and increased p27 expression in the distal colon of AOM treated rats. **mNO-ASA** and **GT-094** are both antiproliferative agents and both have generated positive results in animal models of CRC chemoprevention; clearly, a further similarity is that the “linker” group (disulfide in **GT-094** and hydroxybenzyl in **mNO-ASA**) contributes to biological activity.

NO-ASA mechanism of action

An important first point to be made is that the biological activity of the quinone donors, **oNO-ASA** and **pNO-ASA**, is different in many ways from that of **mNO-ASA**. Indeed, in some respects, the activity of **mNO-ASA** is closer to that of **GT-094**. The differences between quinone forming NO-ASA and **mNO-ASA** have been known in the literature for some time, but have not been correlated with quinone formation. For example, in colon cancer cells, **pNO-ASA** and **oNO-ASA** are potent cytotoxins, inducing apoptotic and atypical cell death, in contrast to **mNO-ASA** and the butyl nitrate NO-ASA derivative, NCX 4215 [48]. The rapid release of NO_x (NO₃⁻ + NO₂⁻) by **oNO-ASA** and **pNO-ASA** was correlated with cytotoxic/apoptotic activity, leading to the conclusion that the rate of bioactivation of NO-ASA to NO correlated with the observed bioactivity. Although bioactivation is responsible for observed differences in activity in vitro, it is bioactivation to a quinone that is key, not bioactivation to NO; **pBr-ASA** and **pCl-ASA** replicate the in vitro activity of **pNO-ASA**. The use of scavengers specific for NO would assist in defining mechanistic roles for NO, however, commonly used traps such as oxyhemoglobin and PTIO, are not specific nor inert in a biological milieu [49];

50]. Nevertheless, the effect of PTIO on induction of NQO1 was studied, revealing no significant inhibition by PTIO (Fig. 7).

Excellent and important papers continue to support the exploration of hybrid nitrates for cancer therapy and prevention, particularly from Rigas and co-workers. Unfortunately, the three NO-ASA isomers are often discussed as a single family, when clearly the chemical structure of **mNO-ASA** results in biological activity that sets it apart from its two isomeric counterparts. An important recent paper, reported the suppression by NO-ASA of microsatellite instability (MSI) in mismatch repair-deficient and hereditary nonpolyposis CRC cells [51]. Again, the different properties of the quinone donor NO-ASA isomers were apparent in every aspect of this paper. **pNO-ASA** and **oNO-ASA** were potent antiproliferative agents ($0.1 < IC_{50} < 2 \mu M$) in contrast to **mNO-ASA** ($60 < IC_{50} < 150 \mu M$); in the suppression of MSI, the order of potency was consistent (**pNO-ASA** ~ **oNO-ASA** > **mNO-ASA**), with **mNO-ASA** 10–100 fold less potent than the quinone forming NO-ASA isomers. It would be of interest to assay the activity of quinone forming X-ASA drugs towards MSI instability.

There is a likely role for quinone induced depletion of cellular GSH in eliciting the biological activity of quinone donor NO-ASA derivatives, at least in cell cultures. The in vitro bioactivity of NCX 4040 and NCX 4016 has been reported to result from induction of oxidative stress, beginning with the depletion of GSH, although this was attributed to the release of sustained amounts of NO [52;53]. The rapid depletion of GSH by **pNO-ASA**, **pBr-ASA**, and **oBr-ASA** in liver cells has been discussed above. Inevitably, cysteine residues of sensor proteins are likely to be discovered as targets for the quinone methide and benzyl electrophiles generated by NO-ASA and X-ASA.

Anti-inflammatory activity

The murine macrophage-like RAW 264.7 cell line is routinely used to examine the ability of agents to block cellular inflammatory response, and represents a model for anti-inflammatory action towards 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 (NO_2^-). Although not a direct measure of NO production, NO_2^- is a major product of NO oxidative metabolism which can readily be quantified using the Griess assay. Controlling for NO_2^- production from nitrate denitration, permits the assay to be used as a reliable measure of iNOS activity and hence to assay anti-inflammatory agents.

A number of cancer chemopreventive agents explored in vivo and in vitro are Michael acceptor electrophiles. Induction of phase 2 enzymes, including NQO1, has been used to define the Chemopreventive Index of such agents [13]. In addition to ARE activation, several of these electrophiles have been shown to have anti-inflammatory activity, including inhibition of induction of COX-2 and iNOS in response to agents such as LPS. One proposed mechanism is covalent modification of p65, inhibiting transcription of NF-kB-dependent genes and hence expression of NF-kB-dependent gene products including cytokines, COX-2, and iNOS [54]. As quinone donors, it is therefore predicted that both **pNO-ASA** and **pBr-ASA** will reduce iNOS activity in LPS-induced macrophages. The same prediction for **mNO-ASA** and for **mBr-ASA** is less certain because of the lower reactivity of the benzyl electrophiles produced on bioactivation. In RAW 264.7 cells, all four compounds were observed significantly to reduce iNOS activity (Fig. 8); in the case of the quinone donors and **mBr-ASA**, iNOS activity was reduced almost to basal levels. These data are entirely compatible with the results presented above, in which **mBr-ASA** is seen to produce a benzyl electrophile with more reactivity and activity than that from **mNO-ASA**. Figure 8 also demonstrates that the therapeutic index for each of **pNO-ASA**, **pBr-ASA**, and **mBr-ASA** provides separation of cytotoxic and anti-

inflammatory activities. Further work is in progress to explore and control the therapeutic index for such agents.

Summary

The NO-ASA family of NO-NSAIDs, notably NCX 4016 (**mNO-ASA**) and NCX 4040 (**pNO-ASA**), have been extensively researched; the search terms NO-ASA or nitroaspirin generate 160 hits in PubMed. These two isomeric hybrid nitrates differ only in the substitution of the “linker” an hydroxybenzyl group assumed to be biologically inert. It is only over the last year that this “inert” linker has been recognized as being the dominant contributor in vitro to biological activity of potential therapeutic value in cancer chemoprevention or in cancer therapy. In this paper, evidence is presented that: (1) the antiproliferative; (2) the chemopreventive (ARE activation – phase 2 enzyme induction); and (3), the anti-inflammatory activity of NO-ASA is replicated by X-ASA conisogeners that are incapable of acting as NO donors.

With respect to antiproliferative/cytotoxic, genotoxic, chemopreventive, and anti-inflammatory actions in cell cultures:

1. **pBr-ASA** and **mBr-ASA** are conisogenic with NO-ASA, but are not NO donors.
2. The biological activity of **pNO-ASA** (NCX 4040) is replicated by **pBr-ASA**.
3. Both **pNO-ASA** and **pBr-ASA** are bioactivated to the same quinone methide.
4. The biological activity of **mNO-ASA** (NCX 4016) is replicated by **mBr-ASA**.
5. **mNO-ASA** and **mBr-ASA** are bioactivated to different benzyl electrophiles.
6. The observed activity is likely initiated by the quinone and benzyl electrophiles trapping thiol biomolecules; leading to depletion of GSH; and modification of Cys-containing sensor proteins.
7. The corollary is that all NO-NSAIDs containing a hydroxybenzyl linker will manifest biological activity resulting from bioactivation to electrophilic metabolites; the corollary does not extend to NO-NSAIDs that contain different linker moieties.

The quinone forming ASA derivatives generated quinone methides that rapidly react with GSH and presumably thiol-rich sensor proteins to activate ARE and induce NQO1. For these compounds, the Chemoprevention Index was relatively low, however, such compounds may be exploited for their cytotoxicity in cancer therapy, if quinone release can be targeted to tumor cells. These quinone donors are cytotoxic and pro-apoptotic, and bear similarity to other quinoid drugs known to inhibit cell growth and induce apoptosis via reaction with GSH and protein thiols [55;56].

Hulsman et al. concluded that for **pNO-ASA** the “presumed invisible” linker is in fact solely responsible for the antitumor effect, and that both the NO-donating group and ASA are passive bystanders [9]. In an in vivo context, this conclusion is not entirely safe, because it assumes that esterase action on intact drug will always precede nitrate reductase action. Nevertheless, for quinone donor NO-NSAIDs, the dominant NO_x metabolite will be NO₃⁻, the least likely of all NO_x species to exert meaningful biological activity. The situation for **mNO-ASA** is more complex, since the action of esterase yields NCX 4015, which can act as both an electrophilic effector, and a substrate for nitrate reductase activity, producing NO₃⁻, NO₂⁻, and potentially NO. Hybrid and related nitrates have provided very positive results in vivo [47]. Importantly, nitrates require reductive, metabolic bioactivation to liberate NO bioactivity, which is often poorly replicated in vitro [30]. The cytotoxic and cytoprotective characteristics observed in cell cultures are common for NO-ASA and conisogenic agents without NO-donating

properties, however, it must be remembered that the NO bioactivity provided by hybrid nitrates in vivo provides proven therapeutic benefits; especially in mitigation of NSAID gastrotoxicity. Finally, it is worth noting a key feature shared by both quinones and NOx: the capacity to modify cysteine thiols in a biological milieu; thereby initiating or inhibiting signaling cascades and biological function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Chemical structures of selected NO-NSAID and NO-ASA derivatives, showing parent aliphatic nitrates and control compounds reported in the literature. R-ASA derivatives are inert control compounds that do not mimic the chemical reactivity of NO-ASA; whereas, X-ASA are biologically reactive control compounds that do mimic the reactivity of NO-ASA. The biological activity of neither R-ASA, nor X-ASA derivatives can be attributed to donation of NO. NCX terminology reflects compound codes assigned by Nicox SA; both pCl-ASA and **pBr-ASA** are reported in the literature to recapitulate the cell killing activity of NCX 4040 (**pNO-ASA**).

Figure 2.

NO-ASA are a subset of X-ASA derivatives. X-ASA possess a good leaving group (X = Cl, Br, MsO, ONO₂,) that allows formation of a quinone methide or benzyl electrophile; subsequent reaction with biomolecules is fast for reactions of quinone methides or slower for reactions with a benzyl electrophile. R-ASA compounds have no good leaving group and therefore do not form a quinone or benzyl electrophiles and do not covalently modify biomolecules (including GSH) directly.

Figure 3. ARE-luciferase reporter induction in HepG2 cells after 4, 8, 12, and 24 h incubation with drug (20 μ M): UPPER **pBr-ASA**; LOWER **pNO-ASA**. Fold induction is relative to the DMSO vehicle.

Figure 4.

Activity of X-ASA derivatives in liver cell cultures. UPPER: Correlation of GSH depletion after 10 min incubation with potency for inhibition of cell growth measured at 48 h. **mMs-ASA** was observed not to inhibit cell growth < 100 μ M. LOWER: Correlation of antiproliferative potency (at 48 h) with potency for induction of NQO1 (concentration required to give 1.5-fold induction of NQO1 at 48 h). Correlations are derived from primary data reported in ref [10].

Figure 5. Single strand DNA damage as measured by Comet assay in hepa 1c1c7 cells after incubation with X-ASA test compounds for 1h. Solid bars are *p*-derivates; open bars are *m*-derivatives; data shows mean and s.d.

Figure 6.

Concentration response curves for inhibition of cell growth by **GT-094** in (squares) HT-29 cells incubated with **GT-094** for 48 h, $\text{IC}_{50} = 59 \pm 1 \mu\text{M}$; (triangles) Hepa 1c1c7 cells incubated with **GT-094** for 48 h, $\text{IC}_{50} = 38 \pm 3 \mu\text{M}$; (circles) RAW 264.7 cells incubated with **GT-094** for 24 h, $\text{IC}_{50} = 51 \pm 1 \mu\text{M}$. Cell growth is represented as % relative to vehicle control; whereby 0% growth is the cell count at time zero and 100% is the cell count at $t = 24$ or 48 h in the vehicle control cultures. Sigmoidal dose-response curves (shown) were used to obtain IC_{50} values.

Figure 7.

Induction of NQO1 activity in hepa1c1c7 cells by **mNO-ASA** with (solid) and without (open bars) PTIO (50 μ M) preincubation. The NQO1 activity is expressed as fold induction relative to the DMSO solvent control. Induction of NQO1 activity in hepa1c1c7 cells by **pNO-ASA** with (solid) and without (open bars) PTIO (50 μ M) preincubation. The NQO1 activity is expressed as fold induction relative to the DMSO solvent control.

Figure 8.

Anti-inflammatory and cytotoxic activity of **mNO-ASA**, **pNO-ASA**, **mBr-ASA**, and **pBr-ASA** in RAW 264.7 cells. Inhibition of LPS-induced induction of iNOS as detected by NO_2^- measurement using Griess assay (solid bars). Cells were pre-incubated for 30 min with drugs before treatment with 1 $\mu\text{g}/\text{ml}$ of LPS and incubation for 20 h. iNOS activity is represented as percentage relative to vehicle control (DMSO); whereby 100% is in presence of 1 $\mu\text{g}/\text{ml}$ LPS and 0% is in absence of LPS. Cell viability is measured in the same experiment in parallel to NO_2^- detection using MTT assay (open bars). Cell viability is represented as percentage relative to vehicle control where 100% is cell density in presence of 1 $\mu\text{g}/\text{ml}$ LPS.

Table 1

NOx release from NO-ASA after 10 min incubation with esterase

Substrate	[substrate], μM	[NO], μM	[NO ₂ ⁻], μM	[NO ₃ ⁻], μM
mNO-ASA	100	nd	nd	nd
mNO-ASA	2200	0.26 ± 0.02	3.0 ± 0.05	nd
pNO-ASA	100	nd	nd	96.6 ± 3.51
pNO-ASA	2200	nd	4.13 ± 0.03	>300

From ref [10]; nd = not detectable above detection limits