Comparison between NO-ASA and NONO-ASA as safe anti-inflammatory, analgesic, antipyretic, anti-oxidant prodrugs

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Abbreviations: NO, nitric oxide; NSAIDs, nonsteroidal anti-inflammatory drugs; ASA, aspirin; NO-ASA, 3-nitrooxyphenyl acetylsalicylate; NONO-ASA, O\(^2\)- (acetylsalicyloxymethyl)-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate; LPS, Lipopolysaccharide; PGE\(_2\), Prostaglandin E\(_2\); MDA, malondialdehyde; SOD, Superoxide dismutase.
Abstract

Chronic inflammation is an underlying etiological factor in carcinogenesis; NSAIDs and their chemically-modified NO-releasing prodrugs (NO-NSAIDs) are promising chemopreventive agents. Head-to-head comparison between two NO-aspirins possessing different NO-donor groups: an organic nitrate (NCX-4016, NO-ASA) or N-diazeniumdiolate (CVM-01, NONO-ASA) as anti-ulcerogenic, analgesic, anti-inflammatory, and anti-pyretic agents. All drugs were administered orally at equimolar doses. Anti-ulcerogenic: 6h post-administration, number/size of hemorrhagic lesions in stomachs from euthanized animals were counted. Tissue samples were frozen for PGE₂, SOD, and MDA determination. Anti-inflammatory: 1h after drug administration, the volume of carrageenan-induced rat paw edemas was measured for 6h. Anti-pyretic: 1h after dosing, fever was induced by LPS (ip) and body core temperatures measured for 5h. Analgesic: time-dependent analgesic effect of prodrugs was evaluated by carrageenan-induced hyperalgesia. Drugs were administered 30 min after carrageenan. NCX-4016 and CVM-01 were better analgesic and anti-inflammatory agents compared to aspirin, but equipotent to each other. Despite a drastic reduction of PGE₂ in stomach tissue, both prodrugs were devoid of gastric side effects. Lipid peroxidation induced by aspirin was higher than that observed by prodrugs. SOD activity induced by both prodrugs was similar, but about 2-fold higher than that induced by aspirin. CVM-01 is as effective as NCX-4016 in anti-inflammatory, analgesic, and anti-pyretic assays in vivo, and it showed an equivalent safety profile in stomach. These results underscore the use of N-diazeniumdiolate moieties in drug design.
Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are a diverse group of compounds used worldwide to treat predominantly pain, fever, and inflammation. However, every day there are new studies supporting the fact that NSAIDs and, more importantly, their chemically-modified prodrugs, are expanding the repertoire of medical applications to include the prophylactic prevention of a wide variety of human diseases such as atherosclerosis (Yasuda et al., 2008; Zhao et al., 2008) thrombosis (FitzGerald, 2003; Yasuda et al., 2008) cancer (Chan et al., 2007; Flossmann and Rothwell, 2007; Orido et al., 2008; Spitz et al., 2009) Alzheimer’s disease (Davies et al., 2001; Szekely et al., 2008; Vlad et al., 2008) and any other disease for which chronic inflammation is an etiological factor. The major anti-inflammatory and analgesic mechanism of action of NSAIDs is the inhibition of cyclooxygenase enzymes (COX-1 and COX-2). As a result of differences in cellular localization and tissue expression, COX-1 and COX-2 produce a distinct set of prostaglandins, which depending on their type and tissue localization operate as “normal” physiologic regulators or as proinflammatory molecules. Therefore, NSAIDs (including COX-2 selective inhibitors, coxibs) inhibit not only those prostaglandins involved in the inflammatory response, but also those responsible for maintaining homeostasis (Cryer and Feldman, 1998). This is one of the main reasons why the use of NSAIDs is often correlated with a relatively high incidence of adverse gastrointestinal (Aalykke and Lauritsen, 2001; Fiorucci and Del Soldato, 2003; Schaffer et al., 2006) and/or cardiovascular (Scheiman and Fendrick, 2007) side-effects, and it is the main reason behind the withdrawal of highly selective COX-2 inhibitors such as Vioxx® and Bextra® (Jaksch et al., 2008).

To overcome this problem, comprehensive research studies have been carried out to modify the chemical structure of classical NSAIDs by forming hybrid (mixed) prodrugs, which upon metabolism, release the parent NSAID and a second biologically active molecule which decreases or counteracts its mechanism-based toxicity (Wallace, 2008). In this regard, there are
three main classes of hybrid NSAIDs; a) the nitric oxide-releasing NSAIDs (NO-NSAIDs) (Davies et al., 2001; Fiorucci et al., 2007; Stefano and Distrutti, 2007) b) the hydrogen sulfide-releasing NSAIDs (HS-NSAIDs) (Fiorucci et al., 2007; Wallace et al., 2007) and c) the phosphatidylcholine-conjugated NSAIDs (Kurinets and Lichtenberger, 1998; Anand et al., 1999; Lichtenberger et al., 2001) by far, the most studied type of hybrid NSAIDs are the NO-NSAIDs.

Nitric oxide-releasing NSAIDs were designed based on the assumption that NO (a potent vasodilator and inhibitor of leukocyte adherence to the gastric vascular endothelium) released from them would mimic most of the beneficial biological effects attributed to prostaglandins in the gastrointestinal tract (Martin and Wallace, 2006; Stanek et al., 2008). This approach effectively yielded safer anti-inflammatory, analgesic, anti-pyretic, and chemopreventive prodrugs in which the NO-donor moiety is organic nitrate (-ONO₂) (Wallace et al., 1994). There are many other types of NO-donors described in the literature, but just a few have been employed to form new NO-NSAIDs. Recent publications have described the use of N-diazen-1-ium-1,2-diolates (Velazquez et al., 2007; Velazquez et al., 2008) which represent improved NO-donors considering that unlike organic nitrates, they do not require metabolic activation to release NO, they release twice as much NO compared to organic nitrates, and they can be synthesized from a wide variety of secondary amines, which gives N-diazen-1-ium-1,2-diolates a lot more versatility over nitrates. Another advantage is that esterase-mediated hydrolysis of CVM-01 (NONO-ASA) generates both NO and ASA simultaneously in the same compartment, whereas the nitrates require two different activation steps to accomplish this: an ester hydrolysis and a separate nitrate reduction, which can occur in different places. This is a potential disadvantage for the nitrates if the NO-mimetic effect is most needed at the site where the freshly formed aspirin is irritating the tissue.

In the present study, we investigated and compared the anti-inflammatory, analgesic, anti-pyretic, and chemopreventive properties of one NO-aspirin possessing an organic nitrate (NCX-
4016, NO-ASA) with those of a NONO-aspirin possessing a N-diazen-1-ium-1,2-diolate (CVM-01, NONO-ASA) (Fig 1). This head-to-head comparison was aimed to determine if there is a statistically significant difference between hybrid NSAIDs possessing distinct NO-donor groups, and to find possible mechanistic differences that might offer additional evidence to support the use of one over the other.
Methods

Chemicals: 3-nitrooxyphenyl acetylsalicylate (Li-yuan et al., 2004) (NCX 4016), and O^2- (acetylsalicyloxy)methyl-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (Velazquez et al., 2005) (CVM-01) were synthesized following procedures described in the literature. Lipopolysaccharide (LPS) from *E. coli*, and carrageenan were purchased from Sigma (St. Louis, MO, USA). Kits used for determination of PGE_2, lipid peroxidation, superoxide dismutase, and total nitrate were from Cayman Chemical (Ann Arbor, MI).

Experimental groups and treatments:

Animals: Male Wistar rats (at least 5 per group) weighing 180–200 g were obtained from Charles River Laboratories International (Wilmington, MA). The rats were fed standard laboratory chow and water. All experimental procedures described below were approved by our institutional animal research committees and were performed in accordance with nationally approved guidelines for the treatment of laboratory animals. Rats were fasted for 48 h with free access to drinking water. Prodrugs were administered orally by gavage suspended in 1% carboxymethylcellulose solution, at equimolar doses: ASA (180 mg/kg), NCX-4016 (NO-ASA, 331 mg/kg) and NONO-ASA (323 mg/kg); the vehicle was 1% carboxymethylcellulose. Six hours post-administration, animals were euthanized by suffocation in a CO_2 chamber; stomachs were removed immediately after, cut along the greatest curvature, and rinsed with ice cold distilled water. The ulcer index (UI) was determined as described by Best et al (Best et al., 1984). Tissues from stomachs were excised and processed for measurement of Prostaglandin E_2 (PGE_2), malondialdehyde (MDA) and Superoxide dismutase (SOD) activity. Blood samples of the rat were taken by cardiac puncture into heparin-containing vials and used for determination of plasma TNFα and total nitrite/nitrate level.
Determination of PGE$_2$ levels: tissue from each rat stomach was removed and weighed (around 1g), placed in a test tube containing 5 mL of 0.1 M phosphate buffer (pH7.4), 1 mM EDTA, and 10 μM indomethacin. The tissue was homogenized and centrifuged for 10 min at 12,000 r.p.m. at 4°C. PGE$_2$ content in supernatant was determined in duplicate by an enzyme immunoassay kit following the protocol described by the manufacturer (Cayman Chemical, Ann Arbor, MI). Briefly, standard (50 μL) or homogenate (50 μL), enzymatic tracer (50 μL) and specific antiserum (50 μL) were mixed. After incubation for 17 h (overnight) at 4°C, the plates were washed with wash buffer and Ellman’s reagent (200 μL) was added into each well. The absorbance at 412 nm was measured after 1 h incubation at room temperature. Results are expressed as pg of PGE$_2$ per mg of protein. Proteins were determined by Biorad assay.

Index of lipid peroxidation: Stomach tissue (25 mg) was snap frozen and sonicated for 15 seconds at 40V over ice with 250 μL of radioimmunoprecipitation (RIPA) buffer (Contents: 25 mM TrisHCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) with PMSF (phenylmethylsulphonyl fluoride) as protease inhibitor. Homogenates were centrifuged for 10 minutes at 1,600 r.p.m. at 4°C. Thiobarbituric acid reactant substances (TBARS) content was measured in the supernatant stored on ice by a colorimetric kit following the protocol described by the manufacturer (Cayman Chemical, Ann Arbor, MI). Briefly, reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) at high temperature (90-100°C) in acidic conditions produced an adduct with a chromophore which absorbed visible light at 530-540nm. The results were expressed as picomoles of malondialdehyde per gram protein. Proteins were determined by Biorad assay.

Antioxidant enzymes: Superoxide dismutase (SOD) activity in the gastric mucosa was assayed using a colorimetric kit following the protocol described by the manufacturer (Cayman Chemical,
Ann Arbor, MI). Mucosal tissue (1 g) was homogenized with 5 mL of 20 mM HEPES buffer (pH 7.2) containing 1mM EGTA and 300 mM of sucrose solution. Homogenates were centrifuged at 1,500 r.p.m. for 10 minutes at 4°C. The supernatant was then removed and stored at -80°C until assayed. SOD activity was measured spectrophotometrically at 460 nm. As indicated in Cayman’s SOD assay kit, “this procedure utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine”. SOD activity is expressed as the amount of the SOD standard showing activity equivalent to the determined activity. The results are expressed as units (U) of SOD activity/mg protein. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutaion of the superoxide radical.

*Determination of plasma TNF-α*: Fresh samples of blood from the animals were taken by cardiac puncture into heparin-containing vials. The determination of plasma TNF-α was carried out by an enzyme immunoassay kit from R&D systems (Minneapolis, MN) following the protocol described by the manufacturer. Briefly, each sample (50 μL) was incubated with antibodies specific for rat TNF-α and washed three times with assay buffer. An enzyme-linked polyclonal antibody specific for rat TNF-α conjugated to horseradish peroxidase was then added to the wells. Following washing of unbound antibody-enzyme reagent, a substrate solution (containing tetramethylbenzidine, TMB, plus hydrogen peroxide) was added to the wells. The enzyme reaction yielded a blue product (oxidized TMB) that turned yellow when the stop solution (dilute hydrochloride acid) was added. The intensity of the color was determined by measuring the OD of the yellow color in a standard ELISA plate reader at 450 nm. Sensitivity of this TNF-α assay was determined by adding two standard deviations to the mean optical density value of 20 x zero standard replicates and calculating the corresponding concentration. The kit contains all reagents and standards needed for the TNF-α sensitivity assay. We also employed the
technical support services of R & D system to evaluate our raw data in their analysis tool. The results are expressed as pg/mL. sensitivity for TNF-α is estimated to be around 1.6 pg/mL.

**Determination of plasma NO content:** Plasma concentration of NO was quantified indirectly as the concentration of nitrate (NO$_3^-$) and nitrite (NO$_2^-$) levels in plasma, by the Griess reaction using an assay kit and following the protocol described by the manufacturer. Rat plasma was filtered using a 10 KD molecular weight cut-off filter from Millipore (Bedford, MA) before each analysis, to reduced background absorbance due to the presence of haemoglobin. After centrifugation for 10 min at 3000 rpm, samples (40 μL/well) were mixed with 10μL nitrate reductase mixture and incubated for 3 h after which Griess reagents 1 and 2 (50 μL each) were added. Absorbance was read after 10 min at 540nm using a plate reader. The concentration of nitrate/nitrite was calculated graphically from a calibration curve prepared from NaNO$_2$ standard solution, and it is expressed as micromolar nitrate.

**Anti-pyretic activity:** Fever was induced in animals as described previously (Pinto et al., 1998). Briefly, LPS (50 μg/kg, Sigma, St. Louis, MO, USA) was administered intra-peritoneally to the animals an hour before the administration of test drugs. Rectal temperature was measured by inserting a lubricated thermistor probe (external diameter: 3 mm) 2.8 cm into the rectum of the animal. The probe was linked to a digital reader, which displayed the temperature at the tip of the probe (± 0.1°C). The values displayed were manually recorded. Rectal temperatures were taken every hour for 5 h.

**Inflammatory Oedema Models:** Carrageenan (1%, 100μL, suspended in sterile saline solution, type IV lambda, from Sigma Chemicals (St. Louis, MO) was subcutaneously injected into the plantar surface of the right hind paw in rat following the protocol described by Winter et al
Paw volume was measured using a water displacement plethysmometer (Model 520, IITC/Life Sciences Instruments, Woodland Hills, CA) before carrageenan injection and thereafter at 1 h intervals for 6 h. The paw volume measured just prior to carrageenan injection was used as the control volume. Data are expressed as the change in paw volume (mL) at each time point.

*Determination of PGE$_2$ in rat paw exudates:* Rats were euthanized by asphyxiation in a CO$_2$ chamber. After cutting each hind paw at the level of the calcaneus bone, exudates (oedema fluid) were collected and processed for measurement of PGE$_2$, as described above.

*Induction and assessment of carrageenan-evoked hyperalgesia:* Rats were housed for 1 week prior to the experiment. They were weighed, marked for identification, and allowed to habituate to the thermostatically controlled test room (22°C) for at least 1 h prior to commencement of the experiment. Hindpaw inflammation was produced by intraplantar injection of carrageenan (100 μL of 1% carrageenan in sterile saline solution) into either hindpaw chosen at random. Suspensions of aspirin (180 mg/kg b.w.), NO-ASA (331 mg/kg b.w.), NONO–ASA (323mg/kg b.w.) or 0.5% w/v carboxymethylcellulose (vehicle, 10 mL kg/b.w.) were administered orally 1 h after carrageenan injection, and the mechanical nociceptive threshold determined 30 min after this and thereafter every 1 h for up to 5 h. The paw hyperalgesia was measured with an electronic pressure-meter. Each hindpaw was positioned in turn under a conical probe surface (tip radius approx. 1 mm) and gradually increasing pressure applied to the hindpaw surface until the animal vocalized at which point the measurement was terminated. Mechanical nociceptive threshold for both the injected and contralateral (i.e. non-injected) hindpaw were determined. The animals were tested before and after treatments and the results are expressed by the delta reaction force (g).
**Statistical Analysis.** All data are presented as the mean ± SEM, with sample sizes of at least 5 rats/group (unless otherwise specified). Comparisons between groups were performed using a one-way analysis of variance followed by the Student-\(t\) test.
Results

Gastric mucosal lesions: we conducted two different assays to determine the ulcerogenic properties of aspirin and its NO-releasing prodrugs. According to the gastric damage score (also described in the literature as “ulcer index”, or UI), animals receiving the vehicle (1% CMC solution) showed a normal glandular region on the surface of the stomach, and no ulcerative damage was observed (UI = 0). However, administration of aspirin (1 mmol/kg) resulted in extensive mucosal injury (UI = 50) to the glandular portion of the gastric fundus. Unlike aspirin, both NO-releasing aspirin prodrugs (NCX-4016 and CVM-01) did not produce significant ulcerative damage (UI = 2 and 3 respectively) compared to the parent NSAID at equimolar doses (1 mmol/kg), which represents a remarkable reduction (P < 0.01) in gastrointestinal toxicity (see Fig 2 A). The second screening assay we carried out to compare the gastroprotective effects of NO released from aspirin prodrugs was the erosion index (EI). In this assay, we found a similar trend to that obtained in the ulcer index test; NO-aspirin and NONO-aspirin produced a significant reduction in gastric ulceration (EI roughly around 3) compared to aspirin (EI = 11).

Gastric mucosal and paw exudate prostaglandin E2 content: we investigated the effect of aspirin and aspirin prodrugs NCX-4016 (NO-aspirin) and CVM-01 (NONO-aspirin) on prostaglandin E2 (PGE2) content in gastric mucous (Fig 3 A) and paw exudates (Fig 4 B). Animals treated with aspirin (1 mmol/kg p.o.) produced about 78% less PGE2 than rats in the control group. Similar reductions in PGE2 levels were observed when NO-aspirin and NONO-aspirin were administered orally (1 mmol/kg, suspension in 1% CMC solution). Prostaglandins are the main product of cyclooxygenase-mediated arachidonic acid metabolism in gastric mucosa, therefore, comparison of PGE2 content between control and drug-treated groups showed a clear and significant COX inhibition by aspirin and its two NO-releasing prodrugs (Fig 3 A). Subsequently, we tested whether NO-releasing aspirins exerted a similar decrease in PGE2 levels in the
carrageenan-induced paw edema model in rats. In this assay, aspirin (1 mmol/kg) induced a considerable decrease in PGE$_2$ levels (5 pg/mg protein) compared to control group (75 pg/mg). Treatment with NO-aspirin (1 mmol/kg) caused a similar decrease in PGE$_2$ content (9 pg/mg), whereas NONO-aspirin (1 mmol/kg) was about three-fold less potent than aspirin, since it only reduced PGE$_2$ levels to about 14 pg/mg protein (Fig 4 B).

**Lipid peroxidation:** Oxidative stress in gastric tissue was assessed by measuring the concentration of MDA in intact mucosa 6 h post administration of drugs (1 mmol/kg, po). MDA levels were 10.1 ± 4.3, 58.3 ± 2.2, 10.3 ± 1.2, 13.6 ± 1.9 nmol/mg protein, for vehicle, ASA, NO-ASA, and NONO-ASA treated animals, respectively (Fig 3B). ASA caused a 5.8-fold induction in MDA levels, whereas NO-ASA and NONO-ASA treated animals had MDA levels that were comparable to the vehicle treated animals.

**SOD activity:** In intact gastric mucosal (control group) SOD activity was 2.4 ± 0.5 U/mg protein. Following administration of ASA, a significant decrease in SOD activity (1.1 ± 0.2 U/ mg protein) was observed (P < 0.05). Treatment with 1 mmol/kg of NO-releasing aspirins had no effect on SOD activity, the respective values for NO-ASA and NONO-ASA being 2.7 ± 0.3 and 2.1 ± 0.4 U/mg protein (Fig 3C).

**Carrageenan-induced paw swelling:** The most common use for NSAIDs (including aspirin) is the treatment of inflammatory conditions. We wanted to compare the COX-dependent anti-inflammatory activity of ASA to that obtained with NO-ASA and NONO-ASA. After inducing inflammation, animals receiving vehicle showed a fast time-dependent increase in paw volume ($\Delta V = 0.8$ mL) after 2 h, and decreased gradually every hour thereafter until the end of the experiment (6 h) (Fig 4 A). In contrast, animals receiving ASA showed a weak inflammatory response ($\Delta V = 0.3$ mL) which was maintained 2-4 h after carrageenan injection, decreasing to
about $\Delta V = 0.2$ mL after 6 h. The anti-inflammatory effect registered in animals dosed with NO-releasing prodrugs was similar but a bit weaker ($\Delta V$ about 0.4 mL) compared to that observed with aspirin (Fig 4 A).

**Plasma TNFα levels:** We determined the inhibitory effect of ASA, NO-ASA, and NONO-ASA on proinflammatory cytokine tumor necrosis factor-α in plasma obtained from control and drug-treated animals. Administration of ASA (1 mmol/kg) increased TNFα concentration by about 20-fold (10 ± 0.4 control and 200 ± 2 pg/mL ASA); however, this rise was considerably lower in the NO-ASA (52 ± 2 pg/mL) and NONO-ASA (95 ± 2 pg/mL) treated animals, (Fig 5).

**Antipyretic activity:** It is well known that aspirin exerts a moderate antipyretic effect when administered orally; therefore, we wanted to determine the decrease in body temperature induced by NO-ASA and NONO-ASA prodrugs compared to that obtained with the parent drug ASA. Experimental drugs (1 mmol/kg) were administered (po) 30 minutes before injecting LPS (50 μg/kg ip) in experimental animals. In this regard, control animals showed a time-dependent increase in body temperature ($\Delta T = 1.5$ °C) up to 4 h and maintained it until the end of the screen (5 h). However, prodrug-treated animals showed only about half degree increase in body temperature 1 h after LPS injection and preserved it within this range throughout the experiment (Fig 6 A). Aspirin was more potent ($\Delta T = 0.3$ °C) than either prodrug, but all three drugs were effectively capable of reducing LPS-induced fever.

**Carrageenan-induced mechanical hyperalgesia:** This assay measures the ability of the test drugs to reverse hyperalgesia (decreased threshold to a painful stimuli) produced by injection of carrageenan reagent. The mechanical pain threshold was increased upon time by administering aspirin or its two NO-releasing prodrugs at an equimolar dose. Pain threshold was markedly reduced from 80 to about 10 g in animals receiving vehicle (control group), indicating a higher
sensitivity to mechanical stimuli (non-painful at normal conditions). Hyperalgesia was decreased in animals receiving NO-ASA (1 mmol/kg po) which produced a decrease in mechanical pain threshold to about 40 g (50% reduction compared to the initial response). The response obtained with NONO-ASA (1 mmol/kg p.o.) was a reduced hyperalgesia (mechanical pain threshold around 30 g), similar to that observed with NO-ASA. However, according to this assay, the analgesic effect exerted by aspirin was weaker (pain threshold in the 15-20 g range) than that observed with either NO-ASA or NONO-ASA, (Fig 6 B).

*Nitric oxide release:* It was essential for us to evaluate the extent of nitric oxide release from aspirin prodrugs possessing different NO-donor groups, namely an organic nitrate (present in NO-ASA) and a N-diazen-1-ium-1,2-diolate (present in NONO-ASA). The NO-release profile from these drugs was monitored every hour (up to 6 h) using the Griess reaction to measure NO as total NO$_3^-$/NO$_2^-$ levels (Fig 7). Both prodrugs showed a time-dependent release of NO. After administration of NO-ASA (1 mmol/kg, po) NO levels increased up to 120 ± 3 µM after 2 h, but decreased thereafter to about 25 ± 2 µM at the end of the assay (6 h). In the case of NONO-ASA, plasma NO levels peaked to 160 ± 3 µM at 4 h post-administration (1 mmol/kg, po) and at the end of the experiment (6 h), the NO level was about 4-fold higher than that observed in animals receiving NO-ASA (Fig 7).
Discussion

Hybrid anti-inflammatory prodrugs possessing organic nitrates are described in the literature as effective anti-inflammatory, analgesic, antipyretic, and chemopreventive drugs (Wallace and Vong, 2008). We conducted an extensive head-to-head comparison between two NO-releasing aspirins, one of them (NCX-4016) possessing an organic nitrate, the other one (CVM-01) possessing a N-diazeniumdiolate. The replacement of organic nitrates by N-diazeniumdiolates, and its biological evaluation under identical experimental conditions, offered us the opportunity to assess physicochemical and pharmacological differences inherent to each drug, and to evaluate unique mechanistic features required for the release of the active components from the prodrug. Furthermore, the use of aspirin as parent NSAID was used as starting point for comparison purposes.

We initiated our comparison by measuring the extent of gastric protection exerted by the two hybrid prodrugs. As described previously, nitric oxide release from hybrid prodrugs was based on the fact that NO is one of the gastric mucous membrane protecting factors (Brown et al., 1992; Brown et al., 1993). Continuous release of NO (through nitric oxide synthase expression) contributes to the physiologic gastrointestinal mobility, tonus, permeability and blood flow to the vessels of gastric wall (Kato et al., 2001) which protects the epithelial layer against the mechanism-based toxicity of aspirin. However, the mechanism of NO release from these prodrugs was expected to be different and therefore, constitute a pharmacological difference between them. In this regard, organic nitrates require an in vivo three-electron reduction to release NO, whereas N-diazeniumdiolate ions spontaneously release NO in physiologic media. Furthermore, N-diazeniumdiolates release two equivalents of NO (hence their name NONOates) per molecule (Davies et al., 2001). Both prodrugs were significantly less toxic than aspirin, which is consistent with prior evidence describing the role of NO in reducing the gastric damage produced by NSAIDs (Fiorucci and Del Soldato, 2003) (Fiorucci et al., 2003).
Nevertheless, there was no statistically significant difference between groups of animals receiving the NO-ASA (NCX-4016) and rats dosed with the NONO-ASA (CVM-01). This was confirmed by the ulcer and erosion indexes. The first assay measures clearly visible ulcers (elongated, hemorrhagic lesions varying in length), whereas the second one accounts for less noticeable micro-hemorrhagic lesions, which are only observed using a magnifying lens. It appears that gastroprotection provided by both hybrid prodrugs was not entirely dependent on the amount of NO released from them. Despite the observed higher levels of NO released from the N-diazeniumdiolate ion present in CVM-01 (confirmed by the measurement of plasma nitrite/nitrite levels), the extent of gastric protection was practically identical for both prodrugs. For this reason, it might be speculated that NO-dependent protection from hybrid prodrugs reaches a “saturation point”, where the excess of NO (about 2 equivalents) released from CVM-01 does not produce additional gastric protection. We also found that an increase in the concentration of TBARS, an index of lipid peroxidation, and a decrease in TNF-α, while the antioxidative marker (SOD) increased in NO-ASA and NONO-ASA treated rats. All changes in the gastric mucosal tissue may be as the result of the antioxidative effects of the prodrug.

In addition to reducing gastric damage, it was essential for us to evaluate and compare the anti-inflammatory profile of aspirin, NO-aspirin, and NONO-aspirin. We conducted this evaluation by measuring the in vivo carrageenan-induced rat paw oedema assay, and direct measurement of cyclooxygenase-dependant production of prostaglandin E$_2$ (PGE$_2$) in gastric tissue as well as plasma. When administered at equimolar doses (1 mmol/kg p.o.), all test drugs showed significant anti-inflammatory activity, by decreasing the inflammatory response (paw volume) and reducing the amount of PGE$_2$ rats.

In the rat paw oedema model, however, there were some differences noted among the three compounds. Both aspirin and NO-aspirin produced a significant reduction (approximately 60%)
in the inflammation of paw compared to the vehicle control group. NONO-aspirin on the other hand had 40% reduction in the rat paw swelling. One possible reason for this might be found in the PGE$_2$ measurements in the paw exudates. ASA and NO-aspirin both produced a 90% or greater reduction in PGE$_2$ levels, whereas NONO-aspirin produced approximately a 70% reduction in PGE$_2$ levels. It may be necessary therefore to inhibit PGE$_2$ levels almost completely in order to have a significant effect on inflammation (Wallace et al., 1999). In models of inflammatory pain (hyperalgesia), NCX 4016 and CVM-01 showed a significant analgesic activity for 6 h after treatment and had a longer duration of action as compared with ASA treated rats. The analgesic effect was demonstrated by aspirin, NO-aspirin and NONO-aspirin, with NO donating compounds being more potent than aspirin on a molar basis. These two compounds also provide anti-pyretic activity as the potential to decrease body temperature induced by LPS (50 μg/kg).

In conclusion, we have demonstrated that the hybrid aspirin possessing $N$-diazen-1-iium-1,2-diolate (CVM-01) has equipotent anti-inflammatory, analgesic, and antipyretic activity compared to the hybrid aspirin possessing an organic nitrate (NCX-4016). Furthermore, it was demonstrated that gastric protection exerted by the release of NO from hybrid NO-releasing aspirins appears to be independent of the extent and mechanism of NO release, since replacement of $N$-diazen-1-iium-1,2-diolates for organic nitrates did not produce a statistically significant difference in the ulcerogenic profile measured for both prodrugs.
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Footnotes
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Figure Legends

Figure 1: Chemical Structures of aspirin and NO-releasing aspirins, NCX 4016 (NO-ASA) and CVM-01 (NONO-ASA).

Figure 2. NO-ASA and NONO-ASA do not cause gastric damage. Drugs were administered orally at equimolar doses (1 mmol/kg) and effects on the stomach were evaluated as indicated in Methods. ASA caused severe gastric damage, UI = 50 ± 7 mm, whereas both NO-ASA and NONO-ASA were gastric damage-sparing, UI = 2 ± 0.2 mm and 3 ± 0.3 mm for NO-ASA and NONO-ASA, respectively (panel A). All three drugs also caused erosions of the gastric mucosa, but the damage was significantly less in the NO-ASA and NONO-ASA groups compared to the ASA group (panel B). Results are mean ± SEM for 9-12 rats in each group, *P < 0.01 compared to the ASA group.

Figure 3. Effects of ASA, NO-ASA and NONO-ASA on gastric PGE$_2$ level, lipid peroxidation (MDA) and superoxide dismutase (SOD). Four groups of rats were treated with vehicle, ASA, NO-ASA, and NONO-ASA and their stomachs removed and processed as described in Methods. All three drugs caused a significant reduction in gastric mucosal PGE$_2$ levels (panel A). Results are mean ± SEM of 9-12 rats in each group, *P < 0.05 vs vehicle group. ASA caused an almost 6-fold increase in MDA levels, for NO-ASA and NONO-ASA treated rats, MDA levels were comparable to that in the vehicle treated rats (panel B). Results are mean ± SEM for 5-7 rats in each group, †P < 0.01 vs ASA group. ASA caused a significant reduction in SOD activity, whereas NO-ASA and NONO-ASA did not have an effect (panel C). Results are mean ± SEM of 5-7 rats, *P < 0.05 vs vehicle group.
Figure 4. Anti-inflammatory properties of ASA, NO-ASA and NONO-ASA. Rat paw edema was induced by carrageenan injection as described in Methods. All 3 drugs caused a significant reduction in paw volume at all time points (panel A). Results are mean ± SEM of 5 rats in each group, *P < 0.05 vs vehicle treated rats at all time points. All 3 drugs also caused a significant reduction in PGE\(_2\) levels in the paw exudate (panel B). Results are mean ± SEM for 5 rats in each group, †P< 0.01 vs vehicle, *P<0.05 vs NO-ASA and NONO-ASA.

Figure 5. Effect of ASA and its NO-releasing prodrugs on plasma TNF-α. Rats were treated with equimolar amounts (1 mmol/kg) of each drug and plasma TNF-α was measured as described in Methods. ASA caused a significant rise in plasma TNF-α, however, this rise was significantly less in the NO-ASA and NONO-ASA treated rats. Results are mean ± SEM for 3 rats in each group, *P < 0.01 vs vehicle, †P < 0.01 vs ASA, §P < 0.05 vs NO-ASA.

Figure 6. ASA, NO-ASA and NONO-ASA reduce LPS-induced fever and raise the threshold for hyperalgesia. Panel A: LPS (50 μg/kg, ip) was administered to the animals one hour before administration of the test drugs. Core body temperature was recorded at 30 min and thereafter hourly for 5 h. Results are mean ± SEM for 5 rats in each group, *P < 0.05 vs vehicle for all three drugs from 1-5 h. Panel B: Mechanical pain threshold was increased in a time-dependent manner by all three drugs, however, both NO-ASA and NONO-ASA were better than ASA especially during the last 2h of the experiment. Results are mean ± SEM for 5 rats in each group. §P < 0.05 vs vehicle for all three drugs from 1-5 h; †P < 0.05 for NO-ASA vs ASA and NONO-ASA from 2-5 h; *P < 0.05 for NONO-ASA vs ASA from 4-5 h.

Figure 7. Time course of NO\(_x\) levels in plasma after NO-ASA and NONO-ASA administration. Plasma concentration of NO was quantified indirectly as the concentration of NO\(_3^-\)/NO\(_2^-\) using
the Griess method. Results are mean ± SEM of 3 rats at each time point, $P < 0.01$ vs zero time at all time points.
Aspirin

NCX 4016, NO-ASA

CVM-01, NONO-ASA
Gastric Damage Score, “Ulcer Index” (mm)

Treatment Groups

Vehicle ASA NO-ASA NONO-ASA

Fig 2
Fig 3

(A) PGE₂, pg/mg protein
(B) MDA, nmol/mg protein
(C) SOD Activity, U/mg protein

Legend:
- Vehicle
- ASA
- NO-ASA
- NONO-ASA

Significance:
- * p < 0.05
- † p < 0.01
Fig 4

(A) Graph showing the change in volume (Δ Volume, mL) over time (0-6 hours) for different treatment groups: Vehicle, ASA, NO-ASA, and NONO-ASA. The graph indicates a time-dependent increase in volume for different treatments with some variability indicated by error bars.

(B) Bar graph showing the PGE$_2$ levels (pg/mg protein) for different treatment groups: Vehicle, ASA, NO-ASA, and NONO-ASA. The graph shows the levels at different time points with asterisks and daggers indicating statistical significance.

Treatment Groups:
- Vehicle
- ASA
- NO-ASA
- NONO-ASA
Plasma TNFα, pg/mL

Vehicle ASA NO-ASA NONO-ASA

Fig 5
Fig 7